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Research Article

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Study on the diversity of microbial communities and chemical constituents in Sichuan pickles

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ABSTRACT

Natural fermented pickled vegetables were traditional food in Sichuan province about three thousand years ago. Microbial communities and chemical constituents of some pickled vegetable samples with different fermented period which collected from Chengdu area had been investigated. The results showed as following: firstly, strains that belonged to genus Lactobacillus and Leuconostoc were the main groups in all samples by the traditional fermentation process; all samples have been polluted by strains that belonged to Enterobacteriaceae to a certain extent. Secondly, the biogenic amines and nitrite content in pickled vegetable sample with 10 years fermented period were the highest among all samples; Thirdly, combining with pickled vegetable's taste and food safety, we believe that the natural pickled vegetables which is not pickled too long may be better for human's health.

Keywords: Pickled vegetables; Bacterial community structure; Chemical Constituents

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INTRODUCTION

As traditional natural fermented food, Sichuan pickled vegetable has a long history of over 3000 years. Because of its crisp, refreshing and as appetizer, Sichuan pickled vegetable has been loved by people. Traditional Sichuan pickled vegetables were soaked by fresh vegetables, mostly using brine to pickle, fermented by lactic acid bacteria from natural environment, and accompanied by alcohol and acetic acid fermentation [1, 2]. In the process of soaking, in order to make Sichuan pickles have the unique flavor and taste, pickled vegetable water is irreplaceable. Pickled vegetable water is usually rich in the probiotics that may help to support the intestinal microflora balance of human beings, and can cure infantile diarrhea, promoting the function of stomach in children [3]. In recent years, the researchers have screened probiotics from pickled vegetables to develop probiotic foods.

Compared to other vegetable processing, after the fermentation by lactic acid bacteria, Sichuan pickled vegetables are rich in minerals, vitamins, carbohydrates, amino acids, protein, fat and other nutrients. What's more, it contain lots of active lactic acid bacteria, which can adjust the human intestinal micro-ecological balance, promoting the absorption of nutrients. In the spontaneous fermentation process of making Sichuan pickled vegetables, along with seasonal vegetables are soaked and nutritional components dissolve into the brine, these nutrients can provide conditions for microbial growth and metabolism, and the soak vegetables show different quality according to different fermentation period. The quality control of pickled vegetables is mainly based on its sensory aroma, color, morphology and physicochemical required parameters (such as pH, salinity) to evaluate [4]. In this experiment, we

attempt to investigate microbial community and chemical constituents of soaked pickled vegetables with different fermentation period and provide a consult for healthy of Sichuan pickled vegetables.

EXPERIMENTAL SECTION

Traditional fermented pickled vegetable water samples preparation

The pickled vegetable water samples were collected from several farmhouses in Chengdu, which have been fermented for 1 years, 5 years and 10 years respectively by soaked vegetables, the corresponding marks of samples for different period are SC1, SC5 and SC10.

DNA extraction and MiSeq sequencing of 16S rRNA gene amplicons

Total DNA of samples was extracted using a method previously described [5] and its quality was checked using a NanoDrop Spectrophotometer. Extracted DNA was diluted to 10 ng/μL and stored at −20 °C for downstream use.

PCR amplification was conducted with primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) designed to be universal for bacteria V4 region in 16S rRNA gene [6]. In order to minimize PCR bias, two PCR reactions were set up for each sample, and the PCR products in the replicate reactions were pooled. The details in PCR and related procedures were described previously [7]. The amplicons from each sample were pooled with an equimolar concentration and sequencing with an Illumina Miseq system (Illumina Inc., San Diego, CA).

