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Interphase microbial community characteristics in the fermentation cellar of Chinese *Luzhou*-flavor liquor determined by PLFA and DGGE profiles

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ABSTRACT

In view of the equilibrium of fermentation rate in the cellar mainly depends on interphase microflora, it is essential to exploit interphase microbial community characteristics to understand the metabolic regulation mechanism. The objective of this study was to investigate interphase microbial community structure in the fermentation cellar of Chinese *Luzhou*-flavor liquor via combining phospholipid fatty acids (PLFAs) and denaturing gradient gel electrophoresis (DGGE). The total microbial biomass analyzed by PLFA in the pit mud (PM: 25.52–103.38 nmol/g) and *Zaopei* (ZP: 29.96–64.50 nmol/g) was obviously higher than *Huangshui* (HS: 4.35–7.82 nmol/g), and was strongly correlated with pit age, which was validated by DGGE results. Among the specific microbial groups, 'gram-positive bacteria, anaerobe bacteria and fungi' dominated in the PM, while 'gram-positive bacteria, aerobe bacteria and fungi' predominated in the ZP and HS, respectively. Principal component analysis (PCA) based ordination of the relative PLFA abundance data segregated PM, ZP and HS. Eubacterial PCR-DGGE illustrated that a total of 31 bands belonged to five families (*Lactobacillaceae*, *Clostridiaceae*, *Porphyromonadaceae*, *Synergistaceae* and *Acetobacteraceae*) were identified, and 41.52% eubacteria were affiliated with *Lactobacillaceae* in the ZP, while 44.67% and 40.41% eubacteria were affiliated with *Clostridiaceae* in the PM and HS, respectively. Archaeal PCR-DGGE analysis demonstrated that *Methanosaeta*, *Methanocorpusculum*, *Methanobrevibacter*, *Methanobacterium* and *Methanoculleus* were the majority of archaea. Particularly, *Methanosaeta*, as acetrophic methanogens, increased gradually in the PM and HS with pit age, and decreased in the ZP, and no family *Methanobacteriaceae* was detected in the 30-year-PM. Furthermore, fungal community was dominated by genus *Pichia*.

1. Introduction

Luzhou-flavor liquor, also known as strong-flavor liquor, is one of the main Chinese distilled spirits and has hundreds of year history. Fermentation cellar, constructed with special clay, provides a suitable habitat for the growth of brewing microbes, and there are three indispensable phases during the fermentation process, including PM, ZP and HS. PM, a solid phase, is domesticated for many years and contains a lot of organics and mineral components to offer mainly anaerobic microbes nutrient substances. ZP is a mixture of *Daqu*, streamed grains, fermented grains and rice husk, and the major biochemical reaction, such as converting starch into alcohol and other ingredients, takes place in this phase (Shi et al., 2011). In view of the physical feature of its constituents, there are a large number of voids in the ZP when filled into the cellar, thus ZP is regarded as a micro-aerobic phase. Moreover, during the process, the liquid metabolites and free water through ZP leach into the bottom of cellar, which are called as HS, a liquid phase. Importantly, interphase mass transfer affects not only material metabolism but community diversity. Therefore, it is essential to exploit interphase microbial community structure and differences in the cellar for understanding the liquor brewing mechanism and regulations.

Culture-dependent method has been used to reveal microbial communities in the PM, ZP or HS alone since 1960s. For example, Wu et al. (1993) and Wu et al. (1980) confirmed that microbial diversity was closely connected with pit age, while the synergistic relationship

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between methanogens and caproate acid bacteria was crucial for liquor brewing. Previous research suggested that *Bacillus*, *Sporolactobacillus*, *Pseudomonas* and *Clostridium* were predominant in the PM, and the quantity of facultative anaerobic bacteria increased with pit age (Yue, Zhang, Lu, Hu, & Zhang, 2007). However, only a small proportion (0.1%–1%) of microbes in complex matrix can be determined by culture-dependent approach, and this method is laborious and time consuming (Amann, Ludwig, & Schleifer, 1995; Rappé & Giovannoni, 2003). In the last two decades, various culture-independent methods, such as PCR-DGGE (Cocolin et al., 2011, 2013), PLFA (Frostegård & Bååth, 1996), fluorescence in situ hybridization (Zarda et al., 1997) and genomics (Riesenfeld, Schloss, & Handelsman, 2004), have been widely used to study community characteristics in various environmental samples. Nevertheless, due to inherent disadvantages for each method, no integrated information on community features was obtained. PCR-DGGE, one of the conventional culture-independent approaches, was only applied to determine the communities genetically and qualitatively. As an established and complementary method, PLFA technology had been used to quantitatively determine microbial compositions in various environmental samples on several levels, from the whole-community profile to specific group abundance, total biomass, and physiological stress conditions. Yet, until now, no investigation about interphase communities in the cellar by PLFA and DGGE was conducted.

Consequently, this study aimed to investigate interphase microbial community structure and differences in the cellar of different pit ages. To the best of our knowledge, this is the first report to uncover the discrepancies and shifts of interphase communities in the cellar by using two culture-independent methods.

