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Biogenic amines content and assessment of bacterial and fungal diversity in stinky tofu – A traditional fermented soy curd

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ABSTRACT

Microbial profiles of eighteen soy curd (stinky tofu) samples as well as their brines, collected from six different regions, were assessed. In addition, biogenic amines were also quantified using HPLC to determine their levels in Chinese fermented soy curd. A total of 444 bacteria, comprising 80 species, and 116 fungal isolates, comprising 26 species, were identified using 16 S rDNA gene and internal transcribed spacer (ITS) rDNA gene sequencing, respectively. In all samples, the average number of bacteria in tofu and brine was 7.00 log cfu/g and 6.99 log cfu/mL, respectively, while the average value of fungal cells in tofu and brine were 2.58 log cfu/g and 3.55 log cfu/mL, respectively. The results revealed the predominance of *Streptococcus lutetiensis*, *Lactococcus lactis* and *Yarrowia lipolytica* in the samples tested. In case of biogenic amines, tyramine was detected in all samples while spermine could not be detected. β-phenethylamine was only detected in the tofu of Xi'an. The microbial composition and biogenic amines content are the main factors affecting the safety of stinky tofu products. This study indicates that a large microbial diversity is associated with the fermentation of stinky tofu which may have a key role in the production of biogenic amines.

1. Introduction

Stinky tofu is one of the traditional fermented soy curds in China which is particularly popular in oriental diets. It has a long and extensive history dating back to hundreds of years. It is also called as Chinese cheese and like some varieties of cheese it smells bad but tastes good. The manufacturing process of stinky tofu varies from region to region in China. The preparation of the stinky brine is considered as the first step in the manufacturing process of stinky tofu. Stinky brine is made by a natural fermentation of the brine containing a mixture of vegetables, meats or herbs to obtain a unique and strong stinky odor (Chao et al., 2008; Chao, Tomii, Watanabe, & Tsai, 2008; Liu, Wei et al, 2011). The maturation process of the brine may take several months. After maturation, tofu, in the form of cubes, is soaked in fermented stinky brine for few hours to several days to obtain the final product enriched with stinky odor.

The process of natural fermentation involved in the production of stinky brine make the process susceptible to microbial contamination from environmental sources and thus renders the process less or unhygienic. This also threatens the safety of this fermented food because of more contamination possibilities from environment harboring pathogenic bacteria. Therefore, it is hard to control the quality of stinky tofu when it is processed in an open environment (Lee, Wang, & Chang, 1996).

Previous studies aiming to explore the microbiota of stinky tofu have reported the dominance of lactic acid bacteria (LAB) and species belonging to the genus Bacillus (Chao et al., 2008; Chao, Tomii et al., 2008; Sun, Zhang, Wang, Wang, & Xie, 2010; Yu, Hu, & Li, 2012). Lactic acid bacteria of the tofu play an important role in determining the overall flavor profile of stinky tofu (Liang, Deng, & Lin, 2013; Liu, Han, & Zhou, 2011; Tripathi & Misra, 2005; Li, Tian et al., 2014; Li, Xu, Jiang, & Li, 2014). Apart from lactic acid bacteria, fungi also contribute positively towards improving its flavor (Tripathi & Misra, 2005; Li, Tian et al., 2014; Li, Xu et al., 2014). However, the microbial diversity of stinky tofu, especially fungi

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(molds and yeasts), has not been explored well.

According to the existing reports, stinky tofu does not pose a health risk to humans (Liu et al., 2010; Liu, Wei et al, 2011; Hu et al., 2013). However, an increase in total biogenic amines (BAs), as undesirable substances in stinky tofu, may be harmful (EFSA, 2011; Santos, 1996). Excessive intake of biogenic amines from foods can lead to various physiological and toxicological problems in humans such as nausea, sweating, migraine, respiratory distress, hot flushes, bright red rash, oral burning, heart palpitation, and hyper- or hypo-tension (Guan et al., 2013; Karovicova & Kohajdova, 2005). Biogenic amines may have more serious implication as its presence in foods may lead to death in certain cases. In addition, biogenic amines have also been reported to have correlation with the spoilage of food products (Karovicova & Kohajdova, 2005; Kim, Mah, & Hwang, 2009; Li, Tian et al., 2014; Li, Xu et al., 2014; Tian et al., 2013). However, currently there is no established standards or regulation for stinky tofu or fermented soybean products to limit biogenic amines levels.

Stinky tofu is rich in amino acids which can be transformed into biogenic amines by microbial metabolism via the activity of specific amino acid decarboxylases. In addition, biogenic amines can also be synthesized by spontaneous chemical reactions during extended fermentation (Beneduce et al., 2010; Liu, Wei et al, 2011). Biogenic amines accumulation in fermented foods is a complex process affected by multiple factors and their interactions (Schirone et al., 2013). Several food fermenting lactic acid bacteria are able to produce biogenic amines and genes of diverse BAs-producing pathways have been identified in lactic acid bacteria (Elsanhoty & Ramadan, 2016; Lonvaud-Funel, 2001; Schirone et al., 2013). Interestingly, the presence of genes seemed to be more strain-dependent than species-specific, suggesting that horizontal gene transfer may be accountable for their dissemination in lactic acid bacteria (Beneduce et al., 2010).

