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Culture-Dependent Analysis on Microbial Dynamics during Suan Cai Fermentation in Northeast China

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In this study, in order to analyze the microbial dynamics during Chinese suan cai fermentation, seventy-three strains were isolated using culture-dependent method. DNA extraction, PCR amplification of 16S rDNA and sequencing were used to identify the strains. At day 6 and day 12 of fermentation, strains were mainly spherical bacteria, including *Leuconostoc mesenteroides* and *Lactococcus lactis*. At day 26 of fermentation, bacteria mainly belonged to *Lactobacillus*. In addition, *Paenibacillus* sp. and uncultured *bacterium* were also detected. Biochemical results showed that the pH value decreased from 7.3 to 4.1 after 8 d fermentation. Lactic acid bacteria and other bacteria dramatically proliferated in the first 6 d fermentation. The accumulation of lactic acid and acetic acid continued to the 26th day of fermentation, and the values were 6.5 and 0.64 g/L, respectively. Lactic acid was the main volatile product. When fermentation temperature was 16 ºC, suan cai was mature after 18 d fermentation. The results of this study provide the fundamental data for screening the effective inoculant used in suan cai fermentation.

Key words: Sauerkraut, Suan cai, Fermentation, Lactic acid bacteria, Microbial dynamic.

In China, suan cai, a Chinese fermented vegetable is a popular food for its crisp taste, appetite, nutrient and unique flavor. In the past, fresh vegetables couldn't be stored for a long time due to the longer winter. Fermentation method was widely used for making suan cai[1]. Currently, the demand for suan cai in market is increasing rapidly. The production scale of suan cai becomes greater. However, traditional fermentation is generally used in the enterprise of producing suan cai until now. The problems such as unstable quality and long-time fermentation are common[2].

Vegetable fermentation involves in a variety of physical and chemical changes. Microorganisms are the origin of these changes[3, 4]. For fermenting the vegetables with good flavour and high quality, it is vital to know about the microbial dynamic systematically[5]. Microbial investigation during natural fermentation is essential for conversion from natural fermentation to artificial fermentation[6-8].

Microbiological studies about suan cai fermentation began in the 1990s. *Klebsiella*, *Halobacterium*, *Acetobacter*, *Lactobacillus* and *Micrococcus* have been detected by the combination of isolation and morphological identification[9]. Lactic acid bacteria such as *Lact. plantarum*, *Lact. brevis*, *Lact. minor*, *Lact. fermentum*, *Lact. harbinensis* and *Pediococcus acidilactici* are often isolated[10-12], and lactic acid bacteria were proved to play a key role during suan cai fermentation[13]. Traditional isolation and molecular identification were used for analyzing microbial diversity of fermented suan cai at the latter fermentation in China recently, and the results showed that the lactic acid bacteria are composed of *Lactobacillus*, *Pediococcus*, *Leuconostoc* and

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Weissella[14]. *Lactobacillus* includes *Lact. plantarum*, *Lact. brevis*, *Lact. reuteri* and *Lact. sakei*[15]. From the previous reports, the studies of microbial diversity focused on some time-point of the latter fermentation. The analyses of microbial dynamic during suan cai fermentation, especially at the early stage, are lack.

In the present study, microbial dynamic during suan cai fermentation was analyzed using the plate-isolation method. Moreover, the biochemical compositions of the suan cai fermentation system were detected. The present study will provide the fundamental data for screening the effective inoculant used in suan cai fermentation.

MATERIALS AND METHODS

Fermentation

The Chinese cabbages used in this study were the same species. Suan cai was pickled using the traditional fermentation method. After harvest, the cabbages were air-dried for two days and were layered into a 750 L tank after cleaning. Then, salt and water (mass ratio of 1:100) were added into the fermentation system. The cabbages were pressed with a stone and fermented for 30 days. The fermentation was carried out in triplicate. Sampling time was set at 0 d, 6 d, 12 d and 26 d of fermentation.

