Comparison of Bacterial Diversity Between Two Traditional Starters and the Round-Koji-Maker Starter for Traditional Cantonese Chi-Flavor Liquor Brewing

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Xiaoqu is a traditional fermentation starter that is used for Chinese liquor production. Although microorganisms in the starters are closely associated with the quality and flavor of liquor, knowledge of the microbiota in xiaoqu is still far from complete, let alone the starters produced by new processes. Here, Illumina MiSeq high-throughput sequencing was applied to study bacterial composition in three types of xiaoqu used in Cantonese soybean-flavor (Chi-flavor) liquor, namely two traditional starters (Jiu Bing and Bing Wan) and a Round-Koji-maker starter (San qu) produced by the automatic starter-making disk machine. The results showed bacterial diversity in traditional starters was similar and higher than that in the Round-Koji-maker starter. Lactobacillus and Pediococcus were the dominant genera in all starters, while other different dominant genera also existed in different starters, which were Weissella, Acetobacter, and Gluconobacter for Jiu Bing, Weissella for Bing Wan, and Bacillus, Acetobacter, Acinetobacter and Klebsiella for San qu, respectively. Meanwhile, Cytophagaceae, one particular microbial family, and some pathogens including Klebsiella, Cronobacter, and Enterobacter were also found in San qu, indicating the automatic starter-making disk machine should be ameliorated before applied into industrial production. These results enriched our knowledge on xiaoqu-related microorganisms and might be helpful in industrial Chi-flavor liquor production and the development of fermentation technology.

Keywords: Xiaoqu, bacterial diversity, MiSeq high-throughput sequencing, Round-Koji-maker starter, Jiu Bing, Bing Wan

INTRODUCTION

Many different types of liquor (baijiu), produced by various processes and material in different regions of China, can be distinguished based on the fermentation material, manufacturing techniques, fermentation starters, and product flavors (Zheng and Han, 2016). They were classified into 12 categories by Zheng and Han according to the final flavor of the liquor such as light flavor, strong flavor, sauce flavor, and Chi flavor (Zheng and Han, 2016). Soybean-flavor liquor (also called as Chi-flavor liquor, such as Jiujiang double distilled liquor and Yubingshao liquor),
one of particular Cantonese traditional liquors in Pearl River Delta region and Southeast Asian Chinese, is named for its dense fermented soybean flavor with β-phenethyl alcohol and ethyl ester as the primary aroma compounds made from fermented soybeans, and its culture has been considered as one of the most archaic distilled alcoholic beverages over the world. The archaic liquor stems from cereals such as rice and soybean by complex fermentation processes using a natural blended cultural starter called xiaoqu which is one of three different types of the starters called JiuQu used in Chinese liquor (Gou et al., 2015), and the complex fermentation involved numerous steps, such as making starter, distilling, packing and so on. This kind of starter works as a saccharifying and fermenting agent for the production of soybean-flavor liquor and contains a variety of microorganisms, enzymes, metabolites and degradation products as well as important flavor compounds, which would contribute to the aroma of the final distillate (Gou et al., 2015).

During liquor production, the functional microbes in JiuQu contribute to the production of aromatic compounds (Liu et al., 2017). Meanwhile, the quantity and species of microorganisms such as bacteria, yeast and molds in fermented starters will be altered due to the micro-environmental changes during the fermentation process, coupled with utilization of nutrimental materials and accumulation of metabolic productions, such as pH decrease, increase of alcohol concentration, and so on (Wang H. et al., 2008). Thus, the microbial components of the starters largely determine the flavor and quality of the produced liquor. Several studies have demonstrated the diversity of the microbial community in traditional Chinese liquor daqu, another starter different from xiaoqu, indicating there are striking differences in community structure among different daqu samples due to the differences in grains, the fermentation time, and so on (Wang C. et al., 2008; Wang H. et al., 2008; Wang et al., 2011; Shi et al., 2009; Li et al., 2011; Liu et al., 2017; Zheng et al., 2012). Meanwhile, a few studies reported the microbial diversity in xiaoqu, but mainly focused on Huaxi Xiaoqu, Sichuan Xiaoqu and Hubei Xiaoqu (Gou et al., 2015; Wu H. et al., 2017), suggesting the bacterial community was relatively more complex than the fungal community, and different xiaoqu possessed different bacterial compositions. However, knowledge of the microbiota in xiaoqu is still far from complete, let alone a new type of starter produced by new processes, i.e., the Round-Koji-maker starter produced by using the automatic starter-making disk machine (its diagram shown in Figure 1A) which decreases the influence of operators and reduces labor costs and fermentation time compared with the traditional methods (Cao et al., 2017). To the best of our knowledge, the bacterial composition of a Round-Koji-maker starter named with San qu was analyzed and compared with that of two traditional fermentation starters named with Bing Wan and Jiu Bing by Illumina MiSeq analysis in this study.