Given the significance of biogenic amines to human health and food safety, monitoring their contents in foodstuffs is very important. Presently, high-performance liquid chromatography (HPLC)-based methods are the most suitable for the analysis of fermented foods. The reliability and sensitivity of these methods render them useful as important techniques to determine the concentration of all biogenic amines in fermented food (EFSA, 2011).

In this research, lactic acid bacteria, bacteria other than lactic acid bacteria (here we refer as non-lactic acid bacteria) and fungi populations were isolated and identified from stinky tofu and stinky brine by 16 S and ITS rDNA gene sequencing, respectively. In addition, the changes in biogenic amines in stinky tofu and brine were investigated. To our knowledge, it is the first report regarding the microbial diversity of stinky tofu samples collected from the regions famous for stinky tofu production in China. At the end, a correlation between microbial diversity and biogenic amine levels has been discussed briefly.

2. Materials and methods

2.1. Collection of samples

Eighteen commercial stinky tofu samples and their corresponding stinky brine samples were randomly purchased from six cities (three from each city) in China (Table 1). All samples were aseptically collected in sterile disposable boxes from local night markets or roadside stands. The samples were transported to the laboratory immediately and stored at 4 °C.

2.2. Microbiological analyses

Samples (10 g tofu or 10 mL brine) were homogenized with 90 mL of 0.85% (w/v) sterile NaCl solution for 1 min using a shaker (Tensuc, Shanghai, China). Following homogenization, samples were serially diluted (10^{-1} to 10^{-6}) with the sterile NaCl solution and 200 μl of each dilution was plated onto Potato Dextrose Agar (PDA) supplemented with chloromycetin (0.1 g/L), Nutrient agar (NA) and de Man Rogosa and Sharp (MRS) agar plates supplemented with Tween-80 (1.0 g/L) and cycloheximide (0.1 g/L). PDA Plates were incubated at 28 °C for 72 h. NA Plates were incubated at 30 °C for 48–72 h (depending on the strains). MRS agar plates were incubated at 30 °C for 48 h. Colonies which looked different were chosen as many as possible for further analysis. Isolates were purified on corresponding PDA, NA and MRS agar plates for at least three times, and stored at −80 °C in corresponding liquid culture medium containing 30% (v/v) glycerin.

Table 1
Viable count (log cfu/g or log cfu/mL) of the main microbial groups in the stinky tofu and brine.

Sample code	product	log cfu/g or log cfu/mL sample			
		Bacteria		Fungi	
		Tofu	Brine	Tofu	Brine
Hz(n = 3)	Hangzhou	7.05	7.21	4.03	4.43
Sx(n = 3)	Shaoxing	6.98	6.99	3.52	4.84
Bj(n = 3)	Beijing	7.07	6.94	–	–
Xa(n = 3)	Xian	6.91	6.73	4.25	4.64
Cs(n = 3)	Changsha	7.04	7.12	–	3.60
Gz(n = 3)	Guangzhou	6.94	6.93	3.70	3.78
Average		7.00	6.99	2.58	3.55

2.3. Genotypic identification

The genomic DNA was extracted by a DNA Extraction Kit for bacteria (Axygen, Hangzhou, China) and fungi (Sangon Biotech, Shanghai, China) following the manufacture's protocol.

For identification of bacteria, the pure isolates were subjected to 16 S rDNA gene sequence analysis. The universal primer pairs, 27F and 1492R (Weisburg, Barns, Pelletier, & Lane, 1991) were used to amplify 16 S rDNA genes under the following PCR conditions: preliminary denaturation for 5 min at 94 °C, followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min, and a final step of elongationat 72 °C for 10 min.

The fungal isolates were subjected to ITS rDNA gene sequence analysis. The primers used were ITS1 and ITS4 (Xu et al., 2007). The PCR cycling conditions were as follows: 94 °C for 5 min; 35 cycles consisting of 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min; and 72 °C for 10 min.

The PCR products were sequenced by Sangon Biotech (Shanghai, China), and the obtained sequences were compared with GenBank database using the BLAST algorithm (National Center for Biotechnology Information, USA).

2.4. Biogenic amines determination

Determination of biogenic amines was carried out by acid extraction and derivatization with benzyl chloride using the methods described by Liu (Liu, Wei et al, 2011).

The HPLC systems used were as follows: separations module (LC-2010AHT; Shimadzu, Kyoto, Japan), UV detector (Shimadzu, Kyoto, Japan), LabSolutions LC Workstation System Software, Workstation version 5 (Shimadzu Corporation). The separation of the analytes was carried out using a ZORBAX SB-C18 column (5 μL, 4.6 × 250 mm; Agilent) and a column oven set at 30 °C. The injection volume was 10 μL. Chromatograms were analyzed at 254 nm. The two solvent reservoirs contained the following