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Microbiota biodiversity of traditional fermented products

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Microbiota biodiversity of traditional fermented products

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Editorial: Microbiota biodiversity of traditional fermented products

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Editorial on the Research Topic
Microbiota biodiversity of traditional fermented products

Fermentation stands as one of humanity's most extensively employed traditional techniques for processing, preservation, and introducing diversity to food products, imparting them with distinctive sensory and physical characteristics and extending their shelf life. The varied microbiota prevailing in fermented foods significantly contribute to fostering gut health through their involvement in digestion and nutrient absorption. Furthermore, the fermentation process produces bioactive compounds such as vitamins, peptides, organic acids, exopolysaccharides, amino acids and proteins which have been linked to improved immune function and overall wellbeing. However, despite their cultural significance and potential health advantages, there is a noticeable lack of comprehensive research on the microbial composition and health benefits of these traditional foods. Hence the research focus on "Microbiota diversity of traditional fermented products" was initiated to resonate within the scientific community encouraging further exploration of this uncharted territory by utilizing advanced research methodologies.

Baijiu is an alcoholic food with a long history in China, while its brewing process is extremely complicated. A correlation study on the dynamics of microbial community, physicochemical properties and volatile flavor compounds in the Xiasha round of the cave-brewed sauce-flavor Baijiu (CBSB) was reported by Ren et al. This study established the correlation networks and metabolic maps to understand the relationship between 18 dominant microbial genera and 23 significantly different volatile metabolites in the fermentation process. The investigation established theoretical foundations for CBSB, thereby creating a robust groundwork for subsequent craft optimization and the enhancement of Baijiu quality. Wu et al. conducted a parallel study utilizing multi-omics technology to examine the interplay between microbial community structure and volatile compounds in Cigar stacking fermentation. The study emphasized the interaction of diverse microbiota during the fermentation of various cigar varieties, which also impacted the development of aroma and other microbial agents. Further, Liu et al. conducted a study exploring the impact of extended fermentation time on the microbiota and wine quality in the fermentation process of Luxiang-flavor Baijiu. The research also analyzed the patterns of change in flavor substances and microbial flora.

The utilization of predictive functional profiles of microbial communities in fermented foods is an effective method for annotating the metabolic pathways within the gene sequences of microorganisms. Das et al. employed shotgun-based metataxonomic sequence analysis to report the microbial community structure and functional profiles

of naturally fermented bamboo shoots. The study identified 49 phyla, 409 families, 841 genera, and 1,799 species, with Firmicutes as the dominant phylum (89.28%). Furthermore, it unveiled genes related to amino acid metabolism, pectin degradation, lipid metabolism, and other crucial pathways, suggesting their potential role in enhancing the nutritional and sensory qualities of fermented bamboo shoot products. In a metagenomic investigation, Sessou et al. contrasted bacterial diversity during the spontaneous fermentation of raw cow milk in two geographically distinct regions: Northeast India and West Africa. The study addressed the safety concerns in spontaneous milk fermentation, proposing strategies and technological interventions for controlled fermentation to ensure consistent, high-quality products with desired properties. Moreover, Xu et al. performed a meta-analysis using metagenomic sequencing data to investigate the potential benefits and risks associated with traditional fermented foods. The study underscored the impact of raw materials, regions, and substrates on microbial diversity and taxonomic composition, offering a thorough assessment of fermented food microbiomes. Another metagenomic investigation conducted by Lv et al. examined the microbial diversity of red vinasse acid, a traditional fermented food and identified key metabolic processes and essential enzyme genes related to sugar and amino acid metabolism. The study also delved into the role of dominant strains in primary metabolic pathways, confirmed the expression of crucial enzyme genes, and provided fundamental research data for the improvement of fermenting techniques to enhance the quality of red vinasse acid. Further, a metataxonomic analysis of indigenous and starter microbiota throughout the ripening process of Gouda cheese produced using unpasteurized milk contaminated with *Listeria monocytogenes* was carried out by Salazar et al. The study examined the bacterial community dynamics in Gouda cheese from unpasteurized milk, with and without *L. monocytogenes*, and identified notable differences in microbiomes during ripening. Unique taxa emerged in cheese without *L. monocytogenes* after 90 days, emphasizing the potential impact of *L. monocytogenes* on the microbial community. The findings underscored the importance of accounting for milk source characteristics in future metataxonomic studies on Gouda cheese.

Expanding upon metagenomic insights, metaproteomics delves into the functional aspects of microbial communities by examining the proteins expressed, providing a holistic understanding of the genetic potential uncovered through genomic analysis. The recently emerging data-independent acquisition (DIA) mass spectrometry in metaproteomics has proven valuable in gaining insights into the functions of microbiota, a domain constrained in genomic studies. Zhao et al. utilized quantitative metaproteomics to unveil the composition and metabolic characteristics of microbial communities in Chinese liquor fermentation starters, specifically Daqu. The outcome of the study highlighted unique microbial features in different Daqu types, with seasonal variations anticipated to guide the optimization of yield, quality, and flavor in liquor production

Another significant aspect of the microbiota biodiversity in traditional fermented products involves the strategies for inoculation, crucial for determining the flavor quality of fermented foods. In a study by Ye et al., the effects of the direct injection strategy and traditional inoculation strategy on physicochemical indices and flavor substances were investigated during the acetic acid fermentation process of Zhenjiang aromatic vinegar. The outcomes revealed that the direct inoculation strategy resulted in elevated levels of total acid, organic acid, and amino acid in comparison to the traditional inoculation approach. Additionally, it effectively stimulated acetoin production. While the traditional inoculation method demonstrated greater strain diversity, it exhibited lower relative abundance of major microbial genera throughout the fermentation process compared to the direct inoculation approach. Further a study by Pan et al. explored the impact of inoculating a single strain of *Bacillus licheniformis* and the microbiota comprising *B. velezensis* and *B. subtilis* on the microbial community and enzymatic activities of medium-temperature Daqu (MTD). The findings suggested that the bacterial community in MTD showed higher sensitivity to bioturbation than the fungal community, leading to variations in enzymatic activities. The indigenous microbiota exhibited a more pronounced response to the single strain than to the microbiota, augmenting the composition of functional microbiota related to liquefying activity (LA) and saccharifying activity (SA) in MTD.

Numerous scientific studies consistently demonstrate the potential health benefits of probiotics in humans. Given the global epidemic of diabetes and its rising association with western eating habits, there is a growing concern. In response, Huligere et al. conducted a study to assess the potential probiotic *Lactobacillus* spp. isolated from traditional fermented batters for potential use in diabetes treatment. The probiotic isolates displayed noteworthy probiotic properties, inhibitory activities against α-glucosidase and α-amylase indicating their potential as a source of anti-diabetic properties and, upon purification, could be employed as probiotic supplements. However, additional *in vivo* research is essential for comprehensive assessment.

Furthermore, a study by Bhutia et al. on the assessment of microbiological safety of traditionally processed fermented meat products was incorporated into the research theme. This study involved identifying potential spoilage or pathogenic bacteria, detecting enterotoxins, and screening antibiotic susceptibility patterns. Also, the study emphasized the need for proper hygiene monitoring during preparation to ensure the safety of these culturally accepted meat products.

In summary, the studies have delved into various facets of microbial diversification in fermented food products, demonstrating significant promise through metagenomic and metaproteomic analyses, as well as evaluations of potential health benefits and quality assessments.

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Molecular Characterization of Bacteria, Detection of Enterotoxin Genes, and Screening of Antibiotic Susceptibility Patterns in Traditionally Processed Meat Products of Sikkim, India

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The lesser-known traditionally processed meat products such as beef *kargyong*, pork *kargyong*, *satchu*, and *khyopeh* are popular food items in the Himalayan state of Sikkim in India. The present study aimed to assess the microbiological safety of traditional meat products by identifying the potential spoilage or pathogenic bacteria, detecting the enterotoxins, and screening the antibiotic susceptibility patterns. The pH and moisture contents of the meat products varied from 5.3 to 5.9 and from 1.5 to 18%, respectively. The microbial loads of aerobic bacteria were 10^5 to 10^7 cfu/g, *Staphylococcus* 10^3 to 10^6 cfu/g, *Bacillus* 10^4 to 10^6 cfu/g, and total coliform 10^2 to 10^7 cfu/g, respectively. Based on 16S rRNA gene sequencing, the bacterial species isolated from traditionally processed meat products were *Staphylococcus piscifermentans*, *Citrobacter freundii*, *Enterococcus faecalis*, *Salmonella enterica*, *Staphylococcus aureus*, *Citrobacter werkmanii*, *Klebsiella pneumoniae*, *Macrococcus caseolyticus*, *Klebsiella aerogenes*, *Staphylococcus saprophyticus*, *Pseudocitrobacter anthropi*, *Citrobacter europaeus*, *Shigella sonnei*, *Escherichia fergusonii*, *Klebsiella grimontii*, *Burkholderia cepacia*, and *Bacillus cereus*. The enzyme-linked immunosorbent assay (ELISA) tests detected *Salmonella* spp. and enterotoxins produced by *B. cereus* well as *Staphylococcus* in a few tested samples. However, the PCR method did not detect the virulence genes of *B. cereus* and *Salmonella* in the isolates. Virulence gene (*sea*) was detected in *S. piscifermentans* BSLST44 and *S. piscifermentans* BULST54 isolated from beef *kargyong* and in *S. aureus* PSST53 isolated from pork *kargyong*. No enterotoxins were detected in *khyopeh* samples. The antibiotic sensitivity test showed that all bacterial strains were susceptible toward gentamicin, cotrimoxazole, norfloxacin, and trimethoprim. Gram-positive bacteria showed 100% sensitivity against clindamycin and erythromycin; however, 50% of the resistance pattern was observed against oxacillin followed by penicillin (33%) and ampicillin (27%).

Keywords: meat products, 16S rRNA, ELISA, pathogens, enterotoxin, virulent genes

INTRODUCTION

Meat and meat products are important dietary cultures of many communities in the world, among which some popular meat products such as sausages, hams, salami, etc. have been extensively studied in terms of microbiological safety, health benefits, and process technology (Petit et al., 2014; Santiyanont et al., 2019; Tamang et al., 2020). Culturally and organoleptically acceptable meat products are produced by traditional methods of preservation of perishable animal flesh through fermentation (Tamang et al., 2016), sun drying (Aksoy et al., 2019), smoking (Plavsic et al., 2015), and salting (Uğuz et al., 2011). Fermented meat products are usually considered safe for consumption due to low water activity (aw) and low pH, which prevent the growth of pathogenic organisms during fermentation (Holck et al., 2017). Moreover, the presence of some dominant lactic acid bacteria in fermented meat products such as *Lactobacillus sakei*, *Lb. plantarum*, *Lb. curvatus*, *Enterococcus faecium*, *Pediococcus pentosaceus*, *Leuconostoc carnosum*, *Leuc. gelidum*, *Leuc. pseudomesenteroides*, and *Weissella* spp. (Dias et al., 2015; Laranjo et al., 2017) also inhibit the populations of pathogenic organisms in the final products. However, there is a probability of occurrence of pathogenic organisms such as Shiga toxin-producing enterohemorrhagic *Escherichia coli*, *Staphylococcus aureus*, *Salmonella*, *Campylobacter* spp., and *Listeria monocytogenes* in meat products (Morales-López et al., 2019).

Varieties of lesser-known and region-specific traditionally processed meat products, produced either by smoking/sun drying or fermentation of domesticated animals such as goat, sheep, pig, ox, and yak, are consumed by different ethnic communities in Asia. Traditionally processed meat (beef, sheep, pig, and yak) products in India are *chartayshya*, *jamma*, and *arjia* of Uttarakhand (Oki et al., 2011); *kargyong*, *satchu*, and *suka ko masu* of Sikkim; and *sa-um* of Mizoram (Rai et al., 2009; De Mandal et al., 2018). Few species of bacteria were reported earlier from *kargyong*, *satchu*, and *suka ko masu* of Sikkim, purely based on limited phenotypic and biochemical tests, which included *Lb. sakei*, *Lb. divergens*, *Lb. carnis*, *Lb. sanfransisco*, *Lb. curvatus*, *Leuc. mesenteroides*, *E. faecium*, *Bacillus subtilis*, *B. mycoides*, *B. thuringiensis*, *S. aureus*, and *Micrococcus* (Rai et al., 2010).

In this study, four different types of traditionally processed meat products of Sikkim in India were selected viz. *kargyong* (**Figure 1A**), a traditional sausage-like product prepared from beef and pork meat; *satchu* (**Figure 1B**), a smoked beef meat product; and *khyopeh* (**Figure 1C**), a fermented yak meat product. *Kargyong* is produced by mixing lean meats of beef or pork with salt, garlic, and ginger, and then mixtures are stuffed into the intestine of animals as natural casings. Both ends of casings are then tied up with a rope, put into utensil, and boiled for 30 min, strained out, hooked in a bamboo stick, and smoked above an earthen oven for 10–15 days (Rai et al., 2009). No nitrates and nitrites are added during the preparation of *kargyong*. *Satchu* is prepared by slicing red meat of beef or yak into long thread-like strips; mixed with turmeric, salt, and oil; and smoked for 7–10 days (Rai et al., 2009; Rai and Tamang, 2017). *Khyopeh* is a naturally fermented yak meat product of Sikkim. During its preparation, chopped meats and innards of yak are mixed with salt, and the mixtures are stuffed into the rumen, which is previously removed from the slaughtered yak. Filled up rumen is tied up with twine and hung into bamboo stripes for natural fermentation for 4–6 months above an earthen oven (Bhutia et al., 2020). *Kargyong* and *satchu* are commonly eaten as fried side dish or curry, while *khyopeh* is eaten as soup or curry. However, the presence of pathogenic bacteria in these traditionally processed meat products has not been assessed as food safety measures. Hence, the present study is aimed to isolate and identify the pathogenic and spoilage bacteria, to detect enterotoxin genes, and also to screen antibiotic resistance patterns.

MATERIALS AND METHODS

Sample Collection

A total of 27 samples of traditionally processed meat products viz. beef *kargyong* (eight samples), pork *kargyong* (eight), *satchu* (six), and *khyopeh* (five) were collected from different households of Sikkim in India (**Table 1**). Collected samples were kept in sterile polybags and put into an ice-box carrier and transported to the laboratory. Samples were stored at kept at 4°C for further analysis.

pH and Moisture

The pH was determined with a pH meter (Thermo Scientific, United States) and the moisture contents of samples were measured by a Moisture Analyzer (Ohaus MB, United States). The measurement was taken in triplicates and average values were considered.

Food Sample Preparation

About 25 g of sample was weighed and homogenized with 225 ml buffered peptone water for 2–3 min at medium speed in a Stomacher 400 (Seward, United Kingdom) to obtain 10^{-1} dilution. Serial dilutions were made by pipetting 1 ml of homogenized samples into 9 ml of sterile diluent. Up to 10^{9} decimal dilutions were made from the previous dilution. An aliquot (0.1 ml) of the diluents was further plated on specific media by the spread plate method (Andrews and Hammack, 2003).

Enumeration of Microbial Load

About 0.1 ml of the homogenized sample in Butterfield's phosphate-buffered water was inoculated into Plate Count Agar (M091, HiMedia, India), Nutrient Agar (M001, HiMedia, India), Baird Parker agar (M043, HiMedia, India), *Bacillus cereus* agar (M833, HiMedia, India), and Violet Red Bile Agar (M049, HiMedia, India) using the surface spread method (Feng et al., 2002). The plates were incubated at 35°C for 24 to 48 h and the colonies were counted and results were expressed as colony-forming units per gram (cfu/g). The 3M^{TM} Petrifilm^{TM} aerobic count plate (6400, 3M, United States) was also used to enumerate the microbial load from the collected meat samples, which contained modified standard nutrients, a soluble gelling agent, and a tetrazolium indicator (Nelson et al., 2013).

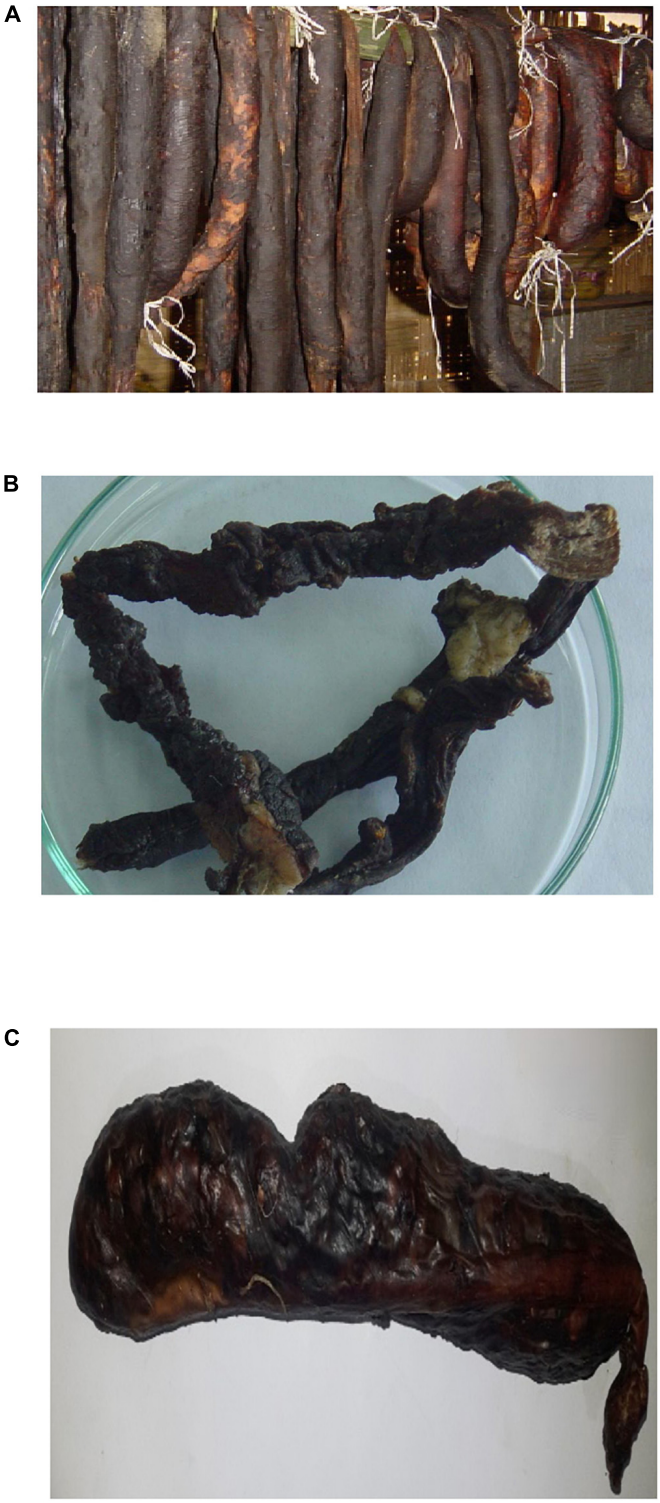

FIGURE 1 | (A) *Kargyong* (beef and pork), **(B)** *Satchu*, and **(C)** *Khyopeh.*

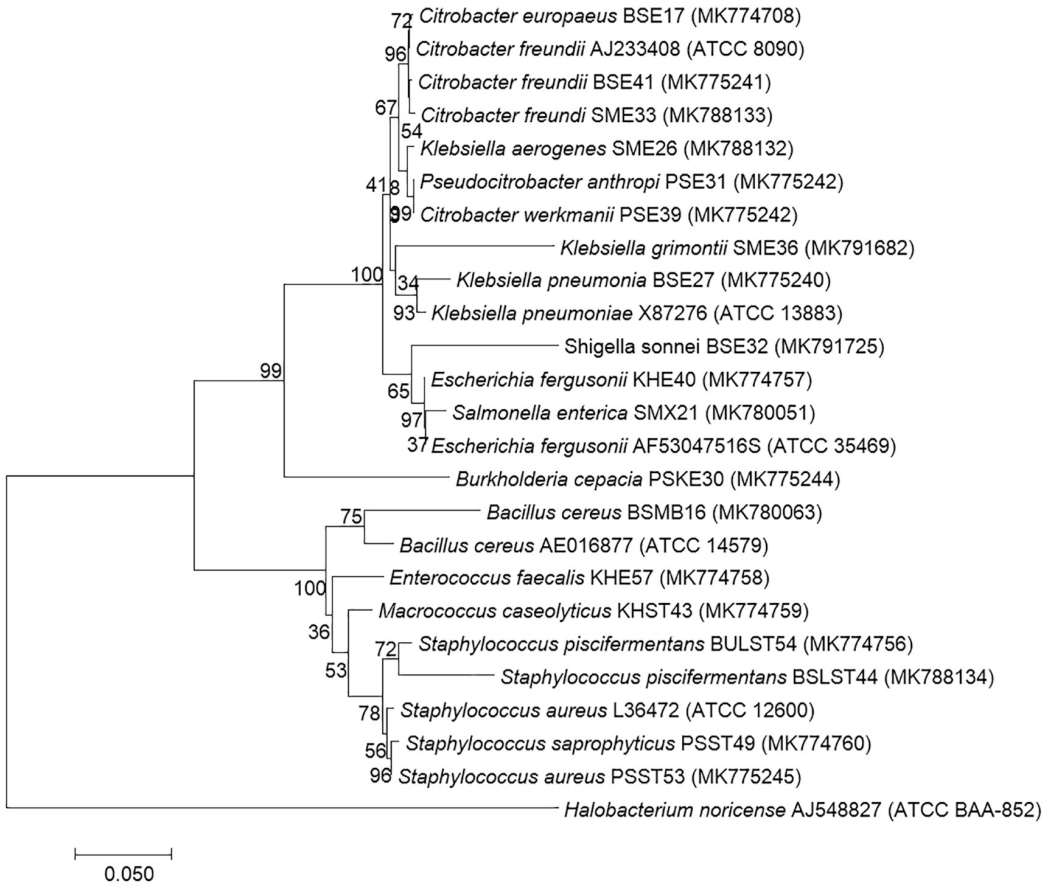

FIGURE 2 | Molecular phylogenetic analysis of 19 bacterial isolates recovered from traditional processed meat products based on 16S rRNA region sequencing. Neighbor-joining phylogenetic tree representation by MEGA 7 with *Halobacterium noricence* AJ548827 as the outgroup with the evolutionary history (Kumar et al., 2016). The optimal tree with a sum of branch length = 1.13658194 and the percentage of replicate trees in which the clustered associated taxa in the bootstrap test (1,000 replicates) are shown next to the branches. The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (Tamura et al., 2004), and are expressed in units of the number of base substitutions per site. Analysis involved 25 nucleotide sequences with a total of 569 positions in the final dataset.

Enumeration of *Staphylococcus*

The presence or absence of *Staphylococcus* in each sample was assessed according to the Food and Drug Administration (FDA) standard method (Tallent et al., 2016). Baird–Parker agar plates were supplemented with Egg Yolk Tellurite Emulsion (FD046L, HiMedia, India). Plates were incubated at 37°C for 36–48 h. Convex, black, shiny colonies with narrow white margin surrounded by a clear zone were regarded as *Staphylococcus* (Tallent et al., 2016).

Enumeration of *Enterococcus*

Ten grams of each sample was diluted in 90 ml of peptone water (0.1% w/v) for homogenization by mechanic stirring. In products with high-fat content, 1% v/v Tween 80 (P1754, Merck, Germany) was added. Then, 0.1 ml aliquots of diluents from homogenized food were spread in Bile Esculin Azide Agar (M493, HiMedia, India) plates and incubated for 24 h at 35°C. Colonies that showed black pigmentation on the BEA agar were regarded as *Enterococcus* (Delpech et al., 2012).

Enumeration of *Bacillus*

Ten grams of each sample was blended in 90 ml of 0.85% sterile saline in using Stomacher 400 (Seward, United Kingdom) for 3 min followed by serial dilution. Aliquots of 0.1 ml of the appropriate dilutions were surface plated on prepoured plates of *B. cereus* agar (M833, HiMedia, India). Blue colonies with positive precipitation were regarded as *Bacillus* (Rai et al., 2010).

Enumeration of *Escherichia* and Coliform Bacteria

Ten grams of each sample was homogenized in a Stomacher 400 (Seward, United Kingdom) for 3 min in 90 ml buffered peptone water and serial dilution was prepared. An aliquot (0.1 ml) of the homogenate was transferred to Violet Red Bile Agar

TABLE 1 | Sequences of primers and PCR reaction conditions.

Target organism	Amplification target	Amplicon size (bp)	Sequence (5′–3′)	Reaction conditions	References
Bacillus cereus	*Nhea*	755	F: GTTAGGACAATCACCGC R: ACGAATGTAATTTGAGTGC	94°C, 2 min → (94°C, 60 s → 56°C, 60 s → 70°C, 120 s) 35 cycles → 72°C, 5 min	Guinebretière and Broussolle, 2002
	Nheb	743	F:TTTAGTAGTGGTCTGTACGC R:TTAATGTTCGTTAATCCTGC	94°C, 2 min → (94°C, 60 s → 54°C, 60 s → 72°C, 120 s) 35 cycles → 72°C, 5 min	Guinebretière and Broussolle, 2002
Staphylococcus aureus	*Sea*	180	F:TAAGGAGGTGGTGCCTATGG R: CATCGAAACCAGCCAAAGTT	94°C, 5 min → (94°C, 1 min → 56°C, 1 min → 68°C, 1 min) 30 cycles → 72°C, 7 min	Cremonesi et al., 2005
Salmonella enterica	*InvA*	275	F: TATCGCCACGTTCGGCAA R: TCGCACCGTCAAAGGAACC	95°C, 1 min → (95°C, 20 s, 55°C, 20 s → 72°C, 2 min) 35 cycles → 72°C for 4 min	Nayak et al., 2004
	Stn	617	F: TTGTGTCGCTATACTGGCAACC R: ATTCGTAACCCGCTCTCGTCC	94°C, 5 min → (94°C, 60 s, 59°C, 60 s → 72°C, 1 min) 35 cycles → 72°C for 10 min	Murugkar et al., 2003

(M049, HiMedia, India) plates and incubated at 18–24 h at 35°C. Colonies that are purple red, 0.5 mm or larger, and surrounded by a zone of precipitated bile acids were regarded as coliforms. The colonies were further transferred to Brilliant Green Bile Broth (M1211, HiMedia, India) and incubated at 35°C for 24–48 h for gas production. Pure culture of isolates was inoculated in Eosin Methyl Blue Agar (M317, HiMedia, India) and MacConkey Agar (M0081B, HiMedia, India) for differentiation of enteric bacteria (Feng et al., 2002).

Enumeration of *Salmonella*

The method for isolation of *Salmonella* was followed as per the protocol described by Santos et al. (2020). About 25 g of the sample was aseptically homogenized and inoculated into 225 ml of buffered peptone water (M614, HiMedia, India) in a sterile jar. The pre-enrichment culture was incubated at 37°C for 18–24 h. Then, 0.1 ml of pre-enrichment culture was inoculated into 10 ml Rappaport–Vassiliadis (RV) medium (M880, HiMedia, India) and incubated at 42°C for 24 h in a circulating, thermostatically controlled water bath (1322, Remi, India). A loopful of cultured broth was streaked onto Xylose-Lysine Deoxycholate Agar (M031, HiMedia, India) plates and incubated at 37°C for 18–24 h. Pink colonies with or without black color were regarded as *Salmonella*. Each isolate was streaked onto Nutrient Agar (M001, HiMedia, India), incubated at 37°C for 24 h, and phenotypically characterized.

Phenotypic Characterization

The isolated bacteria were characterized on the basis of colony morphology (Tallent et al., 2016), Gram's reaction, sporulation test (Hussey and Zayaitz, 2007), motility tests (Shields and Cathcart, 2011), enzymatic reactions, and biochemical tests.

Enzymatic Tests

Catalase test was performed by placing a loopful of bacterial isolates into the test tube containing five to six drops of 3% hydrogen peroxide. A positive reaction was represented by the presence of bubbles and no bubble formation represented catalase negative (Reiner, 2010). For the urease test, a heavy inoculum from 24 h pure culture was streaked into the entire slant surface of a tube containing Christensen's medium and incubated at 35°C. The slant was observed for a color change up to 6 days. Urease production was indicated by a bright pink color on the slant that extends into the butt (Brink, 2010). A gelatin hydrolysis test was performed by stab-inoculating a 24-h culture into the tubes containing nutrient gelatin. The inoculated tubes and uninoculated control tubes were incubated at 25°C for up to 1 week and checked every day for gelatin liquefaction. Liquefaction due to gelatinase activity was confirmed by immersing tubes in an ice bath for 15–30 min, and then tubes were tilted to observe if the gelatin was hydrolyzed. The positive test of hydrolyzed gelatin resulted in a liquid medium after exposure to cold temperature, whereas the negative tests remained solid (dela Cruz and Torres, 2012). For the nitrate reduction test, bacterial cultures were grown in 5 ml nitrate broth for 12–24 h at 35°C. One milliliter of the culture was mixed with three drops of reagents (reagent A and reagent B) and observed for the development of a red/yellow color, indicating the presence/absence of nitrate. A small amount of zinc dust was added to the tube for 5 days to observe for the development of a red color, indicating the absence of nitrate reduction (Buxton, 2011). Coagulase and DNA hydrolysis tests were specifically performed for the characterization of *Staphylococcus*. For the coagulase test, about three to four isolated colonies of suspected *Staphylococcus* were emulsified in 0.3 ml of Brain Heart Infusion Broth (LQ210D, HiMedia, India) and incubated at 35–37°C for 18–24 h. Then, 0.5 ml of reconstituted plasma was added into the BHI culture, mixed thoroughly, and incubated at 37°C. The culture was observed for clot formation at intervals for over the next 46 h. The positive cultures exhibited clotting by the end of 24 h and negative cultures showed the absence of clot formation (Bennett et al., 1986). For the DNA hydrolysis test, pure culture of suspected *Staphylococcus* was streaked on DNase Test Agar w/Methyl Green (M1419, HiMedia, India) and incubated at 37°C

for 24 h. A positive result was indicated by a clear halo around the growth (Kateete et al., 2010).

Biochemical Tests

Carbohydrate Fermentation

Aseptically, two to three drops of the test organism from 18 to 24 h BHI broth culture was inoculated in a Phenol Red Carbohydrate Broth containing Durham's tube and incubated at 37°C for 24 h. A yellow color indicated fermentation of the sugar with lowering of pH to 6.8 or less. A delayed fermentation produces an orange color and bubble trapped within the Durham tube indicated gas production. A reddish or pink color indicated a negative reaction (Reiner, 2012).

IMVic (Indole, Methyl Red, Voges–Proskauer, and Citrate Utilization) and Indole Tests

Pure culture of an isolate was inoculated in the tube of Tryptone Broth (M463, HiMedia, India) and incubated at 37°C for 24–48 h. Five drops of Kovac's indole reagent (R008, HiMedia, India) was added directly onto the cultured tube. A positive indole test was indicated by the formation of pink to red color (cherry red ring) in the reagent layer on top of the medium. A negative test was indicated by yellow color on top of the reagent layer (MacWilliams, 2012).

Methyl Red and Voges–Proskauer Test

The fresh tested culture was inoculated in 5 ml Methyl Red–Voges–Proskauer (MR-VP) broth (LQ082, HiMedia, India) and incubated for 48 h at 35°C. Then, 2.5 ml of culture was transferred into a new sterile tube and five drops of Methyl Red (MR) Reagent (I007, HiMedia, India) was added. The MR-positive test organism showed red coloration of the medium and the negative test organisms showed yellow coloration of the medium due to low acid production (McDevitt, 2009). In the remaining culture grown in MR-VP broth, 0.6 ml of Barritt's reagent A (R029, HiMedia, India) and 0.2 ml of Barritt's reagent B (R030, HiMedia, India) were added. The tubes were then shaken for 30 s to 1 min and the tubes were allowed to stand for 30 min to 1 h. The VP-positive organism showed red coloration on top of the culture and the VP-negative organism showed yellowish color (McDevitt, 2009).

Citrate Test

A Simmon citrate medium (M099, HiMedia, India) was prepared in a tube slant and the fresh pure test organism was inoculated on the surface of the slant, incubated at 37°C for 18–24 h. A positive organism was represented by growth on the surface and change in color from the original green to blue (MacWilliams, 2009).

Genomic Characterization

DNA Extraction

DNA was extracted from each bacterial isolate by the standard phenol/chloroform method of Cheng and Jiang (2006). The quality of DNA was checked by electrophoresis in 0.8% agarose gel and quantified using Nano-Drop ND-1000 spectrometer (Eppendorf, Germany).

PCR Amplification

The PCR of 16S rRNA gene from isolated DNA was amplified using a universal oligonucleotide primer pair 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′) (Lane, 1991) in a thermal cycler (Thermo Fisher, United States). The reaction mixture, conditions, and protocol for the polymerase chain reaction amplification were done following the method of Chagnaud et al. (2001). The PCR amplification was performed in a mixture containing a final volume of 50 μl of GoTaq Green Master Mix (2×) (M7122, Promega, United States), 10 μM of F primer, 10 μM of R primer, and nuclease-free water (NEB). The PCR reaction program was set under the following PCR conditions: 94°C for 10 min; 94°C for 1 min, 65°C for 1 min, and 72°C for 30 s for 35 cycles; and 72°C for 7 min. The PCR products were detected by electrophoresis using 1% agarose, and the bands were stained with 7 μl/100 ml of ethidium bromide and visualized using Gel Doc EZ Imager (Bio-Rad, United States). A standard 100-base pair DNA ladder was used for the verification of amplicon size. The amplified PCR products were purified using the PEG (polyethylene glycol)–NaCl (sodium chloride) (20% w/v of PEG, 2.5 M NaCl) precipitation method of Schmitz and Riesner (2006).

16S rRNA Gene Sequencing

The PCR products were set up in 5 μl volume for a single primer amplification with the same universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′) (Lane, 1991) for separate reactions of each primer. The PCR reaction was set as follows: denaturation (96°C, 10 s), annealing (50°C, 5 s), and elongation (60°C, 2 min) with a stop reaction at 4°C. The amplicons were then precipitated with 1 μl sodium acetate (3 M, pH 5.2) and 24 μl of absolute alcohol, mixed briefly in vortex, and incubated at room temperature for 15 min, centrifuged at 12,000 rpm for 20 min, further washed with 70% ethanol, air-dried, and suspended in 10 μl formamide. Sequencing of the amplicons was performed by the Sanger sequencing method of Heather and Chain (2016) which was carried out in an automated DNA Analyzer (ABI 3730XL Capillary Sequencers, Applied Biosystems, Foster City, CA, United States).

Data Analysis

The quality of sequences was analyzed by Sequence Scanner v.1.0 (Applied Biosystems, Foster City, CA, United States) followed by DNA sequence assembly using ChromasPro 2.1.8[1]. The contigs were subjected to BLAST (Basic Local Alignment Search Tool) (Benson et al., 2014) for nucleotide similarity search. The sequences were then aligned by pairwise alignment using ClustalW and the phylogenetic tree was constructed by the neighbor-joining method using MEGA7.0 software (Kumar et al., 2016). The genera and species were identified based on the lowest *E*-value in BLAST.

[1]http://technelysium.com.au/ChromasPro.html

TABLE 2 | Collection sites, altitude, moisture, pH, and bacterial load of traditionally processed meat products of Sikkim.

Sample	District (number of samples)	Collection site	Altitude (m)	Moisture content (%)	pH	Bacterial load (cfu/g)			
						Aerobic bacteria (10^6)	*Staphylococcus* (10^5)	*Bacillus* (10^5)	Total coliform (10^6)
Beef *kargyong*	East Sikkim (4)	Lalbazaar, Gangtok	1,639	18.0 (16.3–19.7)	5.8 (5.5–6.1)	24.0 (23.0–25.0)	25.0 (23–27)	2.2 (2.1–2.3)	0.82 (0.25–1.4)
		Martam	1,652	15.1 (14.7–15.5)	5.8 (5.8–5.9)	12.3 (2.4–13)	2.0 (1.8–2.2)	1.9 (1.8–2.1)	1.4 (0.21–2.6)
	West Sikkim (2)	Geyzing	1,443	12.8 (12.4–13.3)	5.9 (5.8–6.1)	0.22 (0.18–0.27)	2.0 (2.0–2.1)	2.1 (1.6–2.7)	0.27 (0.26–0.29)
	South Sikkim (2)	Kitam	535	10.8 (10.2–11.4)	5.7 (5.6–5.8)	1.0 (0.23–1.9)	1.9 (1.7–2.1)	10.5 (2.1–19)	0.9 (0.21–1.7)
Pork *kargyong*	East Sikkim (4)	Lalbazaar, Gangtok	1,639	14.8 (8.6–16.5)	5.3 (5.2–5.4)	0.74 (0.28–1.2)	23.0 (22.0–24.0)	11.5 (2.0–21)	0.22 (0.21–2.3)
		Baluakhani Rd	1,722	8.8 (8.6–9.1)	5.4 (5.4–5.5)	0.22 (0.21–0.23)	15.0 (15.0–16.0)	2.4 (2.3–2.6)	2.2 (2.1–2.3)
	West Sikkim (2)	Geyzing	1,443	11.8 (11.3–12.4)	5.8 (5.8–5.9)	1.2 (0.1–2.2)	0.74 (0.18–1.3)	1 (0.2–1.8)	0.15 (0.13–0.18)
	South Sikkim (2)	Kitam	535	9.4 (9.1–9.7)	5.8 (5.8–5.9)	1.8 (1.7–1.9)	2.2 (1.9–2.6)	1.2 (0.15–2.3)	1.0 (0.2–1.8)
Satchu	East Sikkim (4)	Lalbazaar, Gangtok	1,639	7.3 (7.1–7.6)	5.3 (5.3–5.4)	1.2 (0.21–2.2)	10.9 (1.8–20)	1.7 (1.5–1.9)	34.0 (23–45)
		Martam	1,652	7.6 (7.2–8.1)	5.3 (5.3–5.4)	2.8 (2.7–2.9)	19.5 (16.0–23.0)	4.6 (4.5–4.7)	1.5 (0.27–2.8)
	West Sikkim (2)	Geyzing	1,443	10.7 (6.8–7.1)	5.7 (5.7–5.8)	1.4 (1.3–1.6)	2.2 (2.1–2.3)	0.38 (0.17–0.21)	0.16 (0.12–0.20)
Khyopeh	North Sikkim (5)	Lachung	2,625	2.5 (1.5–3.5)	5.9 (5.8–6.1)	1.2 (0.15–2.3)	0.01 (0.002–0.02)	Absent	0.00021 (0.00012–0.00031)

cfu/g, colony-forming units per gram.

TABLE 3 | Phenotypic and biochemical characterizations of bacteria isolated from traditionally processed meat products of Sikkim.

Colony morphology		Enzymatic reactions															Sugar fermentation								IMViC test					
	Media	Gram reaction	Motility	Catalase	Coagulase reaction	Urease reaction	DNase	Nitrate reduction	Gelatin hydrolysis	Lactose	Sorbitol	Arabinose	Glucose	Mannitol	Mannose	Trehalose	Raffinose	Maltose	Xylose	Rhamnose	Sucrose	Ribose	Adonitol	Indole	Methyl red	Voges–Proskauer	Citrate	Tentative identification (total number of isolates)
Pink without sheen	EMB	–	+	+	–	–(6) v(4)	–	+	–	+(5) v(5)	+	+	+	+	+	+	+(4) –(6)	+	+	+	+	+	–(7) v(3)	–(8) v(2)	–	+	+(6) v(4)	*Enterobacter* (10)
Pink, mucoidy	EMB	–	–	+	–	+	+	+	+	+(8) –(7)	+	+	+	+	+	+	+	+	+	+(10) v(5)	–(6) v(9)	+	+	–	+(12) –(3)	+(13) –(2)	+(11) –(4)	*Klebsiella* (15)
Purple with metallic sheen	EMB	–	+	+	–	–	–	+	–	+(10) v(2)	+(10) –(2)	+	+(8) v(4)	+(9) –(3)	–	+	+	–	+	+(9) v(3)	–(8) v(4)	–	–	+(7) v(5)	+	–	–(5) v(7)	*Escherichia* (12)
Red colonies with black centers	XLD	–	+	+	–	–	–	+	–	–(5) v(3)	+	+	+	+	+ –	+	–	+	+	+(4) –(4)	–(3) +(5)	–	–	–	+	–	+(3) –(5)	*Salmonella* (8)
Blackening of media	BEA	+	–	–	–	–	–	+	–	+	+	–	+	+	+	+	–	+	–	–(10) v(6)	+	+	+	–	–	+	–	*Enterococcus* (16)
Blue	BCA	+	+	+	–	+	–	v(3) +(9)	–	–	–	–(3) +(9)	–	+	–	+	+ –	+	–	–	+	+	–	–	–	–(3) +(9)	+	*Bacillus* (12)
Gray-black	BPA	+	+	+	+(15) –(5)	+(12) –(8)	+	+(9) –(11)	+	+	+(3) v(17)	–(12) v(8)	+	+	+	+	–(19) v(1)	+	–	+	+	+	–	–	–	+(11) –(5) v(4)	–	*Staphylococcus* (20)
Purple-blue black	EMB	–	–	+	–	v	–	+	–	v	+	+	+	–	+	+	–	v	+	+	–	–	+(17) –(8)	+(16) –(9)	+	–	+	*Citrobacter* (25)
Pale-green	NA	–	+	+	–	–(8) +(2)	–	+(7) –(3)	+	–	–	+(8) –(2)	+	+(9) –(1)	–	–	–	–	–	+(3) –(7)	–	–	–	–	–	–	+	*Pseudomonas* (10)

IMViC test, indole, methyl red, Voges–Proskauer, and citrate tests; EMB, eosin methylene blue; XLD, xylose lysine deoxycholate; BEA, bile esculin agar; BCA, Bacillus cereus agar; NA, nutrient agar; –, negative; +, positive; v, variable; number in parenthesis indicates number of isolates.

TABLE 4 | Identification of bacterial strains isolated from traditionally processed meat products of Sikkim by 16S rRNA gene sequence based on the Basic Local Alignment Search Tool (BLAST).

Product	Isolate code	Identity	Type species (% similarity)	GenBank accession number	Size (base pair)
Beef *kargyong*	BSE32	*Shigella sonnei*	*Shigella sonnei* CECT 4887 (93.09%)	MK791725	1,073
	BSE27	*Klebsiella pneumonia*	*Klebsiella pneumoniae* subsp. *rhinoscleromatis* R-70s (98.55%)	MK775240	1,442
	BSE41	*Citrobacter freundii*	*Citrobacter freundii* ATCC 8090 (99.16%)	MK775241	1,420
	BSE17	*Citrobacter europaeus*	*Citrobacter europaeus* 97/79 (97.23%)	MK774708	1,037
	BSLST44	*Staphylococcus piscifermentans*	*Staphylococcus piscifermentans* CIP103958 (98%)	MK788134	1,124
	BULST54	*Staphylococcus piscifermentans*	*Staphylococcus piscifermentans* CIP103958 (97.89%)	MK774756	1,088
	BSMB16	*Bacillus cereus*	*Bacillus cereus* ATCC 14579 (97%)	MK780063	1,040
Pork *kargyong*	PSE31	*Pseudocitrobacter anthropi*	*Pseudocitrobacter anthropi* C138 (98.24%)	MK775242	1,420
	PSE39	*Citrobacter werkmanii*	*Citrobacter werkmanii* CDC 0876-58 (97.96%)	MK775243	1,415
	PSKE30	*Burkholderia cepacia*	*Burkholderia cepacia* ATCC 25416 (98.36%)	MK775244	1,460
	PSST49	*Staphylococcus saprophyticus*	*Staphylococcus saprophyticus* subsp. *saprophyticus* ATCC 15305 (98.81%)	MK774760	1,424
	PSST53	*Staphylococcus aureus*	*Staphylococcus aureus* ATCC 12600	MK775245	1,433
Satchu	SME36	*Klebsiella grimontii*	*Klebsiella grimontii* SB73 (92.47%)	MK791682	1,126
	SME26	*Klebsiella aerogenes*	*Klebsiella aerogenes* NCTC10006 (98.28%)	MK788132	1,418
	SMX21	*Salmonella enterica*	*Salmonella enterica* subsp. *arizonae* DSM 9386 (97%)	MK780051	1,432
	SME33	*Citrobacter freundii*	*Citrobacter freundii* ATCC 8090	MK788133	1,423
Khyopeh	KHE40	*Escherichia fergusonii*	*Escherichia fergusonii* ATCC 35469 (99.58%)	MK774757	1,415
	KHST43	*Macrococcus caseolyticus*	*Macrococcus caseolyticus* subsp. *hominis* CCM 7927 (99.16%)	MK774759	1,434
	KHE57	*Enterococcus faecalis*	*Enterococcus faecalis* ATCC 19433 (98.26%)	MK774758	1,433

ATCC, American type culture collection; DSM, Deutsche Sammlung von Mikroorganismen; CCM, Czech Collection of Microorganisms; CECT, Colección Española de Cultivos Tipo; CDC, Centers for Disease Control and Prevention.

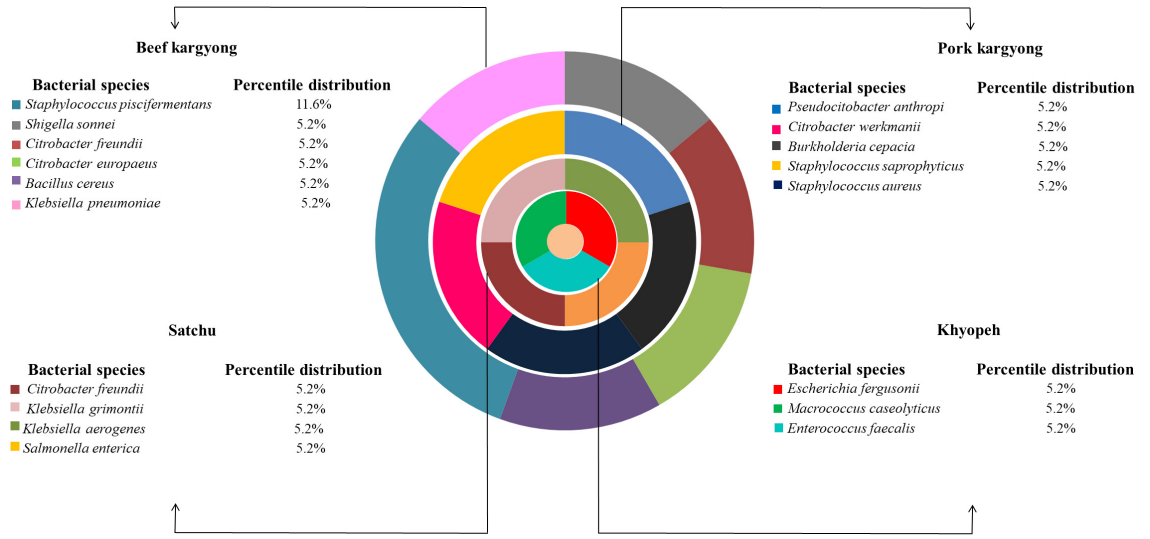

FIGURE 3 | Composition of bacterial species in various meat products of Sikkim.

Detection of Enterotoxin by ELISA

Samples were analyzed for detection of staphylococcal enterotoxin, *Bacillus* diarrheal enterotoxin, and *Salmonella*. For the extraction of *Bacillus* diarrheal enterotoxin, 25 g of the sample was added into 50 ml of 0.25 M Tris buffer (pH 8) and blended for 3 min. The slurry was transferred to a centrifuge bottle and centrifuged for 10 min at 5,000 rpm. The supernatant was then filtered through a prepared syringe and the eluate was adjusted to pH 7.0–8.0. Then, 5 ml of the eluate was mixed thoroughly with 50 µl of the sample additive (Rahmati and Labbe, 2008). The sample was then subjected to an enzyme-linked immunosorbent assay (ELISA) test using Tecra *Bacillus* diarrheal enterotoxin visual immunoassay (BDEVIA48, 3 M, United States) according to the manufacturer's instructions. For the extraction of staphylococcal enterotoxin, 10 g of the sample was added into 15 ml PBS (pH 7.4) and homogenized for about 3 min in a blender. The test sample was centrifuged at 5,000 rpm for 10 min at 10°C and 100 µl of the supernatant was tested with a RIDASCREEN staphylococcal enterotoxin assay (SET Total) kit (R4105, R-Biopharm, Germany) for bulk detection of the enterotoxins *SEA*, *SEB*, *SECl*, *SEC2*, *SEC3*, *SED*, and *SEE* (Bennett, 2005). The test was performed according to the manufacturer's instructions. For detection of *Salmonella* spp., 25 g of the sample was mixed with 225 ml of lactose broth and incubated at 36°C (±1°C) for 22–26 h. Then, 0.1 ml of the primary enriched sample was transferred into 10 mL of Rappaport–Vassiliadis broth and further incubated at 42°C (±1°C) for 18–24 h. One milliliter of the enrichment sample was transferred into 25 µl of sample additive in a tube and heated for 15 min in a water bath and then subjected to ELISA test using the Tecra *Salmonella* Visual immunoassay (SALVIA96, 3 M, United States) according to the manufacturer's instructions (Perez-Montano et al., 2012). Although all positive results were visible with the naked eye, a microplate reader and PC software were also used to measure the results. The absorbance of the samples was measured by determining optical densities at 450 nm using the iMark Microplate Reader (Bio-Rad, United States). The positive control had an absorbance of ≥1.0 and the negative control had an absorbance of ≤0.2 as per the manufacturer's instruction.

Detection of Enterotoxin Genes by PCR

The identified bacterial isolates were tested for the detection of few enterotoxin genes viz. *Staphylococcus* (*sea*), *B. cereus* (*nhea*, *nheb*), and *Salmonella* (*invA*, *stn*) by PCR analysis. These enterotoxin genes were selected based on their prevalence associated with foodborne illness (Kérouanton et al., 2007; Wallin-Carlquist et al., 2010; Tekale et al., 2015; Amor et al., 2019). The PCR amplification was performed in a total volume of 25 µl and the reaction mixture contained GoTaq Green Master Mix (Taq DNA polymerase, dNTPs, $MgCl_2$, and reaction buffers), 10 µM of each primer [Imperial Life Sciences (P) Ltd, India], and nuclease-free water (P1193, Promega, United States). The amplification target genes, amplicon size in bp, primer sequences, and reaction conditions used in this experiment are given in **Table 1**. Ten-microliter aliquot of the PCR product was resolved on 2% agarose gel at 80 V and documented with a camera system (Bio-Rad, United States). For the positive control, a reference strain of *Salmonella enterica* ser. Typhimurium (MTCC 3223), *B. cereus* (MTCC 1272), and *S. aureus* (MTCC 740) obtained from IMTECH, India, was used. *Bacillus nitratireducens* (MK203014), *Enterobacter hormaechei* (MK748263), and *Staphylococcus warneri* (MK203007) were used as a negative control for the test.

Antibiotic Sensitivity Test

Nineteen bacterial isolates were evaluated for antimicrobial sensitivity test using the Kirby–Bauer disk diffusion method according to Clinical and Laboratory Standard Institute (CLSI) guidelines (Patel et al., 2017). The inoculum suspension was

TABLE 5 | Detection of enterotoxins in traditionally processed meat extracts by enzyme-linked immunosorbent assay.

Sample tested	Collection site	Results with the Tecra kit		Results with the Ridascreen set kit
		***Bacillus* diarrheal enterotoxins**	***Salmonella* enterotoxins**	**Staphylococcal enterotoxins**
Beef *kargyong* (*n* = 2)	Martam, East Sikkim	+	–	+
Satchu (*n* = 2)		+	+	–
Beef *kargyong* (*n* = 2)	Lalbazaar, Gangtok	–	–	–
Pork *kargyong* (*n* = 2)		–	–	+
Satchu (*n* = 2)		–	–	–
Beef *kargyong* (*n* = 2)	Kitam, South Sikkim	–	–	–
Pork *kargyong* (*n* = 2)		–	–	–
Beef *kargyong* (*n* = 2)	Geyzing, West Sikkim	–	–	–
Pork *kargyong* (*n* = 2)		–	–	–
Satchu (*n* = 2)		–	–	–
Pork *kargyong* (*n* = 2)	Burtuk, Gangtok	–	+	–
Yak *kargyong* (*n* = 2)	Lachung, North Sikkim	–	–	–
Khyopeh (*n* = 2)		–	–	–
Positive control		+	+	+
Negative control		–	–	–

n = number of sample; results were consistent in duplicate tests with each of the enterotoxins in the tested samples.

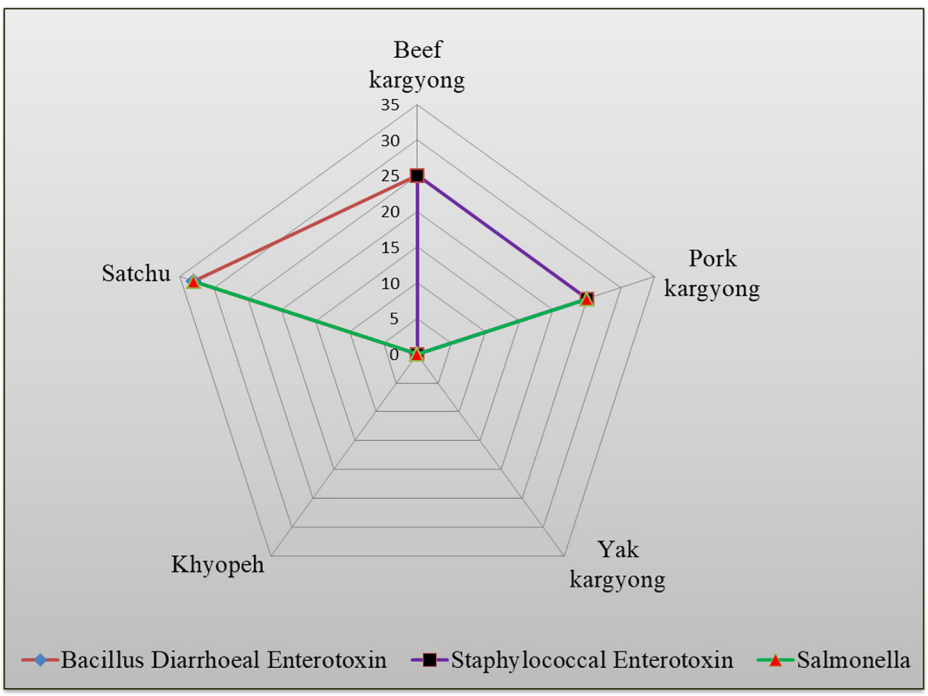

FIGURE 4 | Percentile distribution of different bacterial enterotoxins present in traditional meat products.

prepared by selecting four to five pure isolated colonies from overnight growth (16–24 h of incubation) on a non-selective medium with a cotton swab and suspending the colonies into sterile 0.85% physiological saline water. The density of the suspension was compared to a 0.5 McFarland Standard (R092, HiMedia, India), and turbidity was adjusted to McFarland 0.5 by adding saline or more organisms. Another sterile cotton swab was dipped into an inoculum suspension and the excess fluid was removed to avoid overinoculation of plates by pressing against the wall of the inside of the tube above the fluid level. The inoculum was spread evenly over the entire surface of Mueller–Hinton Agar (M173, HiMedia, India) plates by swabbing in three directions and allowed to stand for 3 to 5 min for drying. The standard antibiotic disks (HiMedia, India) containing amoxicillin/clavulanate (30 μg), ceftazidime (30 μg), cefuroxime (30 μg), cefepime (30 μg),

trimethoprim (5 μg), nitrofurantoin (300 μg), nalidixic acid (30 μg), tobramycin (10 μg), ciprofloxacin (5 μg), erythromycin (15 μg), chloramphenicol (30 μg), penicillin G (10 μg), ampicillin (10 μg), streptomycin (10 μg), tetracycline (30 μg), gentamicin (10 μg), vancomycin (30 μg), oxacillin (1 μg), rifampicin (5 μg), cefoxitin (30 μg), aztreonam (30 μg), clindamycin (2 μg), cotrimoxazole (25 μg), norfloxacin (10 μg), cefotaxime/clavulanic acid (30 μg), and ceftriaxone (30 μg) were dispensed onto inoculated MHA plates using the disk dispenser. The antibiotic disks used were based on their availability at the laboratory at the time of study. The plates were allowed to stand for a few minutes and were incubated for 24 h at 37°C. Antibiotic sensitivity was checked by measuring the zone of inhibition from the back of the plate to the nearest millimeter using a ruler. The zone of inhibition was recorded and compared with the zone diameter interpretative chart to determine whether the bacterial isolates were susceptible, intermediate, or resistant by referring to the CLSI Performance Standards for Antimicrobial Susceptibility Testing (Patel et al., 2017) and journals (Gao et al., 2018; Thung et al., 2018). The bacteria were reported as sensitive (S), intermediate (I), or resistant (R) to each of the antibiotics used in the test. The positive control strains used for the test includes *Escherichia coli* (MTCC 443, IMTECH, India) for Gram-negative bacteria and *S. aureus* (MTCC 96, IMTECH, India) for Gram-positive bacteria. Sterile water was used as negative control.

RESULTS

Microbial Population

A high pH was recorded in *khyopeh* with 5.9 and a lower pH in *satchu* with a mean pH of 5.5, indicating a slightly acidic nature of the meat samples (**Table 2**). We observed a very low percentage of moisture content in *khyopeh* with an average value of 2.5% (1.5–3.5%), and a higher moisture content was observed in beef *kargyong* with a mean value of 14.1%. A higher mean value of aerobic bacterial count was observed in beef *kargyong* with 9.3×10^6 cfu/g and a lower mean value of 1.2×10^6 cfu/g was found in *khyopeh*, respectively. Staphylococcal count was found highest in *satchu* with an average value of 1.8×10^6 cfu/g and least in *khyopeh* with a mean value of 1.0×10^3 cfu/g, whereas *Bacillus* count was found highest in beef *kargyong* with a mean value 4.1×10^5 cfu/g and lowest in *satchu* (2.2×10^5 cfu/g). Interestingly, growth of *Bacillus* was not observed in *khyopeh*. Total coliform count was found highest in *satchu* with a mean value of 1.2×10^7 cfu/g and lowest in *khyopeh* (2.1×10^2 cfu/g) (**Table 2**).

Identification

Selective media were used to isolate some foodborne bacterial pathogens and spoilage bacteria. A total of 128 bacteria were isolated from traditional meat samples. On the basis of cultural characteristics, cell morphology, and carbohydrate fermentation tests, bacterial genera were preliminarily identified using a taxonomical key (Holt et al., 1994) as *Enterobacter*, *Klebsiella*, *Escherichia*, *Salmonella*, *Enterococcus*, *Bacillus*, *Staphylococcus*, *Citrobacter*, and *Pseudomonas* (**Table 3**). Out of the 128 bacterial isolates, 19 representative isolates were selected from each grouped strain having similar phenotypic characteristics (data not shown) for molecular identification.

Based on 16S rRNA gene sequencing (**Figure 2** and **Table 4**), bacterial species were taxonomically confirmed as *Staphylococcus piscifermentans*, *Citrobacter freundii*, *S. aureus*, *Enterococcus faecalis*, *S. enterica*, *Citrobacter* werkmanii, *Klebsiella pneumoniae*, *Macrococcus caseolyticus*, *Klebsiella aerogenes*, *Staphylococcus saprophyticus*, *Pseudocitrobacter anthropi*, *Citrobacter europaeus*, *Shigella sonnei*, *Escherichia fergusonii*, *Klebsiella grimontii*, *Burkholderia cepacia*, and *B. cereus*. The percentile compositions of bacterial species in various meat products of Sikkim are shown in **Figure 3**.

Occurrence of Bacterial Enterotoxins

Enzyme-linked immunosorbent assay tests were performed on 27 samples of meat products, out of which two samples of beef *kargyong* tested positive for *Bacillus* diarrheal enterotoxins and staphylococcal enterotoxins (**Table 5**), two samples of pork *kargyong* tested positive for *Salmonella* spp. as well staphylococcal enterotoxins, and two samples of *satchu* tested positive for *Bacillus* diarrheal enterotoxins and *Salmonella* spp. However, all the tested enterotoxins were absent in the *khyopeh* sample. The overall prevalence of enterotoxins in the meat products detected by ELISA test is shown in **Figure 4**. The optical densities for each extracted sample were also measured at 450 nm with a microtiter reader. The minimum amount of detection limit (LOD) is 0.2 ng/ml of the sample according to the manufacturer's recommendations. We found varied detectable limits with extracts of beef/pork/yak *kargyong*, *satchu*, and *khyopeh* ranging from 0.08 ng/ml in yak *kargyong* to 0.35 ng/ml in beef *kargyong* for *Bacillus* diarrheal enterotoxins, 0.06 to 0.36 ng/ml in *satchu* for *Salmonella*, and 0.08 ng/ml in *khyopeh* to 0.38 ng/ml in beef *kargyong* for staphylococcal enterotoxins (**Table 6**). Additionally, from cultural isolates, molecular detection of a few enterotoxin genes was also performed by the PCR technique using strain-specific primers. *S. piscifermentans* BSLST44 and *S. piscifermentans* BULST54 isolated from beef *kargyong* and *S. aureus* PSST53 isolated from pork *kargyong* were found positive for the *sea* virulent gene (**Figure 5A**). *B. cereus* BSMB16 isolated from beef *kargyong* was found negative for *nhea* and *nheb* genes (**Figure 5B**), and *S. enterica* SMX21 isolated from *satchu* was also tested negative for *stn* and *invA* virulence genes (**Figure 5B**).

Prevalence of Antimicrobial Resistant Bacteria

Bacteria isolated from traditionally processed meat products showed 100% susceptibility against gentamicin, cotrimoxazole, norfloxacin, and trimethoprim. However, a variable resistance pattern was also observed (**Supplementary Tables 1–3**). Overall, the highest level of resistance was observed in amoxicillin–clavulanate (58%) followed by ampicillin (27%). All Gram-positive bacteria were found sensitive against clindamycin and erythromycin, but a resistance pattern was observed in oxacillin (50%) followed by penicillin (33%) and ampicillin

TABLE 6 | Minimum amount of enterotoxins in sample extracts detectable by Tecra and RIDASCREEN kits.

Sample tested[a]	Collection site	Minimum detectable amount (ng/ml)[b] of enterotoxins		
		Bacillus diarrheal enterotoxins	*Salmonella* enterotoxins	Staphylococcal enterotoxins
Beef *kargyong* (*n* = 2)	Martam, East Sikkim	0.35	0.12	0.38
		0.11	0.09	0.17
Satchu (*n* = 2)		0.31	0.36	0.19
		0.28	0.31	0.12
Beef *kargyong* (*n* = 2)	Lalbazaar, Gangtok	0.15	0.16	0.15
		0.13	0.19	0.10
Pork *kargyong* (*n* = 2)		0.16	0.18	0.31
		0.09	0.14	0.12
Satchu (*n* = 2)		0.13	0.16	0.16
		0.11	0.08	0.11
Beef *kargyong* (*n* = 2)	Kitam, South Sikkim	0.13	0.08	0.16
		0.08	0.17	0.09
Pork *kargyong* (*n* = 2)		0.16	0.15	0.13
		0.18	0.11	0.18
Beef *kargyong* (*n* = 2)	Geyzing, West Sikkim	0.09	0.10	0.09
		0.10	0.15	0.18
Pork *kargyong* (*n* = 2)		0.14	0.13	0.13
		0.11	0.17	0.11
Satchu (*n* = 2)		0.12	0.12	0.10
		0.15	0.15	0.16
Pork *kargyong* (*n* = 2)	Burtuk, Gangtok	0.18	0.34	0.12
		0.16	0.13	0.15
Yak *kargyong* (*n* = 2)	Lachung, North Sikkim	0.08	0.18	0.18
		0.12	0.13	0.16
Khyopeh (*n* = 2)		0.16	0.15	0.13
		0.18	0.06	0.08
Positive control		2.81	2.74	3.52
Negative control		0.08	0.12	0.11

[a] *Sample extracts were prepared by using the phosphate-buffered saline described above.*
[b] *The values are averages for duplicate assays.*

(27%) (**Supplementary Tables 3, 4**). Nitrofurantoin was found resistant to *K. pneumoniae* (BSE27) isolated from beef *kargyong*. Cotrimoxazole was found resistant to *E. faecalis* KHE57 isolated from *khyopeh*. The bacterial isolates from different meat samples showed variable patterns of being sensitive, intermediate, and resistant toward various classes of antibiotics which are shown in **Figure 6**.

DISCUSSION

In the present study, we collected four types of traditionally processed meat products (beef *kargyong*, pork *kargyong*, *satchu*, and *khyopeh*) from different places of Sikkim, and they were analyzed for pH, moisture, microbial load, spoilage/pathogenic bacteria, enterotoxins, and antibiotic susceptibility test. The pH value of the samples varied from 5.3 to 5.9 which was similar to the pH value found in traditional dry-cured lacon (5.0–6.0) (Lorenzo et al., 2015), beef and pork jerky (5.5–6.07) (Yang et al., 2018), and biltong (5.0–6.2) (Petit et al., 2014). Preservation of meat by drying has been practiced for centuries and the traditional meat products of Sikkim are preserved either by sun drying or smoking. Among the tested samples, we observed a very less moisture content in *khyopeh* with 2.5 to 18% in beef *kargyong*. The quality and stability of meat products are greatly affected by pH and moisture content (Frazier and Westhoff, 2014). Phenotypic characterization of the 128 bacterial isolates was performed for presumptive identification followed by molecular characterization of representative strains (19) targeting the 16S rRNA gene. The identified bacteria include *S. piscifermentans*, *C. freundii*, *E. faecalis*, *S. enterica*, *S. aureus*, *C. werkmanii*, *K. pneumoniae*, *M. caseolyticus*, *K. aerogenes*, *S. saprophyticus*, *P. anthropi*, *C. europaeus*, *S. sonnei*, *E. fergusonii*, *K. grimontii*, *B. cepacia*, and *B. cereus*. *S. aureus* is usually considered as a food-poisoning bacterium (Hennekinne et al., 2012), but it is frequently underreported since the illness caused by it is mild (Paparella et al., 2018). Foodborne illness caused by *S. aureus* is often associated with the consumption of meat and meat products (Madahi et al., 2014; Hasanpour Dehkordi et al., 2017). The prevalence of *S. aureus* in meat and dairy products has been reported earlier (Guven et al., 2010; Momtaz et al., 2013; Hasanpour Dehkordi et al., 2017;

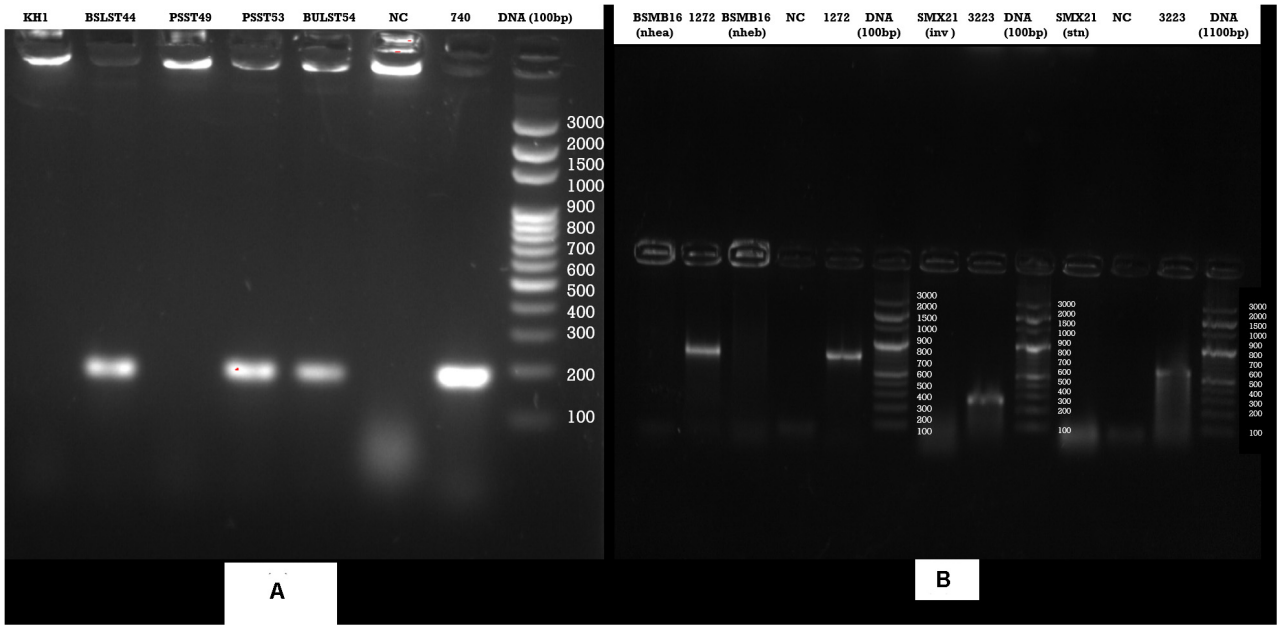

FIGURE 5 | Agarose gel analysis of target strains after PCR amplification of DNA **(A)** *Staphylococcus* enterotoxin gene (*sea*), **(B)** *Bacillus cereus* enterotoxin genes (*nhea, nheab*) and *Salmonella* (*inv, stn*) enterotoxin genes isolated from traditional meat products.

Rahi et al., 2020). Coagulase-negative staphylococci (CoNS) like *S. saprophyticus* and *S. piscifermentans* have been reported as flavoring microorganisms capable of reducing nitrite, enhancing color stability, and preventing rancidity by inhibiting the oxidation of unsaturated free fatty acids (Talon and Leroy, 2006). Many dried/salted/fermented meat products are often reported to be dominated by coagulase-negative staphylococci (Lorenzo et al., 2015), which may contribute to bioprotection against foodborne pathogens by producing bacteriocins (Mainar et al., 2017). We found *sea* enterotoxin gene from isolated strains of *S. aureus* and a few CoNS. Similar studies were reported in ground beef where enterotoxin and biofilm genes in coagulase-negative *Staphylococcus* were detected, indicating it as a potential pathogen (Pavan et al., 2019). Although *Staphylococcus* produces different types of enterotoxins like the classical enterotoxins (sea–see) and the newer enterotoxins (seg–sely) (Fisher et al., 2018), however, strains producing sea are implicated in the majority of cases of staphylococcal food poisoning (Kérouanton et al., 2007; Wallin-Carlquist et al., 2010). The presence of *sea* virulent gene in a few samples of *kargyong* advocates the concern for food safety of the product, which might have been contaminated during traditional processing; however, there has been no official report of food-poisoning cases in Sikkim after the consumption of *kargyong*.

We also identified various bacteria belonging to the family Enterobacteriaceae, which are commonly present in the intestines of human (Martinson et al., 2019) and animal (Schierack et al., 2007) that may survive in diverse environments (Martinson et al., 2019). However, they may also cause a variety of community-acquired (foodborne) and nosocomial infections (Bereket et al., 2012). *Klebsiella* has often been reported in meat products as a contaminant from water, containers, and feces of animal or human origin (Gundogan et al., 2013). Foods are commonly contaminated with *Salmonella* and *Shigella* by an infected food handler who practices poor hygiene and inadequate cooking (Garedew et al., 2016; Liu et al., 2018; Ranjbar et al., 2020). *Salmonella* is one of the most common bacterial pathogens in laboratory-confirmed foodborne illness cases causing gastroenteritis, typhoid fever, and bacteremia (Eng et al., 2015). The major identified virulence genes like *inv*, *stn*, *fimA*, and *spv* responsible for salmonellosis are linked to a combination of plasmid and chromosomal factors (Chaudhary et al., 2015). Dry meat products are traditionally considered as safe food; however, the outbreak of foodborne illness caused by *S. enterica* is often reported due to its ability to withstand the adverse environment leading to cross-protection against abiotic stress (Mutz et al., 2020). Although the ELISA test detected *Salmonella* spp. in a few tested samples, the PCR method showed the absence of *invA* and *stn* virulence genes in the isolated strain. The *invA* gene is primarily targeted for the identification of *Salmonella* in food of animal origin, since it is present in pathogenic serovars and has been demonstrated to be a rapid and effective method for diagnosis of salmonellosis (Karmi, 2013; Carraturo et al., 2016). However, there are few reports on the absence of *invA* gene in certain *Salmonella* strains, which indicate that they are not invasive or may have other invasive mechanisms (Kaushik et al., 2014; Sharma and Das, 2016; Buehler et al., 2019; Kadry et al., 2019; Yanestria et al., 2019).

Enterococci isolated from meat products have the ability to produce enterocins harboring antimicrobial activity against *L. monocytogenes* (da Costa et al., 2019). *B. cereus* is an

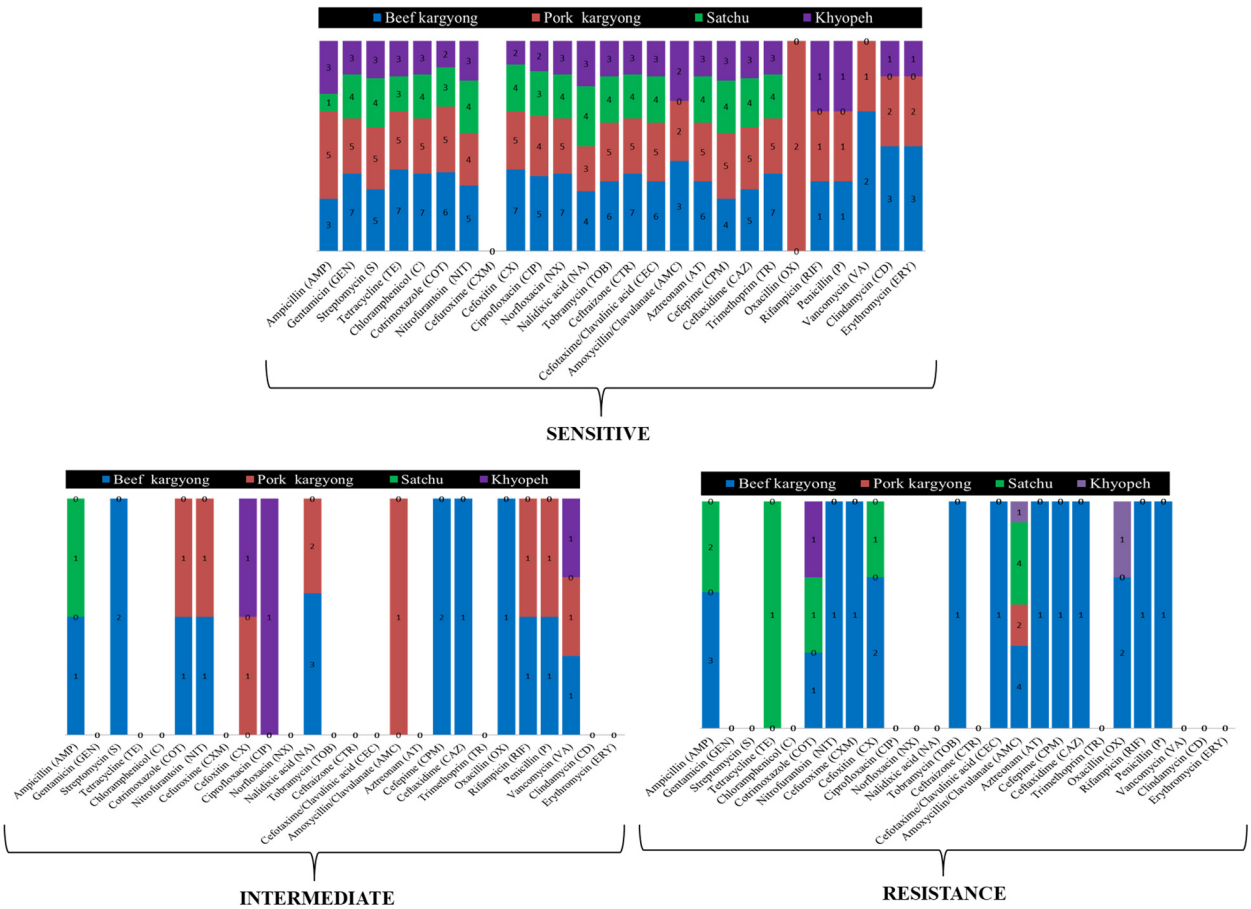

FIGURE 6 | Bacterial isolates from different meat samples showing variable patterns of being sensitive, intermediate, and resistant against the tested antibiotics.

endospore-forming bacterium responsible for foodborne illness in humans and is frequently involved in foodborne outbreaks (Tewari et al., 2015). *Bacillus* enterotoxins have been reported from fermented legume products like *soumbala* and *bikalga* of Africa (Ouoba et al., 2008); doenjang, a fermented soybean food of Korea (Lee et al., 2017); dairy milk (Saleh-Lakha et al., 2017); some street food of Indian Himalayas (Kharel et al., 2016); and meat products (Soleimani et al., 2018). *B. cepacia* is identified as a spoilage microorganism in food (Cunningham-Oakes et al., 2019).

The widespread use of antibiotics in animals for the treatment of diseases and other purposes may develop antibiotic resistance in bacteria resulting into cross-transmission of resistance genes from animals to humans through food (Safarpoor Dehkordi et al., 2017; Tang et al., 2017). The antibiogram of bacterial isolates showed a maximum rate of sensitivity against gentamicin and trimethoprim, which is in accordance with a similar report where 90% of sensitivity was observed in bacterial isolates from street foods (Amor et al., 2019). The majority of isolated strains showed the highest resistance pattern against amoxicillin–clavulanate followed by ampicillin. Amoxicillin–clavulanate-resistant bacteria were also reported from retail sausage in Malaysia which exhibited 100% resistance along with penicillin followed by ampicillin (83.3%) and cefotaxime (71.4%) (Tew et al., 2016). The increasing prevalence of amoxicillin–clavulanate resistance has been reported due to the continued spread of β-lactamase-mediated resistance (White et al., 2004). We also found a strain of bacteria identified as *K. pneumoniae* that showed resistance against nitrofurantoin which is a broad-spectrum antibiotic used for the treatment of uncomplicated urinary tract infections (UTI) (Osei Sekyere, 2018). Most of the Gram-positive cocci were oxacillin resistant; however, the resistance pattern was not observed in any cephalosporin class of antibiotics except for *B. cereus*. Overall, the majority of the isolated strains from traditionally processed meat products of Sikkim showed a maximum percentage of sensitivity against the tested antibiotics.

CONCLUSION

The traditional processing of perishable meat into flavor-enhanced products by drying, smoking, and fermentation is culturally and organoleptically accepted by ethnic people of Sikkim in India. This study showed the prevalence of some spoilage and pathogenic bacteria; however, enterotoxin virulent

genes were not detected in bacterial strains isolated from traditional meat products except for the *sea* virulent gene which tested positive from two samples of *kargyong*. It was interesting to find out the absence of pathogenic bacteria and enterotoxins in *khyopeh*, which is a fermented yak meat product found only in the north district of Sikkim. Most of the bacterial strains in traditionally processed meat products of Sikkim showed a maximum percentage of sensitivity against the tested antibiotics. Some samples of meat products showed the prevalence of spoilage bacteria probably as contaminants during the traditional processing method. Hence, proper monitoring of hygienic conditions during preparation practices is highly recommended for the safety of these culturally acceptable meat products.

DATA AVAILABILITY STATEMENT

The datasets generated for 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

NT and JT: conceptualization, resources, supervision, and writing—review and editing. MB: data curation, formal analysis, methodology, software, visualization, and roles/writing—original draft. JT: funding acquisition and project administration. MB and NT: investigation. MB, NT, and JT: validation. All authors contributed to the article and approved the submitted version.

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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.2020.599606/full#supplementary-material

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Metataxonomic Profiling of Native and Starter Microbiota During Ripening of Gouda Cheese Made With *Listeria monocytogenes*-Contaminated Unpasteurized Milk

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Unpasteurized milk is used to produce aged artisanal cheeses, which presents a safety concern due to possible contamination with foodborne pathogens, especially *Listeria monocytogenes*. The objective of this study was to examine the composition of the bacterial community in unpasteurized milk used to prepare Gouda cheese artificially contaminated with *L. monocytogenes* (~1 log CFU/ml) and assess the community dynamics and their potential interaction with *L. monocytogenes* during a 90-day ripening period using targeted 16S rRNA sequencing. The diversity of bacterial taxa in three batches of unpasteurized milk was not significantly different, and the microbiomes were dominated by species of *Lactococcus*, *Streptomyces*, *Staphylococcus*, and *Pseudomonas*. The highest relative abundances were observed for *Pseudomonas fluorescens* (31.84–78.80%) and unidentified operational taxonomic units (OTUs) of *Pseudomonas* (7.56–45.27%). After manufacture, both with and without *L. monocytogenes*-contaminated unpasteurized milk, Gouda cheese was dominated by starter culture bacteria (including *Lactococcus lactis* subsp. *cremoris*, *lactis*, *lactis* bv. diacetylactis, and *Streptococcus thermophilus*), in addition to unassigned members in the taxa *L. lactis* and *Streptococcus*. During ripening there was an overall decrease in *L. lactis* abundance and an increase in the number of taxa with relative abundances >0.1%. After 90-day ripening, a total of 82 and 81 taxa were identified in the Gouda cheese with and without *L. monocytogenes*, respectively. Of the identified taxa after ripening, 31 (Gouda cheese with *L. monocytogenes*) and 56 (Gouda cheese without *L. monocytogenes*) taxa had relative abundances >0.1%; 31 were shared between the two types of Gouda cheese, and 25 were unique to the Gouda cheese without added *L. monocytogenes*. No unique taxa were identified in the Gouda cheese with the added *L. monocytogenes*. This study provides information on the dynamics of the bacterial community in Gouda cheese during ripening, both with and without the addition of *L. monocytogenes*.

Keywords: microbiome, Gouda cheese, *Listeria monocytogenes*, unpasteurized milk, dairy

INTRODUCTION

Microbes and cheesemaking have been intertwined for hundreds of years, with the first description of cheese microorganisms dating back to 1665 (Donnelly, 2014). Many cheeses that are still consumed today originated hundreds of years ago: Cheddar, Parmesan, and Gouda and Gloucester, for example, were first documented in 1500, 1579, and 1697, respectively. Cheese serves as a long-standing dietary component in many countries around the world. Since the introduction of cheese microorganisms to science, studies have continued to shed light on the identities and roles of the organisms traditionally used in cheesemaking. The knowledge gained from these studies has resulted in a more controlled, standardized, and safe process for producing many of these cheese types (Donnelly, 2014).

Cheeses obtain their unique flavors from defined starter cultures and aging (ripening) conditions in an industrial setting that yields a predictable and replicable result. Artisanal cheesemaking, i.e., small batch, specialty-made, region-specific cheeses, relies on the selection and use of indigenous microorganism in the milk used as the base for the cheese. Supporters of artisanal cheeses argue that the high taxonomic diversity of the native microbiota in unpasteurized milk is important for developing the flavor profile unique to these cheeses. Additionally, it is debated that the diverse microbial profile of these cheeses presents competition and limits foodborne pathogens. Unfortunately, current data contradict this belief: *Listeria monocytogenes* and Shiga-toxin producing *Escherichia coli* have been identified in both unpasteurized milk and cheeses prepared using unpasteurized milk (U. S. FDA, 2016). In several studies, *L. monocytogenes* was identified in a substantial portion of the silos and bulk tanks used to house unpasteurized milk, ranging from 2.3 to 50% of the samples examined (Van Kessel et al., 2004; Jayarao et al., 2006; D'Amico et al., 2008; Jackson et al., 2012). The resident bacteria of unpasteurized milk originate from a variety of sources, including the animal's teat canal and skin, equipment and personnel hygiene, transport and storage, and processing conditions (Verdier-Metz et al., 2009; Braem et al., 2012; Quigley et al., 2013; Kergourlay et al., 2015). Without pasteurization and proper process and post-process handling, the incidence of *L. monocytogenes* contamination may be increased. Pathogenic *E. coli* contamination of Gouda cheese and/or the unpasteurized milk that was used for manufacture has led to multiple outbreaks between 1998 and 2011 (Gould et al., 2014), and in 2013, it was responsible for 23 illnesses, five hospitalizations, and one death (Currie et al., 2018).

As the potential of foodborne illnesses remains a threat, and the consumption of artisanal cheeses increases, the interstate commerce of unpasteurized milk and cheese made with unpasteurized milk remains closely monitored and is illegal except under certain guidelines. Under U.S. Food and Drug Administration (FDA) regulations in the Code of Federal Regulations (CFR) Title 21 (U. S. FDA, 2018), specific standards allow cheese to be made from unpasteurized milk, but must be cured (aged) at a temperature not lower than 1.7°C (35°F) and for a minimum of 60 days, in an effort to reduce pathogen presence. However, multiple studies have indicated that in aged cheese, such as Cheddar and Gouda, the presence of a pathogen can extend well beyond the recommended 60-day ripening period (Reitsma and Henning, 1996; Schlesser et al., 2006; Salazar et al., 2020).

The demand for high-quality artisanal and traditional cheeses likely will not diminish, nor will the incidence of foodborne pathogens in these products. Little knowledge exists on how the resident microbiota impacts pathogen survival and whether altering specific taxa can aid in pathogen prevention measures in the artisanal cheese industry. The present study, complementing previous research (Salazar et al., 2020), assesses the microbial composition of Gouda cheese made with unpasteurized milk during a 90-day ripening process. The goals of this study were to determine the change in abundance of the taxa present (both from the starter culture, as well as those naturally residing in the milk) and to observe dynamic changes in the presence of *L. monocytogenes*, in an effort to provide information that may prove valuable for the development of future guidelines and risk assessments.

MATERIALS AND METHODS

Strains and Inoculum Preparation

A cocktail of four *L. monocytogenes* dairy-related strains were used in this study: LM1240, LM1257, LM-6E, and LM-2F (van der Veen et al., 2008; Wemmenhove et al., 2013). Strains were cultured individually aerobically in Brain Heart Infusion (BHI; Becton, Dickinson and Co., Sparks, MD) broth at 37°C for 16–18 h. Cultures were normalized to an OD_{600} of 0.8, washed twice with phosphate-buffered saline (PBS; pH 7.4), and combined to form a cocktail. The cocktail was serially diluted using PBS and plated onto Brilliance *Listeria* agar (BLA; Thermo Fisher Scientific, Waltham, MA) to verify initial inoculum levels.

Gouda Cheese Manufacture

Three batches of unpasteurized bovine milk (AB, CD, and EF) were sourced from a local dairy in Illinois on different concurrent weeks. Each batch of unpasteurized milk received was comprised of milk from multiple farms in Illinois. A total of three trials and six Gouda cheeses were prepared (labeled A through F). Trial 1 used batch AB unpasteurized milk to manufacture control uninoculated cheese A and *L. monocytogenes*-contaminated cheese B. Trial 2 used batch CD unpasteurized milk to manufacture control uninoculated cheese C and *L. monocytogenes*-contaminated cheese D. Trial 3 used batch EF unpasteurized milk to manufacture control uninoculated cheese E and *L. monocytogenes*-contaminated cheese F.

Gouda cheese was manufactured in a biocontainment pilot plant using a commercial cheese pasteurization vat (V15005, Northwestern Tools, Dayton, OH) as previously described (Salazar et al., 2020). Briefly, 10 gallons (37.9 L) of unpasteurized milk was added to the vat and was left uninoculated (in the case of cheeses A, C, and E) or was

inoculated with 1.05 ± 0.24 log CFU/ml of the *L. monocytogenes* cocktail (in the case of cheeses B, D, and F). The milk was then heated to 30°C, followed by the addition of 2.4 Direct Culture Unit (DCU) of starter cultures consisting of *Lactococcus lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis* bv. diacetylactis, and *Streptococcus thermophilus* (CHOOZIT MA 4001 Lyo 25 DCU, Danisco, Thomson, IL). After 30 min at 30°C, 6.2 ml of rennet (PF 55 coagulant, GetCulture, Madison, WI) was added to the milk, mixed, and the temperature was maintained for 45–55 min. Curds were cut in up and down directions with a 1 $inch^2$ cheese knife, with a 5 min wait between cuts. One third of the whey was drained, followed by the addition of an equal amount of water at 50°C, which aided in increasing the overall temperature of the mixture to 38°C. The curds were "cooked" at 38°C for 30 min with constant stirring. Curd were placed into molds (M19, New England Cheesemaking Supply Co., Deerfield, MA) and pressed with sequential increasing weights as described previously (Salazar et al., 2020). The resulting cheese wheel was brined in 20% (w/v) sodium chloride (Thermo Fisher Scientific) for 48 h, followed by 72 h ambient drying prior to waxing.

Waxing, Storage, and Sampling of Gouda Cheese During Ripening

Gouda cheese was waxed, stored, and sampled as described previously (Salazar et al., 2020). Cheese wheels were waxed with two coats of red cheese wax (New England Cheesemaking Supply Co.) and aged at 10°C for up to 90 days. At 0, 7, 28, 42, 60, 77, and 90 days, a 25-g pie-shaped wedge of cheese was removed from the wheel for sampling. The cheese was re-waxed after each sampling with two coats of cheese wax. All Gouda cheeses prepared in this study conformed to the standard of identity of Gouda cheese (U. S. FDA, 2018; Salazar et al., 2020).

Sample Processing and Sequencing

DNA was extracted from 1 ml of each of the three batches of unpasteurized milk (AB, CD, and EF) prior to the manufacture of the Gouda cheese to determine the composition and relative abundance of bacterial taxa. After the Gouda cheese was prepared, individual 25-g cheese samples, consisting of two 5-g pie-shaped wedges were evaluated. Each 5-g sample was homogenized with 10 ml of Buffered *Listeria* Enrichment Broth (BLEB; Oxoid, Basingstoke, England), and DNA was extracted immediately from 1 ml of each homogenate using the DNeasy PowerFood Microbial Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total DNA was quantified using the Qubit dsDNA BR Assay Kit (Invitrogen, Carlsbad, CA). PCR was conducted with 3 ng of template DNA and one of four 16S rDNA primer pairs to amplify the V4 region as previously described (Salazar et al., 2018). Amplicons were purified using AMPure XP beads (Beckman-Coulter, Indianapolis, IN) and quantified using the Qubit dsDNA BR Assay Kit. Samples were indexed using the Nextera XT Kit (Illumina, San Diego, CA) as described previously (Salazar et al., 2018), pooled, spiked with 10% of 12.5 pM PhiX, and sequenced using an Illumina MiSeq and 600 cycles of V3 chemistry.

Data Analyses

Raw paired-end sequences were quality filtered, merged, and taxonomically profiled using miniKraken and Kraken2 (Wood et al., 2019). Relative abundances were determined using Bracken 2.5 (Lu et al., 2017). Sequence counts were rarefied to 10,000 sequences for each independent sample. NCBI BLAST+ 2.9.0 was used to differentiate between the *L. lactis* subspecies (i.e., *lactis*, *cremoris*, and *lactis* bv. diacetylactis) using the BLAST V5 database. The vegan package 2.5–6 (Oksanen et al., 2019) in R 3.6.2 was used to determine alpha and beta diversity of the microbial population in the three unpasteurized milk batches (AB, CD, and EF), the six Gouda cheeses (A, B, C, D, E, and F), and the two types of cheeses (ACE, uninoculated; BDF, inoculated). Alpha diversity was estimated using Shannon, Simpson, inverse Simpson, and Chao1 diversity indices. Beta diversity was evaluated using Bray-Curtis dissimilarity matrix. A multilevel pairwise comparison using Adonis (~Permanova) was calculated using package pairwiseAdonis (Martinez Arbizu, 2020). A value of p less than 0.05 was considered as significant.

Accession Numbers

Metagenomic sequence data have been deposited in NCBI under Bioproject PRJNA643290, Biosamples SAMN15409189-409233.

RESULTS

Native Bacteria in the Unpasteurized Milk

Three batches of unpasteurized milk were used to manufacture the six Gouda cheeses in this study. From plate count assays conducted previously (Salazar et al., 2020), the average populations of *Enterobacteriaceae*, yeasts and molds, lactic acid bacteria, and mesophilic bacteria in the unpasteurized milk were 0.95, 3.53, 3.33, and 3.09 log CFU/ml, respectively. In this study, a total of 193, 206, and 176 unique operational taxonomic units (OTUs) were identified in unpasteurized milk batch AB, CD, and EF, respectively (**Table 1**). Although differences were observed in the total OTUs, alpha and beta diversity metrics were not significantly different between batches (**Tables 1** and **2**). The bacterial genera *Lactococcus*, *Pseudomonas*, *Polynucleobacter*, *Streptomyces*, and *Staphylococcus* dominated the milk microbiomes (**Figures 1, 2**). A total of 11 different taxa were identified with relative abundances >1% in at least one of the three milk batches and included *L. lactis*, *Pseudomonas fluorescens*, *P. koreensis*, *P. moraviensis*, *Polynucleobacter necessarius*, *Staphylococcus aureus*, *Bacillus thuringiensis*, *Klebsiella pneumoniae*, and *Komagataeibacter rhaeticus*, as well as unclassified members in the genera of *Streptomyces* and *Pseudomonas*. The highest relative abundance was observed for *P. fluorescens* (47.06–73.76%). *Lactococcus lactis* was present in all three milk batches at relative abundances of 1.03–1.81%. Differences in the relative abundances of some taxa were more pronounced than others in the three batches of milk (**Figures 1,**

TABLE 1 | Observed operational taxonomic units (OTUs) and alpha diversity metrics of the three batches of unpasteurized milk (AB, CD, and EF) used for Gouda cheese manufacture.

Milk batch	Observed OTUs	Chao1[a]	Shannon[b]	Simpson[c]	Inverse Simpson[c]
AB	193 ± 51	465 ± 89	1.35 ± 0.11	0.64 ± 0.04	2.89 ± 0.55
CD	206 ± 72	449 ± 52	1.02 ± 0.04	0.55 ± 0.01	2.23 ± 0.04
EF	176 ± 43	486 ± 96	1.04 ± 0.27	0.36 ± 0.24	1.62 ± 0.95

[a]*Chao1 diversity index indicates the microbial richness for the three different batches of unpasteurized milk.*
[b]*Shannon diversity index indicates the richness and evenness of the microbial community in the three different batches of unpasteurized milk.*
[c]*Simpson and Inverse Simpson diversity indices indicate the diversity of the microbial community in the three batches of unpasteurized milk.*

2). *Klebsiella pneumoniae* was present in all three milk batches but at different relative abundances (2.24–34.17%). A high abundance of *Streptomyces* (7.60%) was observed in milk batch AB, whereas this taxon had a relative abundance <1% in the other two batches. *Staphylococcus aureus* was also present in all three milk batches at 2.03–5.60%. No native *Listeria* spp. were detected in the unpasteurized milk *via* 16S rRNA sequencing. In addition, no native *Listeria* spp. were detected in the unpasteurized milk through enrichments (Salazar et al., 2020).

Native and Starter Bacteria in Manufactured Gouda Cheese Prior to Ripening

Two types of Gouda cheese were manufactured in this study: one type was manufactured with unpasteurized milk without addition of *L. monocytogenes* (cheese ACE) and one manufactured with unpasteurized milk containing 1.05 ± 0.24 log CFU/ml of *L. monocytogenes* (cheese BDF). From plate count assays conducted previously (Salazar et al., 2020), the average populations of *Enterobacteriaceae*, yeasts and molds, lactic acid bacteria, and mesophilic bacteria in the manufactured cheeses prior to ripening were 2.07, 3.06, 8.98, and 8.83 log CFU/g, respectively. Relative abundances of native and starter bacteria in the two types of Gouda cheeses (ACE and BDF) were determined at day 0 (after manufacture) and during ripening at 10°C for up to 90 days. At day 0, a total of 85 different taxa were identified in cheese ACE. Similarly, 85 different taxa were also identified in cheese BDF. Excluding starter culture bacteria, only two (*B. thuringiensis* and *P. fluorescens*) of the 11 taxa identified in the unpasteurized milk >1% (**Figure 1**) were present in both types of Gouda cheese at relative abundances >1% (**Figures 3, 4**). *Bacillus thuringiensis* was present in cheese ACE and cheese BDF at relative abundances of 7.97 and 6.58%, respectively. *Pseudomonas fluorescens*, which was identified in the unpasteurized milk at 47.06–73.76%, had reduced relative abundances of 0.23 (cheese ACE) and 0.26% (cheese BDF). *Staphylococcus aureus* was present in cheese ACE at a relative abundance of 2.12%, however, it was <1% in cheese BDF.

