Traceability Analysis of Feng-Flavour Daqu in China

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Abstract. High throughput sequencing was used to analyze the microbial community landscape of Chinese Feng-Flavour Daqu, and to study the specific contribution of different environmental factors to Daqu microorganisms. Taking the microbial population of the raw materials (wheat, pea and barley) and the environmental samples (tools, indoor ground, outdoor ground and air) as the source, and the microbial population of Feng-Flavour Daqu as the receiver, software SourceTracker was used to trace and analyze the microorganisms in Feng-Flavour Daqu. 94.7% of the fungi in the newly pressed Feng-Flavour Daqu come from raw materials, 1.8% from outdoor ground and 3.47% from unknown environment; 60.95% of bacteria come from indoor ground, 20.44% from raw materials, 8.98% from tools, and the rest from unknown environment. The source of main microorganisms in Feng-Flavour Daqu and the influence of environmental factors on the quality of Daqu were clarified, which provided a basis for improving the quality of Feng-Flavour Daqu.

Keywords: Feng-Flavour Daqu · Microorganism · Environment · Traceability

1 Introduction

The flavor is the style characteristics of liquor, which is used to distinguish the difference of the characteristic liquor in China. At present, there are 12 liquor flavor types in China and each one has its unique flavoring characteristics. Different flavor types are mainly due to different production areas and processes. Feng-Flavour Liquor is one of the four famous traditional liquors in China. It has the characteristics of elegant liquor flavor, enjoyable liquor taste, harmonious liquor body and long liquor aftertaste.

Daqu, an undefined starter culture, is one kind of Jiuqu (a sort of equivalence of Koji) [1]. Daqu is commonly known as "Bone of liquor". Daqu contains a variety of microorganisms used for the fermentation of Chinese liquor. Among them, fungi dominated by molds and yeasts are an important functional microorganism, which can secrete amylase, cellulase and other enzymes [2]. The microbes of the Daqu are one of the determinants of the style and taste of liquor. The production process of Feng-Flavour Daqu is divided

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into four stages: crushing of raw materials, pressing and forming of raw materials containing certain moisture by machine, placing the formed Daqu into the culture room for 30 days, and storing it in the warehouse for 3 months. The production of Daqu is a spontaneous solid-state fermentation process with natural inoculation [3]. Raw materials without high-pressure sterilization will be exposed to many environments (such as air, ground, etc.) during the stage from raw materials to mature Daqu [4]. Therefore, microorganisms in the environment, especially in the liquor production area, are also one of the important sources of Daqu flora [5]. Environmental microorganisms in specific areas may be one of the reasons for the specific flavor and types of Chinese liquor in different regions.

Microbial traceability originated in the last century and is mostly used in the study of water pollution to identify pollution sources [6, 7]. At present, microbial traceability analysis has also been widely used in the analysis of microorganisms in soil [8], air [9], coral [10]. Further, the idea of microbial traceability analysis promotes the monitoring of microbial sources in the field of food fermentation. For example, Stellato et al. detected the distribution of microorganisms related to meat product corruption in the environment of meat products processing plant (knife, chopping board and workers' hands) through 16SrRNA amplicon sequencing technology, and detected more than 800 OTUs in the food processing environment, indicating that the microbial composition of food processing environment was complex, and most environmental microorganisms could be detected in meat products [11]. Doyle et al. detected the microbial population distribution in the indoor and outdoor environment of raw milk collection in different seasons through amplicon sequencing technology, the results showed that the microorganisms in the raw milk collected indoors and outdoors were related to the environment (grass, feed, feces, soil and milker) [12]. At the same time, they believed that the milker was the main source of microorganisms in the raw milk. The environmental microecology test of cheese factory also found that the microorganisms on the surface of processing equipment also participated in the fermentation process, due to the differences of cheese types and maturity, the microorganisms on the surface of equipment also formed different microecology [13]. The micro ecology of food fermentation environment is also affected by geographical factors, which have been reported in many foods production. Bokulich et al. discovered that the microbial population in wine grape was related to grape varieties, harvest areas and climate environment, and the microorganisms in grape planting soil and grapes showed the same regional distribution, indicating that soil microorganisms were an important source of wine grape microorganisms [14]. Therefore, different grape planting areas formed different microbial groups in grapes, thus forming a unique wine style in different regions. Knight et al. further proved that the landmark characteristics of wine were related to microorganisms by using different characteristics of Saccharomyces cerevisiae to ferment everlasting longing for each other grapes [15]. The landmark characteristics of this fermented food not only appear in wine. Bokulich et al. found that the type of milk and the origin of milk would affect the microbial population structure in fermented dairy products, while the traditional workshop production method ensured that the microbial characteristics in different regions could be inherited from generation to generation [16].

