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Exploring microbial succession and diversity during solid-state fermentation of Tianjin duliu mature vinegar

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HIGHLIGHTS

- Acetic acid bacteria (AAB) and lactic acid bacteria (LAB) were dominant bacteria.
- Most microorganisms were enriched spontaneously in acetic acid fermentation.
- The abundance and diversity of LAB were more than those of AAB.
- Decrease of AAB might be an indicator for the end of fermentation.

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ABSTRACT

Tianjin duliu mature vinegar was one of famous Chinese traditional vinegars. The unique flavor and taste of vinegar are mainly generated by the multitudinous microorganisms during fermentation. In this research, the composition and succession of microbial communities in the entire solid-state fermentation were investigated, including starter *daqu* and acetic acid fermentation (AAF). Molds and yeasts in *daqu*, including *Aspergillus*, *Saccharomycopsis* and *Pichia*, decreased in AAF. The bacterial compositions increased from four genera in *daqu* to more than 13 genera in AAF. Principal component analysis showed that *Acetobacter*, *Gluconacetobacter*, *Lactobacillus* and *Nostoc* were dominant bacteria that were correlated well with AAF process. In the early fermentation period, lactic acid bacteria (LAB) decreased while acetic acid bacteria and *Nostoc* increased rapidly with the accumulation of total acids. Then, the abundance and diversity of LAB increased (more than 80%), indicating that LAB had important influences on the flavor and taste of vinegar.

1. Introduction

Vinegar is used as flavoring agent and an important feedstock in food industry world widely (Solieri and Giudici, 2009). Tianjin duliu mature vinegar (TDMV), one of famous vinegars in China, is brewed by traditional solid-state fermentation technology. Like many other Chinese traditional vinegars, the fermentation process of TDMV generally includes three steps, starch saccharification, alcohol fermentation and acetic acid fermentation (AAF) (Chen et al., 2009; Xu et al., 2010). But TDMV also has its unique raw materials and fermentation process. *Daqu*, a kind of mixed culture starters, is used as inoculum for the solid-state fermentation and contains a diverse microbial community such as molds, yeasts and bacteria (Zheng et al., 2012). Also, it contains more than 50 types of enzymes produced by microorganisms in it including α-amylase, glucoamylase, acid protease and lipase (Chen et al., 2009). It is usually mixed with raw materials (sticky rice and red sorghum) for starch saccharification. Then raw materials are added with water and go on alcohol fermentation spontaneously. At the end of alcohol fermentation, wheat bran, sorghum hull and rice hull are added to increase the porosity for oxygen intake and heat discharge in AAF process. The mixture (called vinegar *pei* in Chinese) are transport to the bioreactor, a pottery urn, for acetic acid fermentation and the vinegar *pei* is stirred manually every morning to increase the oxygen content. Different from other solid-state fermented vinegar, only the vinegar *pei* in the upper half layer of the urn is stirred in the AAF of TDMV. The vinegar *pei* in the upper half layer and the bottom half layer are exchanged in an empty urn at the 16th day, and then it goes on AAF process in the next 15 days.

The brewing process of Chinese traditional vinegars is usually a spontaneous fermentation, and this fermentation leads to the growth of diverse microorganisms, which have important influences on the quality of vinegar. Due to different raw materials, manufacture process and microorganisms used for fermentation, the taste and flavor of TDMV are distinct from other vinegars. The soft taste and special flavor are decided by the abundant organic acids (lactic acid and malic acid, etc.) and the flavoring

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compounds (esters, aldehydes and ketones, etc.), which are generally from metabolites of microorganisms (Solieri and Giudici, 2009). Thus microorganisms play important roles in the production and characteristic of vinegar. It is very necessary to investigate the microbial communities in fermentation process of Chinese traditional vinegars not only for understanding the relationships between microflora and metabolites but also for proposing a potential approach to monitor the traditional fermentation process. Some studies have focused on microbial community in single fermentation stage of Chinese traditional vinegars with culture-dependent approaches or the culture-independent methods such as denaturing gradient gel electrophoresis (DGGE) (Wu et al., 2004, 2012; Xu et al., 2011). However, the results show that microbial communities are far more complex than initially thought because the vinegar fermentation is a continuous process which goes through three stages, therefore, the dynamic change of microbial community during the entire vinegar fermentation process, especially between *daqu* and AAF process, should be considered together.

In the present study, the microbial composition and succession in the entire solid-state fermentation process (including *daqu* and AAF process) of TDMV were investigated. The relative abundance and dynamic change of bacterial community during solid-state AAF process was further studied using metagenomic approach. The results would be helpful for understanding the structure of microflora and their function on the solid-state fermentation of TDMV from *daqu* to AAF.

