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Diversity of isolated lactic acid bacteria in Ya'an sourdoughs and evaluation of their exopolysaccharide production characteristics

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ABSTRACT

The lactic acid bacterial diversity of wheat sourdoughs collected in Ya'an city, Sichuan province was investigated by culture-dependent methods combined with 16S rRNA gene sequencing, and the exopolysaccharide (EPS) production characteristics of ropy lactic acid bacteria (LAB) strains from these sourdoughs were evaluated. Two hundred nineteen LAB strains were isolated, and genotypic characterization indicated that the isolated LAB strains included *Lactobacillus plantarum* (*L. plantarum*), *L. pantheris*, *Leuconostoc citreum* (*Leu. citreum*), *Weissella viridescens*, *Leu. pseudomesenteroides*, *Lactococcus lactis* (*L. lactis*), *L. raffinolactis*, and *Leu. mesenteroides*. All the isolates were found to harbor genes coding for EPS, and the yield of EPS produced by "ropy" LAB strains ranged between 2.3 and 229.5 mg/g wet cell weight. The present study enhances our knowledge about LAB diversity in Ya'an sourdoughs and provides information about the EPS-producing LAB strains in sourdough samples, which might be favorable for the application of specific LAB strains in sourdough technology.

1. Introduction

Sourdough, which can be traced back to ancient times, is traditionally used to make bakery products. It is widely employed as the "mother sponge" for bread making in Europe, and it is also used as "lao'mian" to produce Chinese steamed bread, a staple food in China (Liu et al., 2016). Sourdough is found to be able to improve the overall quality of bakery goods, including enhancing the nutritional value and sensory properties, extending shelf life, delaying staling and effectively reducing or partially replacing the usage of additives, which has attracted the widespread attention of consumers (Rizzello, Calasso, Campanella, De Angelis, & Gobbetti, 2014; Scarnato et al., 2017).

Sourdough is a mixture of mainly cereal flour (wheat, rice, etc.) and water that is fermented with lactic acid bacteria (LAB) and yeast (Alfonzo et al., 2017; De Vuyst, Vrancken, Ravyts, Rimaux, & Weckx, 2009). In general, the ratio of LAB:yeast in sourdough is found to be approximately 100: 1 (García-Mantrana, Yebra, Haros, & Monedero, 2016; Rizzello et al., 2015). The organic acids, exopolysaccharides (EPSs), enzymes and antibacterial and antimold compounds produced by LAB have been reported to have positive effects on bread quality (Carnevali, Ciati, Leporati, & Paese, 2007; Tieking & Gänzle, 2005). Meanwhile, yeast can convert sugars into alcohol and CO_2 efficiently, which are favorable for aroma formation (Liu et al., 2018). EPSs are natural biopolymers, and their advantages in sourdough, such as improving the viscoelastic properties, increasing loaf volume, reducing crumb hardness and prolonging shelf life, have been confirmed by previous studies (Galle & Arendt, 2014; Torrieri, Pepe, Ventorino, Masi, & Cavella, 2014; Wolter et al., 2014). Therefore, exploring the EPS production characteristics of sourdough LAB has received considerable critical attention. Recently, Dertli, Mercan, Arıcı, Yılmaz, and Sağdıç (2016) reported the EPS production characteristics of Turkish sourdough LAB, and more work could be conducted to enhance our knowledge about LAB diversity in sourdoughs from different countries and regions.

