

Full Paper

Identification and characterization of lactic acid bacteria isolated from traditional pickles in Sichuan, China

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(Received October 19, 2011; Accepted January 7, 2012)

The pickle, a traditional fermented vegetable product, is popular in Sichuan Province of China. The objective of this study was to investigate the diversity of dominant lactic acid bacteria (LAB) in pickles by analyzing 36 samples collected from 6 different regions in Sichuan Province. The LAB counts in these samples varied from 3.90 to 8.40 log cfu ml⁻¹. In total, 185 presumptive LAB with Gram-positive and catalase-negative properties were obtained from these samples using MRS agar, and those strains were identified at the species level by physiological tests, 16S rRNA gene sequencing and multiplex PCR assay. The results revealed that all isolates were accurately identified as *Enterococcus thailandicus* (2 strains), *Lactobacillus alimentarius* (16 strains), *L. brevis* (24 strains), *L. paracasei* (9 strains), *L. plantarum* (81 strains), *L. pentosus* (38 strains), *L. sakei* (8 strains), *L. spicheri* (1 strain), *Leuconostoc lactis* (1 strain) and *Pediococcus ethanolidurans* (5 strains). The predominant LAB in Sichuan pickle was *L. plantarum*, which were isolated from most samples. The results also demonstrated that different regions in Sichuan Province have complex compositions of LAB species, and such a rich resource of LAB strains provides raw data for further studies involving probiotic strain selection.

Key Words—identification; lactic acid bacteria; pickle; 16S rRNA gene sequence

Introduction

Pickles, a type of mildly salted and lactic acid fermented vegetable, have been consumed in Sichuan Province for hundreds of years. Due to the simple method of making and unique flavor, pickles are favored as a side dish with the main meal or appetizer in Sichuan. Homemade pickles are based on spontane-

ous fermentation that is highly dependant on the epiphytic microbes present on the raw materials. For producing acidity and flavor to the desired level, the preparation procedure of pickle is important. Generally, many vegetables, such as cabbage, celery, capsicum and radish are immersed in 6–8% salt solution with red pepper, green onion and garlic in a special jar for at least 5–7 days in summer (about 20–27°C) and 12–16 days in winter (about 8–15°C).

Surfaces of harvested vegetables contain large numbers of miscellaneous microorganisms. Although the number of lactic acid bacteria (LAB) is often relatively low on the surface of vegetables, the anaerobic and low salt conditions engender the rapid growth of LAB at the end of the fermenting stages (Han et al., 2007). Microorganisms that adhere to the surface of

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the freshly harvested vegetables are mainly Gram-negative saprophytes (Seo et al., 2010). At the initial stage of spontaneous fermentation, the number of LAB is low and this engenders the rapid growth of nitrate-reducing bacteria (Yan et al., 2008). In the middle stages, *Enterobacteriaceae* (*Escherichia*, *Enterobacter* and so on) and *Bacillus* are retarded by the salt and anaerobic condition and they gradually die. At the same time, the LAB quickly proliferate, produce acid, become the dominant species under the anaerobic conditions and inhibit the growth of *Enterobacteriaceae* and *Bacillus*. At the end of the fermentation, abundant LAB become the dominate microorganism and the fermentation of pickles remains stable (Karasu et al., 2010; Panagou and Katsaboukakis, 2006). Therefore, it is worth identifying the LAB isolated from pickles for development of industrial starter for pickle fermentation.

This study reports on the isolation and characterization of the dominant LAB isolated from 36 samples of pickles that were collected from different regions in Sichuan Province of China. One hundred eighty-five isolates were initially identified according phenotypic characteristics and further identified by 16S rRNA gene sequence and multiplex PCR assay of the *recA* gene.

Materials and Methods

Collection of pickles samples. Thirty-six samples of pickles were collected from six different regions in Sichuan. The temperature of pickle samples at sampling ranged from 8.7°C to 16.4°C; the average tem-

perature was 13.2°C (Table 1). The pH values of the pickle juice were determined at the sampling site using a calibrated portable pH-meter (pH100, Exttech, USA). The pickle juice samples were collected within 15 min at ambient temperatures, kept on ice during 2–5 h transport and the microbiological analysis was carried out immediately after the samples arrived at the laboratory.