The starter culture used for Gouda cheese manufacture consisted of four different bacteria: *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis* bv. diacetylactis, and *S. thermophilus*. No differences in relative abundances of the starter culture bacteria were observed between the two types of Gouda cheeses (ACE and BDF) on day 0 (**Figures 3, 4**). *Lactococcus lactis* subsp. *lactis* bv. diacetylactis was present at an average relative abundance of 5.73%, followed by *L. lactis* subsp. *cremoris* and subsp. *lactis* at 4.68 (cheese ACE) and 0.78% (cheese BDF). A high abundance of *L. lactis* that was not categorized into subtypes was present in both types of Gouda cheeses at an average abundance of 70.32%. *Streptococcus thermophilus*, another starter culture bacterium, was identified at an average relative abundance of 4.28%.

Influence of *Listeria monocytogenes*-Contaminated Milk on the Gouda Cheese Microbiome During Ripening

During 90-day ripening, beta diversity analysis determined that there were significant differences between the microbiomes of cheeses A and C as well as A and E, all of which were uninoculated (**Table 3** and **Figure 5**). No significant differences were observed between the microbiomes of any of the three inoculated cheeses (B, D, and F). In addition, there was a significant difference between the microbiomes of the two types of cheeses manufactured in this study (ACE and BDF; **Table 3** and **Figure 5**).

The relative abundances of native, starter, and *L. monocytogenes* were determined during the 90-day ripening of the two types of Gouda cheeses (**Figures 3, 4**). *L. monocytogenes* was identified at relative abundances less than 0.001% in Gouda cheese BDF during ripening, which was consistent with previously determined plate count enumeration data (Salazar et al., 2020); after 90-day ripening, the population of *L. monocytogenes* was 1.11, 0.95, and 1.26 log CFU/g in cheeses B, D, and F, respectively. *L. monocytogenes* was not identified in cheese ACE (uninoculated) at any timepoint during ripening using 16S rRNA gene sequencing or via enrichments (Salazar et al., 2020).

Non-starter culture bacteria identified during ripening at relative abundances >1% for at least one timepoint in either type of Gouda cheese included *B. thuringiensis*, *Lactobacillus curvatus*, *Po. necessarius*, *P. fluorescens*, *S. aureus*, and *Str. pyogenes* (**Figures 3, 4**). *Lactobacillus curvatus* was identified at relative abundances of 0.03–2.25% and <0.1–1.26% in cheeses ACE and BDF during ripening, respectively. *Polynucleobacter necessarius* was identified at relative abundances of 0.24–4.35% and 0.15–1.41% in cheeses ACE and BDF during ripening, respectively. *Bacillus thuringiensis* (6.30–9.11%) and *Str. pyogenes* (1.78–4.63%) were the only two taxa present in both ACE and BDF cheeses at all timepoints during ripening. *S. aureus* was also present in both cheeses at all timepoints and increased during ripening at relative abundances of 2.13–10.73% with the exception of 0 and 7 days for cheeses BDF in which the relative abundances were 0.81 and 0.82%, respectively. From initial

TABLE 2 | Pairwise Adonis of the three batches of unpasteurized milk used for Gouda cheese manufacture.

Comparison	Sums of Squares[a]	F.Model[b]	r^{2c}	*p*	Adjusted value of *p*[d]
AB vs. CD	0.41	1.56	0.24	0.07	0.21
AB vs. EF	0.38	1.31	0.25	0.133	0.4
CD vs. EF	0.32	1.28	0.30	0.1	0.3

[a]*Sums of Squares expresses the total variation.*
[b]*F.Model expresses the variation between the two unpasteurized milk batches compared.*
[c]r^2, *coefficient of determination.*
[d]*Adjusted value of p, the value of p obtained using Bonferroni correction.*

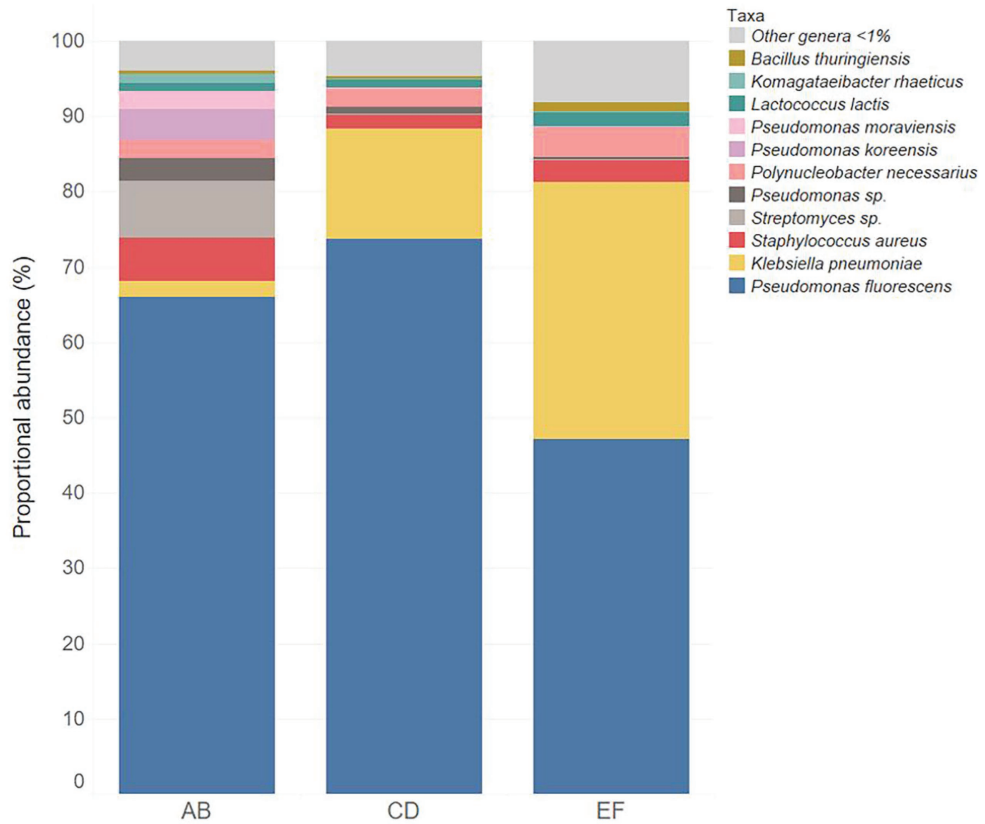

FIGURE 1 | Relative abundance of bacterial taxa in the three batches of unpasteurized milk (AB, CD, and EF) used in this study used to make Gouda cheese.

relative abundances of 2.13 and 0.81% on day 0, the abundance of *S. aureus* after 90 days ripening was 6.61 and 10.73% for cheeses ACE and BDF, respectively.

Less difference was observed in the relative abundances of starter culture bacteria between the two types of cheeses during ripening (**Table 4**). In general, the relative abundance of *L. lactis* subsp. *lactis* remained fairly constant during ripening. The relative abundance of *L. lactis* subsp. *cremoris* was observed to decrease during ripening. Conversely, the relative abundance of *L. lactis* subsp. *lactis* bv. diacetylactis increased over the 90-day ripening period. The greatest increase in relative abundance (2.71%) was observed by *L. lactis* subsp. *lactis* bv. diacetylactis in cheese BDF, whereas the greatest decrease (14.05%) was observed by unassigned *L. lactis* members, also in cheese BDF.

When comparing cheeses ACE and BDF, after 28, 60, and 90 days ripening, a total of 98, 130, and 81 taxa were identified in Gouda cheese ACE, respectively, whereas 95, 77, and 82 were identified in cheeses BDF. Out of the 81 taxa identified in cheese ACE after 90 days, 25, 47, and 9 taxa had relative abundances <0.1%, 0.1–0.5, and >0.5%, respectively. In contrast, out of 82 taxa identified in cheese BDF after 90 days, 51, 26, and 5 taxa had relative abundances <0.1%, 0.1–0.5, and >0.5%. Of the taxa with relative abundances 0.1–0.5%, 26 were shared between both types of cheeses and 21 were only identified in cheese ACE; no taxa were unique to cheese BDF, with the

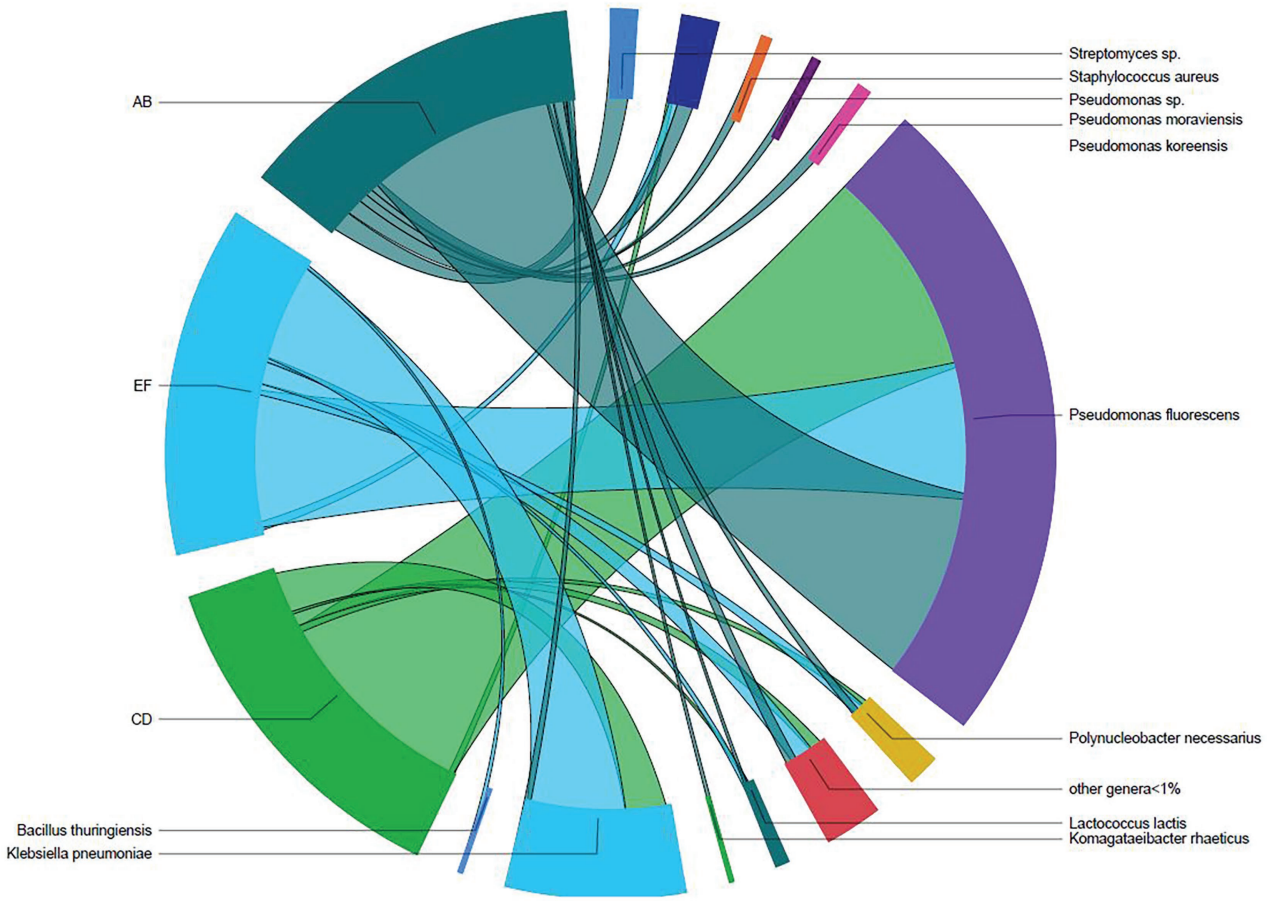

FIGURE 2 | Shared taxa between the three batches of unpasteurized milk (AB, CD, and EF) used to manufacture Gouda cheese.

exception of the inoculated *L. monocytogenes* (**Table 5**). Genera with the most unique species identifications included *Bacteroides* and *Prevotella*.

DISCUSSION

This study used metagenomic sequencing to profile the microbiomes of unpasteurized milk and the resulting Gouda cheese after manufacture and throughout a 90-day ripening period. Although previous studies have examined the resident bacterial populations of unpasteurized cheeses after manufacture and during the ripening process (Delbès et al., 2007; Casalta et al., 2009; Lusk et al., 2012; Quigley et al., 2012; Fuka et al., 2013; Delcenserie et al., 2014; Alessandria et al., 2016; Escobar-Zepeda et al., 2016), this is the first study to assess the community dynamics both with and without the addition of a foodborne pathogen. Contamination of unpasteurized milk can occur on the farm due to poor hygiene or unsanitary conditions, or during processing. *L. monocytogenes* has been identified in unpasteurized milk in various studies (Van Kessel et al., 2004; Jayarao et al., 2006; D'Amico et al., 2008; Jackson et al., 2012). Therefore, in this study *L. monocytogenes* was added to unpasteurized milk used to produce Gouda cheese to understand the dynamics and interactions of this pathogen with the bacterial community present during ripening.

In this study, the microbiomes of the three batches of unpasteurized milk utilized for Gouda cheese manufacture had similar bacterial diversity and were dominated by *Lactococcus*, *Klebsiella*, *Pseudomonas*, *Staphylococcus*, and *Streptococcus* species. These genera have all been previously identified in unpasteurized milk (Correa et al., 2011; Quigley et al., 2013; Sudarwanto et al., 2015; Kable et al., 2016; Rodrigues et al., 2017) and *Streptococcus*, *Staphylococcus*, and *Pseudomonas* have been identified at abundances >30% in unpasteurized milk tanker trucks (Kable et al., 2016). Specifically, *P. fluorescens*, which was identified in the unpasteurized milk batches at abundances of 32–79%, is a known dairy spoilage organism and is responsible for blue discoloration in different types of cheeses (Ternstrom et al., 1993; Martin et al., 2011; del Olmo et al., 2018). These bacteria have also been shown to be inhibitory to *L. monocytogenes* in co-cultures (Buchanan and Bagi, 1999). It is noted that the unpasteurized milk batches used in this study were sourced from only one dairy; however, milk from multiple farms within

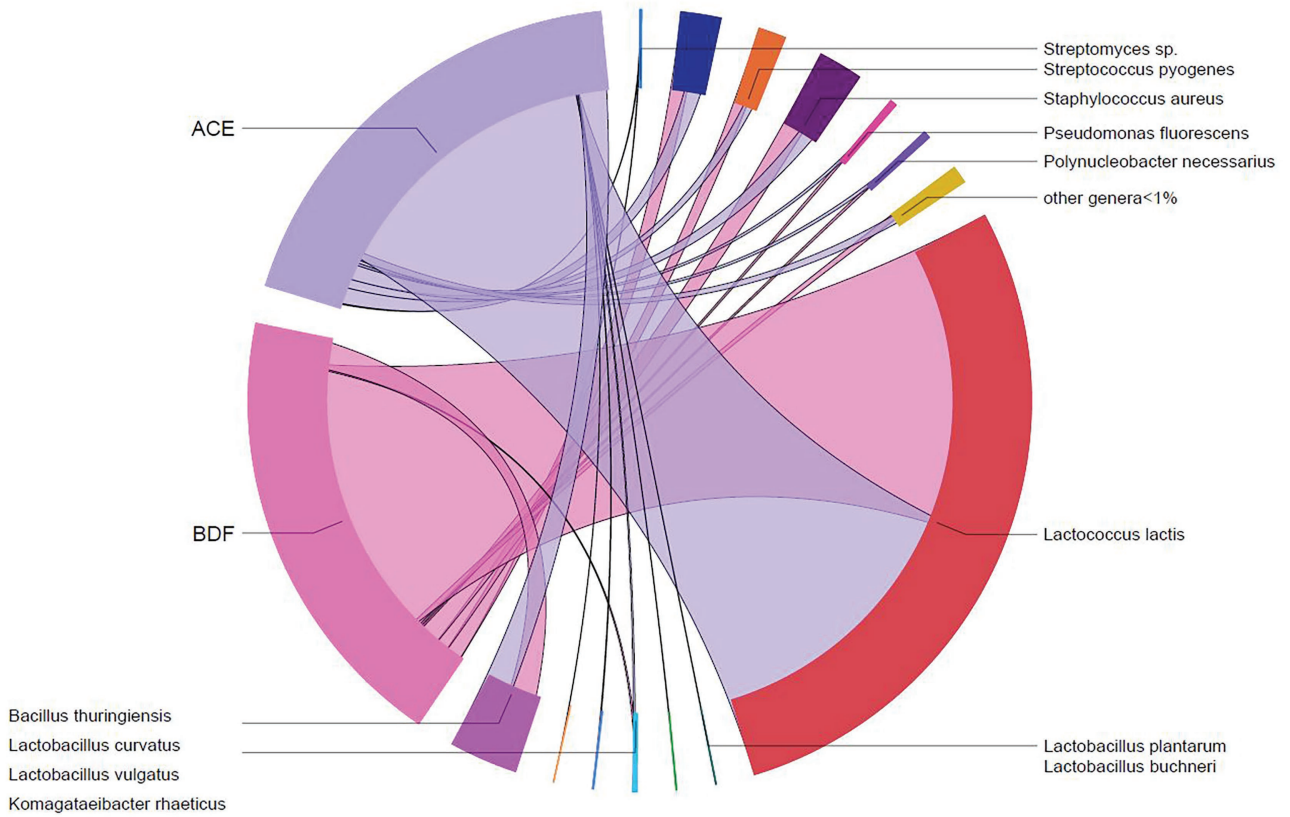

FIGURE 3 | Shared taxa between the two types of Gouda cheeses (ACE, uninoculated and BDF, inoculated) produced in this study.

the same geographic region comprised the batches. Although the microbiome of unpasteurized milk would likely vary based on the types of cattle at each farm, differences attributed to the geographic location were minimized.

After manufacture both with and without the addition of *L. monocytogenes* in the unpasteurized milk, the Gouda cheese bacterial communities were dominated by the starter culture members *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis* bv. diacetylactis, and *Str. thermophilus*, as well as *L. lactis* that was not classified into subspecies and unclassified species in the genus *Streptococcus*. This finding was expected, as high population levels of starter culture bacteria are common in cheeses after manufacture (Montel et al., 2014). The strains in starter cultures play an important role in the cheese manufacturing and ripening process by influencing sensory, flavor, and texture profiles. This study utilized a commercially-available Gouda cheese starter culture. During cheese manufacture, the main contribution of these lactic acid bacteria is in milk acidification. During ripening, these bacteria are also involved in lipolysis, proteolysis, and the conversion of amino acids to flavor compounds (Yvon et al., 1997; Copan et al., 2007). Since the relative abundances of the strains in the starter cultures were similar between cheeses ACE and BDF during ripening, it can be inferred that *L. monocytogenes* is not inhibitory to the starter culture bacteria and vice versa.

After manufacture, the Gouda cheeses made with unpasteurized milk, both with and without the addition of *L. monocytogenes* (cheeses BDF and ACE, respectively), were ripened at 10°C for 90 days. Diversity analysis determined that the microbiomes of these two cheese types were significantly different, indicating that the presence of *L. monocytogenes* may alter the microbial community of Gouda cheese. Overall, the relative abundances of the major identified taxa in both types of cheeses trended similarly during ripening. Starter culture bacteria, along with non-subspecies classified *L. lactis*, decreased in abundance during ripening, yet remained the dominant taxa throughout the 90-day period. Other bacterial taxa identified during ripening with relative abundances >1% included *B. thuringiensis*, *Lb. curvatus*, *S. aureus*, and *Str. pyogenes*. These genera have previously been isolated from Danbo, Stilton, and Gouda cheeses (Antonsson et al., 2003; Ercolini et al., 2003; Hajikhani et al., 2007; Salazar et al., 2018) and have either originated from the milk used to make the cheese (as with this study) or from post-process contamination. *S. aureus* and *Str. pyogenes*, which were identified in both types of cheeses at relative abundances >3.60% after 90-day ripening, are both human foodborne pathogens. Although both of these strains were identified in the unpasteurized milk used to make the Gouda cheese in this study, this finding also highlights the

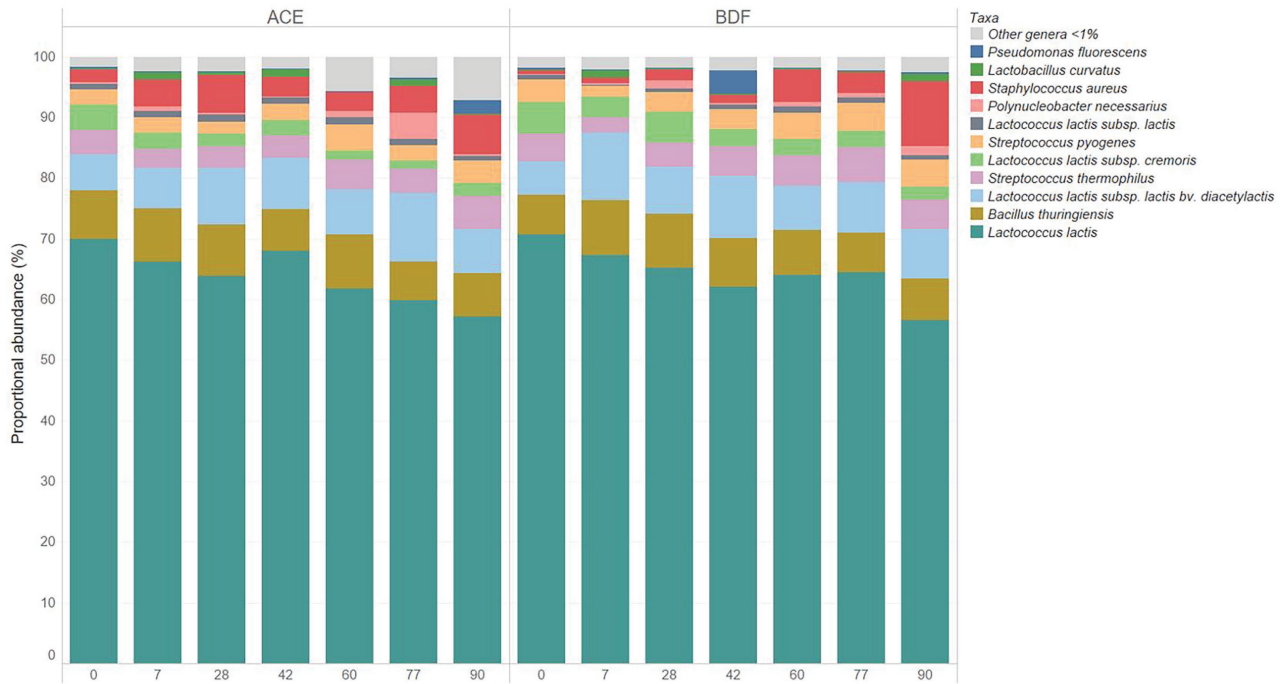

FIGURE 4 | Relative abundance of bacterial taxa during 10°C aging of Gouda cheese manufactured with unpasteurized milk (cheese ACE) or with unpasteurized milk inoculated with 1 log CFU/mL of *Listeria monocytogenes* (cheese BDF).

TABLE 3 | Pairwise Adonis of the six individual Gouda cheeses (A, B, C, D, E, and F) as well as the two types of cheeses (ACE, uninoculated and BDF, inoculated) manufactured in this study.

Comparison	Sums of Squares[a]	F.Model[b]	r^{2}[c]	*p*	Adjusted value of *p*[d]
A vs. B	0.15	2.48	0.17	0.02	0.3
A vs. C	0.30	6.23	0.34	0.001	0.015*
A vs. D	0.19	3.47	0.22	0.008	0.12
A vs. E	0.29	5.98	0.32	0.002	0.03*
A vs. F	0.23	4.98	0.29	0.004	0.06
B vs. C	0.07	1.94	0.14	0.072	1
B vs. D	0.05	1.10	0.08	0.371	1
B vs. E	0.14	3.47	0.21	0.004	0.06
B vs. F	0.07	1.99	0.14	0.071	1
C vs. D	0.05	1.65	0.12	0.128	1
C vs. E	0.06	2.29	0.15	0.033	0.495
C vs. F	0.04	1.80	0.13	0.103	1
D vs. E	0.10	3.06	0.19	0.012	0.18
D vs. F	0.04	1.32	0.10	0.025	1
E vs. F	0.03	1.00	0.07	0.402	1
ACE vs. BDF	0.07	1.96	0.14	0.042	0.042*

*Indicates a significant difference.
[a]Sums of Squares expresses the total variation.
[b]F.Model expresses the variation between the two cheeses compared.
[c]r^{2}, coefficient of determination.
[d]Adjusted value of p, the value of p obtained using Bonferroni correction.

need for proper cheese ripening conditions and personnel hygiene.

Although the abundances of the dominant bacterial community members of Gouda cheeses ACE and BDF were similar during ripening, notable differences in the relative abundances of non-dominant taxa were observed. With relative abundances of 0.1–0.5%, a total of 47 and 26 taxa were identified in cheeses ACE and BDF after 90-day ripening, respectively. Interestingly, no taxa were unique to cheese BDF since all the 26 taxa identified at an abundance of 0.1–0.5% in cheese BDF were also identified in cheese ACE. Twenty-one taxa (species from 11 different genera) having relative abundances 0.1–0.5% were unique to cheese ACE after 90 days (see **Table 3**). The unique taxa included, for example, five species in the genus *Bacteroides*, one species in each of the genera *Flavobacterium*, *Macrococcus*, *Photobacterium*, and *Porphyromonas*, and six species in the genus *Prevotella*.

Within a food matrix, there is a complex interaction between the microbial communities. The addition of a foodborne pathogen, in this case *L. monocytogenes*, may promote growth or hinder the survival of other community members. In contrast, the community members may promote or hinder the survival of *L. monocytogenes*. It is known that certain bacterial taxa, including *Bacillus* spp., *Chryseobacterium* spp., *Enterococcus* spp., *Lactococcus* spp., and *P. fluorescence*, possess anti-listerial properties or can readily out-complete *L. monocytogenes* for nutrients (Carpentier and Chassaing, 2004; Saubusse et al., 2007; Renye et al., 2009). On the other hand, *L. monocytogenes* possesses a bacteriocin, which can target *Prevotella* (Rolhion et al., 2019). In a study assessing the bacterial communities of floor drains in a food production facility both with and without resident *Listeria* spp., *Prevotella* populations were highest in these communities in the absence of *Listeria* spp.

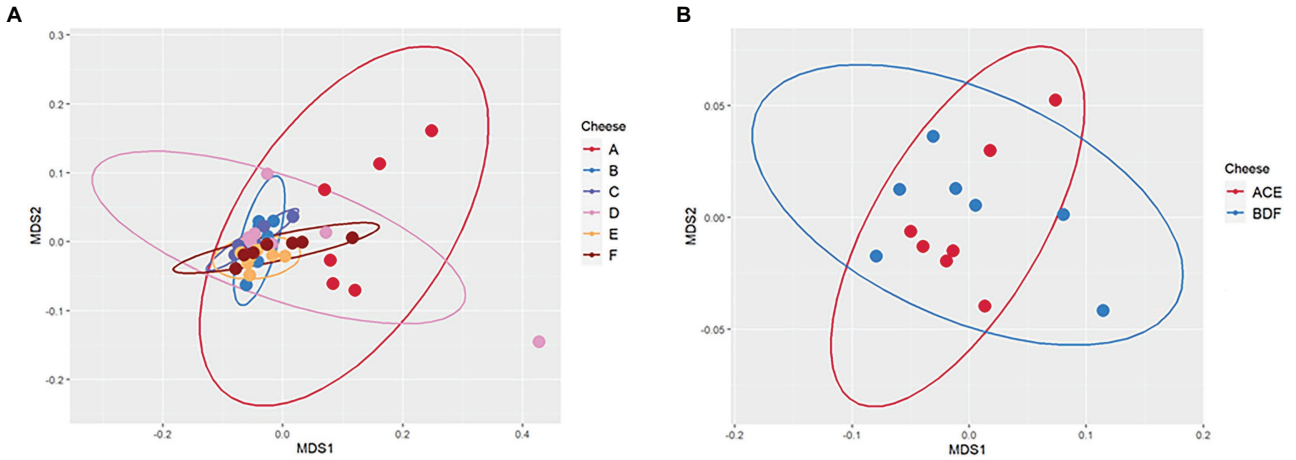

FIGURE 5 | Principal Component Analysis Plot (PCA) using Bray Curtis distance matrix to measure beta diversity of **(A)** the six individual Gouda cheeses (A, B, C, D, E, and F) and **(B)** the two types of Gouda cheeses (ACE, uninoculated and BDF, inoculated) manufactured in this study.

TABLE 4 | Average relative abundances of starter culture bacteria during 10°C ripening of Gouda cheese manufactured with unpasteurized milk (cheeses ACE) or with unpasteurized milk inoculated with 1 log CFU/ml of *Listeria monocytogenes* (cheese BDF).

Starter culture bacteria	Relative abundance (%)			
	0 day	28 days	60 days	90 days
Cheese ACE				
Lactococcus lactis subsp. *cremoris*	4.17	2.11	1.45	2.20
Lactococcus lactis subsp. *lactis*	0.90	1.17	1.14	0.84
Lactococcus lactis subsp. *lactis* bv. diacetylactis	5.97	9.23	7.54	7.29
Lactococcus lactis unassigned OTUs	69.97	63.83	61.78	57.19
Streptococcus thermophilus	3.96	3.64	4.86	5.47
Cheese BDF				
Lactococcus lactis subsp. *cremoris*	5.18	5.08	2.74	2.09
Lactococcus lactis subsp. *lactis*	0.66	0.69	0.99	0.82
Lactococcus lactis subsp. *lactis* bv. diacetylactis	5.50	7.63	7.36	8.21
Lactococcus lactis unassigned OTUs	70.67	65.23	64.08	56.62
Streptococcus thermophilus	4.59	4.05	4.97	4.92

TABLE 5 | Taxa present at relative abundances of 0.1–0.5% in Gouda cheese manufactured with unpasteurized milk (cheese ACE) or with unpasteurized milk inoculated with 1 log CFU/ml of *Listeria monocytogenes* (cheese BDF) after 90 days aging at 10°C.

Taxa unique to ACE	Taxa present in both ACE and BDF
Aerococcus christensenii	*Bacillus thuringiensis*
Amycolatopsis mediterranei	*Bacteroides ovatus*
Anaerotignum propionicum	*Denitrovibrio acetiphilus*
Bacteroides cellulosilyticus	*Faecalibacterium prausnitzii*
Bacteroides dorei	*Fimbriimonas ginsengisoli*
Bacteroides helcogenes	*Haemophilus parainfluenzae*
Bacteroides heparinolyticus	*Ketobacter alkanivorans*
Bacteroides thetaiotaomicron	*Lactiplantibacillus pentosus*
Basilea psittacipulmonis	*Lactiplantibacillus plantarum*
Flavobacterium johnsoniae	*Lacticaseibacillus rhamnosus*
Lactobacillus crispatus	*Levilactobacillus brevis*
Limnohabitans spp.	*Ligilactobacillus acidipiscis*
Macrococcus caseolyticus	*Ligilactobacillus murinus*
Photobacterium damselae	*Limosilactobacillus fermentum*
Porphyromonas gingivalis	*Loigolactobacillus coryniformis*
Prevotella dentalis	*Odoribacter splanchnicus*
Prevotella fusca	*Paenisporosarcina* spp.
Prevotella intermedia	*Propionibacterium freudenreichii*
Prevotella melaninogenica	*Pseudomonas koreensis*
Prevotella ruminicola	*Pseudomonas mandelii*
Prevotella scopos	*Sporosarcina* spp.
	Streptococcus anginosus
	Streptococcus halotolerans
	Streptococcus pyogenes
	Streptomyces spp.
	Syntrophus aciditrophicus

No unique taxa were identified in cheese BDF, with the exception of the inoculated Listeria monocytogenes.

(Fox et al., 2014). The lack of identification of certain taxa in cheese BDF after 90 days may be due to inhibition of these bacteria by *L. monocytogenes* or by other community members.

Optimization of *L. monocytogenes* enrichment techniques for a particular food product is often necessary due to co-enriching and competitor resident microbiota. Rapid and accurate enrichment of *L. monocytogenes* from food matrices is essential to outbreak and recall investigations and public health. Some of the taxa

present in relative abundances >0.1% in the Gouda cheese after 90 day ripening, regardless of *L. monocytogenes* addition, included *Bacillus*, *Ketobacter*, eight species of *Lactobacillus*, two species of *Pseudomonas*, *Streptomyces*, and *Staphylococcus*. *Bacillus*, *Lactobacillus*, *Pseudomonas*, and *Staphylococcus* species are known to co-enrich and, at times, hinder *L. monocytogenes* detection when cultured in typical *Listeria* enrichment broths (Dallas et al., 1991; Millet et al., 2006). The identification of the bacteria present in Gouda cheese during the ripening process can be used to develop appropriate enrichment procedures for *L. monocytogenes* for this food product.

In conclusion, this study examined the dynamics of the bacterial community in Gouda cheese after manufacture and 90-day ripening when made with unpasteurized milk both with and without the addition of *L. monocytogenes*. Although no microbial community differences were observed in the three batches of unpasteurized milk used to make Gouda cheese in this study, there were significant differences between the microbiomes of these two types of cheeses during ripening. While each type of cheese shared community members, a large number of bacterial taxa were unique to cheese ACE (made without *L. monocytogenes*) after 90-day ripening, indicating that *L. monocytogenes* may influence the microbial community. These data are important to understanding the impact of *L. monocytogenes* on the microbiome of Gouda cheese and vice versa. It is noted that these results are specific to the unpasteurized milk sourced in this study and that future studies could examine metataxonomic changes in Gouda cheese based on the seasonality, farm and cattle, and geographic location of the milk utilized.

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

JS, KS, and MT conceived and designed the experiments. JS, LG, and MF performed the experiments and wrote the manuscript. JS, MF, and PR analyzed the data. All authors contributed to the article and approved the submitted version.

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Microbial diversity and functional genes of red vinasse acid based on metagenome analysis

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Red vinasse acid has a distinct flavor and a vivid red color that are directly tied to the intricate metabolic activities of microorganisms that produce it. In this study, metagenomic technology was used to mine its functional genes and examine the microbial diversity of red vinasse acid. The findings revealed the identification of 2,609 species, 782 genera, and 63 phyla of microorganisms, and the dominant genus was *Lactobacillus*. Amino acid metabolism and carbohydrate metabolism were significant activities among the 16,093 and 49,652 genes that were annotated in the evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases, respectively. In gluconeogenesis, red vinasse acid encodes 194 genes controlling the transporter protein systems of different sugars and has key enzyme genes that catalyze the conversion of intracellular sugars into glycolytic intermediates. In amino acid flavor formation, red vinasse acid contains 32 control genes for branched-chain aminotransferase (BCAT), 27 control genes for aromatic-amino-acid transaminase (ArAT), 60 control genes for keto acid invertase, 123 control genes for alcohol/aldehyde dehydrogenase, and 27 control genes for acetyl esterase, which have the basis for the formation of strong flavor substances from amino acids.

KEYWORDS

red vinasse acid, metagenome, microbial diversity, glucose metabolism, functional genes, amino acid

Introduction

Red vinasse acid (Bai et al., 2020) is a kind of unique flavor food in Guangxi, China. It is a well-known regional specialty that is mostly produced in Guangxi Wuxuan and Guangxi Guiping. Bright crimson, crisp., and delectable red vinasse acid is pickled with red rice as seeds. Red yeast rice was fermented at a specified temperature and duration under the combined influence of *Monascus*, *Lactobacillus*, *Yeast*, and other microorganisms to create red vinasse acid (Bai et al., 2020). Its diverse microbial

community, which includes the dominant fungus *Monascus* and the dominant bacteria *Lactobacillus*, is strongly associated with the development of red vinasse acid's distinctive flavor and functional features. Numerous secondary metabolites, such as *Monascus* pigments (MPs), monacolin K, γ-aminobutyric acid (GABA), and dimerumic acid, can be produced by *Monascus* (Aniya et al., 2000; Diana et al., 2014; Chen et al., 2015). In addition to effectively lowering blood pressure, lowering plasma cholesterol, preventing obesity, improving anti-inflammatory activity, preventing cancer, lowering blood sugar levels, and having anti-inflammatory and anti-tumor properties, MPs can replace some synthetic pigments in the food industry (Nam et al., 2014; Zhang et al., 2021). The most important effect of GABA is blood pressure regulation. Numerous studies have shown that GABA can reduce high blood pressure in animals and humans (Yoshimura et al., 2010; Yang et al., 2012; Pouliot-Mathieu et al., 2013; Tsai et al., 2013). Monacolin K can block cholesterol synthesis pathways, reduce cholesterol synthesis, and thus play a role in lowering blood lipids (Hsieh et al., 2013; Ajdari et al., 2014; Dikshit and Tallapragada, 2015, 2016). Lactobacillus has a variety of probiotic functions, such as promoting the absorption of nutrients, improving human immunity, and maintaining the balance of intestinal flora (Arnold et al., 2018; Riaz Rajoka et al., 2018; Sandes et al., 2018; Chen et al., 2020). Therefore, Red vinasse acid thus plays a significant role in healthcare. In recent years, it has been possible to estimate the microbiological diversity of red vinasse acid detected by 16srRNA technology only at the genus level and not with sufficient accuracy at the species level. The discovery of functional genes has some restrictions.

New insights into the roles played by natural microbial communities have been made possible by the introduction of metagenomic methods (Knight et al., 2018). As a result, the integration of the genetic, taxonomic, and functional diversity of the microbial community has drawn more and more attention. A study technique called metagenomics uses gene sequences to analyze the microbial data contained in environmental samples (Mori et al., 2008; Xie et al., 2020). It has developed into a crucial tool for researching microbial diversity and community traits because of its ability to swiftly and reliably gather a wealth of biological data and extensive microbiological information. A metagenomic technique, as opposed to 16srRNA high-throughput sequencing, can not only fully mine the microbial genome information but also study the function and metabolic pathways. Based on this information, it is more convenient to mine the biodiversity, community structure, functional characteristics, and relationships of microflora (Li et al., 2017; Belleggia et al., 2020). Importantly, when compared to conventional rRNA gene sequencing, metagenomic sequencing can identify novel species with greater resolution and accuracy (Chen et al., 2017). Our understanding of the microbial communities in fermented foods has significantly improved over the past several decades as a result of advancements in next-generation DNA sequencing and high-throughput computational methods (Arıkan et al., 2020). Studies on wines (Johansson et al., 2021), kimchi (Kim et al., 2021), fermented Chinese xiaoqu (Su et al., 2020), dajiang-meju (Xie et al., 2019), and Turkish kefir grains (Ilıkkan and Bağdat, 2021) are a few examples.

Studies on the microbiological diversity of red vinasse acid have been conducted recently. Further study is necessary since it is unclear what species of microorganisms exist and because functional genes are nearly at the blank stage. In order to provide a theoretical foundation for the development of the red vinasse acid industry and the development of Monascus resources, metagenomics, and bioinformatics analysis methods were used in this study to identify the core microbial groups at the 'species' level of taxonomic status, analyze the microbial diversity of red vinasse acid, and excavate functional genes in group microorganisms.

Materials and methods

Experimental materials

Composition and production process of red vinasse acid

Fresh edible fruits and vegetables (or edible fruit and vegetable salted billet), red vinasse as the main raw material, then add edible salt, add or not add sugar (sugar or rock sugar, etc.), rice-flavored white wine, garlic, chili pepper, star anise, and other auxiliary ingredients, through the raw and auxiliary materials pretreatment, cleaning, drying, mixing, vinasse fermentation, packaging, and other processes made ready to eat red vinasse acid (Bai et al., 2020).

Source and storage of experimental samples

The samples of red vinasse acid were collected from Guangxi in June, 2021, and then brought back to the laboratory and sent to Biomarker Technologies for metagenome sequencing immediately, and the remaining samples were stored in the laboratory refrigerator at 4°C.

Total DNA extraction

Take about 500 mg of red vinasse acid tissue sample into a domestic 2 ml centrifuge tube, add pre-cooled steel beads, place the taken tissue sample in liquid nitrogen, and grind it thoroughly at 1,500 rpm for DNA extraction.

Complete the nucleic acid extraction using the CTAB procedure, measure the concentration of the extracted nucleic acid using the Qbiut (Invitrogen, model Qubit3.0, reagent Qubit TM dsDNA HS Assay Kit), and assess the integrity using 1% agarose gel electrophoresis. Red vinasse acid sample processing by Biomarker Technologies included DNA extraction and metagenome sequencing.

Metagenome library construction and sequencing methods

Library construction methods

Genomic enzymatic disruption and end repair

1. Take 10 ng of genomic DNA (Qubit quantification) into a 96-well plate, make up to the corresponding volume with Unclease-free Water, finally add the corresponding digestion reagent (on ice), mix well by pipetting, centrifuge instantly, and perform the reaction immediately in a PCR instrument (Eppendorf).
2. Rapid Ligation Butter 3, ligase and index are added to the digestion product, blown, and mixed, centrifuged instantaneously, and placed in the PCR instrument for reaction.

Conjugate product purification

1. Add a certain volume of magnetic beads to the conjugate product, mix thoroughly, incubate at room temperature, and place on a magnetic stand, let stand, and carefully remove the supernatant.
2. Add freshly prepared 80% ethanol to the tube, let it stand on the magnetic rack for 30s, and remove the supernatant.
3. Repeat Step 2 once, after the second wash, centrifuge instantaneously and remove the supernatant with a 10 μl gun.
4. Place the reaction tube on the magnetic rack, open the cap and air dry until the surface of the magnetic beads is lusterless and has fine cracks.
5. Remove the reaction tube from the magnetic rack, add Unclease-free Water to elute DNA, blow and mix well, incubate at room temperature, and then rest on the magnetic rack, carefully aspirate the supernatant into a new PCR tube.

PCR amplification

1. Add various reagents required for PCR reaction, mix well, and centrifuge.
2. According to the PCR reaction conditions, put it into the PCR instrument for PCR amplification.

PCR product fragment screening

1. Instantaneously centrifuge the PCR reaction tube, add the corresponding volume (0.6×) of magnetic beads, and mix thoroughly.
2. Incubate at room temperature, then place on a magnetic stand, and carefully transfer the supernatant to a new PCR tube.
3. Aspirate the corresponding volume (0.12×) of beads into the supernatant of Step 2, gently blow and mix well, incubate at room temperature, then place on a magnetic stand, let stand, carefully remove the supernatant.
4. Add freshly prepared 80% ethanol to the reaction tube, let it stand on the magnetic rack for 30s, remove the supernatant, and repeat the Step 1 time.
5. Open the cap of the tube, air dry until the surface of the magnetic beads is lusterless with fine cracks, add Unclease-free Water to elute the library, blow and mix, let stand at room temperature, let stand on the magnetic stand, carefully aspirate the supernatant into a new 1.5 ml centrifuge tube, and store at −20°C for backup.

Library quality control

The library was quality-checked by Qsep-400 and the library concentration was quantified using Qubit 3.0, and meets the following indicators can be tested on the machine: Concentration ≥ 1 ng/μl, center value of fragment 430–530 bp, average value between 420 and 580 bp, normal distribution of peak pattern, single fragment without spurious peak.

Sequencing methods

The constructed libraries were sequenced using illumina novaseq6000 (San Diego, CA, United States). The kit NovaSeq 6,000 S4 Reagent Kit (San Diego, CA, United States) was used for sequencing.

Data analysis

Sequencing data quality control

The raw sequences (Raw reads) obtained from sequencing contain low quality sequences. To ensure the quality of information analysis, raw reads need to be filtered to obtain clean reads for subsequent information analysis. The main steps of data filtering are as follows.

1. Filtering raw tags to obtain high quality sequencing data (clean tags) using the software Trimmomatic (version v0.33 parameters PE LEADING:3 TRAILING:3 SLIDINGWINDOW:50:20 MINLEN:120).
2. Use bowtie2 (version v2.2.4 parameter—seed 123,456-I 200-X 1000—un-conc) to compare with host genome sequence to remove host contamination (if a host reference genome is provided).

Metagenome assembly

Metagenome assembly was performed using the software MEGAHIT (Li et al., 2015; version v1.1.2 default parameters), filtering contig sequences shorter than 300 bp. The assembly results were evaluated using QUAST (Gurevich et al., 2013; version v2.3 default parameters) software.

Metagenome component analysis

Gene prediction

MetaGeneMark (Zhu et al., 2010; version v.3.26 default parameters) software (http://exon.gatech.edu/meta_gmhmmp.cgi, Version 3.26) was used to identify coding regions in the genome using default parameters (parameters -A -D -f G).

Construction of non-redundant gene sets

Redundancy was removed using MMseq2 software (https://github.com/soedinglab/mmseqs2, Version 11-e1a1c) with the similarity threshold set to 95% and the coverage threshold set to 90%.

Functional annotation analysis

eggNOG functional annotation

Evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG; Powell et al., 2014; evolutionary genealogy of genes: Non-supervised Orthologous Groups) is a database of biological direct homologous gene clusters, which is a continuous update of the COG database. For specific annotation, the protein sequences of non-redundant genes are compared with the eggNOG database by BLAST (diamond v0.9.29 matching threshold *E*-value 1e−5) to find the most similar sequence in the eggNOG database, and the annotation and classification information corresponding to this sequence is the annotation and classification information of the corresponding sequenced genomic genes.

KEGG functional annotation

Kyoto Encyclopedia of Genes and Genomes (KEGG; Kanehisa et al., 2004) is a database for collecting genomes, biological pathways, diseases, drugs, and chemicals. For specific annotation, the protein sequences of non-redundant genes are compared with those included in the KEGG database by BLAST (diamond v0.9.29 matching threshold *E*-value 1e−5) to find the most similar sequence in the KEGG database, and the annotation information, corresponding KO number, and position in the KO corresponding pathway of the sequence is the annotation information of the corresponding gene in the sequenced genome. The annotation information, KO number and position in the biological process pathway of the corresponding gene in the KEGG database is the sequence with KO sequence number.

CAZy function annotation

Carbohydrate-active enzymes database (CAZy; Cantarel et al., 2009) is a database of carbohydrate-active enzymes, formed by the collection and classification of published literature and related proteins, and carefully maintained by experts. For the specific annotation, the hmmer (version 3.0) software was used to compare the protein sequences of the non-redundant genes separately with the Hidden Markov Model of each family of the CAZy database (matching parameters default, default screening threshold "if alignment >80aa, use *E*-value <1e−5, otherwise use *E*-value <1e−3; covered fraction of HMM > 0.3") to find all families that satisfy the filtering threshold, so that the carbohydrate-active enzymes in the genome can be identified and analyzed for the presence of several conserved carbohydrate-related functional domains.

Species annotation and statistics

Based on the species information of the above non-redundant genes compared to the sequences in Nr, the species composition and relative abundance information of the samples were obtained.

Results

Microbial diversity analysis of red vinasse acid

The red vinasse acid sample's genome had a length of 75, 320, and 253 bp, 39,965 sequences, and a G + C content of 50.46 percent. Red vinasse acid gene species abundance annotation revealed 63 phyla, 782 genera, and 2,609 microorganisms. There are three groups of advantage bacteria among them with relative contents above 1% at the phylum level, seven groups at the genus level, and nine groups at the species level (Figure 1).

At the phylum level, *Firmicutes*, *Proteobacteria*, and *Ascomycota* were the dominant phyla, with relative contents of 38.44, 16.90, and 16.78%, respectively, accounting for 72.12% of the total content. At the genus level, the dominant genera include *Lactobacillus*, *Pediococcus*, *Aspergillus*, *Burkholderia*, *Klebsiella*, *Monascus*, and *Penicillium*, with relative contents of 21.59, 16.1, 10.25, 10.02, 2.74, 1.33, and 1.06%, respectively, accounting for 63.13% of the total content. At the species level, there are nine dominant strains with relative content of more than 1%, including *Pediococcus acidilactici*, *Burkholderia multivorans*, *Lactobacillus* sp. *BCRC 12945*, *Lactobacillus panis*, *Lactobacillus fermentum*, *Lactobacillus phage Bacchae*, *Klebsiella pneumoniae*, *Aspergillus wentii*, and *Monascus purpureus*. The relative contents were 15.47, 6.84, 5.19, 4.11, 3.97, 2.08, 1.96, 1.36, and 1.12%, respectively.

Annotation analysis of common database

Annotation analysis of eggNOG database

According to the eggNOG database, 16,093 genes of red vinasse acid showed functional annotation, and these genes can be classified into 23 categories based on their functions. Remove the R (general function prediction only) and S (function unknown) parts with unknown function, in which the number of genes greater than 300 is transcription (1086), intracellular trafficking, secretion, and vesicular transport (589), carbohydrate transport and metabolism (582), replication, recombination, and

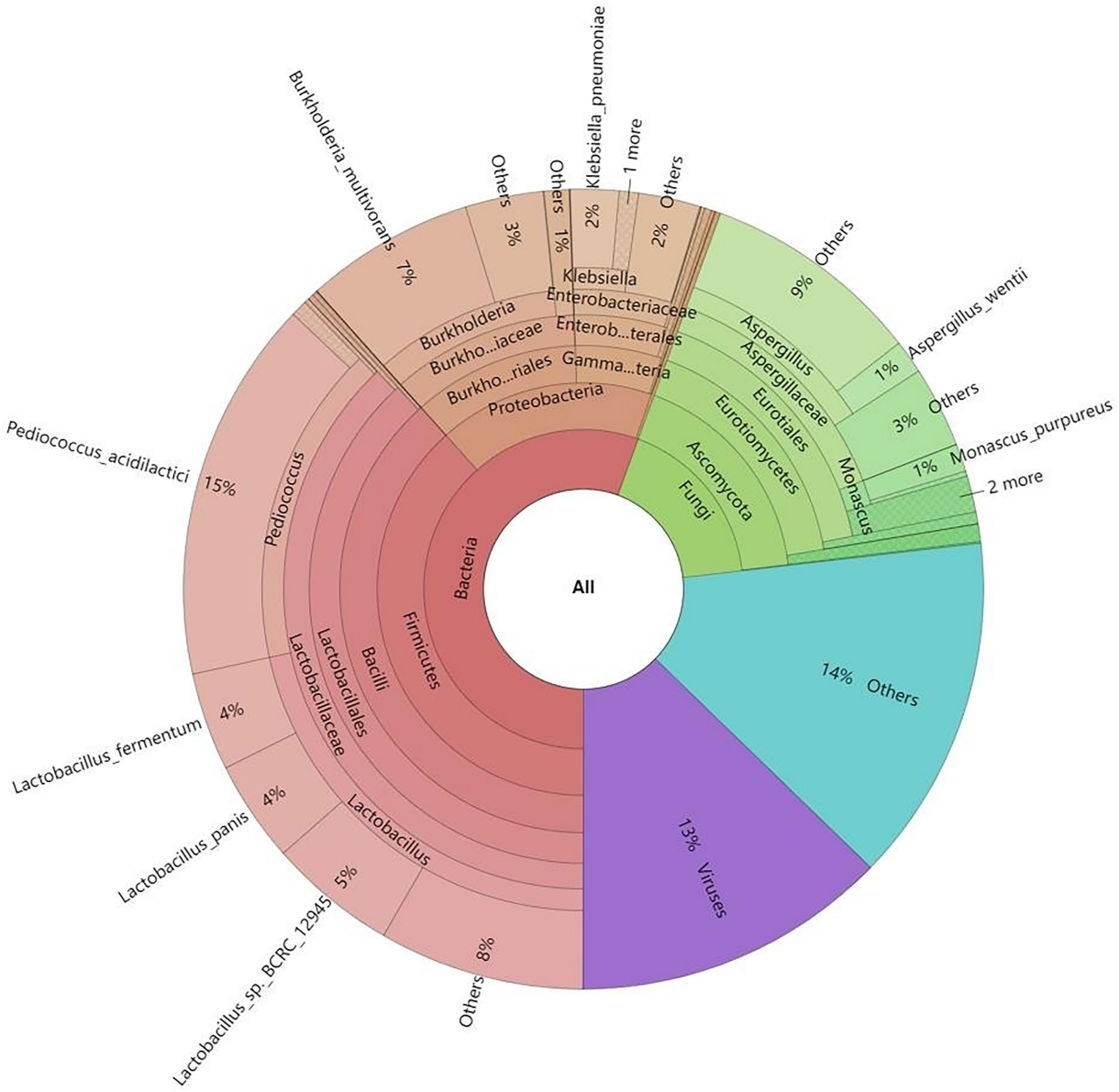

FIGURE 1
Krona diagram of the microbial community of red vinasse acid.

repair (484), posttranslational modification, protein turnover, chaperones (474), signal transduction mechanisms (418), cell wall/membrane/envelope biogenesis (393), inorganic ion transport and metabolism (352), amino acid transport and metabolism (330), and translation, ribosomal structure, and biogenesis (301). In addition to the basic life activities of microorganisms, metabolic activities are mainly carbohydrate metabolism, inorganic ion transport and metabolism, and amino acid transport and metabolism (Figure 2).

Annotation analysis of KEGG database

49,652 different genes in red vinasse acid were annotated, belonging to 21 KEGG pathways in four categories of the KEGG database (Figure 3). The four categories are: metabolism (35,782, 72.07%), genetic information processing (8,272, 16.66%), cellular processes (3,670, 7.39%), and environmental information processing (1,928, 3.88%). Carbohydrate metabolism (10,375), amino acid metabolism (7,057), nucleotide metabolism (4,005), replication and repair (3,516), metabolism of cofactors and vitamins (3,284), energy metabolism (2,849), lipid metabolism (2529), and translation (2,029) are among the eight KEGG pathways that enrich differentially expressed genes (2,029). The two most significant metabolic processes among them are glucose and amino acid metabolism, which are identical to the findings of eggNOG's functional annotation.

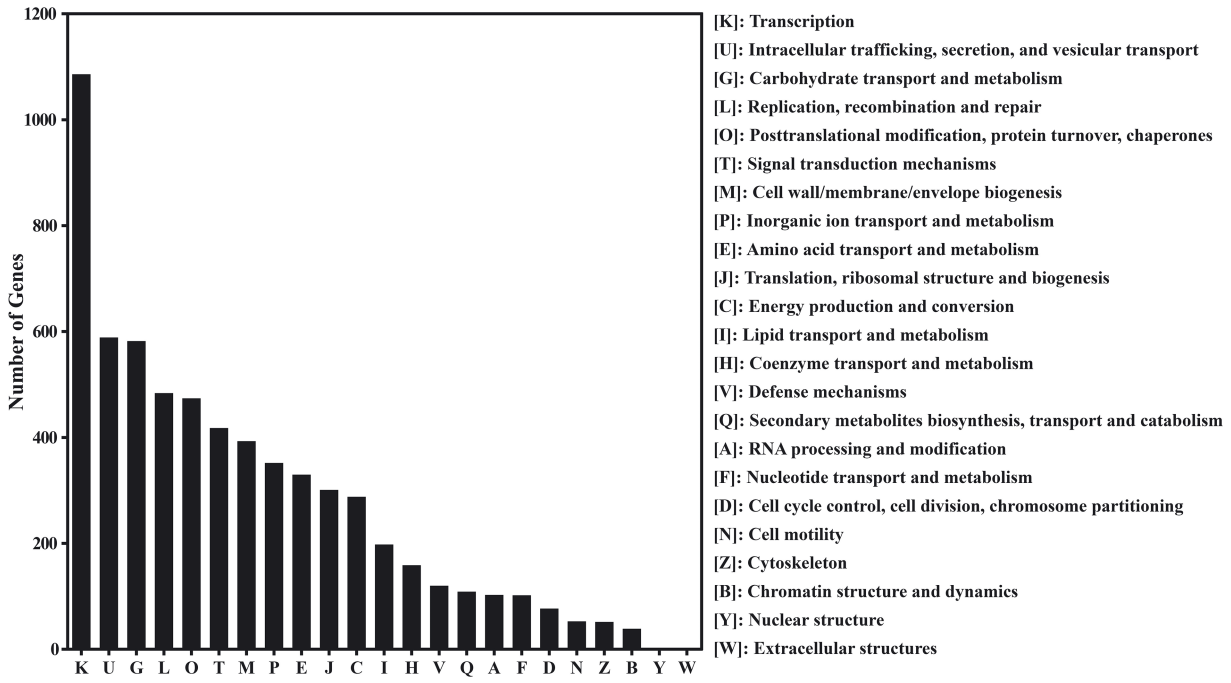

FIGURE 2
The evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG) gene's functional classification in red vinasse acid.

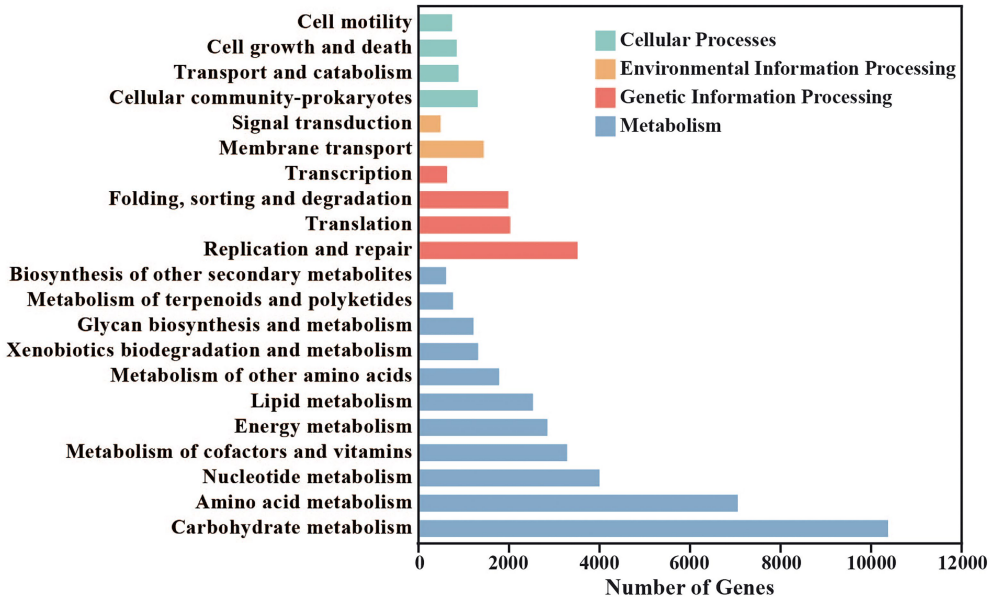

FIGURE 3
Statistics of Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway in red vinasse acid.

Annotation analysis of CAZy database

The CAZy database can divide different types of carbohydrate-active enzymes into six protein families, including glycoside hydrolases (GHs), glycosyl transferases (GTs), polysaccharide lyases (PLs), carbohydrate esterases (CEs), carbohydrate-binding modules (CBMs), and auxiliary activities (AAs). Compared with the CAZy database, a total of 3,328 carbohydrate-active enzymes were identified (Figure 4), of which glycosyl transferases (GTs, 31.3%) and glycoside hydrolases (GHs, 28.3%) were the most. Then, carbohydrate esterases (CEs, 21.4%), auxiliary activities

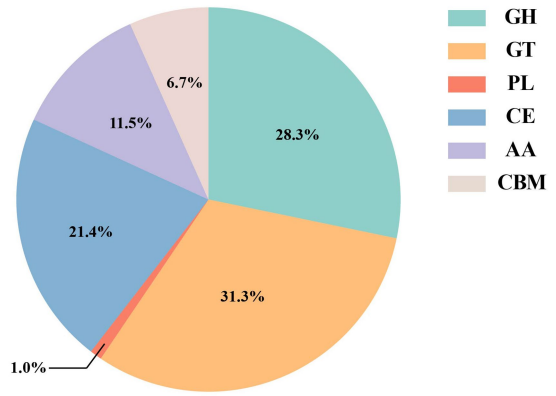

FIGURE 4
Carbohydrate-active enzymes database (CAZy) statistics in red vinasse acid.

TABLE 1 Gene analysis of the sugar transport system in red vinasse acid.

Sugar	Transporter	Gene number
Mannitol	PTS-Gtl-EIIA/B	27
Fructose	PTS-Gfr-EIIA/B	49
Mannose	PTS-Gan-EIIA/B	34
Sucrose	PTS-Scr-EIIA/B	17
Lactose	PTS-Lac-EIIB	1
Maltose	PTS-Mal-EIIB	1
Galactitol	PTS-Gat-EIIA/B	16
Glucose	PTS-Glc/v-EIIA/B	22
Galactose	PTS-Gal-EIIA/B	3
Gluconate	permease protein	10
Oligosaccharides	permease protein	14

(AAs, 11.5%), and carbohydrate-binding modules (CBMs, 6.7%). Polysaccharide lyases (PLs, 1.0%) were the least.

Gene analysis of glucose metabolism

Sugar transport system

In the sugar transport system, mannitol, fructose, mannose, and sucrose can be transported by PTS (phosphotransferase system) simultaneously. A total of 27, 49, 34, and 17 control genes were found in red vinasse acid, respectively, indicating that the ability of red vinasse acid microorganisms to transport different sugars was different during fermentation, and the ability of microorganisms to transport fructose was strong during fermentation. Lactose and maltose can be transported through the PTS transport system, ABC transport protein, and permease. One PTS transport system for lactose and maltose was found in the red vinasse acid gene, but the ABC transport protein and permease gene for lactose and maltose were not found, indicating that the transport of lactose and maltose by microorganisms in red vinasse acid was mainly carried out through the PTS transport system. Galacitol can only be transported through the PTS transport system, and 16 related genes were found in red vinasse acid. Gluconate, fructose, and oligosaccharides can also be used to enter the strain cells by using relevant permeability proteins, and 10, 0, and 14 genes were retrieved respectively, indicating that fructose is mainly transported by microorganisms in red vinasse acid through the PTS transport system (Table 1).

Transformation of D-mannitol 1-phosphate

The PTS transport system phosphorylates sugars before they are delivered to cells. Mannitol was phosphorylated while being transported by the PTS system, and after reaching the cells, D-mannitol 1-phosphate was eventually produced. The latter is often transformed by mannitol-1-phosphate 5-dehydrogenase (EC: 1.1.1.17) into D-Fructose 6-phosphate, an intermediate glycolysis product. In conjunction with Tables 1, 2, red vinasse acid has 14 mannitol-1-phosphate 5-dehydrogenase genes and the mannitol PTS transport system, allowing mannitol to be transformed into the intermediate of the glycolysis pathway known as D-Fructose 6-phosphate. Table 2 demonstrates that there are seven species of bacteria and one species of fungi at the genus level, with *Lactobacillus* being the predominate genus and playing a significant role in the transfer of mannitol (Table 2; Chen et al., 2014).

Transformation of D-fructose 1-phosphate

Fructose was finally transported into cells through the PTS system to form D-Fructose 1-phosphate, which was generally converted into the intermediate product of glycolysis D-glyceraldehyde 3-phosphate through three pathways. There are three combinations of key enzymes, namely, phosphofructokinase (EC: 2.7.1.11) and fructose-2-phosphate aldolase, fructose-bisphosphate aldolase (EC: 4.1.2.13) and triosephosphate isomerase (EC: 5.3.1.1), and fructose-2-phosphate aldolase and triose kinase (EC: 2.7.1.28). Combined with Tables 1, 2, red vinasse acid contains fructose PTS transport system, 36 fructose-bisphosphate aldolase and 33 triphosphate isomerase genes, which can convert intracellular D-Fructose 1-phosphate into D-glyceraldehyde 3-phosphate by the second pathway. Fructose-bisphosphate aldolase (EC: 4.1.2.13) comes in two varieties: fructose-bisphosphate aldolase class I and class II, as indicated in Table 2. Fructose-bisphosphate aldolase and class I annotated five kinds of microorganisms, and the dominant genera were *Franconibacter* and *Cronobacter*. Fructose-bisphosphate aldolase and class II annotated 15 species of microorganisms, and the dominant genus was *Lactobacillus*. Triosephosphate isomerase (EC:5.3.1.1) annotated 15 species of microorganisms, including 11 species of bacteria and four species of fungi. The dominant genera of bacteria were *Lactobacillus* and the dominant genera of fungi were *Millerozyma* and *Aspergillus*. Therefore, *Lactobacillus* plays a major role in the transport of fructose.

TABLE 2 Genes encoding enzymes involved in conversion sugar into intermediates of glycolysis pathway.

	Enzyme	K0 number	EC number	Gene name	Gene number	Annotated microorganisms
Transformation of D-mannitol 1-phosphate	Mannitol-1-phosphate 5-dehydrogenase	K00009	EC: 1.1.1.17	mtlD	14	*Lactobacillus* (*5*), *Franconibacter* (*2*), *Paenibacillus* (*2*), *Unclassified*, *Pantoea*, *Klebsiella*, *Cronobacter*, and *Rasamsonia*
Transformation of D-Fructose 1-phosphate	6-phosphofructokinase 1	K00850	EC: 2.7.1.11	PFK1	24	*Millerozym* (*6*), *Unclassified* (*3*), *Lactobacillus* (*3*), *Paenibacillus* (*2*), *Klebsiella* (*2*), *Pantoea* (*2*), *Aspergillus* (*2*), *Kluyveromyces*, *Pediococcus*, *Penicillium*, and *Chryseobacterium*
	Fructose-bisphosphate aldolase, class I	K11645	EC: 4.1.2.13	ALDO	7	*Franconibacter* (*2*), *Cronobacter* (*2*), *Pantoea*, *Klebsiella*, and *Acetobacter*
	Fructose-bisphosphate aldolase, class II	K01624	EC: 4.1.2.13	FBA	29	*Lactobacillus* (*7*), *Unclassified* (*5*), *Klebsiella* (*3*), *Paenibacillus* (*2*), *Pantoea* (*2*), *Franconibacter* (*2*), *Pediococcus*, *Cronobacter*, *Burkholderia*, *Chryseobacterium*, *Acinetobacter*, *Millerozyma*, *Metarhizium*, *Aspergillus*, and *Byssochlamys*
	Triosephosphate isomerase (TIM)	K01803	EC: 5.3.1.1	TPI	33	*Lactobacillus* (*15*), *Burkholderia* (*2*), *Klebsiella* (*2*), *Millerozyma* (*2*), *Aspergillus* (*2*), *Luteibacter*, *Pantoea*, *Pediococcus*, *Acetobacter*, *Unclassified*, *Franconibacter*, *Acinetobacter*, *Cronobacter*, *Cladophialophora*, and *Zygosaccharomyces*
	Triose kinase	K00863	EC: 2.7.1.28	DAK	13	*Millerozyma* (*7*), *Burkholderia* (*2*), *Aspergillus* (*2*), *Cronobacter*, and *Byssochlamys*
Transformation of D-mannose-6-phosphate	Mannose-6-phosphate isomerase	K01809	EC: 5.3.1.8	manA	26	*Lactobacillus* (*4*), *Franconibacter* (*4*), *Klebsiella* (*4*), *Cronobacter* (*3*), *Pediococcus* (*2*), *Lactobacillus* (*2*), *Aspergillus* (*2*), *Pantoea*, *Unclassified*, *Paenibacillus*, *Penicillium*, and *Millerozyma*
Transformation of sucrose-6-phosphate	beta-fructofuranosidase	K01193	EC: 3.2.1.26	sacA	20	*Lactobacillus* (*9*), *Klebsiella* (*6*), *Franconibacter* (*4*), and *Cronobacter*
Transformation of lactose 6-phosphate	6-phospho-beta-galactosidase	K01221	EC: 3.2.1.85	lacG	0	——
Transformation of gluconate	Gluconokinase	K00851	EC: 2.7.1.12	gntK	28	*Lactobacillus* (*14*), *Klebsiella* (*2*), *Pediococcus* (*2*), *Paenibacillus* (*2*), *Burkholderia* (*2*), *Pantoea*, *Franconibacter*, *Cronobacter*, *Millerozyma* (*2*), and *Aspergillus*
	6-phosphogluconate dehydrogenase	K00033	EC: 1.1.1.44 1.1.1.343	PGD	70	*Lactobacillus* (*14*), *Unclassified* (*23*), *Klebsiella* (*8*), *Paenibacillus* (*4*), *Pediococcus* (*3*), *Millerozyma* (*3*), *Burkholderia* (*2*), *Cronobacter* (*2*), *Pantoea* (*2*), *Chryseobacterium* (*2*), *Acetobacter*, *Luteibacter*, *Franconibacter*, *Kluyveromyces*, *Talaromyces*, *Aspergillus*, and *Rasamsonia*
	Ribulose-phosphate 3-epimerase	K01783	EC: 5.1.3.1	RPE	26	*Lactobacillus* (*8*), *Stenotrophomonas* (*2*), *Burkholderia* (*2*), *Millerozyma* (*2*), *Franconibacter*, *Pantoea*, *Acinetobacter*, *Acetobacter*, *Klebsiella*, *Ideonella*, *Luteibacter*, *Pediococcus*, *Sphingomonas*, *Cronobacter*, *Aspergillus*, and *Kluyveromyces*
	transketolase	K00615	EC: 2.2.1.1	tktA	85	*Lactobacillus* (*21*), *Unclassified* (*14*), *Klebsiella* (*12*), *Franconibacter* (*10*), *Burkholderia* (*6*), *Pantoea* (*3*), *Luteibacter* (*3*), *Acetobacter* (*2*), *Paenibacillus* (*2*), *Acinetobacter* (*2*), *Enterobacter* (*2*), *Cronobacter* (*2*), *Millerozyma* (*2*), *Kozakia*, *Stenotrophomonas*, *Sphingomonas*, and *Aspergillus*

(Continued)

TABLE 2 (Continued)

	Enzyme	K0 number	EC number	Gene name	Gene number	Annotated microorganisms
Transformation of maltose	Maltose phosphorylase	K00691	EC: 2.4.1.8	mapA	18	*Lactobacillus* (*16*), *Paenibacillus* (*2*)
Transformation of lactose	Beta-galactosidase	K01190 K12308	EC: 3.2.1.23	lacZ	65	*Lactobacillus* (*29*), *Burkholderia* (*6*), *Paenibacillus* (*6*), *Franconibacter* (*5*), *Klebsiella* (*4*), *Kluyveromyces* (*3*), *Pantoea* (*2*), *Chryseobacterium* (*2*), *Pediococcus* (*2*), *Aspergillus* (*2*), *Sphingomonas*, *Cronobacter*, *Luteibacter*, and *Unclassified*
Transformation of lactose and galactose	Galactokinase	K00849	EC: 2.7.1.6	galK	20	*Lactobacillus* (*7*), *Pediococcus* (*2*), *Millerozyma* (*2*), *Saccharomyces*, *Aspergillus* (*2*), *Pantoea*, *Enterobacter*, *Franconibacter*, *Chryseobacterium*, *Cronobacter*, and *Klebsiella*
	UDP glucose-hexose-1-phosphate uridylyltransferase	K00965	EC: 2.7.7.12	galT	17	*Lactobacillus* (*6*), *Franconibacter* (*3*), *Unclassified* (*2*), *Pantoea*, *Paenibacillus*, *Cronobacter*, *Pediococcus*, *Millerozyma*, and *Aspergillus*

Transformation of D-mannose-6-phosphate

Mannatose was transported to cells through the PTS system to form D-mannose-6-phosphate, which was directly converted into the intermediate product β-D-Fructose 6 phosphate in the glycolysis pathway catalyzed by mannose-6-phosphate isomerase (EC: 5.3.1.8). Combining Tables 1, 2, it is known that red vinasse acid contains a mannose PTS transport system and 26 mannose-6-phosphate isomerase genes, so mannose can be converted into β-D-Fructose 6 phosphate, which is the intermediate of the glycolysis pathway. As shown in Table 2, nine species of bacteria and three species of fungi were annotated in the transport process. The dominant bacterial genera were *Franconibacter*, *Klebsiella*, and *Lactobacillus*, and the dominant genus of fungi was *Aspergillus*, indicating that the transport of mannose was mainly completed by the above microorganisms.

Transformation of sucrose-6-phosphate

Sucrose was transported to cells by the PTS system to form sucrose 6-phosphate, which was then converted to D-glucose 6-phosphate and β-D-fructose under the catalysis of β-fructofuranosidase (EC: 3.2.1.26). Combined with Tables 1, 2, red vinasse acid contains the PTS transport system of sucrose and encodes 20 genes of β-fructofuranosidase, so red vinasse acid can be transported to intracellular by extracellular sucrose to further produce D-glucose 6-phosphate and β-D-fructose. As shown in Table 2, four kinds of microorganisms are noted. All of them are bacteria, and the dominant genus is *Lactobacillus*.

Transformation of lactose 6-phosphate

The product of lactose transported to cells *via* the PTS system is 6-phosphate lactose, which is catalyzed by 6-phospho-β-galactosidase (EC: 3.2.1.85) to form β-D-glucose and D-galactose-6-phosphate. Combined with Tables 1, 2, the PTS transport system contained lactose, but the gene encoding 6-phospho-β-galactosidase was not found, so the transport of lactose could not be realized by red vinasse acid.

Transformation of gluconate

Gluconate transported to the cells can further form the intermediate substance β-D-fructose 6-phosphate in the glycolysis pathway, but this process needs to be catalyzed by four enzymes: glucokinase (EC: 2.7.1.12), 6-phosphogluconate dehydrogenase (EC:1.1.1.44 1.1.1.343), ribulose-phosphate 3-epimerase (EC: 5.1.3.1), and transketolase (EC: 2.2.1.1). The control genes of four enzymes were found in red vinasse acid, and the number of genes was 28, 70, 26, and 85, respectively, so red vinasse acid can use extracellular gluconate to further produce β-D-fructose-6-phosphate and participate in the pathway of glycolysis. As shown in Table 2, glucokinase annotated 10 species of microorganisms, including eight species of bacteria and two species of fungi, and the dominant genus was *Lactobacillus*. Twelve species of bacteria, with Unclassified and *Lactobacillus* as the most prevalent genera, and five species of fungi, with *Millerozyma* as the most prevalent genus, were found to contain 6-phosphate gluconate dehydrogenase. Ribulose-3-phosphate isomerase annotated three species of fungi and 13 species of bacteria. *Millerozyma* was the main fungus genus, whereas Lactobacillus was the leading genus of bacteria. The predominant genus among the 17 microorganism species for which transketolase was annotated was *Lactobacillus*. As a result, *Lactobacillus* is crucial to the transfer of gluconate.

Transformation of maltose

After entering the bacteria cells, maltose can be converted into D-glucose under the action of maltose phosphorylase (EC: 2.4.1.8) and enter the glycolysis pathway directly. 18 genes of maltose phosphorylase are encoded in red vinasse acid, so maltose can

be converted into D-glucose, an intermediate product of the glycolysis pathway. As shown in Table 2, maltose phosphorylase was annotated to two microorganisms, and *Lactobacillus* was the dominant genus.

Transformation of lactose

Lactose enters the cell through the PTS transport system and produces glycolysis intermediates through two pathways (Table 2). The lactase transferred into the cell was hydrolyzed into α-D-glucose and D-glucose-1-phosphate by pathways I and II. In pathway I, there are 70 genes encoding β-galactosidase (EC: 3.2.1.23), and in pathway II, the number of genes encoding β-galactosidase (EC: 3.2.1.23)/ galactokinase (EC: 2.7.1.6)/UDP- glucose-hexose-1-phosphate uridylyltransferase (EC: 2.7.7.12) is 70, 20, and 17, respectively. Therefore, the intracellular lactose in red vinasse acid can be hydrolyzed into α-D-glucose and D-glucose-1-phosphate by pathways I and II. As shown in Table 2, β-galactosidase, galactokinase, and UDP- glucose-hexose-1-phosphate uridine invertase were annotated to 14, 11, and 9 species of microorganisms, respectively, and all the dominant bacteria were *Lactobacillus*.

Transformation of galactose

Galactose could transform D-glucose 1-phosphate under the catalysis of galactokinase (EC: 2. 7. 1. 6) and UDP-glucose-hexose-1-phosphate uridylyltransferase (EC: 2.7.7.12), and 20 and 17 coding genes were found and annotated for 11 and nine species of microorganisms, respectively. All the dominant bacteria were *Lactobacillus*. And red vinasse acid contains the PTS transport system of galactose, so galactose can be converted into D-glucose 1-phosphate.

Amino acid flavor formation pathway

The pathway of amino acid flavor formation means that amino acids are transformed into corresponding ketoacids by a transaminase. Which are unstable, converted into corresponding aldehydes, further dehydrogenated to alcohols, and then esterified to corresponding esters. These metabolites are important flavor components in food.

Transaminase

The transamination of branched-chain amino acids, aromatic amino acids, and methionine can be catalyzed by different transaminases. Branched-chain amino acid aminotransferase (BCAT) has activity for branched-chain amino acids and methionine, while aromatic-amino-acid transaminase (ArAT) has catalytic activity for aromatic amino acids, leucine, and methionine. As shown in Table 3, 32 BCAT genes (ilvE) were identified, and 15 kinds of microorganisms were annotated with red vinasse acid genes, and the dominant strain was *Aspergillus*. And 27 ArAT genes (tyrB, ARO8, and ARO93) were found, which were, respectively, annotated to seven, six, and two kinds of microorganisms. The dominant bacterial genera were *Burkholderia*, *Debaryomyces*, and *Millerozyma*, respectively. According to the findings, the key players in the fermentation of red vinasse acid were *Aspergillus*, *Burkholderia*, *Debaryomyces*, and *Millerozyma*, which catalyzed the synthesis of branched-chain amino acids, aromatic amino acids, and methionine. Figures 5, 6 respectively depict the metabolic and synthesis processes for aromatic amino acids and branched-chain amino acids.

Ketoacid invertase

Keto acids can be converted in three ways. The first way is that keto acids can be directly converted into carboxylic acids by oxidative decarboxylation. In this pathway, keto acid dehydrogenase (KaDH), phosphate acetyltransferase (PTA), and acetate kinase (ACK) showed activity on the matrix of branched-chain amino acids. As shown in Table 3, there are genes encoding PTA (27) and ACK (33) in the genomic DNA of red vinasse acid, which were annotated to 11 and 7 microorganisms, respectively, indicating that keto acids of the above amino acids can be converted into corresponding carboxylic acids in the process of red vinasse acid fermentation, and the dominant genus was *Lactobacillus*.

The second pathway is that keto acids are decomposed into carboxylic acids by carboxylic acid dehydrogenase (HycDH). HycDH has two stereospecific enzymes, D-carboxylate dehydrogenase (D-HycDH), and L-carboxylate dehydrogenase (L-HycDH). At present, although it cannot be proved that this approach can directly lead to the formation of flavored substances, it can reduce the synthesis of flavored substances by shunting the precursors of flavor substances into non-flavored products. These two carboxylic acid dehydrogenases were not found in the genomic DNA of red vinasse acid, so the overall flavor will not be reduced because of the above pathways.

The third way is the conversion of α-ketoacid to corresponding aldehydes by α-ketoacid decarboxylase (KdcA). The gene encoding keto acid decarboxylase was not found in red vinasse acid, which indicated that the microorganisms in red vinasse acid could not carry out the latter two forms of transformation, and the first keto-acid transformation was mainly used in the fermentation process, and *Lactobacillus* was the main function.

Alcohol and aldehyde dehydrogenase

Aldehyde dehydrogenase and alcohol dehydrogenase can catalyze the conversion of aldehydes (alcohols) into corresponding alcohols and carboxylic acids, and the control genes are aldehyde dehydrogenase (AldDH) and alcohol dehydrogenase (AlcDH), respectively. In the red vinasse acid gene, 83 genes encoding alcohol dehydrogenase (AlcDH) and 40 genes encoding aldehyde dehydrogenase (AldDH) were found. Alcohol dehydrogenase (AlcDH) includes adh, adh2, yiaY, AKR1A1, exaA, adhP, yqhD, and adhC. It is noted that the dominant genus is *Lactobacillus*, and the fungus *Millerozyma* also plays an important role. Aldehyde dehydrogenase (AldDH) includes adhE, feaB, mhpF, and bphJ,

TABLE 3 Genes of encoding enzymes involved in transamination pathway in red vinasse acid.

Abbreviation	Gene name	KO number	EC number	Gene number	Functional description	Annotated microorganisms
BcAT	*ilvE*	K00826	EC: 2.6.1.42	32	Branched-chain amino acid aminotransferase	*Aspergillus* (5), *Burkholderia* (4), *Lactobacillus* (3), *Cronobacter* (3), *Klebsiella* (3), *Acetobacter* (2), *Luteibacter* (2), *Chryseobacterium* (2), *Millerozyma* (2), *Phialosimplex*, *Stenotrophomonas*, *Staphylococcus*, *Pantoea*, *Franconibacter*, and *Acinetobacter*
ArAT	*tyrB*	K00832	EC: 2.6.1.57	17	Aromatic-amino-acid transaminase	*Burkholderia* (5), *Acinetobacter* (3), *Klebsiella* (3), *Unclassified* (2), *Luteibacter* (2), *Stenotrophomonas*, and *Cronobacter*
ArAT	*ARO8*	K00838	EC: 2.6.1.57 2.6.1.27 2.6.1.5	7	Aromatic amino acid aminotransferase I	*Debaryomyces* (2), *Clavispora*, *Wickerhamiella*, *Unclassified*, *Penicilliopsis*, and *Aspergillus*
ArAT	*ARO9*	K05821	EC: 2.6.1.58 2.6.1.28	3	Aromatic amino acid aminotransferase II	*Millerozyma* (2), *Kluyveromyces*
PTA	*pta*	K00625	EC: 2.3.1.8	14	Phosphate acetyltransferase	*Lactobacillus* (8), *Burkholderia* (3), *Pediococcus*, *Acetobacter*, and *Enhydrobacter*
PTA	*pta*	K13788	EC: 2.3.1.8	13	Phosphate acetyltransferase	*Unclassified* (6), *Acinetobacter* (3), *Pantoea*, *Klebsiella*, *Franconibacter*, and *Cronobacter*
ACK	*ackA*	K00925	EC: 2.7.2.1	33	Acetate kinase	*Lactobacillus* (16), *Burkholderia* (6), *Pediococcus* (3), *Unclassified* (2), *Paenibacillus*, *Acetobacter*, *Franconibacter*, *Acinetobacter*, *Aspergillus*, and *Penicillium*
AlcDH	*adh*	K00001	EC: 1.1.1.1	13	Alcohol dehydrogenase	*Lactobacillus* (7), *Burkholderia* (2), *Acinetobacter*, *Klebsiella*, *Enterobacter*, and *Pediococcus*
AlcDH	*adh2*	K18369	EC: 1.1.1.-	3	Alcohol dehydrogenase	*Burkholderia* (2), *Aspergillus*
AlcDH	*yiaY*	K13954	EC: 1.1.1.1	7	Alcohol dehydrogenase	*Cronobacter* (3), *Franconibacter* (2), *Lactobacillus*, and *Acinetobacter*
AlcDH	*AKR1A1*	K00002	EC: 1.1.1.2	3	Alcohol dehydrogenase (NADP+)	*Millerozyma* (3)
AlcDH	*exaA*	K00114	EC: 1.1.2.8	3	Alcohol dehydrogenase (cytochrome c)	*Burkholderia* (2), and *Acetobacter*
AlcDH	*adhP*	K13953	EC: 1.1.1.1	33	Alcohol dehydrogenase, propanol-preferring	*Lactobacillus* (14), *Millerozyma* (4), *Burkholderia* (3), *Pediococcus*, *Asticcacaulis*, *Rhizobium*, *Unclassified*, *Franconibacter*, *Klebsiella*, *Pantoea*, *Fonsecaea*, *Aspergillus*, *Monascus*, *Penicillium*, and *Byssochlamys*,
AlcDH	*yqhD*	K08325	EC: 1.1.-. -	8	NADP-dependent alcohol dehydrogenase	*Klebsiella* (3), *Cronobacter* (2), *Franconibacter* (2), and *Chryseobacterium*
AlcDH	*adhC*	K00121	EC: 1.1.1.284 1.1.1.1	13	S-(hydroxymethyl) glutathione dehydrogenase / alcohol dehydrogenase	*Acetobacter* (2), *Franconibacter* (2), *Unclassified* (2), *Paenibacillus*, *Burkholderia*, *Pantoea*, *Luteibacter*, *Stenotrophomonas*, *Byssochlamys*, and *Millerozyma*,
AldDH	*adhE*	K04072	EC: 1.2.1.10 1.1.1.1	23	Acetaldehyde dehydrogenase / alcohol dehydrogenase	*Lactobacillus* (10), *Franconibacter* (5), *Unclassified* (3), *Pantoea* (2), *Paenibacillus* (2), and *Cronobacter*
AldDH	*feaB*	K00146	EC: 1.2.1.39	9	Phenylacetaldehyde dehydrogenase	*Burkholderia* (3), *Klebsiella* (3), *Unclassified* (2), and *Acinetobacter*
AldDH	*mhpF*	K04073	EC: 1.2.1.10	6	Acetaldehyde dehydrogenase	*Burkholderia* (3), *Unclassified*, *Escherichia*, and *Klebsiella*
AldDH	*bphJ*	K18366	EC: 1.2.1.10 1.2.1.87	2	Acetaldehyde/propanal dehydrogenase	*Burkholderia* (2)

(Continued)

TABLE 3 (Continued)

Abbreviation	Gene name	KO number	EC number	Gene number	Functional description	Annotated microorganisms
EstA	*aes*	K01066	EC: 3.1.1.-	27	Acetyl esterase	*Burkholderia* (*6*), *Klebsiella* (*4*), *Unclassified* (*4*), *Lactobacillus* (*3*), *Citrobacter* (*3*), *Franconibacter* (*2*), *Chryseobacterium*, *Paenibacillus*, *Acetobacter*, *Enterobacter*, and *Acinetobacter*

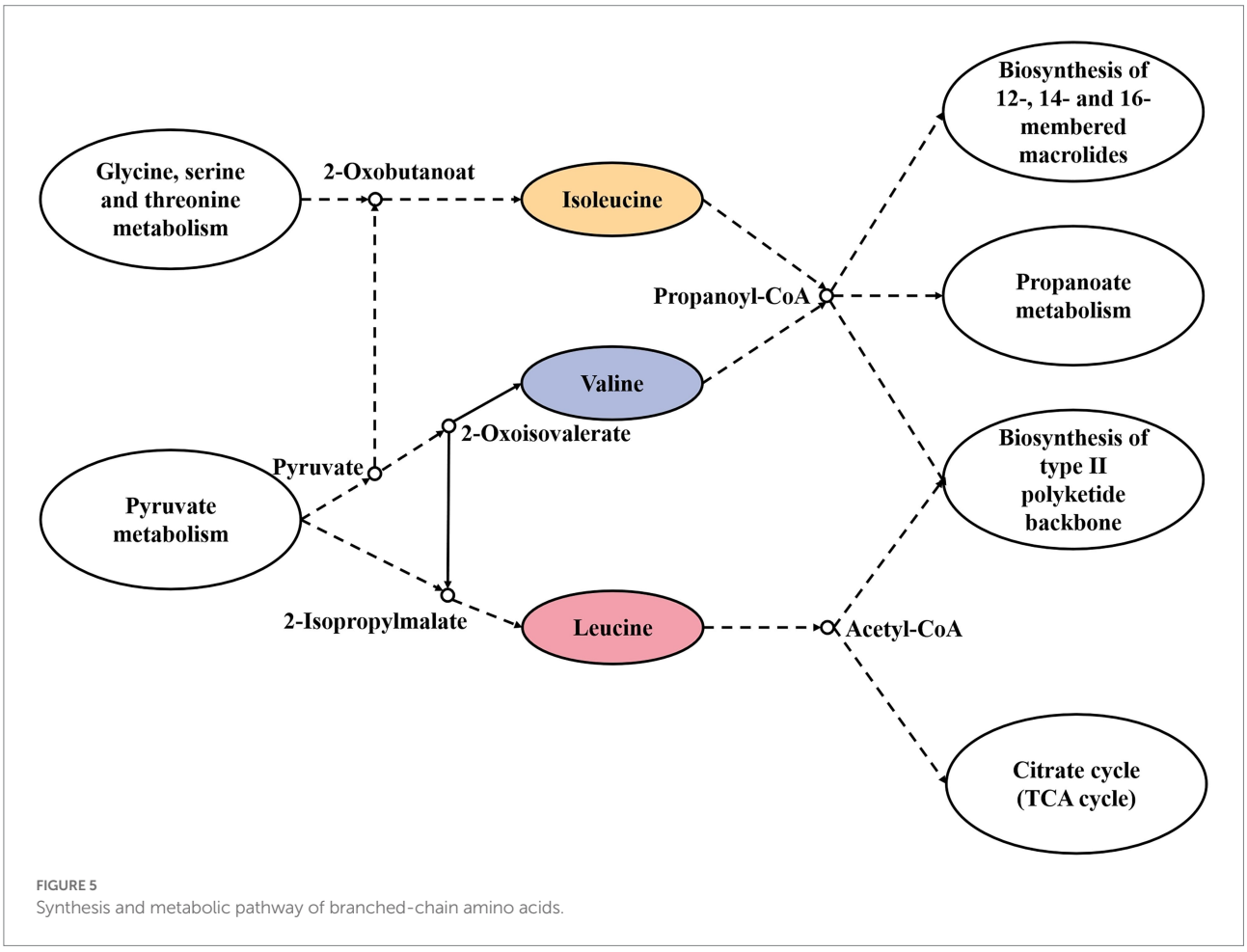

FIGURE 5
Synthesis and metabolic pathway of branched-chain amino acids.

and the dominant genus is *Lactobacillus*. Therefore, in the process of catalyzing aldehydes (alcohols) into corresponding alcohols and carboxylic acids, *Lactobacillus* was the main microorganism.

Discussion

Red vinasse acid has a rich aroma and unique flavor, which is closely related to its complex microbial metabolic activities. It has been reported that in fermented food, the different dynamic patterns of free sugars and amino acids imply different co-metabolisms, for example, free sugars might derive from polysaccharides through hydrolysis and be consumed by lactic acid bacteria, whereas amino acids produced through proteolysis of proteins are consumed by fungi (Mouritsen et al., 2017).

In this study, macrogenomic analysis revealed the microbial diversity and functional properties of the microbial community. We annotated the species abundance of the red vinasse acid gene, and the dominant genera included *Lactobacillus*, *Pediococcus*, *Aspergillus*, *Burkholderia*, *Klebsiella*, *Monascus*, and *Penicillium* (Figure 1). *Lactobacillus* can produce lactic acid and a variety of antibacterial substances. *Lactobacillus* intracellular carbohydrate active enzymes are specific (Swanson et al., 2020). The amount of lactic acid produced during fermentation has an impact on the quality of the fermentation. In addition to improving the nutritional value and quality of food, *Pediococcus* also has great

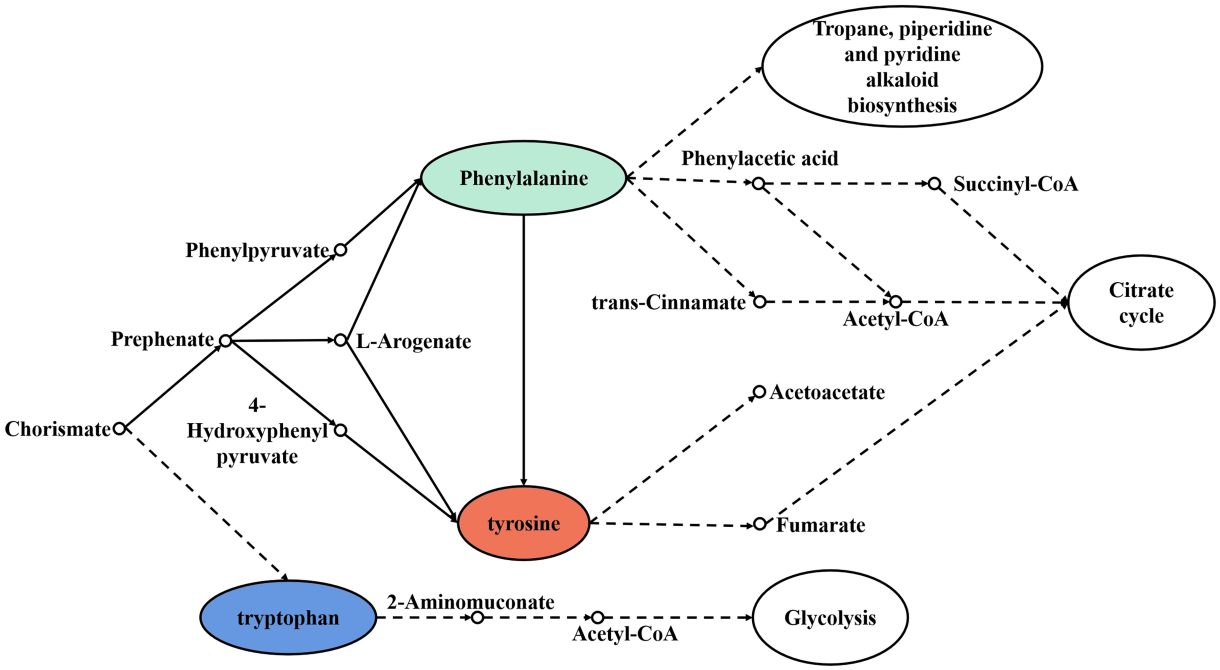

FIGURE 6
Synthesis and metabolic pathway of aromatic amino acids.

resistance to acid and high temperatures. Additionally, *Pediococcus* displays biological properties that fight cancer and lower cholesterol (Zhang et al., 2019). *Monascus* is the main beneficial fungus of red vinasse acid fermentation, which gives red vinasse acid pigment and a unique flavor. Due to the lack of sterilization technology in the production of traditional red vinasse acid, the community information of microorganisms in red vinasse acid is complex and diverse, so in addition to beneficial microbes such as *Lactobacillus and Monascus*, there are still a small number of spoilage microbes and detrimental microbes in the red vinasse acid, such as *Burkholderia* and *Klebsiella*. The comparative analyses that can be performed are limited by the relatively few reports on the microbial diversity of red vinasse acid at the species level, and our macrogenomic analysis of red vinasse acid has enriched the information on the specific category information of microbial diversity of red vinasse acid. Compared with the eggNOG and KEGG databases, 16,093 and 49,652 genes were annotated, respectively. The main metabolic activities were amino acid metabolism and carbohydrate metabolism (Figures 2, 3). Glycosyl transferases and glycoside hydrolases were the most abundant in the CAZy database analysis (Figure 4). Glycoside hydrolases hydrolyze the glycosidic bonds of various sugar-containing compounds such as oligosaccharides and polysaccharides by internal or external digestion and generate monosaccharides, oligosaccharides, or sugar complexes, thus playing an important role in the synthesis of oligosaccharides and aromatic glycosides, and the glycosylation of amino acids and peptides. Glycosyl transferases catalyze the binding of activated sugars to non-receptor molecules, such as proteins, oligosaccharides, lipids, and small molecules (Chen et al., 2013; Cai et al., 2018; Huang et al., 2018). The abundant glycoside hydrolase and glycosyl transferase in red vinasse acid provide the basis for the formation, transfer, and further metabolism of monosaccharides and oligosaccharides.

In the pathway of glucose metabolism, red vinasse acid encodes the sugar transport system genes of mannitol, fructose, mannose, and sucrose, and retrieves the genes of gluconate and oligosaccharide related permeability proteins (Table 1). It also has the key enzyme genes that catalyze intracellular D-mannitol-1-phosphate, D-fructose-1-phosphate, D-mannose-6-phosphate, sucrose-6-phosphate, gluconate, maltose, lactose, and galactose, and has the basis for transforming them into glycolysis intermediates, and the main dominant bacterial genus of the glucose metabolism pathway is *Lactobacillus* (Table 2). *Lactobacillus* uses the relevant transport mechanism to transport various sugar substances from outside the cell to the cell, and then transformed into intermediate products of the glycolytic pathway through the catalysis of various related enzymes. These substances enter the glycolytic pathway and finally produce pyruvate, the precursor substance of lactic acid, through the catalysis of related enzymes. Pyruvate can be catalyzed by L-lactate dehydrogenase or D-lactate dehydrogenase to ultimately produce L-lactate or D- lactate (Kleerebezemab et al., 2000; Wang et al., 2010), resulting in a unique flavor.