Measurement of temperature and pH value

The system temperature was monitored using a thermometer with its probe deep into 10 cm in depth under the surface during the fermentation, and the data was recorded every 7:00 AM. The pH value was measured using a pH meter (B-212, Horiba, Japan) by taking 0.2 mL sample at 5 cm in depth under the surface.

Sampling and preparation

The Chinese cabbage of the third layer under the surface was taken for sampling at every experimental time-point. The fermentated juice (0.5 mL) of cabbage squeezed was used for microbial enumeration and isolation. Volatile products were analyzed using high performance liquid chromatography (HPLC). After centrifugation of fermented juice at 5800 *g* for 10 min, 0.5 mL supernatant was mixed with 0.5 mL acetonitrile (HPLC grade, Merck, Germany). The mixture let stand for 10 min and centrifuged at 10800 g for 10 min. The supernatant was collected and stored at -20 ºC for the following HPLC analyses. The same batch of fermentation juice was measured thrice.

Microbial isolation and cultivation

Microbial enumeration was carried out using plate dilution method. Lactic acid bacteria were cultivated using LAB medium[16]. The main composition of LAB medium (1000 mL) was as follows: beef extract 10 g, yeast extract 10 g, lactose 20 g, Tween 80 1 mL, $CaCO_3$ 10 g, KH_2PO_4 2 g, distilled water 950 mL, pH 6.6. Common bacteria were cultivated using nutrient agar medium[17]. The plates were incubated for 48 h at 30º C for lactic acid bacteria or at 37 º C for common bacteria. After counting, the representative strains were chosen for further streaking and sequencing based on the colony morphology. The isolated strains were stored at -70 ºC using glycerol freezing method[18].

Analyses of volatile products

An HPLC system (Waters, USA) with a dual λ absorbance detector (Waters 2487) was used to determine volatile products (VPs). The Aminex 87H column (Bio-Rad, USA) was equipped. The column temperature was kept at 40 ºC. The mobile phase was 5 mM sulfuric acid. The procedure time was 30 min and the flow rate 0.6 mL/min. Statistical analysis was performed using SPSS software (13.0, SPSS China).

DNA extraction and PCR amplification

Total bacterial DNA extraction was carried out using a kit (OMEGA BIO-TEK, USA) according to the manufacturer's introduction. The extracted DNA was purified using RNA enzyme, and then was used as template for PCR. The 50 µl PCR amplification system included: 15 ng template DNA, 1 × buffer, 0.16 mM dNTP, 1.5 mM $MgCl_2$, 0.45 µM of each primer and 1U Takara rTaq DNA polymerase (Takara, Japan). The gene primers for amplification of 16S rRNA were 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3'). PCR was carried out with an initial denaturation step of 5 min at 94 °C and 1 min at 93 ºC, annealing for 1 min at 50 ºC, extension for 1 min 30 s at 72 ºC, 30 cycles, and 1 min at 72 °C, and a final extension at 72 °C for 3 min [19].

Sequencing

The PCR products were purified using a DNA purification kit (Tiangen, Beijing, China). Sequencing of the PCR products was performed by Sangon Biotech (Shanghai, China). The

sequence similarity was analyzed using GenBank database, and then the phylogenetic tree was constructed using Mega5 software[20].

RESULTS

Changes of temperature, pH and sensory evaluation

Dynamics of temperature and pH during suan cai fermentation were shown as Fig.1. Since the fermentation began in October, the system temperature gradually got lower as the fermentation progressed. It can be observed that pH value mainly decreased (from 7.3 to 4.1) in the first 8 d of the fermentation. During this period, the average temperature of the fermentation system was 18 ºC. After then, the temperature kept decreasing, while the pH value remained around 4.0.

The status of the Chinese cabbage was reflected by Fig. 2. The result indicated that it took 18 days to get the cabbage thoroughly pickled when the average temperature is 16 ºC. The pickled suan cai was crisp with a strong flavor of sourness.