Enumeration and isolation of LAB. The 10^{-1} dilution was made by diluting 1 ml of pickle juice within 9 ml of physiological saline (0.9% NaCl). Further tenfold serial dilutions, ranging from 10^{-5} to 10^{-9} , were prepared and the counts of LAB were determined using Brom Cresol Purple (BCP agar, Nissui Pharmacy, Tokyo, Japan) incubated anaerobically at 30°C for 2 days. Plates were incubated anaerobically at 30°C using anaerobic jars together with the BBL (Baltimore Biological Laboratory, GasPak 100 Anaerobic System, BD Biosciences, Sparks, MD, USA).

The colonies were randomly picked from MRS (Man Rogosa Sharpe broth, Fluka) plates with 30–300 colonies, and transferred into 5 ml of MRS broth. The selected colonies were purified by repeated streaking on the MRS agar. Gram-positive and catalase-negative bacterial isolates were purified and the frozen stocks in 10% (w/v) skim milk broth were stored at –80°C. Lyophilization of isolates was performed for longer storage.

Conventional identification of LAB. Further identification of Gram-positive and catalase-negative isolates was performed by using the following physiological tests: NH_3 production from arginine; CO_2 production

Table 1. General features of pickle samples from six different region of Sichuan.

Sample number	Sampling location	Temperature (°C)	pH value	LAB (log cfu ml ⁻¹)	Main material
1 to 3	Chengdu	15.9±0.5 (15.5–16.4)	3.8±0.3 (3.5–4.0)	5.54±1.49 (4.15–7.11)	summer radish
4 to 7	Chongzhou	13.1±0.5 (12.6–13.5)	3.4±0.2 (3.2–3.5)	6.95±0.71 (5.99–7.69)	celery, cowpea, cabbage, bamboo shoots
8 to 19	Dayi	12.3±1.7 (8.7–15.1)	3.7±0.4 (3.5–4.5)	6.65±1.34 (3.90–8.17)	summer radish, celery, cowpea, cabbage, Chinese cabbage
20 to 25	Pujiang	14.5±1.5 (11.5–15.3)	3.8±0.6 (3.2–4.5)	6.82±1.51 (4.89–8.26)	summer radish, celery, cowpea, cabbage, bamboo shoots
26 to 34	Qionglai	13.2±1.3 (11.9–15.3)	3.5±0.4 (3.0–4.2)	6.26±1.09 (4.36–7.54)	summer radish, celery, cowpea, Chinese cabbage, carrot
35 to 36	Xinjin	11.6±0.8 (11.0–12.2)	3.7±0.9 (3.0–4.3)	7.73±0.95 (7.05–8.40)	summer radish, cowpea, celery, cabbage, capsicum frutescens
Average		13.2±1.7	3.6±0.4	6.58±1.25	

from glucose in MRS broth containing inverted Durham tubes; growth at temperatures of 10°C, 15°C, 45°C and 50°C in MRS broth for 5 days; and growth at pH 3.0, 3.5, 4.0, 4.5, 5.0, 9.0 and 9.6 in MRS broth for 3 days. Salt tolerance was determined in MRS containing 2.0% 3.0%, 4.0%, 6.0%, 6.5%, 8.0% and 10.0% NaCl (w/v) at their isolated temperature for 3 days (Kozaki et al., 1992; Yu et al., 2011). The type and amount of D and L isomers of lactic acid produced from glucose was assayed in modified MRS broth using a commercial kit (Hoffman La Roche Diagnostic, Mannheim, Germany).

Carbohydrate fermentation experiments were conducted according to the method described by Jayne-Williams (1975). Twenty-six kinds of carbohydrates were tested using sugar basal broth with chlorophenol red as the indicator. Different sugars were added to basal broth and adjusted to a final concentration of 0.5% (w/v). The results of carbohydrate fermentation were checked according to the information supplied in Bergey's Manual of Systematic Bacteriology (Kandler and Weiss, 1986).