In the process of amino acid flavor formation, the control genes of 32 branched-chain amino acid aminotransferase (BCAT), 27 aromatic-amino-acid transaminases (ArAT), 60 ketoacid invertase, 123 alcohol/aldehyde dehydrogenases, and 27 acetyl esterases were encoded by red vinasse acid (Table 3). Amino acids can be transformed into corresponding keto acids by transaminase, and then into corresponding aldehydes, further

dehydrogenation to alcohols, and then esterification to corresponding esters, which have the basis for the formation of strong ester flavors, and the main dominant bacteria genus during amino acid flavor formation is *Lactobacillus*. Figure 5 shows the synthesis and metabolic pathway of branched-chain amino acids (Leucine, Valine, and Isoleucine), which is a crosstalk and a simplified process of the KEGG metabolic pathway ko00290 and ko00280. The solid line in the diagram represents the direct process and the dashed line represents the indirect process. The degradation of Valine and Isoleucine to Propanoyl-CoA is catalyzed by a series of enzymes, and Propanoyl-CoA then indirectly enters the following three metabolic pathways: Biosynthesis of 12-, 14-, and 16-membered macrolides, Propanoate metabolism, and Biosynthesis of type II polyketide backbone. Leucine is metabolized to Acetyl-CoA by a series of enzymes, and Acetyl-CoA enters indirectly into the Biosynthesis of type II polyketide backbone and the Citrate cycle (TCA cycle), which further functions in two metabolic pathways. Figure 6 shows the synthesis and metabolic pathway of aromatic amino acids (Phenylalanine, Tyrosine, and Tryptophan), which is a crosstalk and a simplified process of the KEGG metabolic pathway ko00400, ko00360, ko00350, and ko00380. Tryptophan is metabolically involved in the glycolytic process, Tyrosine in the tricarboxylic acid cycle, and Phenylalanine in the Tropane, piperidine, and pyridine alkaloid biosynthesis and Citrate cycle. We show the synthesis and metabolic pathways of branched-chain amino acids and aromatic amino acids in this study in the form of pictures, which are simplified to mainly describe the relationship between metabolic pathways and amino acids compared to the complete metabolic pathways in KEGG, in order to highlight the wide range and importance of amino acids in the process of biological activities, as well as to provide a targeted direction for flavor studies in amino acid metabolism.

The primary metabolic functions of red vinasse acid and the essential enzyme genes for sugar and amino acid metabolism were further discovered based on an examination of the structure and makeup of red vinasse acid bacteria. Analysis of dominant strain roles in the major metabolic pathways and further confirmation of the expression of important enzyme genes. It is anticipated that it will offer a theoretical foundation for the enhancement of red vinasse acid quality and the mining of microbial functional gene pools.

Data availability statement

The data presented in the study are deposited in the National Center for Biotechnology Information repository, accession numbers are SRR21707708, SRR21707707, SRR21707706, SRR21707705, SRR21707704, SRR21707703.

Author contributions

JiL, YY, and YZ contributed to conception and design of the study. JiL and YY organized the database. JiL wrote the first draft of the manuscript. YZ performed the statistical analysis. WL, MC, and HM wrote sections of the manuscript. AG and JuL contributed to funding acquisition, resources, supervision, and writing–review and editing. All authors contributed to the article and approved the submitted version.

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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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Two sides of the same coin: Meta-analysis uncovered the potential benefits and risks of traditional fermented foods at a large geographical scale

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Traditional fermented foods, which are well-known microbial resources, are also bright national cultural inheritances. Recently, traditional fermented foods have received great attention due to their potential probiotic properties. Based on shotgun metagenomic sequencing data, we analyzed the microbial diversity, taxonomic composition, metabolic pathways, and the potential benefits and risks of fermented foods through a meta-analysis including 179 selected samples, as well as our own sequencing data collected from Hainan Province, China. As expected, raw materials, regions (differentiated by climatic zones), and substrates were the main driving forces for the microbial diversity and taxonomic composition of traditional fermented foods. Interestingly, a higher content of beneficial bacteria but a low biomass of opportunistic pathogens and antibiotic resistance genes were observed in the fermented dairy products, indicating that fermented dairy products are the most beneficial and reliable fermented foods. In contrast, despite the high microbial diversity found in the fermented soy products, their consumption risk was still high due to the enrichment of opportunistic pathogens and transferable antibiotic resistance genes. Overall, we provided the most comprehensive assessment of the microbiome of fermented food to date and generated a new view of its potential benefits and risks related to human health.

KEYWORDS

traditional fermented foods, shotgun metagenomics, beneficial microorganism, opportunistic pathogen, antibiotic resistance gene

Introduction

Fermentation is one of the oldest and most economical methods for food storage and processing in the world. It has been widely used to improve food safety, shelf life, and sensory and nutritional properties (Gao et al., 2021). The International Scientific Association of Probiotics and Prebiotics defines fermented foods as "foods made through desired microbial growth and enzymatic conversions of food components" (Marco et al., 2021). Since the Neolithic Age, fermented foods have been produced and consumed as an integral part of the human diet and cultural traditions (Liu et al., 2018; Anyogu et al., 2021). Fermented foods have become very popular in recent years, mainly because they are considered to be beneficial to health. Studies have found that fermented foods have beneficial effects on human health by promoting antioxidation processes, lowering blood lipids, improving immunity, inhibiting tumors, delaying aging, and preventing gastrointestinal diseases (Marco et al., 2017; Melini et al., 2019; Şanlier et al., 2019). However, in most natural fermentation processes, the microbial community is complex, and the fermentation process is difficult to control, which brings many hidden dangers to fermented foods (Anal, 2019). Food safety incidents caused by the consumption of fermented foods occur from time to time, which is not only unfavorable to the development of the fermented food industry but also threatens the safety of consumers (Anal et al., 2020; Wang et al., 2021). Although some literature has reported the health benefits or safety risks of fermented foods, there has been little comprehensive analysis of the potential health benefits and safety risks of fermented foods (Anal, 2019; Dimidi et al., 2019; Şanlier et al., 2019).

Traditional fermented foods usually depend on natural wild colony fermentation as the main production mode. In fermentation, a variety of microorganisms from raw materials and the environment are usually introduced. This process can involve the use of nutrients in the raw materials to change the texture and edible quality of the food through metabolic pathways and produce a unique fermentation fragrance (Kim et al., 2016; Hwang et al., 2017; Licandro et al., 2020). The microbial ecosystem of fermented foods is an important symbol of its typicality, and it is also the decisive factor in the assessment of the function and risk of fermented foods (Tamang et al., 2016). On the one hand, fermented foods contain microorganisms, which are beneficial to consumption. Some microbes in fermented foods can reach the gastrointestinal tract. Although they do not stay in the intestinal tract for a long time, it is sufficient to enable physiological benefits such as the inhibition of intestinal pathogens and the mediation of epithelial regulation and immune regulation in the intestinal tract (Lebeer et al., 2008; David et al., 2014; Dimidi et al., 2019). On the other hand, the natural fermentation process is often accompanied by the introduction of spoilage bacteria or pathogenic bacteria, which will seriously affect the sensory quality, flavor, and edible safety of traditional fermented foods (Anal et al., 2020; Wang et al., 2021). Therefore, it is necessary and urgent to deeply understand the complex microbiome of traditional fermented foods.

Next-generation sequencing technology has completely changed microbial research, including that regarding the microbial community in food, by enabling high-throughput gene analysis of mixed microbial communities (Consortium, 2012; Fierer et al., 2012). Metagenomics, including two different culture-independent sequencing methods, namely, amplicon sequencing, and shotgun genomics, involves the analysis of genomic deoxyribonucleic acid (DNA) isolated from the whole microbial community (Ranjan et al., 2016). To date, most studies related to fermented foods have employed amplicon sequencing to study the composition of bacteria and fungi, which is usually limited by the identification of the genus level (Leech et al., 2020). Shotgun metagenomics has higher resolution, but due to the high sequencing cost, the application of this method in traditional fermented food microbiology research is still lacking.

Here, based on the shotgun metagenome approach, we explored the microbiome of different traditional fermented foods at the species level; revealed the microbial diversity, taxonomic composition, and metabolic pathways of different fermented foods; and comprehensively analyzed the potential benefits and safety risks of the different fermented foods to human health. We hope to provide a scientific basis for consumers to know more about fermented food and provide direction for the development of the traditional fermented food industry.

Materials and methods

Sample collection

A detailed flowchart of the sample collection process is shown in **Figure 1**. The PubMed/Medline and Web of Science bibliometrics databases were used to systematically search peer-reviewed literature, and the retrieved full text and other reviewed references were used for manual search. We used the following keywords in our search: ("fermented food") AND (metagenomic OR metagenome OR microbiota OR shotgun). We found 529 references, 463 of which were retained after deduplication; 422 of these were then excluded from the review of the title and abstracts. Of the 41 reserved articles, 29 were excluded because of the use of 16S gene sequencing technology. Finally, excluding the 12 samples whose data could not be obtained in the NCBI databases, 12 references (including 161 samples) were found to have used shotgun

metagenomic sequencing technology to study the microbiome of fermented food. In addition, we also collected 30 samples of traditional fermented foods from Wenchang, Qionghai, and Lingshui Li Autonomous Region in Hainan Province. The metadata for the samples are presented in **Supplementary Table 1**.

Deoxyribonucleic acid extraction and shotgun metagenomic sequencing

Samples were stored at −20°C until DNA extraction. After homogeneously mixing 10 g of the fermented food sample with 90 mL of sterile NaCl solution (0.85%, w/v), total DNA was extracted from the diluent of the mixture by a QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the instruction manual. After detection for integrity and purity, the shotgun metagenome sequencing was carried out by an Illumina HiSeq 2500 instrument (Novogene, Beijing, China). The whole metagenome library preparation and sequencing process used services from Beijing Novogene Technology Co., Ltd.

Data processing and statistical analysis

MetaPhlan 2 software was used to annotate and classify metagenomic species, and HumanN 2 was used to annotate metagenomic metabolic pathways. By comparing the microbial sequences with the CARD database, the abundance of microbial antibiotic resistance genes (ARGs) was obtained (Hitch et al., 2018). Statistical analysis was performed with the Wilcoxon rank-sum test and Kruskal–Wallis test using R and GraphPad. A p-value < 0.05 was considered to be significantly different.

Results

Literature search and characteristics of included samples

The metadata of the samples are listed in **Supplementary Table 1**. A total of 179 samples were included in this study, of which 149 samples were taken from 12 published articles (Walsh et al., 2018; Kumar et al., 2019; Sirén et al., 2019; Chacón-Vargas et al., 2020; Leech et al., 2020; Li et al., 2020; Yulandi et al., 2020; de C. Lima et al., 2021; Hu et al., 2021; Liu et al., 2021; Zhang et al., 2021; Tamang et al., 2022) and 30 samples were taken from Hainan Province and sequenced. The samples were classified according to the fermentation raw materials, fermentation substrates, and climatic zone in different regions.

The microbial diversity of fermented foods classified by different raw materials, substrates, and climatic conditions was different

The Shannon and Simpson indices were calculated based on relative abundance at the species level to compare microbial alpha diversity in fermented food samples from different raw materials, substrates, and climatic zones. Principal coordinate analysis (PCoA) based on Bray–Curtis distance was used to compare β diversity. The results showed that there were significant differences ($p < 0.05$) in the microbial α diversity of fermented foods with different raw materials (**Figure 2A**). The fermented food with dairy and seafood as raw materials had low alpha diversity, while the fermented food with nuts and vegetables as raw materials had high alpha diversity (**Figure 2A**). The clusters of points representing fermented foods with different raw materials were obviously different in PCoA, which indicates that there were obvious differences in their species structure (**Figure 2B**). Consistently, fermented foods with dairy and marine protein as substrates had low microbial alpha diversity (**Figure 2C**), and fermented foods with different substrates had significantly different microbial beta diversity ($p < 0.05$) (**Figure 2D**). In addition, we found that the α diversity of fermented foods in semitropical regions was significantly higher than that in tropical regions (**Figure 2E**), and β diversity showed aggregation in different climatic zones (**Figure 2F**).

Fermented foods with different substrates differ greatly in microbial composition

To enable the study of more fermented foods, we selected fermented foods with different substrates for more in-depth analysis. Based on the MetaPhlan2 annotation results, as shown in **Figures 3A,B**, we displayed the top ten bacteria with the highest average relative abundance of microbes at the genus level and species level in different substrate fermented foods and defined these bacteria as core microbes. In addition, the heatmap demonstrated differential microbes with significant differences in relative abundance among the different types of fermented foods (kw test, $p < 0.05$) and mean relative abundance greater than 0.05% (**Figures 4A,B**). We found that the types of core microbes in fermented foods from different substrates were quite different, both at the genus level and the species level. In addition, some fermented foods had characteristic species, i.e., the core species in a certain substrate fermented food, that were not present in other types of fermented foods. The bacteria marked with a black box in **Figure 3B** were the characteristic bacteria of various fermented food substrates.

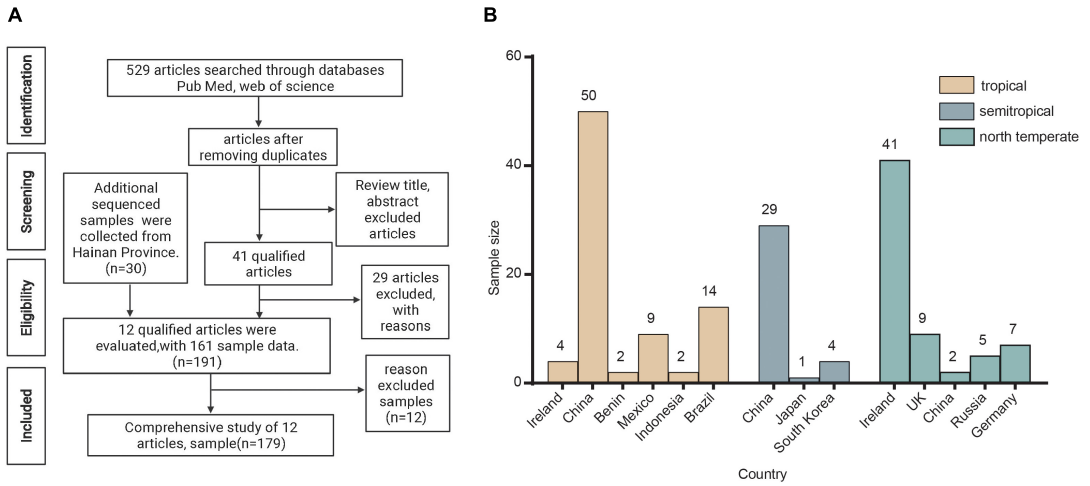

FIGURE 1
Acquisition of samples. **(A)** Flowchart for the identification of studies and samples. **(B)** Geographical distribution of samples.

Analysis of correlation between core microbes and key metabolic pathways

To better understand the important roles of microbes in fermented foods, we investigated the associations of core microbes with key metabolic pathways (the top 20 metabolic pathways in relative abundance) in different substrate fermented foods by calculating the Spearman rank correlation coefficient. Network diagrams with correlation coefficients r greater than 0.4 between core microbes and metabolic pathways of various basal fermented foods are shown in **Figure 5**. The key metabolic pathways of different substrate-fermented foods were significantly different ($p < 0.05$) (**Supplementary Table 2**). The results showed that metabolic pathways related to cellular structure, replication, and translation were prevalent in fermented foods.

Potential benefits and risk analysis of fermented food

To comprehensively analyze the potential benefits and risks of fermented foods to human health, we assessed the beneficial microbes and opportunistic pathogens in fermented foods through extensive literature searches and analyzed the resistance genes of microbes (**Figure 6**). As shown in **Figure 6B**, dairy fermented food had the highest abundance of beneficial bacteria, reaching 83.41%, followed by brine (63.31%), and the lowest abundance of beneficial bacteria was found in fermented soy products (25.5%). The relative abundance of opportunistic pathogens was the lowest in marine protein base fermented foods (10.08%), while the most opportunistic pathogens were found in fermented soy products (35.97%), which exceeded the number of beneficial bacteria in fermented soy products. The compositions of various beneficial bacteria and opportunistic pathogens in different fermented foods are shown in **Figures 6A,C**, respectively. We found that *Leuconostoc mesenteroides* widely existed in other kinds of fermented foods, except fermented soy products. *Lactococcus lactis* was the beneficial bacterium in fermented bean products, and its number accounted for more than 70% of the beneficial microbes in this kind of fermented food. The main opportunistic pathogen in fermented dairy products was *Bacillus amyloliquefaciens*, and *Streptococcus infantarius* was the most important opportunistic pathogen in fermented foods with marine protein. In addition, microbes in fermented soy products had the most types of resistance genes (**Figure 6D**), and the number of antibiotic resistance gene (ARG) reads was the highest (**Figure 6E**). The main resistance genes in fermented soy products were lincosamide antibiotic, tetracycline antibiotic, glycylcycline, nitrofuran antibiotic, diaminopyrimidine antibiotic, fluoroquinolone antibiotic, and tetracycline antibiotic resistance genes (**Figure 6F**). The major resistance genes in other fermented foods were shown in **Supplementary Figure 1**.

Discussion

Food fermentation is a valuable global cultural heritage, a rich and valuable microbial resource, but it is also the key to the economy of many regions (Chen et al., 2017). Exploring the microbiome in fermented foods is beneficial to gain a deep understanding of the commonalities and differences of different fermented foods, to better utilize the beneficial properties and to provide theoretical and data foundations for their effects on

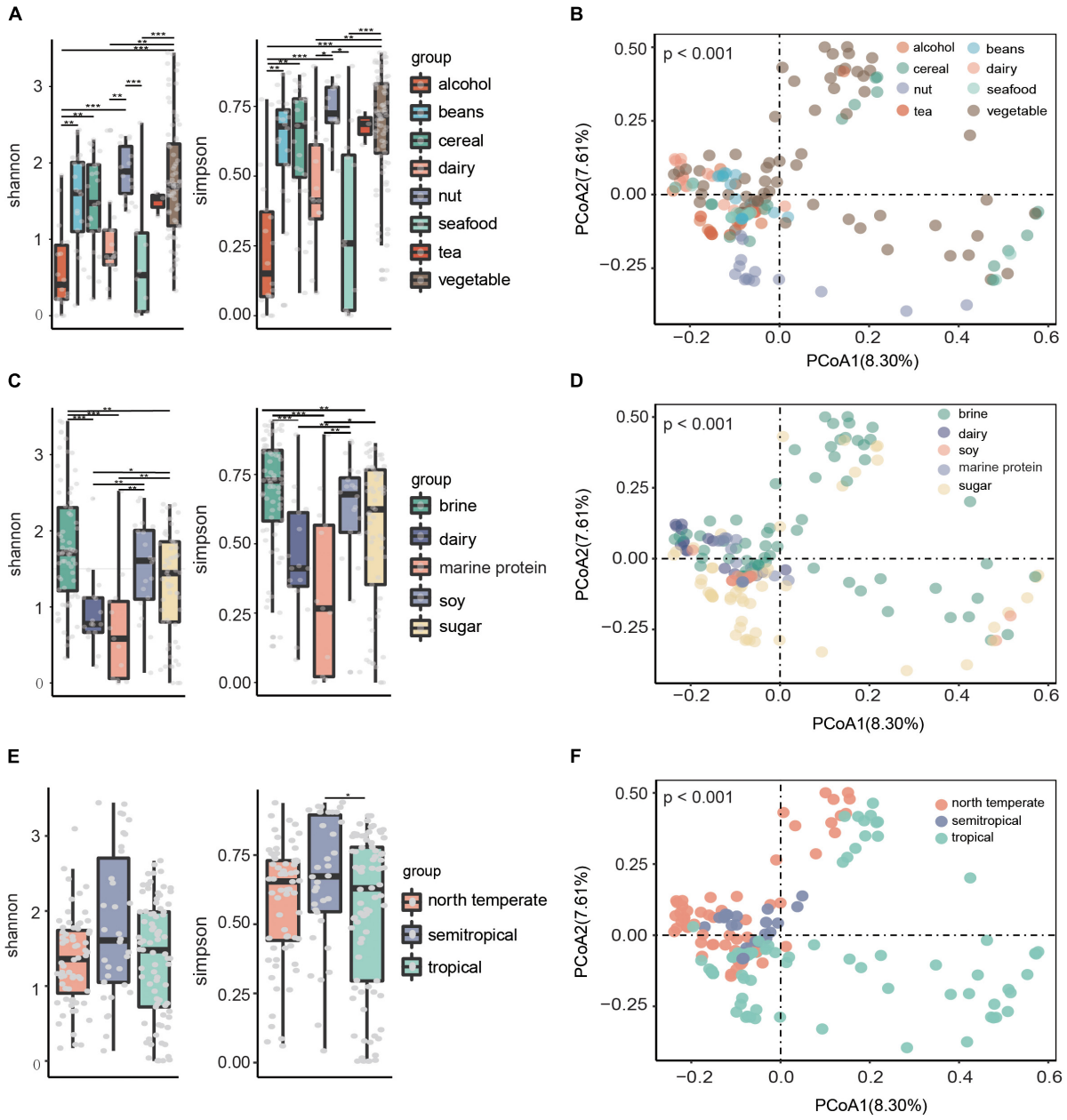

FIGURE 2
Microbial diversity of different fermented foods. **(A)** Microbial alpha diversity in fermented foods with different raw materials. **(B)** Principal component analysis (PCoA) score plot based on Bray–Curtis distance for all samples in fermented food with different raw materials. **(C)** Microbial alpha diversity in fermented foods with different substrates. **(D)** PCoA score plot in fermented food with different raw materials. **(E)** Microbial alpha diversity of fermented foods in different climatic zones. **(F)** PCoA score plot in fermented food with different raw materials. Each point represents the composition of the microbiota of a sample.

human health (Leech et al., 2020; Zhang et al., 2021). The high taxonomic resolution of shotgun metagenome analysis has led to more in-depth microbiome identification of fermented foods, which helps us better understand the properties and functions of different fermented foods.

Environmental heterogeneity has always been considered one of the reasons for the formation of microbial diversity patterns (Yang and Wu, 2020). The non-random distribution of microorganisms is affected by environmental factors and is also related to geographical distance (Xia et al., 2016; Zeng et al., 2019). The regional trend found in our study was based on large-scale data covering tropical, subtropical, and north-temperate regions. The result indicated that optimum conditions for the highest bacterial diversity

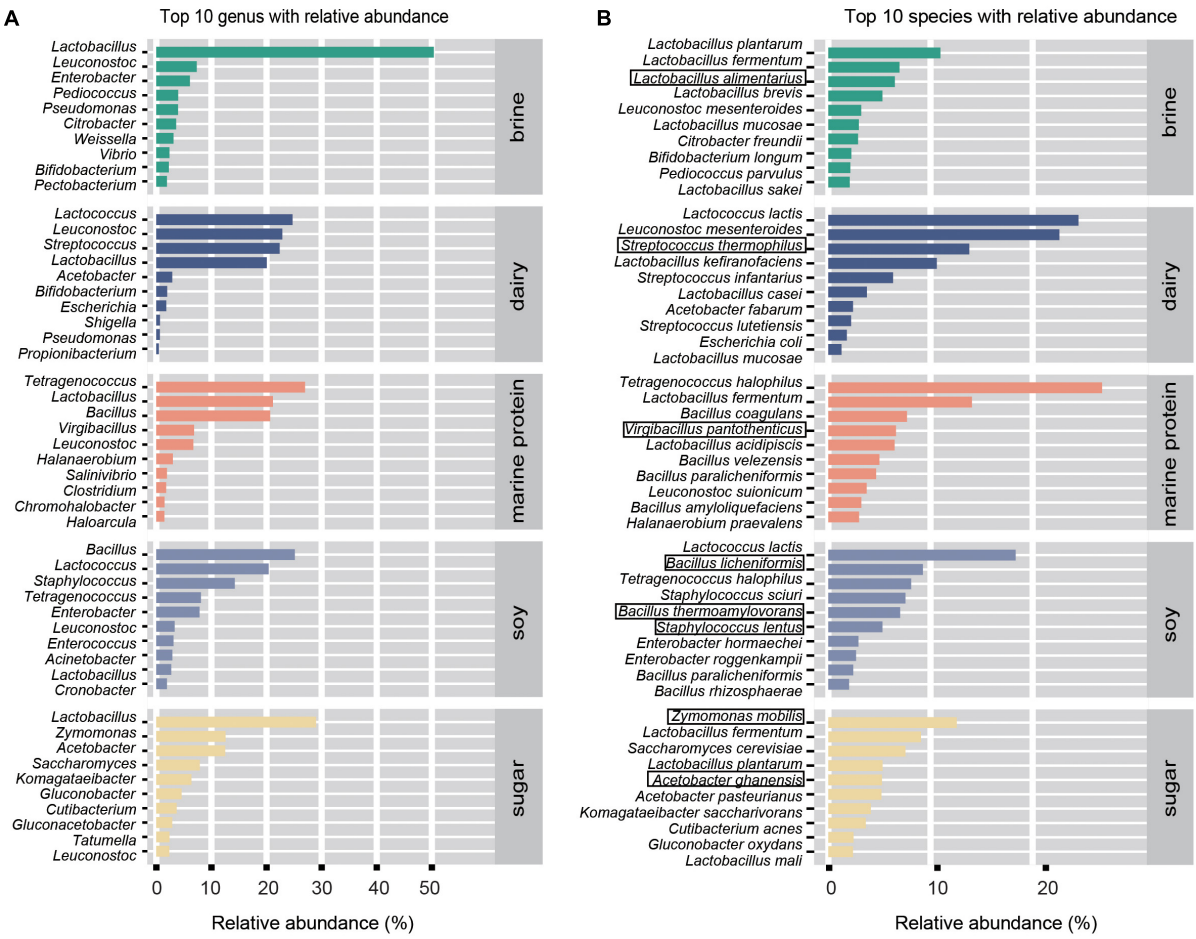

FIGURE 3
Main (core) microbial composition of fermented foods with different substrates. **(A)** Top 10 genera by relative abundance. **(B)** Top 10 species by relative abundance. The bacteria marked by black boxes are the characteristic bacteria.

in our studied areas were located at the semitropical zone (**Figures 2E,F**). Fermentation raw materials and fermentation substrates are important driving factors affecting the microbial composition and metabolic function of fermented foods, and the microbial diversity of fermented foods is largely determined by the types of nutrients available to microbes (Leech et al., 2020; Moonga et al., 2021). Consistently, significant differences in the microbial diversity of fermented foods with different raw materials and substrates were found in our study (**Figure 2**). Furthermore, more than 5,000 fermented foods are consumed around the world (Leech et al., 2020). To make the results of our study more likely to be applied to more fermented foods, we conducted an in-depth analysis of the microbiome of fermented foods under different substrate categories. We found that metabolic pathways related to cellular structure formation, replication, and translation were enriched in all types of fermented foods, implying that microbial metabolism in fermented foods was robust (**Figure 5**). Notably, *Lactococcus lactis* and *Lactobacillus casei*, as core microbes in fermented dairy products, were positively associated with the lactose and galactose catabolism pathways, which might explain the response of people with lactose intolerance to fermented dairy products (**Figure 5B**).

Fermented foods have become the focus of increasing interest as a range of foods claimed to have health benefits. The potential health benefits of fermented foods include a reduced risk of high blood pressure, diabetes, obesity, high cholesterol, diarrhea, blood clots, and more (Marco et al., 2017; Melini et al., 2019). One explanation for the health benefits of fermented foods is related to the beneficial microbes in fermented foods (Orisakwe et al., 2020). In fact, it has been proposed that fermented foods provide a means of safe microbial exposure to compensate for the absence/removal of desirable host microbes to address the "industrialization" of the human microbiota (Chilton et al., 2015; Sonnenburg and Sonnenburg, 2019). *Lactic acid bacteria* (LAB) are an important class of probiotics in the

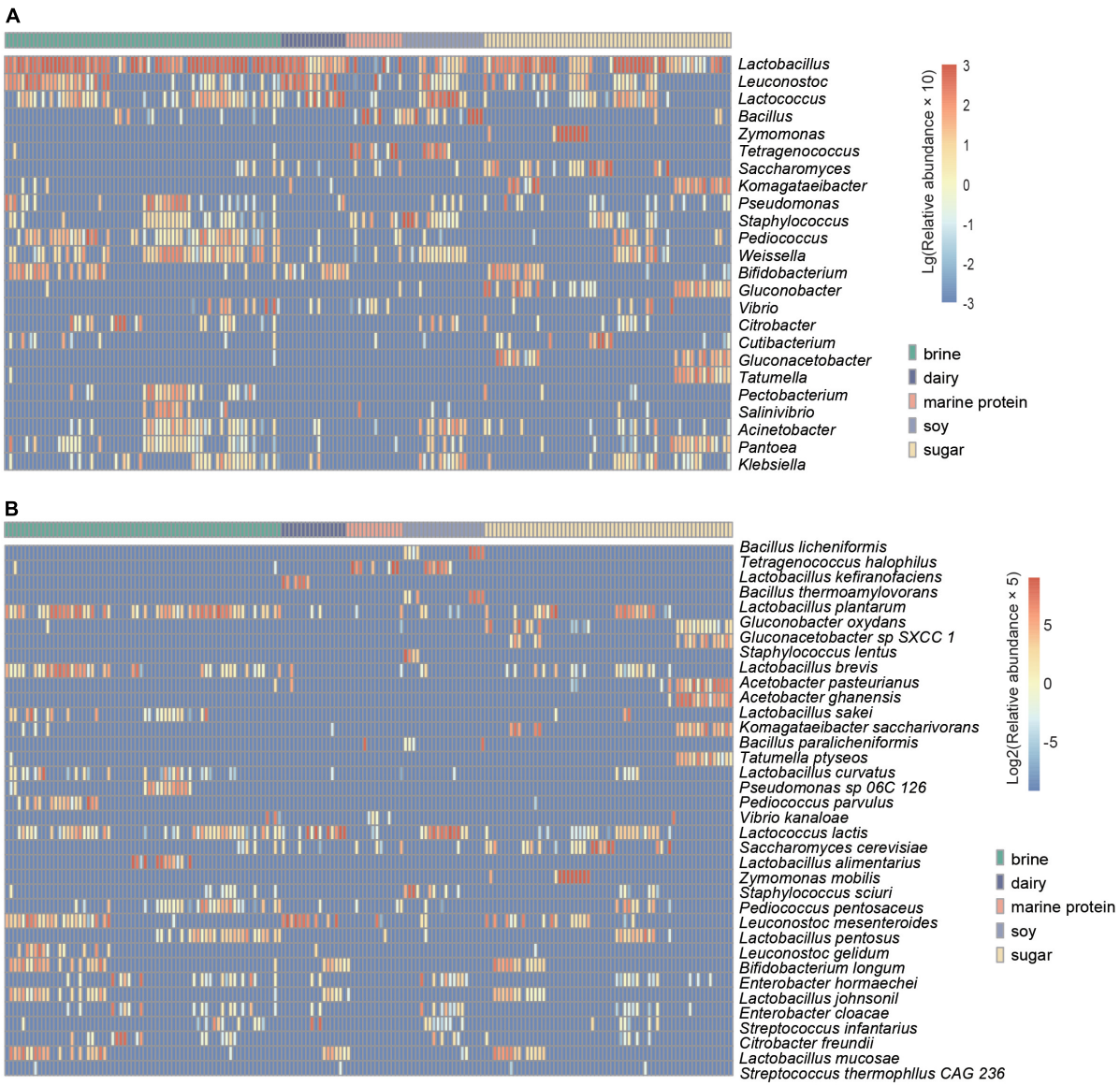

FIGURE 4
Heatmap showing the distribution of the core differential microbes. **(A)** Variations in significantly differentially abundant genera in different substrate-fermented foods screened by Wilcoxon rank-sum test. **(B)** Variations in significantly differentially abundant species in different fermented food substrates screened by the Wilcoxon rank-sum test. The depth degree of color represents the relative abundance (blue indicates a small number, and red indicates a large number).

human body, with immunomodulatory, anti-inflammatory, antioxidative, and antiproliferative functions (Matsuzaki et al., 2017; Xie et al., 2019). LAB can survive and multiply in the human intestinal tract, and their growth and metabolism processes not only maintain their own needs but also help to maintain the health of the host organism (Zhang et al., 2019). *Lactococcus lactis* is the most representative strain of lactic acid bacteria, which is considered a safe microorganism by the US Food and Drug Administration and is commonly used in food fermentation, drug production, and feed addition (Mao et al., 2016). Agarwal and Bhasin (2002) found that *Leuconostoc mesenteroides* was beneficial for shortening the duration of diarrhea. *Lactobacillus fermentum* has a clear role in improving liver steatosis, which has been confirmed in some studies (Rivero-Gutiérrez et al., 2017; Nan et al., 2018). As one of the natural flora of the human digestive tract, *Lactobacillus plantarum* has many probiotic functions, including improving intestinal flora, reducing serum cholesterol content, enhancing immunity, discharging heavy metals, and promoting nutrient absorption (Siezen and van Hylckama Vlieg, 2011). In our study, we found that beneficial bacterial resources were abundant in fermented foods and that LAB

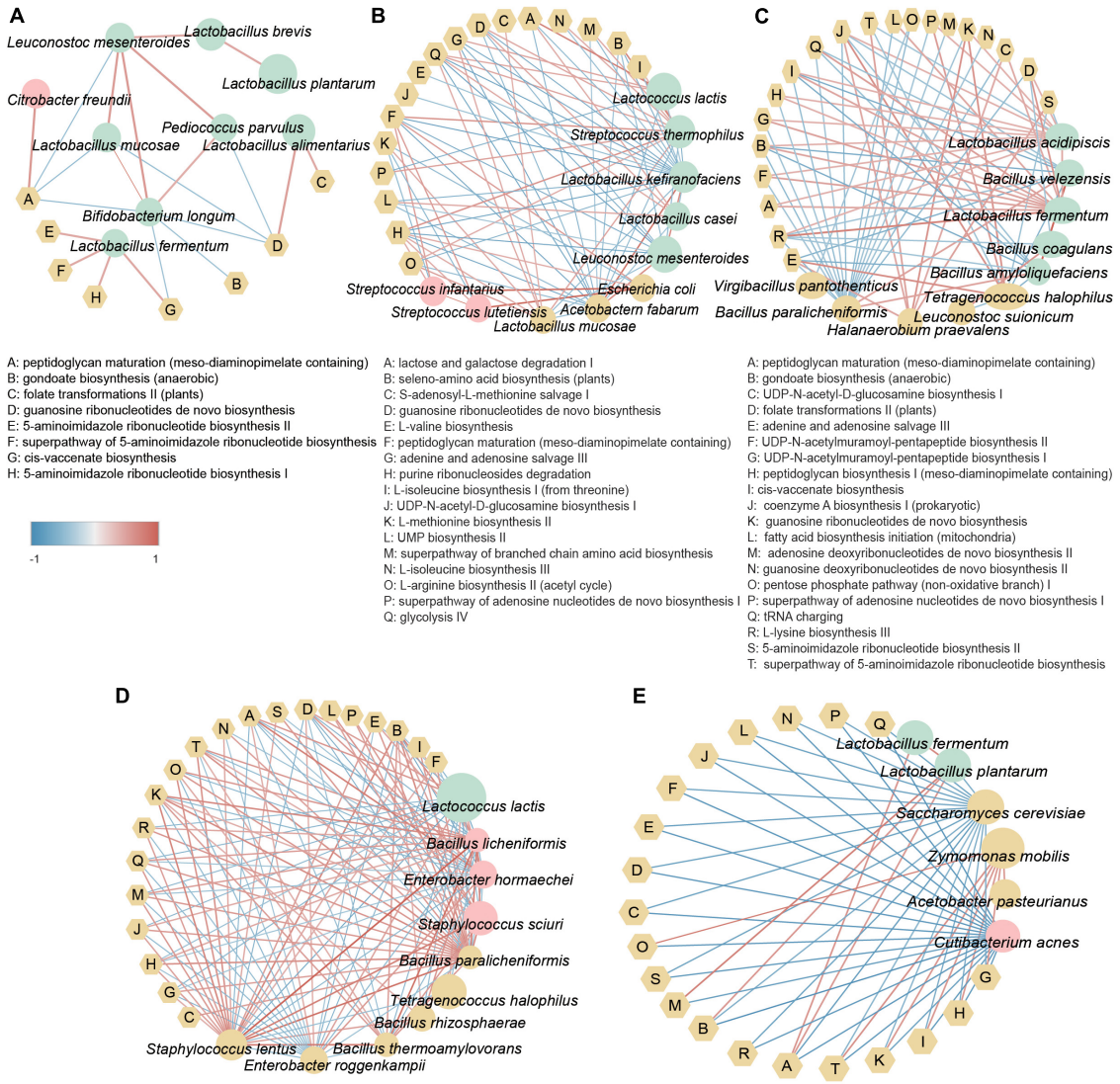

FIGURE 5

Analysis of correlation between core bacterial species and key metabolic pathways in fermented foods with **(A)** brine, **(B)** dairy, **(C)** marine protein, **(D)** soy, and **(E)** sugar. Correlation analysis was performed using the Spearman method. Data with an absolute value of r greater than 0.4 were selected. The edge width and color (red: positive; blue: negative) are proportional to the correlation strength. The size of that node is directly proportional to the average abundance of the respective population. Pink nodes represent opportunistic pathogens, green nodes represent beneficial bacteria, yellow round nodes represent other types of bacteria, and yellow hexagonal nodes represent metabolic pathways.

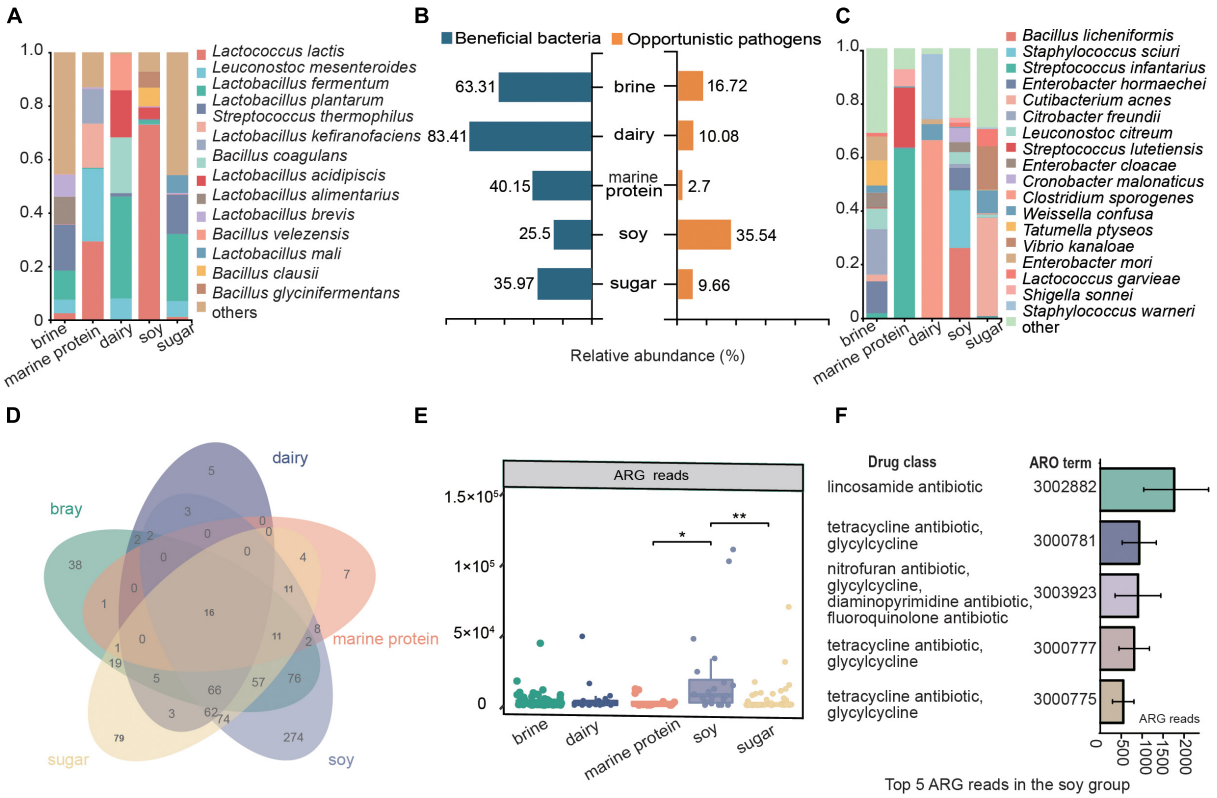

FIGURE 6
Potential benefits and risk analysis of fermented food. **(A)** Composition of beneficial microorganisms in different substrate fermented foods. **(B)** Enrichment of beneficial bacteria and opportunistic pathogens. **(C)** Composition of opportunistic pathogens in different substrate-fermented foods. **(D)** Venn diagram showing the species distribution of antibiotic resistance genes (ARGs) in different fermented foods. **(E)** ARG reads in different fermented foods. **(F)** The top five enriched ARGs and their corresponding antibiotics in fermented soy foods. ARO term, a taxonomic unit in the CARD database.

were enriched in various fermented foods (Figures 6A,B). *Leuconostoc mesenteroides* was widely enriched in fermented foods, except soy fermented foods. *Lactobacillus fermentum* was the main beneficial bacteria in fermented brine, dairy, and marine protein. *Lactococcus lactis* was found to be highly enriched in fermented dairy products and bean products. Moreover, we found that *Lactobacillus kefiranofaciens* was a characteristic bacterium of fermented dairy products, and it has been found that *Lactobacillus kefiranofaciens* has metabolism regulating, immune regulating, and antioxidant functions and improves diabetes, allergies, and brain health (Slattery et al., 2019). *Lactobacillus alimentarius* was abundantly enriched only in the fermented food products using the brine substrate. In addition, the proportion of beneficial bacteria in fermented dairy products was the highest, indicating that fermented dairy products might become the most promising probiotic resource. Thus, our study indicated the enrichment of beneficial bacteria in different fermented foods and provided data support for better utilization of microbial resources in fermented foods.

Unfortunately, the safety of traditional fermented foods has been questioned due to the contamination of pathogenic bacteria and microbes carrying ARGs. The number and types of opportunistic pathogens in different fermented foods were inconsistent (Figure 6). The marine protein-based fermented food had the least opportunistic pathogens (5.92%), followed by fermented dairy products (10.08%), and fermented soybean products (33.63%) had the highest enrichment in opportunistic pathogens, which seemed to indicate that fermented soy products carry a higher potential risk for disease. *Staphylococcus sciuri*, *Enterobacter hormaechei*, and *Bacillus licheniformis* were the core species of fermented soy products and the main opportunistic pathogens (Figures 3B, 6C). *Staphylococcus sciuri* is a human pathogen that causes a wide range of human infections, such as endocarditis, peritonitis, septic shock, urinary tract infection, pelvic inflammatory disease, and wound infection (Li et al., 2011). *Enterobacter hormaechei* causes urinary tract infection, hemorrhagic diarrhea, septicemia, and bloodstream infection (Campos et al., 2007; Kumar and Das, 2016; Parra-Flores et al., 2018). *Bacillus licheniformis*, a characteristic bacterium of fermented soy products, is

considered a pathogen causing infection in humans and can cause skin infection and sepsis (Ameur et al., 2005; Matua et al., 2015). The main conditional pathogen in fermented marine protein products was *Streptococcus infantarius*, which can cause biliary tract infection, endocarditis, colorectal cancer, and hepatobiliary diseases (García-Garrote et al., 2014; Kaindi et al., 2018). *Citrobacter freundii* can cause skin, lung, and systemic diseases (Nakazawa et al., 1983; Chuang et al., 1998) and is enriched in brine-fermented food. *Cutibacterium acnes* was considered to be the main cause of prosthetic joint infection after shoulder arthroplasty (Carabeo, 2011) and was enriched in fermented food based on sugar. We found that *Weissella confusa*, which causes disease in immunocompromised humans and animals, was in content in fermented dairy products (Little et al., 2020). We listed the main opportunistic pathogens in different fermented foods and the diseases that may cause diseases in the human body to attract the attention of the fermented food industry and promote the development of the fermented food industry. In addition, antimicrobial resistance is a global threat, and its major determinant is ARGs (Sanderson et al., 2016). Since bacteria can share genetic components by horizontal gene transfer, even non-pathogenic bacteria can provide ARGs to any pathogen that they are physically close to, including in the human intestinal tract (Tan et al., 2021). The consumption of fermented foods increases the risk of transmission of microbial ARGs in the human intestine (Tan et al., 2021). Therefore, we also analyzed the antibiotic resistance genes in different fermented foods, and the results showed that the types and numbers of antibiotic resistance genes in fermented soybean products were the highest (**Figure 6F**). Our results suggested that the consumption of fermented soy products may be risky.

Taken together, our results uncovered the potential benefits and risks of traditional fermented foods. Fermented dairy products had a high content of beneficial bacteria and a low level of opportunistic pathogens and ARGs, which may make them the most functional fermented foods. However, the risks of consumption were increased due to the enrichment of opportunistic pathogens and ARGs in fermented soy products. Emphatically, potential health promotion attributes were prevalent in fermented foods that benefited from the widespread existence of potential beneficial bacteria, while the potential edible risk of fermented foods should be given more attention. We also analyzed the microbial diversity, taxonomic composition, and metabolic pathways of different fermented foods. In addition, characteristic bacteria were found in different fermented foods, which not only characterized the microbial resources of different fermented foods but also facilitated the development and utilization of microbial resources of fermented foods. The data presented here will provide worthy intelligence or further optimizing the production of fermented foods, utilizing their health promotion potential and reducing their safety risks.

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 below: PRJNA890920.

Author contributions

MX: investigation, project administration, formal analysis, writing—original draft, and data curation. SS, ZZ, SJ, and JZ: project administration, conceptualization, and writing—review and editing. YX and XH: funding acquisition, conceptualization, validation, project administration, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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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.2022.1045096/full#supplementary-material

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Response of microbiota to exogenous inoculation improved the enzymatic activities of medium-temperature *Daqu*

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To explore the potential mechanism of improving enzymatic activities in medium-temperature *Daqu* (MTD) by inoculation functional isolates, we inoculated a single strain of *Bacillus licheniformis*, and the microbiota composed of *Bacillus velezensis* and *Bacillus subtilis* in MTD to investigate the association between the response of the functional microbiota and the enzymatic activity. The results showed that the bacterial community of MTD might be more sensitive to bioturbation than the fungal community, and the indigenous microbiota responded to the single strain more than to the microbiota. Moreover, the differential microorganisms mainly included *Lactobacillales*, *Bacillales*, and *Saccharomycetales* between the conventional and fortified samples. Notably, the composition of functional microbiota related to liquefying activity (LA) and saccharifying activity (SA) were significantly different, changing from *Lactobacillus* and *Rhizomucor* to *Bacillus*, *Weissella*, and *Hyphopichia*. That might be closely related to the effect of the bioturbation on LA (31.33%) and SA (43.54%) associated microorganisms was more tellingly. Furthermore, the relative abundance changes of bioturbation-sensitive modules in the co-occurrence network might also lead to the difference in enzymatic activities. Therefore, the LA and SA of MTD were improved by bioturbation significantly. These results provide diverse insights into the exogenous functional isolates to regulate the MTD microbiota and improve enzymatic activities.

KEYWORDS

medium-temperature *Daqu*, functional isolates, enzymatic activity, sensitive, co-occurrence network

Introduction

Daqu is classified into three main types, namely high-, medium- and low-temperature *Daqu* (Zhou et al., 2022). They are not only an indispensable crude enzyme and starter but also one of the specific raw materials for Baijiu production (He et al., 2020; Xiao et al., 2021). Among them, the medium-temperature *Daqu* (MTD) is

manufactured through spontaneous inoculation and fermentation, which allows its characteristics are endowed by the microbial community inhabiting the raw material and environment (Xiao et al., 2017; Du et al., 2019; Zhang et al., 2021).

In previous studies, the dominant microorganisms, metabolites, and their relationship in MTD have been preliminarily explored (Zheng et al., 2014; Li et al., 2020; Ma et al., 2021). It is a truism that the enzymatic activities of MTD closely lied to the microbial community structure and are manifested as a dynamic succession pattern (Liu et al., 2018; He et al., 2019; Guan et al., 2021). Yet, the succession was nonlinear during the process, which involved the interaction between the functional modules and the nutrition networks (Yi et al., 2019; Mao et al., 2022). The maximum information coefficient (MIC) analysis can capture linear and nonlinear relationships between independent and response variables (Reshef et al., 2011). This method could explore the relationships between microorganisms and environment variables and construct a microbial co-occurrence network (Logares et al., 2020; Lu et al., 2021). Perhaps, it also could explore the relationship between the trophic interaction, functional microbiota, and network modules to the community succession and enzymatic activities of MTD.

Moreover, the technology of inoculating functional isolates to improve the quality of MTD has become a hot topic. For example, the inoculation of *Bacillus licheniformis*, *Bacillus velezensis*, and *Bacillus subtilis* significantly improved the MTD enzymatic activities and flavor profile (Wang et al., 2017; He et al., 2019). Additionally, the content of ethyl caproate in the fermentation process of MTD was increased by inoculating *Saccharomyces cerevisiae*, and/or *Clavispora lusitaniae* (Li et al., 2020). However, the mechanism by which functional isolates drive the succession of indigenous communities and improve the function of MTD is still unclear. The latest works showed that the potential interactions among the different microbiota could be better understood by the co-occurrence network (Faust and Raes, 2012; Tang et al., 2021), and the exogenous species could change the co-occurrence pattern of indigenous microbiota (Carey et al., 2017; Yang et al., 2022). However, the critical role of exogenous strain/microbiota in regulating the network performance of indigenous communities in MTD was still uncertain.

In the present research, taking conventional and two types of fortified MTDs as the objects, the latter were inoculated with *B. licheniformis* and the microbiota composed of *B. velezensis* and *B. subtilis*, respectively, the response patterns of the functional microbiota of the MTD were explored. Then, MIC analysis was performed to determine the functional microbiota related to enzymatic activities and reveal the mechanism of the differences in enzymatic activities caused by different fortified patterns. These results will be beneficial for predicting the characteristics of fortified MTD and optimizing the manufacturing technique of MTD.

Materials and methods

Sources and functional characteristics of functional isolates

The *B. subtilis* D-31 and *B. velezensis* M-14 were isolated from conventional MTD at the famous Baijiu manufacturing enterprises located (Luzhou city and Yibin city, Sichuan Province, China). The *B. licheniformis* C-49 was isolated from the mutated MTD powder which stayed in space for a month and was loaded into the capsule of the Shenzhou 11 spacecraft. These isolates were identified by morphological and biochemical tests as well as 16S rDNA sequence (Fangio et al., 2020). Thereinto, the liquefying and saccharifying activities were significantly higher in MTD by inoculation with *B. subtilis* D-31 and *B. velezensis* M-14 (Xiao et al., 2014; Wu et al., 2017; He et al., 2019). Similarly, the enzymatic activities of MTD were also altered by inoculating *B. licheniformis* C-49 (Chen et al., 2021).

Preparation of isolates suspension

Firstly, the activated isolates were inoculated into the commercial medium (LB, beef extract peptone medium) to prepare seed solution, respectively, and cultured at 37°C with shaking at 120 rpm/min for 24 h. Then, the 3 ml isolates suspension was transferred to 500 ml Aubergine flasks containing 100 ml agar slant of LB medium at 37°C for 24 h. Thirdly, the cultures were eluted with pre-prepared sterile water to prepare the suspension. Finally, the cell quantity of the suspension was counted by hemocytometer and diluted to 2.5×10^6 CFU/ml with tap water.

Experiment procedure and sampling

The manufacturing process of the MTD is shown in Figure 1. Specifically, the crushed wheat was mixed with water containing the suspension (2.5×10^6 CFU/ml) at a ratio of 0.62:0.38 and pressed firmly to shape a cuboid brick (*Daqu Pei*, 30 cm × 20 cm × 7 cm). Subsequently, the *Daqu* bricks were transferred and fermented in a fermentation room (called a *Qu Fang*) for about 12 days. Lastly, these bricks were stacked to another *Qu Fang* for aging to reach maturity. Among them, *Daqu Pei* was inoculated with the microbiota composed of *B. velezensis* and *B. subtilis* at a ratio of 1:1 abbreviated as DD, and with the single strain of *B. licheniformis* abbreviated as the CD. Uninoculated MTD was used as the blank and was called PD. The incubation of the MTD needs to be controlled strictly according to the following procedure. Firstly, the fermentation temperature of MTD increases up to 30–45°C within 2–3 days of the initial phase, gradually. And then, the temperature increases to a maximum of 58–61°C at a rate of 5–8°C/day. Finally, the temperature slowly decreased to below 35°C, indicating that MTD had then entered the mature process. During the entire

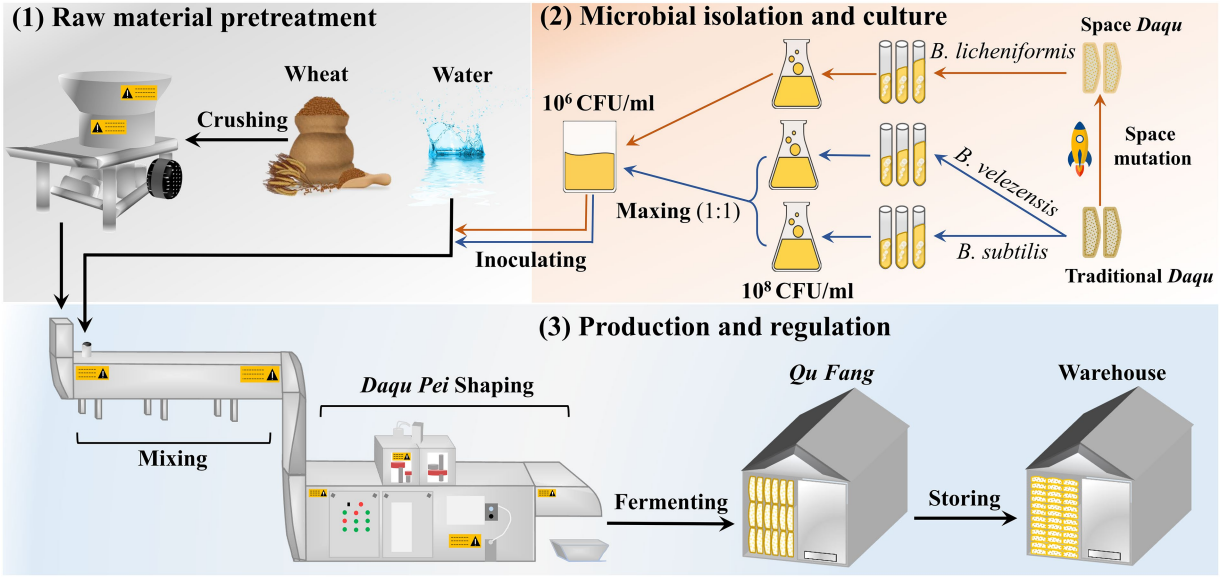

FIGURE 1
The manufacturing process of the medium-temperature *Daqu* (MTD).

fermentation process, the process parameters, such as the room temperature, humidity, and CO_2 concentration, were regulated by opening and closing the doors and windows in time. Samples were collected separately on days 3, 4, 5, 6, 7, 10, 12, 15, 17, and 28 according to the previous method (He et al., 2019). And then, these samples were transported to our Lab *via* cold chain conveyance and stored at −20°C and −80°C, respectively, until analyzed. These samples were conducted biologically in triplicate.

Physicochemical properties analysis

During the fermentation process, the temperature was measured in real-time by randomly inserting thermometers into *Daqu* bricks in each room. The moisture was measured by determining its weight loss after drying 5 g of MTD sample to a constant weight (105°C for 4 h). The content of total acidity was determined by acid–base titration to the endpoint of pH 8.2 with 0.1 mol/l NaOH solution. The liquefying, saccharifying, esterifying, and fermenting activities were detected by the previous method (Tang et al., 2019). All samples were measured in triplicate.

Microbial community analysis

Total genomic DNA extraction was carried out using the Fast DNA SPIN extraction kits (MP Biomedicals, Santa Ana, CA, United States) according to the manufacturer's instructions. For bacteria, the V3–V4 hypervariable region of the 16S rRNA gene was amplified by PCR with the universal primers of the forward 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and the reverse 806R (5′-GGACTACHVGGGTWTCTAAT-3′). For fungi, the ITS1 was amplified by PCR with the primers of ITS5 (5′-GGAAG TAAAAGTCGTAACAAGG-3′) and ITS1 (5′- GCTGCGTT CTTCATCGATGC-3′). After the individual quantification, amplicons were pooled in equal quantities and subjected to high-throughput sequencing with a MiSeq Reagent Kit V3 (Personal, Shanghai, China) for pair-end 2 × 250 bp sequencing.

The sequences were analyzed with QIIME2 (2019.4), and the quality control of the sequences by using DADA2 (Callahan et al., 2016). Specifically, the default parameters of DADA2 were used for filtering and denoising. Secondly, the sequences were automatically calculated and spliced according to the clip length. Thirdly, the sequences with chimerism >8 were removed. Finally, the sequences with low abundance (reads ≤1) were removed. Each de-weighted sequence resulting from quality control using DADA2 is called ASV.

Statistical and bioinformatics analyses

Unless otherwise stated, all statistical analyses were performed in R (v. 3.6.3). The dynamics of fermentation parameters, enzymatic activities, and microbial diversity were fitted *via* OriginPro2019 (OriginLab Corporation, MA, United States). Principal component analysis based on the Bray-Curtis distance was conducted by using the package "FactoMineR." A one-way ANOVA and Stepwise multiple regression analyses were conducted using SPSS 25.0. The dissimilarity of the MTD microbial community and the correlation with fermentation parameters were assessed by principal coordinate analysis (PCoA)

and Mantel tests, both of which were carried out in the "vegan" package. All possible Spearman's rank correlations among α-diversity index, community ASVs and fermentation parameters using SPSS 25.0 and the significant correlations ($|\rho| > 0.60$, $p < 0.05$) were retained.

In this study, all co-occurrence networks were constructed in package "igraph." Utilized the trimmed means of M (TMM) normalized counts per million (CPM) count, then conducted Spearman correlations among filtered ASVs with criteria the reads number >2 and prevalent in >4 samples, and retained the positive, significant correlations ($\rho > 0.65$, $p < 0.001$). To represent microbial groups, identified network modules within the networks (Li et al., 2021). The indicator species analysis was performed in the package "indicspecie" (De Cáceres et al., 2010), marked differential abundant ASVs using likelihood ratio tests (LRT) with FDR $p < 0.05$ in the package "edgeR" (Robinson et al., 2020). The MIC analysis was realized by using the package "minerva" (Reshef et al., 2011).

Results

Dynamics of fermentation parameters and enzymatic activities

As shown in Figure 2A, the dynamic temperature could be divided into three phases, which involved rising in vain (RV, days 0–6), the relative stability of high temperature (RS, days 7–12), and the cooling phase (C, days 13–28). In addition, the moisture and acidity were reduced markedly throughout the RV and RS phases, and in a slow and flat trend in the C phase (Figures 2B,C). The temperature changes of DD and CD were faster than that of sample PD in the RV phase (Figure 2A), while the moisture in the sample CD decreased the slowest (Figure 2B), which might be related to the spatial heterogeneity of solid fermentation and random sampling (Cai et al., 2022; Shi et al., 2022). Moreover, the trends of these parameters changing were similar in different samples ($p > 0.05$). In particular, the RS phase of all MTD samples was relatively stable.

The dynamics of enzymatic activities as shown in Figure 2D. These results suggested that the effect of inoculation functional isolates improved the activity of saccharifying and liquefying significantly ($p < 0.001$). Moreover, the enzymatic activities were different and separated from each other in the different groups of samples (Figure 2E), including the significant contribution of phase (PC1, 44.2%) and MTD type (PC2, 39.1%). These activities were notably related to the moisture for all samples and correlated with temperature and acidity for DD and CD based on multiple regression analyses (Supplementary Table S1).

Microbial community structure and their succession during the process

The effective sequences of bacteria and fungi were 63,806–102,077 and 75,339-103,151, respectively, and the high-quality sequences were 56,612–96,117 and 69,291-97,721, respectively. The proportions of high-quality sequences ranged from 88.73 to 94.16% for bacteria and 88.61 to 95.46% for fungi. The rarefaction curve indicated that the sequencing depth could characterize the diversity of the community (Supplementary Figures S1A,B). As shown in Figure 3A, the Chao1 and Shannon indices of bacteria and fungi were similar for the three types of MTD in the RV phase. However, the indices of community Chao1 and fungal Shannon in DD and CD were significantly higher than that of PD in the C phase ($p < 0.05$). The relationships between α-diversity and fermentation parameters as shown in Figure 3D. The results demonstrated that moisture played a major role in driving the success of α-diversity, which was significantly positively correlated with the bacterial α-diversity of PD, while negatively with the fungal community α-diversity of DD. In addition, the bacterial Shannon index of the DD was also negatively correlated with temperature.

The effect of the different fortification patterns on the microbial community structure in MTD was similar (Supplementary Figures S1C,D). The relative abundance (RA) of *Weissella* was always dominant, although it decreased during the fermentation process, whereas *Bacillus* gradually increased in all samples (Supplementary Figure S1C). In addition, the RA of *Lactobacillus* also increased during the C phase of DD, which was consistent with the results reported by He et al. (2019). The RA of *Pichia* remarkably decreased during the RS and C phases (Supplementary Figure S1D), the succession of *Rhizomucor* in PD was the same, while the *Thermomyces* was the opposite. Furthermore, the RA of *Rhizopus* and *Hyphopichia* in DD and CD dominated during the RV and RS phases rather than PD.

The results based on the PERMANOVA test showed that there were significant differences in the microbial community, including the bacteria ($R^2 = 0.109$, $p = 0.047$), fungi ($R^2 = 0.453$, $p < 0.001$) communities from the phases, and the bacterial community ($R^2 = 0.122$, $p = 0.032$) from MTD types (Figures 3B,C; Supplementary Table S2). Moreover, the fungal communities of all samples were correlated with moisture and acidity by the Mantel test, while the bacteria community of sample DD was primarily related to fermentation temperature (Figures 3E,F; Supplementary Table S3). These results demonstrated that the process parameters play a vital role in community succession, and highlighted that it is necessary to reduce the interference of the process parameters when exploring the effects of functional isolates inoculation on MTD indigenous communities.

Identifying bioturbation respond significantly microorganisms

The linear relationship between fermentation parameters and microbial species (ASVs) was characterize by Mantel test ($p < 0.05$) and Spearman rank correlation analysis ($|\rho| > 0.60$, $p < 0.05$). Defining these microorganisms as those primarily influenced by process parameters, the ASVs for bacteria and fungi were *bp*ASV and *fp*ASV, respectively (Figures 3E,F). The individual bacteria and fungi ASV

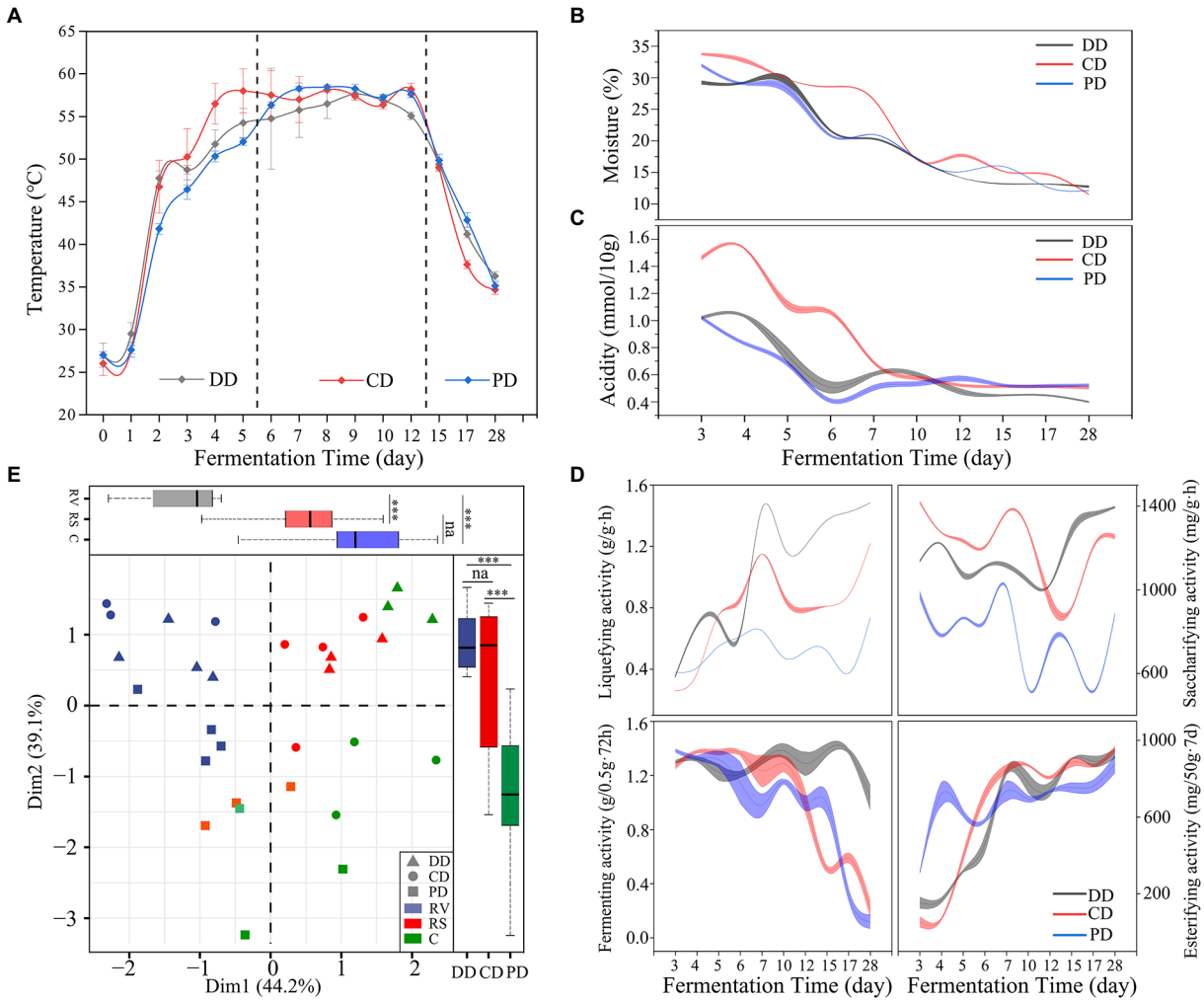

FIGURE 2
Fermentation parameters and enzymatic activity during the fermentation process. **(A–D)** The dynamics of fermentation parameters and enzymatic activity. **(E)** The phase (RV, days 0–6; RS, days 7–12; C, days 13–28) and type division according to principal component analysis based on the Bray–Curtis dissimilarity matrix of enzymatic activity (one-way ANOVA, ***p<0.001).

whose RA varied among the different types of MTD were identified by the indicator species analysis and were named *bi*ASV and *fi*ASV, respectively (Supplementary Tables S4, S5). Notably, DD samples did not include specific *fi*ASV (Supplementary Table S5). As shown in Supplementary Figure S2, ASVs shared between *bp*ASVs and *bi*ASVs were highly limited in DD, while the ASVs between *fp*ASVs and *fi*ASVs were highly coincident in CD (45.45%) and PD (80%). It indicated that the significant difference in the bacterial community among the three types of MTD explained by the process parameters was indistinctive, whereas the differences in the fungal community were more likely to be explained. These results confirmed our previous analysis (Figures 3E,F; Supplementary Table S3).

As mentioned above, the ASVs driven by bioturbation effects might also be influenced by process parameters. Therefore, the remaining *i*ASVs thought removing the *p*ASVs were defined as *br*ASV that were affected by bioturbation, and 151 bacterial and six fungal *br*ASVs were obtained (Supplementary Tables S4, S5). Concretely, bacterial *br*ASVs were mainly composed of *Lactobacillales*, *Bacillales*, and *Enterobacteriales*. While the fungal *br*ASVs were composed of *Saccharomycetales* and prominent associated with the CD (Figure 4A). In addition, there were abundant *br*ASVs in three types of MTD, and various bacterial *br*ASVs were shared between DD and CD (Supplementary Table S4; Figure 4A), which reflected the cluster of these samples in the ordination (Supplementary Table S2). Further, it was defined as *br*ASVs that were supported by the likelihood ratio test (FDR, $p < 0.05$) as bioturbation responding significant ASVs (hereafter: *bs*ASVs), and a total of 102 bacterial (DD: 19, CD: 52, PD: 41) and four fungal *bs*ASVs (CD: 4) were identified (Figure 4B; Supplementary Tables S4, S5). These results showed that the indigenous microbial community of MTD was more responsive to the bioturbation of *B. licheniformis* than to the microbiota composed of *B. velezensis* and *B. subtilis*. As an approximation for the "effect size" of bioturbation on microbial

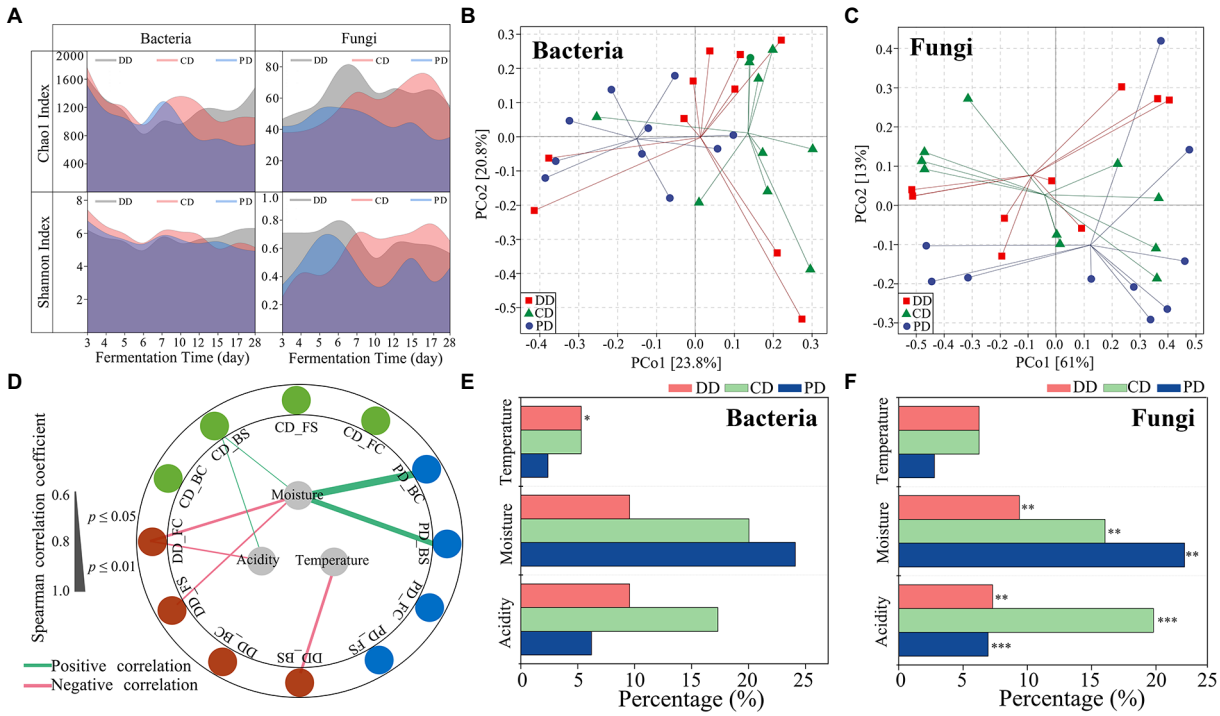

FIGURE 3
Microbial diversity and succession during the process. **(A)** Chao1 and Shannon indexes values for bacterial and fungal communities. **(B,C)** Group division according to principal coordinates analysis based on the Bray–Curtis dissimilarity matrix of microbial communities. **(D)** Correlation network between the α-diversity and fermentation parameters during the process ($|\rho|>0.60$, $p<0.05$). Abbreviations connected with underscores such as DD_BC, DD_BS indicated the bacterial Chao1 and Shannon index of the DD sample. **(E,F)** Percentage of the community turnover associated with different ecological processes as calculated using ASVs (Spearman, $|\rho|>0.60$, $p<0.05$). The significant effects of fermentation parameters on community are determined by mantel tests (***$p<0.001$, **$p<0.01$, *$p<0.05$).

communities by Hartman et al. (2018), these *bs*ASVs accounted for 11.95 and 3.67% of the total bacterial and fungal sequences, respectively, suggesting that the bacterial community of MTD might be more sensitive to bioturbation.

Bioturbation effects on microbial co-occurrence patterns

To further investigate the characteristics of indigenous microbiota in response to bioturbation by network analysis. As shown in Figure 5A, networks of fortified MTD (DD and CD) differed from conventional MTD (PD), and the former had a larger network size (more nodes), which was consistent with an increase in species richness of fortified MTD (Figure 3A). Among them, the DD network was more connected (larger number of links), while the CD network contained more modules. A total of 19, 29, and 27 modules were identified in samples DD, CD, and PD, respectively, suggesting that the response of indigenous microbiota in MTD varied with the fortified pattern.

As shown in Figures 5B, a robust and suitable co-occurrence network was constructed based on the microbiota of all samples (Hartman et al., 2018; Li et al., 2021), and the *bs*ASVs that were closely associated with the core modules were identified. These results showed that the bioturbation changed the co-occurrence network and the RA of core modules (Figure 5B; Supplementary Figure S3). Among the top 10 modules, four modules (M2, M3, M4, and M5) contained abundant nodes of *bs*ASVs and were defined as bioturbation-sensitive modules (hereafter: *bs*Modules), while other modules were the insensitive modules. The distribution of these *bs*Modules in the network reflected the community dissimilarity in PCoA ordinations (Figures 3B,C). For instance, the *bs*ASV nodes of the PD were distributed in M2 and separated from other modules, while the modules of M3 and M4 have abundant *bs*ASV nodes of samples DD and CD, so they were closely connected. Equally, the RA of *bs*Module in different samples had a similar trend, while the modules of M2 and M3 involved in samples CD and DD contained most of *Hyphopichia* and *Bacillus*, respectively (Supplementary Figures S1C,D, S3A). In addition, the RA of all ASVs from the co-occurrence networks was plotted as a function of their degree of co-occurrence, abundant *bs*ASVs were identified among low node degree and lower RA microbiota (Figure 5C).

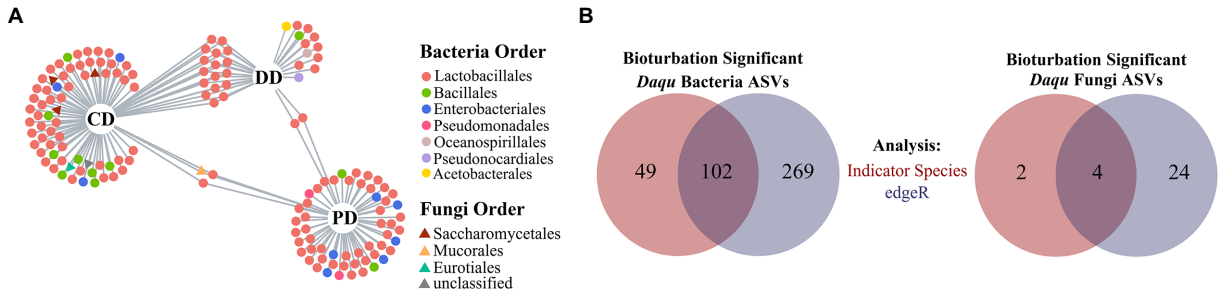

FIGURE 4
Defining the *bs*ASVs in which the MTD microbial communities respond significantly to bioturbation. **(A)** Bipartite networks display the distribution of differential ASVs (*br*ASVs). These ASVs are positively and significantly associated ($p<0.05$) with one or more of the MTD types. **(B)** Venn diagrams show the number of ASVs significantly responding to bioturbation identified with specific ASVs of bioturbation (red) and by edgeR (blue). These ASVs are defined as bioturbation responding significantly ASVs (*bs*ASVs).

Associating enzymatic properties with microbial community

The potential links identified between enzymatic activities and ASVs by MIC analysis (MIC ≥0.35; Reshef et al., 2011), 166, 147, 257, and 168 ASVs were strongly associated with LA, SA, EA, and FA, respectively, and they were defined as *e*ASVs. These *e*ASVs qualitative taxonomic composition by per type sample genus average RA. As shown in Figures 6A,B, the dominant functional microbiota related to enzymatic activities included *Lactobacillus*, *Weissella*, *Bacillus*, *Hyphopicha*, and *Pichia*, which was in agreement with previous studies (Wang et al., 2017; He et al., 2019). Among them, the composition of LA- and SA-related fungal and LA-related bacterial functional microbiota in fortified MTD was significantly different from that of PD (Supplementary Table S6). Specifically, the average RA of *Bacillus*, *Weissella*, and *Wickerhamomyces* were strongly correlated with LA, and that of *Hyphopicha* with SA. These genera were significantly increased in the fortified MTD, while *Lactobacillus* and *Rhizomucor* were the opposite (Supplementary Table S7).

As shown in Figure 6C, the *e*ASVs nodes aggregation in the co-occurrence network. Thereinto, the distributions of *e*ASVs related to LA and SA were similar and highly overlapped with the *bs*ASVs, and the *e*ASVs with MIC ≥0.55 (Chen et al., 2020; Logares et al., 2020) distributed in M4 and M2 were primarily. The distributions of *e*ASVs related to EA and FA in the non-*bs*ASV area of M4 were also similar. The statistical results of *e*ASVs attributes in the network showed that the proportion distributed by the four *bs*Modules was as high as 76–86% (Figure 6D), which implied the change of *bs*Modules significantly affected the enzymatic activities of MTD. Next, we used the ratio of *bs*ASVs in *e*ASVs to characterize the effect intensity of bioturbation on the MTD enzymatic activities (Supplementary Figure S3E). The response of different enzymatic activities to bioturbation was diverse, with the impact on SA (43.54%) being the largest, followed by LA (31.33%) and EA (15.56%). These results revealed the diverse enzymatic activities among different MTD samples.

Discussion

Previous works have proven that temperature, acidity, and moisture could affect the microbial composition of MTD (Deng et al., 2021; Ma et al., 2021). The exogenous inoculation of functional isolates accelerated the metabolism of the indigenous community in the early phase and increased the temperature and acidity rapidly (Figures 2A,C), thereby significantly changing the community structure and enhancing the effect on enzymatic activities (Supplementary Figures S1C,D). Meanwhile, the higher temperature accelerated the decrease in moisture, resulting in the loss of some species and genera that were difficult to survive under low moisture, including *Pichia*, *Rhizopus*, and *Hyphopichia*. Conversely, this change promoted the enrichment of thermophilic microorganisms, namely *Bacillus*, *Thermoascus*, and *Thermomyces*. These results also led to the variation in enzymatic activities (Figure 2D). However, the evolutionary trends of the process parameters were similar across groups, which only partially explained the difference in microbiota between the fortified and conventional MTD. Inoculating function isolates significantly changed the community diversity and structure (He et al., 2019; Li et al., 2020), emphasizing the importance of selecting functional isolates and optimizing fortifying patterns.

The response of the indigenous community in MTD showed partial similarity to different fortifying patterns, while it was more responsive to the inoculation of *B. licheniformis* (Figure 4A; Supplementary Tables S3–S5). In addition, the bacterial community in MTD was more sensitive to bioturbation than the fungal community because *bs*ASVs mainly belonged to bacterial microbiota, which was contrary to the patterns found by Wang et al. (2017). These results indicate that the MTD community might display different responding mechanisms for inoculating different functional isolates. The RA of *bs*ASV in the community of MTD was smaller, and most genera were more likely to be classified as rare rather than abundant taxa (Figure 5C). Previous studies suggested that the rare taxa were more prone to migrating rather than resisting changes (Shade et al., 2014; Jiao et al., 2019), and contain abundant metabolically active lineages (Dohrmann et al., 2013; Xiong et al., 2021). Thus, they might

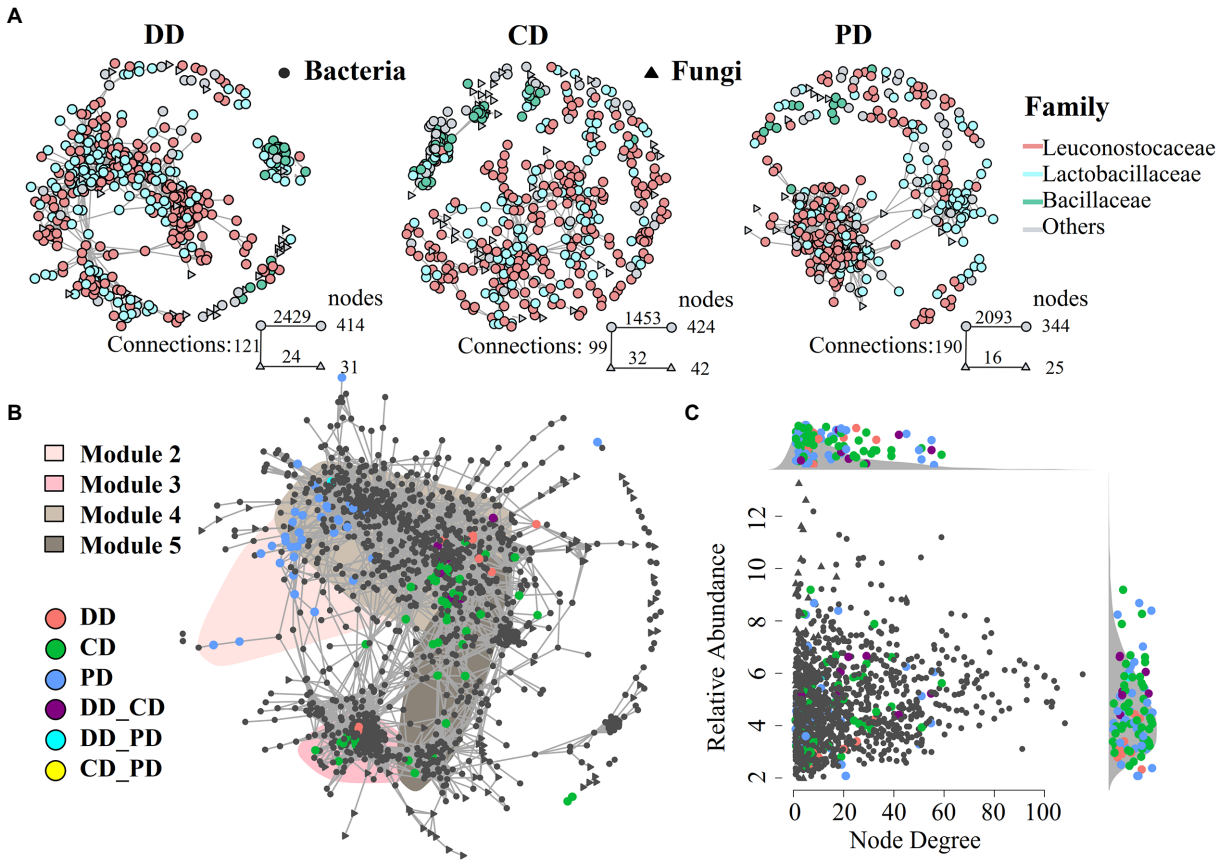

FIGURE 5
The MTD microbial community co-occurrence patterns. **(A)** Co-occurrence network of different types MTD microbial communities (ρ>0.65, p<0.001). **(B)** Full sample co-occurrence network and distribution of bioturbation responding significant ASVs (*bs*ASV) under specific bioturbation, insensitive ASVs were colored in gray. The major network modules containing *bs*ASVs were shaded. Abbreviations connected with underscores such as DD_CD, DD_PD, CD_PD indicated the joint *bs*ASVs of different MTD. **(C)** Degree of co-occurrence and abundance of *bs*ASVs. Relative abundance (as counts per million, CPM) of all ASVs from the MTD microbiome co-occurrence networks is plotted as a function of their degree of co-occurrence. Side panels recapitulate the distributions of co-occurrence degrees and abundance for the *bs*ASVs compared to the density of all.

respond to bioturbation rapidly, resulting in changes in composition and abundance of the microbial community to adapt to external stresses for better survival (Li et al., 2021).

The co-occurrence network has been widely used to explore potential correlations among the communities (Faust and Raes, 2012; Tang et al., 2021). It contributed to exploring how the inoculated functional isolates affect the ecological pattern of indigenous communities in MTD (Yang et al., 2022). In the present study, we found that the bioturbation of the functional isolates could result in a significant change in the abundance of the dominant microorganism in MTD. In addition, the microbiota closely associated with variation in enzymatic activities was also changed (Supplementary Figure S3A). For example, increased *Bacillus* might lead to LA increase, while the improvement of SA may be attributed to the enrichment of *Hyphopichia* (Kurtzman, 2011). The aggregation of *e*ASVs related to the identical enzymatic activity in the co-occurrence network indicated that microorganisms with similar functions cooperated closely because they had niche breadths similarly (Hartman et al., 2018; Li et al., 2021). That also explained the reason by inoculation of the *Bacillus* isolates significantly increased LA and SA in MTD.

The external disturbance could affect interspecies interactions by changing the "accessory microbiome" and embodied in different nutrition patterns (Hartman et al., 2018; Li et al., 2021), which might cause the change of MTD enzymatic activities related to the abundance of *bs*Moudles. For example, the *e*ASVs of LA by bioturbation were mainly distributed in M2 and M4 (Figure 6D), and the average abundance of these *bs*Modules in the RV phase of the DD and CD was higher than that of the PD (Supplementary Figure S3E). Meanwhile, this phase was the main period of enzyme production and accumulation (Li et al., 2017). Therefore, the increased RA of functional microbiota was conducive to accelerating the accumulation of enzymatic activity, thereby enhancing the LA, SA, EA, and FA. These results showed that the changes in the abundance of identified *bs*Modules could partially explain the differences in the enzymatic activities across MTD groups, and also suggested that the *bs*ASVs could promote the variation of accessory ASVs.

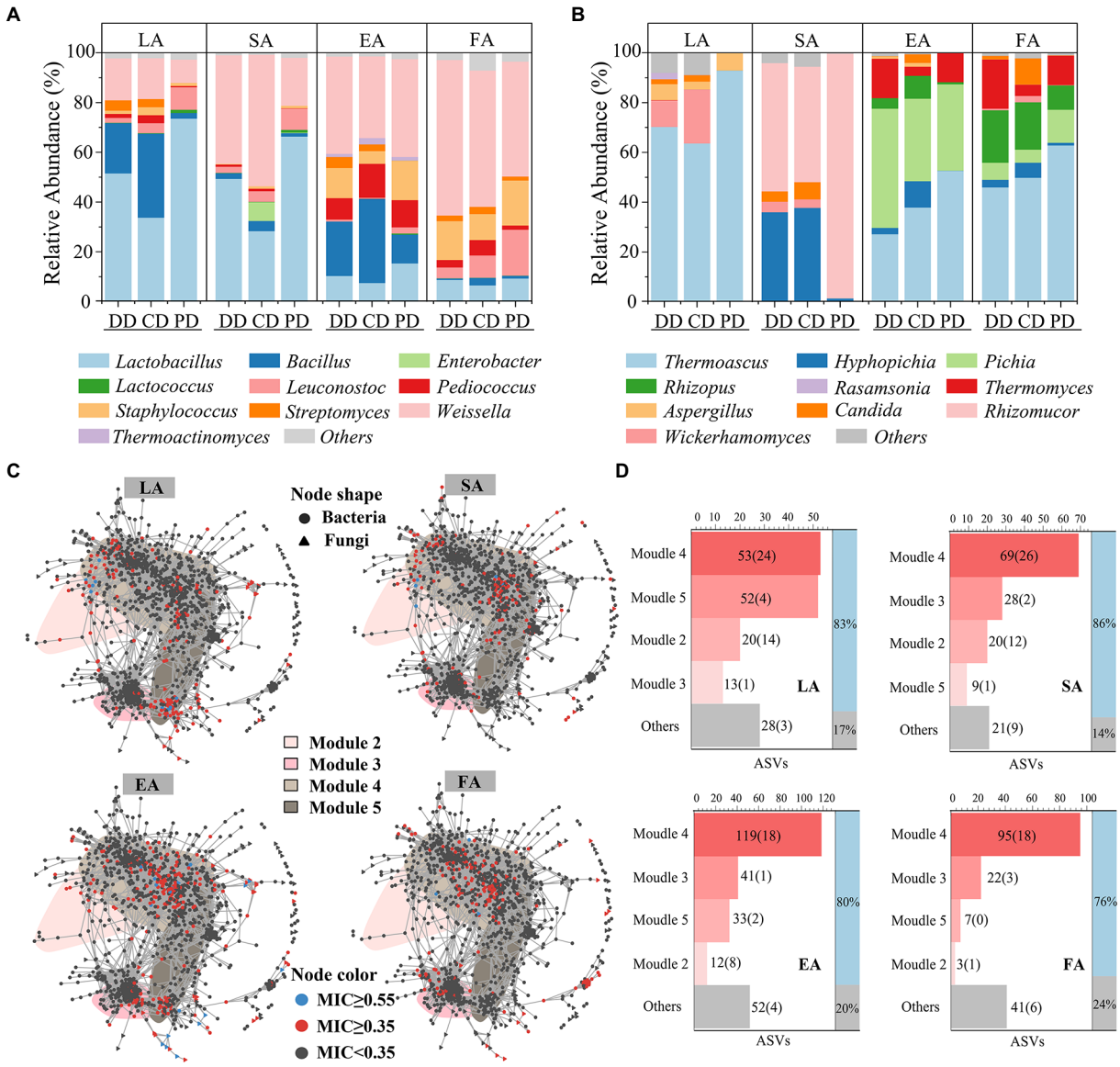

FIGURE 6
Correlation analysis *Daqu* microbial community with enzymatic activity. Community composition of bacterial **(A)** and fungal **(B)** that contribute significantly (*e*ASVs, maximum information coefficient ≥0.35) to MTD enzymatic activity, expressed by the average relative abundance of all samples of each type MTD during the fermentation process. **(C)** Distribution of eASVs in co-occurrence network. LA, SA, EA and FA, respectively, represent liquefying-, saccharifying-, esterifying- and fermenting-activity. **(D)** The number of *e*ASVs (*bs*ASVs) in *bs*Module, "Others" represents the total number of *e*ASVs distributed in insensitive modules. The ratio of *e*ASVs contained in *bs*Module to all detected *e*ASVs is shown in the right histogram.

Conclusion

This study revealed the effects of exogenous inoculation on the microbial community and enzymatic activities of MTD by high-throughput sequencing and ecological statistic method. The results suggested that the enzymatic activities of MTD were closely related to the response patterns of functional microbiota and network modules to inoculation. Briefly, the microbiota related to LA and SA were changed from *Lactobacillus* and *Rhizomucor* to *Bacillus*, *Weissella*, and *Hyphopichia*. Meanwhile, the abundance patterns of *bs*Modules were affected. Ultimately, these changes promote improved enzymatic activity in MTD, especially LA and SA. This work is beneficial for controlling MTD quality by regulating microbiota assembly and provides theoretical insights into the regulation mechanism.

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://www.ncbi.nlm.nih.gov/, PRJNA807119.

Author contributions

QP performed the experiments, analyzed the data and prepared the manuscript. JH guided the experiments. SZ, HQ, and XW provided financial support and sample collection. YM and HT contributed to data curation and manuscript revision. RZ contributed to the experimental design, manuscript revision, and overall support of this study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

SZ, HQ, XW, and RZ were employed by Luzhou Lao Jiao 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. The authors declare that this study received funding from Luzhou Lao Jiao Co., Ltd. The funder had the following involvement in the study: raw materials and site providing, sample collection, and decision to publish.

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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.2022.1047041/full#supplementary-material

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Quantitative metaproteomics reveals composition and metabolism characteristics of microbial communities in Chinese liquor fermentation starters

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Introduction: *Daqu*, the Chinese liquor fermentation starter, contains complex microbial communities that are important for the yield, quality, and unique flavor of produced liquor. However, the composition and metabolism of microbial communities in the different types of high-temperature *Daqu* (i.e., white, yellow, and black *Daqu*) have not been well understood.

Methods: Herein, we used quantitative metaproteomics based on data-independent acquisition (DIA) mass spectrometry to analyze a total of 90 samples of white, yellow, and black *Daqu* collected in spring, summer, and autumn, revealing the taxonomic and metabolic profiles of different types of *Daqu* across seasons.

Results: Taxonomic composition differences were explored across types of *Daqu* and seasons, where the under-fermented white *Daqu* showed the higher microbial diversity and seasonal stability. It was demonstrated that yellow *Daqu* had higher abundance of saccharifying enzymes for raw material degradation. In addition, considerable seasonal variation of microbial protein abundance was discovered in the over-fermented black *Daqu*, suggesting elevated carbohydrate and amino acid metabolism in autumn black *Daqu*.

Discussion: We expect that this study will facilitate the understanding of the key microbes and their metabolism in the traditional fermentation process of Chinese liquor production.

KEYWORDS

***Daqu*, Chinese liquor, microbial community, quantitative metaproteomics, data-independent acquisition**

1. Introduction

Daqu is the fermentation starter for brewing Chinese liquor (*Baijiu*; Zheng et al., 2011). It is produced from carbohydrate-rich raw materials (e.g., barley, wheat, and peas) and aerobically manufactured through a spontaneous solid-state fermentation with inoculation of indigenous microorganisms in the environment (Wu et al., 2016; Yang et al., 2021). High-temperature *Daqu* is usually used as the fermentation starter for sauce-flavore *Baijiu* production (Wang et al., 2021). Factors like the fermentation temperature varying at different positions in the starter brick result in three types of *Daqu* with different colors after fermentation, i.e., white *Daqu* (under-fermented starters), yellow *Daqu* (well fermented starters), and black *Daqu* (over-fermented starters; Gan et al., 2019; Wang et al., 2021). In hot and humid fermentation environment, the Maillard reaction of amino acids and sugars produces brown compounds, which appears to give *Daqu* its color (Zheng et al., 2011). In actual production, the three types of *Daqu* are mixed mainly depends on the workers' experience. The fermentation temperature can potentially contribute to the formation of different microbial communities of *Daqu*. The fermentation yield and quality are closely related to the microorganisms involved in the brewing process (Yang et al., 2018; Wang et al., 2021), and the special microbial compositions in *Daqu* can contribute to the unique aromas and tastes of the produced liquor (Gan et al., 2019). In 2014, Li et al. analyzed the connection between the flavor components of Baijiu and the dominant flavor-producing bacteria isolated from the central black component of *Daqu* (Li et al., 2014). Since then, many studies have reported differences of microbial community structure among the three types of *Daqu* (Gan et al., 2019; Deng et al., 2020; Wang et al., 2021). More recently, Cai et al. reported that the fungal communities in the three types of *Daqu* were significantly correlated with taste of *Baijiu* by high-throughput sequencing and electronic senses (Cai et al., 2021). Hence, it is important to clarify the microbial community structure in different types of *Daqu* and their function in liquor brewing to improve the product quality of *Baijiu*.

Various methods have been applied to explore the microbial composition in *Daqu*. The classical microbiological methods based on bacterial culture or PCR-denaturing gradient gel electrophoresis have identified many common microbes in the *Baijiu* fermentation (Zheng et al., 2011; Lv et al., 2012; Wang et al., 2016). Metagenomics based on high-throughput sequencing has enabled the discovery of the unculturable and low-abundant microbes, revealing the composition and diversity of *Daqu* microbial communities in breadth and depth (Su et al., 2015; Hu et al., 2017; Gan et al., 2019; Wang et al., 2019, 2021). However, knowledge of community structure characterized solely by metagenomics does not necessarily provide useful information on functions such as metabolic capacity and population dynamics (Wu et al., 2016).

