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Effects of temperature on paocai bacterial succession revealed by culture-dependent and culture-independent methods

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ABSTRACT

Paocai is a widely consumed Chinese traditional fermented vegetable product. To understand the effect of temperature on paocai fermentation flora, the bacterial community structure of paocai fermented at 10 °C, 15 °C, 25 °C and 35 °C was analyzed by culture-dependent and culture-independent methods. The results showed that increasing the fermentation temperature in a certain range is beneficial for rapid paocai acid production and shortening of the maturity period. Illumina Miseq sequencing was performed on 56 samples at different fermentation process temperatures using a culture-independent method. A total of 1,964,231 high-quality reads of 16S rRNA V3-V4 regions were obtained, and they were divided into 405 operational taxonomic units (OTUs) and identified as 213 bacterial genera. The bacterial diversity decreased with the progression of fermentation, and some spoiled samples had an increased diversity. The culture-independent method found that at 10 °C, *Lactococcus* appeared at the start of fermentation, *Leuconostoc* and *Weissella* appeared in the middle of fermentation, and *Lactobacillus* and *Leuconostoc* dominated fermentation in the late stage. At 15 °C, *Lactococcus* started fermentation, *Leuconostoc* appeared in the middle stage, and *Lactobacillus* was dominant in the late stage. At 25 °C, *Lactococcus* started fermentation, *Weissella* and *Lactobacillus* appeared in the middle stage, and *Lactobacillus* dominated fermentation in the late stage. Finally, at 35 °C, *Lactococcus*, *Weissella*, and *Lactobacillus* started fermentation, *Weissella* and *Lactobacillus* appeared in the middle stage, and *Lactobacillus* dominated fermentation in the late stage. A total of 647 strains of bacteria were isolated by culture-dependent methods and were divided into 12 genera and 19 species by randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) and 16S ribosomal RNA gene (rDNA) sequencing technology. More types of bacteria were isolated in the early stage of fermentation. At 10 °C, *Lactococcus lactis* began fermentation, and *Lactobacillus brevis* and *Leuconostoc mesenteroides* dominated acid production in the middle and late stages of paocai fermentation. At 15 °C, *L. lactis* initiates fermentation, while *Lactobacillus plantarum* dominates the acid fermentation of paocai. At 25 °C and 35 °C, there were a large number of *Enterobacteriaceae* bacteria in the start-up fermentation stage, and *L. plantarum* was dominant after 1–2 days of fermentation. Redundancy analysis (RDA) found that the lower the temperature, the more bacterial species that are produced, and the higher the temperature and the longer the time, the more obvious are the effects of *L. plantarum* on paocai. The results of dominant bacteria studied by culture-dependent and culture-independent methods are similar. The results indicate that most of the dominant microorganisms in the paocai fermentation system are culturable. This discovery can provide data and physical support for modernization and regulation of different types of paocai production.

1. Introduction

Paocai is a traditional Chinese fermented vegetable product with a long history dating back to the Zhou dynasty > 3000 years ago (Liu et al., 2017). Paocai is popular among consumers because of its fresh and crispy taste and rich supply of active lactic acid bacteria. In the natural fermentation process microbes from raw materials and the environment use nutrients such as vegetable sugars to ferment and

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produce acid. The lactic acid bacteria play an important role in the taste, storage and safety of paocai (Holzapfel, 1997; Jung et al., 2014; Yan et al., 2008). Numerous studies have demonstrated that bacteria are the dominant microorganisms in paocai fermentation (Hong et al., 2013; Park et al., 2012). Usually, the number of bacteria in paocai is much higher than the number of fungi. The number of bacteria in the middle stage of fermentation often exceed 10^8 colony forming units (CFU)/mL (Ahmadsah et al., 2015; Xiong et al., 2012; Xiong et al., 2014), whereas the number of fungi usually does not exceed 10^7 CFU/mL (Pardali et al., 2017). The dominant flora in paocai fermentation is generally considered to be lactic acid bacteria, including *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, and *Lactobacillus brevis* (Kim and Chun, 2005; Wouters et al., 2013; Yu et al., 2012). However, the dominant fermentation microorganisms for different types of paocai are different. In addition to *Lactobacillus*, the acknowledged dominant microorganism in paocai, *Leuconostoc*, *Weissella* and *Lactococcus* also have been reported as dominant microorganisms for the fermentation of vegetables (Jeong et al., 2013a; Jung et al., 2012; Lee and Lee, 2010; Liang et al., 2018; Wang and Shao, 2018), especially in Korean kimchi, Northeast Chinese sauerkraut and other products.

