INTERSPECIES INTERACTIONS WITHIN FERMENTED FOOD SYSTEMS AND THEIR IMPACT ON PROCESS EFFICIENCY AND PRODUCT QUALITY

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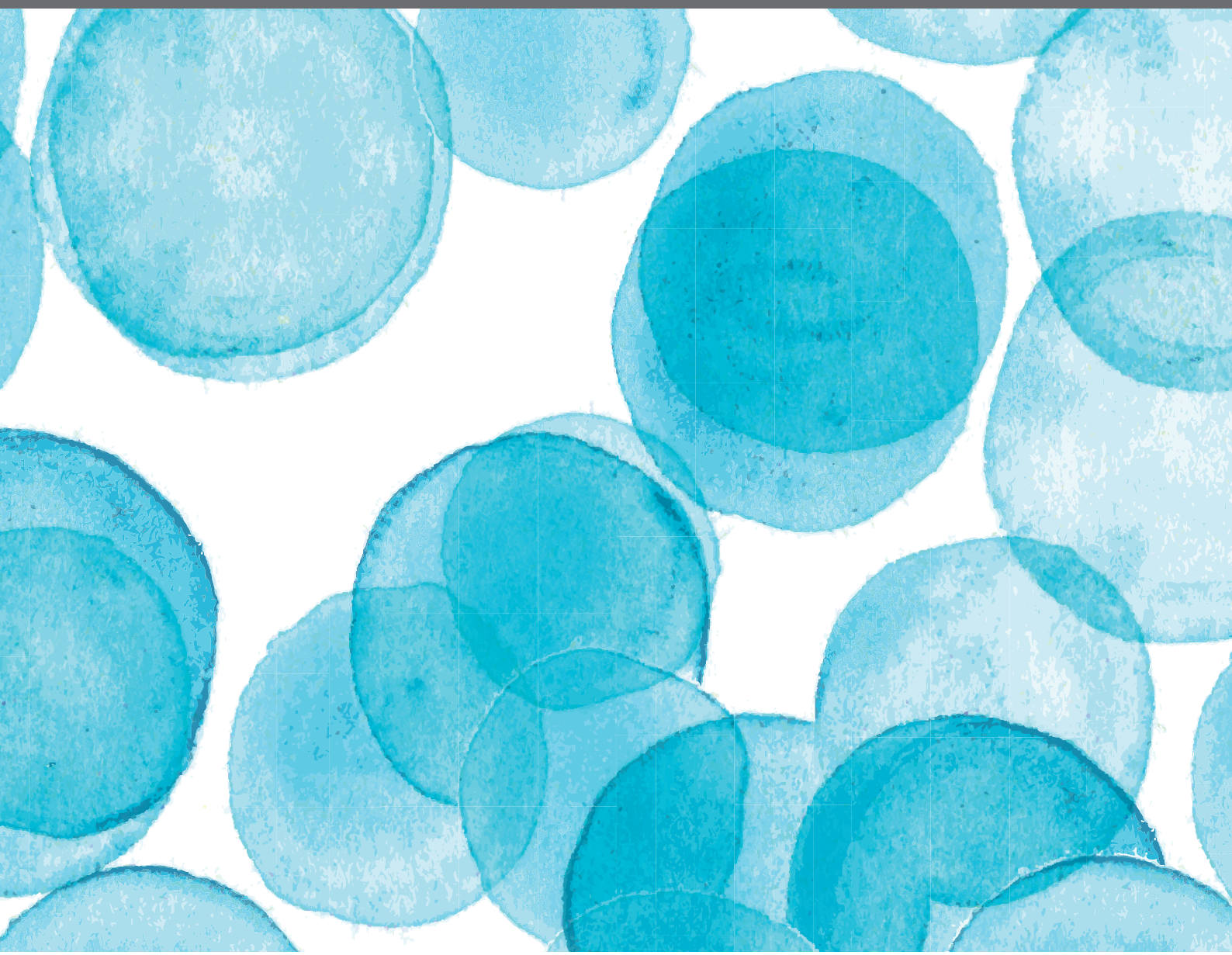

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INTERSPECIES INTERACTIONS WITHIN FERMENTED FOOD SYSTEMS AND THEIR IMPACT ON PROCESS EFFICIENCY AND PRODUCT QUALITY

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EDITORIAL
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Editorial: Interspecies Interactions Within Fermented Food Systems and Their Impact on Process Efficiency and Product Quality

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Editorial on the Research Topic

Interspecies Interactions Within Fermented Food Systems and Their Impact on Process Efficiency and Product Quality

Food is an integral part of human culture. Aside from its nutritional role, it connects us on a daily basis through the act of sitting and eating together, it contributes to feelings of shared identity in populations, it is an essential component of many formative events in our lives, and is a gateway to experiencing new cultures.

While the influence of food in defining human culture is well-understood, there is a growing appreciation of the role of microbial cultures in defining the properties of food. Archaeological evidence points to the use of fermentation over many thousands of years for the production of a range of foods and beverages. Fermentation serves to make foods more digestible, longer-lasting, safer, and more palatable. For our species, the role of fermentation in food production has arguably been as influential as the role of fire for cooking food.

For much of our history, these fermentation processes have been carried out by complex assemblages of organisms, with the use of single strains for fermentation of bread, beer, wine, etc. being a relatively recent development. We have however been seeing a growing interest in the use of microbial communities in food production. In the western world, this revival has been driven in part by the popularity of craft beer, natural wine, fermented non-alcoholic beverages (kefir, kombucha), and sourdough bread. In other parts of the world, complex cultures in fermentation represent continuation of tradition rather than revival.

This Research Topic from Frontiers in Microbiology aims to highlight the beneficial role of microbial complexity in driving fermentation processes and influencing food quality. The issue features ten articles describing investigations into complex microbial fermentation for the production of food, feed, and beverages. Contributions were received from Africa, Asia, Australia, Europe, and South America—highlighting the global importance of fermentation. Demonstrated benefits of particular communities during fermentation include improved flavor and color, greater safety, increased nutritional value, faster fermentation rates, reduced alcohol content, and better industrial applicability.

One of the most direct impacts of fermentation is the creation of specific flavor profiles. We see this in the work of Elhalis et al., who show improved sensory properties of coffee after fermentation with the yeast *Pichia Kudriavzevii*. Likewise, Köhler et al. note how specific yeast and bacteria combinations can improve the flavor of water kefir. Liu et al. describe the

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relationship between bacterial composition and flavor profile of Xifeng liquor (a spirit prepared from fermented sorghum).

Palatability of foods is not the only factor influenced by fermentation. Benefits extend also to improved digestibility and safety. Su et al. for example, note how fermentation of corn by-products with *Saccharomyces* yeast and lactic acid bacteria can improve their suitability for use as feed. Dahunsi et al. provide a comprehensive account of how the presence of lactic acid bacteria within fermentative microbial consortia have a critical role in ensuring the safety and shelf-life of many fermented African foods. Likewise, Huang et al. describe the metabolites produced by the yeast *Debaryomyces hansenii* can prevent the growth of contaminant molds during Danish cheese production.

The composition of species in a fermenting culture is strongly impacted by the environment, a fact that must be considered when trying to control or direct fermentation processes. The effect of environment is clearly seen in the study of Martinez et al. who describe how coffee fermentation is influenced by altitude, and how this is associated with changes in the natural microbiota. Substrate also influences the development of populations, as illustrated by Liu et al. who show how different microbial communities develop depending on the type of sorghum used in Xifeng liquour production. Su et al. likewise show how communities develop differently in different by-products of the corn starch industry. These, and other studies described in this Research Topic, demonstrate the power of high-throughput sequencing in studying microbial communities. This is particularly important for dynamic, complex or under-researched communities. This is exemplified in the studies on dark tea fermentation by Yan et al. or the work of Atter et al. on Ghanian cereal fermentation.

Despite the advantages of complex fermentations with respect to product quality, it should be noted that industrial application of such cultures is not without complications. More complexity may lead to less consistency in fermentation processes. The controlled use of cultures, e.g., supplementing natural microbial consortia with specific species, or rationalizing consortia so that they contain only the keystone species, represents a compromise in this respect. Examples are seen here in the work on cheese production by Huang et al. coffee fermentation by Elhalis et al. and water kefir production by Köhler et al.

Complex fermentations demonstrate great potential for improving food process efficiency, enhancing food quality, and increasing diversity of available foods and beverages. It is clear however that our ability to fully exploit such fermentations is limited by insufficient knowledge of how individuals in mixed populations interact to influence each other and their environment. It is our hope that this Research Topic represents a step forward in our understanding of these complex systems, and will to some extent facilitate the efficient production of a range of high quality food products in the future.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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ORIGINAL RESEARCH
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The Altitude of Coffee Cultivation Causes Shifts in the Microbial Community Assembly and Biochemical Compounds in Natural Induced Anaerobic Fermentations

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Coffee harvested in the Caparaó region (Minas Gerais, Brazil) is associated with high-quality coffee beans resulting in high-quality beverages. We characterize, microbiologically and chemically, fermented coffees from different altitudes through target NGS, chromatography, and conventional chemical assays. The genera *Gluconobacter* and *Weissella* were dominant in coffee's fruits from altitudes 800 and 1,000 m. Among the Eukaryotic community, yeasts were the most dominant in all altitudes. The most dominant fungal genus was *Cystofilobasidium,* which inhabits cold environments and resists low temperatures. The content of acetic acid was higher at altitudes 1,200 and 1,400 m. Lactic acid and the genus *Leuconostoc* (Pearson: 0.93) were positively correlated. The relative concentration of volatile alcohols, especially of 2-heptanol, was high at all altitudes. Bacteria population was higher in coffees from 800 m, while at 1,000 m, fungi richness was favored. The altitude is an important variable that caused shifts in the microbial community and biochemical compounds content, even in coffees belonging to the same variety and cultivated in the same region under SIAF (self-induced anaerobic fermentation) conditions. Coffee from lower altitudes has higher volatile alcohols content, while high altitudes have esters, aldehydes, and total phenolics contents.

Keywords: target sequencing, Caparaó region, microbial community, altitude, coffee fermentation

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INTRODUCTION

The Caparaó is a region located in a mountainous territory shared by two Brazilian states, Minas Gerais and Espírito Santo (Assis et al., 2017; Campanha et al., 2017), and known for producing high-quality coffees. The coffee plants are owned by family farms grown at different altitudes and microclimates (Campanha et al., 2017). Ninety percent of the Caparaó region's production is of *Coffea arabica,* representing 40% of total production in the Espírito Santo state (Santos et al., 2017), and 75% of them are processed by the natural method (Paschoa et al., 2017).

Crop growing environment, plants genetic traits, and post-harvesting processes are among the essential drivers of coffee quality (De Bruyn et al., 2017; Borém et al., 2019), meaning that coffee is a *terroir* product, and care is needed to obtain specialty coffee beverages. Three methods are commonly used to process coffee: natural (dry), wet, and pulped natural (semi-dry). The natural method is the oldest process that uses whole intact fruits, directly placed on cement patios or suspended platforms for fermentation and drying until reaching 11–12% moisture (Schwan et al., 2012). In the wet method, fruits are depulped, then fermented in tanks with water, and placed directly for drying. While the pulped natural is a mixture of both methods where fruits are depulped and placed directly for fermentation and drying. Each method has shown differences in sensory perception and microbiota dynamics (Silva et al., 2000, 2008a; Avallone et al., 2001; Evangelista et al., 2014a,b; Bressani et al., 2018). A more recent method known as self-induced anaerobic fermentation (SIAF) showed promising results in Da Mota et al. (2020) and Martins et al. (2020).

During fermentation, microorganisms consume carbohydrates or other organic compounds and proliferate (Silva et al., 2000; Silva, 2015). Most microorganisms that participate in the process come from the environment like soil, air, plants, and other sources (Silva et al., 2008a; Silva, 2015). Yeasts from the genera *Saccharomyces*, *Pichia*, *Candida*, *Kluyveromyces*, *Hanseniaspora*, and bacteria belonging to *Leuconostoc*, *Lactobacillus*, *Bacillus*, *Flavobacterium*, *Serratia*, *Pseudomonas,* and *Weissella* are often found while fermentation in the different post-harvest processes (Silva et al., 2000; Avallone et al., 2001; Masoud et al., 2004; Vilela et al., 2010; Silva, 2015).

Microbial communities usually change in response to the environmental conditions where fermentations are carried out and affect coffee quality. Those conditions include temperature, moisture, and altitude (Borém et al., 2019; Martins et al., 2020). Bertrand et al. (2006) observed that green coffee beans from the variety Caturra grew at high altitudes and processed via the wet method in Costa Rica have high caffeine and fats and low trigonelline contents. A study with Ethiopian arabica green coffee beans showed that an increase in altitude decrease caffeine and chlorogenic acids contents, while sucrose, acidity, and flavor increase (Worku et al., 2018). In a Brazil, research conducted in the Matas de Minas region showed that yellow and red Catuaí coffee varieties from higher altitudes produce higher quality coffee beans (Silveira et al., 2016).

Further research regarding altitude vs. compounds content variation is needed because they directly influence the beverage flavor. For example, organic acids mainly affect the sweet flavor (Galli and Barbas, 2004) and acidity (Ribeiro et al., 2017). Bioactive compounds trigonelline and chlorogenic acids are precursors of volatile compounds that contribute to roasted coffees taste and aroma (Ribeiro et al., 2016), and volatile alcohol precursors produce rose-like and fruity-like flavors (Lee et al., 2015).

Recent advances in Next-Generation Sequencing (NGS) are now allowing a deep microbiota characterization during the fermentative process under different conditions in several countries (Cao et al., 2017; de Oliveira Junqueira et al., 2019; Zhang et al., 2019; Elhalis et al., 2020; Pothakos et al., 2020), but few studies have been carried out with Brazilian coffees.

The present study aimed to characterize the dominant microbial communities of bacteria and fungi present in self-induced anaerobic fermentations containing different altitudes coffees performed in the Caparaó region through a metataxonomic approach. Moreover, this study aimed to evaluate the effect of altitude and microbiota profile on the biochemical compounds profile (organic acids, bioactives, and volatiles) during the fermentative process.

MATERIALS AND METHODS

Pilot Study On-Farm: Coffee Process and Fermentation

Ripe fruits of *Coffea arabica* cv Catuaí Vermelho IAC 44 were manually collected from different altitudes: 800, 1,000, 1,200, and 1,400 m, at the Caparaó region, located in Minas Gerais and Espírito Santo, Brazil. The coffee fruits were processed using the natural method. Then the fruits were transferred into 20 L bioreactors (polypropylene food buckets with lids), with following the bioreactors were closed for SIAF. Fermentations were performed in triplicate.

The fermentative processes for all coffees from different altitudes were carried out simultaneously in close batches at a farm located at 1,200 m to avoid any environmental interference and favor controlled conditions. The bioreactors were placed under an open storage house built with fences for fermentation and suspended terraces. Before filling the bioreactors with coffee, portable data loggers (INKBIRD) were placed inside the bioreactors to register the mass temperature during fermentation. Fermentation lasted 72 h, and sub-samples of approximately 100 g were taken after 48 h of fermentation for dominant microbiota profiling and metabolites evaluation. Fruits' initial sugar content (Brix degree-°Bx) was measured with a refractometer (Sigma-Aldrich, St. Louis, MO, United States).

Composition and Abundance of Bacteria and Fungi Communities

DNA Extraction

Total DNA was extracted from 48 h fermented coffee fruits collected in fields at 800, 1,000, 1,200, 1,400 m of altitude. One hundred grams of coffee fruits were vortexed in 50 ml of sterilized Milli-Q water for 10 min to detach the fruits' microbial cells. Then the resulted suspension was transferred to another tube and centrifuged (12,745 RCF for 10 min at 4°C) to separate the supernatant and obtain a pellet. After the supernatant was discarded, 30 mg of the remaining pellet was used for DNA extraction with the QIAamp DNA Mini Kit, following the "DNA Purification from Tissues" protocol (Qiagen, Hilden, Germany). The purity of the extracted DNA was checked with a Nanodrop Lite spectrophotometer (Nanodrop Technologies, Wilmington, DE, United States) (260/280 nm ratio), and it was quantified by Qubit® 4.0 fluorometer using the dsDNA HS Assay kit (Invitrogen™) according to the manual. The DNA integrity

was also confirmed by electrophoresis in a 0.8% agarose gel with 1 X TAE buffer.

Illumina High-Throughput Sequencing of Bacterial/Archaeal 16S rRNA Genes and Fungal Internal Transcribed Spacer (ITS)

The NGS Soluções Genômicas performed sample preparation for sequence and sequencing in Piracicaba-São Paulo, Brazil. The V3-V4 regions of the 16S rRNA gene of bacteria and the ITS1 and ITS2 regions of fungi were amplified from the total DNA extracted. We used the primers 341F (5′-CCTACGGGNGG CWGCAG-3′) and 806R (5′-GACTACHVGGGTATCTAATCC-3′) (Klindworth et al., 2013) for bacteria/archaea, and the ITS1f (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′) (Gardes and Bruns, 1993; Smith and Peay, 2014) for fungi. Samples were paired-ended sequenced (2 × 250 bp) on an Illumina MiSeq platform using the V2 kit (Illumina Inc.).

Data Analysis

The raw.fastq files were used to build a table of amplicon sequence variants (ASVs) with dada2 version 1.12 (Callahan et al., 2016). Briefly, using default parameters, the raw data quality was evaluated, filtered, and trimmed. The filtering parameters [maxN = 0, truncQ = 2, rm.phix = TRUE, maxEE = (2,2), and truncLen (235, 230)] were applied before inputting the filtered reads into dada2's parametric error model. The truncLen parameter was not applied for ITS1 and ITS2 reads since the expected sequence length is variable for fungi. Later, the forward and reverse reads were merged to obtain a full denoised sequence, and a higher-resolution table of amplicon sequence variants (ASVs) was constructed. Only ASVs with total abundances higher than 0.1% are reported. Chimeric sequences were detected and removed. Taxonomy was assigned to each ASV using the RDP ribosomal RNA gene database (version 11.5) for the 16S rRNA gene and with UNITE database (version 8.2) for fungal ITS. Sequences were matched the reference sequence with 100% identity.

Biochemical Analysis

Organic Acids Evaluation

Organic acids of coffee fruits were evaluated after 48 h of fermentation. Three grams of coffee fruits were vortexed in Falcon tubes containing 20 mL of 16 mM perchloric acid and Milli-Q water at room temperature (25°C) for 10 min. The resulted suspension (without the fruits) was transferred to another tube, centrifuged at 12,745 RCF for 10 min at 4°C to obtain the supernatant. The supernatant was transferred to a new tube, and then its pH value was adjusted to 2.11 using perchloric acid and recentrifuged under the same conditions. The supernatant from the second centrifugation was filtered through a 0.22 μm cellulose acetate membrane (Merck Millipore, Germany) and directly injected (20 μL) chromatographic column.

The samples were analyzed using a high-performance liquid chromatography (HPLC) system (Shimadzu Corp., Japan) equipped with a detection system consisting of an UV–Vis detector (SPD 10Ai) and a Shimpack SCR-101H (7.9 mm 30 cm) column operating at 50°C, which was used to achieve chromatographic separation of water-soluble acids at a flow rate of 0.6 mL min^{-1}. The acids were identified by comparison with the retention times of authentic standards. The quantification was performed using calibration curves constructed with standard compounds [malic and citric acid were purchased from Merck (Darmstadt, Germany), lactic and tartaric acid were purchased from Sigma-Aldrich (Saint Louis, MO, United States), acetic and succinic acids were purchased from Sigma-Aldrich, isobutyric and butyric acid were purchased from Riedel-deHaen (Seelze, Germany)]. All analyses were performed in duplicate.

Caffeine, Trigonelline, and Chlorogenic Acids by HPLC

The identification of caffeine, chlorogenic acid [5-CGA], and trigonelline was made using a Shimadzu liquid chromatography system (Shimadzu Corp., Japan) equipped with a C18 column, following the protocol proposed by Malta and Chagas (2009). 0.5 g of grounded coffee fruits were placed in tubes containing 50 mL Milli-Q water and boiled for 3 min to extract total compounds. Then the suspension was filtered through a 0.22 μm cellulose acetate membrane (Merck Millipore). Identification and quantitative analysis were performed using caffeine calibration curves, trigonelline, and 5-CGA (Sigma-Aldrich). All analyses were performed in duplicate.

Total Polyphenols and Antioxidant Activity

Coffee samples were defatted following the methodology described by Batista et al. (2016). One hundred fruits were grounded with liquid nitrogen per sample, then 4 g were weighted. Following, 20 mL of n-hexane (Merck) was added into the 4 g, vortexed for 5 min, and centrifuged at 4,200 × g for 10 min/4°C to separate the lipids from the grounded sample left in the supernatant. After discarding the supernatant, the same procedure was repeated three times. The resulted lipid-free samples were air-dried for 24 h to evaporate the residual organic solvent.

The polyphenols and antioxidants were extracted, according to Kim et al. (2018), with minor modifications. Fifty milliliter of distilled water at 90°C were added in a tube containing 2.75 g lipid-free ground coffee. Then, the mixture was left standing at room temperature (25°C) for 20 min. After that period, the mixture was filtered through a Whatman No. 2 filter paper.

Determination of Total Polyphenol Content (TPC)

The total polyphenol content (TPC) was determined by a spectrophotometric assay (UV-VIS Spectrum SP-2000 UV, Biosystems) following the Follin—Ciocalteau methodology (Singleton and Rossi, 1965). In brief, 500 μL of coffee extract, 2.5 mL of Folin–Ciocalteau reagent (10%), and 2.0 mL of Na_2CO_3 (4% w/v) were homogenized and incubated at room temperature (25°C), in the dark for 120 min. The absorbance of the samples was measured at 750 nm. The TPC concentrations were calculated based on the standard curve of gallic acid (ranging from 10 to 100 μg mL^{-1}) and expressed as milligrams of gallic acid equivalents per gram of ground coffee (mg GAE g^{-1}). All analyses were performed in triplicate.

Antioxidant Activity Assays

Two different methodologies were applied to measure the antioxidant activity of coffee extracts. In the first one, the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging assay was performed as follows: 0.1 mL of coffee extract was added to 3.9 mL of the DPPH radical solution (0.06 mM) and incubated at room temperature (25°C), in the dark for 120 min, then the absorbance was measured at 515 nm (Spectrophotometer UV-Vis Spectrum® SP-2000UV, Shanghai, China). Trolox was used as a standard. A calibration curve ($y = -0.0004x + 0.6636$) was assembled using a range of 10, 20, 30, 40, 50 and 60 μM Trolox with linearity $R^2 = 0.9999$ (Batista et al., 2016). The results were expressed as μM Trolox Equivalents (TE) per gram of ground coffee (μM TE g^{-1}).

The second assay was performed with a 2,2′-azinobis (3-ethylbenzothiazoline-6-suslfonic acid) (ABTS) stock solution reaction (7 mM) with potassium persulfate (140 mM). The mixture was left in the dark at room temperature (25°C) for 16 h before use. The ABTS solution was diluted in ethanol to an absorbance of 0.70 ± 0.05 at 734 nm. Thirty microliters of the coffee extracts were added to 3.0 mL of the ABTS radical solution, and after 6 min, the absorbance was measured. Trolox was used as a standard. A calibration curve ($y = -0.0003x + 0.6802$) was assembled using a range of 100, 500, 1,000, 1,500 and 2,000 μM Trolox with linearity of $R^2 = 0.9983$. The results were expressed as μM Trolox Equivalents (TE) per gram of ground coffee (μM TE g^{-1}).

Volatile Compounds

Volatile compounds were extracted from 48 h fermented coffee fruits using a headspace solid-phase microextraction (HS-SPME). Coffee fruits (2 g) were macerated with liquid nitrogen and placed in a 15 ml hermetically sealed vial. After equilibration at 60°C for 15 min, the volatile compounds were extracted at 60°C for 30 min. The desorption time on the column was 2 min.

The compounds were analyzed using a Shimadzu QP2010 GC model equipped with mass spectrometry (MS) and a silica capillary Carbo-Wax 20M (30 m × 0.25 mm × 0.25 mm) column. The operation conditions of analysis consisted of maintaining the oven temperature at 50°C for 5 min, then raised to 200°C at 8°C min^{-1} and maintained for 15 min. The injector and detector were kept at 230 and 200°C, respectively, and He carrier gas was maintained at a flow rate of 1.9 ml min^{-1}. The volatile compounds were identified by comparing their mass spectra against those available in the NIST11 library. The retention Index (RI) for each compound was calculated using an alkane series (C10–C40) compared with those found in the literature.

Statistical Analysis

Alpha and beta diversity analyzes were performed for the evaluated microbial communities. Each altitude richness and abundance were used to calculate the bacterial and fungal Shannon and Simpson diversity indices. Moreover, the relative abundance was calculated, and ASVs profiles were clustered for each altitude using the XLSTAT software (Addinsoft, version 2020.1.3). Bray-Curtis-based non-metric multidimensional scaling (NMDS) was used to evaluate the dissimilarities between the fungal community and organic acids and volatile compounds with the XLSTAT software (Addinsoft, version 2020.1.3).

The raw data normal distribution for statistical analysis was evaluated with the Shapiro-Wilk and Anderson-Darling tests. All values in the figures are expressed as averages. Standard deviations were calculated using the XLSTAT software (Addinsoft, version 2020.1.3). The Tukey test was run with $p \leq 0.05$ in the SISVAR software (Ferreira, 2014) to evaluate the difference in acid concentration, volatiles relative concentration, and antioxidants' concentration and activity. The Pearson correlation coefficient was used to calculate the correlations between the bacterial genera, acids, and volatile compounds, using Origin software (version 2020). The principal component analysis was run on all altitudes, acids, antioxidants, and volatile compounds using XLSTAT software (Addinsoft, version 2020.1.3).

RESULTS

Fruits Initial Sugar Content and Fermentation Temperature

The initial °Brix value from coffee fruits was between 18 and 19.3 (**Table 1**). The coffee mass temperature varied from 18 to 25°C at 48 h (**Table 1**). The environmental temperature varied from 8 to 23.1°C and relative moisture varied from 56.1 to 85% during fermentation.

Microbial Community Profile

A total of 63.966, 16.346, 42.238, and 19.727 filtered 16S rRNA partial gene sequences and 104.719, 194.033, 263.884, and 119.571 filtered ITS sequences were obtained for the altitudes 800, 1,000, 1,200, and 1,400 m, respectively.

Among the altitudes, 800 m had the highest bacterial richness with 18 genera assigned, and 1,000 m had the highest fungal richness with 166 species assigned. **Table 1** shows the bacterial and fungal diversity indices for all evaluated altitudes. In summary, we observed a tendency to decrease the alpha-bacterial diversity indices with the altitude increase (**Table 1**).

The altitudes ASVs profiles were clustered, as illustrated in **Figure 1**, and three groups were obtained for bacteria and fungi. The 800 m bacterial profile was very distant and different from the other altitudes. The 1,400 and 1,000 m profiles were grouped for bacteria and fungi, meaning they were the most similar. On the other hand, the fungal cluster showed that the 1,200 m profile was different from the other altitudes and close to the 800 m profile.

A total of 31 genera were assigned in the bacterial community, as shown in **Figure 2A**. Most sequences in the 800 m sample were assigned to genera *Gluconobacter* (19.8%), *Novosphingobium* (18.9%), and *Sphingomonas* (12.2%). As for the other altitudes, genera *Weissella* (32.7%; 1,000 m), *Sphingomonas* (36.2%; 1,200 m), and *Methylobacterium* (39.4%; 1,400 m) had the highest abundances.

The genera *Actinoplanes*, *Brevundimonas*, *Corynebacterium*, *Roseomonas*, *Phenylobacterium,* *Pseudonocardia*, and *Rhizorhabdus,* were only identified at 800 m, *Arthrobacter*, *Clavibacter*, *Fructobacillus*, and *Pirellula* were only identified

TABLE 1 | Fruits initial brix, coffee mass temperature, and microbial diversity indices.

Coffee altitude (m)	Initial brix (Bx)	Coffee mass temperature (°C)			Bacterial diversity indices		Eukaryotic diversity indices	
		0 h	24 h	48 h	Shannon	Simpson	Shannon	Simpson
800	18.6 ± 0.6	18 ± 0	22 ± 0.06	23 ± 0.15	2.281 ± 0.2	7.973 ± 1.7	3.334 ± 0.1	14.766 ± 2.0
1,000	18.6 ± 1.5	18 ± 0	24 ± 0.12	25 ± 0.06	2.018 ± 0.5	5.574 ± 2.2	2.721 ± 0.1	5.666 ± 0.3
1,200	19.3 ± 0.6	18 ± 0	20 ± 0.10	21.6 ± 0.06	2.005 ± 0.4	5.004 ± 2.0	0.924 ± 0.3	1.417 ± 0.2
1,400	18 ± 3.0	18 ± 0	22 ± 0.12	23 ± 0.15	1.661 ± 0.5	4.330 ± 1.3	2.983 ± 0.2	9.372 ± 1.0

Data are expressed as Mean ± SD.
SD, Standard deviation.

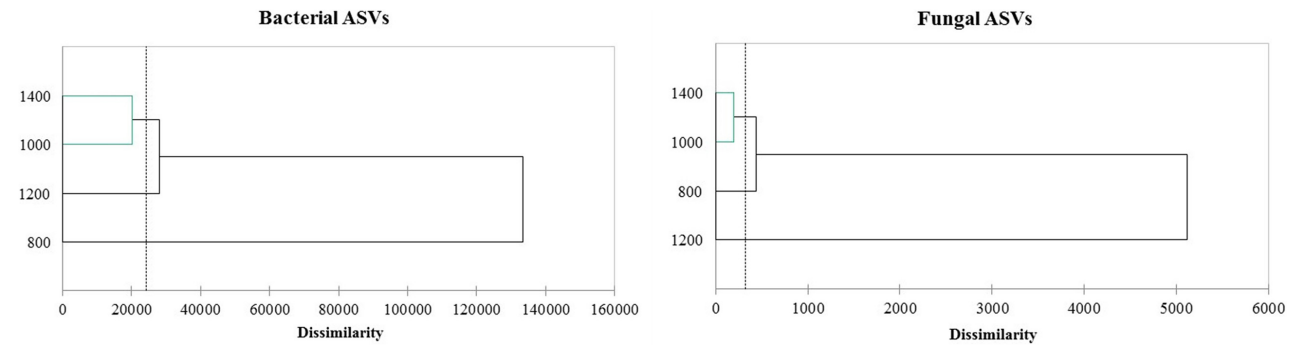

FIGURE 1 | Clustering of ASVs profile from amplicon sequence of the 16S and ITS region.

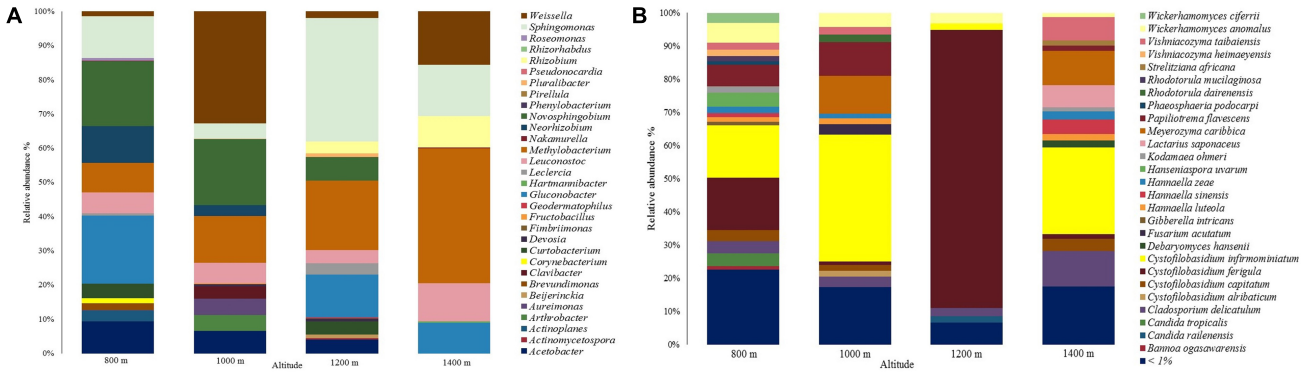

FIGURE 2 | Relative abundance of the microbial communities at different altitudes. **(A)** Bacterial community. **(B)** Fungal community.

at 1,000 m, *Beijerinckia*, *Pluralibacter*, *Actinomycetospora*, *Geodermatophilus*, and *Fimbriimonas* were only identified at 1,200 m, and *Nakamurella* and *Hartmannibacte*r at 1,400 m. *Sphingomonas*, *Methylobacterium*, *Leuconotoc*, and *Weissella* were found in all altitudes. Genus *Gluconobacter* was only identified in samples at 800, 1,200, and 1,400 m with relative abundances of 19.8, 12.5, and 9%, respectively.

Regarding the fungal community, a total of 223 species were assigned, showing a yeast predominance (**Figure 2B**). The most abundant species were *Cystofilobasidium infirmominiatum* (15.831%), *Cystofilobasidium ferigula* (15.700%), and *Papiliotrema flavescens* (6.571%) at 800 m. Following, *Cystofilobasidium infirmominiatum* (38.218%), *Meyerozyma caribbica* (11.445%), and *Papiliotrema flavescens* (10.271%) at 1,000 m, *Cystofilobasidium ferigula* (83.857%), *Wickerhamomyces anomalus* (3.216%), and *Cladosporium delicatulum* (2.539%) at 1,200 m, and *Cystofilobasidium infirmominiatum* (26.187%), *Cladosporium delicatulum* (10.817%), and *Meyerozyma caribbica* (10.216%) at 1,400 m. The species that were below 1% relative abundance are available in **Supplementary Material 1**.

Each altitude had a broad range of distinctive fungal species, which included *Candida sake*, *Sampaiozyma vanillica*, *Apiotrichum laibachii*, and *Citeromyces matritensis* for 800 m, *Fellomyces borneensis*, *Rhodotorula babjevae*, *Rhodotorula taiwanensis*, *Papiliotrema laurentii*, *Candida blattae*, *Wickerhamomyces pijperi*, *Cryptococcus saitoi*, *Rhynchogastrema complexa*, and *Cutaneotrichosporon terricola* for 1,000 m, *Papiliotrema perniciosus*, *Wickerhamomyces sydowiorum*,

Nakazawaea holstii, and *Eupenidiella venezuelensis* for 1,200 m, and *Neoascochyta paspali*, *Euteratosphaeria verrucosiafricana*, *Sporobolomyces johnsonii*, and *Rhodosporidiobolus ruineniae* for 1,400 m. The rest of the distinctive species identified are available in **Supplementary Material 2**.

We also identified frequently described species grouped in the species below 1% of abundance (**Supplementary Material 1**). Those include *Saccharomyces cerevisiae,* which was only identified in altitudes 1,000 (0.006%) and 1,200 m (0.003%), *Candida parapsilosis* in the same altitudes with 0.022 and 0.015%, and *Torulaspora delbrueckii* in altitudes 800, 1,000, and 1,200 m with 0.062, 0.036, and 0.005%. There were other yeasts like *Meyerozyma guilliermondii* (identified at 800 and 1,000 m: 0.904 and 0.130%), *Candida tropicalis* (lower abundances at 1,000, 1,200, and 1,400 m: 0.182, 0.162, and 0.156%), *Debaryomyces hansenii* (lower abundances at 800, 1,000, and 1,200 m: 0.282, 0.398, and 0.869%), *Pichia kluyveri*, *Debaryomyces nepalensis*, *Rhodotorula mucilaginosa* (only in altitudes 1,000, 1,200, and 1,400), *Candida orthopsilosis*, *Candida quercitrusa*, *Fellomyces mexicanus*, *Derxomyces anomalus*, and *Wickerhamomyces lynferdii*.

Filamentous fungi species such as *Aspergillus westerdijkiae*, *Alternaria argyroxiphii*, *Botrytis caroliniana*, *Cladosporium aphidis*, *Cladosporium halotolerans*, *Colletotrichum annellatum*, *Colletotrichum theobromicola*, *Fusarium asiaticum*, *Fusarium delphinoides*, *Fusarium proliferatum*, *Gibberella intricans*, *Lecanicillium antillanum*, *Penicillium kongii*, and *Penicillium solitum* were also identified.

Organic Acids

Effect of Altitude on Acids Content

Acetic, malic, citric, lactic, succinic, and tartaric acid concentrations were statistically different among the altitudes (**Figure 3A**). Acetic, malic, and citric acid concentrations at 1,400 and 1,200 m were higher than 1,000 and 800 m. When concentrations from 1,400 and 1,200 m were compared with the other altitudes in each acid, there were differences of 0.90–2.75 (acetic), 2.66–3.88 (malic), and 1.84–2.16 (citric) g. Kg^{-1}. As for altitudes 1,000 and 800 m, acetic (g. Kg^{-1}: 3.32 and 2.74), lactic (g. Kg^{-1}: 1.12 and 0.92), citric (g. Kg^{-1}: 1.10 and 0.91), and malic acid (g. Kg^{-1}: 0.78 and 0.92) were found in higher concentrations than succinic and tartaric acid. Within the acetic acid results, 1,200 m altitude presented the highest content (5.49 g. Kg^{-1}), and altitude 800 m presented the lowest content with 2.74 g. Kg^{-1}. Malic and succinic acid were significantly higher at 1,200 m (g. Kg^{-1}: 4.66 and 1.02). Citric and lactic acid were higher at 1,400 m (3.07 g. Kg^{-1} and 1.38 g. Kg^{-1}, respectively). Tartaric acid was only detected at 1,000 and 1,200 m with concentrations of 0.04 and 0.11 g. Kg^{-1}, respectively.

The PCA showed that 1,200 m and 1,400 m altitudes were correlated with citric, succinic, malic, and acetic acid (**Figure 3B**). 1,400 m altitude was characterized by lactic acid, while 1,200 m altitude was characterized by tartaric acid (**Figure 3B**).

Acids Correlation With Bacterial Community and Dissimilarity With Fungal Community

The Pearson correlation between acid content and bacterial community is depicted in **Figure 4A**. *Leuconostoc* showed a high positive correlation (0.93) with lactic acid content. Malic acid had the highest positive correlation (0.87) with the *Sphingomonas* genus. Acetic acid was positively correlated (0.87) with *Sphingomonas* and negatively correlated with *Acetobacter* (−0.64). The genera *Pluralibacter*, *Geodermatophilus*, *Fimbriimonas*, *Beijerinckia*, and *Actinomycetospora,* were highly positively correlated with tartaric acid (0.93 for all). Additionally, succinic and citric acid were highly positively correlated

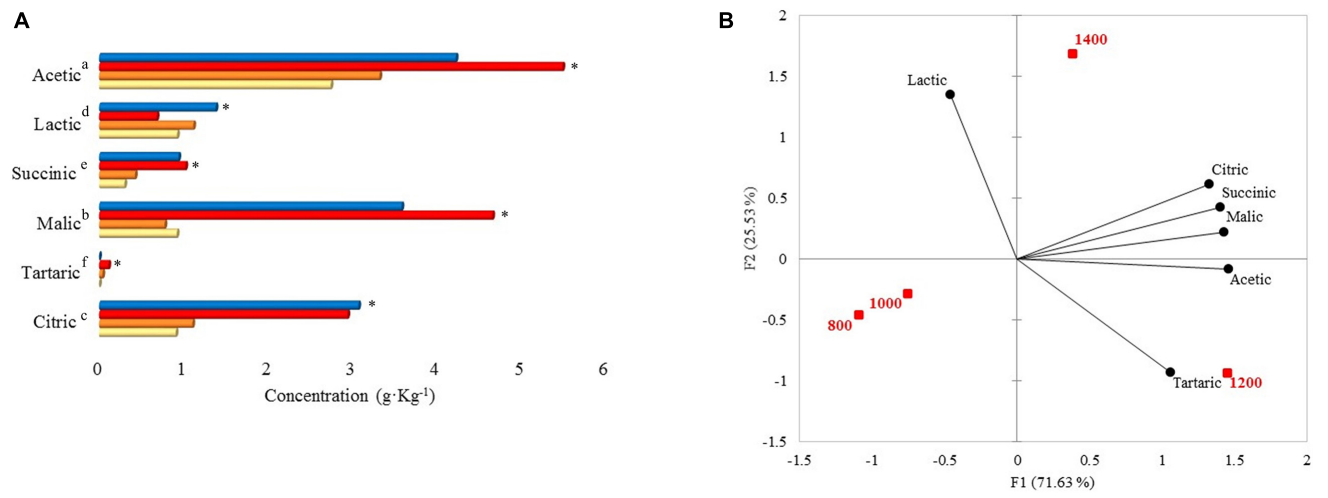

FIGURE 3 | (A) Organic acid concentrations at 48 h of fermentation on different altitudes. Each bar represents an altitude: 800, 1,000, 1,200, and 1,400 m. Significant values ($p \leq 0.05$) are represented in letters, from the highest amount to the lowest. *Altitude that was statistically significant ($p \leq 0.05$) and higher in contrast to the other altitudes. Citric, tartaric, malic, succinic, lactic, and acetic standard deviations: 800 m (0.19, not detected, 0.09, 0.01, 0.43, 0.44), 1,000 m (0.46, 0, 0.37, 0.10, 0.73, 1.57), 1,200 m (1.52, 0, 0.87, 0.41, 0.17, 1.95), 1,400 m (2.55, not detected, 1.97, 0.65, 1.11, 1.65). **(B)** Principal component analysis (PCA) plot of organic acids and altitudes.

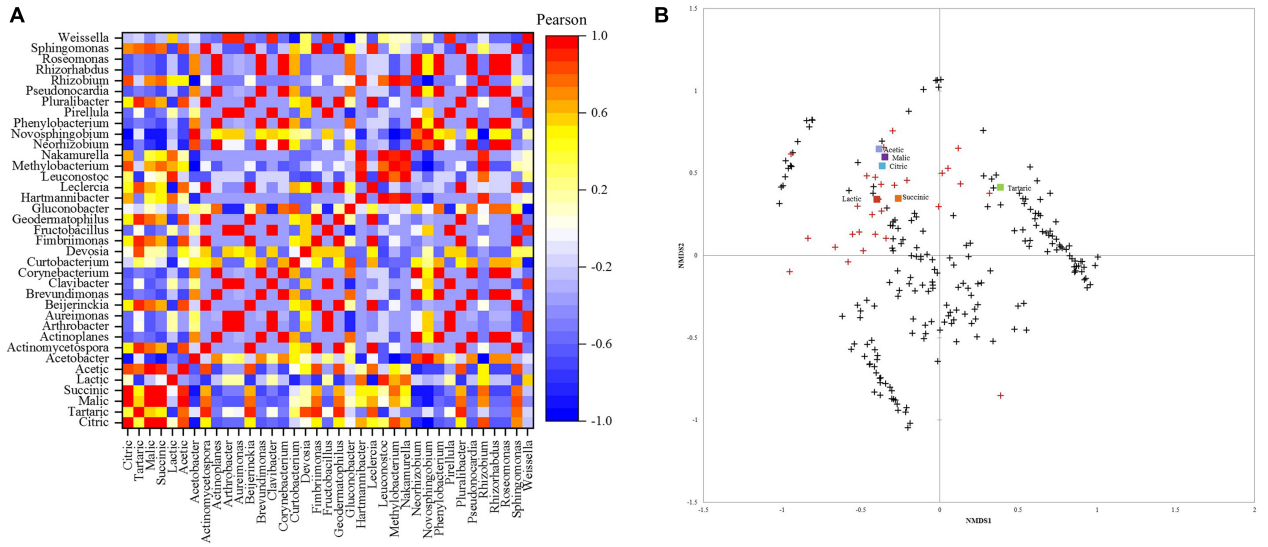

FIGURE 4 | (A) Pearson correlation matrix of bacterial genera and acids. **(B)** Non-metric multidimensional scaling (NMDS) using Bray-Curtis dissimilarity for eukaryotic species and organic acids. **+** species below 1% and **+** abundant species above 1%.

with *Rizhobium* (0.78, 0.87) and *Methylobacterium* with citric acid (0.83).

The NMDS plot (**Figure 4B**) showed that similar acetic, malic, citric, lactic, and succinic acid contents are shared between the fungal community, mainly in greater abundance. Most species with an abundance below 1% were close and different from the high abundance species and did not affect the acids in concentrations.

Volatile Compounds

Effect of Altitude on Volatiles

A total of 67 volatile compounds were detected. These compounds were classified as alcohols (19), esters (13), aldehydes (10), ketones (6), furans (5), phenols (4), pyrans (3), acids (3), alkanes (2), lactones (1), and pyrazines (1).

The total relative concentration of each chemical group was statistically significant. The following chemical groups had the most abundant relative concentrations: alcohols, phenols, aldehydes, lactones, and ketones. The alcohols 2-heptanol and 1,6-octadien-3-ol, 3,7-dimethyl- were the most significant among the other compounds with relative concentrations varying from 70.6 to 26.3 mg. g^{-1} and 37.5 to 11.8 mg. g^{-1} (**Figure 5A**). The relative concentration of these alcohols was higher at 1,400 and 800 m compared to the other altitudes, respectively. Moreover, methyl salicylate and benzoic acid, 2- hydroxy-, ethyl ester were the most abundant within the phenols group and both in altitudes at 800 m (27.4 and 3.3 mg. g^{-1}). Aside from the previous groups, other compounds such as benzeneacetaldehyde, benzaldehyde, 2(3H)-furanone, dihydro-3,5- dimethyl-, acetoin, and 2-propanone, 1-hydroxy- were the most abundant within the aldehydes, lactones, and ketones groups, at altitudes 1,400 (relative concentration: 16.1 and 10.1 mg. g^{-1}), 1,200 (10.8 mg. g^{-1}), 1,000 (20.1 mg. g^{-1}), and 800 (6.9 mg. g^{-1}) m. Some compounds were detected only in certain altitudes: 2-propanone, 1-hydroxy- and phenol, 4-ethyl-2-methoxy- at 800 m, 1-propanol, 2-methyl- and 2-decenal, (E)- at 1,000 m, 2(3H)-furanone, dihydro-3,5-dimethyl- at 1,200 m, and non-anoic acid at 1,400 m. Other compounds like (S)-3-ethyl-4-methylpentanol, benzyl alcohol, phenylethyl alcohol, 2,3-butanediol, [R-(R*, R*)]-, and benzyl acetate were detected.

The PCA graph in **Figure 5B** showed that around 36% (7) of those alcohols characterized 1,000 and 800 m altitudes from the total volatile alcohols. A total of 1,200 m altitude was characterized by the only lactone 2(3H)-furanone, dihydro-3,5-dimethyl-. A total of 1,400 m altitude was primarily characterized by esters (70%-7 from the total).

Volatiles Correlation With Bacterial Community and Dissimilarity With Fungal Community

The Pearson correlation between the volatile compounds and bacterial profile is depicted in **Figure 6A**. Methyl salicylate and 2-propanone, 1-hydroxy- were positively correlated (1) with the genera only found at 800 m altitudes (*Actinoplanes*, *Brevundimonas*, *Corynebacterium*, *Roseomonas*, *Phenylobacterium*, *Pseudonocardia*, and *Rhizorhabdus*). 2(3H)-furanone, dihydro-3,5-dimethyl- was positively correlated (1) with the genera only found at 1,200 m altitude (*Pluralibacter*, *Geodermatophilus*, *Fimbriimonas*, *Beijerinckia*, and *Actinomycetospora*). The genus *Weisella* had a strong positive correlation (0.99) with the ketone acetoin, 11,14,17-eicosatrienoic acid, methyl ester (0.98), and phenylethyl alcohol (0.97). The highest correlation (0.96) for benzeneacetaldehyde was with the species only found at 1,400 m altitude (*Nakamurella* and *Hartmannibacter*).

The NMDS plot (**Figure 6B**) showed that the species with the highest abundance within the fungal community might be producing similar contents of detected volatiles. Though not

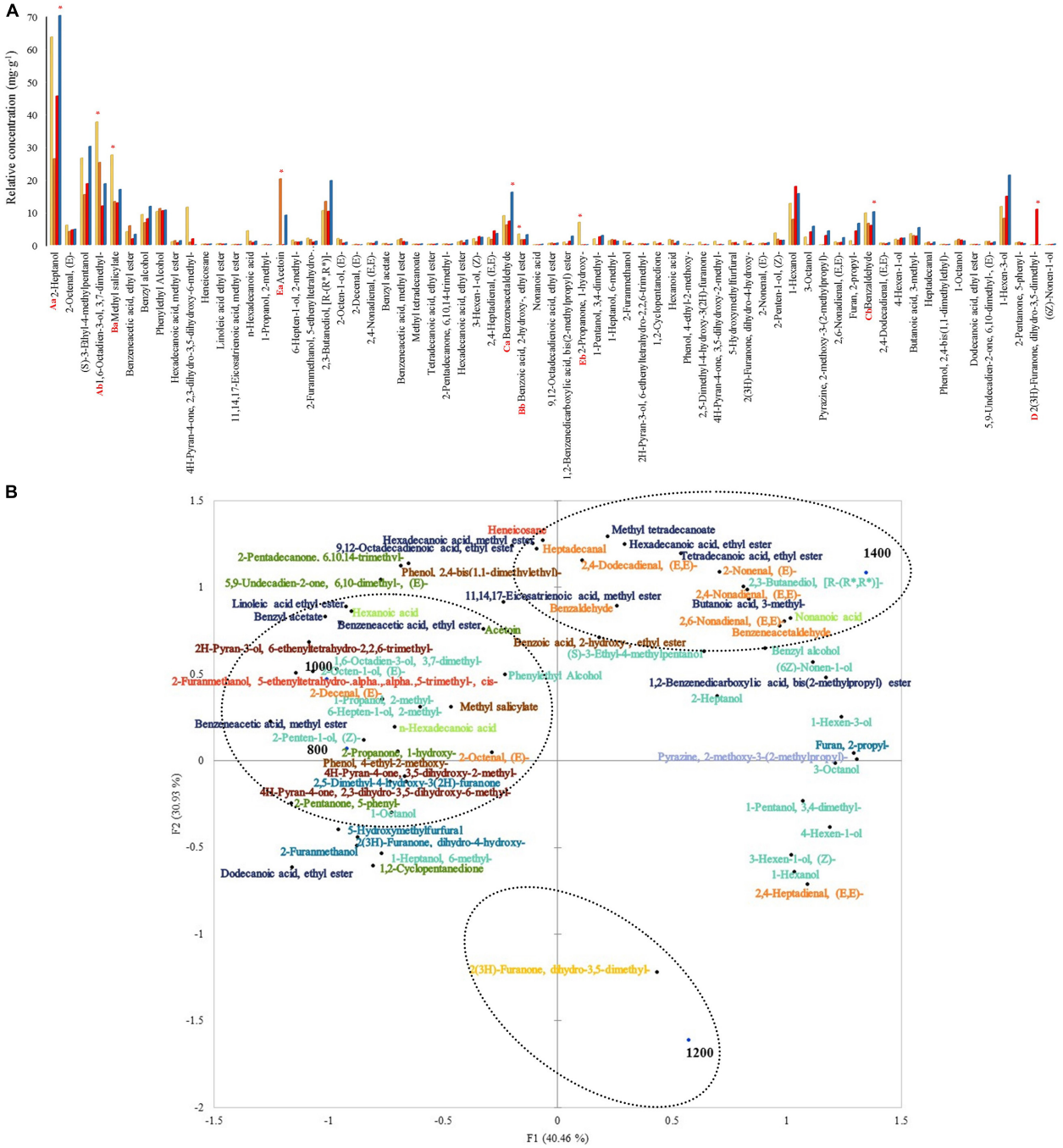

FIGURE 5 | (A) Volatile compounds relative concentrations at 48 h of fermentation on different altitudes. Each bar represents an altitude: 800, 1,000, 1,200, and 1,400 m. Significant values ($p \leq 0.05$) are represented in letters. From the highest amount to lowest, different uppercase letters represent the most predominant groups (A: alcohols, B: phenols, C: aldehydes, D: lactones, and E: ketones), and different lowercase letters represent the two most predominant compounds within the groups. * Altitude that was statistically significant ($p \leq 0.05$) and higher in contrast to the other altitudes. **(B)** Principal component analysis (PCA) plot of volatile compounds and altitudes. Volatile groups: acids, alcohols, aldehydes, alkanes, esters, furans, ketones, lactones, phenols, pyrans, pyrazines.

all the high abundance species affect the same volatile groups, some affect alcohols contents instead of aldehydes contents as observed in the plot. Most fungal species below 1% abundance are close and different from high abundance species and are grouped with low content volatiles (from 0 to 0.4 mg. g^{-1}) phenol, 2,4-bis(1,1-dimethylethyl)-, phenol, 4-ethyl-2- methoxy-, 11,14,17-eicosatrienoic acid, methyl ester, dodecanoic acid, ethyl ester, 2-decenal, (E)-, 1-propanol, 2- methyl-, and (6Z)-nonen-1-ol.

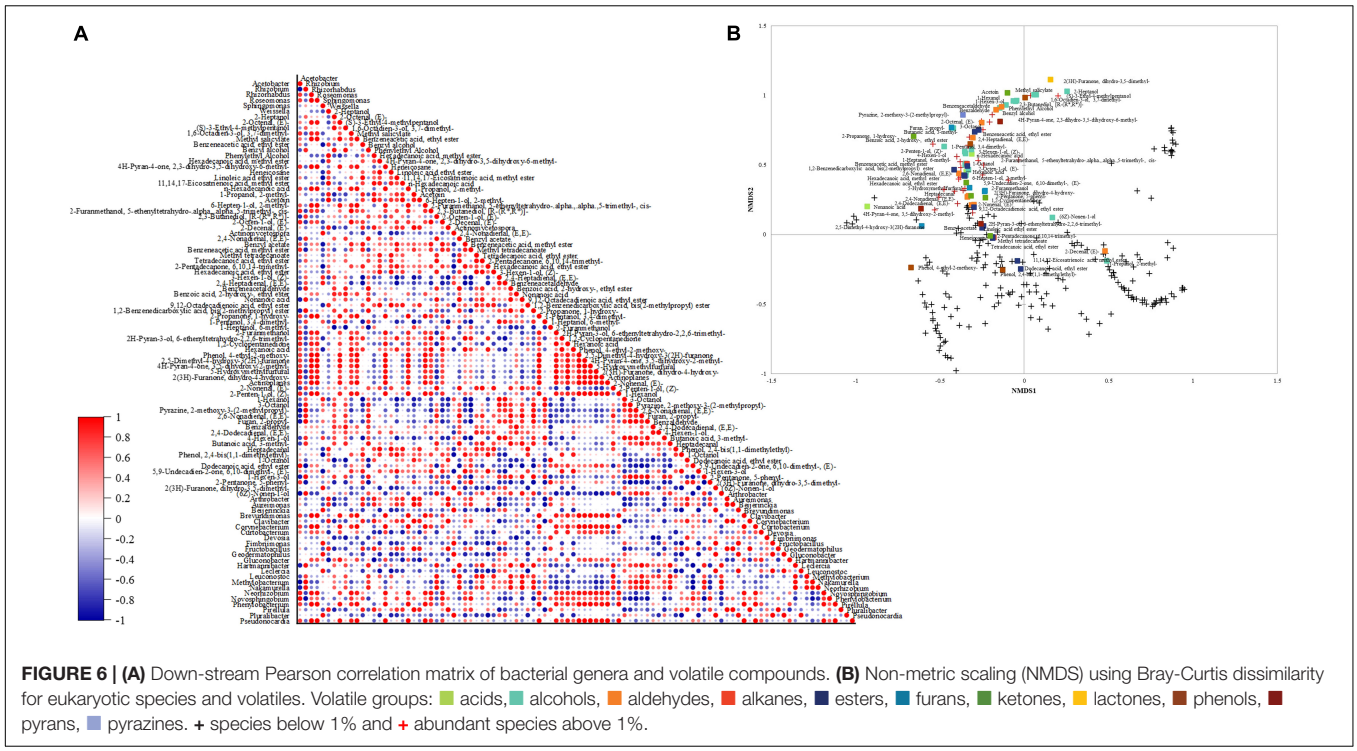

FIGURE 6 | (A) Down-stream Pearson correlation matrix of bacterial genera and volatile compounds. **(B)** Non-metric scaling (NMDS) using Bray-Curtis dissimilarity for eukaryotic species and volatiles. Volatile groups: acids, alcohols, aldehydes, alkanes, esters, furans, ketones, lactones, phenols, pyrans, pyrazines. + species below 1% and + abundant species above 1%.

Effect of Altitude on Caffeine, Chlorogenic Acids, Trigonelline, Total Phenolics Concentration, and Antioxidant Activity

Caffeine total concentration was higher (44.5%) than those of trigonelline (29.9%) and chlorogenic acid (25.6%), mainly at 1,200 m with a significant value of 11.64 g. Kg^{-1} (**Figure 7A**). Among the altitudes, trigonelline concentration was higher at 1,000 m (6.63 g. Kg^{-1}), and chlorogenic acid was higher at 800 m (9.70 g. Kg^{-1}). Total phenolics concentration was higher at 1,200 m with 203.90 mg. g^{-1}, followed by 1,400 m (160.12 mg. g^{-1}). Regarding the antioxidant activity, after the ABTS assay, samples at 1,000 and 1,200 m had the highest activity (331.72 and 340.16 μM TE. g^{-1}) compared to other altitudes, and after the DPPH assay, the highest value was reported at 1,000 m (95.01 μM TE. g^{-1}).

The PCA on **Figure 7B** displays the correlation between altitudes, antioxidants, and their activity. A total of 1,200 m altitude was characterized by ABTS activity, caffeine, and total phenolics due to the high concentrations detected at that altitude. A similar characterization was seen for 1,000 m, however, with DPPH activity. Total phenolics were grouped with caffeine and chlorogenic acid, and trigonelline was grouped with ABTS activity.

DISCUSSION

The aim was to characterize the microbial community and compounds profiles associated with fermented natural coffee from different altitudes. Out of the detected fungal community, yeast species were the most abundant possibly to the region's high relative moisture and temperatures, which were within our ranges. Furthermore, this region has rainy summers (November to January) and cold and dry winters (June to August). The average annual rainfall ranges from 1,000 to 1,500 mm, and the average annual temperature ranges from 19 to 22°C (Campanha et al., 2017). Other factors that probably influenced yeast occurrence were the temperature inside our mass that varied from 21.6 to 25°C and SIAF conditions, becoming beneficial for their growth.

The coffee's microbiota in this work varied at different altitudes. Lower altitudes favored bacterial richness, meaning that altitude is a factor that affects this microbial group. The high *Gluconobacter* abundances found in this work were also reported in a fermented natural processed coffee from Ecuador (De Bruyn et al., 2017). Therefore, the high abundances depend on the processing instead of the altitude. Acetic acid bacteria (AAB) are known to be strictly aerobic and capable of oxidizing alcohols, aldehydes, and sugars into carboxylic acids (Gomes et al., 2018). However, there was no correlation between *Gluconobacter* and acetic acid production in our work, but possibly other AAB, such as *Acetobacter,* were responsible for the high acetic acid production. A food fermentation with similar microbial dynamics as in coffee is cocoa (Schwan et al., 2015). Ho et al. (2018) have confirmed AAB's role in cocoa fermentation, which involves acetic acid production (primary acid involved in cocoa fermentation), pH increase, and volatiles production. A bacterial genus capable of producing acetic acid is *Weissella*, which might have aided with the acetic acid production in this study since the genus was present at all altitudes. However, *Weissella* belongs to

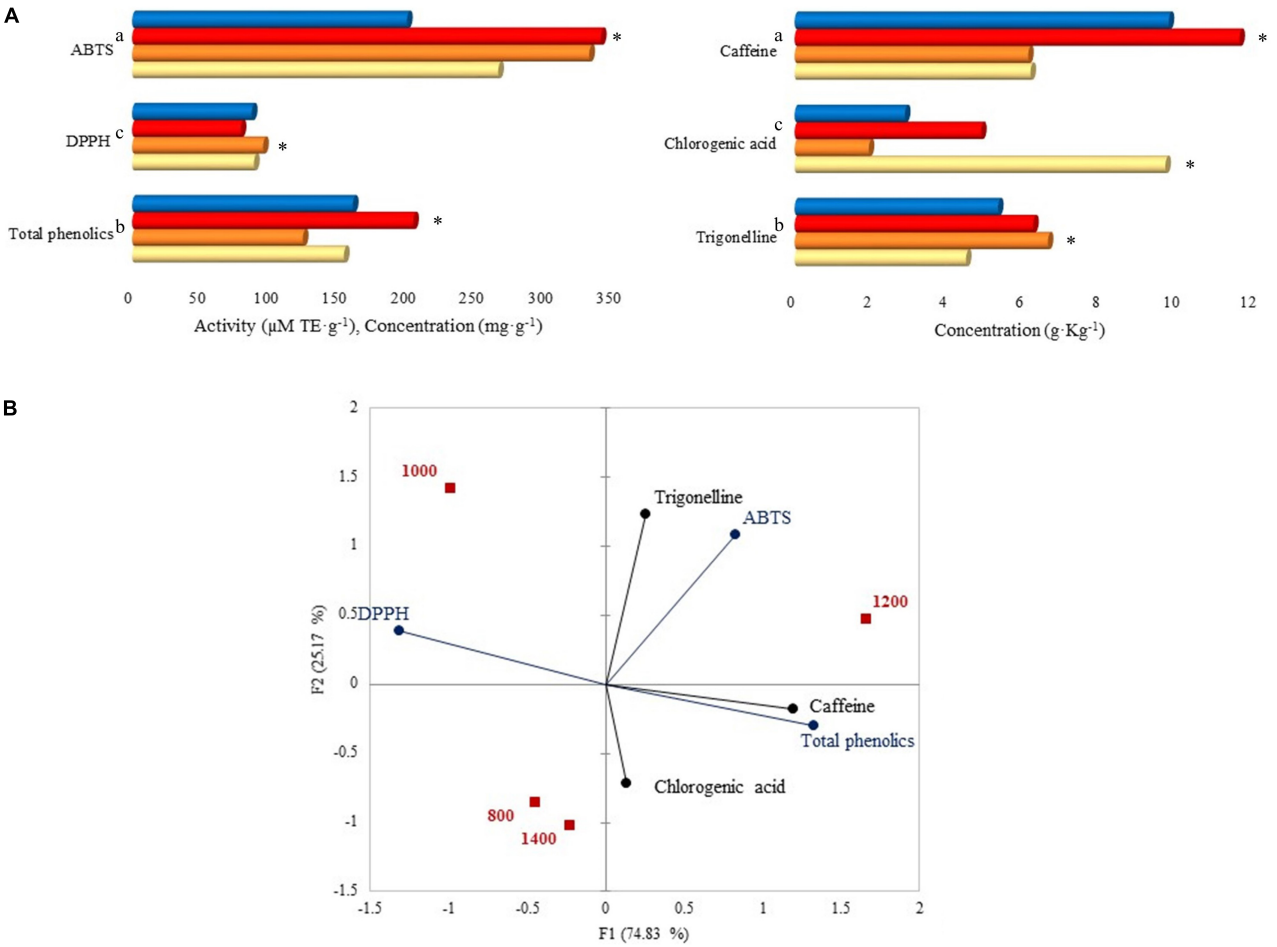

FIGURE 7 | (A) Caffeine, chlorogenic acid, trigonelline, total phenolics concentrations, and antioxidant activity by ABTS and DPPH assays. Each bar represents an altitude: 800, 1,000, 1,200, and 1,400 m. Statistically significant values ($p \leq 0.05$) are represented in letters, from the highest amount to the lowest. *Altitude that was statistically significant ($p \leq 0.05$) and higher in contrast to the other altitudes. Trigonelline, chlorogenic acid, caffeine, total phenolics, DPPH, and ABTS standard deviations: 800 m (1.62, 8.33, 5.21, 0.21, 0, 0.03), 1,000 m (0.78, 0.06, 1.17, 3.72, 0, 21.84), 1,200 m (0.03, 0.01, 0, 3.15, 0, 17.10), 1,400 m (0.08, 0.01, 0.02, 6.60, 0, 0.15). **(B)** Principal component analysis (PCA) plot of antioxidants, antioxidant activity, and altitudes.

the lactic acid bacteria (LAB) group and is heterofermentative (producing acetic acid and lactic acid) (Lorenzo et al., 2018). According to Martins et al. (2020), among the isolated LAB from the Caparaó region, *Weissella paramesenteroides* were more abundant in natural coffees. Moreover, this genus has been found in coffee fruits from Taiwan (Leong et al., 2014), a wet-processed coffee from Colombia (de Oliveira Junqueira et al., 2019), and an Ecuadorian natural processed coffee (De Bruyn et al., 2017).

The LAB *Leuconostoc* was detected at all altitudes and showed a strong positive correlation with lactic acid, suggesting that this genus may be responsible for its production. Prior works testing the *Leuconostoc* genus' potential in coffee have shown that they are incapable of producing pectinolytic enzymes (Avallone et al., 2002). However, they produce lactic acid as a primary compound during fermentation (De Bruyne et al., 2007). In coffee, species of *Leuconostoc* have been isolated from Ethiopian coffee fermentation (De Bruyne et al., 2007) and were abundant in a coffee fermentation performed at 1,329 m in Ecuador (De Bruyn et al., 2017).

For the first time, we report a high abundance of the Proteobacteria *Methylobacterium* in a natural processed coffee fermentation at 1,400 m. Limited information on its function during fermentation is provided, yet they are known as plant growth-promoting bacteria (Ponnusamy et al., 2017). There is no current information about their correlation with citric acid or the contribution to coffee fermentation. Yet, there was a positive correlation between *Methylobacterium* and citric acid, and where the highest content of this acid was detected, this genus was most abundant, possibly to its overproduction during Krebs Cycle. However, further studies must be done to understand their relation.

Similarly, it is the first time *Sphingomonas* (most abundant in 1,200 m fermented coffee), *Roseomonas*, *Fructobacillus*,

and *Nakamurella* are reported in natural coffee processed fermentations. Still, some have already been identified on a wet-processed coffee fermentation (De Carvalho Neto et al., 2018).

According to Martinez et al. (2019), bacteria are the primary acid producers in wet fermentation. Therefore, in this work, the bacterial community from dry-processed coffees was correlated with acids. Acetic acid significantly predominated coffees from the Caparaó region. Bressani et al. (2018) reported similar results in a different region: coffee was fermented with fewer hours and another variety Catuaí at an altitude of 750–800 m. Citric acid is expected to be significant because it is a primary compound produced by any microorganism and enhances fruity flavors. Overall, coffees from 1,200 and 1,400 m favor acetic, malic, and citric acid content (**Figure 3**). Therefore, altitude affects their concentration, and they are expected in higher concentrations because they positively contribute to the beverage acidity (Buffo and Cardelli-freire, 2004). No detection of butyric and propionic acid indicates that 48 h is a proper time for SIAF fermentation and guarantees non-production of off-flavors (Silva, 2015; Haile and Kang, 2019b). As observed in our results, 1,200 m favored tartaric acid production and was correlated with the bacteria genera only found at that altitude and not in the other altitudes, which means that they might be responsible for the tartaric acid production or they might have stimulated the other genera to produce. Until now, no reports have shown their capacity to produce tartaric acid. Detection of tartaric acid in coffee is positively favorable since it produces fruity flavors like wine (Dziezak, 2016).

The bacterial communities were also correlated with volatile compounds because they contribute to their production. In this work, *Weisella* was correlated with Acetoin, and both were abundant at 1,000 m. Also, Acetoin was only detected at that altitude, suggesting that this genus may induce its production or other microorganisms. The same behavior was observed for compounds Methyl salicylate, 2-Propanone, 1-hydroxy-, and Benzeneacetaldehyde in the other altitudes with their respective genera.

Filamentous fungi and yeasts also compose the microbial communities of coffee during fermentation. The fungal diversity varied depending on the altitude and diversity index. Genus *Cystofilobasidium* (yeast) was the most abundant in all altitudes during SIAF conditions. Among the genus, *Cystofilobasidium ferigula* occurrence was at all altitudes with different relative abundances. This species was formerly designated as *Cryptococcus ferigula* and has been previously isolated from leaves submerged in a stream from a natural park in Portugal (Sampaio et al., 2007). C*ystofilobasidium infirmominiatum* is naturally found in cold habitats (Hu et al., 2014), suggesting that its predominance is due to the region's characteristics and capability to resist low temperatures harvesting.

37.7% fungi were not present at all coffee growing altitudes, from which 17.16% represented the species that were only identified in coffee from 800 m, and 25.31, 12.29, and 5.74% in coffees from 1,000, 1,200, and 1,400 m. Therefore, even if coffee belongs to the same region, the altitude's influence on the niches was evident. Since most abundant fungi species were yeast, those who are culturable can be isolated, studied, and use as inoculants for future fermentations in the Caparaó region. For this purpose, yeasts in high abundance such as *Meyerozyma caribbica* and *Wickerhamomyces anomalus* can be further used. The capacity to produce polygalacturonase and pectin lyase enzymes from *Wickerhamomyces anomalus* has already been demonstrated (Haile and Kang, 2019a). *Saccharomyces cerevisiae* was dominant in other coffee-producing regions (Silva et al., 2000; Evangelista et al., 2014b; Bressani et al., 2018), but not in this work.

The species below 1% abundance were clustered together, meaning they were not as influential as higher abundance species. Consequently, the NMSD plots showed that high abundance microbiota influences acids and volatiles contents, which was also confirmed in the Pearson correlations. The same behavior was seen for tartaric acid but with low abundance species.

The relative abundance of most filamentous fungi was within the 1%, which was expected since their populations usually dominate after several drying days due to reduced water activity (Silva et al., 2008b).

As expected, the alcohol group in this work had the highest number of compounds and content, possibly due to the high yeast abundance. Yeast uses the nitrogen compounds from amino acids to produce a pool of volatile alcohols (Dzialo et al., 2017), including phenylethyl alcohol, one of the alcohols detected in all altitudes. Coffees processed via the natural method in Evangelista et al. (2014b) and Bressani et al. (2020) had alcohols as the leading group during fermentation, and most were related to fruity odors. Like Bressani et al. (2020), high contents of 1-hexanol, 2-heptanol, benzyl alcohol, and benzaldehyde were also detected here. These volatiles compounds are essential for tea aroma (Ho et al., 2015).

In coffee, either alcohols or esters are significant because they confer the most sensed odor descriptors. In this study, low altitudes and microbiota are strongly associated with volatile alcohols; these were also the altitudes with the highest bacterial and fungal richness and probably influenced the alcohol quantity. Simultaneously, high altitudes and their microbiota are strongly associated with high contents of aldehydes and esters.

Caffeine, chlorogenic acid, and trigonelline concentrations in our work were in the same range as those previously detected at 800 m in Bressani et al. (2018). Caffeine is crucial because it confers bitterness to the beverage (Sunarharum et al., 2014). As for chlorogenic acids, they are responsible for pigmentation, astringency, and the production of volatile phenols (Duarte et al., 2010; Sunarharum et al., 2014). Trigonelline is responsible for the overall sensory perception. Most importantly, they all exert antioxidant properties. After fermentation, the coffees from higher altitudes contained higher concentrations of caffeine. Total phenolics are mainly composed of tannins and partly chlorogenic compounds (Farah and Donangelo, 2006). With the obtained results, it was observed that the concentration of the chlorogenic acid was only a small part of total phenolics concentration, being supported when correlated (**Figure 7**).

Hence, the antioxidant activity depends on time, temperature, nature of the substance, and concentration of antioxidants or other compounds (Yashin et al., 2013). Concerning our fermented coffees, the altitude that contained the highest content of total phenolics (i.e., 1,200 m) was the altitude with the highest antioxidant activity when measured by ABTS.

CONCLUSION

This work microbial and chemical characterization revealed a new perspective of why coffee from the Caparaó region is different from other Brazilian regions. The altitude and other region characteristics drive shifts in the microbiota profile and abundance, favoring yeast communities during fermentation. Moreover, altitude and high abundance of microbiota affect acetic and citric acid concentration and volatile compounds. 800 m coffee favors bacterial richness, and 1,000 m favors fungal richness during fermentation under SIAF conditions. Yeast that resists low temperatures dominates the Caparaó region coffee's (mainly from genus *Cystofilobasidium*). Dominant microbiota from different altitudes and controlled conditions by SIAF fermentations are the main drivers of biochemical compounds. Coffee from lower altitudes has higher contents of volatile alcohols, while high altitudes have higher esters, aldehydes, and total phenolic contents. Besides, the AAB function in coffee is still unknown; future approaches implementing AAB as inoculants need to be studied.

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: NCBI SRA BioProject, accession no: PRJNA706460, https://www.ncbi.nlm.nih.gov/bioproject/PRJNA706460/.

AUTHOR CONTRIBUTIONS

RS contributed to the experimental design and revision and edition of the manuscript. JS contributed to the study design and data collection. VP supervised and contributed to the manuscript revision. SM collected and analyzed the data, performed the statistical analysis, and wrote the manuscript's first draft. 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.2021.671395/full#supplementary-material

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Debaryomyces hansenii Strains Isolated From Danish Cheese Brines Act as Biocontrol Agents to Inhibit Germination and Growth of Contaminating Molds

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The antagonistic activities of native *Debaryomyces hansenii* strains isolated from Danish cheese brines were evaluated against contaminating molds in the dairy industry. Determination of chromosome polymorphism by use of pulsed-field gel electrophoresis (PFGE) revealed a huge genetic heterogeneity among the *D. hansenii* strains, which was reflected in intra-species variation at the phenotypic level. 11 *D. hansenii* strains were tested for their ability to inhibit germination and growth of contaminating molds, frequently occurring at Danish dairies, i.e., *Cladosporium inversicolor, Cladosporium sinuosum, Fusarium avenaceum, Mucor racemosus,* and *Penicillium roqueforti.* Especially the germination of *C. inversicolor* and *P. roqueforti* was significantly inhibited by cell-free supernatants of all *D. hansenii* strains. The underlying factors behind the inhibitory effects of the *D. hansenii* cell-free supernatants were investigated. Based on dynamic headspace sampling followed by gas chromatography-mass spectrometry (DHS-GC-MS), 71 volatile compounds (VOCs) produced by the *D. hansenii* strains were identified, including 6 acids, 22 alcohols, 15 aldehydes, 3 benzene derivatives, 8 esters, 3 heterocyclic compounds, 12 ketones, and 2 phenols. Among the 71 identified VOCs, inhibition of germination of *C. inversicolor* correlated strongly with three VOCs, i.e., 3-methylbutanoic acid, 2-pentanone as well as acetic acid. For *P. roqueforti*, two VOCs correlated with inhibition of germination, i.e., acetone and 2-phenylethanol, of which the latter also correlated strongly with inhibition of mycelium growth. Low half-maximal inhibitory concentrations (IC_{50}) were especially observed for 3-methylbutanoic acid, i.e., 6.32–9.53 $\times$ 10^{-5} and 2.00–2.67 $\times$ 10^{-4} mol/L for *C. inversicolor* and *P. roqueforti*, respectively. For 2-phenylethanol, a well-known quorum sensing molecule, the IC_{50} was 1.99–7.49 $\times$ 10^{-3} and 1.73–3.45 $\times$ 10^{-3} mol/L for *C. inversicolor* and *P. roqueforti*, respectively. For acetic acid, the IC_{50} was 1.35–2.47 $\times$ 10^{-3} and 1.19–2.80 $\times$ 10^{-3} mol/L for *C. inversicolor* and *P. roqueforti*, respectively. Finally, relative weak inhibition was observed for 2-pentanone and acetone.

The current study shows that native strains of *D. hansenii* isolated from Danish brines have antagonistic effects against specific contaminating molds and points to the development of *D. hansenii* strains as bioprotective cultures, targeting cheese brines and cheese surfaces.

Keywords: *Debaryomyces hansenii*, antagonistic activities, biocontrol, contaminating molds, cheese brine

INTRODUCTION

Mold contamination is a major problem occurring in food processing, which not only leads to severe economic losses and food waste but also may influence food safety (Garnier et al., 2017). In the dairy industry, mold contamination can appear throughout the entire cheese production as cheeses come into contact with processing equipment and air. Mold growth is especially seen during cheese ripening, storage, and distribution which results in a reduction of cheese quality including visible and invisible defects such as mold colonization, off-flavors, and potential risk of mycotoxin formation (Kure and Skaar, 2019). As a consequence, antifungal compounds and potential biocontrol agents have received increasing interest to prevent the growth of contaminating molds on cheeses (Irlinger and Mounier, 2009).