As a powerful mean providing information about protein composition and relevant metabolic pathways, metaproteomics has been applied to investigate enzymatic proteins of microbes in *Baijiu* production (Wu et al., 2016; Wang et al., 2018, 2020; Xia et al., 2022). However, few studies have investigated the metaproteome of microbial communities in *Daqu* with large sample size. Recently, data-independent acquisition (DIA) mass spectrometry has shown the applicability to the analysis of complex metaproteomic samples (Aakko et al., 2020; Long et al., 2020; Pietilä et al., 2022), circumventing the drawbacks of the previously used data-dependent acquisition (DDA) method wherein the stochastic precursor ion selection leads to low reproducibility (Tabb et al., 2010). Moreover, the latest library-free DIA data analysis methods (Muntel et al., 2019; Gotti et al., 2021; Pietilä et al., 2022), such as directDIA (Muntel et al., 2019), no longer require additional DDA data to assist the protein identification and need only a single-shot DIA analysis per sample, enabling identification and quantification of proteins in large sample cohorts.

Herein, we used DIA-based quantitative metaproteomics to analyze a total of 90 samples of white, yellow, and black *Daqu* produced in spring, summer, and autumn. Composition differences of microbial taxa and saccharifying enzymatic proteins were revealed across types of *Daqu* and seasons. Moreover, the functional characteristics of the microbial proteins in different seasons were explored, revealing the varied metabolic features of *Daqu* microbiota across seasons. We expect that this study will aid in uncovering the key microbes and their metabolism in the fermentation process of *Baijiu* and facilitate the deeper understanding of this traditional technique.

2. Materials and methods

2.1. Sample collection

The *Daqu* samples was collected from Kweichow Moutai Liquor Co., Ltd. (Guizhou, China). Ninety samples were collected from *Daqu* finished products in spring, summer, and autumn, including 10 pieces of white, yellow, and black *Daqu* in each season. All *Daqu* samples were produced by the traditional high-temperature *Daqu* production method (Wang et al., 2021). The collected samples were stored in dry ice and transported back to the laboratory within 24 h for storage at −80°C.

2.2. DNA extraction, sequencing, and assembly

For each of the 90 *Daqu* samples, 0.2 g were weighed and then combined into 9 samples according to the seasons and types. The DNA of the pooled samples was extracted using HiPure Bacterial DNA Kits (Magen, Guangzhou, China) according to the manufacturer's instructions. The DNA quality was detected using Qubit and Nanodrop (Thermo Fisher Scientific, MA, United States).

Qualified genomic DNA was firstly fragmented by sonication to a size of 350 bp, and then end-repaired, A-tailed, and adaptor ligated using NEBNext® MLtra™ DNA Library Prep Kit for Illumina (NEB, MA, United States) according to the preparation protocol. DNA fragments with length of 300–400 bp were enriched

by PCR and then purified using AMPure XP system (Beckman Coulter, CA, United States). The libraries were analyzed for size distribution by 2,100 Bioanalyzer (Agilent, CA, United States) and quantified using real-time PCR. Genome sequencing was performed on a NovaSeq 6,000 sequencer (Illumina Inc., CA, United States) using pair-end technology (PE 150).

Raw data from Illumina platform was filtered using FASTP (version 0.18.0; Chen et al., 2018) by the following standards: (1) removing reads with ≥10% unidentified nucleotides (N); (2) removing reads with ≥50% bases having Phred quality scores ≤20; (3) removing reads aligned to the barcode adapter. After filtering, resulted clean reads were used for genome assembly. Clean reads of each sample were assembled individually using MEGAHIT (version 1.2.9; Li et al., 2015). Genes were identified using MetaGeneMark (version 3.38; Tang and Borodovsky, 2013). The output amino acid sequences of the genes were then used as the protein database for metaproteomics analysis.

2.3. Sample preparation for metaproteomics

For each individual *Daqu* sample, 0.5 g was treated with a mechanical grinder (JXFSTPRP-CL, Shanghai Jingxin Industrial Development Co., Ltd., Shanghai, China). After grinding twice with 5 steel balls (−50°C, 70 Hz, on 120 s, off 120 s), 1 ml BPP solution (100 mM EDTA, 50 mM borax, 50 mM vitamin C, 30% sucrose, 10 mM Tris-base, 1% Triton-100, 5 mM 1,4-dithiothreitol, 1% polyvinylpolypyrrolidone) was added to the sample. After vortexing, 1 ml DNA extraction phenol reagent (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) was added. The sample was vortexed (2 min) and centrifuged (12,000 *g*, 20 min, 4°C). The upper phenol phase was taken. Another 1 ml of BPP solution was added to the phenol phase, and the lysis step was repeated once. Then 5 ml of methanol solution containing 0.1 M ammonium acetate pre-cooled at −20°C was added to the upper phenol phase, and the sample was precipitated at −20°C for 4 h. The sample was centrifuged (12,000 *g*, 20 min, 4°C) and the supernatant was discarded. The precipitate was washed with 1 ml of 0.1 M ammonium acetate methanol solution twice. After drying at room temperature, 300 µl of 1% sodium dodecyl sulfate (SDS) solution with 8 M urea was added to dissolve the protein.

Different lysates and cell disruption methods were also benchmarked using 0.5 g summer yellow *Daqu* sample. (1) Sample 1 was mechanical grinded twice with 5 steel balls. Then 1 ml 1% sodium deoxycholate (SDC) lysate containing 1X protease inhibitor cocktail (Thermo Fisher Scientific, MA, United States) was added. After grinding twice, the supernatant solution was obtained by centrifugation. (2) Sample 2 was mechanical grinded same as Sample 1, and treated with 1 ml 1% SDS lysate containing 8 M urea and 1X protease inhibitor cocktail. (3) Sample 3 was treated with mechanical grinding same as Sample 1 and BPP lysate as described above. (4) Sampel 4 was grinded with liquid nitrogen, and then treated with BPP lysate same as Sample 3.

The protein was quantified using Pierce BCA protein assay kit (Thermo Fisher Scientific, MA, United States). For each sample, 8 M urea solution and 1 M Triethylammonium bicarbonate (TEAB) buffer solution was added to 200 µg protein to a volume of 200 µl (the final concentration of TEAB was 0.1 M). Then 4 µl 0.5 M tris-(2-carboxyethyl) phosphine hydrochloride (TCEP) was added. After shaking (600 rpm, 1 h, 37°C), 18 µl 0.5 M iodoacetamide was added and the sample was reacted for 45 min in the dark at room temperature. Then 1.2 ml acetone pre-cooled at −20°C was added, and the sample was precipitated at −20°C for 4 h. After high-speed centrifugation (15,000 g, 15 min, 4°C) to remove the supernatant, the precipitate was washed twice with 90% acetone, dried at room temperature, and fully dispersed in 200 µl 0.1 M TEAB solution. The protein was digested (600 rpm, 16 h, 37°C) with 4 µg trypsin (Beijing Wallis Technology Co., Ltd., Beijing, China). The MonoSpin C18 column (Shimadzu, Tokyo, Japan) was used for desalting, and the Pierce quantitative colorimetric peptide assay kit (Thermo Fisher Scientific, MA, United States) was used for peptide quantification.

2.4. LC–MS/MS analysis

All samples were analyzed by an online nanospray Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, MA, United States) coupled with a Nano ACQUITY UPLC system (Waters Corporation, MA, United States). The peptides (10 µg) were resuspended in 30 µl solvent A (0.1% formic acid in water) spiked with 1X iRT peptides (Biognosys AG, Schlieren, Switzerland). Then 1 µg peptides were loaded to an C18 column (Acclaim PepMap, 75 µm × 25 cm, Thermo Fisher Scientific, MA, United States) and separated with a gradient of 60 min (Supplementary Table 1), from 2 to 95% solvent B (0.1% formic acid, 20% water and 80% acetonitrile). The flow rate was 250 nl/min and the column temperature was 40°C.

The 90 individual *Daqu* samples were analyzed by DIA mode LC–MS/MS. The parameters were (1) MS1: resolution = 120,000; scan range (m/z) = 349.5–1500.5; AGC target = 4e5; (2) MS/MS: resolution = 30,000; AGC target = 50,000; maximum injection time = 30 ms; collision energy = 32%. Sixty variable windows were set for MS2 acquisition (Supplementary Table 2).

The samples for benchmarking different lysates and cell disruption methods were analyzed by DDA mode LC–MS/MS. The parameters were (1) MS1: resolution = 15,000; scan range (m/z) = 350–1,800; (2) MS/MS: resolution = 15,000; collision energy = 30%. Other parameters were the same as those for DIA.

2.5. MS data analysis

The DIA raw data were analyzed by Spectronaut (Bruderer et al., 2015; version 15.4.210913, Biognosys AG, Schlieren, Switzerland). The data were searched by directDIA against the protein sequence database built from metagenomic sequencing (1,614,586 protein entries) without the need of spectral libraries. Trypsin was used for proteolysis and the maximum number of

missed cleavages was 2. Q-value cut-off at both precursor and protein level was set as 1%. Missing value imputation was set to global. Other parameters were default. To save computing resources, raw data of each *Daqu* type (30 files each) were searched in a separate project, and the results were merged using the SNE Combine function in Spectronaut.

The DDA raw data were analyzed by PEAKS Studio (Lin et al., 2013; version X+, Bioinformatics Solutions Inc., Waterloo, Canada). The mass tolerance of precursor and fragment ion was set as 7 ppm and 0.02 Da, respectively. Carbamidomethylation (C) was set as a fixed modification, while Oxidation (M) and acetylation (K) were specified as variable modifications. The false discovery rate (FDR) cut-off on both peptide and protein level was 1% by using a target-decoy strategy. Proteins were identified based on at least one unique peptide. Other parameters were default.

Protein inference was performed by the software for protein identification and quantification. Only the leading protein (with the strongest evidence and ranked first in the result) in each protein group was taken into consideration in all the subsequent analysis.

2.6. Statistical analysis

Partial least squares discriminant analysis was performed on the protein quantified results using MetaboAnalyst (Pang et al., 2022; version 5.0, https://www.metaboanalyst.ca/, accessed in December 2022). Proteins with >50% missing values were removed and missing values were replaced by 1/5 of min positive values of their corresponding variables. Normalization by sum, log transformation, and auto scaling were conducted. The predictive ability of the model was measured *via* leave-one-out cross-validation.

2.7. Taxonomic analysis

The quantified peptides were subjected to Unipept (Gurdeep Singh et al., 2019; version 4.3, https://unipept.ugent.be/, accessed in May 2022) for taxonomic analysis using the lowest common ancestor approach. Leucine and isoleucine were considered equal. If a taxon has only one peptide, it was removed and the peptide was assigned to the parental taxon. Abundance of each taxon was determined by summing the quantities of all peptides corresponding to the taxon, which was then converted to percentages to the total abundance of the "root" taxon. Taxonomic abundance differences across seasons were determined by Kruskal-Wallis test (p-value < 0.01) followed by pairwise Mann–Whitney U test (p-value < 0.01). In an alternative strategy, the abundance of taxon was determined by the number of identified peptides of the taxon.

Co-occurrence patterns of the genera were analyzed by Spearman's rank correlations. Genera with relative abundance >0.05% and significant correlation (Spearman's correlation > 0.8, p-value < 0.05 with Bonferroni adjustment) were taken into consideration. The igraph R package (version 1.2.7, https://igraph.org/r/) was used to build the correlation networks. Nodes were divided into clusters (densely connected subgraphs) by the Walktrap community finding algorithm (Pons and Latapy, 2005).

2.8. Protein functional analysis

The quantified proteins were annotated with eggNOG (Huerta-Cepas et al., 2019; version 5.0, http://eggnogdb.embl.de/, accessed in May 2022). COG and KO annotations, as well as EC number and taxonomic information were extracted from the eggNOG results.

Differential proteins across seasons were determined by fold change (FC > 2 or FC < 0.5) and Kruskal-Wallis test (adjusted p-value < 0.05) followed by pairwise Mann–Whitney U test (adjusted p-value < 0.05) with the Benjamini-Hochberg adjustment. For each of the dominant taxa assigned to the differential proteins, KEGG enrichment analysis was perform using the clusterProfiler R package (Wu et al., 2021; version 4.2.0, https://github.com/YuLab-SMU/clusterProfiler). The KO numbers matched with the differential proteins were used as the gene list of interest. The KEGG entries of all the organisms that belong to the taxon and are available in the KEGG database (Kanehisa et al., 2017; https://www.genome.jp/kegg/, accessed in May 2022) were used as the background genes. The p-values were adjusted using the Benjamini-Hochberg method.

2.9. Data processing and visualization

Data processing and statistics were performed using Python (version 3.9.7, Anaconda distribution version 2021.11, https://www.anaconda.com/) and R (version 4.0.2, Microsoft R Open distribution, https://mran.microsoft.com/open). Gephi (version 0.9.5, https://gephi.org/) was used for network visualization. AntV G2 (version 3.2.7, https://g2.antv.vision/) was used to plot the cladograms illustrating abundance of taxa. The volcano3D R package (Kobayashi et al., 2019; version 1.3.2, https://github.com/KatrionaGoldmann/volcano3D) was used to generate the polar and volcano plots of the differential proteins. The R packages ggplot2 (version 3.3.5), ggpubr (version 0.4.0), and VennDiagram (version 1.6.20) were used for other data visualization.

3. Results and discussion

3.1. Metaproteome profiles of white, yellow, and black *Daqu*

We collected 10 samples in each season (i.e., spring, summer, and autumn) for each type of *Daqu* (i.e., white, yellow, and black *Daqu*), bringing the total to 90 samples (Figure 1). The making process of *Daqu* and the sampling time is illustrated in Supplementary Figure 1. To build a protein sequence database for metaproteomics analysis, the 10 samples of each season and type were mixed into a pooled sample (i.e., 9 pooled samples in total), which was subjected to metagenomics sequencing. The assembled genomes were translated to amino acid

sequences to form a sample-specific protein sequence database (1,614,586 protein entries). It is a challenge to extract proteins from *Daqu* due to the existence of complicated matrices of raw materials. We compared different protein extraction methods. The borax/polyvinylpolypyrrolidone/phenol (BPP) lysate (Wang et al., 2007) and mechanical grinding showed the best performance (Supplementary Note 1; Supplementary Figure 2). Then, the 90 individual samples were treated with the optimized protein extraction method, and analyzed by DIA-based liquid chromatography–tandem mass spectrometry (LC–MS/MS). The DIA data were searched against the sample-specific database using directDIA, resulting in 39,396 peptides of 12,155 proteins identified and quantified in total (Figures 2A,B). The three types of *Daqu* shared 37% of the total proteins. White *Daqu* contained the largest number of proteins (8,304, 68%), while black *Daqu* contained the least number of proteins (7,325, 60%). At the peptide level, the trend of identification numbers across *Daqu* types was consistent with that at the protein level. For each season, PLS-DA was performed using the proteins quantified in ≥50% of the samples. The PLS-DA score plots showed obvious separations among the three types of *Daqu* using the first three components (Supplementary Figure 3). Q^2 was >80% for samples in spring and autumn and slightly lower in summer using the first three components, indicating good predictive ability of the PLS-DA models to distinguish the three types of *Daqu*.

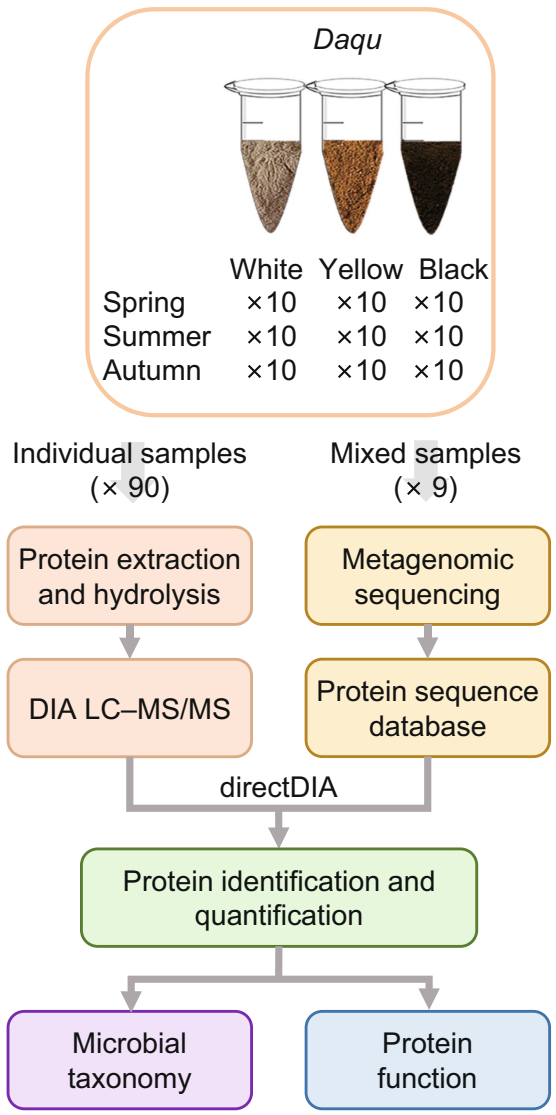

FIGURE 1
Workflow of quantitative metaproteomics analysis of *Daqu* in this study. Metagenomics sequencing was performed on the pooled samples to build the protein sequence database for metaproteomics analysis. The individual samples were characterized by metaproteomics using directDIA.

We further investigated the taxonomy features contributing to the differentiation of the three types of *Daqu*. Taxonomic information was assigned to the quantified peptides using Unipept (Gurdeep Singh et al., 2019). Among the 23,070 annotated peptides, 12,462 were matched to 93 classes, and 7,539 were matched to 449 genera. Relative abundances of taxa were measured by summarizing the (1) intensities or (2) counts of the corresponding peptides annotated to each taxon (Supplementary Data 1), and expressed as percentages to all the annotated peptides (Supplementary Figures 4, 5). Similar results were obtained using the two quantification strategies. The ratio of fungi to bacteria in white *Daqu* was lower than that in yellow and black *Daqu*. White *Daqu* contains more abundant Bacilli, mainly consisting of *Bacillus* and *Oceanobacillus*, than yellow and black *Daqu*. Conversely, Eurotiomycetes, including *Kroppenstedtia* and *Aspergillus*, were less abundant in white *Daqu* (Figure 2C). We further measured the alpha diversity of the genera in *Daqu* microbial communities. The Shannon indexes of white *Daqu* were significantly higher than those of yellow and black *Daqu* in each season (Figure 2D; Supplementary Figure 6). This result was inconsistent with the previous metagenomics study that no significant difference of microbial diversity was observed among the three types of *Daqu* (Wang et al., 2021). It is reasonable that taxonomic abundances based on metagenomics and metaproteomics are different because of divergences between genetic potential and expressed functional activity (Tanca et al., 2017). The quantification based on metagenomics is closely related to the relative cell number, while the quantification based on metaproteomics is closely related to the protein amount of a taxon. The elevated proportion of fungi and the decreased taxonomic diversity in yellow and black *Daqu* is probably a result of the higher fermentation level.

We then explored the co-occurrence patterns of *Daqu* microbial communities based on Spearman's rank correlations (Supplementary Data 2). In the microbial communities of black *Daqu*, 217 significant correlations (edges) were identified from 55 genera (nodes) with an average degree of 7.9 (Supplementary Figure 7). Genera in the Bacillaceae family (e.g., *Oceanobacillus* and *Weizmannia*) exhibited positive correlations. The lactic acid bacteria *Weissella* were positively related with the occurrence of a range of filamentous fungi. Co-occurrence networks of white (188 edges among 44 nodes with an average degree of 8.5) and yellow *Daqu* (217 edges among 58 nodes with an average degree of 8.0) are also shown in Supplementary Figures 8, 9. The largest module of co-occurrence network in white *Daqu* consisted of 22 fungal genera including

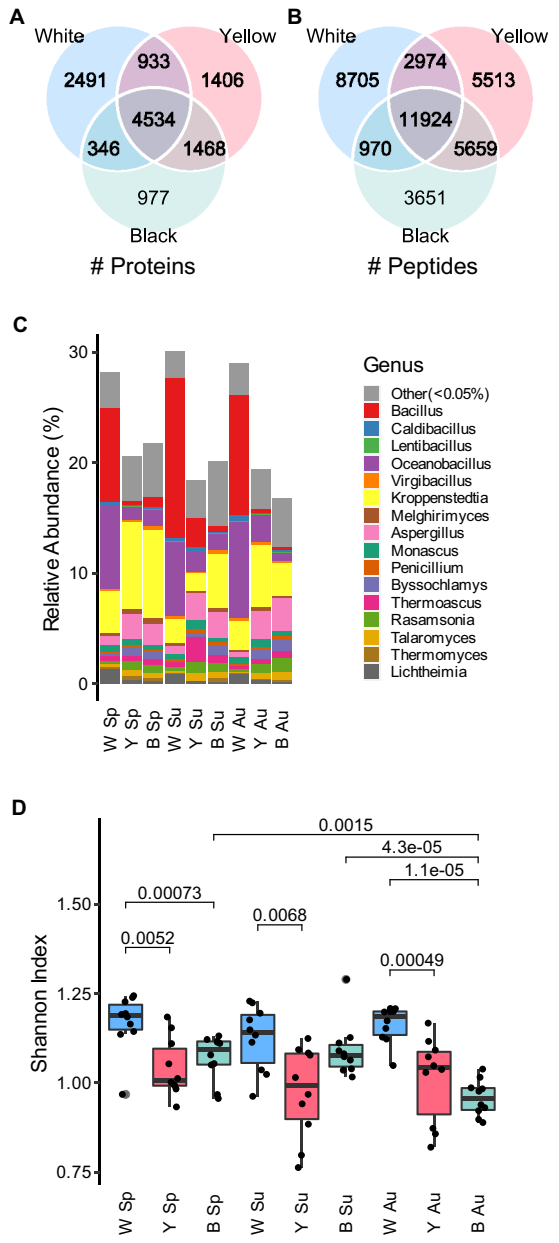

FIGURE 2
Overview of the metaproteomics analysis results of the *Daqu* samples. **(A)** Overlap of the proteins detected in white, yellow, and black *Daqu*. **(B)** Overlap of the peptides detected in the three types of *Daqu*. **(C)** Relative abundances of genera base on peptide quantities in the *Daqu* microbiota. **(D)** Alpha diversity at the genus level. The boxes mark the first and third quantile and the lines inside the boxes mark the median; the whiskers extend from the ends of the inter-quartile range (IQR) to the furthest observations within the 1.5 times the IQR. Individual data points are overlaid as dots. The *p*-values are indicated if they are <0.05. W: white; Y: yellow; B: black; Sp: spring season; Su: summer season; Au: autumn season.

Aspergillus and *Thermoascus*, while that of yellow *Daqu* contained 16 nodes mostly composed of genera in the Bacillaceae family. Despite less nodes and edges, higher average degree in the co-occurrence network of white *Daqu* than those in yellow and black *Daqu*, suggesting closer interconnections and more frequent co-occurrence among microbial taxa in white *Daqu*. White Daqu in the top layer of the starter brick is more sufficiently exposed to air. In contrast, black *Daqu* is distributed in the central part of the starter brick with high temperature and low oxygen tension, inhibiting the growth of non-thermophilic or aerobic microorganisms.

3.2. Taxonomic composition of *Daqu* microbiome across seasons

We next compared the taxonomic abundance of *Daqu* microbial communities in the three different seasons. While no significant change of the Shannon indexes among the three seasons was observed for white and yellow *Daqu*, microbial communities of black *Daqu* presented lower diversity in autumn than the other seasons (Figure 2D).

To explore the taxonomic abundance differences of the microbial communities among the three seasons, fold change (FC) values of the abundances were computed pairwisely between two seasons and Mann–Whitney U test was performed for *p*-value calculation (Supplementary Data 3). The results of black *Daqu* are visualized in Figure 3; Supplementary Figure 10. The abundance of Archaea (summer/spring, FC = 1.52, *p*-value = 0.0073) was higher in summer than that in spring. Bacteria in the phylum Planctomycetes (summer/spring, FC = 2.64, *p*-value = 0.0010; autumn/summer, FC = 0.402, *p*-value = 0.0036), as well as the families Microbacteriaceae (autumn/summer, FC = 0.349, *p*-value = 0.0091), Pseudonocardiaceae (autumn/summer, FC = 0.342, *p*-value = 0.0046), Staphylococcaceae (summer/spring, FC = 2.17, *p*-value = 0.045; autumn/summer, FC = 0.272, *p*-value = 0.0013) and Aerococcaceae (summer/spring, FC = 1.26, *p*-value = 0.0073; autumn/summer, FC = 0.623, *p*-value = 0.038) were more abundant in summer. *Saccharomonospora* and *Saccharomonospora* are two genera in the family Pseudonocardiaceae. *Saccharomonospora* has been reported as one of the main bacteria in *Daqu* (Shang et al., 2022), and can hydrolyze phenolic compounds into a non-toxic form and reduce phytotoxicity (Shivlata and Tulasi, 2015). Previous studies have demonstrated that *Saccharopolyspora* is the third largest dominant microbial clusters in a central black component of *Daqu* (Li et al., 2014), and is essential for generating flavoring substances in the downstream process of liquor production (Gan et al., 2019). *Saccharopolyspora* sp. is known to produce a thermostable alpha-amylase which helps to hydrolyze starch (Chakraborty et al., 2011). *Staphylococcus* in the family Staphylococcaceae has been reported as a biomarker in black *Daqu* in a previous metagenomics study (Wang et al., 2021), and shows the potential to participate in butane-2,3-diol metabolism (Yang et al., 2021).

Yeasts in the family Saccharomycetaceae (autumn/spring, FC = 2.08, *p*-value = 0.00077; autumn/summer, FC = 1.67, *p*-value = 0.011) and Debaryomycetaceae (summer/spring, FC = 2.57, *p*-value = 0.00018; autumn/spring, FC = 1.698959872, *p*-value = 0.0058; autumn/summer, FC = 0.661920859, *p*-value = 0.038) were more abundant in autumn and summer, respectively. *Saccharomyces* in the family Saccharomycetaceae is responsible for the production of ethanol, while non-*Saccharomyces* yeasts are responsible for the production of different kinds of esters as flavoring compounds (Zou

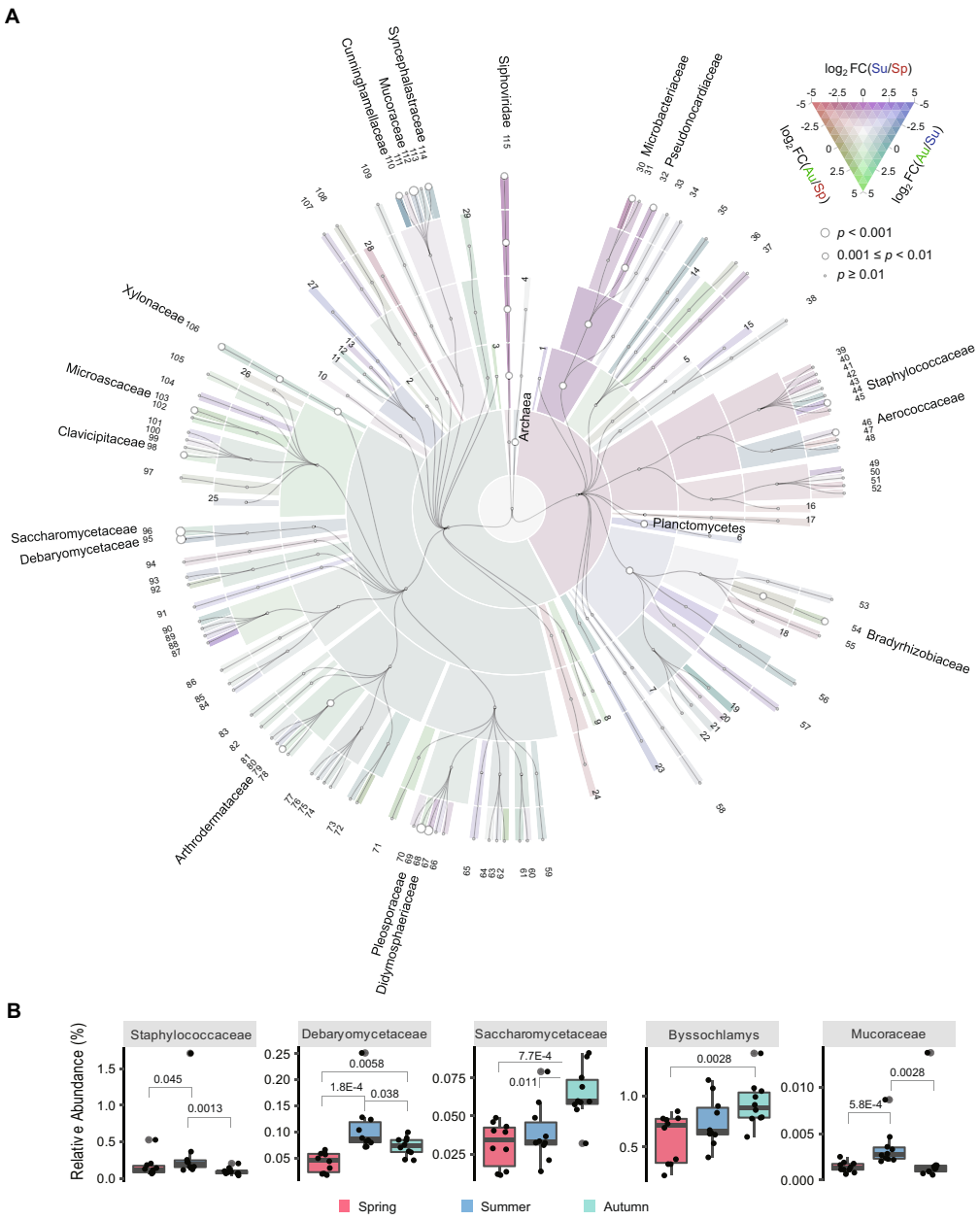

FIGURE 3
Taxonomic abundances of the black *Daqu* microbiota across seasons. **(A)** Cladogram illustrating abundance of taxa (domain to family). Colors indicate the log2 fold change (FC) between each pair of seasons; circle sizes indicate the minimum *p*-value of the three comparisons by pairwise Mann–Whitney U test. Names of the taxa are indicated if their abundance differences were observed (*p*-value<0.01) in any comparison. Information of the taxa with the labeled numbers are shown in Supplementary Data 3. **(B)** Boxplots showing the abundance of the differential taxa. The boxes mark the first and third quantile and the lines inside the boxes mark the median; the whiskers extend from the ends of the inter-quartile range (IQR) to the furthest observations within the 1.5 times the IQR. Individual data points are overlaid as dots. The *p*-values are indicated if they are <0.05.

et al., 2018). *Debaryomyces* sp. has been reported promoting the generation of ethyl hexanoate in liquor producing environment (Tao et al., 2013).

Fungi in Cunninghamellaceae (summer/spring, FC = 5.50, *p*-value = 0.0046; autumn/summer, FC = 0.505, *p*-value = 0.0022), Mucoraceae (summer/spring, FC = 2.45, *p*-value = 0.00058; autumn/summer, FC = 0.694, *p*-value = 0.0028), and Syncephalastraceae (summer/spring, FC = 2.78, *p*-value = 0.0013; autumn/summer, FC = 0.811, *p*-value = 0.0028) were more abundant in summer, while fungi in *Byssochlamys* (autumn/spring, FC = 1.59, *p*-value = 0.0028) were more abundant in autumn. Molds in the family Mucoraceae are known to be strong amylase producers in amylolytic Asian fermentation starters, and have been isolated and identified in *Daqu* in a previous study (Zheng, 2015). *Byssochlamys* is potentially involved in flavor-relevant pathways like biosynthesis of guaiacol (Yang et al., 2021). We also found other fungi with significant abundance differences across seasons, but their roles in fermentation and liquor production await further investigation. Among all the 17

families showed significant difference among seasons in black *Daqu*, 10 were the most abundant in summer and 7 were the most abundant in autumn, indicating that black *Daqu* produced in the two seasons can contain more microbes for fermentation.

Taxonomic abundance differences among the three seasons were also explored for white and yellow *Daqu*. Only a few taxa were significantly changed across seasons in white *Daqu* (Supplementary Figure 11), indicating that the microbial community structure of white *Daqu* was more stable across seasons. Differential taxa of yellow *Daqu* were hardly overlapped with those of black *Daqu* (Supplementary Figures 12; 13). The results demonstrated the distinct differences in seasonal microbial variation across the three types of *Daqu*.

3.3. Quantification of key microbial enzymes related to saccharification process

Based on previous studies on the saccharification process of Chinese liquor brewing (Gan et al., 2019; Fan et al., 2021; Xia et al., 2022), we selected the key enzymes involving in the degradation of biomacromolecules in raw materials, including starches, celluloses, and proteins (Figure 4). Alpha-amylases (EC 3.2.1.1) can break large starches into dextrin and a small amount of maltose during the fermentation process. Alpha-glucosidases (EC 3.2.1.20) and glucoamylases (EC 3.2.1.3) can release glycose from maltose and dextrin. Cellulases are a group of enzymes that can degrade cellulose into glucose, including cellulose 1,4-beta-cellobiosidases (EC:3.2.1.91) breaking cellulose and cellodextrin into cellobiose, and beta-glucosidase (EC 3.2.1.21) releasing glycose from cellodextrin and cellobiose. Proteases like saccharopepsin (EC 3.4.23.25) and aspergillopepsin (EC 3.4.23.18) can carry out protein hydrolysis, which can provide various amino acids as nitrogen sources for the growth of microbes.

The quantified microbial enzymes among the key enzymes were clustered using their abundance patterns measured by quantitative metaproteomics (Figure 4A; Supplementary Figure 14; Supplementary Data 4), where the proteins were annotated using eggNOG (Huerta-Cepas et al., 2019). Except that alpha-amylases of Basidiomycota and glucoamylase of Saccharomyceta were more abundant in white *Daqu*, most of the enzymes were more abundant in yellow *Daqu*. Alpha-amylases and alpha-glucosidases of Eurotiales, as well as alpha-glucosidases of Saccharomyceta were more abundant in spring compared to summer and autumn in yellow and black *Daqu*. The cellulases and proteases of Eurotiales and Onygeles, as well as alpha-amylases, glucoamylases and alpha-glucosidases of other fungi were more abundant in summer yellow *Daqu*. The results suggest that yellow *Daqu* in summer probably has higher saccharifying power for raw material degradation.

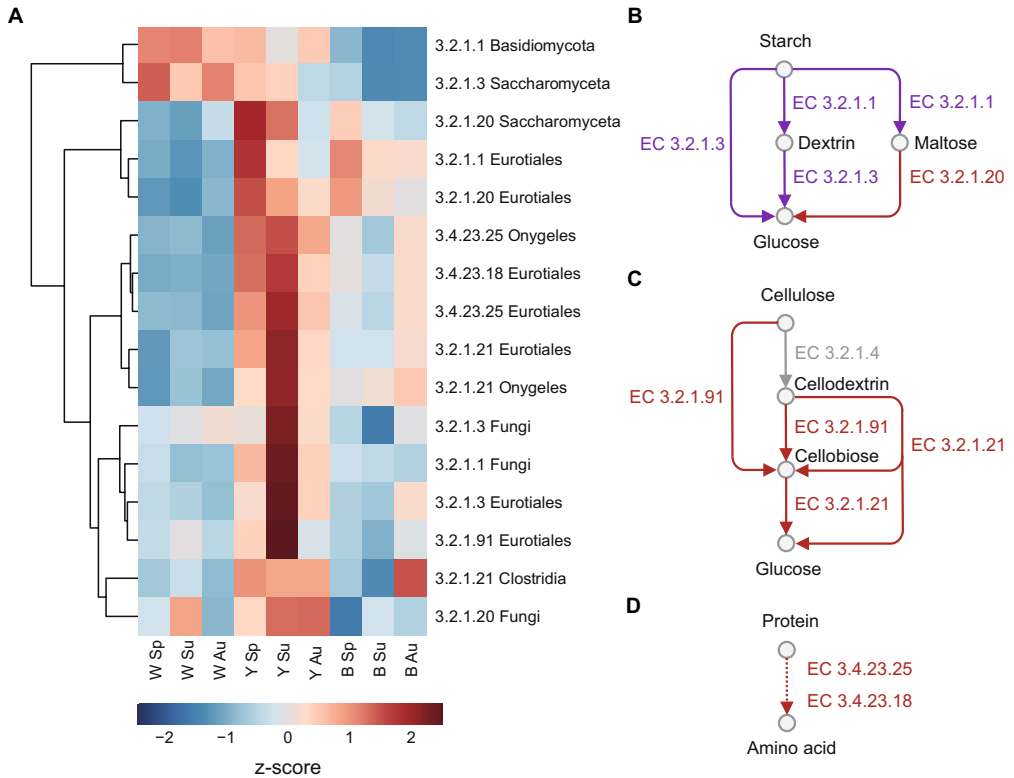

FIGURE 4
Key enzymes related to saccharification process in *Daqu*. **(A)** Heatmap in relative abundance of the enzymes of different taxa. Abundances are normalized to z-scores, which are in the units of standard deviation from the mean. **(B–D)** Enzymes related to the hydrolysis pathway of **(B)** starch to glucose, **(C)** cellulose to glucose, and **(D)** protein to amino acids. The red arrows are enzymes more abundant in yellow *Daqu*, while the purple arrows are enzymes with divergent abundance patterns among taxa. The one in grey was not detected in this study.

3.4. Functional characteristics of altered microbial proteins across seasons

To explore the functional feature of *Daqu* microbiota across seasons, differential proteins between each pair of seasons were determined by abundance FC (>2 or <0.5) and Mann–Whitney U test (p-value < 0.05 with the Benjamini-Hochberg adjustment). While almost no significantly differential protein was observed in white and yellow *Daqu* across seasons, 1,082 proteins in black *Daqu* exhibited significant differences between at least one season pair (Figure 5A; Supplementary Figure 15). Among them, 1,057 were significantly more abundant in autumn, 11 were significantly more abundant in spring, and 7 were significantly more abundant in summer, compared to at least one of the other two seasons. Moreover, 7 proteins were significantly more abundant in both spring and autumn compared to summer. Among the differential

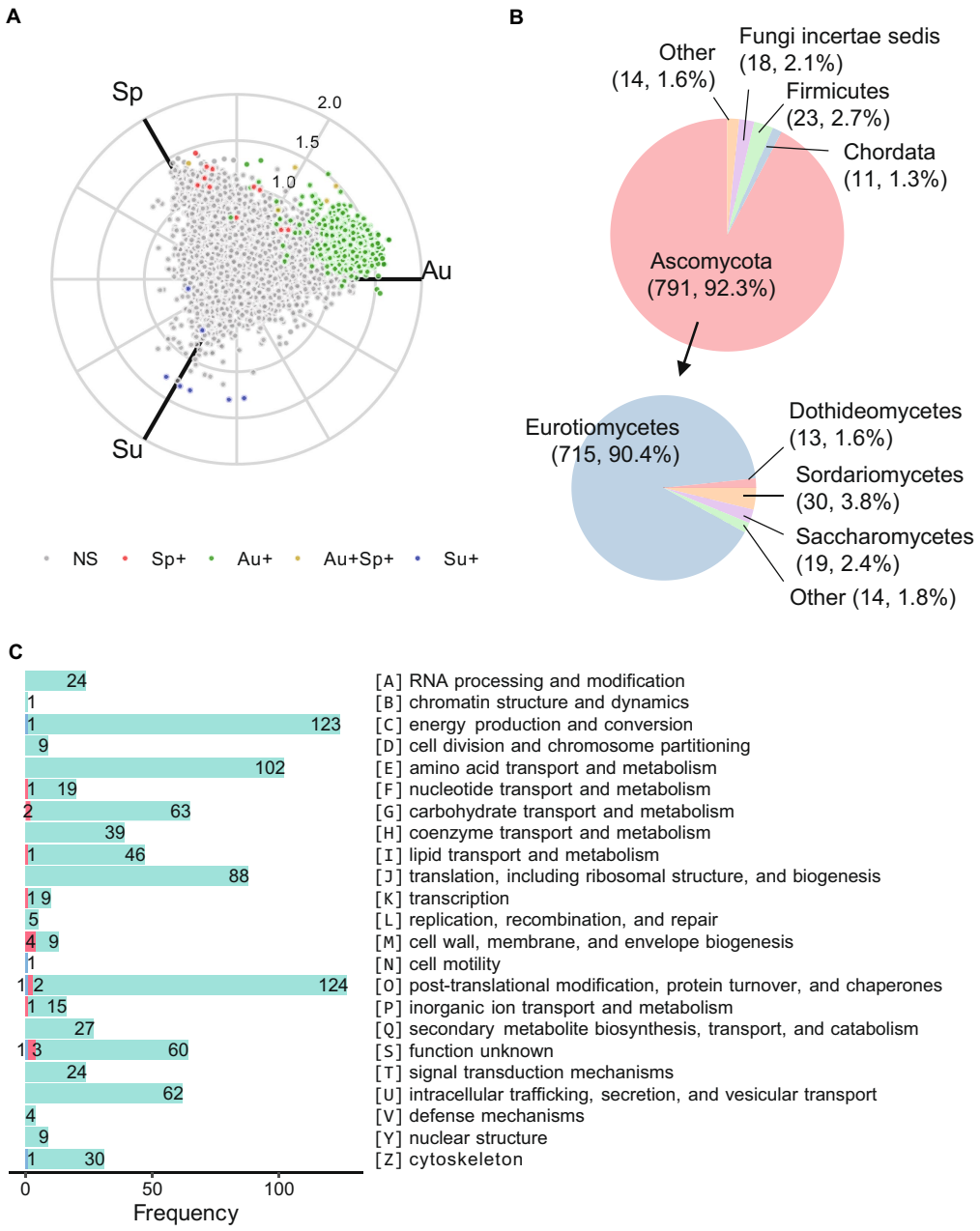

FIGURE 5
Functional annotations of the differential proteins in black *Daqu* microbiota across seasons. **(A)** Three-axis polar plot indicating the differential proteins across seasons. Proteins are color-coded for significant higher abundance in different comparisons with fold change (FC)>2 and adjusted p-value<0.05 by pairwise Mann–Whitney U test. NS: not significant; Sp+: significant higher abundance in spring compared to either of summer and autumn or both; the rest are in the same manner. **(B)** Distribution of taxonomy assigned to the differential proteins. **(C)** Numbers of the differential proteins in each category of clusters of orthologous groups (COG). Proteins more abundant in spring, summer, and autumn are in red, blue, and green, respectively. Sp: spring season; Su: summer season; Au: autumn season.

proteins, 857 were annotated with taxonomy information by eggNOG (Supplementary Data 5). The phylum Ascomycota (92.3%) and Firmicutes (2.7%) accounted for large proportions of the differential proteins, and the differential proteins in Ascomycota mainly belonged to the classes Eurotiomycetes (90.4%), Sordariomycetes (3.8%), and Saccharomycetes (2.4%; Figure 5B). The differential proteins were annotated into 23 categories of clusters of orthologous groups (COG; Figure 5C; Supplementary Figure 15). The top three categories were O (post-translational modification, protein turnover, chaperones), C (energy production and conversion), and E (amino acid transport and metabolism).

The differential proteins were mapped to metabolic pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa et al., 2017) and pathway enrichment analysis was performed (Supplementary Figure 16; Supplementary Data 6). The enriched pathways in autumn black *Daqu* microbiota were mainly involved in carbon metabolism and amino acid metabolism, which is consistent with the COG annotation result. As shown in Figure 6; Supplementary Data 7, enzymes involving glycolysis and the citrate cycle of Eurotiomycetes were more abundant in autumn black *Daqu* to consume glucose released from starch and cellulose degradation. Alcohol dehydrogenases of Saccharomycetes were elevated to produce ethanol. Some enzymes involving the citrate cycle were also increased in Sordariomycetes and Bacilli. The results suggest that autumn black *Daqu* has better potential for carbohydrate degradation and flavor compounds metabolism than the other seasons.

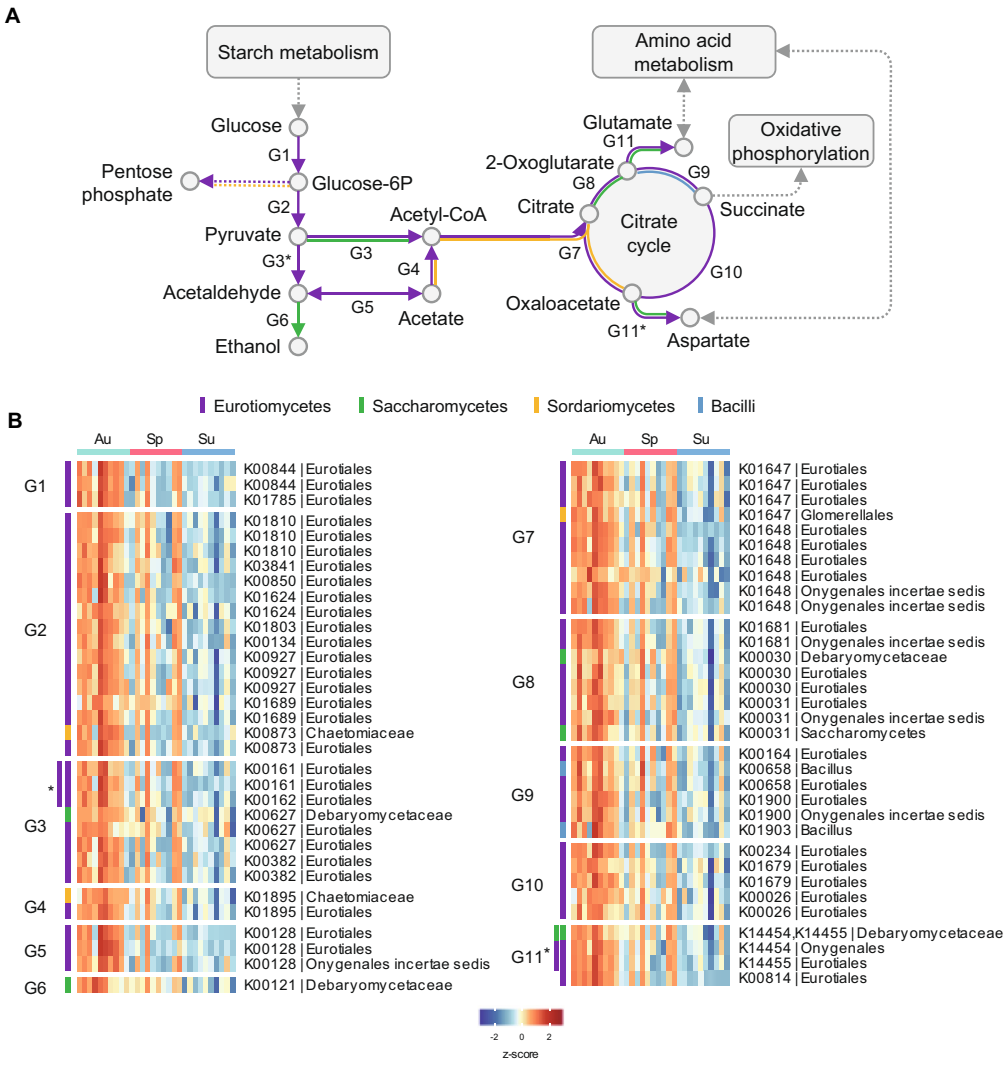

FIGURE 6
Differential proteins related to the carbohydrate metabolism pathway in black *Daqu* microbiota across seasons. **(A)** Pathway diagram of carbohydrate metabolism. **(B)** Heatmap of the significantly changed microbial proteins. Abundances are normalized to *z*-scores. Proteins are grouped by their related metabolic steps (G1, G2, etc.) in **(A)**. Proteins in the subgroup G3* are related to the metabolic steps G3 and G3*, while other proteins in G3 are not related to the metabolic step G3*; the rest are in the same manner. Proteins are color-coded for different taxa.

4. Conclusion

We used DIA-based quantitative metaproteomics to reveal the microbial profiles of a cohort of different types of *Daqu* across seasons. Taxonomic composition varied in the microbial communities of different types of *Daqu*, where the proportion of lactic acid bacteria and microbial diversity reduced in yellow and black *Daqu* compared to the under-fermented white *Daqu*. Higher abundance of key enzymes such as alpha-amylases, cellulases, and proteases suggested the higher saccharifying power for raw material degradation of microbes in yellow *Daqu*, which is indeed the main part of the starter used for sauce-flavor liquor production. Seasonal changes of the microbial communities of different types of *Daqu* were also explored. While the under-fermented white *Daqu* had relatively stable microbial community structure, abundances of many taxa were significantly changed across seasons in yellow and black *Daqu*. In addition, abundance of microbial proteins exhibited considerable variation in the over-fermented black *Daqu*, where the abundance of proteins involved in carbohydrate and amino acid metabolism elevated in autumn. The unique microbial characteristics of different types of *Daqu* and their seasonal features are anticipated to guide the maintenance of yield, quality, and flavor in liquor production. Further studies are needed to profile the *Daqu* microbial communities in time series of the fermentation process and with specific environmental factors, towards a deeper understanding of the traditional *Baijiu* brewing technique. We also expect that the DIA-based quantitative metaproteomics method can boost the study of other, diverse fermenting foods.

Data availability statement

All raw MS data and search results, as well as the protein sequence database generated in this study have been deposited to the ProteomeXchange via the iProX (Ma et al., 2019) partner repository with accession numbers PXD036834 (http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PXD036834) or IPX0005077000 (https://www.iprox.cn/page/project.html?id=IPX0005077000). The raw metagenomic sequence data have been deposited to the Genome Sequence Archive (Chen et al., 2021) in National Genomics Data Center (CNCB-NGDC Members and Partners, 2022), China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences with accession number CRA008200 (https://ngdc.cncb.ac.cn/gsa/browse/CRA00820). Custom scripts for data processing and visualization in this paper are available at Github (https://github.com/lmsac/metaproteomics-utilities).

Author contributions

JZha performed the majority of the experiments, analyzed the data, and wrote the first draft of the manuscript. YY analyzed the data and revised the manuscript. LC and XL collected the *Daqu* sample. JZhe, DL, and ZF assisted in the microbial and proteomic experiments. CS analyzed the MS data. VM and YL performed the whole genome sequencing data analysis. SY provided the MS resources for data collection. FY, LW, and LQ designed the study. LW and LQ supervised all aspects of the study and finalized the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

LC, XL, FY, and LW are employed by Kweichow Moutai Group. CS is employed by Shanghai Omicsolution 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.2022.1098268/full#supplementary-material

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Isolation and characterization of lactic acid bacteria with potential probiotic activity and further investigation of their activity by α-amylase and α-glucosidase inhibitions of fermented batters

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Probiotic microbiota plays a vital role in gastrointestinal health and possesses other beneficial attributes such as antimicrobial and antibiotic agents along with a significant role in the management of diabetes. The present study identifies the probiotic potential of *Lactobacillus* spp. isolated from three traditionally fermented foods namely, jalebi, medhu vada, and kallappam batters at biochemical, physiological, and molecular levels. By 16S rRNA gene amplification and sequencing, the isolates were identified. A similarity of >98% to *Lacticaseibacillus rhamnosus* RAMULAB13, *Lactiplantibacillus plantarum* RAMULAB14, *Lactiplantibacillus pentosus* RAMULAB15, *Lacticaseibacillus paracasei* RAMULAB16, *Lacticaseibacillus casei* RAMULAB17, *Lacticaseibacillus casei* RAMULAB20, and *Lacticaseibacillus paracasei* RAMULAB21 was suggested when searched for homology using NCBI database. Utilizing the cell-free supernatant (CS), intact cells (IC), and cell-free extract (CE) of the isolates, inhibitory potential activity against the carbohydrate hydrolyzing enzymes α-glucosidase and α-amylase was assessed. CS, CE, and IC of the isolates had a varying capability of inhibition against α-glucosidase (15.08 to 59.55%) and α-amylase (18.79 to 63.42%) enzymes. To assess the probiotic potential of seven isolates, various preliminary characteristics were examined. All the isolates exhibited substantial tolerance toward gastrointestinal conditions and also demonstrated the highest survival rate (> 99%), hydrophobicity (> 65%), aggregation (> 76%), adherence to HT-29 cells (> 84%), and chicken crop epithelial cells suggesting that the isolates had a high probiotic attribute. Additionally, the strains showed remarkable results in safety assessment assays (DNase and hemolytic), and antibacterial and antibiotic evaluations. The study concludes that the lactic acid bacteria (LAB) characterized possesses outstanding probiotic properties and has antidiabetic effects. In order to obtain various health advantages, LAB can be utilized as probiotic supplements.

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lactic acid bacteria, gastrointestinal tract, anti-hyperglycaemic, probiotics, α-Glucosidase, α-Amylase

Introduction

Diabetes mellitus (DM) claims more lives than several other disorders known to mankind. It is found that one person dies from DM every 10 s, making it a significant contributor to death from a long-term illness. Due to the staggering rise in diabetes worldwide, it has now been established as a global epidemic causing a huge burden to the healthcare and economy of most developed and developing countries. Recent predictions worldwide indicate a rise in DM in adults reaching nearly 380 million by 2025 from 194 million in 2010, with the most affected countries being India, China, and the United States (Kaul et al., 2012). Diabetic individuals lose control over their blood glucose levels, which leads to both short and long-term complications owing to the presence of glucose in circulation for an extended period of time. DM is a common disorder with little knowledge of its etiology, which is most likely due to a number of genetic and environmental factors contributing to the heterogeneity of type 2 diabetes (Sreepathi et al., 2022). The most prospective targets for the treatment of DM include inhibition of the enzymes α-glucosidase and α-amylase which constitute a means to prevent hyperglycemia. The two enzymes are involved in the breakdown of complex carbohydrates into their constituents in the proximal intestinal brush border. If they are inhibited, it leads to a delay in the absorption of carbohydrates and reduced postprandial glucose excursions. As a result of the increase in blood sugar, there is a reduction in postprandial insulin secretion (Fonseca and John-Kalarickal, 2010). There are several pharmacological drugs available that can be used to intensify the treatment. These include insulin sensitizers-biguanide, metformin and thiazolidinediones, insulin secretagogues-sulfonylureas, and non-sulfonylurea secretagogues, (GLP-1) agonists, DPP4 inhibitors, and pramlintide, an analogue of the peptide amylin that the beta cell co-secretes with insulin, is commonly recommended for use with insulin in both type 1 and type 2 diabetes (Alam et al., 2014). The consumption of these pharmacological drugs as therapy has its own long-term side effects such as renal impairment, cardiovascular diseases, loss of appetite, fluid retention, frequent gastrointestinal (GI) tract infection, etc. In relation to this, the gut microbiota is essential for maintaining a number of diabetic metabolisms (Leu and Zonszein, 2010). Altering the gut flora to attain or maintain a favorable condition is advisable for the improvement of the host's health. When compared to the other commercially accessible medications, using this as therapy has lesser-known negative effects. These live microorganisms that are introduced into the body for their beneficial properties are commonly referred to as probiotics (Kumari et al., 2022a). A change in the GI ecology can be facilitated by the administration of these live microorganisms. There is substantial evidence that probiotics can interact with gut-associated lymphoid tissue, restrict the growth and adhesion of potentially pathogenic organisms, and influence both mucosal immunity and systemic immunity (Qin et al., 2005). The incorporation of such probiotic microbiome through fermented food sources has been demonstrated to be economically efficient worldwide because the raw ingredients used are easily accessible, have low economic value, and appetizing in their raw state (Soni and Dey, 2014). The process of fermentation has been practiced worldwide for enhancing nutrients and making them more accessible while retaining and boosting the amounts of many beneficial bioactive compounds (Guan et al., 2021). It also enhances the product's sensory qualities in addition to eliminating unwanted components. The macro-and micronutrient balance of fermented foods made from cereals could be improved by co-fermenting cereals and legumes to create inexpensive, protein-rich diets (Gupta and Tiwari, 2014). It is interesting to note that indigenous communities and ethnic groups have been practicing lactic acid fermentation to preserve seasonal and perishable vegetables and fruits (Nag and Das, 2013). In developing nations, backslopping and spontaneous fermentation are still used to produce fermented foods and beverages today, while large-scale manufacturing of fermented foods has grown to constitute a significant sector of the global food business in wealthier nations (Wang et al., 2018). The great gourmet attributes of traditionally fermented foods are valued by consumers worldwide for their exceptional health-benefiting qualities (Kariyawasam et al., 2021).

Contrarily, the fermentation of traditionally fermented foods is commonly brought on by naturally occurring, wild varieties of LAB that are derived from the raw material, the processing equipment, or the environment and that start the fermentation process in the absence of a commercial starter (Franz et al., 2014). It is also noted that pure strains isolated from the intricate ecosystems of traditionally fermented foods exhibit a wide range of metabolic activities that greatly differ from those of equivalent strains employed as bulk starters for commercial processes (Silva et al., 2004). Such findings emphasize the significance of the Designation of Protected Origin (DPO) for many of these goods, which is essential from an economic perspective as they let small-scale fermentation units survive in a world of ongoing globalization (da Silva Duarte et al., 2022).

In recent years, it has become popular to separate wild-type strains from conventional products to utilize them as starter cultures for food fermentation as they possess numerous benefiting attributes in enhancing the food grade levels (Carminati

et al., 2010). Indian cuisine on a daily basis consists of a wide variety of traditionally fermented foods made of cereals like rice, wheat, ragi, etc., along with other various legumes that are consumed in large quantities. Idli, and dosa, are the main rice-based overnight fermented south Indian cuisines among the various options (Rao et al., 2013). Where such traditionally fermented food products are majorly fermented by a wild variety of LAB species (Satish Kumar et al., 2013). Lactic acid bacteria (LAB) are well-known probiotics worldwide; when consumed, LAB provides numerous health benefits for the host. Incorporation of these probiotics into the gut through consumption is one of the safest and most modern approaches aiding to their health benefits (Lye et al., 2017). With a long history of use and safe consumption in the development of fermented foods and beverages, the LAB species plays a key role in fermentation. Probiotics are habitually found in dairy products that have been fermented with lactic acid bacteria (LAB), particularly yogurt (Savaiano and Hutkins, 2021). During the production of yogurt, lactose is transformed into lactic acid by a yogurt culture until a final pH of 4.2 to 4.5 is reached. The pH may go to 4.0 after storage. *Lb. delbrueckii subsp. bulgaricus* is implicated in this unfavorable post-acidification, which results in an acidic and bitter flavour. Since these cells can only grow in the presence of actively lactose-fermenting *S. thermophilus* cells due to their protocooperation, lactose-negative mutations of *Lb. delbrueckii subsp. bulgaricus* allow for the creation of mild yogurt (Gilbert et al., 1996). Due to the actions of endogenous milk enzymes as well as the proteolytic and lipolytic activities of LAB present in the cheese, many aromatic compounds are produced during cheese maturation (Fox and McSweeney, 1996). LAB enhances the flavor and aroma of fermented commodities. They create aromatic molecules from amino acids after further bioconversion, acidify the meal, giving it a tart lactic acid flavor, and frequently engage in proteolytic and lipolytic activities (Yvon and Rijnen, 2001).

It has been a technological challenge for the food industry to develop probiotic dairy and fermented products to maintain the stability of LAB and probiotics from manufacturing to consumption. The market preference for foods that are fresh, safe, delicious, low in sugar, fat, and salt, and simple to prepare seems to be in conflict with the demand for less processing and additive use. The connection between food and health is a major concern for consumers today. As a result, the market for foods with health-promoting qualities, often termed functional foods, has experienced an impressive expansion in recent years (Granato et al., 2020). These market shifts put pressure on the food industry to find alternatives. In food fermentation, one of the most important areas for intervention appears to be at the level of the starter culture. The commercial availability of novel intriguing starter cultures is constrained, and industrial starter cultures regrettably lack the required traits for product diversity (Tsuda, 2018). An improved understanding of the food microorganisms' genomes and metabolomics gives up possibilities through molecular techniques. The probiotic LAB isolated from such traditional fermented sources has proven to exhibit anti-diabetic, anti-microbial, and antibiotic activities (Kim and Lim, 2019). In addition to the health-promoting effects, LAB isolates must exhibit certain characteristics to prove their probiotic attributes. Initial traits of the probiotic bacteria include bile and acid tolerance, adhesion to host epithelial cells, and safety assessment. However, the benefits of this probiotic biotherapy have been poorly elucidated (Harun-Ur-Rashid et al., 2007). In this regard, the present study was designed to determine the antidiabetic attributes of the probiotic LAB species isolated from traditionally fermented - jalebi, medhu vada, and kallappam batters (Muganga et al., 2015; Alkalbani et al., 2019; Cai et al., 2019; Jeong et al., 2021). The southern part of India has seen various health benefits from consuming these foods regularly, but the findings are poorly elucidated. Therefore, our study mainly focuses on the evaluation of the probiotic attributes and the ability to inhibit the carbohydrate hydrolyzing enzymes α-glucosidase and α-amylase of such traditionally fermented sources (Kwun et al., 2020). The ideally discovered cultures can also be used as starting cultures in the food fermentation sector which later can improve food safety and/or provide a variety of organoleptic, technical, nutritional, or health benefits. Implementing these chosen strains as starter cultures or co-cultures in fermentation processes can assist in achieving *in situ* expression of the desired attribute, preserving a completely natural and healthy result.

Materials and methods

Materials

Lactobacillus de Man, Rogosa, and Sharpe (MRS) agar and broth, oxgall salt glycerol, phenol, NaCl, xylene, deoxyribonuclease (DNase) agar medium, blood agar medium with 5% (w/v) sheep blood, ABTS, DPPH, and, antibiotic susceptibility disc were procured from HiMedia Laboratories Pvt. Ltd., Mumbai, India. The pathogens namely, *Bacillus subtilis* MTCC 10403, *Escherichia coli* MTCC 4430, *Pseudomonas aeruginosa* MTCC 424, *Micrococcus luteus* MTCC 1809, and *Salmonella typhimurium* MTCC 98 were procured from the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India.

Bacterial isolation from the fermented batters

Rice and pulse-based batters of various traditional fermented foods like jalebi, medhu vada, and kallappam were freshly prepared, in the month of April at room temperature (37°C) in Mysuru, Karnataka, India. For the jalebi batter, a 1:4 proportion of chickpea flour and all-purpose flour was mixed together and a pinch of baking soda was added to enhance the fermentation process. For medhu vada 1:6 proportion of rice flour and soaked black gram was blended in a mixer to batter consistency, whereas for Kallappam a 1:8:4 proportion of toddy,

semolina, and coconut paste, respectively, was mixed into a thick batter consistency. All the above batters were allowed to ferment overnight. No additional cultures were added into the batters for fermentation enhancement. The overnight fermented batters were then serially diluted (phosphate saline 0.1 M, pH 7.2) and pour-plated on a solidified MRS agar plate. Anaerobic incubation was performed at 37°C for 24–48 h. The colonies with various morphological characteristics were selected and pure cultures were streaked onto the MRS agar plate. The colonies were isolated, sub-cultured in MRS broth, and stored at 4°C. For further investigation, the isolated strains were prepared as an extract (CE), and supernatant (CS) as per the methodology mentioned by Jo et al. (2021).

Biochemical characterization

The isolates were preliminarily characterized in accordance with the guidelines from Bergey's Manual of Determinative Bacteriology. All initially screened isolates were tested for tolerance at different temperatures (4, 10, 37, 45, and 50°C), salt (2, 4, 8, and 10%), pH (2, 4, 6, and 8) concentrations, and carbohydrate fermentation against 10 sugars (Table 1) and evaluated (Fitriani et al., 2021).

Evaluation of probiotic attributes *in vitro*

Acid and bile salt

The methodology described by Pan et al. (2009) was used to execute the acid and bile salt tolerance studies, with a few minor adjustments. One hundred microliter of LAB isolates were inoculated into the MRS broth (with pH 2 and (0.3 and 1%) oxgall salt) and incubated at 37°C. The samples were enumerated at 0,2, and 4 h of incubation. The following formula was used to determine the survival rate (%):

$$\text{Survival rate}(\%) = \left[\text{Biomass at time}(t) / \text{Biomass at initial time}(0)\right] \times 100.$$

Simulated gastrointestinal conditions

When ingested, the isolates must withstand the gastric and intestinal conditions for up to 3 and 8 h, respectively in accordance with a healthy digestive process. The assay was performed by the methodology mentioned by Musikasang et al. (2009) with slight modification. Pepsin (3 g/l of PBS, pH 3; 1:3000 AU/mg, Sisco Research Laboratories Pvt. Ltd., Mumbai, India) and trypsin (1 g/l of PBS, pH 8; 2000 U/g, Sisco Research Laboratories Pvt. Ltd., India) were dissolved to prepare simulated gastric and simulated intestinal juice conditions. Under gastrointestinal conditions, the isolates (10^9 CFU/ml) were subsequently inoculated into the juices in an *in vitro* condition. The tolerance of the isolates towards gastrointestinal conditions was evaluated using viable colony counts. The following equation was used to determine the percentage of survival (%):

$$\text{Survival rate}(\%) = \left[\log \text{CFU } N_1 / \log \text{CFU } N_0\right] \times 100.$$

where N_1 = Number of viable cells after treatment and N_0 = Number of viable cells before treatment.

Phenol

The experimental approach by Jena et al. (2013) was used to evaluate the viability and survival rate of the isolates in the presence of phenol solution by inoculating the LAB isolated (10^8 CFU/ml) in MRS broth containing 0.4% phenol (24 h, 37°C). By serial dilution onto the MRS agar plate, the bacterial enumeration was performed at 0 h and 24 h of the experiment.

Cell adherence assays

Cell surface hydrophobicity

The LAB isolates were examined against the polar solvent xylene at 600 nm to understand their cell surface interaction using the previously available approach by Li et al. (2020). In order to aid the separation of the two phases, 1 ml of xylene and 3 ml of LAB cell suspension (10^8 CFU/ml) were mixed in a test tube and allowed to settle for 2 h at 37°C. The absorbance of the aqueous phase was calculated by the equation given below:

$$\text{Hydrophobicity}(\%) = \left[1 - \left(A / A_0\right)\right] \times 100.$$

where, A = final absorbance of the aqueous phase, A_0 = initial absorbance.

Autoaggregation

The autoaggregation of the isolates was performed as per the methods followed by Tareb et al. (2013) at an absorbance of 600 nm. Briefly, the 18 h cultured cells were harvested and resuspended in PBS (10^8 CFU/ml) and evaluated for their ability to aggregate to the cells. Autoaggregation percentage was calculated for time intervals of 0, 2, 4, 6, 10, and 24 h using the equation:

$$\text{Autoaggregation}(\%) = \left[1 - \left(A_t / A_0\right)\right] \times 100.$$

where, A_t and A_0 denote absorbance at the time "t" and "0" (initial), respectively.

Coaggregation

Cell suspension for LAB isolates was taken in the ratio of 2:1 was taken, each of the 5 pathogenic strains [(*Escherichia coli*

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TABLE 1 Characteristics of LAB strains isolated from fermented batters in terms of phenotypic, biochemical, and fermentation capacity.

	Isolates						
Tests	**RAMULAB 13**	**RAMULAB 14**	**RAMULAB 15**	**RAMULAB 16**	**RAMULAB 17**	**RAMULAB 20**	**RAMULAB 21**
Gram staining				Positive			
Catalase				Negative			
Morphology	Short Rod	Rod	Short Rod	Rods	Short Rod	Short Rod	Rod
Biochemical parameters							
Methyl Red	+	+	+	+	+	+	+
Voges Proskauer	–	–	–	–	–	–	–
Indole	–	–	–	–	–	–	–
Citrate	–	–	–	–	–	–	–
Starch	–	–	–	–	–	–	–
Hydrolysis							
Gelatin	–	–	–	–	–	–	–
Liquification							
Probiotic properties							
Temperature-related growth (°C)							
4	–	–	–	–	–	–	–
10	–	–	–	–	–	–	–
37	+	+	+	+	+	+	+
45	–	–	+	–	–	+	+
50	–	–	–	–	–	–	–
Salt–related growth (%)							
2	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+
8	–	–	–	–	–	–	–
10	–	–	–	–	–	–	–
pH-related growth							
2	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+
7.4	+	+	+	+	+	+	+
Carbohydrates fermentation							
Lactose	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+
D-xylose	–	–	+	+	–	–	–
L-xylose	–	–	–	–	–	–	–
Galactose	+	+	+	+	+	+	+
Arabinose	–	–	–	–	–	–	–
Starch	–	–	–	–	–	–	–

"+" indicates presence and "–" indicates absence.

MTCC 4430, *Pseudomonas aeruginosa* MTCC 98, *Bacillus subtilis* MTCC 10403), and *Salmonella typhimurium* (MTCC 98)] were mixed and incubated at 37°C for 2 h. At 600 nm, the combination's absorbance was recorded and analyzed. The methodology was carried out as the early approach mentioned by Tatsaporn and Kornkanok (2020). The coaggregation percentage was expressed as:

$$\text{Coaggregation}(\%) = [(A_L + A_P) - A_{\text{mix}} / (A_L + A_P) \times 100.$$

where A_{mix} signifies [the absorbance of the LAB mixture + pathogen at 4 h], and $A_L + A_P$ denotes [the absorbance of the LAB mixture + pathogen at 0 h].

Adherence to chicken crop epithelial cells

LAB adhesion to crop epithelial cells of chicken was investigated as given by Somashekaraiah et al. (2019) under *in vitro* conditions. LAB isolates and chicken crop epithelial cells (1×10^6 cells/mL) were mixed at a 1:10 ratio and incubated for 1 h at optimum temperature. After incubation, the cells were centrifuged (3,000 rpm, 5 min) for the elimination of non-adherent bacterial cells. The pellets obtained were rinsed and resuspended in 100 µl PBS, stained using crystal violet, and viewed under the bright field microscope at 100X magnification.

HT-29 cell culture and development circumstances

According to the methodology performed by Jeong et al. (2021), the adhesion capacity of the seven isolates to HT29 cells (human colon cancer cell lines) was evaluated. The cells (passage #123–130, National Centre for Cell Science in Pune, Maharashtra, India) were grown in DMEM (25 mM, High media) with GlutaMAX (Gibco, United Kingdom) at 37°C in 5% CO_2. A total of 100 g/ml of penicillin and streptomycin and 10% (v/v) FBS (Gibco, UK) were added to the medium as supplements. In a six-well culture plate, HT-29 cells were subcultured at $1x10^5$ cells/mL and grown at 37°C in a humidified CO_2 atmosphere until they reached 70% confluence in the cell medium. The culture medium was changed every alternate day. As a part of the adhesion test, isolates were grown (16 h, 37°C) in MRS broth. The cells were resuspended in DMEM medium at a concentration of 10^8 CFU/ml and washed twice with PBS. To each well, 1 ml of bacterial suspension [incubated for 30 and 60 min at 37°C (5% CO_2 atmosphere)] was added. The cells were lysed by adding 1 ml of 0.1% Triton-X solution (in PBS) and the non-adherent cells were removed by adding PBS. The solution containing the discharged bacterial cells was serially diluted and plated on MRS agar after 10 min at 37°C and incubated for 24 h. The percentage ratio of the initial number of bacteria implanted to that seeded following washing (CFU/mL) was used to determine its adhesion ability. The experiments were carried out in triplicates. The following equation was used to calculate the adhesion rate of the LAB strains:

$$\text{Adhesion rate}(\%) = (C / C_0) \times 100.$$

where, C = Number of adherent cells, C_0 = Initial number of cells inoculated.

Safety assessment

Antibiotic sensitivity

As per Clinical and Laboratory Standards Institutes (CLSI) guidelines 2018, the antibiotic susceptibility against LAB isolates (10^8 CFU/ml) was evaluated by disc diffusion method for the following antibiotic discs–namely, clindamycin (2 mcg/disc), chloramphenicol (30mcg/disc), ampicillin (10 mcg/disc), gentamicin (10 mcg/disc), tetracycline (30 mcg/disc), kanamycin (30mcg/disc), rifampicin (5mcg/disc), vancomycin (30 mcg/disc), methicillin (5mcg/disc), erythromycin (15 mcg/disc), streptomycin (100 mcg/disc), cefixime (5mcg/disc), and azithromycin (15mcg/disc). The results were elucidated as resistant(R), susceptible (S), or moderately susceptible (MS) by comparison with the diameter of the zone of inhibition.

Hemolytic assay

Hemolytic activity of the isolated LAB strains was examined on blood agar plates (HiMedia, Mumbai, India; Halder et al., 2017). Based on the red blood cell lysis in the medium surrounding the colonies, the hemolytic activity of the isolated strains was assessed. The plates were observed for the appearance of a clear zone surrounding the colony for hemolytic reaction.

DNase activity

To evaluate the isolate's capacity to produce deoxyribonuclease (DNase) enzymes, LAB isolates were streaked onto DNase agar plates (HiMedia, Mumbai, India). DNase activity was evaluated by the formation of the clear zone after incubation for 48 h at 37°C. A distinct zone surrounding the colonies found evidence of positive DNase activity (Boricha et al., 2019).

Antimicrobial activity

The antimicrobial activity of the isolates against pathogenic strains was assessed using the agar well diffusion method as per Arqués et al. (2015) with slight modifications. *Bacillus cereus* MTCC 10403, *Staphylococcus aureus* MTCC 1144, *Salmonella typhimurium* MTCC 98, *Escherichia coli* MTCC 443, *Pseudomonas aeruginosa* MTCC 424, *Klebsiella pneumonia, Micrococcus luteus* MTCC 1809, *Bacillus subtilis* MTCC 10403, *Pseudomonas florescens* MTCC 667, *and Klebsiella aerogenes (Enterobacter aerogenes)* MTCC 2822 were the test organisms. The pathogen (100 µl) was added to Luria Bertani agar (LB agar) plates. Wells were made on the solidified agar medium using a well borer for the treatment of LAB isolates. A 100 µl of 18 h overnight grown LAB isolates were poured into the well, allowed to dry, and incubated at 37°C for 24–48 h.

Molecular identification

Molecular characterization provides a basis for the cultural and biochemical characterization of LAB isolates. The study was performed as per the method described by Agaliya and Jeevaratnam (2013) using the regions of 16S rRNA. Seven LAB isolates obtained in the present study were amplified and sequenced using specific primers targeting the 16S rRNA region. The obtained sequence was evaluated by BLAST analysis and a phylogenetic tree was constructed using MEGA X (Version 10.2.4, CA, United States) 0.1000 bootstrap consensus trees were used to create the phylogenetic tree with the highest likelihood.

The Tamura-Nei model fits the best (Tamura et al., 2007). In order to automatically create the initial tree(s) for the heuristic search, the Neighbor-Joining and BioNJ algorithms were used on a matrix of pairwise distances.

Antioxidant activities

Radical scavenging rate by DPPH assay

The methodology performed by Elfahri et al. (2014) was followed to conduct the assay. The radical scavenging activity of the cells at 10^3, 10^6, and 10^9 CFU/ml was assessed using the DPPH (1,1-diphenyl-2-picrylhydrazyl) assay. To express the radical scavenging activity, the following equation was used.

$$\text{Scavenging rate}(\%) = 1 - \left[\left(A_S / A_b\right) \times 100\right].$$

where A_S = absorbance of the reactants with the sample and A_b = absorbance of the reactants without the sample.

Radical scavenging rate by ABTS assay

The method described by Soleymanzadeh et al. (2016) was used to estimate the radical scavenging rate of the cells at 10^3, 10^6, and 10^9 CFU/ml by 2,2′-azino-bis (3-ethyl benzo-thiazoline-6-sulphonic acid) (ABTS) assay. The below-mentioned equation was used to compute the radical scavenging activity:

$$\text{Scavenging rate}(\%) = 1 - \left[\left(A_S / A_b\right) \times 100\right].$$

where A_S = absorbance of the reactants with the sample and A_b = absorbance of the reactants without the sample.

Inhibitory activity of carbohydrate hydrolyzing enzymes

α-Glucosidase

The α-glucosidase inhibitory activity was performed with slight modifications as described by Reuben et al. (2019). α-glucosidase (ex. yeast, 100 U/mg) was used for inhibitory activity. The test samples (CS, CE, and IC – 100 µl) and 50 mM PBS buffer (pH 6.8) were mixed and incubated for 10 min. α-glucosidase (100 µl, 0.25 U/ml) was added and pre-incubated for 15 min at 37°C. Five millimeter pNPG (p-nitrophenol-D-glucopyranoside-100 µl) was added and re-incubated for 30 min at 37°C. The enzymatic reaction was stopped by adding 1,000 µl of 0.1 M Na_2CO_3 and the absorbance of 4-nitrophenol was measured at 405 nm. The percent inhibition was calculated as per the following formula,

$$\text{Inhibition}(\%) = 1 - \left[\left(A_S / A_c\right) \times 100\right].$$

where A_C denotes the absorbance of the reactants in the absence of the sample and A_S denotes the absorbance of the reactants when combined with the sample.

α-Amylase

The inhibitory potential of CS, CE, and IC against the α-amylase enzyme was evaluated according to the procedure of Ankolekar et al. (2012) with a minor modification. Briefly, porcine pancreatic α-amylase was used in the inhibition assay. CS, CE, and IC (500 µl) obtained from the isolates were mixed with 500 µl of 0.1 M PBS (containing 0.5 mg/ml of α-amylase solution, pH 6.4). The mixtures were incubated at 25°C for 10 min. At specified intervals after the pre-incubation, 1% starch solution (500 µl) prepared in 0.1 M PBS (pH 7.4) was added to each tube. The reaction mixtures were subsequently incubated at 25°C for an additional 10 min. The reaction was terminated using 1.0 ml of DNS reagent followed by incubation in a boiling water bath for 5 min. The tubes were cooled in order to bring it to room temperature. After adding 10 ml of distilled water to the reaction mixture, the absorbance was measured at 540 nm and the percent inhibition was calculated using the equation:

$$\text{Inhibition}(\%) = 1 - \left[\left(As / Ac\right) \times 100\right].$$

where, A_C denotes the absorbance of the reactants in the absence of the sample, and A_S denotes the absorbance of the reactants when combined with the sample.

Statistical analysis

The experiments were carried out in triplicate. The standard deviation is displayed in error bars on graphs. Data examination was done using a one-way analysis of variance (ANOVA). $p \leq 0.05$ was used to determine the significance of the differences.

Results

Preliminary characterization of LAB

A total of 24 isolates were identified from the three batters evaluated in the present study. Seven isolates were classified as LAB in accordance with their phenotypic traits. All of the isolates had a rod-like morphology, were Gram-positive, and did not produce catalase. Biochemical characteristics determined that the isolates were hetero-fermentative, with no gas liberation from glucose fermentation. At an optimum temperature of 37°C, all the isolates showed conventional growth, strains RAMULAB15, RAMULAB20, and RAMULAB21 were able to withstand a temperature of up to 45°C. All the isolates demonstrated optimum growth at salt concentrations of 2 and 4% in the media. At pH 2, 4, and 6, the isolates exhibited mild growth whereas

showed optimum growth at pH 7.4. Lactose, glucose, maltose, sucrose, and mannitol could be fermented by all seven isolates (Table 1).

Evaluation of probiotic attributes

Acid bile salt tolerance

The survival rate of all the LAB isolates tested at pH 2 was assessed under bile conditions (0.3 and 1%) for the evaluation of acid and bile tolerance. Figures 1A,B displays the isolate's capacity to survive at pH 2 and their tolerance to 0.3 and 1% of acid bile, respectively. After 4 h of incubation, for 0.3 and 1% of acid bile, the LAB strains exhibited the highest survival rate of 98 and 93%, respectively. Notably, all seven isolates demonstrated a high survival rate at a bile concentration of 0.3%.

Simulated gastrointestinal juice tolerance assay

The growth of all seven isolates was optimal in both gastric and intestinal conditions. The isolates had a good survival rate up to a period of 8 h (Figures 2A,B). But with time, a slowing in growth was noticed. The plots depict the isolate's tolerance levels for gastrointestinal conditions.

Resistance to phenol

The graph infers the tolerance of all the isolates towards 0.4% phenol (Table 2). The results showed a similar growth after incubation at different time intervals (0 and 24 h) with 0.4% phenol. The viable cell count varied between 6.74 and 7.52 Log CFU/mL. With 7.52 Log CFU/mL, the isolate RAMULAB15 demonstrated greater tolerance than other strains.

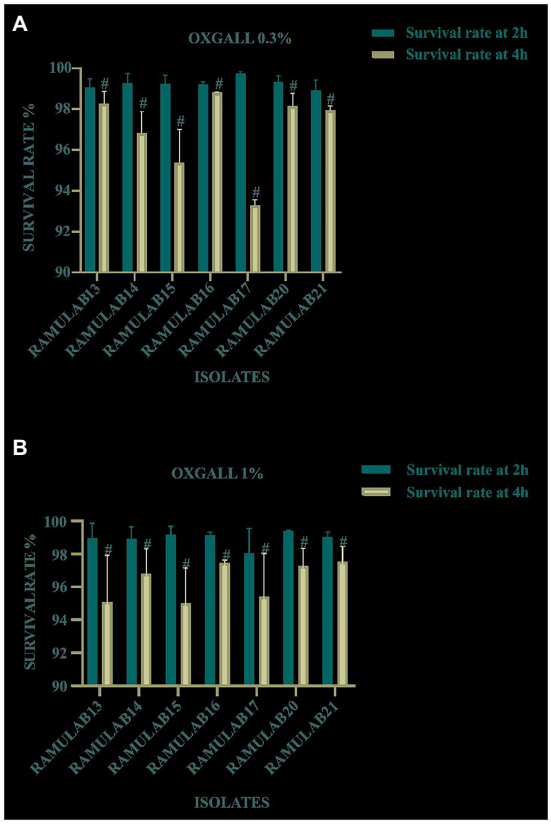

FIGURE 1
Survival of the isolates on MRS agar plates for 2 and 4h at 37°C under circumstances of acidic pH 2 and **(A)** 0.3% and **(B)** 1% bile salt concentration. Data are presented as mean±standard deviation. The Duncan multiple range tests revealed that the means of the survival rate with a 2h time interval and superscripts (#) are substantially different from one another ($p \leq 0.05$).

Adherence assays

Cell surface hydrophobicity

Xylene was the polar solvent used for the determination of cell surface hydrophobicity. In the present study, RAMULAB15 and RAMULAB17 exhibited a maximum of 76.28% and a minimum of 52.13% hydrophobicity (Table 2).

Assay for auto and coaggregation

RAMULAB15 happened to show the highest autoaggregation percentage of 89.47% at 24 h. An exponential increase in the percentage of autoaggregation was observed in all the isolates over time between 2 and 24 h. All the isolates exhibited an autoaggregation activity >77% (Figure 3A). All seven isolates exhibited a high coaggregation percentage with *Micrococcus luteus* MTCC 1809. RAMULAB20 showed the highest coaggregation ability of 42.43% (Figure 3B). Probiotic autoaggregation and coaggregation are crucial for bacterial fortification and colonization and the present study clearly indicates the potential of the isolated LABs as probiotic alternatives.

Adhesion ability to chicken epithelial cells and HT 29 cell lines

The adhesion capacity of the LAB isolated to chicken epithelial cells was determined to be between the minimum (18–25 cells) and (35–60 cells) maximum bacterial cells per epithelial cell (Figure 4). As indicated in Table 3, RAMULAB20 had the highest adhesion rate, whereas RAMULAB13 had the least. Isolates' adherence to the HT-29 cells was greater than 79.82%. Comparatively, RAMULAB16 demonstrated the highest level of adhesion to the other isolates.

Safety assessment

Antibiotic sensitivity

To ascertain the profile of antibiotic resistance, the isolates were screened against 13 different antibiotics. The outcomes were compared to the reference standard chart. One of the basics is the assessment of probiotic qualities and antibiotic sensitivity. Erythromycin, chloramphenicol, rifampicin, gentamicin,

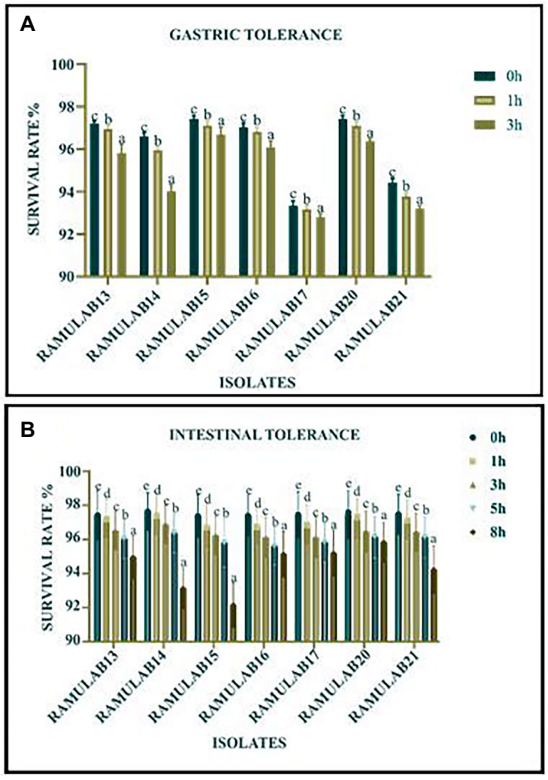

FIGURE 2
Survival rate (%) of gastric **(A)** and intestinal juice **(B)** tolerance of LAB strains after incubation for certain survival time intervals at 37°C. *Data are presented as mean±standard deviation. Duncan's multiple range tests revealed that the means of the survival rate with a 2h time interval and superscripts **(a–e)** are substantially different from one another ($p \leq 0.05$).

TABLE 2 Cell surface hydrophobicity (%) and phenol tolerance (Log CFU/mL) of the isolates.

Isolates	Cell-surface hydrophobicity (%)*	Phenol tolerance (Log CFU/mL)*	
		0 h	24 h
RAMULAB13	$70.82 \pm 7.5^{b,c}$	7.21 ± 0.10^{b}	7.32 ± 0.22^{b}
RAMULAB14	69.17 ± 9.8^{b}	7.12 ± 0.21^{a}	7.26 ± 0.31^{a}
RAMULAB15	76.28 ± 8.4^{d}	7.52 ± 0.60^{d}	7.84 ± 0.41^{e}
RAMULAB16	71.53 ± 2.5^{c}	7.16 ± 0.12^{a}	7.38 ± 0.24^{c}
RAMULAB17	52.13 ± 3.4^{a}	7.28 ± 0.25^{c}	7.46 ± 0.32^{d}
RAMULAB20	$70.99 \pm 5.8^{b,c}$	7.26 ± 0.42^{c}	7.49 ± 0.12^{d}
RAMULAB21	68.75 ± 10.4^{b}	7.17 ± 0.42^{a}	7.36 ± 0.14^{c}

*Data are presented as mean ± standard deviation. Duncan's multiple range tests revealed that the means of the survival rate with a 24 h time interval and superscripts (a-e) are substantially different from one another ($p \leq 0.05$).

ampicillin, tetracycline, streptomycin, clindamycin, and azithromycin were effective against the seven isolates. Conversely, vancomycin, methicillin, kanamycin, and cefixime were resistant (Table 4).

Hemolytic and DNase assay

The six LAB isolates were confirmed safe and categorized under γ - hemolysis after 48 h of incubation at 37°C with no zone surrounding the colonies. This indicates that the isolates were safe to use, which constitutes another indicator of the probiotic formulation's safety. Furthermore, DNase activity also fulfils as an indicator of the safety of the isolates. In the present study, the non-pathogenic nature of the isolates that showed no zone of inhibition was established.

Antimicrobial activity

The antibacterial activity was evaluated against microbial pathogens. Significantly, all of the indicator bacteria were resistant to the isolate's antimicrobial activity. The scale of the zone of inhibition is between 6 and 20 mm. All of the isolates showed an effective antibacterial potential against the opportunistic pathogens *P. aeruginosa* and *M. luteus*. RAMULAB15 had strong antimicrobial efficacy against all the pathogens tested except for *B. cereus, S.typhimurium, K. pneumonia,* and *K. aerogenes*. With the exception of a moderate inhibitory effect on the pathogens *S. aureus, P. aeruginosa*, and *M. luteus*, the isolate RAMULAB17 exhibited negligible activity against all the pathogens (Table 5).

Molecular character and phylogenetic analysis

The amplified 16S rRNA sequences of seven LAB isolates from fermented batters were subjected to evolutionary analyses using the MEGA X software. The homology search revealed that the sequences of strains RAMULAB13, RAMULAB14, RAMULAB15,RAMULAB16, RAMULAB17, RAMULAB20, and RAMULAB21 were > 95% similar to *Lacticaseibacillus rhamnosus, Lactiplantibacillus plantarum, Lactiplantibacillus pentosus, Lacticaseibacillus paracasei, Lacticaseibacillus casei, Lacticaseibacillus casei,* and *Lacticaseibacillus paracasei* respectively, thus validating the homology sequences of the isolates (Figure 5). Table 6 lists the NCBI GenBank accession number of all the isolates.

Antioxidant assay

As the number of cells increased exponentially, all the isolates expressed a higher DPPH free radical scavenging activity in 10^9 CFU/ml, with the results ranging from 26.12 to 76.63% (Figure 6A). RAMULAB15 exhibited the highest radical-scavenging activity (76.63%). At 10^9 CFU/ml, the ABTS radical scavenging activity of the isolates ranged from 31.15 to 84.45%. RAMULAB17 and RAMULAB15 exhibited the lowest and highest inhibition activities, respectively (Figure 6B).

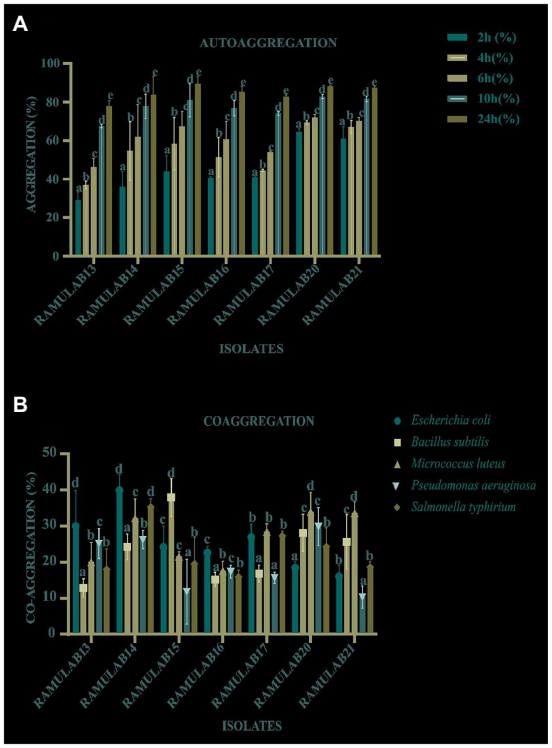

FIGURE 3
The autoaggregation (%) **(A)** and coaggregation **(B)** of LAB strains at different time intervals and 2h incubation at room temperature. *Data are presented as mean±standard deviation. Duncan's multiple range tests revealed that the means of the survival rate with a 24h time interval and superscripts **(a–e)** are substantially different from one another ($p \leq 0.05$).

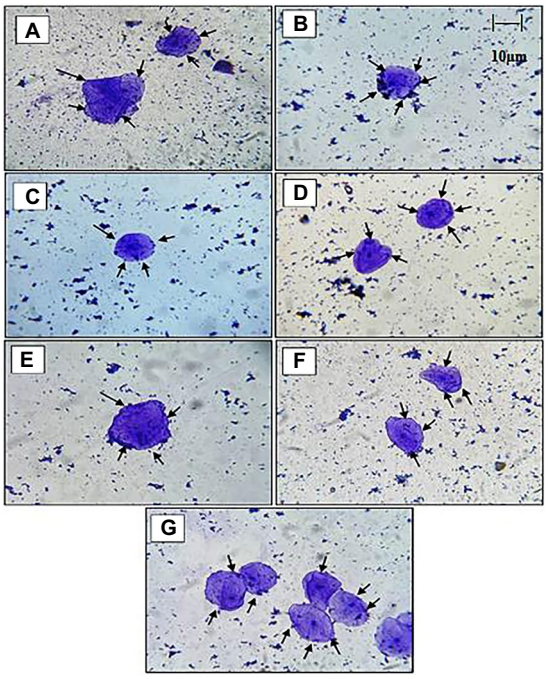

FIGURE 4
Under a light microscope, the adherence of LAB strains to chicken crop epithelial cells, The adhesion of isolates: **(A)** RAMULAB13, **(B)** RAMULAB14, **(C)** RAMULAB15, **(D)** RAMULAB16, **(E)** RAMULAB17, **(F)** RAMULAB20, **(G)** RAMULAB21 to chicken crop epithelial cells. LAB strains are seen adhering to the epithelial cells as indicated by the black arrow.

Inhibitory assay for the carbohydrate hydrolyzing enzymes

The study used CS, CE, and IC obtained from the seven isolates to measure their inhibitory activity against α-glucosidase and α-amylase. For all isolates, the CS and CE exhibited a marked influence on both α-glucosidase and α-amylase. Inhibition of α-glucosidase by the isolates tested using CS, CE, and IC ranged from 15.08 to 59.55% (Figure 7A), whereas α-amylase inhibition ranged from 18.79 to 63.42% (Figure 7B). Strain RAMULAB15 exhibited the highest inhibition rate of 59.55 and 63.42% for both α-glucosidase and α-amylase, respectively. The intact cells from the isolates exhibited the least inhibition compared to the supernatant and pellets (Table 7; Figure 7).

Discussion

A product or food's sensory qualities can be affected by fermentation in addition to its significant potential to improve sustainability, nutrition, and safety leading to health benefits (Lorn et al., 2021). Although traditional spontaneous fermentation typically causes hindrance in certain acceptability features, choosing starters based on particular enzymatic activities can advance a clean label and natural oriented fermentation that results in food with particular sensory properties (Klongklaew et al., 2022). Traditional fermented batters and other types of food involving fermentation were discovered to be rich sources of probiotics based on prior research (Mullish et al., 2021; Grujović et al., 2022; Manovina et al., 2022). The probiotics isolated from these fermented sources have proven to exhibit antihyperglycemic properties (Ayyash et al., 2020; Lakra et al., 2020; Sarkar et al., 2020; Pereira et al., 2021) 0.9 percent of adults worldwide are affected with DM, a systemic chronic disease that can cause difficulties to the majority of the body's organs (Cree-Green et al., 2012). Changes in the pancreatic beta-cell functions lead to insulin deficiency, which in turn results in the hindered cellular response to insulin production. One of the most notable effects of these events is an elevated blood glucose (hyperglycemia), which leads to a number of issues associated with diabetes. These events also induce several anomalies in physiological pathways (Poretsky, 2010). Ineffectiveness and unfavorable side effects are occasionally associated with the use of chemical treatments to regulate blood sugar. As a result, contemporary studies investigated earlier suggest other possible biotherapeutic therapies to be implemented involving probiotics (Akbari and Hendijani, 2016). Recent studies have discovered that diabetic patients had altered gut microbiota;

Blood samples from diabetes individuals revealed the presence of circulating gram-positive gut bacteria (Sato et al., 2014). Through the release of lipopolysaccharides, the altered gut microbiota may cause metabolic endotoxemia, which in turn may promote insulin resistance and inflammation (Bekkering et al., 2014). A positive or negative correlation was found between the quantity of epithelial enteroendocrine L cells and the prevalence of 25 bacterial taxa in the intestine, which play a role in the development of inflammation (Everard and Cani, 2013). The impact of probiotic supplementation on diabetes and its associated ailments was examined by researchers in light of these findings. The therapeutic impact of probiotics on glycaemic control has been the subject of various investigations in humans. Recent clinical studies have produced encouraging outcomes, which emphasize the necessity of a thorough systematic review and meta-analysis of these results (Markowiak and Ślizewska, 2017; Kim et al., 2019; Dudek-Wicher et al., 2020; Montassier et al., 2021; Mullish et al., 2021; Quigley, 2022).