Paocai is influenced by factors such as climatic conditions in different regions, and temperature is the most important factor. Researchers have found that temperature changes systematically alter biodiversity and biomass (García et al., 2018). Temperature is an important indicator that distinguishes paocai processing (Holzapfel, 1997; Lee et al., 2008). For example, Northeast sauerkraut and paocai are mainly fermented at medium and low temperatures (5–15 ˚C), while Sichuan paocai is mainly fermented at medium and high temperatures (15–30 ˚C). Temperature affects the rate of paocai fermentation, which affects the ripening time (Park et al., 2018; Zhang et al., 2016). Under normal circumstances, the fermentation speed is faster under higher temperatures, such as the Sichuan paocai fermentation maturation time of 1–5 days, while Northeast sauerkraut and kimchi need > 10 days to ferment. The temperature difference also affects the growth of lactic acid bacteria, which in turn affects the paocai microbial community composition and fermentation to form different types of paocai. For example, Lee et al. (2006) reported that *Weissella* is a relatively cold-growing strain, that can be grow at −1 ˚C. *Leuconostoc faecalis* also grows at colder temperatures, but not as well as *Weissella sinensis*; when the two are cultured at temperatures higher than 15 ˚C, the growth of *Weissella serrata* is inhibited.

At present, the study of temperature on the paocai fermentation process is mainly focused on the growth of microbial biomass and basic physical and chemical indicators. However, the effects of temperature on the bacterial community structure and dynamics in paocai fermentation based on the environmental system have not been reported. In this study, considering the range of fermentation temperature for the Sichuan paocai, four constant temperatures were set for paocai fermentation. The temperatures were low temperature (10 ˚C), medium temperature (15 ˚C), normal temperature (25 ˚C) and high temperature (35 ˚C). Culture-dependent and culture-independent methods were used to study the paocai bacterial community structure in order to determine the dominant microorganisms, to explore microbial flora changes during fermentation, and to provide guidance for controlling paocai fermentation.

2. Materials and methods

2.1. Paocai preparation

Cabbages (*Brassica oleracea* L.) and salt were purchased from a local supermarket in Meishan, Sichuan Province, China. Cabbages (0.8 kg) were trimmed of outer leaves, washed, cut into 2–3 cm small pieces and set in 2.5 L pottery jars. The brine (1.6 kg) was prepared with cool boiled water containing 3% salt (w/v). The pottery jars were sealed by adding water to the jar edge and stored at 10–15 ˚C for 20 days, 25–35 ˚C for 10 days.

2.2. Measurement of pH and total titratable acidity

The pH of the paocai brine was measured with a pH-3C meter (Yidian, Shanghai, China). Total titratable acid (TTA) was measured according to the AOAC 942.15 (Zhang et al., 2018) International Acidity Titration Standard Analysis Method.

2.3. Total cell counts

One milliliter of paocai fermentation brine was mixed with 9 mL of physiological saline (0.85% NaCl, w/v) and then diluted by gradient dilution. Next, 0.1 mL of the appropriate dilution of bacterial suspension was spread on NA and MRS agar (Land Bridge Biotechnology Co., Ltd., Beijing, China) medium for bacterial counting, and 20 g/L calcium carbonate was added in the MRS medium. Total cell counts were performed in triplicate. After 24–48 h of incubation at 37 ˚C, microbial numbers were calculated as colony forming units per milliliter. Bacteria with different colony morphology were selected for purification by plate scribing, and the number of similar colonies was recorded for subsequent identification and statistics.

2.4. Microbial composition analysis through a culture-dependent method

DNA was extracted and gathered using the Bacterial DNA Isolation Kit (Fuji Biology Co., Ltd., Chengdu, Sichuan, China), and the extracted DNA was subjected to RAPD-PCR. The reaction and thermocycling conditions are referenced in Di Cagno et al. (2010). The PCR product was electrophoresed on a 1% agarose gel and photographed by a gel imaging system. Then, the strips obtained by photographs were converted into data matrices, the electrophoresis results were clustered and classified using NTSYS software, and a similarity of 0.8 or more was classified into one class. Two to three strains were screened for each type for 16S rDNA analysis, and the reaction system and procedure are referenced in Haruta et al. (2002). The positive clones of the PCR products were sequenced by Shanghai Huajin Biotechnology Co., Ltd., Shanghai, China. The detected sequences were analyzed through the GenBank database (http://www.ncbi.nlm.nih.gov).

