Metagenomic analysis reveals microbial community and functional capacity in Kombucha

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Abstract

Kombucha is a traditional beverage obtained from fermented sugar-tea by a community of bacteria and yeasts. Understanding the microbial composition and their functions in Kombucha fermentation is of significance, but most of the studies have relied on the culture-dependent method. In this study, a metagenomic analysis was conducted to obtain a more comprehensive insight into Kombucha fermentation. Results showed that the bacteria of Kombucha were dominated by *Komagataeibacter* (36.24 to 63.35%), *Gluconacetobacter* (10.39 to 26.21%), *Gluconobacter* (6.62 to 27.10%), *Acetobacter* (0.3 to 6.64%), and the fungus *Kluyveromyces* (0.63 to 36.98%) was also identified. Taxonomic composition and abundance of the microbial community were distinct with each Kombucha sample. The carbohydrate active enzyme functions of the communities primarily comprised glycosyltransferase (GT) families (40.6%), glycoside hydrolase (GH) families (32.0%), and carbohydrate-binding module (CBM) families (12.9%). Moreover, functional genes and their KEGG pathways were predicted, which demonstrated that the functional genes present in the bacterial community were enriched in pathways for neurodegenerative disease, amino acid metabolism, metabolism of cofactors and vitamins, carbohydrate metabolism, folding, sorting and degradation, and translation. The results of this study would provide a better understanding of the microbiota and metabolites as well as health-promoting potential of Kombucha, and may facilitate the optimization of the process to produce Kombucha products with desirable qualities.

Keywords: fermentation; Kombucha; metagenomics; microbial community

Introduction

Kombucha is an acidic and sweet beverage obtained from fermented sugared tea, which is widely accepted among consumers worldwide. This beverage is usually fermented by the presence of symbiotic culture of bacteria and yeast (SCOBY) for 10 to 15 days. Kombucha consumption has been associated with many health-promoting properties, such as antimicrobial potential, anti-inflammatory, hepatoprotective, antidiabetic, and antioxidative properties (Hou *et al*., 2021; Jayabalan *et al*., 2014; Morales, 2020).

The enhanced beneficial activities of Kombucha have been brought about by the metabolic interactions between acetic acid bacteria (AAB), lactic acid bacteria (LAB), and yeasts in SCOBY (de Miranda *et al*., 2022). Traditional culture-dependent methods have been applied to describe the microbial communities during the fermentation of Kombucha (Chakravorty *et al*., 2016; Li *et al*., 2022; Torán-Pereg *et al*., 2021). These studies have proved that *Acetobacter*, *Gluconacetobacter*, and *Komagateibacter* were the abundant genus in Kombucha. However, certain species are uncultured on the restricted

culture media, making the description of the whole community unreliable.

In recent years, culture-independent methods have been proved as a good approach to identify microorganisms and reveal their potential metabolic functions in different fermented foods (Chen *et al*., 2020; Song *et al*., 2021; Wang *et al*., 2021; Zhuang *et al*., 2022). The metagenomics analysis provides a higher taxonomic resolution to the species or even strain-level precision with increased accuracy. Moreover, this approach enables functional elucidation of identified microorganisms through nonselective genes annotation of genomic assemblies (Filippis *et al*., 2020). However, the application of metagenomics analysis in characterizing microbial composition and functional microbiota of Kombucha during fermentation is limited.

In this study, the bacterial communities of three traditional fermented Kombucha were characterized by shotgun metagenomics, and the potential metabolic functions of microorganisms were also clarified. The results of this study will facilitate the optimization of process to produce desirable Kombucha products.

Materials and Methods

Kombucha production

Three Kombucha cellulose pellicles with 200 mL starter culture were acquired from three different geographic locations of Guangdong (GD), Anhui (AH), and Shandong (SD) in China. All Kombucha were cultivated under the same condition as previously described by Marsh *et al*. (2014) with slight modifications. For each Kombucha sample, 2 L of water was boiled, and 10 g of black tea (Lipton, UK) was added for the incubation for 20 min at room temperature. After the removal of tea leaves, 200 g of sucrose was added and then stirred to dissolve. Finally, 5% (w/v) of each SCOBY was added once the sugar-tea solution was cooled to room temperature, and then incubated at 30°C for 10 days.

Metagenomic DNA extraction of pellicles

To extract DNA from pellicles of the fermented Kombucha at day 10, 1.0 g of cellulosic pellicle was removed from the surface biofilm, washed twice with sterile water, and then cut into small pieces. Then, the metagenomic DNA of samples was extracted by DNeasy® PowerFood® Microbial Kit (QIAGEN, Germany) according to the manufacturer's instructions. The purity and concentration of DNA were checked by electrophoresis on 1% (w/v) agarose gel and NanoDrop 2000 (Thermo Fisher Scientific, USA).