In the current study, the probiotic *Lactobacillus* spp. isolated from fermented batters (jalebi, medhu vada, and kallappam) are tested for their ability to inhibit the carbohydrate hydrolyzing enzymes α-glucosidase and α-amylase. Seven (07) isolates were chosen for further evaluation out of twenty-four (24) isolates that were retrieved based on their phenotypic traits. The following LAB strains were selected for further testing: 3 LAB strains [RAMULAB (13, 14, and 15)] from jalebi batter, 2 LAB strains [RAMULAB (16, 17)] from kallappam batter, and the rest 2 LAB strains [RAMULAB (20, 21)] from medhu vada batter were retrieved. At various temperatures as well as with regard to salt and acid bile tolerance, the viability of the seven LAB isolates was assessed considering them as important parameters in the probiotic evaluation. The viability rate at various temperatures, salt tolerance, and acid bile tolerance was >93% compared to earlier investigations (Al Kassaa et al., 2014; Reuben et al., 2020). Another key factor for sustaining numerous health benefits is the capacity of the isolates to survive under extreme pH circumstances comparable to that of the gastrointestinal tract. The gastrointestinal assay, which simulates the digestion of food in the stomach and intestine with pH levels of 2 and 8, for durations of 2–3 h and 3–8 h, respectively, was carried out to assess the probiotic potential (Gupta and Tiwari, 2015). For both of the simulated gastrointestinal tolerance conditions, all seven isolates exhibited a > 92% survival rate. A similar survival rate (%) was reported in accordance with the study examined by Kumari et al. (2022a,b). At pH 2, *P. pentosaceus SP2* and *L. paracasei SP5* both strains' viability (%) was observed to be decreased (Mantzourani et al., 2019). In contrast to earlier studies, ours had a consistently high survival rate and a higher tolerance to bile acids, and gastrointestinal conditions. The phenolic conditions that can be created by bacterial deamination of amino acids generated from dietary proteins are able to support the survival of the gut microbiota. Phenol and other hazardous metabolites released during specific digestive processes can be produced by gut microbes. Any bacteria that can endure these conditions can therefore be considered to have probiotic potential (Billah et al., 2010; Padmavathi et al., 2018). After 24 h of incubation with 0.4% phenol, Jena et al. (2013) reported a progressive increase in the cell viability from 5.69 to 7.05 Log CFU/mL was observed previously. Similarly in our study, the cell viability increased from 7.12 to 7.84 log CFU/mL with 0.4% phenolic concentration after 24 h incubation, demonstrating cell viability of LAB with resistance to phenol in the GI tract. With a 7.84 Log CFU/mL viable count, strain RAMULAB15 demonstrated the maximum tolerance to

TABLE 3 Adhesion—measured by the percentage of isolates that adhere to HT-29 cells.

Isolates	Cell Adherence (%)*
RAMULAB13	79.82 ± 07.85 [a]
RAMULAB14	81.17 ± 4.18 [b]
RAMULAB15	83.28 ± 6.74 [c]
RAMULAB16	88.53 ± 4.51 [d]
RAMULAB17	85.13 ± 3.24 [c]
RAMULAB20	80.99 ± 6.98 [b]
RAMULAB21	86.75 ± 1.24 [d]

*Data are presented as mean ± standard deviation. Duncan's multiple range tests revealed that the means of the survival rate with a 24 h time interval and superscripts (a-e) are substantially different from one another ($p \leq 0.05$).

TABLE 4 Antibiotic susceptibility test of the isolates representing resistance and sensitivity based on CLSI, 2018.

Isolates	Antibiotics												
	E	C	RIF	GEN	AMP	TET	STR	CD	AZM	MET	K	CEF	VA
RAMULAB13	S	S	S	S	S	S	S	S	S	R	R	R	R
RAMULAB14	S	S	S	S	S	S	S	S	S	R	R	R	R
RAMULAB15	S	S	S	S	S	S	S	S	S	R	R	R	R
RAMULAB16	S	S	S	S	S	S	S	S	S	R	R	R	R
RAMULAB17	S	S	S	S	S	S	S	S	S	R	R	R	R
RAMULAB20	S	S	S	S	S	S	S	S	S	R	R	R	R
RAMULAB21	S	S	S	S	S	S	S	S	S	R	R	R	R

Erythromycin (E), Chloramphenicol (C), Rifampicin (RIF), Gentamicin (GEN), Ampicillin (AMP), Tetracycline (TET), Streptomycin (STR), Clindamycin (CD), Azithromycin (AZM), Methicillin (MET), Kanamycin (K), Cefixime (CEF), and Vancomycin (V).

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TABLE 5 Antimicrobial activity of the LAB isolates against pathogens.

Pathogens	Isolates						
	RAMU LAB13	RAMU LAB14	RAMU LAB15	RAMU LAB16	RAMU LAB17	RAMU LAB20	RAMU LAB21
M. luteus	+++	+++	+++	+++	+++	+++	+++
P. aeruginosa	+++	++	+++	+++	++	+++	+++
S. aureus	++	++	++	++	++	++	++
B. cereus	+	–	+	+	–	+	+
E. coli	++	+	++	+	+	+	+
B. subtilis	++	+	++	+	–	++	+
K. pneumoniae	+	–	+	+	–	–	–
S. typhimurium	–	+	+	+	–	+	+
K. aerogenes	–	+	+	+	–	+	+
P. florescens	++	++	++	++	+	++	++

Symbols show zones of inhibition (mm): –, no inhibition; +, weak (< 7); ++, good (9–15); +++, strong (> 15).

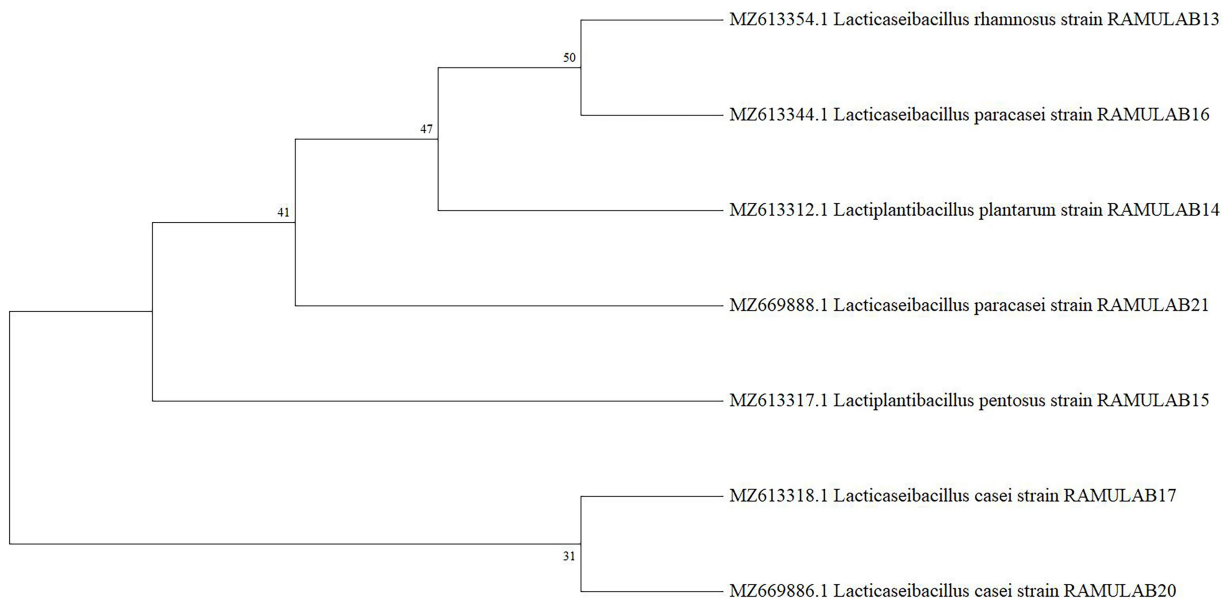

FIGURE 5
Analysis of the phylogenetic relationships among LAB isolates, based on 16S rRNA maximum likelihood bootstrap analysis.

0.4% phenol in our study. RYPR1 and RYPC7 expressed the maximum tolerance to phenol with 7.73 and 7.39 Log CFU/mL (Yadav et al., 2016). In our study, it was discovered that the isolates were highly effective at tolerating phenol, and could survive along the transit of the gastrointestinal tract. In comparison with other investigations mentioned above, all seven isolates from our study demonstrated better tolerance to phenolic conditions.

Colonization due to hydrophobicity, autoaggregation, and coaggregation play a vital role in the probiotic evaluation of LAB. In contrast to coaggregation, where they adhere intercellularly to various strains of bacteria, autoaggregation and hydrophobicity allow the microorganisms to adhere to the intestinal mucosa where they bind to colonies of the same group of microorganisms (del Re et al., 2000; Li et al., 2015). Bacterial attachment to the human intestinal layer is a complex event involving a variety of elements, including the charges of both human and bacterial cells, their hydrophobicity, extracellular polysaccharides, and proteins (cell surface). To acquire proper, potent, or irreversible adhesion, bacterial cells must get through all of these obstacles (Abdulla et al., 2014). The current adherence investigation found that the hydrophobicity, autoaggregation, and coaggregation were above 52.13, 77.48, and 42.43%, respectively. This event contributes to maintaining the favorable environment of the gut. The study investigated the adhesion of LAB to chicken crop epithelial cells and HT29 cells as well, and the outcomes were encouraging >80.99% and a maximum of 35–60 cells/bacterial epithelial cell adhesion were reported by the investigated LAB strains to HT29 cell attachment and chicken crop epithelial cells.

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Thus, aggregation constitutes the defense mechanism against anti-infection for the host (Somashekaraiah et al., 2019; Li et al., 2020; Jeong et al., 2021). Prior studies have revealed the ability of *Lactobacillus* spp. to competitively adhere and minimize inflammatory effects while inducing a higher state of change in the host intestinal epithelial cells' defense system (Dhanani and Bagchi, 2013). Thus from our study, it can be concluded that the adherence capability of the LAB isolates was most efficient than the results which are already available from other earlier investigations.

Additionally, the present study assessed the isolated strains' safety and development. To assess the safety of the LAB, tests for -antibiotic sensitivity, DNase activity, hemolytic assay, and antimicrobial were performed. The antibiotic sensitivity profile was assessed using the CLSI 2018 Scale of Clinical and Laboratory Standard Institute. According to the findings, each of the seven isolates was resistant to methicillin, vancomycin, kanamycin, and cefixime. These LAB isolates are commendable for their positive effects on enhancing intestinal health, particularly when LABs are given concurrently with antibiotics, which can fend off illnesses brought on by other pathogens (Iorizzo et al., 2022). From our findings in the study, it can be deduced that the following LAB strains can be administered along with other known antibiotics in treating illness. The DNase test was also carried out to examine the pathogenicity of bacteria that generate the DNase enzyme, which hydrolyzes DNA. The absence of DNase in the tested isolates was therefore confirmed, indicating their potential safety for use in fermentation. The isolated LAB strains were also found to be harmless after a hemolytic experiment indicated no hemolysis (Saroj et al., 2016). On the other hand, *L. rhamnosus* has been found to compromise the integrity of cellular membranes and cause ATP efflux, which results in pore development and reduces the growth of *M. luteus* (Li et al., 2020). By demonstrating membrane permeabilization activity, the bacteriocin produced from *L. plantarum ZJ316* had a promising antibacterial action against *M. luteus* (Jiang et al., 2018). In the current investigation, all seven isolates demonstrated a strong antibacterial activity when tested against *M. leuteus* MTCC 1809 and effective activity against the other pathogenic organisms. The seven isolates from our study can be used as antibacterial agents against *M. luteus*. All of the seven isolates were sequenced at the 16S rRNA region in an effort to characterize them molecularly. The isolates were identified as LAB based on the sequence data, and phylogenetic analysis using maximum likelihood bootstrap revealed that the isolated strains were RAMULAB13 *L. rhamnosus*, RAMULAB14 *L. plantarum*, RAMULAB15 *L. pentosus*, RAMULAB16 *L. paracasei*, RAMULAB17 *L. casei*, RAMULAB20 *L. casei*, and RAMULAB21 *L. paracasei*. A similar method was used in the identification of LAB strains *L. plantarum subsp. plantarum, L. pentosus, and L. plantarum subsp. argentoratensis.*, by Agaliya and Jeevaratnam (2013), Endres et al. (2021). The bacterial cell surface components are associated with intact cells' capacity to scavenge free radicals. In line with several other earlier investigations, the isolates in our investigation demonstrated a higher scavenging activity. Free radical production has been linked to the pathogenesis and progression of diabetes (Alkalbani et al., 2019; Kim et al., 2021). Inflicting oxidative harm on biomolecules, hydroxyl and related radicals are the most hazardous reactive oxygen species.

TABLE 6 Identified LAB isolates GenBank accession numbers.

Isolates	Sample	Bacteria	GenBank Accession no.
RAMULAB13	Jalebi batter	*Lacticaseibacillus rhamnosus*	MZ613354
RAMULAB14		*Lactiplantibacillus plantarum*	MZ613312
RAMULAB15		*Lactiplantibacillus pentosus*	MZ613317
RAMULAB16	Kallappam batter	*Lacticaseibacillus paracasei*	MZ613344
RAMULAB17		*Lacticaseibacillus casei*	MZ613318
RAMULAB20	Vada batter	*Lacticaseibacillus casei*	MZ669886
RAMULAB21		*Lacticaseibacillus paracasei*	MZ669888

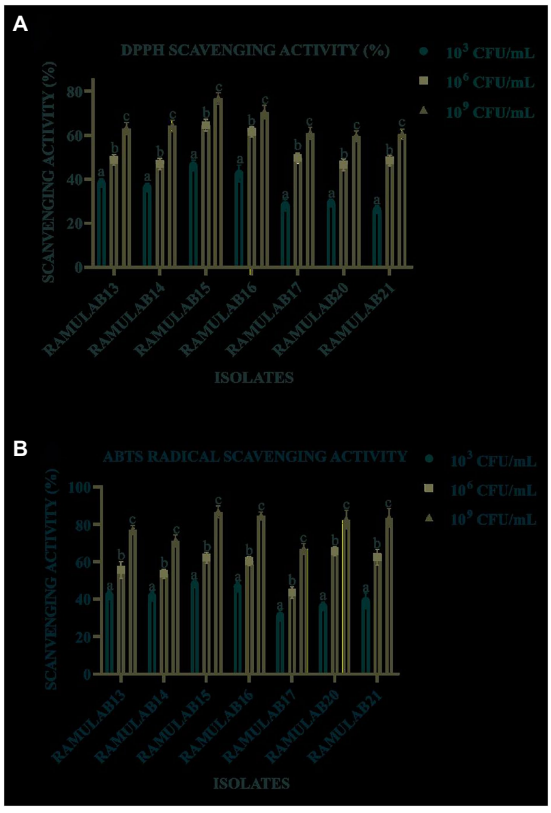

FIGURE 6
Isolates scavenging activity by the isolate on free DPPH **(A)** and ABTS **(B)** radicals. Results are presented as mean±SD. Duncan's multiple range test shows that the means of the same column that are denoted by different letters **(a–d)** are substantially different ($p \leq 0.05$).

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TABLE 7 α-Glucosidase and α-Amylase inhibitory activity of the isolates.

Isolates	α-Glucosidase inhibition*			α-Amylase inhibition*		
	CS	IC	CE	CS	IC	CE
RAMULAB13	49.35 ± 1.97^{b}	21.50 ± 2.23^{b}	45.38 ± 2.34^{b}	59.35 ± 2.34^{c}	32.25 ± 1.97^{d}	52.38 ± 2.23^{d}
RAMULAB14	52.42 ± 2.47^{c}	25.57 ± 2.45^{c}	46.41 ± 3.24^{c}	58.21 ± 3.24^{c}	34.24 ± 2.47^{e}	48.41 ± 2.45^{c}
RAMULAB15	59.55 ± 1.84^{d}	26.35 ± 2.06d	55.24 ± 2.24^{e}	63.42 ± 3.24^{e}	36.12 ± 1.84^{e}	54.24 ± 2.06^{e}
RAMULAB16	45.40 ± 2.45^{a}	15.08 ± 3.20^{a}	42.12 ± 2.14^{a}	53.12 ± 2.14^{b}	18.79 ± 2.45^{a}	46.12 ± 1.42^{b}
RAMULAB17	48.81 ± 3.24^{b}	19.38 ± 1.84^{b}	46.35 ± 3.87^{c}	59.10 ± 2.87^{d}	24.78 ± 3.24^{b}	47.35 ± 2.84^{c}
RAMULAB20	46.07 ± 2.42^{a}	16.08 ± 2.45^{a}	43.81 ± 2.95^{a}	54.07 ± 4.95^{b}	23.45 ± 2.42^{b}	41.81 ± 2.45^{a}
RAMULAB21	54.26 ± 4.24^{c}	25.57 ± 4.21^{c}	51.42 ± 4.98^{d}	52.26 ± 4.98^{a}	28.45 ± 4.24^{c}	46.42 ± 4.21^{b}

*Results are presented as mean ± SD. Duncan's multiple range test shows that the means of the same column that are denoted by different letters (a–d) are substantially different ($p \leq 0.05$).

Antioxidants are converted into molecules that are irreversibly stable by DPPH and ABTS using their electrons or hydrogen atoms. Our findings are consistent with other investigations that showed intact cells from a few LAB isolates, including *P. pentosaceus R1* and *L. brevis R4*, had much stronger ABTS radical scavenging ability than cell-free extract and supernatant. Exopolysaccharides, Mn^{2+}, bioactive compounds, antioxidant enzymes, NADH, NADPH, and other antioxidant molecules are present in LAB strains (Jiang et al., 2018).

It is possible to foretell the inhibition of glucose production and the progressive decrease in postprandial hyperglycemic blood glucose absorption in the small intestine by monitoring the activities of the enzymes α-glucosidase and α-amylase (Ramu et al., 2014; Martiz et al., 2022). The main objective of the study is to assess the ability of the probiotic isolates to inhibit the carbohydrate metabolism enzymes α-glucosidase and α-amylase. According to Muganga et al. (2015) strains CCFM147 and CCFM240 showed strong inhibition of 27.9 and 32.9% for α-glucosidase and α-amylase, respectively. Also, the strains CCFM4 and CCFM240 notably showed the highest levels of inhibition for CE, at 11.8 and 3.3%, respectively. Water-soluble extracts of isolates KX881777, KX881772, and KX881779 reported by Alkalbani et al. (2019) exhibited >34% α-amylase inhibition. The strains indicated 3.9% α-glucosidase inhibitory activity, which is 2.5 times more than the results for acarbose (Kwun et al., 2020). In comparison with our study, the strains isolated from dosa batter exhibited an α-glucosidase inhibitory activity ranging from 7 to 65% (Kumari et al., 2022b). Strain TKSP 24 isolated from Korean fermented soybean sauce-Doenjang exhibited an α-glucosidase inhibitory activity ranging from 58 to 62% (Shukla et al., 2016). *L. brevis* KU15006's CFS and CE had the highest levels of α-glucosidase inhibitory activity when compared to commercially available LAB, at 24.11 and 10.56% percent, respectively (Son et al., 2017). α-glucosidase inhibitory activity for 12 LAB species was examined by Chen et al. (2014), who found that 12 strains showed between 3.42–29.57% inhibition, although MKHA15's activity was substantially stronger at 99.25%. It is noteworthy that HAGB was significantly greater than LPGB produced by the same *L. plantarum* spp. (Jang and Kim, 2021). According to the study findings by Dilna et al. (2015) *Lactobacillus plantarum RJF4* displayed cholesterol lowering property (42.24%) and a-amylase inhibition (40%), However, in our study, the CS of the isolates exhibited 59.55 and 63.42% inhibition potential for α-glucosidase and α-amylase enzymes, respectively. In comparison with earlier research mentioned above, our findings indicate that the isolate from our LAB strain RAMULAB15 exhibited higher inhibition potential towards both α-glucosidase and α-amylase. Therefore, it can be concluded that the

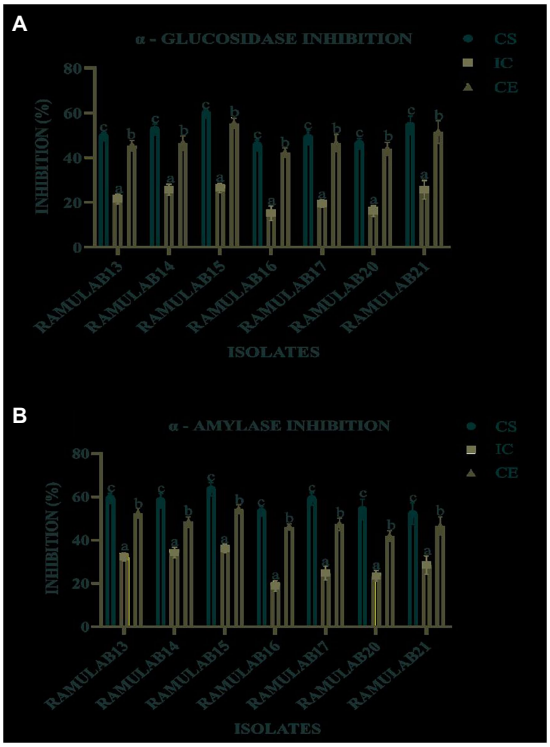

FIGURE 7
α-Glucosidase **(A)** and α-amylase **(B)** inhibitory activity of the isolates. Results are presented as mean±SD. Duncan's multiple range test shows that the means of the same column that are denoted by different letters **(a–d)** are substantially different ($p \leq 0.05$).

inhibition by CS of the isolates could be used as an effective inhibitor of both α-glucosidase and α-amylase enzymes. The inhibition of intestinal α-glucosidase and α-amylase by the CS of the isolates can aid in the treatment of postprandial hyperglycemia and can reduce blood glucose levels. The inhibitory activity of α-glucosidase and α-amylase by the isolates in this regard can delay the onset of diabetes complications.

Conclusion

Diabetes being a global epidemic, a constant increase in the proportion of cases attributed to western eating habits is turning into a serious problem. The goal of the current study is to assess the potential probiotic *Lactobacillus spp.* which was isolated from traditional fermented batters (jalebi, medhu vada, and kallappam), for use in the treatment of diabetes. Based on analysis of the phenotypic, biochemical, and molecular characterization, all seven isolates were identified as LAB. The strains demonstrated noteworthy results in terms of acid-bile, gastrointestinal tolerance, auto-and coaggregation abilities, antibiotic, hydrophobicity, and antibacterial features which are essential parameters to qualify them as probiotics. The seven LAB displayed significant inhibitory α-glucosidase and α-amylase activity. However, the isolates' CS and CE showed more inhibitory action than the isolates' IC when investigated. As a result, the probiotic isolates from fermented batters are a good source of possible anti-diabetic properties. Following purification, the isolates can be utilized as supplements. Additional *in vivo* research is required for further assessment.

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

RR planned and conceptualized the manuscript. VC and SH were involved in data analysis and method development. TA, SK, CC, and RA were involved in supervision, editing, and preparation of the manuscript draft. All authors contributed to the article and approved the submitted version.

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Conflict of interest

SK was employed by Kerry Food Center, Inc. CC was employed by Midwest Veterinary Services, Inc.

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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Microbial community succession patterns and drivers of Luxiang-flavor Jiupei during long fermentation

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Luxiang-flavor Baijiu is the mainstream of Baijiu production and consumption in China, and the microbial composition has a great influence on the flavor and quality of Baijiu. In this study, we combined multi-omics sequencing technology to explore the microbial composition, dynamics and metabolite changes of Luxiang-flavor Jiupei during long fermentation periods. The results showed that based on the interaction between environmental constraints and microorganisms, Jiupei microorganisms formed different ecological niches and functional differentiation, which led to the formation of Jiupei stable core microorganisms. The bacteria were mainly *Lactobacillus* and *Acetobacter*, and the fungi were mainly *Kazachstani* and *Issatchenkia*. Most bacteria were negatively correlated with temperature, alcohol and acidity, and for the fungi, starch content, reducing sugar content and temperature had the most significant effects on community succession. Macroproteomic analysis revealed that *Lactobacillus jinshani* had the highest relative content; microbial composition, growth changes and functions were more similar in the pre-fermentation period (0–18days); microorganisms stabilized in the late fermentation period (24–220days). The metabolome analysis revealed that the metabolites of the Jiupei changed rapidly from 18 to 32days of fermentation, with a significant increase in the relative content of amino acids, peptides and analogs and a significant decrease in the relative content of sugars; the metabolites of the Jiupei changed slowly from 32 to 220days of fermentation, with a stabilization of the content of amino acids, peptides and analogs. This work provides insights into the microbial succession and microbial drivers during the long-term fermentation of Jiupei, which have potential implications for optimizing production and improving the flavor of Baijiu.

KEYWORDS

Jiupei, high-throughput sequencing, microbial community, driving factors, metaproteome

1. Introduction

Baijiu is one of the oldest distilled spirits and has a unique place in traditional Chinese culture (Wang, 2022). It is warmly welcomed by consumers worldwide for its distinctive and evocative flavors, which is mainly divided into three types Luzhou-flavor, Moutai-flavor, and light-flavor (Liu and Sun, 2018; Hong et al., 2020). Among them, Luzhou-flavor is characterized by aromatic and rich

aroma, soft and strong fruit, and long aftertaste, occupying 70% of China's total Baijiu production and playing a pivotal role in Baijiu industry (Fan and Qian, 2006). Luzhou-flavor Baijiu was produced by a cyclic solid-state fermentation process, the essence of which is a complex biochemical metabolic reaction that occurs in the solid, liquid, and gas phases of the brewing flora from the Jiupei, pit mud and the workshop environment (Zhou et al., 2021). The function of brewing flora can be simplified as a community metabolic process driven by microorganisms that convert substances such as starch into ethanol and flavor compounds by fermentation while saccharifying (Wang et al., 2020).

Jiupei (fermented grains) are the raw material for distillation of Baijiu, and the microorganisms in Jiupei mainly come from the Daqu and the mixed old Jiuzao (residue after Baijiu distillation), some of which can decompose the starch and cellulose in the grains, and the rest can use these decomposition products to produce fragrance and acid (Li et al., 2013; Xiang et al., 2013). The flavors in Baijiu are obtained by solid-state distillation of the fermentation metabolites of the microorganisms in the Jiupei at the end of fermentation. During this process, water vapor passes through the Jiupei and condenses, bringing out the flavor components. Therefore, the microorganisms in the Jiupei involved in fermentation are the main driving force in the formation of flavor substances, and the number, community structure, ecological succession and related metabolic activities of microorganisms in Jiupei directly affect the flavor quality of Baijiu (Yang et al., 2021). Jiang et al. (2020) analyzed the bacterial diversity of two Moutai-flavor Baijiu based on high-throughput sequencing, and the results showed high relative abundance of *Proteobacteria* and *Firmicutes*, and increasing comparable abundance of *Lactobacillus* and *Pseudomonas* with increasing fermentation time. Ma et al. (2020) studied Laobaigan-flavor Baijiu at different fermentation times and concluded that the microbiota formed by *Lactobacillus*, *Pediococcus*, *Weissella*, *Saccharomycopsis*, *Issatchenkia*, *Rhizopus*, *Trichosporon*, *Candida*, and *Aspergillus* contributed more to the trace components of Jiupei. Wang P. et al. (2018) studied the core microorganisms and flavor substances in Baijiu and found a highly significant correlation between the core microbiota and the evolution of the flavor profile.

Changes in the physical and chemical indexes of Jiupei during the fermentation process plays a significant role in Baijiu's flavor. It not only affect the normal growth, reproduction, and metabolic activities of brewing microorganisms, but also influence the characteristics of brewing microbial populations and the formation of various aroma substances, which in turn have an impact on the sensory characteristics of Baijiu (Zhang M. et al., 2020). Therefore, studying the change pattern of physicochemical indexes of Jiupei during the fermentation of Luzhou-flavor Baijiu will help to better understand the mechanism of Luzhou-flavor brewing. At present, research on Jiupei microorganisms has focused on the metagenomic, and metaproteomics has been relatively slow (Chai et al., 2019a,b; Du et al., 2021). Because metaproteomics can reveal the nature of microbial function at the protein level, it is increasingly used in studies to assess the functional diversity of microbial communities (Yang et al., 2020). The lack of macroproteomics of Jiupei microorganisms has hindered the in-depth understanding of the fermentation mechanism of Baijiu. Therefore, we used multi-omics techniques, including amplicon sequencing, metaproteomics and metabolomics, combined with physicochemical indicators, to conduct a more in-depth analysis of Luzhou-flavor Jiupei. The study further investigated the changes of microorganisms and metabolites of fermented Jiupei during the long fermentation process, and analyzed the change patterns of flavor substances and microbial flora. The study provided basic data to support the analysis of the relationship between long fermentation time and original wine quality, and laid the foundation for an in-depth understanding of microbial community structures flora during the fermentation of Baijiu.

2. Materials and methods

2.1. Sample collection

We collected Jiupei from five Laojiao distilleries in Luzhou City, Sichuan Province for the long fermentation period, with sampling time points of 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 36, 40, 44, 50, 56, 62, 68, 84, 100, 130, and 220 days. Natural fermentation was taken, with initial fermentation temperature, acidity and moisture content were 22°C, 1.9 mmol/10 g and 59%, respectively. Equal amounts of the Jiupei were collected from the upper and lower layers of the cellar and then mixed and labeled as one sample, and refrigerated at −80°C prior to sequencing.

2.2. Analysis of physicochemical indices

The determination of moisture content refers to "GB 5009.3–2016 Determination of Moisture in Foods." For the determination of acidity, we refer to "Analysis and Detection of Wine Making." The determination of liquefaction power refers to the literature of Sha Junxiang et al. For the determination method of glucoamylase activity, we refer to "General Test Method for Industrial Enzyme Preparations." For the determination of acid protease activity, we refer to the "General Test Methods for Industrial Enzyme Preparations."

2.3. DNA extraction, 16S rDNA amplicon sequencing

Genomic DNA was extracted using a FastDNA spin kit for soil according to the manufacturer's instructions. The concentration and purity of DNA were determined by a NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, United States) and stored at −80°C. To analyze the microbiology community, a target variable region of the small ribosomal subunit RNA gene was PCR-amplified with the KAPA HiFi Ready Mix (Kapa Biosystems CAT#KK2602), the full-length DNA was amplified with universal primers (Supplementary Table S1): then, the PCR product was purified by 0.6x AMPure PB Beads and constructed the library by using the SMRTbell Express Template Prep Kit 2.0 (PacBio) with damage repaired, end repaired, A-tailing, and ligated the sequencing adapters. The SMRTbell library was then purified by AMPure PB beads, and Agilent 2,100 Bioanalyzer (Agilent technologies, USA) was used to detect the size of library fragments. Sequencing was performed on a PacBio Sequel II instrument with Sequencing Primer V4 and Sequel II Binding Kit 2.1 in Grandomics. If the amplicon fragment ≥3 kb, the binding Kit is a 2.0 version (Bengtsson-Palme et al., 2013; Adedire et al., 2022).

The original FASTQ file was processed using QIIME software (Caporaso et al., 2010). After analyzing the sequencing data, the SILVA database was used to compare the 16S rRNA and ITS gene sequences to determine the taxonomic status of the corresponding microbes

(Robeson et al., 2021). QIIME software was used to define sequence similarity >97% as an operational taxonomic unit (OTU), only OTUs containing at least five reads were considered to be valid in this study (Edgar, 2010). The Alpha diversity index was calculated to analyze species richness and uniformity in the samples; after which the Shannon index of the samples was determined to obtain the diversity of the community and the number of OTUs in the samples. Finally, the Beta diversity index was determined to analyze the heterogeneity of the community composition between samples.

2.4. Metaproteomics analysis

2.4.1. Protein extraction and digestion

The collected Jiupei samples were powdered with liquid nitrogen in a sterile mortar. Each sample was accurately weighed 5 g, extracted with acetic acid–sodium acetate buffer (pH 4.6) for 6 h at 4°C, and then the supernatant was centrifuged to extract the supernatant, which was precipitated with 4 times the volume of trichloroacetic acid-acetone solution at −20°C overnight (Figure 1). After centrifugation, the precipitate was taken, followed by two washes with cold acetone, dried and concentrated by nitrogen blowing apparatus to obtain wine lees protein samples. The samples were stored at −80°C. 200 μg of protein was taken for digestion. Dithiothreitol was added to the protein sample to a final concentration of 10 mmol/l and reduced at 56°C for 1 h. Then 55 mmol/L iodoacetamide was added and the reaction was carried out at room temperature for 45 min. Four times the volume of pre-cooled acetone was added overnight at −20°C to precipitate the protein. After centrifugation, the precipitate was washed twice with 90% acetone and concentrated by nitrogen blowing, and then mixed with 1 ml of 50 mmol/L ammonium bicarbonate buffer. Next, trypsin was added in the ratio of protein: trypsin = 20:1, and the samples were incubated at 37°C for 12–16 h. The filtrate was collected and an amount of 0.1% trifluoroacetic acid solution was added, and then the samples were desalted (Zhang Y. et al., 2020).

2.4.2. Peptide fractionation

The Shimadzu LC-20AB liquid phase system was used, and the separation column was a 5 μm 4.6×250mm Gemini C18 column for liquid phase separation of the sample. The dried peptide samples were reconstituted with mobile phase A (5% ACN pH 9.8) and injected, eluting at a flow rate of 1 ml/min by following gradients: 5% mobile phase B (95% ACN, pH 9.8) for 10 min, 5% to 35% mobile phase B for 40 min, 35% to 95% mobile phase B for 1 min, mobile phase B for 3 min, and 5% mobile phase B for 10 min. The elution peak was monitored at a wavelength of 214 nm and one component was collected per minute, and the samples were combined according to the chromatographic elution peak map to obtain 20 fractions, which were then freeze-dried (Rappsilber et al., 2007).

2.4.3. HPLC

The dried peptide samples were reconstituted with mobile phase A (2% ACN, 0.1% FA), centrifuged at 20,000 g for 10 min, and the supernatant was taken for injection. Separation was performed by Thermo UltiMate 3,000 UHPLC. The sample was first enriched in trap column and desalted, and then entered a self-packed C18 column (75 μm internal diameter, 3 μm column size, 25 cm column length) and separated at a flow rate of 300 nl/min by the following effective gradient: 0 ~ 5 min, 5% mobile phase B (98% ACN, 0.1% FA); 5 ~ 45 min, mobile phase B linearly increased from 5% to 25%; 45 ~ 50 min, mobile phase B increased from 25% to 35%; 50 ~ 52 min, mobile phase B rose from 35% to 80%; 52 ~ 54 min, 80% mobile phase B; 54 ~ 60 min, 5% mobile phase B. The nanoliter liquid phase separation end was directly connected to the mass spectrometer (Erickson, 2000).

2.4.4. Mass spectrometry detection

The peptides separated by liquid phase chromatography were ionized by a nanoESI source and then passed to a tandem mass spectrometer Q-Exactive HF X (Thermo Fisher Scientific, San Jose, CA) for DDA (Data Dependent Acquisition) mode detection. The main parameters were set: ion source voltage was set to 1.9 kV, MS1 scanning range was 350 ~ 1,500 m/z; the resolution was set to 60,000; MS2 starting m/z was fixed at 100; the resolution was 15,000. The ion screening conditions for MS2 fragmentation: are charged from 2+ to 6+, and the top 30 parent ions with a peak intensity exceeding 10,000. The ion fragmentation mode was HCD, and the fragment ions were detected in Orbitrap. The dynamic exclusion time was set to 30 s. The AGC was set to MS1 3E6, and MS2 1E5 (Chen and Li, 2019).

2.4.5. Protein annotation and bioinformatics analysis

Tandem library search software was used to perform iterative library searches, and the reduced database files were imported into MaxQuant

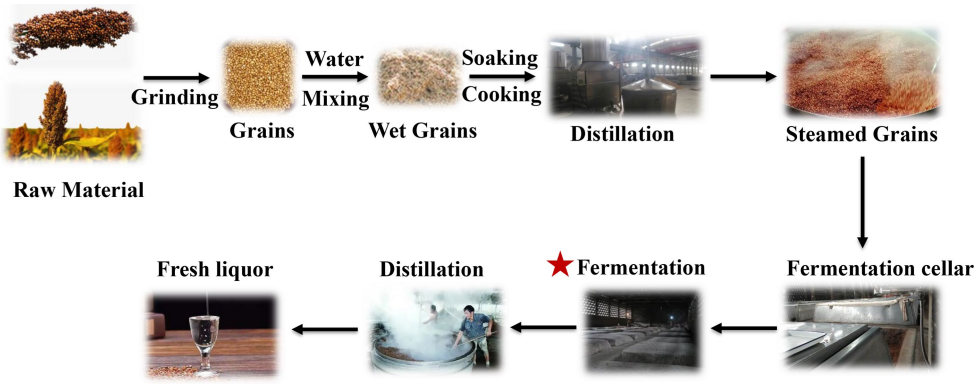

FIGURE 1
Jiupei production process diagram.

(1.5.3.30) for library search identification, followed by quantitative analysis based on information such as peptide peak intensities, peak areas, and liquid chromatographic retention times associated with the primary mass spectra, and a series of statistical analyses and quality control (Cox and Mann, 2008). Then, based on the identification results, functional annotation analysis of proteins such as Gene Ontology (GO) (Ashburner et al., 2000), Kyoto Encyclopedia of Genes and Genomes (KEGG), and species annotation analysis at each taxonomic level were performed (Kanehisa et al., 2014).

2.5. Metabolomic analysis

Untargeted metabolomic analysis to identify the differential metabolites between the different Jiupei samples was done using GC–MS and LC–MS. GC–MS analysis was performed using a 7890A gas chromatograph (Agilent) coupled to a PEGASUS HT mass selective detector (LECO). LC–MS analysis was performed on an Acquity UPLC system (Thermo Fisher Scientific) coupled with a Q Exactive HFX (Thermo Fisher Scientific). The detailed sample preparation and MS analysis methods are described in Text S1 (Sun et al., 2020).

2.6. Statistical analysis

We used Origin and SPSS software for data processing and analysis. Unweighted pair group method with principal coordinate analysis (PCoA) and analysis of similarities (ANOSIM) were carried out in R 4.0.5 (Chan, 2018). Redundancy analysis (RDA) was performed using CANOCO 4.5 software (He et al., 2019). The statistical significance of the difference between the means of samples was tested by one-way analysis of variance (ANOVA) with the Tukey *post hoc* test (McHugh, 2011). Gephi 0.9.2 was used for visualization of correlation analysis between microorganisms (Escandón et al., 2020).

3. Results

3.1. The succession of microbial communities during the fermentation of Jiupei

3.1.1. Bacterial community structure characteristics

The microbial changes of Jiupei during fermentation were analyzed by 16S rDNA amplicon sequencing, and the results showed that the coverage of all samples were above 97%, indicating that the sequencing had sufficient depth and reliable throughput for subsequent analysis. At the genus level, a total of 336 bacterial species were detected in the Jiupei samples. Figure 2A shows the top 30 species in relative abundance, and the other species were merged into others. The top 10 relative abundances were *Lactobacillus*, *Ralstonia*, *Acetobacter*, *Weissella*, *Romboutsia*, *Staphylococcus*, *Bacillus*, unidentified_*Cyanobacteria*, *Comamonas*, and *Leuconostoc*. The relative abundance of *Lactobacillus* did not change much before entering the cellar for 4 days of fermentation. After 6 days of fermentation, the relative abundance of *Lactobacillus* increased rapidly, reaching more than 99%, becoming the dominant genus. After 100 days of fermentation, the relative abundance of *Lactobacillus* showed a downward trend again, and by 220 days of fermentation, *Lactobacillus* decreased by 22.2%. The relative abundance of *Lactobacillus*, which belongs to lactic acid bacteria, increased greatly in the sample as the fermentation progresses, and became the dominant bacteria in the sample. Various *Lactobacillus* occupy an absolute dominant position in the late stage of brewing and fermentation of Luzhou-flavor baijiu, which affects the flavor and quality of Luzhou-flavor baijiu. The *Weissella* genus accounted for a large proportion (12.6%) before entering the pit, and its proportion decreased continuously in the first 2 days of fermentation, and the relative abundance was less than 1%. *Ralstonia* increased in the first 8 days, and then decreased. The relative abundance of *Acetobacter* first increased and then decreased at 8 days before fermentation, and increased again after 130 days. The relative abundance of *Bacillus* decreased continuously 8 days before fermentation.

3.1.2. Fungal community structure characteristics

At the genus level, a total of 654 genus fungal species were detected in the Luzhou-flavor Jiupei samples. Figure 2B shows the top 30 species in relative abundance, and other species were merged into others. It can be seen that the top 10 genera in relative abundance were *Kazachstania*, *Issatchenkia*, *Rhizopus*, *Saccharomyces*, *Pichia*, *Rhizomucor*, *Aspergilus*, *Cladosporium*, and *Thermomyces*. Before entering the cellar, the genus *Issaccharomyces* was the absolute dominant genus, and the genus *Kazachstania* was the absolute dominant fungal genus in the later stage of fermentation. The relative abundance of *Rhizopus* increased continuously in the first 4 days of fermentation, and the relative abundance of *Rhizopus* and *Aspergillus* decreased with the prolongation of fermentation time. The relative abundance of *Issatchenkia* increased after 6 days of fermentation and then decreased with the progress of fermentation. The relative abundance of *Saccharomyces* was increasing.

From the perspective of the succession of the fermentation community structure of Jiupei, the bacterial community was dominated by *Lactobacillus*, *Weissella* and *Bacillus*; the succession direction of the fungal community structure was *Issatchenkia*, *Saccharomyces*, *Rhizopus*, and *Aspergillus* were dominated, with *Kazachstania*, *Saccharomyces* and *Pichia* as the main substitutes.

3.2. Changes of physiochemical indicators during Jiupei fermentation

During the natural fermentation of Baijiu, the diversity and structure of the microbial community are influenced by a number of environmental factors, such as temperature, acidity, moisture and ethanol (Song et al., 2017; Wang X. et al., 2018; Tan et al., 2019). Temperature is the most common fermentation parameter in Baijiu research and affects flavor by influencing the microbial metabolites and content in the fermentation cellar (Zhang et al., 2021; Liu et al., 2022). Therefore, we investigated the temperature of the jiupei fermentation process. From Figure 3A, it can be seen that the fermentation process in Jiupei can be initially divided into four stages depending on the temperature. From 0 to 12 days, the temperature continued to rise, from an initial 21.20 ± 1.02°C rapidly raised to 28.94 ± 1.53°C. On the 12–34 days, the temperature fluctuated in the range of 29.30°C–29.94°C, and then entered the cooling stage, which was basically close to room temperature. On the 100th day, it reached 20.72 ± 1.58°C and then the temperature remained essentially constant. Overall, the parallel fluctuations in temperature were small, and the temperature change curve can be summarized as "slowly rising at the front, rising in the middle, and falling slowly at the back."

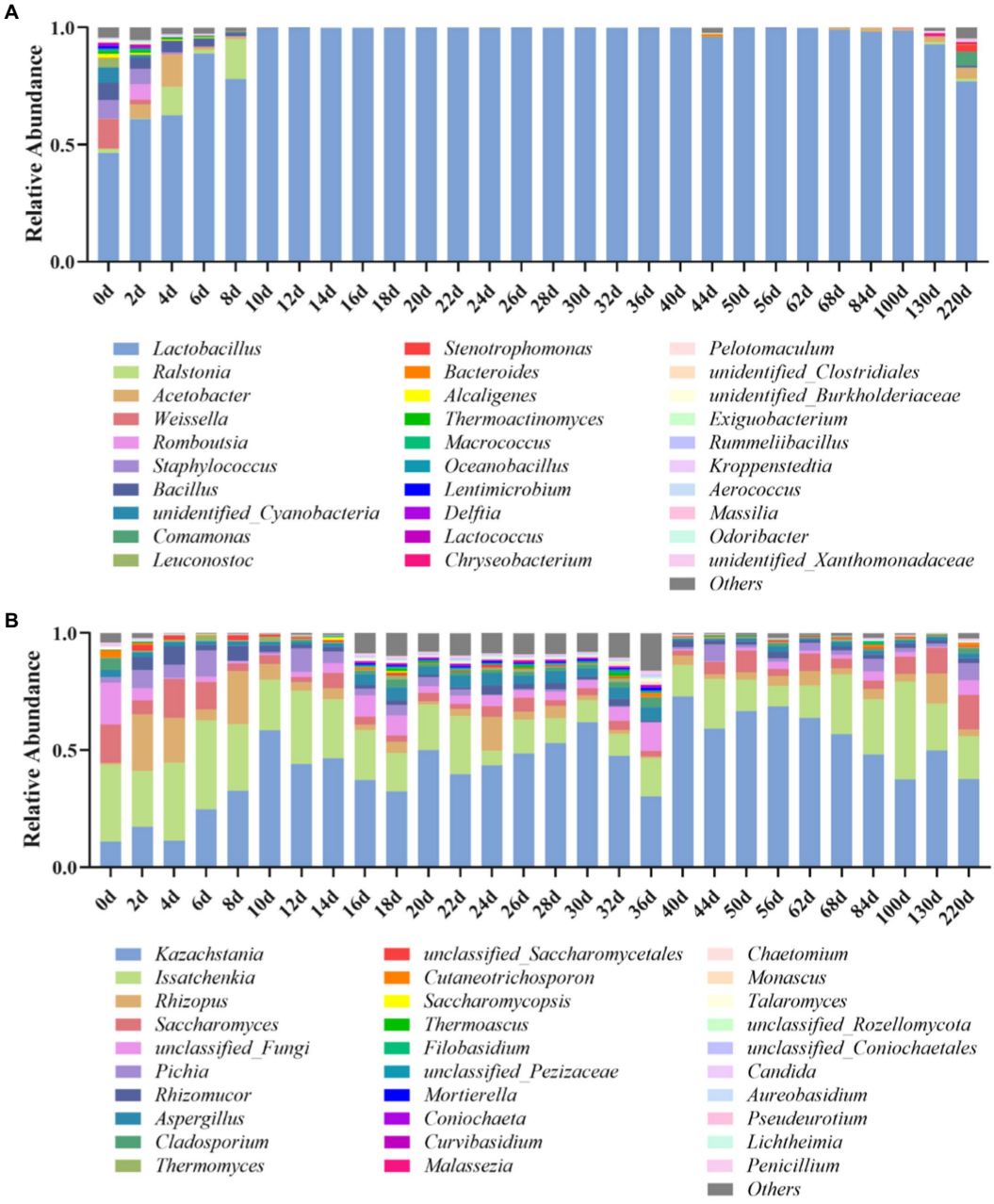

FIGURE 2
Bacterial **(A)** and fungal **(B)** microbial community compositions in Jiupei during fermentation process.

It can be found that the moisture content, alcohol content and acidity of Jiupei varied greatly in the pre-fermentation period, and then presented a relatively stable state throughout the fermentation period. During the fermentation process, the moisture content increased continuously with time, and finally stabilized at 61.63 ± 0.94% (Figure 3B). During the pre-fermentation period (0–10 days), the alcohol content increased rapidly, slowly increased after 10 days, and reached the highest point of 3.52% ± 0.38% on the 32 days. After 36 days, the alcohol content of the fermented grains became flat (Figure 3C). From 0 to 36 days, the acidity increased from 1.87 ± 0.39 (mmol/10 g) to 3.45 ± 0.49 (mmol/10 g), the increase rate was slow in 0–10 days, and the increase rate was fast in 10–36 days, and then the acidity of fermented grains remained basically unchanged, the acidity at the end of fermentation was 3.54 ± 0.25 (mmol/10 g) (Figure 3D). Figure 3E showed the changing trend of protein, starch and reducing sugar contents during the fermentation of fermented Jiupei. It can be seen that the fermentation parameters were significantly different at different time points. The protein content in the fermentation process showed a trend of first increasing and then leveling off, and the protein content of Jiupei in the cellar was 14.88 ± 0.97%. At 130 days, the protein content reached the highest point of 17.18 ± 0.28%. The starch content decreased from 15.35 ± 1.15% to 8.95 ± 0.19%, with a rapid decrease from 0 to 12 days, and then a slow decrease from 12 to 36 days. The reducing sugar content increased slowly from 1.63 ± 0.54% to 3.14 ± 1.06% on the 0–6 days, and then decreased slowly, and the reducing sugar content was 0.46 ± 0.09% in the later stage of fermentation. Our results show that the 0–36 days were an important period for substrate (reducing sugar) utilization and product accumulation.

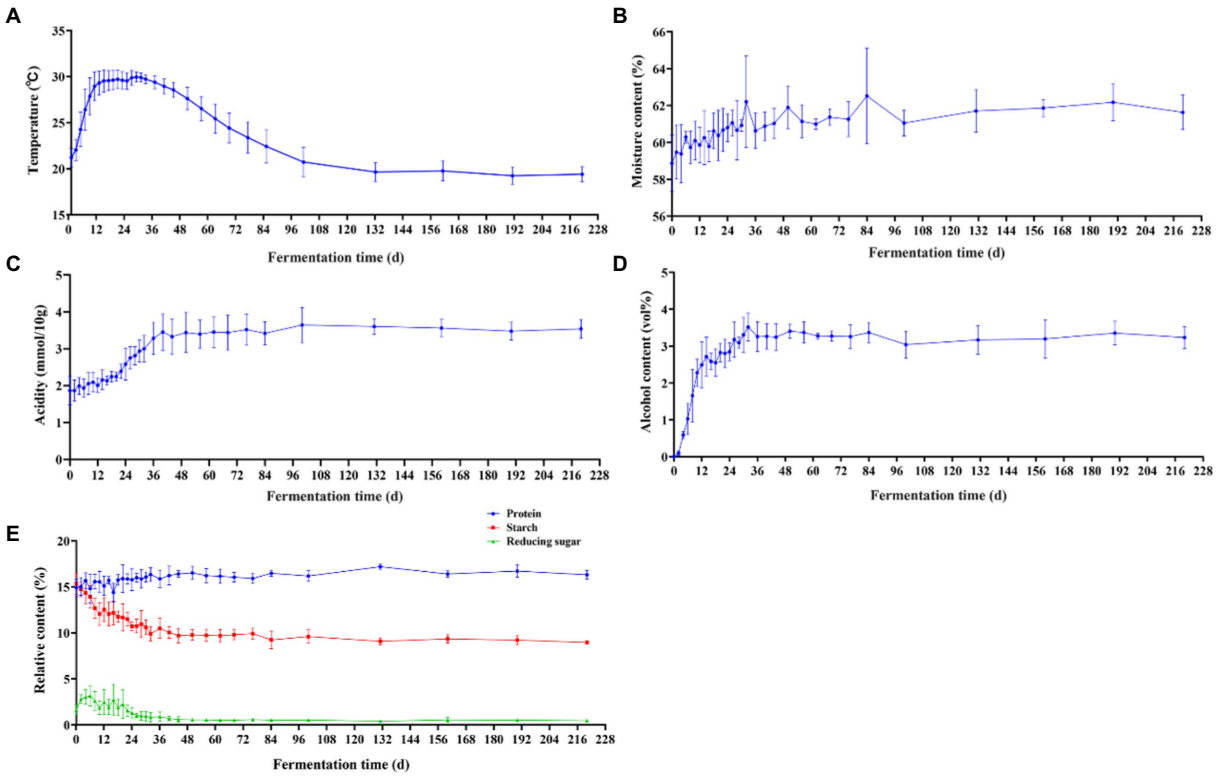

FIGURE 3
Changes in physicochemical parameters in Jiupei during fermentation process. **(A)** Temperature. **(B)** Moisture content. **(C)** Alcohol content. **(D)** Acidity. **(E)** Protein, starch, and reducing sugar content.

3.3. Driving factors of microbial communities during fermentation of Jiupei

We analyzed the driving forces of microbial community succession, including biotic and abiotic factors. During fermentation, temperature, moisture content, alcoholic content, acidity, and protein, starch, and reducing sugar content significant differences throughout the fermentation process, and some of these environmental variables were associated with changes in community dynamics. As shown in Figure 4, the trends of the community at the species level were related to its adaptability to environmental conditions, indicating a gradual stabilization of the microbial community structure under the stress of various environments. For example, most of the bacteria were negatively correlated with temperature, alcoholic content and acidity, and *Lactobacillus homohiochii* was positively correlated with protein content. *Rhizopus microsporus*, *Rhizopus arrhizus* and *Rhizomucor pusillus* were negatively correlated with moisture content, alcoholic content, protein content, and acidity, and positively correlated with starch content and reducing sugar content (Figure 4A). In contrast, *Pichia mandshurica*, *Pichia membranefaciens*, *Pichia others*, *Filobasidium magnum*, *Cutaneotrichosporon moniliiforme,* and *Cladosporium delicatulum* were positively correlated with alcohol content, acidity and protein content related (Figure 4B).

The RDA results shown that the structural succession of the Jiupei bacterial community were highly influenced by the alcoholic content (Figure 4C). In the pre-fermentation, starch content was the main driving force for the succession of the bacterial community, and as fermentation progressed, alcoholic content, moisture content, and temperature became the main driving forces, which had a significant effect on *Lactobacillus*. The extremely small angles between alcohol and acidity, temperature and moisture content, implying a indicating a significant synergistic drive between them. From Figure 4D, we found that starch content and reducing sugar content were the main driving forces from 0 to 10 days of fermentation, while temperature had the most significant effect on fungal community succession from 10 to 36 days. *Rhizomucor*, *Rhizopus* and *Thermomyces* were positively correlated with starch and reducing sugar content in initial stage. The main microorganisms significantly affected by temperature were *Kazachstania*, *Saccharomyces*, *Thermoascus*, *Issatchenkia*, and *Cutaneotrichosporon*.

3.4. Protein and protein peptides characteristics during Jiupei fermentation

3.4.1. Identification of microbial protein peptides and proteins

In this study, mass spectrometry (MS) data were collected for 39 microbial samples, and the statistics of spectra, peptides and protein for each sample were shown in Supplementary Table S2. Principal component analysis showed that there was an obvious succession trend in the fermentation process of Jiupei, which can be divided into three regions. The 0 day after the start of fermentation was located in the lower left corner, and the 4–18 days was the vigorous fermentation period, located in the upper left corner, and entered the stable fermentation period after 24 days

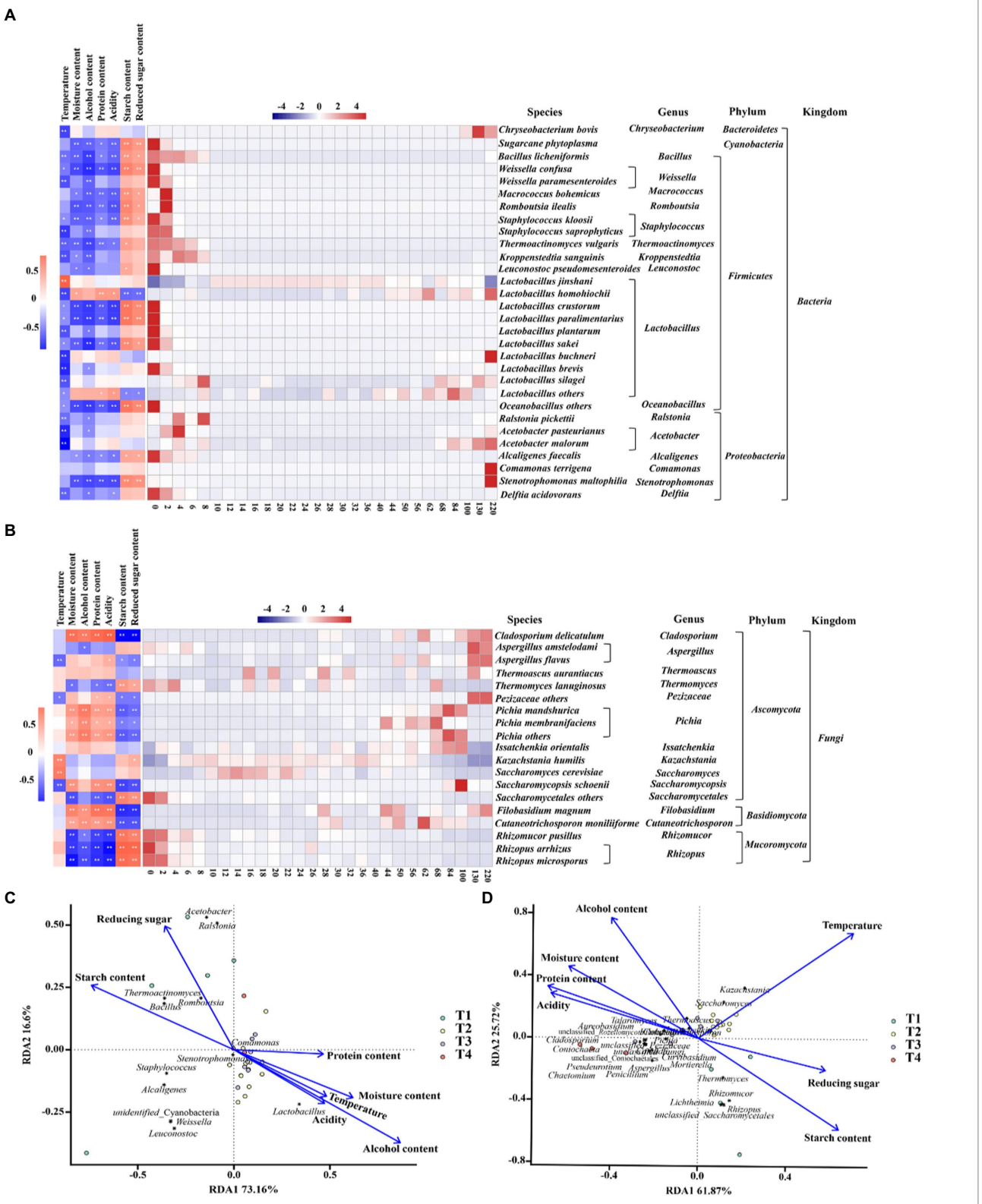

FIGURE 4
Changes in microbial community succession during the fermentation of Jiupei. **(A)** Community succession at the bacterial species level and its correlation with physicochemical parameters. **(B)** Community succession at the fungal species level and its correlation with physicochemical parameters. **(C)** The driving force in the fermentation process of bacterial of Jiupei. **(D)** The driving force in the fermentation process of fungal of Jiupei.

(Supplementary Figure S1). The Pearson correlation clustering also revealed that the protein species were more consistent from 0 to 18 days and from 18 to 220 days (Supplementary Figure S2). After a series of quality control, a total of 1,525 proteins were identified from the microbial proteins obtained from different sampling points during the fermentation of Jiupei, and 646 proteins were

distributed in the range of 10–20 kDa, with the highest content (Supplementary Figure S3).

3.4.2. Protein species composition analysis

We classified the species based on the comparison with the database, and the results showed that a total of 709 proteins were annotated to the species level, accounting for 46.49% (Supplementary Table S3). The proteins with higher relative abundance throughout the fermentation process were mainly derived from the top 10 species with the highest abundance in each sample. The relative abundance of *Lactobacillus jinshani* was the highest, and the relative content remained at 10% to 20% in 0–17 days of fermentation, and reached a peak in 18–24 days, accounting for about 50% of the total content, the abundance decreased slightly and remained stable during the 25–220 days. The relative abundance of *Kazachstania saulgeensis*, *Pichia kudriavzevii*, *Sphingobacteriaceae bacterium*, *Saccharomyces cerevisiae*, *Lactobacillus plantarum* in 0–220 days was greater than that of 25–220 days (Figure 5A).

3.4.3. Prediction of proteins function

Subsequently, the public databases including GO, KEGG, KOG, CAZy and Swiss-Port were annotated to elaborate the functions of the identified proteins. Among them, 82.59% have GO term classification, GO biological process was annotated to 74.91%, GO molecular function was annotated to 79.64%, GO molecular composition was annotated to 52.17%; Approximately 66.29% of proteins have orthologous groups in KOG, 9.26% have CAZy, 73.39% could be mapped to known biological pathways, and Swissprot was annotated to 86.33% in total (Supplementary Table S4). In the KOG database, proteins were divided into 23 entries, with the largest number of 231 corresponding to translation, ribosome structure and biogenesis, implying that there was a large amount of fermented grains in the fermentation process (Figure 5B). Furthermore, the top five functions in terms of protein were posttranslational modification, protein turnover, chaperones, energy production and conversion, carbohydrate transport and metabolism. It showed that microbial proteins mainly perform information storage and processing and metabolism-related functions during fermentation (Figure 5B). Interestingly, it was found that most proteins changed little during fermentation, while proteins performing carbohydrate transport and metabolism and amino acid transport and metabolism increased significantly on the fourth day; proteins produced by membrane biogenesis increased significantly and to a greater extent on the 18th day, indicating that gene expression and metabolism of microorganisms in the system changed significantly on the fourth day of fermented grains (Supplementary Figure S4). Counting the abundance of different functions in each sample, it can be found that succinate-semialdehyde dehydrogenase, elongation factor Tu, trimeric autotransporter adhesin, which were less abundant before 18 days of fermentation and increased significantly after 18 days; serine-type D-Ala-D-Ala carboxypeptidase, actin beta, and L-cystine transport system substrate binding protein showed opposite changes in abundance (Figure 5C). In order to explore the effect of microbial gene expression on Jiupei, KEGG pathway annotation was performed on the detected microbial proteins. The pathways involved in these microbial proteins mainly include carbohydrate metabolism, energy metabolism, translation, signal transduction and folding, sorting and degradation (Supplementary Figure S5). It can be seen that the content gradually increased with the prolongation of fermentation time, and the most rapid growth occurred at 18 days, decreased slightly, and stabilized after 32 days. The obvious changes in the number of proteins involved in these pathways suggested that fermentation for 18 days might be a critical time point for the role of microorganisms in the fermentation process (Figure 5D). A total of 47 glycoside hydrolases (GHs), 36 glycosyltransferases (GTs), 2 polysaccharide lyases (PLs), 12 carbohydrate hydrolases (CEs), 26 carbohydrate binding enzymes (CBMs) and 18 accessory module enzymes (AAs) were annotated in the CAZy database (Supplementary Figure S6A). Further analysis revealed that the contents of *CBM18*, *GH135*, *GH23* and *GT27* showed a significant increase at 18 days of fermentation, while *CBM21* and *CE16* showed a significant decrease (Supplementary Figure S6B).

3.5. Metabolomics study on fermentation process of Jiupei during long fermentation

3.5.1. Principal component analysis

We used LC–MS and GC–MS analysis to further investigate the differences and dynamic changes of metabolites during the fermentation of Jiupei, and sampling time points were 0, 4, 8, 12, 18, 24, 32, 44, 56, 68, 100, 130, 220 days, numbered JZ_0, JZ_4 … JZ_220. PCA analysis reflects the clustering of metabolites in the fermentation process; the closer the metabolic levels, the closer the points on the score plot. PCA was performed based on the obtained data. As shown in PCA score plots (Figure 6A), QC samples were clustered together, indicating good inter-sample repeatability and credible experimental data for the in-depth analysis. The first principal component (PC1) accounted for 25.6% of the variance, and the second principal component (PC2) accounted for 6.8% of the variance. The metabolites during the fermentation of Jiupei changed gradually, and the samples at different times were arranged chronologically on PC1, which also indicated that the changes of the groups had a tendency to move in time, which also implied that the fermentation of Jiupei caused the chemical composition to change continuously.

3.5.2. Metabolite identification

In the untargeted metabolomic analysis, 1,064 metabolites were co-qualified during the fermentation of the Jiupei (Figure 6B), among which organic acids and derivatives were the most types, accounting for 22.27%, followed by lipids and lipid-like molecules (20.02%), phenylopropanoids and polyketide (10.24%), orgaboheterocyclic compounds (10.15%), benzenoids (8.46%) and Organic oxygen compounds (6.86%). The results also showed that a total of 200 amino acids, peptides and their similar substances were identified, mainly including proline, leucine, ornithine, and lysine, the content of which increased rapidly at 18 days of fermentation, reached a maximum at 44 days and remained dynamically stable at the later stage of fermentation (Figure 6C).

3.5.3. Differential expressed metabolites analysis

We used the OPLS-DA model to extract information on the differences of metabolites in Juppei samples with different fermentation times, using JZ_0 as the control group. The differentially expressed metabolites (DEMs) identified if VIP > 1 and p-value < 0.05. The results showed that there were fewer DEMs (100–200) between the first 24 days of fermentation and 0 day, and more than 200 DEMs between the first 32 days of fermentation and 0 day. Combined with PCA score plot (Figure 6A), it could be found that samples from fermentation 4, 8, 12, and 18 days and samples from 32, 44, 56, 68, 100, 130, and 220 days

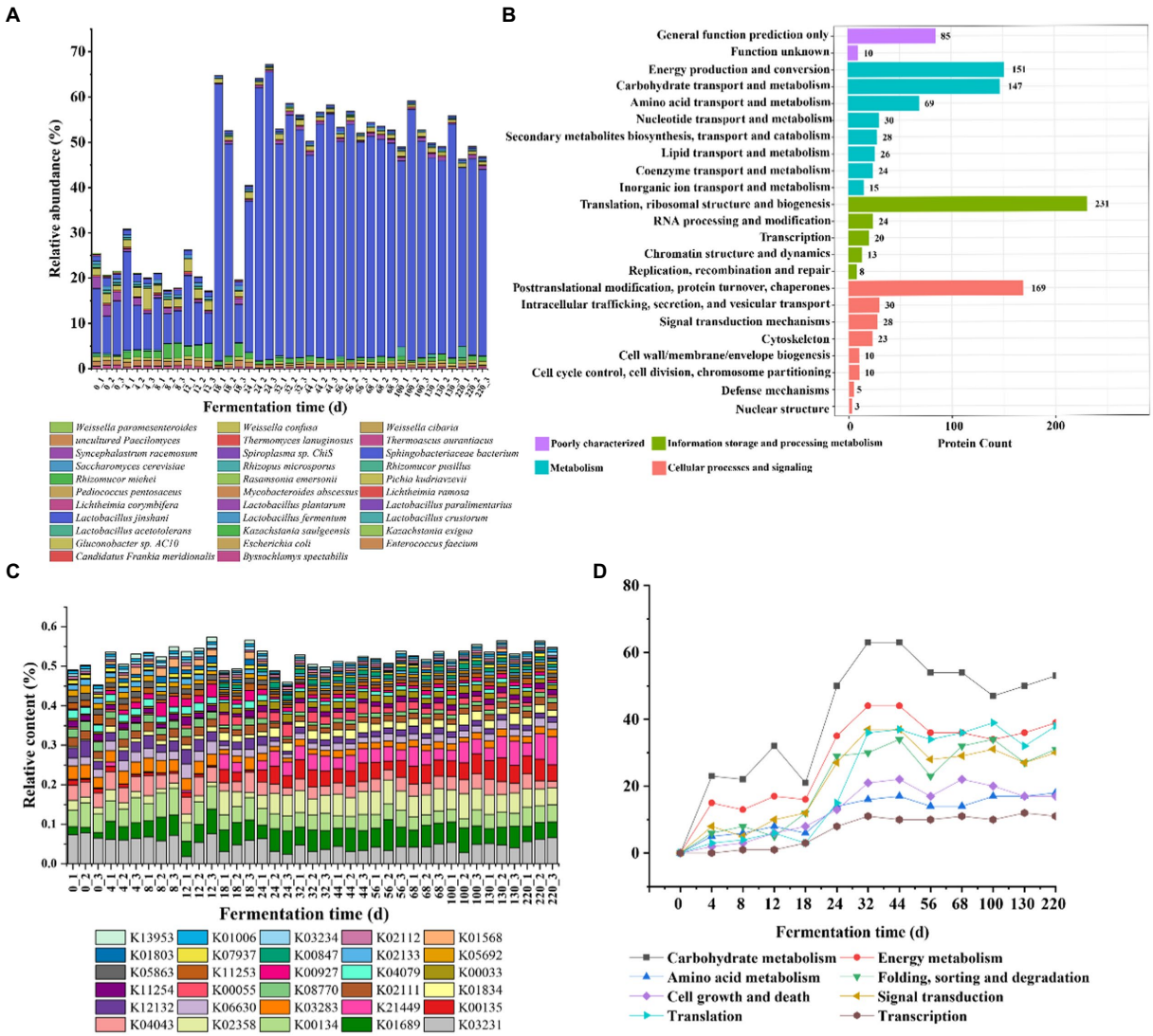

FIGURE 5
Species composition and functional annotation in Jiupei during fermentation process. **(A)** Microbial composition in Jiupei during fermentation process. **(B)** KOG function annotated histogram. **(C)** KO community structure function annotation. **(D)** Changes in the number of major pathways during fermentation.

cannot be well separated, while on the 24 days of fermentation could be well separated from other samples. It was also evident that the content of amino acids, peptides and their analogs increased rapidly from 18 to 32 days of fermentation and then remained in dynamic equilibrium. The results also indicated that the metabolites of the Jiupei changed gradually with fermentation from 4 to 18 and 32 to 220 days. The faster change in metabolites from 18 to 32 days may be due to the higher metabolic activity of microorganisms during this period (Figure 6C).

Subsequently, six samples of Jiupei fermented for 18, 24, 32, 100, 130, and 220 days were selected for differential metabolite analysis with the control group (0 days). At JZ_18 *VS* JZ_0, we identified 115 DEMs (up-regulated: 79, down-regulated: 36), mainly lipids and lipid-like molecules (30.43%) and organic acids and derivatives (11.30%). At JZ_24 *VS* JZ_0, we identified 168 DEMs (up-regulated: 117, down-regulated: 51), mainly organic acids and derivatives (32.75%) and lipids and lipid-like molecules (18.45%). Compared with JZ_18 *VS* JZ_0, the number of amino acids, peptides and analogs increased by 24 and the number of organic acids and derivatives increased by 6 in the DEMs. At JZ_32 *VS* JZ_0, we identified 255 DEMs (up-regulated: 196, down-regulated: 59), mainly organic acids and derivatives (27.84%) and lipids and lipid-like molecules (23.92%). Compared with JZ_24 *VS* JZ_0, the number of amino acids, peptides and analogs increased by 14 and the number of sugar substances and derivatives, organic acids and derivatives increased by 4 and 5 in the DEMs, respectively. We identified 366 (up-regulated: 281, down-regulated: 85), 317 (up-regulated: 226, down-regulated: 91) and 329 (up-regulated: 241, down-regulated: 88) DEMs in JZ_100 *VS* JZ_0, JZ_130 *VS* JZ_0 and JZ_220 *VS* JZ_0 were differentially expressed, correspondingly. The metabolites of the differences were mainly organic acids and derivatives and lipids and lipid-like molecules.

Different metabolites detected in samples with different fermentation times mainly include sugars and their derivatives (sucrose, fructose, sucrose, lactose, ribose, mannose, and glucose, etc.), organic acids (palmitic acid, linoleic acid, 2-hydroxy-4-methylvaleric acid, etc.), amino acids, peptides and their analogs (norleucine, isoleucine, ornithine, protein serine, etc.). In conclusion, the relative content of sugars in the

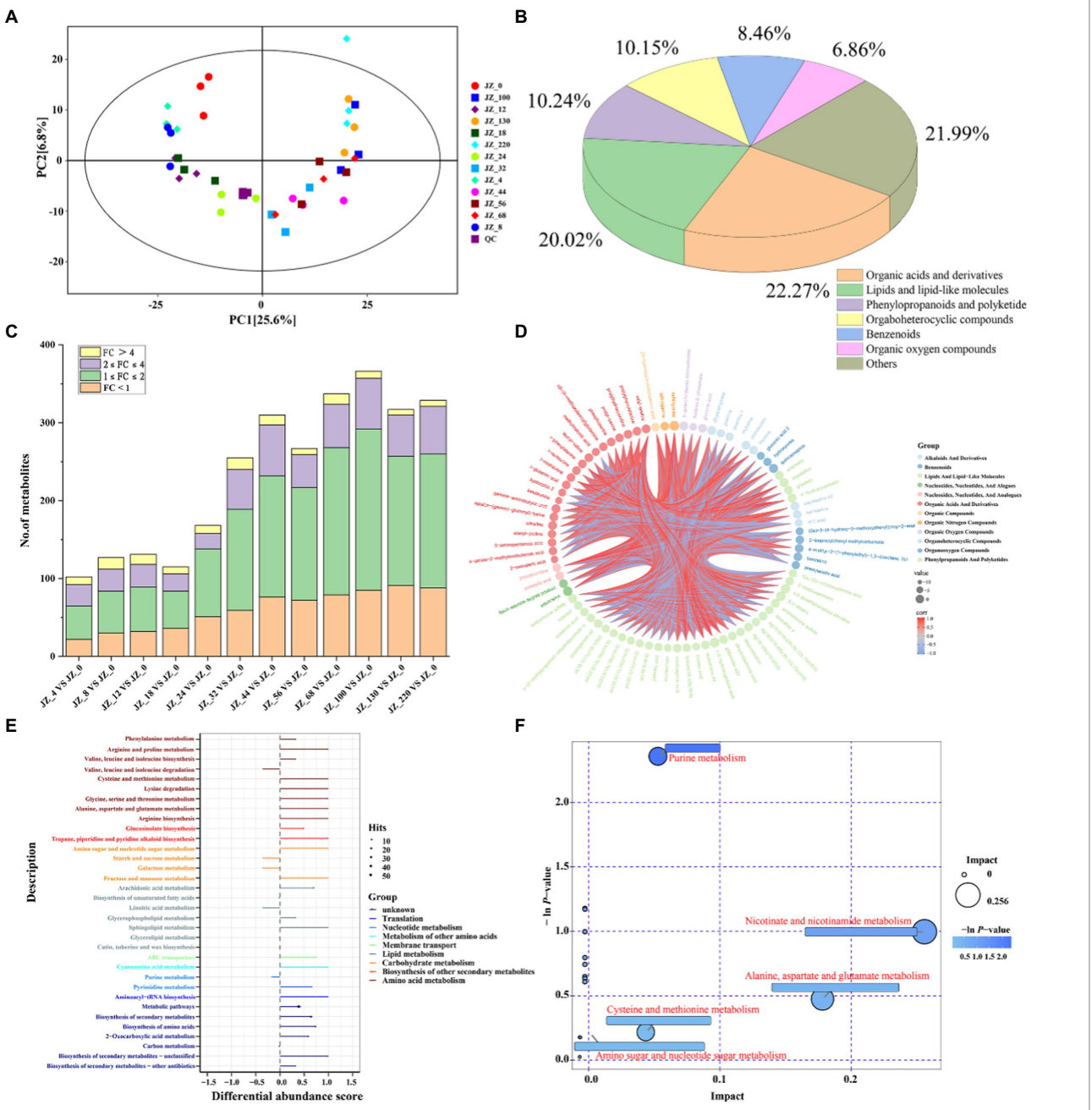

FIGURE 6
Metabolome analysis in Jiupei during fermentation process. **(A)** PCA score plot in Jiupei during fermentation process. **(B)** Classification of fermented Jiupei metabolites. **(C)** Differential metabolite species compared at different fermentation time points and the 0th day of fermentation. **(D)** Differential metabolite analysis of Z_18 *VS* JZ_0. **(E)** KEGG pathway analysis for differential metabolites. **(F)** Pathway bubble diagram of differential metabolites.

Jiupei decreased significantly at 18–32 days of fermentation and then remained dynamically stable, while organic acids and derivatives showed an upward trend throughout the fermentation process, probably due to the strong metabolic activity of microorganisms at 18–32 days, which required large amounts of sugars to meet their growth and reproduction, while the oxygen content in the cellar gradually decreased as fermentation progressed and bacteria began to produce acid by anaerobic fermentation, also indicating that the formation of flavor is a long process.

3.5.4. Analysis of differential metabolic pathways

To further understand the formation mechanism of metabolites during fermentation, metabolic pathways were analyzed. Differential Abundance Score (DAS) is a pathway-based analysis of metabolic changes, which can visualize the up- and down-regulation of metabolic pathways as a whole and the type of metabolism they belong to. As shown in Figure 6E, DAS analysis of the KEGG pathway showed that 17 pathways in JZ_18 *VS* JZ_0, all of which showed up-regulation and were mainly divided into translation, nucleotide metabolism, membrane transport, lipid metabolism, carbohydrate metabolism and amino acid metabolism (please refer to Supplementary Figure S5 for the results of metabolic pathway analysis of other samples). In general, the metabolites of the 18–32 days Jiupei changed rapidly and the metabolic pathways were highly variable, while the metabolites changed slowly and the metabolic pathways did not change significantly in the later stages of

fermentation. Next, we performed RefSeq's pathway database search and metabolic pathway analysis. As shown in Figure 6F, the results of pathway analysis at JZ_18 vs. JZ_0 showed that a total of 20 pathways were obtained, and screening of key metabolic pathways revealed four key metabolic pathways, including purine metabolism, niacin and nicotinamide metabolism, alanine, aspartate and glutamate metabolism, and cysteine and methionine metabolism. In general, the key metabolic pathways changed at 18–32 days of fermentation, showing an increase in the number of amino acid-related metabolic pathways as fermentation progressed, and no significant changes in the key metabolic pathways at 100–220 days of fermentation, indicating that amino acid metabolism was vigorous at 18–32 days of fermentation and slow changes in metabolites in the wine grains at the later stages of fermentation, consistent with the above The trend of the relative content of amino acids, peptides and their analogs with fermentation time is consistent with the above.

4. Discussion

For thousands of years, humans have been optimizing conditions including temperature, humidity and salinity to promote the growth of certain microbial communities and have obtained a variety of fermented foods such as cheese, alcoholic beverages, sourdough bread, soy sauce and vinegar (Pang et al., 2018). Although most modern fermented foods are inoculated with specific starter cultures, it is widely believed that the native microorganisms in traditional spontaneous fermentation add to the aroma of these foods (Petruzzi et al., 2017). Since Daqu preparation and alcohol fermentation are carried out under semi-controlled conditions, their unique ecology and production processes have been well enriched with specific microbiota through long and repeated practice (Pu et al., 2021). Our results showed that the relative abundance of *Lactobacillus* increased considerably in the samples as fermentation proceeded and became the dominant bacteria during the long fermentation of the Luxiang-flavor Baijiu Jiupei. *Lactobacillus* are mostly parthenogenic anaerobic bacteria or anaerobic bacteria, which are the dominant group in the production of many traditional fermented foods and have important biological structure regulation functions (Slattery et al., 2019; Reuben et al., 2020). They can use carbohydrates from raw materials to produce lactic acid and other substances (ethanol, acetic acid, carbon dioxide, etc.) to improve the flavor and storage life of foods (Lynch et al., 2018; Touret et al., 2018). The *Lactobacillus* use sugar to produce lactic acid and acetic acid, which can react with ethanol to produce ethyl lactate and ethyl acetate, of which ethyl acetate is an important flavor substance in Baijiu (Tian et al., 2020). Meanwhile, *Lactobacillus* can produce antagonistic substances, such as bacteriocins, and compete with other microorganisms for substrates and affect the growth of other microorganisms (Aarti et al., 2016). Through the amplicon sequencing technology, this study found that the relative abundance of *Lactobacillus* in fermented Jiupei gradually increased with the fermentation time, and became an absolutely dominant group in the later stage of fermentation. The demise of microorganisms that are not adapted to the brewing environment and some potential pathogenic microorganisms during the fermentation process can be regarded as the "self-purification" of the lactic acid bacteria community in the brewing process. This phenomenon has been reported in some open multi-strain mixed fermentation processes. The microbial community of the brewing bacteria gradually stabilizes and simplifies through the environmental pressure changes (temperature, acid, anaerobic, osmotic pressure and ethanol) caused by metabolic activities (Wang et al., 2015). In a word, various *Lactobacillus* occupy an absolute dominant position in the late stage of brewing and fermentation of Luzhou-flavor baijiu, which affects the flavor and quality of Luzhou-flavor baijiu.

The microbial proteins identified in the long fermentation process of fermented Jiupei were derived from *Lactobacillus jinshani*. The content of *Lactobacillus jinshani* changed significantly at the 18th day of fermentation, and the organic acids and their derivatives were the most species determined by metabolomics, accounting for 22.27%, which also increased from the 18th day. This indicated that *Lactobacillus jinshani* played an important role in the fermentation process of fermented Jiupei, and interestingly, *Lactobacillus* also dominated the microbial communities of other Chinese Baijius with different flavors (Liu and Miao, 2020). This may be due to the use of similar fermentation substrates (sorghum and wheat) and the purpose of the fermentation ecosystem, which is to convert starch feedstocks into ethanol and flavor compounds. Metatranscriptomic analysis showed that the metabolism of flavor compounds and their precursors by *Lactobacillus* plays a key role in Luzhou-flavor Baijiu (Du et al., 2020). *Lactobacillus* was the core functional microorganism for lactic acid accumulation during the production of soy sauce aroma Baijiu (Song et al., 2017b). In the later stage, the oxygen content in the fermented Jiupei decreased, which led to the decrease of *Lactobacillus jinshani* (Wei et al., 2021). The genus *Kazachstania* was the most abundant genus of fungi, and can also affect the fermentation of Luzhou-flavor Baijiu by interacting with other mixed fermenting microorganisms. Studies have found that *K. humilis* can inhibit the production of lactic acid by *L. acetotolerans*, and *L. acetotolerans* can promote the production of *L. acetotolerans* produces ethanol, indicating that yeast of this genus can affect the fermentation process of Luzhou-flavor Baijiu through the interaction with lactic acid bacteria (Wang et al., 2021). Prolamin was the most abundant component in all flavor-type distiller's grains powder, accounting for about 40–45% of the total extracted protein. This is mainly due to the fact that prolamin is insoluble in water and easily soluble in 70% vol. However, during the brewing process of Baijiu, the concentration of ethanol in the environment has not reached a concentration sufficient to dissolve it, so the gliadin cannot be used, so it accumulates in the Jiupei after distillation (Jiang et al., 2020). The metaproteomics analysis of the proteins in the fermented Jiupei after dehosting showed that the proteins involved in carbohydrate transport metabolism and amino acid transport metabolism during Jiupei fermentation increased significantly on the 4th day; membrane biogenesis proteins on the 18th day significantly increased, and the increase range was greater, indicating that the gene expression and metabolism of microorganisms in the system on the 4th day of fermentation of Jiupei had changed a lot. Among them, *Lactobacillus jinshani* had the highest protein expression, and *Lactobacillus jinshani* was found to be widely present in Baijius in various producing areas of the country in the brewing system (Du et al., 2020). It has been reported that *Lactobacillus jinshani* was dominant in the late stage of fermented grains, which may be related to the better acid resistance of the bacteria (Yu et al., 2020). Therefore, *Lactobacillus jinshani* in Luxiang-flavor Baijiu plays a key role in the accumulation of Jiupei proteins and the flavor of Baijiu.

Data availbility 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

JM and CS: designed this experimental subject. XL was involved in the analysis of the entire study and drafted the manuscript. DM, CY, and QY: performed data collection, formal analysis, and data curation. JM, SL, DM, and XL critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

XL was employed by Luzhou Laojiao Group Co. Ltd. SL and JM were employed by Zhejiang Guyuelongshan Shaoxing Wine 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.2023.1109719/full#supplementary-material

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Study on the correlation between microbial communities with physicochemical properties and flavor substances in the Xiasha round of cave-brewed sauce-flavor Baijiu

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The Chishui River basin is the main production area of the sauce-flavor Baijiu. Due to the particularity of sauce-flavor Baijiu technology, a large site of workshops needs to be built for brewing and storage. Therefore, used the natural karst caves of Guizhou province to manufacture the sauce-flavor Baijiu, which has enriched the connotation of sauce-flavor Baijiu and saved valuable land resources. In this study, the fermentation grains in the seven stages during the Xiasha round of the cave-brewed sauce-flavor Baijiu (CBSB) were detected using a combination of physicochemical analysis, Headspace solid-phase microextraction gas chromatography-mass detection, and Illumina HiSeq sequencing methods. The results showed *Unspecified_Leuconostocaceae*, *Weissella*, *Unspecified_Bacillaceae*, *Saccharomycopsis*, *Thermomyces*, and *Unspecified_Phaffomycetaceae* were the main bacterial and fungal genera in the stacking fermentation (SF). In the cellar fermentation (CF), the *Lactobacillus*, *Unspecified_Lactobacillaceae*, *Thermoactinomyces*, *Saccharomycopsis*, *Unspecified_Phaffomycetaceae*, and *Wickerhamomyces* were the main bacterial and fungal genera. A total of 72 volatiles were detected in the fermented grains. Linear discriminant analysis Effect Size (LEfSe) identified 23 significantly different volatile metabolites in the fermentation process, including 7 esters, 6 alcohols, 4 acids, 3 phenols, 1 hydrocarbon, and 2 other compounds. Redundancy analysis was used to explore the correlation between dominant microbial genera and physicochemical properties. Starch was the main physicochemical property affecting microbial succession in the SF. Acidity, moisture, and reducing sugar were the main driving factors of microbial succession in the CF. The Pearson correlation coefficient revealed the correlation between dominant microbial genera and significantly different volatile flavor substances. A total of 18 dominant microbial genera were associated with significantly different volatile metabolites, *Lactobacillus*, *Weissella*, *Wickerhamomyces*, and *Aspergillus* were shown to play crucial roles in metabolite synthesis. On this basis, a metabolic map of the dominant microbial genera was established. This study provides a theoretical basis for the production and quality control of sauce-flavor Baijiu brewed in natural karst caves and lays a foundation for studying the link between flavor formation and microorganisms.

KEYWORDS

cave brewed sauce-flavor Baijiu, Xiasha round, microbial communities, physicochemical properties, volatile flavor substances

1. Introduction

Distilled spirit has been used in China for over 2,000 years and occupies a very unmatched place in traditional Chinese culture (Liu and Sun, 2018). As one of the most complex and typical Baijiu in traditional Chinese Baijiu, sauce-flavor Baijiu is popular with many consumers (Wang Y. R. et al., 2021). The production process is based on a yearly production cycle, with two feeds, nine steams, eight fermentations, and seven distillations (Wang M. Y. et al., 2018). Due to its unique high-temperature fermentation process and particular environment, sauce-flavor Baijiu is distinguished from others by its complex flavors (Wang et al., 2017). The fermentation of sauce-flavor Baijiu is a complex process involving microbial interactions (Jin et al., 2017) that are influenced mainly by the region they are produced (Bokulich et al., 2014). Sauce-flavored Baijiu relies on unrepeatable ecological resources, and its main production areas are concentrated in Renhuai, Xishui, and Jinsha, of the Guizhou Province, China. Meanwhile, the Chishui River basin is the prime production area of the sauce-flavor Baijiu. The distinct manufacturing process of sauce-flavor Baijiu requires a large area for brewing and storage. The unique natural caves formed by the Guizhou karst terrain can be used for this purpose. Located on the banks of the Chishui River, the Guixian Cave is currently the only natural cave in the country for brewing sauce-flavor Baijiu. The cave's relatively stable temperature and humidity throughout the four seasons provide the unique conditions required for manufacturing cave-brewed sauce-flavor Baijiu (CBSB), thereby giving it more connotations.

The first grain-feeding stage of sauce-flavor Baijiu brewing is called the Xiasha round. Sorghum was gelatinized and decomposed to produce various flavors and precursor substances, which lays a foundation for the follow-up brewing process (Hao et al., 2021). The whole fermentation process is divided into two processes: stacking fermentation (SF) and cellar fermentation (CF). The physical and chemical properties (temperature, moisture, acidity, reducing sugar, starch) of the process are essential factors that affect the evolution of the microbial community (Wang H. et al., 2021). The microorganisms in fermented grains come from high-temperature Daqu and the production environment, including air, soils, water, and production tools (Wang et al., 2019; Zhang H. X. et al., 2021a). Bacteria can use protein and starch and contribute significantly to Baijiu's flavor formation (Zuo et al., 2020; Tu et al., 2022). Yeast mainly produces various flavor compounds and alcohol (Hu et al., 2020). Molds can secrete different enzymes to decompose raw materials and provide energy for the growth of other bacterial groups (Wang et al., 2014; Hu Y. L. et al., 2021).

Recent research on Chinese Baijiu has focused on the succession of microbial communities, the screening of functional microorganisms, and the correlation between the microbial communities and the physicochemical properties and flavor substances (Zou et al., 2018a; Jin et al., 2019; Song et al., 2019; Mu et al., 2022). These studies have revealed the core functional microorganisms of the fermentation process of Chinese Baijiu and their contribution to the flavor substances, laying a solid foundation for Chinese Baijiu research. However, these studies are all performed on the fermentation process in a conventional environment distillery, and rare research on brewing Baijiu in natural caves has been reported.

Physicochemical analysis, high-throughput sequencing, and headspace solid-phase micro-extraction gas chromatography-mass spectrometry (HS-SPME-GC-MS) were carried out to analyze the physicochemical properties, the microbial community structure, and the volatile flavor substances. In addition, redundancy analysis (RDA) and the Pearson coefficient analysis were performed to assess the correlation between microbiota and physicochemical properties with the volatile flavor substances. The results enabled metabolic mapping of the dominant genera during the Xiasha round of CBSB. This study can provide a theoretical basis for regulating the CBSB fermentation process.

2. Materials and methods

2.1. Sample collection

The fermented grain samples were manufactured between September 2020 and October 2020 during the Xiasha round at the Dongniangdongcang Winery, Guizhou Province, China. The fermentation process was carried out in two distinct phases: SF and CF. Samples of SF were collected on days 0, 1, 2, and 3 and marked as SF0, SF1, SF2, and SF3, respectively. For the CF stage, the samples were collected at 10-day intervals until the end of fermentation and marked as CF10, CF20, and CF30. The SF and CF sampling diagrams are shown in Figures 1A, B, respectively. The samples were collected from the upper, middle, and bottom layers of the fermentation piles and cellars at each sampling time point. One point was selected in the middle and four points at the edge of each layer, and at each point, 30 g of samples was collected. All samples collected from each point of all three layers were then evenly combined into one mixture to eliminate sampling error (Zhao et al., 2021). After mixing, they were immediately packed in a sterile sealed bag and stored in a low-temperature refrigerator at −80°C until analysis.

2.2. Analysis of physicochemical indicators

To understand the fermentation processes, temperature, moisture, acidity, reducing sugar, and starch were detected. An alcohol thermometer was inserted into the fermented grains using a self-made tool, and the temperature was measured quickly after 5 min. The moisture of fermented grains was determined by a gravimetric method by drying samples to a constant weight at 105°C. The titratable acidity was determined by titration with a standard NaOH (0.1 mol/L) solution. A standard glucose solution (1.0 g/L) was used to titrate reducing sugar and starch in the fermented grains.

2.3. Analysis of volatile flavor substances

The fermented grain sample was ground and mixed, and 1.00 g of it was placed in a 20 ml headspace flask with 2 g of NaCl and 5 ml of purified water. Then, 10 μl of cyclohexanone (20 μg/ml) was added as the internal standard. The SPME fibers were extracted by 50/30 μm DVB/CAR/PDMS fiber (Sigma Aldrich, USA) at 40°C

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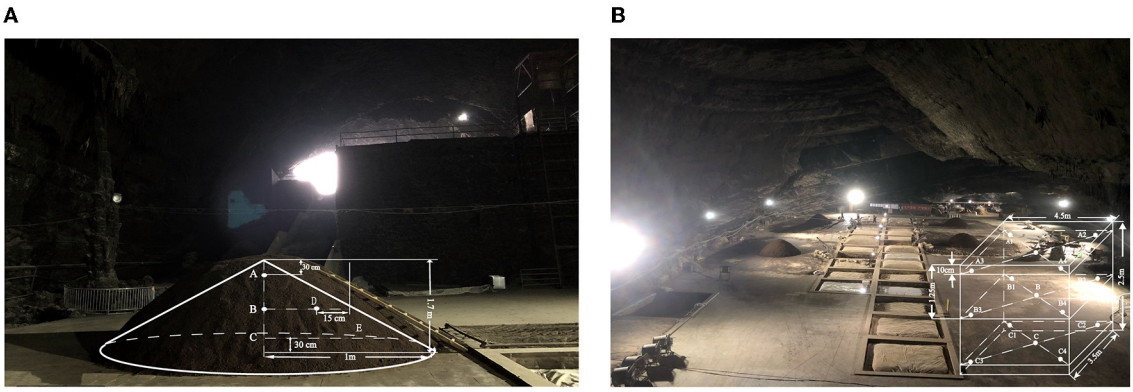

FIGURE 1
Spoil sampling points **(A)** sketch map of stacking fermentation sampling; **(B)** sketch map of cellar fermentation sampling.

TABLE 1 Changes in the physicochemical properties during the fermentation process.

Samples	Temperature (°C)	Moisture (%)	Acidity (ml/g)	Reducing sugar (in glucose, %)	Starch (%)
SF0	27.00 ± 5.00^{b}	41.53 ± 0.15^{e}	0.55 ± 0.08^{e}	0.12 ± 0.01^{e}	40.96 ± 0.04^{a}
SF1	27.33 ± 5.13^{b}	41.46 ± 0.15^{e}	0.57 ± 0.03^{e}	0.13 ± 0.01^{e}	39.24 ± 0.30^{b}
SF2	38.00 ± 8.66^{ab}	40.71 ± 0.27^{f}	0.65 ± 0.04^{e}	0.55 ± 0.03^{c}	38.65 ± 0.03^{c}
SF3	45.67 ± 8.02^{a}	43.41 ± 0.26^{d}	0.83 ± 0.05^{d}	0.82 ± 0.01^{a}	38.45 ± 0.22^{c}
CF10	31.33 ± 3.22^{b}	45.75 ± 0.28^{b}	1.81 ± 0.00^{c}	0.64 ± 0.02^{b}	36.59 ± 0.28^{d}
CF20	31.33 ± 3.22^{b}	45.01 ± 0.08^{c}	2.48 ± 0.07^{b}	0.57 ± 0.03^{c}	35.79 ± 0.03^{e}
CF30	30.33 ± 4.16^{b}	46.30 ± 0.09^{a}	2.77 ± 0.05^{a}	0.50 ± 0.01^{d}	32.52 ± 0.03^{f}

Different letters in the same column indicate significant differences ($P < 0.05$).

for 180 min, inserted into the injection port, and then separated by resolution at 230°C for 5 min.

Chromatographic conditions: A gas chromatography-mass spectrometer was used to analyze the fermented grain (Thermo Fisher Scientific, USA). The capillary column was DB-5MS (30 m × 0.25 mm × 0.25 μm, Agilent, USA) with helium (99.999%) as the carrier gas at a 1.0 ml/min flow rate in the non-split mode. Heating procedure: The column was maintained at 40°C for 5 min, then increased to 150°C at 5°C/min, later maintained at 150°C for 3 min, and finally increased to 240°C at 5°C/min and maintained for 5 min. Mass spectrometry conditions: electron bombardment ion source (EI), electron energy of 70 eV, a transmission line temperature of 280°C, and an ion source temperature of 230°C. The data were collected in the 50–450 amu at a rate of 1 scan/s. The constituents were tentatively identified by matching the mass spectrum with the NIST5 spectrum database and comparing their Kováts retention index (RI) with the RI reported in the literature, calculated using the C7–C40 n-alkanes, for verification.

2.4. Analysis of the microbial community

Total genomic DNA was extracted with the FastDNA® Spin Kit according to the manufacturer's protocol. DNA purity and concentration were checked using a NanoDrop 2000 ultra-micro spectrophotometer, followed by 1% agarose gel electrophoresis at 5 V/cm for 20 min to check DNA integrity. The V3-V4 region of the bacterial 16S rRNA gene was amplified with primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′), and the ITS1 region of the fungi was amplified with the primers ITS1F (5′- CTTGGTCATTTAGAGGAAGTAA-3′) and ITS1R (5′-GCTGCGTTCTTCATCGATGC-3′).