SourceTracker is a tool for quantitative analysis of microbial sources based on Bayesian reasoning [17]. Different from the traditional identification of indicator microorganisms, SourceTracker finds out the source of target microorganisms by comparing the similarity of microbial community structure between samples and pollution sources, and its accuracy is much higher than that of traditional random forest method and Naive Bayes model. The migration direction of microorganisms or genes in the environment can be monitored through SourceTracker, which is widely used in many research areas. Bokulich et al. used SourceTracker to analyze the distribution and migration of microorganisms related to pollution in breweries [14]. Doyle et al. combined with amplicon sequencing technology and source tracker analysis, showed that the microorganisms in raw milk collected indoors and outdoors were related to the environment (grass, feed, feces, soil and milker), and considered that milker was the main source of microorganisms in raw milk [12]. Du et al. used SourceTracker to explore the impact of raw materials and environment on the microbiota of Chinese Daqu, they found that the fungal community in new Daqu mainly comes from the Daqu production environment (mainly tools and indoor ground), most of the bacterial community in Daqu comes from raw materials [18]. Zhou et al. analyzed the source of microorganisms in Gujing tribute liquor Daqu through SourceTracker and found that the bacteria in Daqu at the beginning of fermentation mainly came from raw materials and the fungi came from outdoor ground [5].

This open fermentation of Chinese liquor has brought about beneficial microorganisms fermented by the environment, but also enriched a number of useless or harmful microorganisms. This brings challenges to the quality and safety in production. The batch instability of traditional fermented food also comes from the uncontrollability of environmental microorganisms. Therefore, the traceability of fermented food microorganisms, especially the traceability of environmental microorganisms, is very important to control and improve the quality of liquor.

Different raw materials and processes of Daqu not only affect the source of microorganisms, but also create their unique styles and characteristics. Therefore, we took raw materials (wheat, pea and barley) and environmental samples (tools, indoor ground, outdoor ground, water and air) as the source of microorganisms in Feng-Flavour Daqu, and analyzed them to understand their unique characteristics. In this study, we clarified the influence of environmental factors on microbial changes during the maturation of Feng-Flavour Daqu, which is the basis for further improving the quality of Daqu.

2 Materials and Methods

2.1 Sample Collection

Nine types of samples were collected, including Feng-Flavour Daqu, raw materials for Daqu production (wheat, pea and barley) and environmental samples (tools for Daqu production, indoor ground, outdoor ground and air), as follows (Table 1):

Table 1. Samples in this study.

Sample	Description
Newly pressed Daqu (NDaqu)	Daqu that has just been pressed by machine and has not yet been fermented in the room
Mature Daqu (Daqu)	A Daqu which can be used for Feng-Flavour liquor production after 3 months' storage
Raw materials (RM)	Raw materials from the raw material crushing workshop
Enhanced strains (EDS)	The mixed fortified strains
Tools	The machine for pressing Daqu, the cart for transporting Daqu, the bamboo mat, bamboo and rice bran in contact with Daqu
Indoor ground (ING)	The door, window and middle floor of the room where Daqu is cultivated
Outdoor ground (OUTG)	The sidewalk outside the room where Daqu is cultivated
Air	Air in workshop
Water	Water for Daqu production

For Newly pressed Daqu (NDaqu), Mature Daqu (Daqu), Raw materials (RM) and Enhanced strains (EDS), randomly select three places, and take 50 g of each place as a sample. For tools, Indoor ground (ING) and Outdoor ground (OUTG), randomly select three points, wipe the surface with sterile cotton soaked in 0.1 mol·L^{-1} PBS buffer, and put the sample into a sterile self-sealing bag. For Air, before sampling, the collector and catheter are ultrasonically cleaned and dried, and then 20 mL of 0.1 mol·L^{-1} PBS buffer is filled into the collector. Place the sampler 2 m above the ground and collect at a speed of 10 L·min^{-1} for 2.5 h. The collected PBS solution was filtered with 0.22 μM filter membrane to collect air microbial samples. For Water, take three portions of water for Daqu production, 2000 mL each, and filter them with 0.22 μM filter membrane to collect microbial samples in the water.