Yeasts are considered relevant for biocontrol applications, due to their simple cultivation requirements and limited biosafety concerns (Freimoser et al., 2019). A number of yeast species have been proven to have antagonistic activities against molds, though mostly applied in the control of post-harvest diseases of fruits (Bencheqroun et al., 2007; Liu et al., 2013; Grzegorczyk et al., 2017; Czarnecka et al., 2019). Within food production, antagonistic yeasts have been reported on, e.g., meat and dairy products (Liu and Tsao, 2010; Andrade et al., 2014) but their application is lacking behind. A number of antifungal volatile organic compounds (VOCs) produced by biocontrol yeasts have been associated with fungal inhibition, i.e., several alcohols (2-phenylethanol, ethanol, 2-methyl-1-butanol and 3-methyl-1-butanol, 2-methyl-1-propanol, and isoamyl alcohol) (Fialho et al., 2010; Ando et al., 2012; Núñez et al., 2015; Contarino et al., 2019) and esters (ethyl acetate, isoamyl acetate, phenylethyl acetate, isobutyl acetate, 2-phenyl ethyl acetate, and ethyl propionate) (Masoud et al., 2005; Fialho et al., 2010; Choińska et al., 2020). Although several studies state the production of VOCs involved in the antifungal activities of biocontrol yeasts, only a few of the aforementioned single VOCs have been investigated in depth. Also other antifungal actions of antagonistic yeasts have been reported including the competition for space and nutrients between yeasts and molds (Andrade et al., 2014; Medina-Córdova et al., 2016) as well as killer toxins playing a role in the defense system of yeasts against molds (Santos and Marquina, 2004; Grzegorczyk et al., 2017).

A number of yeasts have been detected and isolated from dairy products and dairy environment (Fröhlich-Wyder et al., 2019). Among these, *D. hansenii* has qualified presumption of safety (QPS) status by the European Food Safety Authority (EFSA) (Koutsoumanis et al., 2020) making it suitable as a biocontrol yeast in dairy products. *D. hansenii* is a halophilic yeasts being dominating among yeast species associated with most cheese varieties (Gori et al., 2012). Previously, we found that *D. hansenii* is by far the most predominant yeast species isolated from Danbo cheese brines reaching $\geq$ 3.5 $\log_{10}$ CFU/mL (Haastrup et al., 2018). Moreover, *D. hansenii* is a highly heterogeneous species showing phenotypic differences at the strain level. Variations among strains include differences in the ability to assimilate and ferment different carbon sources, secretion of dissimilar lipases and proteases, and diverse preferable growth conditions (Petersen and Jespersen, 2004). Consequently, strain variations in antagonistic potential might be expected. Antifungal activities of *D. hansenii* against contaminating molds are reported for several foods including dry-cured meat products (Andrade et al., 2014; Núñez et al., 2015), fruits (Hernández-Montiel et al., 2010), and dairy products (Van Den Tempel and Jakobsen, 2000; Liu and Tsao, 2009; Lessard et al., 2012). In dairy products, *D. hansenii* is reported to reduce the growth of *Penicillium camemberti* (Lessard et al., 2012). Further, *D. hansenii* strains obtained from blue mold cheeses are able to weakly inhibit *P. roqueforti* under aerobic conditions (Van Den Tempel and Jakobsen, 2000). Up to now, several factors have been found to influence the antifungal efficiency of *D. hansenii*, including water activity (a_w), temperature, nutrient availability (Andrade et al., 2014; Núñez et al., 2015), and the concentration of molds (Liu and Tsao, 2009). Further, the production of metabolites varies among *D. hansenii* strains (Núñez et al., 2015; Grzegorczyk et al., 2017; Hernandez-Montiel et al., 2018). In previous studies, *D. hansenii* strains have been shown to exhibit varying antifungal actions (Van Den Tempel and Jakobsen, 2000; Medina-Córdova et al., 2018), however, further in-depth studies are required to explore these actions. We have previously determined the distinctive growth characteristics and NaCl tolerance of *D. hansenii* strains isolated from Danish cheese brines (Zhang et al., 2020), however, their potential abilities to inhibit mold contaminants from dairy environments have not, as yet, been explored.

This paper aims to evaluate the antifungal activities of *D. hansenii* strains isolated from Danish cheese brines against different contaminating molds. For this purpose, the effects of different *D. hansenii* strains were examined for their inhibitory capacity on germination and growth of contaminating molds including determination of the half-maximal inhibitory concentration (IC_{50}) of single VOCs.

MATERIALS AND METHODS

Yeast and Mold Strains, Media, and Growth Conditions

All *D. hansenii* strains used in this study were previously isolated and identified by Haastrup et al. (2018) from three different

Danish dairies; *D. hansenii* strains KU-9, KU-10, KU-11, and KU-12 were isolated from dairy A (vat1); KU-27, KU-28, KU-29, and KU-30 were isolated from dairy A (vat2); KU-72 was isolated from dairy B; KU-78 and KU-80 were isolated from dairy C (vat1 and vat2, respectively). *C. inversicolor*, *C. sinuosum*, and *F. avenaceum* were obtained from a Danish dairy and their identification was verified by Deutsche Sammlung von Mikroorganismen (DSM, Germany). *M. racemosus* DSM5266 (isolated from cheese) and *P. roqueforti* DSM1079 (isolated from cheese) were obtained from DSM. All molds were capable of growing on cheese agar, prepared according to Sørensen et al. (2011) at 25°C for 7 days (**Supplementary Figure 1**). Of these, *C. inversicolor*, *C. sinuosum*, *F. avenaceum*, and *P. roqueforti* reproduce asexually by the formation of conidia, while *M. racemosus* reproduce sexually by the formation of spores. In the following, spores will be used when referring to these reproductive cells of the five molds.

Yeast strains were propagated at 25°C in Malt Yeast Glucose Peptone broth added 4% (w/v) NaCl (MYGP added 4% (w/v) NaCl; per liter, 10 g D (+)-glucose monohydrate (Merck, Darmstadt, Germany), 5 g bactopeptone (BD, Detroit, MI, United States), 3 g yeast extract (BD), 3 g malt extract (BD), 40 g NaCl (Merck) with pH adjusted to 5.3 ± 0.1) or MYGP agar added 4% (w/v) NaCl (adding 20 g bacto agar (BD) to MYGP broth added 4% (w/v) NaCl). Mold species were routinely grown at 25°C in Malt Extract medium (MEB; per liter; 20 g malt extract (BD), 10 g D(+)-glucose monohydrate (Merck), 5 g bactopeptone (BD) with pH adjusted to 5.3 ± 0.1) or on malt extract agar (MEA, adding 20 g bacto agar (BD) to MEB broth components).

Preparation of Spores

Molds were cultured on MEA at 25°C for 7-14 days. Spores were suspended in saline peptone (SPO) solution (per liter; 5 g NaCl (Merck), 0.3 g $Na_2HPO_4{\cdot}2H_2O$ (Merck), 1 g bactopeptone (BD) with pH adjusted to 5.3 ± 0.1) containing 0.01% Tween 80 (Sigma-Aldrich, St. Louis, United States) followed by filtering through six layers of sterilized gauze to remove hyphal fragments. Spore concentrations were estimated by bright field microscopy (Olympus BX40, Japan) using a Neubauer counting chamber and adjusted to a final concentration of 10^4 spores/mL using SPO solution.

Pulsed-Field Gel Electrophoresis and Cluster Analysis of *D. hansenii* Strains

The DNA preparation of yeast strains and the running condition of pulsed-field gel electrophoresis (PFGE) were performed according to Petersen and Jespersen (2004). The gels were visualized with UV transillumination and photographed (alphaeasefc software, Alpha-InnoTec GmbH, Germany). Estimation of the band size was analyzed using the LabImage 1D, ver.7.1.3 software (Kapelan Bio-Imaging, Germany). The cluster analysis was carried out using BioNumerics version 7.1 (Applied Maths, Kortrijk, Belgium). The similarities between profiles of bands were determined using the fraction of shared bands (Dice coefficient), and the cluster analysis was calculated by the unweighted pair group method using arithmetic average linkage (UPGMA method).

Change in pH and Cell Counts of *D. hansenii*

All *D. hansenii* strains were grown in MYGP broth added 4% (w/v) NaCl overnight. $OD_{600\,nm}$ of the overnight cultures were measured using a spectrophotometer (UV 1800, Shimadzu, Japan) followed by dilution with fresh media at initial concentration of 0.01 ± 0.005 (OD_{600nm}). The cell cultures were grown in MYGP broth added 4% (w/v) NaCl for 72 h at 25°C. Measurements of pH were carried out using an electrode (In Lab 426, Mettler-Toledo, Glostrup, Denmark) connected to a pH meter (1120, Mettler-Toledo) and plate counting method on MYGP agar added 4% (w/v) NaCl was used to monitor the growth of *D. hansenii* at the initial and end time point.

Effect of *D. hansenii* Cell-Free Supernatants on the Growth of Molds

Cell-free supernatants were made for each *D. hansenii* strain in the stationary growth phase (cultured in MYGP broth added 4% (w/v) NaCl for 72 h at 25°C) by centrifugation (3000 × *g*, 10 min) followed by filtering the supernatants using 0.22 μm pore size filter (Frisenette ApS, Knebel, Denmark).

Aliquots of 100 μL of double-strength MEB containing 10^4 spores/mL plus 100 μL of the cell-free supernatant collected from each *D. hansenii* strains or 100 μL of Yeast Peptone (per liter, 5 g bactopeptone, 3.0 g yeast extract, 40 g NaCl, pH 5.3) as control were loaded into wells (96 microtiter plates, Corning, New York, America). The antifungal activities of cell-free supernatants against molds were determined by measuring the growth curve using oCelloScope™ Unisensor (Philips BioCell A/S, Denmark) at 25°C for 48 h. The oCelloScope detection system (objective, 4×) was described in detail by Fredborg et al. (2013). The image distance was 4.90 μm and the illumination exposure time was 2 ms. Time-lapse scanning microscopy through a fluid sample was conducted thereby generating a series of 6 images in each well, every 2 h. Growth curves were generated automatically by using the segmentation and extraction of surface areas (SESA) algorithm in UniExplorer (Philips BioCell A/S, Denmark).

Germination ratios of spores were analyzed by ImageJ (v1.51g-v1.51n; Fiji package). A spore with a germination tube longer than the spore itself was considered as a germinated spore. For each image, 90-120 spores were counted and relative germination ratios were calculated as follows:

$$\frac{g_1}{t_1} / \frac{g_0}{t_0} \times 100\%$$

where g_1 is the number of germinated spores added cell-free supernatant, t_1 is the total number of spores added cell-free supernatant, g_0 is the number of germinated spores without cell-free supernatant, t_0 is the total number of spores without cell-free supernatant. The time points for counting germination ratio were chosen according to the method from Trinci (1971).

Growth rate (μ) values were analyzed using the DMfit software available on the Combase website[1] based on the aforementioned growth curve values and the model proposed by Baranyi and Roberts (1994). Relative mycelium growth rate was calculated as follows:

$$\frac{\mu_1}{\mu_0} \times 100\%$$

where μ_1 is the growth rate added cell-free supernatant, μ_0 is the growth rate without yeast cell-free supernatant. The μ values fit with $R^2 > 0.9$ were considered valid data.

Determination of Volatile Compounds (VOCs) Based on GC–MS Spectrometry

Debaryomyces hansenii cell-free supernatants were prepared as described above. VOCs of supernatants were collected in a dynamic headspace sampling (DHS) system. Each sample contained 20 mL supernatant sample plus 1 mL 4-methyl-1-pentanol (5 ppm) as the internal standard. The gas flask equipped with a purge head was equilibrated at 37°C in a water bath with magnetic stirring (200 rpm), and then purged with nitrogen (100 mL/min, 20 min). VOCs were collected by Tenax-TA traps (250 mg, mesh size 60/80, Buchem BV, Apeldoorn, Netherlands). Then the traps were continually purged with nitrogen (100 mL/min, 10 min) to remove excess water. The dynamic headspace collection was carried out in duplicates for all samples. VOCs were analyzed using gas chromatography-mass spectrometry (GC–MS) as described by Liu et al. (2015) and were identified based on the commercial database (Wiley275.L, HP product no. G1035A). The identified compounds were confirmed by comparing with retention indices (RI) of authentic reference compounds or the average of retention indices reported in the literature. All identified VOCs were semi-quantified as peak areas in the total ion chromatogram (TIC).

Evaluation of Inhibitory Effects of Single Targeted VOCs on Molds

Volatile compounds were selected based on correlation between the relative peak area of each compound produced by *D. hansenii* and the inhibition data (relative germination ratio and relative mycelium growth rate obtained from the experiments described in the section "Effect of *D. hansenii* Cell-Free Supernatants on the Growth of Molds") through Spearman correlation analysis. Those with significant strong correlations ($P < 0.05$) were selected for the subsequent experiments and purchased from Sigma-Aldrich. The interpretation of the rank correlation was according to the rules in Akoglu (2018).

Aliquots of 200 μL of MEB containing 10^4 spores/mL supplemented with acetic acid, 3-methylbutanoic acid, acetone, 2-pentanone, and 2-phenylethanol (Sigma-Aldrich, St. Louis, MO, United States) to the final concentrations 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} mol/L were loaded into 96 well plates. Aliquots of 200 μL of MEB containing 10^4 spores/mL were included as controls. The antifungal activities of single identified VOCs were determined by measuring the growth curve using oCelloScope™ Unisensor at 25°C for 48 h. Relative germination ratios and relative mycelium growth rates were calculated as described above. In addition, IC_{50} of single targeted VOCs for each mold was calculated. Long-term impacts of the five single targeted VOCs on the formation of mycelial pellicles were evaluated by prolonged incubation of the 96-well microtiter plates at 25°C for 7 days with the concentrations of the five single targeted VOCs as listed above. By visual inspection inhibition of mycelial pellicle formation was scored.

Quantification of Inhibitory VOCs Produced by *D. hansenii* Strains

Standard curves based on matrix-spiked samples were performed for two acids (acetic acid and 3-methylbutanoic acid), one alcohol (2-phenylethanol), and two ketones (acetone, and 2-pentanone). The peak area of the quantifier ion was used for estimating the concentration of each aforementioned VOCs produced by *D. hansenii* strains.

Data Analysis

Data were expressed as mean ± standard deviation and compared using one-way ANOVA analyzed by SPSS 17.0. Comparison of means was performed using Duncan's multiple range test and the statistical significance was applied at the level $P < 0.05$. IC_{50} of single VOCs was analyzed by GraphPad Prism 5, while the Spearman correlation analysis was analyzed by R software ("Hmisc" package).

RESULTS

Debaryomyces hansenii Strains Isolated From Cheese Brines Had Huge Genetic Diversity

The chromosomal profiles of the 11 *D. hansenii* strains isolated from brines at three different Danish dairies are shown in **Figure 1** and bands sizes are reported in **Supplementary Table 1**. Among the strains, genetic diversity was evident. The numbers of chromosomal bands varied between five and seven with sizes from 0.98 to 3.14 Mb. A cluster analysis based on the chromosomal profiles divided the 11 *D. hansenii* strains into four clusters at a similarity level of 60% (**Figure 1B**). Nine of the *D. hansenii* strains had unique profiles, while the two strains in cluster III (KU-11 and KU-28) isolated from two different vats at dairy A (A1 and A2) had identical profiles. *D. hansenii* strains (KU-9, KU-10, KU-11, KU-12, KU-27, KU-28, KU-29, and KU-30) isolated from the two vats at dairy A (A1 and A2) were divided into three different clusters (cluster II, III, and IV). Further, strains from dairy C were in cluster I (KU-78 and KU-80), clearly separated from the remaining dairies. On the contrary, no clear separation could be observed between dairy A and B as they clustered together in cluster II (KU-9, KU-12, KU-30, and KU-72), though at low similarity.

[1] http://www.combase.cc/index.php/en/

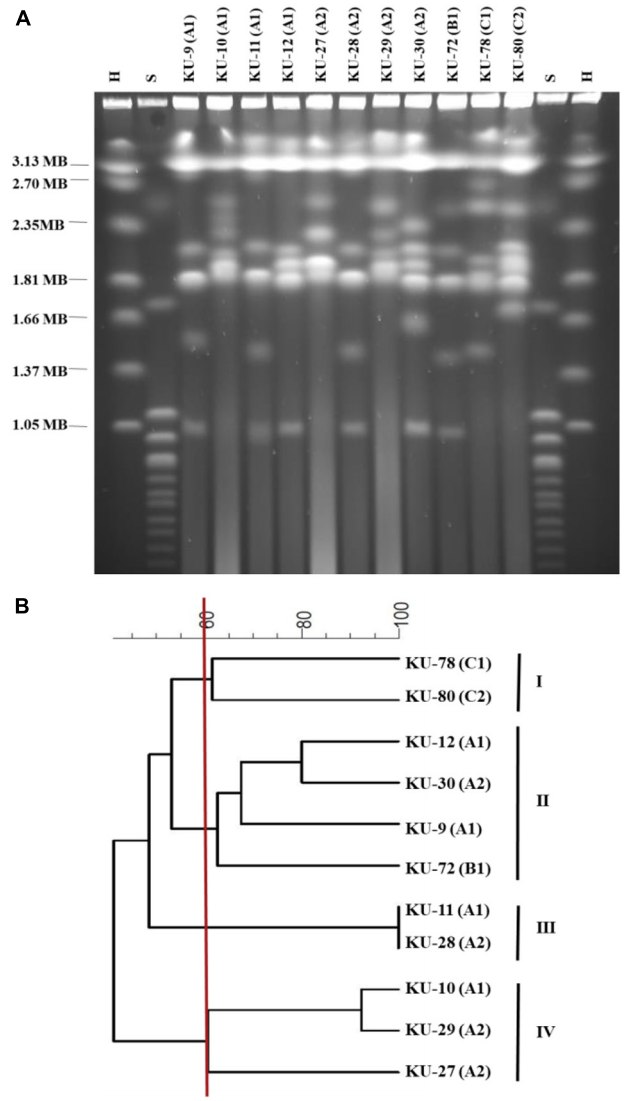

FIGURE 1 | (A) Chromosome polymorphism of *D. hansenii* strains isolated from Danish cheese brines. Pulsed-field gel electrophoresis (PFGE) was carried out at a 200 switch interval at 150 V for 24 h followed by a 700 s switch interval at 100 V for 48 h. Markers: H, *Hansenula wingei*; S, *Saccharomyces cerevisiae*. **(B)** Dice/UPMGA clustering dendrogram of *D. hansenii* strains.

Strain Variations in Acidification and Deacidification Abilities of *D. hansenii* Were Observed

Growth and changes in pH of *D. hansenii* strains grown in MYGP added 4% (w/v) NaCl for 72 h at 25°C are shown in **Table 1**. Examples of growth curves of *D. hansenii* strains are shown in **Supplementary Figure 2**. The initial inocula of the 11 *D. hansenii* strains were $0.7 \pm 0.2 \times 10^4$ CFU/mL and reached $4.2 \pm 1.7 \times 10^7$ CFU/mL after 72 h of growth. Even though all of the 11 *D. hansenii* strains grew to similar levels there were huge variations in their acidification abilities. After 72 h growth, acidification of MYGP from pH 5.3 to a minimum of 4.3 was observed for six *D. hansenii* strains (KU-9, KU-11, KU-12, KU-28, KU-30, and KU-72). Contrary, deacidification of MYGP from pH 5.3 to a maximum of 6.1 was observed for four *D. hansenii* strains (KU-10, KU-27, KU-29, and KU-78), whereas one strain (KU-80) did not significantly change the pH during the 72 h growth. Hence, compared to the cluster analysis, the genetic diversity and acidification/deacidification abilities were partly correlated. The acidifying strains were in clusters II and III, while cluster IV comprised three of the four deacidifying strains. Finally, cluster I contained one deacidifying strain (KU-78) and one strain that did not change pH (KU-80), however, rather high genetic differences between the two strains were detected at 62%.

TABLE 1 | Growth of *D. hansenii* strains and change in pH of MYGP broth added 4% (w/v) NaCl after 72h inoculation at 25°C.

PFGE Cluster	Strain	Growth (CFU/mL) after 72h	pH after 72 h
I	KU-78	$3.9 \times 10^7 \pm 0.5 \times 10^{7a}$	6.1 ± 0.0^{f}
I	KU-80	$8.3 \times 10^7 \pm 0.5 \times 10^{7c}$	5.2 ± 0.1^{d}
II	KU-12	$3.5 \times 10^7 \pm 0.9 \times 10^{7a}$	4.5 ± 0.0^{b}
II	KU-30	$2.7 \times 10^7 \pm 0.7 \times 10^{7a}$	4.8 ± 0.0^{c}
II	KU-9	$4.0 \times 10^7 \pm 0.2 \times 10^{7a}$	4.3 ± 0.2^{a}
II	KU-72	$3.4 \times 10^7 \pm 0.4 \times 10^{7a}$	4.5 ± 0.1^{b}
III	KU-11	$3.7 \times 10^7 \pm 0.6 \times 10^{7a}$	4.5 ± 0.1^{b}
III	KU-28	$2.6 \times 10^7 \pm 0.5 \times 10^{7a}$	4.6 ± 0.1^{ab}
IV	KU-10	$3.9 \times 10^7 \pm 0.7 \times 10^{7a}$	6.1 ± 0.1^{f}
IV	KU-29	$4.3 \times 10^7 \pm 0.6 \times 10^{7a}$	5.9 ± 0.1^{e}
IV	KU-27	$6.2 \times 10^7 \pm 0.6 \times 10^{7b}$	6.1 ± 0.1^{f}

Values in each line marked by different lowercase letters are significantly different using one-way ANOVA with Duncan's test (≥95% confidence, n = 3). Clusters are based on chromosome polymorphism between the stains as determined by PFGE.

Debaryomyces hansenii Cell-Free Supernatants Affected Germination and Mycelium Growth of Contaminating Molds in a Mold Species Dependent Manner

Effects on germination ratios and mycelium growth rates of the five molds when exposed to *D. hansenii* cell-free supernatants collected from stationary phase cultures are shown in **Table 2**. For the germination ratios, the highest significant ($P < 0.05$) inhibition, among the five molds, was observed for *C. inversicolor*, with germination ratios below 5% when exposed to cell-free supernatants of all *D. hansenii* strains. For *P. roqueforti*, cell-free supernatants of *D. hansenii* KU-9 and KU-11 showed significant strong inhibition on the germination ratio to 9.5% and 6.8%, respectively, compared to cell-free supernatants of the other *D. hansenii* strains (13.7-33.8%). Slight inhibition by *D. hansenii* cell-free supernatants was observed on the germination ratio of *C. sinuosum* (82.9%). Contrary, no significant ($P > 0.05$) inhibitory effect on the germination ratios of *F. avenaceum* and *M. racemosus* was observed for any of the *D. hansenii* cell-free supernatants.

For mycelium growth rates, corresponding to exponential growth phase of molds, cell-free supernatants of all *D. hansenii*

TABLE 2 | Germination ratios (%) and mycelium growth rates (μ/h) of molds in presence of *D. hansenii* cell-free supernatants as compared to the control.

		***D. hansenii* strains**										
PFGE cluster		**I**	**I**	**II**	**II**	**II**	**II**	**III**	**III**	**IV**	**IV**	**IV**	
Mold growth	**Mold species**	**KU-78**	**KU-80**	**KU-12**	**KU-30**	**KU-9**	**KU-72**	**KU-11**	**KU-28**	**KU-10**	**KU-29**	**KU-27**	**Average**
Germination ratio	*C. inversicolor*	0.4 ± 0.4^{a}	0.0 ± 0.0	0.8 ± 0.4^{ab}	0.8 ± 0.8^{ab}	4.5 ± 0.6^{d}	0.4 ± 0.4^{a}	1.9 ± 0.4^{bc}	1.9 ± 1.1^{bc}	1.4 ± 0.9^{abc}	1.1 ± 0.0^{ab}	0.4 ± 0.4^{a}	1.2 ± 1.5
	C. sinuosum	74.6 ± 6.6^{ab}	91.6 ± 12.6^{bcd}	83.6 ± 6.5^{abc}	98.3 ± 18.3^{bcd}	82.0 ± 26.0^{abc}	86.7 ± 2.2^{bc}	74.6 ± 11.1^{ab}	88.7 ± 1.1^{bc}	67.3 ± 11.0^{a}	86.9 ± 5.9^{bc}	77.9 ± 11.0^{ab}	82.9 ± 19.6
	F. avenaceum	102.6 ± 2.1^{ab}	96.1 ± 3.6^{a}	99.6 ± 0.5^{ab}	97.3 ± 4.1^{ab}	98.8 ± 4.4^{ab}	98.4 ± 2.3^{ab}	99.4 ± 4.0^{ab}	100.6 ± 2.8^{ab}	102.7 ± 1.1^{b}	99.3 ± 2.8^{ab}	97.5 ± 2.6^{ab}	99.3 ± 4.8
	M. racemosus	103.8 ± 1.2^{bc}	100.8 ± 2.9^{bc}	100.3 ± 1.9^{b}	104.0 ± 1.0^{c}	101.9 ± 1.0^{bc}	104.5 ± 3.3^{c}	92.8 ± 2.3^{a}	101.4 ± 1.8^{bc}	103.6 ± 3.9^{bc}	99.0 ± 2.9^{b}	105.2 ± 3.1^{c}	101.6 ± 4.9
	P. roqueforti	16.2 ± 0.6^{bc}	27.3 ± 5.4^{cd}	14.9 ± 1.3^{b}	20.2 ± 4.1^{c}	9.5 ± 2.9^{ab}	13.7 ± 2.9^{b}	6.8 ± 0.5^{a}	20.0 ± 2.3^{c}	26.1 ± 1.4^{cd}	33.1 ± 6.8^{d}	33.8 ± 4.8^{d}	20.2 ± 11.4
Growth rate	*C. inversicolor*	56.7 ± 14.0^{ab}	50.9 ± 8.2^{a}	79.8 ± 14.5^{bcd}	78.1 ± 8.2^{bcd}	59.7 ± 7.7^{ab}	71.5 ± 9.2^{bcd}	64.1 ± 6.0^{abc}	80.6 ± 11.9^{cd}	63.4 ± 6.3^{abc}	75.5 ± 11.9^{bcd}	78.0 ± 11.4^{bcd}	69.3 ± 13.6
	C. sinuosum	98.0 ± 6.9^{a}	104.2 ± 4.4^{abc}	102.9 ± 3.7^{abc}	106.8 ± 5.5^{abc}	102.9 ± 3.7^{abc}	111.7 ± 7.8^{c}	99.5 ± 5.8^{ab}	100.5 ± 6.3^{abc}	101.3 ± 7.9^{abc}	98.8 ± 4.4^{a}	99.5 ± 5.8^{ab}	102.4 ± 6.6
	F. avenaceum	109.5 ± 4.8^{d}	96.1 ± 5.9^{ab}	102.9 ± 5.9^{abcd}	99.1 ± 4.1^{abc}	104.1 ± 5.3^{bcd}	100.5 ± 2.5^{abc}	92.1 ± 5.9^{a}	100.0 ± 1.0^{abc}	109.6 ± 6.0^{d}	100.6 ± 4.2^{abcd}	103.7 ± 6.0^{abcd}	101.9 ± 6.8
	M. racemosus	90.2 ± 4.6^{bc}	83.1 ± 11.3^{abc}	91.2 ± 4.5^{c}	90.3 ± 2.2^{bc}	82.2 ± 10.3^{abc}	90.2 ± 3.5^{bc}	75.8 ± 9.8^{a}	87.5 ± 1.6^{abc}	89.0 ± 3.9^{bc}	86.8 ± 4.1^{abc}	87.9 ± 4.5^{abc}	86.8 ± 7.4
	P. roqueforti	85.7 ± 3.5^{cd}	84.9 ± 3.7^{bcd}	86.0 ± 4.1^{cd}	86.0 ± 2.7^{cd}	70.0 ± 6.1^{a}	83.0 ± 6.2^{bcd}	77.0 ± 6.3^{ab}	82.0 ± 4.0^{bcd}	85.0 ± 2.3^{bcd}	85.5 ± 3.4^{cd}	88.0 ± 3.1^{d}	83.0 ± 6.3

Values in the same row marked by different lowercase letters are significantly different using one-way ANOVA with Duncan's test (≥95% confidence, n = 6). Clusters are based on chromosome polymorphism between the stains as determined by PFGE.

strains induced inhibition of *C. inversicolor* (69.3%) while slight inhibition of *P. roqueforti* (83.0%) and *M. racemosus* (86.8%) was observed. Mycelium growth rates of *C. sinuosum* and *F. avenaceum* were not affected by any of the *D. hansenii* cell-free supernatants.

The limited effect of cell-free supernatants collected from stationary phase cultures of *D. hansenii* strains were additionally confirmed by the growth curves of the molds (**Figure 2**). Hence, for *C. inversicolor* and *P. roqueforti*, the time point of reaching stationary phase was delayed by the *D. hansenii* cell-free supernatants, while no inhibitory effect was observed for *C. sinuosum*, *F. avenaceum*, and *M. racemosus*. Moreover, at the end of incubation (i.e., 48 h), the *D. hansenii* cell-free supernatants did not lead to reduced mycelium growth of any of the five tested molds. Overall, these results showed that the *D. hansenii* cell-free supernatants predominantly influenced the germination phase in a mold species dependent manner, rather than in a *D. hansenii* strain dependent manner.

Debaryomyces hansenii Produced Antifungal VOCs in a Strain Dependent Manner

To elucidate the mechanisms of action involved in the antagonistic activities, the VOCs produced by stationary phase cultures of the 11 *D. hansenii* strains were determined. In total, 71 metabolites were detected by GC-MS and identified, i.e., 6 acids, 22 alcohols, 15 aldehydes, 3 benzene derivatives, 8 esters, 3 heterocyclic compounds, 12 ketones, and 2 phenols (**Supplementary Table 2** lists all detected VOCs). The relative contents of each metabolite group are shown in **Table 3**. Variations in VOC contents were seen among the *D. hansenii* strains. Especially acids, alcohols, benzene derivatives, esters, ketones, and phenols were among the produced VOCs. Significantly decreased content of aldehydes was detected for all *D. hansenii* strains, except KU-80, while all *D. hansenii* strains had decreased content of heterocyclic compounds. Moreover, significant contents of acids and ketones were produced by *D. hansenii* strains (KU-12 and KU-30). While some *D. hansenii* strains produced significant contents of benzene derivatives (KU-9, KU-11, and KU-27), esters (KU-78, KU-9, KU-10, KU-29, and KU-27), and phenols (KU-11). Hence, strain-dependent VOCs profiles were obtained from the *D. hansenii* strains, however, no correlation between VOC profiles and the genetic clusters from the PFGE could be observed.

To better understand the correlation between the VOCs produced by the *D. hansenii* strains and their antifungal behaviors, Spearman's rank correlation analysis was applied. The results of the correlation and coefficient analysis are shown in **Table 4** and **Supplementary Figure 3**. Among the 71 VOCs, acetic acid, 3-methylbutanoic acid, and 2-pentanone exhibited a strong negative correlation with germination ratios for *C. inversicolor*. For *P. roqueforti*, 2-phenylethanol had a strong negative correlation with both germination ratio and mycelium growth rate, while acetone had a strong negative correlation with germination ratio. Thus, these five VOCs were chosen as targeted single VOCs in the following experiments.

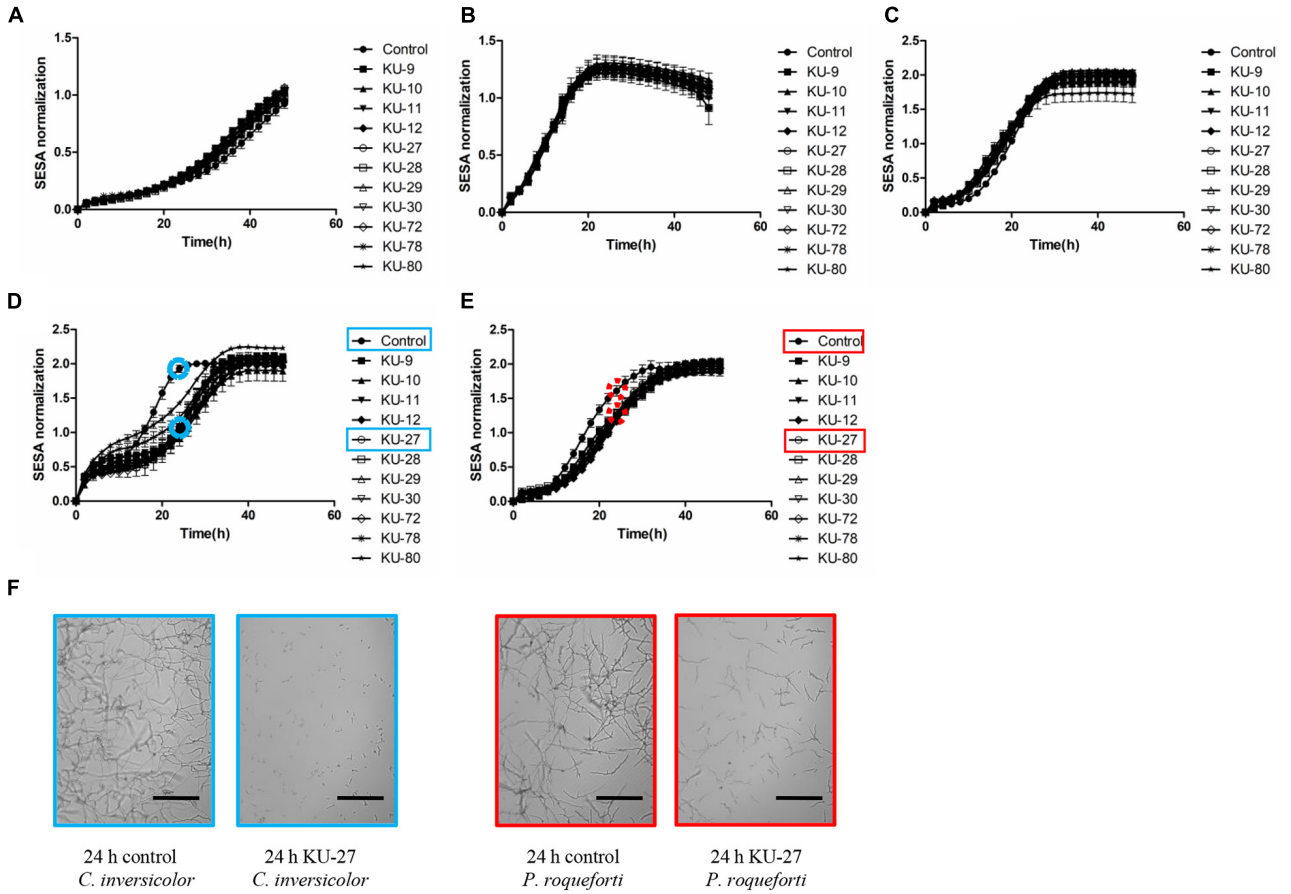

FIGURE 2 | Effects of cell-free supernatants from *D. hansenii* strains KU-9, KU-10, KU-11, KU-12, KU-27, KU-28, KU-29, KU-30, KU-72, KU-78 and KU-80 on the growth of five mold species measured as Normalized SESA units using oCelloScope^{TM}. **(A)** *Cladosporium sinuosum*, **(B)** *Fusarium avenaceum*, **(C)** *Mucor racemosus*, **(D)** *Cladosporium inversicolor*, **(E)** *Penicillium roqueforti*. Each spot represents the mean values from three independent experiments, and the error bars represent the standard deviation. **(F)** Examples of oCelloScope^{TM} images of growth measurements of *C. inversicolor* (blue) and *P. roqueforti* (red) in yeast peptone media (control) or in the presence of cell-free supernatants of *D. hansenii* strain KU-27 at 24 h. Scale bar: 300 μm.

Targeted Single VOCs Inhibited Germination, Mycelium Growth, and the Formation of Mycelial Pellicles of Contaminating Molds

The inhibitory effects of targeted single VOCs were examined successively at different growth stages of *C. inversicolor* and *P. roqueforti*, i.e., on germination ratios and mycelium growth rates within 48 h (**Figure 3** and **Supplementary Figure 4**). Based on germination ratios and mycelium growth rates in **Figure 3**, half-maximal inhibitory concentrations (IC_{50}) were calculated for each growth stage, respectively (**Table 5**).

For the germination ratios, gradually increasing concentration of 3-methylbutanoic acid, 2-phenylethanol, acetic acid, 2-pentanone, and acetone, respectively, were needed to obtain IC_{50} for *C. inversicolor* and *P. roqueforti*. For 3-methylbutanoic acid, IC_{50} were obtained at 9.53×10^{-5} and 2.00×10^{-4} mol/L for *C. inversicolor* and *P. roqueforti*, respectively. For 2-phenylethanol, IC_{50} were 1.99×10^{-3} and 1.73×10^{-3} mol/L for *C. inversicolor* and *P. roqueforti*, respectively. For acetic acid, IC_{50} were 2.47×10^{-3} and 2.80×10^{-3} mol /L for *C. inversicolor* and *P. roqueforti*, respectively. 2-pentanone had an inhibitory effect on germination ratios of *C. inversicolor* and *P. roqueforti* with an IC_{50} of 1.06×10^{-2} and 2.60×10^{-2} mol/L, respectively. Finally, acetone exhibited almost no inhibitory effect on germination ratio of *C. inversicolor* within the experimental period (48 h) but weakly inhibited germination ratio of *P. roqueforti* at IC_{50} of 9.21×10^{-2} mol/L.

For mycelium growth rates, gradually increasing concentrations of 3-methylbutanoic acid, acetic acid, 2-phenylethanol, 2-pentanone, and acetone, respectively, were needed to obtain IC_{50} for *C. inversicolor* and *P. roqueforti*. For 3-methylbutanoic acid, IC_{50} were 6.32×10^{-5} and 2.67×10^{-4} mol/L for *C. inversicolor* and *P. roqueforti*, respectively. For acetic acid, IC_{50} were 1.35×10^{-3} and 1.19×10^{-3} mol /L for *C. inversicolor* and *P. roqueforti*, respectively. For 2-phenylethanol, IC_{50} were 7.49×10^{-3} and 3.45×10^{-3} mol/L for *C. inversicolor* and *P. roqueforti*, respectively. Additionally, 2-pentanone showed a weak inhibitory effect on mycelium growth rate of *C. inversicolor*

TABLE 3 | Relative content of volatile organic compounds (VOCs) in each classification.

PFGE Cluster	Sample	Acids (*n* = 6)	Alcohols (*n* = 22)	Aldehydes (*n* = 15)	Benzene derivatives (*n* = 3)	Esters (*n* = 8)	Heterocyclic compounds (*n* = 3)	Ketones (*n* = 12)	Penols (*n* = 2)
	Control	0.06^{a}	81.47^{a}	14.56^{c}	0.01^{a}	1.90^{ab}	0.15^{e}	1.82^{ab}	0.01^{a}
I	KU-78	0.09^{ab}	87.29^{b}	6.65^{b}	0.05^{abc}	4.80^{cd}	0.08^{cd}	1.02^{a}	0.02^{ab}
I	KU-80	0.06^{a}	81.28^{a}	14.38^{c}	0.05^{abc}	2.67^{ab}	0.05^{a}	1.29^{a}	0.01^{a}
II	KU-12	0.14^{b}	89.68^{bc}	3.22^{ab}	0.04^{abc}	3.32^{abc}	0.08^{cd}	3.50^{c}	0.01^{a}
II	KU-30	0.13^{b}	90.20^{bc}	2.62^{ab}	0.05^{abc}	3.48^{bc}	0.08^{cd}	3.43^{c}	0.02^{ab}
II	KU-9	0.05^{a}	90.50^{bc}	2.47^{ab}	0.06^{bc}	5.36^{d}	0.09^{cd}	1.45^{a}	0.02^{ab}
II	KU-72	0.08^{ab}	92.34^{c}	3.09^{ab}	0.03^{abc}	1.69^{a}	0.08^{cd}	2.66^{bc}	0.02^{ab}
III	KU-11	0.11^{ab}	91.94^{c}	4.53^{ab}	0.06^{bc}	1.89^{ab}	0.10^{d}	1.32^{a}	0.04^{b}
III	KU-28	0.05^{a}	93.19^{c}	2.45^{ab}	0.05^{abc}	3.21^{abc}	0.06^{abc}	1.00^{a}	0.01^{a}
IV	KU-10	0.09^{ab}	90.73^{bc}	3.49^{ab}	0.04^{abc}	4.54^{cd}	0.08^{cd}	1.0^{a}	0.01^{a}
IV	KU-29	0.05^{a}	92.15^{c}	1.58^{a}	0.03^{abc}	5.30^{d}	0.07^{bc}	0.78^{a}	0.03^{ab}
IV	KU-27	0.08^{ab}	88.97^{bc}	3.77^{ab}	0.07^{c}	5.67^{d}	0.05^{ab}	1.40^{a}	0.01^{a}

The relative percentage of relative peak area of each chemical class of samples, (n) refers to the number of volatile compounds in each class. Values in each classification marked by different lowercase letters are significantly different using one-way ANOVA with Duncan's test (≥95% confidence, n = 2). Clusters are based on chromosome polymorphism between the stains as determined by PFGE.

TABLE 4 | Spearman's correlation coefficient analysis between germination ratio (%)/mycelium growth rate (μ/h) and the VOCs detected in the cell-free supernatants of *D. hansenii* strains.

Mold growth	Mold species	Volatile compounds	*R*-value (spearman)[ab]	Strength of association
Germination ratio	*C. inversicolor*	Acetic acid	−0.795	Strong
	C. inversicolor	3-methylbutanoic acid	−0.851	Strong
	C. inversicolor	2-pentanone	−0.731	Strong
	P. roqueforti	Acetone	−0.860	Strong
	P. roqueforti	2-phenylethanol	−0.753	Strong
Mycelium growth rate	*P. roqueforti*	2-phenylethanol	−0.881	Strong

[a] *R = 0 none; −0.3 ≤ R ≤ −0.1 week; −0.6 ≤ R ≤ −0.4 moderate; −0.9 ≤ R ≤ −0.7 strong; −1 perfect.*
[b] *The association was considered significant (P < 0.05), P-values of all compounds mentioned above were below 0.05.*

and *P. roqueforti*, whereas acetone exhibited no inhibitory effect on mycelium growth rates of the two tested molds.

Finally, for confirming the IC_{50} results as well as for evaluating long-term effects of exposure to different concentrations of the targeted VOCs, complete inhibition of mycelial pellicle formation was subsequently scored by visual inspections after 7 days incubation (**Table 6**). As expected, after long-term exposure to the targeted VOCs, inhibition of mycelial pellicle formation confirmed the IC_{50} results for both *C. inversicolor* and *P. roqueforti*. Hence, the highest inhibition was obtained for 3-methylbutanoic acid for both *C. inversicolor* and *P. roqueforti*, for which the concentrations were 10^{-3} and 10^{-2} mol/L, respectively. The concentrations of 2-phenylethanol and acetic acid for preventing the formation of mycelial pellicles were 10^{-2} and 10^{-1} mol/L for *C. inversicolor* and *P. roqueforti*, respectively. Furthermore, the concentration of 2-pentanone was 1 mol/L for *C. inversicolor*, while no inhibition on mycelial pellicle formation was observed for *P. roqueforti* after 7 days. Finally, acetone did not inhibit mycelial pellicle formation of either of the two molds after 7 days.

Among the targeted VOCs, estimated concentrations produced by the *D. hansenii* strains were calculated, when grown under the experimental conditions. The *D. hansenii* strains were found to produce estimated concentrations of 2-phenylethanol at 10^{-3} mol/L (data not shown), which was at the same concentration level as the detected IC_{50} for both *C. inversicolor* and *P. roqueforti*. The strains were additionally found to produce an estimated concentration of 10^{-6} mol/L for 3-methylbutanoic acid and 2-pentanone, and 10^{-4} mol/L for acetic acid and acetone (data not shown), which were all lower than the detected IC_{50} for both *C. invercolor* and *P. roqueforti* for the respective VOCs.

DISCUSSION

In this study, we evaluated *D. hansenii* strains isolated from Danish cheese brines for their antifungal activities against dairy contaminating molds. Our PFGE result confirmed the genetic heterogeneity of *D. hansenii* as previously reported (Petersen et al., 2002; Petersen and Jespersen, 2004). In Petersen et al. (2002), chromosome polymorphism among *D. hansenii* strains could be linked to different pH and NaCl tolerances. The variations in acidification or deacidification capabilities among the *D. hansenii* strains, found in the present study, could to some extend be linked to the genetic variance observed. The

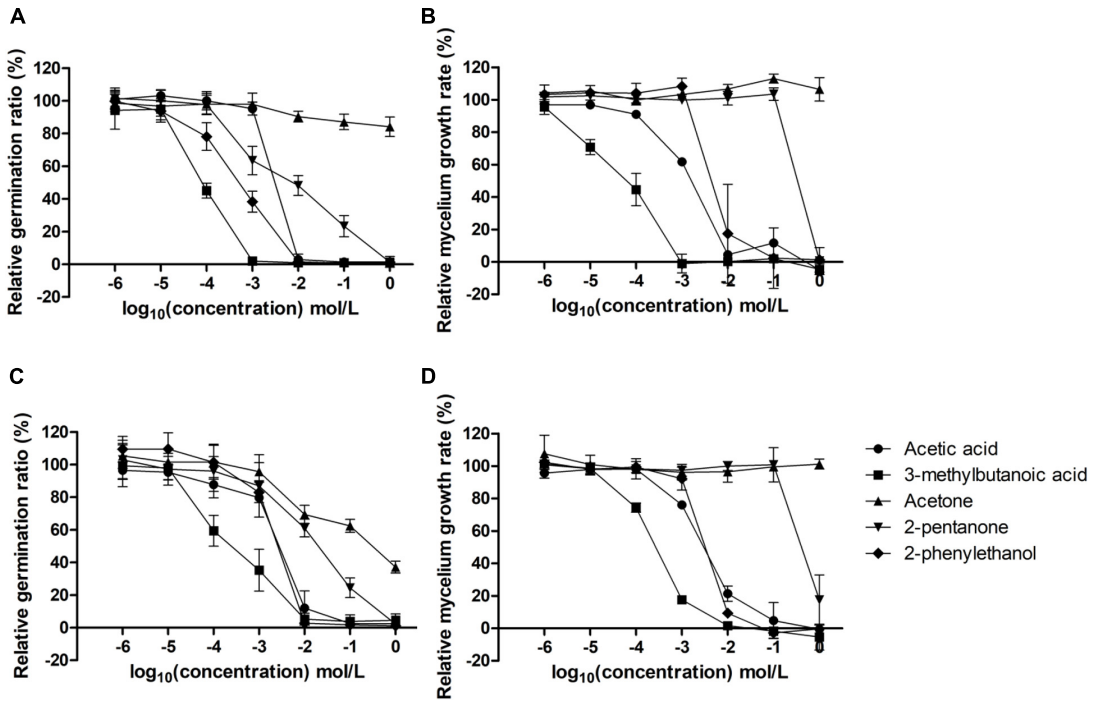

FIGURE 3 | Inhibitory effects of targeted single VOCs on germination ratios (%) of *C. inversicolor* **(A)** and *P. roqueforti* **(C)** and mycelium growth rates (μ/h) of *C. inversicolor* **(B)** and *P. roqueforti* **(D)** at different concentrations (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} mol/L). Each spot represents the mean value from three independent experiments, and the error bars represent the standard deviations.

TABLE 5 | IC_{50} of different VOCs for inhibiting germination and mycelium growth of *P. roqueforti* and *C. inversicolor*.

Mold growth	Volatile compounds	*C. inversicolor*	*P. roqueforti*
		IC_{50} (mol/L)	
Germination ratio	Acetic acid	2.47×10^{-3}	2.80×10^{-3}
	3-methylbutanoic acid	9.53×10^{-5}	2.00×10^{-4}
	Acetone	>1	9.21×10^{-2}
	2-pentanone	1.06×10^{-2}	2.60×10^{-2}
	2-phenylethanol	1.99×10^{-3}	1.73×10^{-3}
Mycelium growth rate	Acetic acid	1.35×10^{-3}	1.19×10^{-3}
	3-methylbutanoic acid	6.32×10^{-5}	2.67×10^{-4}
	Acetone	>1	>1
	2-pentanone	9.45×10^{-1}	9.51×10^{-1}
	2-phenylethanol	7.49×10^{-3}	3.45×10^{-3}

pH differences of the broth media (MYGP added with 4% (w/v) NaCl) might be related to *D. hansenii* producing or assimilating organic acids, proteolytic activity, production of ammonia, cell lysis, as well as several other factors (Gustafsson, 1979; Gancedo and Serrano, 1989; Freitas et al., 1999; Flores et al., 2004; Gori et al., 2007, 2012). Contrary, the VOCs profiles produced by the *D. hansenii* strains, in the present study, could not be linked to the specific chromosomal clusters as observed by PFGE, even though strain dependent differences in VOC production were observed. Strain-dependent VOC profiles have previously been observed among *D. hansenii* strains differing in M13 minisatellites, isolated from traditional ewe's and goat's cheese (Padilla et al., 2014b).

Among the five contaminating molds, *P. roqueforti* is, besides being a contaminant on surface ripened cheeses, a well known starter culture used in the production of blue-veined cheeses. *Mucor* spp. are well known on a variety of mold-ripened cheeses, especially from raw milk hard cheeses such as Saint Nectaire and Tomme de Savoie (Fox et al., 2004), and *M. racemosus* has especially been isolated from French cheeses (Hermet et al., 2015). Further, *F. avenaceum* has predominantly been reported as a mycotoxin producing pathogen on cereals, especially pronounced under cool and wet or humid conditions (Uhlig et al., 2007), however, it has also been isolated as a contaminating mold on Italian cheese (Montagna et al., 2004). Likewise, *F. avenaceum* was isolated at Danish dairies and identified to the species level in the present study. Finally, *Cladosporium* spp. have been isolated from traditional Italian cheese (De Santi et al., 2010). Originally isolated at Danish dairies, *C. inversicolor* and *C. sinuosum* were identified to species level, for the purpose of this study. However, *C. inversicolor* and *C. sinuosum* are so far mostly reported from plant materials (Bensch et al., 2015). Altogether, this indicates a huge species diversity among contaminating molds in dairy environments, far beyond what is usually investigated.

In the present study, the *D. hansenii* cell-free supernatants inhibited spore (conidia) germination and had only minor inhibitory effect on mold growth, indicating a fungistatic

effect, which might be associated with the morphology of the spores or conidia of the five molds. Interestingly, the two *Cladosporium* spp., i.e., *C. inversicolor* and *C. sinuosum* responded very different to the *D. hansenii* cell-free supernatants. Germination and mycelium growth of *C. inversicolor* were significantly inhibited by cell-free supernatants of the *D. hansenii* strains, whereas we only observed a slight inhibition of germination for *C. sinuosum* and no effect on mycelium growth. The *Cladosporium* genus represents one of the larges genera of dematiaceous hyphomycetes and comprises several complexes, which are differentiated morphologically based on especially conidia morphology. *C. inversicolor* is in the *Cladosporium cladosporioides* complex and contains numerous conidia in long chains of up to eight conidia with walls typically being unthickened. *C. sinuosum* is in the *Cladosporium herbarum* complex and contains solitary conidia or short chains of up to four conidia which appear to be thick walled due to surface ornamentation (Bensch et al., 2015). These differences might explain some of the variance in the susceptibility observed between the two *Cladosporium* species.

Among the 71 VOCs produced by *D. hansenii*, five targeted VOCs (acetic acid, 3-methylbutanoic acid, acetone, 2-phenylethanol, and 2-pentanone) had a strong association with inhibition of germination and/or mycelium growth of *C. inversicolor* and *P. roqueforti* and were selected for detailed analyses. Accordingly, *D. hansenii* has been shown to produce acetic acid, acetone, 3-methylbutanoic acid, 2-pentanone, and 2-phenylethanol in cheese-like medium (Padilla et al., 2014a). Moreover, *D. hansenii* increased the amount of 2-pentanone in feta cheese (Bintsis and Robinson, 2004). In our study, 3-methylbutanoic acid had the strongest inhibitory effect and the lowest IC_{50} against germination and mycelium growth of *C. inversicolor* and *P. roqueforti* at 10^{-5} and 10^{-4} mol/L, respectively. The sensory threshold for 3-methylbutanoic acid is reported to 120-700 ppb ($1.17\text{-}6.85 \times 10^{-6}$ mol/L) and at higher concentrations, this branched-chain fatty acid has been described to contribute with unpleasant odors as floor cloth, feet, and sweaty flavors in surface ripened cheeses from France (Lecanu et al., 2002). The estimated concentration of 3-methylbutanoic acid (10^{-6} mol/L) detected in the present study was therefore within the sensory thresholds but lower than the IC_{50} values mentioned above. Even so, the potential use of *D. hansenii* strains producing 3-methylbutanoic acid as a biocontrol agent is still interesting. Higher levels of 3-methylbutanoic acid might potentially be obtained under different processing conditions, as reported by Durá et al. (2004) where NaCl and lactate increased the levels of 3-methylbutanoic acid produced by *D. hansenii*, and detailed analyses still need to be carried out in cheese under relevant maturation conditions.

The VOC 2-phenylethanol is reported as a common inhibitory compound produced by several biocontrol yeasts including *Debaryomyces nepalensis*, *Wickerhamomyces anomalus*, *Metschnikowia pulcherrima*, *Saccharomyces cerevisiae*, and *Pichia manshurica* (formerly, *Pichia galeiformis*) (Zhou et al., 2018; Contarino et al., 2019; Chen et al., 2020). In this study, we found a strong inhibitory effect of 2-phenylethanol on germination and mycelium growth of *C. inversicolor* and *P. roqueforti* at 10^{-3} mol/L. Accordingly, we have previously found that 2-phenylethanol completely impaired conidia germination, hyphal membrane integrity, and growth of another two food spoilage molds, i.e., *Penicillium expansum* and *Penicillium nordicum* at 15×10^{-3} mol/L (Huang et al., 2020). The concentration of 2-phenylethanol produced by *D. hansenii* strains in the

TABLE 6 | Inhibitory effects of five VOCs on the formation of mycelial pellicles of molds after 7 days.

Mold species	Concentration (mol/L)	VOCs				
		Acetic acid	3-methylbutanoic acid	Acetone	2-pentanone	2-phenylethanol
C. inversicolor	10^{0}	+++	+++	–	+++	+++
	10^{-1}	+++	+++	–	–	+++
	10^{-2}	++	+++	–	–	+
	10^{-3}	–	++	–	–	–
	10^{-4}	–	–	–	–	–
	10^{-5}	–	–	–	–	–
	10^{-6}	–	–	–	–	–
P. roqueforti	10^{0}	+++	+++	–	–	+++
	10^{-1}	+++	+++	–	–	+++
	10^{-2}	–	+++	–	–	–
	10^{-3}	–	–	–	–	–
	10^{-4}	–	–	–	–	–
	10^{-5}	–	–	–	–	–
	10^{-6}	–	–	–	–	–

+++ no mycelial pellicles formed.
++ less than or equal to 3 out of 6 replicates forming mycelial pellicles.
+ less than or equal to 5 out of 6 of replicates forming mycelial pellicles.
– no inhibition.

present study was approximately 10^{-3} mol/L and therefore comparable with the IC_{50} (10^{-3} mol/L level), thus highlighting the potential application of the *D. hansenii* strains for biological production of 2-phenylethanol as an antifungal agent. Moreover, 2-phenylethanol is a well-known quorum sensing molecule produced by *D. hansenii*, and is found to upregulate traits involved in adhesion and sliding motility of *D. hansenii* (Gori et al., 2011), increasing the colonization probability on cheese surfaces (Mortensen et al., 2005; Gori et al., 2011), which in turn could contribute to the quality of the final product.

Acetic acid is a common antimicrobial compound and its antifungal activity is well investigated (Cabo et al., 2002; Fernandez et al., 2017; Guimarães et al., 2018; Sadiq et al., 2019). In this study, the IC_{50} for acetic acid was found at 10^{-3} mol/L against *C. inversicolor* and *P. roqueforti*. Quattrini et al. (2019) reported the minimum inhibitory concentration (MIC) of acetic acid for *P. roqueforti* growth to $2.5 \pm 0.6 \times 10^{-2}$ mol/L after 5 days, which is somewhat lower than the concentration of acetic acid for preventing mycelial pellicle formation of *P. roqueforti* (approximately 10^{-1} mol/L) after 7 days, in the present study. In Quattrini et al. (2019), MIC was determined after 5 days in conditions mimicking sourdough using modified MRS, while in the present study inhibition was scored after 7 days in MEB, a medium for optimal mold growth. These differences could explain the different concentrations determined.

In soft cheeses, 2-pentanone has been detected in Camembert, Vacherin, Limburger, Trappist, Gorgonzola and blue cheese above the odor threshold of 0.5-61 ppm contributing fruity, acetone, sweet, and ethereal odor (Sablé and Cottenceau, 1999). In the current study, 2-pentanone showed a weak inhibitory effect on the germination and mycelium growth of *C. inversicolor* and *P. roqueforti*. Further, the estimated concentration of 2-pentanone produced by *D. hansenii* in this study, was lower than the detected IC_{50} but within the ranges of odor threshold reported in previous studies (Sablé and Cottenceau, 1999).

The results of the present study indicate that the antifungal activity of *D. hansenii* is complex and likely results from a synergistic and or additive effect of several inhibitory VOCs, rather than the effect of single compounds. Similarly, growth inhibition of postharvest pathogenic molds in packaged fresh fruit by the proven biocontrol yeasts *W. anomalus* BS91 and *M. pulcherrima* MPR3 has been suggested to arise from a synergistic effect of VOCs and CO_2 (Contarino et al., 2019). Moreover, inhibition of *Fusarium* spp. growth in the presence of cell-free supernatants of *Lactiplantibacillus plantarum* (formerly, *Lactobacillus plantarum*) has been shown not to arise alone from the lactic acid being produced by the lactic acid bacteria (Laitila et al., 2002). Further, additional mechanisms, not investigated in the current study, might also add to the combined antifungal activity of *D. hansenii*, including non-volatile compounds like killer toxins (Banjara et al., 2016), and competition for nutrients and space (Spadaro and Droby, 2016). As nutrients and space are generally limited in cheese processing (Irlinger and Mounier, 2009), this could be especially relevant. Interestingly, *D. hansenii* isolated from dry-cured meat products has been reported to inhibit toxigenic penicillia in co-culture assays on solid media, which could not be fully reproduced by cell-free supernatants or mouth-to-mouth assays. This indicates that efficient mold inhibition relied on additive or synergistic effects of the yeast inhibition factors such as competition for nutrients and space as well as production of soluble compounds or VOCs (Núñez et al., 2015). Similarly, *P. manshurica* has been shown to colonize and amplify quickly in citrus wounds and further to produce eight VOCs, including acetic acid and 2-phenylethanol during growth, which inhibited growth of *Penicillium digitatum*, causing citrus green mold (Chen et al., 2020).

In conclusion, genetic differences of *D. hansenii* together with diverse acidifying or deacidifying capability were confirmed. Cell-free supernatants of *D. hansenii* strains exhibited a strong inhibitory effect on *C. inversicolor* and *P. roqueforti*. Using DHS-GC-MS, 71 VOCs produced by *D. hansenii* strains were identified. Among the VOCs, strong correlation between inhibition of germination of *C. inversicolor* and *P. roqueforti* were obtained for five VOCs (acetic acid, acetone, 3-methylbutanoic acid, 2-pentanone, and 2-phenylethanol), while 2-phenylethanol also correlated strongly with inhibition of mycelium growth of *P. roqueforti*. 3-methylbutanoic acid showed the strongest inhibitory effects on germination and mycelium growth of the two mold species, i.e., IC_{50} of 10^{-5} and 10^{-4} mol/L, respectively. 2-phenylethanol and acetic acid also exhibited strong inhibitory effects on germination and mycelium growth at IC_{50} of 10^{-3} mol/L. Relative weak inhibitory effects of 2-pentanone and acetone were obtained in this study. The results from the current study point to an additive and possibly synergistic effect of the antifungal compounds produced by *D. hansenii*, which needs to be further studied. From an overall perspective, it can be concluded that some native *D. hansenii* strains in the dairy manufacturing environment have a so far neglected role in the natural preservation of cheeses and are eligible for biocontrol of contaminating molds in cheese production. The results additionally point to the coming use of halophilic *D. hansenii* strains as biocontrol agents to be added as bioprotective cultures in the dairy industry, e.g., as an additive to cheese brines.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

CH, LZ, PJ, NA, and LJ conceived and designed the study. MP contributed to the GC-MS analyses. CH performed the

experiments and drafted the manuscript. PJ, NA, and LJ revised the manuscript. 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.2021.662785/full#supplementary-material

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Liquor Flavour Is Associated With the Physicochemical Property and Microbial Diversity of Fermented Grains in Waxy and Non-waxy Sorghum (*Sorghum bicolor*) During Fermentation

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The fermentation process of Chinese Xifeng liquor involves numerous microbes. However, the sources of microbes in fermented grain and the link between liquor flavour and physicochemical properties and microbial diversity during fermentation still remain unknown. Herein, two waxy (JiNiang 2 [JN-2] and JinNuo 3 [JN-3]) and four non-waxy (JiZa 127 [JZ-127], JinZa 34 [JZ-34], LiaoZa 19 [LZ-19], and JiaXian [JX]) sorghum varieties were selected for the comprehensive analysis of the relationship between liquor flavour and the physicochemical properties and microbial diversity of fermented grains. Results showed that ethyl acetate was the main flavour component of JZ-127, JZ-34, and JX, whereas ethyl lactate was mainly detected in JN-2, JN-3, and LZ-19. Ethyl lactate accounted for half of the ethyl acetate content, and JX exhibited a higher liquor yield than the other sorghum varieties. The fermented grains of waxy sorghum presented higher temperature and reducing sugar contents but lower moisture and starch contents than their non-waxy counterparts during fermentation. We selected JN-3 and JX sorghum varieties to further investigate the microbial changes in the fermented grains. The bacterial diversity gradually reduced, whereas the fungal diversity showed nearly no change in either JN-3 or JX. *Lactobacillus* was the most abundant bacterial genus, and its level rapidly increased during fermentation. The abundance of *Lactobacillus* accounted for the total proportion of bacteria in JX, and it was higher than that in JN-3. *Saccharomyces* was the most abundant fungal genus in JX, but its abundance accounted for a small proportion of fungi in JN-3. Four esters and five alcohols were significantly positively related to Proteobacteria, Bacteroidetes, and Actinobacteria; Alphaproteobacteria, Actinobacteria, and Bacteroidia; Bacillales, Bacteroidales, and Rhodospirillales; and *Acetobacter*, *Pediococcus*, and *Prevotella_7*. This positive relation is in contrast with that observed for Firmicutes, Bacilli, Lactobacillales, and *Lactobacillus*.

Meanwhile, *Aspergillus* was the only fungal microorganism that showed a significantly negative relation with such compounds (except for butanol and isopentanol). These findings will help in understanding the fermentation mechanism and flavour formation of fermented Xifeng liquor.

Keywords: alcohols, bacteria, esters, fungi, physicochemical parameters, Xifeng liquor

INTRODUCTION

Sorghum (*Sorghum bicolor* L. Moench) is a widely planted cereal crop found in semiarid regions because of its strong resistance to drought, saline and poor soil conditions. This plant has high nutrient contents, such as phenolic antioxidant compounds, and can be used in livestock feed and biofuel production (Ananda et al., 2011; Xu et al., 2018). Despite the reduced sorghum plantation area in China, sorghum is widely adopted as a raw material for brewing in the wine industry (Tawaba et al., 2013). On the basis of usage, sorghum is divided into four types, namely, grain, sweet, grass and broom sorghum (Lu and Dahlberg, 2001). Brewing liquor, such as Moutai, Wuliangye, Luzhoulaojiao, and Langjiu, and vinegar is based on sorghum grains as raw materials. Grass sorghum is divided into waxy and non-waxy types based on the ratio of amylose and amylopectin (Sang et al., 2008). The selection of raw materials may influence the liquor's quality and value. Therefore, generalised plantation and application of different sorghum varieties are important for the development of the sorghum industry (Elhassan et al., 2015).

Chinese liquor is a famous traditional Chinese drink. It is divided into five main categories, namely, strong aroma, light aroma, soy sauce aroma, sweet honey and miscellaneous type liquors (Fan and Qian, 2006). Although alcohol and water account for 97–98% of liquor, other micro-components determine its flavour and quality (Han et al., 2018). The flavour of Chinese liquor is enhanced by aroma compounds, including esters, alcohols, acids, aldehydes, ketones, acetals, and heterocyclic compounds. Oswald (2003) observed that esters, especially ethyl octanoate and ethyl decanoate, and alcohols are amongst the key aroma compounds formed at the end of winemaking. Aroma compounds have been detected in Chinese liquor. Ethyl hexanoate is believed to be a primary aroma compound in strong aroma-type Chinese liquor (Shen, 1996). By comparing the volatile compounds of Wuliangye with those of Moutai, Kim et al. (2009) found that diethyl succinate only exists in Wuliangye. Therefore, liquor aroma compounds exist in various proportions to create different liquor flavours and types and thus can be used to improve the quality of liquor production.

The quality of liquor made from sorghum is related to the sorghum material, local climate, brewing technology and microbial population (He et al., 2019). The structure and metabolic activities of the microbial community are key factors that determine liquor quality. Microorganisms native to the domain Bacteria and those affiliated with Eukaryota play a crucial role in Chinese liquor fermentation (Wang P. et al., 2017). Jin et al. (2019) reported that Bacillales, Enterobacteriales, and Lactobacillales are dominant bacteria, whereas *Candida*, *Trichoderma*, *Aspergillus*, *Trichosporon*, and *Thermomyces* are predominant fungal communities in Moutai Daqu. Wang C. et al. (2008) discovered different microorganisms in various categories of Chinese liquor and reported that Bacilli, Bacteroidetes and Clostridia are predominant in strong aroma type fermented grains, whereas Bacilli, Flavobacteria, and Gammaproteobacteria are predominant in fermented grains with roasted sesame aroma type. Lactic acid bacteria are also important microbial groups in the Chinese liquor brewing industry; they are used to improve the flavour of liquors (Fang et al., 2015). The organic acids produced by the metabolism of lactic acid bacteria are the main factor affecting the acidity of liquors; these compounds benefit starch saccharification and ethanol fermentation by maintaining the acidity of the brewing environment (Wang et al., 2014). The predominant key microorganisms in fermented grains and the effect of their metabolic products are crucial for the development of liquor flavour. Therefore, analysing the changes in the microbial community structure of fermented grains is important to reveal the mechanism of flavour production, control fermentation and ensure the stability of liquor quality (Cocolin et al., 2000).

Liquors are complex mixtures consisting of hundreds of component substances present in different concentrations (Fan et al., 2012). With the rapid development of sequencing technology, the relationship between microbial community structure and liquor quality has become a research focus (Zheng et al., 2012; Kim et al., 2018). However, limited studies have focused on raw materials that cause differences in the brewing environment (Wu et al., 2015). The mechanism by which microbes change during fermentation and how they affect the flavour of liquors remain unclear. Therefore, this study aimed to (1) examine the changes in the physicochemical parameters of sorghum varieties during fermentation; (2) analyse the microbial (bacterial and fungal) diversity and composition with Illumina sequencing of the 16S rRNA gene and internal transcribed spacer (ITS) gene; (3) determine the volatile compounds in alcohol by liquid-liquid microextraction (LLME) with gas chromatography-mass spectrometry (GC-MS); and (4) evaluate the relationships amongst the physicochemical parameters, microbial community and liquor flavour. Different sorghum varieties were systematically compared to analyse the comprehensive effects of physicochemical parameters and microbial community changes on liquor quality during liquor fermentation and to ascertain the sorghum varieties suitable for brewing liquor. Furthermore, gaining insights into the parameter changes during liquor fermentation and the development of beneficial bacteria are important for ensuring liquor quality.

MATERIALS AND METHODS

Materials and Sample Collection

Six sorghum varieties, namely, JiNiang 2 (JN-2), JinNuo 3 (JN-3), JiZa 127 (JZ-127), JinZa 34 (JZ-34), LiaoZa 19 (LZ-19), and JiaXian sorghum (JX), were used (**Supplementary Figure 1**). These varieties were divided into two genotypes. JN-2 and JN-3 are waxy sorghums with low amylose contents (not exceeding 10%) (Yang et al., 2018). JZ-127, JZ-34, LZ-19, and JX are non-waxy sorghums with high amylose contents. **Supplementary Table 1** shows the quality properties, including starch, protein and fat contents, of the six sorghum grain varieties.

In May 2019, sampling was conducted during fermentation at Xifeng Liquor Limited Liability Company in Fengxiang Town, Shaanxi Province, China (N34°26′6.74″, E107°29′52.51″). Three paralleled fermentation pits were selected for fermented grain sampling. The fermented grain samples were collected with a special sampler (a tweezer-like tool with spoon-like heads and sharp edges) from the middle of the pits and the upper, middle and lower parts of the four pit corners. The samples were then mixed to form one sample. The fermented grain samples were collected after fermentation for 1, 3, 6, 9, 12, 15, 18, 21, 24, and 26 days. Finally, the samples were transported to the laboratory on ice and kept at −80°C.

Measurement of Liquor Quality and Yield

Liquid-Liquid Microextraction-Gas Chromatography-Mass Spectrometry

Exactly 18 mL of 10% (*v*/*v*) finished alcohol sample was aspirated and added with 6 g of sodium chloride until saturation, followed by the addition of 6 μL of 5.85 mg/100 mL butyl hexanoate as the internal standard, 1 mL of re-distilled ether with shaking for 3 min and 1 μL of organic phase after standing and stratification to perform GC-MS analysis. Compound concentrations were calculated based on the ratio of the peak area of a compound relative to the peak area of the internal standard based on the calibration curves.

Gas Chromatography-Mass Spectrometry Analysis Conditions

The inlet temperature was 250°C, the carrier gas was high-purity helium (purity 99.999%), and the column flow rate was 2 mL/min with splitless injection. The program temperature conditions were as follows: holding at 50°C for 2 min, 6°C/min rate to 230°C and holding for 15 min. The MS conditions were as follows: electron ionisation source, ionisation voltage of 70 eV, ion source temperature of 230°C and spectrum scan range of 35–350 amu.

Liquor Yield

The liquor was distilled from the fermented grains obtained from the pit, the comprehensive alcohol content of the original wine was calculated at 65° (Zhou et al., 2015), and the liquor yield was calculated at a specified ratio of grain (900 kg) input to liquor output.

Liquor yield (%) = alcohol output/grain input × 100%.

Analysis of the Physicochemical Properties of Fermented Grains

The moisture content of fermented grains was determined by quarter sampling, which involved weighing 10 g of fermented grain samples and placing them in a petri dish for flattening. The samples were then baked in an infrared oven for approximately 20 min and then weighed after cooling to room temperature. The moisture content was calculated using the equation from the work of Wang P. et al. (2017):

$$Moisture = \frac{W - W0}{W} \times 100\%$$

where W is the initial sample weight and W_0 is the sample weight after drying.

Exactly 5 g of fermented grain samples was weighed and added with 100 mL of 1:4 hydrochloric acid solution to determine the starch and reducing sugars. The bottle was pressed on a reflux condenser, hydrolysed in boiling constant-temperature water bath for 30–60 min, removed from the bath, rapidly cooled and neutralised with sodium hydroxide. The filtrate was collected in a 500 mL volumetric flask after filtering with an absorbent cotton. The residue was washed thoroughly with water to a constant volume of 500 mL. Exactly 5 mL each of Feilin A and B solutions was pipetted to a 150 mL flask, added with 9 mL of 0.1% standard glucose solution from the burette and shaken well. The sample was then heated on an electric stove until boiling and titrated with 0.1% standard glucose solution until the blue colour disappeared immediately, and the solution turned to light yellow. The specific steps were described by Chen et al. (2013).

The acidity of fermented grains was measured by weighing 10 g of fermented grain samples, adding 50 mL of distilled water with stirring and soaking for 30 min in a triangle bottle. After filtering with a filter paper, 5 mL of the supernatant was transferred to a triangular bottle and added with 25 mL of distilled water and two drops of phenolphthalein indicator with stirring. Then, the solution was titrated with 0.1 M sodium hydroxide standard solution until it became reddish. The volume of sodium hydroxide standard solution was recorded to calculate the acidity of the fermented grains (Wang P. et al., 2017).

A total of 100 g of fermented grain samples was weighed and added with 200 mL of distilled water in a 500 mL distillation flask. Then, the mixture was added with 100 mL of effluent in a graduated cylinder to determine the alcohol content of the fermented grains. The distilled liquor was mixed evenly and placed gently on an alcohol meter in a graduated cylinder, and the alcohol degree was corrected to a value of 20°C. After stabilisation, the alcohol degree was read based on the alcohol and temperature correction table.

Fermented Grain DNA Extraction, Polymerase Chain Reaction Amplification, and Illumina Sequencing

The fermented grains of JN-3 and JX sorghum were used for microbial composition analysis. Samples were obtained on days 3, 9, 15, and 26 after fermentation. Microbial DNA was extracted using a PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, United States) following the manual. The purity and quality of the genomic DNA were checked on 0.8% agarose

gels. Primers 16S-F (ACTCCTACGGGAGGCAGCAG) and 16S-R (GGACTACHVGGGTWTCTAAT) were used to amplify the bacterial 16S gene for the V3-V4 hypervariable regions. Primers ITS-F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS-R (5′-TGCGTTCTTCATCGATGC-3′) were used to amplify the fungal ITS region. The ultra-PAGE purified primers were bought from Invitrogen, China. The polymerase chain reaction (PCR) products were purified using an Agencourt AMPure XP Kit. Deep sequencing was performed on MiSeq platform at Allwegene Company (Beijing). The sequence data associated with this project have been deposited at the National Center for Biotechnology Information (accession numbers: PRJNA670598 for bacteria and PRJNA670601 for fungi).

Processing of 16S rRNA and ITS Gene Data

The raw sequences of bacterial and fungal reads were initially trimmed using Mothur, and sequences satisfying the following criteria were considered: (1) precise primers and barcodes, (2) quality score > 30, and (3) length > 200 bp. The Ribosomal Database Project classifier tool was used to classify all sequences into different taxonomic groups (Wang et al., 2007). The qualified reads were separated using sample-specific barcode sequences and trimmed with Illumina Analysis Pipeline version 2.6. Then, the dataset was analysed using QIIME. The sequences were clustered into operational taxonomic units (OTUs) at 97% similarity level to generate rarefaction curves and calculate the richness and diversity indices (Cole et al., 2014).

Statistical Analyses

Taxonomic alpha diversity was calculated as the estimated community diversity by Shannon index using the Mothur software package (v.1.30.1). Non-metric multidimensional scaling (NMDS) was utilised to evaluate the ecological distances of different samples based on the weighted UniFrac distances via EMPeror, and changes in the microbial structure during the fermentation period were considered to indicate the microbial beta diversity. The relationships between the physicochemical properties of fermented grains and liquor quality were determined by Spearman's correlation analysis (SPSS 19.0, SPSS Inc., Chicago, United States). To determine and visualise the correlations, we created networks by using Gephi (Web Atlas, Paris, France) (Bastian et al., 2009). Each node represents a genus, whereas each edge indicates a strong and significant correlation between nodes. All analyses were carried out in R environment with VEGAN and Hmisc (Vanderbilt University, Nashville, TN, United States) packages (Harrell, 2010).

The data, including the physicochemical properties and microbial communities in the fermented grains, were analysed using one-way analysis of variance (ANOVA) of different sorghum varieties and fermentation times ($P < 0.05$). The difference between the mean values was determined using the least significant difference ($P < 0.05$), which was indicated by different letters. Origin 2018 was used to draw the figures, including those for physicochemical properties and microbial communities.