Generally, LAB in sourdough are considered to originate from the flour, other ingredients and the environment (Demirbaş; İspirli, Kurnaz, Yilmaz, & Dertli, 2017). To date, studies on the microflora composition of sourdough originated from different countries are a lot, and techniques such as culture-dependent methods (Palla, Cristani, Giovannetti, & Agnolucci, 2017), polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) (Ruiz Rodríguez et al., 2016), and high-throughput sequencing (Coda et al., 2017; Jiménez et al., 2018) are widely employed to profile LAB diversity. Although sourdough has been widely used in China, very little attention has been paid to the diversity and characteristics of EPS-producing LAB in Chinese sourdough. Ya'an city (102°59′E and 29°59′N), located in the west of Sichuan province, is

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well known as a “rainy city” and “township of the panda”, and has an annual average temperature of approximately 16.1 °C (Zhang, Chen, Li, Xu, & Deng, 2010). People here like eating Chinese steamed bread for breakfast, and the unique environment might contribute to different LAB diversity in the sourdough. The present study aims to investigate the diversity of LAB in traditional sourdoughs collected from Ya'an city using culture-dependent and sequencing approaches. Meanwhile, EPS-producing characteristics of isolated LAB are examined and compared to the report by Dertli et al. (2016).

2. Materials and methods

An A-tailing kit, and a TA cloning kit were purchased from Sangon Biotech (Shanghai, China). PrimeSTAR®HS (Premix) was purchased from Takara Biomedical Technology (Dalian, China). TIANamp Bacteria Genomic DNA purification kit was purchased from Tiangen Biotech (Beijing, China).

2.1. Sampling

Thirteen sourdough samples were collected aseptically between May of 2017 and June of 2017 from 13 small bakeries (Chinese steamed bread manufacturers) in Ya'an city, Sichuan province, and then transported to our laboratory with ice pack in an hour. All sourdough samples were produced from wheat flour with regular propagation by backslopping at room temperature. The samples proceeded to further analysis immediately after arriving.

2.2. pH of sourdough samples, enumeration of LAB and yeast, and the isolation of LAB

The pH value of sourdough samples was determined using a Testo 205 pH probe (Testo AG, Lenzkirch, Germany) inserted about 3 cm deep into the sourdough.

For enumeration of LAB and yeast, samples were removed aseptically, and 25 g of the sample were mixed with 225 mL of sterile saline solution (0.9% NaCl). Serial decimal dilutions were prepared from this mixture, and the number of LAB and yeast were counted via the dilution-plating method. The following incubation conditions were used: MRS agar for LAB such as *Lactobacillus*, *Leuconostoc*, and *Pediococcus* (anaerobic, 2 d at 37 °C); M17 agar for LAB such as *Lactococcus* and *Enterococcus* (anaerobic, 2 d at 37 °C); potato dextrose agar for 5 d at 28 °C for mold-yeast (Liu, Li et al., 2017; Liu, Liu et al., 2017; Mathara, Schillinger, Kutima, Mbugua, & Holzapfel, 2004). All analyses were performed in triplicate and the mean values were calculated.

For isolating LAB, the dilutions prepared above were plated using MRS agar and M17 agar containing 1.5% $CaCO_3$ via the pour plate method. Representative colonies (white, slightly convex, circular, colony diameter of 2–5 mm) with a dissolved calcium circle were randomly picked and re-streaked onto MRS agar plates with 1.5% $CaCO_3$ to obtain pure cultures. Gram-positive, catalase-negative isolates were considered as presumptive LAB (Leite et al., 2015; Liu, Li et al., 2017; Liu, Liu et al., 2017). The isolates were stored at 4 °C after streak inoculation or at −80°C in commercial bacterial frozen buffer (Beyotime Biotechnology, Beijing). These isolates were subcultured twice before use.