Table 1. Microbial enumeration and chemical analyses of during suan cai fermentation

Time (d)	LAB* (Log CFU/g DM #)	Bacteria (Log CFU/g DM #)	Lactic acid (g/L)	Acetic acid (g/L)
0	- $	-	-	-
6	7.8±0.1b	7.9±0.6a	3.5±0.2c	0.31±0.04c
12	8.3±0.1a	6.5±0.2b	4.6±0.2b	0.42±0.03bc
26	8.4±0.2a	3.0±0.1c	6.4±0.5a	0.61±0.11ab

*LAB, lactic acid bacteria; #CFU, colony forming unit; DM, dry matter; $-, not detected; a, b, c, in one column mean significantly different (P<0.05).

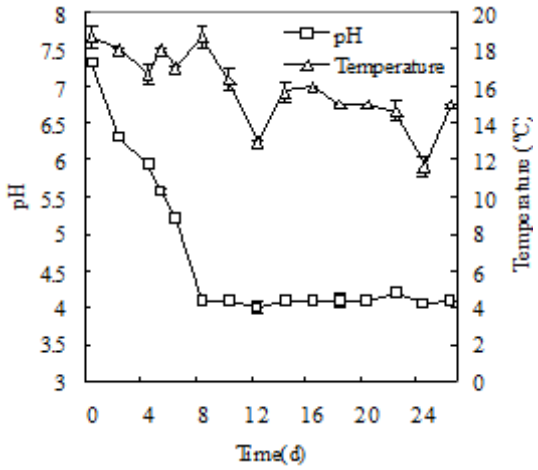

Fig. 1. Dynamics of temperature and pH during suan cai fermentation

Microbial enumeration and volatile products

The dynamics of bacteria are shown in Table 1. Common bacteria and lactic acid bacteria mainly proliferated in the first 6 d. The colony forming unit (CFU)of common bacteria reached its maximum value at 6 d of fermentation. Variation of lactic acid bacteria was obviously different. The CFU of lactic acid bacteria kept increasing after reaching 7.8 at 6 d of fermentation, and remained around 8.3 till the fermentation was completed. Volatile products detected in the fermentation system included lactic acid and acetic acid at 26 d of fermentation. It is evident that lactic acid is the principal product of the volatile products.

Microbial isolation and identification

Representative results of plate streaking

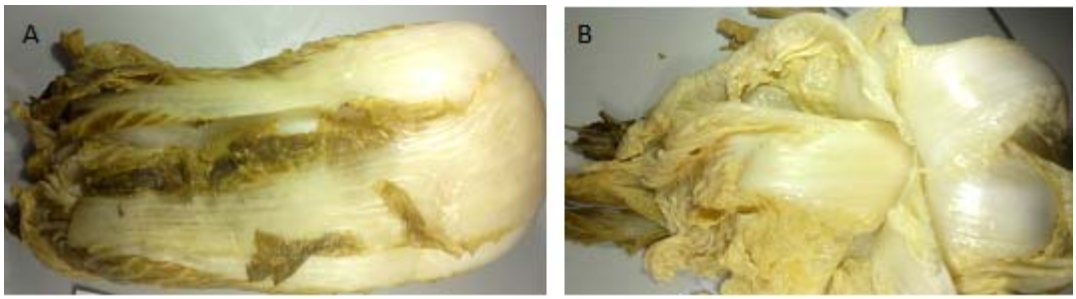

Fig. 2. The status of fermented Chinese cabbage at the 18th d fermentation

and Gram staining are shown in Fig. 3. Seventy-three strains were obtained. All strains were cultivated on LAB medium. As LAB medium contains $CaCO_3$, acid-producing bacteria grown on the LAB agar are able to generate transparent zones. There were 56 strains could generate transparent zones. They were preliminarily identified as lactic acid bacteria. From Gram staining, results the lactic acid bacteria were mainly composed of Gram-positive cocci and bacilli.