RESULTS AND DISCUSSION

Aroma Compound of Liquor

On the basis of aroma characteristics, Chinese liquor can be classified into strong aroma type, light aroma type, soy sauce aroma type, sweet honey type, and miscellaneous types (Fan and Qian, 2006). Liquor aroma compounds exist in various proportions to form different flavours and types of liquor, thus improving the quality of liquor production. Ester is the most abundant and important aromatic component in liquor; the content of esters determines the flavour characteristics of liquor (Lan et al., 2017). To identify and quantify the aroma compounds responsible for the overall aroma profile of liquor, Xifeng liquor was analysed by LLME-GC-MS. Nine aroma compounds including ethyl acetate, ethyl caproate, ethyl lactate, ethyl butyrate, n-propanol, n-butanol, isobutanol, butanol, and isoamylol (**Figure 1A**) were identified and quantitated in Xifeng liquor. The contents of ethyl acetate, ethyl caproate, ethyl lactate

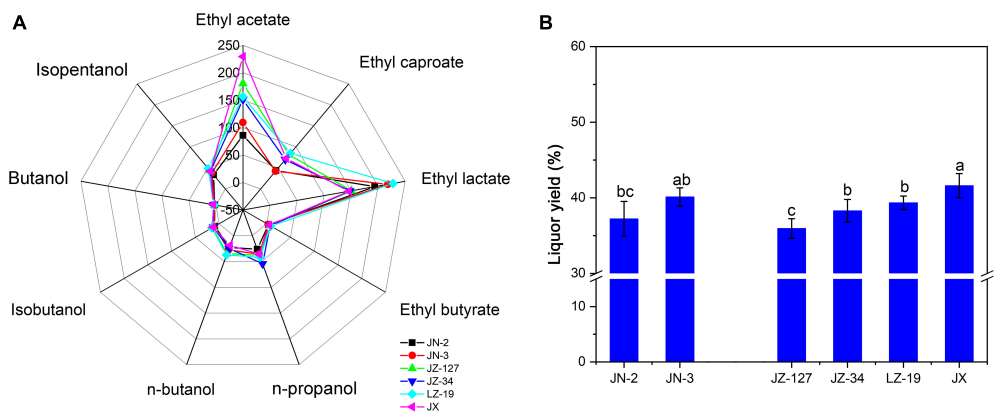

FIGURE 1 | Volatile compounds of liquor **(A)** and liquor yield **(B)** in two waxy (JN-2 and JN-3) and four non-waxy sorghum varieties (JZ-127, JZ-34, LZ-19, and JX). Different letters indicate significant differences (ANOVA, $P < 0.05$) amongst various sorghum varieties.

and ethyl butyrate ranged from 85.86 to 229.29 mg/100 mL, 42.18 to 84.66 mg/100 mL, 146.00 to 227.98 mg/100 mL, and 3.27 to 7.44 mg/100 mL, respectively. Obviously, ethyl acetate had the highest content (229.29 mg/100 mL) in JX, which was commonly regarded as the characteristic aroma compound of Xifeng liquor (Cao et al., 2017). Ethyl esters with a fruity aroma are often considered as the major contributors to the aroma profile of Chinese Baijiu (Li et al., 2021). Apart from ethyl acetate, ethyl lactate accounted for half of the proportion of ethyl acetate in JX (**Figure 1A**), indicating that Xifeng liquor has the characteristic miscellaneous type of mixed flavour liquor and aroma with ethyl acetate as the main aroma compound (Hu et al., 2011; Fan et al., 2019). In addition, JN-2, JN-3, JZ-127, JZ-34, and LZ-19 contained significantly higher amounts of ethyl lactate than JX. A moderate ethyl lactate content is beneficial for the liquor type; however, excessive amounts can cause astringent liquor taste and deteriorated quality (Liu et al., 2011). Although the abundance of amylopectin in waxy sorghum promotes swelling and gelatinisation, it is suitable for liquors with strong aroma and soy sauce aroma types. Therefore, JX as a non-waxy sorghum is suitable for use in the production of miscellaneous type Xifeng liquor in Shaanxi. These results are similar to those of previous studies (Nambou et al., 2014; Corona et al., 2016). Esters are found in high amounts after fermentation, moreover, some major esters have strong relations with fruity/floral/green aromas and are mainly produced by yeasts.

Given that the inherent esters in Chinese liquor are mainly produced during the fermentation stages through the esterification of alcohols and acids, alcohols have a significant influence on the yield and intensity of aroma esters (González-Rompinelli et al., 2013; Wang et al., 2015). In the present study, the contents of n-propanol, n-butanol, isobutanol, butanol and isopentanol ranged from 26.06 to 54.53 mg/100 mL, 18.73 to 37.25 mg/100 mL, 9.31 to 15.23 mg/100 mL, 2.40 to 4.90 mg/100 mL, and 33.42 to 48.37 mg/100 mL, respectively, which are lower than most of the ester compounds (**Figure 1A**), due to their low odour threshold values. Similar to the contents of ester, suitable contents of the five alcohol compounds, especially n-propanol, were detected in JN-3 and JX. Notably, JX had higher contents of isoamylol and isobutanol than JN-3. Given that alcohols have typical fruity and floral aromas, they are used not only as alcoholic sweeteners and flavouring agents but also as precursors of flavouring substances, which have certain effects on liquor flavour (Lan et al., 2017). For example, as expected with any alcoholic beverage, alcohols are amongst the major volatile compounds. During fermentation, yeast can form alcohols from sugars under aerobic conditions and from amino acids under anaerobic conditions (Wondra and Berovic, 2001). However, the alcohol content needs to be controlled within a certain range because slight changes in it will remarkably affect the type of traditional liquor, resulting in spicy, bitter and astringent flavours. Compared with other Chinese Baijiu, the contents of the nine aroma compounds mentioned above in the test Xifeng liquor were different from those of Gujinggong, Jiannanchun, Luzhou Laojiao, Wuliangye, and Yanghe Daqu (Xiao et al., 2014; Zheng et al., 2014a). This discrepancy in the contents of aroma compounds leads to the delicate difference of smell and taste of Xifeng liquor from other Chinese Baijiu. JX and JN-3 exhibited a higher liquor yield than the other sorghums. However, the highest liquor yield was observed in JX (**Figure 1B**).

Physicochemical Parameters in Fermented Grains

Chinese liquor is a traditional distillate fermented from grains. Not only are fermented grains the main body of brewing liquor, but also the microorganisms in fermented grains drive the formation of flavour substances (Jin et al., 2017). Elucidating the dynamic changes of parameters in fermented grains is important for liquor production. The starch in fermented grains can be degraded and converted into reducing sugars, alcohols, acids, esters, aldehydes, and ketones, which affect the quality and type of liquor. Meanwhile, the transmission of environmental information is crucial for the regulation of changes in microbial species, formation and accumulation of metabolites, and determination of the direction of material and energy metabolism. Therefore, after fermentation, the fresh liquor is distilled out and then aged under controlled conditions. The aged distillate is adjusted to the designated alcohol content and blended to ensure the quality of the finished product (Shen, 1996). The physicochemical parameters of fermented grains obtained in this study, including temperature, moisture, starch, reducing sugar, acidity, and alcohol (**Figure 2** and **Supplementary Table 2**), are presented and discussed in the following.

The micro-environment in pits changes during the fermentation process, coupled with the utilisation of nutrients and accumulation of metabolic products, such as decrease in pH and increase of alcohol concentration. In addition, environmental factors, such as temperature and moisture, affect the performance of traditional liquor fermentation. It is crucial to optimise the environmental conditions for co-culture fermentation. Not only are these factors vital indicators of microorganism growth and metabolism; they also affect the bioactivity of microorganisms (Wu et al., 2013).

In the present study, temperature and moisture constantly increased, but the former decreased after 12 days of fermentation. The temperatures of waxy sorghums (JN-2 and JN-3) were higher than those of non-waxy sorghums (JZ-127, JZ-34, LZ-19, and JX) (**Figure 2A**), thereby accelerating the oxygen utilisation, propagation and death of microorganisms (Wang and Xu, 2015; Yan et al., 2015). On the other hand, higher temperature also leads to greater loss of esters due to the increased rates of hydrolysis and volatilisation, whilst a lower fermentation temperature favours the formation of short-chain esters (Fan and Qian, 2006). Other environmental factors such as moisture are not only indispensable to the biochemical reactions in the fermentation process, but also ensure the growth of microbes and provide an effective solvent for their metabolites such as alcohols, acids and esters. Moisture also affects the output rate of the liquor and the extraction of aroma substances existing in fermented grains during the fermentation process. Compared with the temperature, the moisture content was approximately

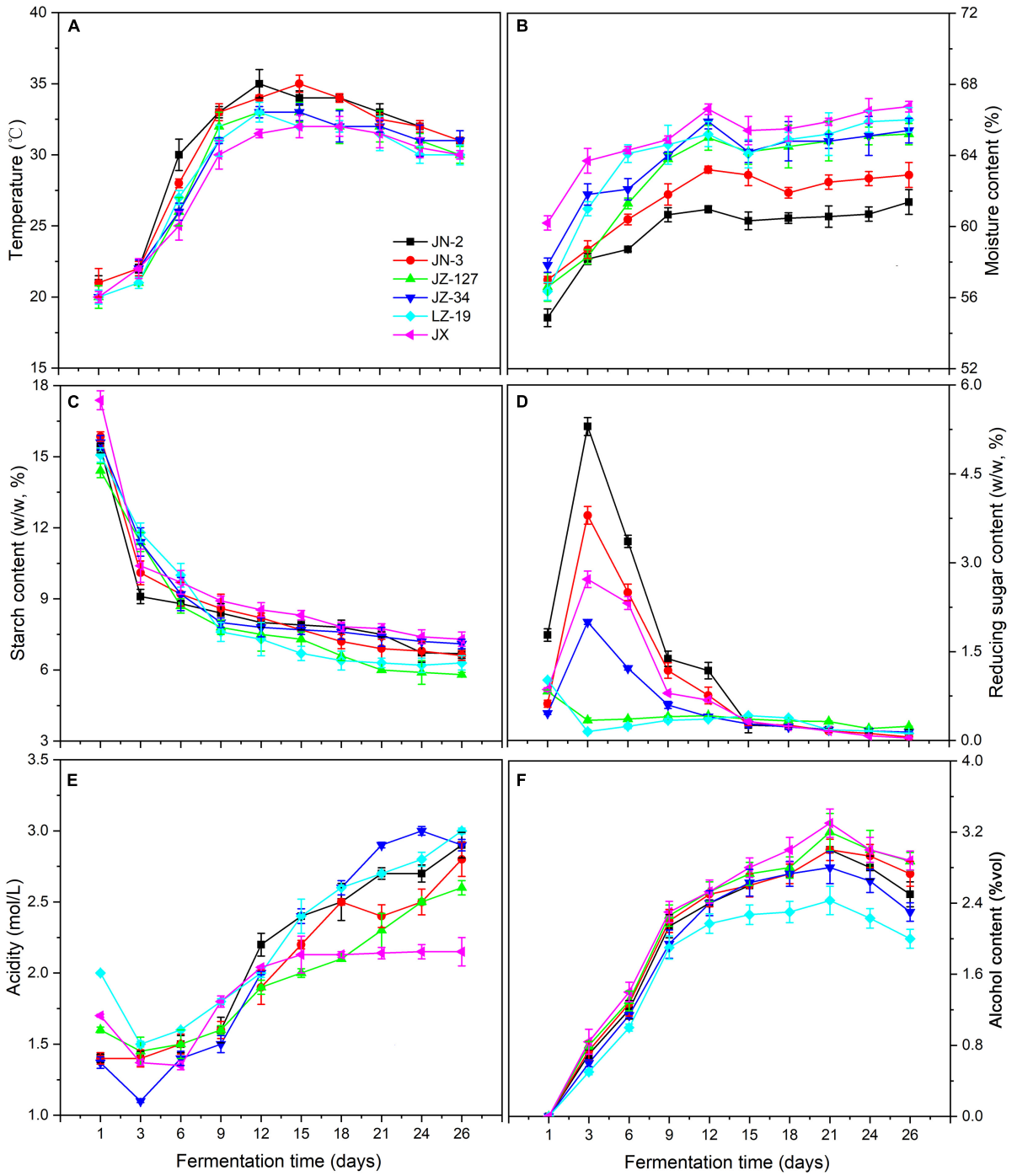

FIGURE 2 | Changes in temperature **(A)**, moisture content **(B)**, starch content **(C)**, reducing sugar content **(D)**, acidity **(E)**, and alcohol content **(F)** of fermented grains during fermentation in two waxy (JN-2 and JN-3) and four non-waxy sorghum varieties (JZ-127, JZ-34, LZ-19, and JX).

60% ($P < 0.05$). The non-waxy sorghums (JZ-127, JZ-34, LZ-19, and JX) exhibited higher moisture contents than their waxy counterparts (JN-2 and JN-3) (**Figure 2B**). Meanwhile, the highest moisture content was maintained in JX fermented grains.

During fermentation, starch as substrate is fermented to sugar and then the sugar forms different compounds (Olofsson, 2008). Starch saccharification rates also produce different sugar concentrations. When starch is less inhibited by reducing sugars in enzymatic hydrolysis, the efficiency of substrate utilisation is increased, thereby significantly influencing cell growth and the metabolism of flavour compounds. The reducing sugar content represents the balance between starch saccharification

and sugar consumption. The sugar consumption rate is regulated by the saccharification rate. These factors ultimately affect the final alcohol yield and quality of the liquor (Wu et al., 2015). In the present study, the starch content of fermented grains exhibited an overall downward trend in all sorghum varieties, and the changes in reducing sugars in waxy (JN-2 and JN-3) and non-waxy (JZ-127, JZ-34, LZ-19, and JX) sorghums were notably different. Moreover, JX sorghum contained the highest starch content amongst the sorghum varieties during fermentation (**Figures 2C,D**). High starch content is beneficial for microbial growth. Starch can be degraded and converted into reducing sugars and other substances. We found that the content of reducing sugars increased 3 days before fermentation and reached a peak, except for that in JZ-127 and LZ-19. This phenomenon was due to the rich oxygen and nutrients in the early stage of fermentation, and microorganisms grew and reproduced rapidly, which resulted in the production of high amounts of amylases and saccharification enzymes and increase in the reducing sugar content (Mcfeeters, 2004). Furthermore, reducing sugars are metabolised by yeast to produce alcohol, thereby affecting the quality and type of liquors (Olofsson, 2008; Chen et al., 2014).

During water kefir fermentation, low nutrient concentrations cause a slow fermentation, resulting in high total residual carbohydrate concentrations and high pH values (Laureys et al., 2018). In liquor production, the environmental factor pH affects the performance of traditional liquor fermentation. Not only is pH an important indicator of microorganism growth and metabolism; it also affects the bioactivity of microorganisms (Wu et al., 2013). In this study, the acidity of fermented grains decreased substantially 3 days before fermentation (**Figure 2E**). The amplification of acidity reached the maximum on the 9th to 12th day of fermentation. The acidity values in JN-3, JN-2, JZ-127, JZ-34, LZ-19, and JX sorghum increased by 37.5, 18.8, 18.8, 33.3, 13.3, and 11.1%, respectively. The acidity of fermented grains showed an upward trend from day 12 to day 26 of fermentation. With the consumption of nutrients such as oxygen, acid-producing bacterial metabolism produced a certain acidity during this stage. A suitable acidity is not only conducive to starch gelatinisation and saccharification but also inhibits the growth of bacteria and facilitates the growth and reproduction of yeast (Wang and Xu, 2015; Yan et al., 2015). Distinctively, we found that JX fermented grains always had low acidity, which is consistent with a previous report (Wang et al., 2014). Wang et al. (2014) demonstrated that in the brewing processes of Chinese liquors, the extreme environment made the dominant bacteria prefer conditions with high ethanol concentrations and low pH values.

Alcohol is the main product of Chinese liquor fermentation, and its concentration is one of the key factors reflecting the fermentation state. During fermentation, yeast can form alcohols from sugars and amino acids under aerobic and anaerobic conditions, respectively (Wondra and Berovic, 2001). Despite the low ethanol content in the fermenting liquid of *Prunus mahaleb* fruit, it contributed to enhancing the sensory characteristics of fermented products and had a preservative function as it inhibited the development of unwanted microorganisms (Swiegers et al., 2005; Gerardi et al., 2019). In this study, the alcohol content showed an upward trend on days 1–21 of fermentation, and alcohol was produced rapidly from day 1 to day 9 (**Figure 2F**). Moreover, the alcohol contents of non-waxy sorghums (JZ-127, JZ-34, LZ-19, and JX) were higher than those of waxy varieties (JN-2 and JN-3); the highest content was observed in JX, indicating that additional substrates for esterification reactions were produced and/or the utilisation of fatty acids was related to the production of alcohol by several microbes (Gong et al., 2017). In the late fermentation stage, the alcohol content showed a decreasing trend, which was possibly due to the inhibited yeast growth under the poor environment following the acid-producing period; subsequently, a portion of the alcohol was converted into acids and esters (Richter et al., 2013). The supply of monosaccharides as a carbon source material for consumption by microorganisms is insufficient.

Microbial Community Diversity in Fermented Grains

Chinese liquor is produced from grains with mixed microbial fermentation technique in solid form, and the metabolic products of the microorganism are important for liquor quality. The micro-environment changes will alter the quantity and species of microorganisms in fermented grains. Therefore, the microorganism community structure of fermented grains not only incarnates the micro-environment in pits, but also affects the formation of liquor flavour components. Studies have shown that the structural diversity and changes of microbial communities are involved in the fermentation of Chinese liquor by multiple analytical methods (Wang X. et al., 2017). To understand the microbial community changes during fermentation, the microbial communities in fermented grains collected from different days were revealed using 16S and ITS Illumina MiSeq technique. After quality sequencing, bacterial (1,623,526 sequences) and fungal communities (761,024 paired-end sequences) were obtained using the 338F/806R (bacterial 16S rRNA) and ITS1F/ITS2 (fungal ITS) primer sets across all fermented grain samples. The numbers of bacterial and fungal sequences varied from 110,328 to 192,689 (mean = 135,586) and from 43,715 to 83,579 (mean = 63,653) per sample, respectively. For downstream analyses of bacterial and fungal sequences, the datasets were rarefied to 108,918 and 41,548 sequences, respectively. From these data, we could conclude that the numbers in bacterial communities were higher than those in fungi.

An OTU level approach was used to calculate the microbial diversity in different sorghum varieties. The Shannon index, which represents the abundance and diversity of microbial communities, was used to reflect the microbial alpha diversity (Gong et al., 2019). As shown in **Figure 3A**, the bacterial alpha diversity had a more remarkable difference in JX than in JN-3, indicating the rich bacterial diversity of the former. Moreover, the microbial diversity (Shannon) indices of the microbial communities in fermented grains decreased significantly from day 3 to day 26. At the beginning of fermentation, a high diversity of microorganisms was observed in fermented grains.

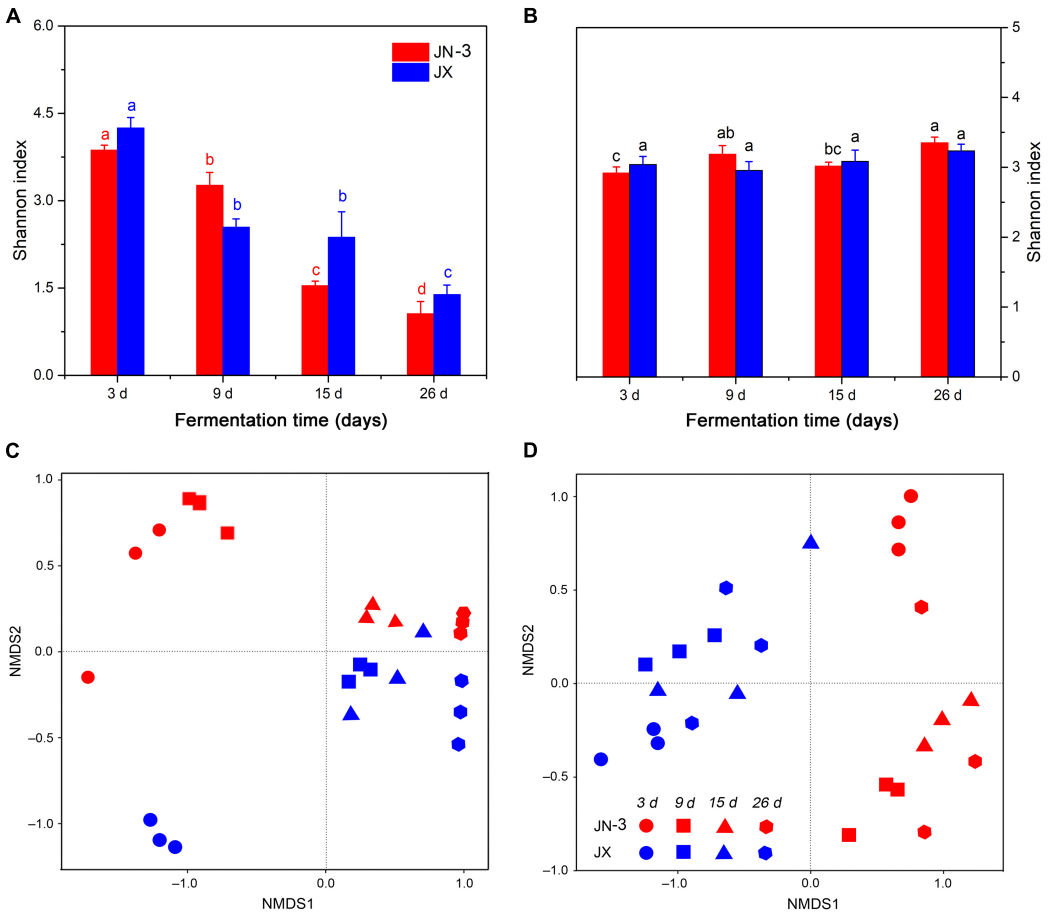

FIGURE 3 | Changes in microbial alpha diversity (Shannon index) [**(A)**, bacteria; **(B)**, fungi] and beta diversity (NMDS) [**(C)**, bacteria; **(D)**, fungi] of fermented grains in one waxy (JN-3) and one non-waxy sorghum (JX). Different letters indicate significant differences (ANOVA, $P < 0.05$) amongst different fermentation times for the same sorghum variety.

With the fermentation time, many microorganisms could not endure the changes of the micro-environment in pits, leading to their death. The fungal alpha diversity showed negligible differences in JN-3 and JX after fermentation (**Figure 3B**). NMDS was used to reflect the microbial beta diversity amongst the sorghum varieties (**Figures 3C,D**). The largest difference in bacterial beta diversity was observed in JN-3 and JX after 3 days of fermentation, revealing that most of the changes occurred in the bacterial community between these varieties. The same trend was not observed for fungi. Our results confirmed that the bacterial alpha diversity was higher in fermented grains of JX, but the fungal alpha diversity for JN-3 and JX fermented grains was difficult to predict, indicating that the bacterial community had higher sensitivity to the Xifeng liquor system than the fungal community.

Bacterial Community Composition in Fermented Grains

As important components of microorganisms for brewing liquor, bacteria can be applied to regulate liquor production and improve liquor quality (Wang X. et al., 2017). In this study, bacterial microorganisms at the phyla level mainly included Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria (**Table 1**). Previous research showed that the environment of the fermentation pit was rich in bacteria mainly belonging to Firmicutes, Actinobacteria and Proteobacteria (Hu et al., 2016). The results of the present study were therefore consistent with those of previous reports. Firmicutes accounted for the total proportion of bacteria in JN-3 and JX, with a consistently increasing trend from day 3 to day 26, whereas JX had a significantly higher bacterial content (46.75 and 15.10%) than JN-3 during the early stage of fermentation (on the 3rd and 9th day of fermentation, respectively). Proteobacteria, Bacteroidetes, and Actinobacteria showed opposite trends during fermentation, and their total proportion in JN-3 and JX consistently declined. Bacilli was the main class observed in all sorghum varieties (**Table 1**). This class has a strong ability to secrete protease, amylase and cellulase and decomposes macromolecular substances to form flavour compounds, such as nitrogen-containing substances. Bacilli also shows high temperature resistance, enzyme production and fragrance production during accumulation (Zhao et al., 2017). In a previous study, the bacterial

TABLE 1 | Relative abundances (average values and standard error) of bacterial compositions across taxonomical classification (phyla, class, and order) of fermented grains in waxy and non-waxy sorghum during fermentation.

Phyla	Class	Order	3 days		9 days		15 days		26 days	
			JN-3	JX	JN-3	JX	JN-3	JX	JN-3	JX
Firm			42.96 ± 1.85d	63.04 ± 3.48d	68.63 ± 0.95c	78.99 ± 0.38c	91.40 ± 0.34b	91.65 ± 1.32b	98.09 ± 0.26a	99.18 ± 0.15a
	Bacil		19.77 ± 0.55d	51.22 ± 1.76d	27.28 ± 0.58c	70.04 ± 0.70c	77.55 ± 0.49b	84.61 ± 1.30b	97.28 ± 0.55a	98.39 ± 0.30a
		Lactob	18.35 ± 0.96d	49.03 ± 2.73d	26.61 ± 0.52c	69.36 ± 0.61c	77.49 ± 0.49b	83.95 ± 1.81b	96.74 ± 0.85a	98.15 ± 0.60a
		Bacill	1.40 ± 0.94a	2.18 ± 1.01a	0.67 ± 0.07ab	0.51 ± 0.11b	0.06 ± 0.02b	0.13 ± 0.05b	0.07 ± 0.04b	0.08 ± 0.02b
	Clost		22.11 ± 1.79b	10.51 ± 1.77a	39.47 ± 1.34a	7.99 ± 0.72b	12.84 ± 0.64c	6.27 ± 0.25b	0.48 ± 0.02d	0.50 ± 0.03c
		Clostr	22.11 ± 1.79b	10.51 ± 1.77a	39.47 ± 1.34a	7.99 ± 0.72b	12.84 ± 0.64c	6.27 ± 0.25b	0.48 ± 0.02d	0.50 ± 0.03c
	Erysi		0.55 ± 0.33b	1.06 ± 0.05a	1.01 ± 0.27a	0.67 ± 0.20b	0.14 ± 0.04b	0.37 ± 0.20c	0.14 ± 0.01b	0.04 ± 0.01c
		Erysip	0.55 ± 0.33b	1.06 ± 0.05a	1.01 ± 0.27a	0.67 ± 0.20b	0.14 ± 0.04b	0.37 ± 0.20c	0.14 ± 0.01b	0.04 ± 0.01c
Prot			23.51 ± 1.48a	21.78 ± 4.08a	5.95 ± 1.05b	11.50 ± 0.17b	1.17 ± 0.06c	2.88 ± 0.94c	1.08 ± 0.02c	0.41 ± 0.03c
	Alpha		21.95 ± 1.13a	20.54 ± 4.07a	3.27 ± 1.05b	11.12 ± 0.14b	0.70 ± 0.04c	2.48 ± 0.93c	0.62 ± 0.02c	0.22 ± 0.01c
		Rhodos	21.23 ± 0.79a	19.94 ± 3.97a	2.27 ± 0.72b	10.97 ± 0.13b	0.42 ± 0.09c	2.09 ± 1.01c	0.24 ± 0.02c	0.17 ± 0.04c
	Gamma		1.19 ± 0.15b	1.08 ± 0.03a	1.88 ± 0.35a	0.34 ± 0.03b	0.42 ± 0.09c	0.35 ± 0.04b	0.36 ± 0.05c	0.17 ± 0.02c
Bact			29.18 ± 1.12a	9.85 ± 1.26a	21.57 ± 1.37b	5.35 ± 0.50b	6.76 ± 0.20c	2.96 ± 0.48c	0.34 ± 0.08d	0.18 ± 0.03d
	Bacte		28.42 ± 1.56a	9.67 ± 1.29a	20.99 ± 1.54b	5.30 ± 0.50b	5.25 ± 0.93c	2.83 ± 0.43c	0.15 ± 0.03d	0.16 ± 0.02d
		Bacter	28.42 ± 1.56a	9.67 ± 1.29a	20.99 ± 1.54b	5.30 ± 0.50b	5.25 ± 0.93c	2.83 ± 0.43c	0.15 ± 0.03d	0.16 ± 0.02d
Acti			4.09 ± 0.56a	4.73 ± 0.60a	3.66 ± 0.33a	3.95 ± 0.18b	0.56 ± 0.03b	2.11 ± 0.48c	0.25 ± 0.03b	0.22 ± 0.09d
	Actin		4.03 ± 0.59a	4.64 ± 0.60a	3.57 ± 0.35a	3.93 ± 0.18a	0.53 ± 0.03b	2.08 ± 0.46b	0.24 ± 0.03b	0.22 ± 0.09c

Phyla level: Firmicutes (Firm), Proteobacteria (Prot), Bacteroidetes (Bact), and Actinobacteria (Acti).
Class level: Bacilli (Bacil), Clostridia (Clost), Erysipelotrichia (Erysi), Alphaproteobacteria (Alpha), Gammaproteobacteria (Gamma), Bacteroidia (Bacte), and Actinobacteria (Actin).
Order level: Bacillales (Bacill), Lactobacillales (Lactob), Clostridiales (Clostr), Erysipelotrichales (Erysip), Rhodospirillales (Rhodos), and Bacteroidales (Bacter).
Different letters indicate significant differences (ANOVA, $P < 0.05$) among different fermentation time for the same sorghum variety.

communities in the fermented grains of two Chinese liquor types were compared (Wang H. Y. et al., 2008), and classes such as Bacilli, Bacteroidetes and Clostridia were dominant in strong flavour type fermented grain. Zheng et al. (2014b) found Lactobacillales as the dominant order in fermented grains, which is consistent with our results. Lactobacillales dominated the fermentation process with relative abundances of 18.35–96.74 and 49.03–98.15% in JN-3 and JX, respectively. Moreover, the proportion of Lactobacillales in JX was higher than that in JN-3 during the whole process of fermentation (**Table 1**). The decreasing trend of prokaryotic diversity and the predominance of *Lactobacillus* may be attributed to the rapid production of Lactobacillales and their tolerance to high concentrations of lactic acid and ethanol in the fermentation process (Li et al., 2011; Martınez-Torres et al., 2016). The inhibition of prokaryotes without acid- and alcohol-resistant properties may also protect the liquor fermentation process from microbiological contamination (Wang X. et al., 2017). Meanwhile, the changes in these bacteria are consistent with those in starch and reducing sugar contents, thus changing the microbial activity to improve the use of nutrients in fermented grains (Sundberg et al., 2013).

At the genus level, four main bacteria, including *Lactobacillus*, *Pediococcus*, *Acetobacter*, and *Prevotella_7*, were detected (**Figure 4A**). In particular, the proportion of *Lactobacillus* gradually increased during fermentation. Our results were consistent with those of a previous study on the bacterial community structures and changes in fermented grain (Li et al., 2011; Wang X. et al., 2017). There into, *Acetobacter* is a strict aerobe, but *Lactobacillus* and *Pediococcus* are facultative aerobes. When fermented for 9 days, the abundance of *Lactobacillus* increased to 77.96% in JX, but it only increased to 23.45% in JN-3. Meanwhile, the abundance of other genera decreased in JX and JN-3 during fermentation, but it was higher in JX than in JN-3. At the end of fermentation, only *Lactobacillus* was abundant in fermented grain. In our study, the highest ethyl acetate content (229.29 mg/100 mL) and highest liquor yield were found in JX. Their findings resulted from bacterial community changes. *Lactobacillus* is the most important bacterium contributing to the fermentation process of producing traditional foods and beverages (Liu et al., 2011). *Acetobacter* is the main functional bacteria used for producing vinegar (Wu et al., 2012). This genus is more competitive under acidic conditions (Jung et al., 2012; Li et al., 2016). In the middle and late stages of fermentation, *Lactobacillus* occupied the majority of microorganisms in the fermented grains, which promoted acidity to rapidly increase the level of esterase and effectively inhibit the metabolic activities of other bacteria (Zheng et al., 2014). *Lactobacillus* can also use lactic acid to form aroma precursors, such as acetic acid, propionic acid and butyric acid, resulting in the synthesis of various esters that increase the aroma components in liquor (Li et al., 2011). However, excessive amounts of *Lactobacillus* in the liquor will result in excessive lactic acid levels and ethyl lactate, resulting in poor taste (Liu et al., 2011). Therefore, the quantity and activity of *Lactobacillus* should be controlled during fermentation to ensure the liquor quality.

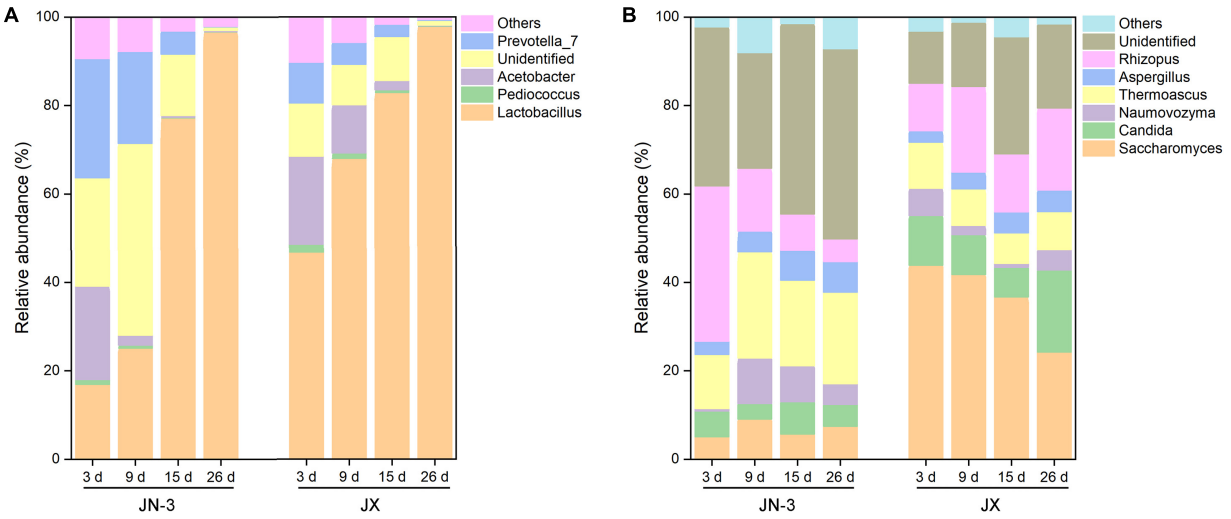

FIGURE 4 | Changes in bacterial **(A)** and fungal **(B)** taxonomic composition of fermented grains at the genus level in one waxy (JN-3) and one non-waxy sorghum (JX). The abundance of each taxon was calculated as the percentage of sequences per gradient for a given microbial group. The taxa represented the class that occurred over 1% abundance in at least one type of sample.

TABLE 2 | Relative abundances (average values and standard error) of fungal compositions across taxonomical classification (phyla, class, and order) of fermented grains in waxy and non-waxy sorghum during fermentation.

Phyla	Class	Order	3 days		9 days		15 days		26 days	
			JN-3	JX	JN-3	JX	JN-3	JX	JN-3	JX
Asco			64.29 ± 1.41d	89.06 ± 0.67a	85.33 ± 0.60c	79.85 ± 1.43b	91.08 ± 1.15b	86.34 ± 1.47a	94.63 ± 0.22a	81.11 ± 2.28b
	Sacch		48.51 ± 3.17c	75.66 ± 0.43a	56.01 ± 3.45b	61.19 ± 4.35c	63.86 ± 1.83a	74.09 ± 1.18a	65.46 ± 2.12a	67.23 ± 3.25b
		Saccha	48.51 ± 3.17c	75.66 ± 0.43a	56.01 ± 3.45b	61.19 ± 4.35c	63.86 ± 1.83a	74.09 ± 1.18a	65.46 ± 2.12a	67.23 ± 3.25b
	Eurot		15.36 ± 1.77b	13.35 ± 0.25b	28.80 ± 3.56a	18.54 ± 0.81a	26.39 ± 2.31a	11.61 ± 0.45c	27.97 ± 1.71a	13.77 ± 1.03b
		Euroti	15.36 ± 1.77b	13.35 ± 0.25b	28.80 ± 3.56a	18.54 ± 0.81a	26.39 ± 2.31a	11.61 ± 0.45c	27.97 ± 1.72a	13.77 ± 1.03b
Muco			35.59 ± 1.43a	10.81 ± 0.60b	14.26 ± 0.74b	19.96 ± 1.61a	8.26 ± 0.97c	13.39 ± 1.50b	5.18 ± 0.09d	18.73 ± 2.23a
	Mucor		35.59 ± 1.43a	10.81 ± 0.60b	14.26 ± 0.74b	19.62 ± 1.61a	8.26 ± 0.97c	13.39 ± 1.50b	5.18 ± 0.09d	18.73 ± 2.23a
		Mucora	35.59 ± 1.43a	10.81 ± 0.60b	14.26 ± 0.74b	19.62 ± 1.61a	8.26 ± 0.97c	13.39 ± 1.90b	5.18 ± 0.09d	18.73 ± 2.23a

Phyla level: Ascomycota (Asco) and Mucoromycota (Muco).
Class level: Saccharomycetes (Sacch), Eurotiomycetes (Eurot), and Mucoromycetes (Mucor).
Order level: Saccharomycetales (Saccha), Eurotiales (Euroti), and Mucorales (Mucora).
Different letters indicate significant differences (ANOVA, $P < 0.05$) among different fermentation time for the same sorghum variety.

Fungal Community Composition in Fermented Grains

Xifeng liquor is produced through a typical simultaneous saccharification and fermentation process. During simultaneous saccharification and fermentation, starch is hydrolysed to fermentable sugar by glucoamylase and α-amylase, which could be produced by many fungal species, such as *Aspergillus*, *Paecilomyces*, *Rhizopus*, *Monascus*, and *Penicillium*. In the meantime, these filamentous fungi have been reported to serve as saccharifying agents (Nahar et al., 2008; Lv et al., 2012; Chen et al., 2014). **Table 2** shows a relatively simple distribution of the taxonomic structure observed at the phyla level. The dominant fungi at the phyla level were Ascomycota and Mucoromycota, accounting for nearly the total proportion of fungi. Moreover, Ascomycota accounted for the total proportion of fungi in JX, which was significantly higher than that in JN-3 during fermentation. Ascomycota was the most dominant fungi at the phyla level, providing the driving force for liquor fermentation. The proportion of Ascomycota consistently increased, whereas that of Mucoromycota gradually decreased. Mucoromycota has strong protease activity, thus allowing the digestion of proteins in dregs into amino acids and further reaction with reducing sugars to form various aroma substances. Saccharomycetes and Saccharomycetales were the main fungal class and order, respectively. Saccharomycetes members play important roles in the synthesis of caproic acid and butyric acid, implying that they also play key roles in brewing liquor (Minnaar et al., 2017).

The changes in fungal communities at the genus level are shown in **Figure 4B**. Six primary fungal genera, including *Saccharomyces*, *Candida*, *Rhizopus*, *Thermoascus*, *Naumovozyma*, and *Aspergillus*, were observed. The starch in sorghum cannot be directly utilised by most yeasts and

bacteria and needs to be hydrolysed into fermentable reducing sugars by α-amylase, β-amylase, glucoamylase and protease. Importantly, these enzymes are mainly produced by a series of microorganisms, such as *Aspergillus*, *Rhizopus*, *Bacillus* spp., *Lactobacillus*, *Wickerhamomyces,* and *Saccharomycopsis* (Beaumont, 2002; Zheng et al., 2011; Chen et al., 2014). Therefore, these genera can effectively decompose starch and increase the content of low-molecular sugars during fermentation to promote liquor fermentation. We found that *Saccharomyces* accounted for one half of the total fungal proportion at the genus level, with the proportion in JX being significantly higher than that in JN-3 during fermentation. *Saccharomyces* is a dominant fungus and has served as a fermenting agent to convert fermentable substrates into alcohol. However, the proportion of *Saccharomyces* in JN-3 was extremely low during the whole fermentation process, which suggests that JN-3 may be unsuitable for brewing liquor. Although several low-abundance genera, such as *Aspergillus* and *Naumovozyma*, showed no significant difference in JX and JN-3, they also played a vital role in the liquor flavour-making process. Wang H. Y. et al. (2008) observed that *Aspergillus* spp. contributed to the saccharification of starch, which accords with the significantly negative relationship between the starch content and *Aspergillus* in this study (**Supplementary Table 4**). Moreover, Vivier et al. (1997) stated that 150 yeast species, expressing glucoamylase in addition to α-amylase with debranching activity, can degrade starch with great efficacy for carbon and energy sources. Thus, fungal communities (*Saccharomyces*, *Candida*, *Rhizopus*, *Thermoascus*, *Naumovozyma,* and *Aspergillus*) are considered as functional microbiota that produce a range of lytic enzymes for synthesising substrates for liquor fermentation and further formation of flavour compounds. Similar results were also reported for *Saccharomyces* and *Candida* in glucose-supplemented minimal medium to understand the role of carbon sources in developing table grape sour rot (Gerardi et al., 2019; Pinto et al., 2019).

Correlation Between Physicochemical Parameters and Microbial Compositions

The differences in the environment also play a key role in the selection of microorganisms used for fermentation. As a result of these multiple variations, Wuliangye, Jiannanchun, and Yanghe Daqu all have unique aroma profiles (Shen, 1996; Fan and Xu, 2000). Therefore, we investigated the interactions between liquor quality and physicochemical property and microbial compositions during fermentation by using Pearson's correlation coefficients (**Figures 5**, **6**). The results exhibited that temperature, moisture and acidity were negatively related to alcohol and ester, whereas starch and reducing sugars showed a positive relation.

These liquor fermentation parameters were also related to the relative abundances of bacterial and fungal communities (**Supplementary Tables 2,3**). Amongst these parameters, temperature, moisture, acidity and alcohol content were negatively related to the relative abundances of bacterial communities, except for Firmicutes, Bacilli and Lactobacillales, in contrast with the relation to starch and reducing sugars. Hence, large amounts of starch and reducing sugar were consumed by

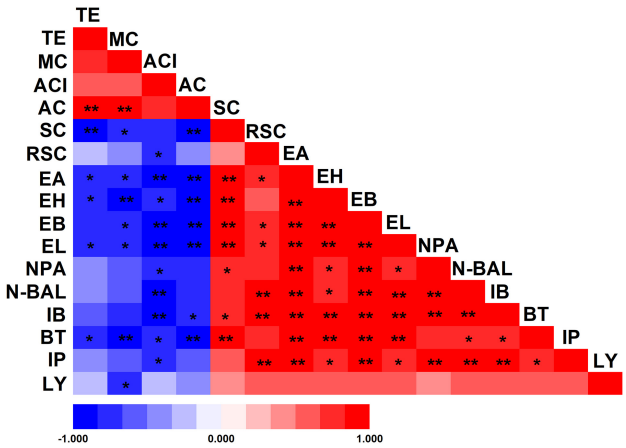

FIGURE 5 | Pearson's correlation coefficients between physicochemical properties of fermented grains and volatile compounds of liquor. *Correlation is significant at the 0.05 level. **Correlation is significant at the 0.01 level. TE, temperature; MC, moisture content; ACI, acidity; AC, alcohol content; SC, starch content; RSC, reducing sugar content; EA, ethyl acetate; EH, ethyl hexanoate; EB, ethyl butyrate; EL, ethyl lactate; NPA, n-propanol; N-BAL, n-butanol; IB, isobutanol; BT, butanol; IP, isopentanol; LY, liquor yield.

Lactobacillales. Temperature, moisture and alcohol content were positively related to the relative abundances of fungal communities, except for Mucoromycota, Mucoromycetes, and Mucorales, in contrast with the relation to starch and reducing sugar.

We found that Proteobacteria, Bacteroidetes and Actinobacteria; Alphaproteobacteria, Bacteroidia, and Actinobacteria; Bacillales, Bacteroidales, and Rhodospirillales; and *Acetobacter*, *Pediococcus,* and *Prevotella_7* were positively related to four esters and five alcohols [the absolute value of Spearman's rank correlation coefficient (ρ) was over 0.6] (**Figure 6**). Despite the low relative abundances of *Acetobacter*, Actinobacteria, Bacillales, and *Pediococcus* in bacterial communities, they played important roles during fermentation. Meanwhile, alcohol and ester were significantly negatively related to Firmicutes, Bacilli, Lactobacillales, and *Lactobacillus* [the absolute value of Spearman's rank correlation coefficient (ρ) was over 0.6]. As lactic acid bacteria, *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Pediococcus,* and *Weissella* are regarded as the main functional genera in the fermentation process of Chinese liquor (Zhang et al., 2005; Li et al., 2011). Previous studies showed that lactic acid bacteria can produce lactic acid, and *Gluconobacter* and *Acetobacter* can produce acetic acid (Jung et al., 2012; Li et al., 2016). High concentrations of lactic acid and acetic acid in fermented grains resulted in acidic stress. Meanwhile, the decreased diversity during fermentation also confirmed the selective action on microbes in fermented grains. Lactic acid is a well-known precursor of ethyl lactate, which can enhance the mellow feeling of Chinese liquor. By contrast, acetic acid is the precursor of ethyl acetate, which contributes to the fruit flavour of Chinese liquor (Gao et al., 2014). These data demonstrate the relationship between alcohol esters and microorganisms.

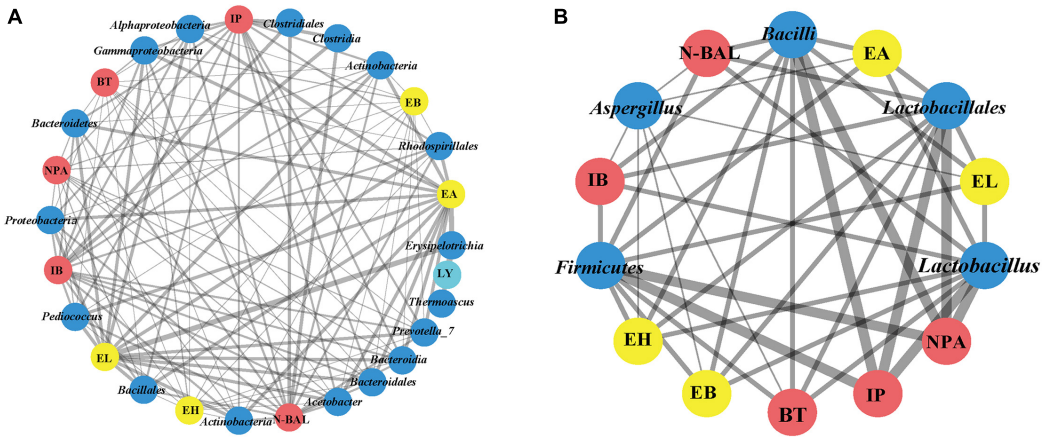

FIGURE 6 | Co-occurrence network of bacterial and fungal communities (phyla, class, order, and genus) of fermented grains and volatile compounds of liquor based on correlation analysis. A connection stands for a strong positive [**(A)**, Spearman's $\rho > 0.6$] or negative [**(B)**, Spearman's $\rho > -0.6$] correlation. The thickness of the line is proportional to the absolute value of Spearman's correlation coefficients.

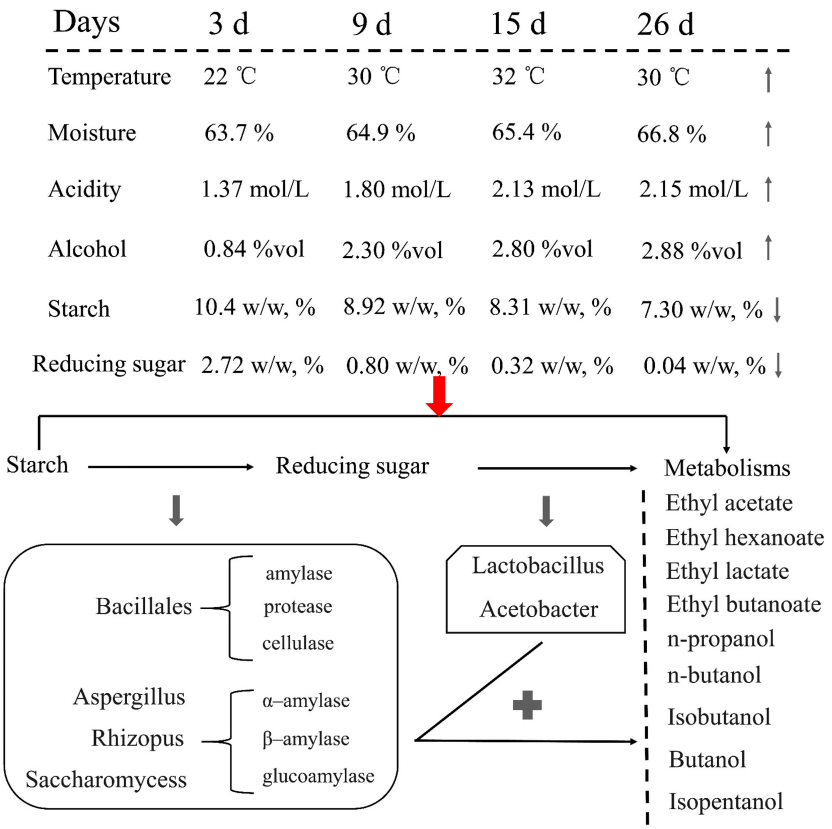

FIGURE 7 | Changes in the physicochemical parameters of JX fermented grains and hypothetical flux of carbon and involvement of key microorganisms during liquor fermentation from starch to the main metabolic products.

For fungal communities, *Saccharomyces* and *Rhizopus* were positively related to four esters and five alcohols, except for Mucoromycota, Mucoromycetes, and Mucorales. However, no significant differences were observed (**Supplementary Table 5**). Notably, only *Aspergillus* was significantly negatively related to four esters (EA, EH, EB, and EL) and four alcohols (NPA, N-BAL, IB, and BT) [the absolute value of Spearman's rank correlation coefficient (ρ) was over 0.6].

Ester and alcohol have a direct relationship with the fermentation environment and microbial communities and are

involved in the fermentation process. In particular, the changes in physical and chemical properties, such as the use of starch and reducing sugars, should be considered during fermentation. Comprehensive analysis showed that JX sorghum is suitable for Xifeng liquor production. Subsequently, the production of Xifeng liquor under the fermentation conditions of JX sorghum as the standard can be promoted (**Figure 7**).

CONCLUSION

This study investigated the dynamic changes in the physicochemical parameters of JX fermented grains. Although the fungal beta diversity remained almost unchanged during fermentation, higher bacterial alpha diversity was found in JX fermented grains. Meanwhile, JX contained the highest ethyl acetate content but the lowest amount of ethyl lactate. We found that Proteobacteria, Bacteroidetes, and Actinobacteria; Alphaproteobacteria, Actinobacteria, and Bacteroidia; Bacillales, Bacteroidales, and Rhodospirillales; and *Acetobacter*, *Pediococcus,* and *Prevotella_7* in bacterial microorganisms were significantly positively related to four esters and five alcohols, whereas only *Aspergillus* in fungal microorganisms was significantly negatively related to four esters and three alcohols. This study provided evidence of the correlation between the physicochemical properties and the bacteria and fungi contributing to the fermentation process.

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: NCBI, prjna670598 and prjna670601.

AUTHOR CONTRIBUTIONS

CL and XG contributed to the experimental design, data analysis, and manuscript writing. GZ, MS, ZJ, and ZY contributed to the experimentation. LL, QZ, TH, XD, and BF contributed to the data interpretation and manuscript preparation. 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.2021.618458/full#supplementary-material

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Microbiological and Chemical Characteristics of Wet Coffee Fermentation Inoculated With *Hansinaspora uvarum* and *Pichia kudriavzevii* and Their Impact on Coffee Sensory Quality

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Hansinaspora uvarum and *Pichia kudriavzevii* were used as starter cultures to conduct inoculated wet fermentations of coffee beans, and their growth, metabolic activities and impact on the flavor, aroma and overall sensory quality of coffee were compared with spontaneous fermentation (control). *H. uvarum* and *P. kudriavzevii* dominated the fermentations, growing to maximum populations of about 10.0 log CFU/ml compared with 8.0 log CFU/ml in the spontaneous fermentation. The dominance of the inoculated yeasts led to faster and more complete utilization of sugars in the mucilage, with resultant production of 2–3 fold higher concentrations of metabolites such as glycerol, alcohols, aldehydes, esters, and organic acids in the fermented green beans. Cup tests showed coffee produced from the inoculated fermentations, especially with *P. kudriavzevii*, received higher scores for flavor, aroma and acidity than the control. The findings of this study confirmed the crucial role of yeasts in the wet fermentation of coffee beans and their contribution to high quality coffee, and demonstrated the potential *H. uvarum* and *P. kudriavzevii* as starter cultures in the process.

Keywords: *Hansinaspora uvarum*, *Pichia kudriavzevii*, yeasts, coffee fermentation, volatiles

INTRODUCTION

The wet process is one of the major primary processing methods for coffee beans and able to produce coffee with high qualities (Mussatto et al., 2011; Brando and Brando, 2014). In the process de-pulped ripe coffee cherries are subjected to underwater fermentation for 6–72 h, and dried (Schwan and Wheals, 2004). The fermentation is performed mainly to remove the mucilage layers still stuck to the coffee beans (Brando and Brando, 2014). Furthermore, it was reported that the process improves coffee flavor and aroma by producing desirable microbial metabolites such as higher alcohols, esters, aldehydes, and organic acids (Mussatto et al., 2011; Elhalis et al., 2020a). The wet fermentation is largely conducted in a traditional, uncontrolled manner where indigenous microflora grow spontaneously, leading to a complex microbial ecology (Avallone et al., 2001; Silva et al., 2008). Conducting the fermentation in this way increases the probability of producing

products with inconsistent and unpredictable quality (Durso and Hutkins, 2003). Transforming the fermentation into a more efficient and controllable industrial process can be achieved by using defined starter cultures. Wild type endogenous isolates originated from the natural ecosystem of wet fermentation can be a good source to search for promising candidates of functional starter cultures (Leroy et al., 2006; Wouters et al., 2013; Pereira et al., 2015).

A wide range of microorganisms have been isolated from coffee fermentations, including yeasts, filamentous fungi, lactic acid bacteria, acetic acid bacteria, *Enterobacteriaceae*, and *Bacillus* spp. (Avallone et al., 2001; Djossou et al., 2011; Elhalis et al., 2020a). Yeasts are one of the frequent isolates from coffee fermentation in multiple locations over the globe with *Hansinaspora uvarum*, *Debaryomyces hansenii*, *Pichia kluyveri*, *P. anomala*, *Saccharomyces cerevisiae*, *Candida*, and *Torulaspora delbrueckii* being the common species (Masoud et al., 2004; Silva et al., 2008; Vilela et al., 2010; Pereira et al., 2014; Elhalis et al., 2020a). Several yeast species isolated from spontaneous coffee fermentations have been reported to possess mucilage degradation capability, produce flavoring compounds, and suppress the growth of mycotoxin producing filamentous fungi (Masoud et al., 2004; Masoud and Jespersen, 2006; Silva et al., 2008; Silva, 2014; Evangelista et al., 2015; Pereira et al., 2015; Elhalis et al., 2020b). These features make yeasts promising candidates as starter cultures in the primary processing of coffee beans. Recently, a few trials of inoculated coffee wet fermentation using yeasts such as *S. cerevisiae*, *Torulaspora*, and *P. fermentans* as starter cultures have been reported, mainly in Brazil, with promising results (Pereira et al., 2014, 2015; Martins et al., 2019). These studies reported increases in essential volatiles such as ethyl acetate, isoamyl acetate, ethanol, and acetaldehyde and improvement in sensory quality of the final products. Previously, we reported the crucial role of yeasts in the wet fermentation of Australian coffee beans and their contribution to the flavor, aroma, and overall sensory perceptions of high quality coffee (Elhalis et al., 2020b, 2021b). Subsequently, we studied the contribution of the isolated yeasts and found among the isolates, *H. uvarum* (MF574306.1) and *P. kudriavzevii* (CP021092.1) have shown high resistance to the stress conditions prevailing in coffee fermentation, and production of desirable flavor and aroma profiles (Elhalis et al., 2021a). In this study, these two yeasts were used, for the first time, as starter cultures to conduct inoculated coffee wet fermentation. The main objective of the study was to investigate the performance of the two yeasts in coffee wet fermentation in terms of their growth, metabolic activities and contribution to the sensory quality of the final coffee product, with a view to evaluating their potential as starter cultures. The second objective of this study was to verify the crucial positive roles of yeasts in coffee fermentation and coffee beverage quality as reported in our previous study where the growth of yeasts was suppressed by the addition of Natamycin and the resultant beans were compared to non-treated spontaneous fermentation (Elhalis et al., 2020b). The levels of key metabolites and volatiles were lower in the yeast suppressed fermented beans in addition to their lower sensory scores than that with yeast growth. Therefore we conclude that yeasts play crucial roles in fermentation for producing high quality coffee beans (Elhalis et al., 2020b).

MATERIALS AND METHODS

Preparation of Yeast Starter Cultures

The yeasts *H. uvarum* and *P. kudriavzevii* were previously isolated from wet fermentations of coffee beans conducted at the laboratory of University of New South Wales, Sydney, NSW, Australia, and their classification was confirmed by sequencing of the 5.8S-ITS rDNA gene region (Elhalis et al., 2020a). Pure cultures of the yeasts were kept on malt extract agar (MEA) slants at 4°C from which they were inoculated into 250 ml of yeast extract broth (YE), incubated in shaking incubator (120 rpm) at 30°C overnight. The cultures were transferred into YE broth (750 ml) and incubated with shaking for 24 h at 30°C. Yeast cells were collected by centrifugation (3,200×*g*, 15 min) and resuspended in 200 ml 0.1% sterile peptone water (Sigma, Sydney, NSW, Australia). Cell counts in the suspensions were performed using a counting chamber (Improved Neubauer, Assistent, Germany) and further confirmed by plate counting on MEA.

Inoculated Coffee Bean Fermentation With Yeasts

Freshly harvested coffee cherries (Coffea arabica var. Bourbon, 40 kg) from Zentveld's Coffee Farm located in Newrybar, NSW, Australia were packed in polystyrene foam containers with ice and immediately airfreighted to UNSW Sydney. Details of sample preparation and fermentation were given in Elhalis et al. (2020a), and only experiments involved the inoculation of the yeast starter cultures are described here. De-pulped coffee beans (5 kg) were transferred to a sterile plastic box (56 × 42 × 33 cm) containing 15 L of tape water, which was inoculated with the suspension of *H. uvarum* and *P. kudriavzevii* separately and together with a 1:1 ratio. The initial population of each species in the ferment was adjusted to a total concentration of about 10^8 cells/ml, which was allowed to ferment for 36 h at room temperature. The fermenting beans were mixed every 12 h. Samples (200 ml) of the liquid fraction of the ferments were collected every 12 h, which were either examined immediately by traditional culture dependent methods or frozen at −20°C until analysis by culture independent molecular methods. Samples of coffee beans (100 g) were also taken and frozen at −20°C for chemical analyses. Several preliminary fermentations were conducted to optimize the fermentation conditions and the initial inoculum counts. Two independent fermentations were conducted for each yeast species and the two yeasts combined together with a total of six inoculated fermentations were conducted. Another 2 × 5 kg of the de-pulped beans were fermented in exactly the same way except without the yeast inoculation (spontaneous fermentation), which was used as the control. After fermentation, coffee beans were dried and roasted as described in Elhalis et al. (2020b). The dried coffee beans were stored at −20°C in vacuumed plastic bags until further analysis. A laboratory dehuller was used to dehull the dried beans and 100 g samples were roasted in a laboratory roaster (IKAWA, London, United Kingdom)

following to the procedures of the Specialty Coffee Association of America (SCAA, 2019). Roasting was performed at 225°C for 7 min and the air flow was set at high, which flowed from the bottom to the coffee beans separating the lightweight undesirable beans. The roasted beans were collected and ground using an electric coffee grinder (Mahlkonig's EK43, Revesby, Sydney, NSW, Australia), which was cleaned between samples. Ground coffee samples were vacuum packed in aluminum bags and stored at −20°C until further analysis.

Sensory Analysis

Sensory evaluation (Cup test) of the coffee was conducted as described previously in Elhalis et al. (2021b) by a panel of three expert coffee testers with Q-Grader coffee certification (Coffee Logic Institute, Sydney, NSW, Australia). In brief, the testing protocol consisted of a three-step process: first, the aroma evaluation of the dry grounded coffee beans by sniffing; second, the aroma evaluation of brewed coffee by sniffing over the cup with stirring three min after its preparation; and third, evaluation of the aroma after 8–10 min of brewing. Each coffee sample was assessed in five cups. Apart from the aroma, coffee characteristics such as acidity, balance, body, aftertaste, uniformity, sweetness, clean cup, and overall impression were also evaluated by tasting the brew. The total score of each sample was estimated by the sum of all the attributes. At the end, the members of the panel were also asked to describe the specific flavor of each sample.

Microbiological Analysis

Culture Dependent Methods

The populations of the inoculums and the endogenous yeasts, as well as total aerobic bacteria count (TABC) and lactic acid bacteria (TLAB) were monitored by traditional plate count methods using appropriate agar media. Details of the methods were given in Elhalis et al. (2020a). Wet mounts were used to differentiate the cellular morphologies of the isolates. LABs were further confirmed by Gram staining and catalase test. The reported population data are the means of duplicate analyses within a standard deviation of ±0.05. For the inoculated yeasts, their identities were initially based on the colony morphology, growth characteristics and the 5.8S ITS rRNA gene region sequencing as described previously in Elhalis et al. (2020a). During the fermentation processes the identity of the inoculated yeasts were further confirmed by restriction fragment length polymorphism PCR (PCR-RFLP) of the 5.8S-ITS rDNA using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) that were further digested using *Cfo*I, *Hae*III, *Cfo*I, and *Hin*fI (White et al., 1990; Esteve-Zarzoso et al., 1999). Specifically, YEA plates with 30–300 colonies were selected and pure colonies were picked randomly to extract DNA (Cocolin et al., 2002). PCR reactions were started by initial denaturation at 98°C for 5 min and 35 cycles of denaturation at 98°C for 30 s, followed by annealing at 56°C for 30 s, extension at 72°C for 50 s, and final extension at 72°C for 7 min. The PCR products were digested using *Cfo*I, *Hae*III, *Cfo*I, and *Hin*fI. The molecular sizes of the PCR and restriction fragment products were estimated by gel electrophoresis on 1.5 and 3% agarose gels, respectively, and compared with yeast profiles reported in the literature to confirm the identity of the yeasts (Esteve-Zarzoso et al., 1999; del Monaco et al., 2014).

Quantitative Real-Time PCR Analysis of Yeasts

Direct DNA Extraction From the Fermentation Mass

Samples (10 ml) were aseptically taken from three different points (surface, middle and bottom) of the fermentation mass and first centrifuged at low speed (200×*g*, 3 min) to precipitate the coarse particles and the supernatants were collected. The precipitates were further washed twice with sterile 50 mM phosphate buffer (pH 6.0) to recover the yeast cells that might precipitate with the coarse particles during the centrifugation. All the supernatants from the first centrifugation and the washing steps were transferred to fresh tubes and centrifuged in an EBA12 Centrifuge (Hettich, Newport Pagnell, Buckinghamshire, United Kingdom) at 13,000 rpm for 10 min to precipitate the microbial cells and the supernatant discarded. DNA from the cells were extracted using a three-step procedure following Elhalis et al. (2020a). In brief, the cell pellet was resuspended in 200 μl of 50 mM phosphate buffer (pH 6.0) containing cell lysis enzymes (chitinase 500 mU/ml and lyticase 0.2 U) and incubated at 30°C for 3 h. The enzymatically digested cells were further lysed using 200 μl of breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM *Tris*–Cl pH 7.6, and 1 mM EDTA pH8) and vortex for 1 min. Finally, the mixtures were sonicated for 1 h at room temperature. The extracted DNA was precipitated by adding an equal volume of isopropanol and purified with the DNeasy blood and tissue kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. The concentrations of extracted DNA and protein contamination were quantified by a spectrophotometer at UV 260 and 280 nm, respectively.

Design of Primers and Determination of Annealing Temperature

The specific primers were created in our laboratory using Primer3 (Rozen and Skaletsky, 2000) and their specificity were check using the BLAST algorithm (National Center for Biotechnology Information, ML, United States[1]). One pair of the specific primers was created for each target yeast species (**Table 1**). The annealing temperature of each primer pair was determined by performing temperature gradient PCR of 50–60°C. PCR was performed in a thermocycler (C1000 Thermal Cycler, Bio-Rad, Hercules, CA, United States) in 25 μl final volume using Hot Start Taq 2X Master Mix (New England Biolabs, Ipswich, MA, United States) according to manufacturer's instructions. The PCR conditions consisted of an initial step at 98°C for 5 min, followed by 35 cycles of denaturation at 98°C for 30 s, annealing at temperature gradient of 50–60°C for 30 s, and extension at 72°C for 50 s. A final extension was conducted at 72°C for 7 min. The PCR products were analyzed by electrophoresis on 3% agarose gel in 1X TBE buffer. The optimal annealing temperatures obtained are given in **Table 1**. The specificity of the primers was further verified using target yeasts and non-target fungal DNA (*S. cerevisiae*) as described above.

[1]http://www.ebi.ac.uk/blastall/nucleotide.html

TABLE 1 | Correlation coefficient, slope, and efficiency of standard curves estimated from serial dilution of the target yeasts grown in YE broth.

Species	Primer	Sequence 5′–3′	R^2	Slope	Efficiency* (%)
Hansinaspora uvarum	H.U.J.F	TTGGTTGTGGGCGATACTCA	0.977	−3.986 ± 0.018	78.187 ± 3.77
	H.U.H.R	CATCACAGCGAGAACAGCGT			
Pichia kudriavzevii	P.K.J.F	TTGAGCCGTCGTTTCCATCTT	0.998	−3.663 ± 0.118	87.508 ± 0.641
	P.K.H.R	CAGCGGGTATTCCTACCTGA			

**Efficiency was estimated by the formula $E = 10^{-1/slope} - 1$ (Higuchi et al., 1993).*

Construction of Standard Curves

The yeast species were cultivated in YE broth at 30°C for 24 h. Final cell counts were determined by plate counting on YEA in duplicate. DNA was extracted as described above from the 24 h culture broth serially diluted with TE buffer (pH 8.0) from 10^{12} to 10^3 cells/ml. PCR assays were carried out using iTaq Universal SYBR Green Supermix (New England Biolabs, Ipswich, MA, United States) according to the manufacturer's instructions. A linear relationship was created by plotting the cell counts against the cycle threshold values (Carrasco et al., 2019). The slope of the curve was used to estimate the amplification efficiency following the equation $E = 10^{-1/\text{slope}} - 1$ (Higuchi et al., 1993) as shown in **Table 1**. The specificity of the primers in qPCR was also confirmed by conventional PCR using target and non-target yeasts as described above. The experiment was repeated twice each with three replicates.

Quantitative PCR of Target Yeast Populations During the Wet Fermentations

The extracted DNA from the fermentation mass were amplified using iTaq Universal SYBR Green Supermix (New England Biolabs, Ipswich, MA, United States) according to the manufacturer' instructions in the same way as described above. The C_t was calculated automatically by the instrument and the cell concentration of each yeast was estimated by using the calibration curve for each target yeast constructed as described above. The experiment was repeated independently two times and each assay was done in triplicates and non-template controls were included in all PCR runs.

Chemical Analysis

Details of the methods used for analysis of sugars, organic acids, glycerol, and mannitol in the green beans were described in Elhalis et al. (2020a). Volatile compounds in the green and roasted beans were analyzed by head space solid phase microextraction gas chromatography mass spectrum (HS-SPME/GCMS) according to Frank et al. (2017) and details of the method was described in Elhalis et al. (2020b). Volatile compounds were identified by matching their electron impact mass spectra with those of reference compounds in the NIST mass spectral library and by comparing linear retention indices with published values in the NIST and PubChem websites. Further confirmation was done in some cases using pure reference standards. The concentration of the volatiles was determined semi-quantitatively using the Shimadzu proprietary software "LabSolutions" (Version 2.53). The extraction and analysis of each sample were performed in triplicates and the results were expressed as an average (±standard deviation).

Statistical Analysis

One-way ANOVA was carried out to compare means between sample treatments, and Tukey's HSD *post hoc* test was used to separate means of significant differences. Differences were statistically significant at $p < 0.05$. All statistical analyses were performed with GenStat® (16th Edition, VSN International, Hemel Hempstead, United Kingdom).

RESULTS

Microbial Ecology

The microbial population showed a total yeast count of 5.5 log CFU/ml during the first hour of the spontaneous fermentation that grew to 7.3 log CFU/ml within 36 h. *H. uvarum*, *P. kudriavzevii* were the most abundant endogenous yeast isolates with an initial population of 5.1 and 4.8 log CFU/ml, respectively (**Figure 1A**). As the fermentation progressed, *H. uvarum and P. kudriavzevii* grew to maximum populations of 7.1 and 6.5 log CFU/ml, respectively. The initial total aerobic bacterial and lactic acid bacteria counts were 5.1 and 5.3 log CFU/ml which subsequently grew to 7.9 and 7.3 log CFU/ml, respectively, in the spontaneous fermentation. The qPCR analysis results for *H. uvarum* and *P. kudriavzevii* of the spontaneous fermentation were similar to the plat counts.

In the fermentation inoculated with *H. uvarum*, all the colonies appeared on YEA agar plates were *H. uvarum* with an initial count of 8.0 log CFU/ml which subsequently grew to a maximum population of 9.6 log CFU/ml within 36 h (**Figure 1B**). The growth pattern of total LAB in the fermentation was similar to that in the spontaneous fermentation. The total aerobic bacterial count at the beginning was 5 log CFU/ml, which grew to a maximum population of 8.5 log CFU/ml within 36 h. The qPCR results showed an initial count of *H. uvarum* of 8 log CFU/ml, which subsequently increased to a maximum population of log 10.1 CFU/ml as the fermentation progressed. The qPCR also detected *P. kudriavzevii* throughout the entire *H. uvarum* inoculated fermentation with an initial count of 5.0 log CFU/ml that grew to a maximum population of 6.3 log CFU/ml at the end.

In the fermentation inoculated with *P. kudriavzevii*, 100% of the colonies grown on YEA agar plates were *P. kudriavzevii*, with the populations ranging from 8.2 to 10 log log CFU/ml, and no other yeast species were detected. The total aerobic bacterial and

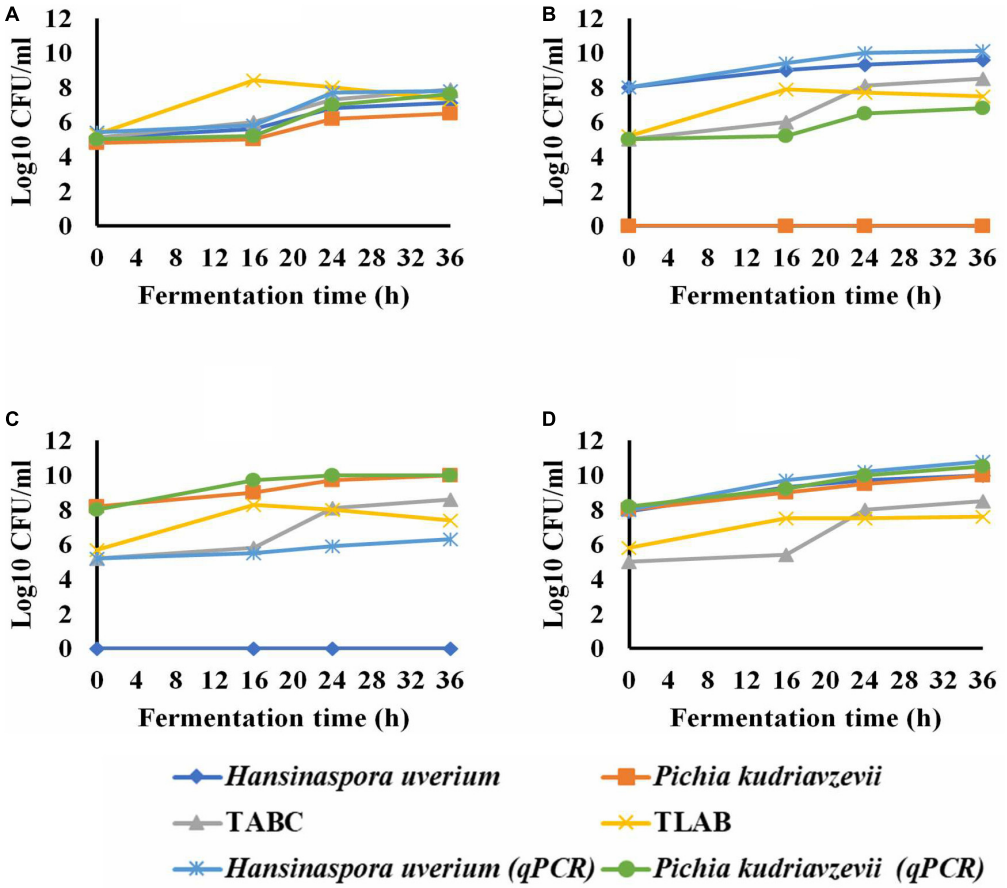

FIGURE 1 | The growth of yeasts during wet coffee beans fermentation; spontaneous (control) **(A)**; *Hansinaspora uvarum* inoculations **(B)**; *Pichia kudriavzevii* inoculations **(C)** and inoculation with *H. uvarum* and *P. kudriavzevii* Combined **(D)**. TABC, total acrobic bacteria count; TLAB, total lactic acid bacteria; qPCR, quantitative real time PCR. Microbial populations were determined by plate coubting with SD of means less than 5% (, , ,) and qPCR with SD of means ranged from 0.006 to 0.09 log10 CFU/ml (,).

LAB populations and their growth dynamics were similar to those in the *H. uvarum* inoculated fermentation. The qPCR identified *P. kudriavzevii* as well as *H. uvarum* in the fermentation with initial populations of 5.2 and 8.0 log CFU/ml, which subsequently grew to maximum populations of 6.3 and 10.0 log CFU/ml, respectively (**Figure 1C**).

In the fermentation inoculated with both yeasts, 100% of the recovered yeasts on YEA were *H. uvarum* and *P. kudriavzevii* sharing about 50% each, which grew to maximum populations of 10.0 log CFU/ml each at the end. The total aerobic bacteria and LAB grew to maximum populations of 8.5 and 7.6 log CFU/ml, respectively. The qPCR results agreed with the plate counts at the start of the fermentations, and then *H. uvarum* and *P. kudriavzevii* grew to 10.8 and 10.5 log CFU/ml in at the end, respectively (**Figure 1D**).

Microbial Metabolisms

Non-volatile Compounds

The concentrations of the major non-volatile compounds found in the mucilage and endosperm of coffee beans are shown in **Tables 2**, **3**. Three main sugars were detected in coffee beans, which were sucrose, glucose and fructose. The levels of these sugars declined during the fermentation which were faster in the inoculated fermentation ($p > 0.05$). Lactic acid and glycerol were the major end metabolites detected during fermentations which were about 2-fold higher in the inoculated beans than the controls ($p > 0.05$).

The concentrations of the major non-volatile compounds found in the mucilage layers of coffee beans are shown in **Table 2**. The mucilage layers contained three sugars, which were fructose (27.0 g/100 g), glucose (19.4 g/100 g) and sucrose (13.0 g/100 g). The levels of these sugars declined during the spontaneous (non-inoculated control) fermentation to 2–7 g/100 g. In the inoculated fermentations, the decreases of sugars were faster and more extensive, leading to either total consumption or lower concentrations than those in the control. Four main organic acids were detected at the beginning of the fermentations, which were malic, succinic, quinic, and citric with concentrations of about 1–2 g/100 g. During the control fermentation, their concentrations declined to about 0.8–1.6 g/100 g. Similar trends were observed in the inoculated fermentations; however, the level of citric

TABLE 2 | Comparison of chemical composition and microbial metabolites in the mucilage of coffee beans undergone spontaneous (control) and inoculated fermentations with *H. uvarum*, *P. kudriavzevii*, and the two yeasts combined.

Compounds	Concentration (g/100 g)							
	Control		*H. uvarum*		*P. kudriavzevii*		Combined yeasts	
	T0	*T36*	*T0*	*T36*	*T0*	*T36*	*T0*	*T36*
Sucrose	13.03 ± 1.50^{a}	2.01 ± 0.17^{b}	13.70 ± 0.75^{a}	1.02 ± 0.20^{c}	13.70 ± 3.10^{a}	1.22 ± 0.02^{c}	13.18 ± 0.17^{a}	ND^{d}
Glucose	19.41 ± 1.20^{a}	7.06 ± 2.07^{b}	21.09 ± 1.01^{a}	ND^{d}	21.80 ± 0.79^{a}	2.01 ± 0.08^{c}	27.20 ± 1.04^{a}	2.03 ± 0.03^{c}
Fructose	27.02 ± 2.01^{a}	4.05 ± 0.08^{b}	27.04 ± 1.33^{a}	3.12 ± 0.07^{c}	27.70 ± 1.22^{a}	3.03 ± 0.05^{c}	27.14 ± 1.14^{a}	2.70 ± 0.04^{c}
Glycerol	ND^{d}	0.90 ± 0.02^{c}	ND^{d}	1.70 ± 0.03^{b}	ND^{d}	1.32 ± 0.02^{b}	ND^{d}	2.10 ± 0.13^{a}
Mannitol	ND^{b}	0.92 ± 0.03^{a}	ND^{b}	1.10 ± 0.05^{a}	ND^{b}	1.00 ± 0.01^{a}	ND^{b}	1.22 ± 0.03^{a}
Citric acid	1.18 ± 0.03^{b}	0.80 ± 0.03^{c}	1.23 ± 0.01^{b}	1.50 ± 0.02^{a}	1.16 ± 0.02^{b}	0.83 ± 0.01^{c}	1.20 ± 0.03^{b}	1.63 ± 0.03^{a}
Malic acid	2.30 ± 0.04^{a}	1.56 ± 0.01^{b}	2.12 ± 0.02^{a}	1.53 ± 0.01^{b}	2.11 ± 0.03^{a}	1.51 ± 0.01^{b}	2.13 ± 0.01^{a}	1.52 ± 0.02^{b}
Quinic acid	1.90 ± 0.03^{a}	1.52 ± 0.02^{b}	1.97 ± 0.01^{a}	1.62 ± 0.02^{b}	2.02 ± 0.08^{a}	1.52 ± 0.02^{b}	1.93 ± 0.04^{a}	1.52 ± 0.01^{b}
Succinic acid	2.14 ± 0.11^{a}	1.32 ± 0.02^{b}	2.05 ± 0.13^{a}	1.43 ± 0.09^{b}	2.09 ± 0.08^{a}	1.36 ± 0.05^{b}	2.14 ± 0.10^{a}	1.53 ± 0.01^{b}
Lactic acid	ND^{c}	0.81 ± 0.02^{b}	ND^{c}	1.63 ± 0.07^{a}	ND^{c}	1.52 ± 0.02^{a}	ND^{c}	1.73 ± 0.06^{a}

Means in each row bearing the same letters are not significantly different ($p > 0.05$) from one another using Tukey's test (mean ± standard deviation).