2.5. Microbial composition analysis through a culture-independent method

Microbial composition was analyzed using Illumina MiSeq sequencing with slight modifications (Zhang et al., 2018). Then, 10 mL of paocai brine was centrifuged at 12,000 rpm for 5 min, and the supernatant was collected. The DNA was extracted and gathered using the universal E.Z.N.A. Soil DNA Kit (Omega, Norcross, GA, USA) according to the manufacturer's instructions. The TransGenAP221-02 kit with TransStart Fastpfu DNA polymerase (TransGen Biotech, Beijing, China) was used in the PCRs. The bacterial 16S rRNA gene was amplified by PCR using forward primer 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′) to determine the microbial community compositions. Electrophoreses of 2 μL of each reaction on 2% agarose gel were used to confirm the products. The triplicate products were pooled and purified using the AxyPrep DNA 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 OTU clustering of non-repetitive sequences was performed at a 97% similarity level. The RDP classifier Bayesian algorithm was used to analyze the 97% similarity level of OTU representative sequences, and data visualization of the microbial community was achieved with GraPhlAn software.

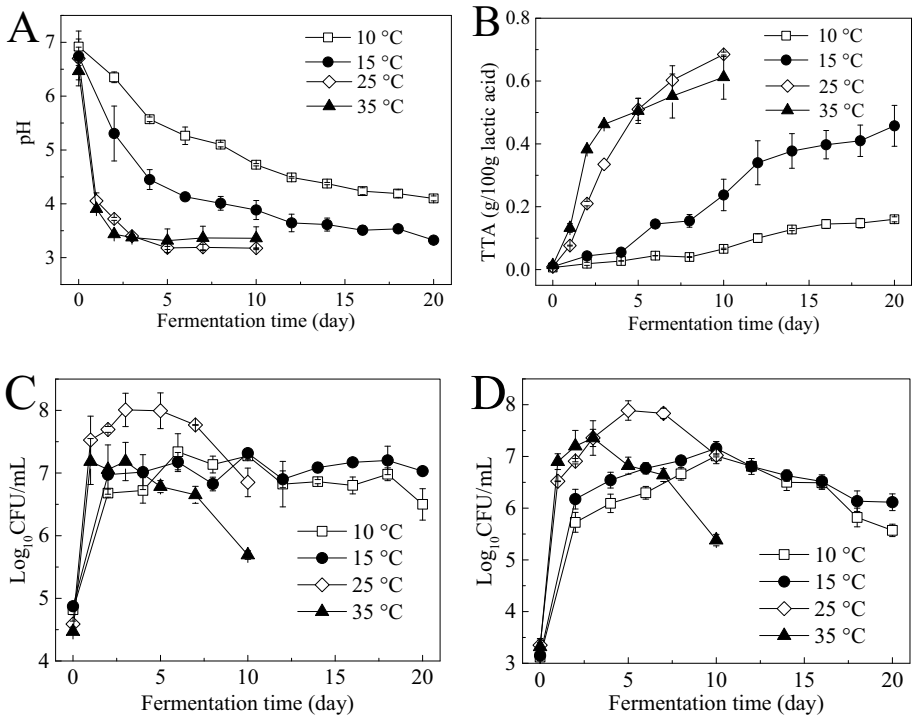

Fig. 1. Microbial and physicochemical changes during paocai fermentation at different temperatures. (A) pH, (B) total titratable acidity (TTA), (C) counts of bacteria by NA agar, (D) counts of lactic acid bacteria by MRS. All analyses were conducted in duplicate and the average values are presented.

2.6. Data analysis

Statistical analysis was performed using the Origin 9.1 software (OriginLab Corporation, USA). RDA was to visualize the relationship between the microbial community and environmental factors using Canoco for Windows v4.5 software (Wageningen UR, Netherlands).