MATERIALS AND METHODS

Sample Collection and Preparation

Bing Wan, Jiu Bing, and San qu (their shapes shown in Figures 1B–D) labeled with W, B and S, respectively, were collected from Guangdong Jiujiang Distillery Co., Ltd., Foshan, China. Bing Wan (traditional), the mother starter used to produce Jiu Bing (traditional) and San qu (the Round-Roji-maker starter), is made of 100 kg of rice, 4 kg of leaves of Isatis indigotica, 0.3 kg of Chinese medicinal herbs, as well as 3 kg of stock culture which was produced by Jiujiang Distillery Co., Ltd., afterward all the materials have been shaped into a ball. Jiu Bing makes up of 2 kg of Bing Wan, 100 kg of rice, 22 kg of soybeans, and 9 kg of leaves of Isatis indigotica, which are later shaped into a brick before the fermentation process. San qu, which was fermented in Round-Koji-maker, contains the same materials as Jiu Bing other than some chaff and lacks the shaping process during the manufacturing procedures.

In order to obtain adequate information, three blocks of each type of starter samples (B, W, and S) were randomly selected from the upper, middle, and lower locations in the storage room, and samples B and W were promptly ground to powder while samples S remained the initial loose condition. All samples were stored in a refrigerator at 4°C and subjected to microbial analysis within 24 h.

DNA Extraction

Total DNA was extracted from five grams of each sample powder without chaff and leaf according to previous reports with slight modification (Schmidt et al., 1991; Li et al., 2011). Briefly, the power was suspended in 20 mL wash buffer (2% β-Mercaptoethanol, 0.2 M Tris, 0.05 M EDTA, 0.25 M NaCl, pH 8.0) with horizontal shaking at 200 rpm for 20 min at 20°C. The supernatant was discarded after centrifugation at 8000 rpm, and the pellet was dissolved in 2 mL modified extraction buffer (3% CTAB, 2% β-Mercaptoethanol, 2% PVPP, 1.5 M NaCl, 100 mM Tris, 100 mM EDTA, pH 8.0) with 20 μL proteinase K (10 mg/mL) and incubated overnight at 37°C in an incubator shaker at 150 rpm. Subsequently, the whole system was mixed with 200 μL of 10% SDS and kept at 37°C for 30 min with mixing gently by inverting the tube every 5 min. The supernatants were collected after centrifugation at 8000 rpm for 20 min at room temperature and the protein and polysaccharide complexes were removed by extraction with an equal volume of chloroform-isoamyl alcohol (24:1), followed by extraction with equal volumes of phenol-chloroform-isoamyl alcohol (25:24:1). The aqueous phase was carefully collected by centrifugation at 8000 rpm for 15 min, and mixed with 0.1 volume of 3 M sodium acetate and 0.7 volume of cold isopropanol for 1 h at −20°C. The resulting nucleic acids were washed with 70% ethanol, and resuspended in sterile deionized water.

DNA Purification and PCR Amplification

The crude nucleic acids were purified with a glass fiber filter tube by High Pure PCR Template Preparation Kit (Roche). The concentrations and quality of purified DNA were determined by using a NanoDrop 2000 spectrophotometer (Thermo Scientific, United States) and PCR amplification, respectively. 20 ng DNA was used to amplify the bacterial 16S rRNA gene using the primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GTTACCTTGTAGGACCTT-3′), and the primers 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and...
FIGURE 1 | The shapes of three different types of starters and the diagram of the automatic starter-making disk machine. (A) Automatic starter-making disk machine. (B) Bing Wan. (C) Jiu Bing. (D) San qu.

