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Sequencing-based screening of functional microorganism to decrease the formation of biogenic amines in Chinese rice wine

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ABSTRACT

As a traditional alcoholic beverage, Chinese rice wine with a unique flavor and high nutritional value has been popular in China for thousands of years. The biogenic amines in Chinese rice wine and other fermented food are attracting much more attention than ever. There are numerous microorganisms accompanying the fermentation of Chinese rice wine. Based on the next-generation sequencing technology, an *in vivo* screening process was carried out to find the strains without biogenic amines formation, and then these strains were screened and back-added into the fermentation mash. It was found that the *Lactobacillus* might not produce biogenic amines by the correlation analysis of bacteria community succession and biogenic amines change. After that, three *Lactobacillus* strains without biogenic amines formation were screened and identified as *Lactobacillus plantarum* and *L. hilgardii*. The *L. plantarum* JN01 showed high lactic acid production, low acetic acid production, and high ethanol tolerance which was preferable for Chinese rice wine brewing. When 0.001 gDCW/t of *L. plantarum* JN01 was used to inoculate the fermentation mash, the biogenic amines concentration was significantly decreased 24%. Although the formation of biogenic amines was further inhibited by increasing the dose of *L. plantarum* JN01, the total acid and the residue sugar would increase resulting an incomplete fermentation. In this paper, we firstly developed an effective method to decrease the formation of biogenic amines in traditional fermentation foods based on the next-generation sequencing technology. This research provided new strategy to decrease biogenic amines in fermentation food.

1. Introduction

As a traditional alcoholic beverage, Chinese rice wine (CRW) with a unique flavor and high nutritional value has been popular in China for thousands of years (Chen & Xu, 2010). The brewing process of CRW is a typical open semisolid-state fermentation process. In the brewing process, there is no sterilization procedure, the adding of wheat qu brings lots of microorganisms (Xu, Wang, Fan, Mu, & Chen, 2010), and the fermentation process is carried in an open-environment fermenters without autoclave process. As a result, there are numerous microorganisms accompanying the fermentation of CRW.

During the brewing, the fungi and *Bacillus* produce amylase saccharifing the starch, the yeasts produce ethanol from sugar, acetic acid bacteria and lactic acid bacteria produce organic acid and reduce pH. The microorganisms are critical for CRW making, while they might also bring negative effects. As the fermentation goes on, the succession of microorganism community forms the sugar, ethanol, organic acid, flavor and nutrition (Wang et al., 2014), while the undesirable metabolism in the community also forms potentially harmful products, especially biogenic amines (Yongmei et al., 2007). Biogenic amines are mainly produced through microbial decarboxylation of amino acids (Ten Brink, Damink, Joosten, & In't Veld, 1990). In the fermentation of CRW, the high concentration of amino acids and vigorous microbial metabolism

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inevitably produce a high biogenic amine concentration (Lehtonen, 1996; Yongmei et al., 2007).

The biogenic amines are not only found in CRW, but also widely exist in nearly all fermentation food like wine (Martuscelli, Arfelli, Manetta, & Suzzi, 2013; Pramateftaki, Metafa, Karapetrou, & Marmaras, 2012), dairy (Herrero-Fresno et al., 2012; Perin, Miranda, Todorov, Franco, & Nero, 2014), and fish (Herrero-Fresno et al., 2012). In recent years, more concerns about food safety together with the consumers' demand for safer and healthier products have promoted the studies for compounds with harmful effects on human health. The methods to decrease biogenic amine formation are attracting much more attention than ever (Alvarez & Moreno-Arribas, 2014; Pineda, Carrasco, Peña-Farfal, Henríquez-Aedo, & Aranda, 2012).

In most fermented foods it is difficult to prevent the accumulation of biogenic amines since the microbiological/chemical/physical conditions of the fermentation cannot be easily modified. An alternative in such cases is the use of food microorganisms that are able to degrade biogenic amines after they have been synthesized in the food matrix (Alvarez & Moreno-Arribas, 2014). However, in this paper we provided a feasible method to decrease the biogenic amine formation in the fermentation process of CRW by *in vivo* screening of *Lactobacillus*.

In the development of next-generation sequencing technology and bioinformatics (Qin et al., 2010; Shendure & Ji, 2008; Turnbaugh et al., 2008), microbial community study has reached a new level. It is faster and cheaper to obtain a complex community and easier for functional analysis. Besides, the metabolic characterization of the microorganisms always showed significant changes from their natural environment to standard laboratory conditions. Based on the development of next-generation sequencing technology and bioinformatics, we designed a reverse process: characterizing the functional microorganisms *in vivo* firstly, then designing feasible medium to screen the target strains, and at last confirming the microbial function in the original environment. The *in vivo* study of the metabolic characterization ensured the screened microorganism would performed its desirable function in the original environment.

2. Materials and methods

2.1. Sample collection and processing

Fermentation broths from a typical semidry-type rice wine production process in Shaoxing (Zhejiang, China) were sampled in the center of fermented mash. The suspension was filtered through four layers of sterile gauze to remove the unliquefied rice and sealed in a sterile plastic bottle. The suspensions were then snap-frozen in liquid nitrogen and transported into the ultra-low temperature chest freezers (Forma 8600, ThermoFisher scientific). All the samples were collected in April 2014, and three samples for each fermentation stage were collected from different tanks.