16S rRNA gene sequencing and phylogenetic analysis. Total genomic DNA was extracted from 5 ml of overnight cultures at 37°C by the previous method (Yu et al., 2009). Purified DNA was diluted to a final concentration of 100 ng/μl for application. The 16S rRNA gene were amplified using primers 16S-FA (GCA GAGTTCTCGGAGTCACGAAGAGTTTGATCCTGGCT CAG) and 16S-RA (AGCGGATCACTTCA CACAGGAC TACGGCTACCTTGTTACGA) described by Sun et al. (2010). Nucleotides 1 to 21 of both 16S-FA and 16S-RA are the specific sequencing primers, respectively. 16S rRNA genes of LAB were amplified in an automatic thermal cycler (PTC-200; MJ Research, Waltham, MA, USA). Each sample contained 1 × Taq buffer (TaKaRa Bio-Co., Shiga, Japan), 1.5 mM MgCl₂, 0.2 μM of each dNTP, 10 pmol of each primer, 10 ng template bacterial DNA and 1.0 U Ex TaqTM polymerase (TaKaRa Bio-Co.). The reaction conditions were as follows: 94°C for 5 min, 94°C for 1 min, 58°C for 1 min, 72°C for 2 min, 30 cycles, and then 72°C for 10 min. Reaction products were resolved by electrophoresis in 1.0% agarose gels and visualized by ethidium bromide staining. The PCR product of interest was isolated from the agarose gel using a Huashun Gel Extraction Kit (Huashun, China). The purified PCR fragments were used for sequencing by the corresponding sequencing primers. DNA sequencing was performed by

Shanghai Sangni Biosciences Corporation. The sequences were analyzed and determined using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/blast>; Altschul et al., 1997) and were submitted to the NCBI (<http://www.blast.ncbi.nlm.nih.gov>). Consensus sequences were imported into MEGA version 4.0 software (<http://www.megasoftware.net>; Tamura et al., 2007), with which a sequence alignment and phylogenetic trees were created based on the neighbor-joining (NJ) method.

Discrimination of *L. plantarum* group. For further discrimination of strains in the *L. plantarum* group, a multiplex PCR assay was performed with the *recA* gene-based primers: paraF (5'-GTCACAGGCATTAC GAAAAC-3'), pentF (5'-CAGTGGCGCGGTTGATATC-3'), planF (5'-CCGTTTATGCGGAACACCTA-3'), and pREV (5'-TCGGGATTACCAAACATCAC-3'). The PCR mixture and amplifications were performed as described by Torriani et al. (2001).

Results

Enumeration and isolation of LAB

The pH value of pickle samples ranged from 3.2 to 4.5 (Table 1). The viable counts of the LAB present in the samples are showed in Table 1. Total counts of LAB in 36 pickle samples on BCP agar varied in the range from 3.90 to 8.40 log cfu ml⁻¹. The average LAB counts of the pickles from Chengdu, Chongzhou, Dayi, Pujiang, Qionglai and Xinjin regions were 5.54 ± 1.49, 6.95 ± 0.71, 6.65 ± 1.34, 6.82 ± 1.51, 6.26 ± 1.09 and 7.73 ± 0.95 log cfu ml⁻¹, respectively. Gram-positive and catalase-negative bacteria growing on MRS agar were considered as presumptive LAB, and 185 presumptive LAB were isolated from 36 pickle samples.

Conventional identification of LAB

According to the morphological, physiological and biochemical properties, the 185 strains were divided into nine groups (Table 2). All of the isolates could grow at pH 5.0, 15°C, 2.0%, 3.0% NaCl and 15°C but not 50°C or pH 3.0, and they were able to ferment D-glucose, D-fructose and D-maltose but not inulin, starch, xylitol or glycogen. Sixteen isolates of group 1 were identified as *L. alimentarius* based on the physiological and biochemical properties (Reuter, 1983). They produced L-lactic acid, but could not produce CO₂ from glucose or NH₃ from arginine. Most of isolates grew well at 10°C, pH 4.0 and 4.5, in 6.0% and

Table 2. Phenotypic characteristic of LAB isolated from natural fermented pickle in Sichuan Province, China.