TABLE 3 | Comparison of chemical composition and microbial metabolites in the endosperm of coffee beans undergone spontaneous (control) and inoculated fermentations with *H. uvarum*, *P. kudriavzevii* and the two yeasts combined.

Compound	Concentration (g/100g)							
	Control		*H. uvarum*		*P. kudriavzevii*		Combined yeasts	
	T0	*T36*	*T0*	*T36*	*T0*	*T36*	*T0*	*T36*
Sucrose	10.80 ± 0.50^{a}	5.00 ± 0.71^{b}	10.90 ± 0.35^{a}	5.06 ± 0.28^{b}	12.13 ± 0.33^{a}	4.93 ± 0.49^{b}	10.71 ± 0.21^{a}	4.50 ± 0.47^{b}
Glucose	0.92 ± 0.01^{a}	0.84 ± 0.01^{b}	0.93 ± 0.03^{a}	0.67 ± 0.01^{c}	0.88 ± 0.02^{a}	0.62 ± 0.01^{c}	0.93 ± 0.01^{a}	0.63 ± 0.02^{c}
Fructose	1.82 ± 0.24^{a}	0.93 ± 0.11^{b}	1.85 ± 0.17^{a}	0.71 ± 0.09^{b}	1.52 ± 0.21^{a}	0.84 ± 0.10^{b}	1.34 ± 0.22^{a}	0.73 ± 0.07^{b}
Glycerol	ND^{c}	0.09 ± 0.01^{b}	ND^{c}	0.20 ± 0.03^{a}	ND^{c}	0.17 ± 0.09^{a}	ND^{c}	0.21 ± 0.03^{a}
Mannitol	ND^{b}	0.10 ± 0.01^{a}	ND^{b}	0.09 ± 0.01^{a}	ND^{b}	0.08 ± 0.02^{a}	ND^{b}	0.09 ± 0.01^{a}
Citric acid	1.40 ± 0.01^{a}	1.21 ± 0.02^{b}	1.31 ± 0.01^{a}	1.12 ± 0.02^{b}	1.29 ± 0.06^{a}	1.00 ± 0.02^{b}	1.36 ± 0.01^{a}	1.20 ± 0.03^{b}
Malic acid	0.42 ± 0.03^{a}	0.23 ± 0.10^{b}	0.44 ± 0.02^{a}	0.15 ± 0.04^{b}	0.40 ± 0.07^{a}	0.21 ± 0.01^{b}	0.41 ± 0.05^{a}	0.20 ± 0.03^{b}
Quinic acid	0.67 ± 0.02^{a}	0.53 ± 0.01^{b}	0.71 ± 0.01^{a}	0.51 ± 0.03^{b}	0.70 ± 0.02^{a}	0.52 ± 0.04^{b}	0.78 ± 0.03^{a}	0.49 ± 0.01^{b}
Succinic acid	0.37 ± 0.02^{a}	0.23 ± 0.01^{b}	0.39 ± 0.04^{a}	0.22 ± 0.02^{b}	0.33 ± 0.07^{a}	0.21 ± 0.03^{b}	0.34 ± 0.03^{a}	0.20 ± 0.01^{b}
Lactic acid	ND^{c}	0.41 ± 0.13^{b}	ND^{c}	0.83 ± 0.17^{a}	ND^{c}	0.78 ± 0.12^{a}	ND^{c}	0.81 ± 0.09^{a}

Means in each row bearing the same letters are not significantly different ($p > 0.05$) from one another using Tukey's test (mean ± standard deviation).

acid increased slightly to a maximum concentration of about 1.6 g/100 g in fermentations inoculated with *H. uvarum* and combined yeasts. Furthermore, lactic acid was not present in any of the fermentations at the start, and was first detected at 16 h with a concentration of about 0.4 g/100 g. The level of lactic acid increased in the control beans to 0.8 g/100 g, while in the inoculated fermentations it was about twofold higher than in the former. Similarly, the mucilage did not contain glycerol at the beginning of the control fermentation and was first detected at 24 h with a value of 0.7 g/100 g which subsequently increased to a maximum concentration of 0.9 g/100 g at the end. In the inoculated fermentations, glycerol was detected earlier at 16 h, which increased to maximum concentrations of 1.3–2.1 g/100 g. For all fermentations, mannitol was not present at the beginning, and was first detected at 16 h with a concentration of about 0.4 g/100 g, then increased to maximum concentrations of 0.9–1.2 g/100 g, without significant differences between the different fermentations.

Less extensive changes in these non-volatile compounds were observed in the endosperm compared to the mucilage layer, **Table 3**. Sucrose, fructose, and glucose were detected in the endosperm at the start of fermentation with the concentrations of 10.8, 1.8, and 0.9 g/100 g, respectively. In the control fermentation, sucrose gradually declined to 5.0 g/100 g, while fructose and glucose reached approximately 0.9 g/100 g each. The sugar metabolism was faster in the inoculated fermentations where they ended up with slightly lower concentrations compared to the spontaneous fermentation. Four main organic acids were detected in the endosperm at the beginning of the fermentations with citric acid being the major one, followed by quinic, malic and to by 0.4–1.4 g/10 g. No significant differences in the concentration of these organic acids were observed in the inoculated fermentations. Although the level of citric acid detected in the mucilage layer increased during the inoculated fermentations, no corresponding change was observed in the endosperm. Lactic acid was first detected at

16 h, which subsequently increased to a maximum concentration of 0.8 g/100 g in the inoculated fermentations, which doubled the level of the control. Glycerol and mannitol were not present at the beginning of all fermentations and was first detected at 24 h, which gradually increased to a maximum concentration of about 0.1 g/100 g each in the control. No significant difference was observed in the concentration of mannitol in the inoculated fermentations; however, glycerol was detected earlier, and its concentration was approximately twice that of the control.

Volatile Compounds

Seventy eight (78) volatile compounds were identified in the coffee beans, which were grouped based on their chemical class into alcohols, aldehydes, ketones, esters, acids, phenols, furans, pyrazines, sulfur containing compounds and miscellaneous (**Supplementary Table 1**).

Alcohols

Nine main alcohols were identified in the green beans with a total concentration of 1,166.5 μg/kg, which represented about 43% of the total volatiles in the beans. Ethanol was the most abundant component with a maximum concentration of 481.7 μg/kg, followed by isopentyl alcohol, 3-methyl-2-buten-1-ol, isoamyl alcohol, 1-non-anol, 3-hexanol, and phenyl ethyl alcohol. In the inoculated fermentations with *H. uvarum* and the two yeasts combined, ethanol concentration was approximately threefold higher than that of the control, while in the *P. kudriavzevii* inoculated fermentation it was fourfold higher than the control. The level of isoamyl alcohol was more than threefold higher in the fermentation inoculated with *H. uvarum* and about fourfold higher with *P. kudriavzevii* and the combined yeasts, compared to that in the spontaneous fermentation ($p < 0.05$). Similarly, the phenylethyl alcohol level was approximately twofold higher in the fermentation inoculated with *H. uvarum*, fivefold with *P. kudriavzevii* and sixfold with the combined yeasts, compared to the control ($p < 0.05$). No significant differences were detected with the remaining alcohols among the different green bean types ($p > 0.05$). As expected, after roasting the concentrations of total alcohol in the various types of beans decreased to about half of those in the green beans; however, the levels of residue ethanol, isoamyl alcohol, and phenylethyl alcohol were significantly higher (2–10 folds) in the inoculated fermentations than those in the control fermentation ($p < 0.05$). Furthermore, four additional alcohols were identified in the roasted beans but not in the green beans, which were 2-methyl-3-buten-2-ol, 2,3 butanediol, 2,3-hexanediol, and 1,4-butanediol, with no significant differences in their concentrations among the different types of beans ($p > 0.05$). Low amounts of furfuryl alcohol and 1-non-anol were detected in green beans, which increased significantly after roasting with maximum concentrations of 115.5 and 289.3 μg/kg in the control beans ($p < 0.05$), respectively. The level of furfuryl alcohol was significantly higher in the inoculated fermentations, especially with combined yeasts, while the 1-non-anol level was similar in all bean types ($p > 0.05$).

Aldehydes

Aldehydes represented about 10% of the total volatiles in the green beans with a maximum total concentration of 256.3 μg/kg, which subsequently increased by about sevenfold after roasting in the control beans. Acetaldehyde, hexanal, and benzaldehyde were the most abundant compounds among this group and their levels were generally higher in the green beans from inoculated fermentations compared to the control ($p < 0.05$). After roasting, the concentration of acetaldehyde remained largely unchanged in all fermentations; however, its level was significantly higher in the beans from inoculated fermentations, especially with *P. kudriavzevii*, than that in the control beans ($p < 0.05$). Similar patterns were also observed for hexanal, while benzaldehyde was not detected in any of the roasted beans Furthermore, neo-formation of aldehydes were detected in the roasted beans and these were furfural and 5-methyl furfural, with no significant differences observed among the different bean types ($p > 0.05$). Low levels of 3-methyl butanal was identified in all green beans with similar concentrations which subsequently increased after roasting with significantly higher concentrations in the inoculated beans, especially with combined yeasts, than those in the control beans ($p < 0.05$).

Esters

About 30% of the total volatiles quantified in the green beans were esters, among which ethyl acetate (52%) and methyl acetate (46%) were the most abundant in the control fermentation. Higher levels of ethyl acetate and methyl acetate were observed in the inoculated fermentations, especially with *H. uvarum* and combined yeasts, compared to the control fermentation ($p < 0.05$). Furthermore, trace amounts of methyl formate was also detected in the green beans, and its levels were significantly higher in the inoculated fermentations than that in the control ($p < 0.05$). After roasting, the levels of the esters declined; however, their concentrations remained significantly higher in the inoculated fermentations than in the control ($p < 0.05$). Furthermore, three additional esters were detected after roasting but not identified in the green beans, namely furfural propionate, furfuryl formate, and furfuryl acetate. Significantly higher levels of furfuryl formate and furfuryl acetate were observed in the inoculated fermentations than that found in the control ($p < 0.05$), while the concentrations of furfuryl propionate were almost the same in all bean types ($p > 0.05$).

Ketones

The fermented green beans contained low levels of ketone compounds, which increased by about 14-fold after roasting in the control fermentation. Three main ketones were detected in the green beans, namely 2-butanone, 3-pentanone, and 2,3-butanedione. No significant differences in the concentration of 2-butanone were found between the fermentations ($p > 0.05$), while the levels of 3-pentanone and 2,3-butanedione were significantly higher in the inoculated fermentations compared to the control ($p < 0.05$). After roasting, the concentration of 3-pentanone declined, while those of 2,3-butanedione and 2-butanone increased, and their levels remained higher in the inoculated fermentations than that of the controls ($p < 0.05$). Furthermore, three additional ketones were formed after roasting, namely 2,3-pentanedione, 2,3-hexanedione, and 2,3-heptanedione. No

significant differences were found in the concentration of 2,3-heptanedione between the fermentations, while the levels of 2,3-pentanedione and 2,3-hexanedione were significantly higher in the inoculated fermentations, especially with the combined yeasts, compared to the control fermentation ($p < 0.05$).

Acids and Phenols

Two main organic acids were detected in the green beans, which were acetic (383.1 μg/kg) and butanoic (20.1 μg/kg) acids in the spontaneous control fermentation. In the inoculated fermentations, the level of acetic acid was approximately double while butanoic acid was half of that found in the control beans ($p < 0.05$). After roasting, the concentrations of both acetic and butyric acids declined, however, the level of the former was slightly higher in the inoculated fermentations, especially with combined yeasts, while the level of butanoic acid was lower than that in the control ($p < 0.05$). Low levels of phenol were detected in the green beans of all fermentations with similar concentrations, which remained relatively unchanged after roasting. In addition, three new phenols were formed during roasting, namely 2-methoxy phenol (guaiacol), 2-methyl phenol, and 2-methoxy-4-vinylphenol but their concentrations were not significantly different among the different types of beans ($p > 0.05$).

Pyrazines, Pyrroles, and Pyridines

Pyrazine compounds were detected in only the roasted beans and not in the green beans from all fermentations. A total of 12 pyrazines, representing about 27% of the total volatiles, were detected after roasting, among which 2-methylpyrazine, 2,5-dimethypyrazine, 2,6-dimethylpyrazine, 2,3-dimethylpyrazine, and pyrazine, 2-ethyl-6-methyl were the most abundant. The concentrations of these volatiles, however, were significantly higher in the inoculated fermentations, especially with *P. kudriavzevii* and the combined yeasts, compared to those in the control. Roasted beans also contained significant amounts of 2-ethyl-5-methylpyrazine; however, no significant differences were detected between the different fermentations ($p > 0.05$). Four main pyrroles were identified in the roasted beans, namely 1-methyl-pyrrole, 2-ethyl-pyrrole, 2-formyl-1-methyl pyrrole, and 2-formyl pyrrole. The concentration of 2-formyl pyrrole was significantly higher in the control and *H. uvarum* inoculated fermentation compared with other fermentations. The levels of 2-ethyl-pyrrole and 1-methyl-pyrrole were significantly higher in the inoculated fermentations compared to the control ($p < 0.05$). No significant differences in the concentration of 2-formyl-1-methylpyrrole were observed among the different fermentations ($p > 0.05$). Three pyridine compounds were also identified in the roasted beans, among which pyridine was the most abundant, and its level was significantly higher in the inoculated fermentations, especially with *P. kudriavzevii* and combined yeasts, than in the control ($p < 0.05$).

Furans and Sulfides

A total of 10 furan compounds were detected after roasting, but not identified in the green beans, representing about 4% of the total volatiles, among which 2-acetylfuran and 2-propionylfuran were the most abundant. The concentration of 2-acetylfuran was higher in the inoculated fermentations than that of the control ($p < 0.05$), while no significant difference was detected in the level of 2-propionylfuran in all sample types. In addition, significant higher levels of 2-methylfuran, 2,5-dimethylfuran, 2-allylfuran, and tetrahydro-2-(methoxymethyl) furan were found in the inoculated beans. Sulfur containing volatiles were not detected in any green beans but identified after roasting. A total of six compounds were detected, among which dimethyl trisulfide was the most abundant followed by furfuryl methyl sulfide, dimethyl disulfide, methanethiol, dimethyl sulfide, and bis-2-(furfuryl)-disulfide with similar concentrations in all fermentations ($p > 0.05$).

Sensory Analysis

Results of cup tests by three Q-Grade coffee masters showed no statistically significant differences between coffees brewed from beans from different fermentations on sweetness, balance, clean cup, uniformity, aftertaste, and overall scores (**Figure 2**). Coffee produced from the inoculated fermentations, especially with *P. kudriavzevii*, was awarded high scores of flavor, aroma and acidity than the control fermentation. The maximum overall score (82.0) was awarded to coffee from the inoculated fermentation with combined yeasts ($p < 0.05$), while the score for *P. kudriavzevii* and *H. uvarum* inoculated fermentations and the control were 81.5, 79.8, and 80.3, respectively, although the differences in these scores were not statistically significant ($p > 0.05$). The panel reported distinctive sensory characteristics for each coffee beverage. The coffee beverage produced from the control fermentation was perceived to be "vanilla," "plummy," and fruity in aroma. The beverage produced from *H. uvarum* inoculated fermentation was characterized by "roasted almond" aroma. The coffee produced by *P. kudriavzevii* inoculated fermentation was described as "malty," "vanilla," peanut in flavor, while the coffee produced inoculated fermentation with combined yeasts were characterized by earthy, apple cider, and walnut notes and a smooth mouthfeel.

DISCUSSION

Primary coffee processing, especially the fermentation stage, is currently conducted overwhelmingly in the traditional way where fermentation occurs spontaneously. Previous studies have demonstrated the critical roles of yeasts in the spontaneous wet coffee fermentation process and their capability to improve the quality of the resultant beans by producing desirable metabolites and aromatic compounds (Elhalis et al., 2020a,b, 2021b). *H. uvarum* is known for its ability to quickly utilize available sugars present in the mucilage, which might help the mucilage degradation process (Pretorius, 2000). In wine fermentation, *H. uvarum* was reported to be present in the initial stage and produce high concentrations of acetic acid and its esters (Heard and Fleet, 1986; Peddie, 1990). *P. kudriavzevii* was reported to resist stresses such as acidic conditions, high temperature and high salt content, making it applicable for industrial fermentation processes (Koutinas et al., 2016; Techaparin et al., 2017; Chamnipa et al., 2018). In addition, *P. kudriavzevii* has

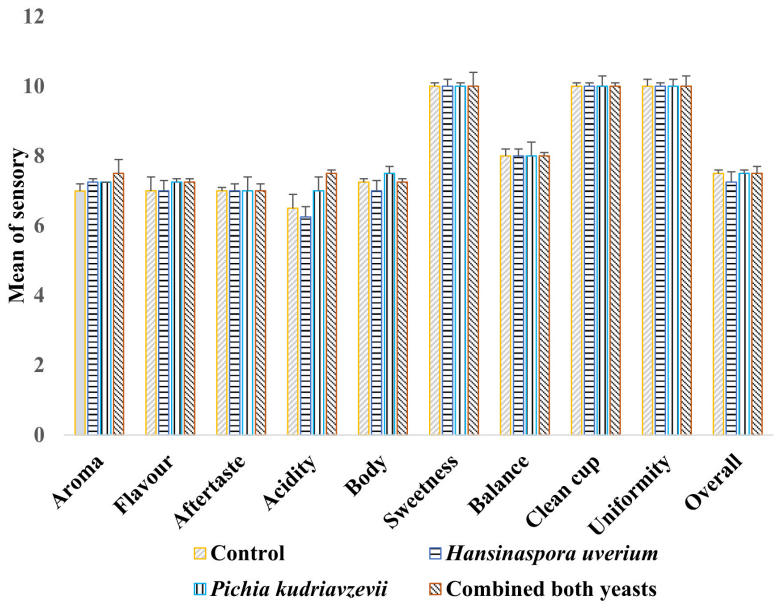

FIGURE 2 | Scores of cup test means (SCAA) by three Q-Graded coffee panelists for coffee beverages produced with roasted beans from the three inoculated (*H. uvarum, P. kudriavzevii*, combined yeasts) and spontaneous (control) fermentations (Mean ± standard deviation).

been shown to potentially improve the quality of products such as cocoa, cheese and alcoholic beverages (del Monaco et al., 2014; Saerens and Swiegers, 2016; Pereira et al., 2017; Zheng et al., 2018). To date, *H. uvarum* and *P. kudriavzevii* have not been reported for inoculation in the wet fermentation of coffee beans.

One of the major differences between the yeast inoculated and the spontaneous fermentations was that the yeast population in the former was about 2 logs CFU/ml higher than in the latter. This occurred in the inoculated fermentations with *H. uvarum*, *P. kudriavzevii* and the combination of both yeasts. This showed that the inoculated yeasts not only can successfully grow but also dominate the fermentation, demonstrating their potential as starter cultures. Both *H. uvarum* and *P. kudriavzevii* were inoculated in this study. As both yeasts species were naturally part of the microflora present in the spontaneous wet fermentation process, their populations in the inoculated fermentations were the sum of both the inoculum and the endogenous yeast counts and, therefore, relatively high. Furthermore, the growth of both yeasts in the combined inoculation illustrated that they can successfully grow together and were not be suppressed by each other, demonstrating the suitability of both species to be used as a mixed species starter culture. In contrast, when *H. uvarum* and other yeasts such as *S. cerevisiae* and *P. kluyveri* were used together in cocoa bean fermentation, *S. cerevisiae* grew to became the dominant species while the growth of *H. uvarum* was largely suppressed (Batista et al., 2009). Previous studies on yeast inoculated fermentations did not obtain consistent results. Pereira et al. (2015) inoculated a single yeast species, *P. fermentans*, in wet fermentation with a concentration of 7.5 log CFU/ml, which increased during fermentation to reach a maximum population of 8.8 CFU/ml after 12 h. However, in another study where *S. cerevisiae* and *Torulaspora delbrueckii* were inoculated together in wet fermentation at an initial count of 4.2–5.3 log CFU/g, the yeast population gradually declined to less than 2 log CFU/g at the end of fermentation after 72 h (Martins et al., 2019).

Surprisingly, the population of TABC also increased substantially in the yeast inoculated fermentations to reach a maximum population of about 8.5 log CFU/ml after 24 h, which was higher than that in the spontaneous fermentation at 7.9 CFU/ml, while the population of LAB was similar in all the fermentations. This showed that the high yeast growth in the inoculated fermentations did not suppress the growth of TABC and LABs, and might even helped the growth of some bacteria, possibly by the production of essential nutrients such as amino acids, vitamins, and purines (Viljoen, 2006). The ability of the yeasts and bacteria to co-exist also points to the potential for using a combination of yeast and bacteria as starter cultures in coffee fermentation.

The microbial population during fermentation was determined by both the traditional plate counting and the qPCR methods, and it was found that the former generally underestimated the population by about one log. This discrepancy could be due to that the plate counting technique only measured viable microbial cells in the samples, while qPCR quantified the DNA of all cells including viable, damaged, stressed, and dead cells. Furthermore, the plating technique was unable to detect the endogenous yeasts present in the fermentations due to the high numbers of the inoculated yeasts, where after necessary serial dilutions, the former fall below the detection limit. Such limitations of the plate counting method demonstrated the value of using molecular methods such as qPCR in the study of complex microbial communities. In

addition, such techniques will enable real time monitoring of the growth of target microorganisms during fermentation, which would be valuable in industrial situations. qPCR techniques have been used to monitor the growth of bacteria and fungi in spoiled and fermented foods (Hein et al., 2001; Blackstone et al., 2003; Bleve et al., 2003; Haarman and Knol, 2005). Because of their specificity, sensitivity as well as speed compared to plate counting, the potential of using such techniques in monitoring industrial fermentation processes have been highlighted (Nakayama et al., 2007; Hagi et al., 2010). However, it should be taking into consideration that overestimation could occur due to possible recovery DNA from dead cells.

The higher yeast populations in the inoculated fermentations were corresponded with a faster sugar consumption in the mucilage. Interestingly, the decrease in the sugar level inside the bean (endosperm) was also faster in the inoculated fermentations than in the control. One possible explanation could be the leaching out of the sugars from the endosperm to the surrounding environment during wet fermentation (Wootton, 1971), and the lower sugar concentrations in the mucilage of the beans in inoculated fermentations could lead to a faster leakage of sugars from the endosperm. Furthermore, yeast population was much higher in the inoculated fermentations, which could cause a faster removal of the mucilage layer from the surface of the beans, which in turn might improve the sugar leaching process. The level of the reducing sugars inside the beans has a potential impact on coffee aroma, flavor and color during subsequent roasting process where sugars were a key participant of Maillard reaction and caramelization (Fischer et al., 2001). The consumption of sugars in the mucilage was accompanied by the accumulation of microbial metabolites such as glycerol, mannitol and lactic acid. As expected, higher concentrations of glycerol and lactic acid were detected in the inoculated fermentations than in the control, which is liked due to the higher populations of yeasts in the inoculated fermentations as yeasts are not only responsible for producing glycerol, but also produces lactic acid (Komesu et al., 2017). Glycerol is commonly detected in yeast fermented products and has a sweet and smooth mouthfeel, which typically has a positive contribution to sensory quality (Swiegers et al., 2005). The presence of relatively high levels of glycerol in beans from inoculated fermentations was likely responsible for the higher scores in mouthfeel of the coffee prepared from them compared to that of the spontaneous fermentation. The level of mannitol was similar in the different fermentations, which is likely due to the similar LAB counts in all the fermentations as mannitol was reportedly produced mainly by LABs through fructose metabolism (De Vuyst et al., 2010; Saha and Racine, 2011). Mannitol is characterized by a favorable cool taste (De Vuyst et al., 2010; Saha and Racine, 2011), which may explain the similar scores of aftertaste awarded to coffees brewed from the different types of beans.

The much higher levels of acetic acid in the inoculated fermentations than in the control were also likely due to the higher yeast populations in the former as yeasts such as *H. uvarum* are known produce acetic acid by direct sugar metabolism (Swiegers et al., 2005). Presence of acetic acid in coffee has a pleasant clean and sweet taste at low concentrations but produces a vinegary and pungent taste when its concentration exceed 1 mg/ml (Bertrand et al., 2012). The presence of the high levels of organic acids, i.e., acetic and lactic acids, in the inoculated beans are likely the cause of the higher acidic scores for coffee brewed from them. In contrast, the level of butanoic acid was lower in the inoculated fermentations. Butanoic acid has an onion like flavor and is produced mainly by undesirable growth of contaminated microorganisms during fermentations (Amorim and Amorim, 1977). These findings indicate the potential role of the inoculated yeasts in suppressing the growth of these undesirable microorganisms and minimizing the formation of off flavor compounds.

Both the green and roasted beans from inoculated fermentations also had significantly higher levels of alcohols than the control, with ethanol, isoamyl alcohol, and phenylethyl alcohol being the main ones. These alcohols are produced by yeasts in a wide range of fermented foods and beverages (Tamang and Fleet, 2009; Ho et al., 2014; Batista et al., 2015; Pereira et al., 2015). Ethanol contributes to the beverage viscosity and solubility of volatiles, while isoamyl alcohol and phenylethyl alcohol are characterized by their desirable sweet and fruity flavors (Tamang and Fleet, 2009; Gamero et al., 2019). Similarly, the inoculated beans (both green and roasted), especially with *P. kudriavzevii*, also had significantly higher concentrations of aldehydes, such as acetaldehyde, than those in the control. Acetaldehyde is a well-known yeast metabolite, as an intermediate of ethanol production, and has an almond, fruity and sweet aroma (Rosca et al., 2016). Interestingly, the concentration of 3-methyl butanal increased almost 100 times after roasting and was significantly higher in the inoculated beans, especially those with *P. kudriavzevii* and the combined yeasts. Branched-chain aldehydes such as 3-methyl butanal were detected in fermented food products and have malty and chocolate-like taste (Smit et al., 2009). Such aldehydes were mainly formed during heat treatment by Streaker degradation of amino acids with reducing sugars (Arnoldi et al., 1987). A substantial increase in the level of aldehydes, mainly acetaldehyde, was also observed with *P. fermentans* inoculated fermentations (Pereira et al., 2015). Regarding the esters, methyl acetate and ethyl acetate were the most important, whose concentrations were significantly higher in the inoculated green beans and remained more than two times higher after roasting in the inoculated beans, especially those with *H. uvarum* and combined yeasts than those in the control. Esters are considered key volatiles in many fermented foods and beverages even with low concentrations, and they are well known yeast metabolites (Peddie, 1990; Cristiani, 2001). Overall, our results showed that higher concentrations of alcohols and aldehydes were correlated with the growth of *P. kudriavzevii*, while higher levels of esters were observed with *H. uvarum.* Those metabolites, and also ketones, were detected with higher concentrations in the beans from inoculated fermentations with combined yeasts. Those volatiles are regarded as key volatiles that potentially contribute to coffee flavor and aroma (Czerny and Grosch, 2000), which may explain the high sensory scores of flavor and the distinct fruity and malty notes for coffee from the inoculated beans.

Most of the ketones, pyrazines, pyrroles, pyridines, furans, phenols, and sulfur containing volatiles were detected after roasting. These volatiles were usually generated by thermal reactions, such as Maillard reactions, of the bean components such as amino acids and sugars (Yaylayan and Keyhani, 1999; Daglia et al., 2007; Baggenstoss et al., 2008). These volatiles confers a wide range of sensory characteristics such as sweet, roast, caramel, buttery and earthy (Mayer et al., 2000). The significantly higher levels of ketones, pyrazines, and furans in the beans from inoculated fermentations suggest that yeast activities may have an impact on the internal components such as sugars and amino acids, which in turn affected the synthesis of these volatiles during roasting. However, as discussed above, beans from the inoculated fermentations had a lower concentration of reducing sugars. It was also reported that fermentation had no significant impact on the protein and peptide content of coffee bean (Ludwig et al., 2000; Elhalis et al., 2020a). Thus, direct yeast activities did not appear to explain their influence on the bean components. Pyrazines such as 2-ethyl-3,5-dimethylpyrazine were also found in coffee beans from *S. cerevisiae* and *T. delbrueckii* inoculated wet fermentation, but not detected in the beans from spontaneous fermentation (Martins et al., 2019). Yu and Zhang (2010) reported that the formation of volatiles during thermal treatments were dependent on the bean acidity. In addition, pretreating the beans with acetic acid to decrease the pH has been shown to facilitate the formation of pyrazines and furans during coffee roasting (Liu et al., 2019). Therefore, the higher concentrations of organic acids, such as lactic and acetic acids, in the inoculated green beans might be the reason for the higher concentrations of these volatiles in the beans from inoculated fermentations formed during roasting. However, further investigations are required to confirm this hypothesis.

CONCLUSION

Results of this study showed that when *H. uvarum* and *P. kudriavzevii* were inoculated, either separately or together, into wet coffee bean fermentations, they were able to dominate the fermentations and became the overwhelmingly dominant microorganisms. Such process was shown to increase the concentrations of the final volatiles fractions of the roasted beans which were correlated with higher sensory scores for coffee brewed from them. The dominance of the inoculated yeasts led to faster and more complete utilization of sugars in the mucilage, with resultant production of higher concentrations of metabolites such as glycerol, alcohols, aldehydes, esters, organic acids, and pyrazines in the fermented green beans, compared with spontaneous fermentation. Although the levels of these metabolites were greatly reduced after roasting, they remained significantly higher in the beans from the inoculated fermentations, which were correlated with the distinct fruity notes reported with coffee brewed from them. Overall, the findings of this study confirmed the crucial role of yeasts in the wet fermentation of coffee beans, and their contribution to high quality coffee. In addition, this study also demonstrated that qPCR is a fast and reliable method for real time monitoring of yeast populations in fermentation. These results suggest that each yeast has distinctive metabolic activities that can be used individually or combined to modulate the coffee quality. Future studies could be directed to examine the potential of mixed cultures of yeast and bacterial species for accelerating coffee fermentation as well as improving product quality.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the HREAP Executives (HC number HC190689). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

HE designed and conducted the experiments, performed the data analysis, and wrote the manuscript. JZ and DF supervised the experiments progress, interpreted the scientific values of the obtained data, and proofread the manuscript. JC contributed to supervision of the work. All authors read and approved the manuscript.

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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.2021.713969/full#supplementary-material

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Microbial Diversity and Metabolite Profile of Fermenting Millet in the Production of *Hausa koko*, a Ghanaian Fermented Cereal Porridge

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Hausa koko is an indigenous porridge processed from millet in Ghana. The process involves fermentation stages, giving the characteristic organoleptic properties of the product that is produced largely at a small-scale household level and sold as a street food. Like many other indigenous foods, quality control is problematic and depends on the skills of the processor. In order to improve the quality of the product and standardize the process for large-scale production, we need a deeper understanding of the microbial processes. The aim of this study is to investigate the microbial community involved in the production of this traditional millet porridge and the metabolites produced during processing. High-throughput amplicon sequencing was used to identify the bacterial (16S rRNA V4 hypervariable region) and fungal [Intergenic Transcribed Spacer (ITS)] communities associated with the fermentation, while nuclear magnetic resonance (NMR) was used for metabolite profiling. The bacterial community diversity was reduced during the fermentation processes with an increase and predominance of lactobacilli. Other dominant bacteria in the fermentation included *Pediococcus*, *Weissella*, *Lactococcus*, *Streptococcus*, *Leuconostoc*, and *Acetobacter.* The species *Limosilactobacillus fermentum* and *Ligilactobacillus salivarius* accounted for some of the diversities within and between fermentation time points and processors. The fungal community was dominated by the genus *Saccharomyces*. Other genera such as *Pichia*, *Candida*, *Kluyveromyces*, *Nakaseomyces*, *Torulaspora*, and *Cyberlindnera* were also classified. The species *Saccharomyces cerevisiae*, *Stachybotrys sansevieriae*, *Malassezia restricta*, *Cyberlindnera fabianii*, and *Kluyveromyces marxianus* accounted for some of the diversities within some fermentation time points. The species *S. sansevieria* and *M. restricta* may have been reported for the first time in cereal

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fermentation. This is the most diverse microbial community reported in *Hausa koko*. In this study, we could identify and quantify 33 key different metabolites produced by the interactions of the microbial communities with the millet, composed of organic compounds, sugars, amino acids and intermediary compounds, and other key fermentation compounds. An increase in the concentration of organic acids in parallel with the reduction of sugars occurred during the fermentation process while an initial increase of amino acids followed by a decrease in later fermentation steps was observed.

Keywords: fermented cereal, *Hausa koko*, Africa, metabolomics, bacteria, fungi, millet

INTRODUCTION

Porridges produced from fermented cereals such as maize, millet, and sorghum are essential diets in many parts of Africa, where they are used mostly as staple, weaning, or complementary foods, providing necessary nutrients (Omemu et al., 2007; Omemu and Omeike, 2010; Owusu-Kwarteng et al., 2010b). In Ghana, fermented thin cereal porridges are called *koko* and are usually eaten at breakfast. Fermented stiff cereal porridges are eaten as main meals mostly at lunch or dinner and include *kenkey*, *banku*, and *tuo zaafi*. One of the most popular of the Ghanaian thin porridges is *Hausa koko*, a spicy, smooth, and free-flowing fermented millet porridge that is mostly produced and sold as street food (Mensah et al., 2002; Haleegoah et al., 2016).

Hausa koko is produced by traditional food processors as a micro or cottage industry in mainly home-based operations using the two main indigenous methods shown in **Figure 1**. Lei and Jakobsen (2004) described and studied Process A while Process B is more commonly used and is the subject of the present investigation. The processes in *Hausa koko* production involve initial steeping of millet grains for 12–24 h, during which fermentation starts. The steeped grains are then washed and milled together with different spices in a plate mill. The resulting flour is mixed with water to prepare a slurry, which is then sieved using a cheese cloth. The slurry is allowed to ferment spontaneously for 8–12 h and becomes sour. During the fermentation of the slurry, it settles into a sediment and a supernatant with a foam sitting on top. The foam is scooped off and discarded, while the supernatant may be consumed in its raw state as *koko sour water* for medicinal purposes, especially for treatment of diarrhea. Indeed, Lei et al. (2006) have confirmed that *koko sour water* has natural probiotic properties. To prepare *Hausa koko*, most processors boil four volumes of water, and while stirring the fermented slurry (the supernatant and sediment together representing one volume), slowly pour in the hot boiled water to obtain the smooth gel-like porridge. A few processors, however, add water to the supernatant and boil the diluted supernatant. The hot diluted supernatant is then poured slowly into the sediment while stirring continuously to obtain *Hausa koko*. *Hausa koko* is sweetened with sugar before consumption and many consumers may add milk to the thin porridge or even roasted groundnuts. Traditionally, *Hausa koko* may be eaten with *koose*, a fried cowpea doughnut or *masa*, a fried sour millet, maize, or rice doughnut; however, *Hausa koko* is often eaten with bread.

Only a few studies have been carried out to study the microbiology of *Hausa koko* processing, notably the work by Lei and Jakobsen (2004). They reported the spontaneous fermentation to be dominated by *Weissella confusa* and *Lactobacillus* (now *Limosilactobacillus*) *fermentum*, showing a pronounced taxonomic biodiversity at sub-species level between stages within the production as well as between production sites. Other species reported in *koko* sour water by Lei and Jakobsen (2004) were *Lactobacillus* (now *Ligilactobacillus*) *salivarius*, *Pediococcus pentosaceus*, *Pediococcus acidilactici*, and *Lactobacillus* (now *Lactiplantibacillus*) *paraplantarum*, and live LAB content of *koko* sour water has been estimated as c. 10^8 cells per ml.

Consumption of *Hausa koko* gives some of the benefits of fermentation. Fermentation of cereals is reported to reduce the levels of some anti-nutrients including tannins, polyphenols, and phytates, some of which can inhibit amylolysis and proteolysis and sequester proteins and valuable minerals in complexes; microbial enzymatic activity can disrupt these complexes and improve the bioavailability of minerals such as iron, calcium, zinc, and phosphorus (Sharma and Kapoor, 1996; Sindhu and Khetarpaul, 2001; Blandino et al., 2003). Fermentation also enhances the nutritional and sensory qualities as well as shelf life of the products, among many other benefits (Holzapfel, 2002; Ibnouf, 2012; Arena et al., 2014). Cereal fermented foods are also reported to provide health benefits such as blood-lowering effects, inhibition of allergies, antimicrobial effects, and control of diarrhea through probiotic mechanisms (Lei et al., 2006; Vasiljevic and Shah, 2008; Das et al., 2012; Zannini et al., 2012; Wang et al., 2014; Ray et al., 2016).

In the production of indigenous African foods, fermentations are carried out mostly spontaneously, i.e., without the use of a traditional inoculum or a starter culture. However, the products attain a low pH. In spite of this, the microbiological quality of *Hausa koko* and other porridges seem unpredictable. According to Yeleliere et al. (2017) indigenous Ghanaian cereal porridges can be contaminated with pathogens including *Bacillus cereus*, *Staphylococcus aureus*, and *Enterobacteriaceae*, which are significant in terms of food safety.

In the past few decades, several successful attempts have been made to upgrade the technologies used in the production of

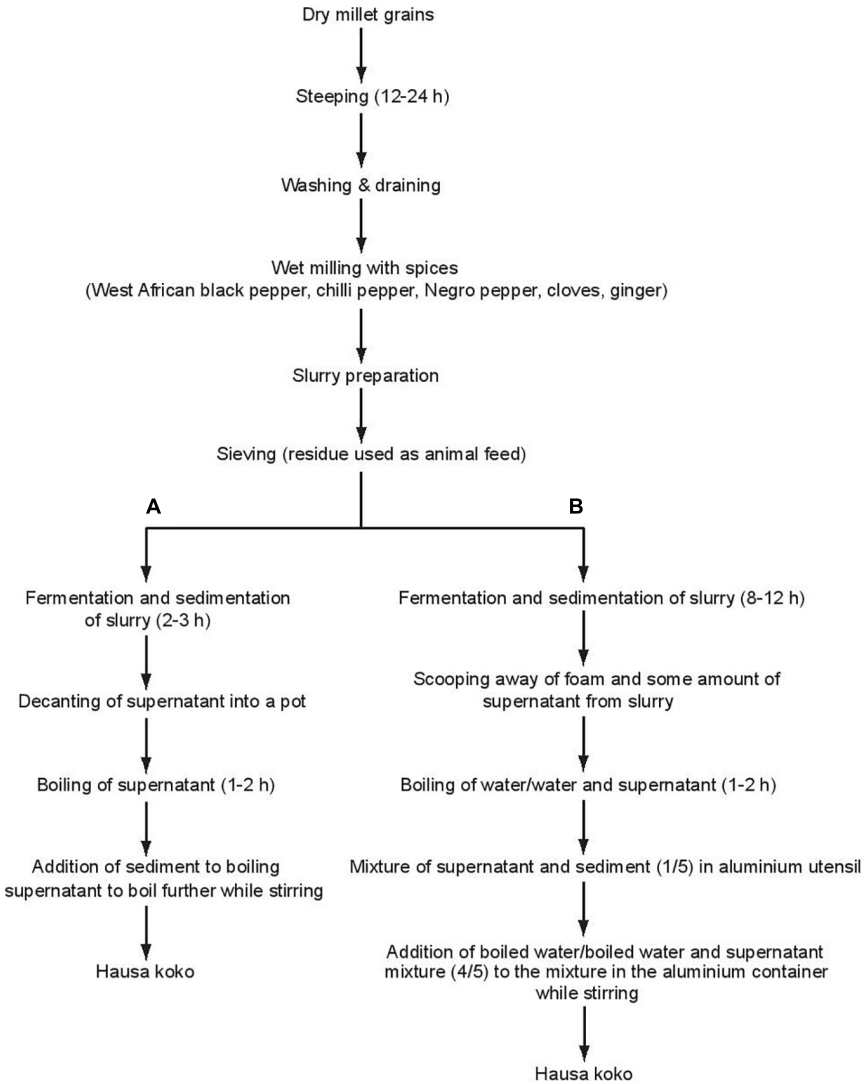

FIGURE 1 | Flow diagram of *Hausa koko* production process. **(A)** Process described by Lei and Jakobson (2004). **(B)** Process described in the current work.

some indigenous African foods, including fermented products. Many of these are now produced by small- and medium-scale enterprise (SME) as convenience foods for both local and foreign markets targeting Africans. Attempts have also been made to use starter cultures at the SME level to gain a greater control over the process, but this is yet to be widely adopted by industry. The use of starter cultures, however, is seen as a prerequisite for standardizing the sensory quality of the products and improving their safety. In Ghana, the use of starter culture to produce *kenkey*, a cooked fermented maize dumpling, was demonstrated at an upgraded traditional food processing facility, but could not be sustained at the facility by the plant owner (Halm et al., 1996; Amoa-Awua et al., 2007). With regard to *Hausa koko*, an in-depth understanding of the microbial community involved in its production is required, if a suitable starter culture is to be developed for the production of *Hausa koko* by the Ghanaian food industry as a high-quality convenience food.

Porridges made from fermented cereals are also reported to have micronutrient deficiencies, hence require some fortification (Owusu-Kwarteng et al., 2010a; Tano-Debrah et al., 2019). In view of this, a food supplement known as KOKO plus was used to improve the nutritional profile of *koko* produced from fermented maize dough, while *Hausa koko* from pearl millet was fortified with soybean for complementary feeding for infants and children (Owusu-Kwarteng et al., 2010a; Tano-Debrah et al., 2019). Unfortunately, there is no information available on the nutritional profile and other metabolites produced during the processing of millet into *Hausa koko*.

This study was carried out to determine the diversity of microorganisms involved in the processing of millet into *Hausa koko* following 15 small-scale processors located in six regions of Ghana, using high-throughput sequencing technology. It also sought to determine the metabolites produced during the process.

MATERIALS AND METHODS

Sampling

Samples of millet-based *Hausa koko* taken during the main steps involved in its production were collected from 15 small-scale processing sites. The sites were located in six regions of Ghana, distributed in three different geographical belts – northern, middle, and southern belts. The sites were distributed as follows: Northern region (Northern belt) – Tamale Central (TAC), Tamale Kalariga (TAK), and Tamale Dabokpa (TAD); Bono East Region (Middle belt) – Techiman Diasempa (TED), Techiman Abourso (TEA), Techiman Pomaakrom (TEP), and Techiman Kenten (TEK); Bono Region (Middle belt) – Sunyani (SUN); Central Region (Southern belt) – Mankessim (MAN) and Winneba (WIN); Eastern Region (Southern Belt) – Dodowa (DOD); Greater Accra Region (Southern belt) – Accra Ashaiman-Tulaku (AAT), Accra Madina Zongo (AMZ), Accra Ashaley Botwe (AAB), and Accra Ashaiman-Fafraha (AAF). The following samples were collected during processing at sites: dry millet (D), 12-h fermented millet (12 h), 24-h fermented millet (24 h), milled millet with spices (M), supernatant of slurry (Su), sediment of slurry (Sd), and *Hausa koko* (K). About 500 g of each sample was aseptically collected in duplicates into sterile bags and containers and transported to the CSIR-Food Research Institute (FRI) Microbiology laboratory under cold storage, where they were preserved at -20°C. The samples were later transported under cold conditions (frozen with iced packs) to the Quadram Institute Bioscience (QIB, Norwich, United Kingdom), where they were also preserved at −20°C and processed for further analysis.

Total Microbial DNA Extraction From Fermented Samples

Microbial DNA from the fermented samples was extracted according to Diaz et al. (2019), with minor modifications. First, 20 g of each sample was homogenized with 10 ml of ice-cold sterile ultrapure H_2O by vortexing and centrifuged (Eppendorf 5810R, Germany) for 1 min at 800 × *g* at 4°C to remove the solid particles of the sample. This process was repeated twice, and the resulting supernatants were pooled together and centrifuged at 3,000 × *g* at 4°C for 20 min to harvest the cells. The pellets were washed three times with 1 ml of phosphate buffered saline (PBS) and centrifuged at 14,000 × *g* for 2 min. Microbial DNA was extracted from the pellets using the FastDNA spin kit for soil (MP Biomedicals, United States). All steps were performed following the manufacturer's instructions, with cell lysis performed mechanically using a FastPrep-24 instrument (MP Biomedicals, United Kingdom) for 60 s at a speed of 6.5 m/s. The process was repeated three times with samples kept on ice for 5 min between each homogenization. DNA was eluted in 50 μl of DES (DNase/Pyrogen free water) pre-warmed at 55°C and quantified using the Qubit 3.0 fluorometer (Invitrogen, Malaysia) using the Qubit dsDNA Broad Range (BR) Assay kit or the Qubit dsDNA High Sensitivity (HS) Assay kit (Invitrogen), depending on the DNA concentration of the sample.

16S rRNA Amplicon Sequencing

Bacterial diversity was analyzed by 16S rRNA high-throughput amplicon sequencing. Amplification and sequencing were performed by Novogene (HK) Company Limited (Hong Kong) as follows. The V4 hypervariable region of the 16S rRNA gene was amplified by PCR using specific primers 515F and 806R (Caporaso et al., 2011) and the Phusion High-Fidelity PCR master mix (New England Biolabs, United States), following the manufacturer's instructions. The amplicons were used to generate libraries using the Illumina NEBNext Ultra II DNA Library Prep Kit (New England Biolabs, United Kingdom) and then sequenced using paired-end Illumina sequencing (2 × 250 bp) on the HiSeq 2500 platform (Illumina, United States).

ITS Amplicon Sequencing

Fungal diversity was analyzed by high-throughput amplicon sequencing of the internal transcribed spacer (ITS). For this analysis, a subset of samples obtained from five processors were included (MAN, SUN, TAD, TEK, and WIN). Amplification and sequencing were performed at QIB (Norwich, United Kingdom). The ITS region was amplified by using primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) (Jackson et al., 1999) and ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′) (White et al., 1990) and the KAPA2G Robust HotStart PCR Kit (Sigma). Amplification was performed at 95°C for 5 min, 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s followed by a final 72°C for 5 min. PCR products were purified using KAPA Pure Beads (Roche) and used to generate a library using the KAPA2G Robust HotStart PCR Kit and the Nextera XT Index Kit v2 index primers (Illumina) following the manufacturer's instructions. The libraries were sequenced on an Illumina MiSeq instrument using MiSeq® Reagent Kit v3 (Illumina) following the Illumina denaturation and loading recommendations.

Metabolite Analysis and Quantification

Metabolites from samples at selected stages (D, 12 h, 24 h, M, Su, and K) were analyzed by ^{1}H-nuclear magnetic resonance spectroscopy (NMR). The solid samples were ground into fine flour with a laboratory mortar and pestle, and 1 g of the product was mixed with 4 ml of ultra-pure H_2O by vortexing for 30 s. Liquid and semi-liquid samples were homogenized by vortexing, and 5 ml was used for further analysis. All samples were centrifuged at 2,000 × *g* for 5 min at 4°C and 400 μl of the supernatant was collected and mixed with 400 μl of NMR buffer (4.2 g $NaH_2PO_4.H_2O$, 3.3 g K_2HPO_4, 17.2 mg of Na_3PO_4, and 20 mg NaN_3 in 100 μl of 100 mM EDTA in H_2O). Six hundred microliters of the mixture was transferred into 5-mm NMR borosilicate glass NMR tubes (Wilmad, Vineland, NJ, United States) and run on a 600-MHz AVANCE^{TM} spectrometer (Bruker, Billerica, MA, United States) with cryoprobe. The recorded spectra were transformed with a 0.3-Hz line broadening, and manually phased, baseline-corrected, and referenced by setting the TSP methyl signal to 0 ppm using the TopSpin software. Metabolites were identified and quantified by computer-assisted manual fitting

with Chenomx NMR suite v 8.12 (Chenomx, Edmonton, AB, Canada), using Chenomx 600 MHz HMDB Compounds library.

Bioinformatic and Statistical Analysis

Bacterial and fungal diversity were analyzed using the Quantitative Insights Into Microbial Ecology 2 (QIIME2) 2020.8 software (Bolyen et al., 2019). The demultiplexed paired-end reads were filtered to remove substitution and chimera errors and merged using DADA2. Bacterial taxonomic assignment was performed at 97% similarity using a Naive Bayes classifier trained on the Silva version 138.1 99% operational taxonomic unit (OTU) database, where the sequences have been trimmed to only include 250 bases from the V4 region bound by the 515 F/806 R primer pair. For fungal characterization, primer and adapter sequences were removed from the reads. Analysis was carried out with the forward reads only to avoid bias caused by amplicons of larger length (i.e., larger than ca. 500 bp) that could not be merged (Taylor et al., 2016). The demultiplexed single-end reads were filtered to remove substitution and chimera errors and merged using DADA2 and taxonomic assignment was performed at 97% using a Naïve Bayes classifier trained on the UNITE Community (2019) fungal classifier version 8_99. Bacterial and fungal alpha diversity was analyzed using observed OTUs, Shannon, Pielou's evenness, and Faith's phylogenetic diversity indexes. Rarefaction curves were computed using observed OTUs. Significant differences in alpha diversity were calculated using the alpha-group-significance script in QIIME2, which performs the Kruskal–Wallis test. Jaccard, unweighted, and weighted UniFrac distances were used to generate beta diversity PCoA biplots, which were visualized using the Emperor tool. Differences in beta diversity between groups were analyzed using PERMANOVA including pairwise test (Anderson, 2017). Significant differences in the bacterial community structure among the groups were evaluated by Analysis of Composition of Microbiomes (ANCOM) (Mandal et al., 2015). For metabolites, statistical analysis using two-way ANOVA with Tukey's multiple comparisons test was applied using GraphPad Prism version 8.4.3. A p-value ≤ 0.05 was considered statistically significant.

RESULTS

Bacterial Diversity

DNA sequencing of the V4 amplicons by the Illumina HiSeq platform resulted in 8,361,221 paired-end sequence reads with an average of 89,905.60 $\pm$ 5932.20 sequences per sample. Of these, 18.5% were discarded due to poor quality, reads not merging, or after being identified as chimeras; as a result of these steps, 6,813,680 high-quality sequences were retained and analyzed, with an average of 73,265.37 $\pm$ 6301.12 sequences per sample. Distribution of reads per sample can be found in **Supplementary Table 1**. Background DNA was removed by filtering out sequences assigned to chloroplast and mitochondrial taxonomic groups. Data were rarefied to 49,824 sequences per sample to avoid bias. As shown in **Figure 2A**, the rarefaction curves for the observed OTUs were enough to capture the diversities within all the samples.

Alpha diversity indexes (observed OTUs, Shannon, Faith's phylogenetic diversity, and Pielou's evenness) were compared based on the fermentation stages. Significant differences were detected between the observed OTUs at the various time points (p-value = 0.026), a reduction in the observed OTUs was detected between the grain samples and the 12-h fermentation, and the lower diversity was maintained during the production stages (p-values: vs. 12 h = 0.003; vs. milled = 0.004; vs. supernatant = 0.004; vs. sediment = 0.001; vs. koko = 0.012) (**Figure 2B**). Significant differences in the alpha diversity were also found between grain samples and other fermentation stages when using Faith's phylogeny diversity index (**Figure 2C**) (p-value = 0.030 for all groups, p-values of grain vs.: 12 h = 0.005; 24 h = 0.033; milled = 0.021; supernatant = 0.002; sediment = 0.005; koko = 0.036). No statistical differences were observed when evenness and Shannon indexes were used.

Changes in the bacterial populations through the fermentation process and between different regions were analyzed by principal coordinate analysis (PCoA) (**Figure 3**) based on Jaccard (**Figures 3A,C**) and unweighted Unifrac (**Figures 3B,D**) distances. Differences between groups were mainly caused by OTUs within the genera *Weissella*, *Pediococcus*, and *Acetobacter* as well as the genus *Lactobacillus* and the species *L. fermentum* and *L. salivarius*. For Jaccard distances, statistical differences were found between samples based on the region in which they were produced (p-value = 0.001). Pairwise comparison showed statistically significant differences between all regions except between Central and Bono or Eastern regions. For unweighted Unifrac distances, statistical differences were observed between samples based on the region of production (p-value = 0.001). Samples produced in Northern and Greater Accra regions were the only ones showing significant differences with all other regions (p-values Northern region vs.: Bono East = 0.001; Bono = 0.002; Central = 0.001; Eastern = 0.001; Greater Accra = 0.001; p-values Central vs.: Bono East = 0.001; Bono = 0.002; Central = 0.001; Eastern = 0.001; p-values Greater Accra region vs.: Bono East = 0.001; Bono = 0.001; Central = 0.001; Eastern = 0.002; Northern = 0.001). Statistically significant differences in unweighted Unifrac distances were also found between the different fermentation steps (p-value = 0.024). Particularly, grain samples were different from the rest of time points (p-value of grain samples vs.: 12 h = 0.001; milled = 0.005; supernatant = 0.002; sediment = 0.002; koko = 0.023), except for 24 h, although this could be caused by the smaller sample size of the 24-h group.

Over 400 different bacteria were profiled in this study. The samples from the different processors across the six regions showed that OTUs at the genus level in grain samples were mainly dominated by *Sphingomonas*, *Clostridium*, *Staphylococcus*, *Pseudomonas*, *Bacteroides*, *Chryseobacterium*, *Enterobacteriaceae*, and *Escherichia-Shigella*. Fermentation time points were, however, dominated by the genera *Lactobacillus* and *Acetobacter*. *Pediococcus*, *Weissella*, *Pantoea*, *Leuconostoc*, *Gluconobacter*, *Streptococcus*, and *Lactococcus* were also notable components. ANCOM at the genus level showed significant differences in the relative abundance depending on the processing step: the genus *Pantoea* (W = 842) decreased during

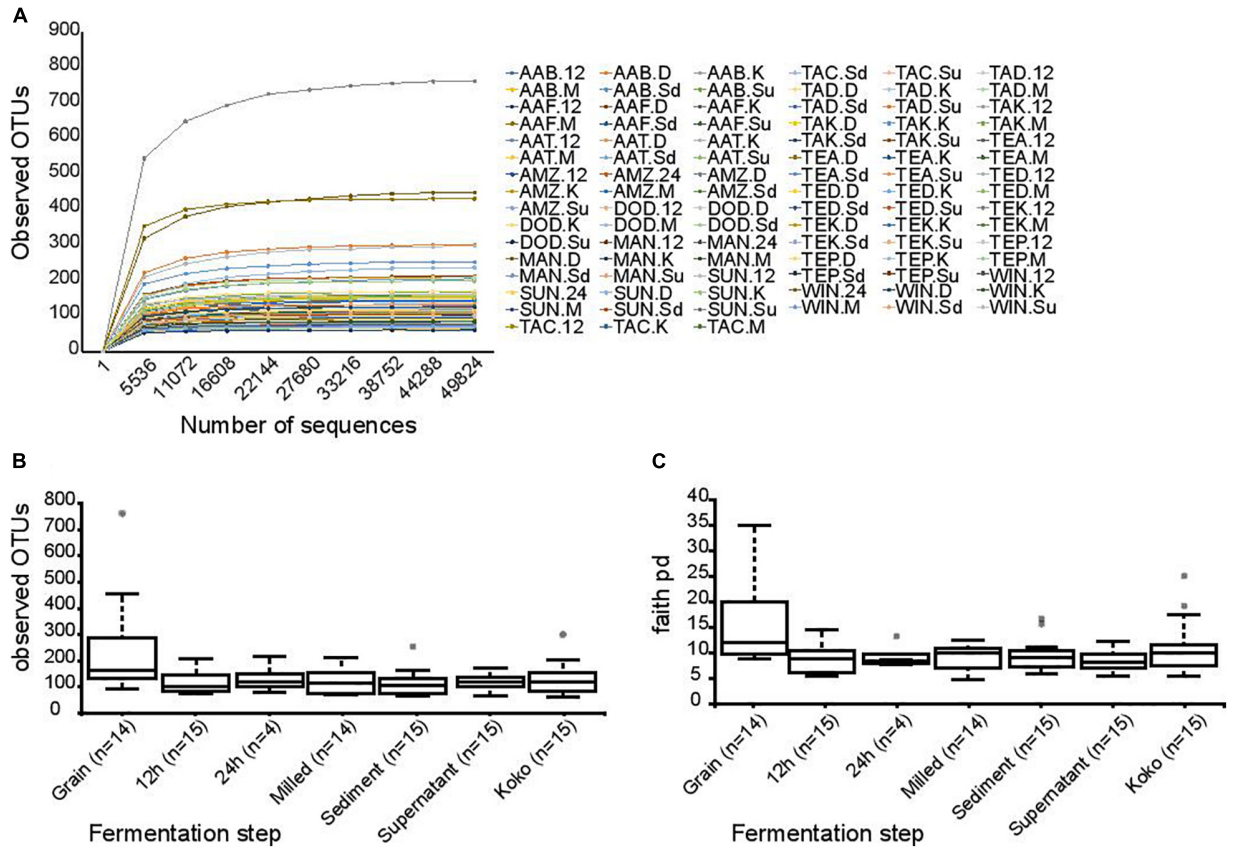

FIGURE 2 | Bacterial alpha diversity indexes. **(A)** Rarefaction plots of observed OTUs depending on the number of sequences. Each curve represents a sample. Observed OTUs **(B)** and Faith's phylogeny **(C)** diversity box plots for each fermentation step. Dots represent outliers.

the first fermentation step, and then was maintained at low levels, while the genus *Limosilactobacillus* increased during the fermentation process ($W = 781$) (**Figure 4**).

Fungal Diversity

Fungal diversity was profiled in a subset of samples obtained from five of the processing locations. DNA sequencing of the ITS amplicons by the Illumina MiSeq platform resulted in 4,792,254 single-end sequence reads with an average of 145,219.8 ± 56,955.31 sequences per sample. Of these, 34.36% were discarded due to poor quality or after being identified as chimeras; as a result of these steps, 1,646,961 high-quality sequences were retained and analyzed, with an average of 49,907.90 ± 44,668.62 sequences per sample. As observed in **Supplementary Table 2**, the distribution of reads per sample was very variable. Prior to alpha and beta diversity analysis, the data were rarefied to 4,640 sequences per sample. Although not all the observed OTUs were captured (**Figure 5A**), this sampling depth enabled us to analyze most of the samples (all except SUN-D, MAN-D, WIN-D, WIN-M, WIN-24, and MAN-K). For alpha diversity indexes (**Figure 5B**), significant differences (p-value = 0.025) in observed OTUs were detected between groups based on fermentation time points. Although grain samples seem to contain more observed OTUs, differences were not statistically significant ($p = 0.051$), probably due to the small number of samples that could be retained after removing samples with less than 4,640 reads per sample. Pairwise comparison showed significant differences between the following fermentation stages: 12 h vs. milled ($p = 0.014$); 12 h vs. sediment ($p = 0.009$); grains vs. sediment ($p = 0.051$); grain vs. supernatant ($p = 0.051$); milled vs. supernatant ($p = 0.049$); and supernatant vs. sediment ($p = 0.016$).

Analysis of the beta diversity indexes showed significant differences (p-value = 0.001) for weighted Unifrac distance based on fermentation time points (**Figure 5C**). Pairwise comparison showed significant differences between the following fermentation time points: grain vs. 12 h ($p = 0.039$), grain vs. supernatant ($p = 0.042$), and 12 h vs. koko ($p = 0.01$). Differences in weighted Unifrac distances were caused mainly by OTUs within the species *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, *Cyberlindnera fabianii*, *Stachybotrys sansevieriae*, and *Malassezia restricta*.

Analysis of Composition of Microbiomes at the genus level showed significant differences in the relative abundance depending on the processing step: the genera *Saccharomyces* ($W = 123$) and *Pichia* ($W = 111$) were more abundant in samples after the fermentation started than in the grain samples. **Figure 5D** shows the relative abundance across the fermentation steps.

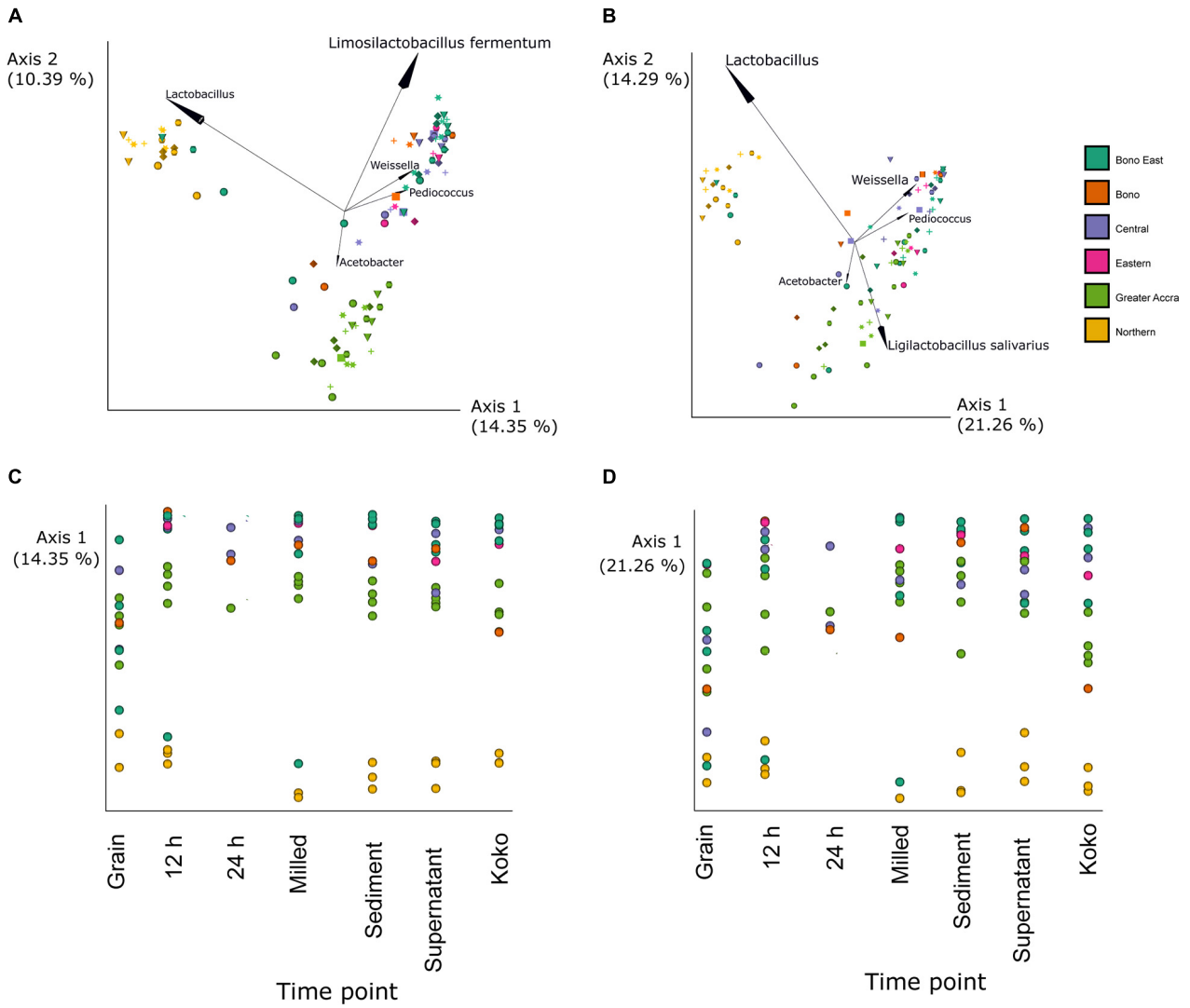

FIGURE 3 | Bacterial beta diversity analysis. Principal coordinate analysis (PCoA) showing Jaccard **(A,C)** and unweighted UniFrac **(B,D)** distances. Samples produced in different regions are represented by different colors as indicated in the legend. In panels **(A,B)**, fermentation time points are represented by different shapes: grain (circle), milled (triangle), 12 h (hexagon), 24 h (square), sediment (cross), supernatant (star), and *Hausa koko* (diamond). The arrows indicate the five taxonomic groups (at genera or species level) that contribute most to the differences. In panels **(C,D)**, fermentation time points are represented along the *x*-axis. The percentage of variation explained by the plotted principal coordinates is indicated in the *y*-axis.

The grain samples were characterized by fungi of the genera *Kazachstania*, *Aspergillus*, *Penicillium*, *Talaromyces*, *Eremothecium*, *Anthracocystis*, *Moesziomyces*, *Sarocladium*, and *Dirkmeia*; a limited population of *Saccharomyces*; and a number of unidentified fungi. The 12-h and 24-h fermentation time points recorded a shift from this diverse population to a less diversified community, dominated by *Saccharomyces* and *Pichia*. *Candida*, *Kluyveromyces*, *Nakaseomyces*, *Torulaspora*, *Stachybotrys*, and *Cyberlindnera* were also present. The milled samples with spices also showed a diverse profile made up of OTUs including yeasts *Saccharomyces*, *Pichia*, *Kazachstania*, *Kluyveromyces*, and *Torulaspora*. The sediment and supernatant fermentation time points were mainly dominated by *Saccharomyces*. Other fungi such as *Kluyveromyces*, *Pichia*, *Kazachstania*, *Candida*, *Stachybotrys*, *Moesziomyces*, *Claviceps*, and *Malassezia* were also associated with this time point. *Hausa koko* samples were highly diversified with yeast communities dominated by *Pichia*, *Malassezia*, *Cyberlindnera*, *Kluyveromyces*, and *Saccharomyces*. The *Hausa koko* sample from Winneba particularly was highly diversified compared to the others. Other fungal genera identified included *Aspergillus*, *Meyerozyma*, *Eremothecium*, *Candida*, *Claviceps*, *Bjerkandera*, *Kondoa*, *Malassezia*, *Moesziomyces*, and some unidentified communities.

Metabolic Composition

The metabolites present in the samples that are consumed (Su and K) as well as the different time points (D, 12 h, 24 h, and M) during *Hausa koko* production by 15 producers within six

FIGURE 4 | Relative abundance of the bacterial communities of the different processing steps during *Hausa koko* production. Each horizontal piled bar represents a single sample. Taxonomic groups at genera (g) level are identified where possible. Where genera could not be determined, order (o) or domain (d) are shown. Taxonomic groups with abundance <1% were included in the group "others."

regions were analyzed. A total of 33 metabolites were detected and quantified (**Figure 6**) along the fermentation stages with varying trends in similar patterns in all the samples, irrespective of the geographical location the sample was obtained from. Two-way ANOVA test showed significant differences between time points with regard to organic acids. Among them, *post hoc*

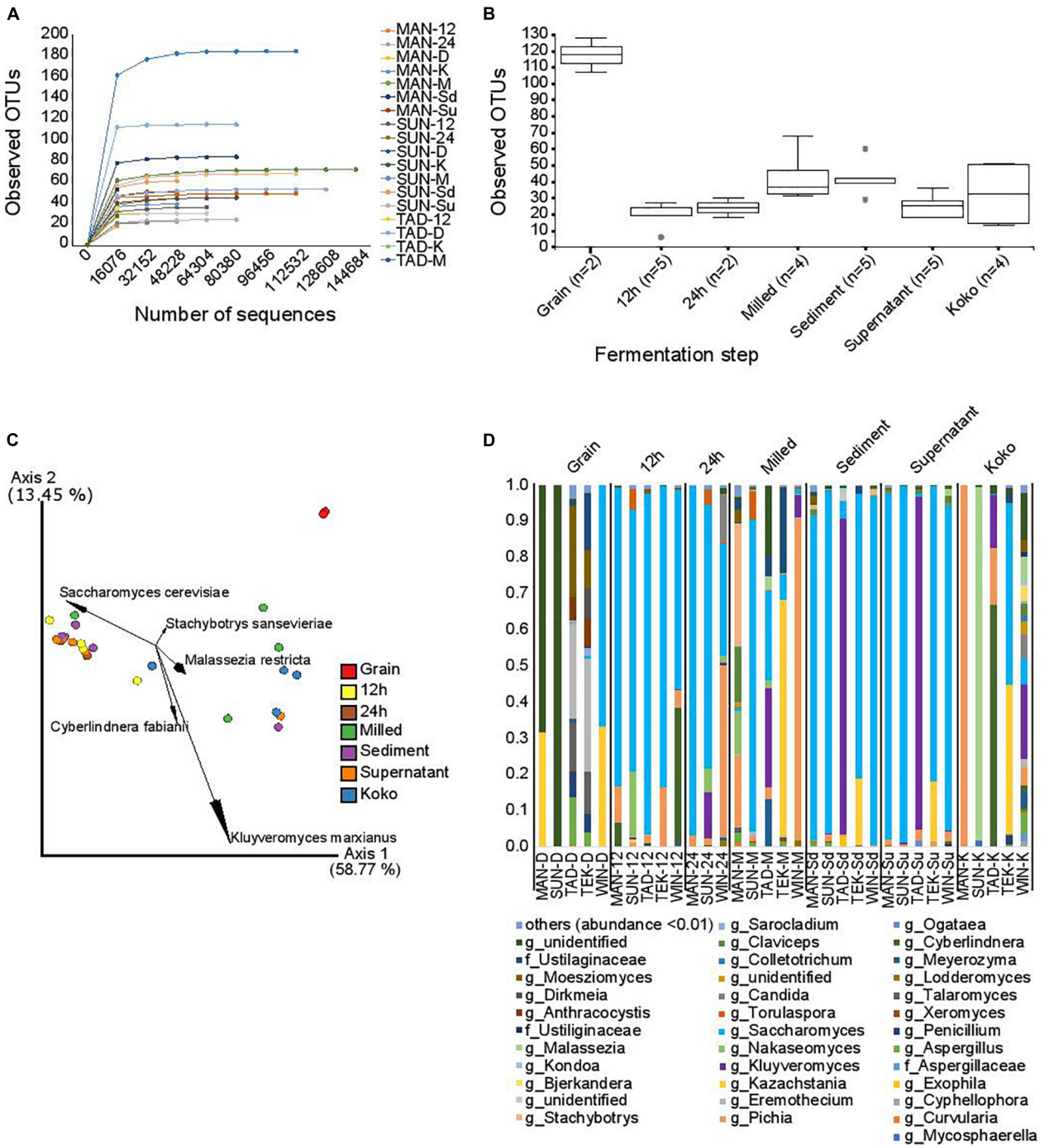

FIGURE 5 | Fungal diversity. **(A)** Rarefaction plots of fungal observed OTUs depending on the sequencing depth. Each curve represents a sample. **(B)** Fungal alpha diversity based on the observed OTUs in the different fermentation steps. Dots represent outliers. **(C)** Fungal beta diversity represented by principal coordinate analysis based on weighted Unifrac distances. Fermentation time points are represented by different colors as indicated in the legend. The arrows indicate the five taxonomic groups that contribute most to the differences. **(D)** Relative abundance.

analysis showed significant increase in the concentration of lactate between grains and all the fermentation steps except *koko* (p-value grain vs. 12 h, 24 h, milled, and supernatant was <0.0001). Although no significant differences were detected, the concentration of lactate in *koko* was slightly higher than that in the grains. Similar changes were observed for acetate: statistical increases in acetate concentration between grains and 12 h ($p < 0.0001$), 24 h ($p = 0.035$), and milled samples

($p < 0.0001$) were detected. Again, statistical differences were found for ethanol between grains and 12 h ($p < 0.0127$), 24 h ($p = 0.0342$), and M ($p < 0.0001$). There were no significant differences for the other organic acids produced at the different time points. The changes observed in organic acids were inverse to the changes observed in sugar levels: glucose, the most abundant sugar found in the samples, decreased significantly in the first step of the fermentation ($p < 0.0001$) and increased slightly in the milled sample, although with a high variation between samples, to then decrease again in the supernatant and koko samples ($p < 0.0001$). Significant differences also existed in the concentration of fructose between grains and all the fermentation time points ($p < 0.0001$).

Another group of metabolites of importance identified at the various stages were amino acids (**Figure 6C**). The amino acid concentrations generally increased from the dry grains to the fermenting time points and reduced in the later stages of production, particularly in both supernatant and *Hausa koko* samples. Significant increase between grains and the 12- and 24-h fermentation points were detected for almost all of them ($p < 0.0001$ for leucine, isoleucine, aspartate, alanine, threonine, valine, and phenylalanine; $p = 0.0007$ for glutamate, $p = 0.0002$ for glutamine, and $p = 0.0137$ for methionine), although the concentration of glycine only increased significantly after 24 h ($p = 0.0003$) and asparagine decreased between these time points ($p = 0.0001$). Between the fermentation and milling of the samples, the concentration of some amino acids decreased significantly ($p < 0.0001$ for leucine, alanine, and valine; $p = 0.0239$ for glutamine), while for others, the decrease in concentration was observed in the supernatant or koko samples ($p < 0.0001$ for glycine, isoleucine, aspartate, alanine, threonine, and phenylalanine; $p = 0.026$ for tyrosine; $p = 0.0005$ for aspartate; $p = 0.002$ for glutamine; $p = 0.02$ for methionine).

Other metabolites not included in previous groups were detected at very low concentrations: betaine, trigonelline, uracil, choline, and succinate. Betaine and choline decreased after the first 12 and 24 h of fermentation ($p < 0.0001$) while uracil and succinate increased ($p < 0.0001$). The concentration of these compounds was maintained in the milled samples and dramatically decreased in supernatant and *koko* samples ($p < 0.0001$).

DISCUSSION

Hausa koko is one of the most popular fermented foods consumed in Ghana. It is an affordable and nutritious porridge for all people. Understanding the microbial community dynamics and the metabolic changes during the production of this fermented porridge is needed to design starter cultures to perform safer, standardized, and scalable production batches. In this study, we used culture-independent high-throughput sequencing of the 16S rRNA gene to study the bacterial diversity of *Hausa koko*, showing a high species richness at the different time points from the different geographical sampling locations. Significant differences were observed between the different time points during the production process.

The grain samples, used as the raw material to produce the *Hausa koko*, recorded more OTUs and higher relative abundance of the genus *Pantoea* compared to the other time points. This genus of Gram-negative bacteria of the Enterobacteriaceae family is usually isolated from several sources and ecological niches including plants (Kini et al., 2018; Azizi et al., 2020) and they are most probably associated with the original plant material and the soil environment (Sarita and Singh, 2016). The grain samples were also dominated by other Gram-negative microbes including *Pseudomonas*, *Enterobacteriaceae*, and *Escherichia-Shigella*, some of which may be potential pathogens and are commonly associated with feces, soil, and intestinal tracts of humans and warm-blooded animals and may cause gastroenteritis, diarrhea, vomiting, and nausea (Gadaga et al., 2004, 2008).