2.2. Genomic DNA extraction, PCR amplification and sequencing

Genomic DNA of the microorganisms in fermentation broth was extracted using OMEGA D5625 E.Z.N.A.® Soil DNA Kit) according to the manufacturer's protocol. The V4 hypervariable region of bacterial 16S rDNA was chosen for PCR amplification. The degenerate primers XXXXXXX- AYTGGGYDTAAAGNG and XXXXXXX-TACNVGGGTATCTAATCC were used for V4 amplification. The unique heptad-nucleotide sequences were used as barcodes to differentiate each sample. The concentration of DNA in the purified PCR products was further quantified with PicoGreen (Invitrogen, USA). The amplicons were pooled into a single tube in equimolar ratios. Sequencing of the PCR-amplifications was carried out by next-generation sequencing platforms Illumina MiSeq (Shendure & Ji, 2008) according to the manufacture's protocol.

2.3. Sequence processing and microbial community succession

Sequencing reads from different sample were separated by specific barcode and analyzed with mothur (version v.1.30.1) (Schloss et al., 2009). To reduce sequencing noise from raw data, a pre-clustering step (Huse, Welch, Morrison, & Sogin, 2010) was performed to minimize the effects of random sequencing errors and avoid overestimating the phylogenetic diversity (Kunin, Engelbrektson, Ochman, & Hugenholtz, 2010). The normalized reads were assigned to different clusters using Usearch5.2 (http://www.drive5.com/uclust/) with 97% identify and classified taxonomy by RDP database. The relative abundance of operational taxonomical units (OTUs) with 97% identity between pair-wise samples or between groups of samples was compared. The OTUs defined by a 3% distance level were phylogenetically classified using the “classify.otu” command in Mothur (Schloss et al., 2009) with the RDP database and a taxonomy file describing the complete taxonomic information of each sequence in the database from domain to genus.

2.4. Lactobacillus screening, culture and preparing

The fresh fermentation broths of 0 h, 144 h, 432 h, and 576 h were diluted and inoculated on MRS medium plates. The plates were cultured both in Whitley DG250 Anaerobic Workstation (Don Whitley Scientific, England) and constant temperature incubator. The clones on the plate were purified twice. About 20 strains of gram-positive, rod-shaped bacteria and without spore forms, were screened. To detect the generation of biogenic amines, the bacteria pre-cultured in biogenic amine production medium (Landete, Ferrer, & Pardo, 2007) and sub cultured for three times in this medium at 37 °C. Their abilities of producing biogenic amines were conformed using a biogenic amine detecting medium at 37 °C for 4 h (Bover-Cid & Holzapfel, 1999).

The 16s rDNA sequences of the bacteria without biogenic amine formation was amplified using the primer AGAGTTTGATCCTGGCTCAG nd CGGTTACCTTGTTACGACTT, and then ligated with pMD18-T vector (TaKaRa). The cloned genes were sequenced with a dye terminator cycle-sequencing Fs ready reaction kit and a model ABI 3130 automatic DNA sequencer (Applied Biosystems). A homology search was performed with NCBI. A phylogenetic tree was built through neighbor-joining methods by MEGA5.0 with 1000 rounds of bootstrapping.

The *Lactobacillus* in MRS was incubated at 37 °C for 24 h under anaerobic condition, then about 1% was used to inoculate a fresh MRS liquid medium and incubated at 37 °C for 15 h. The culture was centrifuged at 4000 × g for 10 min to pellet the cell. The *Lactobacillus* was washed twice by sterile phosphate buffered saline (pH 7.0, 20 mM).

The MRS medium composition (g/L): peptone 10.0, meat extract 8.0, yeast extract 4.0, glucose 20.0, K_2HPO_4 2.0, sodium acetate trihydrate 5.0, Tween 80 1, triammonium citrate 2.0, $MgSO_4$ 0.2, $MnSO_4$ 0.05, final pH 6.2 at 25 °C. When preparing solid medium, 20 g/L agar was added. Biogenic amine production medium (g/L): tryptone 5 g, meat extract 8 g, yeast extract 4 g, glucose 1.5 g, fructose 1 g, Tween 80 0.5 g, $MgSO_4$ 0.2 g, $FeSO_4$ 0.04 g, $MnSO_4$ 0.05 g, $CaCO_3$ 0.1 g, tyrosine 2 g, histidine 2 g, ornithine 2 g, lysine 2 g, tryptophan 2 g, pyridoxal phosphate 0.25 g and pH 5.5. Biogenic amine detecting medium (g/L): tryptone 5 g, meat extract 8 g, yeast extract 5 g, glucose 0.5 g, Tween 80 1 g, triammonium citrate 2.0, NaCl 2.5 g, $MgSO_4$ 0.2 g, $MnSO_4$ 0.05 g, $FeSO_4$ 0.04 g, K_2HPO_4 2 g,