3. Results

3.1. Microbial and physicochemical changes during paocai fermentation

The pH and TTA are significant physical and chemical parameters, showing not only paocai quality but also microbial growth (Cheigh and Park, 1994; Zhang et al., 2016). As shown in Fig. 1A, the pH of paocai fermentation decreases. Within a certain temperature range, the higher the temperature is, the faster the pH decreases; it then slows to steady-state fermentation in the middle and late stages. When the pH was lower than 4 and TTA was higher than 0.3 g/100 g, the paocai reached maturity with the disappearance of a raw vegetable smell (Zhang et al., 2016). For paocai to reach maturity, 20 days or more is required at 10 °C, 12 days at 15 °C, 2 days at 25 °C, and 1 day at 35 °C. Predictably, increases in fermentation temperatures dramatically reduced fermentation times and thereby shortened fermentation cycles. Effect of temperature on the growth and acid production of lactic acid bacteria, resulting in different pH values for the paocai at different temperatures, consistent with the results of Xiong et al. (2012) and others.

As shown in Fig. 1B, the TTA of paocai increases with fermentation progression and the higher the temperature is, the faster the increase. This study found that fermentation speed under low-temperature conditions was significantly slower than that under high-temperature conditions, indicating that low-temperature storage in the late stage of paocai fermentation helps prevent rancidity. Interestingly, the TTA content after paocai fermentation at 25 °C for 5 days is higher than that of fermented paocai at 35 °C. This result indicates that the influence of temperature is limited in the paocai fermentation system, and that the higher the temperature is, the faster the fermentation speed, which may be related to the microbial community structure and the characteristics of the strain itself (Lee et al., 2006).

The paocai fermentation process is accompanied by changes in the number of microorganisms. At the initial stage of fermentation, the number of bacteria gradually increases, and the higher the temperature is, the faster the growth rate of the bacteria. The number of bacteria in the middle stage of fermentation was relatively stable, showing a downward trend in the later stage. As shown in Fig. 1C, the changes in the number of bacteria in the paocai at 10 °C and 15 °C were consistent. The number of bacteria in the fermented paocai at 25 °C and 35 °C reached a peak and then decreased rapidly, and the number of bacteria 35 °C decreased faster than at 25 °C. This result indicates that high temperature may be not beneficial for the growth of microorganisms in the paocai system.

Paocai fermentation is mainly caused by lactic acid bacteria, and the change in the number of lactic acid bacteria reflects the degree of paocai fermentation (Hamady et al., 2008). As shown in Fig. 1D, the number of bacteria in the initial stage of paocai fermentation was higher than that of the lactic acid bacteria, indicating that a large number of contaminating bacteria grew in the early stage of fermentation. The change in the number of lactic acid bacteria in the middle and late stages of fermentation was consistent with the change in bacterial number overall. At this time, lactic acid bacteria became the dominant bacteria. The higher the temperature is, the faster the number of lactic acid bacteria changes, indicating that paocai is not beneficial for the maintenance of lactic acid bacteria at high temperatures.

3.2. Microbial composition analysis through a culture-independent method

MiSeq sequencing was used to analyze the bacterial diversity of 56 paocai brine samples collected in four temperature fermentation processes. After removing the low-quality sequences and chimera, 1,964,231 bacterial sequences were obtained, and the average sequence length was 447.25 bp. All optimized sequences were mapped to the OTU representative sequence, sequences with 97% similarity to the OTU were selected, and 405 OTUs were obtained by bacterial division.

There are detailed data regarding the sequence information and the microbial diversity indices in the Supplemental materials (Supplemental file). All samples belong to 14 phyla, namely, Firmicutes, Proteobacteria, Bacteroidetes, Acidobacteria, Tenericutes, Cyanobacteria, Verrucomicrobia, Planctomycetes, Deinococcus-Thermus, Saccharibacteria, Gemmatimonadetes, Fusobacteria, Elusimicrobia, and Chloroflexi, but > 89% of the OTUs were assigned to Firmicutes, Proteobacteria and Bacteroidetes. A total of 213 genera were detected, and all samples had an average abundance of ≥1% of 11 genera in the fermentation process, including *Lactobacillus*, unclassified_*Enterobacteriaceae*, *Lactococcus*, *Acinetobacter*, *Leuconostoc*, *Weissella*, *Raoultella*, unclassified_*Lactobacillales*, *Bacillus*, *Erwinia*, and *Pseudomonas*.