Microbial diversity

Microorganisms isolated at 6 d, 12 d and 26 d of fermentation were shown in Fig. 4, Fig. 5 and Fig. 6, respectively. At 6 d of the fermentation, there were 10 strains belonging to *Leuconostoc*, with *Leuconostoc mesenteroides* as their relative species. *Lactococcus* (10 strains, *Lactococcus lactis* as relative species), *Enterococcus* (3 strains,), and *Kluyvera cryocrescens* (1 strain) were also isolated. The result from Fig. 5 showed that the strains isolated at 12 d of the fermentation belonged to *Leuconostoc*, *Lactococcus*. and *Enterococcus*. The microorganisms isolated belonged to cocci.

As Fig. 6 indicated, microorganisms isolated at 26 d of fermentation included *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus sakei*, *Lactobacillus curvatus*, *Lactobacillus casei*, *Lactobacillus paracasei* and *Leuconostoc mesenteroides*. *Paenibacillus* sp. and uncultured bacteria were also isolated. Most of the obtained lactic acid bacteria at 26 d of fermentation were lactobacilli.

DISCUSSION

Temperature and pH

The average temperature was 16 ºC during the whole process of fermentation. The pH value mainly decreased at the primary stage of fermentation, when the temperature was relatively high (Fig. 1). During this period, lactic acid bacteria and common bacteria also increased rapidly (Fig. 1). It is obvious that at the primary stage of fermentation, the high temperature helped the microbial growth. At the same time, carbohydrates

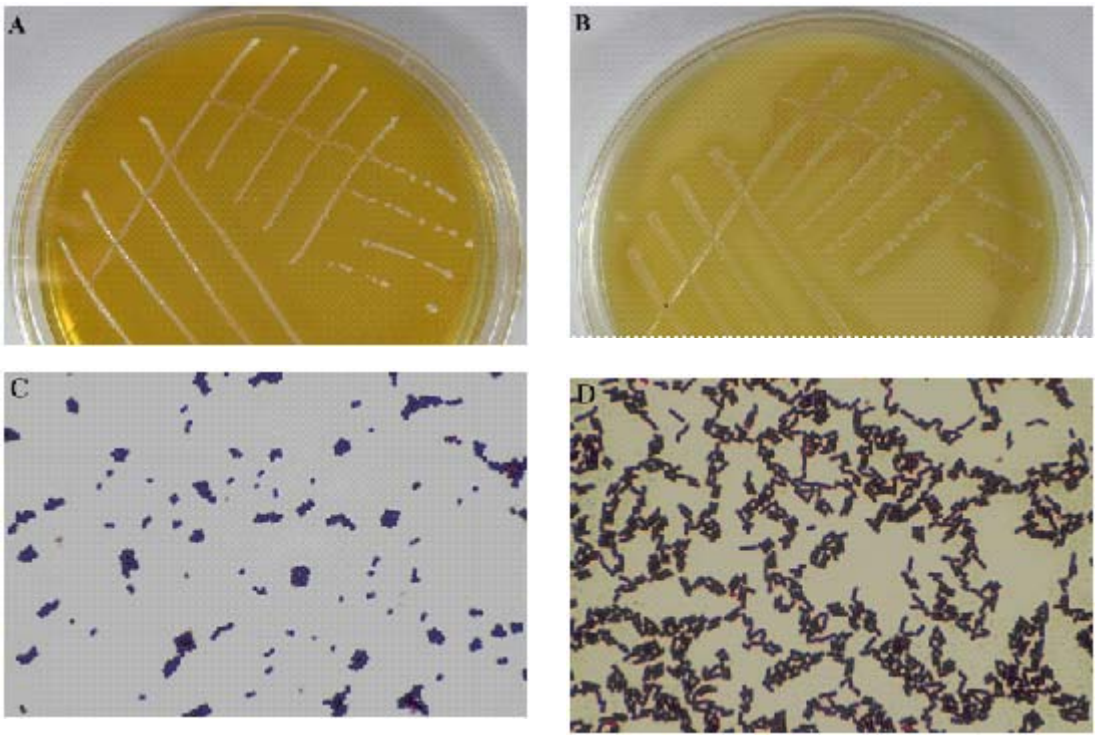

Fig. 3. Colonial morphology and Gram-staining results of typical strains. A, B: colonies growing at the media; C, D: Gram-staining results