Kombucha samples were subjected to sequencing on an Illumina HiSeq Xten instrument (San Diego, CA, USA). Library preparation was carried out according to the NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) per manufacturer's instructions. Sequencing was carried out using a 300 cycle High Output V2 kit, following standard Illumina sequencing protocols as described by Doyle *et al*. (2017). Raw whole-metagenome shotgun sequencing reads were trimmed in each sample dataset based on sequence length and quality by using SeqPrep and Sickl. MetaPhlan3 was used to determine the specie-level taxonomic profile of microbiome of each sample using default settings. High-quality Illumina metagenomic samples were assembled using metaSPAdes (ver. 3.13.0). Melonnpan v. 0.99 was used to predict the metabolites produced from each microbiome. Metagenome assembly was carried out to assemble contigs. MetaBAT 2 was implemented for genome binning, with default settings. CheckM was then used to check the quality of metagenome assembled genomes (MAGs), as well as to assign taxonomic classifications. High-quality MAGs (completeness >80% and contamination <10%) were selected for downstream analysis. FastANI was used to assign taxonomy to the MAGs. The annotated amino acid sequences were functionally annotated by comparing against the Kyoto Encyclopedia of Genes and Genomes (KEGG) using diamond. Putative secondary metabolites were encoded by antiSMAH. Amino acid sequences were also compared against the Carbohydrate-Active Enzymes database (CAZy) using HMMER (Chen *et al*., 2020; Villarreal-Soto *et al*., 2020).

Statistical analysis

The statistical significance was calculated by using one-way analysis of variance (ANOVA) in SPSS (version 20, IBM, USA), and only the P value < 0.05 was considered statistically significant.

Results and Discussion

Microbial composition of Kombucha

Detailed insights into both microbial community and their metabolic functions are necessary to obtain Kombucha products of a desired quality. In this study, metagenomics was applied to examine the microbiota of Kombucha after 10 days. A total of 74,262,320, 78,324,270, and 80,119,548 reads were generated for GD, AH, and SD, respectively. There was significant difference

in the read number among three groups ($P < 0.05$). Following assembly, 9912, 10,450, and 10,437 contigs were obtained in GD, AH, and SD, respectively (Table 1). Significant difference ($P < 0.05$) was also observed in species-level α diversity (Shannon, Simpson, and ACE) among the three Kombucha (Table 1).

The sequences comprising the total reads corresponded to 7 phyla, 39 genera, and 77 species in Kombucha. The overall microbial diversity was higher than the findings of previous studies (Jafari *et al*., 2022; Torán-Pereg *et al*., 2021). The results indicated that the metagenomic approach may generate a more complete microbiota profile with enhanced taxonomic resolution and precision in analyzing microbial community in foods when compared with culture-dependent methods (Zhang *et al*., 2022).

At the phylum level, Proteobacteria was the dominant phylum and in all Kombucha samples with a relative abundance of 96, 62.92, and 98.1% in GD, AH, and SD, respectively (Figure 1A). Other studies also reported this as the most abundant phyla in Kombucha (Fabricio *et al*., 2022). Ascomycota is another prevailing phylum in sample AH with an abundance of 36.95% (Figure 1A).

The most abundant genus *Komagataeibacter* accounted for 36.24, 45.61, and 63.35% of microbial composition in GD, AH, and SD, respectively, followed by *Gluconacetobacter and Gluconobacter* (Figure 1B). These genera were also reported to be dominant in previous studies (Lee *et al*., 2022; Subbiahdoss *et al.*, 2022; Villarreal-Soto *et al.*, 2020). *Komagataeibacter* has the ability to produce cellulose, and organic acids integral to sweet and sour flavor profiles of Kombucha, and shows higher resistance to acetic acid than other AAB (Subbiahdoss *et al.*, 2022). *Gluconacetobacter* participated in producing gluconic and glucuronic acids, and Dsaccharic acid-1,4-lactone during the fermentation process (Li *et al*., 2022). Besides these three genera, *Acetobater* proved to be another prevailing genus in GD and SD samples (Figure 1B). The genus of *Acetobacter* produces acetic acid from ethanol via alcohol dehydrogenase and aldehyde dehydrogenase, which enters the Krebs cycle obtaining water and carbon dioxide as end products. Other genera like *Pediococcus*, *Bacillus*, and *Burkholderia* represented less than 1% of the communities found in all samples (Figure 1B). *Pediococcus* strains from Kombucha proved to have high inhibitory activity against a large range of foodborne bacteria and fungi (Diguta *et al.*, 2020).

