

Dynamic changes of bacterial communities and nitrite character during northeastern Chinese sauerkraut fermentation

Qi Zhou¹ · Shizhu Zang¹ · Zinan Zhao¹ · Xinli Li¹

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Abstract Northeastern Chinese sauerkraut is a well-known traditional fermented vegetable in China. Incomplete identification of the microorganisms' (bacteria in spontaneous fermentation) diversity and accumulation of nitrite make it difficult to normalize the fermentation process and product qualities of northeastern Chinese sauerkraut. Conventional culturing and polymerase chain reaction-denaturing gradient gel electrophoresis methods were combined to describe microbial structure and diversity. *Lactobacillus*, *Leuconostoc*, *Enterobacter*, *Accumulibacter*, *Thermotoga*, *Pseudomonas*, *Clostridium*, *Rahnella* and *Citrobacter* were predominant microorganisms in different fermentation periods. The pH value and nitrite concentration presented a certain relevance to the amount of lactic acid bacteria. *Lactobacillus* and *Leuconostoc* had the ability to decrease nitrite by inhibiting nitrate-reducing bacteria such as *Enterobacter*. Therefore, Northeastern Chinese sauerkraut should not be eaten until 4 weeks of fermentation for the safety and quality of fermented foods. Northeastern Chinese sauerkraut is rich in lactic acid bacteria, which demonstrate its ability as an excellent probiotic for applications in functional foods.

Keywords Northeastern Chinese sauerkraut · Microbial diversity · Nitrite · DGGE

Introduction

Northeastern Chinese sauerkraut (NCS), one of the well-known fermented vegetable foods in the northeastern part of China, has been widely consumed over centuries. In the past, Chinese cabbages were pickled for food preservation in winter; its nutritional value and taste were improved by a traditional fermentation technology [1]. Nowadays, the basic methods for NCS fermentation, which are mostly based on spontaneous lactic acid fermentation both in homemade and industrial processes, have not been changed to a great extent [2]. Unlike the raw material and fermentation process of PaoCai in the Sichuan Province [3], Dongbei SuanCai only use Chinese cabbages as raw material in winter, which is immersed in 2% (w/v) salt solution and undergoes lactic acid fermentation by naturally occurring lactic acid bacteria (LAB) present on the Chinese cabbages, such as *Leuconostoc mesenteroides* and *Lactobacillus plantarum* [4] for almost 2 months at room temperature.

NCS is popular because of its special flavor, however, the accumulation of nitrite is an important problem [5]. During fermentation, nitrate present in the vegetable due to the large use of chemical fertilizers is reduced to nitrite by nitrate-reducing bacteria, such as *Enterobacteria*. Rabie et al. [6] and Wu et al. [7] have reported that starter cultures inoculation may be more effective in lowering nitrite concentration.

NCS is an important source of LAB. LAB have exhibited excellent probiotic properties [8]. Besides, various kinds of naturally occurring microorganisms have also participated in the whole homemade process of NCS. Incomplete identification of the microorganisms makes it difficult to normalize the fermentation process and product qualities.

✉ Xinli Li
lixinlibio@hotmail.com

¹ Department of Biotechnology, Dalian Medical University, Dalian, China

In this study, the microbial structure in the fermentation process was investigated by conventional culturing and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) methods; the diversity of bacteria communities was assessed. NCS is rich in LAB, which demonstrate its ability as an excellent probiotic for applications in functional foods.

Materials and methods

Preparation and sampling

Fresh Chinese cabbages were purchased from the farmer's market in Dalian, Liaoning Province, China. Chinese cabbages (1.5 kg) were put into a 20 L jar, and cool boiled water with 2% salt was added to the jar until the materials were submerged. The jars were kept at room temperature (approximately 20 °C) for 2 months. Four jars were prepared under the same conditions. Fifty milliliters of brine and five grams of cabbage leaves were aseptically sampled every week (samples 1–8).

