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Dynamics and diversity of microbial community succession during fermentation of Suan yu, a Chinese traditional fermented fish, determined by high throughput sequencing

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ABSTRACT

The main goal of this study was to investigate the dynamics, diversity and succession of microbial community present during the preparation of Suan yu (fermented fish), with and without starter cultures by high-throughput sequencing of 16S rRNA and ITS1 genes. *Firmicutes* and *Ascomycota* were the predominant phyla of bacteria and fungi, respectively, in all samples. At the genus level, *Lactobacillus*, *Macrococcus* and *Staphylococcus* were the predominating bacteria throughout the fermentation process, regardless of the inclusion of starter cultures. *Saccharomyces* was the predominating fungal genus in the early-fermentation stage of samples that inoculated starter cultures (MS), while the final product was dominated by *Candida* and *Wickerhamomyces*. Compared with naturally-fermented samples (NS; no starter cultures), *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Vibrio*, *Fusicolla* and *Torulaspora* were inhibited and *Aureobasidium* emerged in samples inoculated with starter cultures ($P < .05$). Unweighted pair-group and principal component analyses of bacterial and fungal compositions revealed that microbiota structures differed between NS and MS samples. Redundancy analysis indicated that water content and pH might be important factors influencing the dominant bacterial and fungal community. Results indicated that microbial community were dynamic during fermentation process and the inoculation of mixed starter culture inhibited the growth of many organisms associated with food spoilage and contributed to the improvement of the quality of Suan yu products.

1. Introduction

Fish fermentation is a traditional preservation technique that relies on spontaneous fermentation or starter cultures under controlled conditions. Thousands of fermented fish products are consumed for their distinct flavor, texture, and extended shelf life, and are popular throughout the world in countries such as Japan (Kuda, Kawahara, Nemoto, Takahashi, & Kimura, 2014; Kuda, Mihara, & Yano, 2007), Togo and Ghana (Anihouvi, Sakyi-Dawson, Ayernor, & Hounhouigan, 2007), Egypt (Rabie, Simon-Sarkadi, Siliha, El-seedy, & El Badawy, 2009), Thailand (Kopermsub & Yunchalard, 2010; Paludan-Müller, Madsen, Sophanodora, Gram, & Møller, 2002), India (Aarti et al., 2017; Devi, Deka, & Jeyaram, 2015), and Korea (Lee, Choi, Hwang, Hong, & Lee, 2016).

In fact, a variety of microorganisms present in fermentation processes and contribute to complex biochemical changes, resulting in a great diversity of product properties (Alkema, Boekhorst, Wels, & van Hijum, 2016; Smid & Kleerebezem, 2014). For instance, lactic acid bacteria (LAB), which are mainly involved in accelerating lactic acid and acetic acid formation via the fermentation of available carbohydrates, are largely responsible for the safety and flavor of fermented products (Gänzle, 2015; Waters, Mauch, Coffey, Arendt, & Zannini, 2015). Besides that, strains of certain LAB species have potential health benefits and nutritional properties, as they display probiotic activity and release bioactive molecules (Pessione & Cirrincione, 2016). Meanwhile, staphylococci and yeasts have great lipolytic and proteolytic capability and ferment residual lactose, contributing to the aroma and flavor development of fermented products (Padilla, Belloch, López-Díez, Flores, & Manzanares, 2014; Zeng, Xia, Jiang, & Yang, 2013b). However, undesirable aspects of the metabolism of the microbiota may have adverse effects and produce potentially harmful substances (Yongmei et al., 2007). Previous studies have demonstrated that *Pediococcus*, *Klebsiella*, and *Streptococcus* tend to develop biogenic amines that are precursors of N-nitrosamines and are considered to be carcinogenic amines (Rabie, Simon-Sarkadi, Siliha, El-seedy, & El Badawy, 2009). Therefore, in order to produce high-quality and safe products, it is of primary importance to monitor the composition and behavior of the microbial community present in fermented products.