As expected, the fermentation stages presented a major shift to mainly fermentation-related genera dominated by lactic acid bacteria (LAB), which were detected already after 12 h of fermentation. A significant increase in the relative abundance of the genus *Limosilactobacillus* (formerly included in the *Lactobacillus* genus) (Zheng et al., 2020) was observed and other genera became dominant, including *Pediococcus*, *Weissella*, *Lactococcus*, *Streptococcus*, *Leuconostoc*, and other genera within the lactobacilli group, such as *Lactobacillus* and *Ligilactobacillus*. Consequently, an increase in the concentration of organic acids produced by the fermenting microbes was observed and a subsequent decrease in the population of *Pantoea* spp. was noticed. Shifts in the acidity after 24 to 48 h of fermentation of maize dough, used in the preparation of three popular Ghanaian traditional foods, have been reported (Halm et al., 2004). These organic acids, lactic acid in particular, are key fermentation features, important for both organoleptic qualities and pathogen reduction (Oguntoyinbo and Narbad, 2015). Sour foods are popular in Ghana, and in all such sour foods, the role of LAB has been demonstrated (Amoa-Awua et al., 1996; Obilie et al., 2004; Obodai and Dodd, 2006; Atter et al., 2014; Annan et al., 2015). The relative abundance of LAB populations increased along the fermentation stages from 12 to 24 h, M, Su, and Sd. The population, however, was reduced in the final porridge, which may be attributed primarily to application of heat. Acetic acid bacteria, *Acetobacter* and *Gluconobacter* genera, were also present in varying relative abundances at the different time points in the *Hausa koko* production process. Although not significant, some of the *Hausa koko* samples contained a higher relative abundance of acetic acid bacteria. Acetic acid bacteria produce mainly acetic acid, some vitamin C, and cellulose during spontaneous fermentation of foods and beverages (De Roos and De Vuyst, 2018). Their presence has been reported in other African cereal fermented foods including *kunu* and *burukutu* (Oguntoyinbo, 2014; Ezekiel et al., 2019). They are responsible for oxidation of ethanol produced during fermentation to acetic acid (Gómez-Manzo et al., 2010; Ezekiel et al., 2019).

Generally, significant differences did not occur between most of the samples within the same time point from the different regions or within different processing facilities of the same region, although some significant differences were detected in samples from the Northern and Greater Accra region, which could be attributed to the microbial communities of the millet substrates,

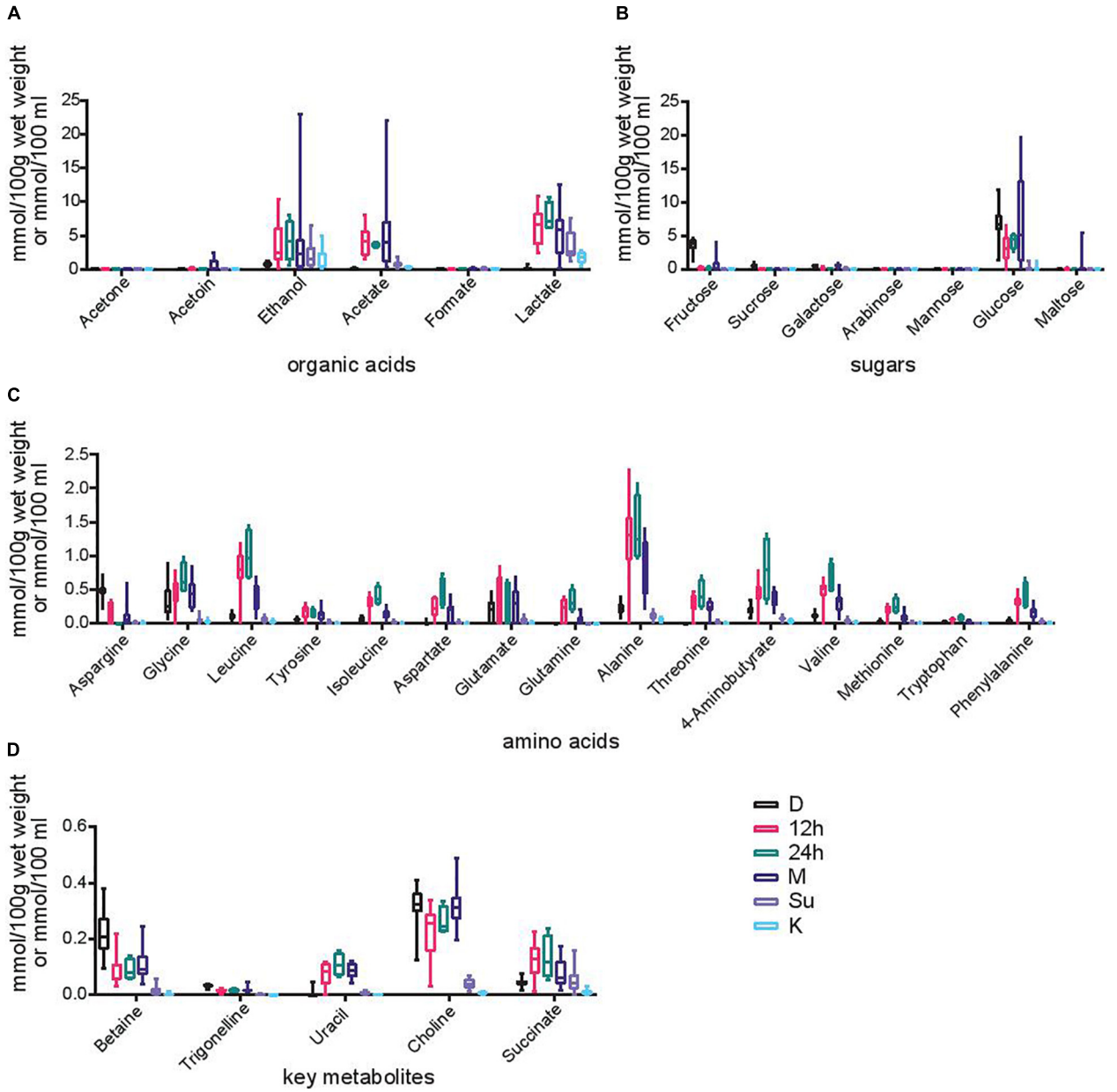

FIGURE 6 | Metabolite profiles. Box plots of the concentration of various metabolites expressed in mmol per 100 g of wet weight for grains, 12 h, 24 h, and milled samples or mmol per 100 ml for supernatant and *Hausa koko* samples. Each box represents the 5th to 95th percentile. Each panel represents a different category of metabolites produced at different time points (dry, 12 h, 24 h, milled, supernatant, and koko) during *Hausa koko* production. **(A)** Organic acids, **(B)** sugars, **(C)** amino acids, and **(D)** key metabolites.

as they may vary depending on the source. Additionally, the quality of water, spices, utensils, contact surfaces, and hands may influence these differences (Gadaga et al., 2008). The significant difference in the samples from the Northern region and the other five regions was due to OTUs within the genus *Lactobacillus* while the differences in the samples from the Greater Accra region seem to be attributed to OTUs within the *Acetobacter* genus. This study did not find any previous observations of regional differences in African cereal fermented foods. Therefore, further studies would be needed to determine what is causing those differences.

In this study, we selected a subset of five samples from three of the main regions, to characterize the fungal populations. Yeasts are the most common microorganisms apart from bacteria in spontaneous cereal fermentations (Achi and Ukwuru, 2015). Synergism occurs between bacteria and yeast in fermentation niches, with acidification of the medium by bacteria supporting yeast growth and subsequent release of amino acids and

vitamins (Stadie et al., 2013). Factors such as the raw materials used, processing operations including duration and temperature of fermentation, hygienic practices, interactions between the microbes, and their successions, which are influenced by intrinsic and extrinsic growth factors, may influence the diversity of yeast in *Hausa koko* (Jespersen, 2003; Achi and Ukwuru, 2015; Johansen et al., 2019). The fungal community at the different stages of *Hausa koko* production was profiled using the ITS sequence data. Although most of the grain samples had to be removed in the diversity analysis due to the low number of reads obtained in the sample, the two samples that could be retained showed a highly diverse population of molds and yeast genera in the grains that are likely to be soil and grass inhabitants with very little *Saccharomyces*. In some of the grain samples, there was a high abundance of OTUs that could not be classified. The dominance of soil- and grass-associated fungi was drastically reduced in the fermentation time point samples at 12 h, 24 h, supernatants, and sediments. There was an increase in the relative abundance of the genera *Saccharomyces* and *Pichia*. Some of the samples were dominated by the genus *Kluyveromyces*. This trend may be attributed to acidification of the samples at these time points and microbial succession, which may have led to the inhibition of growth of some of these fungi while promoting the growth of others (Achi and Ukwuru, 2015). These time points were significantly different from the others due to OTUs classified as *S. cerevisiae*, which are predominant in indigenous African fermented cereal foods (Achi and Ukwuru, 2015; Johansen et al., 2019). The presence of *S. sansevieriae* and *M. restricta* in some of the milled samples have not been reported in indigenous African fermented foods yet. The genus *Pichia* is another frequently occurring yeast in fermented cereals and reported extensively. *Pichia kudriavzevii* was reported in another spontaneously fermented pearl millet product, *fura*, in West Africa (Pedersen et al., 2012), *ogi* (Omemu et al., 2007), and *gowé* (Greppi et al., 2013). *K. marxianus* is one of the promising yeast species isolated from fermented foods with beneficial characteristics and has been associated with spontaneous fermentation in West Africa (Karim et al., 2020; Motey et al., 2020). All the yeasts profiled at the different time points may contribute to production of aroma compounds from different carbon sources, mycotoxin degradation, increase in shelf life, safety, and nutritional value of *Hausa koko* (Forti et al., 2018; Johansen et al., 2019). Although significant reads from both bacteria and fungi were obtained from the K samples, cooking of the final *Hausa koko* porridge by addition of boiling water is expected to affect the number of live microbes in the final product. This could potentially reduce the level of most contaminants, potential pathogens, and aflatoxin-producing fungi that started the fermentation, making *Hausa koko* safer. It has been established that even though cooking of fermented cereal porridges actually reduces the antimicrobial effect of LAB and yeast on pathogens, significant inhibition of these pathogens still occurs (Mensah et al., 1991; Mensah, 1997).

The presence and interactions between the wide array of microbes at the various stages of *Hausa koko* production yielded different metabolites following the conversion of carbohydrates in accordance to the principles of fermentation (Hagman and Piškur, 2015). These metabolites were produced by the various fermenting microbes, predominantly LAB and yeast during this mixed fermentation process (Ohimain, 2016). Inconsistent trends in the sugar concentrations with a general increase in metabolic products of LAB and yeast was observed. There was a general reduction in the sugar concentrations along the processing time points. The milling breaks down the grains and makes the carbohydrates more accessible to the enzymes produced during fermentation. These enzymes then break down the complex carbohydrates into simple sugars that are utilized by the fermenting microbes for energy (mainly glycolysis). The spices may also have contributed to the increase in sugars. The high concentrations of sugars dominated by glucose in the milled samples make them ideal substrates and carbon source for use by fermenting microbes for growth (Charalampopoulos et al., 2002; Di Stefano et al., 2017). The production of organic acids such as lactate, acetate, and ethanol progressed steadily along the fermentation stages peaking in the milled millet samples. Dilution of the milled millet with water resulted in a decrease in their concentrations in the supernatants and *Hausa koko* samples. Other organic acids occurred in small concentrations. The presence of such organic metabolites is indicative of the involvement of heterofermentative LAB and yeast (Michodjèhoun-Mestres et al., 2005) and contributes to the flavor, taste, and sensorial properties of the final product (Sripriya et al., 1997; Onyango et al., 2000; Akpinar-Bayizit et al., 2010; Weldemichael et al., 2019). Amino acid concentrations in the grain samples were generally low but increased marginally in the 12-h fermented millet and milled samples (Mbithi-Mwikya et al., 2000; Adebiyi et al., 2017). This could be attributed to increased hydrolytic enzyme activities from the grains as well as breakdown of complex proteins to amino acids (Saleh et al., 2013). The concentration of some amino acids was reduced in the milled samples, but most of them decreased in the supernatant samples, which could be due to a combination of factors: the consumption by the microorganisms in order to multiply and the dilution with water in the supernatant and *Hausa koko* samples. Other important metabolites including betaine and trigonelline were profiled at the various time points of *Hausa koko* production. Whole grain cereals are excellent dietary sources for betaine and its precursor choline, which are associated with amino acid and lipid metabolism (Bruce et al., 2010; Ross et al., 2014). Trigonelline has been described as possessing anti-diabetic properties and decreasing blood cholesterol level, and is used for treating migraine, cancer, and other conditions (Basch et al., 2003; Bahmani et al., 2016).

The different metabolites profiled in the current study as a result of fermentation by the various microbes may contribute to the flavor and aroma of *Hausa koko*, similar to compounds developed during fermentation of other products (Salmerón et al., 2014; Weldemichael et al., 2019). These metabolites, including organic compounds, are antimicrobial substances involved in inhibiting the proliferation and survival of potential pathogens and other contaminants. Their presence contributes to the safety of the product and reduces incidence of diarrhea and other food borne diseases (Achi and Ukwuru, 2015; Adebo et al., 2017).

CONCLUSION

Significant differences were observed in bacterial diversity at the different time points of *Hausa koko* production across the six regions. The grain samples were more diverse with high abundance of the genus *Pantoea*. Lactobacilli, however, increased steadily as fermentation progressed during the production stages, peaking in the sediment samples but reduced in the final *Hausa koko* as a result of dilution with water and application of heat. There were few differences in diversity between regions. The Northern region samples were different from the other regions due to OTUs within the genus *Lactobacillus*. ITS sequence data also revealed a high population of fungal genera in the grains including known contaminants, and soil and grass inhabitants with many unidentified. In general, the most abundant yeast during *Hausa koko* fermentation was the genus *Saccharomyces*. This may be the first time *S. sansevieriae* and *M. restricta* are reported in indigenous African fermented foods. *Hausa koko* samples recorded OTUs of the genera *Pichia*, *Malassezia*, *Cyberlindnera*, *Kluyveromyces*, and *Saccharomyces* and were differentiated from other time points by *C. fabianii* and *M. restricta*. The metabolomics study using NMR unveiled the profile of metabolites that were produced during *Hausa koko* fermentation. From the profile obtained, it can be suggested that traditional fermentation of *Hausa koko* undergoes the typical shifts from the fermentation process but the concentration of most of the organic acids and amino acids is drastically reduced in the final product that is consumed.

DATA AVAILABILITY STATEMENT

The sequencing datasets generated in this study can be found in the SRA database under the BioProject accession number PRJNA70037.

AUTHOR CONTRIBUTIONS

AA collected the samples, performed the DNA and metabolite extractions, interpreted the data, and drafted the original manuscript. MD supervised the work, performed bioinformatic and statistical analysis, interpreted the data, and contributed to the manuscript writing. KT-D and AK contributed to supervision and review and editing. MM and AN contributed to conceptualization, funding acquisition, supervision, and review and editing of the manuscript. IC performed nuclear magnetic resonance spectroscopy. DB sequenced the yeast samples. DN reviewed and edited the manuscript. WA-A contributed to conceptualization, supervision, and review and editing. 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.2021.681983/full#supplementary-material

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Analysis of the Fungal Diversity and Community Structure in Sichuan Dark Tea During Pile-Fermentation

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The fungi present during pile-fermentation of Sichuan dark tea play a pivotal role in the development of its aroma and physical characteristics. Samples of tea leaves were collected on days 0 (YC-raw material), 8 (W1-first turn), 16 (W2-second turn), 24 (W3-third turn), and 32 (W4-out of pile) during pile-fermentation. High-throughput sequencing revealed seven phyla, 22 classes, 41 orders, 85 families, 128 genera, and 184 species of fungi. During fermentation, the fungal diversity index declined from the W1 to W3 stages and then increased exponentially at the W4 stage. A bar plot and heatmap revealed that *Aspergillus*, *Thermomyces*, *Candida*, *Debaryomyces*, *Rasamsonia*, *Rhizomucor*, and *Thermoascus* were abundant during piling, of which *Aspergillus* was the most abundant. Cluster analysis revealed that the W4 stage of fermentation is critical for fungal growth, diversity, and the community structure in Sichuan dark tea. This study revealed the role of fungi during pile-fermentation in the development of the essence and physical characteristics of Sichuan dark tea. This study comes under one of the Sustainable Development Goals of United Nations Organization (UNO) to "Establish Good Health and Well-Being."

Keywords: Sichuan dark tea, pile-fermentation, fungal community, high-throughput sequencing, 16S rRNA (16S rDNA)

INTRODUCTION

Sichuan dark tea is manufactured by processing a mixture of fresh leaves and mature branches collected from tea plants (*Camellia sinensis*; Wang et al., 2018b). Dark tea is one of the six famous tea types produced in the northwest and southwest borders of China, and the production of this tea is a significant contributor to the livelihood of ethnic minorities in China (Wang et al., 2016). Sichuan dark tea is a post-fermented tea with a unique flavor, can be stored for long-time, and can be brewed multiple times. It has demonstrated health benefits including anti-hyperglycemia, anti-hypertension, and anti-hyperlipidemia effects (Wang et al., 2016, 2018b). Dark tea is one of the richest sources of biologically active compounds including vitamins, amino acids, organic acids, polyphenols, and trace elements (Chen et al., 2017; Xu et al., 2020). It is also a widely used medicinal plant as a preventive, especially in traditional

Chinese medicine (Peng et al., 2014; Liu, 2016). However, the over-use of dark tea may have some negative health effects, such as caffeine causes heartburn, nausea, dizziness, and poor sleep quality (Srinivasan et al., 1997), while tannins intercalate with iron and refrain, it being absorbed by human digestive system (Walch et al., 2012).

The pile-fermentation process of dark tea comprises the following steps: fixation, rolling, piling, and drying (Wang et al., 2016). During fixation, high temperature causes oxidase inactivation, dehydration, soften leaves, and develop aroma. In rolling, tea leaves are chopped into small strips and squares and finally milled to disrupt cell walls of tea cells. During piling, the tea leaves are piled under warm, humid conditions, during which fermentation by associated microbes occurs, called as pile-fermentation. Finally, unique dark color and aroma are developed by baking during drying process. The microorganisms involved in the pile-fermentation process of dark tea are unique among tea types. Notably, although a considerable number of microbes in raw tea is killed after fixation, many microbes retained during subsequent processing (Li et al., 2017a). Piling is a key determinant of the color, aroma, taste, and shape of dark tea. The unique flavor and essence of dark tea are predominantly developed through polyphenol oxidation, the metabolic activities of microbes, biochemical processes, such as those catalyzed by extracellular enzymes, and physicochemical properties, such as heat and humidity, during pile-fermentation (Zhang et al., 2016a; Wang et al., 2018b). For example, *Penicillium* can hydrolyze the fiber in the tea during piling, increase its sugar content, and contribute to the taste of dark tea. *Aspergillus niger* and yeasts produce a variety of hydrolytic enzymes that participate in mutual transformation reactions among the various substances in tea. In piling, the raw tea materials undergo enzymatic reactions and fermentation, which are driven by a series of microorganisms to develop the characteristics of quality dark tea, that is, a dark leaf color and pure aroma (Wang et al., 2016).

Recently, the processing techniques and technical equipment used in the Sichuan tea industry have improved substantially. However, the piling process used to develop the characteristic essence and flavor of dark tea is still based on traditional natural fermentation. Different successive microbial populations contribute to the process of natural fermentation of dark tea (Zhang et al., 2016b; Li et al., 2018b), and variations in environmental conditions can significantly alter the process that supports different populations in the microbial community. In short, the qualitative characteristics of dark tea, such as its essence and aroma that develop during the pile-fermentation process, are entirely dependent on the populations in the microbial community. Operational taxonomic units (OUTs) are variations among homologous sequence clusters of 16S rDNA of different microbial species with 97% identity threshold, which are employed to distinguish different microbial species present in samples (Schloss and Westcott, 2011). Principal component analysis (PCA) is a Euclidean based ordination technique that is used to express correlation in genomic sequencing data of microbes in the simplest way by removing noise and redundancy. While principal co-ordinate analysis (PCoA) is an independent algorithm non-constrained data dimensionality reduction method to express the correlation in genomic sequencing data of microorganisms (Minchin, 1987).

Yibin city in Sichuan province is situated on the bank of the Yangzi River, and dark tea cultivated here retains a special aroma due to the characteristic environmental conditions, unique pile-fermentation techniques used, and microbial resources. Although dark tea has been extensively studied for its growth, bioactive ingredients, and health benefits, little is known about the microbes involved in its qualitative improvement (Chen et al., 2013; Fu and Liu, 2015; Zhang et al., 2016a; Ge et al., 2019). To improve the unique qualities of dark tea, it is imperative to carefully analyze and effectively use these diverse microbial communities and their metabolic activities during pile-fermentation. The purpose of this study was to investigate the presence of different fungal species at different stages of pile-fermentation, so that beneficial fungal species can be artificially inoculated to improve aesthetic and nutritional value of dark tea. In this study, high-throughput sequencing was employed to investigate the composition of the diverse microbial communities present in Sichuan dark tea during pile-fermentation to improve processing.

MATERIALS AND METHODS

Experimental Materials

Samples of Sichuan dark tea raw ingredients prepared from fresh leaves were obtained from the Sichuan Tea Industry Group Co., Ltd.[1] No specific permission was required for sample collection for academic research. During fermentation, the leaves were mixed to ensure homogeneity; tap water was added as needed to maintain the solids content at 65-75% (w/v), and the temperature was maintained at 45-71°C. Samples were collected on days 0 (YC), 8 (W1), 16 (W2), 24 (W3), and 32 (W4) during fermentation. Three replicates were set up, and the collected samples were subjected to microbial analysis. The temperature at the center of the fermented tea pile, at a depth of 40 cm, was measured each day.

DNA Extraction

For DNA extraction, 5 g of sample was weighed and immediately soaked in 25 ml of ddH_2O. After stirring thoroughly, the sample was filtered through three layers of sterile gauze to remove large particles and then centrifuged at 12,298 × *g* for 10 min at 4°C. The supernatant was discarded, and the precipitate was used for genomic DNA (gDNA) extraction. We used the E.Z.N.A™ Fungal DNA Miniprep Kit (OMEGA, United States) according to the standard protocol to extract gDNA from the fungal tissues. The quality of the extracted gDNA was confirmed by running 2 μl of each sample on 1% agarose gel, which was visualized under a UV light in a gel documentation system (iBright imaging system, iBright 1500, ThermoFisher, United States; Wang et al., 2018a). The concentration of extracted

[1]http://www.scteag.com/

DNA was recorded on spectrophotometer (Nanodrop 2000) at OD260/280, which was 2.1-42.5 ng/μl in each sample with triplicate manners.

PCR Amplification and Sequencing Analysis

The primer pair ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2R (5'-GCTGCGTTCTTCATCGATGC-3') was used to amplify the ITS1 region of the fungal ITS rDNA sequence (Li et al., 2017b) by PCR. Each reaction contained 4 μl of 5× FastPfu Buffer, 2 μl of 2.5 mmol/L dNTPs, 0.8 μl each of 5 μmol/L forward and reverse primer, 0.4 μl of FastPfu DNA Polymerase (TransStart®, AP221-01, China), 10 ng of DNA template, and ddH_2O to 20 μl. The PCR conditions were as follows: an initial denaturation step at 95°C for 3 min, followed by 32 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 45 s, and amplification at 72°C for 45 s, with a final amplification step at 72°C for 10 min, and storage at 10°C. To confirm amplification and amplicon size, 2 μl of each PCR product was separated by 2% agarose gel electrophoresis. The PCR product was used to construct a gDNA library, which was evaluated by high-fidelity Illumina MiSeq™ PE300 sequencing. Raw reads were trimmed, duplicate reads were merged according to PE read overlap, the quality of the reads was assessed, and splicing events were controlled. To distinguish samples, barcodes were assigned to each sample at the start and end of the sequencing based on the primer sequence. Finally, we obtained sufficient sequences, and their directions were corrected (Mukherjee et al., 2014).

Sequencing and Phylogenetic Analysis

Phylogenetic clustering analysis was conducted using the Uparse OTU clustering software tool at a 97% identity threshold to identify representative sequences of operational taxonomic units (OTUs; Ye et al., 2017; Peng et al., 2019).[2] The RDP classifier Bayesian algorithm was used to perform a taxonomic analysis of the OTUs at 97% identity threshold. The community composition and scientific classifications of each sample were established at the kingdom, phylum, class, order, family, genus, and species levels (Chen et al., 2016). We calculated the α-diversity of one sample of each using the Chao 1, ACE, Shannon, and Simpson indices to evaluate sequencing depth and coverage and to compare the abundance and diversity of the microbial communities in dark tea at different stages (Rogers et al., 2016). The number of common and unique OTUs in all samples was counted and a Venn diagram was constructed (Fouts et al., 2012). Variation in the composition of the OTUs in every sample was calculated using 97% identity threshold through PCA and principal coordinate analysis (PCoA; Calderón et al., 2017). The taxonomic analyses of all samples were compared at each classification level, and the R tool was used to construct community structure diagrams and histograms as combined analysis diagrams (Lu et al., 2016; Zhou et al., 2017).

[2]http://drive5.com/uparse/

Statistical Analysis

All data were explained in mean values of standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA). A Duncan multiple-comparison test was applied to detect variations among means of all the samples at $p < 0.05$ level of significance. All correlation and path coefficient analyses were performed with SPSS Statistics 20.0 (SPSS Inc., Chicago, IL, United States) and Excel 2019.

RESULTS

Statistical Analysis of Sequencing Data

Illumina next generation DNA sequencing (NGS) of all five samples of Sichuan dark tea was performed in triplicate, and the results were analyzed to identify the identity threshold level among the fungal populations present in each sample using ITS metagenomics. The ITS1F/ITS2R universal primer pair was used to amplify the genomes of the different populations of fungi present in the microbial community of the dark tea (**Supplementary Table S1**). Incomplete and poor quality reads were eliminated. Ultimately, we obtained 334,593 high-quality fungal genomic sequences, with read lengths ranging from 249 to 278 bp (**Supplementary Figure S1; Table 1**).

Rarefaction curves reflect the richness and uniformity of the microbial species present in each sample of dark tea. The relative abundance curves of samples collected at W1 and W4 at 97% identity threshold appeared to have a small span and a steep decline (**Figure 1**). These results showed that the relative abundances of the OTUs between samples were remarkably different, the uniformity was very low, the fungal composition was relatively single, and diverse fungal species were present. On the other hand, the relative abundance curves of the YC and W2 samples were wider, and the curves were flat with a gradual decline, indicating that the species composition of the YC and W2 samples is richer, and the uniformity among species was higher (**Figure 1**). Except for the W2 samples, the microbial strains were not obvious and were relatively concentrated.

Operational Taxonomic Unit Cluster Analysis

During the OTU analysis, all non-repetitive single sequences were separated from the optimized sequences, and redundant

TABLE 1 | Characteristics of ITS sequences of the fungal populations in samples collected from Sichuan dark tea and the five time points during pile-fermentation.

Sample	Reads	Total bases	Average length (bp)
YC	69,242	16,428,484	276.6316
W1	65,769	16,436,511	249.9127
W2	71,262	19,845,494	278.4863
W3	65,242	17,154,692	262.9394
W4	63,078	15,839,408	277.6428

Column 1 is the sample name, column 2 is the number of sequencing reads in each sample, column 3 is the total number of bases, and column 4 is the average length, in base pairs, of each run.

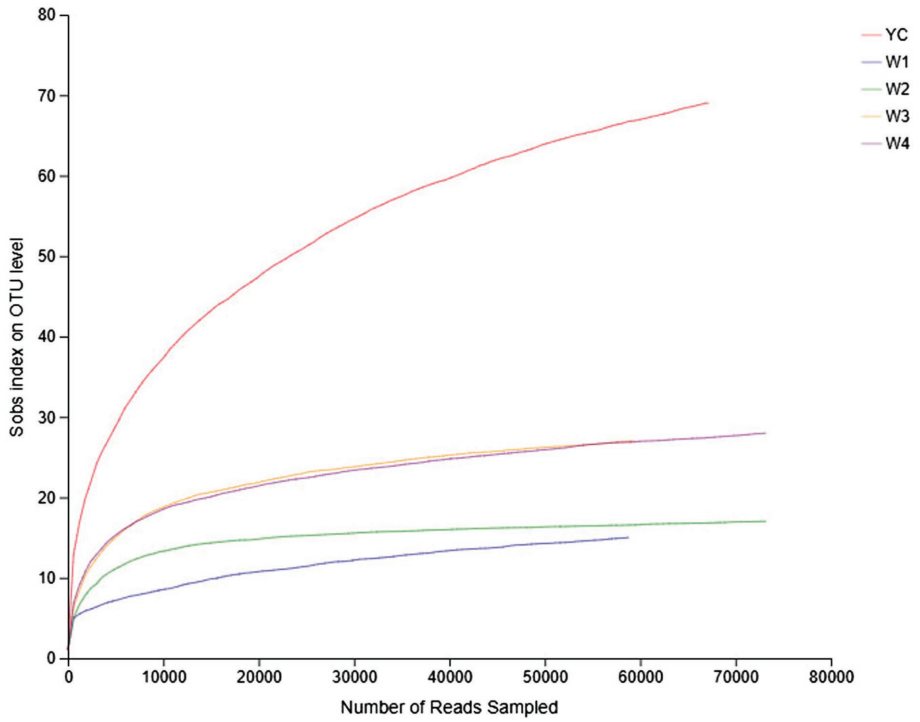

FIGURE 1 | Rank abundance curves of all the samples. The abscissa represents the rank of the number of operational taxonomic units (OTUs) at a certain taxonomic level, and the ordinate represents the relative percentage of the number of species at that classification level. The position of the abscissa of the extension end point of the sample curve is the number of species in each sample. A smooth decline indicates higher species diversity in all the samples, while a rapid and steep decline indicates a high proportion of the major bacterial strains and low diversity.

sequences were eliminated. Except for single sequences, OTU clustering analysis of non-repetitive sequences was performed at 97% identity threshold. Subsequently, all the chimeric sequences were removed, and only a single representative sequence for each OTU was included in the analysis. Clustering analysis of valid data yielded 270 fungal OTUs, which were classified into seven phyla, 22 classes, 41 orders, 85 families, 128 genera, and 184 species (**Supplementary Table S2**). Only 11 fungal OTUs were common among all five groups, 14 OTUs were common between W1 and YC, 53 OTUs were common between W2 and YC, 23 OTUs were common between W3 and YC, and 34 OTUs were common between W4 and YC (**Figure 2**).

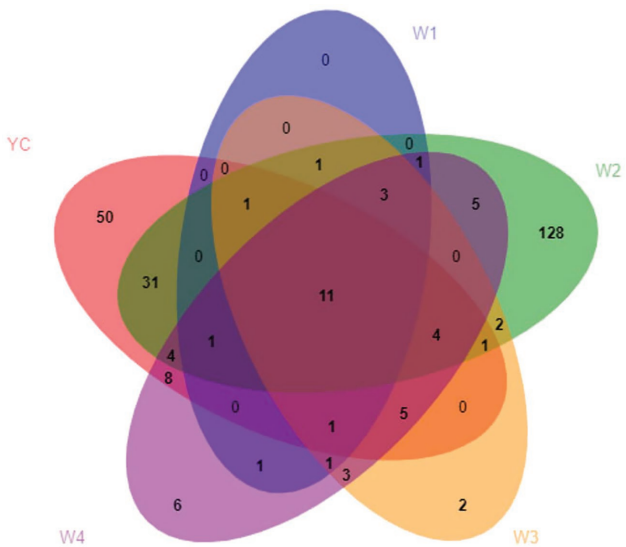

FIGURE 2 | Venn analysis of the OTUs. Different groups are shown in different colors, and the numbers in the overlapping portions represent the number of species common to all the groups.

Microbial Diversity Analysis

The coverage threshold for the sequences from the dark tea samples collected at all five time points was >99%, indicating that it represents the true fungal populations in the microbial community of each sample. To estimate the species richness or total number of microbial species in each sample, the sequencing results were analyzed using ACE and Chao 1 indices (Chernov et al., 2015; Wang et al., 2017). The samples with the highest abundance levels, according to the ACE and Chao1 values (at 89.27 and 84.57%, respectively), were observed in the YC group (**Table 2**). In contrast, the samples with the lowest abundance levels, according to the ACE and Chao1 values (at 28.16 and 20.00%, respectively), were observed in group W1. The remaining groups had intermediate abundance levels according to the ACE and Chao 1 values, which were 45.03 and 44.43 in W2, 33.40 and 36.00 in W3, and 49.53

TABLE 2 | Indices of fungal community richness and diversity of Sichuan dark tea.

Sample	Shannon	Simpson	ACE	Chao1	Coverage
W1	0.84	0.49	28.16	20.00	0.999915
W2	0.77	0.55	45.03	44.43	0.999928
W3	0.60	0.70	33.40	36.00	0.999927
W4	0.94	0.49	49.53	46.27	0.999825
YC	1.09	0.46	89.27	84.57	0.999650

The first column is the sample names, and columns 2-4 are the Shannon, Simpson, ACE, and Chao 1 diversity index values of each sample. The last column is average coverage diversity index values.

and 46.27 in W4, respectively. The overall microbial species abundance in the Sichuan dark tea samples collected at the tested time points was in descending order: YC > W4 > W2 > W3 > W1 (**Table 2**).

The Shannon and Simpson indices are comprehensive indicators that reflect the proportion of different species in a diverse community (Chernov et al., 2015; Wang et al., 2017). The Shannon-Weiner index is directly proportional to the richness and diversity of different populations in a community and is negatively correlated with the Simpson index. An Elevated Shannon-Weiner index value indicates higher species diversity in a microbial community, whereas an elevated Simpson index value indicates low species diversity. The Shannon-Weiner values of the samples were, in descending order: YC >W4 > W1 > W2 > W3. Similarly, the Simpson values of the samples were in descending order: W3 > W2 > W1 > W4 > YC. The highest Shannon-Weiner index value and lowest Simpson index value of the YC samples showed both high fungal species richness and diversity. With continued piling time, the fungal diversity first decreased and then exponentially increased in YC samples, and the highest diversity was observed before piling.

Fungal Community Structure Analysis

Comprehensive qualitative or quantitative compositional analysis of the 40 most frequent populations in the microbial communities of Sichuan dark tea at the genus level were used to construct a histogram (**Figure 3**) and heatmap (**Figure 4**). The analysis showed that *Aspergillus* was the most abundant in all the tested samples. The abundance of *Aspergillus* increased exponentially to 99.3% at W1 and then decreased over time. In addition to *Aspergillus*, four other genera, *Thermomyces*, *Candida*, *Debaryomyces*, and *Rasamsonia*, also displayed higher abundances in the YC group, at 19.1, 16.9, 12.4, and 7.8%, respectively. In the W2 group, the genus with the highest abundance was also *Aspergillus*, although *Rhizomucor* (13.4%) and *Thermomyces* (3.1%) were also frequent (**Figure 3**). The possible reason of higher fungal species during early stages could be the presence of fungal spores in raw material used in pile-fermentation.

Other highly abundant fungal genera in W3 were *Thermomyces*, *Rasamsonia*, and *Thermoascus*, while the highly abundant fungal genera in group W4 were *Rasamsonia* and *Thermomyces* (**Figure 3**). The highest abundance of *Aspergillus* species was obvious in the heat map (**Figure 4**). Notably, although information about unclassified *Trichocomaceae* and *Ascomycota* could not clearly be distinguished at the genus level, these likely are mostly *Aspergillus cristatum*. In addition, *Candida* and *Debaryomyces* were also detected in the dark tea samples after pile-fermentation, but their abundance was less than 1% (**Figure 4**).

Fungal Community Structure

The OTU composition of all Sichuan dark tea samples was analyzed *via* PCA and PCoA to undermine the Euclidean distances and dissimilarity index. The highly abundant fungal genera in all the samples were used to generate PCA and PCoA maps (**Figures 5, 6**). PC1 and PC2 were drawn along the *x*- and *y*-axes, respectively, and their combined total contribution to the PCA score was 98.07% (**Figure 5**) and that to the PCoA score was 95.42% (**Figure 6**). Notably, YC and W2 were closely clustered but distant from the rest of the samples collected at W1, W3, and W4. In contrast, the YC and W4 samples disintegrated along PC1 and PC2. These results showed obvious differences in the fungal communities of Sichuan dark tea at the different stages of pile-fermentation.

The fungal communities in the dark tea samples collected at pile-fermentation stages YC, W1, W2, and W3 were remarkably similar, but quite different from those in sample collected at W4. The community at W4 was separate from the communities in the other samples. These results led us to conclude that the late stage of pile-fermentation (a critical stage of dark tea production; W4) contained prominent factors that affected the population structure in the fungal community of Sichuan dark tea. Note that the total number of samples and the unique results of individual samples have implications for the results of the entire analysis, which could be minimized by increasing the total number of samples.

Functional Classification of the Fungi in Sichuan Dark Tea

Guild/ecological guild refer to the relationship between closely or distantly related species inhabiting similar or different environments in similar ways (Schmidt et al., 2019). FUNGuild (Nguyen et al., 2016) is a sequencing and analysis platform-independent Python-based tool that was employed for functional classification of the fungal OTUs in each sample of Sichuan dark tea.[3] The relative abundance of two ecological guilds, undefined saprotrophs and endophytes/plant pathogens, inhabiting the Sichuan dark tea samples collected at 8-day intervals during piling obtained by FUNGuild was more than 99% (**Figure 7**). The population abundance information for each OTU was also obtained, which is a prerequisite for understanding the sources and pathways of different microbes. These results showed that a considerable portion of the fungal species was assimilated with the harvested raw materials for the piling of Sichuan dark tea. Herein, the detection of a higher number of endophytes/plant pathogens is an indicator of the existence of a wide range of fungal communities present in the raw material for dark tea (**Figure 7**).

[3]http://www.funguild.org

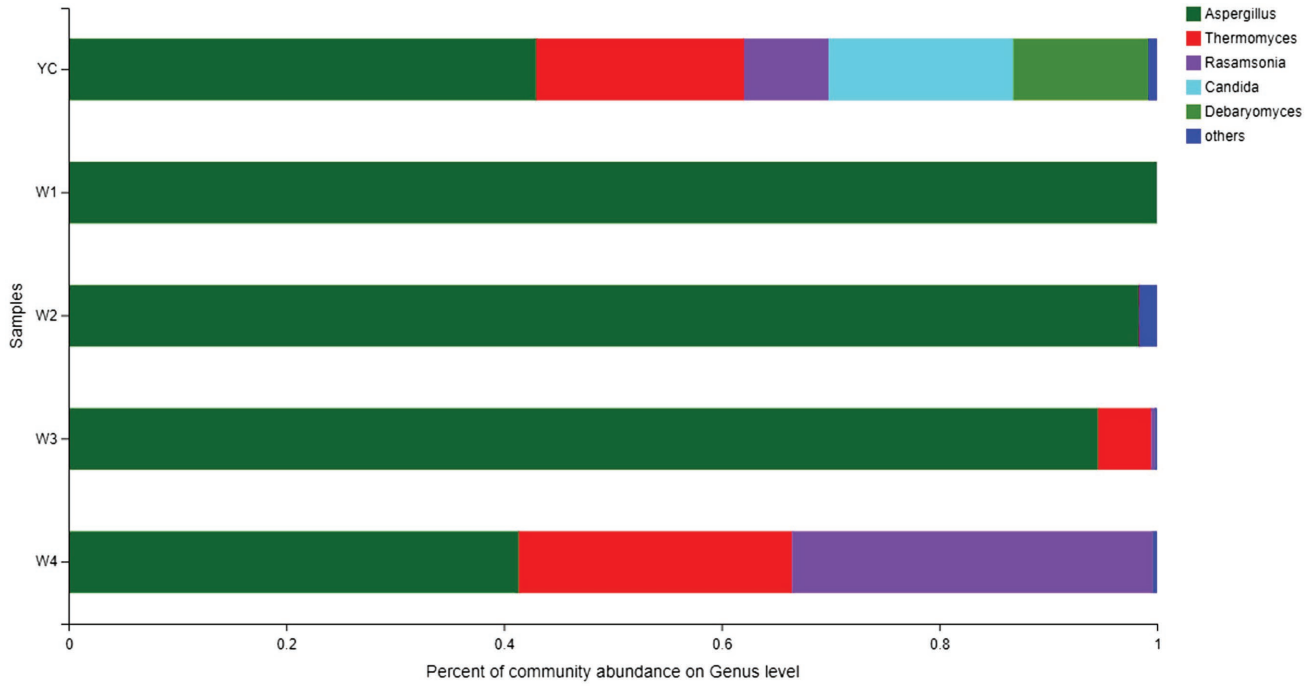

FIGURE 3 | Fungal community structure bar plot at the genus level. Each ordinate is the sample name, and the abscissa is the proportion of each species in a sample. Different species are shown as columns of different colors, and the size and proportion of each species is represented by the length of the column.

DISCUSSION

The length of the piling cycle required to develop the unique flavor of Sichuan dark tea depends predominantly on the composition of the microbial community in the pile. A variety of microorganisms are involved in the pile-fermentation of Sichuan dark tea. In the past, due to technical limitations, determining the composition of the microbial community responsible for the essence/flavor development of dark tea required traditional separation and culture-dependent methods, which are time-consuming, laborious, costly, and ultimately non-robust, because of the failure to cultivate several microbes. Therefore, only fungi which can be cultured were explored, and it was impossible to fully elucidate the unique role of microbes during pile-fermentation (Li et al., 2017b). In this study, we used a robust high-throughput sequencing method and obtained 334,593 high-quality fungal sequences, with an average length of 269.12 bp, with an aim to determine the composition of the fungal community during pile-fermentation of dark tea (**Table 1**). All the sequences were classified into OTUs at 97% identity threshold, and 270 fungal OTUs were obtained by clustering valid data, which were divided into seven phyla, 22 classes, 41 orders, 85 families, 128 genera, and 184 species.

Based on diversity indices, the abundance of the fungal community changed during pile-fermentation, which first decreased and then increased. The highest fungal diversity was observed at the end of pile-fermentation, which was higher than that in the raw materials, indicating that the highest number of fungal species was present at the end of fermentation. A possible reason for this could be a higher rate of bacterial growth over fungal growth at the initial stage of pile-fermentation due to temperature conditions that were more suitable for bacteria. Bacterial growth appeared to be reduced at the middle and final stages due to the increase in temperature. Investigations on the possible role of bacteria during pile-fermentation are underway. Notably, at the final stages of pile-fermentation, a series of catabolic reactions, such as anaerobic bacterial respiration and decay of the vegetative parts of tea plants, provided a suitable temperature and essential nutrients to support the growth and reproduction of fungi. A gradual increase in piling time resulted in an increase in the diversity index of the fungal populations, indicating that piling time and fungal diversity are directly proportional; a similar result was obtained in a previous study (Li et al., 2018a).

The similarity of the fungal community structure was lower in the YC samples and then gradually increased in samples collected at stages W1-W3, which is consistent with previous findings (Zhang et al., 2016a), regarding the structural variations in the microbial community during the fermentation of Puer tea (Chen et al., 2013). The abundance of the fungal community increased exponentially in all W4 samples when compared to that of the W1, W2, and W3 samples, indicating that the W4 step of pile-fermentation is a key time point for flavor and quality development. The fungal population was relatively rich at the YC time point, indicating that the raw materials used in the pile-fermentation of dark tea is a secondary factor affecting the variation in fungal community structure (Zhao et al., 2017).

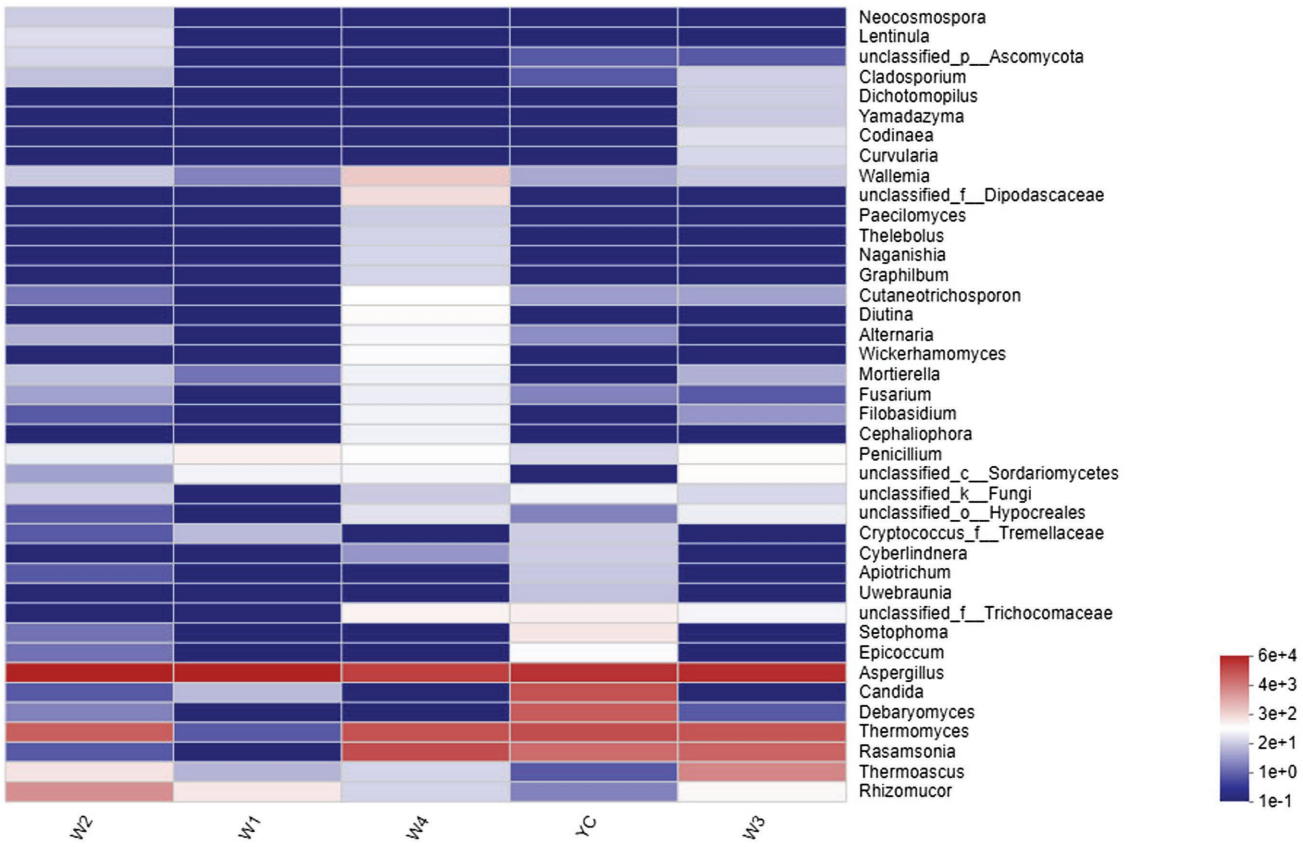

FIGURE 4 | Community heatmap at the genus level. The abscissa represents the group name, and the ordinate represents the species name. The changes in the abundance of different species in each sample are displayed as a color gradient in a color block. The bar on the right side of the figure shows the abundance as a color gradient.

During pile-fermentation of dark tea, the growth and reproduction of microbes are interdependent, and a balanced and stable mechanism will eventually develop to generate the conditions necessary for post-fermentation processing. This study revealed that the most abundant fungal genera involved in the pile-fermentation of Sichuan dark tea are *Aspergillus*, *Thermomyces*, *Candida*, *Debaryomyces*, *Rasamsonia*, *Rhizomucor*, and *Thermoascus* (**Figures 3, 4**). Given their abundance, the abovementioned abundant fungal species likely play pivotal roles in development of the unique aroma and active ingredients of dark tea. Numerous *Aspergillus* species, such as *A. cristatum* (*Eurotium cristatum*) and *A. carbonarius*, are economically important and are widely employed in food biotechnology to enhance the nutritional value of food products, such as tea and coffee, due to the various metabolites they produce during fermentation (Samson et al., 2014; De Souza et al., 2021). Many *Aspergillus* species produce and secrete various enzymes, such as α-amylase, glucoamylase, cellulase, pectinase, xylanase, hemicellulase, and protease, which are applied on an industrial scale to improve the taste of food items by breaking down proteins or lipids and developing unique flavors (Ward et al., 2005). Additionally, *A. cristatum* is widely used in making crimson soup from dark tea, and lovastatin, a chemical secreted by this genus, is a statin that lowers cholesterol levels (Shi et al., 2005; Zhao et al., 2013). Further studies are needed to explore the probiotic properties of individual fungal species and their role in nutrition enhancement.

Some species of *Candida*, such as *Candida etchellsii*, *C. milleri*, *C. rugosa*, and *C. tropicalis*, can grow on liquor waste and are used in the food and feed industries to improve nutritional value and taste and as cell factories for the production of single-cell proteins (Bourdichon et al., 2012). *Debaryomyces hansenii* (anamorph *C. famata*) is also employed in the food industry for surface ripening of cheese and meat products, the production of riboflavin (vitamin B2), bioconversion of xylose into xylitol sweetener, and the biosynthesis of arabinitol and pyruvic acid (Breuer, 2010). During dark tea pile-fermentation, some fungi, such as *Rhizomucor*, can secrete antibacterial substances that inhibit the growth of bacteria, which makes the tea safer to drink. *Rhizomucor* is also involved in the degradation of bio-waste and carbon uptake for the biosynthesis of various useful enzymes, such as 1,4-β-xylosidase, endo 1,4-β-glucanase, phosphatase, chymosin, protease, and alcohol dehydrogenase, which are important for the flavor development of dark tea (Zhang et al., 2013). *Rasamsonia* produces cellulase, hemicellulase, pectinase, and starch-degrading enzymes, and

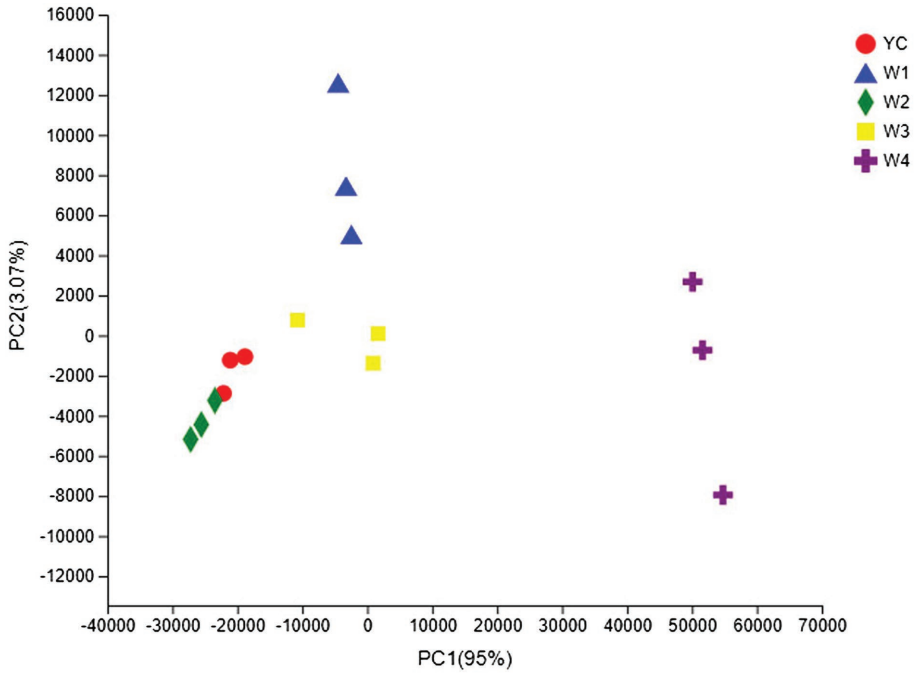

FIGURE 5 | Multiple sample principal component analysis (PCA) of the OTUs. Both selected principal component axes are represented by the *x*- and *y*-axes, and the percentage represents the difference in sample composition as determined by the principal component; the scales of the *x*- and *y*-axes represent the relative distances. Samples are shown as different color points or shapes in different groups. The closeness of two points or shapes represents the similarity between the species composition of two samples.

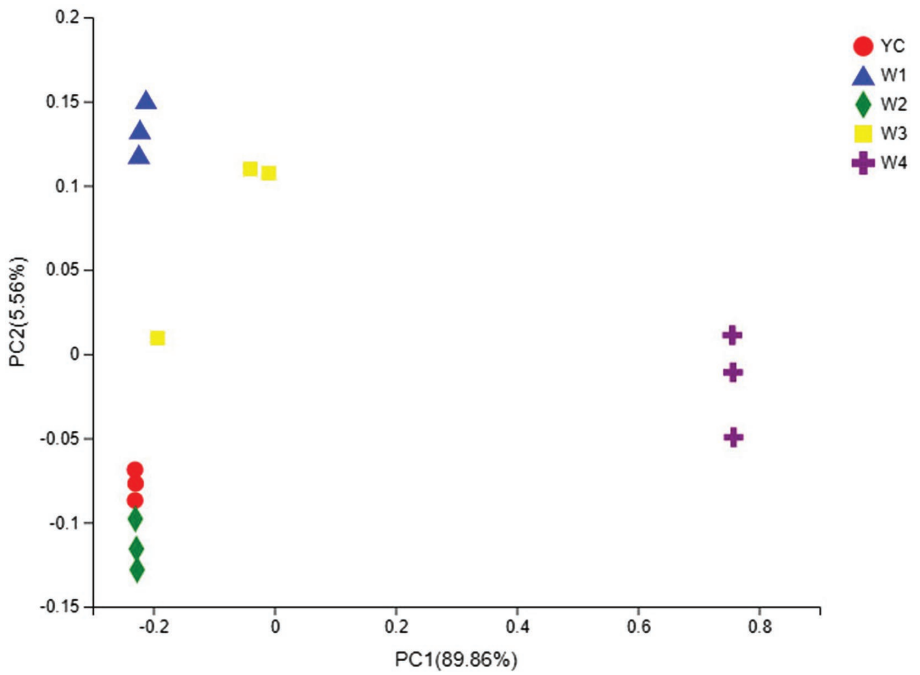

FIGURE 6 | Multiple sample principal coordinate analysis (PCoA) of OTUs. Both selected principal component axes are represented by the *x*- and *y*-axes, and the percentage represents the difference in sample composition as determined by the principal component; the scales of the *x*- and *y*-axes are the relative distances. Samples are shown as different color points or shapes in different groups. The closeness of two points or shapes represents the similarity between the species composition of two samples.

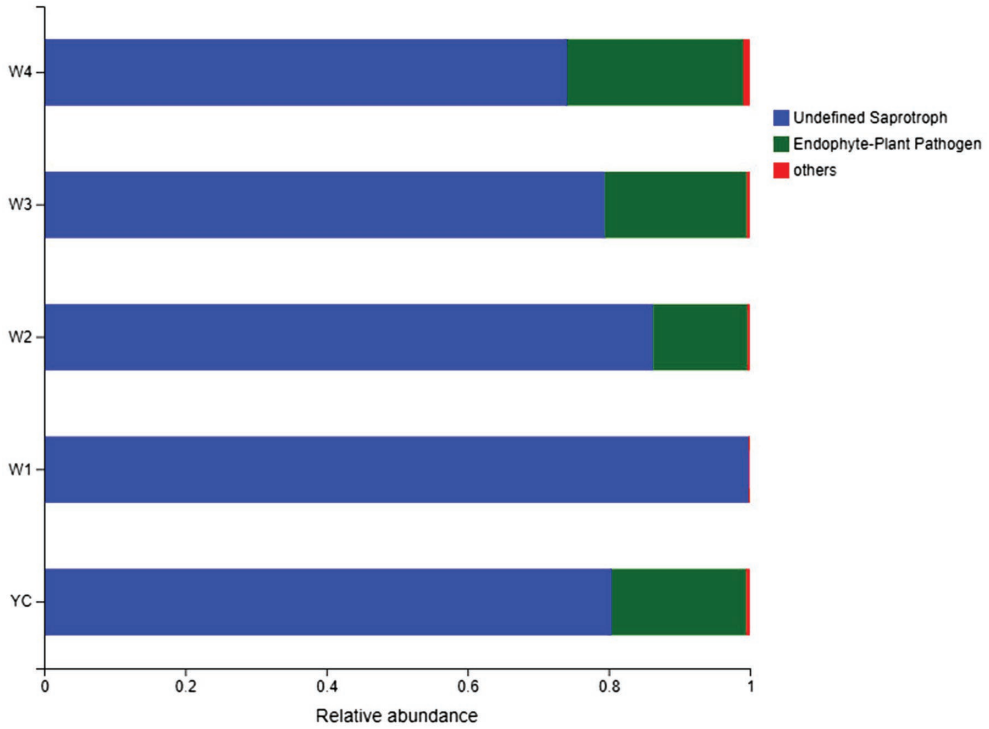

FIGURE 7 | FUNGuild analysis of fungal functional groups. The relative abundance of the Guild in different groups or samples is on the *x*-axis, and groups or samples are on the *y*-axis. According to the variation in functional groups, FUNGuild can calculate the abundance of each fungal species and their functional classification in each sample.

catalyzes various reactions, such as oxidation, oxido-reduction, and proteolysis, which are important for dark tea fermentation (Waters et al., 2010). The *unclassified_Trichocomaceae* and *unclassified_Ascomycota* reads identified in this study are probably from *A. cristatum*, commonly known as "golden flower," which has a major impact on the pile-fermentation process of dark tea (Ge et al., 2016; Li et al., 2017a). Several indole alkaloids and indole diketopiperazine alkaloids can be extracted from a culture of *A. cristatum*, which possess brine shrimp killing activity, antibacterial activity against *E. coli*, radical scavenging activity against DPPH radicals, and marginal attenuation of 3T3L1 pre-adipocytes (Du et al., 2017). Therefore, an artificial inoculation of dark tea with these fungi could improve its health benefits and could be used to produce probiotic dark tea.

It is of immense interest to determine the functional classification, abundance, source, and metabolic pathways of the microbes involved in the flavor development of dark tea. The source of the external fungi was the spores present on the raw material, and their relative abundance was high in each pile-fermentation. The high number of endophytes shows the broad range and diversity of the fungi in the raw materials of dark tea (Crous et al., 2006). Our findings provide deep insights in fungal community structure, which can be applied in piling stage-specific fungal inoculation that may improve the nutritional quality and aesthetic value of dark tea (Bressani et al., 2021) to overcome the challenge of malnutrition, which is a key point for one of the "Sustainable Development Goals (SDGs)" of UNO, to "Establish Good Health and Well-Being."

CONCLUSION

The Sichuan dark tea is being processed *via* pile-fermentation to improve its nutritional value and aroma. It is believed that microorganisms, such as fungi and bacteria, present during each piling cycle play crucial role in the development of nutritional value and aroma during fermentation, but little is known about their composition. A deep insight in composition of these microbes will definitely pave way to artificially and pile specific inoculation of symbionts to improve the nutritional and aesthetic values of dark tea. Robust next generation sequencing analysis revealed that following fungal genera; *Aspergillus*, *Thermomyces*, *Candida*, *Debaryomyces*, *Rasamsonia*, *Rhizomucor*, and *Thermoascus* were highly abundant during piling process, of which *Aspergillus* was the most abundant. Notably, the highest number of fungal species were observed before piling stage (YC), which were gradually decreased in subsequent piling stages (W1 and W2), and then gradually increased again at piling stages W3 and W4. Among all, the highly abundant fungal genus was *Aspergillus* present at W4 piling stage, which is of medical and commercial importance. In conclusion, W3 and W4 are suitable stages for inoculation of symbiotic fungal species to develop essence and nutritional value of Sichuan dark tea.

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: European Nucleotide Archive (ENA), Accession number PRJEB46392. Our study unique name is ena-STUDY-Manzar Abbas-16-07-2021-16:22:51:147-260.

AUTHOR CONTRIBUTIONS

KY and MA designed the experiments. KY, MA, LM, ZP, QL, XZ, and HC performed the experiments. KY, MA, AE-S, LY, and XZ analyzed the data. MA and KY wrote the manuscript. 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.2021.706714/full#supplementary-material

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Variations of Soybean Meal and Corn Mixed Substrates in Physicochemical Characteristics and Microbiota During Two-Stage Solid-State Fermentation

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Corn germ meal (CGM) and corn gluten feed (CGF) are the two main corn byproducts (CBs) obtained from corn starch extraction. Due to their high fiber content, low protein content, and severe imbalance of amino acid, CBs are unable to be fully utilized by animals. In this study, the effect of microorganism, proteases, temperature, solid–liquid ratio, and time on nutritional properties of CB mixture feed (CMF) was investigated with the single-factor method and the response surface method to improve the nutritional quality and utilization of CBs. Fermentation with *Pichia kudriavzevii*, *Lactobacillus plantarum*, and neutral protease notably improved the nutritional properties of CMF under the fermentation conditions of 37°C, solid–liquid ratio (1.2:1 g/ml), and 72 h. After two-stage solid-stage fermentation, the crude protein (CP) and trichloroacetic acid-soluble protein (TCA-SP) in fermented CMF (FCMF) were increased ($p < 0.05$) by 14.28% and 25.53%, respectively. The *in vitro* digestibility of CP and total amino acids of FCMF were significantly improved to 78.53% and 74.94%, respectively. In addition, fermentation degraded fiber and provided more organic acids in the CMF. Multiple physicochemical analyses combined with high-throughput sequencing were performed to reveal the dynamic changes that occur during a two-stage solid-state fermentation process. Generally, *Ascomycota* became the predominant members of the community of the first-stage of fermentation, and after 36 h of anaerobic fermentation, *Paenibacillus* spp., *Pantoea* spp., and *Lactobacillales* were predominant. All of these processes increased the bacterial abundance and lactic acid content ($p < 0.00$). Our results suggest that two-stage solid-state fermentation with *Pichia kudriavzevii*, *Lactobacillus plantarum*, and protease can efficiently improve protein quality and nutrient utilization of CMF.

Keywords: corn byproducts, two-stage solid-state fermentation, nutritional value, microbiota, *in vitro* digestibility

Abbreviations: SSF, solid-state fermentation; CGM, corn germ meal; CGF, corn gluten feed; CBs, corn byproducts; CMF, CBs mixture feed; FCMF, two-stage solid-state fermented CBs mixture feed; DM, dry matter; EE, ether extract; AA, amino acid; CP, crude protein; TCA-SP, trichloroacetic acid soluble protein; CF, crude fiber; ADF, acid detergent fiber; NDF, neutral detergent fiber; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

INTRODUCTION

The competition for food between humans and livestock has become a topic with great concern in the past few years. The shortage of feed resources led to a sharp rise in the prices of conventional feed ingredients, such as corn, soybean meal, fish meal, etc. In order to reduce feed costs, less-expensive, alternative agricultural and industrial byproducts are increasingly included in livestock diets (Li et al., 2018). Corn is one of the most important cash crops in the world. Global corn production exceeded one billion tons per year, and the corn-processing industry produced large quantities of byproducts (Stein et al., 2016).

Corn germ meal (CGM) and corn gluten feed (CGF) are two main by-products obtained from corn starch extraction by wet milling (**Figure 2B**). CGM, the remaining portion of corn germ after oil removal, contains 30% protein, 18% starch, 12% cellulose, 2% ash, and 0.7% fat (Nielsen et al., 1979). CGF is produced by combining concentrated steepwater with the fiber during wet milling separation process, which typically contains 60% fiber and 20% protein. The low solubility and unbalanced AA composition of protein limit the application of CBs in the feed industry (Anderson et al., 2012). Solid-state fermentation in feedstock processing is an efficient biotechnological approach to improve nutritional value and increase nutrient bioavailability (Decimo et al., 2017; Wang et al., 2018a). *Bacillus subtilis*, *Pichia kudriavzevii*, and *Lactobacillus plantarum* were widely used SSF strains (Ni et al., 2017). *Bacillus subtilis* can degrade large molecular proteins to produce small peptides in SSF due to their capacity to secrete extracellular protease (Soares et al., 2005). Yeast (such as *Pichia kudriavzevii* and *Saccharomyces cerevisiae*) is most commonly used for agricultural byproduct SSF to obtain single-cell proteins (S) due to their low nutritional requirements, rapid growth, and high protein content (Hezarjaribi et al., 2016; Deng et al., 2020). The organic acids produced by *Lactobacillus plantarum*, mainly lactic acid, can reduce the pH of the fermentation system and inhibit the proliferation of some pathogenic bacteria and fungi (Missotten et al., 2015). In recent years, there have been many reports on the use of single bacteria to SSF agricultural byproducts, and a few study on the use of the abovementioned bacteria pairwise-combination fermentation to compare the fermentation effect. Our previous research shows that solid fermented soybean meal–corn mixture feed and distilled dried grain with soluble *Bacillus subtilis* and *Lactobacillus plantarum* can effectively improve their nutritional value and increase the content of small peptides and lactic acid (Shi et al., 2017; Wang et al., 2018b; Zhang et al., 2018; Zong et al., 2020). In addition, protease is widely used in SSF to degrade large molecular proteins to produce small peptides (Bedrosian and Kung, 2019). We hypothesized that using protease instead of extracellular protease-producing *Bacillus subtilis* to ferment with *Lactobacillus plantarum* and *Pichia kudriavzevii* would have a better fermentation effect.

In this study, a solid-state fermented feed system containing CBs, corn, soybean meal, and wheat bran was performed to gain a sufficient usage of CBs. To degrade fibers and produce digestible protein, *Bacillus subtilis*, *Lactobacillus plantarum*, and *Pichia kudriavzevii* were used for pairwise fermentation of CMF and reinforced by adding protease. Fermentation time, liquid ratio, and temperature would be traced to find better fermentation conditions and form a fermentation strategy.

MATERIALS AND METHODS

Microorganisms and Enzymes

Bacillus subtilis ZJU12-1 (CGMCC No: 12825) and *Lactobacillus plantarum* CWLP (CGMCC No: 1.510) were obtained from Chinese traditional pickled vegetables. *Pichia kudriavzevii* PKWF was obtained from grains of a Chinese local distiller. *Bacillus subtilis* ZJU12-1, *Lactobacillus plantarum* CWLP, and *Pichia kudriavzevii* PKWF were maintained on Luria broth (LB), de Man, Rogosa, and Sharp (MRS), and yeast extract peptone dextrose (YPD) plates preserved at 4°C. Neutral protease was from *Bacillus* spp. (P3111; Sigma-Aldrich Corp.). CMF contains 20% CGM, 30% CGF, 30% corn, 15% soybean meal, and 5% wheat bran, which were obtained from the Cofine Bio-tech Co., Ltd. (Jiaxing, China).

Preparation of Fermented Mixed Substrates

The fermentation substrates were set as follows to obtain the optimal fermentation combination: *B. subtilis* ZJU12-1 + *P. kudriavzevii* PKWF, *P. kudriavzevii* PKWF + *L. plantarum* CWLP, *B. subtilis* ZJU12-1 + *L. plantarum* CWLP, *P. kudriavzevii* PKWF + neutral protease, *L. plantarum* CWLP + neutral protease, and *L. plantarum* CWLP + *P. kudriavzevii* PKWF + neutral protease. Before fermentation, *B. subtilis* ZJU12-1 was cultured in LB liquid medium at 37°C for 12 h. *L. plantarum* CWLP was cultured in MRS liquid medium at 37°C for 18 h. *P. kudriavzevii* PKWF was cultured in YPD liquid medium at 37°C for 12 h. The 100 g CMF was mixed and placed in a 500-ml Erlenmeyer flask, and sterile water was added to achieve a solid–liquid ratio (1.5:1 g/ml). The wet mixed CMF was inoculated with microbes (7.0 log cfu/g) or protease (50 U/g) according to the fermentation combinations listed above. Then the flask was covered with a membrane (aerobic condition) and fermented at 37°C. The control group of CMF was treated without different bacteria and neutral protease, and other fermentation conditions were consistent. Moist samples (approximately 100 g) at 0, 36, and 72 h were collected to determine the numbers of microorganisms and microbial metabolites, and for 16S rRNA and ITS gene high-throughput sequencing, and the remaining samples were treated at 105°C for 30 min to prevent continuous fermentation. Then, the samples were dried at 65°C for 24 h, cooled, and ground, and subjected to physicochemical analysis, SDS-PAGE.

Optimization of Fermentation Conditions

Based on the restriction of the fermentation conditions of SSF in actual scale production in China, the solid–liquid ratios (1.8:1, 1.5:1, and 1.2:1 g/ml), fermentation

temperatures (27°C, 32°C, and 37°C), and fermentation times (24, 48, and 72 h) were investigated with response surface analysis to optimize the parameters of the optimal fermentation, and the Design Expert software (Version 8.0.6, Stat-Ease Inc., Minneapolis, MN, United States) was used for the regression and graphical analysis of the experimental data obtained.

$M = a0 + a1 \times A + a2 \times B + a3 \times C + a4 \times A^2 + a5 \times B^2 + a6 \times C^2 + a7 \times A \times B + a8 \times A \times C + a9 \times B \times C$. M is the predicted response; $a0$ is the intercept term; $a1$, $a2$, and $a3$ are the linear coefficients; $a4$, $a5$, and $a6$ are the squared coefficients; and $a7$, $a8$, and $a9$ are the interaction coefficients.

To further study the effect of anaerobic condition on CMF fermentation quality, we used the two-stage SSF method. Briefly, the two-stage SSF process includes two stages; at the first stage, CMF was used as the main fermented substrate, which was inoculated with an effective combination of *Pichia kudriavzevii* and neutral protease at aerobic fermentation for 36 h; then at the second stage, *Lactobacillus plantarum* was inoculated into the first stage-fermented substrate at anaerobic fermentation for 36 h.

Chemical Analyses

The dried samples of CMF were ground, sieved through a 1-mm sieve, and then the DM, CP, AA, EE, CF, NDF, and ADF were analyzed as described by AOAC (2005). Determination of TCA-SP in samples was performed using the method proposed by Ovissipour et al. (2009).

Microorganisms and Microbial Metabolites

The pH and microbial counts were analyzed by the method of Wang et al. (2018b) with minor modifications. In brief, 5 g of wet samples was dissolved in 45 ml of sterile water and placed on a shaker at 150 rpm for 20 min. The pH of the supernatant was measured with a pH meter (Mettler Toledo, Switzerland). The samples were diluted 10-fold with sterile water for microbial counts. The viable count of *B. subtilis* was counted after culturing on LB agar for 24 h at 37°C. The viable count of *L. plantarum* was counted after culturing on MRS agar for 48 h at 37°C. The viable count of *P. kudriavzevii* was counted after culturing on YPD agar for 48 h at 37°C.

The concentration of organic acids (acetic acid, propionic acid, butyric acid, and lactic acid) in each sample was separated and quantified using a gas chromatograph (GC; GC−14B, Shimadzu, Japan; capillary column 30 m × 0.32 mm × 0.25 μm film thickness/VARIAN CP−3800, Varian, Palo Alto, CA, United States) as described by Franklin et al. (2002). In brief, the samples (1 g) were thawed and suspended in 2 ml of distilled water in a screw−capped tube. After being vortexed, each sample was centrifuged (12,000 × *g*) at 4°C for 10 min. The supernatant (1 ml) was transferred into a 2-ml centrifuge tube and mixed with 0.2 ml of metaphosphoric acid and kept at 4°C for 30 min. The mixtures were then centrifuged (12,000 × *g*) again at 4°C for 10 min. Aliquots of the supernatant (1 μl) were analyzed by GC.

In vitro Digestibility

In vitro two-stage enzymatic hydrolysis process was performed by the method of Sakamoto et al. (2008) with minor modifications. In short, CMF or FCMF (2 g) was added to a 150-ml Erlenmeyer flask, containing 50 ml, 10,000 U/ml of pepsin (activity: 3,000 U/mg, Sigma) solution (0.05 mol/L KCl-HCl buffer, pH 2.0), and incubated on a shaker at 37°C, 100 rpm for 5 h. The pH of the mixture was then adjusted to 6.8 with 1 mol/L NaOH and 1 mol/L HCl, and 150 mg of trypsin (activity: 250 U/mg, Sigma) was added to the mixture and incubated on a shaker at 37°C, 100 rpm for 5 h. After digestion, 5 ml of 20% sulfosalicylic acid was added to the mixture and settled for 30 min. The digested slurry samples were centrifuged at 3,000 × *g* for 15 min. Then the precipitate, washed with doubled-distilled water for several times and collected, dried at 105°C, was used to analyze the content of CP and AA. *In vitro* CP (AA) digestibility (%) = [original CP (AA) amount − residual CP (AA) amount]/original CP (AA) amount × 100%.

Microscopic Inspection

Changes in the physical properties of the substrates before and after fermentation were examined by SEM according to the protocol of the Electronic Microscopy Center of Zhejiang University. The microstructures of CMF and FCMF were observed using a field-emission scanning electron microscope (KYKY-EM3200, China) at ×100, ×1,000, and ×3,000 magnifications.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

The proteins in CMF and FCMF were extracted using the procedure described by Faurobert (1997). The gel running conditions were chosen according to the report of Meinlschmidt et al. (2016). In brief, 12% polyacrylamide-separating gels were used for electrophoresis. Approximately 5 g of the protein sample was placed in each well, and the sample was separated at 55 mV for 200 min. The gel was stained with Coomassie Brilliant Blue (CBB) R-250 (Bio-Rad, United States) for 60 min and destained with 7% acetic acid.

DNA Extraction and Illumina MiSeq Sequencing, and Metabolic Function Prediction

Total microbial genomic DNA, including bacterial and fungal genomic DNA, was extracted from the 18 samples using the E.Z.N.A soil DNA kit (Omega Bio-Tek, Norcross, GA, United States). A NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, DE, United States) and 1% agarose gel electrophoresis were used to analyze DNA content and quality.

The V3–V4 gene regions of the bacterial 16S rRNA gene were amplified with primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3=) and 806R (5′-GGACTACHVGGGTWTCTAAT-3=). The primer sequences for the ITS2 region of the fungal ITS gene were ITS1F

(5′-CTTGGTCATTTAGAGGAAGTAA) and ITS2R (5′-GCTGCGTTCTTCATCGATGC). PCR was conducted as follows: 3 min of denaturation at 95°C; 27 cycles of 30 s at 95°C, 30 s of annealing at 55°C, and 45 s of elongation at 72°C; and a final extension at 72°C for 10 min. The AxyPrep DNA gel extraction kit (Axygen Biosciences, Union City, CA, United States) and QuantiFluor-ST instrument (Promega, United States) were used to further extract, purify, and quantify the PCR products. The MiSeq platform (Shanghai Majorbio Biopharm Technology Co., Ltd.) was used to describe the bacterial community based on the gene segment from the V3–V4 and ITS2 portion of the rRNA gene. Subsequently, raw illumina FASTQ files were demultiplexed, quality filtered, and analyzed using Quantitative Insights into Microbial Ecology (QIIME v1.9.1). Raw fastq files were quality filtered by Trichromatic and merged by FLASH. Operational taxonomic unit (OTUs) were clustered with 97% similarity cutoff using UPARSE (version 7.1). The taxonomy of each 16S rRNA gene sequence was analyzed using the RDP Classifier algorithm[1] against the Greengenes 16S rRNA database using a confidence threshold of 70%. The assembled MiSeq sequences were submitted to the NCBI's Sequence Read Archive (SRA BioProject no. PRJNA730509) for open access. Estimates of diversity values for these samples using the Chao1, Shannon, and Simpson indexes for diversity estimation were calculated by rarefaction analysis. Good's coverage analysis was also performed. PCA and cluster analysis with the Ward method were conducted using the web server tool METAGENassist based on unweighted UniFrac distances. The main differentially abundant genera were selected by the LEfSe method[2]. To predict metabolic genes during the process, PICRUSt (see text footnote 2) was applied to obtain a functional profile from the 16S rRNA data. Prior to metagenome prediction, the OTUs of 16S rRNA sequences were analyzed using PICRUSt. PICRUSt and KEGG were used to obtain functions for the genes that were predicted to be present in the samples and to assign the genes into metabolic pathways. Fungal communities were analyzed and classified by the FUNGuild online tool[3].

Statistical and Bioinformatics Analysis

All assay data were analyzed using SPSS 20.0 software (SAS Inc., Chicago, IL, United States). One-way ANOVA and Duncan test were used to determine the difference between the mean values, and data are expressed as mean value ± standard deviation (SD). The differences between the means of the treatments were considered significant at $p < 0.05$. The heatmap package of R (R Core Team, 2014) was applied to generate heat maps of genera and L3-predicted microbial gene functions. Bar plots were generated in GraphPad Prism 8 (San Diego, CA, United States). Multiple testing corrections of distinguished species and predicted metabolic functions during fermentation were employed using Welch's test and the Benjamini–Hochberg false-discovery rate (FDR) method for statistical analysis of metagenomic profiles (STAMP version 2.1.3).

[1]http://rdp.cme.msu.edu/

[2]https://huttenhower.sph.harvard.edu/galaxy/

[3]http://www.funguild.org/

RESULTS

Selection of the Strain and Enzyme

Based on the results of the single-factor experiment (**Table 1**), CMF fermented with *P. kudriavzevii* PKWF, *L. plantarum* CWLP, and neutral protease (CMFPLN) has the highest content of CP, TCA-SP, and EE compared with the other fermentation combinations, which were 9.5%, 22.1%, and 38.1% higher than that of CMF, respectively. Furthermore, CF of CMFPLN is significantly lower than that of CMF ($p < 0.05$). These results indicate that the process of fermentation with *P. kudriavzevii* PKWF, *L. plantarum* CWLP, and neutral protease significantly improved the nutritional composition of CMF. In addition, CMFPLN has a more viable count of *P. kudriavzevii* PKWF and *L. plantarum* CWLP. The results above all suggest that CMFPLN may have the best fermentation potential.