Characteristics	Groups ^a								
	1	2	3	4	5	6	7	8	9
Number of isolates	16	24	9	119	8	1	1	2	5
Shape	R	R	R	R	R	R	C	C	C
Gas from glucose	–	+	–	–	–	–	+	–	–
Lactic acid isomer ^b	L	DL	L	DL	DL	DL	D	L	DL
NH ₃ from arginine	–	+	–	–	–	+	–	+	–
Growth at pH value									
3	0 ^c	0	0	0	0	0	0	0	0
3.5	0	0	6	89	0	0	0	0	3
4	15	24	9	119	4	1	1	0	5
4.5	16	24	9	119	5	1	1	0	5
5.0	16	24	9	119	5	1	1	2	5
9.0	0	0	9	110	5	1	1	2	0
9.6	0	0	0	0	0	1	0	2	0
Growth in NaCl (w/v)									
2.0%	16	24	9	119	5	1	1	2	5
3.0%	16	24	9	119	5	1	1	2	5
4.0%	16	24	9	119	5	1	1	2	1
6.0%	16	10	9	119	5	1	1	2	0
6.5%	10	0	7	119	5	1	0	2	0
8.0%	3	0	0	89	5	0	0	0	0
10.0%	0	0	0	0	2	0	0	0	0
Growth at temperature (°C)									
10	14	24	9	119	0	1	0	0	1
15	16	24	9	119	5	1	1	2	5
45	0	0	3	92	5	0	1	2	5
50	0	0	0	0	0	0	0	0	0
Acid from									
D-Arabinose	16	24	0	0	5	0	0	0	0
L-Arabinose	16	24	0	43	5	0	0	0	0
D-Cellobiose	16	0	9	119	5	0	1	2	5
Esculin	16	12	9	119	5	0	1	2	5
Galactose	16	24	9	119	5	0	1	2	5
Gluconate	16	24	9	119	5	0	0	2	0
D-Lactose	0	22	8	119	5	0	0	2	4
D-Mannitol	0	0	8	119	0	0	0	2	0
D-Mannose	16	0	9	119	5	0	1	2	5
D-Melezitose	0	0	8	113	0	0	0	0	0
D-Melibiose	0	24	0	119	5	0	1	0	0
D-Raffinose	0	21	0	119	0	0	1	0	0
L-Rhamnose	0	0	0	13	0	0	0	0	4
Ribose	16	24	9	119	5	1	1	2	0
Salicin	16	0	9	119	5	0	1	2	5
D-Sorbitol	0	0	9	119	5	0	0	0	0
L-Sorbose	0	0	9	0	0	0	0	0	0
D-Sucrose	16	14	9	119	5	0	1	2	5
D-Trehalose	16	0	9	119	5	0	0	0	5
D-Xylose	0	24	0	38	0	1	1	0	0

All strains fermented D-glucose, D-fructose and D-maltose. No strains fermented inulin, starch, xylitol or glycogen.

^aGroups 1 to 9 were identified as *Lactobacillus alimentarius*, *L. brevis*, *L. paracasei*, *L. plantarum*, *L. sakei*, *L. spicheri*, *Leuconostoc lactis*, *Enterococcus thailandicus*, and *Pediococcus ethanolidurans*, respectively. ^bL: L-lactic acid, DL: DL-lactic acid, D: D-lactic acid. ^cNumber of positive strains.

6.5% NaCl, but only 3 strains could grow in 8.0% NaCl. Those strains could utilize D-arabinose, L-arabinose, ribose, galactose, D-mannose, esculin, salicin, D-cellobiose, D-sucrose, D-trehalose and gluconate. Group 2 included 24 rod-shaped isolates, which produced DL-lactic acid, and could produce CO₂ from glucose and NH₃ from arginine. Strains could grow at 10°C, pH 4.0 and 4.5, and in 4.0% NaCl. All but 10 isolates failed to grow in 6.0-10% NaCl. Most isolates could ferment D-arabinose, L-arabinose, ribose, D-xylose, galactose, esculin, D-lactose, D-melibiose, D-sucrose, D-raffinose, and gluconate. Those strains were identified as *L. brevis* due to their characteristics. Strains of group 3 produced L-acid but could not produce CO₂ or NH₃, and grew well at pH 3.5, 4.0, 4.5 and 9.0, in 4.0%, 6.0% and 6.5% NaCl, at 10°C. Those isolates could produce acid from all the sugars except D-melibiose, D-arabinose, L-arabinose, L-rhamnose, D-raffinose and D-xylose. Considering all the factors, this group was identified as the *L. casei* group. One hundred nineteen isolates of group 4 were found to be closely related to *L. plantarum*. They produced DL-lactic acid but could not produce CO₂ from glucose or NH₃ from arginine. The majority could grow at 10°C and 45°C, pH 3.5, 4.0 and 4.5, and grew well in 6.0%, 6.5% and 8.0% NaCl. Those strains could utilize most sugars except D-arabinose, L-arabinose, D-xylose, L-rhamnose and L-sorbose; therefore a minority of isolates could ferment L-arabinose, L-rhamnose and D-xylose. Eight strains of group 5 were assigned to *L. sakei*. Those isolates produced DL-lactic acid but could not produce CO₂ from glucose or NH₃ from arginine, and they could grow at pH 4.0, 4.5 and 9.0, in 4.0-6.5% NaCl, and 45°C. It is worth noting that two strains could grow in 8.0% NaCl. All isolates could produce acid from D-arabinose, L-arabinose, ribose, galactose, D-mannose, D-sorbitol, esculin, salicin, D-cellobiose, D-lactose, D-melibiose, D-sucrose, D-trehalose and gluconate. One strain belonging to group 6 was classified as *L. spicheri* based on its characteristics (Meroth et al., 2004), which included producing DL-lactic acid, and gas from arginine but not from glucose. This isolate could grow at 10°C, pH 4.0, 4.5, 9.0 and 9.6, and in 4.0%, 6.0% and 6.5% NaCl, but only could ferment ribose and D-xylose. The strain of group 7 was considered to belong to genus *Leuconostoc*, which produces D-lactic acid and produces gas from glucose, but not hydrolyze arginine. The strain could grow at pH 4.0, 4.5 and 9.0, in 4.0% and 6.0% NaCl at 45°C. It utilized ribose, D-xylose, galactose, D-