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Suan yu, a traditional Chinese fermented freshwater fish product, are well-accepted by consumers in China due to their characteristic flavor and nutrition, which is attributed to a process of variable microorganisms (Xu et al., 2018; Zeng, Xia, Jiang, & Yang, 2013a). In our previous study, *Lactobacillus plantarum* 120, *Staphylococcus xylosus* 135, and *Saccharomyces cerevisiae* 31 were isolated from Chinese traditional low salt fermented whole fish products as the most appropriate strains, according to their technological properties and antimicrobial characteristics (unpublished). Moreover, products with these added starter cultures had better flavor, safety properties, and overall acceptability compared with samples prepared without the addition of starter cultures (Gao, Wang, Jiang, Xu, & Xia, 2016). However, so far, only traditional microbiological methods based on cell culturing and colony counting, have been used in studying microbes present in Suan yu and these cannot accurately discriminate all strains of microbes (Li et al., 2011; Zeng, Chen, & Zhang, 2016; Zeng, Xia, Jiang, & Yang, 2013). Furthermore, the microbial community structure and succession and the effect of inoculation on microbial community are still unknown. With the development of molecular biology technology, high-throughput sequencing (HTS) methods are becoming indispensable tools for the detection of microbial community structure and composition, which cannot be obtained from traditional culture-based analysis (Sekse et al., 2017). Moreover, HTS methods provide deeper and more precise information on the microbiota compared with PCR-denaturing gradient gel electrophoresis (PCR-DGGE) (Ogier et al., 2004; Soto Del Rio, Dalmasso, Civera, & Bottero, 2017). Recently, such methods have been successfully used to detect microbial community in ecosystems (Claesson et al., 2009) and food matrixes (Lusk et al., 2012; Masoud et al., 2011).

Therefore, the present study aims to: 1) investigate microbial community diversity and dynamics during Suan yu fermentation for the first time using high-throughput sequencing; and 2) examine the influence of mixed starter culture inoculation on the microbial community during the fermentation of Suan yu.

2. Materials and methods

2.1. Starter cultures

Strains of *Lactobacillus plantarum* 120 (L.p120), *Staphylococcus xylosus* 135 (S.x135) and *Saccharomyces cerevisiae* 31(S.c 31) were isolated from traditional Suan yu and cultivated according to the method of Zeng, Zhang, and Zhu (2016). The L.p120 strain was cultivated twice in DeMan-Rogosa-Sharpe (MRS) broth at 30 °C for 2 days; S.x135 was cultivated in nutrient broth at 30 °C for 3 days; and S.c 31 was cultivated twice in yeast extract peptone dextrose medium (YPD) broth at 30 °C for 24 h. Subsequently, all the cell pellets were centrifuged in a high-speed refrigerated centrifuge (Sigma Laborzentrifugen, Model 4K15, Osterode, Germany) at 10,000*g* for 15 min at 4 °C and the resuspended cell pellets were washed using sterile saline water (0.9% NaCl). Finally, the cell concentrations of L.p120 strain, S.x135 and S.c 31 were approximately 9 log cfu/mL, 9 log cfu/mL and 7 log cfu/mL, respectively.

