Microbiota succession and metabolite changes during the traditional sourdough fermentation of Chinese steamed bread

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ABSTRACT
This study was conducted to evaluate the changes in microbial communities and metabolites during the Chinese traditional sourdough fermentation. A gradual decrease in pH values was observed throughout the fermentation process. A considerable increase in LAB and yeasts counts was noticed during the primary stage of fermentation with the corresponding increase in amino acids, glucose, lactic acid and acetic acid. During the secondary fermentation stage, LAB count started decreasing slowly, while yeast count still increased, and reached to the maximum value. However, glucose, amino acid, lactic acid and acetic acid contents decreased slightly. PCR-DGGE results identified nine bacteria, six yeasts and one mold species during the whole fermentation process. Saccharomyces cerevisiae and Lactobacillus sanfranciscensis were the predominant species. These results would help to understand the LAB and yeasts influence on the metabolic process during the fermentation of steamed bread by Chinese traditional sourdoughs.

1. Introduction

Chinese steamed bread (CSB) is a fermented product produced from wheat flour and cooked by steaming. It is viewed as a distinct feature of Chinese culture and its first appearance in China as a staple food dates back to the 3rd century AD (Su, Ding, Li, Su, & Zheng, 2005). Compared to the baked bread, it is considered as safer and healthier food due to having lower fat and sodium contents and the absence of toxic Maillard reaction products such as acrylamide (Hou & Popper, 2006; Zhu, 2014). This is the reason why it is gaining more popularity around the world, especially in Asian countries.

The basic ingredients of CSB are wheat flour, water and leavening agent (sourdough or commercial yeast). It is well established that the quality parameters of CSB are affected by the milling process of wheat flour as well as its main components like protein, starch, and lipids (Lin, Liu, Bi, & Li, 2012; Sun, Zhou, Zhi, & Li, 2007). Apart from wheat flour, the addition of sourdough plays a key role in improving the overall quality of CSB. The main characteristic feature of sourdough is the microbial community, mainly yeasts and lactic acid bacteria (LAB) and a unique symbiosis that exists between certain hetero- and homo-fermentative LAB and certain yeasts. The unique advantages of sourdough are mainly related to the metabolic activities of LAB such as lactic acid fermentation, acidification, proteolysis and synthesis of flavor compounds which positively influence nutritional, functional and sensory properties (Chavan and Chavan, 2011; Banu & Aprodu, 2012; Yazar & Şebnem, 2012). During the fermentation process, sourdough microbiota produces carbon dioxide, which makes the steamed bread rise and is responsible for the crumb structure of CSB. Therefore, studying the diversity of microbial communities in sourdough system has been the focus of many studies (Luangsakul, Keeratipibul, Jindamorakot, & Tanasupawat, 2011; Zhang et al., 2011).
Microbial diversity in different sourdough samples, belonging to different areas of China, has been investigated in our previous studies (Zhang & He, 2013; Zhang et al., 2015). There are great advancements in understanding the microbial communities in sourdough for making CSB. Until now, most of the studies have focused on the wheat flour and different processing parameters involved in making CSB. To the best of our knowledge, however, there is no such study conducted to elucidate the microbial community dynamics and metabolites changes during various stages of CSB making process.

The aim of this study is to investigate the changes in the microbial communities and to evaluate the production of metabolites during the CSB making. The results of this research would help to understand the microbial ecosystem and fermentation mechanism of sourdough in making CSB.

2. Materials and methods
2.1. Wheat flour and sourdough samples
The flour used in this study to make CSB was purchased from a local market. It had 12.57% moisture, 7.56% protein, 18.50% wet gluten and 0.05% ash. The five Chinese traditional sourdough samples were aseptically collected from private households and steamed bread shops in different provinces of China. They were named as, Hn (from Henan province), Sx (from Shanxi province), Gs (from Gansu province), Hf (from Anhui province) and Hr (from Heilongjiang province). All samples were kept at 4 °C during the transportation and before use.