Bacterial diversity showed that the Shannon index were high in the early stages of fermentation and low in later stages, indicating that the bacterial diversity decreased with paocai fermentation. At the beginning of paocai fermentation, the bacteria are mainly derived from the environment and the original microorganisms, including unclassified_*Enterobacteriaceae*, *Lactococcus*, *Acinetobacter* and *Bacillus*. With increasing temperature and fermentation progression, the proportion of each bacterium changed differently. Interestingly, the late stage of paocai ripening at different temperatures is dominated by *Lactobacillus*, and the higher the temperature is, the earlier the emergence of *Lactobacilli*. As shown in Fig. 2, the structures of the paocai community at 10 °C and 15 °C are similar. In the middle stage, *Leuconostoc*, *Weissella*, and *Lactococcus* are the dominant bacteria and ferment to produce acid, which inhibits unfriendly microorganisms such as *Bacillus*, *Raoultella* and *Acinetobacter*. The rapid growth of *Lactobacillus* is accompanied by the disappearance of weak-resistance bacteria, especially *Enterobacteriaceae*, which decreases rapidly with the growth of *Lactobacillus*, indicating that *Lactobacillus* is potent at inhibiting *Enterobacteriaceae* in paocai. A large number of *Lactococcus* and *Weissella* appeared in the paocai at 25 °C and 35 °C, respectively, and *Lactobacillus* dominated fermentation at the later stage. However, a large amount of *Acinetobacter* appeared in the late stage of maturity, and the sensory evaluation of the sample (the results are not shown) also showed obvious paocai soft rot and off-flavor.

3.3. Microbial composition analysis through a culture-dependent method

A total of 647 strains of bacteria were isolated and purified from different temperature paocai fermentation processes. The RAPD-PCR clustering and 16S rRNA identification showed that the bacteria belonged to 12 genera and 19 species. The specific results are shown in Table 1, including five gram-positive bacteria (*Kytococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Staphylococcus*) and seven gram-negative bacteria (*Acinetobacter*, *Hafnia*, *Enterobacter*, *Klebsiella*, *Kluyvera*, *Lelliottia*, and *Raoultella*). Among them, more kinds of bacteria were isolated in the early stage of fermentation.

The bacterial succession patterns of fermented paocai at four temperatures were relatively similar. The changes in the bacteria were due to different fermentation temperatures. At the beginning of paocai fermentation, the bacteria were mainly dominated by gram-negative organisms such as *Enterobacteriaceae*. With increasing temperature and fermentation, the number of bacterial species gradually decreased, and gram-positive bacteria such as lactic acid bacteria gradually became dominant. The results of traceability and identification were based on the number of similar strains, and the effects of temperature on the number of dominant bacteria in the paocai fermentation process were analyzed. As shown in Figs. 3 and 4, in the initial stages of paocai fermentation at 10 °C, *Acinetobacter calcoaceticus*, *Lelliottia amnigena*, and *Raoultella ornithinolytica* accounted for the majority of bacteria, of which *R. ornithinolytica* had an absolute quantitative majority in the middle stage of fermentation. The number of bacteria reached 6.22 Log_{10}CFU/mL. After 14 days of fermentation, the abundance of *R. ornithinolytica* rapidly decreased as that of *L. mesenteroides* and *L. brevis* increased. The initial stage of paocai fermentation at 15 °C was dominated by *A. calcoaceticus*, *Enterobacter cloacae*, and *R. ornithinolytica*. On the 4th day of fermentation, *Lactococcus lactis* reached its maximum abundance. After 12 days, the counts of *L. lactis* decreased rapidly with the increase in *L. plantarum* abundance. For paocai fermentation at 25 °C, the dominant bacteria in the initial stage were *A. calcoaceticus*, *Enterobacter kobei* and *R. ornithinolytica*. After 1 day, *Kluyvera georgiana* was dominant, and *L. plantarum* was present for 3 days of fermentation. In the fermented paocai at 35 °C, *E. kobei* fermentation was initially dominant but disappeared after 1 day of fermentation. *L. plantarum* accounted for the quantitative advantage in the middle and late stages of fermentation.

3.4. Multivariate analysis of the microbial community during fermentation

The genus level of the culture-independent flora of the fermented paocai and the species level of the culture-dependent flora under different temperature conditions were analyzed by RDA with temperature.