mannose, esculin, salicin, D-cellobiose, D-melibiose, D-sucrose and D-raffinose. Two cocci of group 8, identified as genus *Enterococcus*, produced L-lactic acid but could not produce gas from glucose. They could grow at 45°C, pH 4.5, 9.0 and 9.6, in 4.0%, 6.0% and 6.5% NaCl. Those strains could utilize ribose, galactose, D-mannose, esculin, salicin, D-mannitol, D-cellobiose, D-lactose, D-sucrose and gluconate. Five cocci of group 9 were identified as genus *Pediococcus* based on their sugar fermentation test. They produced DL-lactic acid but could not produce gas from glucose or arginine. Most of strains grew well at 45°C and pH 3.5, 4.0 and 4.5, and only one strain could grow in 4.0% NaCl at 10°C. They could utilize galactose, D-mannose, L-rhamnose, esculin, salicin, D-cellobiose, D-lactose, D-sucrose and D-trehalose.

16S rRNA gene sequencing and phylogenetic analysis

To confirm the species, the nucleotide sequences of the 16S rRNA gene of all the tested strains were analyzed and determined by the BLAST program at NCBI (<http://www.ncbi.nlm.nih.gov/>). The obtained sequences (approximately 1,400 bp) were deposited in GenBank and assigned the following accession numbers: GU125423, GU125424, and GU125427-GU125609. Phylogenetic tree analysis was performed to show the relationship of 16S rRNA gene sequences between the representative isolates and related type strains by using MEGA software (Fig. 1). Strain IMAU80014 in group 1 was closely related to *L. alimentarius* DSM 20249^T (M58804) as it showed 99.7% homology to *L. alimentarius* DSM 20249^T (M58804). Strain IMAU80001 of group 2 and type strain *L. brevis* ATCC 14869^T (GU125423) were clustered into a group with a similarity of 99.8%. Strain IMAU80040 of group 3 was placed in the cluster of *L. paracasei* JCM 8130^T (D79212) with a similarity of 100%. Representative strains IMAU80002 and IMAU80005 of group 4 appeared to be equally linked to both *L. plantarum* ATCC 14917^T (AJ965482) and *L. pentosus* JCM 1558^T (D79211), and their 16S rRNA gene sequences showed a similarity of 99.9% to *L. plantarum* ATCC 14917^T (AJ965482) and 99.8% to *L. pentosus* JCM 1558^T (D79211). Strain IMAU80073 of group 5 was closely related to *L. sakei* DSM 20017^T (AM371184) and they shared 99.8% homology. Strain IMAU80039 of group 6 was closely related to *L. spicheri* LTH 5753^T (AJ534844) in 100% of bootstrap analyses. Strain IMAU80137 of group 7 and IMAU80024 of group 8 grouped with *Leu.*