2. Methods

2.1. Sampling

The *daqu* and vinegar *pei* were collected from Tianjin Tianliduliu Mature Vinegar Co., Ltd. (Tianjin, China). For the purpose of monitoring the change of microbial community during solid-state AAF process, fifteen samples of vinegar *pei* (1d, 3d, 5d, 7d, 9d, 11d, 13d, 15d, 16d, 18d, 20d, 22d, 24d, 26d, 28d) were collected. All samples were collected at approximately 30 cm depth from the upper surface in the urn.

2.2. Physicochemical property analysis of vinegar pei

The physicochemical property of vinegar *pei*, including pH, total acidity and concentration of amino nitrogen, was analyzed. Ten grams of samples were homogenized with 30 mL of distilled water, and then the pH was measured using a pH meter (Mettler Toledo, USA). Total acidity was evaluated by titration using standardized solution (0.1 M sodium hydroxide) with phenolphthalein as indicator. Amino nitrogen was determined according to previous method (Thomas and Ingledew, 1990).

2.3. Genomic DNA extraction

Samples were homogenized using liquid nitrogen (flash-frozen in liquid nitrogen and then rapidly thawed in a water-bath at 65 °C for 2 min, repeated three times), and then approximately 500 mg of each sample was used for genomic DNA extraction according to the previous method (Vainio and Hantula, 2000).

2.4. PCR-DGGE analysis of microbial community

The V3 region of the 16S rDNA gene amplified using primers 338f and 518r was selected for bacterial community analysis (Muyzer et al., 1993). Two pairs of universal primers of 18S rDNA and 26S rDNA were chosen for fungal community analysis. zAmplification of the 18S rDNA gene was performed using primers NS3 and YM951r (Haruta et al., 2006), and primers NL1 and LS2 were used to amplify the D1 region of the 26S rDNA gene (Cocolin et al., 2002). The primers 338f, NS3 and NL1 had a GC clamp, CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG, at the 5′ end, and a TouchDown-PCR protocol was applied (Haruta et al., 2006; Cocolin et al., 2002). PCR products were run on 2% agarose gel electrophoresis and the DNA band with the correct size was excised and purified using QIAquick Gel Extraction Kit (Qiagen, Shanghai, China).

Preparation of the 8% polyacrylamide denaturing gradient and gel electrophoresis was done according to the manufacturer instructions for the D-Code Universal Detection System (BioRad, Hercules, CA). For determining the structure of bacterial community, a 30–60% gradient of urea and formamide was used. For fungal community, 20–50% (for 18S rDNA) and 30–50% (for 26S rDNA) denaturant gradients were applied. The electrophoresis was performed at a constant voltage of 80 V for 11 h (16S rDNA or 18S rDNA) and 130 V for 4.5 h (26S rDNA) at a constant temperature of 60 °C. The DGGE gels were visualized by UV irradiation following stain with SYBR Green I (Invitrogen, USA). All bands were excised and reamplified using the same primers but without the GC clamp and common PCR program. PCR products were purified and sequenced. The sequence analysis was carried out using the BLAST algorithm in GenBank nucleotide database (http://www.ncbi.nlm.nih.gov/blast/).

2.5. Illumina sequencing for bacterial community and data analysis

The relative abundance and dynamic change of bacterial community in AAF process were systematically investigated using Illumina sequencing. V3 hypervariable regions of 16S rDNA were amplified with primers 338f and 518r, and PCR products were purified for Illumina high-throughput sequencing (BGI-Shenzhen, China). The V3 tags of 16S rDNA from all sequences were compared against the Ribosomal Data Project II database. All V3 tags were aligned together and pre-clustered at 98% similarity by single-linkage preclustering (SLP) tool (Huse et al., 2010). The mothur software package v1.11.0 (Schloss, 2009) and SLP methodology were used for the analysis of the number of sequences assigned to each cluster (regarded as Operational Taxonomic Unit, OTU). To indicate the microbial diversity in AAF process, the α diversity indices (including Chao1, ACE, Simpson, and Shannon–Weaver indices) were quantified in terms of OTU richness, and β diversity of different samples was analyzed using Whittaker's method (Whittaker, 1960). Principal components analysis (PCA) of normalized OTU abundance was performed using the *prcomp* function of the *stats* package of R statistical language (www.r-project.org), and the *pvclust* package of R with the default settings was used for clustering analysis. The clustering analysis was carried out with the cluster method of ward.

2.6. Nucleotide sequence accession numbers

For DGGE fingerprinting analysis, the sequences obtained in this research have been deposited in the GenBank database under accession numbers KC820009 to KC820048. The Illumina sequencing data of the 16S rDNA genes are publicly available in the NCBI Short Read Archive under accession No. SRA073391.

3. Results and discussion

3.1. Physicochemical features of vinegar pei

Organic acids including acetic acid and lactic acid were dominant flavor components of vinegar and the major sources of total