Determination of pH and nitrite concentration

The pH values of samples 1–8 were measured with a pH meter (FE20-FiveEasy Plus, Mettler Toledo, Shanghai Toledo Instrument, Co., Ltd., China).

Nitrite concentration was determined by a previous method [4]. Five grams of NCS was chopped, deproteinated, and defatted by 10 mL ZnSO₄ (0.42 mol/L) followed by filtration. The supernatant was treated with 0.2% sulfanilamide, 0.1% N-1-naphthyethylene diamine dihydrochloride and 44.5% HCl (1 mL each of three reagents) sequentially. The extracts were kept at room temperature for 5 min. OD was detected at 540 nm against the reagent blank. To build a standard curve, 0–12.5 µg sodium nitrite solutions (5 µg/mL) were used by the same color development and measurement steps.

Enumeration of culturable *Lactobacillus*

Samples 1–8 were withdrawn every week and microbial counts of *Lactobacillus* were performed according to a previous report [9]. Serial decimal dilutions were prepared in the sterile PBS (81 mL, 0.2 M Na₂HPO₄; 19 mL, 0.2 M NaH₂PO₄; pH 7.4) inoculated in triplicate by surface spreading on MRS agar (Difco, Sparks, MD, USA) at 37 °C for 48 h under anaerobic incubation.

Deoxyribonucleic acid (DNA) extraction

First, 50 mL of samples 1–8 were filtered and centrifuged (HC-3018 R high speed refrigerated centrifuge, Zhongke Zhongjia Scientific Instrument Co., Ltd. Anhui, China) at 12,000 rpm for 6 min at 4 °C to remove supernatant. The crude bacteria precipitate was washed with 0.2 M sterile PBS twice, collected by centrifugation, and preserved at – 20 °C. DNA was extracted with Fore Genen Bacterial DNA Isolation Kit (Foregene, China) according to the manufacturer's instructions.

Polymerase chain reaction (PCR) amplification

The primer pairs GC-357f (CGCCCGGGCGCGCCCGGGCGGGGCGGGGACGGGGGCCTACGGGAGGCAGCAG) and 518r (ATTACCGCGGCTGCTGG) [10] (Dalian TaKaRa Biotechnology Co., Ltd., China) were used. The PCR mix (25 µL) contained the following: 2 µL of purified genomic DNA as template (approximately 200 ng), 12.5 µL of 2× Easy Taq PCR SuperMix (Beijing TransGen Biotech Co., Ltd., China), 0.5 µL of forward primer (10 µM), and 0.5 µL of reverse primer (10 µM), and filled up to 25 µL with sterile water. PCR was performed with a PCR amplifier (Thermo, USA): an initial denaturation step at 94 °C for 5 min, 30 cycles at 94, 54, and 72 °C for 30 s each, and annealing temperature of 72 °C for 7 min [11].

DGGE analysis

DGGE was performed with D-CodeTM universal mutation detection system (Bio-Rad, Hercules, CA). PCR amplified products were electrophoresed on 8% polyacrylamide gels containing a denaturant gradient ranging from 30% to 60%. Thereinto, 100% denaturant contained 7 M urea and 40% (v/v) deionized formamide. Electrophoresis was performed for 6 h at 70 V in a 1× TAE buffer at 60 °C [10]. DGGE analysis experiments were repeated three times. Gels were stained with AgNO₃ [12].

Sequence analysis

The separated and strong bands were cut out by a sterile scalpel. Gel fragments were steeped in 20 µL sterile water at 95 °C for 10 min. The solution (4 µL) was reamplified using 357f (CCTACGGGAGGCAGCAG) and 518r primers with the same PCR program. Subsequently, sequences were detected by TaKaRa Biotechnology (Dalian) Co., Ltd, and compared directly with those in GeneBank by BLAST (NCBI).