806R (5'-GGACTACHVGGGTWTCTAAT-3') (Anahtar et al., 2016) in 50 µL reaction mixtures with 25 µL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), respectively. PCR products using the primers 27F and 1492R were used to quality testing of DNA samples. Then PCR products using the primers 338F and 806R were analyzed by 1.0% agarose gels electrophoresis and purified and quantified with an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) and QuantiFluor™-ST (Promega) according to the manufacture's direction, respectively.

High-Throughput Sequencing and Sequence Analysis

Purified amplicons were pooled in equimolar volumes and sequenced on Illumina MiSeq platform (Majorbio Bioinformatics Technology Co., Ltd., Shanghai, China) using standard protocols. The raw Illumina sequences were deposited to the NCBI Sequence Read Archive1 with the project accession number of SAMN07336319-07336327 and quality-filtered by an open-source soft package named Quantitative Insights into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010). Operational taxonomic units (OTUs) were assigned with a 97% similarity cut-off using the UPARSE pipeline and classified taxonomically using RDP classifier against the RDP database2, where chimeric sequences were removed using UCHIME (Wang et al., 2007; Edgar, 2013). Rarefaction curves and the estimates of Chao1, Ace, and Simpson/Shannon, representing the species abundance, the amount of unique OTUs found in each sample, and the microbial diversity, respectively, were generated by the Mothur software (Schloss et al., 2011). Bray–Curtis distances were calculated by QIIME pipeline and the similarity and difference between the communities were depicted by Hierarchical cluster analysis and Venn diagram. Meanwhile, Principal components analysis (PCA) was performed in the Fluidigm SINGuLAR Analysis Toolset 2.0 R package, which calls the princomp R package3. Additionally, abundant features consistent with biologically meaningful categories were identified differentially on the OTU level by LEfSe, an algorithm for high-dimensional biomarker discovery and explanation which identifies genomic features and characterizing the differences in different conditions, on the website https://huttenhower.sph.harvard.edu/galaxy/, and the threshold on the logarithmic LDA score for discriminative features is > 2.0 (Zhang et al., 2013).

1http://www.ncbi.nlm.nih.gov/Traces/sra
2http://rdp.cme.msu.edu/
3http://stat.ethz.ch/R-manual/R-patched/library/stats/html/princomp.html
Plate Counting
Ten grams of the samples were homogenized in 90 mL sterile phosphate buffered saline and shaken for 10 min at normal speed. Then ten twofold serial dilutions were made and 1 mL of the dilution was mixed with molten medium consisting of 5 g/L beef extract, 10 g/L peptone, 5 g/L NaCl and 15 g/L agar for pour-plating. After incubation, the colonies appearing on the plates were counted and selected randomly for cultivation in order to extract DNA and amplify the bacterial 16S rRNA gene. Obtained sequences were sequenced in Majorbio Bioinformatics Technology Co., Ltd., and blasted in NCBI website. All counts were repeated three times for each sample and results were reported as the means.

Volatile Acids Analysis
Volatile acids, such as formic acid, acetic acid, propionic acid, and so on, were analyzed via ion-exchange chromatography on a DIONEX ICS-3000 ( Dionex Corporation, Sunnyvale, CA, United States). Briefly, the samples after fermented with three starters were filtered through Dionex OnGuard™ II RP and H cartridge (Thermo, Sunnyvale, CA, United States) to remove interfering substances and was loaded into the instrument according to the instructions (Zhang and He, 2010). Volatile acids in samples were the sum of the content of each acid calculated with the standard solution.

Data Analysis
Statistical analysis was subjected to one-factor analysis of variance performed using SPSS 18.0 (SPSS Inc., Chicago, IL, United States) and p < 0.05 was considered to be statistically significant differences.