2.2. Fermentation of steamed bread by sourdough
The preparation of sourdough steamed bread was carried out by secondary fermentation, as described previously by Zhang et al. (2016). First, 140 g of wheat flour, 20 g of each conventional sourdough dough samples, and 70 mL of distilled/deionized (dd) water were mixed for 5 min and then fermented in a controlled fermentation tank (LXX24, Shandong, China) at 28 ± 2 °C and 75% relative humidity. Second, 60 g wheat flour and 20 mL dd water were remixed with the full fermented dough for 15 min. Then, the dough was rolled into round shape manually and the fermentation continued for 40 min. Third, the proofed dough samples were steamed for 30 min in a steamer (Joyong, Hangzhou, China). The control sample was prepared in the same way as described for sourdough samples but without any leavening agent.

2.3. Determination of pH and total titratable acidity (TTA) measurement
The pH and TTA (total titratable acidity) values of each sample were determined by standard method (American Association of Cereal Chemists [AACC], 2000) using a pH meter PB-10 (Sartorius Scientific Instruments Co., Ltd. Beijing, China) (Zhang et al., 2015). All experiments were performed in triplicate and replicates on three different occasions every 3 h.

2.4. Microbial population dynamics by culture-dependent method
For the mold, yeast and LAB culture-dependent methods were consistent with previous research (Zhang et al., 2015). Ten grams of sourdough were homogenized with 90 mL sterile sodium chloride solution (the mass fraction is 0.85%) to a homogenous suspension, and then a tenfold serial dilution in the same solution was carried out.

For mold, 0.1 mL of a suitable serial dilution (10⁻¹ to 10⁻³) was added to a Petri dish containing 20 mL of Potato Dextrose Agar (PDA, Difco, Detroit), which contained 150 mg/L chloramphenicol, and incubated at 28°C for 48 h.

For yeasts, 0.1 mL of a suitable serial dilution (10⁻¹ to 10⁻⁵) was added to a Petri dish containing 20 mL of Yeast extract-Peptone-Dextrose agar (YPD, Difco, Detroit, Mich., USA) followed by incubation at 28°C for 48 h.

For LAB, 0.1 mL of a suitable serial dilution (10⁻¹ to 10⁻⁹) was added to a Petri dish containing 20 mL of de Man-Rogosa-Sharpe (MRS) agar (Difco, Detroit) and sourdough bacteria (SDB) agar (Kline & Sugihara, 1971), and incubated anaerobically at 30 °C for 72 h.

The dishes with colonies between 30 and 300 were counted and changes of microorganisms during sourdough fermentation were recorded.

2.5. Microbial population dynamics by PCR-DGGE
PCR-DGGE methods were performed according to the previously described protocol (Zhang et al., 2015). For LAB, 16S rRNA gene was amplified with the primers 341f (5’-CCTACGGGAGGCAGCAG-3’) and 534r (5’-ATTACGGGCAGCTGCTG-3’). The variation in the banding profiles was achieved by the conjunction of GC clamp (5’-CGCCCGCGGCCCGGCCGCGCGCGCG-3’) with the forward primer. A touchdown PCR program was used to obtain a broad-range bacterial amplicons in sourdough samples for further DGGE analysis, which was carried out on the D-CodeTM Universal Mutation Detection System (Bio-Rad, Hercules, Calif., USA). PCR was performed in a total volume of 25 μL, including 200 μM dNTP, 0.2 μM each primer, 0.625 U of Ex Taq polymerase (Takara, Shanghai, China) and 2 μL of genomic DNA (20 ng/μL). Template DNA was denatured for 4 min at 94 °C followed by 20 cycles touchdown of denaturation at 94 °C for 1 min, annealing at 72 °C for 1 min. The initial annealing temperature was 65 °C and was decreased by 0.5 °C per cycle. Then, at 55 °C, five additional cycles were performed. The final extension was carried out at 72 °C for 10 min. PCR products were loaded onto a mass fraction of 8% polyacrylamide gel with a 30% to 60% urea-formamide gradient. Electrophoresis was performed at 50 V for 12 h at 60 °C.

For yeasts and molds, the 5.8S and 28S-1 rRNA genes of the ITS region were amplified using primers 5.8Sf (5’-GGGCGCACCGACCCGGCCGCGCTHGG-3’) and 534r (5’-ATATTGCTTAAAGTCAGCGG-3’). The same GC clamp was used to ligate to the second primer to obtain 285-1r-GC. A touchdown PCR program was similar to the above, but the annealing temperature was changed from 60 °C to 50 °C with an extension time of 2 min each cycle. PCR products were loaded onto a mass fraction of 6% polyacrylamide gel with a 35% to 65% urea-formamide gradient. Electrophoresis was performed at 50 V for 14 h at 60 °C.