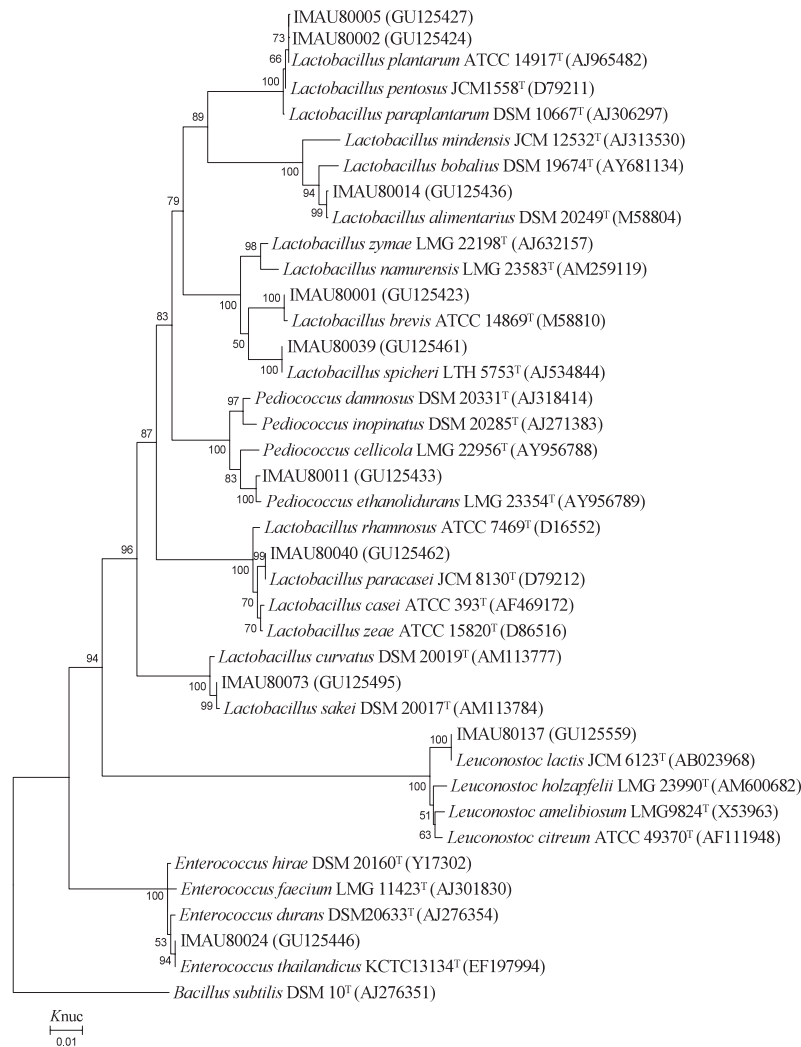


Fig. 1. Neighbor-joining tree based on 16S rRNA gene sequences of representative strains and the reference strains.

Bacillus subtilis was used as the outgroup. Bootstrap values based on 100 replications are given at the nodes.

lactis JCM 6123^T (AB023968) and *Enterococcus thailandicus* KCTC 13134^T (EF197994), and their 16S rRNA gene sequences showed a similarity of 100% to their type strains. Strain IMAU80011 of group 9 was close to *Pediococcus ethanolidurans* LMG 23354^T (AY956789) and they shared 100% homology. Based on 16S rRNA gene sequences analysis, 177 rods isolates from pickles were accurately assigned to 5 species and 1 group, namely *L. brevis* (24 strains), *L. paracasei* (9 strain), *L. alimentarius* (16 strain), *L. sakei* (8 strains), *L. spicheri* (1 strain) and *L. plantarum* group (119 strains); those results are consistent with the results of phenotypic characteristics. Moreover, 8 cocci were characterized as *E. thailandicus* (2 strains), *P. ethanolidurans* (5 strains), and *Leu. lactis* (1 strain);

however, those strains were difficult to identify at the species level by phenotypic characteristics.

Multiplex PCR results

Multiplex PCR was used to distinguish the closely related species of the *L. plantarum* group. The expected sizes of the amplicons were 318 bp for *L. plantarum*, 218 bp for *L. pentosus* and 107 bp for *L. paraplantarum*. 81 strains in group 3 and type strain *L. plantarum* ATCC 14917^T produced 318 bp products, while 38 strains and type strain *L. pentosus* JCM 1558^T produced 218 bp products (Fig. 2).