RESULTS

Overall Analysis of Illumina MiSeq Sequencing
To assess the bacterial diversity, Illumina MiSeq sequencing was applied and a total of 19,222 valid reads and 497 OTUs were obtained from the nine samples. The samples from Jiu Bing, Bing Wan and San qu contained 52 to 57 OTUs, 46 to 56 OTUs and 57 to 66 OTUs, respectively (Table 1). All of the rarefaction curves based on the OTUs (Figure 2) tended to reach saturation, indicating that obtained sequenced reads were sufficient to represent the bacterial diversity in different samples.

The total number of OTUs calculated by Chao 1 estimator was 61 for Jiu Bing on average, 67 for San qu on average and 67 for Bing Wan on average, respectively, indicating that Jiu Bing had the lowest richness than the other two samples where there was no significantly difference (p > 0.05), which was validated by ACE indices (Table 1) and consistent with the differences in bacterial counts. The abundance of each species distributed in a community was assessed by Shannon diversity index which is positively correlated with Alpha-diversity and Simpson index which is negatively correlated with Alpha-diversity. The smallest Shannon index was 1.44 for San qu on average, followed by 1.64 for Bing Wan on average and 1.96 for Jiu Bing on average, indicating the diversity in San qu was lower than that in Bing Wan and Jiu Bing, which also demonstrated by Simpson indexes in Table 1. All samples had a high Good’s coverage (99.9%).

Bacterial Community Profiles
There were dissimilar bacterial communities at the phylum and class level for three types of starters (Figure 3). Firmicutes was the domain phylum in Bing Wan, Jiu Bing and San qu, accounting for 98.3, 75.4, and 90.3% of the sequences on average in each library, respectively, while Proteobacteria, another domain phylum in three types of samples, accounted for 24.1% of the sequences on average in Jiu Bing, but only for 8.9 and 1.2% in San qu and Bing Wan, respectively (Figure 3A). Among 18 classes detected in the nine samples, three of them were dominant (>1% abundance at least one type on average) as shown in Figure 3B. Bacilli was shared by the three types of samples with higher relative abundances than Alphaproteobacteria and Gammaproteobacteria, but Bacilli was the only dominant class

| Sample | OTU | Ace | Chao | Shannon | Simpson | Coverage |
|--------|-----|-----|------|---------|---------|----------|
| B1     | 52  | 57  | 56   | 1.73    | 0.2786  | 0.9996   |
| B2     | 57  | 67  | 68   | 2.07    | 0.1581  | 0.9994   |
| B3     | 52  | 58  | 58   | 2.07    | 0.1636  | 0.9995   |
| S1     | 66  | 72  | 72   | 1.41    | 0.4768  | 0.9995   |
| S2     | 57  | 67  | 65   | 1.47    | 0.3499  | 0.9994   |
| S3     | 60  | 66  | 63   | 1.45    | 0.3243  | 0.9995   |
| W1     | 56  | 74  | 76   | 1.69    | 0.2225  | 0.9992   |
| W2     | 46  | 58  | 62   | 1.61    | 0.2351  | 0.9993   |
| W3     | 51  | 63  | 64   | 1.62    | 0.2525  | 0.9993   |

FIGURE 2 | Rarefaction analysis of the different samples. Rarefaction curves of OTUs clustered at 97% sequence identity for different samples.
Bacterial Composition in Jiu Bing, Bing Wang and San qu

for Bing Wan, and Gammaproteobacteria was dominant only for San qu.

The Venn diagram with common and unique OTUs was adopted to describe the difference and similarity among Jiu Bing, Bing Wan and San qu (Figure 4A). All three types of starters had a total of 105 OTUs observed and 38 of them were in common. The shared OTUs were Firmicutes, Proteobacteria and Bacteroidetes, accounting for 45.9%, 37.8%, and 11.2% of the shared OTUs, respectively. Bing Wan and San qu had more shared OTUs (61, 54.1% of total) than any of them with Jiu Bing (B/S, 44, 41.9%; B/W, 48, 45.7%). The number of OTUs unique to individual starters was 13 for Jiu Bing, 12 for San qu, and 3 Bing Wan, respectively.