The total PCR amplification volume was 20 μl, including 0.8 μl of the forward primer (5 μm), 0.8 μl of the reverse primer (5 μm), 2 μl of 2.5 mm dNTPs, 2 μl of 10 × buffer, 0.2 μl of rTaq polymerase, 0.2 μl of BSA, 10 ng of template DNA, and 20 μl of ddH_2O. The PCR conditions were as follows: initial denaturation at 95°C for 3 min, denaturation at 95°C for 30 s, annealing at 53°C and 55°C for 30 s for bacteria and fungi, respectively, followed by extension at 72°C for 45 s, 29 and 35 cycles for bacteria and fungi, respectively, and final extension at 72°C for 10 min and cooling to 10°C to end the reaction. All PCR products were tested for concentration and purity using NanoDrop2000, and 2% agarose gel electrophoresis was used for integrity testing.

The libraries were quantified and tested by Qubit. Then, the purified amplicons were pooled in equimolar amounts and paired-end sequenced (2 × 300) by using an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocols. Each sample was quality filtered, trimmed, denoised, and merged. Then, the chimeric sequences were identified and removed using the QIIME2 dada2 plugin to obtain the feature table

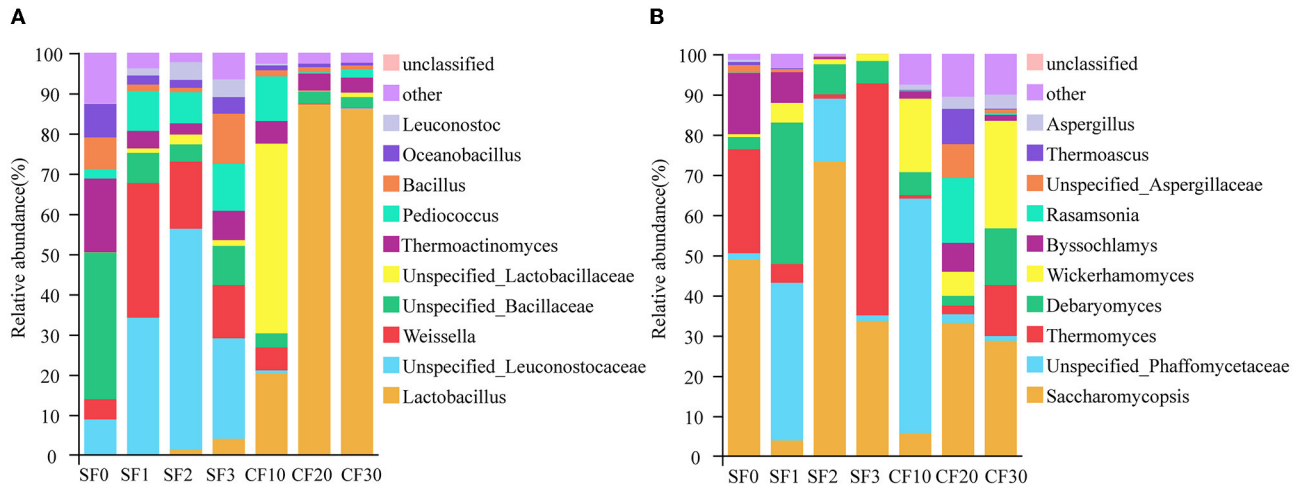

FIGURE 2
Relative abundance of bacterial **(A)** and fungal **(B)** genera in the Xiasha round of the cave-brew sauce-flavor Baijiu.

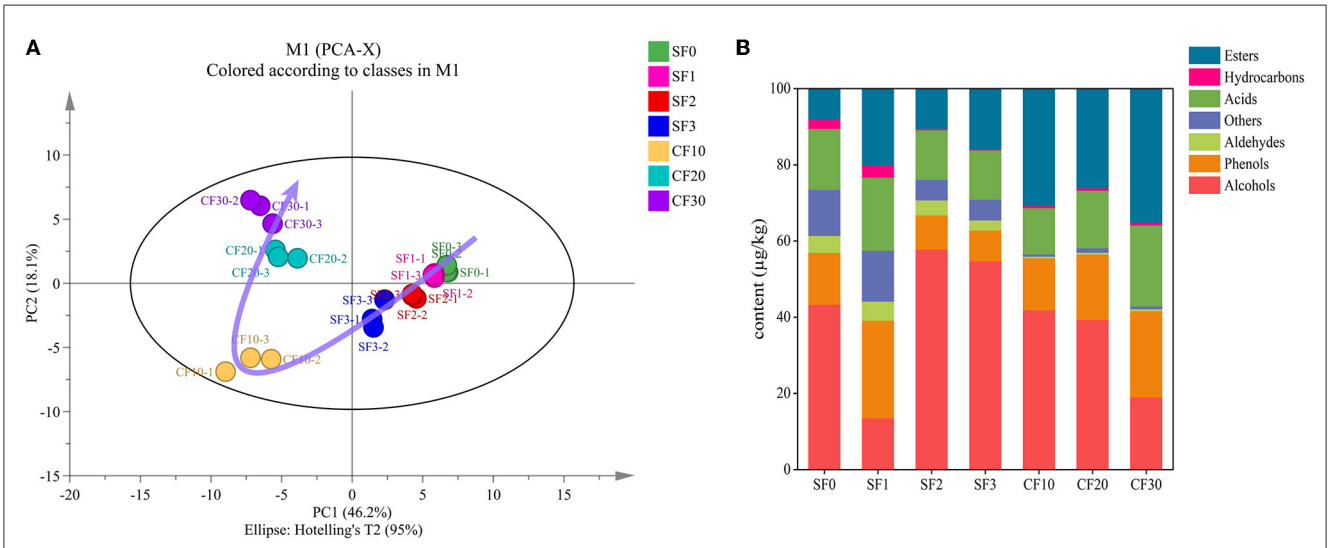

FIGURE 3
Principal component analysis of volatile compounds **(A)** and percent stacked column chart of volatile flavor substances **(B)**.

of the amplicon sequence variant (ASV) (Callahan et al., 2016). Taxonomic annotation was carried out on the obtained ASVs based on the SILVA (bacteria) and UNITE (fungi) taxonomic databases.

2.5. Statistical analysis

The experiments were repeated three times; data analyses were performed using IBM SPSS statistics 26.0, and the obtained data were analyzed by ANOVA with a *P*-value of <0.05 as the significance level. A principal component analysis was performed using SIMCA (version 14.1), and the graphs were drawn using Origin (version 9.8). Visualized correlation data painting was performed using the Cytoscape software (version 3.8.2).

3. Results and analysis

3.1. Changes in the physicochemical properties

As shown in Table 1, due to the enrichment and rapid growth of microorganisms, the temperature reached a maximum of 45.67 ± 8.02°C on day 3 of the SF process. We observed the time required by the sample to reach the highest temperature in the SF stage and found that it was consistent with that of the sauce-flavor Baijiu brewed in a conventional environment distillery (Hao et al., 2021). The acidity increased significantly ($P < 0.05$) on day 3 of SF, and then, the increase was more significant in the CF stage, which may be due to the accumulation of acid produced by the

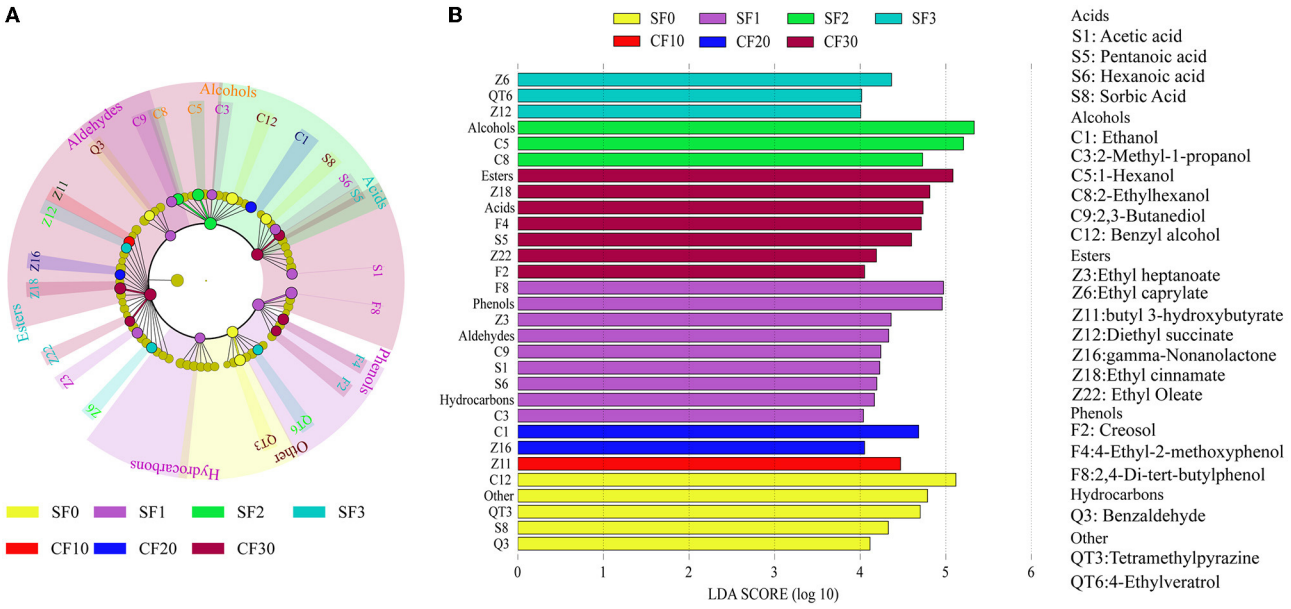

FIGURE 4
The LEfSe analysis of the flavor components in the Xiasha round of cave-brew sauce-flavor Baijiu. Cladogram **(A)** and LDA bar graph **(B)** results for flavor metabolite contents. Flavor compounds were evaluated by linear discriminant analysis coupled with effect size (LDA = 4, $P < 0.05$).

microorganisms during anaerobic fermentation (Cai et al., 2019). The starch content decreased significantly ($P < 0.05$) on day 1 of SF from 40.96 ± 0.04% to 39.24 ± 0.30%, with a decreasing trend in the CF stage. The reduced sugar content increased significantly ($P < 0.05$) on day 2 in the SF stage and significantly decreased in the CF stage ($P < 0.05$). The rapid growth and multiplication of microorganisms in the SF stage consumed starch to produce monosaccharides, which were further used to produce more flavors during the CF stage (Wu et al., 2013; Hu X. L. et al., 2021). The growth and reproduction of microorganisms produced a large amount of biological heat (Cai et al., 2019). Therefore, the moisture content tended to decrease during the SF process. The increased moisture content during CF was related to the interaction between microorganisms (Huang et al., 2020; Hu Y. L. et al., 2021).

3.2. Differences in the microbial community structure during the fermentation process

The changes in the microbial community structure of the 21 samples from 7 time points were detected. The effective sequences of the bacteria and fungi ranged from 23,824 to 38,841 and 16,487 to 39,450, respectively. Although the number of fungal sequences in the samples was greater than that of bacteria, the total number of bacterial ASV was much greater than that of fungi (Supplementary Table S1). In addition, the sparsity curve reached a saturation plateau, indicating that the sequencing depth can represent the microbial diversity of the samples (Supplementary Figure S1). The samples were analyzed for α-diversity, and Supplementary Table S2 lists the species richness and diversity parameters, including the Chao1, Shannon, and Simpson indices. The diversity indices were consistent with the ASV results. Bacterial richness was greater than fungi in the SF, while the opposite was true in the CF stage. During the CF stage, bacterial diversity decreased but fungal diversity increased.

The results showed that, among 19 bacterial phyla and 185 bacterial genera, Firmicutes was the dominant bacterial phylum (90.18–99.73%). We defined the top 10 genera in relative abundance as the dominant microorganism genera to study community succession during fermentation (Figure 2). In all samples, the dominant bacterial genus contributed to 95.28% of the bacterial sequences, as shown in Figure 2A. The dominant microbial genera include *Lactobacillus* (28.46%), *Unspecified_Leuconostocaceae* (17.63%), *Weissella* (10.65%), *Unspecified_Bacillaceae* (9.60%), *Unspecified_Lactobacillaceae* (7.63%), *Thermoactinomyces* (6.64%), *Pediococcus* (6.43%), *Bacillus* (3.83%), *Oceanobacillus* (2.79%), and *Leuconostoc* (1.62%).

The bacterial community in the fermented grains changed significantly during the fermentation process (Figure 2A). *Unspecified_Bacillaceae* and *Thermoactinomyces* were the dominant genera during the SF1, which might be derived from high-temperature Daqu (Zuo et al., 2020). As SF proceeded, *Unspecified_Leuconostocaceae* and *Weissella* gradually replaced *Unspecified_Bacillaceae* and *Thermoactinomyces* as the dominant genera between days 1 and 3. The reason for the change in *Weissella* abundance may be due to the floor, tools, etc., in the operating environment (Wang X. S. et al., 2018). After the end of SF and into the cellars, which was anaerobic fermentation, the dominant bacterial genera were *Lactobacillus*, *Unspecified_Lactobacillaceae*, and *Pediococcus* at day 10 of CF. These acid-tolerant microorganisms gradually replaced the non-acid-tolerant ones as the acidity of the anaerobic fermentation system increased. During days 20–30 of CF, *Lactobacillus* became

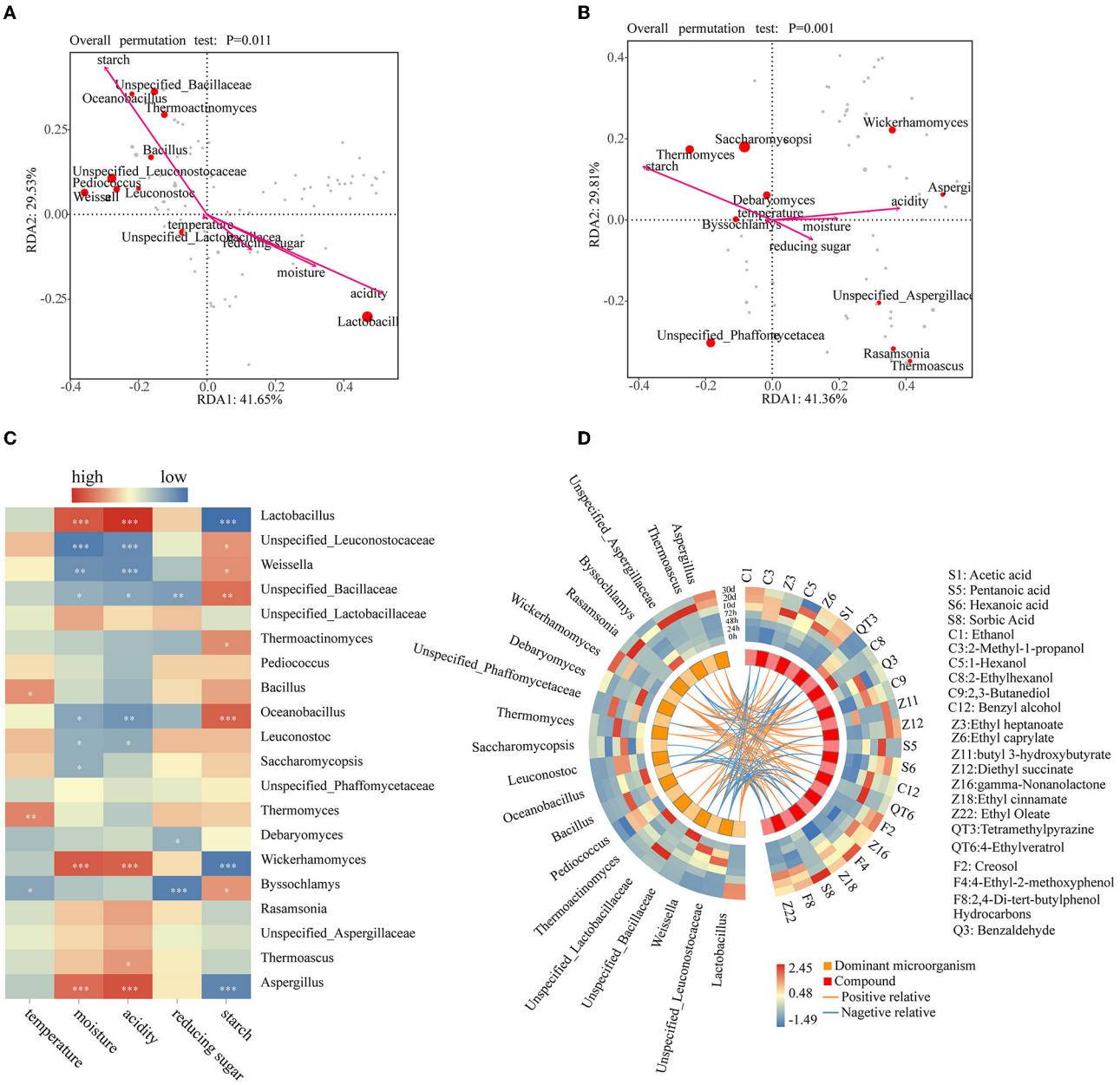

FIGURE 5
RDA analysis of the physicochemical properties and the bacterial **(A)** and fungal genera **(B)**, the heat map of the correlation index between the top 10 microbial genera in relative abundance and physicochemical properties **(C)**, and circos visualization figure between significantly different volatile compounds and dominant microbial genera in the Xiasha round of the cave brewing sauce-flavor Baijiu based on the Pearson correlation coefficient **(D)** [when the Pearson correlation coefficient is more significant than 0.5, it means that the absolute value of the Pearson correlation coefficient is positively correlated (orange line) or negatively correlated (blue line) with statistically significant differences ($P < 0.05$)].

the dominant microbial genus. *Lactobacillus* can produce lactic acid and a variety of antimicrobial substances to inhibit the growth of pathogens and microorganism spoilage during brewing (Galati et al., 2014). Therefore, the abundance of some non-acid-tolerant and pathogenic bacteria, such as *Enterococcus* spp. and *Klebsiella* spp., was gradually decreased as *Lactobacillus* became the dominant bacteria genus during the fermentation process.

In the previous study of the conventional environment distillery, Wang et al. (2015) found that the main bacterial groups of sauce-flavor Baijiu SF and CF were *Bacillaceae* and *Lactobacillales*. In this study, we found that the dominant bacteria family was *Leuconostocaceae*, which was different from the conventional environment distillery of the SF. However, in the anaerobic fermentation, the dominant bacteria order also was *Lactobacillales*, which was the same as the conventional environment distillery in the CF. This indicates that, with the change in the environment, there is a change in the dominant microorganisms in the SF but has little impact on the CF.

A total of six fungal phyla and 102 fungal genera were identified, and Ascomycota was the dominant phylum

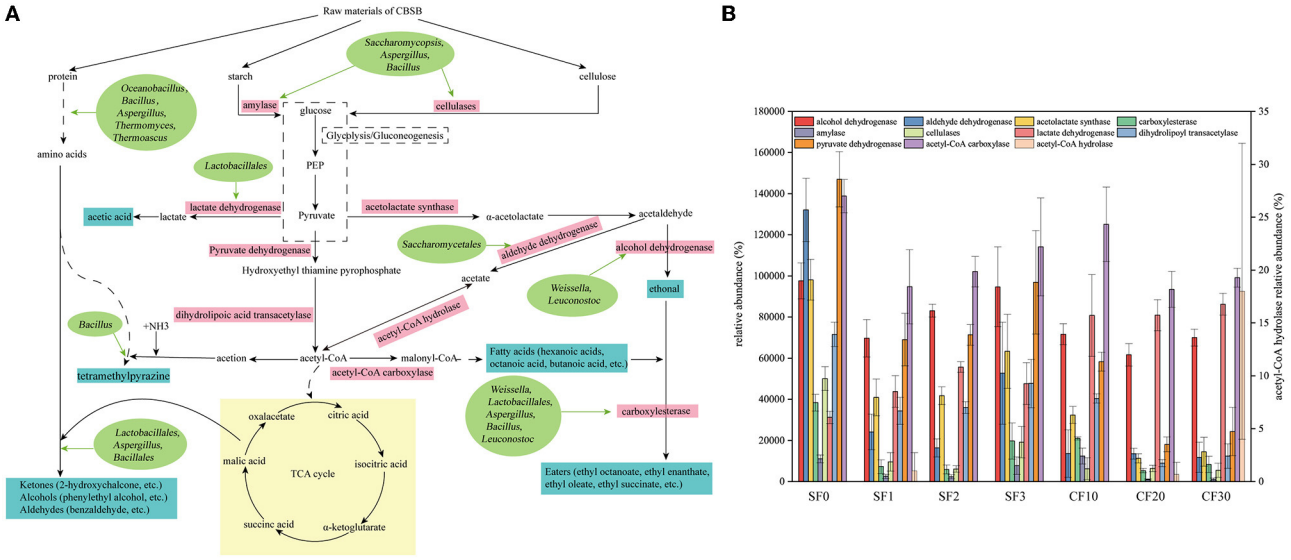

FIGURE 6
Metabolic profile of the dominant microorganisms **(A)** and relative abundance of enzymes **(B)** during the Xiasha round of the cave brew sauce-flavor Baijiu.

(96.80–100.00%). Meanwhile, *Saccharomycopsis* (32.44%), *Unspecified_Phaffomycetaceae* (17.05%), *Thermomyces* (15.00%), *Debaryomyces* (10.52%), *Wickerhamomyces* (8.51%), *Byssochlamys* (4.86%), *Rasamsonia* (2.40%), *Unspecified_Aspergillaceae* (1.74%), *Thermoascus* (1.44%), and *Aspergillus* (1.22%) were the dominant fungal genera. During the SF, *Saccharomycopsis*, *Thermomyces*, *Unspecified_Phaffomycetaceae,* and *Debaryomyces* were predominant, accounting for 89.49% of the total abundance. The CF's main genera were *Saccharomycopsis*, *Wickerhamomyces,* and *Unspecified_Phaffomycetaceae*, accounting for 60.01% of the total abundance. The dominant fungi during SF and CF of sauce-flavor Baijiu in conventional environments were *Pichia*, *Saccharomyces,* and *unidentified* (Hao et al., 2021). The Xiasha round is the non-wine-producing round in the production cycle of CBSB. The role of microorganisms is mainly focused on the process of aroma production and precursor substance production (Wang M. Y. et al., 2018). For example, *Saccharomycopsis*, which is more abundant, although it is not a significant brewing yeast, produces a variety of enzymes to break down starch and is vital in terms of flavor determination and aroma complexity of the final product (Padilla et al., 2016; Boro et al., 2022). Wang H. et al. (2021) analyzed microbiome diversity and evolution during SF for seven rounds in conventional environment distilleries and found that the dominant fungal were *Thermomyces*, *Byssochlamys*, *Thermoascus*, *unclassified_o_Eurotiales*, and *Aspergillus*. Previous studies showed that the fungi in fermentation mainly come from the ground of the fermentation environment (Zhang H. X. et al., 2021a). Therefore, we suppose that the environment is the most crucial factor that affects the fungal community structure in the fermentation process of sauce-flavor Baijiu.

The community structure of microorganisms is closely related to the interactions between microorganisms (Wang Y. R. et al., 2021). Pearson correlation coefficients and *p*-values were calculated to evaluate the interactions at the microbial genus level whose relative abundance was >1%. We found 20 nodes and 33 edges (Supplementary Figure S2). The results of the correlation analysis between bacterial genera showed that *Lactobacillus* was significantly negatively correlated with *Oceanobacillus* and *Unspecified_Leuconostocaceae* ($P < 0.01$, $r < -0.5$). *Lactobacillus* is often found as the dominant bacteria in Baijiu fermentation, which has a regulatory function on the microbial flora in the Baijiu fermentation system and has a positive role in the production of Baijiu flavor substances (Cai et al., 2021; Wang H. et al., 2021). It can produce acetic acid by heteromorphic lactic acid fermentation during anaerobic metabolism (Sifeeldein et al., 2018). *Bacillus* was significantly positively correlated with *Thermoactinomyces*, *Saccharopolyspora*, *Oceanobacillus*, and U*nspecified_Bacillaceae* ($P < 0.05$, $r > 0.5$). *Bacillus* was identified as the dominant bacteria in the previous studies on the sauce-flavor Baijiu (Hao et al., 2021; Zhang H. X. et al., 2021b). In this study, it is not surprising that the average relative abundance of *Bacillus* has yet reached the maximum at the end of the SF. *Bacillus* can secrete various enzymes to degrade the fermentation substrate to meet its growth and produce Baijiu flavor substances (Cai et al., 2021; Xiao et al., 2021). The correlation results between fungi showed that *Aspergillus* was significantly positively correlated with *Wickerhamomyces* ($P < 0.05$, $r > 0.5$). In Baijiu fermentation, *Aspergillus* is a functional microorganism with a good saccharification effect (Wu et al., 2021) and is critical for flavor compound production (Jin et al., 2019; Liu et al., 2021). *Saccharomycopsis* was significantly negatively correlated with *Unspecified_Phaffomycetaceae* ($P < 0.01$, $r < -0.5$). *Saccharomycopsis* was the dominant fungal genus in high-temperature Daqu and plays an essential role in the formation of the unique flavor of Baijiu (Jiang et al., 2021; Zeng et al., 2022).

The results of the correlation between bacteria and fungi showed that *Wickerhamomyces* and *Aspergillus* were significantly

positively correlated with *Lactobacillus* ($P < 0.01$, $r > 0.5$). *Wickerhamomyces* and *Aspergillus* can produce multiple enzymes to decompose raw materials and create conditions for the rapid growth of *Lactobacillus*. *Thermomyces* was significantly positively correlated with *Saccharopolyspora* and *Bacillus* ($P < 0.05$, $r > 0.5$). It can survive high temperatures and produce enzymatic decomposition materials (Liu and Miao, 2020). *Saccharopolyspora* and *Bacillus* can produce important biologically active substances to make the flavor more complex (Jiang et al., 2021). *Aspergillus* was significantly negatively correlated with *Weissella* and *Unspecified_Leuconostocaceae* ($P < 0.01$, $r < -0.5$), indicating that *Aspergillus* had an antagonistic effect on both. The flavor and quality of Baijiu can be affected by microbial interaction and the regulation of microbial structure (Zou et al., 2018b; Gao et al., 2022). In summary, these microorganisms coordinate and control each other during the fermentation process in the Xiasha round of CBSB. These mutual biological relationships constitute the primary biological control mechanism of the fermentation process of the CBSB, which is also the fundamental component of flavor formation.

3.3. Dynamic changes in flavor substances in fermented grains

In total, 72 volatile flavor substances were detected, including 23 esters, 13 alcohols, 11 acids, 8 phenols, 6 hydrocarbons, 4 aldehydes, and 7 other compounds (Supplementary Table S3). As shown in Figure 3A, the clusters on days 0, 1, 2, and 3 in SF were clearly different from those on day 10 of CF. This result is mainly due to changes in the oxygen level of the fermentation environment that eventually led to changes in the microbiota (Figure 2) and rapid changes in the metabolites in the fermented grains. Moreover, in the CF stage, the fermentation progressed very rapidly for the first 20 days and then changed slowly. This rapid metabolic change may be related to the favorable temperature and sufficient oxygen content available during CF for the first 20 days.

The changes in the contents of volatile compounds during fermentation are shown in Figure 3B and Supplementary Table S4. Esters, acids, and phenols showed an increasing trend during the SF stage. Meanwhile, the alcohol content decreased significantly on day 1, which is closely related to the increase in other flavor substances. During SF, the alcohol content increased with the extension of fermentation time (Wei et al., 2020). While the high phenol content in the early period may be associated with the raw material sorghum (Rao et al., 2018; Li et al., 2021), the higher content of esters in the later period might be related to the accumulation of ester precursors and the production of metabolites by microorganisms (Xu et al., 2022). The significant increase in alcohol content in the CF10 might be caused by the rapid growth and reproduction of alcohol-producing yeast such as *Saccharomyces* (Cai et al., 2019). As oxygen depletion and the available substances inhibited the production of alcohol, the alcohol content decreases significantly. With the rapid growth of acid-producing microorganisms, the growth of ester-producing microorganisms is inhibited (Wu et al., 2017). Therefore, the content of esters decreased significantly in CF20. The contents of esters, phenols, and aldehydes were increased in CF30, which might be due to the transformation of alcohols into other substances by microorganisms and enzymes (Dai et al., 2020; Hong et al., 2020).

LEfSe analysis was performed to identify significantly different metabolites in each stage in the Xiasha round of the CBSB. A total of 23 compounds were significantly different in the Xiasha round of the CBSB (LDA = 4, $P < 0.05$) (Figure 4).

Ethyl heptanoate (Z3), ethyl caprylate (Z6), butyl 3-hydroxybutyrate (Z11), diethyl succinate (Z12), gamma-nonalactone (Z16), ethyl cinnamate (Z18), and ethyl oleate (Z22) were the differential eaters (Figure 4A). Among them, diethyl succinate, gamma-nonalactone, and ethyl oleate were detected throughout the fermentation process. Esters in Baijiu were usually synthesized by microbial metabolic pathways, with a small percentage coming from environmental and spontaneous chemical reactions (Xu et al., 2021). The levels of ethyl heptanoate, ethyl octanoate, and ethyl cinnamate gradually increased with increasing fermentation time during the Xiasha round, reaching a maximum on day 10 of CF and then decreased. Butyl 3-hydroxybutyrate was detected on day 0 and day 1 of SF and on days 20 and 30 of CF, which was not detected the rest of the time. Among them, ethyl heptanoate, ethyl octanoate, and ethyl cinnamate were not detected in the SF0, and their content increased with fermentation. This was sufficient to prove that these three esters were produced by microbial metabolism and esterification during the fermentation process (Dong et al., 2022). Moreover, because of the high content of ethanol produced during the fermentation of Chinese Baijiu, ethanol and acid undergo biochemical reactions to produce esters, which may also account for the great variety and concentration of ethyl esters. It can add fruit and floral aromas to Baijiu and make Baijiu taste soft, sweet, and astringent (Xu et al., 2022).

Fatty acids are crucial for the flavor of Chinese Baijiu, and microorganisms can synthesize low-molecular-weight fatty acids such as butyric, valeric, hexanoic acid, and octanoic acids, which are precursors of ester compounds (Wei et al., 2020). During the Xiasha round of CBSB, acetic acid (S1), pentanoic acid (S5), hexanoic acid (S6), and sorbic acid (S8) are the differential metabolites (Figure 4). While acetic acid and hexanoic acid were detected throughout the fermentation process, pentanoic acid was detected only on day 2 of SF and on day 10 of CF. In contrast, sorbic acid was detected only at the beginning and end of fermentation. Strong-flavored Baijiu is rich in hexanoic acid, which is a volatile flavoring substance that contributes much to the flavor (Fan et al., 2019). The acetic acid content was the highest throughout the fermentation stage. Although acetic acid produced a pungent odor, it could increase the flavor of Chinese Baijiu upon its combination with alcohol to produce esters, such as ethyl acetate (Cai et al., 2019).

Alcohols are auxiliary agents that coordinate Baijiu aroma and taste (Zhang M. Z. et al., 2021). In the differential alcohol metabolites during the Xiasha round of CBSB (Figure 4), the ethanol content showed an increasing trend during SF and CF, and it is an essential indicator of the fermentation performance of Baijiu (Wei et al., 2021). Furthermore, the higher alcohols 2-methyl-1-propanol (C3), benzyl alcohol (C12), 1-hexanol (C5), 2,3-butanediol (C9), and 2-ethylhexanol (C8) are present in many alcoholic beverages. Moderate amounts of higher alcohols increase the flavor and taste of Baijiu (Han et al., 2014).

Creosol (F2), 4-ethyl-2-methoxyphenol (F4), 2,4-di-tert-butylphenol (F8), tetramethylpyrazine (QT3), 4-ethylveratrol (QT6), and benzaldehyde (Q3) were also differential metabolites found during the Xiasha round of CBSB (Figure 4A). Creosol, 4-ethyl-2 methoxyphenol, and 2,4-di-tert-butylphenol showed an increasing trend during fermentation, while tetramethylpyrazine showed a decreasing trend. Benzaldehyde was detected throughout the fermentation stages, whereas 4-ethylveratrol was detected only in a few fermentation stages. Although these substances are a small part of the fermentation process, like phenolics, they are antioxidants and have beneficial effects on human health (Rao et al., 2018).

3.4. Correlation of microorganisms with physicochemical properties and volatile compounds

The effect of physicochemical characteristics on the dominant bacterial and fungal genera was revealed by the redundancy analysis (RDA), with the first two axes explaining 71.18 and 71.17% of the variations in community composition, respectively (Figures 5A, B). As shown in Figure 5C, among the bacterial communities, *Unspecified_Leuconostocaceae*, *Weissella*, *Unspecified_Bacillaceae*, *Oceanobacillus,* and *Thermoactinomyces* were significantly positively correlated with starch but negatively with acidity. Only *Lactobacillus* showed a highly significant negative correlation with starch ($P < 0.001$) but showed a highly significant positive correlation with acidity and moisture ($P < 0.001$). *Bacillus* and *Unspecified_Bacillaceae* were significantly positively and negatively correlated with temperature and reducing sugar, respectively. It shows that high moisture content and acidity can inhibit the growth of other microorganisms. Regarding fungi, *Wickerhamomyces* and *Aspergillus* showed a significant negative correlation with starch ($P < 0.001$) and a positive correlation with moisture and acidity ($P < 0.001$) (Figure 5C). Temperature showed a significant positive correlation and a significant negative correlation with *Thermomyces* and *Byssochlamys,* respectively. Only *Saccharomycopsis* has a negative correlation with moisture. Both *Debaryomyces* and *Byssochlamys* showed a significantly negative correlation with reducing sugar. Only *Byssochlamys* showed a significant positive correlation with starch. *Lactobacillus* and *Aspergillus* had a highly significant positive correlation with moisture and acidity, consistent with the research result of nong sauce–flavored Baijiu in a conventional environment (Zhuansun et al., 2022). They have a highly significant negative correlation with starch. These illustrate that they may all be related to the breakdown of starch. *Aspergillus* is an important filamentous fungus that produces a variety of enzymes to degrade starchy material into fermentable sugars during the fermentation of Chinese Baijiu (Chen et al., 2014). The RDA showed that microorganisms producing amylase have a significant influence on starch in the SF. At the same time, after entering the CF, the monosaccharide produced by starch decomposition provided the conditions for the growth of other microorganisms and influenced the acidity changes. Based on these results, starch was the main physicochemical property factor that affects microbial succession in the SF stage. At the same time, acidity, moisture, and reducing sugar were the main driving factors of microbial succession in the CF stage. This is different from the research results of the traditional brewing environment, in that reducing sugar is the key factor affecting the microbial succession during the SF process of sauce-flavor Baijiu, while acidity, alcoholicity, and temperature are the main factors affecting the microbial succession during the CF process (Hao et al., 2021). Previous research results of light flavor Baijiu showed that acidity, reducing sugar, and temperature were the decisive factors affecting its bacterial succession (Shen et al., 2021). During the fermentation of strong-flavor Baijiu, starch, moisture, and pH significantly affected microbial succession (Guan et al., 2020). These dominant microorganisms were regulated by the physicochemical properties by influencing the physicochemical properties.

Based on Pearson correlation analysis between the dominant microbial genera and the significantly different volatile metabolites visualized (Figure 5D), regarding the bacterial genera, *Lactobacillus* and *Weissella* were the genera with the maximum quantity of positive and negative correlations with 11 and 12 significantly different volatile metabolites, respectively. For fungi, *Wickerhamomyces* and *Aspergillus* were positively associated with 10 significantly different volatile metabolites, respectively, while *Byssochlamys* was negatively associated with four significantly different volatile metabolites.

Acetic acid (S1) and ethanol (C1) were the basis for the formation of various complex compounds, both of which showed significant negative correlations ($P < 0.05$, $r < -0.5$) with *Weissella*, *Unspecified_Leuconostocaceae*, *Unspecified_Bacillaceae,* and *Oceanobacillus* but highly significant positive correlations ($P < 0.001$, $r > 0.6$) with *Wickerhamomyces*, *Lactobacillus,* and *Aspergillus*. *Wickerhamomyces* and *Lactobacillus* were positively correlated with 10 and 11 significantly different metabolites, respectively, indicating that they were in the direction of anabolic products under anaerobic conditions with high acidity (Hu X. L. et al., 2021; Lin et al., 2022). *Aspergillus* was positively correlated with 10 significantly different metabolites. *Aspergillus* can secrete various enzymes to convert raw materials into small molecules and supply a material basis for the growth and metabolism of the microbial community (Ali et al., 2018; Zhao G. Z. et al., 2020). To the best of our knowledge, the contents of esters, phenols, alcohols, and acids are higher in CF. Previous studies indicated that *Lactobacillus* and *Aspergillus* vitally contribute to the synthesis of esters (He et al., 2022; Xu et al., 2023). Ethyl caprylate (Z6), diethyl succinate (Z12), gamma-nonalactone (Z16), and ethyl oleate (Z22) showed a positive correlation with *Aspergillus* ($P < 0.01$, $r > 0.5$). Diethyl succinate (Z12), gamma-nonalactone (Z16), ethyl cinnamate (Z18), and ethyl oleate (Z22) showed a positive correlation with *Lactobacillus* ($P < 0.01$, $r > 0.5$). These esters were significantly negatively correlated with *Weissella*. Unsurprisingly, this correlation is not consistent with the previous report that *Weissella* has the function of synthesizing esters (Hasan et al., 2006). *Wickerhamomyces* and *Unspecified_Lactobacillaceae* have a significant positive correlation with four esters separately. Compared with the SF stage, these microorganisms with many positive correlations with ester substances have higher relative abundance in the CF

stage than in the SF stage. It shows that they have significant contributions to the synthesis of esters. *Wickerhamomyces*, *Unspecified_Phaffomycetaceae*, *Unspecified_Lactobacillaceae*, *Thermoascus*, *Rasamsonia*, *Aspergillus*, and *Lactobacillus* were significantly positively correlated with acids (acetic acid, pentanoic acid, hexanoic acid, and sorbic acid). This is consistent with the previous statement that *Aspergillus* and *Lactobacillus* have the ability to produce organic acids (Tang et al., 2019). *Wickerhamomyces*, *Thermoascus*, *Rasamsonia*, *Lactobacillus*, and *Aspergillus* have a significantly positive correlation with alcohols (ethanol, 1-hexanol, 2-ethylhexanol, and benzyl alcohol). *Lactobacillus* can produce ethanol by heterofermentation (Du et al., 2021). The microorganisms with a significant positive correlation with phenols (creosol, 4-ethyl-2-methoxyphenol, and 2,4-di-tert-butylphenol) were *Wickerhamomyces*, *Unspecified_Lactobacillaceae*, *Thermoascus*, *Lactobacillus*, and *Aspergillus*. The research results from a conventional environment showed that phenols are mainly related to *Kroppenstedtia*, *Staphylococcus*, and *Thermoactinomyces* (Zhuansun et al., 2022). The relative abundance of these microorganisms in the CF stage is higher than that in the SF stage. It shows that the CF stage is the main period for the accumulation of flavor substances, and the production of flavor substances is likely the result of the cooperation of microorganisms and biochemical reactions.

3.5. Metabolic mapping of dominant microorganism genera during the fermentation

To better understand the relationship between microorganisms and volatile flavors, metabolic mapping of dominant microorganisms during the Xiasha round of the CBSB was performed based on microbial annotated related enzymes and combined with the KEGG database and relevant literature (Figure 6A). Starch and cellulose as raw materials are metabolized by *Saccharomycopsis*, and *Aspergillus* and *Bacillus* secreted amylase and cellulase to form monosaccharides (Gopinath et al., 2017; Jin et al., 2019; Huang et al., 2020). Subsequently, monosaccharides are converted into the critical intermediate metabolite pyruvate, which is involved in the downstream of sugar metabolism. Pyruvate is converted into acetyl-CoA by the action of aldehyde dehydrogenase secreted by *Saccharomycetales*. Acetyl-CoA enters the TCA cycle with the participation of *Lactobacillales, Bacillales*, and *Aspergillus* to form alcohols, aldehydes, and ketones with amino acids. Among them, amino acids are derived from protein degradation by proteases secreted by *Thermoascus*, *Thermomyces*, *Aspergillus*, *Bacillus,* and *Oceanobacillus* (Ali et al., 2018; Jin et al., 2019; Park et al., 2019; Zhao G. Z. et al., 2020). Meanwhile, acetyl-CoA is converted into malonyl-CoA through the action of acetyl-CoA carboxylase to generate fatty acids and then forms an ester with ethanol with the participation of *Weissella*, *Lactobacillales*, *Bacillus*, *Leuconostoc*, and *Aspergillus* (Zhao C. et al., 2020). *Lactobacillale* is the main microorganism responsible for the production of lactic acid, which not only inhibits harmful microorganisms but also acts as a precursor for the formation of ethyl lactate (Jin et al., 2017; Zhang et al., 2018). In addition, *Bacillus* is associated with the synthesis of tetramethylpyrazine (Zhu and Xu, 2010a,b). The changes in the corresponding enzyme abundance are shown in Supplementary Table S5 and Figure 6B. Regarding the raw materials for Baijiu, the three main types of substances initially provided for microbial utilization are starch, cellulose, and protein. The average relative abundance of cellulase in SF was much higher than that in CF and increased significantly on day 3, which might be related to the significantly increased *Bacillus* during this period as it was the primary cellulase producer (Huang et al., 2020). The highest average relative abundance of amylase was observed on day 10 of CF, indicating that the content of starch computation was the largest on day 10, which was consistent with our aforementioned research result that significantly reduced the starch content on day 10 of the CF stage. The relative abundance of lactate dehydrogenase and acetyl CoA hydrolase in CF was higher than in the SF stage. We observed that the acid content increased significantly after entering the CF. Lactate, (S)-isopropyl lactate, and isobutyl lactate also have the same change trend, and *Lactobacillus* was the absolute dominant bacteria in the CF. As shown in Figure 6A, the average relative abundance of microorganisms producing carboxylesterase in CF was greater than that in the SF stage, which was consistent with the significant increase in ester content in the previous CF process.

4. Conclusion

We investigated the dynamic changes of a microbial community, physicochemical properties, and volatile flavor compounds during the Xiasha round fermentation process of the CBSB. During the Xiasha round of the CBSB, the SF progress enriched the microflora necessary for flavor formation, and the flavor substance accumulation was mainly concentrated in the CF progress. This study clarified the microbial community structure in the fermentation process of Xiasha round in the CBSB. We revealed the correlation between microbial community, physicochemical properties, and flavor substances and established the correlation networks and metabolic maps to better understand the relationship between dominant microbial genera and critical flavor compounds in the fermentation process. We have demonstrated the potential role of dominant microorganism genera in flavor formation. In this study, most of the dominant microorganisms we detected also appeared in the traditional environmental fermentation process, which fully demonstrated the feasibility of brewing sauce-flavor Baijiu in the natural caves. Thus, these findings will provide theoretical bases for CBSB and thus lay a solid foundation for craft optimization and Baijiu quality improvement. Meanwhile, we are providing technical support for the further development of microbial resources for Baijiu brewing.

Data availability statement

The data presented in the study are deposited in the National Center for Biotechnology Information (NCBI) repository, accession number of bacteria and fungi are PRJNA928402 and PRJNA928447, respectively.

Author contributions

TTR and WS contributed the experimental design. TTR performed the statistical analysis and wrote the manuscript. WS and DWZ provided expertise and insight relating to Baijiu microbiology. TTR, WS, YCM, and QQ contributed to manuscript revision, read, and approved the submitted version. All authors contributed to the article and approved the submitted version.

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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.2023.1124817/full#supplementary-material

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Inoculation strategies affect the physicochemical properties and flavor of Zhenjiang aromatic vinegar

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Inoculation strategy is a significant determinant of the flavor quality of Zhenjiang aromatic vinegar. Herein, the comparative analyses of the effects of various inoculation strategies on the physicochemical properties, microbial community structure, and flavoring characteristics of Zhenjiang aromatic vinegar were performed. The results showed that the contents of total acid (6.91g/100g), organic acid (2099.63±4.13mg/100g) and amino acid (3666.18±14.40mg/100g) in the direct inoculation strategy were higher than those in the traditional inoculation strategy (6.21±0.02g/100g, 1939.66±4.16mg/100g and 3301.46±13.41mg/100g). At the same time, it can effectively promote the production of acetoin. The diversity of strains under the traditional inoculation strategy was higher than that under the direct inoculation strategy, and the relative abundance of major microbial genera in the fermentation process was lower than that under the direct inoculation strategy. In addition, for two different inoculation strategies, pH was proved to be an important environmental factor affecting the microbial community structure during acetic acid fermentation. The correlation between main microbial species, organic acids, non-volatile acids, and volatile flavor compounds is more consistent. Therefore, this study may help to develop direct injection composite microbial inoculants to replace traditional starter cultures in future research.

KEYWORDS

Zhenjiang aromatic vinegar, inoculation strategy, fermentation, flavor quality, correlation analysis

1. Introduction

Zhenjiang vinegar is one of the four famous vinegar in China, and it is always a popular acidic condiment with a long history (Jin et al., 2017). Due to the seasonal changes in the process of open fermentation, the diversity of raw materials and the complexity of microorganisms, the internal and external environmental factors of vinegar fermentation have changed, so the flavor quality of traditional solid-state fermentation vinegar is unstable (Jin et al., 2017). Acetic acid fermentation is the most important stage in the process of vinegar brewing, and also the decisive factor of vinegar flavor quality (Wu et al., 2021). Traditional solid-state vinegar fermentation is carried out through spontaneous fermentation of raw materials and complex microbial communities in the environment. Under open conditions, a series of complex microbial groups produce rich metabolic products through fermentation to create Zhenjiang aromatic vinegar

flavor with unique characteristics, such as smooth, aromatic, mild sweetness, rich color, and fresh taste.

Because the quality of vinegar produced by traditional inoculation is mostly adjusted by experience, the metabolic mechanism and environmental parameters of complex fermentation microbial population cannot be controlled. This leads to low output and unstable quality of products, which seriously hinders technological progress and expansion of production scale. Therefore, the study of the relationship between microbiota and the formation of flavoring substances is important for understanding, controlling and effectively improving vinegar fermentation to improve food quality, productivity and safety. The rapid development of molecular biology and modern detection technology has gradually revealed the contribution of core microorganisms and functional microflora to the flavor of fermented products. In addition, breeding excellent functional strains and adjusting microbial community function through direct inoculation to improve fermentation efficiency are one of the most effective measures to improve yield and quality. Compared with traditional inoculation strategies, microbial community with selective species proportion is simplified and fermentation is easier to control (Wolfe and Dutton, 2015).

The direct inoculation strategy refers to the use of core microorganisms or functional microorganisms as a direct-injection starter (DVS) to replace the traditional fermented and mature seed grains in fermentation production. Under the same conditions, compared with the traditional inoculation strategy, the direct injection inoculation strategy can strengthen the adaptability and stability of the functional bacteria directly inoculated in the fermentation system, and effectively improve the product quality. Moreover, it can be applied in food and pharmaceutical fields (Shi et al., 2022). Relevant research indicates that using *Aspergillus niger*-enhanced gluten meal in the production of Baoning Vinegar can increase the total acid and organic acid contents as well as the utilization rate of starch and maintain relatively stable quality throughout the three cycles of fermentation (Liu et al., 2020). Lactobacillus sp. and *Acetobacter pasteurianus* were used as direct-dose microbial inoculum for *in-situ* biological coculture, and they could synergistically promote the accumulation of key components including acetoin (Shi et al., 2022). Additionally, non-rich species *Komagataeibacter europaeus* is applied to the fermentation of Zhenjiang aromatic vinegar, which can effectively regulate the composition of bacteria, strengthen the *in situ* symbiotic network, and up-regulate the abundance of genes related to sugar and alcohol metabolism (Lu et al., 2018; Peng et al., 2021). Therefore, the direct inoculation strategy can theoretically stabilize the flavor quality of the product and preserve the richness of flavoring substances in the traditional solid-state fermented vinegar, which is an effective strategy to regulate the microbial community and its functions and improve the quality of traditional Chinese grain vinegar.

Therefore, acetic acid and lactic acid bacteria, the key microorganisms in the production of Zhenjiang vinegar, were introduced into the solid-state fermentation production as direct inoculants. The effects of two different inoculation strategies on the physicochemical properties (total acid, reducing sugar and non-volatile acid), microbial community structure and flavor characteristics (organic acid, free amino acid and aroma) of Zhenjiang vinegar were compared. In addition, the effects of two inoculation strategies on microbial communities and metabolic components, and their differences in contribution to vinegar flavor were studied. The purpose of this study was to explore the effects of two inoculation strategies on the physicochemical indexes of Zhenjiang vinegar during acetic acid fermentation, and to provide theoretical support for the combination of traditional and modern vinegar brewing methods and the adjustment of technological parameters in large-scale production.

Abbreviations: AAF, Acetic acid fermentation; HPCLC, High pressure liquid chromatography; OTU, Operation taxonomical unit; NMDS, Non-metric multidimensional scaling; RDA, Redundancy analysis.

2. Materials and methods

2.1. Preparation of samples

In this study, Zhenjiang aromatic vinegar was brewed by traditional solid-state fermentation process. The natural seed fermented grains inoculated on the seventh day of fermentation (provided by Jiangsu Hengshun Vinegar Co., Ltd.) and artificially pure cultured strains (*Acetobacter* A-1 and Lactic Acid Bacteria Y-14) of Zhenjiang aromatic vinegar were used as research subjects. The marinade samples collected on days 0, 2, 4, 6, 8, 10, 12, 14, 16, and 18 of the fermentation were sampled daily before the rotation of the fermented grains, with three replicates for each sample. The vinegar was extracted from the fermentation tank from top to bottom, and 100 g of it was weighed in a self-sealing bag after uniform mixing. A 30-mL sample of marinade was transferred from the bottom of the cylinder to a 50-mL centrifuge tube and stored at −20°C. Samples were thawed in a water bath at room temperature (26°C to 30°C) before analysis.

2.2. Reagents

Sodium hydroxide, potassium hydrogen phthalate, glucose monohydrate, 99% anhydrous sodium phosphate monohydrate, potassium hexacyanoferrate trihydrate, zinc sulfate heptahydrate, ethanol, phosphoric acid, phenolphthalein indicator, high-performance liquid chromatography grade methanol, boric acid, lithium chloride, lithium hydroxide-monohydrate, 4-methyl-2-pentanol, citric acid, hydrochloric acid, octanoic acid, potassium acetate, sodium acetate trihydrate, acetic acid, sodium chloride, ninhydrin crystal, phenol, and ascorbic acid were purchased from Guoyao Group Chemical Reagent Co., Ltd (Shanghai, China). Citric acid (≥99.5% purity), L-pyroglutamic acid (≥99% purity), lactic acid (≥99% purity), acetic acid (≥99% purity), succinic acid (≥99% purity), and malic acid (≥99% purity) were purchased from McLean Biochemical Technology Co., Ltd (Shanghai, China).

2.3. Analysis of physical and chemical properties

The temperature during the Fermentation was monitored using a thermometer. A total of 10 g of the vinegar *pei* was added into 30 ml distilled water, agitated at 100 rpm for 3 h at room temperature (20°C), centrifuged for 10 min at 6000 g, and the supernatant collected for the

following analysis. The total acid, pH, amino nitrogen, and reducing sugar contents were determined following the previous methods (Huang et al., 2022). The contents of nine organic acids were analyzed by an Agilent 1,260 high-performance liquid chromatography (HPLC) system (Agilent Corp., Karlsruhe, Germany) with Aminex HPX-87H ion exclusion column (7.8 × 300 mm, i.d., 5 μM) according to the previous research (Wu et al., 2017). The amino acid content was measured using a Sykam S-433D amino acid analyzer (Sykam GmbH, Bavaria, Germany) (Yang et al., 2021). The reducing sugar content was measured by the 3,5-dinitrosalicylic acid (DNS) method (Yang et al., 2018). The contents of volatile compounds were detected by headspace-solid phase microextraction (HS-SPME)/gas chromatography–mass spectrometry (GC–MS) using an Agilent 7890B-5977B GC–MSD system equipped with a DB-wax capillary column (30.0 m × 0.25 mm × 0.25 μm, Agilent Technology, Santa Clara, CA, United States; Jia et al., 2020). All measurements were carried out in triplicates.

2.4. Microbial community analysis

Samples of vinegar *pei* and the precipitates was prepared on the basis of the previous method and the DNA of the sample was extracted (Wu et al., 2017; Huang et al., 2022). The qualified DNA was submitted to Majorbio Biopharm Technology Co., Ltd (Shanghai, China) for Illumina MiSeq amplicon sequencing (2 × 300 bp). Primers 338F and 860R were used to amplify the V3-V4 region of bacterial 16S rRNA gene. For fungi, the internal transcribed spacer (ITS) region was amplified with primers ITS1F and ITS2 (Nie et al., 2013; Huang et al., 2022). After sequencing, the raw sequences were quality-filtered by removing sequences as previously described (Zhang et al., 2020). Qualified reads from all samples were removed chimera sequences and assigned to operational taxonomic units (OTUs) at a 97% sequence similarity threshold with QIIME (version 1.9.1) pipeline (Caporaso et al., 2010). The representative OTU sequences were aligned against the bacterial 16S rRNA gene database Greengenes (version 13.8) (DeSantis et al., 2006) and the fungal ITS database UNITE (version 7.1) (Abarenkov et al., 2010) for taxonomic classification. The analysis of alpha-diversity and betadiversity were conducted after rarefying all samples to the same sequencing depth (Huang et al., 2022).

2.5. Data analysis

2.5.1. Statistical analysis

SPSS statistical software was used to analyze and report the results as mean standard deviation. The significance level for the independent *t*-test used to compare the differences between the two inoculation strategies was set at 0.05. One-way analysis of variance and Tukey HSD inspection were performed at a significance level of 0.05 to determine whether the two inoculation strategies had a significant effect on quality.

2.5.2. High-throughput sequencing data analysis

The onboard data of PacBio SMRT were exported, the CCS was identified through the barcode using Lima V1.7.0 software, and the barcode-CCS sequence data were obtained. Subsequently, barcode-CCS was filtered to obtain an effective sequence. Finally, the chimeric sequences were identified and eliminated using UCHIME V4.2 software to obtain the optimized ccs sequences. The valid sequences of the samples were clustered using USEARCH software, and the sequences were clustered into the operational taxonomical unit (OTU) under the condition of 97% similarity. Moreover, the most abundant sequence (MOST ABUNDANT) was selected as the OTU representative sequence. Using SILVA 132 as the reference database, the naive Bayesian classifier was combined with the alignment method for taxonomic annotation of the sequences, with a 70% confidence threshold. Sample α diversity index was evaluated using the Mothur V1.30 software. Furthermore, β diversity was analyzed with QIIME software, and the similarity of species diversity among various samples was compared.

2.5.3. Correlation analysis

Spearman correlation analysis was performed on the relative abundance of >1% and the difference or key strain with the physicochemical factors and main volatile flavor substances in vinegar-fermented grains, respectively, to calculate the correlation coefficient R-value and significant difference *p* value, which were analyzed and plotted using GraphPad Prism 7.0 and R language and other software.

3. Results and discussion

3.1. Differences in physicochemical properties of different inoculation strategies during fermentation

To study the dynamic changes in physicochemical parameters and flavoring substances during acetic acid fermentation of Zhenjiang aromatic vinegar under different inoculation strategies, the total acid (Figure 1A), reducing sugar content (Figure 1B), nonvolatile acid content (Figure 1C), fermented grain temperature (Figure 1D), organic acid content (Figures 1E,F), and free amino acid content (Figures 1G,H) were measured during the fermentation process. These parameters were important physicochemical indices related to microbial growth during fermentation (Lu et al., 2018).

The total acid content of vinegar-fermented grains exhibited a fluctuating upward trend throughout the acetic acid fermentation process, which was a key indicator of vinegar acidity. On days 0–7 of acetic acid fermentation, the total acid content continued to increase owing to the mass propagation of microorganisms such as lactic acid bacteria. Aerobic microorganisms such as acetic acid bacteria proliferate in a large number and generate a large amount of organic acids including acetic acid and lactic acid for 7 to 18 days after the fermented grains are completely turned over for fermentation in order that the total acid content increases rapidly (Wang et al., 2015). At the end of fermentation, the total acid content using the traditional inoculation strategy was as high as 6.21 ± 0.02 g/100 g, the highest dose achieved *via* direct inoculation was 6.91 g/100 g.

The sweet flavor of Zhenjiang aromatic vinegar is primarily a result of the saccharification of food. These saccharides can enhance the flavor of vinegar. Simultaneously, the reducing sugar of vinegar can react with amino acids to produce flavor substances (Fang et al., 2021). On the whole, the change trend of reducing sugar content

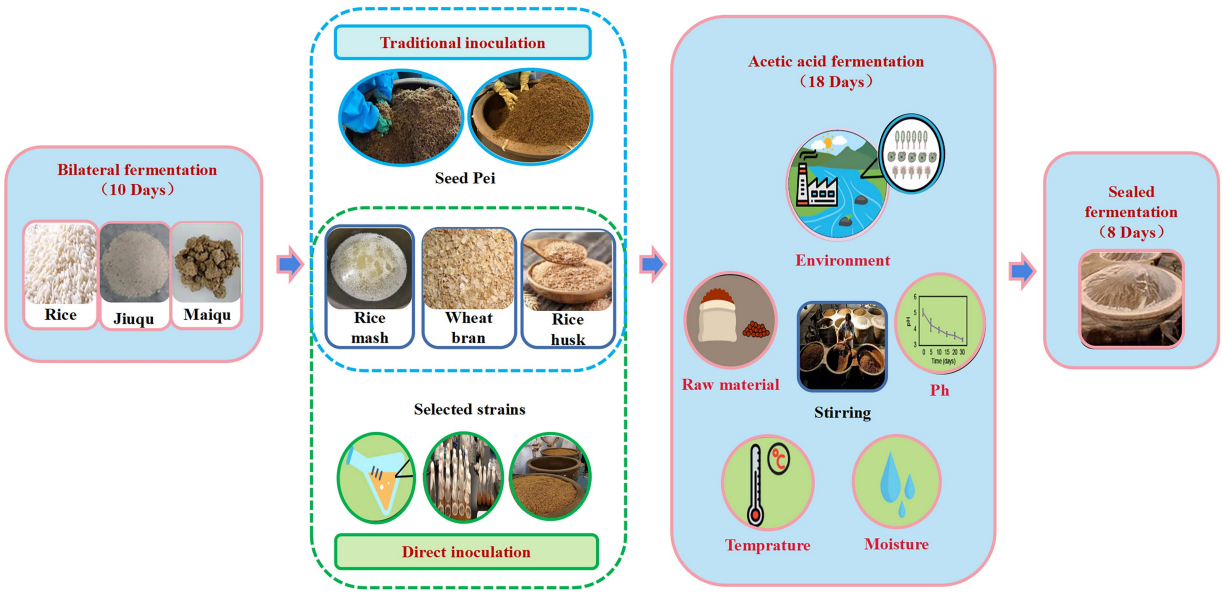

FIGURE 1
Fermentation process of Zhenjiang aromatic vinegar under the traditional inoculation strategy and the direct-injection inoculation strategy.

under the direct inoculation strategy and the traditional inoculation strategy is roughly the same, both of which show a trend of increasing first and then decreasing (Figure 1B). The main difference between the two inoculation strategies in the acetic acid fermentation process is that the traditional inoculation strategy is in the rising period of reducing sugar content in 4 ~ 8 days, reaching the maximum value of 2.27 ± 0.03 g/100 g on the 8th day of fermentation, and gradually decreasing in the later stage. The direct inoculation strategy is that the content of reducing sugar is in the rising stage on 6 ~ 14 days and reaches the maximum value of 3.27 ± 0.01 g/100 g on the 14th day of fermentation. There may be two reasons for the increase of reducing sugar content in the early stage. First, reducing sugar mainly comes from bran during acetic acid fermentation. The content of reducing sugar in wine mash and rice husk is relatively low. In the early stage, as the acidity of vinegar *pei*, the macromolecular sugar contained in raw material bran is hydrolyzed into small molecular sugar by acid, such as monosaccharide and other reducing sugar. At the same time, the temperature reached the optimum temperature of saccharifying enzyme (35°C-40°C), the activity of the enzyme increased, and the reducing sugar produced by hydrolysis of raw materials was accumulated. Because the activity and acid-producing ability of acetic acid bacteria under the direct inoculation strategy is stronger than that of the traditional inoculation strategy, it can fully acidolysis macromolecular sugar and produce more reducing sugar. In the late stage of acetic acid fermentation, microorganisms proliferate, nutrients are rapidly consumed, and reducing sugar content is significantly reduced.

The non-volatile acids can neutralize the strong acidity of acetic acid and make the finished vinegar taste soft and delicate, which is the key to the quality of Zhenjiang balsamic vinegar (Wang et al., 2015). Lactic acid is the most important nonvolatile acid (Wang et al., 2015). Under the traditional inoculation strategy, the nonvolatile acid content was maximum in the fermentation process after 12 days, reaching up to 0.41 ± 0.01 g/100 mL, whereas it reached up to 1.58 g/100 mL under direct inoculation.

Temperature affects the metabolic activity of microorganisms and the changes of alcohol and water during the fermentation process, making it a comprehensive indicator used to determine the fermentation quality of vinegar-fermented grains. Under optimal conditions, the rapid propagation and metabolism of microorganisms not only rapidly converted the alcohol in vinegar-fermented grains into acetic acid but also metabolized a large number of organic acids, amino acids, esters and other substances. The fermentation temperature ranged from 38°C to 46°C, which was the most active phase of acetic acid bacterial metabolism. A large number of products were accumulated, and the primary flavoring substances of aromatic vinegar were generated, alongside a large amount of heat. During the final phase of fermentation, owing to the consumption of nutrients and large accumulation of metabolites, the metabolism of microorganisms such as acetic acid bacteria was inhibited, and the temperature was reduced and maintained at 35°C (Shi et al., 2022). Using the traditional inoculation strategy, the fermented grain temperature of vinegar reached a maximum of 46.07°C ± 1.10°C on the eighth day of fermentation and then decreased to 27.33°C ± 2.48°C. however, the temperature of vinegar *Pei* under the direct inoculation strategy continued to rise until the fermentation temperature reached 48.0°C on the twelfth day and then began to decrease to 35.33°C ± 0.35°C. It was possible that the direct inoculation of acetic acid bacteria contained a high concentration of lactic acid bacteria and produced more acid and heat, which inhibited the growth of other bacteria.

Overall, there were considerable differences in total acid, nonvolatile acid, reducing sugar, the temperature of fermented vinegar, organic acid, and organic acid content between the two inoculation strategies (Figure 2). Moreover, the total acid of fermented vinegar exhibited an upward trend. The total acidity of fermented vinegar inoculated using the direct inoculation strategy was considerably greater than that of vinegar inoculated using the traditional strategy. In the first 12 days, the total acid of acetic acid fermentation using the traditional inoculation strategy increased

faster than that using the direct inoculation strategy, but then the total acid of acetic acid fermentation using the direct inoculation strategy surpassed that using the traditional inoculation strategy and reached 6.91 g/100 g dry fermented grains. The reducing sugar content in the acetic acid fermentation process using the two inoculation strategies increased continuously for 8 days before fermentation, but after 8 days of fermentation, the reducing sugar content in the vinegar-fermented grains using the traditional inoculation strategy gradually decreased, whereas that in the vinegar-fermented grains using the direct inoculation strategy continued to increase. The concentration of nonvolatile acid first increased and then decreased. The amount of nonvolatile acid in fermented grains fermented using the direct inoculation strategy was considerably greater than in those fermented using the traditional inoculation strategy. The temperature of vinegar-fermented grains exhibited a fluctuating upward trend 8 days before fermentation, and the fermentation temperature of vinegar-fermented grains using the direct inoculation strategy was higher than that using the traditional inoculation strategy. These physical and chemical parameters are not only the indices of normal fermentation, but also the potential environmental drivers of microbial community accumulation in fermented foods (Zheng et al., 2014; Xiao et al., 2017).

According to the results, the total amino acid content in the acetic acid fermentation stage tended to increase first and then decrease (Figures 1G,H). The total amino acid content in acetic acid fermentation (AAF) reached a maximum of 3301.46 ± 13.41 mg/100 g dry fermented grains on the 12th day using the traditional inoculation strategy, whereas the total amino acid content of the direct inoculation strategy reached the highest of 3666.18 ± 14.40 mg/100 g dry fermented grain on the 10th day (Figures 1G,H). The traditional inoculation strategy produced more His, Arg, Ile, and Lys, whereas the direct inoculation strategy produced more His, Arg, Ile, and Tyr. Organic acid is the primary source of the sour taste of vinegar and the most important flavoring substance of vinegar (Zhu et al., 2016). Vinegar primarily contained volatile acids such as formic acid, acetic acid, propionic acid, and butyric acid, and nonvolatile acids such as lactic acid, tartaric acid, citric acid, malic acid, and succinic acid (Nie et al., 2017). Using the traditional inoculation strategy, the total organic acid content increased continuously *via* fermentation and reached 1939.66 ± 4.16 mg/100 g dry fermented grains at the end of AAF, among which acetic acid, lactic acid, and citric acid were the highest produced. Using the direct inoculation strategy, the organic acid content increased to 2099.63 ± 4.13 mg/100 g dry fermented grains on the 14th day of AAF and then decreased slightly, with acetic acid, citric acid, and tartaric acid accounting for most (Figures 1E,F). Acetic and lactic acids is the primary organic acids in grain vinegar, with acetic acid being the volatile acid with the highest concentration and possessing the characteristics of strong irritability and short aftertaste. Lactic acid, citric acid, tartaric acid, and other nonvolatile acids can adjust the sour taste, endowing vinegar with a soft flavor, which is conducive to the equilibrium of acetic acid sour taste stimulation

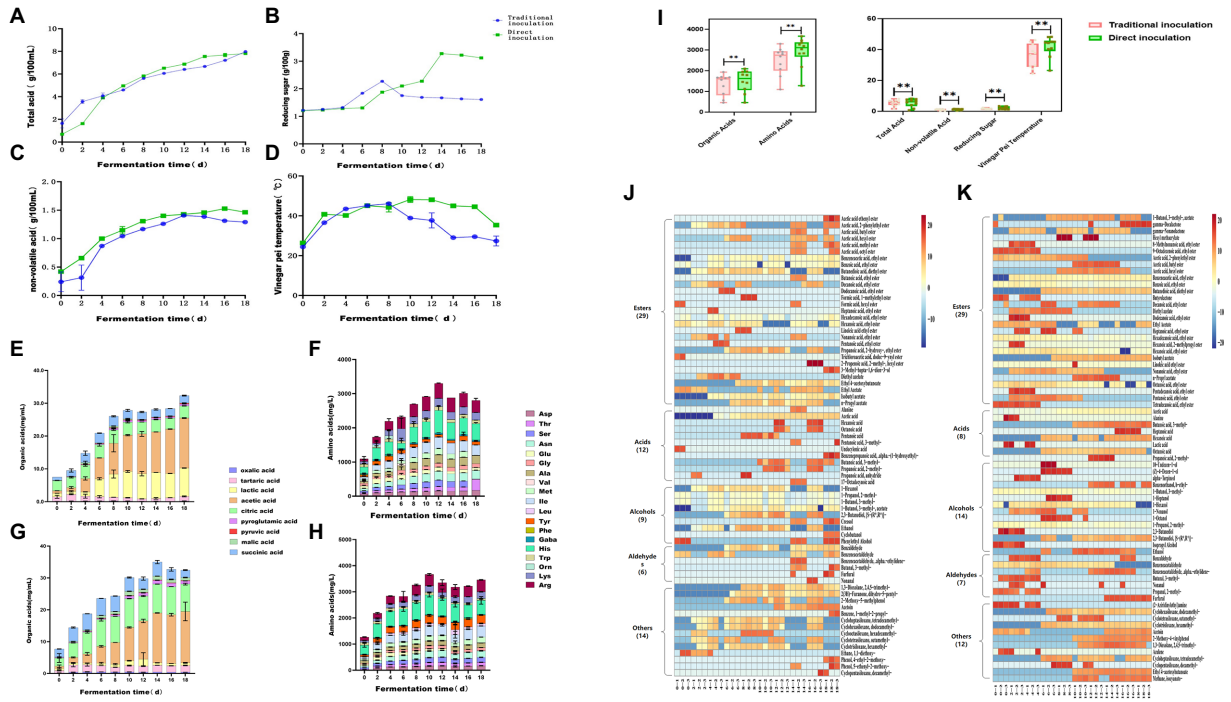

FIGURE 2
Dynamic changes in physicochemical parameters and flavoring substances during the fermentation of Zhenjiang aromatic vinegar using different inoculation strategies. **(A)** Total acid, **(B)** reducing sugar content, **(C)** nonvolatile acid content, **(D)** fermented grain temperature, **(E)** organic acid content using the traditional inoculation strategy, **(F)** organic acid content using the direct inoculation strategy, **(G)** free amino acid content using the traditional inoculation strategy, **(H)** free amino acid content using the direct inoculation strategy, **(I)** total acid, nonvolatile acid, reducing sugar, vinegar-fermented grain temperature, and total content of organic acid and organic acid in the fermentation process of Zhenjiang aromatic vinegar using different inoculation strategies, **(J)** volatile flavoring substance content using the traditional inoculation strategy, and (K) volatile flavoring substance content using the direct inoculation strategy.

(Lv et al., 2016). Lactobacillus and Acetobacter are the high-abundance microorganisms in the AAF process of traditional grain vinegar (Ai et al., 2019; Kou et al., 2022). Due to the participation of these microorganisms, the metabolic flavor substances produced by fermentation are more abundant (Wu et al., 2017). Overall, the content of organic acid, amino acid and total acid in fermented grains under the direct inoculation strategy was slightly higher than that under the traditional inoculation strategy.

Compared with organic and amino acids, the volatile substances in fermented vinegar were considerably low in concentration, but they played a substantially important role in vinegar, as the majority of them possessed unique aromas that contributed to a more balanced and diversified flavor. According to previous research reports, the characteristic flavoring substances of Zhenjiang aromatic vinegar mainly include acetic acid, lactic acid, phenethyl alcohol, 3-methylbutyraldehyde phenylacetic acid, 2,3-butanedione, 3-hydroxy-2-butanone, ethyl acetate, and acetoin (Yu et al., 2012). In conclusion, 70 volatile compounds were identified in AAF vinegar-fermented grains using traditional and direct inoculation strategies (Figures 1I,J). Twenty-nine distinct esters of volatile substances were identified using both the inoculation strategies. The concentrations of acids (12 types) and other volatile substances (14 types) such as ketones and phenols using the traditional inoculation strategy were higher than those of acids (8 kinds) and other flavor compounds (12 kinds) using the direct inoculation strategy. However, the numbers of alcohols (14 kinds) and aldehydes (7 types) using the direct inoculation method were higher than those of alcohols (9 types) and aldehydes (6 types) using the traditional inoculation strategy. Furthermore, 13 identical esters, 5 identical acids, 6 identical alcohols, 6 identical aldehydes, and 5 identical ketones, phenols, and other volatile substances are shown in Table 1 for both the inoculation strategies.

With the development of fermentation, most volatile substances in fermented grains showed an increasing trend. Acetoin was produced in the acetic acid fermentation process during both inoculation strategies. Acetoin is the precursor of ligustrazine (2,4,5,6-tetramethylpyrazine (TTMP), nut and baking aroma). According to reports, TTMP is the primary bioactive component in vinegar (Wang et al., 2016). As fermentation progressed, the concentration of acetoin steadily increased. On the 18th day of fermentation, the amount of acetoin directly inoculated with *Pei* was 5.88 ± 2.81 mg/100 g dry *Pei*, whereas the amount of acetoin using the traditional inoculation strategy was only 3.35 ± 0.42 mg/100 g dry *Pei*. Therefore, this result indicated that the direct inoculation strategy could effectively promote the production of acetoin.

Esters are the most abundant volatile flavoring substances, and ethyl acetate and ethyl lactate had the highest concentrations in the entire process, which was directly correlated with the higher concentrations of the synthetic precursors of acetic acid and lactic acid (Wang et al., 2016). The environment of solid vinegar fermented grains is extremely conducive to the proliferation and fermentation of ester-producing yeast, making a large number of ester substances produced and accumulated in acetic acid fermentation. In addition to the common ester flavor substances, there are 16 kinds of esters unique to the traditional vaccination strategy, such as 2-phenylethyl acetate, butyl ester and methyl ester of acetic acid and valeric acid. There are 15 direct injection vaccination strategies, such as acetate, gamma decalac, and gamma nonlac. The production of alcohols in the fermentation process, such as hexanol, 3-octanol (nuts, mushrooms) and phenylethanol (flowers, honey), brings richer flavor. Acetic acid, methylbutyric acid, caproic acid, ethanol and phenylethanol are the main acids and alcohols in the production of Zhenjiang vinegar. There are four kinds of alcohols unique to traditional vaccination strategies, including 1-butanol, 3-methyl-acetate and creosote. The direct injection vaccination strategy includes nine kinds, such as 10-undecen-1-ol, (Z) - 4-deca-1-ol, etc. There are 7 kinds of acid specific to traditional vaccination strategy, including 3-methylundecanoic acid. The direct injection vaccination strategy includes three kinds, such as alanine and heptane.

3.2. Analysis of differences in the structural composition of microbial communities using different inoculation strategies during fermentation

The structure and succession pattern of bacterial and fungal communities throughout the entire fermentation process for the two inoculation strategies was investigated by using amplicon sequencing analysis. Significant differences were found between the Chao1 and Shannon indices of acetic acid fermentation bacteria and fungi for the direct inoculation strategy and those for the traditional inoculation strategy (Figure 3A). The Chao1 and Shannon indices of bacterial and fungal communities were greater for the traditional inoculation strategy than for the direct inoculation strategy. Inoculated acetic acid bacteria and lactic acid bacteria produced a large amount of acid, which affected the internal environment of fermented grains and inhibited the growth and reproduction of other microorganisms. Moreover, the vinegar-fermented grain communities for the traditional inoculation strategy (blue) and the direct inoculation strategy (red) in acetic acid fermentation gathered into a cluster, and the distribution distance between the communities was wide (Figure 3A), indicating that the inoculation strategy considerably affected the community structure of bacteria and fungi.

Throughout the entire process of acetic acid fermentation, bacteria predominate the metabolic activities in vinegar culture, and their biomass in vinegar-fermented grains is higher than that of fungi (Wang et al., 2016; Chai et al., 2020). Fungi are rare and diverse species in vinegar culture. Their presence provides a biological buffer against environmental changes, but their intrinsic growth rate is low (Huang et al., 2022). The species distribution maps at the phylum (Figure 3C) and genus levels (Figure 3D) revealed that the microflora in vinegar-fermented grains under the two inoculation strategies consisted primarily of bacteria such as Bacteroides, Actinomyces, Sclerotinia, and Proteus as well as fungi such as Myxomycetes, Basidiomycetes, and Ascomycetes, with lactic acid bacteria and Acetobacter as the predominant bacteria in general (Figure 3C). Yeasts and Alternaria were the predominant fungi.

During AAF, the relative abundance of Lactobacillus increased initially and then decreased, whereas the relative abundance of *Acetobacter* continue to increase. During this stage, the predominant microorganisms were lactic acid bacteria and *Acetobacter*, including *Komagataeibacter* and *Gluconacetobacter*. In addition to oxidizing alcohol substrates such as ethanol and sugar alcohols *via* the respiratory chain to organic acids such as acetic acid and lactic acid, *Acetobacter* could promote the production of certain ketone and

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TABLE 1 Same flavoring substances and special flavoring substances under different inoculation strategies.

Kind	Common flavoring substances	Flavoring substances peculiar to traditional inoculation strategies	Flavoring substances peculiar to direct inoculation strategy
Esters	Acetic acid ethenyl ester, Acetic acid, hexyl ester, benzeneacetic acid, ethyl ester, benzoic acid, ethyl ester, Decanoic acid, ethyl ester, dodecanoic acid, ethyl ester, heptanoic acid, ethyl ester, hexanoic acid, ethyl ester, linoleic acid ethyl ester, nonanoic acid, ethester, pentanoic acid, ethyl ester, ethyl acetate, Isobutyl acetate and n-Propyl acetate	Acetic acid, 2-phenylethyl ester	1-Butanol, 3-methyl-, acetate
		Acetic acid, butyl ester	Gamma-decalactone
		Acetic acid, methyl ester	Gamma-nonanolactone
		Acetic acid, octyl ester	Hexyl methacrylate
		Butanedioic acid, diethyl ester	8-Methylnonanoic acid, ethyl ester
		Butanoic acid, ethyl ester	9-Octadecenoic acid, ethyl ester
		Formic acid, 1-methylethyl ester	Acetic acid, 2-phenylethyl ester
		Formic acid, hexyl ester	Butanedioic acid, diethyl ester
		Hexadecanoic acid, ethyl ester	Butyrolactone
		Propanoic acid, 2-hydroxy-, ethyl ester	Diethyl azelate
		Trichloroacetic acid, dodec-9-ynyl ester	Hexadecanoic acid, ethyl ester
		2-Propenoic acid, 2-methyl-, hexyl ester	Hexanoic acid, 2-methylpropyl ester
		3-Methyl-hepta-1,6-dien-3-ol	Octanoic acid, ethyl ester
		Diethyl azelate	Pentadecanoic acid, ethyl ester
		Ethyl 4-acetoxybutanoate	Tetradecanoic acid, ethyl ester
		Alanine	
Acids	Acetic acid	Pentanoic acid	Alanine
	Hexanoic acid	Pentanoic acid, 3-methyl	Heptanoic acid
	Octanoic acid	Undecylenic acid	Lactic acid
	Butanoic acid, 3-methyl-	Benzenepropanoic acid, .alpha.-(1-hydroxyethyl)	
	Propanoic acid, 2-methyl-	Propanoic acid, anhydride	
		17-Octadecynoic acid	
Alcohol	1-Hexanol	1-Butanol, 3-methyl-, acetate	10-Undecen-1-ol
	1-Propanol, 2 – methyl-	Creosol	(Z)-4-Decen-1-ol
	1 — Butanol, 3 – methyl-	Cyclobutanol	alpha—Terpineol
	2,3-Butanediol, [S—(R*,R*)]	Phenylethyl alcohol	Benzeneethanol, b-ethyl-
	Ethanol		1-Heptanol
	Acetoin		1-Nonanol
			1-Octanol
			2,3-Butanediol
			Isopropyl Alcohol
Aldehydes	Benzaldehyde	–	Propanal, 2-methyl-
	Benzeneacetaldehyde		
	Benzeneacetaldehyde, .alpha.-ethylidene-		
	Butanal, 3-methyl-		
	Furfural		
	Nonanal		
Other	1,3 — Dioxolane, 2,4,5-trimethyl-	2(3H)-Furanone, dihydro-5 – pentyl-	(2-Aziridinylethyl)amine
	Cycloheptasiloxane, tetradecamethyl-	2-Methoxy-5-methylphenol-	2-Methoxy-4-vinylphenol
	Cyclohexasiloxane, dodecamethyl-	Benzene, 1-methyl-2-propyl-	Azulene
	Cyclotetrasiloxane, octamethyl-	Cyclooctasiloxane, hexadecamethyl-	Cyclopentasiloxane, decamethyl-
	Cyclotrisiloxane, hexamethyl-	Ethane, 1,1-diethoxy-	Ethyl 4-acetoxybutanoate
		Phenol, 4-ethyl-2-methoxy-	Methane, isocyanato-
		Phenol, 5-ethenyl-2-methoxy-	
		Cyclopentasiloxane, decamethyl-	

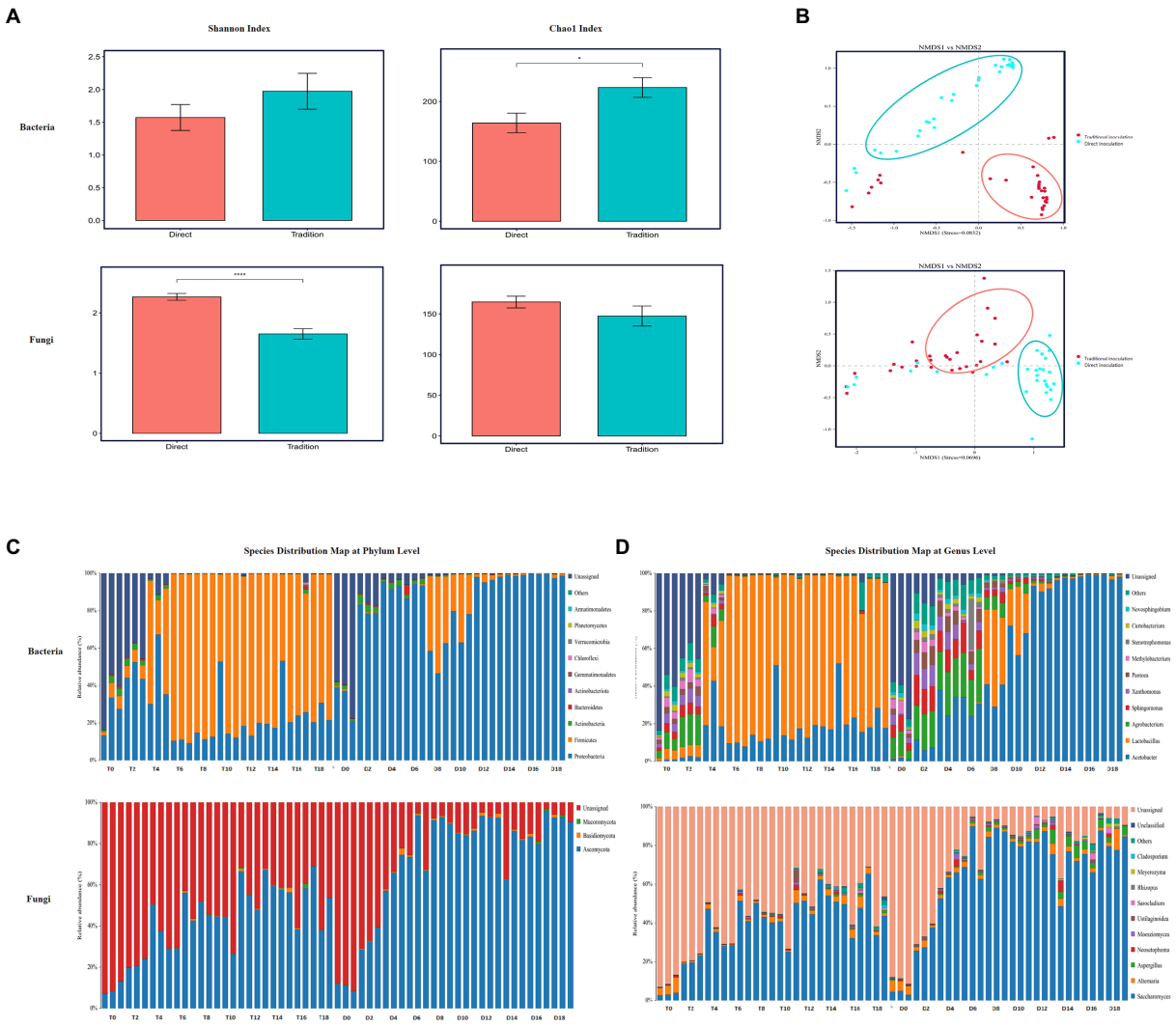

FIGURE 3
Analysis of microbial community diversity during the fermentation of Zhenjiang aromatic vinegar for different inoculation strategies. **(A)** α diversity of acetic acid fermentation microbial community for different inoculation strategies, including shannon index and Chao1 index. **(B)** NMDS map of microorganisms for different inoculation strategies. **(C)** Diversity analysis of microbial community during the fermentation of Zhenjiang aromatic vinegar for different inoculation strategies at the gate level. **(D)** Diversity analysis of microbial community during the fermentation of Zhenjiang aromatic vinegar for different inoculation strategies at the genus level.

aldehyde flavoring substances (Huang et al., 2022). Lactic acid bacteria play an important role in the formation of vinegar taste by producing organic acids such as acetic acid, malic acid, and lactic acid, and by synthesizing 2,3-butanediol and acetoin as precursors of tetramethylpyrazine (Chai et al., 2020). Additionally, during the fermentation process, bacillus generates organic acid *via* a tricarboxylic acid circulation pathway to improve the pungent sour taste caused by acetic acid and soften the mouth. Simultaneously, the flavor of vinegar is improved by the secretion of protease to decompose protein and generate amino acids (Nie et al., 2013). Owing to open fermentation, some bacteria in the environment participate in the esterification reaction, producing esters (Xu et al., 2011). As the acetic acid fermentation process progressed, the dominant microbial population for fermentation also changed (Figure 3D). During AAF, the relative abundance of acetic acid bacteria under the direct inoculation strategy was significantly higher than that under the traditional inoculation method, while lactic acid bacteria was on the contrary, because the direct inoculation strategy used biologically enhanced acetic acid bacteria as a bacterial agent to inoculate in the fermented grains, and acetic acid bacteria inhibited the activity of lactic acid bacteria. However, *Agrobacterium*, *Sphingomonas* and other major bacterial genera showed a trend of increasing first and then decreasing in the fermentation process, and the relative abundance of bacteria under the direct inoculation strategy was slightly higher than that under the traditional inoculation strategy.

The proportion of fungi in the two inoculation strategies was considerably lower than that of bacteria. Fungi are well known to play an important role in fermenting foods (Venturini, 2019). However, there are few studies on the changes in fungal communities during vinegar fermentation. Studies have proven that fungi are important contributors to flavor production in AAF and are rare communities (Huang et al., 2022). During AAF, *Saccharomyces*, *Alternaria* and

Aspergillus were the dominant fungi under the two inoculation strategies. Like the above bacteria, the relative abundance of bacteria under the direct inoculation strategy is slightly higher than that under the traditional inoculation strategy. The unidentified microorganisms in vinegar need further study. In addition to produce beneficial nutrients and flavor substances, some microorganisms may also produce harmful substances and their precursors, such as biogenic amines, urea, carbamate and other substances (Gao et al., 2023). Studies have shown that rare microflora may considerably affect the local microbial interactions during environmental disturbance (Jiang et al., 2019). The future research should focus more on the role of fungi in the structure of the fermentation microbial community and the generation of vinegar flavor (Huang et al., 2022).

3.3. Correlation analysis between internal environment of fermented grains and main microorganisms of different inoculation strategies during fermentation

In the AAF process, the formation of solid vinegar is the result of the continuous reproduction and metabolism of flavor substances by a large number of microbial communities using fermentation raw materials. The environment has a substantial effect on the metabolism of microbial communities. Once fermentation begins, the rapidly changing environmental conditions of the niche (e.g., the high ethanol content and anaerobic environment of fermented rice milk, and the high acidity of vinegar fermentation) provide selective strength for the 'systematic self-domestication' of microbial communities, thus shaping the decision-making process of microbial succession (Lu et al., 2018).

Environmental factors had a significant impact on the fermentation of vinegar in the solid state. In addition to the indoor temperature, humidity, and other external environments of fermented grains, the total acid, nonvolatile acid, and reducing sugar of vinegar-fermented grains were not only metabolites of acetic acid fermentation but also substrates of vinegar production, making them crucial environmental variables in vinegar-fermented grains. Redundancy analysis (RDA) and Spearman were used to conduct correlation analysis on seven bacterial and seven fungal groups (with average relative abundance >10%) and four environmental parameters to determine the relationship between the succession of microbial communities during the acetic acid fermentation stage and environmental changes caused by different inoculation strategies (Figure 4).

According to the RDA results, a significant correlation existed between prevalent microorganisms and environmental parameters (Figures 4A,B). Various environmental factors influence the survival and reproduction of various microorganisms. The cumulative interpretation of the relationship between major microorganisms and environmental factors on Axis I and Axis II was as high as 47.48 and 40.49%, respectively, in vinegar-fermented grains after traditional and direct inoculation (Figures 4A,B). In conjunction with the Spearman correlation coefficient and Figures 4C,D, the heat map of correlation between the main bacteria and fungi (average relative abundance >10%) and the environmental parameters in the fermentation process, for the two inoculation strategies, *Acetobacter pasteurianus*, *Lactobacillus acetotolerans*, and *Saccharomyces* sp (unclassified) were significantly positively correlated with reducing sugar, nonvolatile acid, and total acid. *Acetobacter pasteurinus* has strong ethanol resistance and can convert ethanol in fermented grains into lactic acid, acetic acid and other substances (Jiang et al., 2019). *Lactobacillus acetotolerans* prefers high-acid environment (growth pH 3.0–5.0, optimum pH 4.0), and its relative abundance will increase with acetic acid fermentation (Yu et al., 2020). *Saccharomyces sp.*, the main fungus in vinegar fermented grains, was brought into the fermented grains during the alcohol fermentation stage. *Saccharomyces sp*. decomposes polysaccharides to produce more reducing sugars, and decomposes monosaccharides to produce alcohol. *Saccharification* is accompanied by alcoholization and acetic acid. The content of non-volatile acid and volatile acid in vinegar fermented grains increases rapidly (Zhang et al., 2017). *Xanthomonas* is a common bacteria brought in from the fermented mash (Zhang et al., 2017). With the continuous reduction of acetic acid fermentation abundance, *Xanthomonas* is a small number of bacteria that can finally adapt to the acidic environment and remain in the microbial community of fermented mash with relatively low abundance (Wang et al., 2016). *Alternaria* is the dominant fungus genus of Zhenjiang vinegar (Wang et al., 2016). These bacteria were negatively correlated with reducing sugar, non-volatile acid and total acid. Under the traditional inoculation strategy, *Acetobacter Pasteuri*, *Acetyl-tolerant Lactobacillus* and *Saccharomyces cerevisiae (unclassified)* were significantly negatively correlated with pH. *Xanthomonas*, *Lactobacillus* and *Alternaria (unclassified)* were significantly positively correlated with the pH value of the direct inoculation strategy. Therefore, under different inoculation conditions, pH has the most significant effect on microorganisms. The pH value will affect the activity of microorganisms such as *Acetobacter* and *Lactobacillus* during acetic acid fermentation. When the pH value in the fermentation broth is too high or too low, the growth of microorganisms will be inhibited. In addition, pH value can also affect the accumulation of nutrients in the fermentation broth and the stability of the metabolic substances of acetic acid bacteria, thus reducing the acid production capacity of acetic acid bacteria (Wang et al., 2015).

3.4. Correlation analysis between flavoring substances and main microorganisms for various inoculation strategies during fermentation process

To fully understand the correlation between flavoring substances and the predominant microorganisms for different inoculation strategies, the Spearman correlation coefficient was used to investigate the correlation between important microorganisms (average relative abundance >1%) and flavoring substances formed during the fermentation process, The red line represents positive correlation, and the blue line represents negative correlation (Figures 5A,B, 6A,B). *Acetobacter Pasteurianus*, the main bacteria under the traditional inoculation strategy, is positively correlated with active acid, acid acid, pyruvic acid, lAsn, Thr, Ala, a-ABA, Asp., Ile, Cys, Arg, P-ser, His and Acetin. *Lactobacillus Acetotolerans,* the main bacteria under the traditional inoculation strategy, is positively correlated with oxalic acid, citric acid, pyroglutamic acid, successive acid, Leu, Cys, Arg, P-ser, Lys, PEA, 2-Methoxy-5-methylphenol, propanoic acid, 1-butanol, 3-methyl-, acetate (Figures 5A, 6A). While, in the direct

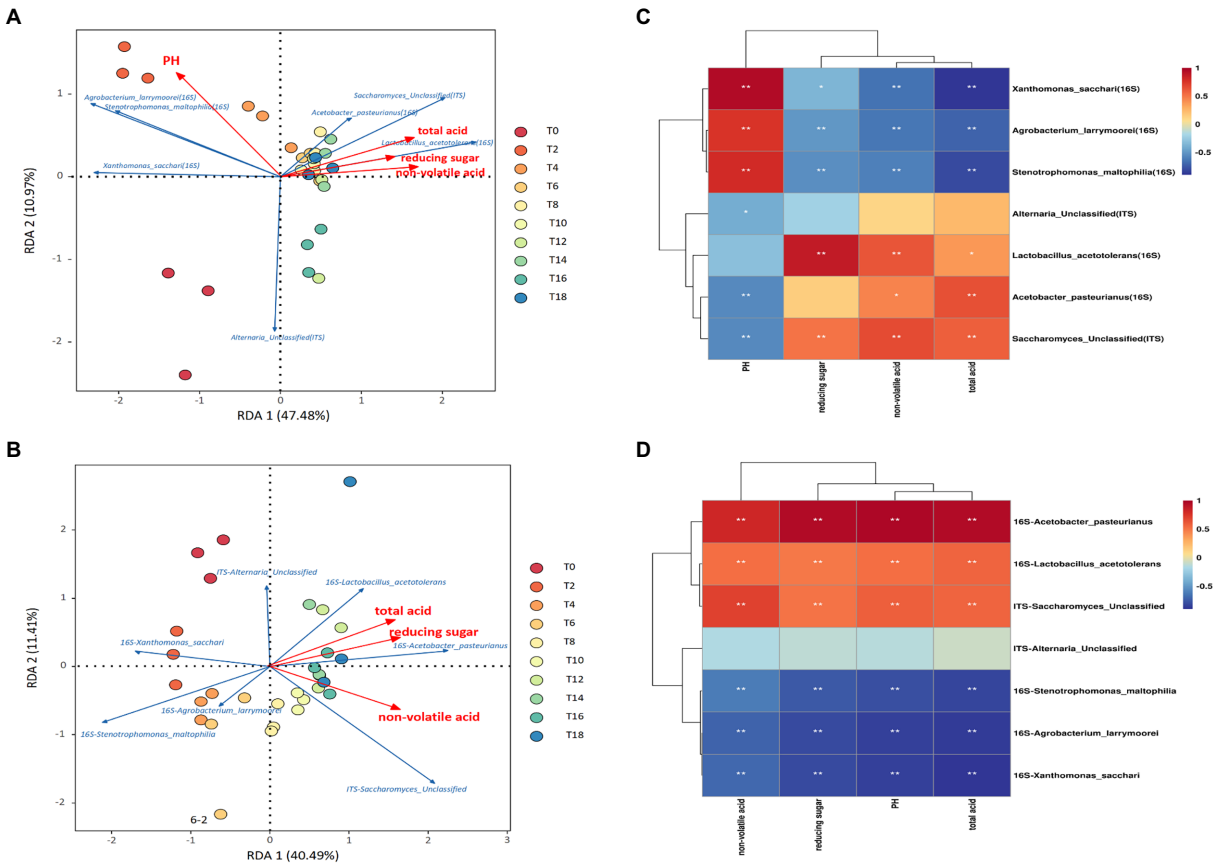

FIGURE 4
Correlation analysis between main bacteria and fungi for different inoculation strategies and environmental parameters during fermentation. **(A)** RDA diagram of main microorganisms and environmental parameters for the traditional inoculation strategy. **(B)** RDA of main microorganisms and environmental parameters for the direct-injection inoculation strategy. **(C)** Correlation heat diagram of main microorganisms and environmental parameters for the traditional inoculation strategy. **(D)** Correlation heat diagram of main microorganisms and environmental parameters for the direct inoculation strategy.

inoculation strategy, the main bacteria, *Acetobacter Pasteurianus,* is positively correlated with acid acid, pyroglutamic acid, citric acid, malic acid, ethyl 4-acetoxybutyloate, butanoic acid, 3-methyl-, 2-Methoxy-4-vinylphenol, acid, isobutyl acetate, hexanoic acid, methane, isocyanato-, 1,3-Dioxolane, 2,4,5-trimethyl-, benzeneethanol, b-Ethyl-, and negatively correlated with hexadecanoic acid, ethyl ester. Meanwhile, *Acetobacter Pasteurianus* is positively correlated with Ile, Met, Asp., a-ABA, Gly, Tyr, Asn, Ser, Thr, Lys, Arg, PEA, Ala, Orn. *Lactobacillus Acetotolerans* under the direct inoculation strategy is positively correlated with ethyl 4 – acetoxybutanoate, butanoic acid, 3 – methyl–, 2 – methoxy–4 – vinylpheno, acetic acid, benzeneethanol, b – ethyl–, and negatively correlated with acid and pyroglutamic acid (Figures 5B, 6B). This shows that the function of acetic acid bacteria under the direct inoculation strategy is strong, which is more conducive to the generation of organic acids, non-volatile acids and volatile flavor compounds than the traditional inoculation strategy. Jia et al. (2020) cultivated *Acetobacter Pasteuri* and *Lactobacillus plantarum*, which are conducive to the formation of flavor substances in Shanxi aged vinegar, so as to improve the content of flavor substances that contribute to the flavor of Shanxi aged vinegar, such as benzyl alcohol, ethyl acetate, phenylethyl acetate, 2,5-dimethylpyrazine, which are aroma-active compounds with floral, fruity, sweety and chocolate-like notes (Gao, 2021; Zhang et al., 2022).