After 1 h of staining, the DNA bands were observed with SYBR Green I (5-S5767, Invitrogen, Carlsbad, CA, USA) and photographed under UV (Gel Doc XR, Bio-Rad). Selected DGGE bands were excised from polyacrylamide gels using a sterile scalpel blade. The fragments were transferred in 25 μL sterile water and allowed the DNA to diffuse into the water at 4 °C overnight. Two microliters of the eluted DNA
was used for re-amplification and was examined by DGGE together with appropriate sourdough DNA as a control. The PCR products were cloned into pGEM-T Easy vector (Promega, Milan, Italy) and sequenced by a commercial sequencing facility (Shanghai Sangni Biosciences Corporation, Shanghai, China). The sequence of the measured gene was uploaded and then compared to the sequence in the NCBI database (Standard Nucleotide BLAST).

2.6. Metabolite analysis

Concentration of glucose in fermented dough samples was determined by Glucose Assay Kit (Sigma-Aldrich, USA). Concentrations of lactic acid and acetic acid were determined by high-performance liquid chromatography (HPLC) analysis with a Waters chromatograph (Waters Corp., Milford, MA), equipped with a 2414 differential refractometer. An ICsep ICE ORH-801 column (Interchim, Montlucon, France) was used with 5 mmol/L H$_2$SO$_4$ as mobile phase at a flow rate of 0.4 mL/min. The column temperature was kept at 30 °C. Sample preparation was performed as described above.

The composition of amino acids during sourdough fermentation was determined using amino acid analyzer (L-8900 Hitachi-hitech, Japan). Samples containing 0.10 g of dough were acid hydrolyzed with 4.0 mL of 6 mol/L HCl in vacuum-sealed hydrolysis vials at 110 °C for 22 h. The ninhydrin was added to the HCl as an internal standard. Hydrolysates were suitable for analysis of all amino acids. The tubes were cooled after hydrolysis, opened and placed in a desiccator under vacuum until dry. The residue was dissolved in a suitable volume of a sample dilution with 0.02 mol/L HCl, filtered through a millipore membrane (0.45 μm pore size) and analyzed for amino acids.

3. Results and discussion

3.1. Changes in pH and TTA values

A gradual decrease in pH values was observed during the whole process of sourdough fermentation, and TTA values slightly increased accordingly (Figure 1). The initial pH and TTA values of all sourdough samples were in the range of 4.15(Gs)-5.28(Hf) and 7.61(Hf)-13.66(Sx), respectively. However, the dough gradually became more acidic as the pH decreased during the fermentation process. The final pH values were in the range of 3.66 (Gs) to 4.7 (Hf) while the TTA values were in the range of 10.9 (Gs) to 14.35 (Hf). The yeast in sourdough can convert the sugar in the flour into CO$_2$ and alcohol, aldehydes. LAB use the fermentable sugar in the dough to produce organic acids such as lactic acid, acetic acid and propionic acid (Paramithiotis, Gioulatos, Tsakalidou, & Kalantzopoulos, 2006). The production of these substances causes a decrease in pH and an increase in TTA. It was reported that the low pH and high TTA values achieved during the LAB fermentation enhances the capacity of dough to retain the gas produced by yeast or LAB. Our results of changes in pH and TTA are in corroboration with the studies of (Gobbetti, 1998). Lower pH has many effects along with retaining gas such as phytic acid (antinutritional factor) is also degraded by low pH and many autonomous enzymes are also activated by low pH which confer beneficial effects on sourdough bread. For example, cereal proteinases liberate amino acids that are converted to flavor volatiles by the metabolic activities of sourdough yeasts and lactobacilli, or by thermal reactions during baking (Brandt, Hammes, & Ganzle, 2004). Furthermore, the cereal enzymes in the sourdough can degrade the protein and promote the formation of exopolysaccharides by the lactobacilli to affect the dough rheology and bread texture (Thiele, Ganzle, & Vogel, 2003).