All sequence reads were trimmed and assigned to each sample based on their barcodes. Multiple steps were required to trim the sequences by using QIIME Pipeline–Version 1.7.0 (http://qiime.org/tutorials/tutorial.html), such as removal of sequences less than 220 bp. The aligned 16S rRNA gene sequences were used for a chimera check using the Uchime algorithm [8], sequences were clustered into operational taxonomic units (OTUs) at a 97% identity threshold. Taxonomy was assigned using the Ribosomal Database Project classifier [9]. The phylogenetic affiliation of each sequence was analyzed by Ribosomal Database Project (RDP) Classifier at a confidence level of 80%. To assure the accuracy of RDP Classifier results, the representative sequences of dominant bacteria was subjected to BLAST homology search against the non-environmental sequences and non-metagenomes in the NCBI Nucleotide Database (http://blast.ncbi.nlm.nih.gov).

Biogenic amines, nitrite and physicochemical indicators (salinity, sugar content and pH)

The concentration of biogenic amines in samples was determined using acid extraction according to Saarinen [10], followed by the dansyl chloride derivatization reaction according to Dugo et al. [11] with slight modifications. Samples were detected and quantified by reverse-phase high performance liquid chromatography (model 1100; Agilent Technologies, Santa Clara, CA) according to Dugo et al [11].

Nitrite content of pickled vegetable water was determined using a colorimetric nitrite assay as described by Yan et al [12].

pH was measured using a micro computerized pH meter Crison 2001 (Crison Barcelona, Spain), inserting the electrode directly in the pickled vegetable water. Sugar content (mainly glucose and sucrose) was measured with Brix spindke and the analysis of salt content using the classical Mohr's method titration procedure [13].

The aromatic compounds of pickled vegetable water

The extraction of volatiles was carried out according to Qian and Reineccius [14] with some modifications. Briefly, pickled vegetable water samples (0.1mL) with different fermented period were exactly added into a 5mL headspace bottle. After the equilibrium time of 15 min, the SPME fiber was exposed to the headspace of pickled vegetable water samples for the extraction time (45min) at the extraction temperature (60 °C), and then the SPME fiber was desorbed into the injection port at 250 °C in splitless mode (2–3min).

Samples were analyzed in triplicate on a Trace GC Ultra gas chromatograph-DSQ II mass spectrometer (Thermo Electron Corp.) equipped with a TR-5MS capillary column (30.0 m×0.25 mm i.d., 0.25 μm film thickness; Thermo Electron Corp.). Gas chromatographic analyses were performed under the following conditions: an inlet temperature of 250 °C, splitless mode, and a helium (purity: 99.999%) carrier gas flow of 1mL min^{-1}. The oven was temperature programmed from 40 °C (5 min) to 220 °C (5 min) at 5 °C min^{-1}.

Mass spectrometric conditions: the transfer line and ionization source were thermostated at 250 °C and 200 °C, respectively. Mass spectra were recorded in electron impact (EI) mode at 70 eV within the *m/z* range 40–500.

Volatile compounds were identified by comparing their mass spectrum with the NIST2005 library database. Quantitative data were obtained by calculating the peak areas of mass spectrum in relation to that of the IS.

RESULTS AND DISCUSSION

The constitution of bacteria in pickled vegetable samples with different pickled period

The most abundant families were *Lactobacillaceae*, *Leuconostocaceae*, *Enterobacteriaceae*, *Pseudomonadaceae*, and the family *Lactobacillaceae* was prominent among all samples (Fig. 1). As the most abundant family, the relative abundance of family *Lactobacillaceae* in samples SC1 pickled for 1 year and samples SC10 pickled for 10 years were 19.28% and 79.43%, respectively. In addition, *Leuconostocaceae* accounted for 4.98% of total bacteria in SC1, and decreased to 0.25% and 0.08% in SC5 and SC10, respectively. However, the relative proportion of *Enterobacteriaceae* in SC10 (8.66%) was more higher than SC5 (6.46%) and SC1 (0.08%), suggesting that SC10 may had been polluted more seriously than other samples [15, 22].

