Microbiota and metabolic compounds of traditional sweet fermented highland barley from Chinese Qinghai

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Summary
Sweet fermented highland barley, a traditional whole grain food with abundant nutrients, is commonly consumed in Qinghai of China. However, little is studied about its microbial composition and metabolic compounds, limiting the development and industrialization. The study explored the microbial communities and metabolic compounds of the representative sweet fermented highland barley. According to the results, core operational taxonomic units (OTUs) mainly belong to *Lactobacillus, Pediococcus, Issatchenkia* and *Sacchromycopsis*. Firmicutes and Ascomycota dominated in sweet fermented highland barley. Significant biomarker taxa were found. With network analysis, *Bacillus*, *Weissella* and *Leuconostoc* had more positively correlations with other taxa, while *Pediococcus*, *Lactobacillus* and *Acetobacter* had more negatively correlations with other taxa. The most enriched Kyoto Encyclopedia of Genes and Genomes (KEGG, Kyoto University, Kyoto, Japan) pathways in the sweet fermented highland barley mainly included metabolism of different amino acids, nucleotide, starch and saccharose, and vitamin. The microorganisms were closely related with the chemical substances. The findings offer valuable insights into the microorganisms and their interactions with metabolic compounds, paving the way for further research and industrial applications. The identification of core microbial communities and their metabolic activities underscores the potential health benefits and nutritional value of sweet fermented highland barley.

Keywords
sweet fermented highland barley; microbiota; illumina sequencing; metabolic analysis

Highland barley, known as naked barley or hull-less barley as well, is classified as *Hordeum vulgare* Linn var. *nudum*. It is named as "Qingke" in Chinese [1]. It is mainly cultivated at highlands regions including Tibet and Qinghai, highly resistant to cold, and widely adaptable [2, 3]. Highland barley possesses superior nutritional functions [1]. Compared with other grains, highland barley has high dietary fibre, rich in β-glucan, arabinoxylan and polyphenols [4], result in the antioxidant, antitumor and antibacterial abilities. As a rich resource of dietary fibre and nutrients, regular consumption and intake of highland barley is vital in keeping dietary balance, and has important functions on preventing colon cancer, cardiovascular disease and lowering cholesterol, reducing the content of blood lipid and delay the glucose absorption by small intestine, and prevent the occurrence of diabetes [1, 5, 6]. It is used for research and specialty food development, such as gluten-free foods.

Fermentation can extend shelf life, conferring beneficial properties of flavour, texture, and nutrition for food [7]. It also represents an economically efficient method for preserving and processing food. Sweet fermented highland barley represents a traditional whole-grain food made from highland barley, popular in Chinese Qinghai regions. Due to the cold and scarcity in Qinghai region, geographical environments such as oxygen lack and high altitude, traditional fermented foods are popular among local populations [8]. A significant proportion is occupied in the dietary structure, and fermented foods contain functional microorganisms that could have a close relationship with the gut microbiota balance of local popula-

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tions [8]. Traditional sweet fermented highland barley undergoes a complex fermentation process, resulting in a diverse microbial community. However, limited information is available regarding the microbial communities and metabolic compounds of this traditional food product.

To address this knowledge gap, high-throughput sequencing and liquid chromatography-mass spectrometry (LC-MS) analysis were used in this study to provide a comprehensive understanding of sweet fermented highland barley. It was expected that the microbial communities in different samples of sweet fermented highland barley would vary, but some core microbial groups would be shared among them. Additionally, given the distinct flavour and quality of sweet fermented highland barley, it was hypothesized that the microbial community would closely correlate with the metabolic compounds present. This investigation would explore the microbial diversity and metabolic compounds of sweet fermented highland barley samples, as well as their relationships. Findings from this study could contribute to the microbial basis of sweet fermented highland barley and offer valuable insights for other fermented foods, as well as potential applications to enhance food properties.

MATERIALS AND METHODS

Microbial diversity analysis

Three representative Chinese sweet fermented highland barley samples (XN, HFX and BHS) from Qinhai province were chosen and brought from local families. One gram of each sweet fermented highland barley sample was weighted and the total DNA of the sweet fermented highland barley was prepared with DNA Kit (Omega Biotek, Norcross, Georgia, USA), with three biological replicates.