2.2 Microbial High-Throughput Sequencing Analysis of Traceable Samples

Extraction of total DNA from microbiota with DNA kit (Omega Bio-Tek, USA). Nanodrop were used to quantify DNA and the quality of DNA was determined by 1.2% agarose gel electrophoresis. The extracted DNA from samples were stored at −80°C for amplicon sequencing.

Amplicon sequencing: The fungal sequencing region was ITS_V1, using primers ITS5F (GGAAGTAAAAGTCGTAACAAGG) and ITS2R (GCTGCGTTCTTCATCGATGC).

The bacterial sequencing region was 16S v3–v4, using primers F (ACTCCTACGGGAGGCAGCA) and R (GGACTACHVGGGTWTCTAAT). After amplification, the purified 16S rRNA gene and ITS1 sequences were sequenced by Illlumina MiSeq platform, respectively, at BioNovoGene Co., Ltd. (Suzhou, China).

Sequence analysis: Microbiome bioinformatics were performed with QIIME2 2019.4 [19] with slight modification according to the official tutorials. Briefly, raw sequence data were demultiplexed using the demux plugin following by primers cutting with cutadapt plugin. Sequences were then quality filtered, denoised, merged and chimera removed using the DADA2 plugin. Non-singleton amplicon sequence variants (ASVs) were aligned with mafft and used to construct a phylogeny with fasttree2. Alpha-diversity metrics (Chao1, Observed species, Shannon, Simpson, Faith's PD, Pielou's evenness and Good's coverage), beta diversity metrics (weighted UniFrac, unweighted UniFrac, Jaccard distance, and Bray-Curtis dissimilarity) were estimated using the diversity plugin with samples were rarefied to 16166 (bacterial) and 2018 (fungal) sequences per sample. Taxonomy was assigned to ASVs using the classify-sklearn naïve Bayes taxonomy classifier in feature-classifier plugin against the SILVA Release 132/UNITE Release 8.0 Database.

Bioinformatics and statistical analysis: Sequence data analyses were mainly performed using QIIME2 and R packages (v3.2.0). ASV-level alpha diversity indices, such as Chao1 richness estimator, Observed species, Shannon diversity index, Simpson index, Faith's PD, Pielou's evenness and Good's coverage were calculated using the ASV table in QIIME2, and visualized as box plots. ASV-level ranked abundance curves were generated to compare the richness and evenness of ASVs among samples. Beta diversity analysis was performed to investigate the structural variation of microbial communities across samples using Jaccard metrics, Bray-Curtis metrics and UniFrac distance metrics and visualized via nonmetric multidimensional scaling (NMDS) and unweighted pair-group method with arithmetic means (UPGMA) hierarchical clustering. The taxonomy compositions and abundances were visualized using MEGAN [20] and GraPhlAn [21].

2.3 Traceability Analysis of Brewing Microorganisms

Venn diagram can directly reflect the common and unique microbial populations of Daqu and the environment. Venn diagram was generated to visualize the shared and unique ASVs among samples or groups using R package "VennDiagram", based on the occurrence of ASVs across samples/groups regardless of their relative abundance. In this study, each type of sample contains three parallels. We only select the bacteria that appear in all three parallels as valid data, and then conduct genus level Venn analysis through the screened data.

Traceability analysis of fermentation microorganisms: according to the microbial population structure of Daqu and fermentation environment, this study uses SourceTracker software to analyze the source of microorganisms in Daqu, and sets the microbial population of Daqu raw materials (wheat, pea and barley) and environmental samples (tools, indoor ground, outdoor ground and air) as the source, the microbial population of Feng-Flavour Daqu as the receiving end, running 1000 times, and other parameters are default.

3 Results

3.1 Analysis of Fungal Community Diversity in Feng-Flavour Daqu and Its Environment

The dilution curve shows that all samples have reached the platform stage, indicating that this sequencing can cover the vast majority of fungal population information in the samples (Fig. 1a). New pressed Daqu and raw materials share the highest ASV (194), while mature Daqu and air share the highest ASV (31) (Fig. 1b). The Venn diagram results of ASV of each sample show that the number of unique ASV in the air is the most and the mature Daqu is the least, there are 7 ASVS common to all samples (Fig. 1c).