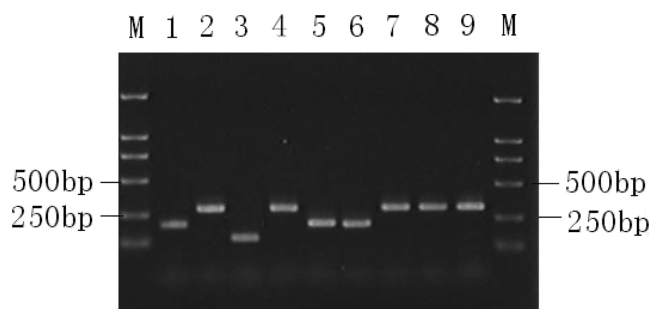


Fig. 2. Multiplex-PCR amplification patterns.

Lanes: M, DL 2000 DNA Marker; 1, *Lactobacillus pentosus* JCM 1558^T; 2, *L. plantarum* ATCC 14917^T; 3, *L. paraplantarum* DSM 10667^T; 4, IMAU80002; 5, IMAU80159; 6, IMAU80005; 7, IMAU80142; 8, IMAU80151; 9, IMAU70042.

Discussion

Pickles are produced using a traditional and ancient method of natural fermentation. This unique method preserves beneficial microorganisms and that indigenous microbiota plays a major role in pickle production, especially the unique flavor and probiotic characteristics. In this study, we determined the composition of LAB and described the phenotypic and genotypic characteristics present in pickles from Sichuan Province of China.

Slight variations in the viable counts of LAB were observed among pickle samples from different regions in Sichuan Province. The average LAB counts of pickle samples from Chongzhou, Dayi, Pujiang and Qionglai were lower than that of samples from Xinjin, and slightly higher than that of samples from Chengdu, and the mean value of LAB in Sichuan pickle juice was $6.58 \pm 1.25 \log \text{cfu ml}^{-1}$. Because the raw material and temperature of pickles are different in the six regions (Table 1), so we considered that this difference in LAB counts may be related to the type of vegetables, source of vegetables and temperature of pickles.

One hundred eighty-five LAB were isolated from pickle samples, and all strains were divided into 9 groups by conventional methods based on physiological properties, nutritional requirements and growth conditions. However, genotypic analysis showed that all these isolates were identified to 10 species. According to physiological properties, strains in group 7, 8 and 9 were identified belong to genus *Enterococcus* (2 strains), genus *Leuconostoc* (1 strain) and genus *Pediococcus* (5 strains), and the 16S rRNA gene sequence analysis showed the representative strains

form a well-defined cluster with their type strains in a phylogenetic tree. Moreover, strains of group 4 were identified as belonging to the *L. plantarum* group (119 strains) based on phenotypic characteristics, and their 16S rRNA gene sequence showed 99.8% similarity to *L. plantarum* and *L. pentosus*. Ennahar et al. (2003) reported that *L. plantarum* and *L. pentosus* have very similar 16S rRNA gene sequences that differ by only 2 bp. In the present study, multiplex PCR assay was applied to discriminate the two species and the result displayed 81 isolates from group 4 identified as *L. plantarum*, while 38 isolates were *L. pentosus*. Traditional phenotypic characteristics often yield variable results for the identification of closely related LAB (Yanagida et al., 2007); therefore phenotypic properties and molecular techniques should combine to accurately identify LAB at the species level.

The distribution of isolates among different samples is presented in Table 3. The predominant isolates of *L. plantarum* (43.8%) were isolated from all sampling sites and most samples, and *L. pentosus* (20.5%), *L. brevis* (13.0%) and *L. alimentarius* (8.6%) were isolated from most sampling sites. Other species isolated with relatively low frequencies, including *L. paracasei*, *L. sakei*, *L. spicheri*, *P. ethanolidurans*, *E. thailandicus*, *Leu. lactis*, *Lactobacillus brevis* and *L. plantarum*, have been reported to be involved in many lactic acid fermented vegetable foods, including sauerkraut and cucumbers (Randazzo et al., 2004). *Leuconostoc mesenteroides* and *L. plantarum* are the most frequently isolated bacteria in kimchi in Korea (Lee and Lee, 2010). Tamang et al. (2005) reported that the major representatives of LAB involved in lactic-fermented vegetable products in India were identified as *L. brevis*, *L. plantarum*, *P. pentosaceus*, *P. acidilactici* and *Leu. fallax*. Park et al. (2009) used a culture-independent technique to confirm that several LAB species, such as *Leu. mesenteroides*, *L. brevis*, *P. pentosaceus* and *L. plantarum*, contribute to the complex pickles fermentation process. Yan et al. (2008) indicated that *L. plantarum* (43.6%), *L. pentosus* (19.1%), *Leu. mesenteroides* (11.0%), *L. brevis* (7.3%) were the predominant species in pickles in China. Similar to our result, *L. plantarum*, *L. pentosus* and *L. brevis* usually predominated in the different fermented vegetables. *Lactobacillus sakei* is usually isolated from fermented vegetables and meat (Andrighetto et al. 2001; Kim and Chun 2005); the versatility can partly be explained by its ability to survive and grow under adverse condi-