Table 1. Sample sequencing information and α-diversity indices.

Group	Reads	Contigs	Shannon	Simpson	ACE
GD	74,262,320	9912	3.280023	0.841363	44.0
AH	78,324,270	10,450	2.206987	0.696792	45.0
SD	80,119,548	10,437	2.750050	0.751996	43.0

GD: sample collected from Guangdong; AH: sample collected from Anhui; SD: sample collected from Shandong.

Fungus diversity was considerably lower in Kombucha compared to bacteria due to the pH effect, although bacteria and fungus are both necessary for the fermentation of Kombucha. In this study, *Kluyveromyces* and *Malassezia* were the dominant fungus in all Kombucha samples, while *Aspergillus* and *Pichia* were present in low concentration (Figure 1B). However, other common yeasts in Kombucha (*Dekkera*, *Zygosaccharomyces*, and *Candida*) were undetected in this study. Such distinction in the fungus diversity is rather interesting and needs to be further studied. Yeasts would produce ethanol through the glycolysis pathway by the hydrolysis of sucrose into fructose and glucose (Hou *et al*., 2021).

At the species level, *Komagataeibacter intermedius*, *Gluconacetobacter sp.*, *Gluconobacter oxydans*, *Komagataeibacter hansenii*, and *Komagataeibacter rhaeticus* were among the most abundant species in GD and SD samples. However, the abundant species in the AH sample were *Komagataeibacter saccharivorans*, followed by *Kluyvromyces marxianus*, *Gluconacetobacter* sp, *K. hansenii*, and *Gluconobacter oxydans* (Figure 1C). *Listeria monocytogenes* was identified in Kombucha. This may be due to the strong tolerance to low pH in Kombucha (Duze *et al*., 2021). This foodborne pathogen may cause a life-threatening disease in humans. Other pathogenic microorganisms, including *Salmonella enterica* and *Penicillium* spp, were also identified from Kombucha prepared in unhygienic environments (Villarreal-Soto *et al*., 2020).

LAB are not always isolated from Kombucha. *Lactococcus lactis*, *Lactobacillus parakefiri*, *Lactobacillus* sp. wkB10, and *Bifidobacterium* sp. were identified in this study. LAB were also reported to be used in Kombucha fermentation to enhance its biological function or added as probiotics (Fu *et al*., 2014; Yang *et al*., 2022). The presence of LAB is interesting due to their potential to confer probiotic properties and promote health through production of prebiotics (Amiri *et al*., 2021; Milićević *et al*., 2021).

A significant difference in bacterial community of different Kombucha samples at the genus level was observed in this study, due to the weather, geographical location, and different fermentation conditions (Leal *et al*., 2018). In general, Kombucha is homemade with poor control of the microorganisms in the starter culture, resulting in a final product with heterogeneous properties (Noronha *et al*., 2022).