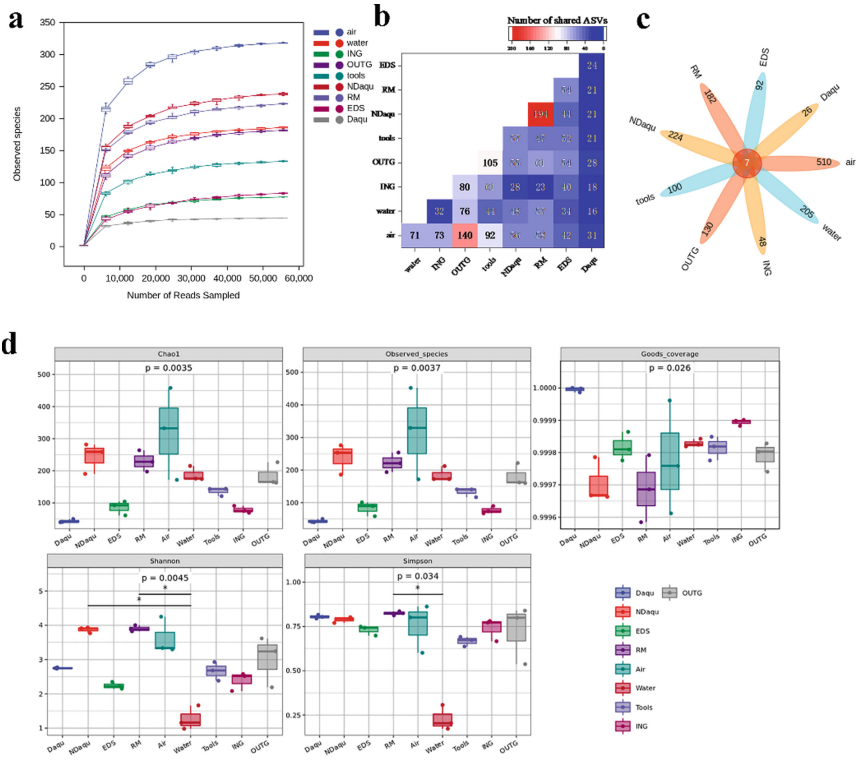

Fig. 1. (a) Dilution curve of fungal population. This sequencing can cover the vast majority of fungal population information in the samples. (b) Amplicon sequence variants (ASVS) distribution of fungi in each sample. NDaqu and RM share the highest ASV, while Daqu and air share the highest ASV. (c) Venn diagram is used to represent the ASV shared by fungi in the experimental sample, there are 7 ASVS common to all samples. (d) The fungal population diversities of Daqu, RM and environment were evaluated by richness index (Chao1 and observed species), good's coverage index (good's coverage) and diversity index (Shannon and Simpson). The richness and diversity of fungi is the highest in the air and the lowest in Daqu. Note: For the sake of concise expression, in to ground, out ground, new Daqu, raw materials and enhanced trains in this article are abbreviated as ING, OUTG, NDaqu, RM and EDS respectively.