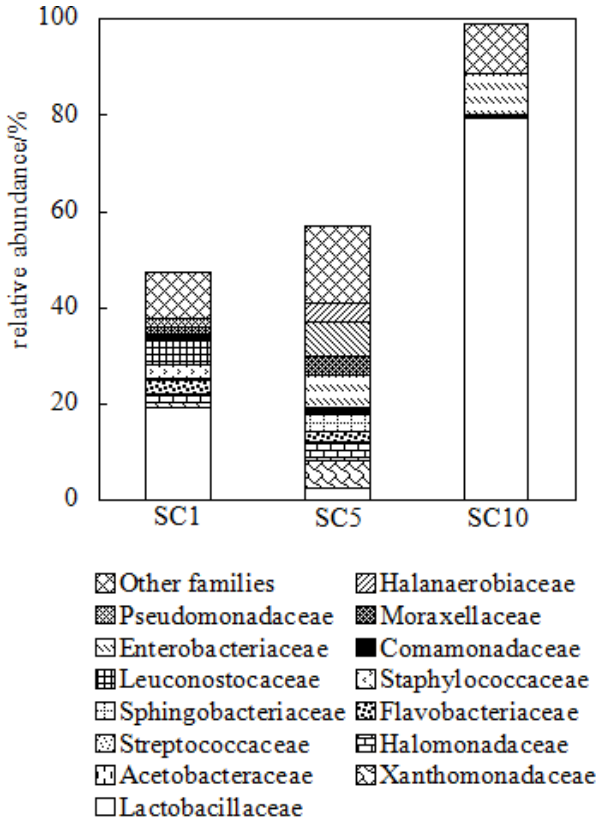

Fig. 1 Prokaryotic community structure at family level in pickled cabbage water

At genus level, no matter the difference of samples' fermentation period, genus *Lactobacillus* was always the main group in the structure of the microbial community (Fig. 2), this was corresponded to some reports about Sichuan pickled vegetables [19]. In addition, population of genera *Leuconostoc* and *Pediococcus* decreased significantly along with duration of pickled vegetables, from 3.90% and 1.25% of samples SC1 to 0.07% and 0.02% of samples SC10. This may because the *Leuconostoc* growth can be affected by pickled cabbage raw accessories, temperature and salinity [16, 17]. On the other hand, much higher proportion of genus *Lactobacillus* was detected in pickled vegetable water: 11.24% in samples SC1, 52.54% in samples SC10, which is consistent with some reports that only after *Leuconostoc* started with pickled vegetable fermentation [18, 19], could *Lactobacillus* actually become the predominant genera with the accumulation of fermentation metabolites, probably due to its distinct features of high acid tolerance as well as high production of lactic acid [20].

In samples SC5, the relative abundance of family *Lactobacillaceae* was 2.73%, and its genus *Lactobacillus* was 1.89%. The lower abundance of family *Lactobacillaceae* in comparison with SC1 and SC10 may be due to the raw materials which were not cleaned thoroughly, the hygienic condition of manufacturing process and the contaminant bacteria in storage space.