2.3. Molecular identification of presumptive LAB isolates

The presumptive LAB isolates were further identified by 16S rRNA gene sequencing. First, the isolates were cultured in MRS/M17 broth overnight. Second, the genomic DNA of these isolates were extracted using the TIANamp Bacteria Genomic DNA purification kit (Tiangen Biotech, Beijing, China). Third, the 16S DNA fragments were amplified by PCR with the primers 27F (5′- AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′- GGTTACCTTGTTACGACTT-3′). The PCR amplification solution (25 μL) included 12.5 μL of PrimeSTAR® HS (Premix), 1 μL of DNA template (5–20 ng), 2 μL of primers (1 μL of 27F and 1 μL of 1492R, 10 μmol), and 10.5 μL of deionized water. The PCR program was as follows: (1) 94 °C for 4 min; (2) 27 cycles of 94 °C for 1 min, 60 °C for 30 s, and 72 °C for 1 min; and (3) 72 °C for 7 min. The resulting material from the PCR was analyzed using 1.2% agarose gel electrophoresis. Subsequently, the PCR products were purified, and a Poly (A) + tail was added using an A-tailing kit with reference to the manufacturer's protocol. For each, the resultant gene was ligated into the pUCm-T vector and transformed into competent *Escherichia coli* DH5α cells using a TA Cloning Kit following the manufacturer's procedure. White transformed clones were selected and confirmed by PCR with M13 + and M13-primers. Positive clones were sent for sequencing (Sangon Biotech, Shanghai, China), and sequences of all the isolates were deposited in GenBank (nucleotide accession number: MG754504–MG754722). The sequences were examined for homology using BLAST and sequences with a similarity of ≥99% were considered as the same species. Typical data were then selected to execute phylogenetic analysis with Mega 5.0 software (http://www.megasoftware.net/) using the neighbor-joining method.

Table 1
The pH, the number of LAB and yeast of the sourdough samples.

Sourdough sample	pH	Number of LAB (lg CFU/g)	Number of yeast (lg CFU/g)
A	4.11 ± 0.04	8.16 ± 0.04	5.91 ± 0.03
B	4.15 ± 0.04	8.02 ± 0.04	6.06 ± 0.05
C	4.36 ± 0.05	8.25 ± 0.01	5.78 ± 0.06
E	3.98 ± 0.04	8.36 ± 0.01	6.29 ± 0.01
F	4.24 ± 0.03	8.15 ± 0.03	5.92 ± 0.03
G	4.36 ± 0.02	8.08 ± 0.05	6.19 ± 0.04
H	4.06 ± 0.05	8.41 ± 0.03	6.35 ± 0.03
J	4.64 ± 0.06	8.03 ± 0.03	6.22 ± 0.03
K	4.07 ± 0.06	8.32 ± 0.05	6.02 ± 0.04
L	4.67 ± 0.05	8.26 ± 0.02	6.35 ± 0.01
M	4.74 ± 0.04	8.11 ± 0.01	6.25 ± 0.02
N	4.08 ± 0.04	7.99 ± 0.02	6.01 ± 0.05
S	4.74 ± 0.04	8.13 ± 0.04	5.98 ± 0.04

2.4. PCR detection of EPS-coding genes in LAB isolates

The presence of EPS-coding genes indicated an essential step of EPS biosynthesis. PCR detection of EPS-coding genes in all 219 LAB isolates were conducted using primer sets mentioned in the report by Dertli et al. (2016). These EPS-coding genes included epsA (putative transcriptional regulator), epsB (putative polymerization and chain length determination protein gene), gtf (glucansucrase), lev (levansucrase) and p-gtf (putative priming glycosyltransferase gene).

2.5. Isolation of strains capable of producing high levels of EPS

For evaluating the EPS-producing capacity of LAB strains, LAB were cultured on modified MRS agar plates, into which glucose was reduced to 0.5% and 5% sucrose was added, and EPS was detected by observing whether long ropy strands could be observed when touching the colonies with a toothpick (Kojic et al., 1992; Nakajima et al., 1990). Afterward, the EPS yield of the isolates with clear long ropy strands (> 1 mm) was determined using the method published by Li et al. (2015) with modifications. Briefly, a single colony of each isolate was cultured in MRS broth to achieve an OD_{600nm} of approximately 0.6–0.8, and then the culture (4%, v/v) was employed to inoculate a 50 mL batch modified MRS broth. After incubation at 30 °C for 24 h, the cells and fermentation broth were collected by centrifugation (4000 g, 15 min, 4 °C). The cells were transferred to a weighted 1.5 mL tube, washed two times by sterile saline solution, and weighed in the tube, obtaining the weight of the cells by deduction of the weight of the tube.

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