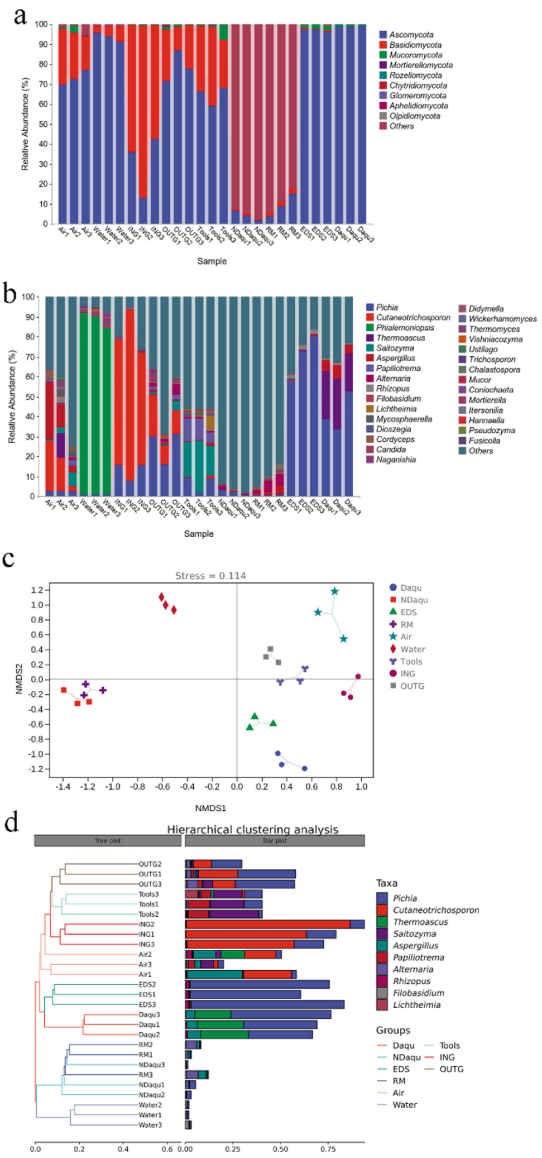

Fig. 2. (a) Analysis of fungal population structure of each sample at phylum level, 9 fungal phyla were detected, the dominant fungi phyla were *Ascomycota* and *Basidiomycota*. (b) Analysis of fungal population structure of each sample at genus level, 301 fungal genera were detected, the dominant fungi in different samples were vary greatly. (c) Nonmetric multidimensional scaling analysis (NMDS) of fungal population suggests that NDaqu and RM are close, and Daqu and EDS are close. (d) Hierarchical cluster analysis (HCA) of fungal population of different samples.

In this study, the fungal population diversity of Daqu, raw materials and environment was evaluated by richness index (Chao1 and observed species), good's coverage index (good's coverage) and diversity index (Shannon and Simpson) (Fig. 1d). The results of diversity analysis showed that the fungal richness of mature Daqu was lower than that of newly pressed Daqu, and the fungal richness of mature Daqu was the lowest in all samples. In environmental samples, the indoor ground soil fungal richness was the lowest and the air was the highest. The sequencing depth of all samples in the figure is greater than 0.9995, indicating that the sequencing depth has basically covered all species in the samples. According to Shannon index and Simpson index, the fungal diversity of mature Daqu is lower than that of newly pressed Daqu, and the fungal diversity of raw materials is basically the same as that of newly pressed Daqu. In environmental samples, the fungal diversity in air is the highest and in water is the lowest.

A total of 301 genera were detected at the genus level, of which 85 genera could be detected in newly pressed Daqu, only 33 genera in mature Daqu, and 202 genera could only be detected in raw materials or environmental samples (Fig. 2). The relative abundances of three ASVs in the new pressed Daqu were $> 1\%$, and the relative abundances of the top two were $63.58 \pm 6.11\%$ and $31.51 \pm 7.33\%$ respectively, but the two ASVs were not annotated to the genus level, and *Pichia* ranked third. The dominant genera in mature Daqu are *Pichia, Thermoascus* and *Aspergillus.*

In order to further explain the relationship between Daqu raw materials, environment and Daqu microorganisms, the nonmetric multidimensional scaling (NMDS) and hierarchical clustering analysis (HCA) based on Jaccard distance were applied to analyze the data. The analysis results of NMDS and HCA (Fig. 2c–d) show that the newly pressed Daqu is closest to the raw materials, the mature Daqu is closest to the enhanced strains, the water and other samples are far away, and the air, tools and ground samples are close.

3.2 Diversity Analysis of Bacteria and Environmental Communities in Feng-Flavour Daqu

The dilution curve shows that all samples have reached the platform stage, indicating that this sequencing can cover the vast majority of bacterial population information in the samples (Fig. 3a). The number of new pressed Daqu and fortified strains was the highest (283), followed by raw materials (200) and tools (214). The number of ASVs shared by mature Daqu and tools is the highest (86), followed by indoor ground (75) (Fig. 3b). The results of ASV Wayne diagram of each sample show that the unique ASV in the tool is the most (3127), and the unique ASV in the newly pressed Daqu is the least, only 341, the number of ASV detected in all samples is 0 (Fig. 3C).