For bacterial 16S rDNA amplification, primers of 338F and 806R were used [7]. For fungal internal transcribed spacer (ITS) rDNA amplification, the primers ITS1F and ITS2R targeting the ITS1 region of fungi were employed [7, 9]. Polymerase chain reaction (PCR) amplification was carried out using an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany) with a 25 μl reaction system. The reaction mixture comprised of 2×Taq PCR Mix (12.5 μl), bovine serum albumin (BSA, 3 μl), each primer (1 μl, 10 μmol·l^{-1}) respectively, 2.5 μl of metagenomic DNA, and 5 μl double distilled H_2O. The PCR products underwent purification, and the sequencing libraries were then constructed, and Illumina Novaseq 6000 PE250 (Illumina, San Diego, California, USA) was employed for sequencing in Beijing Allwegene Technology (Beijing, China) following its pipeline.

Quantitative insights into microbial ecology QIIME (University of Colorado, Boulder, Colorado, USA) was applied to dissect the sequences, and count the richness and diversity indices according to the operational taxonomic units (OTUs) [10]. Qualified sequences were categorized into OTUs with 97% similarity. To characterize the differences among multiple samples, principal component analysis (PCA) was executed using R software (R Foundation for Statistical Computing, Vienna, Austria), as well as linear discriminant analysis effect size (LefSe) analysis, Spearman test.

Metabolites extraction and analysis

50 mg of sample was introduced into the 300 μl pre-cooled solution of methanol-acetonitrile-H_2O in a volumetric ratio of 2:2:1, which included two steel balls. The tissue grinder Tissuelyser-48 (Shanghai Jingxin Technology, Shanghai, China) was applied at 60 Hz for 120 s and ultrasound with the ultrasonic instrument PS-60AL (Shenzhen Leidebang Electronics, Shenzhen, China) for 10 min. Subsequently, the sample was incubated at –20 °C for 1 h, followed by centrifugation at 12 000 ×g for 10 min. The supernatant was gathered and subjected to freeze-drying. For mass spectrometry (MS) analysis, the freeze-dried sample was reconstituted in a 100 μl solution of acetonitrile-H_2O (1:1), vortexed for 30 s, and ultrasonicated for 10 min. The reconstituted solution was aspirated into the injection bottle. Moreover, 10 μl of each sample was pooled to create a quality control, also loading into an injection vial. The samples were processed by the LC-MS system according to the manufacturer's instructions. All chromatographic separations were made by an ultra-performance liquid chromatography (UPLC) system from Shimadzu (Kyoto, Japan). The separation was made by Waters Acquity UPLC T3 column (Waters, Milford, Massachusetts, USA). It was held at 50 °C, while the flow rate was 0.3 ml·min^{-1}. Metabolic analytes eluted from the column were collected in positive and negative mode. The specific parameters could refer to Chen et al. [11]. The extraction, processing and analysis also consulted the records of Alseekh et al. [12] and Dunn et al. [13]. The internal MS databases (Allwegene, Beijing, China) were used for metabolite annotation. Each LC-MS/MS sample was processed in 6 replicates.

RESULTS AND DISCUSSION

Microbiota diversity analysis

527 590 bacterial and 904 711 fungal clean sequences were generated from the sweet fermented highland barley samples. Distribution analysis revealed that the majority of bacterial sequences had a length of 420–440 bp (90.0 %), while fungal sequences had a length of 260–320 bp (63.9 %).

Sequences were classified to 164 OTUs of bacteria and 407 OTUs of fungi. The values of Goods coverage were 1.00 for all the samples. Among bacteria, all three α-diversity indices, i.e. Chao 1, Observed species, and Shannon index, exhibited the highest values in sample HFX (Tab. 1). Regarding fungi, sample XN showed the highest fungal richness in terms of Chao 1, observed species, and Shannon index (Tab. 1). The lowest values of Chao 1, observed species, and Shannon index were found in sample BHS for both bacteria and fungi. These results suggest that the sample BHS has the lowest diversity of both bacteria and fungi, the sample HFX exhibited the highest bacterial diversity and XN had the highest fungal richness.

Principal component analysis (PCA) demonstrated that each type of bacterial and fungal community in sweet fermented highland barley formed individual clusters (Fig. 1).

Analysis of similarities (ANOSIM) confirmed significant differences in microbial compositions among the sweet fermented highland barley samples with 999 permutations (R statistic = 1.000, $p = 0.003$ for all group).