In addition, non-metric multidimensional scaling (NMDS) and hierarchical clustering analyses, which examine relationships between bacterial communities such as those of the microbial flora, were used to determine whether those OTUs identified differentiated among different types of starters. The comparisons by NMDS (genus level similarity) using the Bray–Curtis similarity metric indicated that the identified taxa significantly partitioned the starter samples into two distinct groups, with samples B clustering with samples W whereas samples S were separated by the second non-metric multi-dimensional scaling (nmds2) (Figure 4B). These results suggested that the new technique applied to produce San qu resulted in the distinction of the microbial community of the starters, and there would be similarity between Jiu Bing and Bing wan as the same traditional technique was used in their production.

**Genus-Level Distribution**

A hierarchically clustered heatmap analysis based on the bacterial community profiles at the genus level demonstrated that samples W grouped together at first, and then clustered with the group of sample B2 and B3, and sample B1 in order, which ultimately clustered with the group of samples S where the sample S1 clustered with sample S3 and S2 in order (Figure 5), indicating the distinction of bacterial genus in samples S significantly differed from those in samples B and W, which was consistent with the results obtained from NMDS and hierarchical clustering analyses. There were a total of seventy-two taxa observed in three types of starters. For samples S there were six dominant genera including Lactobacillus, Pediococcus, Bacillus, Acetobacter, Acinetobacter, and Klebsiella, while three dominant genera (Lactobacillus, Pediococcus, and Weissella) exited in both samples B and W, but samples B harbored two other dominant genera (Acetobacter and Gluconobacter) which accounted for a small proportion in samples W. Specifically, the relative abundance of four dominant genera including Pediococcus (48.7% on average), Bacillus (28.3% on average), Acinetobacter (4.3% on average) and Klebsiella (1.6% on average) in samples S was clearly higher than that in samples B and W, and seven special genera including Cronobacter, Enterobacter, Flavobacteriaceae, Klebsiella, Paenibacillus, Pantoaea, and Rickettsia existed in samples S but not or few in samples B or W, while the relative abundance of Weissella and Lactobacillus in samples S was apparently lower than those in samples B and W, and the absence of Synergistaceae was apparent in samples S. Compared with samples W, samples B possessed lower abundance of Lactobacillus (36.0% on average), one of three shared dominant genera in samples B and W and Leuconostoc, but higher abundance of Acetobacter (22.1% on average), Gluconobacter (1.7% on average), and Acinetobacter (0.1% on average). Moreover, Carnimonas and Kurthia are peculiar to samples B. Meanwhile, principal component analysis (PCA) was performed to reveal the relationships among the different samples (Figure 6). On the right side of the graph along the first principal component axis (PC1) accounting for 54.86% of the total variations, were samples S which were gathered together. Although samples B were close to samples W, they were distinctly separated by the second component (PC2) which accounted for 19.65% of the total variation. Overall, the PC1 and PC2 axes explained 74.42% of the variations between the differences of the bacterial communities. These data indicated that three types of starters had different characteristic bacterial communities, which was in agreement with heatmap analysis.

Linear discriminate analysis (LDA) effect size (LEfSe) was used to find biomarkers from metagenome data to identify potential distinguishable taxa in three types of starters. A total of 181 bacterial groups were well-defined to at least one type of starter using the default logarithmic (LDA) value of 2.0, and Cladograms showed taxa with LDA values higher than 2.0 for lucidity (Figures 7A,B). We detected that seven groups of bacteria were enriched in Jiu Bing (B), namely, Proteobacteria, Gluconobacter,
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Kurtia, Planococcaceae, Halomonadaceae, Oceanospirillales, and Carnimonas. Within these, one conspicuous species, Proteobacteria, had an LDA value higher than five (Figure 7A). In San qu (S), the major bacterial families were Cytophagaceae, Moraxellaceae, Enterobacteriaceae, Alcaligenaceae, Rickettsiaceae, Hyphomicrobiaceae, Caulobacteraceae, Paenibacillaceae, and Bacillaceae (Figure 7B), and Only Bacillaceae and Pseudomonadales showed LDA values higher than five (Figure 7A). Meanwhile, the Firmicutes phylum and Synergistia phylum were enriched, particularly the Bacilli and Synergistales, respectively, and another group, Rickettsiales, was also rich in Bing Wan (W) (Figure 7B). At precise taxonomy levels, Bing Wan only had Lactobacillus and Firmicutes showing LDA values higher than five (Figure 7A).

