International Journal of Food Microbiology 211 (2015) 23–31

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Changes in flavour and microbial diversity during natural fermentation of suan-cai, a traditional food made in Northeast China

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ARTICLE INFO

Article history:
Received 27 November 2014
Received in revised form 8 May 2015
Accepted 27 June 2015
Available online 2 July 2015

Keywords:
Natural fermentation
Suan-cai
Flavour
Microbial diversity

ABSTRACT

We measured changes in the main physical and chemical properties, flavour compounds and microbial diversity in suan-cai during natural fermentation. The results showed that the pH and concentration of soluble protein initially decreased but were then maintained at a stable level; the concentration of nitrite increased in the initial fermentation stage and after reaching a peak it decreased significantly to a low level by the end of fermentation. Suan-cai was rich in 17 free amino acids. All of the free amino acids increased in concentration to different degrees, except histidine. Total free amino acids reached their highest levels in the mid-fermentation stage. The 17 volatile flavour components identified at the start of fermentation increased to 57 by the mid-fermentation stage; esters and aldehydes were in the greatest diversity and abundance, contributing most to the aroma of suan-cai. Bacteria were more abundant and diverse than fungi in suan-cai; 14 bacterial species were identified from the genera *Leuconostoc*, *Bacillus*, *Pseudomonas* and *Lactobacillus*. The predominant fungal species identified were *Debaryomyces hansenii*, *Candida tropicalis* and *Penicillium expansum*.

1. Introduction

Suan-cai is a low-salt, fermented foodstuff produced through fermentation of vegetable products by lactic acid bacteria. Its unique flavour and texture make it very popular in Northeast China. The fermentation method influences the diversity of microbes (bacteria and fungi) present, and different groups of microorganisms are responsible for fermentation at different stages of production; this produces its unique flavour (Zhang et al., 2011; Kim and Chun, 2005).

Researchers studying the diversity of bacterial populations in suan-cai usually adopt traditional methods, such as separation, purification and identification. These methods are not only time-consuming, but can also be highly subjective because they are sometimes unreliable, inaccurate, and seriously affected by researcher experience and culture conditions; this makes them unlikely to reflect the true diversity of bacteria in suan-cai (Xiong et al., 2014; Lucilla et al., 2009). Using culture dependent methods, Zhang et al. (2009) isolated 97 isolates of *Lactobacillus* sp. and 2 exopolysaccharide (EPS)-producing isolates; *Lactobacillus casei* and *Lactobacillus plantarum* were the most abundant. *Lactobacillus brevis*, *L. plantarum* and *Leuconostoc mesenteroides* have also been isolated from naturally fermented suan-cai liquid and identified using classical methods (Liu et al., 2009). In recent years methods based on 16s rRNA in combination with the degeneration gradient gel electrophoresis method (DGGE), have proved to be effective techniques for quantifying microbial community structure and diversity (Hong et al., 2014; Water et al., 2000). In DGGE analysis PCR amplification from different bacteria that are in the same size but have different sequences can be separated (Hong et al., 2013). Using these techniques microbial diversity has been quantified, without the need for isolation, in soil (Pan et al., 2013), sea (Bowman et al., 2003; Yiğittürk and Uzel, 2015), sludge (Xia et al., 2005) and particularly in fermented food, such as kimchi (Lee et al., 2005) and sausages (Rantsiou et al., 2005; Cecilia et al., 2005). Diversity determined using these techniques was much greater than that identified using traditional methods (Sekiguchi et al., 2001); the greatest advantage of DGGE is that it can identify the presence of bacteria and fungi that cannot be isolated and cultured *in vitro*.

With changes in horticultural approaches, including increased pesticide use, there have been reports of nitrate accumulation in fresh vegetables (Eichholzer and Gutzwiller, 1998). Nitrate can be reduced into nitrite and when nitrate, nitrite or other nitrogen-based compounds are ingested they can form nitrosamines, which are carcinogenic in the stomach (Deb et al., 2007). Consequently people are becoming increasingly concerned about the content of nitrite in Chinese suan-cai and its safety.

Amino acids are the main contributors to the delicate flavour of fermented vegetables and, when present in different combinations,

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http://dx.doi.org/10.1016/j.ijfoodmicro.2015.06.028
0168-1605/

affect the colour and aroma of these products (Cha et al., 1998). Some amino acids can be synthesized by the body and do not need to be ingested in food. However, some cannot be synthesized, and must be acquired from food to meet the needs of the human body; these are termed essential amino acids (Galili and Amir, 2013). Amino acids are relatively abundant in fermented vegetable products, and provide people with essential amino acids necessary for health (Depree et al., 1998). Fresh vegetables contain a number of different kinds of aroma components. After fermentation some original flavour components may disappear, while other new flavour components develop; ultimately mature fermented vegetable products contain a complex blend of flavour compounds (Kim and Sohn, 2001; Wu and Wang, 2000).

Understanding the changes in flavour of suan-cai during natural fermentation, and their relationship with microbial diversity will provide a theoretical basis for understanding the traditional fermentation technology of Chinese suan-cai. In this study we determine the physicochemical properties (pH, soluble protein, nitrite, amino acid content, volatile profiles) of suan-cai fermented for different periods of time and relate this to the microbial diversity present. This could aid selection of the most appropriate inoculants and accelerate the safe industrialization of suan-cai production in Northeast China.

2. Materials and methods

2.1. Materials

Naturally fermented, homemade suan-cai, at different stages of fermentation was collected from local producers in Shenyang, Liaoning Province, China, and then transported to the Food Analysis Laboratory at Shenyang Agricultural University on the day of collection.