Microbial compositions

Nine bacterial phyla and ten fungal phyla were identified in total. Firmicutes was predominant bacterial phylum (all > 80 %), followed by Proteobacteria. Phyla abundance varied among sweet fermented highland barley samples. As to fungi, Ascomycota constituted the highest abundance (XN 57.0 %, HFX 85.8 %, BHS 84.5 %), followed

Tab. 1. Microbial α-diversity indices of sweet fermented highland barley.

Microbial type	Sample	α-diversity indices		
		Chao1	Observed species	Shannon
Bacterial	XN	95.7 ± 2.1	85.7 ± 1.4	1.7 ± 0.1
	HFX	108.1 ± 3.9	90.7 ± 2.0	2.6 ± 0.1
	BHS	63.3 ± 3.7	54.3 ± 1.7	0.7 ± 0.1
Fungal	XN	174.7 ± 6.8	150.7 ± 3.2	2.4 ± 0.2
	HFX	172.8 ± 14.2	143.7 ± 8.9	2.3 ± 0.4
	BHS	151.6 ± 5.8	126.0 ± 3.1	2.1 ± 0.1

XN, HFX, BHS – samples of sweet fermented highland barley.

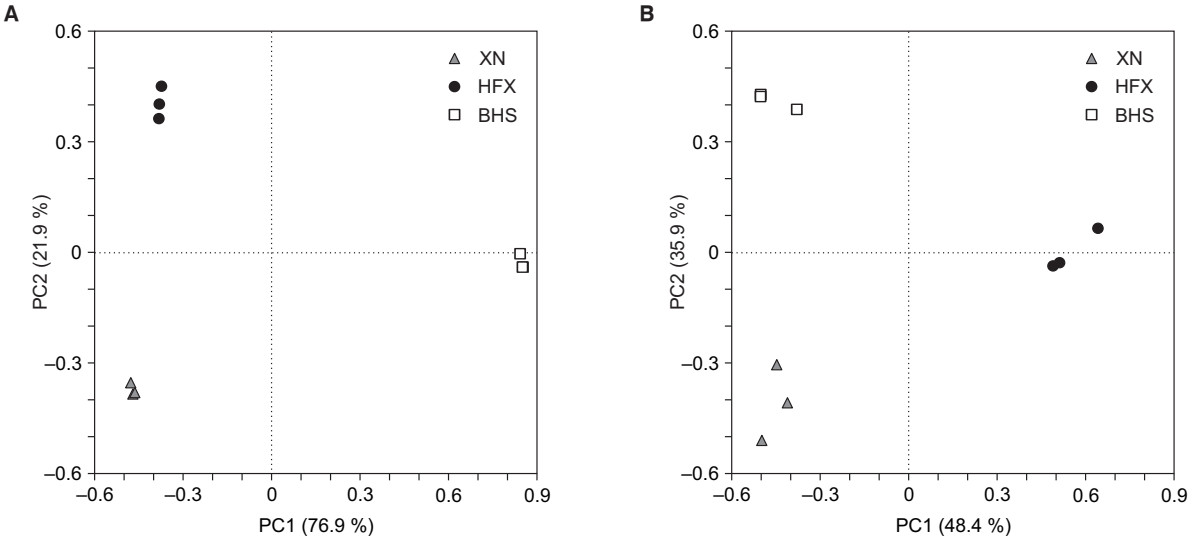

Fig. 1. Principal component analysis of microbial communities β-diversity of sweet fermented highland barley.

A – bacterial community, B – fungal community.
XN, HFX, BHS – samples of sweet fermented highland barley.

by Mucoromycota (XN 42.5 %, HFX 12.3 %, BHS 15.0 %) and Basidiomycota (XN 0.2 %, HFX 1.2 %, BHS 0.2 %).

At the genus level, the sweet fermented highland barley samples exhibited different relative abundance and composition. For XN samples, a prokaryotic genus *Bacillus* was most abundant, followed by *Acetobacter*. *Lactobacillus* was most abundant in HFX samples. *Pediococcus* predominated in BHS samples, followed by *Lactobacillus*. Eukaryotic genera *Rhizopus* and *Aspergillus* were most abundant in XN. *Issatchenkia* and *Rhizopus* were most abundant in HFX. *Sacchromycopsis* and *Rhizopus* were most abundant in BHS.