Optimal Fermentation Conditions of Corn Byproduct Mixture Feed Fermented With *Pichia kudriavzevii* PKWF, *Lactobacillus plantarum* CWLP, and Neutral Protease

Box–Behnken design (BBD) was employed to optimize three variables: fermentation temperature (A), fermentation time (B), and solid–liquid ratio (C) of CMFPLN. Based on the response surface results (**Figure 1**) and multiple regression analysis of the experimental data, the following second-order polynomial equation between the viable count of *P. kudriavzevii* PKWF, *L. plantarum* CWLP, and the three variables during fermentation were found: the viable count of *P. kudriavzevii* PKWF $= 6.56 + 0.27 \times A + 0.38 \times B + 2.20 \times C + 0.64 \times A \times B + 0.025 \times A \times C - 0.015 \times B \times C - 3.08 \times A^2 - 2.72 \times B^2 - 1.31 \times C^2$, the viable count of *L. plantarum* CWLP $= 28.80 - 1.3 \times A + 8.89 \times B + 27.36 \times C + 3.38 \times A \times B + 13.83 \times A \times C + 7.10 \times B \times C - 6.55 \times A^2 - 0.13 \times B^2 + 15.42 \times C^2$. Similarly, second-order polynomial equation between the content of CP, TCA-SP, and the three variables during fermentation were found: the content of CP $= 23.41 + 0.47 \times A + 0.34 \times B + 0.65 \times C - 0.046 \times A \times B + 0.52 \times A \times C + 0.66 \times B \times C - 0.60 \times A^2 + 0.20 \times B^2 - 0.22 \times C^2$, the content of TCA-SP $= 40.63 - 1.45 \times A - 1.35 \times B - 1.46 \times C - 0.59 \times A \times B - 2.76 \times A \times C - 1.14 \times B \times C + 1.73 \times A^2 + 2.06 \times B^2 - 0.91 \times C^2$.

By solving the regression equations above, the optimal condition of the three variables to obtain the maximum point of the model were calculated to be fermentation temperature at 32°C, fermentation time of 50 h, and a solid–liquid ratio of 1.2:1 g/ml, with the corresponding viable count of *P. kudriavzevii* PKWF at 7.50×10^7 CFU/g. Similarly, under the optimal conditions of fermentation temperature at 37°C, fermentation time of 72 h, and a solid–liquid ratio of 1.2:1 g/ml, the viable count of *L. plantarum* CWLP reached the maximum value of 96.75×10^7 CFU/g. The content of CP reached the maximum value of 25.86%, which was the same as the optimal conditions for the maximum viable count of *L. plantarum* CWLP. In addition,

TABLE 1 | Nutrient composition and microorganisms of corn byproducts mixture feed (CMF) and CMF fermented with different microbes and neutral protease (as air-dry basis).

Items	CMF	CMFBP	CMFPL	CMFBL	CMFPN	CMFLN	CMFPLN
DM, %	90.33 ± 0.40	91.46 ± 0.48	91.08 ± 0.42	90.84 ± 0.56	90.80 ± 0.36	91.96 ± 0.22	91.04 ± 0.17
EE, %	2.23 ± 0.04e	2.51 ± 0.08d	2.59 ± 0.03cd	2.72 ± 0.06c	2.88 ± 0.08b	2.36 ± 0.08e	3.08 ± 0.14a
CP, %	22.27 ± 0.35d	23.60 ± 0.40b	23.42 ± 0.48bc	22.85 ± 0.25c	23.08 ± 0.14bc	23.29 ± 0.28bc	24.39 ± 0.24a
TCA-SP, %	37.45 ± 0.47e	44.22 ± 0.31bc	42.78 ± 0.27d	43.65 ± 0.39c	44.00 ± 0.29c	44.72 ± 0.24b	45.74 ± 0.13a
CF, %	6.11 ± 0.32a	5.33 ± 0.39bc	5.51 ± 0.42b	5.18 ± 0.14bc	5.37 ± 0.32*bc*	5.12 ± 0.10bc	4.91 ± 0.16c
pH	6.40 ± 0.03a	6.22 ± 0.10a	4.67 ± 0.09b	4.69 ± 0.17b	6.21 ± 0.12a	4.55 ± 0.07b	4.52 ± 0.18b
Microorganism, $\times 10^7$ CFU/g							
Bacillus subtilis ZJU12-1	–	82.00 ± 5.13	–	5.33 ± 8.19	–	–	–
Pichia kudriavzevii PKWF	–	1.80 ± 0.61	2.10 ± 0.44	–	1.71 ± 0.61	–	2.73 ± 0.65
Lactobacillus plantarum CWLP	–	–	16.67 ± 4.51	14.67 ± 4.51	–	16.00 ± 0.44	23.00 ± 6.25

CMFBP, CMF fermented with B. subtilis ZJU12-1 and P. kudriavzevii PKWF; CMFPL, CMF fermented with P. kudriavzevii PKWF and L. plantarum CWLP; CMFBL, CMF fermented with B. subtilis ZJU12-1 and L. plantarum CWLP; CMFPN, CMF fermented with P. kudriavzevii PKWF and neutral protease; CMFLN, CMF fermented with L. plantarum CWLP and neutral protease; CMFPLN, CMF fermented with P. kudriavzevii PKWF, L. plantarum CWLP, and neutral protease.
Values are mean ± SD, n = 3. Means followed with different superscript letters (a, b) within each line are significantly different ($p < 0.05$).

under the optimal conditions of fermentation temperature at 37°C, fermentation time of 24 h, and solid–liquid ratio of 1.8:1 g/ml, the content of TCA-SP reached the maximum value of 46.93%. In order to verify the optimization results, a verification experiment was conducted to show that the viable count of *P. kudriavzevii* PKWF, *L. plantarum* CWLP, the content of CP, and TCA-SP in their optimum conditions were 7.82×10^7 CFU/g, 90.21×10^7 CFU/g, 25.52%, and 46.21%, respectively, which indicated that the model was satisfactory and practicable.

Two-Stage Solid-State Fermentation

Two-stage solid-stage fermentation was conducted and the process is presented in **Figure 2A**. The nutrient contents of CMF and CMF by two-stage solid-stage fermentation (FCMF) are presented in **Table 2**. Compared with CMF, the fermented CMF contained more CP, TCA-SP, and EE, which were augmented ($p < 0.05$) by approximately 14.28%, 25.33%, and 42.119%, respectively. Furthermore, the content of CF, ADF, and NDF were decreased ($p < 0.05$) by 29.10%, 10.43%, and 18.15%, respectively. In this study, the content of amylose and total starch were decreased dramatically after fermentation ($p < 0.05$). In addition, fermentation with inoculated microorganisms and neutral protease also affected the AA composition in CMF. In the present research, three indispensable AA (His, Ile, and Phe), two dispensable AA (Ser and Ala), and total AA significantly increased in FCMF compared with CMF. In addition, except for Asp, most AA showed an increase trend after fermentation. These results of the two-stage SSF were similar to the response surface analysis, which indicated that the oxygen was exhausted at the aerobic fermentation process and formed an anaerobic condition in the mixed substrates. This process is familiar with the two-stage SSF.

Microorganisms and Microbial Metabolites of Fermented Corn Byproduct Mixture Feed

To further evaluate the nutritional properties of FCMF, we determined the microorganisms and microbial metabolites after fermentation, and the results are presented in **Table 3**. After fermentation, the viable counts of *P. kudriavzevii* PKWF and *L. plantarum* CWLP in FCMF reached 6.95×10^7 and 90.21×10^7 CFU/g, respectively. The pH of CMF decreased from 6.42 to 4.54, which was mainly caused by organic acids produced by *L. plantarum* CWLP. The acetic acid, propionic acid, butyric acid, and lactic acid in FCMF were increased by 6.72, 20.83, 21.00, and 5.64 times, respectively.

In vitro Digestibility of Fermented Corn Byproduct Mixture Feed

The results of the digestibility of CMF and FCMF are presented in **Table 4**. The *in vitro* digestibility of DM and CP in FCMF were notably improved by 18.98% and 16.62%. In addition, the *in vitro* digestibility of 14 AA, including 9 essential AAs (Arg, His, Ile, Leu, Met, Lys, Thr, Phe, and Val) and 5 dispensable AA (Ser, Glu, Ala, Tyr, and Cys) were significantly enhanced. Furthermore, after 72 h of fermentation, the digestibility of the average indispensable AA, average dispensable AA, and total AA were enormously improved by approximately 1.19, 1.17, and 1.13 times, respectively.

Electrophoresis and Microscopic Observation

In the present study, the protein profiles of CMF were distributed in the range of 20–100 kDa (**Figure 2C**). However, in the fermentation with *P. kudriavzevii* PKWF, *L. plantarum* CWLP, and neutral protease for 72 h, the protein profile corresponding to multiple bands in the range of 55–100 kDa in FCMF were completely degraded. After 72 h of fermentation, the protein profile corresponding to multiple bands in the range of 30–50 kDa in FCMF were obviously degraded. In addition, the content of small peptides (<25 kDa) was significantly increased in FCMF compared with CMF.

Scanning electron microscopy (SEM) was applied to investigate the physical structures of CMF and FCMF. **Figure 2D** shows the surface images of CMF and FCMF at magnification factors of ×100, ×1,000, and ×3,000. After 72 h of fermentation, more fragmental structures were detected. At the same

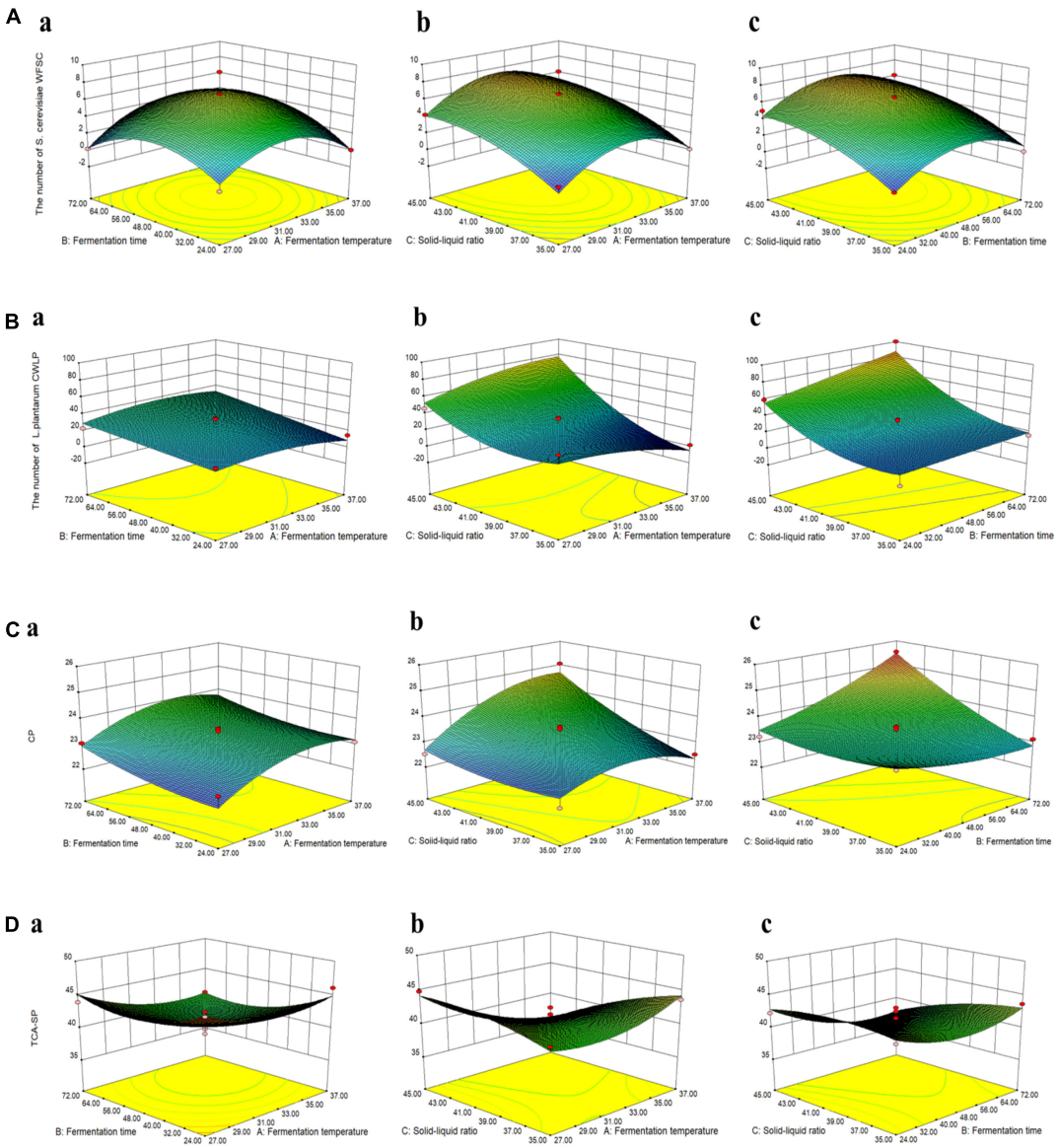

FIGURE 1 | The response surface and contour plots showing the interactive effects of fermentation conditions on the viable count of *Pichia kudriavzevii* PKWF **(A)**, the viable count of *Lactobacillus plantarum* CWLP **(B)**, crude protein (CP) **(C)**, and trichloroacetic acid-soluble protein (TCA-SP) **(D)** of corn byproduct mixture feed fermented with *P. kudriavzevii* PKWF, *L. plantarum* CWLP, and neutral protease (CMFPLN) [**(a)** fermentation time and fermentation temperature; **(b)** solid–liquid ratio and fermentation temperature; **(c)** solid–liquid ratio and fermentation time].

magnification factor, CGM and CGF in CMF had relatively large, compact, and smooth-faced structures, while the two main CBs in FCMF had smaller cracked structures and large holes. Besides, the surface of FCMF gathered more inoculated microbes.

Changes in the Bacterial Community

Overall, 229,415 high-quality sequences were collated. Additionally, the general 16S rRNA OTU numbers reached 798 based on 97% sequence similarity (**Table 5**). Combined with Good's coverage index (99.6% ± 0.00%, data not shown), the results suggested that the samples exhibited abundant OTU coverage and that the sequencing depth was sufficient for analysis of the actual structure of the bacterial community during SSF. **Figure 3A** shows that the number of OTUs increased after the addition of *P. kudriavzevii* PKWF during the initial 36 h. In contrast, the number of OTUs decreased after the following 36 h of anaerobic fermentation. A Venn plot (**Figure 3B**) shows the common and unique OTUs in the groups. Fifty-eight OTUs as core genera were shared by all of the groups. The principal component analysis (PCA) plot (**Figure 3C**) shows that samples at 0, 36, and 72 h were well resolved and obviously distinct, while the OTU sample at 36 h exhibited fewer differences than others of the same group. In general, more than four bacterial phyla were found in all the samples (**Figure 3D**). In

TABLE 2 | Nutrient composition of CMF and fermented CMF (FCMF) (as air-dry basis).

Items	CMF	FCMF	Items	CMF	FCMF
DM, %	90.33 ± 0.02a	91.67 ± 0.02a	Indispensable AA,%		
EE, %	2.28 ± 0.14b	3.24 ± 0.11a	Arg	1.74 ± 0.02a	1.75 ± 0.01a
CP, %	22.33 ± 0.13b	25.52 ± 0.18a	His	0.59 ± 0.02b	0.66 ± 0.02a
TCA-SP, %	37.26 ± 1.00b	46.70 ± 0.78a	Ile	1.38 ± 0.01b	1.55 ± 0.06a
CF, %	6.70 ± 0.17a	4.05 ± 0.09b	Leu	0.74 ± 0.01a	0.76 ± 0.00a
ADF, %	10.64 ± 0.08a	9.53 ± 0.17b	Lys	0.74 ± 0.01a	0.76 ± 0.00a
NDF, %	28.71 ± 0.13a	23.50 ± 0.25b	Met	0.16 ± 0.01a	0.18 ± 0.01a
Total starch, %	14.49 ± 0.11a	13.96 ± 0.05b	Phe	0.75 ± 0.01b	0.83 ± 0.03a
Amylopectin, %	10.40 ± 0.14a	10.34 ± 0.07a	Thr	0.38 ± 0.01a	0.41 ± 0.01a
Amylose, %	4.09 ± 0.05a	3.62 ± 0.06b	Val	0.78 ± 0.01a	0.85 ± 0.05a
			Dispensable AA,%		
			Asp	1.74 ± 0.00a	1.71 ± 0.01a
			Ser	1.41 ± 0.01b	1.54 ± 0.00a
			Glu	4.61 ± 0.07a	4.76 ± 0.07a
			Gly	0.62 ± 0.01a	0.64 ± 0.00a
			Ala	1.32 ± 0.01b	1.48 ± 0.00a
			Cys	0.21 ± 0.01a	0.23 ± 0.00a
			Tyr	1.19 ± 0.03a	1.26 ± 0.05a
			Pro	1.41 ± 0.00a	1.37 ± 0.03a
			Total AA	19.61 ± 0.13b	20.67 ± 0.12a

Values were mean ± SD, n = 3. Means followed with different superscript letters (a, b) within each line are significantly different ($p < 0.05$).

TABLE 3 | Microorganism and microbial metabolites of CMF and FCMF.

Items	CMF	FCMF
Organic acids, mg/100 g		
Acetic acid	22.07 ± 0.04b	148.22 ± 0.52a
Propionic acid	0.06 ± 0.01b	1.25 ± 0.03a
Butyric acid	0.18 ± 0.01b	3.78 ± 0.09a
Lactic acid	26.53 ± 0.08b	149.61 ± 0.09a
pH	6.42 ± 0.11a	4.54 ± 0.15b
Microorganism, 10^7 CFU/g		
P. kudriavzevii PKWF	–	6.95 ± 0.60
L. plantarum WCLP	–	90.21 ± 8.40

Values were mean ± SD, n = 3. Means followed with different superscript letters (a, b) within each line are significantly different ($p < 0.05$).

TABLE 4 | *In vitro* crude protein (CP) and amino acid (AA) digestibility (%) of CMF and FCMF.

Items	CMF	FCMF
DM, %	48.89 ± 0.37b	58.17 ± 1.64a
CP, %	67.34 ± 0.70b	78.53 ± 0.51a
Indispensable AA, %		
Arg	64.50 ± 1.02b	73.71 ± 4.75a
His	66.11 ± 3.13b	79.37 ± 3.18a
Ile	52.58 ± 1.30b	66.33 ± 4.15a
Leu	57.25 ± 1.19b	64.92 ± 2.19a
Lys	50.67 ± 1.53b	60.67 ± 3.06a
Met	52.81 ± 1.51b	58.66 ± 0.60a
Phe	63.61 ± 0.54b	76.81 ± 1.92a
Thr	50.4 ± 1.48b	62.21 ± 1.15a
Val	51.86 ± 1.47b	65.91 ± 2.59a
Dispensable AA, %		
Asp	85.53 ± 0.19a	81.76 ± 1.59b
Ser	62.57 ± 1.76b	74.65 ± 1.10a
Glu	79.75 ± 1.51b	91.08 ± 1.94a
Gly	61.44 ± 0.99a	60.44 ± 1.97a
Ala	56.46 ± 1.13b	67.61 ± 1.96a
Cys	49.86 ± 1.29b	59.75 ± 4.25a
Tyr	55.35 ± 3.34b	63.55 ± 2.96a
Pro	66.91 ± 1.41a	68.83 ± 1.56a
Total AA, %	66.20 ± 0.31b	74.94 ± 1.27a

Values were mean ± SD, n = 3. Means followed with different superscript letters (a, b) within each line are significantly different ($p < 0.05$).

the CMF samples, *Cyanobacteria* and *Proteobacteria* accounted for 96.96% ± 0.10% of the sequences. However, as fermentation progressed, *Firmicutes* rapidly became the primary members of the community, accounting for approximately 99% of the sequences. With regard to the changes in bacterial community structure, the results at the gene level were similar to those at the phylum level (**Figure 3E**). Unfermented materials contained various native bacteria, including pathogens such as *Enterobacter* spp. and *Clostridium* spp. As the overall fermentation progressed, the predominant bacteria changed from *Cyanobacteria* and *Proteobacteria* to *Lactobacillus* spp.

Furthermore, the linear discriminant analysis (LDA) effect size (LEfSe) results showed significantly different taxonomy among different fermentation time points (**Figure 3F**). After 36 h of aerobic fermentation, the abundances of the genera *Bacillus* and *Xanthobacteraceae* increased significantly. After 36 h of anaerobic fermentation, *Paenibacillus* spp., *Pantoea* spp., and *Lactobacillales* were predominant.

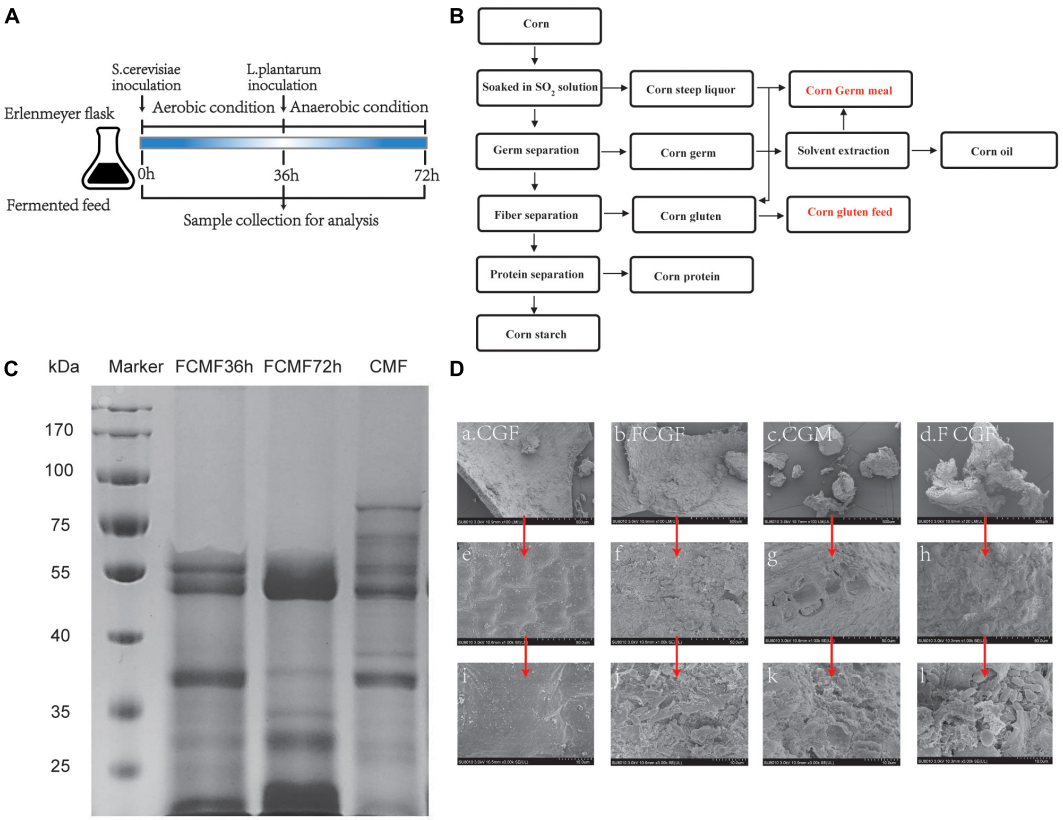

FIGURE 2 | Experimental design, electrophoresis, and scanning electron microscopy (SEM) image of corn byproduct mixture feed (CMF) and fermented CMF (FCMF). **(A)** Experimental design. **(B)** Simplified process of corn wet milling for starch production and its byproducts. **(C)** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of FCMF at fermentation times of 0, 36, and 72 h. **(D)** SEM images of CMF and FCMF after 72 h of fermentation at ×100 **(a–d)**, ×1,000 **(e–h)**, and ×3,000 **(i–l)** fold magnifications.

Changes in the Fungal Community

Full of 644,350 high-quality sequences were collated byITS Genes Amplification, and the general operational taxonomic unit (OTU) numbers reached 547 based on 95% sequence similarity (**Table 6**). Combined with Good's coverage index (99.7% ± 0.00%, data not shown), the results indicated that the samples exhibited abundant OTU coverage and that the sequencing depth was adequate for analysis of the actual structure of the fungal community during SSF. **Figure 4A** shows that the number of observed OTUs decreased after the addition of *P. kudriavzevii* PKWF during the first stage, then the number of observed OTUs decreased continuously after the following 36 h of anaerobic fermentation. A Venn plot (**Figure 4B**) shows the common and unique OTUs in the groups. Twenty-three OTUs as core genera were presented by all of the groups. The PCA plot (**Figure 4C**) illustrates that the samples between 0 and 36 h were significant resolved and obviously distinct, while the OTUs sample at 36 and 72 h exhibited fewer differences than those of the other samples. In general, more than three fungal phyla were found in all the samples (**Figure 4D**). In the CMF samples, *Ascomycota*, *unclassified_k_Fung*, and *Basidiomycota* occupied 97.96% ± 0.10% of the sequences. As fermentation progressed, *Ascomycota* became the predominant member of the community, accounting for approximately 99% of the sequences. When it comes to the changes in fungal community structure, the results at the gene level were similar to those at the phylum level (**Figure 4E**). Unfermented feed contained various native fungi, including pathogens such as *Colletotrichum* and *Fusarium*. As the overall fermentation progressed, the predominant fungi were kept unchanged with *Ascomycota*.

Furthermore, the linear discriminant analysis (LDA) effect size (LEfSe) results showed significantly different taxonomy among different fermentation time points (**Figure 4F**). After 72 h of fermentation, the abundances of the OTU8 (*P. kudriavzevii*) increased significantly.

Bacterial Metabolism of Fermented Mixed Substrates

The microbial metabolic functions presented in **Figure 5** were obtained based on the Clusters of Orthologous Groups of proteins (COG) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. **Figure 5A** shows the changes of COGs in three different fermentation time points, with the fermentation process, proteins related to metabolism functions (G,F) and information storage and processing functions (K) improved significantly; however, the functions of cellular

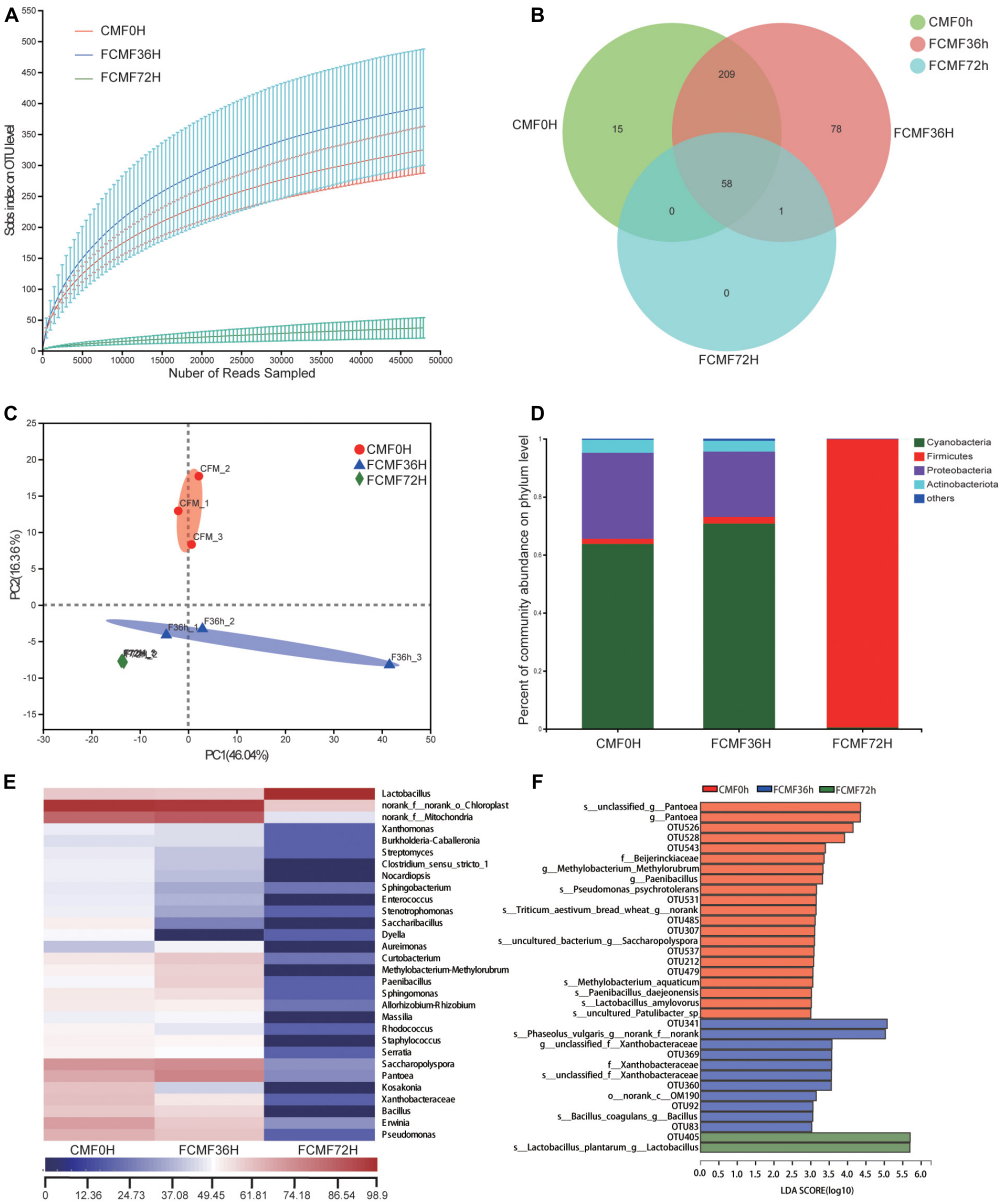

FIGURE 3 | Microbial diversity and bacterial community structure during two-stage solid-state fermentation (SSF) (n = 3). **(A)** Observed operational taxonomic unit (OTU) line chart. **(B)** Venn diagram representing the common and unique OTUs found at each fermentation time point. **(C)** Principal-component (PC) analyses of samples conducted based on unweighted UniFrac distanced. **(D,E)** Phylum-level **(D)** and genus-level **(E)** compositions of the bacterial community in FCFM. **(F)** Linear discriminant analysis (LDA) effect size (LEFSe) histogram showing the LDA scores (>3.0) computed for features at the OTU level. Letters indicate the taxonomy of the bacteria: p, phylum, c, class; o, order; f, family; g, genus.

processes and signaling (O,T,U,N,Z) decreased from 0 to 72 h. A majority of the predicted protein sequences ranged from 17.34% ± 0.02% to 0.05% ± 0.00% at the three time points among six different metabolic functions (**Figure 5B**), which represented different pathways (**Figure 5C**). Notably, carbohydrate metabolism, energy metabolism, and membrane transport accounted for more than 10% of the enriched pathways throughout the fermentation period. Furthermore, the sequences related to membrane transport, carbohydrate metabolism, energy metabolism, metabolism of cofactors and vitamins, nucleotide metabolism, and environmental adaptation were significantly enriched during intact fermentation process ($p < 0.00$).

At level 3 of the microbial gene functions of bacteria, some differences in efficiency were observed during SSF (**Figure 5D**). The abundance of a majority of the genes assigned to AA metabolism (cysteine and methionine metabolism, phenylalanine, tyrosine, and tryptophan biosynthesis) and carbohydrate metabolism (glycolysis/gluconeogenesis pyruvate metabolism, amino sugar and nucleotide sugar metabolism, fructose and mannose metabolism, pentose phosphate pathway,

TABLE 5 | Characteristics of amplicon libraries in the bacteria community.

Characteristic	Data for samples at time (h)			Total no.
	CFM 0 h	FCMF 36 h	FCMF 72 h	
No. of sequences	59, 862 ± 9, 815	54, 342 ± 5, 696	57, 857 ± 6080	229,415
No. of operational taxonomic units (OTUs)	405 ± 29*A*	406 ± 105*A*	41 ± 20*B*	798
Chao1 index	456 ± 64*A*	489 ± 69*A*	89 ± 21*B*	
Shannon index	1.69 ± 0.19*A*	1.75 ± 0.57*A*	0.06 ± 0.01*B*	
Simpson index	0.39 ± 0.11*A*	0.32 ± 0.11*A*	0.98 ± 0.01*B*	

Means with different letters in each row differ at $p < 0.05$.

TABLE 6 | Characteristics of amplicon libraries in the fungal community.

Characteristic	Data for samples at time (h)			Total no.
	CFM 0 h	FCMF 36 h	FCMF 72 h	
No. of sequences	69, 999 ± 1, 237	72, 531 ± 2, 410	72, 252 ± 1, 752	644350
No. of OTUs	136 ± 35*A*	31 ± 25*B*	14 ± 8*B*	547
Chao1 index	148 ± 28*A*	40 ± 27*B*	23 ± 62*B*	
Shannon index	2.26 ± 0.31*A*	0.10 ± 0.04*B*	0.81 ± 1.09*B*	
Simpson index	0.17 ± 0.05*B*	0.96 ± 0.01*A*	0.97 ± 0.00*A*	

Means with different letters in each row differ at $p < 0.05$.

and propanoate metabolism) increased dramatically during the fermentation process ($p < 0.05$). Similarly, the genes associated with membrane transporter, such as ABC transporters and phosphotransferase system (PTS), were markedly enriched by the fermentation ($p < 0.05$). All these gene functions that improved were attributed to using *L. plantarum* during the second stage of fermentation. In contrast, the abundances of most genes related to glycan biosynthesis and metabolism and energy metabolism decreased with fermentation. Interestingly, the abundance of genes involved in global and overview maps (carbon metabolism) decreased during the aerobic fermentation period, while a considerable increase was observed following anaerobic fermentation.

As expected, the gene functions related to *P. kudriavzevii* were improved after the addition of *P. kudriavzevii* PKWF and reduced during the second-stage fermentation. The gene function prediction of the fungal community is presented in **Figure 6**. Fungal communities were analyzed by the FUNGuild online tool (see text footnote 3). In general, more than six fungal function groups were inferred by FUNGuild (**Figure 6A**). After adding *P. kudriavzevii*, the main fungal function group was Saprotroph, and it continued to the end of fermentation. In addition, the fungal functional groups inferred by FUNGuild, indicated to us that the unfermented CMF contain the animal pathogen and plant pathogen; in contrast, the fermented CMF could inhibit these pathogenic microbes and improve the safety and quality of CMF, and the level 3 KEGG ortholog functional predictions of the relative abundances exhibited several changes of key enzymes (**Figure 6B**). Adenosine triphosphatase, ubiquitinyl hydrolase 1, benzoate 4-monooxygenase, xenobiotic-transporting ATPase, and tetrahydrofolate synthase were significantly enriched during the intact fermentation process ($p < 0.00$), while peptidylprolyl isomerase, DNA-directed RNA polymerase, proteasome endopeptidase complex, L-arabinose isomerase, glucan 1,4-alpha-glucosidase, H(+)-transporting two-sector ATPase, histone acetyltransferase, and beta-glucosidase were decreased by adding *L. plantarum* CWLP.

DISCUSSION

In recent years, there have been many reports on the positive effects of bacteria or fungi (such as *P. kudriavzevii* and *L. plantarum*, etc.) and their metabolites in fermented feed. On the one hand, these microbes secrete a series of enzymes that effectively degrade anti-nutritional factors to improve the nutrient value of the feed materials (Arevalo-Villena et al., 2017). Besides, microorganisms and their functional metabolites, such as organic acids, cell wall polysaccharides, etc., can significantly improve the immune function of animals and inhibit the proliferation of pathogenic microorganisms, thereby maintaining the health of the animals (Fukuda et al., 2011; Zhu et al., 2014; Chen and Stappenbeck, 2019). *Bacillus* spp., *Lactobacillus* spp., and yeast were widely used in feed fermentation. However, the organic acids produced by *Lactobacillus* spp. would limit the activity of *Bacillus* spp. and, thus, inhibit its secretion of proteases (Wong and Chen, 1988). In the present study, we found that fermentation of *P. kudriavzevii* PKWF, *L. plantarum* CWLP, and neutral protease had the positive effect on improving CP and TCA-SP of CBs through single-factor experiments, which indicated that the role of *Bacillus* spp. in degrading proteins into TCA-SP could be replaced by neutral proteases.

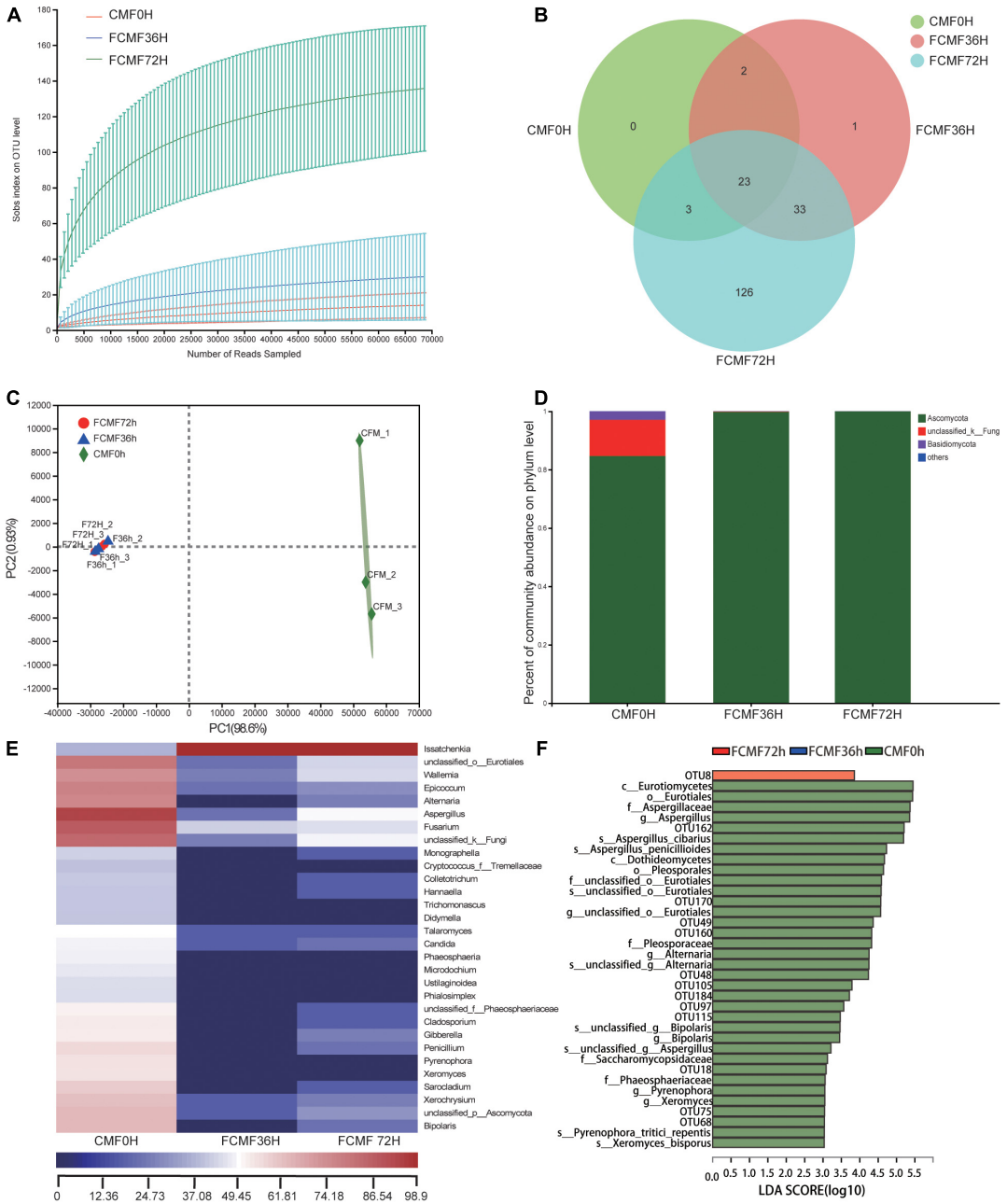

FIGURE 4 | Microbial diversity and fungal community structure during two-stage SSF (n = 3). **(A)** Observed OTU line chart. **(B)** Venn diagram representing the common and unique OTUs found at each fermentation time point. **(C)** Principal component analyses (PCA) of samples conducted based on unweighted UniFrac distanced. **(D,E)** Phylum-level **(D)** and genus-level **(E)** compositions of the fungal community in FCFM. **(F)** LEFSe histogram showing the LDA scores (>3.0) computed for features at the OTU level. Letters indicate the taxonomy of the bacteria: p, phylum, c, class; o, order; f, family; g, genus.

Response surface analysis is an effective way to investigate the interaction between different factors during fermentation. Compared with the previous report (Li et al., 2019), the optimized fermentation conditions of soybean meal by response surface analysis were fermentation temperature (30°C), fermentation time (72 h), and solid–liquid ratio (1:3.5 g/ml); the protein hydrolysis of fermented soybean meal could reach to 10.05% by *Neurospora crassa* under the fermentation conditions. In our research, under the fermentation conditions of 32°C, solid–liquid ratio (1.2:1 g/ml), and 50 h, the proliferation of *P. kudriavzevii* PKWF reached its maximum. The rapid growth of *P. kudriavzevii* PKWF increased the consumption of oxygen, which provided an anaerobic environment for *L. plantarum* CWLP (Han et al., 2017). The viable count of *L. plantarum* CWLP reached its maximum value under the fermentation conditions of 37°C, solid–liquid ratio (1.2:1 g/ml), and 72 h. In

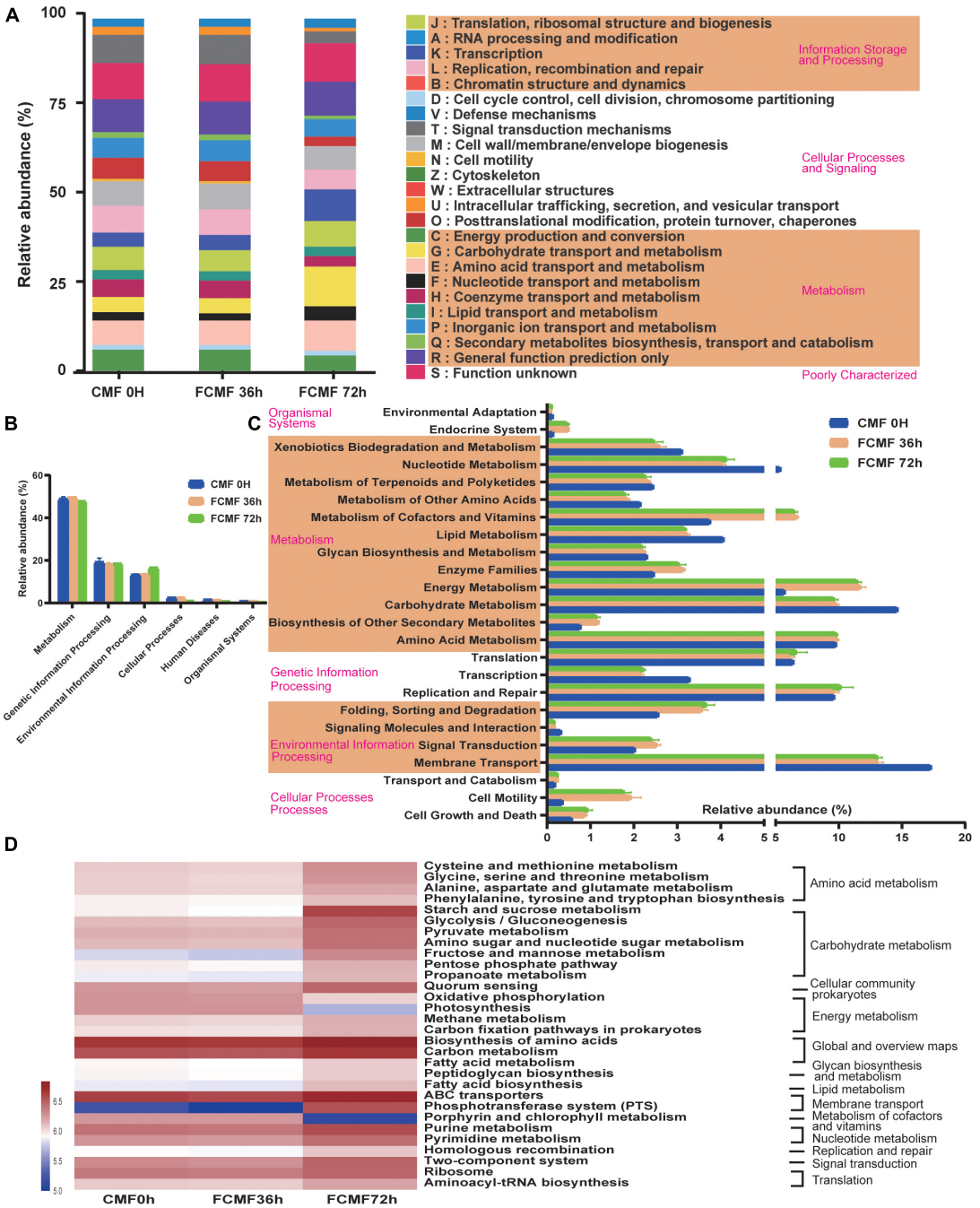

FIGURE 5 | Dynamics of bacterial functional profiles during CMF fermentation process analyzed by PICRUSt (n = 3). **(A)** Clusters of Orthologous Groups of protein (COG) function classification. **(B)** Level 1 metabolic pathways. **(C)** Level 2 Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog functional predictions. **(D)** Level 3 KEGG ortholog functional predictions of the relative abundances of the top 30 metabolic functions.

addition, neutral protease may have the optimal enzyme activity to degrade macromolecular proteins into TCA-SP at 37°C. Accumulation of single-cell protein produced by microorganisms may lead to the increase in CP (Aggelopoulos et al., 2014; Mekoue Nguela et al., 2016), which reached its maximum under the fermentation conditions of 37°C, solid–liquid ratio (1.2:1 g/ml), and 72 h. In summary, we recommend the fermentation conditions of 37°C, solid–liquid ratio (1.2:1 g/ml), and 72 h for SSF of CMF.

The content of CP, TCA-SP, and AA in CMF was significantly increased after the fermentation in our research. In addition to the accumulation of single-cell proteins produced by microorganisms, the loss of DM (mainly carbohydrates) in the fermentation substrate may be another reason for the relative

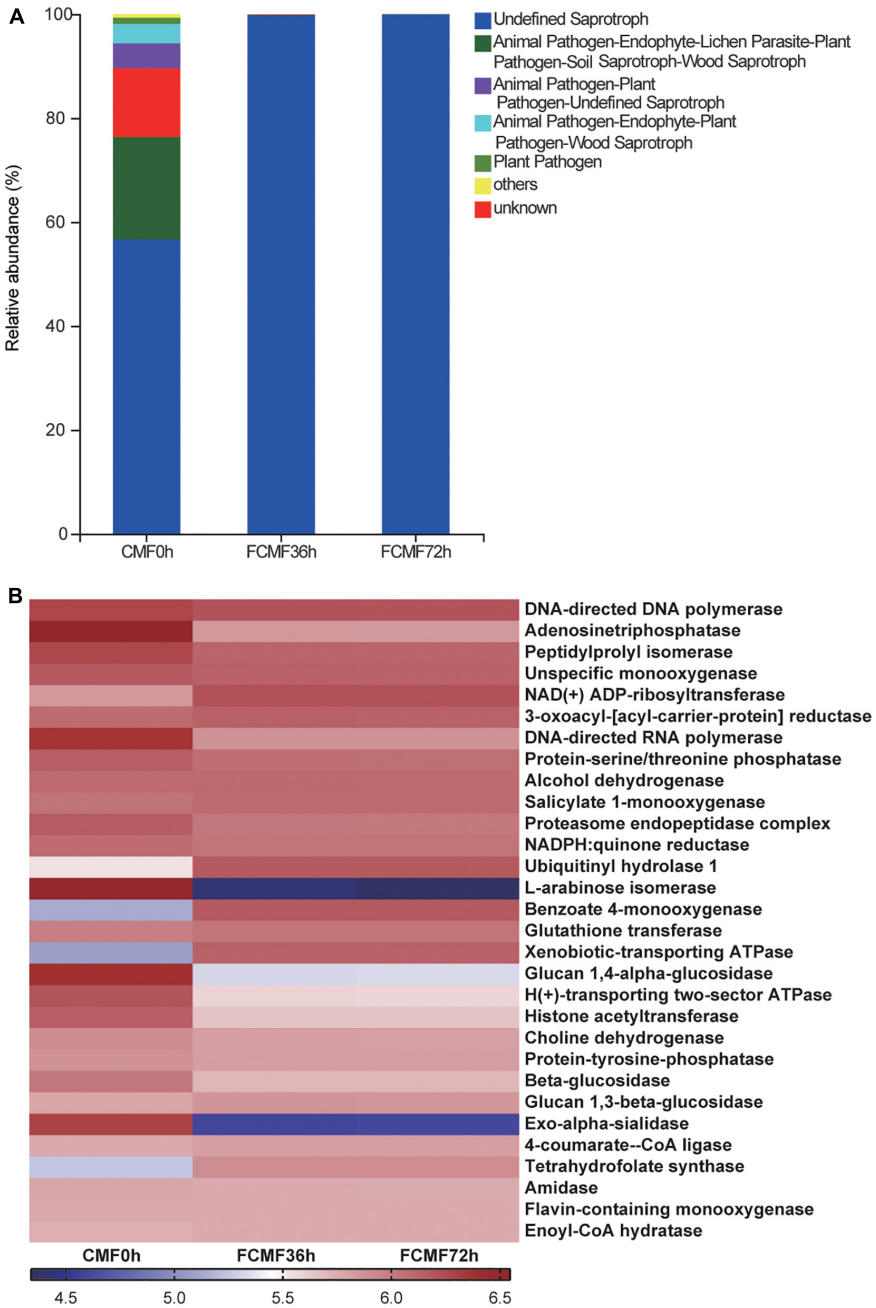

FIGURE 6 | Dynamics of fungal functional profiles during CMF fermentation process analyzed by FUNGuild and PICRUSt (n = 3). **(A)** Variations in composition of fungal functional groups inferred by FUNGuild. **(B)** Level 3 KEGG ortholog functional predictions of the relative abundances of the top 30 enzymes.

increase in the concentration of CP (Stokes and Gunness, 1946). TCA-SP consists of small peptides and free AA, most of which can be directly absorbed by the gastrointestinal tract (Gilbert et al., 2008). In addition, AA composition pattern changes during fermentation may be related to microbial protein synthesis and decomposition (Metges, 2000). Therefore, the increase in the content of TCA-SP and the change in AA composition can improve the nutritional value of CMF. Furthermore, the lignocellulosic components and amylose is poorly digested in the upper gut of monogastric animals (Regmi et al., 2011). CF, ADF, and amylose are effectively degraded after fermentation in this study, which might be due to the cellulase and amylase secreted by *L. plantarum* (Lee et al., 2019; Xu et al., 2020).

In contrast to CMF, FCMF exhibited small, cracked structures and large holes. The change in the surface structure of CMF after fermentation may be associated with extracellular enzymes (especially protease and carbohydrase) secreted during the process. The cracked and porous structure may provide increased

access to enzymes for nutrient hydrolysis and may make the substrates considerably easier to utilize (Zheng et al., 2017), suggesting that FCMF had higher digestibility than CMF. Additionally, Tang et al. (2009) reported that smaller protein aggregates may result in a higher solubility. Zhao et al. (2015) found that soybean proteins with loose networks and diffuse structures have higher emulsification activity and solubility. Thus, the physicochemical properties of CMF may also have been affected by the changed microstructure in this study.

Digestibility is an essential method to evaluate the nutritional value of protein. The increase in CP and TCA-SP and the optimization of AA composition pattern in FCMF may be the main reasons for improving the *in vitro* digestibility of CP and AA. Some macromolecular proteins induce allergic reactions in humans and animals (Holzhauser et al., 2009; Wu et al., 2016). In the present study, CMF, fermented with *P. kudriavzevii* PKWF, *L. plantarum* CWLP, and neutral protease, contained less macromolecular proteins and more small peptides compared with CMF; the result was consistent with the previous report (Chi and Cho, 2016). In addition, the degradation of viscous-resistant starch and cellulose in FCMF leads to the exposure of internal proteins to the environment of pepsin and trypsin (Agama-Acevedo et al., 2005; Yang et al., 2010), which possibly contributes to the *in vitro* digestibility of CP and AA. Furthermore, the low pH value of FCMF is more effective to promote the function of pepsin (Mat et al., 2018).

High-throughput sequencing was first applied to analyze the changes in microbial community, including bacterial and fungal communities, structure, and metabolic functions during the fermentation process. The increase in OTU number during first-stage fermentation was because of the aerobic condition. In contrast, the second-stage fermentation process suggested that *L. plantarum* CWLP decreased OTU number, inhibited other bacteria and fungi, and became the dominant bacterium. The main phyla (*Cyanobacteria*, *Proteobacteria*, *and Firmicutes*) found in the present study were also obtained in some other studies related to SSF (Wang et al., 2020).

As fermentation progressed, *unclassified_k_Fung* and *Basidiomycota* became the predominant members of the community; *Ascomycota* were identified as the core genera during first stage of SSF. This type of fungi could consume oxygen in the first stage of fermentation, which may inhibit other pathogenic aerobic microorganisms. Meanwhile, *Paenibacillus* spp., *Pantoea* spp., and *Lactobacillales* spp. were predominant during the later 36 h, and members of the heat-tolerant genus *Paenibacillus* spp. are effective at degrading proteins and cellulose during SSF due to their strong hydrolytic abilities (de Gannes et al., 2013). *Lactobacillales* spp. is a mesophilic genus whose members generate acid products (Yuan et al., 2018). Thus, these dominant genera indicated a selected community categorized by typical large-molecule catabolism characteristics, achieved by the addition of the two inoculated microbes. The evolution of bacterial and fungal structure during the process demonstrated that the artificially added inoculated microbes not only increased the number of added microbes but also boosted some other functional microbes that could develop a form of symbiosis with the inoculated microbes.

The results of KEGG levels 1 to 3 gene function analysis were generally consistent. As fermentation progressed, the abundances of both carbohydrate metabolism and AA metabolism genes gradually increased. Metabolism of cellulose and hemicellulose can produce many compounds that support bacterial growth (Toledo et al., 2017). AAs are also an energy and carbon source for bacteria (Sanchez et al., 2017). These results indicated that the degradation of large carbohydrate and proteins resulted in increased levels of saccharides and AAs, which could be utilized by the microbiota in FCMF. The gene abundances of membrane transporter, ABC transporters, and phosphotransferase system (PTS) increased during fermentation. These metabolic functions were associated with compound production and membrane transport, suggesting the mechanism of enzyme synthesis by, and activity of, the core bacteria in FCMF. In contrast, glycan biosynthesis and metabolism, and energy metabolism were inhibited by the core genera, indicating that these genes may be involved in native bacterial gene functions of CMF. Although the addition of *L. plantarum* inhibited the growth of other CMF-native microbes, it did not decrease the abundances of enzyme families. Additionally, the differences in the abundances of genes involved in global and overview maps (carbon metabolism) between the aerobic and anaerobic stages suggested the different metabolic roles of *P. kudriavzevii* and *L. plantarum*.

Various bacteria and fungi were correlated with different metabolic pathways, revealing that multiple metabolic pathways were active during the two stages of the SSF. Two inoculated microbes were enriched in environmental information processing and cellular processes throughout fermentation. These metabolic functions allowed the microbe to grow, proliferate, and respond to the environment (Kausar et al., 2011). The results demonstrated the superior adaptation of the inoculated microbes in response to fermentation.

CONCLUSION

In summary, two-stage SSF with *P. kudriavzevii* PKWF, *L. plantarum* CWLP, and neutral protease under the conditions of fermentation temperature of 37°C, fermentation time of 72 h, and solid-liquid ratio of 1.2:1 g/ml effectively improved protein digestibility in CMF through degrading macromolecular protein into TCA-SP, improving AA composition patterns and degrading lignocellulose to expose internal nutrients. Therefore, this study provides a novel method for improving the nutritional quality of CMF and provides a basis for demonstrating that the inoculated microbes dynamically change the physicochemical features, microbiota, and metabolic functions during the two-stage SSF, which could serve as a valuable resource for industrial feed-based practices and metabolomic research on SSF systems; besides, this study also provides a strategy for the utilization of CBs as feed materials. Further studies should focus on the use of additional enzymes and inoculation with other bacteria during fermentation to further reduce the ANF content of FCMF and produce various types of organic acids.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

WS: investigation, data curation, and writing-original draft. ZJ: investigation, software, and writing original draft. LH: visualization. WL: project administration. TG: resources. YZ: formal analysis. SD and CW: software and validation. ZL and MJ: conceptualization, supervision, and writing review and editing. YW: supervision, funding acquisition, methodology, software, and writing review and editing. 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.2021.688839/full#supplementary-material

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Comprehensive Analysis of Bacterial Community Structure and Diversity in Sichuan Dark Tea (*Camellia sinensis*)

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Bacteria and fungi present during pile-fermentation of Sichuan dark tea play a key role in the development of its aesthetic properties, such as color, taste, and fragrance. In our previous study, high-throughput sequencing of dark tea during fermentation revealed *Aspergillus* was abundant, but scarce knowledge is available about bacterial communities during pile-fermentation. In this study, we rigorously explored bacterial diversity in Sichuan dark tea at each specific stage of piling. Analysis of cluster data revealed 2,948 operational taxonomic units, which were divided into 42 phyla, 98 classes, 247 orders, 461 families, 1,052 genera, and 1,888 species. Certain members of the family *Enterobacteriaceae* were dominant at early stages of fermentation YC, W1, and W2; *Pseudomonas* at middle stage W3; and the highest bacterial diversity was observed at the final quality-determining stage W4. Noticeably, probiotics, such as *Bacillus*, *Lactobacillus*, *Bifidobacterium*, and *Saccharopolyspora* were also significantly higher at the quality-determining stage W4. Our findings might help in precise bacterial inoculation for probiotic food production by increasing the health benefits of Sichuan dark tea. This research also falls under the umbrella of the "Establish Good Health and Well-Being" Sustainable Development Goals of the United Nations Organization.

Keywords: bacterial diversity, high-throughput sequencing, Sichuan dark tea, pile-fermentation, qPCR

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INTRODUCTION

Tea can be considered a medicinal plant with a lot of health benefits, and it is consumed as a beverage across the world. China is the origin of the tea plant (*Camellia sinensis*) and has great importance in Chinese culture. In China, plentiful tea products are found, relying upon growth areas, processing techniques, and polyphenol oxidase contents (Jie et al., 2006; Lee et al., 2015). Dark tea is one of the six major tea types found in China, and it is cultivated in Sichuan, Yunnan, Guangxi, Hunan, and Hubei (Peng et al., 2014; Liu, 2016). Dried green tea leaves are the raw material that is processed to make dark tea *via* microbial fermentation and solid-state fermentation (SSF). Growth of microbes, such as bacteria and fungi, is a key step in the biosynthesis of dark tea during the wet piling technique, which determines the aesthetic value of dark tea, such as stale or mellow taste and reddish brown wine (Li et al., 2017).

Sichuan province is a prominent dark tea production area due to its unique and favorable climatic conditions for the growth of raw material and specialty of processing technology in pile-fermentation. It is hypothesized that the potential microbial resources of the Sichuan region are

entirely different as compared with other dark tea production areas. Traditional bacterial culture techniques are employed for undermining structural variations among microbial communities in dark tea, in which only a few bacterial strains can be isolated because selective bacterial strains can grow on available mediums (Ge et al., 2019). The probability of fake results of microbial diversity in the fermentation of dark tea is significantly high by culturing techniques.

Contrastingly, culture-independent methods have the advantage over culture-dependent methods because of its potential for microbial community structure detection in any kind of medium even with low abundance. Recently, culture-independent methods are being widely used in the food industry to undermine the composition of microbes more accurately and comprehensively (Li et al., 2017). The principal of culture-independent methods is based on direct diversity analysis *via* sequencing of ribosomal RNA (rRNA) genes without any involvement of a culture-medium step; that is why it is robust, fast, and cost-effective and provides high resolution of microbial communities. However, culture-independent methods are scarcely applied to investigate the microbial community structure in dark tea (Chen et al., 2013; Fu and Liu, 2015; Zhang et al., 2016; Ge et al., 2019).

In this study, high-throughput sequencing was employed to undermine the entire diverse bacterial community structure and to specifically reveal dominant bacterial taxa at each stage of pile-fermentation present in Sichuan dark tea. Our study provides a preliminary exploration of the dynamic characteristics of the bacterial community during the piling process and reveals the actual modulator behind the development of the unique aesthetic values of Sichuan dark tea. A better understanding of the microbial community structure and their abundance at each aesthetic value-determinant stage paves the way to regulate their concentration and develop new techniques to precisely develop unique tastes and probiotic foods for human beings.

MATERIALS AND METHODS

Experimental Materials

In order to conduct this research, samples of Sichuan dark tea were provided by the Sichuan Tea Industry Group Co., Ltd., and these were prepared from fresh leaves of tea plants collected in summer and autumn. During the fermentation process, the leaves of tea plants were mixed thoroughly to ensure homogeneity, and tap water was sprinkled in an adequate quantity to maintain 65–75% (w/v) solid contents and the temperature at 45–71°C. Samples were collected from tea piles at 15-day intervals and subjected to sensory evaluation as described by GB/T 23776-2009 (Gong et al., 2009). The fermentation process is over as the tea mass becomes reddish-brown and free of its stringent taste. Samples were collected during the fermentation process in triplicate and at the following time intervals; days 0 (YC), 8 (W1), 16 (W2), 24 (W3), and 32 (W4). The temperature of each fermentation tea pile was recorded from the center at the depth of 40 cm almost every day.

DNA Extraction

For DNA extraction, 5 g of each sample was suspended in 50 mL sterile Tween-NaCl buffer [0.9% NaCl (w/v), 0.05% Tween 20 (v/v) and 2% polyvinylpolypyrrolidone (w/v)] and homogenized by mixing thoroughly for 30 min *via* sonication at 4°C. The solution was further passed through a sterile gauze and subsequently centrifuged at 2,000 rpm for 2 min at 4°C to remove course material. To collect the microbial communities, the supernatant was shifted into a new tube and centrifuged at 10,000 rpm for 10 min at 4°C. Microbial genomic DNA was extracted from the precipitate using the E.Z.N.A.^{TM} HP Plant DNA Kit (Omega Bio-Tek Inc., GA, United States) according to the manufacturer's protocol. Finally, DNA was purified using the E.Z.N.A.^{TM} Soil DNA Kit to remove impurities, such as polyphenol, that may have a negative effect on the PCR reaction (Yan et al., 2021).

PCR Amplification and Sequencing Analysis

The primer pair 338F: 5′-ACTCCTACGGGAGGCAGCAG-3′ and 806R: 5′-GGACTACHVGGGTWTCTAAT-3′ was employed to amplify the V3+V4 region of the bacterial 16S rRNA gene (Zeng et al., 2011; Apprill et al., 2015). The PCR reaction conditions were as follows: initial denaturation at 95°C for 3 min, denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 45 s, 30 cycles in total; final extension was at 72°C for 10 min and held at 10°C for 10 min. The total PCR reaction system was 20 μL with the following ingredients; 4 μL of 5 × PCR buffer (with Mg^{2+}), 2 μL of 2.5 mmol/L dNTP, 0.8 μL of 5 μmol/L P1 (338F), 0.8 μL of 5 μmol/L P2 (806R), 0.4 μL of 5 U/μL Taq Enzyme, 2 μL of DNA template, and 10 μL of ddH_2O. PCR products of all the same samples were mixed together and electrophoresed by running on a 2% agarose gel. Gel cutting was performed with a sterile sharp edge blade, and subsequently, the AxyPrepDNA Gel Recovery Kit (AXYGEN®) was used for gel elution to recover the correct DNA band. Furthermore, PCR products with good quality were subjected to library construction and sequenced by using the robust Illumina MiSeq^{TM} PE300. Pair reads were merged into a single sequence by following the overlap relationship between PE reads and filtered for quality control to a avoid splicing effect. Finally, reads obtained by sequencing were checked for their quality, and sequence direction was corrected (Mukherjee et al., 2014; Yan et al., 2021). According to primer use, proper barcoding/labeling was performed on each step to avoid any error.

Taxonomic Assessment and Data Analysis

The USEARCH manual version 7.1[1] was deployed to extract non-repetitive sequences from optimized sequences, which is very useful to remove redundant calculations during data analysis[2], and to remove non-repetitive single sequences[3]. Similarly,

[1]http://drive5.com/uparse/

[2]http://drive5.com/usearch/manual/dereplication.html

[3]http://drive5.com/usearch/manual/singletons.html

UPARSE software (see text footnote 1) was deployed for clustering analysis at a 97% similarity index to get representative sequences of operational taxonomic units (OTUs) (Ye et al., 2017). The RDP classifier Bayesian algorithm was deployed to perform taxonomic analysis of OTUs at the 97% similarity index. Bacterial community composition present in every dark tea sample was classified and counted at each level of phylogeny, such as kingdom, phylum, class, order, family, genus, and species (Chen et al., 2016).

To construct a rarefaction curve, a specific number of individuals from each sample were randomly selected to evaluate their OTUs, and a number of individuals with their relevant OTUs were used to construct the curve. The rarefaction curve is used to compare the abundance of each specie in given samples with variable sequencing reads and to validate the amount of sequencing data. Rarefaction analysis of OTUs at the 97% similarity index obtained from 16S rRNA gene sequence reads *via* illumina MiSeq platform was performed at the mothur website[4], and the curve was made using the R tool (Amato et al., 2013). To evaluate the sequencing depth index, α-diversity, such as Chao 1, ACE, Shannon, and Simpson of each sample, were individually calculated, and the abundance as well as the diversity of microbial communities in dark tea were analyzed at different fermentation stages (Rogers et al., 2016). A Venn diagram was constructed to count the number of common and unique OTUs in all samples (Fouts et al., 2012). According to the taxonomic analysis results, single or multiple samples were compared at each classification level, and the R tool was employed to construct community structure component diagrams, histogram combined analysis diagrams, and RDA principal coordinate analysis diagram (Lu et al., 2016; Zhou et al., 2017).

Statistical Analysis

All data is explained in mean values of standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA). A Duncan multiple-comparison test was applied to detect variations among means of all samples at a p-value < 0.05 level of significance. All correlation and path coefficient analyses were performed with SPSS Statistics 20.0 (SPSS Inc., Chicago, IL, United States) and Excel 2019.

RESULTS

Sequencing Data Analysis

We analyzed the diverse bacterial communities found in Sichuan dark tea at five different stages during the fermentation process by large-scale sequencing-based analysis of 16S rRNA gene sequences. The sequencing data of the bacterial colonies found in all samples collected at five different stages of fermentation are given in **Table 1**. The total number of sequencing reads of sample YC are 62,456, the total number of nitrogenous bases are 26,118,006 bp, and the average sequence length is 418.59 bp. Similarly, the total number of effective sequencing reads of sample W1 are 46,909, the total numbers of nitrogenous bases are 19,638,346 bp, and the average sequence length is 420.24 bp. Sequencing analysis revealed 40,399 full-length sequence reads in sample W2, the total number of nitrogenous bases is 17,285,142 bp, and the average read length is 427.75 bp. In sample W3; 43,362 full length sequencing reads were obtained with a total number of 18,361,869 bp nitrogenous bases, and the average read length is 423.51 bp. Finally, after elimination of raw and incomplete reads, 60,400 total reads were obtained in sample W4, containing 25,535,185 bp total number of nitrogenous bases, and the average length of sequence reads was 422.57 bp (**Table 1**).

TABLE 1 | Sequencing analysis of 16S rRNA gene of bacterial communities in all five samples.

Sample	Reads	Total bases	Average length
YC	62,456	26,118,006	418.59
W1	46,909	19,638,346	420.24
W2	40,399	17,285,142	427.75
W3	43,362	18,361,869	423.51
W4	60,400	25,535,185	422.57

Column 1 represents sample name, column 2 represents reads obtained after elimination of raw and incomplete reads, column 3 is comprised of total number of nitrogenous bases, and column 4 represents average read length in base pair.

The rarefaction curve was drawn to show the sampling depth of each sample, which can be used to evaluate either sequencing date and is sufficient to represent all bacterial strains present in each sample. The flatness of each rarefaction curve representing all five samples proved that the sampling process had adequately collected the experimental material (**Figure 1**). The bacterial diversity in the amount of each randomly collected sample was enough to construct an individual DNA library for each sample. The confidence level about the amount of each bacterial community structure in Sichuan dark tea samples was very high to accurately reflect the bacterial community.

OTUs Cluster Analysis

The bioinformatics and statistical analyses were performed at the OTU level with a 97% similarity index. Furthermore, a Venn diagram was constructed to display compositional similarity among the different bacterial communities present in all samples, and the overlapping of different samples was intuitively displayed at different classification levels. A total number of OTUs obtained by clustering the valid data were 2,948, and these were further divided into 42 phyla, 98 classes, 247 orders, 461 families, 1,052 genera, and 1,888 species (**Supplementary Table 1**). Similarly, OTUs in each stage were 2,082, 2,116, 381, 399, and 580, respectively. Among all samples, YC and W1 samples shared the highest number of 1,211 OTUs, YC and W2 samples shared 15 OTUs, YC and W3 samples shared 25 OTUs, and YC and W4 samples shared 50 OTUs. The unique OTUs that were not common or overlapping in each sample were 360 in YC, 399 in W1, 70 in W2, 94 in W3, and 151 in W4, which represented 17.3, 18.9, 18.4, 23.6, and 26.0% of total OTUs, respectively (**Figure 2**). The total number of common OTUs among all five samples collected at five different stages of pile-fermentation were 103, which were 3.6% of the total number of OTUs in all samples. These results indicate that the bacterial community structure

[4]https://mothur.org/wiki/miseq_sop/

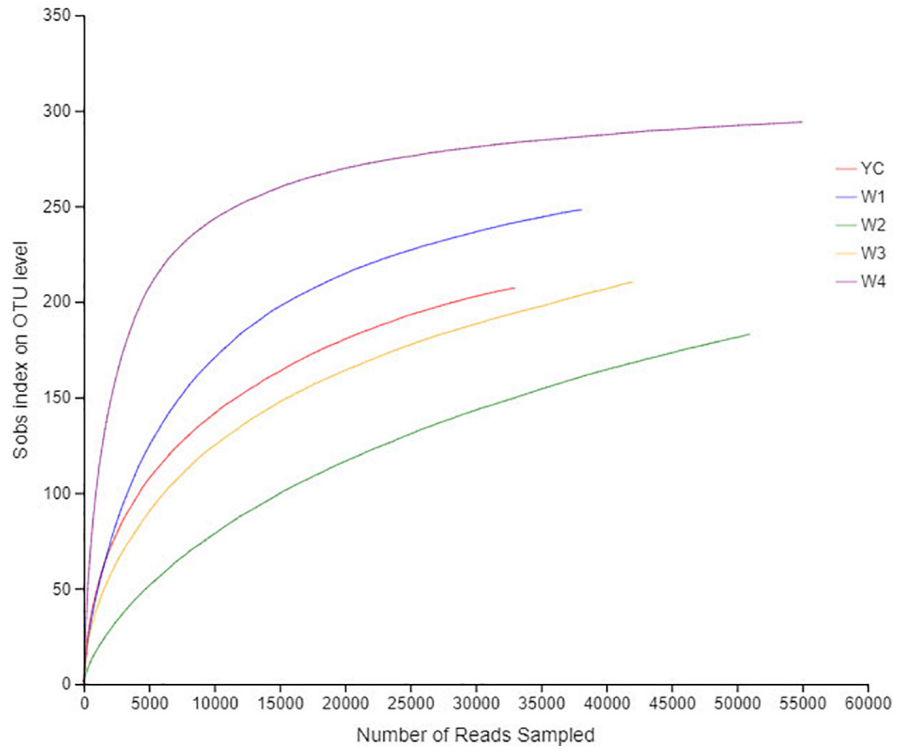

FIGURE 1 | Rarefaction curves of all samples. The abscissa represents the amount of randomly selected sequencing data; the ordinate represents the number of species observed. The flat curve shows that the amount of sequencing data for this study is sufficient.

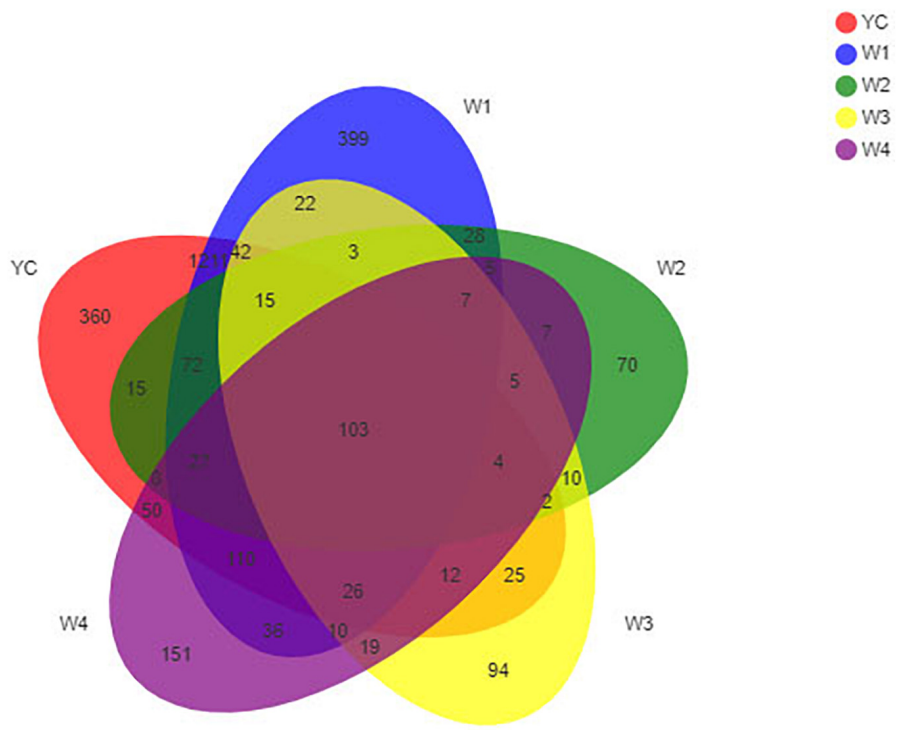

FIGURE 2 | OTU Venn analysis. Different groups are represented by different colors, and numbers in overlapping portions represent the number of species common among all groups.

of Sichuan dark tea has undergone significant changes during pile-fermentation.

Microbial Abundance and Diversity Analysis

Bacterial community richness and diversity was explored in Sichuan dark tea samples collected at different stages of pile-fermentation. Noticeably, the sequencing coverage of all samples was higher than 0.9984, which represents the actual conditions of bacterial communities present in Sichuan dark tea samples collected at different stages of fermentation (**Table 2**). In general, the higher Shannon and lower Simpson indices represents the higher bacterial community diversity index in a sample (Huang et al., 2018). We observed the highest bacterial community diversity in Sichuan dark tea samples collected at YC and W4 stages, followed by the W1 period, and the lowest bacterial community diversity was observed in samples collected at the W2 stage.

To investigate the abundance of the bacterial community structure in Sichuan dark tea collected at different stages of fermentation, the Chao and ACE indices were measured. We observed the highest bacterial community abundance in dark tea samples collected at the YC and W4 stages, followed by samples collected at the W1 stage, and the lowest bacterial community abundance was observed in samples collected at the W2 and W3 stages. Our results show that the abundance and diversity of the bacterial community present in Sichuan dark tea samples was quite different at different stages of fermentation.

Bacterial Community Structure Analysis

All Sichuan dark tea samples collected at different stages of pile-fermentation were analyzed to disclose the structure of the bacterial communities. Bacterial communities with higher abundance were annotated and clearly classified into 18 genera, and bacterial communities with relatively low abundance were merged together into a single category expressed by others (**Figure 3**). For example, bacterial communities with relatively high abundance at the genus level were *Pseudomonas*, unclassified_f_Enterobacteriaceae, *Bacillus*, *Kocuria*, *Ralstonia*, *Lactobacillus*, *Staphylococcus*, etc. (**Figure 3**). At the YC stage of fermentation, the significantly higher bacterial communities were unclassified_Enterobacteriaceae (26.38%), and the relatively high abundant bacterial communities at the genus level were *Kocuria* (15.72%), *Streptomyces* (5.92%), *Pseudomonas* (5.02%), *Bacillus* (4.73%), and *Staphylococcus* (4.19%).