The results of bacterial diversity analysis showed that the bacterial richness of mature Daqu was higher than that of newly pressed Daqu, and the bacterial richness of raw materials was the lowest in all tested samples; Except for tools, the sequencing depth of other samples is greater than 0.99, indicating that the sequencing depth has basically covered all species in the samples. The bacterial diversity in mature Daqu is higher than that in newly pressed Daqu. Among all tested samples, the bacterial diversity in raw materials is the lowest, that in tools is the highest, and that in indoor ground is the second (Fig. 3d).

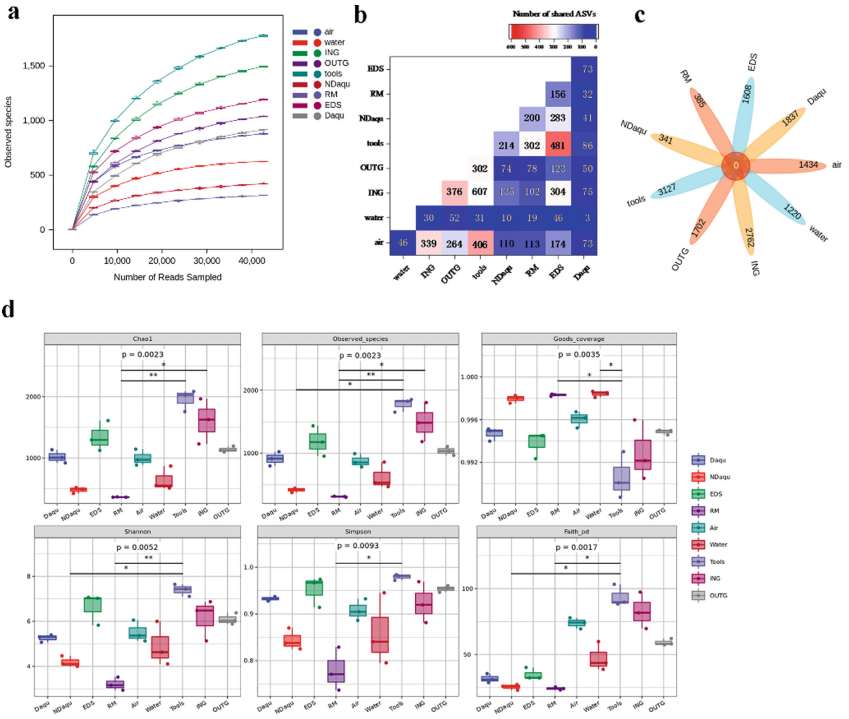

Fig. 3. (a) Bacterial population density curve. This sequencing basically covers the majority of fungal population information in the sample. (b) ASV distribution of bacterial in each sample shows that NDaqu and EDS, Daqu and tools share the highest ASV respectively. (c)Venn diagram is used to represent the AVS shared by bacteria in the experimental sample. Nine samples had no shared ASV. (d) The bacterial population diversity of Daqu, RM and environment was evaluated by richness index (Chao1 and observed species), good's coverage index (good's coverage) and diversity index (Shannon and Simpson). The richness and diversity of fungi in the tools were the highest, and the richness and diversity of fungi in Daqu were higher than those in NDaqu.

A total of 682 genera were detected at the genus level, of which 105 genera could be detected in newly pressed Daqu, only 50 genera in mature Daqu, and 555 genera could only be detected in other samples such as environment (Fig. 4a-b). The dominant bacteria in the newly suppressed Daqu are *Pantoea, Chloroplast, Leuconostoc* and *Erwinia.* The dominant genera in mature Daqu are *Bacillus, Streptomyces, Saccharopolyspora, Lactobacillus, Kroppenstedtia, Pseudonocardiaceae, Weissella, Staphylococcus* and *Acetobacter*.

The results of NMDS analysis and HCA analysis show that the microbial population of newly pressed Daqu is closest to the raw material, while the mature Daqu is far away from other samples (Fig. 4c–d). Therefore, we speculate that raw materials are the main source of bacterial population in newly pressed Daqu. Through the process of Daqu Culture and storage, the bacterial population in newly pressed Daqu gradually succession to the bacterial population structure in mature Daqu.