3.2. Changes of microbial communities by culture-dependent method

Changes of LAB and yeast communities are shown in Figure 2-A. At the first fermentation stage (after 6 h), colony counts rapidly increased for both LAB and yeast populations. For Hn sample, LAB counts increased from 7.82 log/(cfu/g) to 8.91 log/(cfu/g) and yeast count increased from 6.66 log/(cfu/g) to 8.76 log/(cfu/g). However, LAB counts decreased slowly at the secondary fermentation stage (after 12 h), while yeast counts increased slowly and reached to a maximum number. Generally, growth rate and microbial yield are regulated by myriad of ecological factors. In case of sourdoughs, these factors are usually temperature, pH, oxidation-reduction potential, ionic strength, dough yield, and products formed because of microbial action such as lactate, acetate, CO$_2$ and ethanol (Freeman & Shelton, 1991). As the composition of compounds present in the dough changes, the composition of microbial communities also undergoes changes. Such as depletion of some compounds in dough depletes the specific microorganism.

Figure 1. Changes of pH and TTA values during the sourdough fermentation (1: 3 h; 2: 6 h; 3: 9 h; 4: 12 h).

Figura 1. Cambios en los valores de pH y TTA durante la fermentación de la masa madre (1: 3 horas; 2: 6 horas; 3: 9 horas; 4: 12 horas).
which wholly depends on that due to their specific metabolic activity.

3.3. Changes of microbial communities by PCR-DGGE method

The dynamics of bacterial and fungal communities during the whole sourdough fermentation of steamed bread was investigated periodically by the DGGE band patterns. A total of 16 bacterial and 13 fungal DGGE bands were isolated and sequenced, respectively (Figure 3). The results of DGGE revealed nine bacteria, six yeasts and one mold species during the whole fermentation process (Table 1).

For bacterial community, bands 1, 5, 6, 7 and 12 representing Lactococcus garvieae, Enterococcus faecium, Lactobacillus delbrueckii, Enterococcus cecorum, and Lactobacillus sanfranciscensis, respectively, emerged at the initial stage and throughout the entire period of sourdough fermentation and were deemed as the main bacteria during the steamed bread process. Band 2 (Lactobacillus plantarum) was detected in Gs and Hn samples at an initial stage, and disappeared in Gs but its intensity increased in Hn sample at primary stage, however, L. plantarum decreased in intensity at secondary fermentation stage in Hn sample. Bands 3, 4 and 8 (Lactobacillus alimentarius and Lactobacillus sp.) were only found in Hn sample, and disappeared along with the fermentation process, indicating either the substrates were not suitable as energy sources for these bacteria to thrive or their growth was inhibited by the changes induced by the sourdough fermentation process, such as decrease in pH.

Figure 2. Changes of microorganisms (A) and metabolites (B, C, D) (0: initial fermentation stage; 1: first fermentation stage; 2: secondary fermentation stage) during sourdough fermentation.

Note: Fig.2-A: compared with initial fermentation stage, **: p<0.01.

Figura 2. Cambios en los microorganismos (A) y metabolitos (B, C, D) (0: etapa inicial de fermentación; 1: primera etapa de fermentación; 2: etapa de fermentación secundaria) durante la fermentación de la masa madre.

Nota: Figure 2-A: comparado con la etapa de fermentación inicial, **: p<0.01.
value. Band 11 (Lactobacillus crustorum) existed in Hn sample and an obvious increase in its intensity was observed during the sourdough fermentation. Band 13 (L. sanfranciscensis) were found in Sx sample, Band 14 (L. sanfranciscensis) existed in Hr sample, its increased at primary stage and disappeared at secondary fermentation stage.