In addition to the most important *Acetobacter and Lactobacillus,* other major microorganisms, such as *Agrobacterium Larrymorei* under the traditional inoculation strategy, have a positive correlation with tartaric acid *Agrobacterium Larrymoorei* was positively correlated with tartartaric acid, and negatively correlated with pyroglutamic acid, lactic acid and pyruvic acid. *Stenotrophomonas Maltophilia* was negatively correlated with pyroglutamic acid and pyruvic acid. *Xanthomonas Sacchari* was positively correlated with tartartaric acid and negatively correlated with acid. *Agrobacterium larrymoorei*, *Stenotrophomonas maltophilia*, *Xanthomonas Sacchari* were positively correlated with 3mehis, dimethyl ether and ethane, 1,1-diethoxy-and negatively correlated with lAsn, Thr, Ala, a-ABA, Asp., Ile, Cys, Arg, P-ser and Acetoin. *Agrobacterium larrymoorei*, *Stenotrophomonas maltophilia, Xanthomonas Sacchari* under the direct inoculation strategy, were positively correlated with 11 alcohols, 22 esters, 6 aldehydes and 3 acids, and negatively correlated with 6 alcohols, 7 esters, 1 aldehyde and 7 acids, and these strains are negatively correlated with ethyl 4-acetoxybutyloate, Butanoic acid, 3-methyl-, 2-methody-4-vinylphenol, and with hexadecanoic acid, ethyl ester, pexanoic acid, ethyl ester, octanoic acid, ethyl ester, benzoic acid, ethyl

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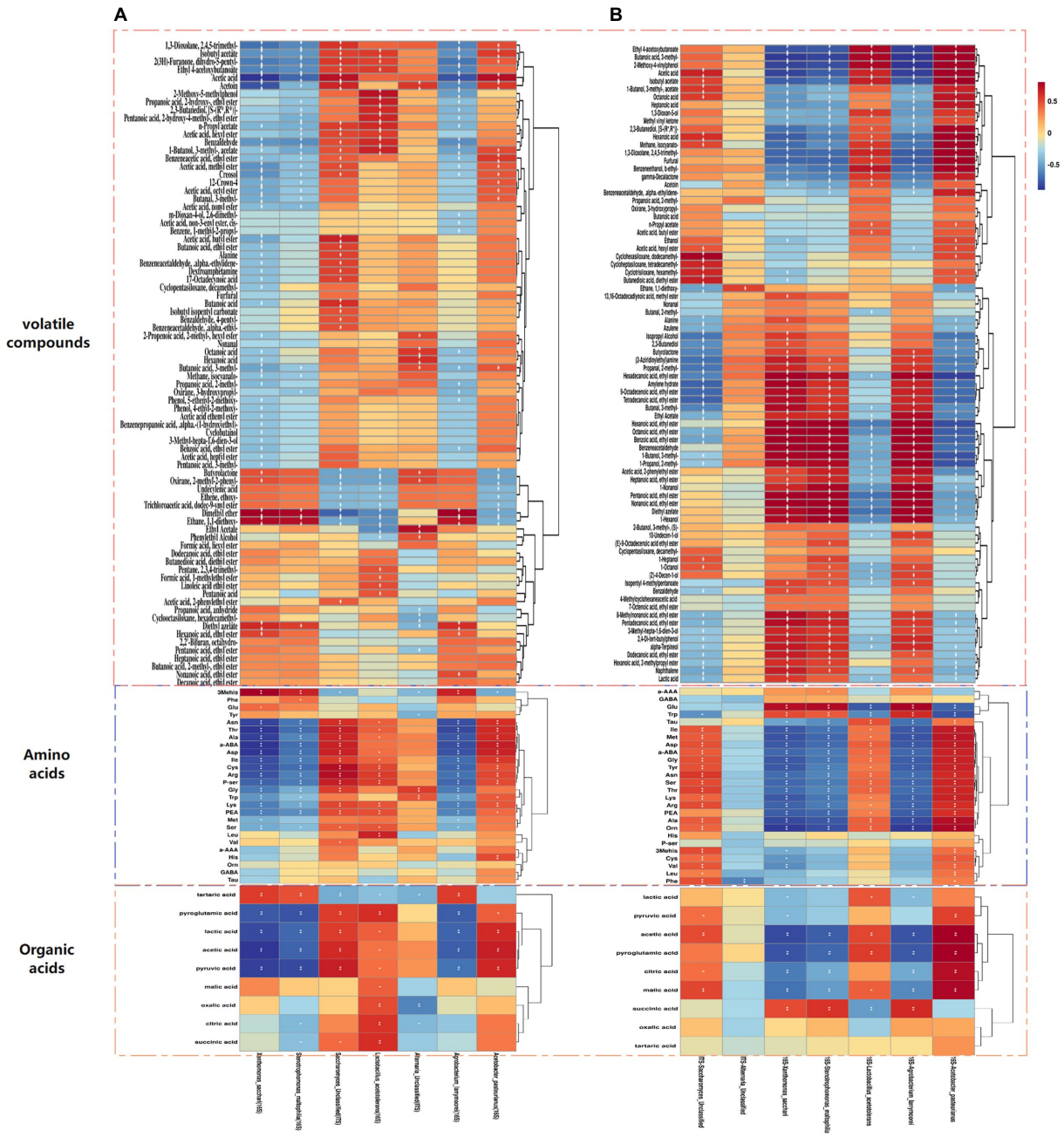

FIGURE 5
Correlation analysis between main bacteria and fungi and metabolites (volatile compounds, amino acids, and organic acids) during fermentation for different inoculation strategies. **(A)** A correlation heat map of microorganisms and flavor substances produced in the acetic acid fermentation process for the traditional inoculation strategy. **(B)** A correlation heat map of microorganisms and flavoring substances produced in the acetic acid fermentation process for the direct inoculation strategy.

ester, heptanoid acid, ethyl ester, 1-Nonal, Pentanoic acid, ethyl ester, Nona Noic acid, ethyl ester and diethyl azelate are positively correlated.

It was well known that fungi play an important role in fermented food (Venturini, 2019). *Saccharomyces Unclassified*, the main fungus under the traditional inoculation strategy is positively correlated with acetic acid, pyruvic acid, Asn, Thr, Ala, a-ABA, Asp., Ile, Cys, Arg, P-ser, Gly, Lys, PEA, and negatively correlated with tartaric acid. While, *Saccharomyces Unclassified*, the main fungus under direct inoculation strategy is positively correlated with both malic acid and acetic acid. Yang et al. (2017) used excellent *Saccharomyces cerevisiae* strains with strong fermentation capacity and strong fragrance production capacity for pure and mixed fermentation, and found that the content of volatile flavor substances could be increased when two excellent yeast strains were mixed for fermentation (Yang et al., 2017). *Alternaria Unclassified* is positively correlated with Gly and Trp. These main fungi are positively correlated with 5 alcohols, 27 esters, 7 aldehydes and 9 acids, among which *Alternaria Unclassified* is positively correlated with phenol alcohol, octanoic acid, exanoic acid, *Saccharomyces Unclassified*

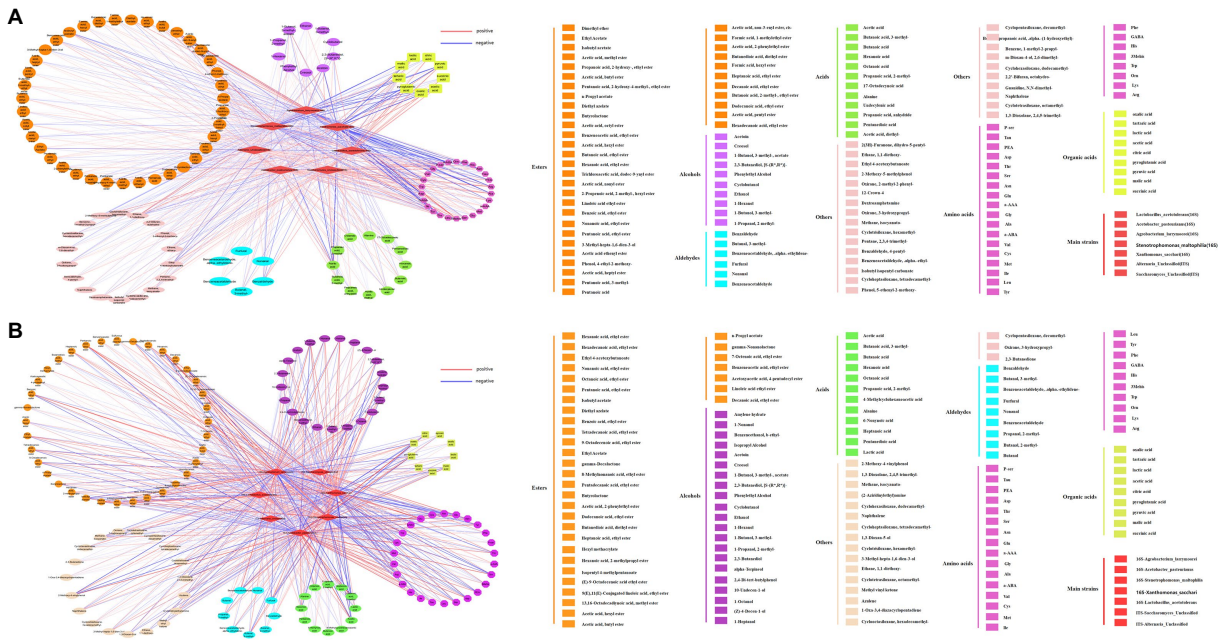

FIGURE 6
(A) A related network diagram of microorganisms and flavoring substances produced in the acetic acid fermentation process for the traditional inoculation strategy. **(B)** The relevant network diagram of microorganisms and flavoring substances produced in the acetic acid fermentation process for the direct inoculation strategy.

is positively correlated with acetic acid, acetoin and ethyl acetate. Although there are few studies on the changes of fungal community during vinegar fermentation, fungi have made a significant contribution to the production of flavor substances in vinegar fermentation. Therefore, in the future research, the role of fungi in the microbial community structure of fermentation and the formation of vinegar flavor quality cannot be ignored (Huang et al., 2022).

4. Conclusion

To fully understand the effects of different inoculation strategies on the physicochemical quality and flavor of Zhenjiang aromatic vinegar, we studied the effects of various physicochemical indexes, differences in the composition of flavor substances and microorganisms, and environmental factors on the main microorganisms of Zhenjiang aromatic vinegar for different inoculation strategies during the entire fermentation process. By comparing the physicochemical properties and flavor substances of different inoculation strategies in the fermentation process, it can be found that the content of total acid, organic acid, amino acid and key flavor substances in acetic acid fermentation under the direct inoculation strategy is slightly higher than that under the traditional inoculation strategy. At the same time, the direct inoculation strategy can effectively promote the production of acetoacetate.

The structure and succession pattern of bacterial and fungal communities in the whole fermentation process of two inoculation strategies were studied by using amplified sequence analysis. It was found that inoculation strategies significantly affected the structure of bacterial and fungal communities. The future research should focus more on the role of fungi in the structure of the fermentation microbial community and the generation of vinegar flavor.

Through the correlation analysis between the internal environment of fermented grains and main microorganisms (relative abundance greater than 10%) under different inoculation strategies in the fermentation process, it was found that pH had the most significant effect on microorganisms under different inoculation strategies.

Finally, the Spearman correlation coefficient was used to study the correlation between important microorganisms (average relative abundance>10%) and flavoring substances formed during fermentation under different inoculation strategies. It was found that in the direct inoculation strategy, the correlation between major microbial species and organic acids, non-volatile acids and volatile flavor compounds was more consistent.

The results revealed the effects of different inoculation strategies on the microbial composition and flavor quality during the fermentation of Zhenjiang aromatic vinegar, which will provide useful information for the future development of a direct-injection composite microbial inoculum to replace the traditional starter.

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

XY conducted experiments and made significant contributions to the acquisition, analysis, and interpretation of data. YZ and JL participated in the experiment and revised and discussed the

manuscript. KW and PL participated in the revision of the manuscript. YY participated in the experiment and reviewed and agreed to publish this article. ZY and YW designed the study and participated in the drafting and revision of the manuscript. All authors contributed to the article and approved the submitted version.

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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.2023.1126238/full#supplementary-material

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First report on metagenomics and their predictive functional analysis of fermented bamboo shoot food of Tripura, North East India

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Moiya pansung, *mileye amileye*, *moiya koshak*, and *midukeye* are naturally fermented bamboo shoot foods of Tripura. The present study aimed to reveal the whole microbial community structure of naturally fermented *moiya pangsung, mileye amileye, moiya koshak,* and *midukeye* along with the prediction of microbial functional profiles by shotgun metagenomic sequence analysis. The metataxonomic profile of *moiya pangsung, mileye amileye, moiya koshak,* and *midukeye* samples showed different domains, viz., bacteria (97.70%) followed by the virus (0.76%), unclassified (0.09%), eukaryotes (1.46%) and archaea (0.05%). Overall, 49 phyla, 409 families, 841 genera, and 1,799 species were found in all the fermented bamboo shoot samples collected from different places of Tripura. Firmicutes was the most abundant phylum (89.28%) followed by Proteobacteria (5.13%), Bacteroidetes (4.38%), Actinobacteria (1.02%), and Fusobacteria (0.17%). *Lactiplantibacillus plantarum* was the most abundant species in *moiya pangsung, mileye amileye, moiya koshak,* and *midukeye* followed by *Lactococcus lactis, Levilactobacillus brevis, Leuconostoc mesenteroides, Weissella paramesenteroides, Leuconostoc kimchii, Pediococcus pentosaceus, Leuconostoc gasicomitatum,* and *Lacticaseibacillus casei*. A few phyla of fungus were found, viz., *Ascomycota, Basidiomycota,* and *Glomeromycota*, where *Ascomycota* was present in high abundance. Functional analysis of *moiya pangsung, mileye amileye, moiya koshak,* and *midukeye* metagenome revealed the genes for the synthesis and metabolism of a wide range of bioactive compounds including, various essential amino acids, and conjugated amino acids. The abundance profile and predictive analysis of fermented bamboo shoots revealed a huge plethora of essential microorganisms and KEGG analysis revealed genes for amino acid metabolism, pectin degradation, lipid metabolism, and many other essential pathways that can be essential for the improvement of nutritional and sensory qualities of the fermented bamboo shoot products.

KEYWORDS

fermented bamboo shoot, *meloncana baccifera*, shotgun metagenomics, microbial community analysis, functional gene analysis

1. Introduction

Bamboo is a long-living, robust, adaptable, and highly renewable species of grass that grows as woody stems and is primarily found in moist deciduous, semi-evergreen, tropical, subtropical, and temperate regions of forests (Tewari et al., 2019). There are more than 75 genera and 1,250 species of bamboo documented on a global scale. Bamboo shoots are a healthy vegetable that is frequently harvested, consumed, and sold by the rural tribes of North Eastern India (Singhal et al., 2018). Bamboo shoots have a great deal of promise as a food resource with a high protein and fiber content but with little fat, making them a wonderful source of nourishment. They include vital antioxidants and therapeutic ingredients that can help delay the onset of metabolic problems in addition to being a storehouse of nutritious materials (Hussain et al., 2020). The product becomes acidic and digestive due to the action of lactic acid bacteria at large and some yeast species (Ferreira and Mendes-Faia, 2020). The fermentation of bamboo shoots not only makes them very nutritious and increases their shelf life but also makes them pleasant in terms of flavor, aroma, texture, and appearance (Behera and Balaji, 2021).

Fermented bamboo shoots are commonly consumed in North East India and go by a variety of regional names, including *ekung* and *eup* in Arunachal Pradesh (Tamang and Tamang, 2009), *miyamikhri* and *khorisa* in Assam, *lung-siej*, *tuaithar,* and *tuairoi* in Mizoram, *soidon* and *soibum* in Manipur, *bastangapani* in Nagaland and *mesu* in Sikkim (Tamang et al., 2008). Bamboo shoots are a rich source of nutrients, powerful antioxidants, and healing compounds that can postpone the onset of certain metabolic diseases (Yu et al., 2021). Colossal diversity of microorganisms has been reported from different types of fermented bamboo shoots, including the species of bacteria. *Lactobacillus johnsonii, Lb. plantarum, Lb. brevis, Lb. fermentum, Lb. curvatus, Leuconostoc mesenteroides, Leuconostoc lactis, Pediococcus* sp., *Micrococcus* sp., *Bacillus* sp., and the yeasts species, viz., *Saccharomyces cerevisiae, Zygosaccharomyces rouxii, Yarrowia lipolytica,* and *candida* sp. (Tamang et al., 2012; Nongdam, 2015; Voidarou et al., 2021).

A total of 21 species of bamboo are reported in Tripura among which *muli bans* [*Meloncana. baccifera* (Roxb.) Kurz] is the most important bamboo of the state while other species contribute only 5–7% of total production. About 708 metric tonnes of young bamboo shoots are harvested from Tripura, of which *M. baccifera* constitutes about 80–85% of the total harvest (Banik, 2010). Different ethnic communities, viz., Chakma, Debbarma, and Uchoi use young tender shoots of *muli bans* to prepare fermented products like *moiya pangsung, mileye amileye, moiya koshak,* and *midukeye* (Uchoi et al., 2015). All the fermented samples collected from Tripura are still prepared at a small household level by the ethnic group using rudimentary tools, so the microbiota of fermented foods is very essential to be studied to better understand the dominating microbes responsible for fermentation as well as to know the impact on the health of the consumers. This fermented bamboo shoot is usually prepared by aged women of the community.

The microbiology of fermented bamboo shoots has been rarely studied through culture-independent techniques except in *tuaithar* of Mizoram (Deka et al., 2021), *khorisa* of Assam (Behera et al., 2020), *suansun* of China (Hu et al., 2021). Moreover, there are no culture-independent studies present on the fermented bamboo shoot products of Tripura.

Since *moiya pangsung, mileye amileye, moiya koshak,* and *midukeye* are traditionally prepared from the young bamboo shoot by natural fermentation, diverse species of bacteria and fungi present in the local environment may appear in the final product. Moreover, there is no report on the predictive functionalities of microbial genes present in this fermented bamboo shoot. Predictive functional profiles of microbial communities in fermented foods are an appropriate approach to annotating the predictive metabolic pathways in gene sequences of bacteria and fungi (Sun et al., 2022) using different pipelines of bioinformatics tools such as Metagenomic Rapid Annotations using Subsystems Technology (MG-RAST) (Keegan et al., 2016), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa and Goto, 2000) for the bacterial gene. Hence, we aimed to study the bacterial and fungal community structure by shotgun sequencing tool during the natural fermentation of *moiya pangsung, mileye amileye, moiya koshak,* and *midukeye* of Tripura in India. We also aimed to predict the functional profiles of bacterial and fungal genes by using the bioinformatics pipelines, MG-RAST, during the natural fermentation of these fermented bamboo shoots.

2. Materials and methods

2.1. Sampling and site description

A total of 6 samples corresponding to four different types of fermented bamboo shoots were sampled in duplicates from different households and marketplaces in Tripura. A sample of *mileye amileye* was collected from the Panisagar district (PBMA) market (24.263307, 92.152439), 2 samples of *midukeye* were collected from the Dharmanagar household (DBMD) and Panisagar district (PBMD) (24.380504, 92.166556, and 24.269956, 92.150036, respectively), 2 samples of *moiya pangsung* were collected from Manubazar (MBMP) market place and Dharmanagar household (DBMP) (24.393275, 92.166073, and 23.074567, 91.645094, respectively) and 1 sample of *moiya koshak* was collected from Dharmanagar household (DBMK) (24.379511, 92.165093). All collected samples were kept in an ice box carrier and immediately transported within 24 h, where they were stored at −20°C for further analysis. Before fermentation, the sheaths of the young tender shoots were removed and then washed with water. *Moiya pangsung* is prepared from a top tender shoot and fermented for 2 days at room temperature in a closed container completely submerged in water (**Supplementary Figure 1A**), while *mileye amileye* is also fermented in a similar way but the middle portion of the bamboo shoot is used (**Supplementary Figure 1B**). Similarly, for *midukeye* and *moiya koshak*, the middle portion of the tender shoot is cut into pieces, then wrapped in banana leaves and left at room temperature for 2 days (**Supplementary Figure 1C**). These bamboo shoot products are sour and with a sweet and earthy after-taste. The type of samples and code name assigned to the samples are mentioned in **Supplementary Table 1**.

2.2. DNA extraction and sequencing

In a stomacher (400 Circulator, Seward, UK) ten grams of each sample were homogenized with 90 mL of sterile 0.1 M phosphate buffer saline (pH 6.4). The homogenate was filtered after homogenization, and the filtrate was utilized to extract genomic DNA using the Maggenome XpressDNA plant kit (Maggenome, USA) as per the manufacturer's protocol. Using 0.8% agarose gel electrophoresis, the DNA samples were separated to check for deterioration and contaminants and to assess quality (voltage 100 V, period 40 min). A precise measurement of the total DNA mass concentration was obtained utilizing nanodrop (Eppendorf Biospectrometer@ basic, Germany), to confirm that the parameters for the library creation adhered to. Following the manufacturer's instructions, total DNA was submitted to DNA library construction and amplification using the TruSeqTM DNA Sample Prep Kit (Illumina, United States) and the cBOT TruSeq PE Cluster Kit v3 (Illumina, United States) reagents. The TruSeq SBS Kit v3-HS (Illumina, United States) was used to sequence the DNA libraries utilizing the Illumina Novaseq 6,000 technology. According to the established Illumina methods, the sample DNA libraries were submitted to a paired-end (2 × 150 bp) sequencing platform. Fastq format was used to store the raw reads.

2.3. *De novo* metagenome assembly

High-quality filtered paired-end libraries of the samples of raw reads were assembled separately using MetaSPAdes assembler with default parameters (Nurk et al., 2017). The metagenomic data obtained from MetaSPAdes was run through the Galaxy platform[1] (Afgan et al., 2018) using the default parameters.

2.4. Taxonomic classification and abundance

The putative microbial population reads from six samples were classified using Kraken2 against the reference database containing all Refseq bacterial, archaeal, fungal, and viral genomes with a 0.1 confidence threshold (Marcelino et al., 2020). After the classification by Kraken2, Bracken was used to re-estimate the different microbial abundances at taxa levels from species to phylum using a read length parameter of 150 (Terrón-Camero et al., 2022).

2.5. Predictive functionality

The gene prediction for protein-coding genes (ORFs) was predicted using Prodigal (Hyatt et al., 2010). ORFs shorter than 180 nucleotides were excluded from further analysis by default. tRNA genes were predicted with the ARAGORN program (Laslett and Canback, 2004), and Ribosomal RNA genes (5S, 5.8S, 16S, 18S, 23S, 28S) were identified and classified using rRNAFinder (Dong and Strous, 2019). All ORFs were searched against the evolutionary genealogy of genes, non-supervised Orthologous Groups (eggnog) database (Muller et al., 2009), for Clusters of Orthologous Groups/Non-supervised Orthologous Groups (COG/NOG) annotation (Galperin et al., 2018) and Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa and Goto, 2000) for KEGG ID annotation.

2.6. Statistical analysis

2.6.1. Pooled sequences

Nucleotide diversity (pi) analysis and different indices of neutrality test based on sequence polymorphism in DnaSP software version 6 (Rozas et al., 2017) were performed to justify the pooling of DNA from each duplicate sample in terms of intra-sample (within the sample) diversity. Among the different indices of neutrality tests, Tajima's D value and Fu's Fs statistics were performed in the same software. To check the intra-sample diversity in terms of species level and functional profiles, one sample *T*-test was performed in IBM SPSS version 20 for all the samples. Statistical relations among the samples were performed using the Mann-Whitney test (Fong and Huang, 2019) in terms of species level and level 3 functional profiles in PAST version 4.0 software (Martino et al., 2019).

2.6.2. Alpha and beta diversity indexes

Non-parametric Shannon index, Simpson's index of diversity (1-D), Chao-1, and evenness were calculated using PAST software version 4.0. The Bray-Curtis index of beta diversity was also calculated using past version 4.0 and visualized *via* a Principal Coordinates Analysis (PCoA) plot (Martino et al., 2019).

2.6.3. Functional profiling

The clustering pattern among the samples was checked through the PCoA plot using the level 3 sub-pathways. The PCoA plot was constructed after the log transformation of data and then visualized in the ClustVis web tool (Metsalu and Vilo, 2015). Non-parametric Spearman's rank correlation was performed using IBM SPSS software version 20 and PAST version 4.0 (Martino et al., 2019) software to check the correlation between the microorganisms in samples and different levels (super-pathways) functional profiles. Heatmap was constructed *via* the ClustVis web tool to visualize the correlation profiles. Correlation between different bacteria and the amino acid profiles obtained from KEGG annotation was also performed.

3. Results

3.1. Pooled sequences

As mentioned earlier, each sample was collected in duplicates and was pooled on the basis of their geographical origin and we hypothesized that the samples from the same origin possess minimal diversity differences. Nucleotide diversity (pi) per site was 0.712 (PBMA), 0.713 (DBMD), 0.713 (PBMD), 0.733 (DBMK), 0.721 (DBMP), and 0.712 (MBMP), respectively. However, the

1 https://usegalaxy.org/

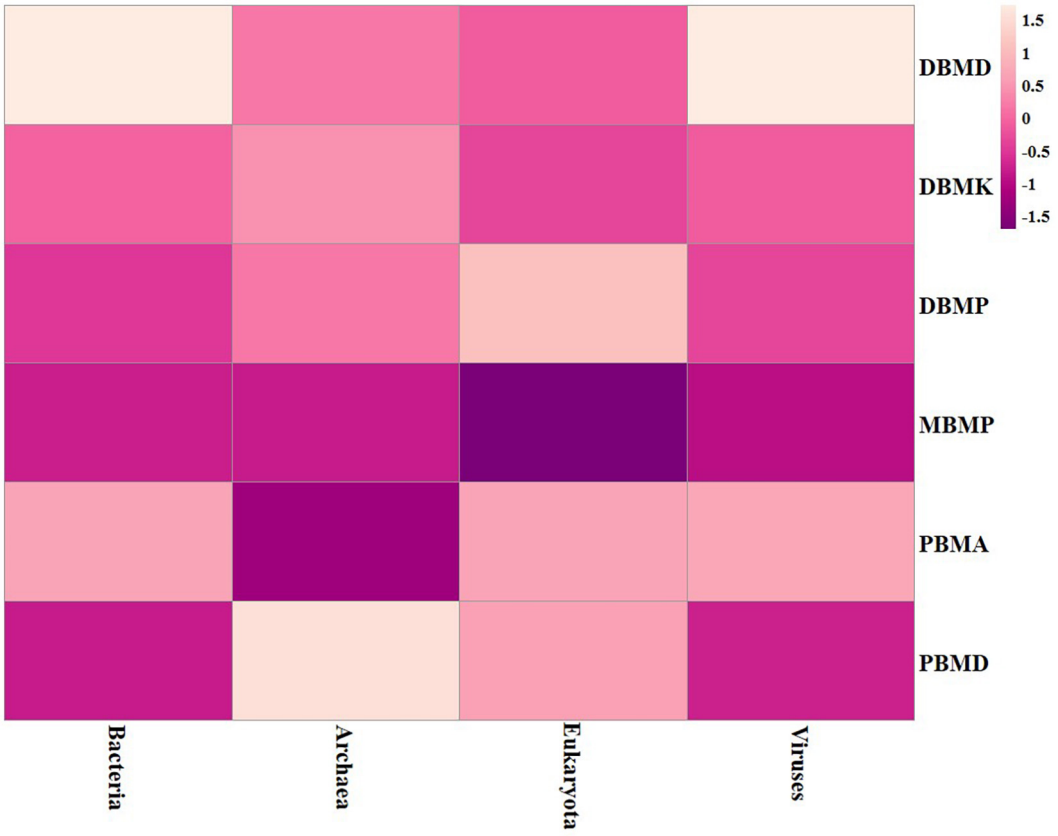

FIGURE 1
Heat map representation of major domain in *moiya pangsung, mileye amileye, moiya koshak,* and *midukeye*.

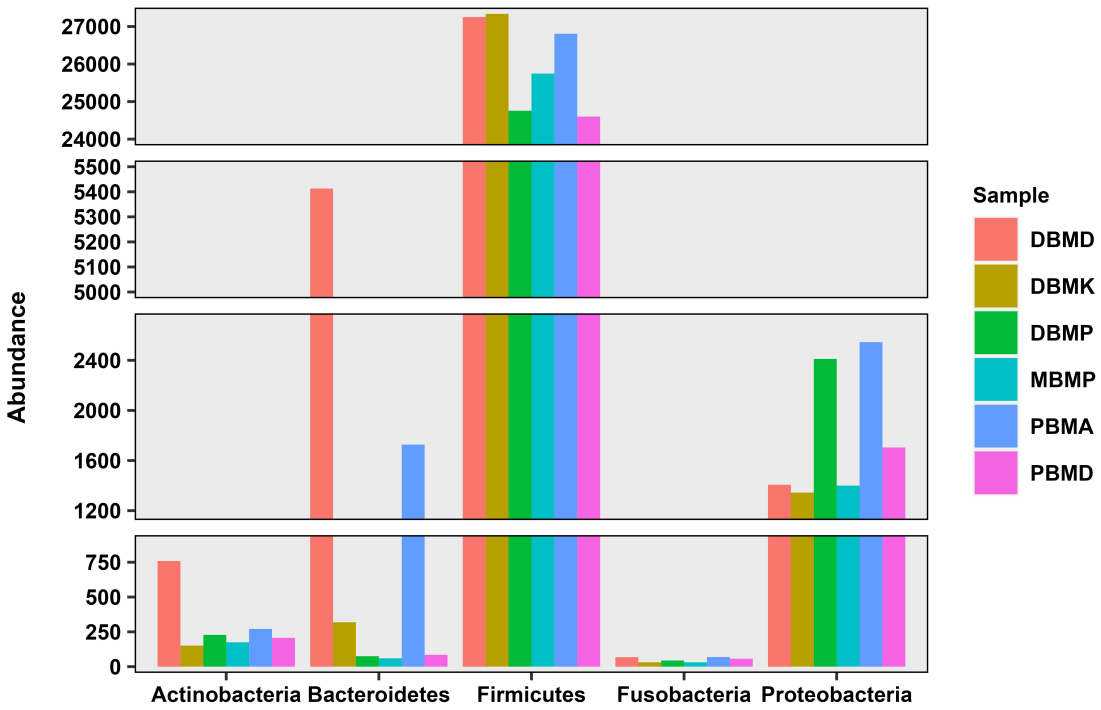

FIGURE 2
Bar plot for major phylum in bacteria.

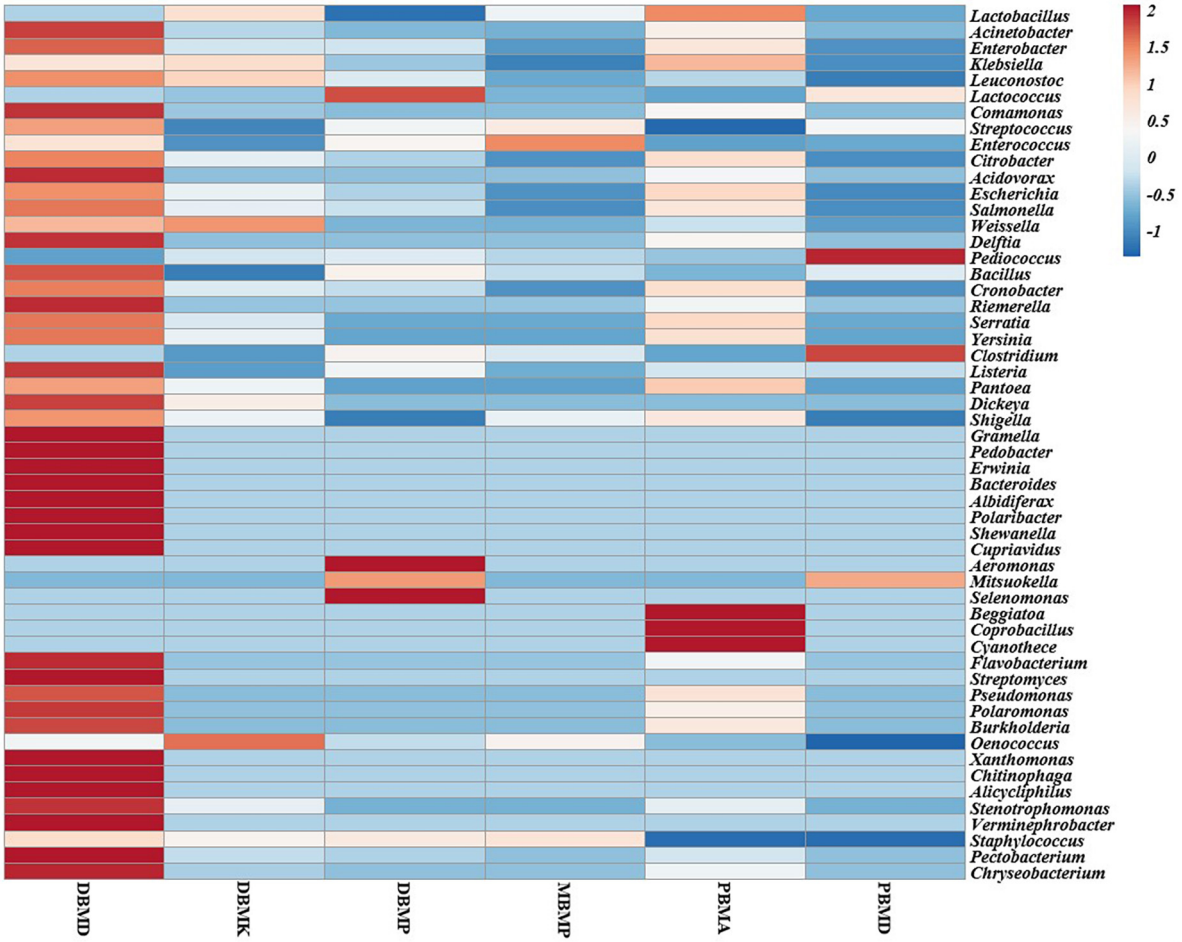

FIGURE 3
Heat map representation of major bacterial genera in *moiya pangsung, mileye amileye, moiya koshak,* and *midukeye*.

theta values (nucleotide difference) found for each of the samples were 0.72, 0.73, 0.72, 0.72, and 0.73, respectively. First, we checked Tajima's D value among the different indices of the neutrality test. Each sample showed a negative value for Tajima's D, which were −0.21 (PBMA), −0.19 (DBMD), −0.19 (PBMD), −0.20 (DBMK), −0.21 (DBMP), and −0.19 (MBMP), respectively. The second neutrality test indices were Fu's F_S statistics (FST); FST values found for all the samples were −5.30, −3.24, −3.24, −3.88, −5.21, and −3.90.

3.2. Taxonomic classification and abundance profiling

A total of 1,570,915 reads were obtained from the samples with an average of 261,820 reads per sample. The average length of the reads was found 583 bp. The total number of bases recovered from the samples was 311,915,402 (DBMP), 285,708,731 (DBMD), 93,242,563 (MBMP), 71,456,540 (DBMD), 61,889,925 (PBMA), and 55,160,324 (DBMK), respectively. Shotgun metagenomic sequence analysis of *moiya pangsung, mileye amileye, moiya koshak,* and *midukeye* samples showed different domains, viz., bacteria, archaea, viruses, and eukaryotes. The total relative abundance was calculated in all the samples where bacteria were the most abundant domain (97.70%) followed by the virus (0.76%), unclassified (0.09%), eukaryotes (1.46%) and archaea (0.05%). Among samples, DBMD was seen to have the highest bacterial abundance (28.84%), PBMD had the highest archaeal abundance (24.46%), DBMP had the highest eukaryotic abundance of (0.41%), and DBMD had the highest viral abundance (31.34%) (**Figure 1**). A total of 49 phyla, 409 families, 841 genera, and 1,799 species were recorded after abundance analysis. At the bacterial phylum level, Firmicutes was the most abundant phylum (**Figure 2**). Across all the bacterial families, *Lactobacillaceae* was found the most abundant family followed by *Enterobacteriaceae* (**Supplementary Table 2**). *Lactobacillus was* found to be the most abundant genera of all, among them PBMA had the highest abundance (**Figure 3**). No cluster was found among the samples except PBMD and MBMP in PCoA analysis according to the abundance/distribution of bacterial genera from different samples (**Figure 4**). At the species level, *Lactiplantibacillus plantarum, Levilactobacillus brevis, Lactococcus lactis, Pediococcus pentosaceus, Leuconostoc mesenteroides,* and *Leuconostoc citreum* (**Supplementary Figure 2**), non-LAB, archaea, yeasts, filamentous moulds, other phages, microeukaryotes, and parasites were also detected in < 1% abundance from *moiya pangsung,*

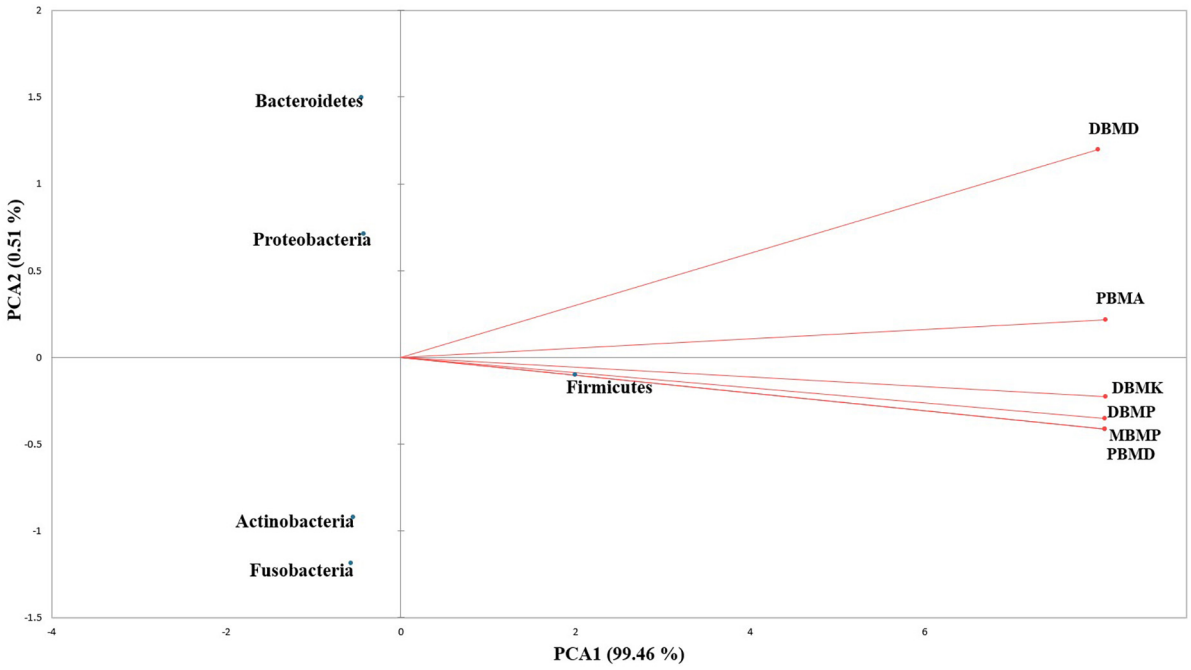

FIGURE 4
PCA biplot analysis of samples and microbial diversity at the genus level.

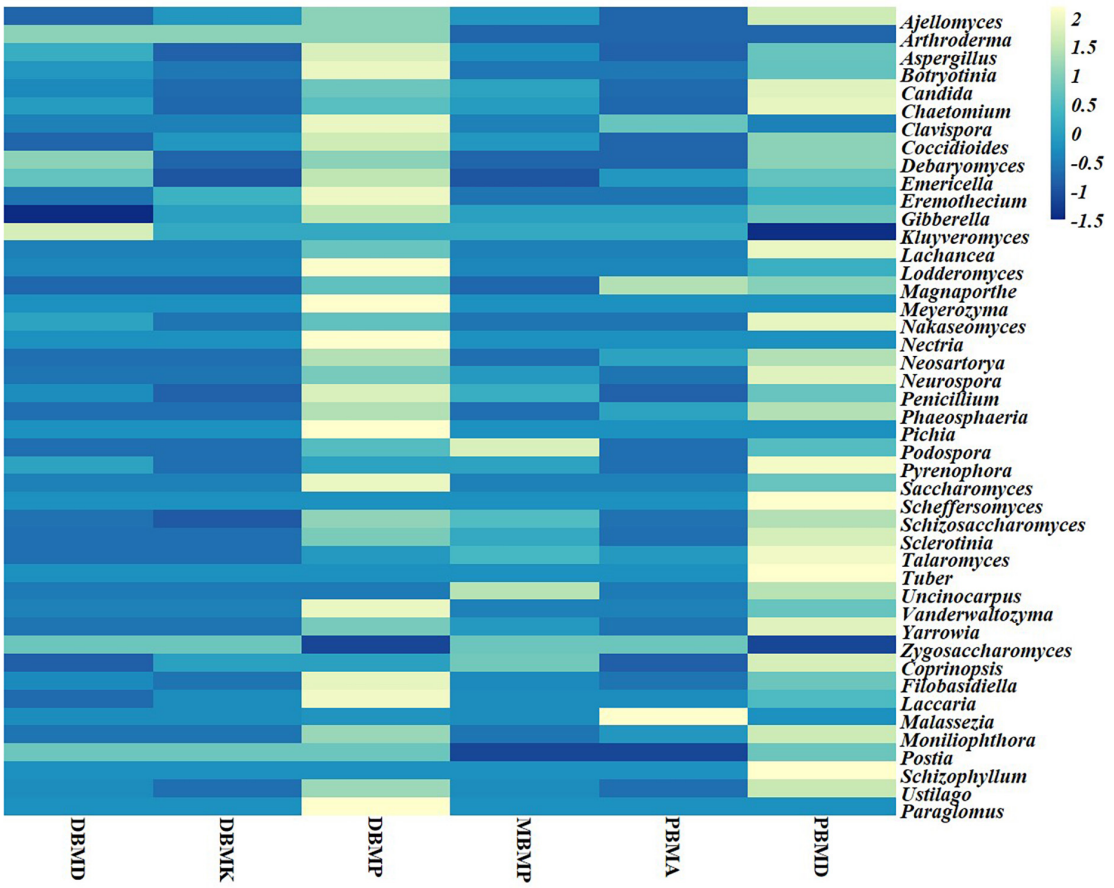

FIGURE 5
Heat map representation of major fungal genera communities in *moiya pangsung, mileye amileye, moiya koshak,* and *midukeye*.

mileye amileye, moiya koshak, and *midukeye* samples. From the Eukaryota domain, 3 phyla of fungus were found *Ascomycota, Basidiomycota,* and *Glomeromycota*, where *Ascomycota* was present in abundance (Supplementary Figure 3A), *Saccharomycetaceae* was overall the most abundant family (Supplementary Figure 3B), *Schizosaccharomyces* was overall the most abundant fungal genera (Figure 5), and *Schizosaccharomyces pombe* was found to be the most abundant species on an overall basis (Supplementary Figure 3C).

3.3. Diversity indices

The non-parametric Shannon, Simpson, Chao-1, and Evenness indices were highest in the DBMD sample (Table 1). At the genus level, the beta diversity of DBMP and PBMD were completely different from other samples as visualized in principal component analysis obtained through Jaccard indices (Figure 6). Similarly, Bray-Curti's beta diversity at the genus level also revealed DBMD and PBMD were completely different from the other 4 samples (Figure 7). The shared and unique species were visualized between 4 samples using a venn diagram elaborated that 61, 8, 1, and 85 species were unique to *moiya pangsung, moiya koshak, mileye amileye*, and *midukeye,* respectively, whereas 306 species were common in all the fermented products (Supplementary Figure 4).

3.4. Functional profile

Different enhanced functional pathways were observed after mapping metagenomic ORFs against eggNOG and KEGG databases. The number of prodigal ORFs identified from *moiya pangsung, moiya koshak, mileye amileye*, and *midukeye* samples were 23,615 (DBMD), 14,447 (DBMK), 25,596 (DBMP), 13,483 (MBMP), 18,329 (PBMA), and 21,710 (PBMD), respectively. About 49.65% of the total mapped ORFs were assigned to the COG functional genes, 49.82% were assigned to the KEGG functional pathways, and the rest 0.53% were assigned to the NOG pathway. In this study, we focused more on the KEGG functional pathways, COG mapping, and NOG pathways. The functional pathways were categorized into three sub-classes *via* KEGG mapping. In level 1, metabolism was the most abundant category followed by genetic information processing, environmental information processing, cellular process, human disease, and organismal system (Figure 8). At level 2, 50 super-pathways, and at level 3, 238 sub-pathways (terminal) from different categories were identified (Supplementary Table 3). Spearman's rank correlation was performed between some major microorganisms in *moiya pangsung, moiya koshak, mileye amileye*, and *midukeye* samples and different major level 2 super-pathways obtained from KO annotation. *Lactiplantibacillus plantarum, Latilactobacillus curvatus,* and *Lentilactobacillus hilgardii* showed a positive correlation with amino acid metabolism. While, *Lactobacillus jensenii*, and *Lacticaseibacillus casei* showed a positive correlation with lipid metabolism. *Latilactobacillus curvatus* showed the highest correlation with the membrane transport system (Figure 9).

One sample *T*-test was performed to check the intra-sample and inter-sample significance in terms of functional profiles of *moiya pangsung, moiya koshak, mileye amileye*, and *midukeye* samples metagenome. No intra-sample significance was found in any of the pooled samples in terms of functional profiles. The *p-value* for six samples were 0.98 (DBMD), 0.98 (DBMK), 1 (DBMP), 1 (MBMP), 0.99 (PBMA), and 0.99 (PBMA), respectively.

In total, 15 pathways for amino acid metabolism were visualized and annotated to microbial functional genes in the few microbial species in all samples. The most abundant amino acid metabolic pathway visualized in all the fermented bamboo shoot samples was Alanine, aspartate, and glutamate metabolism; ko00250 (16.98%), the next most abundant was glycine, serine, and threonine metabolism; ko00260 (15.97%) as well as cysteine and methionine metabolism; ko00270 (14.97%).

The second most abundant pathway found in all the fermented bamboo shoot samples related to the impartment of flavor and aroma were glycerolipid metabolism (ko00561) (25.33%), fatty acid biosynthesis (ko00061) (25.22%), and glycerophospholipid metabolism (ko00564) (24.02%) (Table 2). A few pathways like the metabolism of terpenoids and polyketides were related to the color and aroma of the fermented bamboo shoot such as carotenoid biosynthesis (ko00906). Pathways of secondary metabolism related to the impartment of flavor and aroma to the fermented bamboo shoots were also annotated such as phenylpropanoid biosynthesis (ko00940), flavonoid biosynthesis (ko00941), flavone and flavonol biosynthesis (ko00944), stilbenoid, diarylheptanoid and gingerol biosynthesis (ko00945), and tropane, piperidine, and pyridine alkaloid biosynthesis (ko00960), etc. Some metabolic pathway related to pectin degradation were identified within carbohydrate metabolisms such as pentose and glucuronate interconversions (ko00040). Pathways related to vitamins metabolism were also annotated such as thiamine metabolism (ko00730), riboflavin metabolism (ko00740), retinol biosynthesis (ko00830), nicotinate and nicotinamide metabolism (ko00760), pantothenate and CoA biosynthesis (ko00770), biotin biosynthesis (ko00780), folate biosynthesis (ko00790), and lipoic acid biosynthesis (ko00785).

Comparing the dataset of our samples with the NOG database, it was found that all the samples had the maximum number of amino acid transport metabolism unigenes, followed by cell wall/membrane/envelope biogenesis, transcription, and carbohydrate transport metabolism unigenes, respectively (Supplementary Figure 5). COG dataset yielded 97,635 unigenes, from which carbohydrate transport metabolism had the highest number of unigenes in all samples, followed by amino acid transport metabolism and replication, recombination, and repair, respectively. The sample bearing code DBMD has the

TABLE 1 Alpha diversity indexes.

Sample	Simpson	Shannon	Chao-1	Evenness
DBMD	0.9232	3.009	49	0.4104
DBMK	0.8332	2.318	28	0.3626
DBMP	0.8957	2.285	23	0.4271
MBMP	0.7258	1.782	17	0.3495
PBMA	0.8698	2.626	38	0.3657
PBMD	0.7679	1.881	16	0.4102

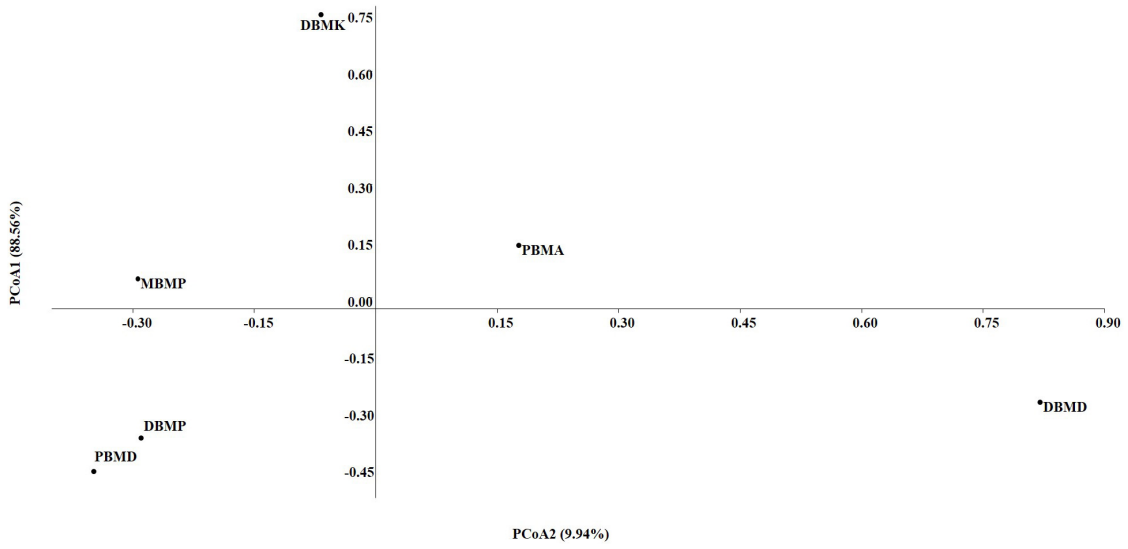

FIGURE 6
PCA plot for beta diversity analysis *via* Jaccard at the bacterial genus level.

highest number of unigenes as seen in the COG database than DBMK, PBMA, PBMD, MBMP, and DBMP, respectively (**Supplementary Figure 6**).

In our bamboo shoot samples, according to the KEGG pathway, there were 80 unigenes for cyano amino acid metabolism among which 46 unigenes from *moiya pangsung,* 3 unigenes *moiya koshak,* 1 from *mileye amileye* and 30 unigenes belonged to *midukeye*. Cyanogenic glycoside can be hypothesized to be metabolized by the cyano amino metabolic pathway *via* tyrosine metabolism.

4. Discussion

Moiya pangsung, moiya koshak, mileye amileye, and *midukeye* are one of the oldest fermented bamboo shoot gastronomies of Tripura and are prepared by natural fermentation (Uchoi et al., 2015). Profiling of microbial community in naturally fermented *moiya pangsung, moiya koshak, mileye amileye*, and *midukeye* by the shotgun metagenomic method has not been reported yet. Hence, we applied the shotgun metagenomic method to profile the whole microbial community structure in naturally fermented *moiya pangsung, moiya koshak, mileye amileye*, and *midukeye.* In our study, we collected samples from three places in duplicate; *mileye amileye* and *midukeye* were collected from Panisagar and Dharmanagar, *moiya pangsung* was collected from Manubazzar and Dharmanagar, and *moiya koshak* was collected from Dharmanagar. The shotgun sequencing was performed after pooling the DNA of the samples which were all collected in duplicates, because the pooled sequence can also represent the actual sequences from the pooled DNA of the individual samples (Anand et al., 2016) to maintain adequate diversity within and between the samples. We anticipated the microbial community structure to vary within a single representative pool because these samples are naturally fermented. Nucleotide differences and the neutrality test were used to interpret the pooled sample using statistics, and the results clearly showed that there was little polymorphism and variation within the pooled sample, indicating that samples from the same origin have very little diversity differences (Hivert et al., 2018). Negative FST values are also unequivocal proof that there are more alleles than necessary in a single pooled sample, which is truly more than one sample (de Meeûs, 2018). These two tests demonstrate that each of our pooled samples truly has combined data from multiple samples and that there is very little intra-sample variations in the pooled sample, which can also support our pooling method.

Firmicutes was the most abundant phylum in *moiya pangsung, moiya koshak, mileye amileye*, and *midukeye*, like in other Asian fermented bamboo shoot foods like *suansun* of China (Hu et al., 2021), and *tuaithar* of Mizoram, India (Deka et al., 2021). *Lactobacillaceae* was the abundant family in *moiya pangsung, moiya koshak, mileye amileye*, and *midukeye* as reported earlier in other ethnic bamboo shoot fermented foods (Ghosh et al., 2019). *Lactiplantibacillus plantarum* was the most abundant species in all the samples followed by *Lactococcus lactis, Levilactobacillus brevis*, and *Leuconostoc mesenteroides,* which was consistent with other plant-based fermented foods (Lappe-Oliveras et al., 2022) *L. plantarum* attracted many researchers because of its wide applications in the medical field with antioxidant, anticancer, anti-inflammatory, antiproliferative, anti-obesity, and antidiabetic properties (Arasu et al., 2016). *Lactococcus lactis* has been found to be major bacterial species to be found in different fermented bamboo shoot foods as well as other fermented plant-based products (Swain et al., 2014). *Lacticaseibacillus brevis* has been seen to have probiotic and antifungal attributes (Somashekaraiah et al., 2021). *Pediococcus pentosaceus* which has been found in all the samples of the fermented bamboo shoots has been seen to pose a partial characterization of heat-stable, antilisterial, and cell lytic bacteriocin (Halami et al., 2011). Though in a minor abundance, the detection of amino acid-producing bacteria *Corynebacterium glutamicum* (Hahne et al., 2018) and *Bacillus thuringiensis* (Hibi et al., 2011) is an indication of nutritive

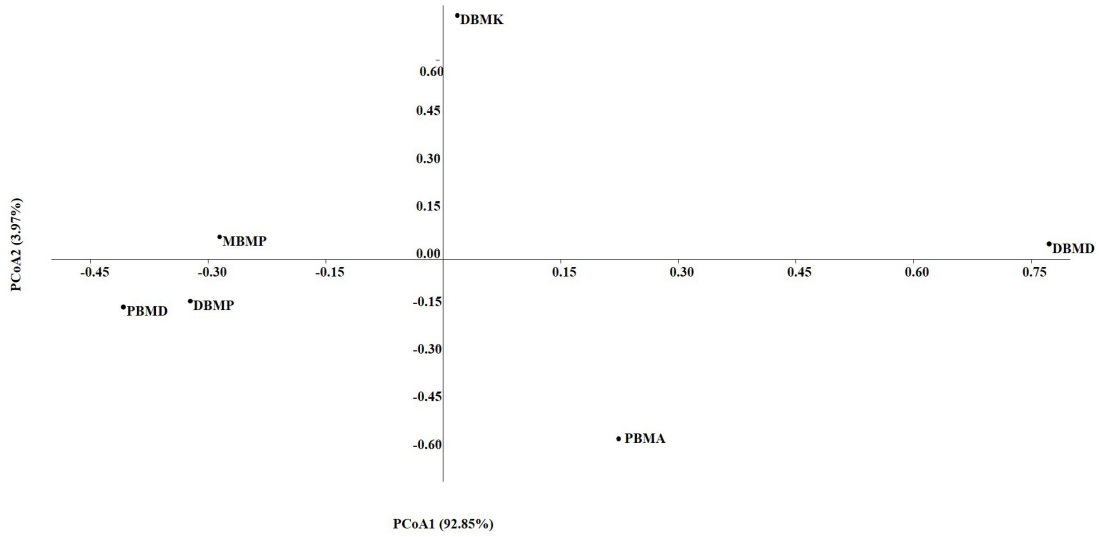

FIGURE 7
PCA plot for beta diversity analysis *via* Bray-Curtis at the bacterial genus level.

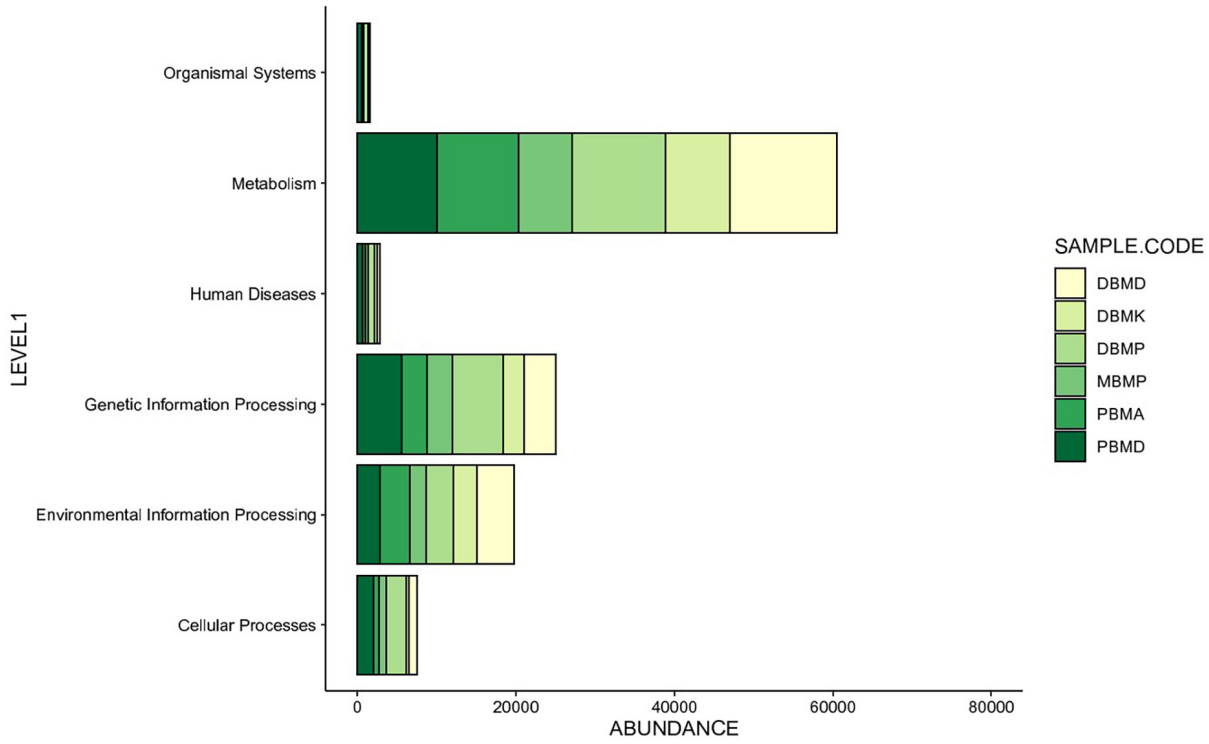

FIGURE 8
KEGG (level 1) showing the highest number of genes annotated to pathways.

significance in *moiya pangsung, moiya koshak, mileye amileye*, and *midukeye*.

Though the cumulative abundance of lactic acid bacterial species was > 1‘%, while other genera at lesser abundance than 1% were also detected such as *Bacillus, Cronobacter, Riemerella, Serratia, Yersinia, Clostridium, Stenotrophomonas, Pseudomonas, Listeria, Burkholderia, Oenococcus*, and *Xanthomonas* in all the samples. After bacteria, eukaryota was the most abundant domain found in the metagenome analysis of *moiya pangsung, moiya koshak, mileye amileye*, and *midukeye*. The most abundant eukaryota found in the samples were Ascomycota, Basidiomycota, and Glomeromycota in all the fermented bamboo shoot samples which is consistent with other samples like *Suansun* from China, *tuaithar* from Mizoram, India (Deka et al., 2021), fermented shrimp and sauerkraut (Park et al., 2020). We observed a co-existence of *Lactobacillus* and *Saccharomyces* in all the fermented bamboo

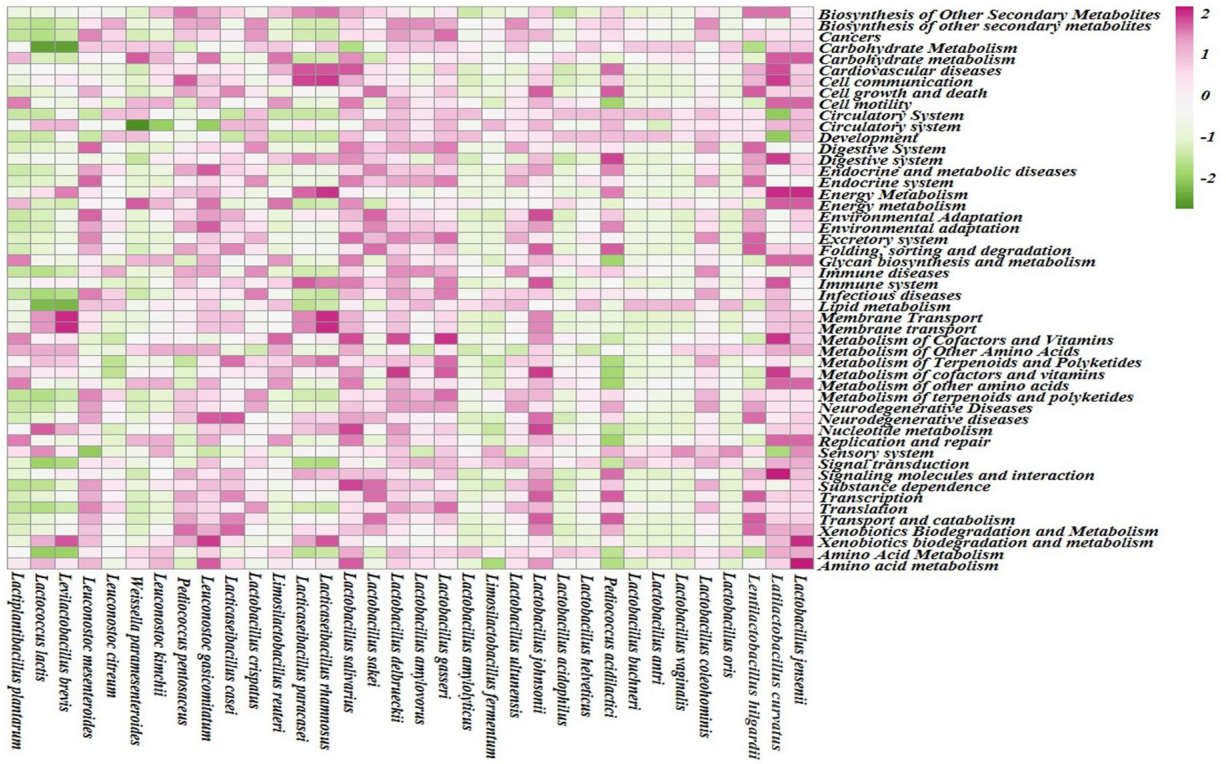

FIGURE 9
Spearman RS correlation of major bacterial species with KEGG (level 2) pathways.

shoot samples. Various *non-Saccharomyces* yeasts (*Torulaspora, Lachancea,* and *Starmerella*) were also found. These yeasts are responsible for the addition of aroma (Djeni et al., 2020), glycerol concentration, volatile compounds, and for increasing the concentration of terpenes in fermented foods (Das and Tamang, 2021). Acinetobacter phages were more abundant than other phages in *moiya pangsung, moiya koshak, mileye amileye*, and *midukeye* metagenome. Bacteriophages generally play a critical role in determining bacterial abundance over time (Shapiro et al., 2010). In our study, the abundance of *lactobacillus phages* was found much lower than the abundance of *Lactobacillus* species. However, the presence of phages against the hosts like *Staphylococcus* sp., *Salmonella* sp., and *Streptococcus* sp., indicates the bio-control activity of bacteriophages in food fermentation (Kumar et al., 2019).

Few species of haloarchaea were detected in *moiya pangsung, moiya koshak, mileye amileye*, and *midukeye,* though their abundance was very low. The exact role of archaea in *moiya pangsung, moiya koshak, mileye amileye*, and *midukeye* is still unknown. Halophilic archaea have been reported as the producer of halocin, a compound that infers anti-microbial activity (Corral et al., 2020). Hence the presence of haloarchaea again may be linked with the bio-enhancement of the fermented bamboo shoot food (Mota de Carvalho et al., 2018). Low abundances of the domain eukarya, which included yeasts, filamentous moulds, several species of algae, protozoa, and parasites, were observed in *moiya pangsung, moiya koshak, mileye amileye*, and *midukeye*. Protozoa and parasites are among the other microeukaryotes that are believed to have a detrimental effect on consumers' health and wellbeing, albeit a comprehensive safety assessment of fermented bamboo shoot cuisine has not yet been conducted. A robust approach that can identify the whole microbial population in a sample with both high and low abundances is the shotgun metagenome sequence (Kumar et al., 2022), may help to understand the safety measure of foods on the basis of their abundances level.

The KEGG annotation showed that metabolic pathways were common in *moiya pangsung, moiya koshak, mileye amileye*, and *midukeye*. One of the predominant predictive functional super-pathways within the metabolism category was amino acid metabolism. During fermentation, alanine, aspartate, and glutamate metabolism and biosynthesis increase the creation of flavor and aroma compounds (Sulaiman et al., 2014). The abundance of genes involved in the synthesis of branched-chain amino acids (valine, leucine, and isoleucine) was also detected from the metagenome analysis of *moiya pangsung, moiya koshak, mileye amileye*, and *midukeye*, which are involved in energy metabolism of *Lactobacillus* sp. (Neinast et al., 2019). The functional study of the *moiya pangsung, moiya koshak, mileye amileye*, and *midukeye* metagenome was used to predict the synthesis and metabolism of glutathione. The cellular processes of anti-oxidant defense, drug detoxification, and cell signaling involved in the regulation of gene expression and apoptosis all involve the metabolism, production, and transport of glutathione (Kennedy et al., 2020). Additionally, computational methods were used to predict the presence of the taurine and hypotaurine production genes, which have been linked to a reduced risk of cardiovascular disease (Xu et al., 2008). According to the COG database, carbohydrate metabolism, RNA

TABLE 2 KEGG metabolic pathways annotated to different microbial species.

Ko number	KEGG pathway	Microorganism annotated to KEGG pathway
Ko00250	Alanine, aspartate, and glutamate metabolism	*Lactiplantibacillus plantarum, Lactiplantibacillus pentosus, Pediococcus pentosaceus, Lactococcus Lactis, Lactiplantibacillus buchneri, Levilactobacillus brevis, Weissella paramesenteroides*
Ko00270	Cysteine and methionine metabolism	*Lactiplantibacillus plantarum, Lactiplantibacillus fermentum, Levilactobacillus brevis, Weissella paramesenteroides*
Ko00260	Glycine, serine, and threonine metabolism	*Lactiplantibacillus amylolyticus, Lacticaseibacillus casei, Lactiplantibacillus buchneri, Lactiplantibacillus plantarum, Lactiplantibacillus fermentum, Lactiplantibacillus vaginalis Levilactobacillus brevis, Weissella paramesenteroides*
Ko00300	Lysine biosynthesis	*Lactiplantibacillus amylolyticus, Lactiplantibacillus casei, Lactiplantibacillus buchneri, Lactiplantibacillus plantarum, Lactiplantibacillus fermentum, Lactiplantibacillus vaginalis, Levilactobacillus brevis, Weissella paramesenteroides*
Ko00330	Arginine and proline metabolism	*Pediococcus pentosaceus, Lactiplantibacillus plantarum, Lactiplantibacillus brevis, Lactococcus lactis*
Ko00350	Tyrosine metabolism	*Lactiplantibacillus buchneri, Lactiplantibacillus amylolyticus, Lactiplantibacillus plantarum, Lactiplantibacillus pentosus, Lactiplantibacillus hilgardii, Levilactobacillus brevis, Weissella paramesenteroides*
Ko00340	Histidine metabolism	*Lactiplantibacillus buchneri, Lactiplantibacillus plantarum, Lactiplantibacillus fermentum,*
Ko00290	Valine, leucine, and isoleucine biosynthesis	*Lactiplantibacillus pentosus, Lactiplantibacillus casei, Lactiplantibacillus buchneri, Lactiplantibacillus plantarum, Levilactobacillus brevis, Weissella paramesenteroides*
Ko00280	Valine, leucine, and isoleucine degradation	*Lactiplantibacillus buchneri, Lactiplantibacillus amylolyticus, Lactiplantibacillus plantarum, Lactiplantibacillus pentosus, Levilactobacillus brevis, Weissella paramesenteroides*
Ko00360	Phenylalanine metabolism	*Lactiplantibacillus buchneri, Lactiplantibacillus amylolyticus, Lactiplantibacillus plantarum, Lactiplantibacillus pentosus, Levilactobacillus brevis, Weissella paramesenteroides*
Ko00380	Tryptophan metabolism	*Lactiplantibacillus buchneri, Lactiplantibacillus amylolyticus, Lactiplantibacillus plantarum, Lactiplantibacillus pentosus, Lactiplantibacillus hilgardii, Levilactobacillus brevis, Weissella paramesenteroides*
Ko00400	Phenylalanine, tyrosine and tryptophan biosynthesis	*Lactiplantibacillus buchneri, Lactiplantibacillus amylolyticus, Lactiplantibacillus plantarum, Lactiplantibacillus pentosus*

processing and modification, chromatin structure and dynamics and cytoskeleton were predominant in all the samples when correlated between COG data and major species which is consistent with the results obtained by Hu et al. (2021). KEGG pathway annotation of samples revealed the catabolism of cyanogenic glycosides, viz., dhurrin, and linamarin through the cyano amino metabolic pathway. It is possible that taxiphyllin may follow the same catabolic process thereby reducing its amount in the fermented bamboo shoots (Appenteng et al., 2021). Terpenoids are also known to be the aroma compound in wine fermentation (Belda et al., 2017). The presence of flavonoids and triterpenes in fermented bamboo shoots has been documented by Lin et al. (2018) to impart flavor to the bamboo shoots. The genes for flavonoid metabolism were also detected from our samples through the culture-independent method. The presence of polygalacturonase enzyme which is referred to as the key enzyme in the pectin degradation pathway may suggest or predict that the bacteria present in fermented bamboo shoots have pectinase degrading ability and soften the texture of the fermented bamboo shoots (Buergy et al., 2020).

The presence of the thiamine metabolic pathway not only indicates that thiamine acts like a coenzyme for various enzymes but also aids in the production of more flavoring substances as opposed to off-flavoring substances (Labuschagne and Divol, 2021). Lactic acid bacteria were the dominant microorganism in the fermented bamboo shoots and previously have been seen to produce riboflavin which is a strong candidate for food biofortification, besides its role in red blood cell production and also aids in the release of energy from proteins (Spacova et al., 2022). The vitamin A component retinol has become an increasingly sought-after cosmetic ingredient (Hu et al., 2022). The presence of a nicotinamide biosynthesis pathway ensures the good absorption of tryptophan and niacin when fermented food is consumed (Faivre et al., 2021). Pantothenate and biotin metabolic pathway help in the growth of various lactic acid bacteria (Yao et al., 2018). The water-soluble vitamin B group member folate, particularly in the forms of tetrahydrofolate (THF) and methyl-tetrahydrofolate (MTHF), is crucial for the carbon-1 transfer processes required for the creation of DNA, nucleic acids, amino acids, and other vitamins (Mahara et al., 2021). These metabolic pathways are attributed to microorganisms fermenting the food, lactic acid bacteria have been found to possess the above KEGG

metabolic pathways as previously described in the correlation heat map of major lactic acid bacteria species and KEGG pathways (Cui et al., 2023).

This present study may also provide theoretical and technical support for search and application of the key enzymes produced by the fermenting microbes, and various essential amino acids with health benefits predicted in the fermentation of *moiya pangsung, moiya koshak, mileye amileye*, and *midukeye* for other Asian fermented bamboo shoot. The metataxonomic and predictive functional features of the *moiya pangsung, moiya koshak, mileye amileye*, and *midukeye* metagenome may be used to design the starter culture with enhanced nutritional quality under controlled and hygienic fermentation conditions.

5. Conclusion

Moiya pangsung, moiya koshak, mileye amileye, and *midukeye* is a delicacy of the Tripura ethnic tribe's diets and presents one of the most consumed foods of Tripura. There have been no studies on microbiology and nutritional aspects of *moiya pangsung, moiya koshak, mileye amileye*, and *midukeye*. Our study revealed different beneficial microorganisms associated with *moiya pangsung, moiya koshak, mileye amileye*, and *midukeye* fermentation along with their beneficial predictive functional profiles. We hope that the information obtained from this study may help the commercial producers and consumers to be aware of the functional microbial community, the health benefits, and importance of hygiene and food safety during preparation and handling of *moiya pangsung, moiya koshak, mileye amileye*, and *midukeye*. We believe this is the first report on the shotgun-based metataxonomic profile of naturally fermented *moiya pangsung, moiya koshak, mileye amileye*, and *midukeye*.

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

RD carried out sampling, investigation, data curation, formal analysis, and preparation of original draft. BT conceptualized the work and helped in writing and supervision. IN helped in data analysis. NT helped in writing and editing. KM helped in sampling. All authors contributed to the article and approved the submitted version.

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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.2023.1158411/full#supplementary-material

SUPPLEMENTARY FIGURE 1A
Preparation of *moiya pangsung* a traditional fermented bamboo shoot of Tripura **(a)** tender shoots of *Melanconna baccifera*, **(b)** cutting, **(c)** top portion of the bamboo shoot, and **(d)** soaked in water for fermentation.

SUPPLEMENTARY FIGURE 1B
Preparation of *mileye amileye* a traditional fermented bamboo shoot of Tripura **(a)** tender shoots of *Melanconna baccifera*, **(b)** cutting, **(c)** middle portion of the bamboo shoot, **(d)** soaked in water for fermentation, and **(e)** fermented product.

SUPPLEMENTARY FIGURE 1C
Preparation of *midukeye*, and *moiya koshak* a traditional fermented bamboo shoot of Tripura **(a)** tender shoots of *Melanconna baccifera*, **(b)** cutting, **(c)** middle portion of the bamboo shoot, **(d)** washed with water, **(e)** wrapped in banana leaves for fermentation, and **(f)** fermented product.

SUPPLEMENTARY FIGURE 2
Heat map representation of major species in *moiya pangsung, mileye amileye, moiya koshak*, and *midukeye*.

SUPPLEMENTARY FIGURE 3A
Stacked bar plot representation of major fungal phylum communities in *moiya pangsung, mileye amileye, moiya koshak*, and *midukeye*.

SUPPLEMENTARY FIGURE 3B
Heat map representation of major fungal family communities in *moiya pangsung, mileye amileye, moiya koshak*, and *midukeye*.

SUPPLEMENTARY FIGURE 3C
Heat map representation of the major fungal species communities in *moiya pangsung, mileye amileye, moiya koshak*, and *midukeye*.

SUPPLEMENTARY FIGURE 4
The shared and unique species were visualized between samples using a venn diagram.

SUPPLEMENTARY FIGURE 5
NOG pathways annotated.

SUPPLEMENTARY FIGURE 6
COG pathways annotated.

SUPPLEMENTARY TABLE 1
Fermented bamboo shoots samples collected from different locations in Tripura, India.

SUPPLEMENTARY TABLE 2
Abundance of bacteria at family level in *moiya pangsung, mileye amileye, moiya koshak*, and *midukeye*.

SUPPLEMENTARY TABLE 3
KEGG gene abundance in each sample in levels 2 and 3.

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Interaction analysis of tobacco leaf microbial community structure and volatiles flavor compounds during cigar stacking fermentation

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Introduction: Cigar stacking fermentation is a key step in tobacco aroma enhancement and miscellaneous gas reduction, which both have a great influence on increasing cigar flavor and improving industrial availability.

Methods: To analyze the effect of cigar tobacco leaf (CTLs) microbial community on volatiles flavor compounds (VFCs), this study used multi-omics technology to reveal the changes in microbial community structure and VFCs of different cigar varieties during stacking fermentation, in addition to exploring the interaction mechanism of microbiome and VFCs.

Results: The results showed that the dominant microbial compositions of different CTL varieties during stacking fermentation were similar, which included *Staphylococcus*, *Corynebacterium 1*, *Aerococcus*, and *Aspergillus*. These dominant microbes mainly affected the microbial community structure and characteristic microorganisms of CTLs through microbial interactions, thereby influencing the transformation of VFCs. Characteristic microorganisms of different CTLs varieties such as *Trichothecium*, *Trichosporon*, *Thioalkalicoccus* and *Jeotgalicoccus*, were found to positively correlate with characteristic VFCs like megastigmatrienone 4, pyrazine, tetramethyl-, geranyl acetone, and 2-undecanone, 6,10-dimethyl-, respectively. This in turn affected the aroma and sensory quality of the CTLs.

Discussion: This study provides theoretical support for the analysis of the mechanism of microorganisms on VFCs and aroma, and development of microbial agents during cigar stacking fermentation.

KEYWORDS

cigar, stacking fermentation, microbial community, volatile flavor compounds, interaction, aroma

1. Introduction

Cigars are tobacco products rolled from cigar tobacco leaves (CTLs), and are made through a long process of preparation, fermentation, and aging, ultimately creating a unique and complex flavor (Krusemann et al., 2018; Spatarella et al., 2018). Cigars have various aromas such as nutty, flowery, fruity, and chocolate, and the production of these complex aromas involves the transformation of many substances related to the microbial community and enzymes in CTLs (Gupta, 2015; Krusemann et al., 2020). The content of macromolecular substances and their

related enzyme activity change significantly during the growth and agricultural fermentation stages of CTLs (Banozic et al., 2020). Starch, cellulose, and pectin accumulated during CTLs growth and maturation processes are then gradually degraded during the agricultural fermentation stage and subsequently converted into the aroma precursors and VFCs of CTLs (Zhang et al., 2021). At this point, the primary aroma of CTLs has been formed, although the smoke gas remains relatively rough, and miscellaneous gas, bitterness, and other bad flavors need to be further mellowed, in addition to reducing irritation through stacking fermentation to further enrich the aroma of CTLs and improve their quality (Liu F. F. et al., 2022). Stacking fermentation is the industrial fermentation stage in the production process of cigars, and compared with the rapid degradation of macromolecular substances in the growth and agricultural fermentation process, stacking fermentation primarily transforms small molecular substances and VFCs. The content of VFCs changes with the function of microorganisms and related enzymes, although there is little variation in type, and to achieve the effect of increasing aroma, reducing miscellaneous gas, and mellowing the smoke gas (Liu F. F. et al., 2022; Liu T. et al., 2022; Zheng et al., 2022a).

In recent years, the study of aroma type, microbial community composition, and VFCs in cigar stacking fermentation has increased (Liu F. F. et al., 2022; Zheng et al., 2022a), yet the evolution of the microbiome structure during stacking fermentation and its mechanism for characteristic aroma formation remain poorly understood. Therefore, it is necessary to use high-throughput sequencing technology to study the changes in microbial community composition during stacking fermentation, since this is conducive to the mining of functional microorganisms and the analysis of the evolution mechanism of microbial communities, and to further improve the level to which the industrial fermentation production process can be controlled. This is very important for improving the production and process standards related to cigar fermentation (Zhang et al., 2020; Wu et al., 2021; Zhou T. et al., 2021; Zheng et al., 2022b). In this study, different varieties of high-quality CTLs were used as the research objects, whilst the changes in microbial community composition and volatile changes during stacking fermentation were dynamically analyzed. The correlation network between microorganisms and volatile aroma was first established, with the changes in microbial community structure during stacking fermentation and the influence of this on VFCs and aroma subsequently analyzed. This study provides guidance for analyzing the mechanism of stacking fermentation and the excavation of functional microorganisms involved in this.

2. Materials and methods

2.1. Sample collection

Eight high-quality CTL samples were collected from China Tobacco Hubei Industry Co., Ltd., including different varieties of CX14, DX4, CIOLLO 98, N-Jalap HABANA, HVA, BESUKI, MATA FINA, and E-HABANO 2000. The entire stacking fermentation process consists of a total of five nodes, for every 7 d of fermentation, or temperature reached 45°C for sampling and re-stacking. Five nodes were named, as raw materials (T0, 0–7 d), pre-fermentation (T1, 7–14 d), mid-fermentation (T2, 14–21 d), post-fermentation (T3, 21–28 d), and end of fermentation (T4, 28–35 d). Further information regarding these samples is presented in Supplementary Table S1.

2.2. CTLs sampling

CTL samples of 5 g were added to a sterile pre-chilled mortar, before including liquid nitrogen, and this mixture was then ground into a powder. The CTL powder was put into a 500 mL shaker flask containing 100 mL of sterile normal saline and was subsequently treated at 10°C and 220 rpm for 2–3 h. After the elution was filtered, CTL powder was removed by filtering through four layers of gauze, before the filtrate was centrifuged at 4°C and 7,000 rpm for 20 min. The precipitate was then collected to represent the total microorganism content of the CTLs. These collected microbial cells were extracted using the DNeasy PowerSoil Pro Kit to show CTL microbial genomic DNA. The extracted DNA samples were then amplified through PCR using the primers ITS1F (5'-GGAAGTAAAAGTCGTAACAAGG-3')-ITS2R (5'-GCTGCGTTCTTCATCGATGC-3') and 515F (5'-GTGCCAGCMGCCGCGGTAA-3')-907R (5'-CCGTCAATTCMTTTRAGTTT-3') for fungi and bacteria, respectively. The amplification products were then electrophoresed on 1% agarose gel, with the corresponding products being recovered for further analysis.

2.3. Amplicon sequencing

Here, the Illumina platform was used to sequence the DNA fragments of the microbial community using the two-ended sequencing method, silva_132 and unite_8, whilst the sequence analysis method used was DADA2 (Callahan et al., 2016).

2.4. Profiling of VFCs by HS-SPME-GC-MS during stacking fermentation

Using the DB-5MS column with headspace solid-phase microextraction (50/30 DVB/CAR/PDMS fiber), the helium flow rate was constant at 1.0 mL/min. The temperature procedure was as follows: at the beginning of injection, the column temperature was 40°C (hold for 2 min) and then increased to 250°C (hold for 5 min) at a rate of 10°C/min. The ion source temperature was maintained at 210°C and the transfer line temperature was maintained at 280°C. The system operated in EI mode with an EI voltage of 70 eV. Full scan mode used a mass scan range of 33–400 m/z with an acquisition rate of 10 scans per second (Zheng et al., 2022a). Compared with chemical standards in National Institute of Standards and Technology spectral library WILEY 8.0 and NIST14, VFCs with similarity above 800 and flavor characteristics were selected for analysis, and the relative content was calculated by the area normalization method (Wu et al., 2022).

2.5. Sensory quality analysis

The sensory evaluation of CTL samples was carried out by China Tobacco Hubei Industry Co., Ltd. The indexes of the samples were scored 1–5 points, whilst the non-existent aroma and bad flavors were

scored 0 points. Aromas were divided into woody, bean, flower, honey sweet, mellow sweet, burn sweet, light sweet, pollen, milk, hay, roasted, resin, fruity, herb, nutty, leather, coffee and pepper. Bad flavor was divided into green miscellaneous gas, burning, woody gas, earthy, regional gas, and protein.

2.6. Data analysis

The correlations between microorganisms and VFCs, and between VFCs and aroma were calculated using the psych package for R software (version 4.2.2) (Yu S. et al., 2021). Furthermore, the pheatmap package was used to create a heatmap of the VFCs, whilst Gephi (version 0.9.4) and Cytoscape (version 3.9.1) software were used for the network of interactions (Shannon et al., 2003), the (Partial Least Squares Discriminant Analysis) PLS-DA model of SIMCA (version 14.1) was used to screen CTLs characteristic VFCs (Ruiz-Perez et al., 2020), and Origin 2021 and Excel 2021 software were used for data processing and analysis.

3. Results

3.1. Microbial community changes during stacking fermentation

The changes in microbiota structure during cigar stacking fermentation were analyzed by 16S and ITS amplicon sequencing, the bacterial and fungal phylum with relative abundances in the top 10 were selected for subsequent analysis, as shown in Figure 1. *Firmicutes*, *Actinobacteria* and *Proteobacteria* were the dominant bacterial phylum in the different varieties of CTLs. During stacking fermentation, the relative abundance of *Firmicutes* was gradually increased in DX4 CTLs; *Actinobacteria* increased to 46.30%, 51.70%, 20.63%, 72.70% and 43.59% in CX14, CRIOLLO 98, BESUKI, MATA FINA and E-HABANO 2000 CTLs, respectively. In HVA and N-Jalap HABANA CTLs, relative abundance of *Firmicutes* had changed, with 78.82% and 97.38% at the end of fermentation. *Ascomycota* was the primarily dominant fungi at the phylum level during stacking fermentation, the relative abundance of different CTLs varieties from 40.54% to 99.46%.

The bacterial and fungal genera with relative abundances in the top 20 were selected for subsequent analysis, as shown in Figure 2. The results showed that the compositions of the dominant bacterial genera in the different varieties of CTLs were similar. The three dominant bacterial genera, *Staphylococcus*, *Corynebacterium 1* and *Aerococcus*, were the main components of the bacterial communities in different CTL varieties. The total relative abundance of all samples was between 67.15% and 99.10%; however, the relative abundance changes during stacking fermentation differed. The relative abundances of *Corynebacterium 1* and *Aerococcus* gradually increased from the CX14, BESUKI, and MATA FINA CTLs, while the sum of the relative abundances of *Corynebacterium 1* and *Aerococcus* at the end of fermentation were 79.59%, 67.68%, and 72.30%, respectively. The CTLs from DX4 and N-Jalap HABANA had *Staphylococcus* as the dominant bacteria in the stacking fermentation process, with its relative abundance exceeding 90% at the end of fermentation. Furthermore, the CTLs from CRIOLLO 98, HVA, and E-HABANO 2000 showed *Staphylococcus* as the main dominant bacteria, although the relative abundance of *Staphylococcus* gradually decreased, whilst the relative abundances of *Halomonas*, *Atopostipes*, *Corynebacterium 1*, and other genera showed a slight increase. The high relative abundance of dominant bacteria *Staphylococcus*, *Corynebacterium 1* and *Aerococcus* in stacking fermentation might be due to the decrease of tobacco leaf water content and the increase of pH with the fermentation process, and the strains had better alkali and salt resistance, making them in a dominant position of staking fermentation (Di Giacomo et al., 2007; Li et al., 2020).

During stacking fermentation, the fungal community with *Aspergillus* as the dominant fungal genus showed the highest proportion of relative abundance among different varieties of CTL fungi, whilst the relative abundance of *Aspergillus* in different CTLs varied greatly. Among them, CX14, CRIOLLO 98, HVA, BESUKI, and E-HABANO 2000 CTLs *Aspergillus* decreased during the fermentation process, with the community composition being richer at the end of fermentation. While *Aspergillus* was the most dominant fungus in the CTLs from DX4, N-Jalap HABANA, and MATA during stacking fermentation, at the end of fermentation, the relative abundances of *Aspergillus* were 73.98%, 97.83%, and 88.88%, respectively. *Aspergillus*, the dominant fungus genus of stacked fermentation, is a common high-abundance fungus genus in solid-state fermentation, which can produce a variety of enzymes such as protease and amylase, and has the functions of increasing flavor and regulating flora (Zhou J. et al., 2021; Zhu et al., 2022).

The above results showed that the dominant microorganisms of cigar stacking fermentation were *Staphylococcus*, *Corynebacterium 1*, *Aerococcus* and *Aspergillus*, which were similar to the results of the research results of Zheng L. L. et al. (2022), under the influence of the chemical composition of tobacco leaves, tobacco endophytes and fermentation environment, the relative abundance of dominant microorganisms of different varieties changes were different, which further affected the changes of microbial communities of different CTLs varieties.

3.2. Characteristic microorganisms from different varieties of CTLs

The results showed that the dominant genera and community structures of different samples affected the metabolism of microbial communities, whilst the characteristic microorganisms were significantly correlated with the characteristic VFCs. For example, *Staphylococcus* and *Bacillus*, were found to have been positively correlated with sclareolide and isophorone, respectively (Liu et al., 2021; Liu T. et al., 2022; Yan et al., 2022; Zheng T. F. et al., 2022), therefore the characteristic microorganisms of different CTL varieties may have had an important influence on the composition of the characteristic aroma. Linear discriminant analysis effect size (LEfSe) was used to identify the characteristic microorganisms from different varieties of CTLs (LDA score > 2), and the results of this are shown in Figure 3. Among these, DX4 CTLs had the largest number of characteristic bacterial genera, whilst BESUKI CTLs had the fewest characteristic bacterial genera. Additionally, CX14 CTLs showed the largest number of characteristic fungal genera, whilst N-Jalap HABANA CTLs had the fewest characteristic fungal genera.

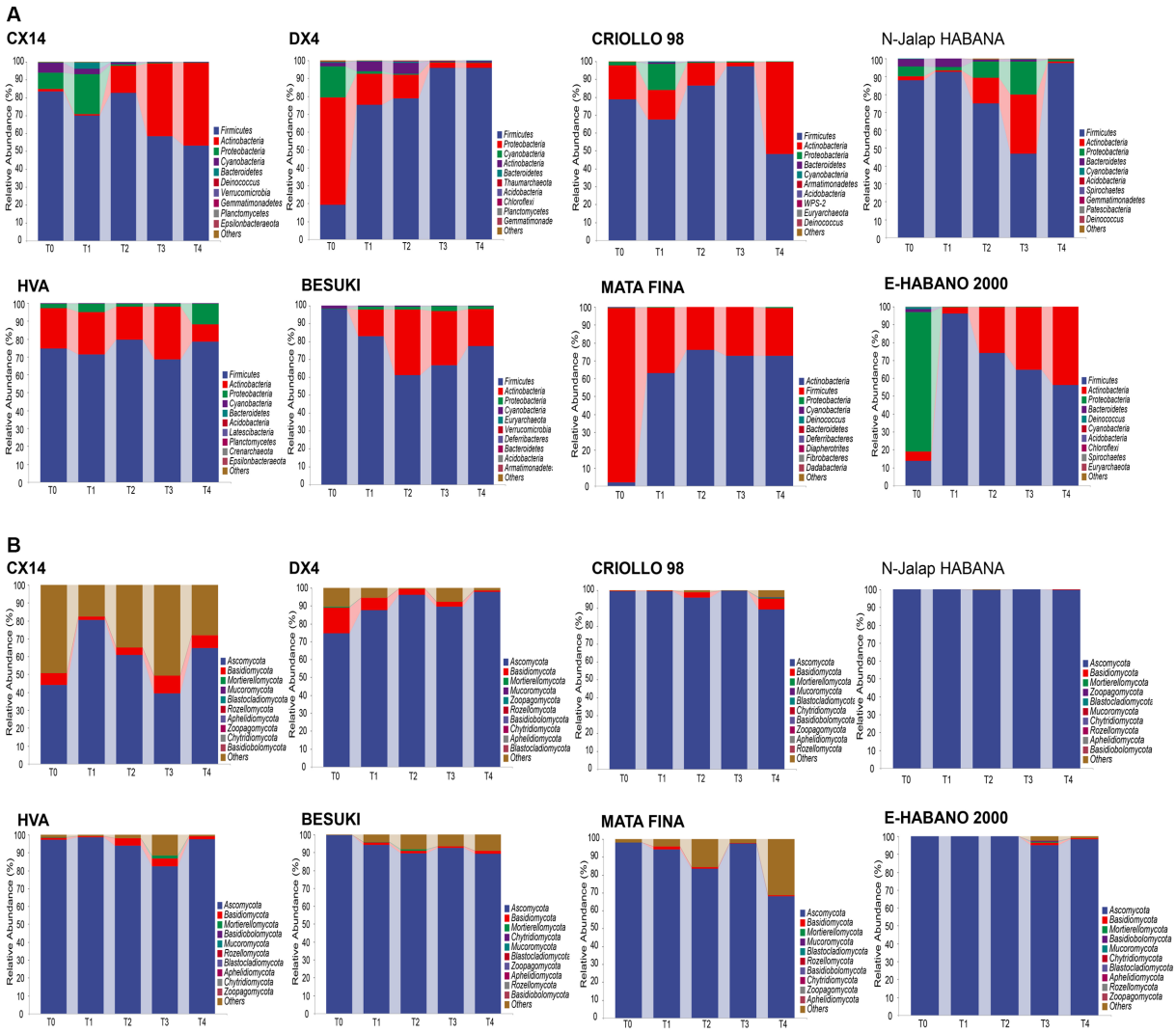

FIGURE 1
Microbial community changes of CTLs from different varieties at the phylum level. **(A)** Composition of bacterial community during stacking fermentation. **(B)** Composition of fungal community during stacking fermentation.

The different microbial genera of different varieties of tobacco leaves are as follows: CX14 CLTs *Sphingobium*, *Pseudomonas*, *Acinetobacter*, *Penicillium*, *Diaporthe*, etc.; *Halomonas*, *Trichosporon*, etc. in DX4 CTLs; *Falsirhodobacter*, *Septoria*, etc. in CRIOLLO 98 CTLs; N-Jalap HABANA CTLs of *Sphingobacterium*, *Solitalea*, *Thioalkalicoccus*, etc.; *Enteractinococcus*, *Lactobacillus*, *Jeotgalicoccus*, *Talaromyces*, etc. in HVA CTLs; *Carnimonas*, *Debaryomyces*, etc. in BESUKI CTLs; *Brachybacterium*, *Brevibacterium*, *Glutamicibacter*, *Yaniella*, etc. in MATA FINA; E-HABANO 2000 CTLs of *Ralstonia*, *Deinococcus*, *Sphingomonas*, etc. The different microorganisms of CTLs, such as *Pseudomonas*, *Sphingomonas*, *Acinetobacter*, *Lactobacillus*, *Trichothecium*, *Nigrospora*, were common genus in tobacco endophytes. Within strong salt tolerance, they could better adapt to the alkaline environment of CTLs, and had the effects of promoting growth, resistance to diseases and pests, and pollutant degradation. They had great economic value and application development potential (Li et al., 2019; Gushgari-Doyle et al., 2021; Gibot-Leclerc et al., 2022).

3.3. Interaction relationship between microbial community

Using Spearman to analyze the interaction relationship between CTL microbial communities, $\rho < 0.05$, $r > 0.6$ were defined as significant positive correlations, whilst $\rho < 0.05$, $r < -0.6$ were defined as significant negative correlations, with the results being shown in Figure 4. During stacking fermentation, among the dominant bacteria, *Staphylococcus* were negatively correlated with *Corynebacterium 1* and *Aerococcus*, whilst *Corynebacterium 1* and *Aerococcus* interacted positively. Dominant bacterial genera from different CTL varieties were significantly related to characteristic microorganisms, for example, *Corynebacterium 1* was positively correlated with *Facklamia*, *Jeotgalicoccus*, *Brachybacterium*, *Geomicrobium*, *Glutamicibacter*, *Yaniella*, *Salinicoccus*, *Solibacillus*, and *Natribacillus*. Additionally, *Aerococcus* was positively correlated with *Carnimonas*, *Facklamia*, *Yaniella*, whilst *Staphylococcus* was shown to have been positively correlated with *Halomonas* and *Solitalea*.