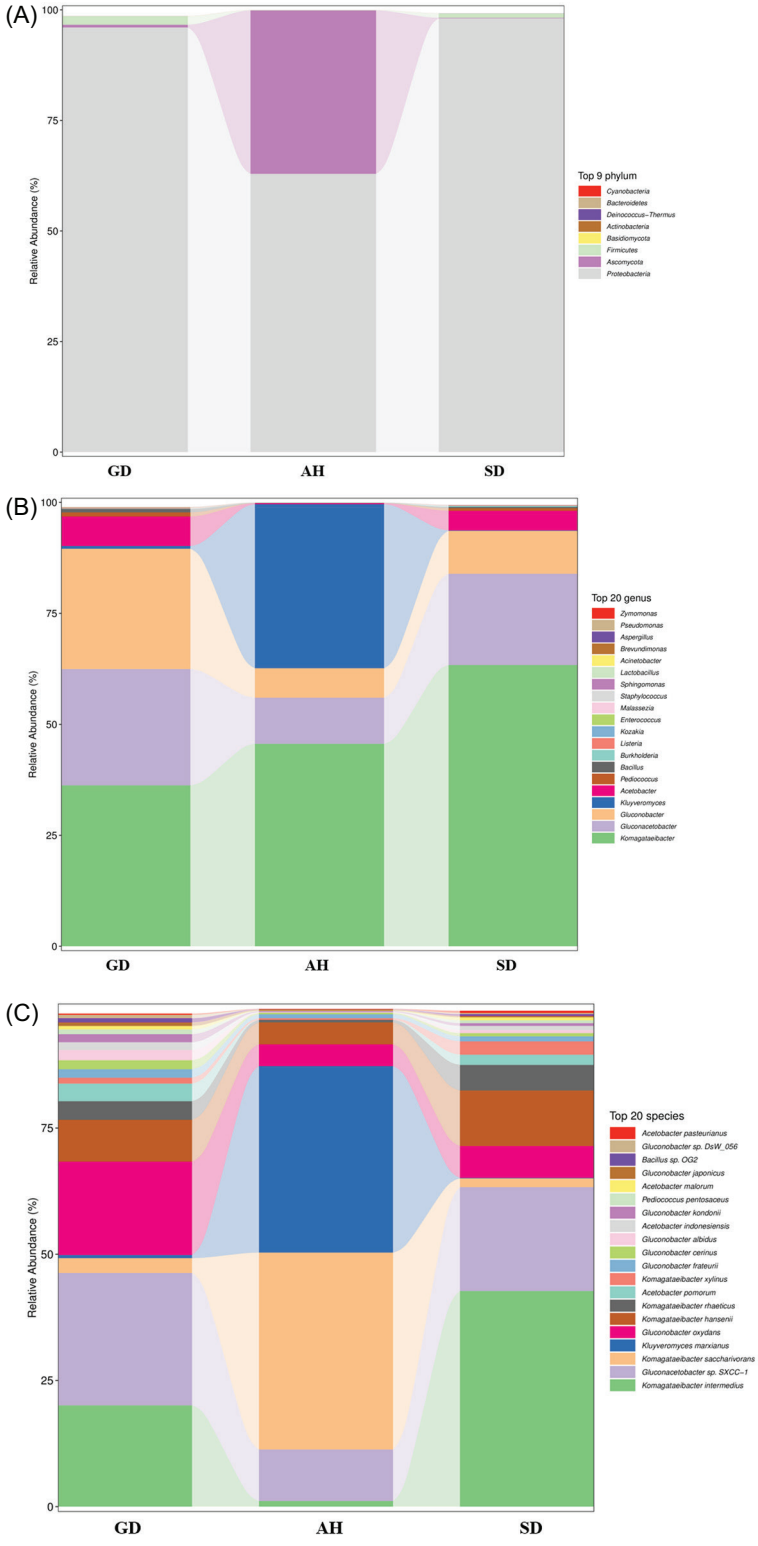

Figure 1. Classification of microbial population of three different Kombucha after 10 days of fermentation at the phylum level (A), genus level (B), and species level (C). GD, AH, and SD indicate the samples collected from Guangdong, Anhui, and Shandong provinces, China..