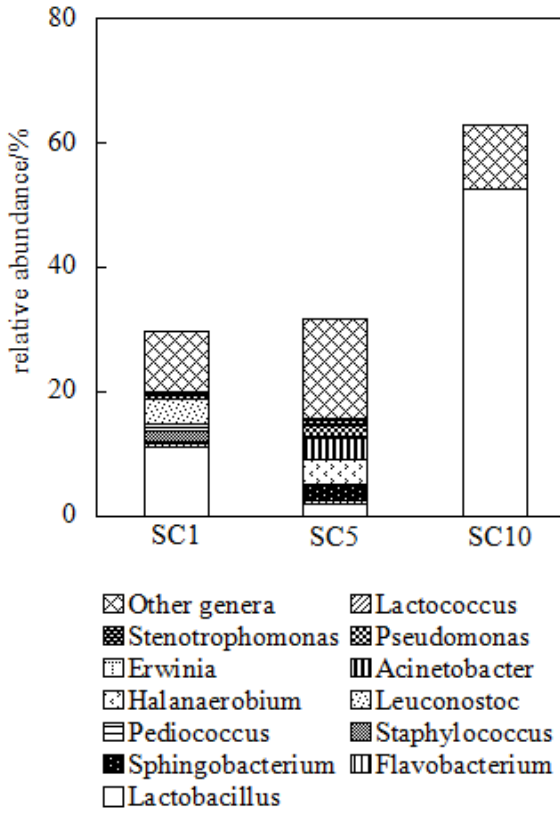

Fig. 2 Prokaryotic community structure at genera level in pickled cabbage water

Analysis of biogenic amines

Table1 Regression equation, R^2 and detection limit of biogenic amine were detected by HPLC

Biogenic amine	Regression equation	R^2	Detection limit (mg/L)
SPD	Y=106X+545	0.9104	0.050
PU	Y=676X+741	0.9974	0.010
CA	Y=164X+444	0.9976	0.011
HI	Y=559X+199	0.9957	0.010
SPM	Y=29X+128	0.9977	0.019

SPD: spermidine; PU: putrescine; CA: cadaverine; HI: histamine; SPM: spermine

The peak area of 5 biogenic amines and their corresponding concentration showed a linear relationship in a linear range of 0.1–10 mg/L; putrescine, cadaverine, histamine and spermine were less than 0.99, spermidine within 0.9 (Table 1); signal to noise ratio (S/N) was 3:1, the lowest detection limit was 0.010 mg/L.

Table 2 Concentration of BA in pickled vegetables (mg/L)

Biogenic amine	(mg/L)	SC1	SC5	SC10
SPD		1.393	0.199	ND
PU		1.137	0.657	0.924
CA		ND	1.485	4.386
HI		ND	0.203	0.319
SPM		ND	0.516	1.865

SPD: spermidine; PU: putrescine; CA: cadaverine; HI: histamine; SPM: spermine
ND, Not determined, as biogenic amine was below the detection limit

The types and concentration of biogenic amines in different samples are distinguishing (Fig. 3; Table 2). Among 5 kinds of biogenic amines which have been tested, cadaverine and histamine showed increased concentration with the duration of pickled vegetables. In samples SC10, which had been pickled for 10 years, the cadaverine content reached 4.386 mg/L. Furthermore, its total amounts of biogenic amine were the highest (7.494 mg/L), comparing with samples SC1 and SC5. The content of cadaverine seems to be strongly linked to some bacteria (such as *Enterobacteria*), as reported Latorre-Moratalla [21] and Bover-Cid [22] who showed that *Enterobacteria* are the

main factor responsible for cadaverine formation. Besides, the type and concentration of sugar (mainly glucose and sucrose) were useful for reducing cadaverine [23]. Therefore, the largest proportion of *Enterobacteria* among all samples was observed in SC10 (Fig. 1, Table 2, Table 3).

Fig.3 shows biogenic amines' HPLC chromatogram in pickled vegetables and its concentrations are listed in Table 2

