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Prevalence and diversity of lactic acid bacteria in Chinese traditional sourdough revealed by culture dependent and pyrosequencing approaches

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ABSTRACT

The aim of this study was to investigate the microbiota of lactic acid bacteria (LAB) in fifteen different samples of Chinese traditional sourdough collected from different regions through culture dependent and pyrosequencing approaches. The median value of cell density of lactic acid bacteria was found to be 9.60 log cfu/g and the median values of pH and total titratable acidity were 3.69 and 11.77 ml, respectively. A total of 246 presumptive LAB strains were isolated and subjected to a clustering analysis based on $(GTG)_5$-PCR fingerprinting profiles. Eleven species were found with an overwhelming predominance of *Lactobacillus sanfranciscensis* by culture dependent method. On the other hand, pyrosequencing revealed that more than 90% of the bacteria belong to LAB in thirteen sourdough samples out of the fifteen. A total of twenty-four species were found by pyrosequencing and seventeen of them belonged to lactobacilli. Pyrosequencing indicated that *Lactobacillus sanfranciscensis* was the only species found existing among all the fifteen sourdoughs and that other species most frequently encountered in the samples included *Weissella confusa*, *Lactobacillus plantarum* and an unclassified *Lactobacillus* species. We conclude that LAB dominate in Chinese traditional sourdough and *Lactobacillus sanfranciscensis* is the predominant species in this specific niche.

1. Introduction

Sourdough has been playing a significant role in making flour-based fermented food for a very long time (Hammes & Gänzle, 1997). It is widely used as a starter for bread making throughout Europe and likewise, it is also employed as an inoculum in making Chinese steamed bread, a staple food in China for more than 2000 years. Sourdough fermentation not only improves dough properties, in terms of texture, flavor and nutrition, but also retards the staling process and prolongs the shelf life of the resulting products by inhibiting microbial growth. However, like other traditional fermented food matrices, constant quality between batches is hard to be achieved because of the complexity of microbial consortia, which is liable to be influenced by endogenous or exogenous factors (De Vuyst et al., 2014). Extensive research efforts have been exerted towards the elucidation of the microbiota involved in sourdough fermentation originated from different countries (Bessmeltseva, Viiard, Simm, Paalme, & Sarand, 2014; Elizaquivel et al., 2015; Lattanzi et al., 2013; Weckx et al., 2010) and there is no doubt that lactic acid bacteria (LAB) are the predominant bacteria in this specific niche. More than seventy species of LAB have been isolated from sourdough ecosystem (Gobbetti & Gänzle, 2012). The most frequently encountered LAB mainly belong to heterofermentative lactobacilli, such as *Lactobacillus sanfranciscensis*, *Lactobacillus plantarum* and *Lactobacillus fermentum*. However, *Lactobacillus sanfranciscensis* is regarded as the key sourdough LAB species (Gobbetti & Corsetti, 1997).

Different approaches aiming to determine the microbiota of sourdough have their own pros and cons and there is no universally

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accepted strategy to investigate the biodiversity of a complex fermented food matrix like sourdough. Culture dependent method can successfully provide the taxonomic and metabolic analysis of any material to a certain depth and has been widely used to analyze microbial communities. This classical method, however, remains unsuccessful in providing the broad coverage required to study complex microbial niches. On the contrary, pyrosequencing, a culture independent high throughput technique, has great advantages over traditional culture dependent approaches and offers an attractive avenue to explore sprawling microbial communities dwelling in natural habitats. It involves high throughput sequencing and can efficiently unravel unparalleled taxonomic diversities of microbial communities associated with fermented foods (Elizaquivel et al., 2015; Lattanzi et al., 2013).

To date, a large number of studies have been conducted to explore the microflora of sourdough originated from different countries, but the microbial composition of Chinese sourdoughs has been scarcely investigated. All kinds of sourdoughs are widely used and distributed in China, but only sourdough of certain area and in a small number has been investigated and the actual profile of microbial composition of Chinese traditional sourdough remains unclear. The aim of this study is to investigate the detailed information concerning prevalence and diversity of LAB in Chinese traditional sourdough collected from different regions of China by high-throughput sequencing technology combined with culture dependent approaches, through which we could get a deeper insight into this microecosystem and lay a foundation for further standard manufacturing of Chinese steamed bread.

2. Materials and methods

2.1. Sample collection

Fifteen sourdough samples were collected from nine different provinces (Table 1) in northern China. Samples were chosen for their specific origin. Most of the provinces belong to the Yellow River basin, the typical wheat-growing area in China. Once in the laboratory, all the samples were immediately refrigerated until analysis.