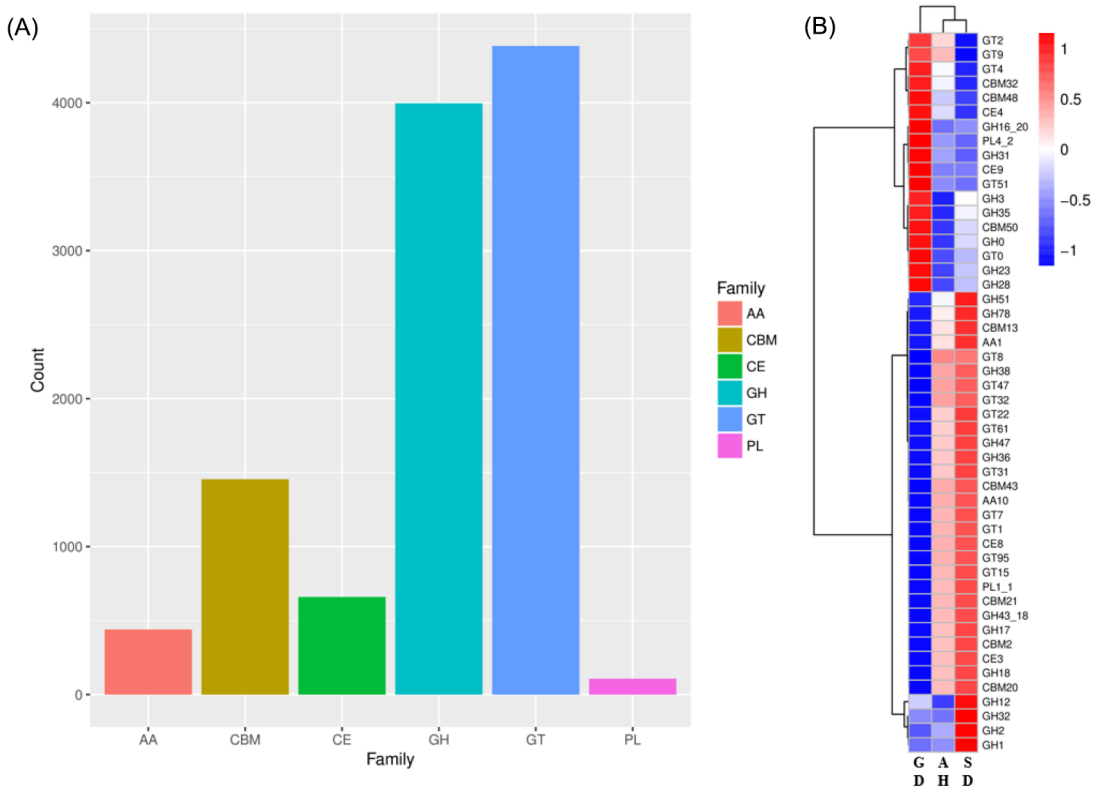

Figure 2. Relative abundances of carbohydrate-active enzymes at the family level (A) and their distribution in different Kombucha samples (B).

Functional annotation of microbial communities in Kombucha

Since various active enzymes are involved in the degradation of sucrose during the Kombucha fermentation, the potential genes were predicted to be involved in carbohydrate metabolism based on comparisons of genes against the CAZy database. It is interesting to identify the differences in the metagenomic potential of carbohydrate utilization capacity between the Kombucha samples. At the class level, glycosyltransferases (GT) families GT2, GT8, GT4, GT1, and GT0 are the abundant enzymes in Kombucha, followed by glycoside hydrolases (GH) families GH18, GH2, GH23, and GH28; carbohydrate-binding modules (CBM) families CBM48, CBM50, and CBM13; carbohydrate esterases (CE) families CE9, CE8, and CE3; and auxiliary activity (AA) families AA1 and AA6 (Figure 2A). Comparison of the metagenome-encoding microbial carbohydrate metabolism genes across the three Kombucha revealed that: seven CAZy families (GT2, GT4, CBM48, PL4_2, GT1, GH23, and CBM50) were significantly enriched in GD; three CAZy families (GT2, GT4, and CBM48) were significantly enriched in AH; and GT2, GT4, CBM48, GT1, and CE8 were significantly enriched in SD (Figure 2B). GTs catalyze the formation of glycosidic bonds by transferring a sugar residue from a donor to an acceptor, which could include carbohydrates, proteins, lipids, and other molecules. Cellulose synthase of the GT2 family is a known metabolic activity of *K. xylinus* (Villarreal-Soto *et al*., 2020). GHs and PLs are enzyme families that cleave glycosidic bonds between carbohydrates or between carbohydrate and noncarbohydrate moieties.

Yet, it is not fully understood which metabolic pathways are preferably used to generate the appropriate metabolites resulting in a successful Kombucha fermentation, and how the different functionalities are distributed among them. In this study, genes encoding metabolism were dominant, followed by those involved in genetic information processing, human disease, and organismal systems (Figure 3A).

The heatmap of KEGG metabolic pathways showed that the functional profile of the three Kombucha samples collected from different regions were relatively homogenous, but some differences were observed (Figure 3B). Three samples collected from different regions were mainly involved in 42 metabolic pathways. Among them, neurodegenerative disease, amino acid metabolism, metabolism of cofactors and vitamins, carbohydrate metabolism, folding, sorting and degradation, and

Figure 3. Predicted genes in KEGG pathways within the microbial community. (A) Counts of genes associated with KEGG pathways at level 1. (B) Relative abundances of KEGG pathways in different Kombucha samples at level 2.

translation were identified in all samples. In the category of metabolism, pathways that were enriched in carbohydrate metabolism, signal transduction, amino acid metabolism, translation, cell growth and death, metabolism of cofactors and vitamins, and energy metabolism were included. Carbohydrate metabolism is the most

abundant pathway, which accounted for an average of 6.4% of the total microbial gene abundance in Kombucha (Figure 3B). This pathway has also proved to be important in Kombucha fermentation (Villarreal-Soto *et al.*, 2020).

Conclusion

The in-depth characterization of the microbial community and functional capacity in Kombucha collected from different regions of China were revealed by metagenomic analysis in this study. Some similarities were observed between the three different microbial populations, and the abundant species were limited to *Komagataeibacter*, *Gluconacetobacter*, *Gluconobacter*, and *Acetobacter.* Considerable differences were exhibited in microbial communities and metabolic functions of various Kombucha samples, but whether the difference in biological profiles of obtained Kombucha beverage needs further studies. The results provide a new insight into the microbiota and metabolites as well as the health-promoting potential of Kombucha, and may facilitate the optimization of the process to produce Kombucha products with desirable qualities.

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