At stage W1, the dominantly higher bacterial communities were unclassified_Enterobacteriaceae (26.38%), and the relatively high abundant bacterial communities at the genus level were *Pseudomonas* (4.91%), *Lactobacillus* (3.18%), *Bifidobacterium* (2.90%), and *Bacteroides* (2.10%). Similarly, the highest abundance of bacterial community at stage W2 was unclassified_ Enterobacteriaceae (51.55%), followed by *Pseudomonas* (41.50%), unclassified_Bacteria (2.15%), and the relative abundance of other bacterial genera were all less than 1%. *Pseudomonas* (61.61%) was an absolutely dominant bacterial strain at the W3 stage, and with relatively high abundance were unclassified_ Enterobacteriaceae (8.98%), *Kocuria* (6.86%), and unclassified_Bacteria (6.97%). At stage W4, the highest relative abundance was of *Bacillus* (33.20%), followed by *Kocuria* (18.63%), *Lactobacillus* (13.74%), *Staphylococcus* (7.92%), and *Ralstonia* (7.39%). We also observed that *Pseudomonas* and unclassified_Enterobacteriaceae displayed higher abundance at the YC, W1, W2, and W3 stages, and *Bacillus*, *Kocuria*, and *Lactobacillus* have higher abundance in the W4 period (**Figure 4**). Our results show that the bacterial diversity has obvious differences with the different piling-fermentation stages of Sichuan dark tea.

TABLE 2 | Bacterial community richness and diversity indices of Sichuan dark tea.

Sample	Shannon	Simpson	ACE	Chao	Coverage
W1	1.65	0.3649	287	284	0.9984
W2	0.78	0.6875	242	214	0.9988
W3	1.67	0.4025	260	267	0.9987
W4	2.47	0.1857	289	293	0.9995
YC	2.21	0.1854	329	315	0.9988

First column consists upon sample name, columns 2–4 represent Shannon, Simpson, ACE and Chao 1 values of diversity index in each sample. Last column contains average of values of diversity index.

Effect of Bacterial Community Composition on Active Ingredients

Redundancy analysis (RDA) is a type of PCA analysis that is constrained by environmental factors. For the pictorial representation of the relationship between bacterial flora at the genus level and environmental factors, samples and environmental factors were drawn on the same 2-D sequence diagram (**Figure 5**; Huhe et al., 2017). Among the four active compounds of Sichuan dark tea, polysaccharides have a positive correlation with total flavonoids, and caffeine has a positive correlation with amino acids. Furthermore, correlation was analyzed among the active compounds of dark tea, bacterial taxa, and different stages of pile-fermentation. We observed that effective formation of caffeine and amino acids predominantly exists at the following stages of fermentation: YC, W1, and W2.

Noticeably, the polysaccharides and total flavonoid contents were increased significantly during pile-fermentation stage W4. Similarly, the following bacterial genus *Bacillus*, *Ralstonia*, and *Saccharopolyspora* were significantly correlated with total flavonoid contents, and *Kocuria* was closely related with the formation of polysaccharides. The dominant bacterial genus at stage W3 was *Pseudomonas*, which displayed no relationship with any of the abovementioned four active ingredients of dark tea. These results prove that the biosynthesis of active ingredients of Sichuan dark tea were significantly affected by the composition of the bacterial community found in pile-fermentation.

DISCUSSION

The time required to accomplish the pile-fermentation cycle of dark tea and development of flavor, color, fragrance,

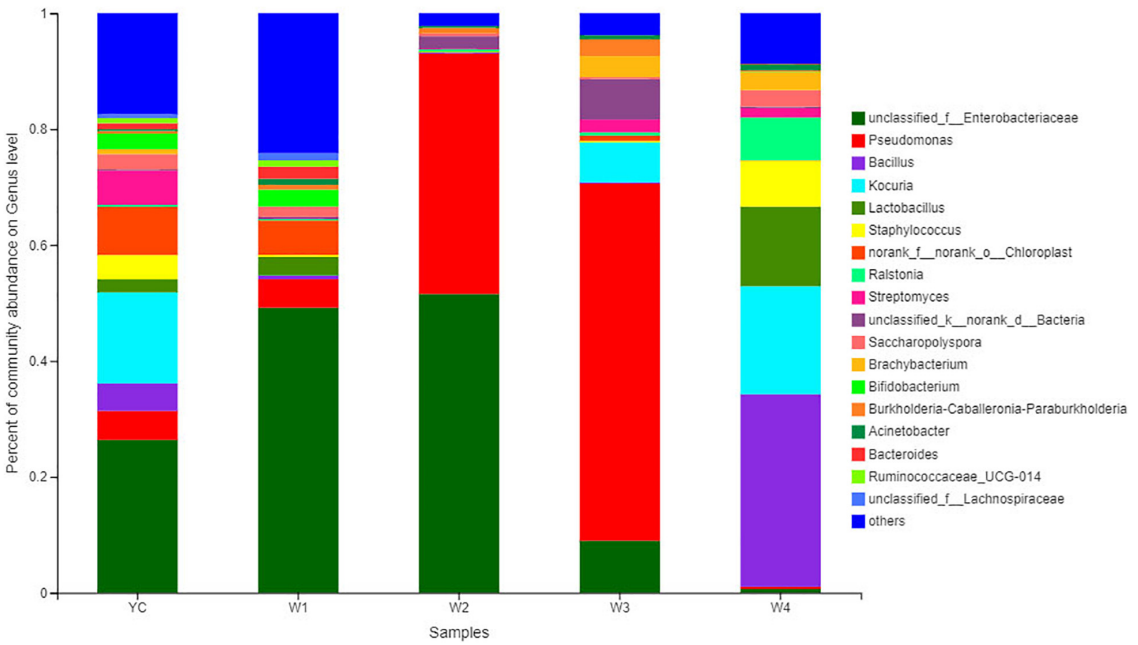

FIGURE 3 | Bacterial community structure bar plot analysis at the genus level. The ordinate is the sample name, and the abscissa is the proportion of different taxa at the genus level in a sample. Different taxa are represented by columns with different colors, sizes, and proportion of species.

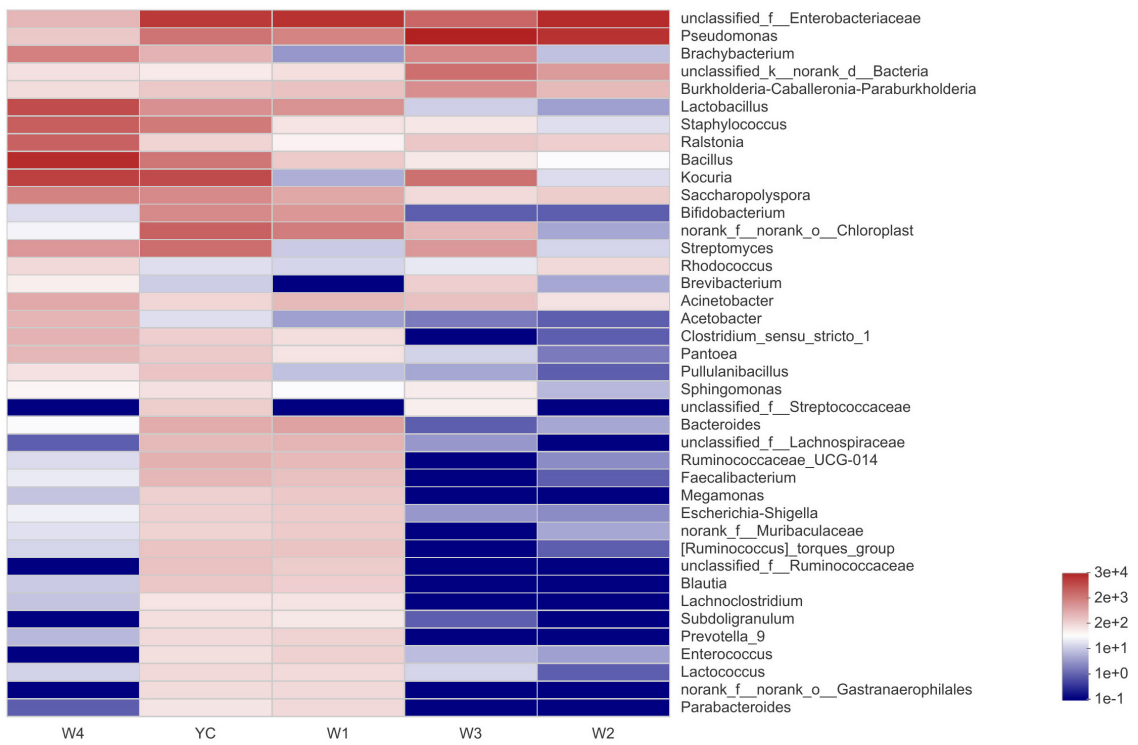

FIGURE 4 | Community heat map analysis at the genus level. The abscissa represents the group name, and the ordinate represents the taxa name. Abundance changes of different taxa in each sample are displayed by color gradient of the color block. The bar on the right side of the figure represents the abundance value by color gradient.

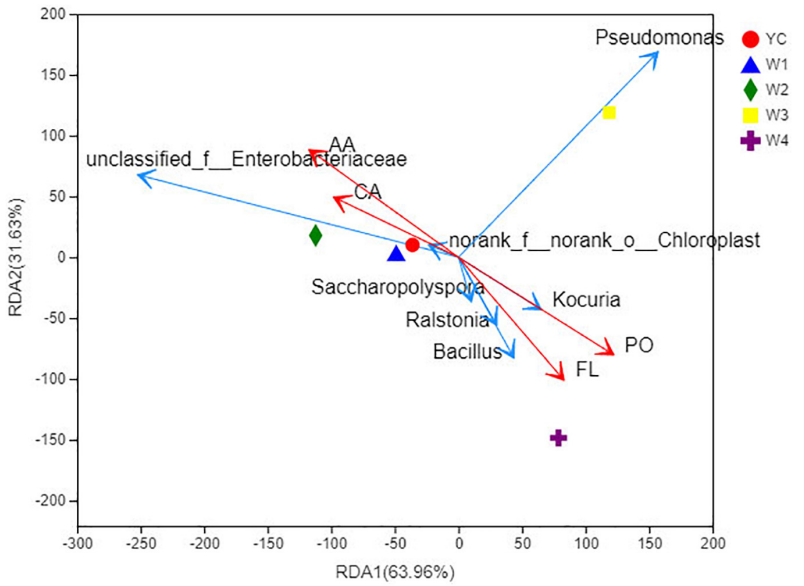

FIGURE 5 | RDA at the genus level. Points of different colors or shapes represent sample groups under different environments or conditions in the figure; taxa are represented by green arrows in the RDA diagram; quantitative environmental factors are represented by red arrows. The length of the environmental factor arrow can represent the degree of influence of the environmental factor on the taxa data; the angle between the environmental factor arrow represents the positive and negative correlation (acute angle: positive correlation; obtuse angle: negative correlation; right angle: no correlation). Projection from the sample point to the arrow of the quantitative environmental factor, the distance between the projection point and the origin, represents the relative influence of the environmental factor on the distribution of the sample community. PO, polysaccharides; FL, flavonoids; CA, caffeine; AA, amino acids.

and nutritional value are predominantly determined by the composition and fermentation stage-specific variations in the microbial community present in the fermentation reaction (Chai et al., 2015). Better understanding of the microbial community in the piling process is a prerequisite for precise manipulation of microbial flora to improve the aesthetic value of Sichuan dark tea. On the other hand, traditional culturing methods to explore microbial flora in dark tea samples is time-consuming, labor-intensive, and unsustainable because all bacterial species cannot be cultivated. We employed robust high-throughput Illumina Miseq^{TM} sequencing technology, which revealed 253,526 high-quality bacterial sequence reads with an average length of 422.53 bp. Furthermore, the clustering of valid sequencing data revealed 2,948 OTUs at a 97% identity threshold of 16S rRNA, which were classified as 42 phyla, 98 classes, 247 orders, 461 families, 1,052 genera, and 1,888 species. These findings lead us to conclude that bacterial flora present in the fermentation process are a result of inoculation from tea leaves (epiphytic and endophytic microbes) and the surrounding environment.

In recent years, only fungi have been the focus of researchers exploring the composition of the microbial community in dark tea, such as *Eurotium cristatum* (Yan et al., 2021), but knowledge about the bacterial community is scarce (Zeng et al., 2020). Bacterial flora displayed a lagging growth phase at the early flowering stage of *Camellia sinensis* due to the exponential growth of *E. cristatum*, which transformed into bacterial exponential growth at the late flowering stage to establish a symbiotic relationship with *E. cristatum* (Liu et al., 2014). Noticeably, the bacterial flora present in the dark tea of different regions is unique (Zhao et al., 2017). In Sichuan dark tea, two relatively abundant bacterial genera were unclassified_f_Enterobacteriaceae and the *Pseudomonas* sequence analyzed during the YC to W3 stages of fermentation. Certain members of the *Enterobacteriaceae* family retain disease-causing potential, encompassing beneficial commensal microbiota opportunistic pathogens that can inflict considerable morbidity and mortality on compromised hosts, and a few are harmless and are widely distributed in the environment, including soil, water, plants, insects, and animals (Ito et al., 2019); that is why members of this genus were abundantly found (51.55%) during the early stages of pile-fermentation (W2). *Pseudomonas*, *Vibrio*, and *Staphylococcus* are generally considered to be conditional pathogens in dark tea (Williams et al., 2010). Comparatively, *Pseudomonas* was higher, 61.61%, in the dark tea samples collected at the W3 stage, and further studies are invited to explore its specific role.

The highest diversity in the bacterial community was observed at the quality-determining W4 stage of Sichuan dark tea. For example, *Bacillus* was highest, 33.20%, at the W4 stage, probably due to the decrease in temperature, which shows that the composition of the bacterial community during pile-fermentation is temperature-dependent (Endres et al., 2011). We can control the distribution of the bacterial community by regulating the temperature during dark tea pile-fermentation and, ultimately, the quality of dark tea. For example, the quick drying of dark tea is performed at 50°C during the final stage, W4, and at which abundance of *Bacillus* is also highest. The genus *Bacillus* are probiotics that have beneficial health effects on the intestines of animals and humans, play a role in the frequency and characteristics of feces, and skin properties (Endres et al., 2011). For example, *B. coagulans* is a health beneficial food

ingredient, included in the qualified safety presumption (QPS) list by the European Food Safety Agency (2008), and approved by the United States Food and Drug Administration (Pozen et al., 2011) recognized safety standards (GRAS) (Zhao et al., 2013). Because the probiotic bacterial strain *B. coagulans* is dominant during the pile-fermentation process of dark tea, it can also be employed to improve the flavor and shelf life of different foods.

Other probiotic genera, *Lactobacillus* and *Bifidobacterium*, were also abundantly found at the final stage, W4, and these are also found in the human intestine and are widely used in the production of various types of fermented foods (Handjiev and Kuzeva, 2018). For example, the relative abundance of *Lactobacillus* was higher (13.74%) at the final stage, W4, and it helps in digestion, is beneficial for human and animal intestines, and is being widely used in the biosynthesis of yogurt and sausage (Cavalheiro et al., 2019). *Lactobacillus* present in black tea is also beneficial for intestinal flora and the health of mice (Arellano et al., 2020; Gao et al., 2020). The share of *Saccharopolyspora* was 2.85% at the W4 stage, which is an antitumor agent (Liu et al., 2005); therefore, it can be used in food processing to increase the health benefits of dark tea. Additionally, Butenyl-spinosyn is produced by *Saccharopolyspora*, which is a strong insecticidal agent with a broad pesticidal spectrum (Rang et al., 2021).

Biological active compounds present in Sichuan dark tea, such as polysaccharides, flavonoids, caffeine, and amino acids, are highly correlated with the abundance of bacterial genus during pile-fermentation. During the process of pile-fermentation, the concentration of these biologically active components declined due to microbial metabolic activities, and our findings are in accordance with Pu'er tea (Li et al., 2018). Similarly, cellulase, hemicellulase, and protease enzymes secreted by microbes during metabolic activities also have a catalytic effect on the main metabolites (Abe et al., 2008). Further studies are invited to unravel the relationship between biologically active ingredients of dark tea and the microbial diversity during the pile-fermentation process of Sichuan dark tea so that the quality of dark tea can be improved.

CONCLUSION

Sichuan dark tea is economically important due to its unique aroma, and it has been prepared *via* pile-fermentation for decades. Microbes play a crucial role in the development of its unique aroma, nutritional value, and physical characteristics. In-depth knowledge about piling stage-specific bacterial composition will provide benchmarks for precise inoculation of probiotic bacteria for biofortification. High-throughput sequencing of the bacterial 16S rRNA gene revealed that certain members of the family *Enterobacteriaceae* were dominantly present in early piling stages YC, W1 and W2; *Pseudomonas* was dominant at stage W3; and the highest bacterial diversity was at stage W4. We observed that probiotic bacterial genera, such as *Bacillus*, *Lactobacillus*, *Bifidobacterium*, and *Saccharopolyspora* were in abundance at the final piling stage W4. In conclusion, precise inoculation of members of these bacterial genera in dark tea might improve its nutritional value and health benefits.

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: European Nucleotide Archive, accession no: PRJEB46897.

AUTHOR CONTRIBUTIONS

KY, MA, AE-S, and XZ: conceptualization. KY and MA: writing—original draft. KY, LY, LM, AD, LW, AE-S, and HC: drawing figures. XZ, QL, AE-S and MA: editing and proofreading. KY and XZ: supervision. KY: project administration. 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.2021.735618/full#supplementary-material

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Tradition as a Stepping Stone for a Microbial Defined Water Kefir Fermentation Process: Insights in Cell Growth, Bioflavoring, and Sensory Perception

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A process development from a traditional grain-based fermentation to a defined water kefir fermentation using a co-culture of one lactic acid bacterium and one yeast was elaborated as a prerequisite for an industrially scalable, controllable, and reproducible process. Further, to meet a healthy lifestyle, a low ethanol-containing product was aimed for. Five microbial strains—*Hanseniaspora valbyensis*, *Dekkera bruxellensis*, *Saccharomyces cerevisiae*, *Liquorilactobacillus nagelii*, and *Leuconostoc mesenteroides*—were used in pairs in order to examine their influence on the fermentation progress and the properties of the resulting water kefir products against grains as a control. Thereby, the combination of *H. valbyensis* and *L. mesenteroides* provided the best-rated water kefir beverage in terms of taste and low ethanol concentrations at the same time. As a further contribution to harmonization and reduction of complexity, the usage of dried figs in the medium was replaced by fig syrup, which could have been proven as an adequate substitute. However, nutritional limitations were faced afterward, and thus, an appropriate supplementation strategy for yeast extract was established. Finally, comparative trials in 5-L scale applying grains as well as a defined microbial consortium showed both water kefir beverages characterized by a pH of 3.14, and lactic acid and aromatic sensory properties. The product resulting from co-culturing outperformed the grain-based one, as the ethanol level was considerably lower in favor of an increased amount of lactic acid. The possibility of achieving a water kefir product by using only two species shows high potential for further detailed research of microbial interactions and thus functionality of water kefir.

Keywords: water kefir, co-culture, lactic acid bacteria (LAB), yeast, bioflavoring, process development, yeast extract

INTRODUCTION

Traditionally fermented beverages enjoy increasing popularity against the background of a conscious and healthy lifestyle (Kandylis et al., 2016). Especially sour fermented beverages, such as kombucha or kefir, promise health benefits, e.g., antimicrobial, anticancer, or probiotic, by their indigenous microorganisms (Prado et al., 2015; Tamang et al., 2016; Ghosh et al., 2019). Thereby, water kefir (WK), which is based on sugary water (usually sucrose is used) and a fruit component, offers a vegan and lactose-free alternative. The characteristic microorganisms of WK are lactic acid bacteria (LAB) and different yeasts, but also acetic acid bacteria can be involved (Bourrie et al., 2016). The microorganisms are organized in so-called grains that are based on exopolysaccharides mainly produced by the LAB (Nielsen et al., 2014).

Generally, the so-called cross-feeding occurs when LAB and yeast are fermented in co-culture meaning that both groups of organisms can profit from the metabolic products of one another (Bader et al., 2010; Smid and Lacroix, 2013; Stadie et al., 2013; Zhang et al., 2017). This is especially important in the case of WK, where the nutrients in general and nitrogen in particular are limited and may induce sulfurous off-flavors (Vardjan et al., 2013; Laureys et al., 2018). Lactobacilli produce lactate in the course of their carbohydrate metabolism, which yeast can absorb and break down. This prevents a sharp drop in pH and aids the lactobacilli in continuing their usual metabolic activities. The yeasts in turn produce a large number of amino acids and also some vitamins, such as vitamin B6 (Stadie et al., 2013), which are essential nutrients for lactobacilli (Ponomarova et al., 2017; Zhang et al., 2017). Due to this interaction, stimulated growth of lactobacilli is often observed in a co-culture compared with the pure culture (Stadie et al., 2013; Vardjan et al., 2013; Bechtner et al., 2019). Further studies describe culture-related differences in the expression of genes that affect the metabolism of carbohydrates and amino acids (Yamasaki-Yashiki et al., 2017; Bechtner et al., 2019), as well as exopolysaccharide formation (Yamasaki-Yashiki et al., 2017) and aggregation factors (Bechtner et al., 2019) involved. In addition, during co-cultivation, the aerobic metabolism of the yeasts reduces the oxygen content of the nutrient medium, which in turn can be beneficial for the growth of lactobacilli (Mendes et al., 2013; Nejati et al., 2020). Although some general modes of interactions between different groups of microorganisms are known (Nejati et al., 2020), the relationship between different strains and whether the interactions are synergistic or counteractive is still not fully understood and remains a topic of current research. Additionally, the mixture of the specifically involved organisms results in a distinct profile of organic acids and exopolysaccharides that have an important influence on the sensory properties of the final beverage (Bader et al., 2010; Bertsch et al., 2019; Jin et al., 2019).

Besides the consortium of microorganisms, also the fruit component is rather complex. Traditionally, e.g., figs, dates, raisins, and/or lemons, were used to produce WK (Açik et al., 2020). However, as with every natural ingredient, quality can vary; and there may occur seasonal effects that influence the industrial production. Moreover, the choice of the fruit component significantly influences the fermentation process by the provision of nutrients for the involved microorganisms as well as the final sensory properties of the beverage.

In summary, industrializing the production of WK with a complex mixture of raw materials and microorganisms is very difficult in terms of final product properties and especially reproducibility (Laureys and de Vuyst, 2017; Nejati et al., 2020). To the knowledge of the authors, successful industry-scale water kefir products are scarce because the management of the fermentation process to obtain reproducible products is similarly complex as the microbial consortia themselves. Therefore, the companies keep their recipes and control strategies guarded. A systematic understanding of mutual interactions aids in the specific control of the production process resulting in safe, defined, and reproducible products. Consequently, efforts to develop defined fermentations applying specific starter cultures are undertaken, whereby a reasoned selection of production strains is of utmost importance. Thereby, one can assume that the level of interactions in a defined co-culture is lower than a complex microbial community, posing the risk of also resulting in a less complex product. However, the application of defined co-cultures has the potential to be better controllable due to its lower level of complexity resulting in less "adjustment screws" and thus lower product deviations.

In order to get a deeper insight which microorganisms originating from natural WK grains contribute synergistically to the fermentation progress resulting in an organoleptic characteristic beverage, systematic experiments to reduce the microbial complexity were conducted. Furthermore, the main focus was the production of a lactic acidic beverage by co-culture fermentation, which should preferably contain a low amount of alcohol meeting the consumer demands for a healthy drink. Thereby, the influence of the available nutrients, such as amino acids and trace elements, on the microbial interactions and the sensory properties represented by different microbially built chemical compounds as well as possible limitations were considered. This paper finally presents a comparison of a traditionally produced complex WK and a fermented beverage product produced with specific starter cultures mimicking WK regarding the fermentation parameters as well as the final product properties.

MATERIALS AND METHODS

Microorganisms

In the present studies, WK grains were used. These have been used successfully for many years for the traditional production of WK via back-slopping processes. The microbial strains used as defined cultures were isolated and identified from these mentioned grains. Therefore, grains were homogenized by using the ULTRA-TURRAX® T25 basic (IKA® -Werke GmbH & CO. KG, Staufen, Germany), and different dilutions of the homogenized liquid were plated on yeast extract (YE) dextrose agar as well as MRS agar according to De Man et al. (1960). Incubation took place at 26, 30, and 37°C under aerobic and anaerobic conditions. Pure cultures were gained by repeating the plating step of single colonies several times. From those single colonies, material was taken for DNA extraction (Phire™ Plant

Direct PCR Master Mix, Thermo Fisher Scientific Inc., Waltham, MA, United States) followed by PCR amplification using primers 8(F) (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492(R) (5′-GGTTACCTTGTTACGACTT-3′) for bacteria (Turner et al., 1999) and ITS1(F) (5′)-TCCGTAGGTGAACCTGCGG-3′) ITS4(R) (5′-TCCTCCGCTTATTGATATGC-3′) for yeast (Op De Beeck et al., 2014) followed by sequencing and blasting the resulting PCR products for species identification. Selected isolates of LAB and yeast were transferred in the VLB strain collection and further examined in fermentation studies: *Hanseniaspora valbyensis* Hs-0302 (Han), *Dekkera bruxellensis* Br-0115 (Dek), *Saccharomyces cerevisiae* Sa-07366 (Sac), *Liquorilactobacillus nagelii* La-3804 (formerly *Lactobacillus nagelii*; Liq), and *Leuconostoc mesenteroides* Le-0304 (Leu). Every yeast strain was combined with every bacterium in co-culture, which led to six applied combinations.

Media Composition and Preparation

Two different media, herein referred to as basis medium and modified medium, were used for the fermentation studies. The basis medium was established on a common recipe for WK production. In detail, it was composed of the following (per liter): 1 dry fig (Alesto, Lidl Dienstleistung GmbH & Co. KG, Neckarsulm, Germany), 60 g of brown cane sugar (Fairglobe, Lidl Dienstleistung GmbH & Co. KG), and 7.5 mL of lemon juice concentrate (Solevita Zitrone, Lidl Dienstleistung GmbH & Co. KG). The modified medium was composed of the following (per liter): 9 mL of fig syrup (Schoeneberger, Salus Pharma GmbH, Bruckmühl, Germany), 60 g of brown cane sugar, 7.5 mL of lemon juice concentrate, and a defined concentration of either X-SEED KAT YE or X-SEED Peptone (Ohly GmbH, Hamburg, Germany), a yeast peptone. Both yeast derivatives were of food grade quality. For media preparation, sugar, dry fig/fig syrup, and YE when applicable were dissolved in boiling tap water (80% of needed volume). After being cooled down to room temperature (20°C), lemon juice concentrate was added, and batches were filled up to desired volume with lukewarm tap water.

Experiments

Pre-cultivation

The strains were pre-cultured in two steps. In both steps, yeast strains were pre-cultured in 250-mL shaking flask at 130 rpm and 30°C for 72 h in YE dextrose (YED) medium containing 50 g/L of glucose (AppliChem GmbH, Darmstadt, Germany) and 10 g/L of YE (SERVABACTER®, SERVA Electrophoresis GmbH, Heidelberg, Germany), pH 5.5. LAB were pre-cultured as stand culture in closed 250-mL bottles at 30°C for 72 h in MRS medium according to De Man et al. (1960) from Difco™ (Becton Dickinson GmbH, Franklin Lakes, NJ, United States). Pre-culture 1 was inoculated with 1% (v/v) of cryo-culture stocks, and pre-culture 2 was inoculated with 1% (v/v) of pre-culture 1. Before the main cultures were inoculated, the microbial suspensions were washed in 0.9% sodium chloride solution to reduce the influence of media components. WK grains were cultivated in a typical matrix [60 g of sucrose (AppliChem GmbH, Darmstadt, Germany), 100 g of pre-washed grains, and two dried figs per liter, 26°C] over several 3-day cycles in the back-slopping process.

Testing of Different Combinations of Microbial Strains

In the first part of the studies, fermentations with different combinations of two microbial strains in co-culture were investigated. Thereby, $1 \cdot 10^6$ yeast cells/mL and $1 \cdot 10^7$ LAB/mL were applied in co-culture. The ratio yeast/LAB (1:10) was derived from the respective cell concentrations in the supernatant of WK fermented by grains. For comparison, fermentations with WK grains were investigated simultaneously. Thereby, 100 g of WK grains per liter was used for the fermentation. For the investigations, the basis medium, which contained dried fruits, was used. The fermentations were conducted in 1-L glass bottles with loosely sealed caps at 22°C (±2°C) (room temperature) as stand cultures. At the start of the fermentation and before sampling, the samples were mixed by mild horizontal shaking. The sampling was executed once per day for offline analyses, which included determination of cell count, high-performance liquid chromatography (HPLC) analysis, pH measurement, and sensory analysis. After 7 days of fermentation, the products showed the best obtainable characteristics and were analyzed toward a wide range of volatile components (see section "Analyses of Volatile Components"). The fermentation studies were executed in biological duplicates. The most appropriate combination of microorganisms was then applied in further trials.

Testing the Impact of Different Yeast Extracts and Concentrations During Fermentations With Leu + Han

In further experiments, the components of the basis medium were adapted to make the composition more defined, further called modified medium. Thereby, the impact of the supplementation of either X-SEED KAT YE or X-SEED Peptone (Ohly GmbH), a yeast peptone, was tested in the concentration of 0.2 and 1.0 g/L each. Roughly, these two yeast supplements differed in their protein content (75% in X-SEED KAT vs. 67% in X-SEED Peptone) and composition [higher proportion of short-chain peptides and amino acids in X-SEED KAT (available at X-SEED product sheets)]. Furthermore, the modified medium contained fig syrup in a defined volume (cf. section "Media Composition and Preparation") to avoid the usage of whole dried fruits. The sampling was executed once per day for offline analyses, which included determination of cell count, HPLC analysis, pH measurement, and sensory analysis. After 7 days of fermentation, aroma analyses of the final products took place. The fermentation studies were executed in biological duplicates.

Five-Liters Fermentation With Water Kefir Grains or the Co-culture of Leu + Han Under Defined Conditions

Finally, fermentations in 5-L bioreactors with WK grains as well as the co-culture combination Leu + Han with the modified medium were performed. For this purpose, 4.5 L of modified medium including 1.3 g/L of X-SEED KAT and 4.5 L of modified medium without YE was used in the case of the co-culture Leu + Han and WK grains, respectively. Fermentations took place in 5-L Biostat® B Twin bioreactors (Sartorius AG, Göttingen, Germany), at 26°C with a low

stirring rate of 20 rpm and no aeration. The pH value and dissolved oxygen (pO_2-%-saturation) were measured online via EASYFERM PLUS VP PH/RX 325 and VISIFERM DO ECS 325 H0 (Hamilton Germany GmbH, Gräfelfing, Germany) probes. The sampling was executed three times a day for offline analyses, which included determination of cell count, HPLC analysis, pH measurement, and once-per-day sensory analysis. Fermentations were carried out in biological triplicates. Data from identical sampling times were shown as the mean value of a triple, double, or single sampling and were marked accordingly.

Analytical Methods

Determination of Total Cell Concentration by Coulter Counter

The total cell concentration was analyzed by using impedance measurement (Multisizer^{TM} 3, Beckman Coulter GmbH, Brea, CA, United States). In the case of samples including grains, only the liquid fraction was analyzed. Ten microliters of the sample was diluted in 10 mL of Isoton II, and 50 μL thereof was analyzed using a 30 μL capillary. With the use of the Multisizer^{TM} 3 Software Version 5.53, the pulse data were converted to size features. In the case of co-culturing, LAB and yeast strains were used, which differed in their cell sizes; and thus, a discrimination of the respective populations was possible. Particles with a size of 0.6–2.0 μm were considered as LAB cells, whereas particles with a size of 2.0–10.0 μm were considered as yeast cells based on cell size distribution in pure cultures, respectively. Their concentration per mL was calculated. A potential overlap of the LAB and yeast populations by crossing cell sizes was in neither case significant and thus was neglected for the determination of the real population distribution ratio, which was a good approximation. For the strains used, a chain formation could not have been microscopically verified in the conditions applied in the experiments.

Determination of Low-Molecular Sugars, Ethanol, and Organic Acids

The analyses of low-molecular sugars (in detail glucose, fructose, and sucrose) and ethanol and organic acids (more detailed lactic acid and acetic acid) were conducted via HPLC (Knauer Wissenschaftliche Geräte GmbH, Berlin, Germany) applying 10 μL of sample on a Nucleogel® Ion 300 OA column (Macherey-Nagel GmbH & Co. KG, Düren, Germany) at 40°C column temperature. The separation of the target compounds was achieved using an isocratic elution with 5 mmol/L of H_2SO_4 at a flow rate of 0.4 mL/min. The sugar and ethanol detection was performed in a refractive index detector. Organic acids were detected via multiple wavelength detector at a wavelength of 210 nm. The residual sugar was defined as the sum of glucose, fructose, and sucrose.

Analyses of Volatile Components

The final fermented products as well as the respective unfermented beverage bases were tested for a number of volatile components and fatty acids, which are described in detail as follows.

Quantification of Acetaldehyde, Higher Alcohols, and Acetate Esters

The quantification of acetaldehyde, higher aliphatic and aromatic alcohols, and acetate esters was determined by static headspace gas chromatography with flame ionization detection (HS-GC-FID) according to method 9.39 outlined in the European Brewery Convention (EBC) (Analytica-EBC, 1997). The GC-FID instrument used was a Shimadzu GC-2010 (Shimadzu Corp., Kyôto, Japan) equipped with a DB-Wax (60 m × 0.32 mm × 0.5 μm film thickness) from Agilent (Santa Clara, CA, United States). Quantification of higher alcohols and acetate esters was reached using butan-1-ol and phenol as internal standards added at a concentration of 15 mg/L. The calibration ranges of the compounds differed and were as follows: acetaldehyde (1.3–75 mg/L), ethyl acetate (1.3–75 mg/L), propanol (2.5–75 mg/L), 2-methylpropan-1-ol (isobutanol) (2.5–75 mg/L), 3-methylbutyl acetate (isoamyl acetate) (0.2–10 mg/L), 2-methyl-1-butanol (2.5–75 mg/L), 3-methyl-1-butanol (2.5–100 mg/L), phenyl ethanol (2.5–75 mg/L), and phenylethyl acetate (0.05–5 mg/L). The lowest concentration of the calibration range was defined as limit of quantification for each compound.

Determination of Ethyl Esters

Ethyl butanoate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, and ethyl dodecanoate were determined by headspace–solid-phase microextraction (HS-SPME) gas chromatography–mass spectrometry (GC-MS). The GC-MS system consisted of a Shimadzu GC 2010 interfaced with a MS-QP2010 Plus (Shimadzu Corp.) equipped with a Gerstel MPS 2XL auto sampler (Gerstel, Linthicum Heights, MD, United States) for automated HS-SPME sampling. Data evaluation was done using the GCMSsolution software Version 4.45 SP1 (Shimadzu Corp.). Esters were extracted from 2 mL of liquid sample using a 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber (Supelco, Bellefonte, PA, United States). The column used for chromatographic separation was a HP-5MS UI column [30 m × 0.25 mm i.d. × 0.25 μm film thickness from Agilent (Santa Clara)]. The extraction and GC parameters were used as described by Dennenlöhr et al. (2020a). The mass spectrometer was operated in selected ion monitoring (SIM) mode, using the following qualifier and quantifier ions: ethyl butanoate (m/z = 71 and m/z = 60), ethyl hexanoate (m/z = 88 and m/z = 101), ethyl octanoate (m/z = 88 and m/z = 127), ethyl decanoate (m/z = 88 and m/z = 73), and ethyl dodecanoate (m/z = 88 and m/z = 101). The calibration ranges for all ethyl esters were 5–1,000 μg/L. The lowest concentration of the calibration range (5 μg/L) was defined as limit of quantification. Isotopically labeled d_5-ethyl hexanoate (m/z = 93 and m/z = 106) was used as internal standard at a concentration of 100 μg/L.

Determination of Volatile Sulfur Components

The analyses of volatile sulfur components were accomplished by application of two separate HS-SPME GC-MS/MS methods that were both run on an Agilent Technologies 7890B gas chromatograph interfaced to a 7000C Triple Quadrupole mass spectrometer (Agilent). This GC-MS/MS setup was equipped with a Gerstel MPS 2XL sampler (Gerstel) for

automated HS-SPME sampling, and the column used for chromatographic separation was a HP-5ms Ultra Inert GC Column (30 m × 0.25 mm, × 0.25 μm film thickness from Agilent). Agilent MassHunter Workstation Software—Qualitative Analyzes (ver. B.07.00) was used for data analyses. Method 1 covered methanethiol, ethanethiol, propane-1-thiol, and butane-1-thiol. The second method covered the thioesters and sulfides, namely, *S*-methyl thioacetate, ethyl thioacetate, carbon disulfide, diethyl sulfide, dimethyl disulfide, and dimethyl trisulfide. Sample preparation for the analyses of volatile thiols was done using the on-fiber derivatization (OFD) assay as published by Dennenlöhr et al. (2020b). The conditions during sample preparation and the GC separation were also as previously published. The mass spectrometer was operated in electron impact ionization (70 eV) multiple-reaction ion monitoring (MRM) mode using the following transitions for quantification and qualification: methanethiol (m/z = 228 → 181 and m/z = 181 → 161), ethanethiol (m/z = 242 → 181 and m/z = 213 → 45), propane-1-thiol (m/z = 256 → 181 and m/z = 214 → 181), and butane-1-thiol (m/z = 270 → 89 and m/z = 213 → 181). Analytes were calibrated in a range from 5 to 500 ng/L (methyl mercaptan: 100–10,000 ng/L) and the lowest concentration of the calibration range (5 ng/L for ethanethiol, propane-1-thiol, and butane-1-thiol and 100 ng/L for methanethiol) was defined as limit of quantification. 1-Hexanethiol was used as an internal standard (m/z = 117 → 83 and m/z = 298 → 117), and it was added to the samples at a concentration of 100 ng/L at the very beginning of the sample preparation. Sample preparation for the analysis of thioesters and sulfides was done under similar conditions as described for the thiols, while the OFD step was skipped. The GC temperature program used to separate the components of interest was as follows: start at 30°C, ramp with 2°C/min to 41°C, then ramp with 40–140°C, then ramp with 60–300°C. The mass spectrometer was operated in MRM except for carbon disulfide that was analyzed in SIM with selected ions m/z = 76 (quantification) and m/z = 78 (qualification). The MRM transitions used for quantification and qualification of the remaining components were *S*-methyl thioacetate (m/z = 90 → 43, no qualifier transition), ethyl thioacetate (m/z = 104 → 43 and 43 → 42), diethyl sulfide (m/z = 90 → 62 and 75 → 47), dimethyl disulfide (m/z = 122 → 94 and 94 → 66), and dimethyl trisulfide (m/z = 126 → 61 and 79 → 45). Isotopically labeled d_6-dimethyl trisulfide (m/z = 132 → 82 and 114 → 50) was used as an internal standard and was added to the samples at a concentration of 1 μg/L at the very beginning of the sample preparation. The calibration ranges of the components differed and were as follows: carbon disulfide (0.005–0.1 μg/L), *S*-methyl thioacetate (0.025–20 μg/L), ethyl thioacetate (0.005–4 μg/L), diethyl sulfide (0.005–2 μg/L), dimethyl disulfide (0.005–4 μg/L), and dimethyl trisulfide (0.005–1 μg/L). The lowest concentration of the calibration range was defined as limit of quantification for each component.

Quantification of Short- and Medium-Chain Fatty Acids

Short- and medium-chain fatty acids, herein referred to as fatty acids, were determined by HS-SPME-GC-FID. The GC-FID system used was a Shimadzu GC-2010 (Shimadzu Corp.) equipped with an Agilent CP Wax 58 FFAP (50 m × 0.32 mm × 0.32 μm). HS-SPME sampling was done using a Gerstel MPS 2XL auto sampler (Gerstel) equipped with an 85 μm polyacrylate fiber (Supelco). To extract the fatty acids, 2 mL of aliquots of the liquid samples was acidified by addition of 80 μL of 1 M HCl in 10-mL amber headspace vials. To enrich components in the headspace above the sample, sealed headspace vials we incubated for 15 min at 50°C (500 rpm), followed by an extraction for 15 min at 50°C. The loaded fiber was then desorbed for 1 min at 250°C using a split ratio of 2. A temperature program starting at 60°C, followed by a ramp of 17°C/min to 150°C, and followed by a ramp of 8°C/min to 220°C was used to separate the target analytes. The calibration ranges were as follows: butanoic acid (0.15–12 mg/L), 3-methyl butanoic acid (0.05–4 mg/L), pentanoic acid (0.03–2.4 mg/L), hexanoic acid (0.05–4 mg/L), octanoic acid (0.1–8 mg/L), decanoic acid (0.04–3.2 mg/L), and dodecanoic acid (0.03–2.4 mg/L). The lowest concentration of the calibration range was defined as limit of quantification for each component. 4-Methyl pentanoic acid was used as internal standard; it was added to the samples at a concentration of 3 mg/L prior to acidification with HCl.

Sensory Analyses

Three trained experts in tasting of sour fermented beverages analyzed sensory properties of the fermentation products. For this purpose, a descriptive and evaluative analysis scheme for sour fermented beverages established at the VLB was used. After sampling, all beverages were stored in closed screw-cap tubes for a maximum of 2 days at 4°C and brought to room temperature shortly before tasting. Samples were tasted in a non-blinded manner.

RESULTS

In the following, the fermentation progress of the trials examining different microbial combinations (section "Different Lactic Acid Bacteria–Yeast Combinations for Producing Water Kefir Beverages") as well as adapted media (sections "Using Fig Syrup Compared With Dry Figs Based on Fermentation With Water Kefir Grains" and "Effect of Different Yeast Extracts and Concentrations on the Performance of Leu + Han During Water Kefir Fermentation") aiming for a more defined WK production process is shown. Finally, 5-L bioreactor fermentations applying the appropriate microorganism combination compared with fermentations with original WK grains were performed (section "Five-Liters Bioreactor Fermentation With Water Kefir Grains and Defined Co-culture Leu + Han").

Different Lactic Acid Bacteria–Yeast Combinations for Producing Water Kefir Beverages

Firstly, systematic experiments to reduce the microbial complexity were conducted. Therefore, combinations of one lactic acid bacterium and one yeast strain were applied in basis medium against the usage of grains as a control. The focus was on the production of a lactic acidic beverage, which should

preferably contain a low amount of alcohol besides an overall balanced sensory perception.

Figure 1 demonstrates the differences of cell growth and pH changes during 7 days of fermentation caused by different microorganism combinations. Firstly, the usage of WK grains led to different courses in pH and the concentrations of cells in the liquid phase, metabolites, and volatile components compared with defined combinations (cf. **Figures 2**, **3**). After at least 4 days, the residual sugar was close to zero, and the alcohol content increased to a mean value of finally 24 g/L (3.0 Vol.-%) by the usage of grains, which is shown in **Figure 2**. In mean, 2 g/L of lactic acid and 1 g/L of acetic acid were produced. The acid concentrations were higher than those produced by the co-cultures. This led to the fastest pH decrease resulting in a final pH value of 3.5 displayed in **Figure 1C**.

The grains showed the fastest rise of bacterial cell growth compared with the defined co-cultured bacteria cells (1 vs. 2 days to end exponential growth phase). However, at the end of the fermentation, similar bacterial cell concentrations were reached ($\sim 1 \cdot 10^8$ cells/mL). In contrast, lower final yeast cell counts of $7.64 \cdot 10^6$ cells/mL than in co-culture ($\sim 2.5 \cdot 10^7$ cells/mL on average), which showed similar progress of the yeast cell counts, were observed. In the co-culture, the bacteria cell growth depended on the combined yeast. Consequently, the combinations including Dek showed the lowest yeast cell growth compared with the other combinations but the fastest bacteria growth in case of co-culture, which resulted in an amount of 1.8 g/L of lactic acid (cf. **Figure 2**) and led to low pH values of 3.81. In conjunction with a low sugar consumption (1.2% observed sugar reduction), a low amount of ethanol was produced during the fermentation processes including Dek (<2.0 g/L; ∼0.25 Vol.-%). The low cell growth of Dek in the beginning allowed a faster growth of the LAB. These observations indicate an interaction between the used LAB and Dek regarding the competition for nutrients and differ from the interaction of other used yeasts, Han and Sac.

Combinations with Han and Sac led to ethanol concentrations of ∼6 g/L (∼0.76 Vol.-%), which was sensory recognizable in the case of Liq + Han. The combination of Leu + Sac showed the lowest pH decrease to finally 4.3 (cf. **Figure 1**). This is in accordance with the lowest observed acid production of ∼1 g/L of lactic acid (cf. **Figure 2**), and the sensory evaluation, in which only a weak acid was perceptible (cf. **Figure 4**). **Figure 4** demonstrates less pronounced typical WK-like sensory attributes—low sourness, carbonization (in case of combinations with Leu), and fruitiness for instance. Sac seemed to have been inhibiting or have less promoting effects on Leu, which could be concluded from the lower bacteria cell concentration ($6.57 \cdot 10^7$ cells/mL) compared with that of the other co-cultures ($>1 \cdot 10^8$ cells/mL) and the resulting lower acidity due to less produced lactic acid.

Figure 3 shows the concentration of different volatile components in the unfermented basis medium as well as the final fermented WK products after 7 days. The unfermented basis medium containing dried figs, sugar, and lemon juice concentrate contained a few volatile components in a detectable range. These were smaller fatty acids as well as low amounts of methanethiol and related sulfurous components (cf. **Figures 3C,E,F**). In the fermented products, concentrations of the volatile components were generally higher but varied from product to product. The volatile profile of WK fermented by usage of grains differed from that observed for WK from co-cultures. Within the WK resulting from co-cultures, volatile profile differed, and this is attributed to the combination of different microorganisms. Ethyl acetate was the most dominant form of volatile component and was detected in the products containing the yeasts Han and Sac in combination with Liq and Leu, respectively, but did not occur in a detectable range in the product using grains. The detected amounts of higher aliphatic alcohols and esters differed depending on the used combination (cf. **Figures 3A,B**). It is conspicuous, that either grains or microbial co-cultures produced certain components of these higher aliphatic alcohols and esters. The combination of Dek + Liq as well as Dek + Leu led to no formation of those alcohols. It is of particular note that the latter combination led to the highest amount of fatty acid ethyl esters, which led to the fruity characteristics of the products and which were produced in a much lower quantity by the other combinations. **Figure 3C** displays a much lower

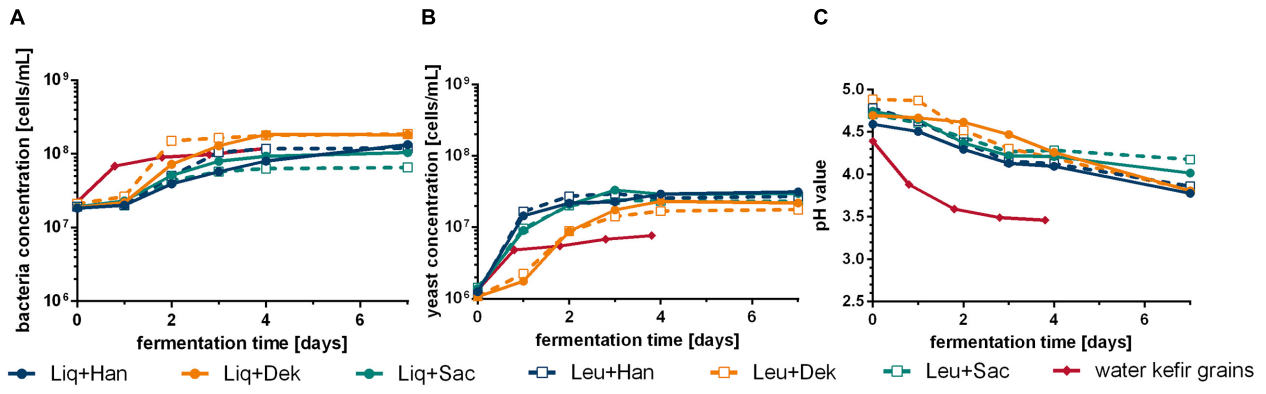


FIGURE 1 | Course of bacteria **(A)** and yeast **(B)** cell concentration and pH change **(C)** during WK fermentation with different starter culture combinations. Data are the mean value of biological duplicates (LAB–yeast combinations) or triplicates (grains). WK, water kefir; LAB, lactic acid bacteria.

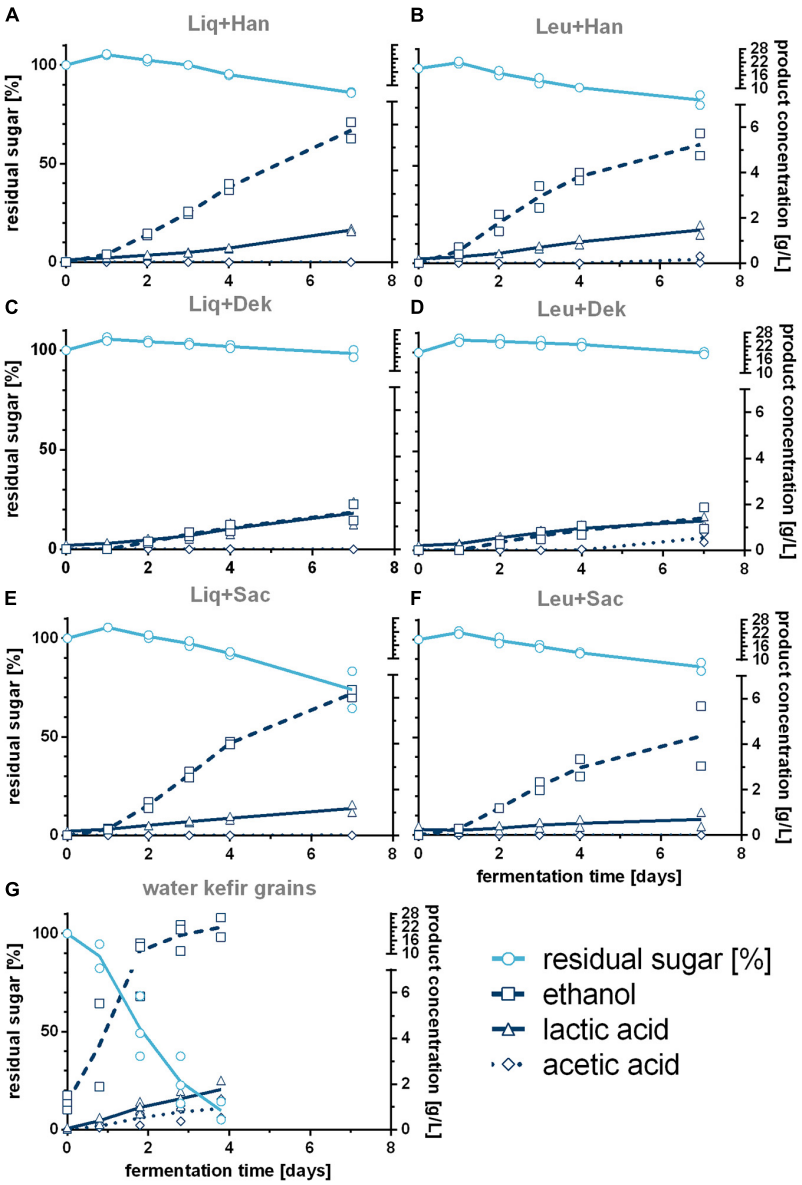


FIGURE 2 | Course of produced ethanol, lactic, and acetic acid and consumed sugar during the WK fermentations with different LAB–yeast combinations **(A–F)** and complex grains **(G)**. Shown are the data of biological duplicates (LAB–yeast combinations) or biological triplicates (complex grains) and the corresponding trend lines. WK, water kefir; LAB, lactic acid bacteria.

amount of fatty acids, especially hexanoic acid as well as octanoic acid, in the fermented products after 7 days compared with the unfermented basis medium. These components have been metabolized by the cells. Although the components butanoic acid and 3-methylbutanoic acid are the most dominant fatty acids, their concentration was either lower than or the same as concentration in the medium.

In contrast to fruity aroma components, thiols and sulfurous components were found in a detectable range in all combinations as well as in the basis medium, which is displayed in **Figures 3E,F**. Thereby, the most abundant component was methanethiol, which acts as a precursor for further sulfurous components such as dimethyl disulfide and dimethyl trisulfide (Kinzurik et al., 2020), and was formed by the microbial combination consisting of Dek + Liq as well as Dek + Leu mostly. Other sulfurous components were produced by the usage of Liq in combination with the yeast Han as well as Sac in the highest amount, e.g., ethanethiol as well as dimethyl disulfide. The specific aroma profile of the products led to a specific sensory taste as well, which is demonstrated in **Figure 4**.

Although the combinations of Dek with Liq and Leu (orange lines in **Figure 4**) showed a high potential in the expression of aimed WK-like attributes, the usage of these combinations was evaluated as not appropriate for beverage production.

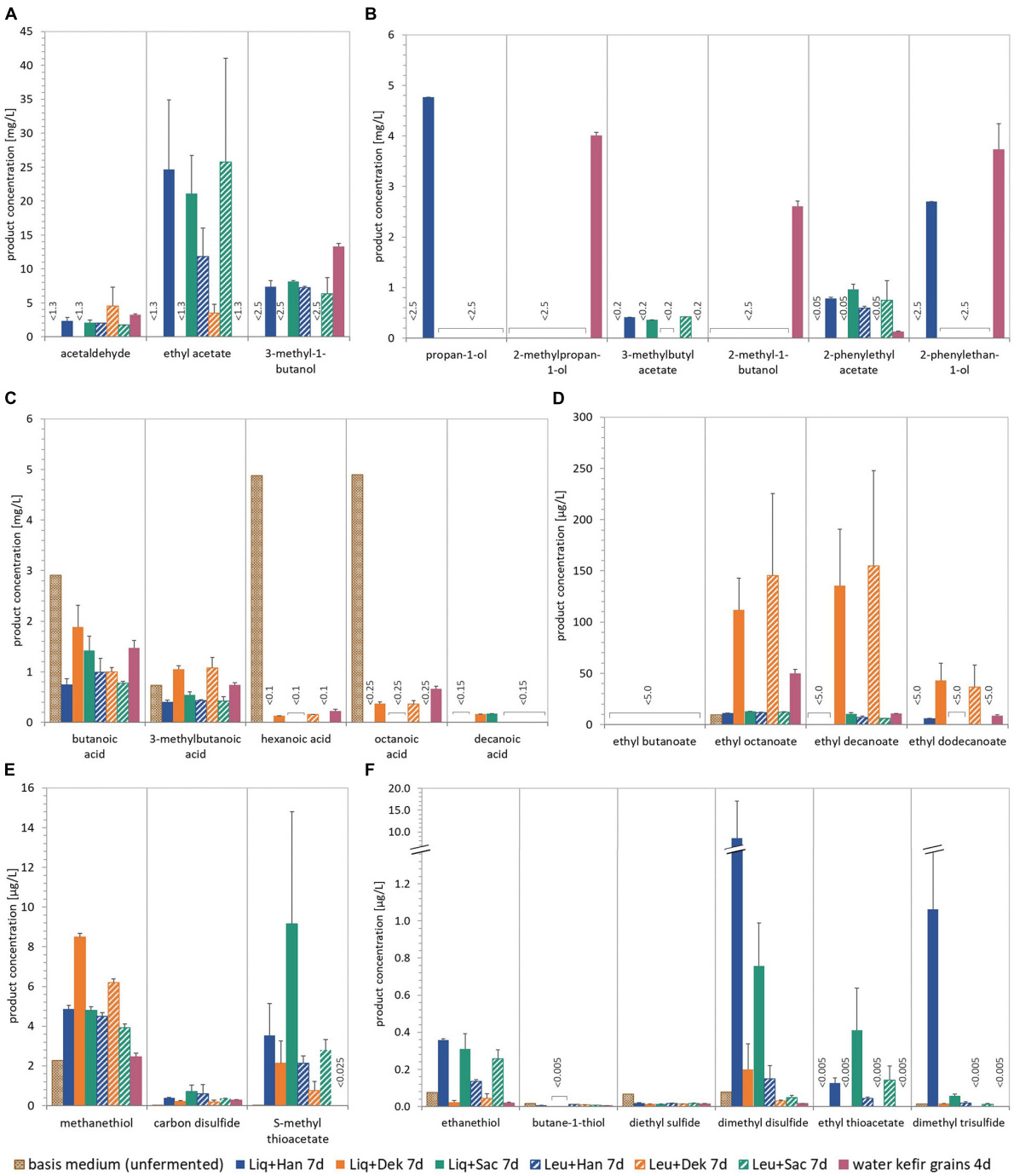
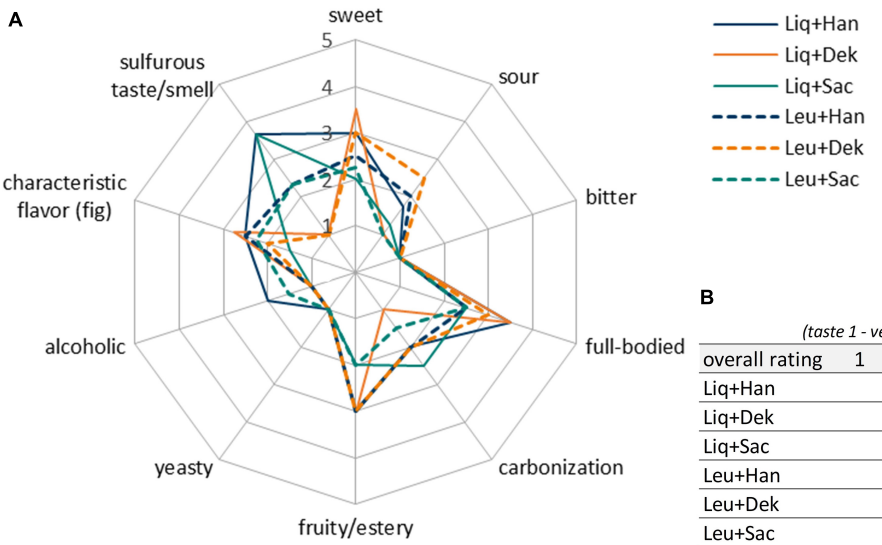


FIGURE 3 | Concentrations of different selected volatile components in the unfermented basis medium and beverages produced with different LAB–yeast combinations and WK grains after 7 and 4 days of fermentation, respectively. The quantified aroma-relevant analytes included higher aliphatic alcohols and esters **(A,B)**, fatty acids **(C)**, fatty acid ethyl esters **(D)**, and thiols and other sulfur components **(E,F)**. The detection limit is specified for the components for which no detection was possible. The analytes pentanoic acid, dodecanoic acid, ethyl hexanoate, and propane-1-thiol (all not shown) were not detected in any sample. Data of the fermented products are the mean value of biological duplicates ± mean deviation. WK, water kefir; LAB, lactic acid bacteria.

After 7 days, the final products were lactic and citric acidic, full-bodied, mildly fruity, and having a dominant fig flavor. However, these products showed a strong off-flavor, which was describable as "mustily," "stuffy," and "stale" (not shown in the sensory panel). This can be attributed to the applied yeast, Dek.

Further, the blue lines in **Figure 4** display the well-balanced composition between sourness and sweetness of the



B

(taste 1 - very good; 5 - bad)

overall rating	1	2	3	4	5
Liq+Han			X		
Liq+Dek				X	
Liq+Sac				X	
Leu+Han		X			
Leu+Dek			X		
Leu+Sac					X

FIGURE 4 | Sensory properties of products fermented with different LAB–yeast combinations after 7 days of fermentation. Shown are the mean data of two samples produced in biological duplicates. The rating scale of the descriptive analysis **(A)** ranges from 1 (imperceptible) to 5 (very pronounced). The overall rating of the taste **(B)** ranges from 1 (very good) to 5 (bad taste). LAB, lactic acid bacteria.

final products fermented by Han + LAB after 7 days. These results determined the final fermentation duration of 7 days. In contrast, the fermented products by using the yeast Sac showed both lactic and acetic acid taste represented by green lines in **Figure 4**. Apart from the desired product properties, a highly pronounced sulfurous taste and smell occurred during fermentation, especially in combinations with Liq. The products smelled and tasted like rotten eggs. The most promising products were fermented by the yeast Han. Thereby, the combination with Liq showed stronger alcoholic and sulfurous taste as well as less sourness than in combination with Leu.

Summarizing, the fermentations with the co-cultivation of LAB and yeasts lasted longer to get a final product than the fermentations with grains. The fermentation with WK grains was faster and led to a more complex taste and a higher diversity in perceptible sensory properties but resulted in a higher content of alcohol in the final product. By using defined starter cultures in a co-cultivation, the formation of ethanol was lower and might be more controllable than by fermentations with grains.

Sensory-wise, the combination of Leu + Han was preferred by the panelists, which was based on a well-balanced characteristic between sourness (lactic acid) and sweetness and the presence of desired attributes such as fruity and carbonized. Based on the results, the combination was chosen for further trials.

Using Fig Syrup Compared With Dry Figs Based on Fermentation With Water Kefir Grains

In order to implement more defined media components in the production process, investigations with fig syrup were executed. For this purpose, different volumes of fig syrup were tested to get the most appropriate concentration equal to the usage of one dry fig per liter. In a test row, basis media were investigated, which differed in the volumes of liquid syrup. Sugar and lemon juice concentrate, as was used above, were added, and sensorial tests were performed. In conclusion, 9.0 mL of fig syrup per liter corresponded to the usage of one dry fig per liter regarding the taste as the basis. Based on this, fermentations with WK grains with the chosen amount of fig syrup compared with the usage of dry figs were performed, and results can be seen in **Supplementary Figure 1**. In this context, the fermentations showed similar behavior in cell growth, pH value decrease, and metabolic activity as well as sensory properties. Thus, fig syrup was used in following studies.

Effect of Different Yeast Extracts and Concentrations on the Performance of Leu + Han During Water Kefir Fermentation

In order to avoid the noticeable presence of sulfurous components in the final product when applying defined starter cultures, two different YEs, developed for LAB cultivation especially, were used as a sufficient supplement of nutrients. The fermentation performance of the microorganism combination Leu + Han without the supplementation of YE was not as good as in the first study (cf. **Figures 1**, **2**). This might be because of the replacement of dry fig (used in experiments shown in **Figure 1**) with fig syrup (experiments shown in **Figure 5**) and an associated lack of nutrients. It was concluded that fig syrup was not sufficient as the only source of nutrition in the case of the here-applied co-cultivation for the WK production. On the contrary, a positive effect of adding YE to the medium for the WK production with two microbial strains was apparent, which was demonstrated by a higher concentration of LAB compared with

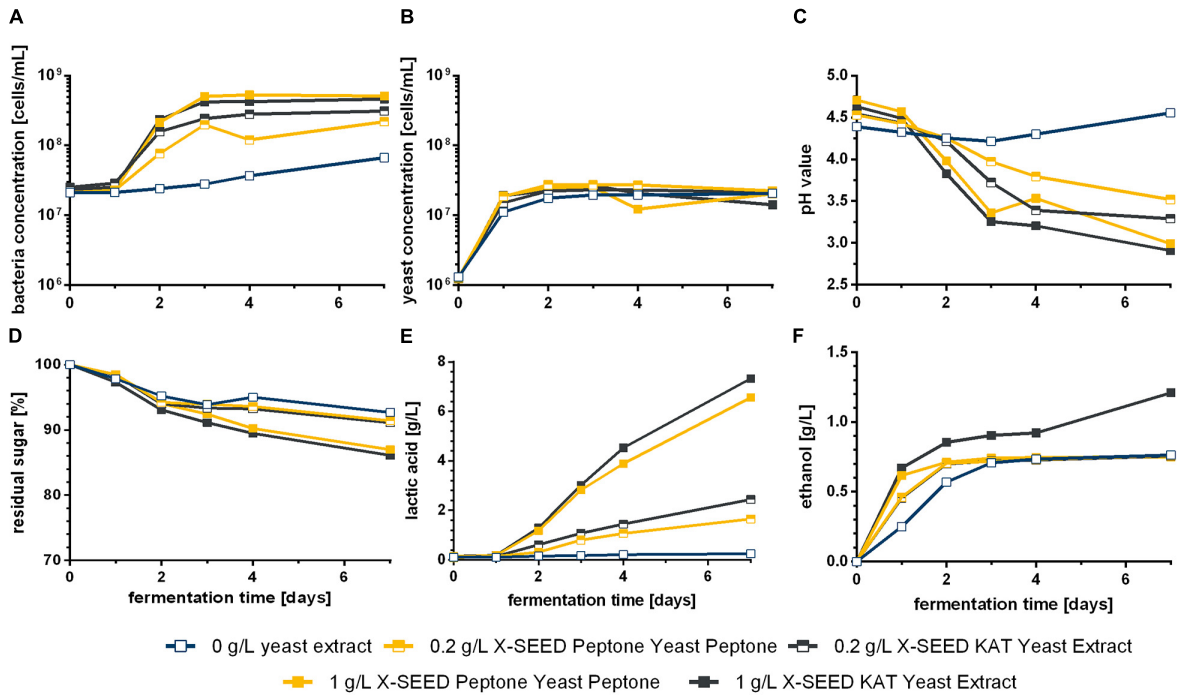


FIGURE 5 | Course of cell concentrations **(A,B)**, pH value **(C)**, residual sugar **(D)**, produced lactic acid **(E)**, and ethanol **(F)** during the WK fermentation by Leu + Han with different types and concentrations of supplemented YE. Data are the mean value of biological duplicates. WK, water kefir; YE, yeast extract.

the fermentation without YE (**Figure 5A**). Thereby, increasing the YE concentration led to a faster growth of LAB. Accordingly, **Figure 5C** shows a faster decrease of the pH based on the added YE, especially 1 g/L of X-SEED KAT. Furthermore, the final pH was lower than the values observed during the fermentation with grains. These high pH decreases are because of the high yield of lactic acid concentrations and can be seen in **Figure 5E**. Lactic acid reached values higher than 7 g/L using 1 g/L of YE, whereas the application of 0.2 g/L of YE led to ∼2 g/L. Concluding, the amount of produced lactic acid differed between different applied concentrations of YE. In contrast, the yeast cell growth was not influenced by different applied YEs and their concentrations (**Figure 5B**); thus, the yielded ethanol concentration differed remarkably after 3 days of fermentation except for the usage of 1 g/L of X-SEED KAT YE with a slightly higher concentration (**Figure 5F**).

Like previously shown, the fermentations with the combination Leu + Han without adding YE differed when using fig syrup or dried fruits. These results (cf. **Figure 5**) were confirmed by the analyses of the volatile components (**Figures 3**, **6**). That is why further results are compared with those of the previously described trials, in which dried figs were used.

The comparison of the volatile components, displayed in **Figure 3** (basis medium) and **Figure 6** (modified medium, 0 g/L of YE), in the two main media largely showed similarities. Differences occurred in the amount of thiols and sulfurous components. Methanethiol was twice as high in the modified medium with fig syrup regardless of the additional usage of YE media compared with the medium with dried figs, and the associated component ethanethiol was 10-fold higher, which is displayed in **Figures 6E,F**, respectively.

The analyses of the volatile components of the products fermented by Leu + Han with the usage of different concentrations of YE and the replacement of dried figs by fig syrup showed a smaller amount of esters and higher aliphatic alcohols, e.g., acetaldehyde and ethyl acetate and can be seen in **Figure 6**, compared with the former experiments (cf. **Figure 3**). The formerly solely detected aliphatic alcohol—2-phenylethyl acetate—was under the range of detection without the supplementation of YE, whereas with the addition of YE, half as much was formed in maximum than observed as shown in section "Different Lactic Acid Bacteria–Yeast Combinations for Producing Water Kefir Beverages." Thereby, the higher the YE concentration, the higher the formed amount was. In contrast to the lower amount of some fermentation by-products, in detail, higher aliphatic alcohols as well as esters (part A and B), the amount of fatty acid ethyl esters was similar or increased. This was associated with the usage of 1 g/L of KAT YE, compared with the former experiments without YE and dried figs, and comparable with the amounts in the unfermented media.

The fatty acid concentration in the products during the supplementation of YE decreased similarly to the product previously described, and no influence of different YEs as well as concentrations was observed. Interestingly, the concentration of butanoic acid of the fermented product without YE was the highest in all experiments at about 24 mg/L. The associated rancid

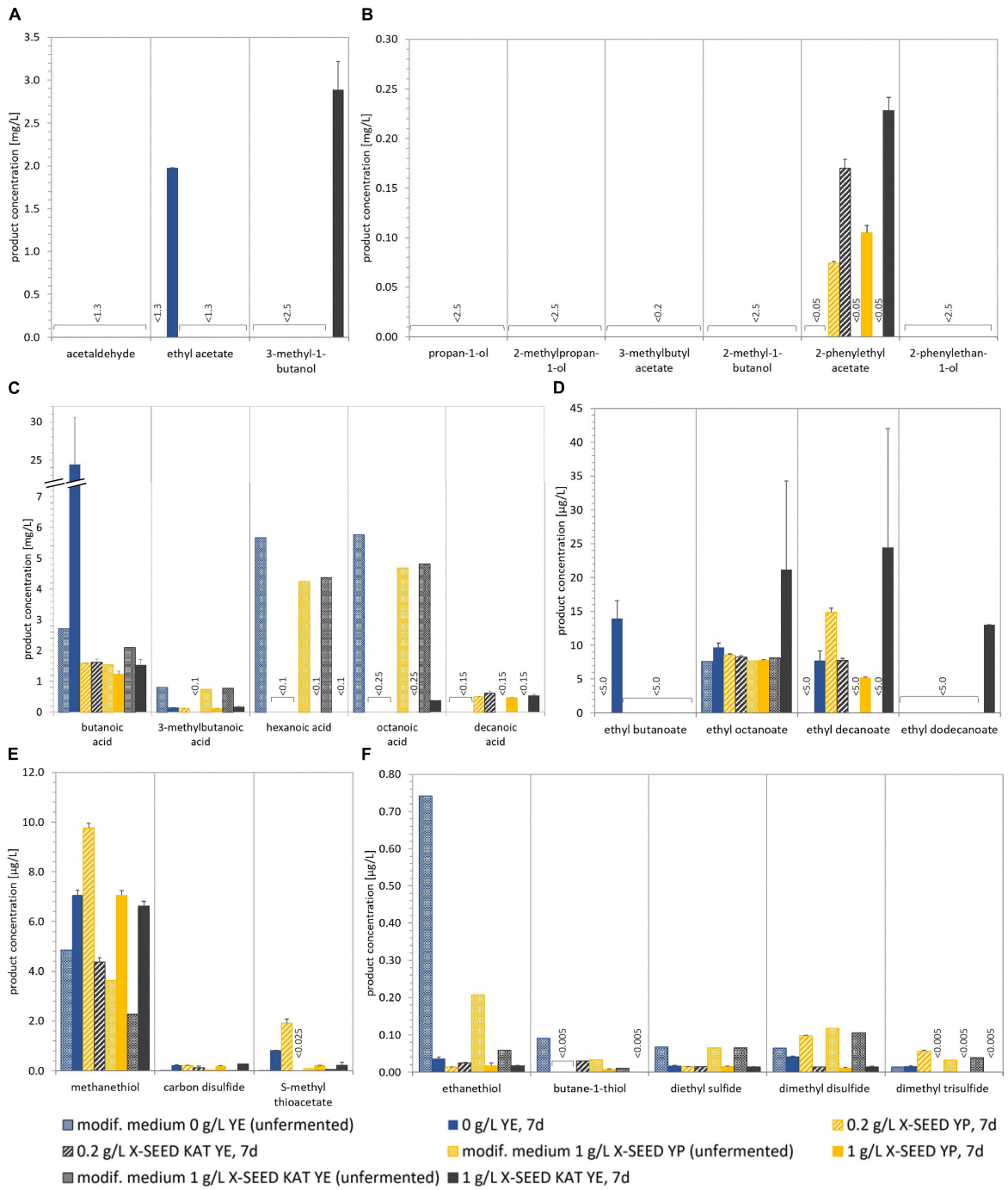
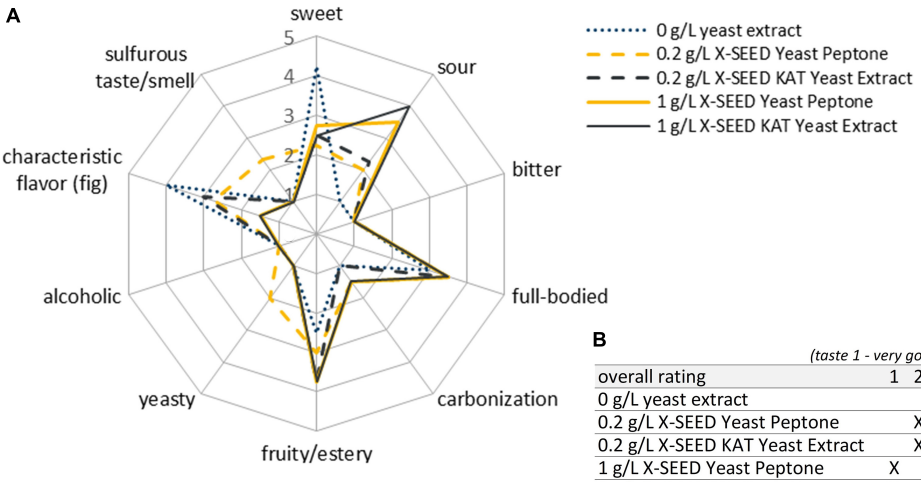


FIGURE 6 | Concentrations of different volatile components in modified media (references) and in 7-day fermented beverages produced with the Leu + Han combination and different types and amounts of YE. The quantified aroma-relevant analytes included higher aliphatic alcohols and esters **(A,B)**, fatty acids **(C)**, fatty acid ethyl esters **(D)**, and thiols and other sulfur components **(E,F)**. The detection limit is specified for the components for which no detection was possible. The analytes pentanoic acid, dodecanoic acid, ethyl hexanoate, and propane-1-thiol (all not shown) were not detected in any sample. Data are the mean value of biological duplicates ± mean deviation. YE, yeast extract.

bad taste was clearly identified in the sensory evaluation as well (cf. **Figure 7**).

Of particular note during these experiments was the presence of sulfurous components, recognizable in detectable volatile components (**Figures 3E,F**, **6E,F**) as well as smell and taste (cf. **Figures 4**, **7**). Although most of the analyzed sulfurous components in the unfermented modified media were concentrated higher than in the basis medium, their



B

(taste 1 - very good; 5 - bad)

	1	2	3	4	5
overall rating					
0 g/L yeast extract				X	
0.2 g/L X-SEED Yeast Peptone		X			
0.2 g/L X-SEED KAT Yeast Extract		X			
1 g/L X-SEED Yeast Peptone	X				
1 g/L X-SEED KAT Yeast Extract	X				

FIGURE 7 | Sensory properties of the products fermented with the microbial combination Leu + Han under the investigation of 0, 0.2, and 1 g/L of two different YEs after 7 days of fermentation. Shown are the mean data of two samples produced in biological duplicates. The rating scale of the descriptive analysis **(A)** ranges from 1 (imperceptible) to 5 (very pronounced). The overall rating of the taste **(B)** ranges from 1 (very good) to 5 (bad taste). YE, yeast extract.

amount was reduced by the addition of YE, except for methanethiol, which was in the same range. The concentrations of ethanethiol were 5- to 10-fold lower as well as the formed amount of dimethyl disulfide, which was about 10-fold lower, except for the addition of 0.2 g/L of X-SEED Yeast Peptone. Thereby, the highest amount of dimethyl disulfide was produced during the trials, which was still ∼30% lower than those without YE. The detected concentration of dimethyl trisulfide in the product without YE was similar to the former experiments, and reduced to levels under the range of detection with the addition of YE, except for the addition of 0.2 g/L of X-SEED Yeast Peptone, which led to 3-fold higher concentrations of this. This reduction in sulfurous components was also observed in the sensory analysis of the products and is displayed in **Figure 7**.

The final products showed improved sensory properties, recognizable in a higher pronounced sourness, more full-bodied products, and more fruitiness as compared with the study without YE in co-cultivation (dotted blue line in **Figure 7**). Thereby, the expression depended on the applied YEs and their concentration. The supplementation of the tested YEs avoided the production of noticeable sulfurous components at the end of the fermentation except for the usage of 0.2 g/L of X-SEED Peptone Yeast Peptone, as shown in the dashed yellow line in **Figure 7**. Concluding, the usage of 1 g/L of YE of both X-SEED KAT and X-SEED Peptone showed the best final products after 7 days of fermentation. That is why the next investigations were performed under the supplementation of X-SEED KAT YE with the microorganism combination Leu + Han. Through the fact that no YE off-flavor was detectable in taste and smell, a slightly higher concentration amounting to 1.3 g/L of YE was used in following studies.

