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Microbial composition of spoiled industrial-scale Sichuan paocai and characteristics of the microorganisms responsible for paocai spoilage

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ABSTRACT

The microorganisms of spoiled industrial-scale Sichuan paocai (ISSP) were isolated using six types of media, and 16S rRNA and 26S rRNA gene sequence analyses were used to identify the isolates. Meanwhile, the microbial composition was investigated using a culture-independent method through 16S rRNA and ITS sequencing on an Illumina MiSeq platform. The results obtained by these two methods were compared. Furthermore, characteristics of the isolated microorganisms responsible for ISSP spoilage were studied. Sixty-two strains belonging to twenty-three species, including three ammonia-producing genera, two gas-producing genera, two pectinase-producing genera, two cellulase-producing genera, three film-producing genera and one slime-producing genus, were isolated. *Lactobacillus*, *Bacillus*, *Debaryomyces* and *Kazachstania* were the dominant genera as confirmed through both culture-dependent and culture-independent methods. *Bacillus*, *Paenibacillus*, *Pichia* and *Debaryomyces* could be the main microorganisms responsible for ISSP spoilage. *Bac. licheniformis* was closely correlated with the off-flavour of ISSP. *Pae. peoriae*, *Bac. stratosphericus*, *Bac. toyonensis* and *Bac. cereus* were responsible for tissue softening, and *Bac. subtilis*, *Bac. methylotrophicus*, *Pic. membranifaciens* and *Deb. hansenii* caused film formation.

1. Introduction

Sichuan paocai is a type of traditional Chinese fermented vegetable immerged typically in salt concentration of 4–15% (*w/v*), for which the dominant microorganisms are lactic acid bacteria (Li et al., 2014). To data, Sichuan paocai has always been popular as a cooking material and breakfast necessity in southwestern China. The production output of ISSP reached 3.6 million tons in 2016, and its corresponding economic output was approximately $ 3100 million. The salting of raw vegetables in a saline pool is an important process for the production of ISSP; it contributes to the appropriate fermentation and long-term storage of paocai. However, spoilage phenomena of some ISSP appear easily in the surface of saline pools and they include film formation, tissue softening, off-flavour, which might be due to the effects of a hostile environment, and improper operation. This spoilage can observably lower the sensory quality of ISSP and cause a potential food safety issue, thereby bringing great economic loss to paocai factories.

The spoilage of fermented vegetables is ascribed to the undesired composition and metabolism of microorganisms existing in fermentation systems. Medina et al. (2016) found that *Pichia manshurica*, *Issatchenkia occidentalis*, *Lactobacillus buchneri*, *Lactobacillus parrafaraginis*, *Pediococcus ethanolidurans*, *Clostridium* sp. and *Propionibacterium* sp. had an important relationship with the spoilage of fermented cucumber in the United States. Moon et al. (2014) isolated *Kazachstania bulderi* and *Pichia membranifaciens* from spoiled Korean kimchi, and these two strains were considered to be the microorganisms responsible for tissue softening and film formation, respectively. He et al. (2017) found that *Bacillus subtilis*, *Citrobacter freundii*, *Staphylococcus cohnii* subsp. *cohnii*, *Providencia vermicola*, *Klebsiella* sp. and *Enterobacter cloacae* were the corresponding microorganisms for pellicle formation in fermented vegetables. Pectinolytic yeasts, such as *Saccharomyces cerevisiae*, *Pic. manshurica* and *Candida boidinii*, can contribute to olive softening (Golomb et al., 2013). The findings reported above indicate that the spoilage of fermented vegetables is due to the combined effects of multiple types of microorganisms. Therefore, it is important to study the microbial properties of the microorganisms for ISSP spoilage, thereby preventing fermented vegetables from spoiling.

In this study, the aerobic and anaerobic microorganisms of spoiled ISSP were first isolated with six types of media, and 16S rRNA and 26S rRNA gene sequence analyses were used to identify the isolates. The

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Table 1
Primers used in this study.

Primer	Primer sequences (5′ to 3′)	References
27F	AGAGTTTGATCCTGGCTCAG	Frank et al., 2008
1492R	CTACGGCTACCTTGTTACGA	Frank et al., 2008
NL1	GCATATCAATAAGCGGAGGAAAAG	Kurtzman and Robnett, 1998
NL4	GGTCCGTGTTTCAAGACGG	Kurtzman and Robnett, 1998
338F	ACTCCTACGGGAGGCAGCAG	Liu et al., 2016
806R	GGACTACHVGGGTWTCTAAT	Liu et al., 2016
ITS1F	CTTGGTCATTTAGAGGAAGTAA	Bokulich and Mills, 2013
ITS2-2043R	GCTGCGTTCTTCATCGATGC	Huang et al., 2016

culture-independent method via Illumina MiSeq based on 16S rRNA and ITS sequencing was then applied for investigation of the microbial composition. Furthermore, the dominant microorganisms for ISSP spoilage were determined by evaluating their characteristics, such as ammonia-producing, gas-producing, pectinase-producing, cellulose -producing, slime-producing and film formation capacities.

2. Materials and methods

2.1. Paocai samples and chemicals

Spoiled ISSP was obtained from the surface layer of a saline pool in a pickle factory in Meishan, Sichuan, China. It showed serious spoilage, including a brown or black colour, softening in texture, white film on the ISSP surface and off-flavour. Normal ISSP, with a bright colour, firm tissue and good fermentation flavour, was obtained from the middle layer of the same saline pool. The salt concentration of these two ISSP samples was measured as 12.63 ± 0.12%. Nessler reagent was purchased from Land Bridge Biotechnology Co., Ltd., Beijing, China. The primers used in this study, as shown in Table 1, were purchased from Qingkezixi Biotechnology Co., Ltd., Chengdu, Sichuan, China. Other chemical reagents were of analytical grade.