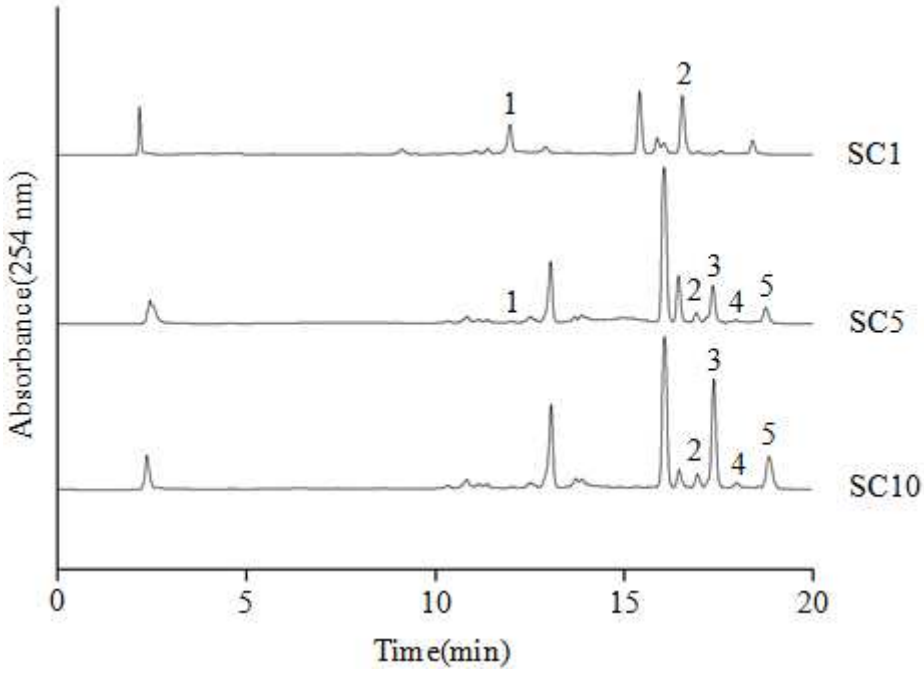

Fig. 3 HPLC chromatograms of pickled vegetable water. 1.spermidine; 2.putrescine; 3.cadaverine; 4.histamine; 5.spermine.

Analysis of physicochemical parameters

Table3 Physicochemical parameters of pickled vegetable water

Samples	pH	Sugar (% w/w)	NaCl (% w/v)	Nitrite (mg/L)
SC1	4.10	4.13	9.06	0.232
SC5	3.98	3.91	7.01	0.177
SC10	3.84	2.22	11.28	0.235

The samples' pH value, sugar content and salinity have been tested, and the results are shown in Table 3, pH value is lower with longer period of pickled vegetables. Salinity of samples SC1 and SC5 were 9.06% and 7.01% respectively, but concentration of NaCl of SC10 was 11.28%. Combining with prokaryotic microbial community of these samples (Figs. 1,2), we find that the high concentration of NaCl may has certain antibacterial effect, and it is the same as previous reports [24]. All these results suggested that halo-tolerant and acid-resistant strains of genus *Lactobacillus* had dominated in sample SC10. In addition, the nitrite concentration in pickled vegetables were very low, and lower than the Chinese national standard for food [25]. What's more, the concentration of nitrite and salt in SC5 is the lowest (Table 3), which is in agreement with previous reports that during the process of making pickled vegetables, the producing of nitrite was inhibited effectively with treatment of low content of salt [26], so lower content of sodium chloride could restrain generating of nitrite. Besides, the concentration of biogenic amines and nitrite content in SC10 were the highest among samples (Table2; Table3).

Characterization of the volatile composition of pickled vegetable water

The SPME GC-MS method was applied to the characterization of pickled vegetable water samples. Percent quantitative data for a total of 52 volatiles identified in all samples under study are listed in Table 4.

Volatile compounds have a great influence on the flavor of pickled vegetables. From Table 4, all samples have lots of volatile compounds, samples SC1,SC5 and SC10 have been identified with 39, 29 and 27 aromatic compounds (including the same 13 kinds of aromatic compounds) respectively. Sample SC1 have 9 alcohols, 15 organic acids, 4 esters, and 4 aldehydes; Sample SC5 also have 9 alcohols, 7 organic acids, 4 esters and 2 aldehydes; Sample SC10 have 11 alcohols, 4 organic acids, 4 esters, 1 aldehyde and 1 ketone. Table 5 shows that the shorter of period for

vegetable pickling, the more volatile compounds produced. But the longer for pickling, the ratio of alcohols, organic acids and esters were higher.