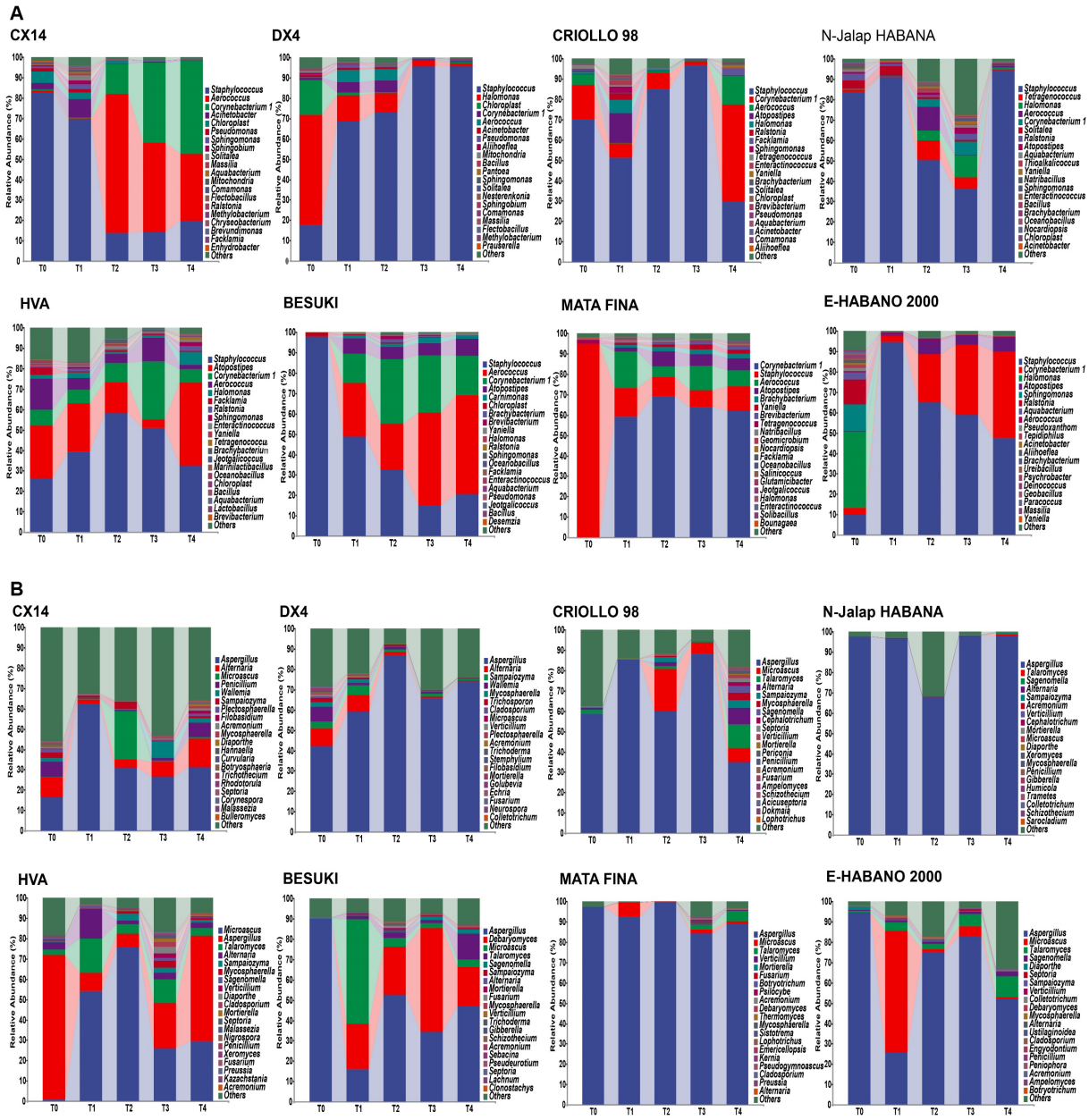

FIGURE 2
Microbial community changes of CTLs from different varieties at the genus level. **(A)** Composition of bacterial community during stacking fermentation. **(B)** Composition of fungal community during stacking fermentation.

During stacking fermentation, the dominant bacterial genera were found to have affected the growth of the corresponding characteristic bacterial genera, such as *Aerococcus* and *Corynebacterium 1*, which in turn increased in the pre-fermentation period. This promoted the proliferation of *Sphingobium*, *Aquabacterium*, *Halomonas*, etc., and decreased when *Staphylococcus* increased during the middle and late fermentation stages.

Aspergillus, the dominant fungi of CTLs, was negatively correlated with characteristic fungal genera, including *Alternaria*, *Curvularia*, *Fusarium*, *Gibberella*, *Mycosphaerella*, *Plectosphaerella*, *Pseudeurotium*, *Penicillium*, *Sampaiozyma*, *Septoria*, and *Trichothecium*. During stacking fermentation, *Aspergillus* inhibits the growth of characteristic fungi, such as *Alternaria*, *Trichosporon*, and *Mycosphaerella* which was opposite to the change observed in *Aspergillus*. The abundance of *Aspergillus* was found to have been higher during the early stage of fermentation, whilst the characteristic fungus was then inhibited from growing and increased during the middle and late stages of fermentation with a decrease in *Aspergillus*. The above results showed that the abundance and interactions of dominant microbes during stacking fermentation affected the changes in characteristic microorganisms in the microbial community and subsequently influenced the microbiota structure of CTLs during the stacking fermentation process.

Characteristic bacteria such as *Aquabacterium*, *Brachybacterium*, *Tetragenococcus*, *Ralstonia*, *Enteractinococcus*, *Jeotgalicoccus*, *Lactobacillus*, *Bacillus*, and *Yaniella* all interacted in a positive

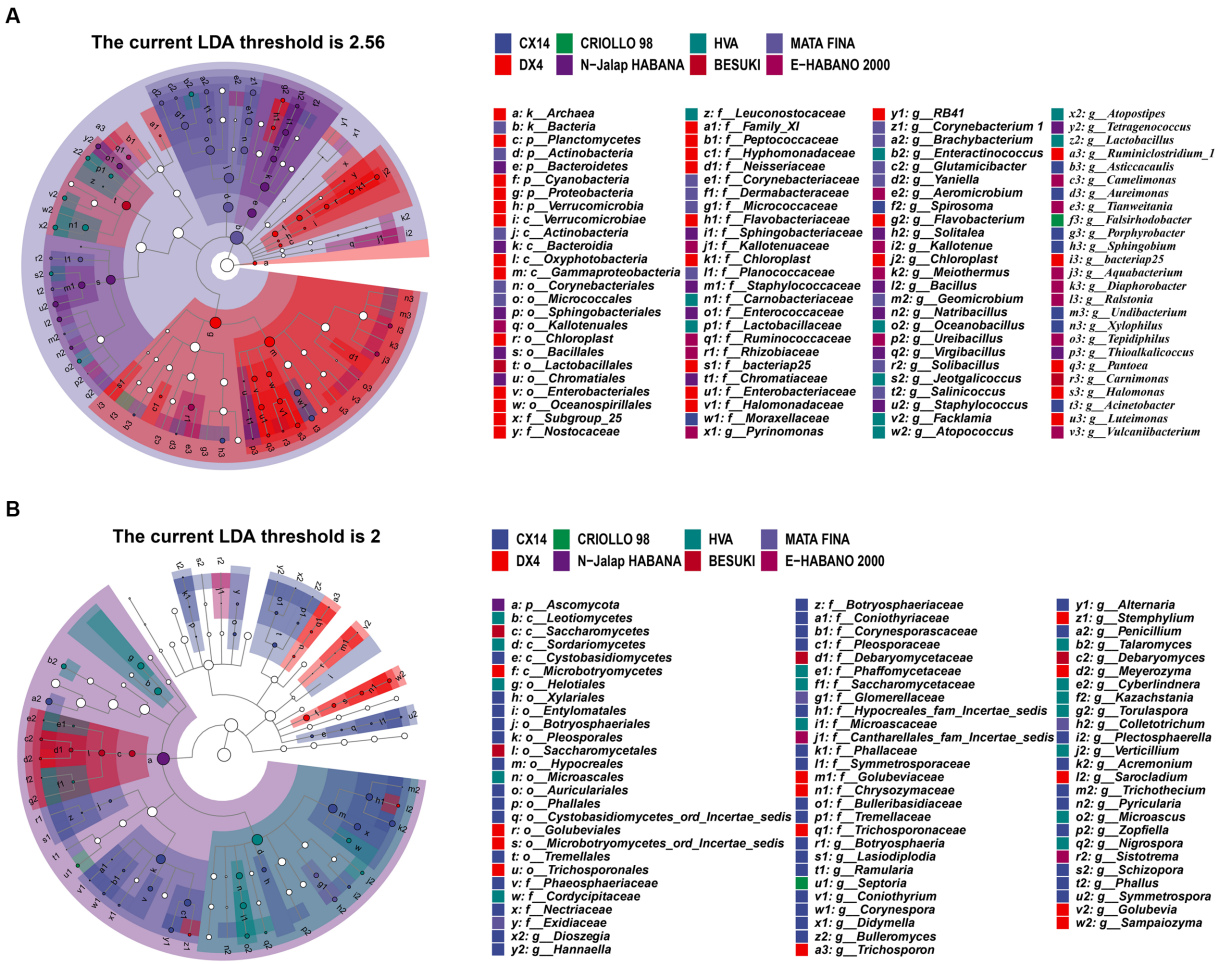

FIGURE 3
Characteristic microorganisms from different varieties of CTLs. **(A)** Characteristic bacterium. **(B)** Characteristic fungus.

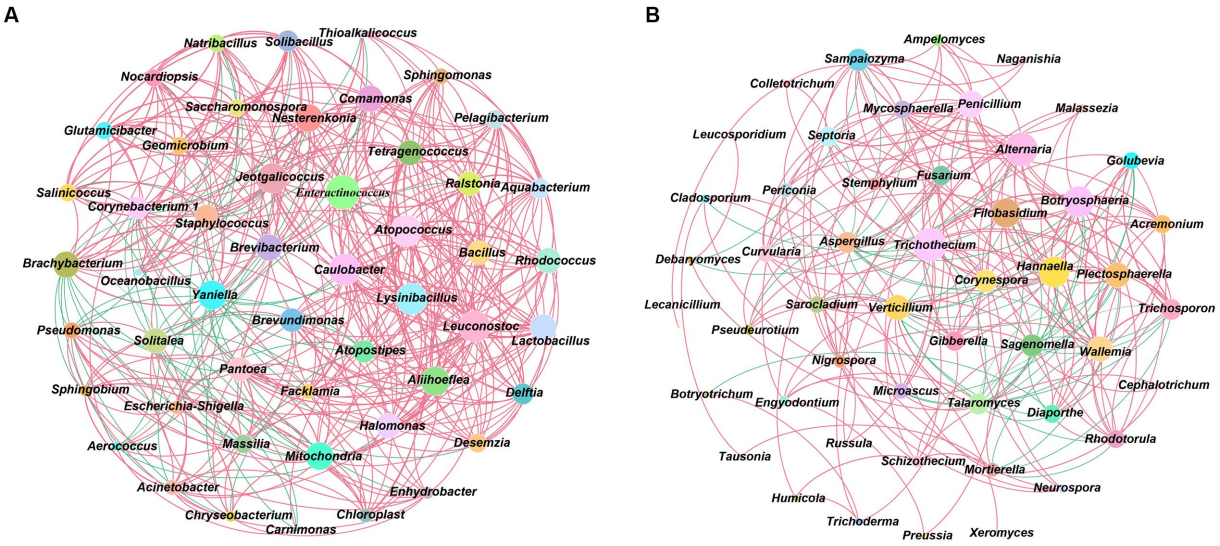

FIGURE 4
Microbial community interaction networks. **(A)** Interaction network between CTLs bacterial communities. **(B)** Interaction network between CTLs fungal communities. Red represents positive correlation and blue represents negative correlation.

direction, whilst characteristic fungi such as *Sampaiozyma*, *Wallemia*, *Penicillium*, *Trichosporon*, *Nigrospora*, *Septoria*, and *Plectosphaerella* also all interacted in a positive direction. The interactions between characteristic microorganisms had an important impact on the evolution of microbiota structure during stacking fermentation (Wang et al., 2019). Zhou J. et al. (2021) defined *Mycosphaerella*, *Plectosphaerella*, *Bacillus*, *Methylobacterium* and *Fusarium* as symbiotic flora microorganisms that have a greater impact on the structure and function of microbial flora. Wang et al. (2019) defined *Lactobacillus, Saccharomyces*, *Pichia*, *Geotrichum* and *Candida* with aroma production ability as the symbiotic microorganisms of simulated artificial fermentation flora in the liquor mixing system. This were similar to the natural fermentation system in the direction of succession in the artificially constructed fermentation system. Therefore, the abundance and interaction between microorganisms of the dominant genus during stacking fermentation affect the changes of characteristic microorganisms in the community, and as a symbiotic group in the stacking fermentation microbial community, the characteristic microorganisms can affect the succession of tobacco leaf flora structure during stacking fermentation.

3.4. Changes of VFCs in the stacking fermentation

The results showed that the types of VFCs increased during stacking fermentation with the VFCs detected including 2-undecanone, 6,10-dimethyl-, geranyl acetone, β-ionone, phytone, farnesyl acetone, ethanone, 1-(3-pyridinyl)-, myosmine, and nicotyrine (Liu F. F. et al., 2022). However, the transformation mechanism of stacking fermentation on CTL aroma and sensory quality remained unclear. The results are shown in Figure 5, with the VFCs at the end of fermentation for different CTLs and the VFCs of CTLs between raw materials and the end of fermentation being found to have been quite different. During stacking fermentation, carotenoid degradation products, Maillard reaction products, and nicotinic degradation products increased, which was consistent with the past results found by Liu's study, indicating that these three types of substances were the key VFCs for the quality improvement of CTLs (Liu F. F. et al., 2022).

In addition to β-ionone, dihydroactinidiolide, 6-methyl-3,5-heptadien-2-one, geranyl acetone, and isophorone, the carotenoid degradation products in different varieties of CTLs also contained β-ionone-5,6-epoxide, menthol, and α-terpineol, among other aroma intermediates. Carotenoid degradation products are important components of tobacco aroma, the threshold is low, and their type and content both have an important impact on improving tobacco aroma and mellowness (Popova et al., 2019; Liang et al., 2021). For example, β-ionone is an aroma ingredient of CTLs flowery and woody aroma; dihydroactinidiolide has a sweet and woody aroma, which could mellow the smoke gas; 6-methyl-3,5-heptadien-2-one and geranyl acetone have a rosy, leafy, and fruity aroma (Liang et al., 2021). The content of Maillard reaction products, such as pyrazine, 2,5-dimethyl-, pyrazine, 2,6-dimethyl-, pyrazine, tetramethyl-, furfural, 2-propanone, 1-hydroxy-, ethanone, 1-(3-pyridinyl)-, and indole, all increased during stacking fermentation. Maillard reaction products are mainly generated by carbohydrates and amino acids via the production of dicarbonyl compounds and amino ketones, primarily creating nutty, roasted, chocolate, and other aromas for CTLs (Mortzfeld et al., 2020). Nicotine, the most abundant and important alkaloid in CTLs, is irritating and bitter when its content is too high, whilst there is insufficient smoke and a bland flavor when its content is too low. CTLs can reduce irritation and increase mellowness through degrading nicotine to produce myosmine and nicotine during stacking fermentation (Lin et al., 2016). Yao et al. (2022) inoculated *Saccharomycopsis fibuligera* and *Hanseniaspora uvarum* in cigar core tobacco leaves, the content of nicotine in fermented CTLs was significantly reduced, the content of carotenoid degradation products such as phytone, farnesyl acetone and limonene increased, and the Maillard reaction products such as 3-acetylpyridine, pyridine and furan increased, and the aroma of CTLs has been improved to varying degrees. Therefore, in stacking fermentation, the transformation of carotenoid degradation and Maillard reaction products alongside the degradation of nicotine played a role in mellowing smoke gas and enhancing the aroma of CTLs (see Figure 6).

3.5. Characteristic VFCs from different CTLs varieties

The characteristic VFCs of different CTL varieties were analyzed using partial least squares discriminant analysis (PLS-DA), and the results are shown in Figure 7. The model Q^2 value was 0.809, indicating a better model prediction effect. VFCs with a VIP value greater than 1 were defined as characteristic VFCs, which contributed more to the difference between samples. The characteristic VFCs at the end of the fermentation of CTL varieties are shown in Table 1.

3.6. Correlation analysis between microorganisms-VFCs and VFCs-aroma

The interaction between CTLs microorganisms and VFCs during stacking fermentation was analyzed by Spearman correlation analysis, with $\rho < 0.05$ and $r > 0.6$ being defined as significant positive correlations, and $\rho < 0.05$ and $r < -0.6$ being defined as significant negative correlations, as shown in Figure 8. The correlation between bacteria and VFCs during stacking fermentation was more complex than that between fungi and VFCs whilst also being mainly positively correlated. Characteristic VFCs of different varieties of CTLs were also positively correlated with characteristic microorganisms, such as in CX14 CTLs, *Penicillium* was positively correlated with dimethyl phthalate, whilst *Diaporthe*, *Trichothecium* were positively correlated with ethanone, 1-(2-pyridinyl)-, tigelic acid, megastigmatrienone 4, and bourgeonal. This provides CTLs with a fresh flowery and light sweet aroma. In DX4 CTLs, *Chloroplast* was positively correlated with ethanone, 1-(1-cyclohexen-1-yl)-, and *Trichosporon* was positively correlated with pyrazine, tetramethyl-, which could increase the roasted and burnt sweet aroma of CTLs. Among N-Jalap HABANA CTLs, *Thioalkalicoccus* was positively correlated with geranyl Acetone and α-curcumene, and *Solitalea* was positively correlated with acetic acid, which could increase the light sweet and flowery aroma of CTLs. Furthermore, *Enteractinococcus*, *Jeotgalicoccus*, and *Talaromyces* in the HVA CTLs were positively correlated with 2-undecanone, 6,10-dimethyl-, and *Lactobacillus*, whilst *Atopococcus* was positively correlated with butanoic acid and isophorone, which could increase

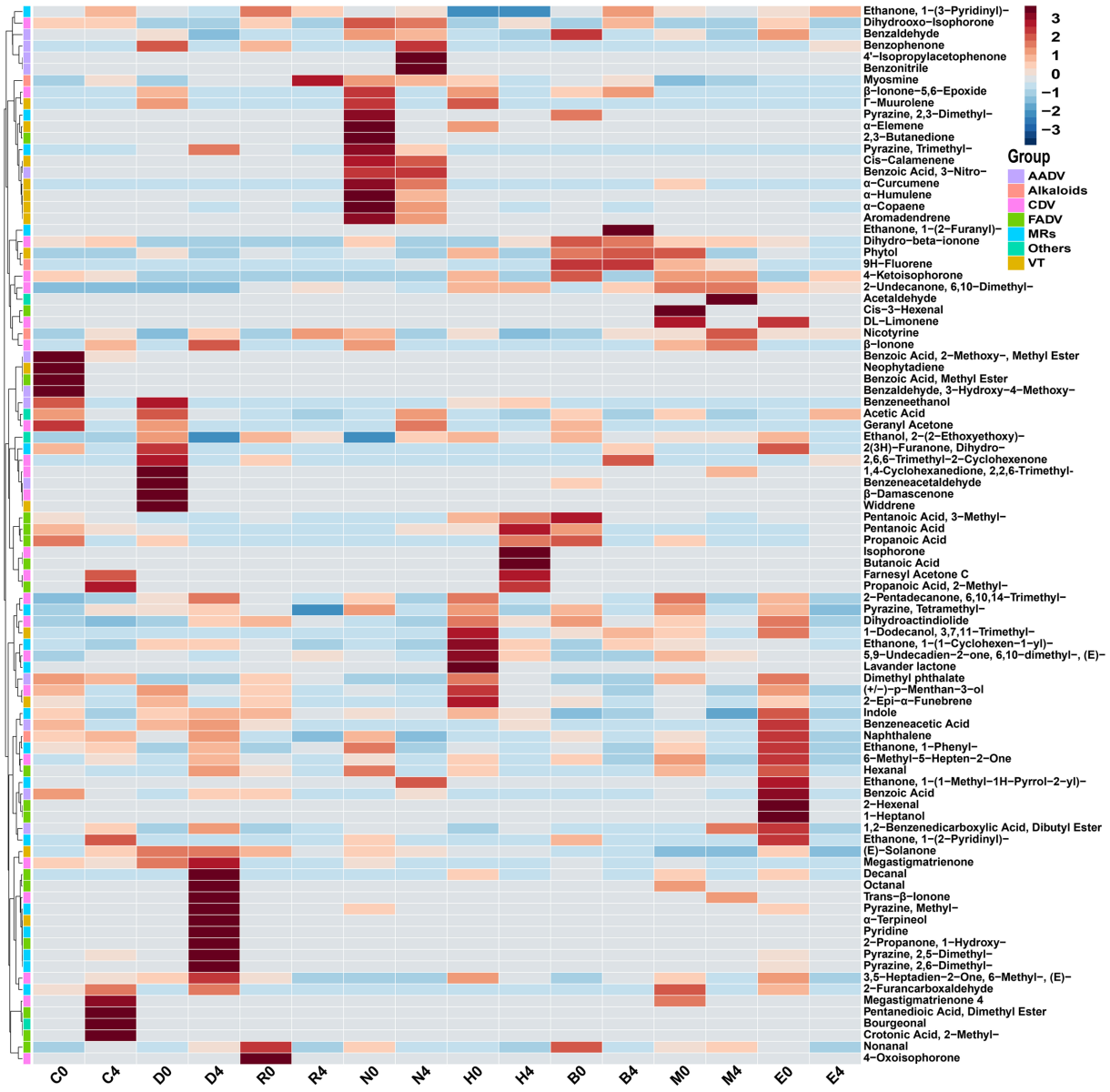

FIGURE 5
Cluster analysis of VFCs from different varieties of CTLs between raw materials and the end of fermentation. AADV, aromatic amino acid degradation products; Alkaloids, nicotinic degradation products; CDV, carotenoid degradation products; FADV, fatty acid degradation products; MRs, Maillard reaction products; VT, terpenoids; Others, Other VFCs.

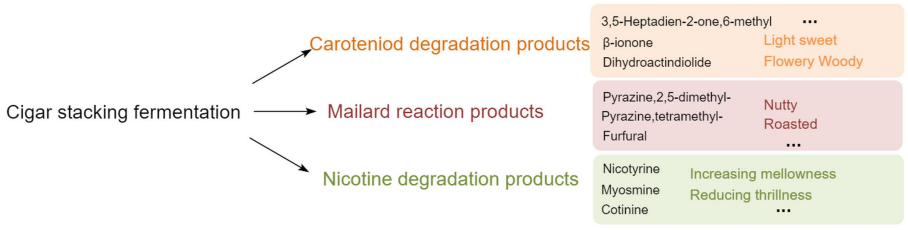

FIGURE 6
Effects from VFCs of stacked fermented CTLs on the aroma and quality.

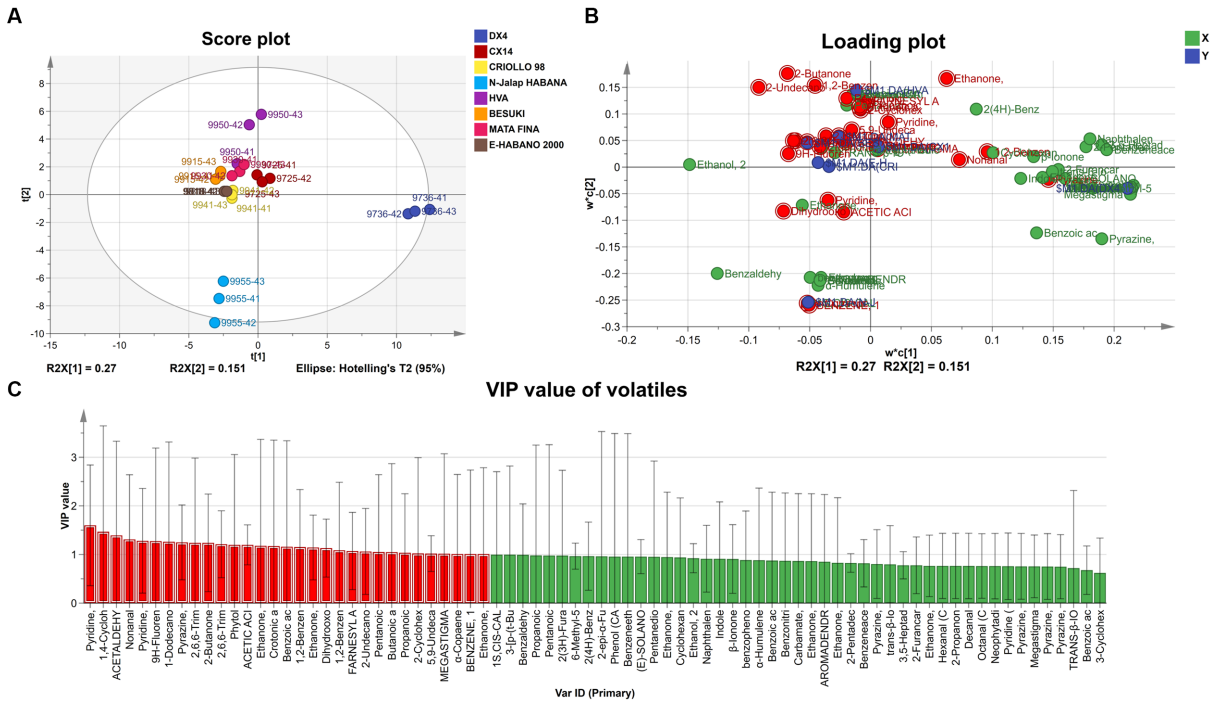

FIGURE 7
PLS-DA Analysis of VFCs at the end of fermentation. **(A)** PLS-DA score plot of VFCs. **(B)** Loading plot of VFCs. **(C)** VIP value of VFCs.

TABLE 1 Characteristic VFCs of different varieties of CTLs.

Varieties	Characteristic VFCs
CX14	Ethanone, 1-(2-pyridinyl)-, Tiglic acid, Benzoic acid, 2-methoxy-, methyl ester, Dimethyl phthalate, Megastigmatrienone 4, Bourgeonal
DX4	Nonanal, Pyrazine, tetramethyl-, Ethanone, 1-(1-Cyclohexen-1-yl)-
CRIOLLO 98	Myosmine
N-Jalap HABANA	Acetic Acid, Geranyl Acetone, α-Curcumene
HVA	Farnesyl acetone C, 2-Undecanone, 6,10-dimethyl-, Pentanoic acid, 3-methyl-, Butanoic acid, Propanoic acid, 2-methyl-, Isophorone, α-Copaene
BESUKI	9H-Fluorene, 1-Dodecanol, 3,7,11-Trimethyl-, 2,6,6-Trimethyl-2-Cyclohexenone, Dihydro-beta-ionone, Phytol, Dihydrooxo-isophorone, Ethanone, 1-(2-furanyl)-
MATA FINA	1,4-Cyclohexanedione, 2,2,6-Trimethyl-, Acetaldehyde, Nicotyrine, 1,2-Benzenedicarboxylic acid, dibutyl ester, 4-Oxoisophorone

the fruity and mint aroma. *Carnimonas* and *Debaryomyces* in BESUKI CTLs were also positively correlated with 1-dodecanol, 3,7,11-trimethyl- and dihydro-beta-ionone, which could increase the flowery and hay aroma. Additionally, *Yaniella* in MATA FINA CTLs was positively correlated with nicotine, when *Salinicoccus*, *Nocardiopsis*, *Natribacillus*, *Solibacillus*, *Geomicrobium*, *Salinicoccus* and *Glutamicibacter* were positively correlated with 4-oxoisophorone and 1,4-cyclohexanedione, 2,2,6-trimethyl-, which could increase the sweet, hay, nutty, and woody aroma.

Radar charts were used to identify the aroma of different CTL varieties at the end of stacking fermentation. The results are shown in Figure 9A, where the woody aroma was found to be the most prominent among the different CTL varieties, with each sample being supplemented with another aroma. Burned sweet aroma was the most prominent aroma in CX14 CTLs, supplemented by hay and roasting aromas. DX4 CTLs had the highest scores in woody and mellow sweet, supplemented by resin aroma and nutty flavor. CRIOLLO 98 had the highest scores in woody, supplemented by mellow sweet, resin and bean aroma. N-Jalap HABANA with mainly woody aroma, supplemented by baking and honey sweetness. HVA had the highest scores in woody, supplemented by roasting, coffee and nutty aroma; BESUKI had the highest scores in woody and hay, supplemented by honey sweet and burned sweet aroma; MATA FINA had the highest scores in woody aroma, supplemented by honey, roasted and nutty aroma. E-HABANO 2000 had the highest scores in woody and honeysweet, complemented by roasted, resin and nutty aromas.

Using Spearman correlation analysis, the interaction network between volatile aroma was established; $\rho < 0.05$ and $r > 0.6$ were defined as significant positive correlations, whilst $\rho < 0.05$ and $r < -0.6$ were defined as significant negative correlations, with the results being shown in Figure 9B. The characteristic VFCs of CTLs showed a stronger positive correlation with many aromas, such as bean aroma, which was positively related to nonanal, decanal, pyrazine, 2,5-dimethyl-, furfural, pyrazine, 2,6-dimethyl-, and α-terpineol. Meanwhile, the coffee aroma was positively correlated with farnesyl acetone C and propanoic acid, 2-methyl-, whilst the flowery aroma was positively correlated with geranyl acetone, benzophenone, benzonitrile, and α-curcumene. Hay aroma was positively correlated with dihydro-beta-ionone; Honey sweet aroma was positively correlated with benzophenone; Mellow sweet aroma was positively

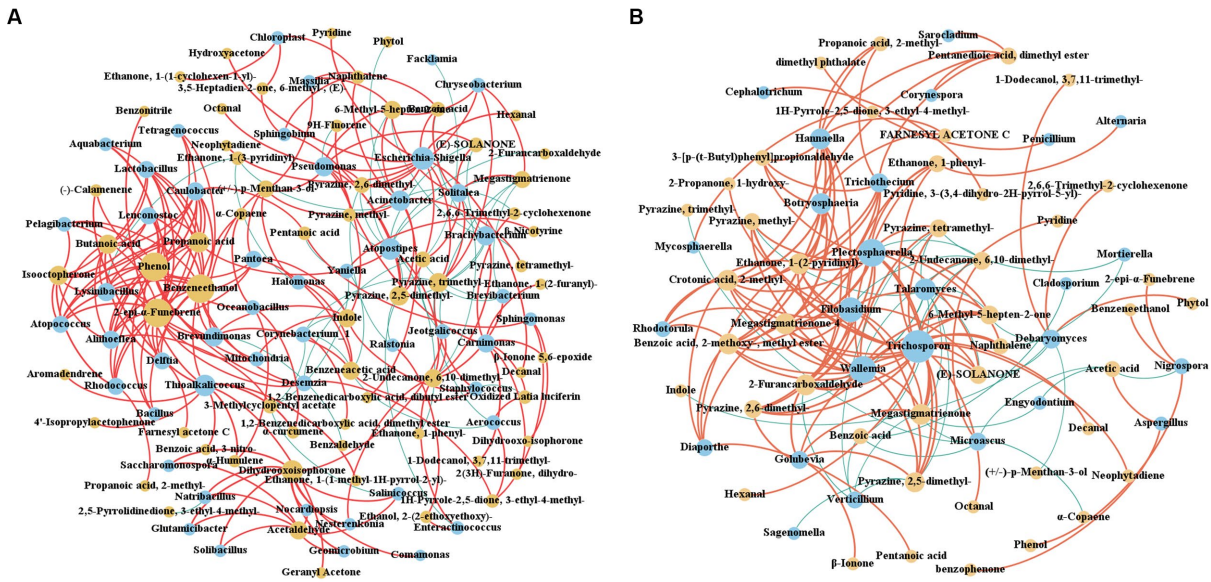

FIGURE 8
Interaction network between CTLs microorganisms and VFCs. **(A)** Interaction network between bacteria and VFCs. **(B)** Interaction network between fungi and VFCs. Red represents positive correlation and blue represents negative correlation.

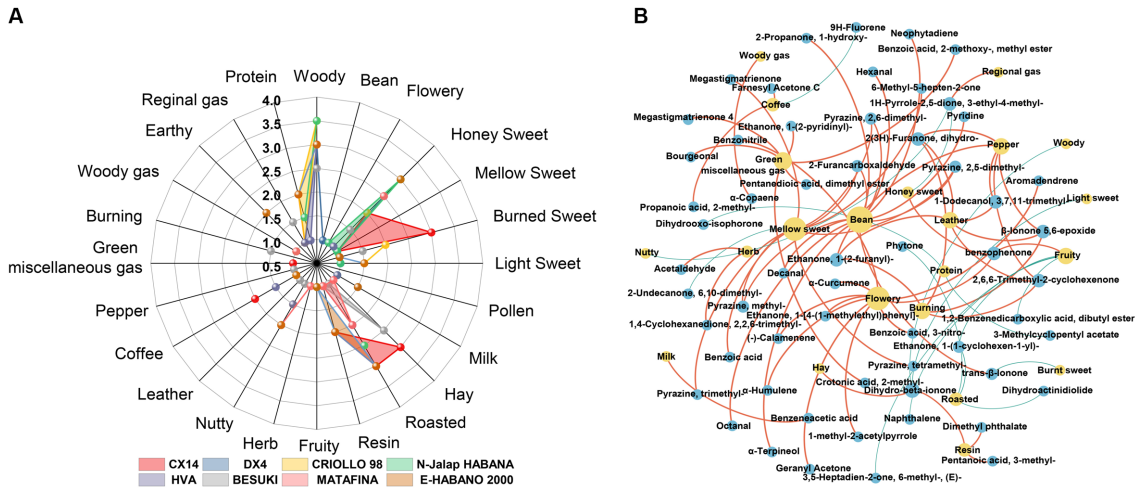

FIGURE 9
Aroma composition of CTLs and interaction network between VFCs and aroma. **(A)** Radar chart of the aroma from different varieties of CTLs. **(B)** Interaction network between the VFCs and aroma. Red represents positive correlation and blue represents negative correlation.

correlated with megastigmatrienone, pyrazine, tetramethyl-, and furfural; Pepper aroma was positively correlated with 1-dodecanol, 3,7,11-trimethyl-, 2,6,6-trimethyl-2-cyclohexenone and ethanone, 1-(2-furanyl)-. The resin aroma was positively correlated with dimethyl phthalate and dihydro-beta-ionone.

The characteristic VFCs of different CTLs varieties were positively correlated with the aroma of CTLs, such as the characteristic VFCs of DX4 CTLs, including nonanal and pyrazine, tetramethyl, which corresponded to sweet, roasted, and nutty honey aromas, respectively. In N-Jalap HABANA CTLs, geranyl acetone and α-curcumene were positively correlated with the honey sweet aroma. The characteristic VFCs farnesyl acetone C and propanoic acid, 2-methyl-, were positively correlated with coffee and roasted aroma in HVA CTLs. In BESUKI CTLs, dihydro-beta-ionone was positively correlated with hay aroma; In MATA FINA CTLs, acetaldehyde and 4-oxoisophorone were positively correlated with honey sweet and roasted aroma, respectively. In CX14 CTLs, characteristic VFCs were not strongly correlated with the aroma, but the VFCs with a pleasant aroma, such as the characteristic VFCs ethanone, 1-(2-pyridinyl)-, tigelic acid, benzoic acid, 2-methoxy-, methyl ester, and bourgeonal, corresponded to the burn sweet, roasted and hay aromas, respectively. Furthermore, the higher contents of ethanone, 1-(3-pyridinyl)-and pentanoic acid, 3-methyl- in E-HABANO 2000 CTLs were positively correlated with featured

roasted and resin aromas, respectively. The above results showed that the characteristic VFCs of CTLs had an important contribution to the aroma.

4. Discussion

CTLs that go through stacking fermentation could better improve their aroma quality, aroma amount, and mellowness, whilst decreasing irritation; therefore, it is important to study the influence of the microbial community on aroma changes and the mechanism of this. In this study, through the analysis of the microbial community composition and VFCs of CTLs, the interaction network between microorganisms, volatile and aroma was established, as shown in Figure 10. This which provides theoretical support for the analysis of the mechanism of microorganisms and the changes in VFCs during stacking fermentation as well as the development of cigar stacking fermentation microbial agents.

The results of amplicon sequencing showed that the microbial community structures of different CTLs varieties were quite different during stacking fermentation, whilst the dominant microbial structures remained similar. The dominant bacterial genera here were *Staphylococcus*, *Corynebacterium 1*, *Aerococcus*, and *Aspergillus*, which was similar to the results found previously by Zheng L. L. et al. (2022) and Zheng et al. (2022a). The results of microbial interaction analysis showed that during stacking fermentation, the dominant microorganisms were strongly related to the CTLs characteristic microorganisms, whilst the abundance of characteristic microorganisms was affected by the correlation and abundance of dominant microorganisms. These characteristic microorganisms also showed a positive correlation with the aroma and could be defined as functional microorganisms in the stacking fermentation process. The characteristic bacterial genera *Jeotgalicoccus*, *Geomicrobium*, *Glutamicibacter*, and *Yaniella* were positively correlated with *Corynebacterium 1*. Furthermore, *Carnimonas* and *Yaniella* positively correlated with *Aerococcus*. Among them, *Carnimonas* was positively correlated with Dihydro-beta-ionone; *Yaniella* was positively correlated with nicotine, and *Geomicrobium*, *Salinicoccus*, *Glutamicibacter* were positively correlated with VFCs such as 1,4-cyclohexanedione, 2,2,6-trimethyl-, and 4-oxoisophorone. Among CTLs, *Jeotgalicoccus*, *Glutamicibacter*, *Yaniella*, and other characteristic microorganisms have good salt tolerance, can better adapt to the alkaline environment of CTLs, and have functions including enzyme production and volatile transformation (Mokashe et al., 2015; Xiong et al., 2019; Zhang et al., 2022). Therefore, during the stacking fermentation process, CTL-dominant microorganisms may have affected the succession changes of functional microorganisms and microbial communities through microbial interactions, thereby affecting the transformation and aroma composition of CTLs (Zhou J. et al., 2021; Zheng et al., 2022a). Jia et al. (2023) found that *Candida* only accounted for 0.42% of the total microbial abundance of the community, but it was used as a symbiotic flora in the microbial community and characteristic microorganisms in the late fermentation period. Not only did it degrade nitrogenous compounds, but it also improved cigar flavor and shortened fermentation period.

Stacking fermentation is an important part of mellowing smoke gas and improving tobacco permeability and aroma, with different varieties of CTLs having their own aroma and characteristic VFCs. The analysis of VFCs between raw materials and the end of fermentation showed that VFCs were mainly increased during stacking fermentation, including Maillard reaction products such as nonanal, pyrazine, tetramethyl-, furfural and ethanone, 1-(3-pyridinyl)-. This is in addition to, carotenoid degradation products such as dihydroactinidiolide, geranyl acetone, 2-pentadecanone, 6,10,14-trimethyl-, 2-undecanone, 6,10-dimethyl-, 1,4-cyclohexanedione, 2,2,6-trimethyl-, 6-methyl-3,5-heptandien-2-one, 2,6,6-trimethyl-2-cyclohexenone, as well as nicotine degradation products such as nicotyrine and myosmine. The above results showed the transformation of small molecular substances such as carotenoid degradation, Maillard reaction, and nicotine degradation during the stacking fermentation process, causing the reduction of miscellaneous gas and the improvement of quality and aroma of CTLs (Lin et al., 2016; Popova et al., 2019; Yu H. et al., 2021). Carotenoid degradation products are considered to be an important source of flavor in CTLs, among which geranyl acetone and dihydroactinidiolide have a woody aroma, green flavor, and can mellow, and flue smoke gas whilst increasing the smoke concentration. 2,6,6-trimethyl-2-cyclohexenone, 2-undecanone, 6,10-dimethyl- and 1,4-cyclohexanedione, 2,2,6-trimethyl- usually have sweet and violet flavors (Gupta, 2015). Maillard reaction products are important aromagenic components of CTLs that burn with a sweet and roasted

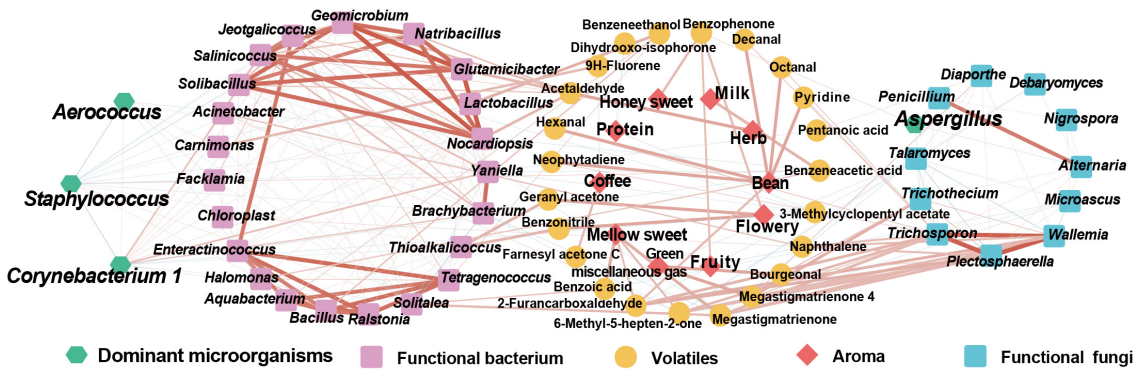

FIGURE 10
Interaction network among microorganism-VFCs-aroma. Red represents positive correlation, blue represents negative correlation.

aroma, and studies have shown that microorganisms can synthesize aminoketones, which are the precursors of pyrazines, and further convert them into pyrazines (Zhang et al., 2019; Mortzfeld et al., 2020; Yu H. et al., 2021). pyrazine, tetramethyl-, pyrazine, 2,5-dimethyl-, furfural and pyrazine, 2,6-dimethyl- have a roasted aroma and burn sweet aroma, commonly used as CTLs flavor additives (Gupta, 2015). Compared with other tobacco products, nicotine had relatively low content in CTLs. Due to the dependence, nicotine exposure and subsequently risk of lung, laryngeal and oral cancers, the degradation of nicotine during cigar stacking fermentation helped to reduce the risk (Henningfield et al., 1996; Baker et al., 2000). This may be useful to control the nicotine content and for potential research and regulatory efforts as well as appropriate product for consumers (Koszowski et al., 2018).

Through the correlation analysis of aroma components and tobacco leaf aroma characteristics, it was found that the characteristic aroma components of different varieties of CTLs corresponded to the prominent aroma of tobacco leaves. For example, *Diaporthe* and *Trichothecium* in CX14 CTLs are positively correlated with ethanone, 1-(2-pyridinyl)-, tigelic acid, megastigmatrienone 4, and bourgeonal, which can enhance the flowery and light sweet aroma of CTLs. In DX4 CTLs, *Chloroplast* were positively correlated with ethanone, 1-(1-cyclohexen-1-yl)-, whilst *Trichosporon* was positively correlated with pyrazine, tetramethyl-, which was positively correlated with a roasted and burnt sweet aroma. The above results showed that the characteristic microorganisms from different varieties of CTLs during stacking fermentation might be functional microorganisms enhancing the characteristic aroma of CTLs, which had an important impact on aroma transformation and microbial community interactions. These results have theoretical significance for screening functional strains and producing cigar products with characteristic aromas.

5. Conclusion

In this study, multi-omics technology was used to analyze different CTL varieties in the stacking fermentation process. The commonality and difference changes in microbial communities and VFCs during stacking fermentation were investigated, with the correlation between microorganisms and VFCs was revealed. This provides technical support for the optimization of the stacking fermentation process whilst improving the quality of cigars. This study found that the dominant microorganisms in the process of cigar stacking fermentation were *Staphylococcus*, *Corynebacterium 1*, *Aerococcus*, and *Aspergillus*, which mainly affected the composition and community succession of stacking fermentation through microbial interactions, thereby influencing the aroma composition and aroma of CTLs. Characteristic microorganisms such as *Jeotgalicoccus*, *Geomicrobium*, *Glutamicibacter*, and *Yaniella* had strong correlation with dominant microorganisms. Meanwhile, characteristic microorganisms were probably the functional microorganisms with commensal and aroma-producing ability during stacking fermentation. Based on the analysis of VFCs between raw materials and the end of fermentation for different varieties of CTLs, it was found that Maillard reaction products, carotenoid degradation products, and nicotine degradation products were used as the key aroma components for sensory quality improvement and aroma enhancement during the stacking fermentation process. This provides a basis for improving the quality of cigars and the controllability of cigar proportioning in industrial production. In this study, the interaction network analysis of characteristic microorganisms and VFCs was used for the screening of functional microorganisms and the development and application of microbial agents to improve the aroma quality and characteristic aromatic cigar production.

Data availability statement

The data presented in the study are deposited in the NCBI Sequence Read Archive database, accession number PRJNA937179.

Author contributions

QW and ZP: conceptualization, data curation, formal analysis, methodology, software, and writing—original drafting. QW, ZP, and LnL: investigation, methodology, and resources. JZ, YP, JW, and LpL: methodology, resources, and project administration. JZ, YP, and JW: funding acquisition, supervision, and writing—reviewing and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

YP, LpL, LnL, and JW were employed by China Tobacco Hubei Industrial Co., Ltd, Wuhan, China.

The authors declare that this study received funding from China Tobacco Hubei Industrial Co., Ltd. The funder had the following involvement in the study: investigation, resources, project administration and funding acquisition.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1168122/full#supplementary-material

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Comparative analyses of the bacterial communities present in the spontaneously fermented milk products of Northeast India and West Africa

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Introduction: Spontaneous fermentation of raw cow milk without backslopping is in practice worldwide as part of the traditional food culture, including "*Doi*" preparation in earthen pots in Northeast India, "*Kindouri*" of Niger and "*Fanire*" of Benin prepared in calabash vessels in West Africa. Very few reports are available about the differences in bacterial communities that evolved during the spontaneous mesophilic fermentation of cow milk in diverse geographical regions.

Methods: In this study, we used high throughput amplicon sequencing of bacterial 16S rRNA gene to investigate 44 samples of naturally fermented homemade milk products and compared the bacterial community structure of these foods, which are widely consumed in Northeast India and Western Africa.

Results and discussion: The spontaneous milk fermentation shared the lactic acid bacteria, mainly belonging to *Lactobacillaceae (Lactobacillus)* and *Streptococcaceae (Lactococcus)* in these two geographically isolated regions. Indian samples showed a high bacterial diversity with the predominance of *Acetobacteraceae (Gluconobacter and Acetobacter)* and *Leuconostoc*, whereas *Staphylococcaceae (Macrococcus)* was abundant in the West African samples. However, the *Wagashi* cheese of Benin, prepared by curdling the milk with proteolytic leaf extract of *Calotrophis procera* followed by natural fermentation, contained *Streptococcaceae (Streptococcus* spp.) as the dominant bacteria. Our analysis also detected several potential pathogens, like *Streptococcus infantarius* an emerging infectious foodborne pathogen in *Wagashi* samples, an uncultured bacterium of *Enterobacteriaceae* in *Kindouri* and *Fanire* samples, and *Clostridium* spp. in the *Doi* samples of Northeast India. These findings will allow us to develop strategies to address the safety issues related to spontaneous milk fermentation and implement technological interventions for controlled milk fermentation by designing starter culture consortiums for the sustainable production of uniform quality products with desirable functional and organoleptic properties.

KEYWORDS

spontaneously fermented milk products, MiSeq amplicon sequencing, *Lactobacillus, Lactococcus, Gluconobacter, Acetobacter, Macrococcus caseolyticus, Streptococcus infantarius*

1. Introduction

Fermented milk products are an essential component of the traditional food cultures of different ethnic communities worldwide. These fermented milk products are prepared from the raw or boiled milk of cow, buffalo, yak, camel, goat, and sheep through backslopping or spontaneous fermentation. *Kefir* of Russia, *Koumiss* and *Tarang* of Mongolia and China, *Dahi* of India, *Suero Costeno* of Colombia, and *Lait caillé* of sub-Saharan countries are a few examples of well-known traditional fermented milk products (de Melo Pereira et al., 2022). *Dahi* is an analog of *yogurt*, a semi-solid ready-to-drink Indian food generally prepared from boiled milk by backslopping a part of the previous batch of successful fermentation as a starter (Dewan and Tamang, 2007; Rai et al., 2016; Mudgal and Prajapati, 2017; Mallappa et al., 2021). Unlike thermophilic *yogurt* making, *Dahi* is usually incubated at room temperature (15–30°C) for 1–3 days in mesophilic fermentation (Mudgal and Prajapati, 2017; Mallappa et al., 2021; de Melo Pereira et al., 2022). The Mongoloid ethnic communities in Northeast India mostly prefer the spontaneous fermentation of fresh cow milk in earthen wares without backslopping (Joishy et al., 2019). The local vernacular names of these products are *Doi*, *Dei*, *Dahi*, and *Mishti-dahi* in Assam, Tripura, and Meghalaya, or *Sangom afamba* in Manipur (Joishy et al., 2019; Barooah et al., 2020; Wahengbam et al., 2020; Mallappa et al., 2021). Similarly, Fulani communities in Western African countries practice spontaneous fermentation of unpasteurised raw milk in calabash vessels without backslopping, such as *Kindirmou* in Niger and *Fanire* in Benin (Agyei et al., 2020; Fagbemigun et al., 2021). Backslopping naturally selects well-adapted microbes, resulting in consistent-quality products, whereas spontaneous fermentation results in highly heterogeneous products. Moreover, the microbes present in raw milk of animal origin also influence fermentation, often resulting in poor-quality products (Sun and D'Amico, 2021).

Recent next-generation sequencing (NGS)-based cultivation-independent studies showed a ubiquitous presence of beneficial bacteria, mainly *Lactobacillaceae* (*Lactobacillus delbrueckii*, *Lactobacillus kefiranofaciens*, *Lactobacillus helveticus*, and *Leuconostoc mesenteroides*) and *Streptococcaceae* (*Streptococcus thermophilus* and *Lactococcus lactis*) in most of the naturally fermented milk products worldwide (Jayashree et al., 2013; Oki et al., 2014; Bokulich et al., 2015; Motato et al., 2017; Shangpliang et al., 2018; Mallappa et al., 2021; de Melo Pereira et al., 2022). The NGS-based studies also highlighted the dominance of acetic acid bacteria, *Acetobacteraceae* (mainly *Gluconobacter* and *Acetobacter*), in several spontaneous fermented milk products (Shangpliang et al., 2018; de Melo Pereira et al., 2022). Among yeast, *Kluyveromyces marxianus*, *Geotrichum candidum*, and *Saccharomyces cerevisiae* are recorded across naturally fermented milk products (Bokulich et al., 2015; Sessou et al., 2019; Gastrow et al., 2020; Tenorio-Salgado et al., 2021). The substrate-specific adaptive evolution during backslopping of boiled milk and mesophilic fermentation shapes a particular group of bacterial communities, resulting in uniform quality products, whereas spontaneous fermentation of raw milk results in high variability in taste, flavor, and texture due to variations in geography, milk source, local climatic conditions, quality, and composition of milk (Zhong et al., 2016; Peng et al., 2021; Zhang et al., 2021; Zhao et al., 2021; Tamang, 2022). At the same time, such spontaneous fermentation also results in products displaying unique tastes and aromas, such as *Khoormog* and *Airag* of Mongolia and China (Oki et al., 2014; de Melo Pereira et al., 2022), *Churpii* of India (Shangpliang et al., 2018), and *Wagashi* cheese of Benin (Sessou et al., 2019), which deserve geographical indicator tagging. Therefore, there is a need to study the bacterial community structure and safety of these products by culture-independent NGS analysis to understand the presence/absence of beneficial microbes and unwanted potential pathogens (Tamang et al., 2021; de Melo Pereira et al., 2022; Rai and Tamang, 2022). This understanding will allow us to design starter culture consortia for sustainable industrial production of quality and safe fermented milk products with health benefits (Agyei et al., 2020).

In this study, we aimed to understand how spontaneous fermentation of raw cow milk without backslopping shapes the bacterial diversity in two geographically separated regions of two continents by analyzing homemade milk products, namely, *Doi*, *Sangom afamba*, and *Mishti dahi* of Northeast India; and *Kindirmou* and *Fanire* of Western Africa, by using 16S rRNA gene amplicon sequencing. *Doi* is traditionally prepared from fresh cow milk without backslapping by keeping it in an earthen pot wrapped with banana leaves and allowing it to ferment for 2 days (Joishy et al., 2019). *Sangom afamba* is also prepared from fresh cow milk, similar to *Doi*, in a traditional earthen pot, but a spoonful of the previous fermented batch is added and covered with a muslin cloth and allowed to stand for 2–3 days. In the *Mishti dahi*, cow milk is boiled with up to 10% sugar, cooled, and poured into small earthen pots. A starter from the previous batch was added and kept at room temperature for 1–2 days. Traditionally, *Doi*, *Sanggon afamba*, and *Misthi dahi* are consumed raw and taken along with steamed rice. *Sangom afamba* is essential for performing rituals in certain traditional religious ceremonies, such as the *na-hutpa* (ear-piercing ceremony of children) in Manipur. Fulani communities of Western African countries practice spontaneous fermentation of unpasteurised fresh cow milk without backslopping in calabash vessels for 1 day at room temperature to allow spontaneous fermentation (Sessou et al., 2019), such as *Kindirmou* in Niger and *Fanire* in Benin, which are commonly used as a dessert or refreshment by people in these countries. In addition, we studied a unique West African traditional cheese, *Wagashi*, with no report available on its associated bacterial communities. *Wagashi* (Gassire in the local Fulfulde language) is a soft, fresh cheese from Benin produced from cow milk. In the traditional preparation, 1 L of boiled cow milk is mixed with ~0.5 L of fresh milk with the extract of *Calotropis procera* leaves (10–15 g). The mixture is kept at a warm temperature (60–70°C) until coagulation is achieved, and then the curd and whey are separated. The curd portion is drained, molded without pressing, and incubated at room temperature. The pink-colored Wagashi cheese is prepared by soaking in a Sorghum (*Sorghum bicolor*) leaf extract brine for the pink color formation. Unlike conventional cheeses, *Wagashi* is prepared by curdling cow milk with proteolytic *Calotrophis procera* leaf extract (Akogou et al., 2018; Sessou et al., 2019). We compared the bacterial community structure of both uncoloured and colored *Wagashi* cheese samples from Benin using 16S rRNA gene amplicon sequencing and related

its bacterial community to similar cheeses reported from earlier studies (Shangpliang et al., 2018; Zhao et al., 2021). In addition, we aimed to assess the safety of these spontaneously fermented milk products by detecting potential foodborne pathogens.

2. Materials and methods

2.1. Sampling and homogenisation

The spontaneously fermented milk product samples from Northeast India, Niger, and Benin were collected under aseptic conditions (Table 1). Samples of uncoloured and colored *Wagashi* cheese prepared traditionally at home and locally marketed in different towns in Benin were also collected. The samples were transported in ice-cooled boxes and stored in the laboratory at −80°C for further analysis. A volume of 10 mL of each fermented milk sample was homogenized with 90 mL of 2% sodium citrate solution, while 25 g of each cheese sample was homogenized with 45 mL of buffered peptone water (Bio-Rad, pH 7.0 ± 0.2) at 200 rpm for 2 min using a Stomacher 400 Circulator (Seward, United Kingdom). After allowing the large debris to settle down, the resulting clear homogenate was used for metagenomic DNA extraction.

2.2. Metagenomic DNA extraction

Two different extraction methods were independently used to extract DNA from fermented milk and cheese samples. Metagenomic DNA of fermented milk samples was extracted according to method V, described earlier by Keisam et al. (2016). Briefly, 1.5 mL of homogenate was transferred to a sterile 2-ml screw-cap tube containing zirconia/silica beads and centrifuged. The resulting pellets were treated with enzymes (50 KU lysozyme and 25 U mutanolysin) and incubated at 37°C for 1 h, incubated with proteinase-K at 65°C for 1 h, and treated with GES reagent (5 M guanidine thiocyanate, 100 mM EDTA, and 0.5% sarkosyl). The samples were further treated with ammonium acetate, purified with chloroform:isoamyl alcohol (24:1), and precipitated with ethanol. The precipitated DNA pellets were dissolved in 50 µl of TE buffer. The metagenomic DNA of *Wagashi* cheese samples was extracted as described earlier (Anihouvi et al., 2021) using the NucleoSpin® Food (Macherey-Nagel GmbH&Co.) following the manufacturer's instructions. Qualitative (A260/280) and quantitative estimations of the extracted DNA of both food types were performed using a spectrophotometer (NanoDrop ND-1000, United States). The DNA was stored at −20°C for subsequent 16S rRNA gene amplicon sequencing.

2.3. Barcoded illumina MiSeq sequencing and data processing

Barcoded Illumina MiSeq amplicon sequencing was used for in-depth bacterial community structure analyses. Two independent approaches were performed for the spontaneously fermented milk products and traditional *Wagashi* cheese. For fermented milk products, the V4-V5 region of the 16S rRNA gene was targeted with the forward primer F563–577 (5′-AYTGGGYDTAAAGNG-3′) and reverse primer R924–907 (5′-CCGTCAATTCMTTTRAGT-3′) with barcodes for sample multiplexing as described earlier (Romi et al., 2015). The PCR-amplified DNA was purified using a QIAquick gel extraction kit (Qiagen, New Delhi, India) and quantified with a Qubit dsDNA BR Assay Kit in a Qubit 2.0 fluorometer (Invitrogen) for multiplexing in equimolar proportion. The DNA pool was sequenced on the Illumina MiSeq platform (Xcelris, Ahmedabad), and the sequence data were processed through the QIIME v1.8.0 bioinformatics pipeline (Caporaso et al., 2012) for adapter sequence removal, paired-end read generation, and sample de-multiplexing. A further MG-RAST pipeline was used at a 97% similarity threshold against the M5RNA database for the generation of OTU tables at different taxonomic levels. For the *Wagashi* cheese samples of Benin, the V1-V2 region of the 16S rRNA gene was targeted using the forward primer 28F (5′-GAGTTTGATCNTGGCTCAG−3′) and reverse primer 388R (5′-TGCTGCCTCCCGTAGGAGT-3′) with Illumina adapter and barcodes (Anihouvi et al., 2021). The target was PCR amplified in an ABI Verti-thermocycler, purified, pooled in equimolar proportion after quantification using a Qubit assay, and sequenced on Illumina MiSeq at RTL Genomics (Lubbock, TX, United States) as described earlier (Anihouvi et al., 2021). The sequence data were processed by PEAR sequence merger, USEARCH clustering and alignment algorithm, and UPARSE algorithm for OTU generation at different taxa levels.

2.4. Eubacterial-specific qPCR assay

A SYBR green-based qPCR assay targeting the SSU rRNA gene V3 region was performed for total bacterial load quantification. The PCR reaction containing 0.25 µM of each primer, with forward primer 338f 5′-ACTCCTACGGGAGGCAGCAG-3′ and reverse primer 518r 5′-ATTACCGCGGCTGCTGG-3′ (Ampe et al., 1999), and 1 × EXPRESS SYBR GreenER qPCR Supermix (Invitrogen), was used according to the manufacturer's instructions. The Applied Biosystems 7500 was used to carry out the PCR amplification at 95°C, 5 min, which consisted of 40 cycles of denaturation, annealing, and extension at 95°C for 15 s; 62°C for 30 s, and 68°C for 45 s, respectively (Keisam et al., 2016). A melt curve was generated for each assay from 60°C to 95°C using the default conditions to check the assay's specificity. The SSU rRNA gene (1×10^1 to 1×10^8 copies) derived from the strain type *Lactiplantibacillus plantarum* ATCC 8014 was used for the standard curve preparation.

2.5. Statistical analysis

The relative abundance data of the bacterial OTUs at the different taxonomic positions were used for statistical analysis. The significant differences in the relative abundance (%) of taxa between the study groups were calculated by Student's two-tailed *t*-test and ANOVA. Principal coordinate analysis (PCoA) was performed using the Bray-Curtis dissimilarity (PAST v3.22) (Hammer and Harper, 2008). The PERMANOVA test with 10,000 permutations was used to observe the significance of the difference in the bacterial taxa in the samples between two geographical

TABLE 1 Features of naturally fermented cow milk product samples collected in Northeast India and West Africa.

Region	Local name	Place of collection	Coordinates	Altitude (msl)	Temperature range (°C)	Pre-treatment	Backslopping	Number of samples
Northeast India	*Doi*	Guwahati, Assam	26.1792° N, 91.7533° E	55	11–33	No	No	3
	Doi	Silchar, Assam	24.8207° N, 92.8018° E	22	11–33	No	No	2
	Doi	Jorhat, Assam	26.7477° N, 94.2132° E	116	11–31	No	No	3
	Doi	Agartala, Tripura	23.8419° N, 91.2820° E	13	10–34	No	No	4
	Doi	Shillong, Meghalaya	25.5811° N, 91.8865° E	1525	4–23	No	No	3
	Sanggom afamba	Imphal, Manipur	24.8072° N, 93.9341° E	786	5–30	No	Yes	3
	Mishti Dahi	Agartala, Tripura	23.8419° N, 91.2820° E	13	10–34	Addition of 10% sugar and boiling	Yes	3
West Africa	*Kindirmou*	Niger (Tillaberi, Dosso, Niamey)	14.2061° N, 1.4580° E; 13.0505° N, 3.2081° E 13.5116° N, 2.1254° E	180–228	18–40	No	No	8
	Fanirè	Benin (Agouna, Pehunco, Parakou Djougou, Houeyogbe)	9.3467° N, 2.6090° E 10.2283° N, 2.0019° E 9.3466° N, 2.609° E 6.5321° N, 1.8708° E	234–444	17–40	No	No	5
	Uncoloured *Wagashi* cheese	Benin (Abomey-Calavi, Cotonou)	6.3719° N, 2.4348° E 6.4503° N, 2.3468° E	51–54	24–32	Yes, boiling and *Calotropis* leaf extract addition	No	5
	Colored *Wagashi* cheese	Benin (Abomey-Calavi, Cotonou)	6.3719° N, 2.4348° E 6.4503° N, 2.3468° E	51–54	24–32	Yes, boiling, *Calotropis* leaf extract addition and Sorghum leaf extract coloration	No	5

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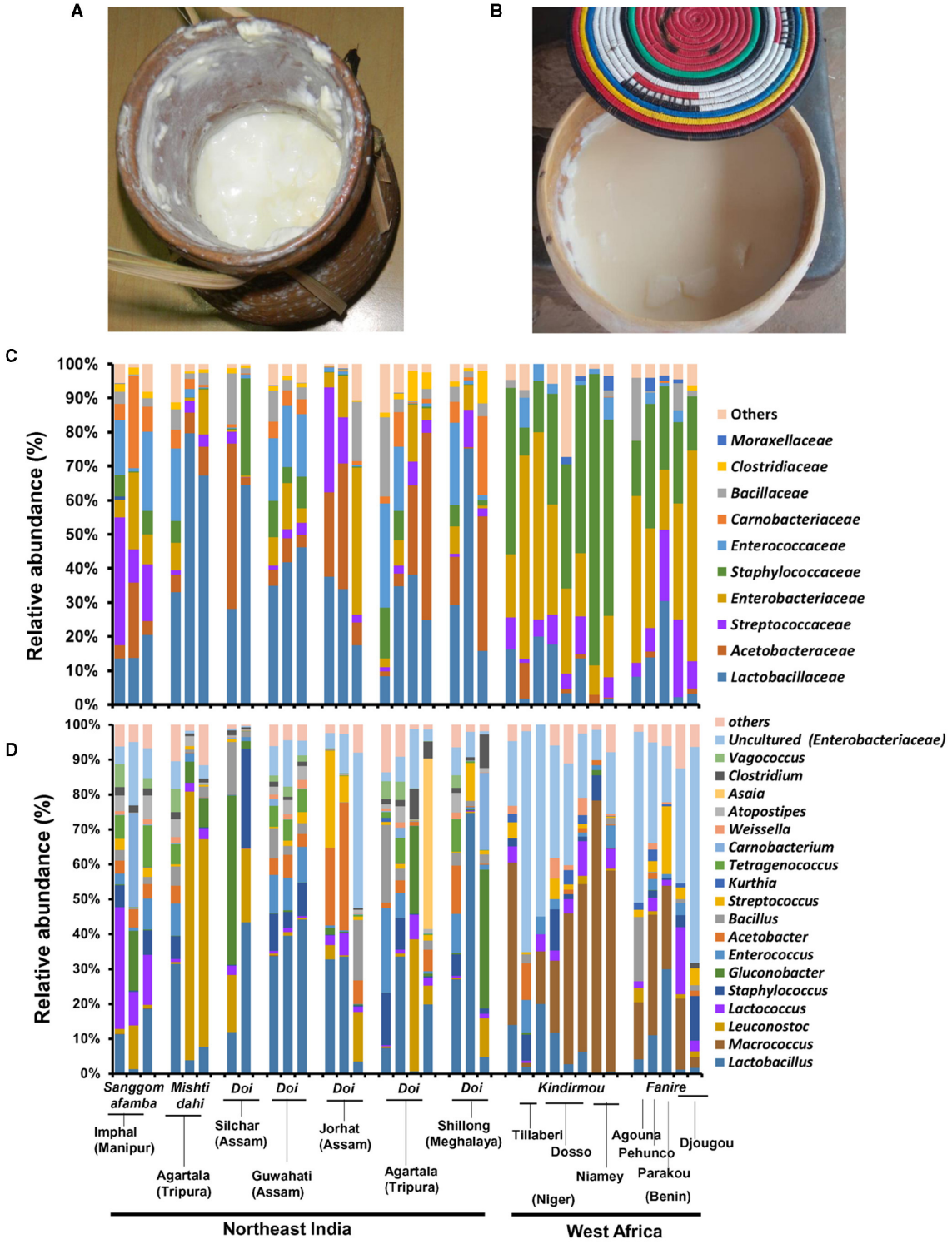

FIGURE 1
The bacterial community compositional difference in the spontaneously fermented milk products of Northeast India and West Africa. The spontaneously fermented milk products *"Doi"* in the traditional earthen pot **(A)** in Northeast India and *"Fanire"* in the traditional calabash vessel **(B)** in the Fulani camp of Benin are shown here. The taxon bar chart shows the family-level **(C)** and genus-level **(D)** differences in the relative abundance (%) of predominant bacteria present in the fermented milk products of Northeast India and West Africa. The sample details are available in Table 1.

regions and express it as a Bonferroni-corrected *p*-value. For calculating the alpha diversity indices (Chao species richness and Shannon diversity index) between two geographical regions, the compare_alpha_diversity.py script was used in the QIIME pipeline (Morris et al., 2014). The observed differences were visualized as boxplots using BoxPlotR (http://shiny.chemgrid.org/boxplotr/). The Wilcoxon test using "svDialogs" in the R package (v3.5.2) was conducted to show the significance of the difference in the

bacterial species between two geographical regions and express it as a Benjamini-Hochberg (BH)-corrected *p*-value (Tuikhar et al., 2019). The hierarchically clustered heat map to show the species-level significant difference between two geographical regions was visualized using "gplots" in R. The OTU data, with a relative abundance of more than 0.1% and a significance of $p > 0.001$, was used for the heatmap generation.

2.6. Data availability

The sequence data associated with this study are available on the MG-RAST server: https://www.mg-rast.org/mgmain.html?mgpage=project&project=mgp87174 and MG-RAST ID mgp104874.

3. Results

3.1. Spontaneously fermented milk products from Northeast India and West Africa display distinct bacterial diversity

We performed a cultivation-independent bacterial community analysis of spontaneously fermented milk products from Northeast India ($n = 21$, *Doi/Sangom afamba/Mishti dahi*) and West Africa ($n = 13$, *Kindrmou* of Niger and *Fanire* of Benin) by Illumina MiSeq amplicon sequencing of the 16S rRNA gene from the DNA of fermented milk samples. Bacillota (relative abundances of 72 and 61%) and Pseudomonadota (relative abundances of 17 and 3%) were the dominant bacterial phyla present in these naturally fermented milk products of India and West Africa, respectively. The mesophilic spontaneously fermented milk products of India analyzed in this study comprised *Lactobacillaceae* (34.7%), mainly *Lactobacillus* (*L. delbrueckii*) and *Leuconostoc* (*L. mesenteroides*); *Acetobacteraceae* (14.7%), mainly *Gluconobacter* and *Acetobacter* spp.; and *Streptococcaceae* (8.3%), mainly *Lactococcus* (*L. lactis*) and *Enterobacteriaceae* (8.3%, uncultured bacterium related to *Enterobacter* spp.) as core bacteria (Figure 1). However, the spontaneously fermented milk products of West Africa studied here contained *Staphylococcaceae* (34.6%), mainly *Macrococcus* (*Macrococcus caseolyticus*), *Enterobacteriaceae* (32.6%, uncultured *Enterobacter* sp.), *Streptococcaceae* (8.4%, *L. lactis*), and *Lactobacillaceae* (8%, *L. delbrueckii*) as the dominant bacteria.

An unweighted principal coordinate analysis (PCoA) based on the Bray-Curtis distance matrix using species-level relative abundance showed a distinct separation of the fermented milk samples of India from West Africa (PERMANOVA, $p < 0.0001$) (Figure 2A). The PCoA biplot displayed that *M. caseolyticus*, uncultured *Enterobacteriaceae*, *L. mesenteroides*, *G. frateurii*, and *Tetragenococcus halophilus* were the key ecological drivers that shaped up the overall bacterial community structure difference in the fermented milk products of two geographical regions.

The fermented milk products of India had significantly higher bacterial species richness (Chao1) and diversity (Shannon index) than the West African products ($p = 2.34$E-07, Student's *t*-test, two-tailed) (Figure 2B). The total bacterial load of fermented milk samples analyzed using a eubacteria-specific qPCR assay resulted in a 9.47–11.02 log10 bacterial load per gram of the samples, without any significant difference between Indian and African samples.

While comparing the two geographical regions, *Acetobacteraceae* (*G. frateurii* and *Acetobacter* spp.) ($p = 0.007$) and *L. mesenteroides* ($p = 0.014$) were abundant in fermented milk samples from India, while *Staphylococcaceae* (*M. caseolyticus*) ($p = 9.72$ E-07) and uncultured *Enterobacteriaceae* were present abundantly in West African fermented milk samples ($p = 1.23$ E-05). In addition, *Tetragenococcus*, *Atopostipes*, and *Vagococcus* were present only in Indian fermented milk products (Figure 3). Among the beneficial Actinobacteria present in fermented milk products (Parker et al., 2018), *Bifidobacterium* was detected in only two samples from India. Within Indian samples, *Streptococcaceae* (21.4%, mainly *L. lactis*) were abundant ($p = 0.014$, Student's *t*-test, two-tailed) in the *Sangom afamba* samples, where backslopping was practiced with unpasteurised raw milk.

3.2. Bacterial community differences in the colored and uncoloured *Wagashi* cheese

The bacterial community structure of uncoloured and colored *Wagashi* cheese of Benin ($n = 10$) prepared by spontaneous fermentation of boiled cow milk was analyzed by Illumina amplicon sequencing and compared with other reported traditional cheeses. At the phylum level, Bacillota (relative abundance of 85.1 and 76.2%) and Pseudomonadota (14.8 and 23.9%) were dominant in the uncoloured and colored *Wagashi* cheese samples, respectively. *Lactobacillaceae* (55.4%), mainly *Lactobacillus* (*L. delbrueckii*), and *Streptococcaceae* (31.9%), mainly *Streptococcus* spp., were dominant in the uncoloured *Wagashi* cheese. However, the colored *Wagashi* cheese was detected mainly with *Streptococcaceae* (72.9%) and low *Lactobacillaceae* (1.6%). Although no significant difference in the overall bacterial diversity (Chao species richness and Shannon diversity index) was visible between these two cheese types, we noticed a drastic change in the abundance of *Lactobacillus* (*L. delbrueckii*, $p = 0.01$, Students *t*-test, paired two-tailed) after soaking with Sorghum (*Sorghum bicolor*) leaf extract in the colored Wagashi cheese (Figure 4).

3.3. Safety of the spontaneously fermented milk products of Northeast India and Western Africa

In addition to the fermenting beneficial bacteria, the 16S rRNA gene amplicon sequencing-based in-depth analysis effectively detected the presence of several unwanted potential pathogens in the fermented milk samples. Our study detected *Clostridium* spp. in all the *Doi* samples up to 4% of relative abundance, particularly in the samples collected from Tripura and Meghalaya of Northeast India, whereas none of the West African fermented milk samples detected *Clostridium*. The *Wagashi* cheese samples from Benin contained *Streptococcus infantarius*, with up to 80% relative abundance in some samples. In addition, we found an uncultured *Enterobacteriaceae* bacterium with

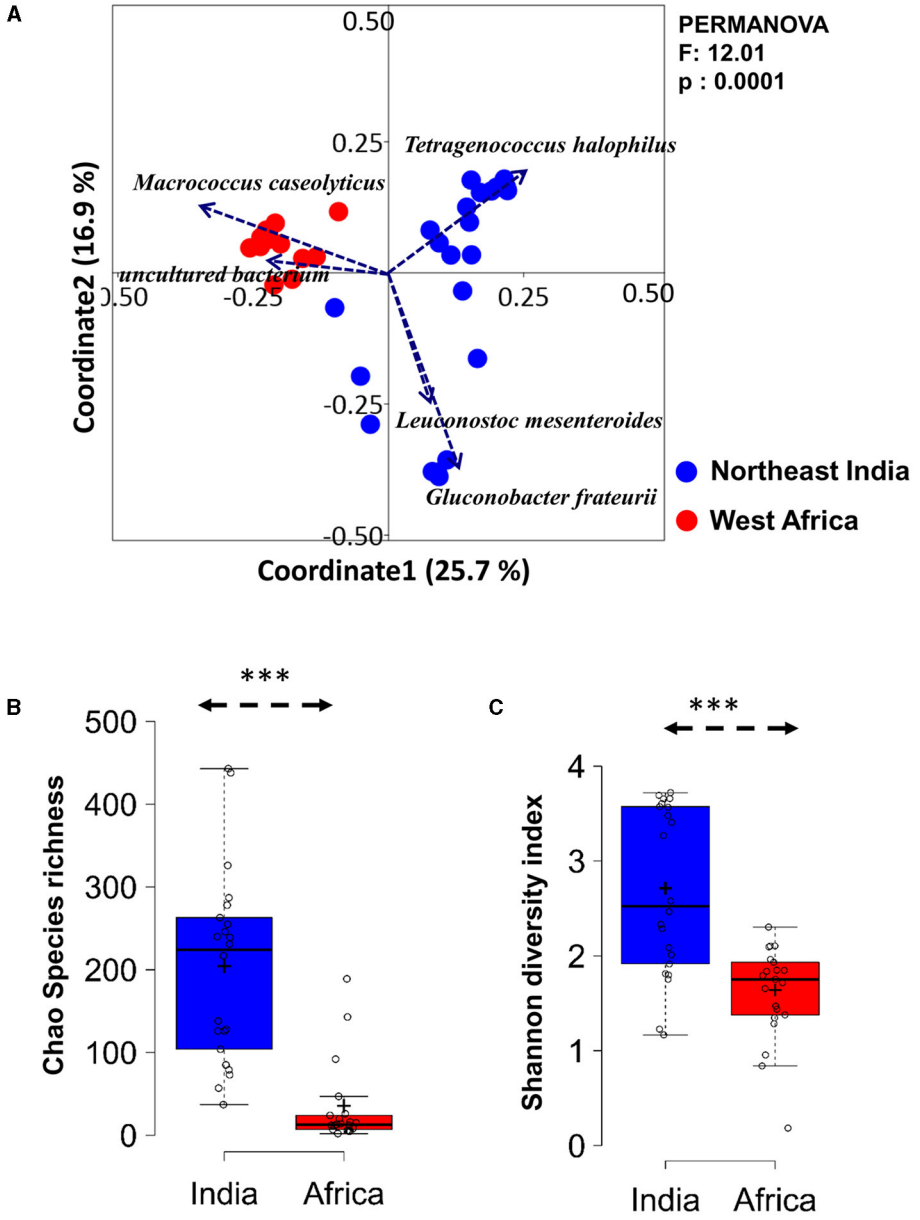

FIGURE 2
Differences in bacterial diversity in the spontaneously fermented milk products of Northeast India and West Africa. **(A)** PCoA biplot based on Bray-Curtis dissimilarity of the species-level OTUs shows a significant difference in the overall bacterial community structure between the spontaneously fermented milk products of the two continents. The significance of the difference is expressed as a Bonferroni-corrected *p*-value ($q = 0.0001$, $F = 12.0$, PERMANOVA). **(B, C)** The boxplot shows higher bacterial diversity in Indian fermented milk products (Chao species richness and Shannon diversity index) than in West African samples. The significance of the difference was calculated using Student's *t*-test and indicated as ***$p<0.0001$.

<96% similarity with the 16S rRNA sequence of *Enterobacter* spp. in West African and Indian fermented milk products. On average, *Kindrmou* and *Fanire* samples contained the uncultured bacterium related to *Enterobacter* with a 32% relative abundance, and *Wagashi* cheese samples had a 15% relative abundance of *Enterobacter*. In addition, *Wagashi* cheese contained, on average, more than 4% relative abundance of *Escherichia coli*. Among the members of *Staphylococcus*, we detected *Staphylococcus aureus* in West African fermented milk products and *Staphylococcus epidermis* in Indian *Doi* samples. While compared to the uncoloured Wagashi cheese, *Bacillus cereus* was abundantly present, with more than 5% average relative abundance in the colored, processed Wagashi cheese samples.

4. Discussion

Unlike thermophilic *yogurt* fermentation, in which *L. delbrueckii* and *S. thermophilus* dominate (Bokulich et al.,

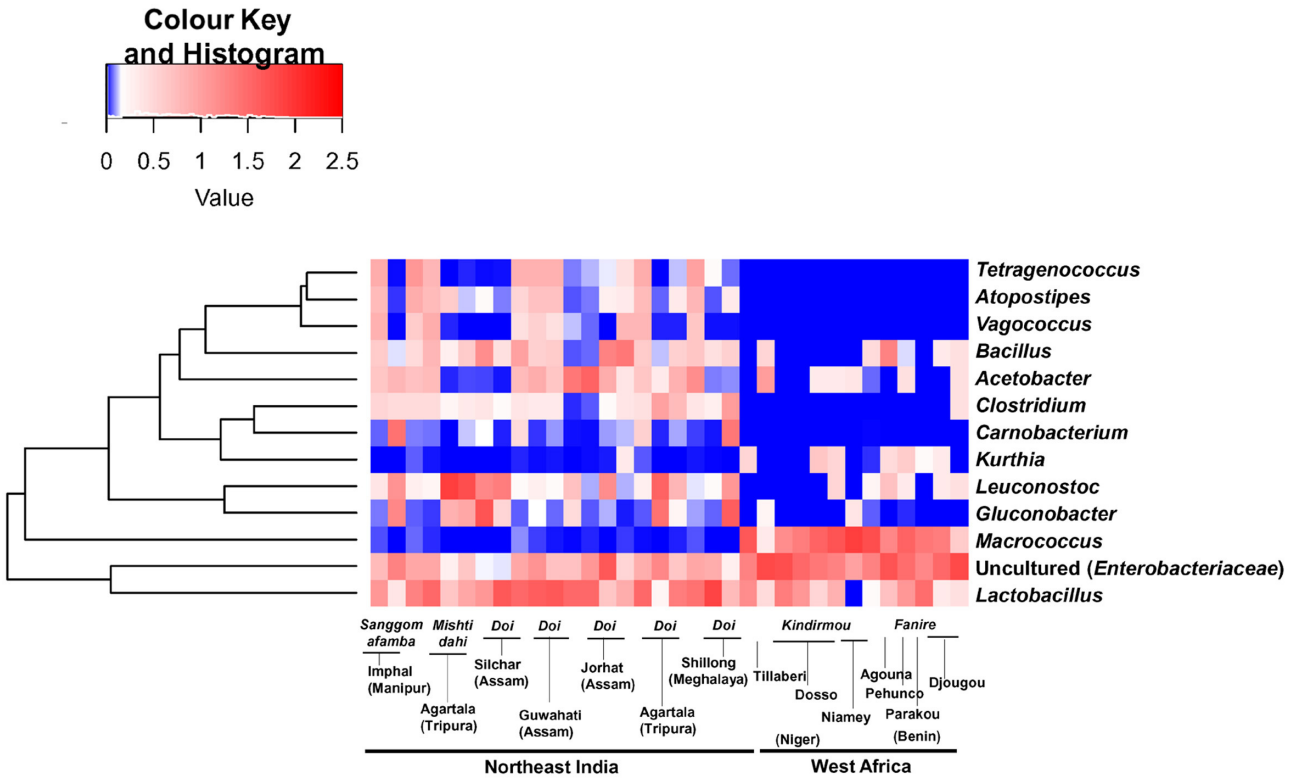

FIGURE 3
A hierarchically clustered heat map shows the significantly differing bacterial genera between the spontaneously fermented milk samples of Northeast India and West Africa. The bacterial genus with a relative abundance of more than 1% and significantly different between the two continents (Wilcoxon test, $q < 0.001$, BH corrected) is listed here. The abundance difference is shown as a red and blue colour gradient key.

2015; Parker et al., 2018; de Melo Pereira et al., 2022), the mesophilic spontaneously fermented milk products of Northeast India comprise mainly *Lactococcus*, *Leuconostoc*, *Gluconobacter*, *Acetobacter*, and *Enterobacter*, in addition to *Lactobacillus*. Most of the NGS-based earlier studies reported the dominance of *Lactococcus* in naturally fermented milk products prepared by backslopping (Jans et al., 2017; Shangpliang et al., 2018; Gastrow et al., 2020; Moonga et al., 2020). Similarly, *L. mesenteroides*, which can tolerate high concentrations of sugar compared to other lactic acid bacteria (Fellows, 2017), was abundantly present in the *Mishti Dahi*, prepared by boiling milk with 10% sugar, and allowed mesophilic fermentation by backslopping (Mallappa et al., 2021).

The NGS-based earlier studies showed the dominance of *L. lactis*, *L. helveticus*, *L. mesenteroides*, and *Acetobacter* spp. in the *Dahi* prepared by backslopping in the high-altitude Himalayan region (Shangpliang et al., 2018; de Melo Pereira et al., 2022). At the same time, the *Dahi* prepared in southern India had *L. delbrueckii* as the dominant bacteria (Jayashree et al., 2013; Joishy et al., 2019; Mallappa et al., 2021). However, *Streptococcus* spp. and *Enterococcus* spp. were the most abundant in the *Dahi* samples of Bangladesh (Nahidul-Islam et al., 2018) and Bhutan (Shangpliang et al., 2017), respectively. *Enterococcus durans* predominates in the naturally fermented milk of cow and yak products of Arunachal Pradesh in India, such as mar, chhurpi, and churkam (Shangpliang and Tamang, 2021). Among the beneficial Actinobacteria reported in the fermented milk products (Parker et al., 2018), only two *Doi* samples were detected with *Bifidobacterium*.

On the contrary, the spontaneously fermented raw milk products of West Africa studied here contained *Macrococcus* and *Enterobacter* as the dominant bacteria. Unlike other species of *Staphylococcaceae*, *M. caseolyticus*, abundantly present in the fermented milk products of West Africa, is not considered a human pathogen and has been used as a starter culture for aroma and flavor production in several fermented foods (Ramos et al., 2021). The unique characteristic property of Fulani fermented milk products prepared in calabash containers may be linked with such bacterial associations (Groenenboom et al., 2020; Moonga et al., 2020; Fagbemigun et al., 2021). However, earlier NGS studies did not report *M. caseolyticus* as a primary bacterium in other African fermented milk products such as *Nono* (Fagbemigun et al., 2021) and *Mabisi* (Moonga et al., 2020). The bacterial diversity of fermented foods inferred from metagenomic studies differs depending on the method of DNA extraction used. In this study, we used an enzymatic lysis method that recovers a high bacterial diversity from fermented milk products (Keisam et al., 2016). We speculate the DNA extraction method adopted here may be one reason for the above differences, in addition to the environmental factors during the sampling (Zhao et al., 2021).

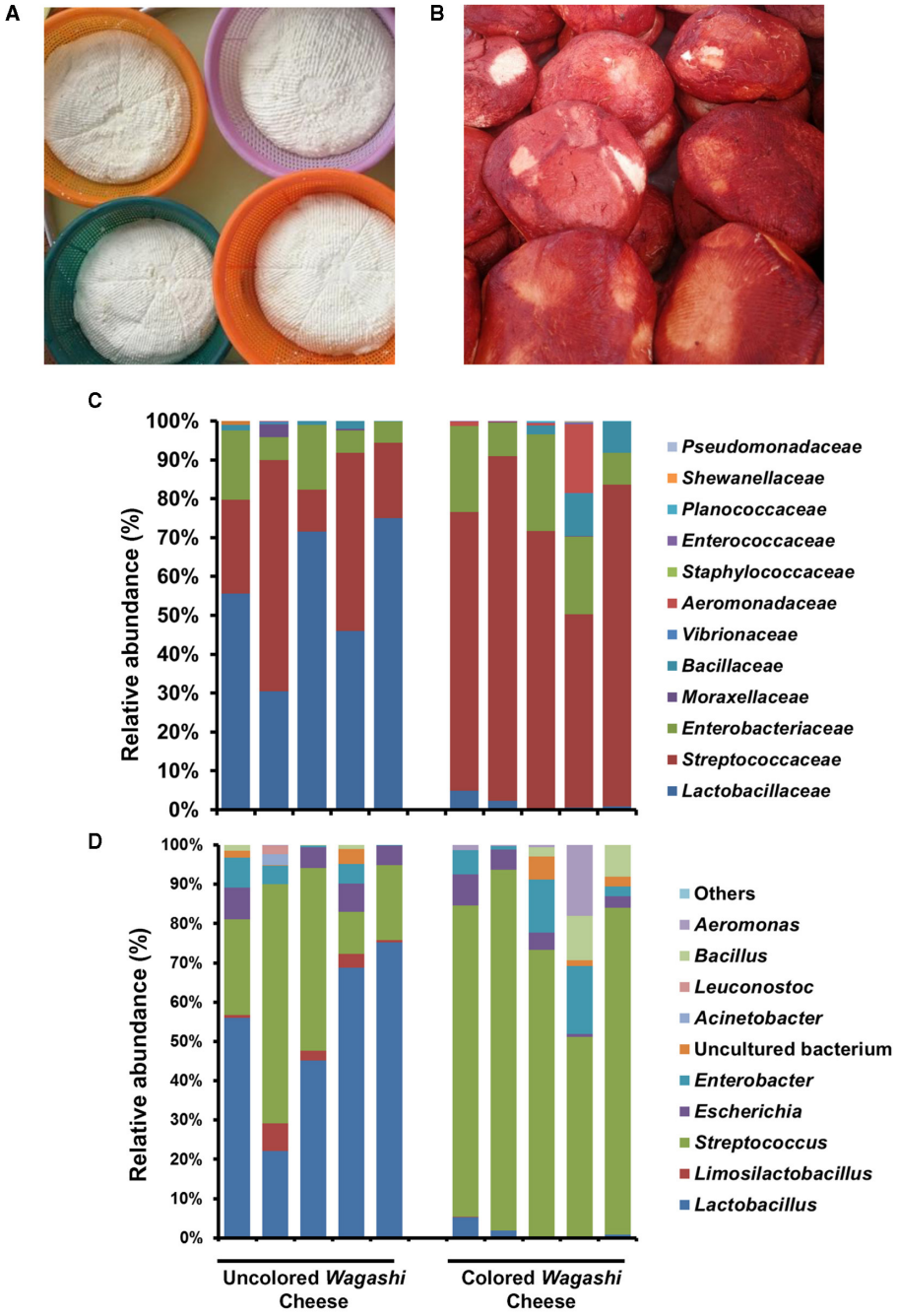

FIGURE 4
The difference in the overall bacterial community structure in the uncoloured **(A)** and coloured *Wagashi* cheese **(B)** marketed in Benin. The taxon bar chart shows the difference in the relative abundance (%) of predominant bacteria present in the uncoloured *Wagashi* cheese and coloured *Wagashi* cheese at the family level **(C)** and genera level **(D)**.

Earlier NGS analysis of similar spontaneously fermented unpasteurised cow milk "*Nono*" prepared in Nigeria by mesophilic fermentation in a calabash container without backslopping contained mainly *Lactobacillus* spp. and *Acetobacter* spp. (Fagbemigun et al., 2021). Another Zambian traditionally fermented milk, "*Mabisi*," prepared similarly, was dominated by *L. lactis* (Groenenboom et al., 2020; Moonga et al., 2020). On the contrary, *S. thermophilus* and *L. helveticus*, reported worldwide as dominant bacteria in several naturally fermented milk products (Oki et al., 2014; Bokulich et al., 2015; Shangpliang et al., 2018; Peng et al., 2021; Yu et al., 2021), were rare (<2% relative abundance) in the spontaneously fermented milk products of this study. The mesophilic spontaneous fermentation, without backslopping, might be the reason for such a difference in the predominant bacterial communities. Another observation of total bacterial load analyzed by culture-independent qPCR assay is much higher than those reports based on the cultivation-dependent analysis of similar fermented milk products with 6.0–9.0 log 10 population

load per gram (Dewan and Tamang, 2007; Shangpliang et al., 2017), which support the possible presence of unculturable strains of dominant bacterial species in natural milk fermentation.

The presence of *Lactobacillus* (*L. delbrueckii*) and *Streptococcus* as dominant bacteria in the uncoloured *Wagashi* cheese is similar to the Mexican Poro Cheese (Aldrete-Tapia et al., 2014) and traditional cheeses of China, Mongolia, and Russia (Zhong et al., 2016; Zhao et al., 2021). The drastic change in the main bacterial abundance in the colored Wagashi cheese is linked with the Sorghum leaf extract used for coloring and further drying. Sorghum leaf extract mainly contains apigeninidin pigment and phenolic compounds, normally used for prolonging shelf life (Akogou et al., 2018). The antimicrobial activity of sorghum leaf extract (Akogou et al., 2018; Schnur et al., 2021) and further processing, such as drying, may have favored *Streptococcus* spp. Contrary to the cheese types produced worldwide from cow milk through natural fermentation, *L. lactis* and *L. mesenteroides* (Delcenserie et al., 2014; Shangpliang et al., 2017; Rocha et al., 2021; Sun and D'Amico, 2021; Zhang et al., 2021) were not predominant in *Wagashi* cheese, while *Streptococcus* spp. dominated. The traditional production method of curdling with proteolytic leaf extract of *Calotrophis procera* and thermophilic incubation at 60–70°C (Akogou et al., 2018; Sessou et al., 2019) might have favored *Streptococcus* spp. in *Wagashi* cheese of Benin.

Clostridium in naturally fermented milk products is a safety concern in Northeast India, where the Indian Council of Medical Research (ICMR) has already reported high incidences of foodborne diseases and intestinal infections (ICMR Report, 2017). Earlier NGS-based studies also detected a similar presence of *Clostridium* in the *Dahi* samples of Bangladesh (Nahidul-Islam et al., 2018). Our study detected *Clostridium* in the *Doi* samples collected from Tripura and Meghalaya, which are Indian states bordering Bangladesh. Several earlier reports also showed the presence of *Clostridium* in other fermented foods (particularly in fermented pork, fish, and soybean products) marketed in Northeast India (De Mandal et al., 2018; Keisam et al., 2019; Deka et al., 2021).

The *Wagashi* cheese samples from Benin contained *Streptococcus infantarius*, possibly related to *S. infantarius* subsp. *infantarius* (Sii), belonging to the *Streptococcus bovis*/*Streptococcus equinus* complex (SBEEC), which has recently been reported as an emerging infectious foodborne pathogen in African countries (Jans et al., 2017). Attempts on selective isolation and characterization of an uncultured *Enterobacteriaceae* bacterium found in West African and Indian fermented milk products could result in novel species. The earlier NGS studies also reported the dominance of *Enterobacter* spp. in the African naturally fermented milk *Mabisi* (Moonga et al., 2020) and *Kefir* of Turkey (Dertli and Çon, 2017). Similar to our results, several reports based on 16S rRNA gene sequencing account for the presence of *E. coli* in traditional cheese produced by natural fermentation (Fuka et al., 2013).

Most West African fermented milk products are prepared from raw, unpasteurised milk by spontaneous fermentation (Leone et al., 2022). Usually, unpasteurised raw milk contains *Staphylococcaceae* members abundantly (Joishy et al., 2019; Sun and D'Amico, 2021), which supports the dominance of *M. caseolyticus* in spontaneously fermented raw cow milk in West African countries. Moreover, several traditionally fermented milk products have been reported to contain *M. caseolyticus* (Fuka et al., 2013). Few other studies have detected *M. caseolyticus* in foods of animal origin (Aldrete-Tapia et al., 2014; Ramos et al., 2021). However, it is not prioritized as a human pathogen and has been used as a starter culture in several fermented foods (Ramos et al., 2021). The traditional milk curdling with proteolytic leaf extract of *Calotrophis procera*, thermophilic incubation at 60–70°C, and further processing by colouration and drying might have favored the *B. cereus* growth in the colored Wagashi cheese. Lowering the moisture content by drying and adding 3% NaCl to the colored Wagashi cheese would reduce the presence of *B. cereus* (Raevuori and Genigeorgis, 1975; Rukure and Bester, 2001). These findings will allow us to understand the safety issues related to spontaneous milk fermentation and to develop strategies to overcome them by identifying the critical control points of pathogen entry. Moreover, technological intervention by designing the starter culture consortiums for a controlled fermentation of boiled or pasteurized milk will improve the safety and quality of the fermented milk products in both regions studied (Agyei et al., 2020; Mallappa et al., 2021; de Melo Pereira et al., 2022).

Natural milk fermentation is highly reproducible across geographical regions under similar processes and conditions (Wolfe et al., 2014). Such substrate- and condition-specific adaptive evolution of microbiota is responsible for the unique properties of different fermented foods (Tamang et al., 2021). In our study, natural mesophilic fermentation of unpasteurised cow milk without backslopping in two geographically separated regions shaped different bacterial community structures. In contrast to the thermophilic spontaneous milk fermentation by backslopping commonly practiced worldwide, the mesophilic milk fermentation without backslopping favored a combination of lactic acid bacteria (*Lactobacillus* and *Lactococcus)* and acetic acid bacteria (*Gluconobacter* and *Acetobacter*) in Indian *Doi* samples. Meanwhile, *Macrococcus* and an uncultured bacterium of *Enterobacteriaceae* were found in Fanire and Kindirmou samples of Benin and Niger, respectively. Such differences in the microbiota evolving during natural fermentation may relate to the microbiota of raw cow milk used and the indigenous production process (in traditional earthen pots or calabash containers) in practice in both regions. Moreover, we report for the first time the bacterial communities of Wagashi cheese in Benin and their differences during processing (coloring). This understanding will allow us to implement controlled production through technological intervention by designing the starter culture consortiums, which improve the safety and quality of the traditional fermented milk and cheese products in India and Africa, where natural fermentation is in practice.

Data availability statement

The data presented in the study are deposited in the MG-RAST repository, MG-RAST accession numbers are mgp104874 and mgp87174. The sequence data associated with this study are available on the MG-RAST server: https://www.mg-rast.org/mgmain.html?mgpage=project&project=mgp87174 and MG-RAST ID mgp104874.

Author contributions

KJ, PS, and SK were involved in conceiving the research idea and designing the work plan. The experiments were performed by SK (Indian fermented milk), PS, MG (fermented milk of Benin and Niger), and GK (*Wagashi* cheese of Benin). KJ and SK analyzed the data and interpreted the results. KJ and PS wrote the manuscript. GK, SF, and JM revised the manuscript. All authors contributed to the article and approved the submitted version.

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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

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