2.2. Acidity and microbial loads of the samples

To measure pH and total titratable acidity (TTA) of the samples, 5 g of each sample was added into 50 ml of distilled water and homogenized for 10 min with a magnetic stirrer (IKA basic 2 RH, Germany) until the sample was thoroughly suspended. The pH of the samples was measured using a pH meter (PB-10, Sartorius, Germany) and acidity was determined by titration with 0.1 mol/L NaOH to final pH 8.5. TTA value was defined as the amount (milliliters) of a 0.1 mol/L NaOH solution required for 10 g of sourdough to reach a pH value of 8.5. LAB was counted as previously reported (Di Cagno et al., 2014) with some modification, 5 g of each sourdough was suspended in 45 ml sterile physiological saline (0.85%, wt/vol) and decimally diluted, and then 100 μl of the 10^{-5} to 10^{-7} dilutions was plated onto sourdough bacteria (SDB) agar (Kline & Sugihara, 1971) containing 0.1 g of cycloheximide (Sigma–Aldrich St. Louis, MO, USA) per liter and incubated anaerobically at 30 °C for 48 h in an anaerobic incubator (YQX-II, Shanghai CIMO medical Instrument Co. Ltd., China).

2.3. Isolation of lactic acid bacteria

To isolate LAB, decimal dilutions were plated onto both SDB agar and de Man Rogosa and Sharp (MRS) agar (Hangzhou microbial reagent Co. Ltd, China) both supplemented with cycloheximide (0.1 g/L). For each sample, fifteen to twenty isolates were picked from the highest three dilutions based on colony morphology and purified by successive streak plate method. After Gram staining and catalase test, the Gram-positive and catalase-negative isolates were presumptively considered as LAB and stored at 4 °C for further analysis.

2.4. Grouping of LAB isolates by rep-PCR genomic fingerprinting

The rep-PCR was performed to cluster the initial collection of LAB isolates into some genotypically related groups for subsequent identification. The genomic DNA was extracted using a DNA Extraction Kit (Axygen, Hangzhou, China) following the manufacture's protocol. For rep-PCR, the oligonucleotide primer $(GTG)_5$ (5′-GTGGTGGTGGTGGTG-3′) was employed under the previously described conditions (Versalovic, Schneider, De Bruijn, & Lupski, 1994) with a minor modification: preliminary denaturation for 5 min at 95 °C, followed by 30 cycles of 95 °C for 60 s, 40 °C for 60 s and 65 °C for 8 min, and terminated with an elongation step at 65 °C for 16 min. PCR amplification was conducted with a PCR thermocycler instrument (Bio-Rad Inc., Hercules, CA, USA). The PCR products were electrophoresed on 1.5% agarose gel (15 by 20 cm) containing the DNA dye GelRed Nucleic Acid Gel Stain (Biotium Hayward, Ca, USA) for 15.5 h at a constant voltage of 55 V in 1 × TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 4 °C. The rep-PCR

Table 1
Determination of acidity and enumeration of LAB and yeast of the sourdough samples.

Sample number	Sources	pH	TTA (ml/10 g)	LAB (log cfu/g)	Shannon index
1	Gansu province	3.65 ± 0.00^{f}	11.70 ± 0.00^{fg}	9.78 ± 0.04^{ab}	1.115842
2	Gansu province	3.64 ± 0.01^{f}	19.33 ± 0.81^{a}	9.2 ± 0.07^{f}	0.867269
3	Shandong province	3.54 ± 0.02^{h}	13.05 ± 0.21^{d}	9.68 ± 0.03^{bcd}	0.236602
4	Shandong province	3.55 ± 0.03^{h}	11.75 ± 0.07^{fg}	9.48 ± 0.04^{e}	0.689891
5	Shandong province	3.73 ± 0.01^{c}	11.77 ± 0.21^{fg}	9.83 ± 0.04^{a}	1.099837
6	Shandong province	3.59 ± 0.01^{g}	11.37 ± 0.12^{g}	9.69 ± 0.03^{bc}	0.379234
7	Shanxi province	4.07 ± 0.01^{a}	12.67 ± 0.12^{e}	8.75 ± 0.04^{h}	2.059253
8	Shanxi province	3.66 ± 0.01^{f}	13.53 ± 0.13^{c}	9.39 ± 0.03^{e}	0.188632
9	Inner Mongolia	3.84 ± 0.02^{b}	9.73 ± 0.06^{j}	9.60 ± 0.04^{d}	1.060975
10	Inner Mongolia	3.69 ± 0.01^{e}	10.00 ± 0.10^{ij}	9.09 ± 0.09^{g}	1.675394
11	Heilongjiang province	3.72 ± 0.00^{cd}	10.33 ± 0.06^{i}	9.44 ± 0.09^{e}	0.156853
12	Henan province	3.69 ± 0.02^{e}	12.67 ± 0.35^{e}	9.63 ± 0.01^{cd}	1.825608
13	Hebei province	3.71 ± 0.01^{de}	12.00 ± 0.04^{f}	7.81 ± 0.14^{i}	0.701741
14	Shannxi province	3.61 ± 0.02^{g}	14.07 ± 0.12^{b}	9.75 ± 0.03^{ab}	0.918395
15	Anhui province	3.69 ± 0.01^{e}	10.93 ± 0.06^{h}	9.64 ± 0.05^{cd}	1.283195

Values are expressed as averages of three independent experiments ± SD.
Values marked with different letters in the same column are significantly different ($p < 0.05$).