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Dynamics and diversity of microbial community succession in traditional fermentation of Shanxi aged vinegar

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The traditional fermentation of Shanxi aged vinegar (SAV), a well-known traditional Chinese vinegar, generally involves the preparation of starter *daqu*, starch saccharification, alcoholic fermentation (AF) and acetic acid fermentation (AAF). Dynamics and diversity of microbial community succession in *daqu* and other fermentation stages were investigated by denaturing gradient gel electrophoresis (DGGE). Results showed that eight bacterial genera and four fungal genera were found in *daqu*. However, *Staphylococcus*, *Saccharopolyspora*, *Bacillus*, *Oceanobacillus*, *Enterobacter*, *Streptomyces*, *Eurotium*, *Monascus* and *Pichia* in *daqu* were eradicated during AF. Four bacterial genera and three fungal genera were found in this stage. *Weissella*, *Lactobacillus*, *Streptococcus*, *Saccharomyces*, and *Saccharomycopsis* were the dominant microorganisms in the late stage of AF. During AAF, four bacterial genera and four fungal genera were found. *Weissella*, *Streptococcus*, *Klebsiella*, *Escherichia*, and *Cladosporium* gradually disappeared; the dominant microorganisms were *Acetobacter*, *Lactobacillus*, *Saccharomycopsis*, and *Alternaria* in the late stage of AAF. Alpha diversity metrics showed that fungal diversity in *daqu* was greater than that in AF and AAF. By contrast, bacterial diversity decreased from *daqu* to AF and increased in the first three days of AAF and then decreased. Hence, these results could help understand dynamics of microbial community succession in continuous fermentation of traditional Chinese vinegars.

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1. Introduction

Traditional vinegars are typical fermented foods produced by various microorganisms. This type of fermented food is usually produced by spontaneous fermentation technology to yield various metabolites, which are accounted for the unique flavor and taste of vinegar. Fermentation involved in traditional vinegar production creates a unique microbial ecosystem in which microbial communities are enriched and changed naturally. Dynamics and diversity of microbial community succession in traditional vinegars are key factors to understand the mechanism of traditional fermentation technology.

Vinegars have been produced in China for centuries (Liu et al., 2004). Among the most well-known Chinese traditional vinegars with a history of approximately 3000 years, Shanxi aged vinegar (SAV) is selected as the national intangible cultural heritage of China (Chen et al., 2013). Fermented with multiple species of fungi and bacteria, SAV is generally produced from sorghum, pea, wheat bran, millet bran and rice hull through open-style and solid-state fermentation (SSF). The traditional fermentation of SAV generally involves the preparation of *daqu* (made from barley and pea by spontaneous growth of the microorganisms on/in it, take about 60% of the raw material), starch saccharification (SS), alcoholic fermentation (AF), and acetic acid fermentation (AAF) (Chen et al., 2009; Wu et al., 2012a). Traditional SAV fermentation is controlled empirically to facilitate spontaneous microbial growth and enrichment. A starter *daqu* containing various microorganisms and enzymes is used in starch saccharification in which starch is immediately hydrolyzed (Chen et al., 2009; Nie et al., 2013). SS and AF are normally conducted simultaneously after steamed sorghum is mixed with *daqu* powder and water (Wu et al., 2012a). The addition of water establishes an anoxic environment for AF in which sugars are converted to ethanol. The proportions of water and sorghum are 4:1 (w/w). This process continues for approximately 13 days. In the last phase of AF, high amounts of wheat bran and rice hull (the proportion with sorghum is 1.2–1.5:1, w/w) are

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mixed with alcoholic samples (called *jiulao* in Chinese) to increase porosity for oxygen uptake and heat discharge. Vinegar seeds (about 10%–15%, w/w) produced from the last batch of vinegar samples (called *cupei* in Chinese) are also added to the urn for AAF, which generally lasts 7 days. AAF is anoxic environment suitable for the growth of acetic acid bacteria (AAB), which could oxidize ethanol to acetic acid. AAB, lactic acid bacteria (LAB), and other cultivatable microorganisms in single or double fermentation processes of SAV have been extensively investigated using culture-dependent and culture-independent approaches (Hao et al., 2008; Hu and Hao, 2004; Wu et al., 2010, 2012a,b). However, whole fermentation, particularly dynamic changes in bacterial and fungal community in starter daqu and the continuous fermentation processes of SAV, has been rarely explored. Culture-dependent approaches exhibit limitations in terms of reproducibility and challenges in the culturability of some uncultured microorganisms (Jung et al., 2011). Thus, microbial community composition and succession are difficult to be reflected by cultured isolates. Denaturing gradient gel electrophoresis (DGGE) fingerprinting has been applied to understand microbial structures in different kinds of vinegars; DGGE results have shown that microbial communities are more complex than initially thought (Gullo et al., 2009; Haruta et al., 2006; Seo et al., 2010; Solieri et al., 2006; Wu et al., 2012b; Xu et al., 2011).