45 bacterial and 97 fungal OTUs were classified in the samples of sweet fermented highland barley. The range of unique OTUs was from 12 bacterial OTUs (BHS) and 48 fungal OTUs (BHS) to 28 bacterial OTUs (XN) and 87 fungal OTUs (XN). Across all the samples, core OTUs mainly belonged to *Lactobacillus, Pediococcus, Weissella, Bacillus, Issatchenkia, Sacchromycopsis, Candida* and *Penicillium*.

Among sweet fermented highland barley samples, significant biomarker taxa from phylum to species were identified by LEfSe analysis. Based on the linear discriminant analysis score, *Bacillus* and *Weissella* in XN, *Lactobacillus*, *Leuconostoc* and *Acetobacter* in HFX, and *Pediococcus* (with species *P. acidilactici*) in BHS were most important biomarkers for bacterial community. As to fungi, abundance of *Suillus*, *Millerozyma*, *Moniliella* and *Morchella* in XN, abundance of genus *Issatchenkia* in HFX and *Sacchromycopsis*, *Wickerhamomyces*, and *Saccharomyces* in BHS might account for the differences in fungal composition and could serve as important biomarkers.

The microbial communities of sweet fermented highland barley samples exhibited variations on relative abundance and composition, despite the consistent presence of bacterial taxon Firmicutes and fungal taxon Ascomycota as the richest phyla, which is consistent with previous reports on microbial communities in fermented foods like soybean paste [7]. The most abundant groups included lactic acid bacteria, *Bacillus*, *Acetobacter*, yeasts, *Rhizopus* and *Aspergillus*. It is worth noting that different samples can host distinct microbial communities due to their unique microenvironments [7].

Our study found that common shared core OTUs for the samples mainly belong to *Lactobacillus, Pediococcus, Weissella, Bacillus, Issatchenkia, Sacchromycopsis, Candida* and *Penicillium*. These taxa likely play significant roles in the fermentation process of sweet fermented highland barley. *Lactobacillus, Pediococcus* and *Weissella*, members of the lactic acid bacteria group, are frequently detected in various foods [7, 8, 14].

Lactobacillus spp. and their metabolites are utilized as biopreservatives to prevent pathogens for ensuring food safety and quality [15].

Pediococcus spp. possess the capability to produce flavours, linked to the biosynthesis of excessive diacetyl, exopolysaccharides, and/or biogenic amines [16].

Weissella spp. could be found in a wide range of habitats, including various fermented foods. Some *Weissella* strains have garnered attention as potential probiotics and have been investigated for their therapeutic potential, such as controlling periodontal disease [17].

Certain *Bacillus* species are generally regarded as safe (GRAS) by Food and Drug Administration [22]. Some *Bacillus* species are recognized for their capability to produce a diverse array of extracellular enzymes, making them valuable industrial enzyme producers [18].

Issatchenkia, Sacchromycopsis and *Candida* are all yeasts that are widely used in fermentation processes. *Issatchenkia* has been found to exhibit notable tolerance to various stresses, such as low pH, making it a potential candidate for organic acid production [19]. *Sacchromycopsis* has been reported to participate in the fermentation of rice protein [20], while *Candida* fermentation may enhance the food quality [21].

Penicillium is a diverse genus with members that play significant roles in the decomposition of organic materials. Some *Penicillium* species are considered as enzyme factories, while others can cause detrimental rotting in food products [22].

Significant biomarker taxa were found by LEfSe analysis, *Pediococcus* (with species *P. acidilactici*) in BHS was most important biomarkers of bacteria, and fungal abundance of *Suillus, Millerozyma, Moniliella* and *Morchella* were found in XN. *Pediococcus* (with species *P. acidilactici*) predominated in BHS samples. *P. acidilactici* showed beneficial properties as potential probiotic, starter culture and could be bacteriocin-producing [23].

Millerozyma and *Moniliella* were yeast. *Millerozyma* could be xylitol-producing thermotolerant yeast, isolated from traditional Korean fermentation initiators [24]. *Moniliella* members have been characterized for their role in beer fermentation [25].