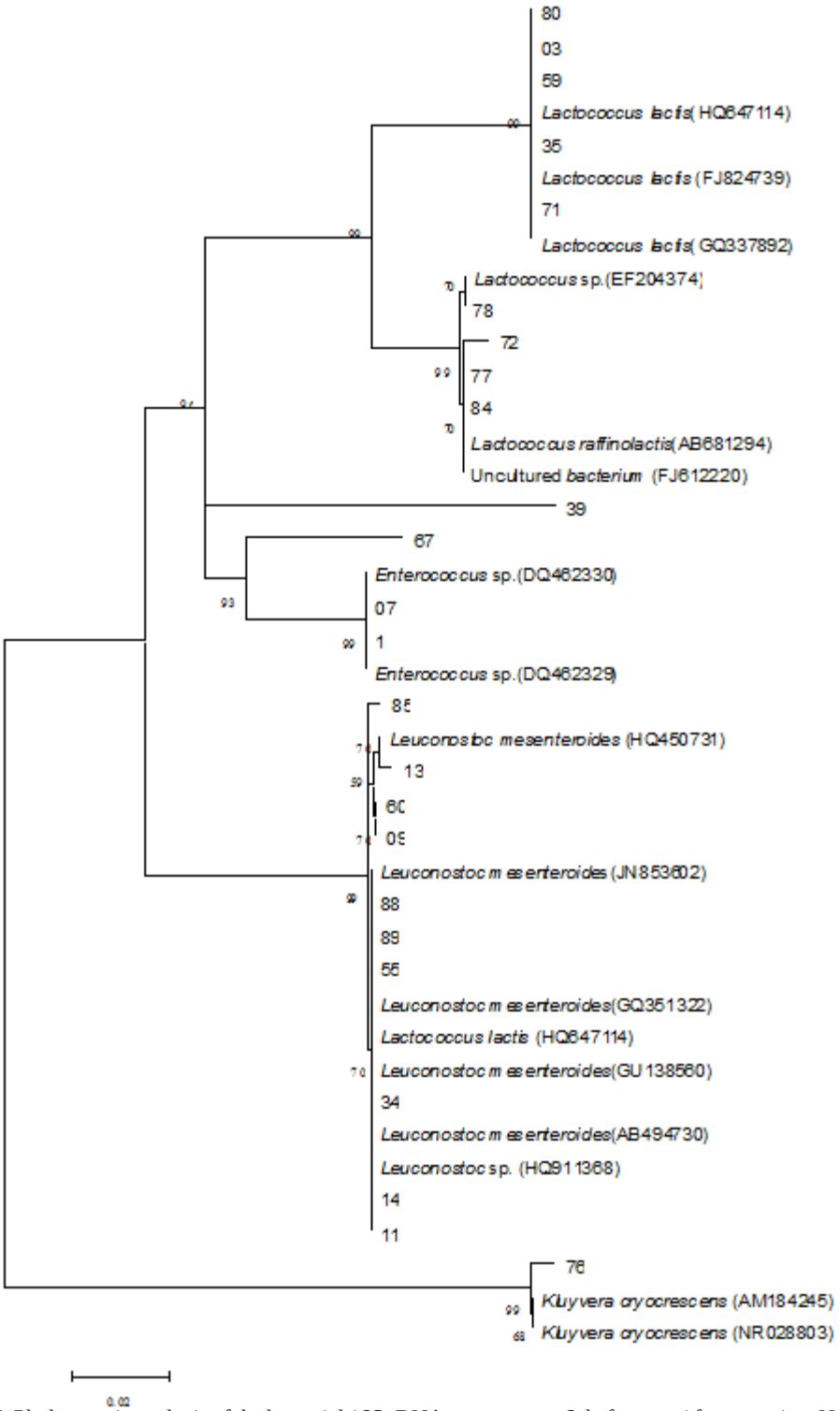

Fig. 4. Phylogenetic analysis of the bacterial 16S rDNA sequences at 6 d of suan cai fermentation. Neighbor-joining method was used. The bar represents 2% sequence divergence. The number in parentheses shows the GenBank accession number. 80, 03 59… in the phylogenetic tree expressed the ID of strain