Statistical analyses

DGGE profiles were analyzed with Quantity One 4.6.2 (Bio-Rad). The similarity clustering analysis was conducted with an unweighted pair group method with arithmetic average (UPGMA) method [13]. Software R version 3.2.3 (R is an open access program for statistical computing, downloadable from <https://www.r-project.org/>) was used for principal component analysis (PCA). Hierarchical Clustering Explorer version 3.5 was used for hierarchical cluster analysis. SPSS version 17.0 was used for viable counts and Shannon–Wiener index (H'). P values were determined using the t test, $P < 0.01$ was considered significant.

Results and discussion

pH and nitrite concentration during NCS fermentation

Throughout the fermentation process, the mean pH value (Fig. 1A) declined from 6.15 at the beginning to 3.91 at the fifth week and then increased to 6.69. The changes were not significant ($P > 0.05$).

The nitrite concentration (Fig. 1B) increased rapidly from 0 to 21.0 mg/kg during the first 7 days, with the peak concentration appearing on the seventh day followed by a sharp decline during the following 21 days. On the 28th day, the nitrite concentration had decreased to 2.1 mg/kg; it continued to decrease until the 35th days and remained almost unchanged thereafter.

Enumeration of *Lactobacillus*

The LAB population (Fig. 1C) altered significantly ($P < 0.01$) during the fermentation process, ranging from 5.49 to 6.35 log cfu/mL. The total amount of LAB increased sharply at the beginning (0–6.1 log cfu/mL). After 6 weeks, this population decreased slightly.

DGGE analysis

The dynamic changes in the microbial community were periodically investigated (Fig. 2A). Lanes 1–8 were samples from 1 to 8 weeks of fermentation, respectively. Obviously, *A*, *F*, *J*, *K*, and *M* existed in the entire fermentation processes (lanes 1–8). *C* and *L* existed at the beginning and middle of the fermentation processes (lanes 1–6). *B*, *D*, *E*, *G*, *H*, and *I* existed at the end of the fermentation processes (lanes 7–8).

Similarity clustering analysis was visualized in an UPGMA dendrogram. There were three main clusters (Fig. 2B) in the dendrogram. The first was lane 1 related to the first week of NCS fermentation, the second was lanes 6–8 related to the 6–8 weeks of fermentation, and the last cluster was lanes 2–5 related to the 2–5 weeks of fermentation. The maximum bacterial similarity index between weeks 7 and 8 of fermentation was 0.83 and that for 6 or more weeks of fermentation was 0.66. For 1 week of fermentation, the similarity was the lowest (0.18) compare to other groups, which suggested that the microbial community of early fermentation was clearly different from the others. The end of the fermentation periods (7 and 8 weeks) shared a relatively high similarity.

In DGGE profiles, each band possibly derived from one phylogenetically distinct community. Hence, an estimation