In the present study, the microbial community succession in starter *daqu* and the other fermentation processes of SAV was systematically investigated by DGGE. This study aimed (1) to reveal the dynamics in bacterial and fungal communities succession in starter *daqu* and the continuous processes of fermentation and (2) to compare bacterial and fungal diversity in different fermentation stages. Our results would help understand the unique microbial ecosystem and fermentation mechanism of traditional Chinese vinegars.

2. Materials and methods

2.1. Sampling and genomic DNA extraction

Daqu, *jiulao*, and *cupei* samples of SAV were collected from Qingxu, China. To monitor the dynamic changes in microbial community, we periodically collected five *jiulao* (1d, 4d, 7d, 10d, and 12d) and five *cupei* (0d, 1d, 3d, 5d, and 7d) from the same location. All of the *cupei* samples were collected at a depth of approximately 30 cm from the upper surface in the urn. Approximately 200 g of samples were collected and placed into sterile blue cap bottles and immediately stored in an ice box. The samples were stored at -80 °C until used for genomic DNA extraction and physicochemical analysis.

Before DNA was extracted, all of the samples were pretreated and semi-liquid *jiulao* samples were centrifuged ($8000 \times g$ for 10 min at 4 °C) to collect pellets. Approximately 1 g of solid samples was homogenized and grinded using liquid nitrogen. Approximately 500 mg of samples were then added to the extraction buffer [100 mM Na_3PO_4, 100 mM Tris–HCl, 100 mM ethylene diamine tetraacetic acid (EDTA), 1.5 M NaCl, 1% cetyltrimethyl ammonium bromide (CTAB, w/v), 2% polyvinyl-polypyrrolidone (PVPP, w/v), pH 8.0]. CTAB was used to remove polysaccharides from the vinegar samples. PVPP was used for polyphenolic component adsorption. Total gDNA was extracted according to previously described methods (Mamlouk et al., 2011; Nie et al., 2013).

2.2. Physicochemical analysis

The concentration of alcohol in *jiulao* samples was determined by high-performance liquid chromatography (HPLC) according to a previously reported method (Wu et al., 2013). Approximately 10 g of *cupei* samples were homogenized with 30 mL of distilled water and pH was determined using a pH meter (Mettler Toledo, USA). Total acid (TA) and total soluble solids (TSS) of *cupei* samples were measured according to previously described methods (Chen et al., 2012; Nie et al., 2013).

2.3. DGGE analysis of bacterial and fungal community

The DGGE fingerprinting analysis of microbial community was performed according to a previously described method (Nie et al., 2013). The V3 region of the 16S rDNA gene (primes 338f/518r) was selected for bacterial community analysis, and the D1 region of 26S rDNA gene (primers NL1/LS2) was used for fungal community analysis. All of the bands were excised and reamplified. Afterward, the PCR products were purified and sequenced. Sequence analysis was conducted using BLAST algorithm in GenBank nucleotide database. (http://www.ncbi.nlm.nih.gov/blast/).

2.4. Multivariate statistical analyses

The DGGE profiles were evaluated using Quantity One version 4.6.2 (BioRad, Hercules, CA) to determine the relative quantities of DGGE bands. Considering relative quantities, we calculated the relative abundance of each microorganism at a genus level; hierarchical cluster was then analyzed using Hierarchical Clustering Explorer (HCE) version 3.5 (Seo et al., 2006). To describe microbial diversity (including dominance, richness, and equitability), we calculated biodiversity indices, including Margalef index (Magurran, 2004; Margalef, 1958), Simpson index (Simpson, 1949), Shannon–Wiener index (Shannon and Weaver, 1949), and Pielou evenness index (Pielou, 1966), based on the relative quantities of DGGE bands. Principal component analysis (PCA) was then performed to group microorganisms using Canoco for Windows v4.5 software (Wageningen UR, Netherlands).

2.5. Nucleotide sequence accession number

The sequences obtained in this research were deposited in GenBank database under Accession Nos. KC961553–KC961621.

3. Results and discussion

3.1. Changes in physicochemical characteristics during whole fermentation

The changes in alcohol in AF were analyzed (Fig. 1I). The alcohol concentration gradually increased to 8.9% (v/v) until AF was completed. At alcohol concentration of 8.9% (v/v), AF was terminated, and *jiulao* samples were used in AAF. AAF is a critical stage to produce organic acids, which are the dominant compounds in vinegar. Temperature, pH, TA, and TSS were found as key physicochemical metrics to monitor AAF (Fig. 1II). AAF was maintained at approximately 40 °C–45 °C. The TA concentration accumulated as fermentation continued and increased to 5.64 ± 0.67 g/100 g culture at 7 d of AAF. In AAF, pH changed closely with TA and was generally maintained between 3.5 and 4.5.

3.2. Dynamics and succession of microbial community during whole fermentation

The dynamic changes in bacterial community during whole fermentation of SAV were investigated periodically (Fig. 2I and Table 1). *Daqu* was used as a starter of starch saccharification. The bacterial community of *daqu* was composed of eight genera,

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