2.2. Suan yu manufacturing and sampling

A total of 20 fresh live carps (*Cyprinus carpio* L.; average weight: 2 ± 0.2 kg;) were purchased from Vanguard Market (Wuxi, Jiangsu, China) in April and were killed by a sharp blow to the head by professionally-trained personnel according to the Guidelines for the Treatment of Animals and Experimental Animal Management Regulations issued by Ministry of Science and Technology of the People's Republic of China. Immediately after death, the entrails and the scales were removed and the bellies of the fish were stuffed with crushed ice. The fish were then packed in ice-filled bubble boxes and transported to the laboratory within 20 min. The fish were cleaned with iced water and cut into 300 blocks of approximately 3 cm × 2 cm × 2 cm. Suan yu was prepared according to the method described by Zeng, Xia, Jiang, and Yang (2013) with small modifications. Briefly, the carp pieces (approximately 260 carp blocks) were cured with 2% (*w*/w) sucrose and 3% (w/w) salt at 4 °C for 48 h, and then oven dried for 3 h at 50 °C and 65% RH before being mixed with roasted corn flour (25% w/w) by hand. Subsequently, the fish mixtures were randomized into two groups (approximately 130 carp blocks in each group). One group was further inoculated with mixed starter cultures (L.p120, S.x135 and S.c 31[1:1:1 by volume]) (1% inoculation) and named Group MS. The other group, without any starter cultures, was used as the control (Group NS). The MS and NS groups were placed in small oxygen-free jars and fermented at ambient temperature (25 °C) for 4 weeks. Samples (approximately 20 carp blocks in each batch) were taken from two groups for the physicochemical analysis and samples (approximately 10 carp blocks in each batch) were taken from two groups for total DNA extraction and PCR amplification after 1, 2, 3 and 4 weeks of fermentation.

2.3. Physicochemical analysis

pH and water content of 9 groups of samples (fresh, MS1, MS2, MS3, MS4, NS1, NS2, NS3, NS4) measurements were performed. Each sample (10 g) was homogenized in 90 ml of distilled water at 12,000 rpm for 1 min. Their pH values were recorded using a digital pH meter (Mettler Toledo 320-s, Shanghai, China). Water content was calculated according to weight loss; a 5 g sample was dried at 105 °C for 4 h by which time its weight was constant. TBARS were measured by the method of Gao, Wang, Xia, Xu, and Jiang (2016). Analyses were performed in triplicate for each sample.

2.4. DNA extraction and PCR amplification

Total genomic DNA was extracted from 9 groups of samples (fresh, MS1, MS2, MS3, MS4, NS1, NS2, NS3, NS4) with a PowerSoil DNA Isolation Kit (MO BIO Laboratories, USA) according to the manufacturer's protocol. The quality and quantity of the extracted DNA were assessed by the ratios of 260 nm/280 nm and 260 nm/230 nm measured by a Nanodrop spectrophotometer (ND-1000, NanoDrop Technologies, USA).

The V3-V4 region of the bacterial 16S rRNA gene was amplified with the universal primers 338F(5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The ITS1 region of the fungi was amplified with the forward primer ITS1F (5′-CTTGGTCATT TAGAGGAAGTAA-3′) and the reverse primer ITS1R (5′-GCTGCGTTCT TCATCGATGC-3′) (Sun, et al., 2014). PCR amplification was performed in a total volume of 50 μl, which contained 10 μl Buffer, 0.2 μl Q5 High-Fidelity DNA Polymerase, 10 μl High GC Enhancer, 1 μl dNTP, 10 μM of each primer and 60 ng genome DNA. Thermal cycling conditions were as follows: an initial denaturation at 95 °C for 5 min, followed by 15 cycles at 95 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 7 min. The PCR products from the first step PCR were purified through VAHTSTM DNA Clean Beads. A second round PCR was then performed in a 40 μl reaction which contained 20 μl 2 × Phμsion HF MM, 8 μl ddH2O, 10 μM of each primer and 10 μl PCR products from the first step. Thermal cycling conditions were as follows: an initial denaturation at 98 °C for 30s, followed by 10 cycles at 98 °C for 10s, 65 °C for 30s min and 72 °C for 30s, with a final extension at 72 °C for 5 min. The amplified products were purified and recovered using a 1.8% agarose gel electrophoresis method. The 16S rRNA and ITS1 gene amplicons were sequenced using Illumina HiSeq deep sequencing at Biomarker Bioinformatics Technology Co., Ltd., Beijing, China. To obtain the raw tags, paired-end reads were merged by FLASH v1.2.7. Raw tags were then quality filtered under specific filtering conditions and clustered in the next steps. The merged tags were compared to the primers, and the tags with more than six mismatches