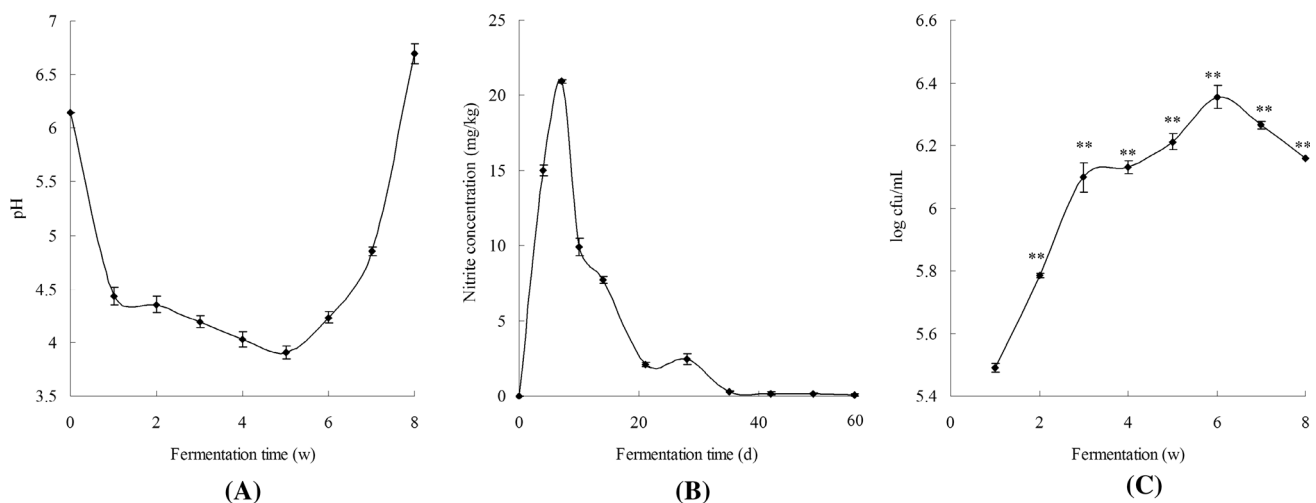


Fig. 1 pH (A), nitrite concentration (B) and number (log cfu/mL) of total *Lactobacillus* (C) during NCS fermentation ($n = 4$)

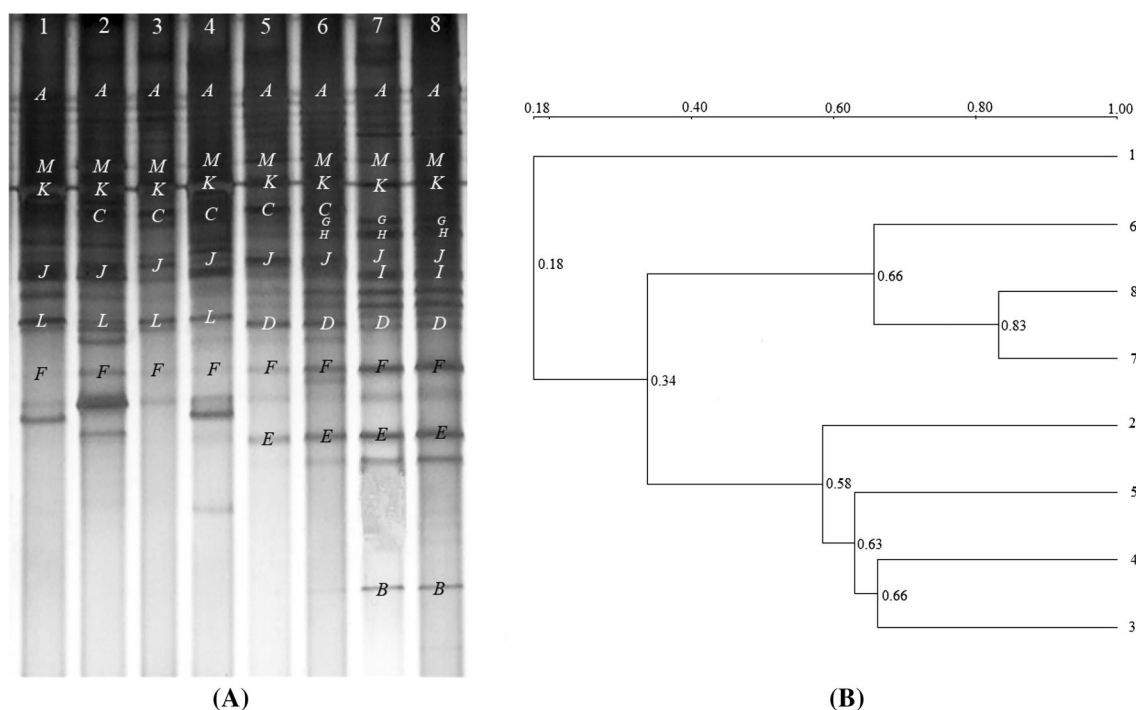


Fig. 2 Representative DGGE profiles (A) and UPGMA dendrograms (B) of different fermentation periods (1–8 weeks) by universal primers

of species number could be based on the total number of the bands in the profile [14].