Interestingly, *Suillus* and *Morchella* are large edible fungi known for their unique flavour. *Suillus* mushrooms are conspicuous, typically found above-ground, and contribute significantly to ectomycorrhizal sporocarp production in conifer forest

ecosystems. *Morchella* mushrooms are highly regarded for their special flavour and health-promoting benefits. They encompass diverse bioactive components such as polysaccharides, polyphenolic compounds, proteins, and protein hydrolysates, contributing to their range of biological activities [26]. Grain fermentation with edible fungi, including *Suillus* and *Morchella*, has gained attention due to their beneficial effects [27].

Further studies can be conducted in the future to explore and understand the roles and potential applications of these taxa in grain fermentation processes.

Correlation network analysis

Spearman test was performed to explore the correlation among the top 30 genera abundances of all the sweet fermented highland barley samples, and the related phylum was used as legend, the results filter out those with p values greater than 0.05. The results revealed that among the most abundant genera, *Bacillus, Weissella, Leuconostoc*, and others exhibited predominantly positive correlations with other taxa, while *Pediococcus, Lactobacillus, Acetobacter* and others showed predominantly negative correlations with other taxa. The correlations among fungal genera appeared to be simpler compared to bacterial genera. Key genera such as *Issatchenkia, Rhizopus, Sacchromycopsis* and others were found to be correlated with other genera. The thickest line was observed between *Mortierella* and *Ambrosiozyma*, indicating a high level of correlation.

These results help explain the structural differences observed in the network analysis, suggesting that each microbe in sweet fermented highland barley forms its own unique network with others, eventually leading to different microbial community compositions as a whole.

Tab. 2. Principal components analysis model parameter of metabolites.

A	*N*	R^2X(cum)	Title
2	21	49.6 %	Total sample
2	12	43.0 %	XN vs HFX
2	12	62.7 %	XN vs BHS
2	12	60.8 %	HFX vs BHS

A – the number of principal components in the model; *N* – the number of observations in the model; R^2X(cum) – represent the model's explanatory power for variable *X*; Title – the data objects corresponding to this model.
XN, HFX, BHS – samples of sweet fermented highland barley.

Metabolic data analysis

LC-MS was utilized to analyse the metabolites of sweet fermented highland barley. PCA was conducted to assess the samples' metabolic profiles (Tab. 2). The results indicated that samples within each group fell within the 95% confidence intervals, demonstrating good within-group reproducibility. The XN vs BHS group and HFX vs BHS group exhibited significant differences, as reflected by the longer distances between their confidence intervals. While, the confidence intervals for the XN and HFX groups partially overlapped with relatively small differences.

The super-classes included lipids and lipid-like molecules, benzenoids, phenylpropanoids and polyketides, organoheterocyclic compounds, alkaloids and derivatives, nucleosides, nucleotides, and analogues, organic acids and derivatives, and organic oxygen compounds.

The different metabolic components among sweet fermented highland barley were measured by Kruskal-Wallis H test ($p < 0.05$) (Fig. 2).

Among top 10 chemicals, *cis*-8,11,14-eicosatrienoic acid, taxa-4(20),11(12)-dien-5alpha-acetoxy-10beta-ol, indoline, 7-O-acetylaustroinulin, and various amino acids were included. Among them, *cis*-8,11,14-eicosatrienoic acid, taxa-4(20),11(12)-dien-5alpha-acetoxy-10beta-ol, indoline, 7-O-acetylaustroinulin were significantly abundant in BHS samples. Conversely, nicotinurate and caffeic acid showed significantly higher levels in XN samples. Additionally, some beneficial compounds like cordycepin were also detected.

The detected compounds are known for their bioactive potential. *cis*-8,11,14-Eicosatrienoic acid is a major constituent of peanut oil and has been reported to possess antibacterial activity [28]. Taxa-4(20),11(12)-dien-5alpha-acetoxy-10beta-ol is an important precursor of the anticancer drug paclitaxel. 7-O-acetylaustroinulin is a diterpene compound isolated from *Stevia rebaudiana*, which is a major source of high-potency sweeteners. Nicotinurate could be one of the diabetes biomarkers and positively related with body mass index and blood pressure [29]. Caffeic acid is known for its powerful antioxidant and antimicrobial activities. It also promotes collagen generation and helps prevent premature aging, making it promising for the treatment of dermal diseases [30]. Cordycepin has garnered attention since its medicinal potential and biological value in interacting with various therapeutic targets involved in cancer, tumors, inflammation, and other conditions [31]. More research is warranted for the health benefits in sweet fermented highland