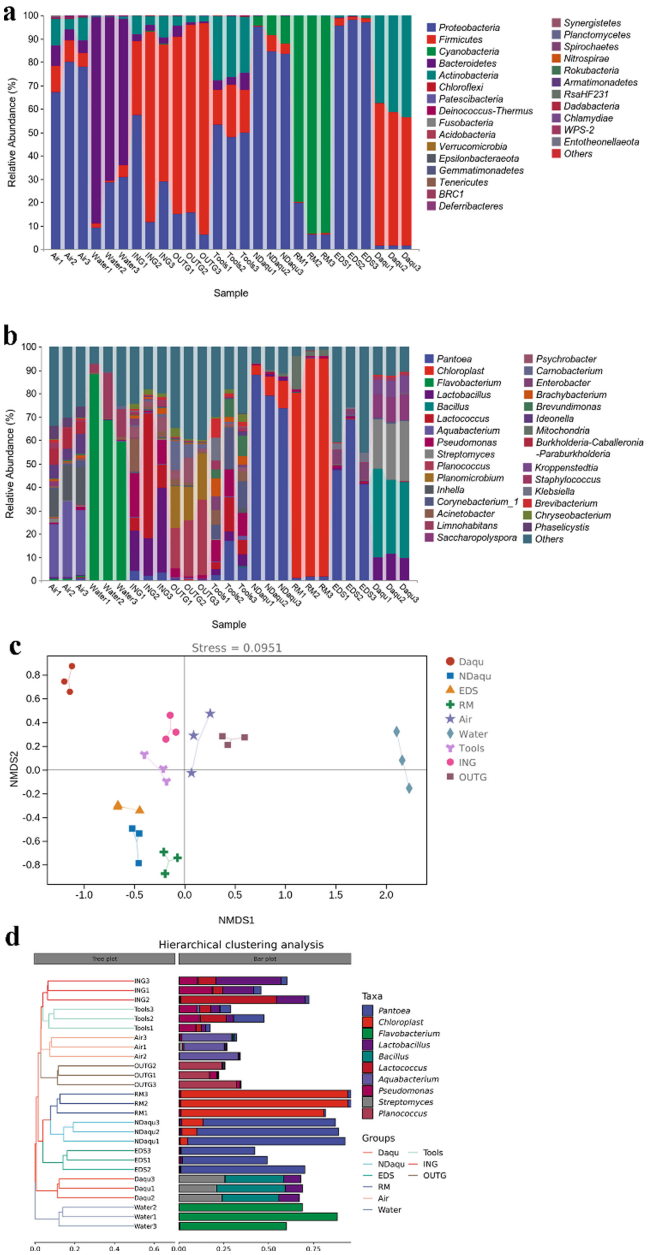

Fig. 4. (a) Analysis of bacterial population structure of each sample at phylum level. 27 bacterial phyla were detected and the dominant bacterial phyla were *Firmicutes, Proteobacteria, Actinobacteria, Bacteroidete*s and *Cyanobacteria.* (b) Analysis of bacterial population structure of each sample at genus level. 682 bacterial genera were detected, and the dominant bacteria in different samples were vary greatly. (c) Nonmetric multidimensional scaling analysis (NMDS) of bacterial population shows that NDaqu and RM, Daqu and ING shares the nearest distance. (d) Hierarchical cluster analysis (HCA) of bacterial population suggests that NDaqu and RM shares the nearest distance.

3.3 Microbial Traceability Analysis of Feng-Flavour Daqu

In this study, SourceTracker software was used to track the sources of microorganisms in Daqu with potential source microorganisms (raw materials and environment) of Daqu as the source end and newly pressed Daqu microorganisms as the receiver end. The results showed that the fungi in the newly pressed Daqu mainly came from raw materials (94.7%), followed by outdoor ground (1.8%) and unknown environment (3.47%); Bacteria mainly came from indoor ground (60.95%), followed by raw materials (20.44%), tools (8.98%) and unknown environment (9.63) (Fig. 5a).