Table 1 shows that there are some differences in the genotypes of L. sanfranciscensis isolated from different areas. It can be seen that during the whole sourdough fermentation, lactobacillus species (L. sanfranciscensis, L. Delbrueckii and L. plantarum) remained prominent. There are various factors which are responsible for the overwhelming dominance of lactobacillus during the sourdough fermentation. Firstly, sourdough is an ideal substrate for carbohydrate metabolism of lactobacillus as it possesses high quantity of fermentable carbohydrates. Secondly, the growth requirements of L. sanfranciscensis in terms of temperature and pH match the conditions attained during sourdough fermentation (Gänzle, Ehrmann, & Hammes, 1998). Thirdly, lactobacilli present in sourdough are known to possess several stress response mechanisms that help them to thrive extreme conditions of acidity, temperature, dehydration and starvation (De Angelis, Bini, Pallini, Cocconcelli, & Gobbetti, 2001). These factors contribute to competitiveness and adaptability of these strains to this peculiar environment. Also, the production of antimicrobial compounds including both organic as well as proteinaceous substances further enhance their competitiveness and promote their stable persistence during sourdough fermentation (Hammes & Gänzle, 1998).
For fungal communities in sourdoughs, only band 5 (S. cerevisiae) emerged in all samples throughout the whole fermentation process, and other bands showed varied existence in samples at different fermentation stages (Figure 3). Band 1 (Cyberlindnera jadinii) in Hn sample increased gradually in intensity along with the fermentation. Band 2 (Wickerhamomyces anomalus) in Hr and Hn sample increased at the major phase of fermentation, however, appeared as the major band of secondary fermentation, but it disappeared at the last stage in Hr sample. On contrary, in Hn sample increased gradually during the whole fermentation process. Band 6 (Rhizopus oryzae) decreased in intensity throughout the fermentation process and ultimately disappeared at the last phase of fermentation.

It is well known that, S.cerevisiae is the most prevalent strain of sourdough and a major leavening agent of bread (Luangsakul et al., 2011; Vrancken et al., 2010; Zhang et al., 2011). It produces CO₂ for the dough leavening and also gives the unique flavor and distinguished properties to CSB. Rhizopus oryzae, existed in sourdough at first fermentation stage, however, disappeared after 12 h. It can produce various enzymes particularly glucoamylase which improves the degradation of starch in wheat flour (Chi, Liu, Wang, Ju, & Zhang, 2009).

3.4. Changes of metabolites

Glucose is a primary source of energy for the growth of yeasts and LAB in sourdough. Glucose concentration was found varied in fermented and unfermented doughs (Figure 2-B). The results show that glucose concentration in control sample increased gradually from 0.43 mg/g to 1.42 mg/g. However, the glucose content in sourdough fermented dough increased rapidly at the first fermentation stage and decreased notably at the secondary phase due to utilization of glucose by the sourdough microbiota. There may be two reasons for the increased glucose concentration in dough. It may either be originated from the hydrolysis of starch or sucrose due to the action of indigenous amylases (α-amylase) on damaged starch granules (Martinez-Anaya, 1996). On the other hand, it can be hydrolyzed due to yeast invertase activity. In addition, amylolytic activity of LAB can also increase glucose content through partial hydrolysis of starch granules (Calderon, Loiseau, & Guyot, 2002). It was reported that L. sanfranciscensis can hydrolyze maltose to release glucose. So due to the microbial activities, the concentration of glucose in fermented dough increased quickly as compared to the control sample. At the secondary stage, the microbial communities reached a peak, and the glucose content decreased due to increased consumption of glucose by microbial communities (Martinez-Anaya, 1996).

Lactic acid and acetic acid are the major metabolites produced during the sourdough fermentation. In sourdough ecosystem, homofermentative LAB mainly produce lactic acid while heterofermentative LAB (L. sanfranciscensis) produce acetic acid, besides lactic acid, CO₂, acetic acid and/or ethanol, through 6-phosphogluconate/phosphoketolase (6-PG/PK) pathway (Corsetti & Settanni, 2007). The changes in lactic acid and acetic acid contents in different fermented doughs are shown in Figure 2-C,D. It can be observed that lactic acid and acetic acid were absent at the initial fermentation stage in all samples. Along with the sourdough fermentation process, lactic acid and acetic acid concentrations increased up to 0.91 mg/g and 0.35 mg/g, respectively, in Hn sample. At the secondary fermentation stage, contents of lactic acid and acetic acid reached at maximum value. The highest contents of lactic acid (1.19 mg/g) and acetic acid (0.57 mg/g) were detected in Hn and Sx samples, respectively.