Table 1 shows that diversity in the 2–8 weeks of fermentation increased as compared to the first week of fermentation with statistical significance. Compared to the first week of fermentation, the number of bands was richer in other time points of fermentation except the third and the fourth weeks of fermentation ($P < 0.01$). Moreover 5–8 weeks of fermentation produced a relatively high evenness score. Based on these results, it appeared that the

Table 1 The diversity indexes analysis^a ($\bar{x} \pm s$, $n = 4$) of the entire microbial community

Group	S	H^b	E^c
1	22.5 ± 0.58	2.485 ± 0.130	0.798 ± 0.047
2	25.26 ± 0.96**	2.780 ± 0.306	0.861 ± 0.091
3	18.25 ± 0.50**	2.523 ± 0.096	0.869 ± 0.029
4	16.25 ± 0.96**	2.630 ± 0.255	0.946 ± 0.113
5	21.50 ± 0.58*	2.825 ± 0.150*	0.921 ± 0.046*
6	26.75 ± 0.96**	2.855 ± 0.130**	0.869 ± 0.032*
7	25.25 ± 0.96**	3.243 ± 0.345**	1.004 ± 0.101*
8	26.25 ± 0.96**	2.995 ± 0.146**	0.917 ± 0.045*

^aCompare to group 1, respectively, ** $P < 0.01$, and * $P < 0.05$

^b $H' = -\sum (pi) (\ln pi)$, where pi is the proportion of the bands in the track; $pi = ni/\sum ni$, where ni is the average density of peak i in the densitometric curve

^c $E = H'/\ln S$, where S is the number of bands

whole microbial community was changed during the fermentation, and the richness, diversity index, and evenness score increased at the end of the fermentation process.

Table 2 shows the closest relatives based on BLAST with DNA sequences obtained from DGGE gel. A, B, E, and F were sequenced and identified as *Lactobacillus sakei*, *Lactobacillus rhamnosus*, *Lactobacillus hokkaidonensis*, and *L. plantarum* with the similarity of 100, 99, 96 and 99%, respectively, all belonging to *Lactobacillus* genus. *L. sakei* and *L. plantarum* existed in the entire fermentation process, *L. rhamnosus* and *L. hokkaidonensis* increased at the end of the fermentation process, whereas there was nearly no band at the corresponding position of other time points. C and L were sequenced and identified as *Leuconostoc carnosum* and *Enterobacter cloacae* with the similarity of 98 and 99%, belonging to the *Leuconostoc* and *Enterobacter* genera, respectively; they only existed at the beginning of the fermentation process. G and I were sequenced and identified as the *Rahnella aquatillis* with the similarity of 93 and 92%, respectively, all belonging to *Rahnella* genus. D, H, J, K and M were sequenced and identified as *Clostridium saccharobutylicum*, *Citrobacter freundii*, *Ca. Accumulibacter phosphatis*, *Thermotoga* sp. and *Pseudomonas rhizosphaerae* with the similarity of 100, 93, 90, 93 and 98%, belonging to the *Clostridium*, *Citrobacter*, *Accumulibacter*, *Thermotoga*, and *Pseudomonas* genera, respectively.

During the entire fermentation process, *L. sakei*, *L. plantarum*, *Ca. Accumulibacter phosphatis*, *Thermotoga*

Table 2 Sequences of PCR amplicons derived from DGGE fingerprints of bacteria and identities based on the BLAST database