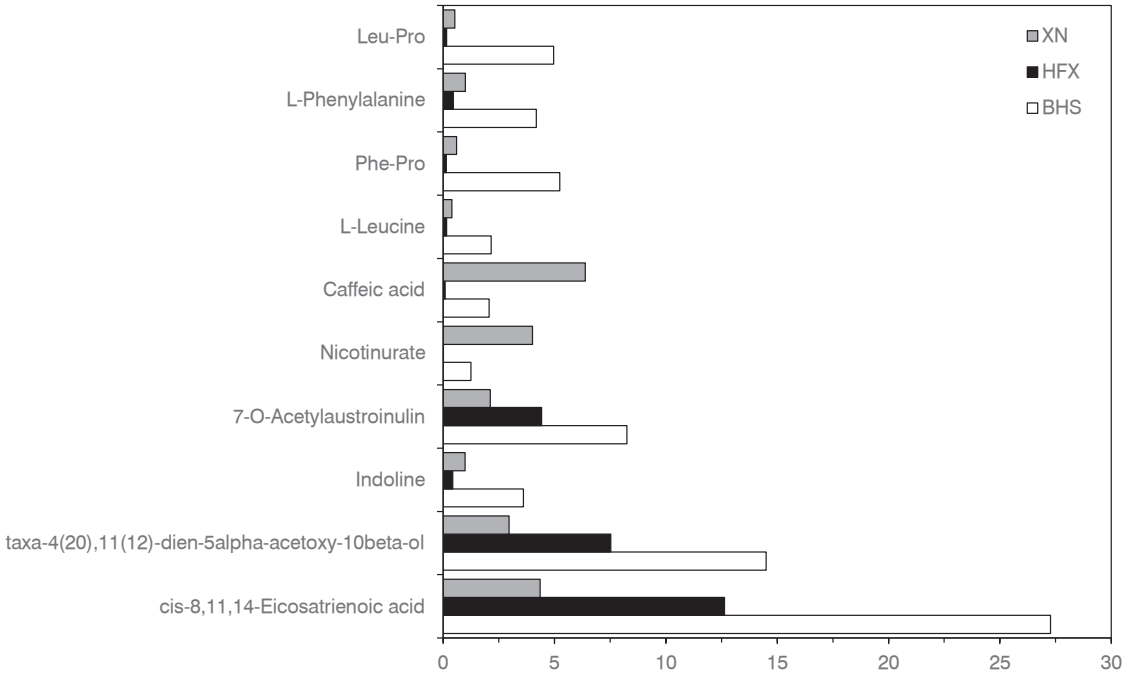

Fig. 2. Diagram of Kruskal-Wallis H test to detect the different metabolic components among sweet fermented highland barley samples.

XN, HFX, BHS – samples of sweet fermented highland barley.

Tab. 3. Top 20 enriched KEGG pathways for the sweet fermented highland barley samples.

KEGG pathway term	Impact rich factors [%]	Number of hits
Nucleotide metabolism	17.2	10
Valine, leucine and isoleucine biosynthesis	17.4	4
Pyrimidine metabolism	10.9	7
Aminoacyl-tRNA biosynthesis	11.5	6
Pantothenate and CoA biosynthesis	13.3	4
Phenylalanine metabolism	10.2	5
D-Amino acid metabolism	7.2	5
Cyanoamino acid metabolism	11.1	5
ABC transporters	6.5	9
Valine, leucine and isoleucine degradation	7.1	3
Alanine, aspartate and glutamate metabolism	7.1	2
Purine metabolism	5.9	6
Pyruvate metabolism	6.3	2
Biosynthesis of amino acids	5.5	7
Starch and saccharose metabolism	8.1	3
Vitamin B6 metabolism	6.9	2
Streptomycin biosynthesis	4.2	1
Cysteine and methionine metabolism	4.5	3
2-Oxocarboxylic acid metabolism	4.5	6
Phenylalanine, tyrosine and tryptophan biosynthesis	5.7	2

KEGG – Kyoto Encyclopedia of Genes and Genomes (Kyoto University, Kyoto, Japan).

barley. Cordycepin-producing microorganisms, such as *Cordyceps*, usually thrive in highland regions. It raises the question of whether the compound's presence in sweet fermented highland barley is related to its geographical distribution. Regional environmental factors could influence and make the samples host different microbial communities [7], meticulous researches on their effects for our samples should be conducted.