Table 4 Volatile composition and contents in pickled vegetable water

no.	Compound	Relative data (%)[a]		
		SC1	SC5	SC10
1	3-methyl-1-butanol	1.83	0.29	2.06
2	cineole	-	0.29	0.79
3	3-methyl-1-pentanol	0.99	0.12	0.13
4	hexyl alcohol	0.32	0.04	0.12
5	1-isopropyl-2-methylbenzene	-	0.05	0.03
6	1-nonanal	0.58	0.08	-
7	ethyl lactate	0.8	0.55	0.92
8	3-hydroxy-2-butanone	-	-	0.08
9	acetic acid	7.14	2.08	-
10	dodecyl aldehyde	0.23	-	-
11	3-methyl-2-heptanol	-	-	0.12
12	benzaldehyde	0.31	-	-
13	linalool	0.88	2.28	0.95
14	2-ethylhexanol	0.08	-	0.11
15	isobutyric acid	0.69	0.05	0.52
16	α,4-dimethyl-3-cyclohexene -1- aldehyde	0.24	0.24	0.11
17	2(2-ethoxyethoxy)ethanol	0.42	-	-
18	4-carvomenthenol	-	2.52	1.31
19	butyric acid	1.17	0.15	-
20	2-methyl butyric acid	4.56	-	-
21	3-methylthiopropanol	0.15	-	-
22	benzyl acetate	0.03	-	-
23	thymol	-	2.82	1.98
24	benzyl alcohol	2.42	0.74	0.59
25	valeric acid	0.34	-	-
26	ethanol	20.28	5.04	8.69
27	methyl salicylate	0.74	-	-
28	3- hydroxy -4- methoxy toluene	0.38	0.4	0.21
29	4-methyloctanoic acid	4.37	-	-
30	hexanoic acid	1.0	-	-
31	α-amyl-γ-butyrolactone	-	-	0.3
32	guaiacol	0.38	-	-
33	phenol	0.3	0.13	-
34	terpin monohydrate	-	0.84	0.87
35	2-methoxy-4-propyl-Phenol	-	2.05	0.16
36	4-ethylphenol	-	1.36	0.81
37	2-methyl-2-pentenoic acid	2.91	-	-
38	2,4-di-tert-butylphenol	0.15	0.15	0.05
39	decanoic acid	0.41	0.29	-
40	benzoic acid	8.58	0.11	0.12
41	cyclopentadecanol	1.02	-	-
42	methyl oleate	-	0.09	0.68
43	lauric acid	0.11	0.1	0.02
44	diisobutyl phthalate	-	0.14	0.05
45	3-phenyl-1-propanol	0.07	-	-
46	octanoic acid	0.26	-	-
47	palmitic acid	0.72	0.11	0.12
48	nonanoic acid	0.38	-	-
49	sorbic acid	3.57	-	-
50	dibutyl phthalate	-	0.12	-
51	dehydroacetic acid	5.87	-	-
52	dimethyl phthalate	0.15	-	-

[a] The data were the mean value of triplicate measurements, and calculated on the basis of peak area
-: Not determined or peak area of <0.01%

All samples contained volatile compounds linalool, ethyl lactate and α, 4-dimethyl-3- cyclohexene-1-aldehyde, these compounds were the main flavors in Sichuan pickled vegetables [27]. However, we haven't detected sulfur compounds in Sichuan pickled vegetables, which was main substance contributed to Korean kimchi's flavor [28, 29, 30]. This difference of flavors may be caused by the different process and pickled materials of these food. The other reasons due to specific geographical environment in Sichuan.

CONCLUSION

Sichuan traditional pickled vegetables are popular fermented foods in southwest of China. Bacterial community and chemical constituents produced during natural ferment process have effect on quality of pickled vegetables and health of people. This investigation suggested: (1) The strains of genus *Lactobacillus* and *Leuconostoc* are main groups in traditional fermented pickled vegetables. With the extension of the pickling time, genus *Lactobacillus* will be dominant. (2) The longer period for vegetables pickling, the concentration of biogenic amines and nitrite were higher, because nitrosamines, biogenic amines and nitrite can generate carcinogenic, so long-term consumption of pickled vegetables soaked for many years may lead certain harm to human health. (3) Considering the edible safety and tasted favor of pickled vegetables, we believe that the pickled vegetables should not pickled for long time.

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