2.2. Preparation of media

Plate count agar (PCA) medium, nutrient broth (NB) medium, potato dextrose agar (PDA) medium, Gifu anaerobic agar medium (GAM), and other reagents including NaCl, CMC-Na, KH_2PO_4, $MgSO_4$, glucose, peptone, yeast extract, pectin, arginine and bromocresol purple were purchased from Land Bridge Biotechnology Co., Ltd., Beijing, China. Potato and skimmed milk were purchased from a local supermarket in Chengdu, Sichuan, China.

The liquid potato dextrose (LPD) medium, ammonia-producing medium, glucose fermentation (GF) medium, yeast extract peptone dextrose (YPD) medium, pectinase-producing medium, and CMC-Na medium were prepared as described in supplementary materials. The high-salt media of the above media were prepared by adding extra NaCl to reach the salt concentration of 10% (*w*/w) except for in the ammonia-producing medium.

2.3. Determination of pH and titratable acidity

A 20 g aliquot of ISSP cut previously into small pieces (approximately 1 cm × 1 cm) was put into 20 mL of deionized water and mixed. The pH of the mixture was determined by a pH-3C meter (Yidian, Shanghai, China), and the titratable acidity (TTA) was determined according to the titration method of AOAC 942.15 (Horwitz, 2000). Each test was performed three times in parallel, and the results are expressed as the mean ± standard deviation.

2.4. Microbial composition analysis through culture-dependent method

2.4.1. Isolation of microorganisms

A 25 g aliquot of ISSP was cut into small pieces (approximately 1 cm × 1 cm), and 225 mL of sterile saline was added and mixed. After a serial dilution (10^{-1} to 10^{-4}) in sterile saline, every 0.1 mL of diluted bacterial suspension was inoculated into six types of media. Aerobic bacteria in the PCA medium and halophilic bacteria in the high-salt PCA medium were incubated at 37 °C for 48 h. Fungi in the PDA medium and halophilic fungi in the high-salt PDA medium were incubated at 28 °C for 48 h. Anaerobic bacteria in the GAM medium and halophilic bacteria in the high-salt GAM medium were incubated in an anaerobic jar with an AnaeroPack (MGC, INC, Japan) at 37 °C for 48 h. The media without inoculation were used as blanks. A single colony of every colony morphology type identified in each medium was selected from plates with low counts and purified by repeated streaking on corresponding agar plates. For long-term storage, purified aerobic bacteria, fungi and anaerobic bacteria were preserved at −80 °C in the PCA, PDA or GAM media containing glycerol (20%).

2.4.2. Identification of microorganisms

16S rRNA gene sequence analysis of bacterial isolates: bacterial DNA was extracted according to the instructions of a bacterial DNA extraction kit (Fuji Biology Co., Ltd., Chengdu, Sichuan, China). The primer used for PCR amplification was 27F/1492R, and the reaction procedure was performed according to the method of Haruta et al. (2002). 26S rRNA gene sequence analysis of fungal isolates: fungal DNA was extracted according to the instructions of a fungal DNA extraction kit (Shenggong Biology Co., Ltd., Shanghai, China). The primer used was NL1/NL4, and the reaction procedure was performed according to the method of Tofalo et al. (2013). The positive clones of the PCR products were sequenced by Shenggong Biotechnology Co., Ltd., Shanghai, China. A representative 16S rRNA/26S rRNA gene sequence from each phylotype was archived in the GenBank database (http://www.ncbi.nlm.nih.gov).

2.5. Microbial composition analysis through culture-independent method

A 10 g aliquot of spoiled ISSP was cut into small pieces (approximately 1 cm × 1 cm), and 90 mL of sterile saline was added and mixed. After centrifugation at 12,000 rpm for 5 min, the supernatant was collected. The DNA was extracted and gathered by the universal E.Z.N.A. Soil DNA Kit (Omega, Norcross, GA, USA) according to the manufacturer's instructions. The TransGen AP221-02 kit with TransStart Fastpfu DNA polymerase (TransGen Biotech, Beijing, China) was used in PCR reactions. To analyse the taxonomic composition of microbes, bacteria 16S rRNA gene was amplified by PCR using primer 338F/806R, and fungi ITS was amplified by primer ITS1F/ITS2-2043R. Then, all PCR products were visualized on agarose gels (2% in TBE buffer). For each sample, three independent PCRs were performed. The triplicate products were pooled and purified using the AxyPrepDNA Gel Recovery Kit (Axygen Scientific, Inc., CA, USA). The DNA concentration of each PCR product was determined using a QuantiFluo-ST Blue Fluorescent Quantitative System (Promega, Madison, Wisconsin, USA). The amplified product was sequenced through the Illumina MiSeq sequencer (Illumina, Inc., CA, USA) by Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China. The Operational Taxonomic Unit (OTU) clustering of non-repetitive sequences was performed according to 97% similarity. The RDP classifier Bayesian algorithm was used to analyse the 97% similar level of OTU representative sequences, and data visualization of the microbial community was achieved with GraPhlAn software (Asnicar et al., 2015). The Illumina MiSeq sequencing data generated for this study were submitted to the Sequence Read Archive (SRA), and the SRA accession number is SRP128624.