2. Materials and methods

2.1. Sampling

PM, ZP and HS were sampled from different cellars in liquor manufacture (*Xufu* Co., Ltd, Yibin City, Sichuan Province, China), which were used uninterruptedly for 2, 10 and 30 years, respectively. The sampling method was according to the stratified random method documented in the literature (Carter & Gregorich, 2006), and sampling sites were shown in Fig. 1. For the PM and ZP, each sample plot was divided into four different positions (upper layer of cellar wall, middle layer of cellar wall, under layer of cellar wall and the bottom of cellar), and two subsamples were collected in each layer of cellar wall, and one subsample was collected from the center of the bottom of cellar, and well mixed, then transferred to sterile polyethylene bags and stored at −20 °C until analyzed. For each HS sample, approximately 100 mL was collected from the bottom of cellar after fermentation and immediately poured into glass bottles, then sealed storing at 4 °C until analyzed. In addition, the basic physicochemical properties were detected according to the previous literature (Darias-Martín et al., 2003; Shen, 2007).

2.2. PLFA analysis

2.2.1. Pretreatment of samples

Ten grams of each sample was suspended in 25 mL of citrate buffer (0.1 mol/L, pH 4.0), and eddied for 5 min (Frostegård & Bååth, 1996). The suspension liquid was centrifuged (12,000 ×*g*, 4 °C) for 10 min, and the precipitate was washed thrice by the same citrate buffer, and then the sediment was harvested for PLFA extraction.

2.2.2. Extraction of PLFAs

The phospholipid extraction was accomplished using the procedure described by Bligh and Frostegård (Bligh & Dyer, 1959; Frostegård & Bååth, 1996) with some modifications. Briefly, a single-phase mixture of chloroform:methanol:citrate buffer (19 mL at a 1:2:0.8, v/v/v) was added into the pretreated sample, and then shocked for 2 h in the dark, at the room temperature. Following this, it was split into two phases by adding chloroform (10 mL) and citrate buffer (5 mL), and stood overnight in darkness to separate. The $CHCl_3$ layer was then transferred to a new tube and dried under a stream of N_2 at 40 °C. The lipid extraction was fractionated on a silica column (200 mg/3 mL, Hardwee, Shanghai, China), which was first conditioned with $CHCl_3$ (3 mL). Next, neutral lipids and glycolipids, phospholipids were eluted with chloroform (3 mL), acetone (3 mL) and methanol (3 mL), respectively. Finally, the methanol fraction was collected and evaporated to dryness under nitrogen at 40 °C.

The PLFA fractions were trans-esterified by a mild alkaline methanolysis with a methanol:toluene (1 mL at a 1:1, v/v) solution and potassium hydroxide methanol solution (0.2 mol/L, 1 mL), and heated in the water bath at 37 °C for 30 min. After methylation, acetic acid solution (0.6 mL, 0.6 mol/L) was added. Fatty acid methyl esters (FAMEs) were extracted in hexane (2 mL) and dried under N_2 at 40 °C.

2.2.3. Detection of PLFA

FAMEs were analyzed with Trace GC Ultra gas chromatograph-DSQ II mass spectrometer (Thermo Electron Corporation, Waltham, USA) equipped with an HP-INNOWAX capillary column (30.0 m × 0.25 mm × 0.25 μm, Agilent Technology, USA) and a flame ionization detector (FID). Mass spectrum was generated in the electron impact

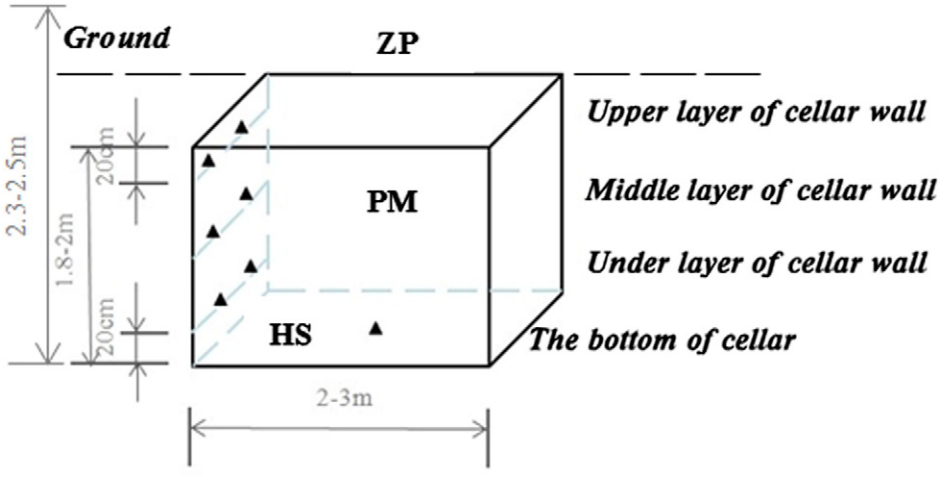

Fig. 1. Sampling sites of PM, ZP and HS. PM and ZP samples were collected from four different positions of cellar, and two subsamples were collected in each layer of cellar wall, and one subsample was collected from the center of the bottom of cellar; while HS sample was collected from the bottom of cellar.