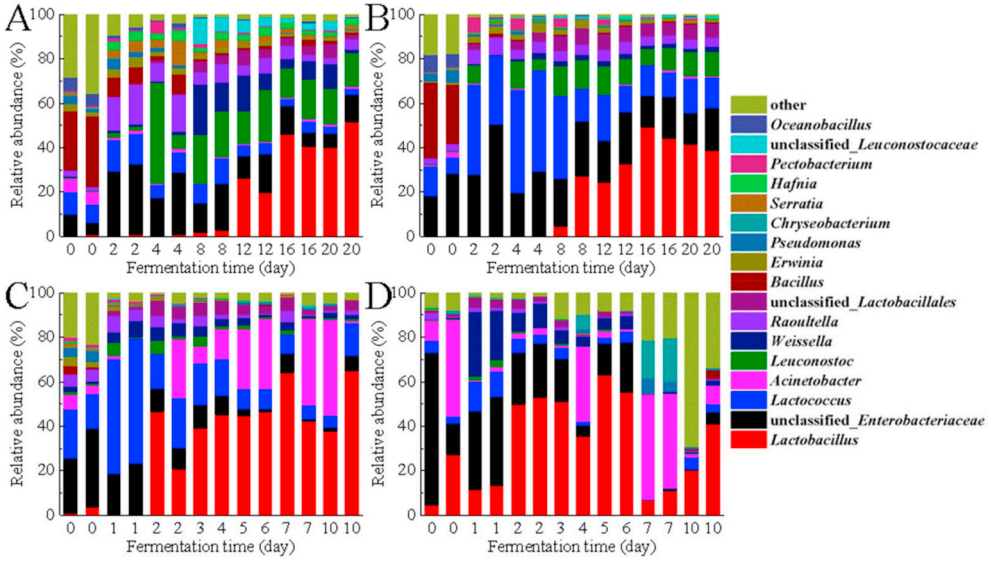

Fig. 2. Relative abundance analysis of paocai bacterial community structure at different temperatures by culture-independent methods. Select the average abundance ≥0.5% in all samples. (A) 10 °C, (B) 15 °C, (C) 25 °C, (D) 35 °C.

Table 1
Molecular identification of strains isolated from paocai through 16S rRNA gene sequence homology.

Strain	Length (bp)	Accession number	Closest relative sequence (accession number)	Similarity (%)
Acinetobacter sp.10B0D1-C1	1408	MN250320	*Acinetobacter baumannii* ECAn10 (JF911352)	100
Acinetobacter sp.10B0D2-C2	1422	MN250321	*Acinetobacter calcoaceticus* YR-9 (KY753249)	99
Acinetobacter sp.15B0D1-A2	1402	MN250319	*Acinetobacter junii* DWS3 (MK418695)	100
Enterobacter sp.10B2D2-D4	1395	MN249619	*Enterobacter aerogenes* JC4 (KF263568)	100
Enterobacter sp.15B0D2-B1	1367	MN249614	*Enterobacter cloacae* 72231 (MH304301)	100
Enterobacter sp.10B0D1-A2	1364	MN249624	*Enterobacter kobei* (LT547821)	100
Hafnia sp.10B8D1-A2	1349	MN249621	*Hafnia alvei* 95-3 (KY400228)	100
Klebsiella sp.25B1D2-C4	1393	MN249623	*Klebsiella oxytoca* YNB101 (JQ039993)	99
Kluyvera sp.10B2D1-A5	1416	MN249617	*Kluyvera cryocrescens* (LC060917)	99
Kluyvera sp.25B1D1-A4	1410	MN249622	*Kluyvera georgiana* CTD639-K16 (JQ917919)	99
Kytococcus sp.10B23D1-B2	1328	MN249610	*Kytococcus aerolatus* 02-St-019/1 (NR116931)	100
Lactobacillus sp.10B23D2-C5	1423	MN249611	*Lactobacillus brevis* G TIP (MK530232)	100
Lactobacillus sp.10B33D2-B2	1406	MN249613	*Lactobacillus plantarum* IMAU20959 (MK369875)	100
Lactococcus sp.10B0D2-A3	1422	MN249620	*Lactococcus lactis* CAU:175 (MF369834)	100
Lelliottia sp.10B0D1-C2	1418	MN249616	*Lelliottia amnigena* Lmb019 (KT986089)	99
Leuconostoc sp.10B28D1-A1	1413	MN249612	*Leuconostoc mesenteroides* CAU:714 (MF369940)	100
Raoultella sp.10B0D1-A1	1394	MN249615	*Raoultella ornithinolytica* CAU10171 (MF429129)	99
Raoultella sp.10B2D1-B5	1419	MN249618	*Raoultella terrigena* TGRB3 (MK102091)	99
Staphylococcus sp.10B23D1-A1	1416	MN249609	*Staphylococcus arlettae* 109 (MH910213)	100