Table 3. The distribution of lactic acid bacteria in Sichuan pickles.

Sampling location	Sample number	Strains ^a									
		A	B	C	D	E	F	G	H	I	J
Chengdu	1				5	1					
	2	4									
	3				2	1	1				
	4		1		3						
Chongzhou	5		1		3						
	6						7				
	7			2	1	4					
	8		1		2						
	9		1			5					
	10					2				2	
	11	1				6					
	12				6						
Dayi	13	5									
	14	1		1		5		1			
	15			3		6					
	16				5						
	17		1		1	5					
	18		2	1	1	2					1
	19	1	1		3	2					
	20					3					
	21		5								
	22					6					
Pujiang	23		2			3					
	24					9					
	25	1									
	26			1		4					
	27										1
	28										1
	29	2				4					
Qionglai	30										2
	31		3			1					
	32		4		1				1		
	33	1		1	3	4					
	34				2	5					
	35		2			2					
Xinjin	36					1					
Total		16	24	9	38	81	8	1	1	2	5

^aA = *Lactobacillus alimentarius*, B = *L. brevis*, C = *L. paracasei*, D = *L. pentosus*, E = *L. plantarum*, F = *L. sakei*, G = *L. spicheri*, H = *Leuconostoc lactis*, I = *Enterococcus thailandicus*, J = *Pediococcus ethanolidurans*.

tions, such as low temperature and pH, high salt concentration, ethanol and low water activity (Sorvig et al., 2005). *Lactobacillus spicheri* is typically isolated from sourdoughs (Meroth et al., 2004), and it is rare in fermented vegetables. Only one strain was isolated from the Sichuan pickle of Chengdu City in our study. To our knowledge, *P. ethanolidurans*, *E. thailandicus*, and *Leu. lactis* have never been reported in natural fer-

mented pickles, probably due to their complex nutritional requirements, as well as weaker adaptation to the pickles environment. However, we isolated a small amount of those strains in this study.

The complexity of the microbial composition in pickle products appears to vary by region. We isolated two species of LAB in Xinjin City, three species Pujiang City, four species in Chengdu City, five species in

Chongzhou City, seven species in Qionglai City, and eight species in Dayi City. The presence of LAB with such variation of strains is not surprising because other factors may contribute to the variability of strains, such as sample number, different production methods, and raw materials. Nine and twelve samples were collected from Chongzhou and Dayi, respectively, so more species were isolated from those regions. Furthermore, the environmental temperature and regional differences may also cause some variation in strains.

In conclusion, this report describes the microbiological study and detailed identification of the LAB involved in pickle samples. *L. alimentarius*, *L. brevis* and *L. plantarum* are the dominant LAB species in Sichuan pickles. Other species including *E. thailandicus*, *L. paracasei*, *L. sakei*, *L. spicheri*, *L. plantarum*, *Leu. lactis* and *P. ethanolidurans*, were identified at lower frequencies. The species distribution depends on the manufacturing processes, as well as on the specific ecological locality where the pickle products were manufactured. This study provides raw data and a LAB strain resource for further studies.

Acknowledgments

This research was supported by National Natural Science Foundation of China (Grant No. 31025019), the Earmarked Fund for Modern Agro-industry Technology Research System (Grant No. nycyt-0501), the Prophase Research Program of the 973 Project of China (Grant No. 2010CB134502), the Innovation Team Development of the Ministry of Education of China (Grant No. IRT0967), and the Hi-Tech Research and Development Program of China (863 Planning, Grant No. 2011AA100901, 2011AA100902).

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