Overall, two phyla, two classes, five orders, nine families, and two genera were significantly higher in San qu, compared with other two starters (Figure 7B). Cytophaga and Gammaproteobacteria were enriched from class to genus level, while Caulobacteriales and Burkholderiales were enriched from order to family level. In contrast, the enrichment of Firmicutes and Synergistetes could be observed in Bing Wan. Moreover, members from Proteobacteria were enriched in Jiu Bing. Notably, at the class level, microbes from Cytophaga were unique in San qu, and those from Synergistetes were only determined in Bing Wan, with those from Oceanospirillales significantly more prominent in Jiu Bing. More specifically, the overrepresented genera in San qu included Pediococcus, Bacillus, Acinetobacter, Klebsiella, and Cronobacter, and in particular Adhaeribacter, was also detected as the unique microbe. On the other hand, Lactobacillus, Weissella, and Pediococcus were found in all three types of the starters despite the level of Weissella in San qu being significantly lower than those in the other two starters, while Acetobacter and Gluconobacter were distinctly abundant in Jiu Bing but not in Bing Wan.

Plate Count, Isolated Bacteria and Volatile Acid Analysis

Bacterial counts were detected by plating serial dilutions of samples, and the number was 4.15 × 10⁶ on average for Bing Wan, 1.56 × 10⁵ on average for Jiu Bing, and 1.1 × 10⁵ on average for San qu, respectively, indicating the bacterial diversity and/or compositions may be quite different among three starters. Moreover, a total of 20 bacterial strains in each of three starters were randomly selected and identified by sequencing the 16S rRNA. Bacillus tequilensis, Bacillus amyloliquifaciens, and Pediococcus lolii were found and accounted for 66.67, 16.67, and 16.67%, respectively, in San qu. Lactobacillus pentosus and Undibacterium oligocarboniphilum were found in Jiu Bing, accounting for 33.33 and 66.67%, respectively. However, only Pediococcus pentosaceus was encountered in Bing Wan. The obtained results testified that Lactobacillus, Bacillus, Pediococcus, and Undibacterium (belonging to Proteobacteria phylum) are the most dominant genera for Bing Wan, San qu, and Jiu Bing, respectively, which coincided with the heatmap (Figure 5) and the LDA scores (Figure 7A). Furthermore, volatile acids after fermentation using three starters were measured by ion chromatography and the contents were 0.23 g/L on average for Bing Wan, 0.52 g/L on average for Jiu Bing, and 0.89 g/L on average for San qu, respectively, which might be due to the different bacterial counts and/or compositions among three starters.

DISCUSSION

The present study is the first report to compare the bacterial diversity between the traditional starters and the Round-Koji-maker starter. The results indicated the traditional starters (Jiu Bing and Bing Wan) showed higher bacterial richness and diversity than that in the Round-Koji-maker starter.
FIGURE 5 | Double hierarchical dendrogram showing the bacterial distribution among nine samples. The bacterial phylogenetic tree was calculated using the approximately-maximum-likelihood method, and the relationship among samples was determined by the Bray distance and the complete clustering method. The heatmap plot depicts the relative percentage of each bacterial genus (variables clustering on the y-axis) within each sample (x-axis clustering). The relative values for bacterial genus are depicted by color intensity with the legend indicated at the bottom of the figure. Clusters based on the distance of the nine samples along the X-axis and the bacterial genera along the Y-axis are indicated in the upper and left of the figure, respectively.
(San qu), and bacterial composition in the three xiaoqu starters mainly included Lactobacillus, Pediococcus, Weissella, Bacillus, Acetobacter, Acinetobacter, Gluconobacter, which was different from that in Huaxi xiaoqu, Sichuan xiaoqu and Hubei xiaoqu, where the dominant bacteria are Weissella, Staphylococcus, Empedobacter, and Corynebacterium for Huaxi xiaoqu, Weissella, Staphylococcus, Corynebacterium, Bacillus, and Leuconostoc for Sichuan xiaoqu, and Weissella, Empedobacter, Sphingobacterium, Acinetobacter, Bacillus, Pseudomonas, and Flavobacterium for Hubei xiaoqu, respectively (Gou et al., 2015; Wu H. et al., 2017). Meanwhile, slight differences existed in three samples of San qu (Figures 4B, 6), which might be caused by instability of quality during storage. The bacterial composition in tested xiaoqu starters was also significantly different from that in daqu where different samples consisted of different bacteria, for example, the dominant bacterial species were Bacillus, Virgibacillus, Lactobacillus, and Trichococcus in Baiyumbian liquor fermentation starters, Bacillus, Thermonactinomycetes, Staphylococcus, Lactobacillus, Micrococcae, Brachybacterium, Enterobactera, Penioccocus, and Brebivacterium in the starter of Fen Liquor, and Bacillus, Acetobacter, Lactobacillus, Clostridium in the starter of Maotai-flavor liquor, respectively (Wang C. et al., 2008; Wang H. et al., 2008; Shi et al., 2009; Zheng et al., 2012; Xiong et al., 2014). The dominant genera detected in all three types of starters were Pediococcus, Lactobacillus, and Weissella, all of which are main lactic acid bacteria (LAB) typically found in fermenting matter (Nout, 2009), and which were also separated from the alcoholic fermentation stage of Shanxi aged vinegar (Wu et al., 2012). Lactic acid was identified as a precursor of ethyl lactate, contributing to the major flavor compounds in strong aromatic liquor and affecting both the technological