Selected band	Most similar sequence relative (GeneBank accession number)	Bacteria genus	Identity (%)
A	<i>Lactobacillus sakei</i> (NC 007576.1)	<i>Lactobacillus</i>	100
B	<i>Lactobacillus rhamnosus</i> (NC 021725.1)		99
E	<i>Lactobacillus hokkaidonensis</i> (AP014680.1)		96
F	<i>Lactobacillus plantarum</i> (NC 021514.1)		99
C	<i>Leuconostoc carnosum</i> (NC 018673.1)	<i>Leuconostoc</i>	98
D	<i>Clostridium saccharobutylicum</i> (NC 0225717.1)	<i>Clostridium</i>	100
G	<i>Rahnella aquatillis</i> (NC 017047.1)	<i>Rahnella</i>	93
I	<i>Rahnella aquatillis</i> (NC 016818.1)		92
H	<i>Citrobacter freundii</i> (CP007557.1)	<i>Citrobacter</i>	93
J	<i>Candidatus Accumilibacter phosphatis</i> (NC 013194.1)	<i>Accumulibacter</i>	90
K	<i>Thermotoga</i> sp. (NZ CP007633.1)	<i>Thermotoga</i>	93
L	<i>Enterobacter cloacae</i> (NC 014618.1)	<i>Enterobacter</i>	99
M	<i>Pseudomonas rhizosphaerae</i> (CP009533.1)	<i>Pseudomonas</i>	98

sp., and *P. rhizosphaerae* were dominant bacteria according to PCR-DGGE analysis. At the fifth week of fermentation, *L. carnosum* and *E. cloacae* were rapidly eradicated, whereas *L. rhamnosus*, *C. saccharobutylicum*, *L. hokkaidonensis*, *R. aquatillis*, and *C. freundii* grew fast and became the dominant bacteria.

The correlation between microorganisms and fermentation periods was analyzed by PCA (Fig. 3A). PC1 and PC2 were two factors, which accounted for 43.0 and 34.0% of variability, respectively. PCA demonstrated that the

three types of communities could be separated using bands abundance data set from PCR-DGGE analysis, indicating that early, middle and late fermentation periods had significantly different bacterial species. *L. sakei* and *E. cloacae* were highly correlated with early 4 weeks of fermentation; *L. plantarum*, *L. carnosum* and *L. hokkaidonensis* were highly correlated with the middle period of fermentation. *L. rhamnosus* was highly correlated with the late period of fermentation. These results suggested that LAB gradually became the dominant