in the fermented system also consumed rapidly. When the proportion of lactic acid and acetic acid increased (Table 1), the pH in the system decreased dramatically. As the fermentation progressed, the acidic environment aid the proliferation of lactic acid bacteria. The common bacteria reached its peak at 12 d of fermentation. After then, the growth of common bacteria was restricted. This restriction was partly due to accumulation of volatile organic acids in the fermentation system[21], and secretion of antibacterial substances from the lactic acid bacteria in the system[22].

Lactic acid bacteria during suan cai fermentaiton

In this study, 73 strains were isolated and DNA extraction, PCR amplification and 16S rDNA sequencing were applied to identify the microorganism. At 6 d of fermentation, the isolated microorganism included *Leuconostoc*, *Enterococcus* and *Lactococcus*. These strains are all heterofermentativ and belong to cocci. Lactic acid bacterial species isolated at 12 d of fermentation were the same as those at 6 d of fermentation. Lactic acid bacteria that were isolated at 26 d of fermentation mainly included lactobacilli. And a large number of homofermentative lactic acid bacteria were found. The results indicated that heterofermentative lactic acid bacteria were predominant during the primary stage of Chinese

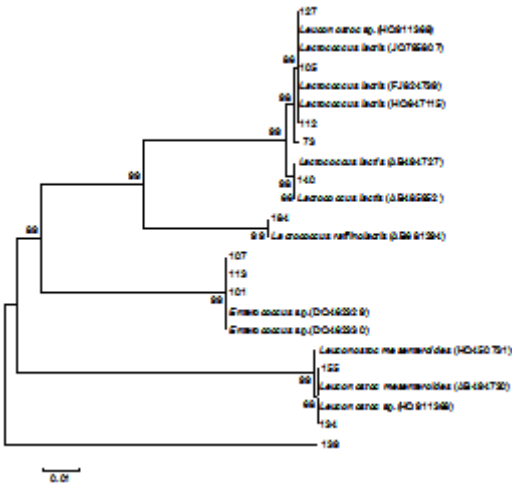

Fig. 5. Phylogenetic analysis of the bacterial 16S rDNA sequences at 12 d of suan cai fermentation. Neighbor-joining method was used.The bar represents 1% sequence divergence. The number in parentheses shows the GenBank accession number. 127, 105, 112…in the phylogenetic tree expressed the ID of strain