2.2. Determining the main physical and chemical properties of suan-cai at different stages during fermentation

2.2.1. Determining the pH values, soluble protein and nitrite at different stages during fermentation

The pH, soluble protein concentration and nitrite concentration were determined from samples (n = 3) of suan-cai that had been fermenting for 0, 10, 20, 30, 40, 50, 60, 70, 80 or 90 days (d). The pH of the suan-cai liquid was determined directly using a pH meter; three sequential readings were taken and the average value was determined for each sample. The suan-cai samples were pounded to form homogenates, weighed and the soluble protein concentration and nitrite concentration were determined. The Coomassie brilliant blue G250 method was used to determine the concentration of soluble protein per 100 g (Sedmak and Grossberg, 1977). Hydrochloride naphthodiamide was used to determine the concentration of nitrite per kg according to GB 5009.33-2010 (Du et al., 2013).

2.2.2. Determining the concentration of free amino acids at four stages of fermentation

Samples (30 g) of suan-cai were taken from collected material that had been fermented for 0 d (zero fermentation stage), 18 d (initial fermentation stage), 30 d (mid-fermentation stage) or 40 d (end-fermentation stage). Three samples were tested from each fermentation stage. The juice was extracted from each sample by squeezing and then filtered through sterile gauze and centrifuged; the supernatant was reserved. In a centrifuge tube 12 mL of 10% sulfosalicylic acid was added to 12 mL of suan-cai juice and, following oscillation blending, it was centrifuged at 120,000 *g* for 15 min. A 1 mL aliquot of the resulting supernatant was filtered through a 0.22 μm micropore filter and evaluated in a High Speed Amino Acid Analyzer (Model Hitachi L-8800). The chromatographic column was a Hitachi 2622 c type with cation exchange resin, 4.6 mm × 6.0 m; the injection volume was 20 μL; citric acid–sodium citrate buffer was introduced at a rate of 0.4 mL/min while the column temperature and pressure were maintained at 50 °C and 90 kgf/cm^3 respectively; ninhydrin solution was introduced at a rate of 0.35 mL/min while column temperature and pressure were maintained at 135 °C and 11 kgf/cm^3 respectively; an ultraviolet detector was used to detect proline at a wavelength of 440 nm and other amino acids at 570 nm. Three aliquots were evaluated from each of the three samples taken from each fermentation stage and a mean was determined. External standard methods were used to calculate the quantity of each free amino acid (Zhou and McFeeters, 1998).

2.2.3. Determining the volatile flavour components present in suan-cai at four stages of fermentation

Suan-cai juice was extracted from replicate 30 g samples (n = 3) from each of the same four fermentation stages, using the same methods as described in Section 2.3. To 10 mL of suan-cai juice that had been placed in headspace vials (The United States Supelco company), 3.2 g NaCl was added and the vial sealed. Vials were heated in a water bath at 40 °C and agitated every 5 min for 40 min. A solid phase microextraction (SPME) head was then inserted into the vial by direct penetration through the cap and the fibre head exposed to the volatiles within. The fibre head was not allowed to contact the suan-cai juice directly. After 40 min the fibre head was retracted. The SPME head was then inserted into the injection port of a gas chromatographic mass spectrometer (GC-MS) (The United States Thermo Fisher company), and the fibre desorbed for 6 min. This was repeated five times for each sample. The chromatographic column used was a HP-5 elastic quartz capillary column with a size of 25.0 m × 0.25 mm × 0.25 m; the injection port was operated in a splitless mode with helium as the carrier gas at a flow rate of 1 mL/min. The temperature programme was an initial temperature of 35 °C for 3 min followed by 6 °C/min increases until 160 °C was achieved, and then 10 °C/min increases until 250 °C was achieved. The injection port temperature was 230 °C. The mass spectrometer was operated in the electron ionization mode at 70 eV, a source temperature of 230 °C, quadrupole at 150 °C in the scan range from 20 u to 500 u. Data were recorded through a G1701BA chemical workstation system processing. Identification of unknown compounds was achieved by matching with data from the Wiley Spectral library and the NIST98 library. The quantity of each compound was determined using the area normalization method (Zhao et al., 2006).

2.3. Determining the microbial diversity in suan-cai at four stages of fermentation

Microbial diversity was determined using polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE). Suan-cai juice was extracted from samples that had been fermenting for 0, 6, 12, 22, 28, 34 or 40 d, as described in Section 2.3. To 50 mL of suan-cai juice from each fermentation stage, 5 mL of PBS buffer was added, and then vortexed to mix for 30 s. This was centrifuged at 350 *g* for 5 min and the supernatant decanted. The supernatant was then centrifuged at 12,000 *g* for a further 5 min. The supernatant from this second centrifugation was discarded and 800 μL TE buffer solution added to the precipitate, blended with an oscillator and placed in a centrifuge tube with 0.5 g of glass beads. Total DNA was rapidly extracted using the Fast Prep method in combination with CTAB. Briefly: The treatment sample was placed in the Fast Prep nucleic acid fast extraction apparatus to oscillate at 6.0 m/s for 30 s. To this 50 μL SDS was added and incubated at 37 °C for 1 h. Then 80 μL NaCl/100 μL CTAB was added and incubated at 65 °C for 20 min. Equal volumes of phenol–chloroform–isoamyl alcohol were added (25:24:1), mixed well, allowed to settle for 1 min and then centrifuged at 12,000 *g* for 10 min. Equal volumes of chloroform–isoamyl alcohol (24:1) were rapidly added to the resulting supernatant, mixed well, allowed to settle for 1 min and then centrifuged at 12,000 g for a further 10 min. Finally 500 μL of isopropyl alcohol and 50 μL 3 M NaAc were added to the upper phase and incubated at −20 °C to precipitate the DNA. After centrifugation at 12,000 g for 10 min and washing the precipitate twice in 70% ethanol, the resulting DNA was