![FIGURE 6](image-url) Principal component analysis of nine metagenomes from three different types of starter made by two different processes, showing the relatedness of the bacterial community in the different samples.

![FIGURE 7](image-url) Key phylo-types of bacteria analysis in three types of starters by using LEfSe. (A) Indicator bacterial groups with the three types of starters with LDA values higher than 2.0. (B) Cladogram indicating the phylogenetic distribution of microbial species associated with the three types of starter. Species with LDA values of 2.0 or higher determined by LEfSe are displayed. Differences are represented in the color of the most abundant class. The diameter of each circle is proportional to the abundance of the taxon. The strategy of multiclass analysis is nonstrict (at least one class differential). Circles represent phylogenetic levels from domain to genus inside out. Labels are shown of the phylum, class, and family levels.
properties and microbial stability of the final products (Yousif et al., 2010; Wang et al., 2011; Ding et al., 2014). Also, as they can yield lactic acid from glucose or starch and provide substrates for esterification of yeasts, LAB cannot be absent from Chinese liquor (Li et al., 2011). It was also pointed out that lactic acid is able to further react with ethanol to yield the ethyl lactate (Ding et al., 2015), which constitutes the major flavor components of Jiujiang soybean-flavor liquor (Zhang and He, 2010). However, compared with two traditional starters, Lactobacillus was significantly lower and Weissella was even absent in the Round-Koji-maker starter (San qu), which resulted in both lactic acid ethyl ester and ethyl ester are noticeably lower in liquor produced by Round-Koji-maker starter (data not shown). Moreover, Bacillus became one of major genera in San qu, but was extremely sparse in both Jiu Bing and Bing Wan, which may be due to the richness of Lactobacillus that might synthesize antibiotics and bacteriocins resulting in growth suppression of Bacillus in the two traditional starters (Pal et al., 2010), and the competition and consumption of nutrition between two genera in both traditional starters, as well as the non-compact, i.e., loose, condition allowing the Round-Koji-maker starter to be exposed to air which provided Bacillus with sufficient oxygen for the robust proliferation. Compared with the two traditional starters, the richness of Bacillus in San qu may lead to the increase of volatile acids, which are the synthetic precursors of esters, because Bacillus was the important source of proteases and amylases and resulted in a precursor-rich environment which is useful for subsequent reactions leading to flavor production (Pal et al., 2010). which might cover the shortage of lactic acid ethyl ester and ethyl ester in San qu and so the flavor components of final liquor products using all three types of starters to ferment are not significantly different according to the information provided by Jiujiang Distillery Co., Ltd. In conclusion, the relatively low abundance of Lactobacillus and Weissella and high abundance of Pediococcus and Bacillus can potentially be used to distinguish the Round-Koji-maker starter from the traditional starters.