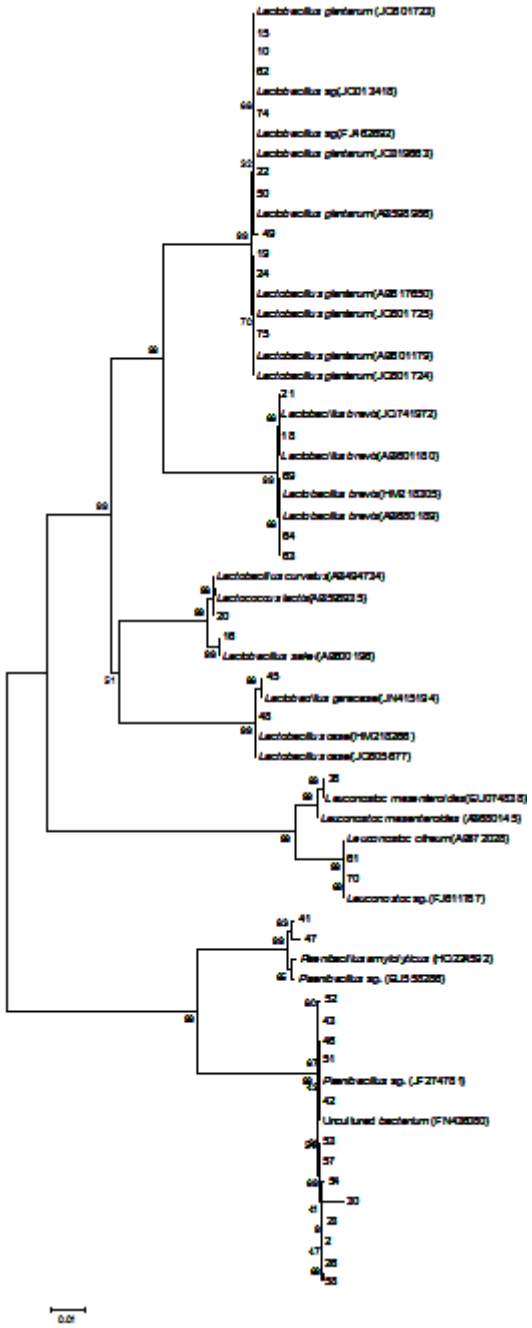

Fig. 6. Phylogenetic analysis of the bacterial 16S rDNA sequences at 26 d of suan cai fermentation. Neighbor-joining method was used.The bar represents 1% sequence divergence. The number in parentheses shows the GenBank accession number. 15, 10, 62…in the phylogenetic tree expressed the ID of strain

cabbage fermentation. While homofermentative lactic acid bacteria were predominant during the latter stage of fermentation. The result is also consistent with previous findings concerning microbial isolation from fermented vegetables[23].

Microorganisms except for lactic acid bacteria

In this study, there were 17 strains were not lactic acid bacteria. At 6 d of fermentation, *Kluyvera cryocrescens* was isolated. *Kluyvera* is an opportunistic pathogen, which is Gram-negative and can be isolated in various environments[24]. *Paenibacillus* isolated at 26 d of the fermentation. This kind of microorganisms are gram-positive, facultative anaerobic, nitrogen-fixing. They can generate endospores and grow in various environments[25]. There are no reports about these microorganisms isolated from the fermented vegetables.

Possible microbial inoculant for suan cai fermentation

The microflora during the fermentation has been systamatically studied in American sauerkraut[4]. Fleming et al have indicated that the sauerkraut fermentation can be divided into two stages, that is primary heterofermentation and latter homofermentation. Lactic acid bacteria became the predominant microbes. At the primary stage of the fermentation, *Leuc. mesenteroide* was the main bacterial species, while *Lact. pantarum* dominated in latter stage of fermentation. They also found that the extention of early homofermentation could help prevent excessive sourness and generate more flavor substances. Base on these results, microbial inoculants were selected to serve for the industrial production of the American sauerkraut[26,27]. The inoculants were made from *Leuc. mesenteroides* or mixed microorganisms mainly composed of *Leuc. mesenteroides*.

The natural fermentation is generally being employed in present production of suan cai. In order to accelerate the conversion of natural suan cai fermentation into artificial fermentation, selecting effective microbial inoculant is the key point in further study. In this study, bacterial diversity was analyzed using isolation, especially at the early stage of fermentaion. A variety of heterofermentative lactic acid bacteria and homofermentative lactic acid bacteria were obtained. The following work is use the current strains to clear the effects of homofermentative lactic acid bacteria and/or heterofermentative lactic acid bacteria on suan cai fermentation. The results will provide technical support for the selection of the effective and stable microbial inoculant.

CONCLUSION

Microbial dynamic of bacteria during the fermentation of traditional suan cai using isolation and identification. The result showed that at the primary stage of the fermentation heterofermentative lactic acid bacteria were predominant. *Leuconostoc*, *Enterococcus* and *Lactococcus* were isolated. In the latter stage of fermentation, homofermentative lactic acid bacteria (mainly *Lactobacillus*) were predominant. Meanwhile, common bacteria including *Kluyvera* sp. and *Paenibacillus* sp. were also isolated during the fermentation. The results provide basic data for further selection of microbial inoculant using for suan cai fermentation.

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