Main metabolic pathways, and correlation analysis

The top 20 enriched Kyoto Encyclopedia of Genes and Genomes (KEGG, Kyoto University, Kyoto, Japan) pathways for the sweet fermented highland barley samples mainly included various amino acids like valine, leucine, isoleucine, alanine, nucleotide metabolism, starch and saccharose metabolism, and vitamin metabolism (Tab. 3). Nucleotide metabolism, ABC transporters, and amino acids biosynthesis were among the pathways with the highest number of hits.

The relationship between the microbes and metabolic components was analysed. Spearman correlation uncovered a close correlation between the microorganisms and chemical substances. Specifically, it was found that bacterial genus *Pediococcus* and fungal genus *Saccharomyces* exhibited a notable positive correlation with the abundance of 7-O-acetylaustroinulin, *cis*-8,11,14-eicosatrienoic acid, and taxa-4(20),11(12)-dien-5alpha-acetoxy-10beta-ol. Conversely, bacterial genera *Weissella* and *Bacillus*, as well as fungal genus *Millerozyma*, showed a negative correlation with these compounds. Bacterial genus *Leuconostoc* and fungal genera *Candida* and *Ambrosiozyma* exhibited a negative correlation with the middle five compounds (Phe-Pro, L-Phenylalanine, Leu-Pro, Indoline, L-Leucine). *Saccharomycopsis*, on the other hand, displayed a positive correlation with these five compounds.

The correlation analysis indicated that the detected compounds were closely correlated with several bacterial and fungal genera. These findings suggest the complex interactions between bacterial and fungal microbes in determining the chemical composition and properties of sweet fermented highland barley.

Significance and limitations of the study

The sweet fermented highland barley is a popular flavour snack in northwest region of China. It is renowned for its rich nutrients, sweet and sour taste, and various health benefits such as aiding digestion, relieving thirst, and promoting stomach health [1, 8]. It is rich in nutrients and well-balanced, abundant in protein and fibre, with low fat. According to our lab-made sweet fermented highland barley, the fat content is even lower after fermentation.

However, there have been limited studies conducted on sweet fermented highland barley, with some focusing on its production process and optimization technology [1, 8]. This has resulted in a lack of comprehensive understanding of the microbiota and their functions in sweet fermented highland barley. Given the health benefits of highland barley and the advancements in fermentation technology, there has been an increasing interest in highland barley fermentation and related products [32–35]. We investigated the microbiota and metabolic compounds of representative sweet fermented highland barley samples from Qinghai region in China using Illumina sequencing and LC-MS technologies in this study. The investigation provided valuable insights into the relatively little explored microbiota and metabolic compounds of traditional sweet fermented highland barley.

The results provide important information and directions for further research. This study also has some limitations including sampling bias, the cross-sectional nature of the study, and lack of direct functional validation of metabolites. Relevant future experiments are needed to address these limitations. It is also essential to explore the specific interactions between microbes and metabolic components, clarify the relationship between the presence of certain compounds and the geographical origin of the barley, and further explore the potential health benefits and therapeutic applications of bioactive compounds like cordycepin in sweet fermented highland barley.

The findings of this study could be utilized as a reference for subsequent research on the microbes and metabolites of other fermented foods. Additionally, they can provide theoretical foundations for improving the quality and future industrialization of sweet fermented highland barley products.

CONCLUSIONS

This study clarified the microbiota and metabolic compounds of Qinghai's traditional sweet fermented highland barley. Core OTUs were dominated by *Lactobacillus, Pediococcus, Issatchenkia* and *Sacchromycopsis*, with Firmicutes and Ascomycota as the main phyla. LEfSe analysis identified biomarker taxa, and network analysis revealed inter-taxa relationships. KEGG path-

way analysis showed that microbiota were actively involved in amino acid, nucleotide, starch, saccharose, and vitamin metabolism, contributing to the food's nutrition and flavour. The close link between microorganisms and chemical substances underscored the microbiota's crucial role. These results filled knowledge gaps, provided insights for future research, and laid a foundation for exploring health benefits and promoting industrialization. Future studies could focus on the functions of specific microorganisms and metabolites, and optimize the fermentation process for better product quality.

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