Five-Liters Bioreactor Fermentation With Water Kefir Grains and Defined Co-culture Leu + Han

In order to estimate the reproducibility of the WK production by co-cultivation compared with WK grains, fermentations in 5-L bioreactors were performed (**Figure 8** and **Supplementary Figure 2**). For the fermentation, fig syrup was used, and in case of co-cultivation, 1.3 g/L of X-SEED KAT YE was used additionally as tested before. **Figure 8** shows the averaged values of triple, double, or single sampling.

It is of particular note that the grain-based fermentations gave desirable sensorially similar products after 3, 6, and 5 days, whereas beverages produced by co-cultures were sensorially similar after 7 days of fermentation each. This was not obvious from the measured data but is of importance for later industrial application. The reached cell concentrations of yeast and bacteria were higher in co-cultivation than during the fermentation with grains (∼3-fold for bacteria and 10-fold for yeast). Incidentally, the yeast concentration decreased after 3 days of fermentation by using grains, which is most probably reasoned by the low stirring rate and the impeded homogenization caused by an increasing grain mass during fermentation. The gray lines in **Figure 8** show a similar pH progress in fermentation by using grains and co-cultivation of the two microorganisms until the fourth day of fermentation. Afterward, the pH of the grain fermentation stagnated, whereas the pH in co-culture further decreased to a final value of 3.14.

The usage of grains led to higher ethanol concentrations (23 g/L at 3 days; ∼3 Vol.-%) than the co-cultivation of Leu + Han, accompanied by a nearly complete consumption of the sugar at the fourth day of fermentation, which is comparable with the fermentations in section "Different Lactic

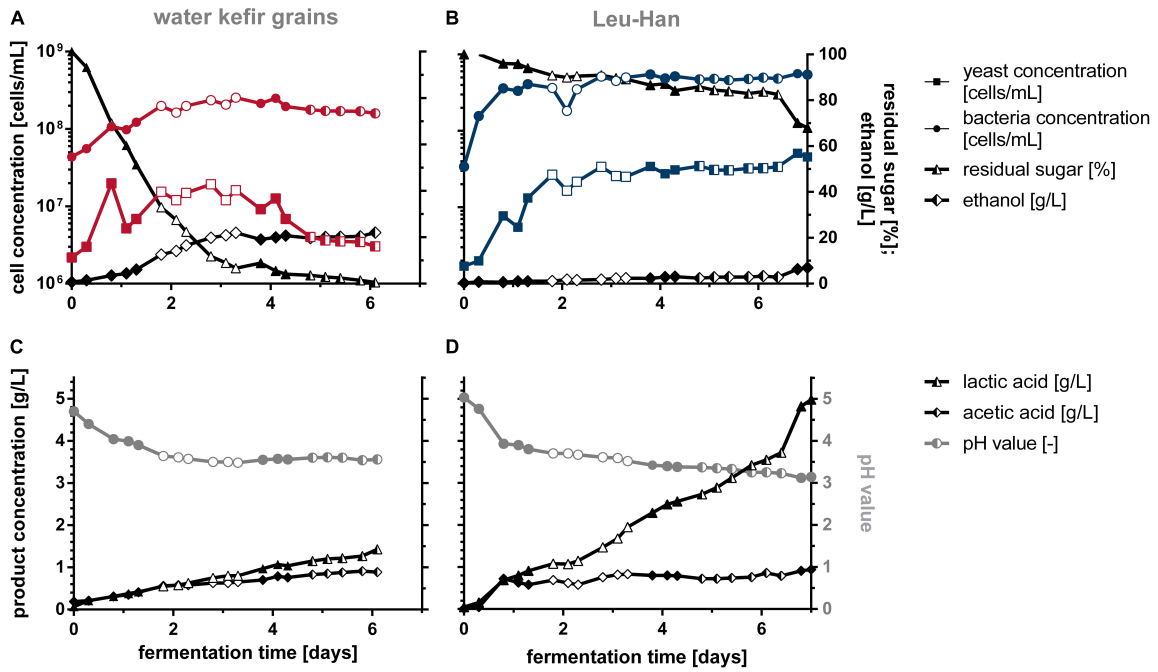


FIGURE 8 | Off-line data of WK fermentations in 5-L bioreactors with grains **(A,C)** and the defined co-culture Leu + Han **(B,D)**. Fermentations were conducted in biological triplicates. Data from identical sampling times are shown as the mean value of a triple (full-filled mark), double (half-filled mark), or single (unfilled mark) sampling. WK, water kefir.

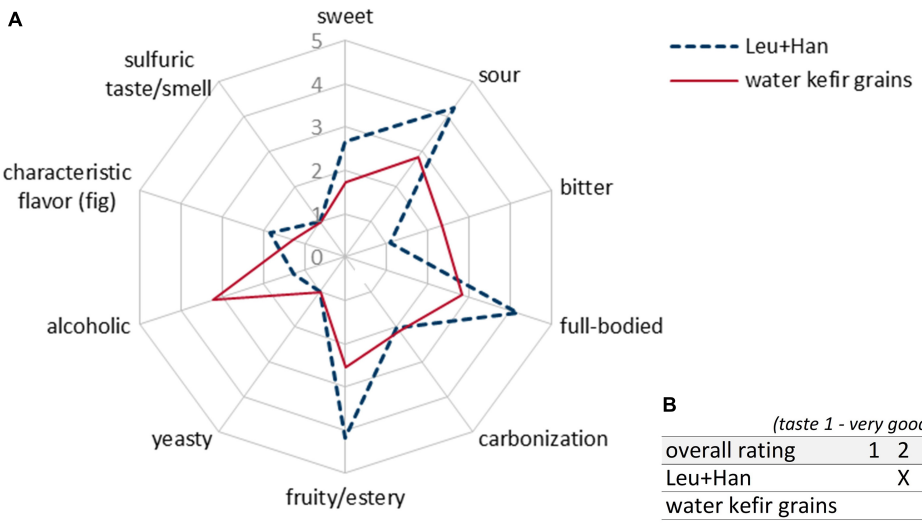
Acid Bacteria–Yeast Combinations for Producing Water Kefir Beverages" in 1-L scale. After 6 days of fermentation with co-cultures, the amount of ethanol increased to 3.3 g/L (0.42 Vol.-%), and to a final value of 7 g/L on average after 1 week of fermentation rapidly. When comparing the three fermentations with co-cultures, the sugar consumption as well as the ethanol content in the end deviated (14.8 vs. 2.7 and 3.5 g/L), although staying below 3 g/L until 6 days of fermentation, which is why the mean value of ethanol was that high. The lactic acid concentration reached higher values by co-cultivation than by the fermentation with grains (4.98 vs. 1.33 g/L), which was recognizable in the taste of the final products.

Additional online analyses of the pH, dissolved oxygen, and redox value are illustrated in **Supplementary Figure 2**. In summary, these data confirm the fermentation processes shown in **Figure 8**. The essential differences, namely, that the sugar is consumed faster when using grains and proportionally fewer organic acids are formed from it, is confirmed by the online curves. It also illustrates the rapid setting of anaerobic conditions in both approaches. The dissolved oxygen was consumed within the first 2.5 h when applying grains, whereas the usage of Leu + Han resulted in full oxygen consumption after 8 h of fermentation. This was largely due to the activity of the yeasts in the beginning of the fermentation, whereby the defined one only contained one yeast strain.

Figure 9 shows the mean scores of sensory properties of biological triplicates at the best time of each single fermentation depending on the best achieved sensory properties as stated above (3, 6, and 5 days for grain fermentations; 7 days for co-culture fermentations). The final products of the fermentation with the co-cultivation of Leu + Han (blue dashed line in **Figure 9**) showed more desirable sensory properties in line with the study aim of a balanced low-alcoholic beverage than the product produced by the fermentation with grains (red line in **Figure 9**). Thereby, a higher value of sourness as well as a fruitier taste was observed (**Figure 9**). Furthermore, the products were more full-bodied and not that much alcoholic than the products fermented by using grains. Further, no off-flavor based on sulfurous taste or smell was observed in any of the products.

DISCUSSION

The aim of this study was to expand the knowledge about the impact of different microbial consortia representing different degrees of complexity on the characteristics of WK. Therefore, LAB and yeasts isolated from WK grains were used for fermentations as defined starter cultures in co-cultivation against the original grains as a control. The focus of the trial was firstly to reduce the complexity of WK fermentations in order to provide a more controllable and reproducible process and secondly to monitor differences in cell growth, the corresponding metabolization of sugars, production of organic acids, and the perceptible taste in combination with chemical analysis of the microbial volatile components.



B

(taste 1 - very good; 5 - bad)

	1	2	3	4	5
overall rating					
Leu+Han		X			
water kefir grains			X		

FIGURE 9 | Sensory properties **(A)** and rating **(B)** of products fermented with the combination of Leu + Han as well as WK grains in 5-L bioreactors. Shown are the mean data of biological triplicates at the best time of each single fermentation depending on the best achieved sensory properties: Leu + Han at 7 days of fermentations. Grain trial 1 (f1) at 3 days, trial 2 (f2) at 6 days, and trial 3 (f3) at 5 days of fermentation. The rating scale of the descriptive analysis **(A)** ranges from 1 (imperceptible) to 5 (very pronounced). The overall rating of the taste **(B)** ranges from 1 (very good) to 5 (bad taste). WK, water kefir.

Water Kefir Production With Complex Grains and Defined Starter Cultures

Based on previous internal studies regarding isolated defined strains from traditional WK grains (data not shown), combinations of two different LAB, *L. nagelii* (Liq) and *L. mesenteroides* (Leu), as well as three different yeasts, *H. valbyensis* (Han), *D. bruxellensis* (Dek), and *S. cerevisiae* (Sac), were applied within the present study. Comparative fermentations with six microorganism combinations in co-cultivation as well as kefir grains were performed.

In summary, the fermentations with grains were much faster than in co-cultivation, which could be explained by a high adaptation of the higher number of microorganisms in grains among one another in contrast to only two species that might not be able to fully compensate for all microbial interactions (**Figure 1**). This is supported by data from Laureys et al. (2021), who also found that WK liquor as a fermentation starter to progress slower as compared with the usage of grains. Metabolic exchanges, which can act as a power source as well as a supply of microbial growth factors, were reported before (Prado et al., 2015), whereby the mutual interactions can appear directly as well as indirectly. In a consortium of several different microbial strains, a possible undersupply of nutrients can be compensated, whereas in co-cultivation, the nutrition exchange is limited to the present microbial strains. That is why the production of WK by using a preferably small number of microbial strains is possible but is less self-regulating and thus requires more process knowledge. However, the conducted fermentations with WK grains resulted in a final product characterized by ethanol levels of around 20 g/L as well as higher amounts of higher alcohols compared with co-culture trials, which could be observed by Laureys and de Vuyst (2014) as well. In line with Chen et al. (2009), who declared that the application of co-culture processes for the WK production could sidestep the intense formation of alcohols, the beverages produced with defined co-cultivations showed significantly lower ethanol formation in the present study. The resulting effects could not be traced back to the performance of one single microorganism in the applied co-cultivations, but rather to microbial interactions.

Combinations including *S. cerevisiae* showed the lowest growth of the investigated LAB in co-cultivation, especially in the case of co-cultivated *L. mesenteroides*, compared with the other applied yeasts, which resulted in the lowest produced amount of lactic acid in association with the lowest observed pH drop (**Figures 1C**, **2**). This resulted in less pronounced WK typical characteristics. In accordance with this, Stadie et al. (2013) observed a poorer growth of *L. nagelii* in co-cultivation with *S. cerevisiae* compared with the co-cultivation with the yeast *Zygotorulaspora florentina* in their investigations of metabolic activity and symbiotic interactions of LAB and yeasts isolated from WK. Similar observations were described by Bechtner et al. (2019). They investigated proteomic analyses of *L. nagelii* in the presence of *S. cerevisiae* isolated from WK. In contrast, the study of Jin et al. (2019) did not demonstrate a negatively affected growth rate of *Lactobacillus plantarum* by the presence of *S. cerevisiae* but a possible inhibition of yeast by *L. plantarum*, which is recognizable in a lower growth rate of the population compared with the single culture of the yeast. It is obvious that a generalization of the microbial interactions during the co-cultivation of a lactic acid bacterium with *S. cerevisiae* as well as other yeast species is not possible and has to be examined individually.

Different gas chromatographic methods were conducted to quantify volatile components in the fermented products. Laureys and de Vuyst (2014) found ethyl acetate, isoamyl

acetate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate as the most prevalent volatile components in their investigations of the microbial species diversity, community dynamics, and metabolite kinetics of WK fermentation with the usage of grains. Except for ethyl hexanoate, these components were produced in different amounts during their fermentations with the tested combinations, whereby ethyl acetate was the most prevalent component (Laureys and de Vuyst, 2014). Walsh et al. (2016) pointed out a correlation between *Saccharomyces* spp. and esters' production during their investigations regarding microbial succession and flavor production in the fermented dairy beverage kefir. In accordance with Walsh et al. (2016), *S. cerevisiae* combined with *L. mesenteroides* led to the highest amount of ethyl acetate in the present study. The usage of *D. bruxellensis* showed a different behavior in the formation of ethyl acetate, whereby in combination with *L. nagelii* no formation and in combination with *L. mesenteroides*, a low formation of ethyl acetate occurred. The presence of higher aliphatic alcohols after 7 days of fermentation was observed, whereby a high variety especially during the usage of grains occurred. The combination of *H. valbyensis* and *L. nagelii* led to the highest detectable amounts of these followed by grains and the usage of *S. cerevisiae*. The combination containing Dek formed a lower amount of higher aliphatic alcohols and no amount of esters in a detectable range. Compared with the other co-cultures, the lowest degradation of fatty acids occurred, which may be attributed to a lower fermentation activity of Dek. In contrast, the associated ethyl esters were present in the highest amount, which led to fruity characteristics of the products. This could be explained by a high noticeable fig taste after 7 days and, consequently, less pronounced sensory properties of these products due to a lower fermentation performance of *D. bruxellensis*. This could be due to slightly lower growth rates of *D. bruxellensis* in the microbial combinations with *L. nagelii* and *L. mesenteroides* compared with the other tested combinations. Former studies including *D. bruxellensis* showed a similar behavior regarding a slow growth rate in the beginning of fermentation (e.g., Abbott et al., 2005), which might promote the LAB growth rate and increase the beginning acidification. Thus, the combinations including LAB and Dek had a specific effect on the fermentation performance favoring LAB growth and acidification. Furthermore, a strong-off flavor, which was describable as "stuffy" and "stale," was observed in these products. In the wine industry, Dek is well-known for the production of the observed flavors and thus considered as a wine-spoilage yeast. Among other studies, this observation was mentioned by Lambrechts and Pretorius (2019) in their investigations of yeasts' importance to wine aroma in context of wine contamination with *D. bruxellensis*. Whereas in other contexts the combination of *Dekkera* and LAB was advantageous [e.g., in a commercial alcohol production process described by Passoth et al. (2007)], in the case of the present study, the usage of this yeast in co-culture was not appropriate for WK production.

In contrast to the presence of fruity notes, in all products except for the combinations with *D. bruxellensis*, a sulfurous off-flavor in taste and smell was observed. This was more pronounced when using *L. nagelii* than *L. mesenteroides*, which led to the conclusion that the production of these components might have been influenced by the presence of the lactic acid bacterium in co-culture. Accordingly, this observation was confirmed by the volatile component analysis. The unfermented medium already contained small amounts of methanethiol, ethanethiol, diethyl sulfide, and dimethyl disulfide most probably originating from the figs. In the fermented products, however, high amounts of methanethiol as well as ethanethiol and dimethyl disulfide, ethyl thioacetate, and dimethyl trisulfide occurred, which share the thiol precursor methanethiol (Landaud et al., 2008; Kinzurik et al., 2020). Landaud et al. (2008) described the formation of these thiols due to the degradation of sulfur/carbon bonds of methionine or cysteine derivates during their investigation of the formation of volatile sulfur components and metabolism of methionine and other sulfur components in fermented food. Particularly hydrogen sulfide, methanethiol and ethanethiol as volatile sulfurous components are often sensed as garlic, rotten eggs, onion, and fermented cabbage (Moreira et al., 2002; Landaud et al., 2008). These components have a negative impact on the beverage aroma, when they are present in an amount higher than their perception thresholds. This is especially observed in wine and often is being traced back to enzymatic formation resulting from yeast metabolism (Moreira et al., 2002). The component H_2S as an undesirable possible by-product during the alcoholic fermentation of *S. cerevisiae* caused by the conversion of cysteine, can further be converted from ethanol to ethanethiol (Landaud et al., 2008), which was in a detectable range during all co-cultivations as well as during the usage of grains. The quantities of the components methanethiol, ethanethiol, dimethyl disulfide, and dimethyl trisulfide were the highest of all tested sulfurous components, whereby the combination of *L. nagelii* and *H. valbyensis* as well as *S. cerevisiae* showed the highest formation.

Irrespective of the occurred off-flavors, the combination of the yeast *H. valbyensis* and the lactic acid bacterium *L. mesenteroides* showed the highest potential in order to develop a water kefir beverage based on the usage of defined microbial strains. This combination in co-cultivation led to the best final product with high-pronounced attributes, such as lactic acidic, full-bodied, fruity, and little carbonized. In this context, the sensory characteristic of the final products played a crucial role in the evaluation of an appropriate combination of microorganisms. Overall, no undesirable microbial interactions were evidenced during this microbial combination, which affected the growth rates as well as microbial metabolisms of these participating microorganisms adversely; thus, this combination was used for further investigations.

Adaption of the Fermentation Basis Medium by Supplementation of Yeast Extract and Substitution of Dried Figs by Fig Syrup

Regardless of the type of the chosen starter cultures, in addition to a carbon source (mostly raw cane sugar or pure sucrose), most dried fruits (e.g., figs or cranberries) are used for the production of WK. Although the latter serves as a source of amino acids, vitamins, and minerals, the small applied amount of dried fruits

compared with the richness in nutrients of fresh fruits leads to a WK medium relatively poor in nutrients (Laureys et al., 2018). One step in the process development in this study aiming for a more defined product was the replacement of dried figs with liquid fig syrup that is well-defined (see Manna-Feigen-Sirup product sheets). Pretests were used to set the concentration in such a way that it corresponded to the previously proven use of dried figs in terms of sensorial and chemical parameters during fermentation, in which 9 mL of fig syrup per liter sugar water was considered as appropriate. The executed fermentations with grains and syrup did not differ to the usage of dried figs in their main performances (**Supplementary Figure 1**). However, the sole use of sucrose and fig extract in co-cultivation led to a nutritional undersupply of the starter cultures and in particular of the applied lactic acid bacterium (*L. mesenteroides*), which was expressed in a reduced bacterial growth rate and acid formation (see **Figure 5**; 0 g/L of YE). This led to an unbalanced product with poor sensory ratings, for instance, high sweetness as well as a high-pronounced characteristic fig flavor and low acidity, which were perceived as unpleasant in this combination (**Figure 7**; 0 g/L of YE). Consequently, in the course of the investigations, two essential factors could be identified that are crucial for the production process of this beverage under defined conditions: efficient nutrient supply and prevention of the formation of sulfurous metabolic products, which can be caused by yeast metabolism, especially in the case of an undersupply of assimilable nitrogen components (Song et al., 2020).

In order to overcome nutritional limitations in co-culture approaches, the supplementation of YE in different concentrations was investigated. Vardjan et al. (2013) described a decreased biochemical activity as well as no growth of various microorganisms, which were separated as pure cultures for milk kefir production, in their studies on the characterization and stability of lactobacilli and yeast microbiota in kefir grains. In order to overcome these issues, they mentioned an improvement by the addition of YE to the growth medium, which was done in the present study as well. The X-SEED KAT YE contains a high content of free amino acids and is rich in B vitamins and minerals, whereas the X-SEED Peptone yeast peptone contains almost 80% of the available amino acids in the form of small peptides. The application of YEs as shown here (**Figures 5**, **6**) promoted growth rates of LAB, which led to higher acidity, and was associated with a higher lactic acid formation and pH drop of the products as compared with fermentations without the supplementation of YE. This was also recognizable in well-balanced sensorial properties of the final products, e.g., a higher acidity and more full-bodied profiles (**Figure 7**). Thereby, the extent of the improved performances was dependent on the concentration of the applied YE: the higher the concentration, the better the fermentation performance of the LAB as well as the yeast. However, there was no clear preference in either of the yeast derivatives, concluding that *L. mesenteroides* was able to use the nitrogen in the form of free amino acids as well as in the form of peptides equally well. Contrarily, no promotion of the growth rate of *H. valbyensis* by adding YE during the co-cultivation with *L. mesenteroides* was detectable at all.

Laureys et al. (2018) investigated the influence of different fruits or YE/yeast peptone on the WK fermentation process using grains. During their studies, different fermentation characteristics, indicated by formation of ethanol (21.3 g/L using figs; 26.1 g/L applying a mixture of YE and peptone) and lactic acid production (2.83 g/L using figs; 2.06 g/L applying a mixture of YE and peptone) among others were observed (Laureys et al., 2018). Interestingly, within this study, no excessive production of ethanol was observed by the supplementation of YE applying the co-cultivation of *H. valbyensis* and *L. mesenteroides* (e.g., ∼1.2 g/L of ethanol using 1 g/L of X-SEED KAT YE). A lower production of fermentation by-products as well as sulfurous metabolic components was observed, which is most likely based on the yeast metabolism. The concentration of methanethiol was in the same range comparing fermentations without YE and with the usage of dried figs (**Figures 3**, **6**). However, the amount of the associated metabolites, for instance, ethanethiol as well as dimethyl disulfide, has been reduced by about 10-fold (**Figures 3**, **6**). This was recognizable in smell and taste. The final products including YE exhibited no sulfurous aroma in smell and taste, except for the usage of 0.2 g/L of X-SEED Peptone yeast peptone. Accordingly, this product showed the highest amount of thiols and sulfurous components detected via gas chromatographic analysis after fermentation.

Concluding, the addition of at least 1 g/L of YE, especially the applied X-SEED KAT YE, promoted a nutritional supply and might balance a lack of nutrition, which avoids the production of high amounts of sulfurous components when defined starter cultures are used. Furthermore, this effect allowed the replacement of dried figs with fig syrup. These steps led to a more defined process of the WK production.

However, the concentration of YE could be increased in order to decrease the fermentation time and therefore the production process bearing in mind avoiding noticeable yeasty flavor components in smell or taste. The usage of X-SEED KAT YE showed slightly higher lactic acid concentrations (**Figure 5E**) in the development of a WK-like beverage by co-cultivation consisting of one LAB and yeast, respectively, and thus, further investigations with 1.3 g/L of X-SEED KAT YE were conducted.

Characterization of Water Kefir Fermentation Based on Grains and Defined Co-cultures in 5-L Bioreactors

The development of a defined process for WK production instead of using complex grains enables the possibility to produce a WK-like beverage by using the co-cultivation of one LAB and yeast. In order to compare the production of WK with grains with the usage of defined co-cultivation under well-monitored and scalable conditions, 5-L bioreactor fermentations were performed in triplicates (see **Figure 8** and **Supplementary Figure 2**). Fermentations performed by a small number of participated microorganism cultures are aimed for because of their higher opportunity to control and guide the process and the properties of the final product (Chen et al., 2009). Further, for a large-scale production, a stable microbial community,

which does not change over time, is crucial (Vardjan et al., 2013). Additionally, possible contaminations might be much earlier detectable as compared with a non-defined complex consortium, allowing for early counteractions as well. Although the fermentation with two defined starter cultures is still a complex process, and interactions within the processes are still not fully understood, the fermentation of co-cultures showed a better-defined and more controllable process. This led to a more reproducible production process compared with the fermentation with WK grains, which showed high fluctuation regarding the time when the beverage had the best sensory properties (3, 5, and 6 days; cf. section "Five-Liters Bioreactor Fermentation With Water Kefir Grains and Defined Co-culture Leu + Han"). The usage of WK grains has been a traditional method for many years, which is similar to the milk kefir production. However, the industrial production of WK by using grains is challenging. There are only few opportunities to control the process (Chen et al., 2009; Laureys and de Vuyst, 2017), which leads to a lower reproducibility and might end in high alcoholic products, which were observed in the present study. The fermentations with grains showed a high formation of ethanol during the first 3 days of the fermentation with a final amount of 23 g/L. Laureys and de Vuyst (2014) also noted a high production of ethanol in WK (around 20 g/L), which was produced by using grains. In comparison, with the co-culture, only 0.49 Vol.-% ethanol was produced until the time the beverage was "ready," which consequently complied with the legislation in the regions of Germany, Austria, and Switzerland, which permit an alcoholic content of 0.5 Vol.-% as a maximum in beverages declared as "alcohol-free" (Das Eidgenössische Department des Innern, 2005). In contrast, the product fermented by Leu + Han showed a significantly higher residual sugar content than did the grain fermentation. As illustrated in **Figure 8**, ∼80% of the originally inserted amount of sugar (60 g/L) was found after 6 days of fermentation corresponding to ∼48 g/L. The World Health Organization recommends a sugar uptake of free sugars to <10% of total energy intake corresponding to ∼50 g/L of free sugars for an adult having a diet of 2,000 kcal (World Health Organization [WHO], 2015). However, in comparison with common soft drinks, such as Coca-Cola (14 FO bottle; 111 g/L of sugars), Pepsi (20 FO bottle; 118 g/L of sugars), or Red Bull (8.4 FO can; 109 g/L of sugars) (Ventura et al., 2011), the WK produced by co-culturing within the present study would be considered as a low-sugar-containing beverage according to the classification of Bandy et al. (2020). With regard to further potential health benefits associated with fermented drinks in general, the herein presented product would be competitive on the beverage market.

Summarizing, the WK beverage gained by co-cultivation with *L. mesenteroides* and *H. valbyensis* differed in the determined cell count, acidity, fermentation duration, ethanol content, and consequently its sensory profile as compared with the grain-based WK. Wang et al. (2016) encapsulated yeast, LAB, and acetic acid bacteria in liquid core capsules and achieved a volatile aroma profile close to that of the grain-based product in milk kefir. WK produced by Laureys and de Vuyst (2014) by using grains contained 4.9 g/L of lactic acid and 1 g/L of acetic acid, which is in accordance with the metabolite concentrations of the developed fermented beverage without using grains in this study. However, two different products were gained in the present study, whereby both products showed typical attributes of WK. In addition, the co-cultivated products performed slightly better in sensory terms than those made with grains (cf. **Figure 9**) regarding a low alcoholic balanced sour and fruity drink. These first results are therefore very promising for further beverage developments to be based on. Under this aspect, the studies carried out here can be understood as a piece of the puzzle in order to establish knowledge-based production processes for complex fermentation products such as WK, so that these are more controllable and reproducible, especially for industrial production. The first studies were conducted by Stadie et al. (2013) as well as Bechtner et al. (2019), which made symbiotic interactions of co-cultures for WK production subjects of discussion.

CONCLUSION

The work presented herein described steps from a traditionally produced process to a defined water kefir fermentation process applying a co-culture of only one LAB and one yeast, respectively. It could be shown that the main characteristics of WK—a fruity, aromatic, and acidic beverage made by fermentation of characteristic strains—were achieved by the use of two microbes *L. mesenteroides* and *H. valbyensis*, although the chemical analyses revealed differences compared with those of the undefined grain origin. However, the defined consortium outperformed the grains, as considerably lower levels of ethanol were formed. These results proved the possibility to reduce the complexity of the fermentation process by keeping the aimed product characteristics at the same time. The here-examined defined microbial consortia for the production of WK caused different product characteristics, opening up further space for detailed research and in which remaining challenges, e.g., a possible optimization of the fermentation time, should be addressed. Especially, the usage of one LAB and one yeast each is a promising approach in order to get deeper insights into microbial interactions during fermentation and simultaneously avoiding blurring effects due to an uncontrollable number of associated actors.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

AT, MSc, ML, and SK conducted the experiments. NR was responsible for the aroma analyses. MSe and SK performed the

data analyses. MSe, MSc, and SK drafted the manuscript. ML reviewed and contributed to the structure and content of the manuscript. 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.2021.732019/full#supplementary-material

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Co-occurrence of *Lactobacillus* Species During Fermentation of African Indigenous Foods: Impact on Food Safety and Shelf-Life Extension

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The benefits derived from fermented foods and beverages have placed great value on their acceptability worldwide. Food fermentation technologies have been employed for thousands of years and are considered essential processes for the production and preservation of foods, with the critical roles played by the autochthonous fermenting food-grade microorganisms in ensuring food security and safety, increased shelf life, and enhanced livelihoods of many people in Africa, particularly the marginalized and vulnerable groups. Many indigenous fermented foods and beverages of Africa are of plant origin. In this review, the predominance, fermentative activities, and biopreservative role of *Lactobacillus* spp. during production of indigenous foods and beverages, the potential health benefit of probiotics, and the impact of these food-grade microorganisms on food safety and prolonged shelf life are discussed. During production of African indigenous foods (with emphasis on cereals and cassava-based food products), fermentation occurs in succession; the first group of microorganisms to colonize the fermenting substrates are lactic acid bacteria (LAB) with the diversity and dominance of *Lactobacillus* spp. The *Lactobacillus* spp. multiply rapidly in the fermentation matrix, by taking up nutrients from the surrounding environments, and cause rapid acidification in the fermenting system *via* the production of organic compounds that convert fermentable sugars into mainly lactic acid. Production of these compounds in food systems inhibits spoilage microorganisms, which has a direct effect on food quality and safety. The knowledge of microbial interaction and succession during food fermentation will assist the food industry in producing functional foods and beverages with improved nutritional profiling and technological attributes, as *Lactobacillus* strains isolated during fermentation of several African indigenous foods have demonstrated desirable characteristics that make them safe for use as probiotic microorganisms and even as a starter culture in small- and large-scale/industrial food production processes.

Keywords: Africa, fermented foods, starter culture, food security, *Lactobacillus* species

INTRODUCTION

Fermented foods have been in existence since antiquity and are consumed by different regions globally because of the numerous nutritional values conferred on humans. For decades, the physiology and the functionalities of many important microorganisms associated with fermented foods have been studied, and this has assisted in the development of foods with improved flavor, taste, texture, and consistency (Marco et al., 2020). In Africa, fermented foods constitute the main dietary components, as many foods are fermented spontaneously before consumption with the predominance of lactic acid bacteria (LAB). These fermented foods and beverages serve as a vehicle for important microorganisms, thus playing essential roles in humans when consumed. Traditional fermentation of food is a natural method used in preserving different foods and beverages produced and thus extends the food shelf life, improves food palatability and food digestibility, and inhibits undesirable microorganisms, which results in improvement of food nutritional values. All these positive characteristics that impacted the fermented foods are dependent on the functional and technological roles displayed by autochthonous microorganisms present in the food substrates. Yeast and LAB are the groups of microorganisms that perform fermentative roles and flavor production during food fermentation, with LAB being the predominant microbes isolated from African indigenous fermented foods (cereals and tubers), although other microorganisms such as *Bacillus* spp., acetic acid bacteria, and molds have been reportedly isolated during production of some other fermented foods: legumes, wine, etc. (Adesulu-Dahunsi et al., 2017a, 2020; Chaves-López et al., 2020). Studies have shown that during fermentation of African fermented cereal-based and cassava-based foods, growth is firstly initiated by the LAB.

In Africa, production of several foods consumed by the populace relies on the fermentation processes; food security and shelf life are improved because of the biopreservative role played by the autochthonous microorganisms, which is advantageous to the community where there is poor access to electricity and refrigerating systems (Adesulu and Awojobi, 2014). Fermentation in food production is simply brought about by different activities of microorganisms and their enzymes to enhance the bio-accessibility and bioavailability of nutrients present in the raw food substrates, thus improving the organoleptic properties, improving digestibility, extending the food shelf life, inhibiting undesirable microorganisms, and serving as a vehicle for delivery of probiotics in humans (Diaz et al., 2019; Marco et al., 2020). Fermentation causes changes in both the physical and chemical properties of foods. It is described as the natural way of enhancing vitamin contents, essential amino acids, and sensorial properties of foods (Sharma et al., 2020). Food fermentation technologies are carried out at the household level *via* spontaneous fermentation consisting of mixed cultures of different species of autochthonous microbes, and back-slopping (re-inoculation of a previous successful fermentation batch into the new set to be fermented, which results in the dominance of best-adapted strains) has been carried out for several decades. In the last 2–3 decades, with increasing research and development, discoveries in the use of industrial-scale technology are preferred, i.e., inoculation of "defined starter culture" (pre-cultured single or mixed strains of microorganisms with functional and probiotics characteristics) in controlled fermentation processes; the latter is becoming a necessary under-regulated process to increase automated production, leading to improved and consistent fermented food quality for commercial purposes.

Several advantages of these fermented food products have been reviewed by various researchers (Melini et al., 2019; Şanlier et al., 2019; Sharma et al., 2020; Tsafrakidou et al., 2020), including the following:

- Production of safe food products.
- Reduction in cooking time.
- Production of foods with extended shelf life.
- Improvement of the organoleptic properties of fermented food products/enhancement of food sensorial characteristics.
- Increases mineral and trace element bioavailability in food/enrichment of food nutritional contents and digestibility.
- Elimination of cyanide compounds in cassava tubers and other harmful substances that may be present in cereals.
- Prevention of diseases or infections such as diarrhea and salmonellosis.

The population dynamics of microbes during fermentation using phenotypic and culture-dependent techniques have resulted in the understanding of different important roles that exist in the microbial consortia (Oguntoyinbo and Narbad, 2015; Adesulu-Dahunsi et al., 2017b). The invention of meta-omic methods (metagenomics, metatranscriptomics, metaproteomics, and metabolomics) has greatly complemented the culture-dependent techniques for strain-level characterization of food microbiota during fermentation processes and has also assisted in the studying of strains' performances and microbial interactions of the important microorganisms within the food matrix and the systematic analysis of microbial metabolism and responses to the external/environmental factors (Liu et al., 2005; Makarova et al., 2006; Walsh et al., 2017; Taylor et al., 2020). The understanding of different roles displayed by food-grade microorganisms present in spontaneously fermented food products is crucial and has helped in the optimization of the final food quality, bringing about improved food safety. This review gives an overview of the impact of the activities and predominance of *Lactobacillus* microbiota during the production of African indigenous foods and the industrial importance of their interactions in cereal-based and cassava-based fermentation, their application in optimized or controlled food fermentation over mixed culture/uncontrolled fermentation, and the impact of *Lactobacillus* species co-occurrence in the fermenting food matrix on product development and safety.

PREDOMINANCE OF *Lactobacillus* spp. DURING FERMENTATION OF AFRICAN INDIGENOUS FOODS

Lactic acid bacteria perform the function of converting carbohydrate in food substrates into organic acids (principally lactic acid) and can also produce a wide range of metabolites (Rodríguez et al., 2019). Lactic acid bacteria is a common name given to the family *Lactobacillaceae* (Pfeiler and Klaenhammer, 2007). These groups of microorganisms are Gram-positive, acid-tolerant, mainly lactic acid producers, non-sporulating groups of important bacteria widely reported to perform fermentative roles in several African fermented foods and beverages, and some genera, most importantly the genus *Lactobacillus*, have a "Generally Recognized As Safe" (GRAS) status; these characteristics displayed by LAB affect the storage quality, thereby extending the shelf life and safety of many fermented foods and beverages (Oguntoyinbo and Narbad, 2015; Adesulu-Dahunsi et al., 2018). The most common LAB genera frequently reported to be associated with African indigenous fermented foods are *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus,* and *Weissella. Lactobacillus* constitute an important genus within the phylum Firmicutes, class Bacilli, order Lactobacillales, and family Lactobacillaceae. Recently, the International Scientific Association for Prebiotics and Probiotics (Marco et al., 2021) reported that the genus *Lactobacillus* comprises over 250 species; the species that are more closely related (i.e., those that share similar physiological traits) are grouped under the same genus, *Lactobacillus* spp., with desirable probiotic attributes, and are now renamed, including the following: *Lactobacillus casei* (renamed as *Lacticaseibacillus casei*), *Lactobacillus paracasei* (renamed as *Lacticaseibacillus paracasei*), *Lactobacillus rhamnosus* (renamed as *Lacticaseibacillus rhamnosus*), *Lactobacillus plantarum* (renamed as *Lactiplantibacillus plantarum*), *Lactobacillus brevis* (renamed as *Levilactobacillus brevis*), *Lactobacillus salivarius* (renamed as *Ligilactobacillus salivarius*), *Lactobacillus fermentum* (renamed as *Limosilactobacillus fermentum*), and *Lactobacillus reuteri* (renamed as *Limosilactobacillus reuteri*) (Marco et al., 2021). Zheng et al. (2020) also proposed the reclassification of the genus *Lactobacillus* into 25 genera; the classification includes the genus *Lactobacillus* (*Lactobacillus delbrueckii* group), *Paralactobacillus*, and 23 other novel genera. *Lactobacilli* are predominant organisms involved in the fermentation of cereal-based foods and beverages in Africa. During lactic acid fermentation, LAB at the initial stage of fermentation improve the flavor, shelf life, nutritional values, and digestibility of fermented foods. The predominance and the involvement of *Lactobacilli* especially [*L. plantarum* (*Lactiplantibacillus plantarum*) and *L. fermentum* (*Limosilactobacillus fermentum*)] during food fermentation have been reported by several researchers (Oguntoyinbo and Narbad, 2015; Adesulu-Dahunsi et al., 2017a; Naghmouchi et al., 2019).

Lactobacillus species are widely applied in food production and are known for their fermentative ability, health, and nutritional benefits. The role of LAB in the safety and quality of fermented foods around the globe has been reviewed by several researchers (Antara et al., 2019; Chaves-López et al., 2020; Goel et al., 2020). Strains of *Lactobacillus* play key roles during food fermentation by contributing immensely to the microbiological safety and extension of food shelf life. During fermentation, *Lactobacilli* usually outcompete other microorganisms through the production of organic acids, which are inhibitory to potential competitors, leading to the elimination of pathogenic microorganisms. The co-occurrence and activities of the LAB species in the fermenting food matrix and their functional and technological roles during food production have a major impact on the food safety, nutrition, sensory characteristics, and shelf life extension, as these LAB species have been found to inhibit pathogenic microorganisms that may be present in the fermentation through increased pH and production of lactic acid, and removal of toxic compounds (Behera et al., 2018; Douillard and de Vos, 2019; Virdis et al., 2021). In Africa, diverse foods are produced from plant sources and animal milk, and these foods are naturally fermented into different types of edible products. Some of these plant-based fermented foods have their sources from cereal (maize, sorghum, and millet), cassava (tubers), African oil palm, fruits, and leafy vegetables (African locust beans, melon, castor oil seed, sesame, cotton seeds, fluted pumpkin bean, ripe plantain, the fruit of sand apple, fresh leaves of *Cassia obtusifolia,* etc.).

Although other types of microorganisms may be involved during the production of foods and beverages, the predominance of *Lactobacilli* during indigenous food fermentation is commonly associated with cereal-based and cassava-based foods; these substrates are processed into different types of foods and beverages. Most of the fermented cereal-based foods are consumed as complementary infant food and as cereal beverages in adults, while the fermented cassava-based foods are consumed as main course meals. Different fermented foods from cereals and cassava tubers include *mawé* (Beninese fermented sour dough); *gowé* (Beninese malted and fermented cereal-based beverage); *tchoukoutou* (Beninese home-brewed beer from sorghum); *ben-saalga* (Burkinabé fermented cereal gruel); *dolo* (Burkinabé fermented sorghum beer); Bikédi (Congolese fermented cassava tubers); *poto poto* (Congolese fermented maize dough); *cingwada* (East African retted cassava); *kisk* (Egyptian fermented cereal mixture); Bikédi, *foufou*, *chikwangue, mbalampinda, tutu, tsaba, minsela,* and *tsiya borde* (Ethiopian fermented beverages)*; injera* (Ethiopian sour fermented flatbread); *kocho* (Ethiopian fermented bread); *koko* and *koko* sour water (Ghanaian fermented millet porridge and drink)*; agbelima* and *kenkey* (Ghanaian fermented cassava-based food); *kisra* dégué (Ivorian fermented cereal gruel); *wômi, baca,* and *doklu* (Ivorian fermented cereal-based foods); *attiéké, placali,* and *attoupkou* (Ivorian fermented cassava-based foods); *uji* (Kenyan millet-based fermented porridge); *ogi* (Nigerian fermented cereal gruel); *gari, fufu,* and *lafun* (Nigerian fermented retted cassava); *pito* and *kunun zaki* (Nigerian fermented cereal-based beverages); *ikigage* (Rwandan fermented sorghum beer); *hussuwa* (Sudanese fermented sorghum food); (Sudanese fermented sorghum bread); *mbege, togwa*, and *kivunde* (Tanzanian retted cassava); *komé* (Togolese fermented cereal-based foods); *koko* and

bushera (Ugandan fermented cereal beverage); and *mahewu* (Zimbabwean fermented cereal beverages) (Aboua et al., 1989; Keleke, 1996; Olasupo et al., 1997; Mestres et al., 1999; Kimaryo et al., 2000; Blandino et al., 2003; Lei and Jakobsen, 2004; Abriouel et al., 2006; Sawadogo-Lingani et al., 2007; Ndiaye et al., 2008; Hama et al., 2009; Ray and Sivakumar, 2009; Mukisa et al., 2012; Owusu-Kwarteng et al., 2012; Greppi et al., 2013; Sanni and Adesulu, 2013; Soro-Yao et al., 2013; Obinna-Echem et al., 2014; Djeni et al., 2015; Adesulu-Dahunsi et al., 2017b; Aka-Gbezo et al., 2017). Several examples of these African indigenous fermented foods and beverages (AIFFBs) ranging from cereal-based to cassava-based foods and beverages and the predominant *Lactobacillus* spp. associated are shown in **Table 1**.

Diversity of LAB intraspecies interaction was reported to be associated with Ghanaian spontaneously fermented millet porridge and drink (*koko* and *koko* sour water), with *L. fermentum* as the predominant organisms. Assohoun et al. (2012) revealed the succession and predominance of LAB species during spontaneous fermentation of maize for the production of *doklu* (Ivorian maize dough). It was reported that after 24 h of fermentation, the percentage occurrence of *L. plantarum* was 64%, which was the highest, followed by other LAB: *Weissella cibaria* (22%), *Pediococcus acidilactici* (7%), and *L. fermentum* (7%); at 48 h fermentation time, *L. plantarum* spp. still predominate, having 56% of occurrence, and at the end of fermentation, the percentage of occurrence of *L. fermentum* was 100%. The predominance of LAB species especially *L. plantarum* in Nigeria traditional fermented foods and beverages is well documented (Oguntoyinbo and Narbad, 2015); this *Lactobacillus* strain plays significant roles during fermentation, and has been selected as a potential probiotic and starter culture due to the different properties it displayed, such as good acidification, hydrogen peroxide production, and variation in carbohydrate fermentation patterns (Rozos et al., 2018). From our previous study, the intraspecies differentiation of *L. plantarum* strains isolated from indigenous fermented foods was carried out using different molecular techniques for the selection of strains that can be used to produce foods with desirable functional properties or as an adjunct and/or starter culture during food fermentation processes. It is well established that the predominance of *Lactobacillus* spp. and their enzyme production capacity in African fermented foods have assisted in the breaking down of molecules of high-molecular-weight organic acids and compounds, bacteriocins, and hydrogen peroxide (Mokoena et al., 2016).

Lactic acid bacteria have been reportedly isolated at different fermentation stages, and researchers have recommended that the quality of the final food products is dependent on the microbial diversity, dynamics, and frequency of occurrence of these LAB species. Oyedeji et al. (2013) isolated and characterized the predominant LAB species during spontaneous fermentation of Nigerian cassava-based and cereal-based foods, *fufu* and *ogi*; the dominance in the population of the *L. plantarum* strains was observed, and the use of these LAB strains as a starter culture for production of fermented foods was suggested. Karaca et al. (2017) reported the predominance of *Lactobacillus* spp. (*Lactobacillus gasseri, L. fermentum, L. brevis,* and *L. casei*) during fermentation of *Dégué* (fermented millet beverages popularly consumed in some parts of African countries, namely, Burkina Faso, Ivory Coast, Senegal, Mali, Guinea, and Benin Republic).

ROLES OF *Lactobacillus* spp. IN FOOD FERMENTATION INDUSTRY AS FUNCTIONAL STARTERS OR CO-CULTURES

In food processing industries, *Lactobacilli* constitute an important group of LAB, and the industrial importance of this *Lactobacillus* spp. cannot be overemphasized; they are commonly recommended as a starter culture for controlled fermentation of African indigenous fermented foods (Giraud and Cuny, 1997; Olasupo et al., 1997). The increase in demand for consistent and quality fermented products has resulted in the use of a starter culture for a more controlled fermentation process (Adebo et al., 2018; Masebe and Adebo, 2019). Controlled fermentation using a well-defined starter culture guarantees fast, consistent fermentation, the introduction of specific strains with exceptional functional properties, and food safety, though this technology is not widely practiced/used in many developing countries. *Lactobacillus* spp. associated with African indigenous fermented foods are known to exhibit many interesting beneficial traits and functional properties such as the production of the desired flavor, and antimicrobial production results in improved nutritional quality and safety of the food products and ultimately promotes the health status of the consumers. The roles and applications of *Lactobacillus* spp. are shown in **Figure 1**.

The use of a carefully selected functional starter culture with added nutritional benefits during microbial fermentation will enhance the production of fermented foods with improved quality (Nkhata et al., 2018). The technological effectiveness of LAB during food fermentation must be taken into consideration before selection as a functional starter culture or co-culture; some of these attributes include rapid acidification, development of antimicrobial compounds, probiotic features, and ability to improve the nutritional quality of fermented foods (Soro-Yao et al., 2014; Banwo et al., 2021). Many plant-based food substrates fermented with *lactobacilli* are reported to have high percentages of rhamnose (a naturally occurring deoxy sugar), and these sugars possess antioxidant properties that can be used as a substrate during *Lactobacillus* metabolism in cereal fermentation to produce vitamin-enriched foods and in combating nutritional deficiency in many African fermented cereal-based foods. During cereal-based fermentation, the dominance of *Lactobacilli* and other LAB with extracellular amylase production has resulted in the provision of metabolizable starch *via* hydrolysis and can be utilized for industrial fermentation processes, because of its acid production, which simultaneously improves the preservation and safety of fermented foods.

In cereal fermentation, the enzymatic activity of amylase displayed by *Lactobacillus* spp. in catalyzing the hydrolysis of amylose and amylopectin to yield fermentable maltose makes each strain desirable, important, and useful as a starter culture.

TABLE 1 | Africa indigenous cereal-based and cassava-based fermented foods and beverages and the predominant *Lactobacillus* spp. associated.

Products	Country of production	Substrates	Form	Predominant *Lactobacillus* spp. associated with the fermented products
Aklui	Bénin	Maize	Porridge	*Lactobacillus* spp.
Poto-poto	Congo	Maize	Dough	*L. plantarum, L. paraplantarum, L fermentum, L. gasseri, L. delbrueckii, L. reuteri, L. casei, L. brevis*
Mawè	*Côte d'Ivoire, Togo*	Maize	Basis for preparation of many dishes	*L. fermentum, L. reuteri, L. brevis*
Banku	Ghana	Maize	Dough	*Lactobacillus* spp.
Kenkey	Ghana	Maize	Staple food/Weaning food	*L. fermentum, L. reuteri*
Masa	Nigeria	Maize	Snacks	*L. fermentum, L. plantarum*
Ben-saalga	Burkina-Faso	Millet	Porridge as weaning food	*L. fermentum, L. plantarum*
Dégué	Burkina-Faso	Millet	Porridge	*Lactobacillus* spp.
Dagnan	*Côte d'Ivoire*	Millet	Dough	*Lactobacillus* spp.
Womi	*Côte d'Ivoire*	Millet	Fried cake	*Lactobacillus* spp.
Busa	Egypt	Millet	Beverage	*L. helveticus, L. salivarius, L. casei, L. brevis, L. plantarum, L. buchneri*
Fura	Ghana, Nigeria	Millet	Weaning food/Beverage for adult	*Lactobacillus* spp.
Dalaki	Nigeria	Millet	Porridge	*Lactobacillus* spp.
Eko	Nigeria	Millet	Staple food	*Lactobacillus* spp.
Cere	Senegal	Millet	Used for preparation of many dishes	*L. plantarum*
Bushera	Uganda	Millet	Beverage	*L. plantarum, L. paracasei* ssp. *paracasei, L. fermentum, L. brevis, L. delbrueckii* ssp. *delbrueckii*
Gowé	Bénin	Sorghum	Beverage for adult	*L. fermentum*
Tchoukoutou	Bénin	Sorghum	Beer	*L. fermentum, L. divergens, L. fermentum, L. fructuvoans, L. casei, L. acidophilus*
Burukutu	Bénin, Ghana, Nigeria	Sorghum	Beer	*Lactobacillus* spp.
Dolo, Pito	Burkina Faso, Ghana, Nigeria	Sorghum	Beverage	*L. fermentum, Lactobacillus delbrueckii* ssp. *delbrueckii, Lact. delbrueckii* ssp. *bulgaricus*
Tchapalo	Côte d'Ivoire	Sorghum	Beer	*L. fermentum, L. cellobiosus, L. brevis, L. coprophilus, L. plantarum.*
Kome	Togo	Sorghum	Dough as staple	
Baca, Coco-baca	*Côte d'Ivoire*	Maize, Millet	Porridge as weaning food	*Lactobacillus* spp.
Kunun-zaki	Nigeria	Millet or Sorghum	Beverage for adult	*L. plantarum, L. fermentum*
Koko	Ghana	Maize, Sorghum, Millet	Porridge	*L. fermentum, L. reuteri*
Koko sour water	Ghana	Maize, Sorghum, Millet	Weaning food/ Dough as porridge	*L. fermentum, L. salivarius*
Ogi	Ghana, Nigeria	Maize, Sorghum, Millet	Cereal gruel	*L. plantarum, L. fermentum, L. brevis*
Agidi	Ghana, Nigeria	Maize, Sorghum, Millet	Food staple	*Lactobacillus* spp.
Akamu	Nigeria	Maize, Sorghum, Millet	Cereal gruel as staple, Weaning food	*L. fermentum, L. plantarum, L. pentosus, L. cellobiosus, L. mesenteroides, L. acidophilus, L. delbrueckii, L. lactis, L. casei*
Kaffir	South Africa	Malt of Sorghum, Maize	Beer	*Lactobacillus* spp.
Bounganda, Loutoko, Biyoki	Congo	Maize, Cassava tuber	Aqueous mixture maize flour and cassava flour	Not studied
Banku	Ghana	Maize, or Maize and Cassava	Dough as staple	*Lactobacillus* spp.
Ubuswage, Imikembe	Burundi	Cassava	Food staple	*Lactobacillus* spp.
Ikivunde	Burundi, Rwanda	Cassava	Food staple	*L. plantarum, L. brevis, L. fermentum*
Myondo and Bobolo	Cameroon	Cassava	Food staple	*Lactobacillus* spp.
Mangbele	Central Africa	Cassava	Food staple	*Lactobacillus* spp.
Meduame-Mbong, cossette	Central Africa	Cassava	Food staple	*Lactobacillus* spp.

(Continued)

TABLE 1 | (Continued)

Products	Country of production	Substrates	Form	Predominant *Lactobacillus* spp. associated with the fermented products
Chikwangue	Congo	Cassava	Food staple	*L. plantarum*
Ntobambodi	Congo	Cassava (leaves)	Food staple	*L. fermentum, L. plantarum, L. lactis diacetylactis*
Bikédi	Congo	Cassava tubers	Food staple	*L. delbrueckii, L. fermentum*
Mbalampinda, Tutu	Congo	Cassava tuber	Starched and gelled cassava flour	Not studied
Kokondé, Kokonte, Crueira, Alebo	Côte d'Ivoire,	Cassava	Snack	Not studied
Placali	Côte d'Ivoire,	Cassava	Staple	Not studied
Attoupkou	Côte d'Ivoire	Cassava	Starchy cake	Not studied
Kokondé (Kokonte)	Côte d'Ivoire	Cassava	Food staple	Not studied
Agbelima	Côte d'Ivoire, Ghana, Togo	Cassava	Basis for preparation of many dishes	*L. brevis, L. plantarum, L. salivarius, L. fermentum*
Attiéké	Côte d'Ivoire, Burkina Faso, Bénin, Togo, Mali, Senegal	Cassava	Food staple	*L. plantarum, L. fermentum, L. cellobiosus, L. brevis*
Inyange, Ivunde, Mokopa	East Africa	Cassava	Food staple	Not studied
Mboung	Gabon	Cassava	Food staple	Not studied
Akeyke	Ghana	Cassava	Food staple	*L. plantarum, L. salivarius, L. brevis, L. fermentum*
Abacha	Nigeria	Cassava	Snack	*Lactobacillus* spp.
Lafun	Nigeria	Cassava	Food staple	*L. fermentum, L. plantarum*
Gari	Nigeria, Central and East Africa countries	Cassava	Staple	*L. plantarum, L. fermentum, L. brevis, L. pentosus, L. acidophilus, Lactobacillus* spp.
Kivunde	Tanzania	Cassava	Food staple	*Lactobacillus* spp.
Mokopa	Uganda	Cassava	Food staple	*Lactobacillus* spp.

Muyanja et al., 2003; Lei and Jakobsen, 2004; Yousif et al., 2005; Vieira-Dalodé et al., 2007; Assohoun et al., 2012; Oguntoyinbo and Narbad, 2012; Greppi et al., 2013; Sanni and Adesulu, 2013; Adesulu-Dahunsi et al., 2017a; Bationo et al., 2019; Pswarayi and Ganzle, 2019.

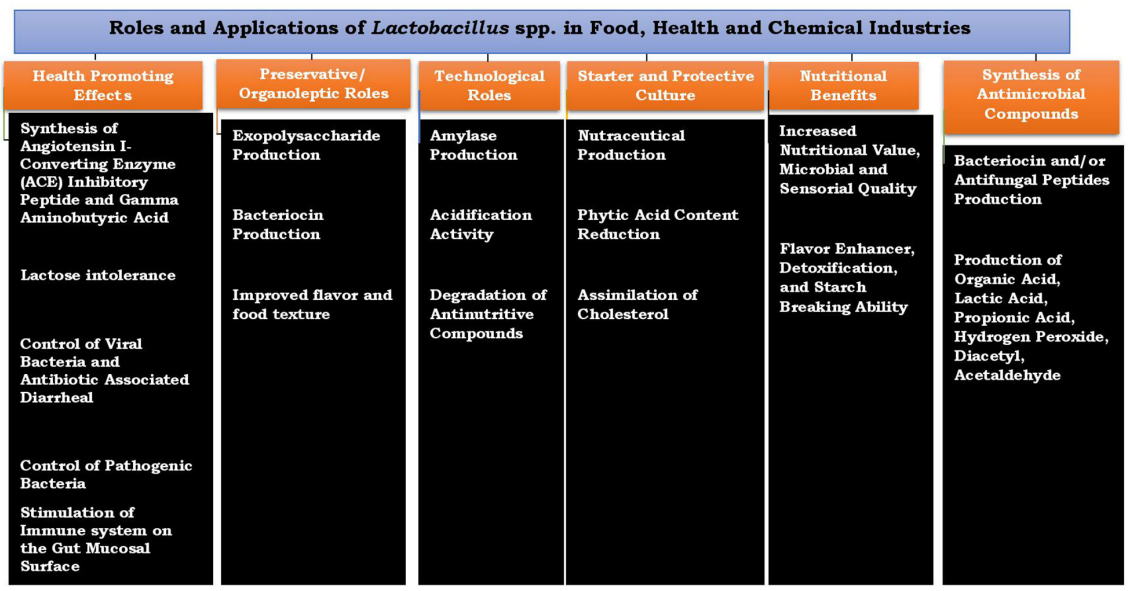

FIGURE 1 | Roles and applications of *Lactobacillus* species in food, health and chemical industries.

In traditional food fermentation, using amylolytic lactic acid bacteria as a starter culture offers an advantage by combining both amylase production and acidification properties in a single strain. The raw starch binding ability of the α-amylase producing gene (*amyA*) of *L. plantarum* A6 (amylolytic strain isolated from retted cassava) with an unusual structure of the 3′ end of the

α-amylase gene and the ability to break down raw starch has been successfully cloned and sequenced (Giraud et al., 1991; Giraud and Cuny, 1997). The *L. plantarum* A6 strain can synthesize large amounts of α-amylase that can be used as a starter culture during fermentation processes, resulting in the production of more lactic acid in the fermentation matrix.

Though beneficial starter cultures have not been fully utilized during the traditional fermentation of many cereal- and cassava-based products, fermented foods have probiotic potentials due to the *Lactobacillus* species present in the fermenting matrix. There are reports on the quality of some African fermented products that have been greatly improved upon using beneficial cultures (Nyanzi et al., 2013). *Ogi* was enhanced with a lactic acid starter culture to produce a functional food called *Dogik*. Nyanzi et al. (2013) reported the use of LAB associated with fermented foods to produce a starter culture possessing antimicrobial potentials; this starter culture has antimicrobial activities against diarrheagenic bacteria. During spontaneous fermentation of cereals to produce African fermented beverages, *akamu* and *ogi*, several LAB species such as *L. plantarum, Lactobacillus pentosus, Lactobacillus cellobiosus, Pediococcus pentosaceus,* and *Leuconostoc mesenteroides* were associated with *L. plantarum*, displaying the potential of being used as a starter culture (Nwachukwu et al., 2010). Oguntoyinbo and Narbad (2012) reported the multifunctional properties displayed by two strains of *L. plantarum* isolated from Nigerian cereal-based beverages (*L. plantarum* ULAG11 and *L. plantarum* ULAG24) and the potential of using these LAB strains for industrial processing of West African cereal-based foods and beverages, due to the probiotic function, amylase enzyme, and bacteriocin-producing ability exhibited by the strains (Mokoena et al., 2016).

BIOPRESERVATIVE ROLE OF *Lactobacillus* spp. IN FERMENTED FOOD

The protective functions of many food-grade LAB in terms of improved food safety and food quality, against pathogenic and spoilage microorganisms during food fermentation, have attracted the attention of researchers. Biopreservatives are naturally occurring compounds that can be produced from microorganisms, plants, or animals. Some of the compounds produced by food-grade *Lactobacillus* spp. can be used to extend the food's shelf life, because the antimicrobial metabolites released by these microorganisms may have some potential usage as natural preservatives that can be used to control or inactivate spoilage or pathogenic microorganisms in the fermenting food matrix; inhibition of the pathogenic or spoilage organisms that may be present in foods will result in increased food functionality and food quality (Pisoschi et al., 2018). Biopreservation using LAB and their metabolites connotes an alternative for improving food shelf life and safety (Reis et al., 2012). Many studies on the bacteriocinogenic effect of *Lactobacillus* spp. and its several applications in food preservations have been carried out. Many probiotic *lactobacilli* are reported to display inhibitory effects, which makes them useful as biological preservatives (Naghmouchi et al., 2019). The shelf life and safety of fermented foods can be enhanced through the biopreservative's role as demonstrated by several LAB species in the form of competition to obtain nutrients during fermentation; this antagonistic form of interaction produces antimicrobial substances. Bacteriocin production by LAB is of great interest to the fermentation industry as some well-characterized bacteriocins have been reported to show a broad spectrum of activity on pathogenic and spoilage microorganisms (Cebrián et al., 2012; Naghmouchi et al., 2019; Vieco-Saiz et al., 2019).

Bacteriocins can be used as "biologically derived" preservatives; thus, food shelf life can be extended, and pathogenic organisms are inhibited without altering the product's nutritional quality (Hugas et al., 2002; Ross et al., 2002). Lactic acid bacteria isolated from African fermented food are reported to demonstrate antimicrobial activity toward many pathogenic organisms, by the production of lactic acid and reduction of pH *in vivo* (Sanni et al., 2002). Many *Lactobacillus* species are known to be bacteriocin producers, and their presence in food inhibits the growth of pathogenic microorganisms. Olsen et al. (1995) reported the occurrence of antimicrobial compounds displayed by *Lactobacillus* species during fermentation of maize dough; the antagonistic interactions against pathogenic organisms in the fermented dough showed that the inhibitory compounds produced by the synergistic relationship between the strains of *L. plantarum* and *L. fermentum* were effective against both Gram-positive and Gram-negative bacteria, because of their ability to produce acids and bacteriocins. Adebayo et al. (2014) reported the use of bacteriogenic *Lactobacillus* strains (*Lactobacillus fermentum* and *Lactobacillus casei*) isolated from naturally fermented foods in Nigeria for effective control of spoilage and pathogenic microorganisms; the strains showed a broad range of activities and had a significant effect on the selected pathogenic microorganisms. Adedokun et al. (2016) demonstrated the use of a *Lactobacillus* strain as a food preservative agent. The antifungal activity of *L. fermentum* YML014 isolated from cassava-based fermented foods against food spoilage molds using tomato puree was investigated by Adedokun et al. (2016). It was found that the *Lactobacillus fermentum YML*104 showed strong antifungal activity against *Aspergillus niger, Aspergillus flavus*, and *Penicillium expansum*, and thus caused an increase in the shelf life of the tomato puree.

Production of antimicrobial compounds such as lactic acid, propionic acid, and diacetyl by LAB during fermentation results in a decrease in pH and inhibition of many pathogenic microorganisms. Rattanachaikunsopon and Phumkhachorn (2010) reported the inhibitory activity of reuterin produced by *Lactobacillus reuteri* against protozoa and fungi. Other inhibitory compounds such as organic acids, bacteriocins, and antifungal peptides produced by *L. plantarum* have been reportedly isolated from silage (De Vuyst and Leroy, 2007). Ben Omar et al. (2008) isolated 135 *Lactobacillus* species during the production of *poto poto* (Congolese fermented maize product) using species-specific PCR and 16S rRNA gene sequencing; 31 strains were identified as bacteriocin producers, and the bacteriocins produced by these LAB strains, *L. plantarum* (28) and *L. fermentum* (3), were identified as plantaricin and

were found to display a broad spectrum of inhibition against the following pathogens: *Escherichia coli, Salmonella enterica, Enterobacter aerogenes, Bacillus cereus, Staphylococcus aureus, Listeria monocytogenes,* and *Enterococcus faecalis*. *Lactobacillus* spp. (*L. plantarum* F1 and *L. brevis* OG1) isolated from Nigerian fermented foods were reported to produce bacteriocins, which have a broad spectrum of inhibition against pathogenic and spoilage organisms; the *Lactobacillus* strains exhibited activities of 6,400 and 3,200 AU/ml, respectively, against *Escherichia coli* NCTC10418 and *Enterococcus faecalis* EF1, but did not inhibit *Candida albicans* ATCC10231 and *Klebsiella* sp. UCH15 (Ogunbanwo et al., 2003).

In another study, one *L. rhamnosus* and several *L. plantarum* strains isolated from *sha'a* (fermented maize beverage produced in Cameroon) produced bacteriocins that were able to inhibit both Gram-positive and Gram-negative bacteria, including species of the genera *Lactobacillus*, *Streptococcus*, *Bacillus*, *Salmonella*, *Shigella*, *Pseudomonas*, and *Klebsiella* and multidrug-resistant strains of the pathogens *E. coli* and *S. aureus* (Kaktcham et al., 1995). During food fermentations, *Lactobacillus* species have been reported to perform protective functions. Kaktcham et al. (1995) investigated the antimicrobial properties and protective functions of *Lactobacillus* spp. isolated during the fermentation of Cameroonian traditional cereal-based beverages and cow milk (*Sha'a* and *Kossam*). It was observed that 12 strains out of the 21 LAB species were reported to display inhibitory substances to proteolytic enzymes (Trypsin and Proteinase K) and the bacteriocins produced showed broad inhibitory activity against pathogenic microbes.

In cereal-based foods, LAB generally produces enzymes that assist in the breaking down of polysaccharides and other high-molecular-weight substances such as bacteriocins and hydrogen peroxide, thereby making it inhibitory for pathogenic organisms to survive (Mokoena et al., 2016). These LAB in fermented cereals also cause an increase in free amino acids and vitamin B groups, by breaking down antinutritional compounds, thus leading to the availability of iron, zinc, and calcium and production of gas and other volatile compounds and resulting in improved sensorial properties of the cereal-based foods and beverages (Blandino et al., 2003). Production of bacteriocins by LAB has also provided a good alternative to synthetic drugs and antibiotics. *Lactobacillus* produces antimicrobial ribosomally synthesized peptides, or "bacteriocins." The LAB-producing bacteriocin has been reported to possess biopreservative characteristics, which makes them ideal food biopreservatives; some of these characteristics include the bacteriocin proteinaceous nature, *in vivo* non-toxicity and non-immunogenicity, inactivity against eukaryotic cells, thermoresistance against heat treatment, and broadened bactericidal activity (Naghmouchi et al., 2019; Vieco-Saiz et al., 2019). Several classes of LAB bacteriocins have their application in foods as food additives and control undesirable microorganisms in food (Heng et al., 2007). Nisin is the only FDA-approved bacteriocin produced from LAB species, and *Lactococcus lactis* is a widely studied biopreservative and has numerous applications as a food additive effective against foodborne pathogens and Gram-positive spoilage organisms during the production of cheese and dairy products. The effectiveness and application of bacteriocin in the food system have been widely reviewed (Cleveland et al., 2001; Naghmouchi et al., 2019). In the selection of bacteriocin-producing strains in food applications, the following must be put into consideration:

- The bacteriocin-producing strain should possess a "GRAS" status.
- The strain must display a broad spectrum of inhibition.
- The strain must be thermostable.
- The strain must possess beneficial effects and improve safety.

POTENTIAL BENEFITS OF *Lactobacillus* spp. AS PROBIOTICS

Probiotics are live microbes that impart health benefits to humans when an adequate amount is consumed (Hill et al., 2014). The significant effect of *Lactobacillus* as probiotic strains was first established at the beginning of the 20th century (1908) by the Russian scientist and Nobel Prize winner Elie Metchnikoff while working in Bulgaria; he hypothesized that the longevity of the Bulgarian centenarians was a result of the improved health benefits conferred upon daily intake of fermented yogurt containing *Lactobacillus bulgaricus* (Metchnikoff, 1908). Several indigenous fermented foods and dairy products have been studied to contain live microorganisms with potential health benefits (Lei and Jakobsen, 2004; Salmerón et al., 2015). *Obiolor* and *kunu-zaki* are examples of African fermented beverages with probiotic potentials and have indirectly impacted beneficial effects on the host; several attempts have been made to introduce probiotic drinks and beverages containing *L. rhamnosus* GR-1 and *Streptococcus thermophilus* in the form of a starter culture in the production of yogurt in Uganda, Tanzania, and Kenya (Salmerón et al., 2015; Nath et al., 2016; Westerik et al., 2020). The importance of probiotic microorganisms is so enormous to the development of the gastrointestinal tract (GIT), as probiotics have been described as a live microbial feed supplement that beneficially affects the host *via* improving the intestinal microbial balance (Steinkraus, 1995; Rezac et al., 2018). *Lactobacillus* has been well researched and is reported to be the most common microorganism associated with fermented foods, and most strains of this LAB species can reasonably confer a health benefit when an adequate amount is ingested (Hill et al., 2014; Sanders et al., 2018).

Lactobacilli have been reported as potential probiotics or employed as a starter culture, because of the essential roles they played during food fermentation. Up to 20 LAB species, consisting mainly of the genus *Lactobacillus*, were recognized by Canadian Food Inspection Agency [CFIA] (2013). Health Claims. Probiotic Claims. Summary table of acceptable non-strain specific claims for probiotics and eligible species for the claims. in Section 8.7.3 (Canadian Food Inspection Agency [CFIA], 2013). Many African fermented foods have been reported to confer probiotic attributes and enormous health benefits when

consumed. These foods have probiotic potentials as so many isolated and characterized LAB are capable of surviving at low pH (2.5) and tolerating 0.3% bile salt for several hours. Application of viable cultures in controlling diarrhea among infants and their use as functional foods have been reported. Lei and Jakobsen (2004) reported the ameliorative effect in consuming *koko* sour water (Ghanaian spontaneously fermented millet drink) among diarrhea patients (diarrhea is the main cause of infant morbidity and mortality in developing countries). The *koko* and *koko* sour water millet porridge and drink were reported to contain an abundance of viable LAB, which are responsible for the beneficial effect conferred when these beverages are consumed. A similar report was obtained among Tanzanian children that were fed with *togwa* (lactic acid fermented cereal gruel) (Svanberg et al., 1992). A large number of LAB strains of the genus *Lactobacillus* and *Bifidobacterium* from fecal or vaginal sources are frequently reported compared to LAB of food origin, as many food-origin LAB are likely to be recovered from fecal sources.

Lactobacillus have been reported to have the ability to adhere to intestinal cells *via* coaggregation to form normal balanced flora and also produce compounds such as organic acids, hydrogen peroxides, and bacteriocins that are inhibitory to pathogenic organisms (Adeyemo et al., 2018; Ajayeoba and Ijabadeniyi, 2019). Several benefits are associated with the consumption of foods containing sufficient amounts of probiotic-containing well-defined and viable food-grade microorganisms (Dicks and Botes, 2010; Yadav et al., 2011; Furtado-Martins et al., 2013; Pasolli et al., 2020); these benefits include the following:

- Regulation of gut microbiota, which, in turn, leads to the improvement of the intestinal health of the host.
- Immune system development and prevention of infectious diseases.
- Improved nutritional quality of fermented products.
- Enhancement of the bioavailability of nutrients.
- Alleviation of allergies and of symptoms that may be associated with the lactose-intolerant individuals.
- Development of nutraceutical and/or functional foods.
- Production of bioactive compounds that enhances the functionality of foods and beverages.
- Lowering of serum cholesterol levels.
- Reduction in the risk of contracting some diseases.
- Prevention of cancer.

To harness the full LAB potentials of food origin, daily consumption of fermented foods is recommended. In the consensus statement on fermented foods issued by the International Scientific Association for Probiotics and Prebiotics (Marco et al., 2021), the panelists recommended that the term "Probiotic" should be used when demonstrated health benefits are conferred on humans by well-defined and characterized live and active microbes. From the conclusion, it is not enough that fermented foods contain viable and active microbes, but foods that will be labeled as probiotics must possess additional health benefits with proven safety and should ensure that such strain-specific products must be able to confer the required benefits on the host when the adequate amount is consumed.

FERMENTED FOODS AND HUMAN GUT HEALTH

The inclusion and activities of bacteria as part of the human gut microbiota play valuable roles in the general health and wellbeing of man. The consumption of fermented foods is very crucial to gut health, as the LAB associated with these foods assist in boosting the good/beneficial microflora in the intestinal tract, resulting in the increased gut microbiome in the digestive system through which the human immune system is enhanced. Several investigators have recommended the consumption of traditional fermented foods as a veritable source of probiotics in humans (Mahasneh and Abbas, 2010; Jafari et al., 2011). Daily consumption of fermented foods containing live microorganisms will aid the delivery of substantial beneficial microorganisms to the GITs, as the presence of microorganisms associated with fermented foods in the GIT is sparsely documented (Zhang et al., 2016; Rezac et al., 2018). Some probiotic LAB isolated from the human gut have displayed their functionality in health improvement and have also been found to display some characteristics such as immune system improvement, inhibition of pathogenic bacteria, and modulation of epithelial cell permeability, which is relevant to therapeutic and/or prophylactic treatments against various diseases (Anderson et al., 2010; Ashida et al., 2011; Diaz Heijtz et al., 2011; Ahern et al., 2014; Wang et al., 2018).

Investigations have shown that fermented foods are the source of LAB represented in the gut microbiome. The predominance of these LAB in the human gut is a result of different contributing factors, age, lifestyle, geographical location, diet, and use of antibiotics, as the gut microbial composition is directly influenced by main dietary composition (Brewster et al., 2019). Several LAB species associated with fermented foods have been reported to have similar physiological traits with the strains known for improving gut health. The increasing interest in the use of probiotic LAB as a vehicle for drug delivery and treatment of GIT diseases has also been well documented (Mokoena et al., 2016; Wang et al., 2018). The probiotic microbes associated with fermented foods have immensely contributed to the protective roles in the gut as they are found to exhibit a strong inhibitory effect *in vitro via* organic acid production. *Lactobacillus* spp. also contribute to the healthy microbiota of human mucosal surfaces.

Gut bacteria perform several functions in the maintenance of human health, including production of vitamins, regulation of gut motility and development, maintenance of epithelial integrity by regulating tight junction permeability, inhibition of pathogenic microorganisms, and development of the central nervous system (CNS; Ashida et al., 2011; Erny et al., 2015; Mangalam et al., 2017). It has been proposed that gut bacteria are required to maintain epithelial integrity by regulating tight junction permeability. Karczewski et al. (2010) reported the ability of the LAB strain *L. plantarum* WCFS1 in enhancing the intestinal barrier of the host with intestinal disorders; the experiment showed the ability of the *L. plantarum* strain to regulate tight-junction proteins and to protect against chemical-induced disruption of the epithelial barrier. *L. rhamnosus* species have also been reported to demonstrate adherence to the gut

epithelial tissue, resulting in the colonization of human GIT (Collins et al., 1998; Dunne et al., 2001).

Factors such as excessive antibiotic usage, stress, and diseases can alter the gut microbiota and may lead to dysbacteriosis. Consumption of fermented foods has been proven to be the easiest way of introducing potentially beneficial microorganisms to the GIT, though persistency of the probiotic LAB of food origin in the gut may not occur for a long time; it is recommended that regular consumption of these fermented foods will enhance the ability of the food-associated microorganisms to confer probiotic potentials in human (Derrien and Vlieg, 2015; Zhang et al., 2016). Thus, daily consumption of fermented foods is recommended, as it will enhance a healthy gut because of the innate beneficial microorganisms present in these foods.

CONCLUSION

In Africa, the biotechnological application, development of bioreactor technology, and the use of a starter culture for food fermentation processes should be prioritized through technical skills training, provision of infrastructures, and government willingness in funding research in the area of upgrading fermentation processes and fortification of these foods with bio-enriched vitamins. Also, synergism among research institutes, universities, and manufacturing sectors will play a major role in bringing starter culture development and usage to the limelight in the food industry; this will guarantee consistent and safe food with improved shelf life. Several foods produced in Africa are fermented before consumption, and it is highly recommended that among the household food producers, good manufacturing practices should be effectuated from the point of collection of the raw materials/substrates because of the risks that are associated with improperly/unhygienically produced foods during the production of the final food products. The use of safe water should also be emphasized throughout the production/fermentation process; food should not be manufactured under poor hygienic conditions, and all these can be achieved by properly educating the food producers. The activities of fermentative LAB and most especially *Lactobacillus* strains have played substantial roles in ensuring food security and extension of food shelf life. Production of antimicrobial substances (most especially bacteriocins) by *Lactobacillus* spp. and the spectrum of antibacterial activity displayed by these species can find their wide applications in the food industry. As applications of *Lactobacillus* are enormous and wide usage as a probiotic and/or as a starter culture due to their ability to improve the product's nutritional and technological features cannot be neglected, this LAB group occupies a central role during the fermentation process and significantly possesses many benefits as discussed in this review, with long and safe usage in the food industry. *Lactobacillus* species (those associated with African fermented foods) are described as key players during fermentation technology and have been reportedly involved in the enhancement of flavor, texture, and improved rheology with health-promoting characteristics, which can be applied in the production of functional foods, thus conferring beneficial attributes and consistency in food quality and thereby making the African food market/industry attractive. However, the use of appropriate metagenomic tools that will provide insights into the gene, structure, and functions of microbial strains with multi-functional properties during controlled fermentation will guarantee product quality and consistency, which will be accessible to all. In developing products with improved quality and safety, the inclusion of technologically relevant microorganisms (such as *Lactobacillus* strains) is crucial and will serve as sustainable interventions for the development of African-specific starter cultures. Development of starter culture technology for both small-scale and industrial-scale food production will lead to products of greater consistency, safe quality, and global acceptance, and all these should be the focus of African researchers and scientists. It can be observed that despite the rich diversity of food-grade microorganisms present in many African indigenous fermented foods, most of the well-defined starter cultures available in the shops and markets are not manufactured in Africa; therefore, there is a need to produce well-defined and well-characterized microbial cultures from autochthonous microorganisms peculiar to African foods.

AUTHOR CONTRIBUTIONS

AA-D: conceptualization and writing—original draft preparation. TA: writing part of the primary draft. AA-D and SD: technical editing of the manuscript and reviewing the manuscript for submission. All authors have read and agreed to the published version of the manuscript.

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