Acetobacteraceae, ubiquitous in nature on various plants such as fruits, cereals and herbs, can oxidize many types of sugars and alcohols to organic acids as final products during the fermentation process with an obligate aerobic metabolism (Sengun and Karabiyikli, 2011). It was reported that acetic acid is abundant in the strong-flavor liquors and could contribute positively to the flavor by yielding esters such as isoamyl acetate and 2-phenylethyl acetate (Sun et al., 2016). Here, a great abundance of acetic acid bacteria (AAB), Acetobacter, and Gluconobacter, appeared only in Jiu Bing among all types of starters while Gluconobacter was absent in San qu.

In addition to Bacillus and LAB genera, other bacteria such as Enterobacter, Klebsiella, Cronobacter, and Acinetobacter were also detected and occupied a noticeable position in the bacterial community in Round-Koji-maker starter but were rarely detected in the traditional starters, which may be attributed to the loose states caused by the production process lacking shaping of koji, which provided these microbes with suitable conditions for incubation. On the other hand, the difference in the abundance of Cronobacter might result from the raw ingredients, such as the grain chaff, which may be a vital source of Cronobacter in the food chain (Baumgartner et al., 2009). Pathogenicity of these bacteria is widely known and most of them are regarded as food-borne bacterial pathogens, but some pathogens could actually participate in flavor formation, for instance, Enterobacter can produce amylases and lipases (Li et al., 2015). As the existence of these food-borne bacterial pathogens, the mechanically prepared Round-Koji-maker starter could not be applied directly into industrial liquor production like the mechanically prepared daqu (Xiong et al., 2014) and the present technique should be improved in the future.

The most unique bacteria in the Round-Koji-maker starter samples was Cytophagaceae, whose members are widely distributed in nature and could be isolated from aquatic, terrestrial, and air samples but also represent Gram-negative chemoorganotrophic with primarily respiratory metabolism. Previous studies suggested that many members of Cytophagaceae played a crucial role in digesting macromolecules such as proteins and polysaccharides (McBride et al., 2014), so we inferred that the presence of Cytophagaceae in the Round-Koji-maker starter may be beneficial to utilize the grain chaff by digestion of the coarse fiber of the chaff. Also, the certain Adhaeribacter, which was previously isolated from forest soil (Zhang et al., 2009), was found in a small amount in San qu, but its effects in liquor production were still unknown, which would be further investigated. So far, the San qu has not been used in actual production process including but not limited to the reasons mentioned above, but Jiu Bing and Bing wan are used as starter for making of Jiujiang Double Distilled and Jiujiang Twelve Square (Wu Z. et al., 2017), two traditional Chinese Cantonese soybean-flavor liquor (Cantonese rice liquor) for more than six decades.

CONCLUSION

This is probably the first study to compare the bacterial diversity between the traditional starters and the Round-Koji-maker starter by using Illumina MiSeq sequencing platforms. The main bacteria were Lactobacillus, Pediococcus, Weissella, Bacillus, Acetobacter in three different types of xiaoqu, but different types of starters harbored different and specific microbial communities and abundances, which might be due to the differences in the raw materials and the conditions of their incubation. On the other hand, the Round-Koji-maker starter contained a high proportion of sample-specific bacteria which were regarded as candidate biomarkers to distinguish from traditional starters. Furthermore, some pathogens were also detected in the Round-Koji-maker starter, which suggested that the automatic starter-making disk machine should be ameliorated from the perspective of the bacterial results before being applied into industrial liquor production. Accordingly, our findings provide useful information for liquor production and process improvement based on xiaoqu fermentation. Additionally, further investigation will be needed to have a deeper insight into xiaoqu, as well as the
Round-Koji-maker starter regarding other aspects, such as the fungal communities, and the development of microbes in the xiaoqu preparation and ripening process as well as production in different regions using different culture conditions.

**AUTHOR CONTRIBUTIONS**

JW analyzed the data and prepared the manuscript. QZ contributed to manuscript discussion. YY and HL designed and performed the experiments. LW and YT contributed to manuscript revision. XF and ZL contributed to the experimental design, manuscript revision, and overall support of this study.

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

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