Raw materials contributed most of the main fungi in the newly pressed Daqu, among which the most contributing species were not classified to the genus level. In addition,

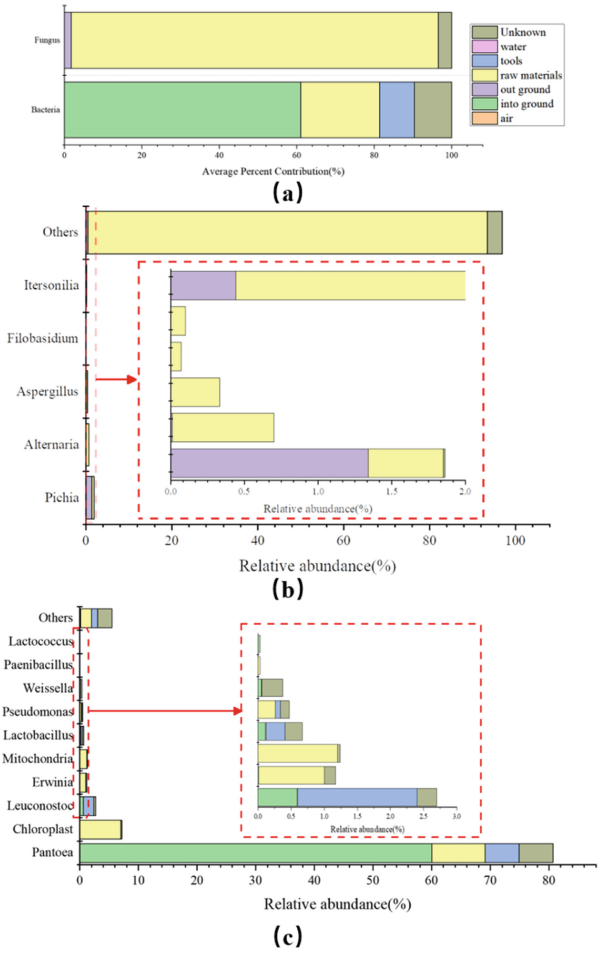

Fig. 5. (a) Microbial traceability analysis of NDaqu to determine the contribution rate of different sources. 94.7% of fungi come from RM and 60.95% of bacteria come from ING. (b) Fungal traceability shows that RM contribute to the most. (c) Bacterial traceability shows that *Pantophytic* are the dominant bacteria, which mainly come from ING.

they also contributed a small amount of *Pichia, Alternaria, Aspergillus, Filobasidium, Itersonia*, etc., and the outdoor ground contributed 1.34% of *Pichia* (Fig. 5b). *Pantophytes* are the dominant bacteria in the newly pressed Daqu, with 60.02% coming from the indoor ground (Fig. 5C).

4 Discussion and Conclusion

The main sources of fungi in the newly pressed Feng-Flavour Daqu were raw materials (94.7%), and bacteria were mainly from indoor ground (60.95%) and raw materials (20.44%).

This is different from the source of microorganisms in Fen-Flavour and Luzhou-flavor Daqu. The main sources of fungi in Fen-Flavour new pressed Daqu are tools (55.18%) and indoor ground (15.97%). At the beginning of Luzhou-Flavor Daqu fermentation, 53.7% of fungi came from outdoor ground and indoor roof contributed 23.0%. The main source of bacteria in Fen-Flavour and Luzhou-Flavor new pressed Daqu is raw materials.

This may be related to the different sampling of new pressed Daqu. Feng-Flavour Daqu is directly taken, just pressed and formed, and the new Daqu that has not entered the room has relatively little contact with tools and environment. Fen-Flavour Daqu and Luzhou-Flavor Daqu are taken from Daqu that has entered the room and has not yet started cultivation, and have been fully contacted with transportation and cultivation tools; The different sources of three kinds of Daqu fungi may also be caused by different raw materials. The raw materials of Fen-Flavour Daqu are barley and pea, the raw materials of Feng-Flavour Daqu are barley, pea and wheat, and the raw materials of Luzhou-Flavor Daqu are wheat. Different raw materials have selectivity for microbial enrichment; Of course, the microbial community structure in different regions is different, which may also lead to the differences of Daqu microorganisms.

The yeast in Daqu plays a role in saccharification, liquefaction and fermentation in wine production, and also plays a certain role in the production of flavor substances, while the bacteria in Daqu are mostly related to the production of flavor substances. Different flavor types of Daqu have different microbial sources. Raw materials and environment determine the quality of Daqu. Specific production areas have specific microbial communities, forming a unique microbial structure in Daqu and giving Daqu a specific flavor type. In addition to these natural factors, the quality of Feng-Flavour Daqu also depends on our artificially cultured enhanced strains. In previous experiments, we also tried to use non enhanced strains, but the quality is poor. Therefore, the quality of enhanced strains is also one of the key factors affecting the quality of Feng-Flavour Daqu.

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