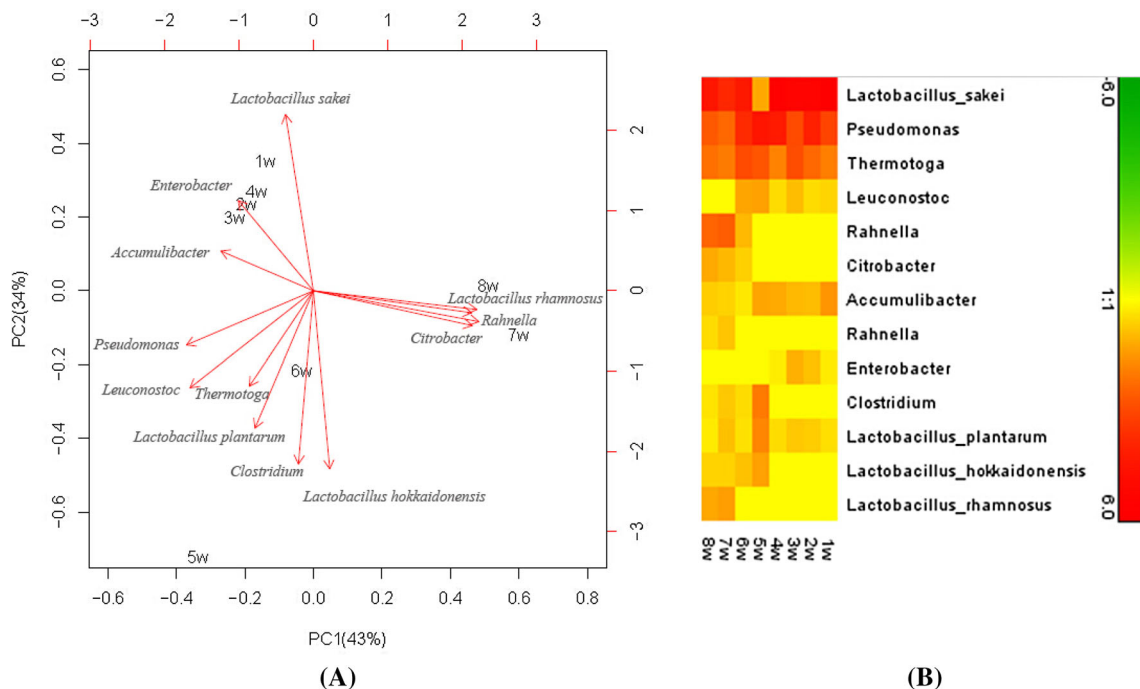


Fig. 3 PCA (A) and hierarchical cluster analysis (B) based on dominant bacteria PCR-DGGE profiles

organisms; by contrast, *E. cloacae* (harmful bacteria) decreased as the fermentation continued. Figure 3B shows the heat map of bacterial genera during fermentation. *L. sakei* was the most abundant. *L. rhamnosus* and *Rahnella* rapidly grew in the late period of fermentation.

NCS fermentation is a continuous process with a spontaneous growth of microbial community. Continuous changes in fermentation periods influence the microbial diversity and structure. *Lactobacillus*, *Enterobacter* and *Leuconostoc* were identified as dominant bacteria in NCS by the culture-dependent method [15]. In the present study, *Lactobacillus* and *Leuconostoc* were both found; even more bacterial genera of *Enterobacter*, *Pseudomonas*, *Accumulibacter*, *Rahnella*, *Clostridium*, *Thermotoga* and *Citrobacter* were detected simultaneously by DGGE. *Leuconostoc* and *Lactobacillus*, possessing probiotic potential, have been used in the spontaneous fermentation [16]. In the early fermentation period, *Leuconostoc* gradually grew and increased rapidly. *Leuconostoc* has started the fermentation as a starter cultures, and then gradually eradicated at the end. *L. sakei* and *L. plantarum* grew spontaneously, and *L. rhamnosus* and *L. hokkaidonensis* rapidly increased until the fermentation was completed. Our results showed that LAB was the dominant microbial community, which played an important role in NCS. During spontaneous fermentation, *Enterobacter* was considered as the nitrate-reducing bacteria, which rapidly grew in the early period of fermentation and disappeared in the late period. Meanwhile, the nitrite concentration increased, reached the peak (at the 7th day of fermentation) and decreased to 0.075 mg/kg thereafter. These results showed that LAB had ability to decrease nitrite and inhibit the growth of nitrate-reducing bacteria such as *Enterobacter*. As we all know, vegetables, fruits, and cured meats with high nitrate can cause a risk of gastrointestinal cancer and methemoglobinemia in humans [7, 17]. Therefore, NCS should not be eaten until 4 weeks of fermentation. Bacteria belonging to the *Lactobacillus* genera were the most dominant organisms during the fermentation of NCS; they have a significant potential to be used as probiotics for conferring health benefits on the host, more specifically, improving growth performances in animals, lowering serum cholesterol level, increasing the utilization of nutrients, decreasing the use of antibiotics, etc. [18], [19].

NCS, a traditional fermented food, plays an important role in human health. Our results showed that *Lactobacillus*, *Leuconostoc* and *Enterobacter* are the predominant microorganisms in the different fermentation periods of NCS. LAB gradually increased; by contrast, *E. cloacae* (harmful bacteria) decreased as the fermentation continued. The pH value and nitrite concentration presented a certain relevance to the amount of LAB. *Lactobacillus* and *Leuconostoc* had the ability to decrease

nitrite by inhibiting nitrate-reducing bacteria during the fermentation of NCS.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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