Fermentation quotient (FQ), molar ratio between lactic acid and acetic acid, is an important factor that plays a key role in
deciding the aroma profile of sourdough (Table 2). Also, these two acids contribute their role in determining the overall structure of final product by affecting the structure of gluten, making the gluten either more elastic (lactic acid) or acetic acid.

The changes in the concentrations of amino acids, including total amino acids, essential amino acids, aromatic amino acids, branched-chain amino acids, sulphuric amino acids and glutamic acid in control and five sourdough fermented doughs are shown in Figure 4. For the control sample (100 g), total amino acids contents were 4.16 g that increased up to 4.23 g, and decreased to 4.05 g. However, the contents of amino acids in sourdough fermented samples increased more as compared to the control sample. For instance, in Sx sample, total amino acids increased from 4.46 g/100 g to 5.12 g/100 g sample after 6 h (first fermentation stage) and then decreased to 4.45 after 12 h (second fermentation phase) (Figure 4). The levels of amino acids may be affected by the microorganisms in sourdough. They are released from proteins by the enzymes produced by the proteolytic strains in sourdough ecosystems through the Ehrlich pathway (Gänzle, Loponen, & Gobbetti, 2008). The activation of cereal proteases also results in the degradation of proteins thus producing amino acids (Loponen & Mikola, 2008).

Table 2. Fermentative quotient (FQ; molar ratio between lactic to acetic acid) values during sourdough fermentation (0: initial fermentation stage; 1: first fermentation stage; 2: secondary fermentation stage).

| Samples | Fermentation stage |
|---------|-------------------|
|         | Before 6 h (1st stage) | After 12 h (2nd stage) |
| Hn      | 1.5               | 2.0               | 2.2               |
| Sx      | 1.4               | 1.6               | 1.7               |
| Gs      | 1.4               | 1.5               | 1.9               |
| Hf      | 1.7               | 1.9               | 2.5               |
| Hr      | 1.5               | 1.6               | 1.9               |

Figure 4. Changes of amino acids of different sourdoughs fermented dough during sourdough fermentation (0: initial fermentation stage; 1: first fermentation stage; 2: secondary fermentation stage).

Note: compared with control, *: p<0.05; **: p<0.01. Cys: Cysteine; Met: Methionine; Leu: Leucine; Ile: Isoleucine; Val: Valine; Phe: Phenylalanine; Tyr: tyrosine.

Figura 4. Cambios en los aminoácidos de diferentes masas madre, con masa fermentada durante la fermentación de masa madre (0: etapa de fermentación inicial; 1: primera etapa de fermentación; 2: etapa de fermentación secundaria).

Nota: comparado con el control, *: p<0.05; **: p<0.01. Cys: cisteína; Met: metionina; Leu: leucina; Ile: isoleucina; Val: valina; Phe: fenilalanina; Tyr: tirosina.
2004). These amino acids are further used by yeast or transformed into aromatic volatiles during fermentation. LAB in sourdough utilizes amino acids as an energy reservoir to synthesize proteins or regenerate co-substrates (Årđö, 2006). So, the contents of amino acids can be slightly decreased during fermentation.

Protein degradation that occurs during sourdough fermentation is of crucial importance for the overall quality of CSB. The volatile flavor compounds produced because of microbial catabolism of amino acids (Årđö, 2006; Procopio, Krause, Hofmann, & Becker, 2013). For example, Branched-chain amino acids (Leu, Ile, Val) can change into specific aldehydes with fatty acids, alcohols with fruity flavors and acids with sweet, sour, rancid, rotten, fruity and butyric flavors. Aromatic amino acids (Phe, Tyr, Trp) are catabolized into compounds that contribute to a variety of flavors like rose, flowers and bitter almond. Sulphur compounds (Met, Cys), catabolized by the LAB, may follow the same pattern as branched-chain and aromatic amino acids, but methanethiol may also be released directly from the Methionine. Aminotransferases of LAB, especially Lactobacillus strains, produce glutamic acid. Glu contents, contribute to umami flavor, a savoury taste. It may decrease considerably by the activity of glutamate decarboxylase (Årđö, 2006). Some of these flavors are commonly considered as off-flavors, and strains in sourdough can be selected to avoid the production of these amino acids.

Disclosure statement
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