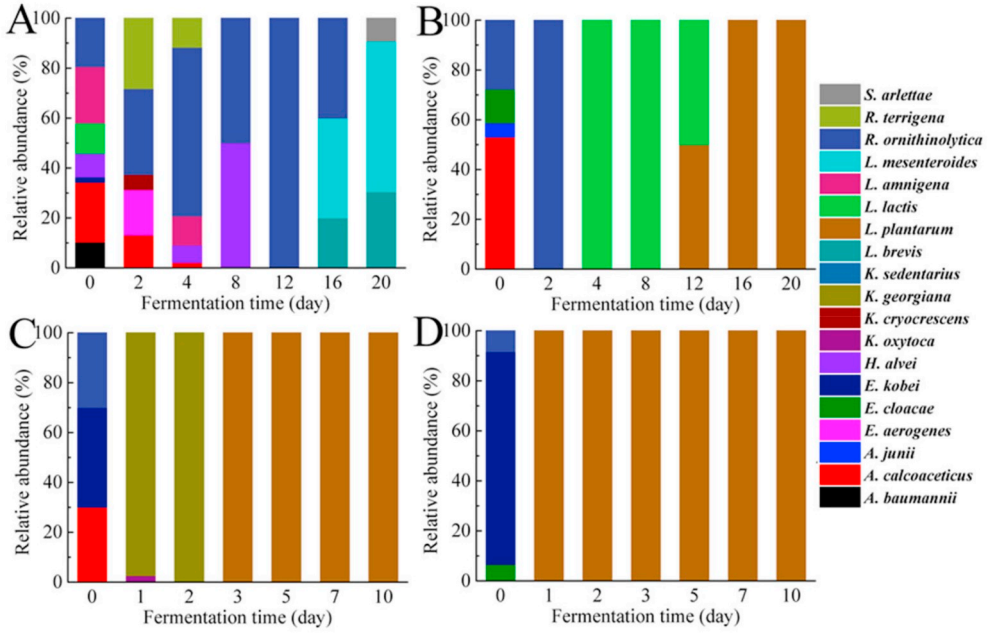

Fig. 3. Relative abundance analysis of paocai bacterial community structure at different temperatures by culture-dependent methods. (A) 10 ˚C, (B) 15 ˚C, (C) 25 ˚C, (D) 35 ˚C.

As shown in Fig. 5A and B, the RDA showed that the cumulative contribution rate of temperature to the structure of the paocai flora was 34.6% and 37.1%, respectively, and there were significant differences in the microbial flora at the genus and species levels of the fermented paocai at four temperatures. The paocai samples fermented at 10 ˚C and 15 ˚C were similar to each other, indicating that there were more microorganisms with similar community structures and a high correlation. The paocai samples fermented at 25 ˚C and 35 ˚C were similar to each other, but fewer microorganisms had a high correlation, and the microbial difference compared to that of the paocai fermented at 10 ˚C and 15 ˚C was large. The temperature at the genus level was positively correlated with *Lactobacillus*, *Acinetobacter*, *Chryseobacterium*, and *Weissella*. The species level of discovery was positively correlated with *L. plantarum*, *R. ornithinolytica*, *Klebsiella oxytoca*, and *K. georgiana* and negatively correlated with other microorganisms; the higher the fermentation temperature was, the greater the correlation with *L. plantarum*. The discovery time at the genus level was positively correlated with *Lactobacillus*, unclassified_*Lactobacillales*, unclassified_*Lactobacillales*, and *Leuconostoc*. The species level of discovery was positively correlated with *L. plantarum*, *Kytococcus aerolatus*, *Staphylococcus arlettae*, *L. brevis*, and *L. mesenteroides* and negatively correlated with other microorganisms. *L. plantarum* has the greatest impact on fermentation time, indicating that it has a more pronounced effect as fermentation progresses, and that it plays a leading role in the late stage of normal high-temperature paocai.

4. Discussion

A difference in temperature leads to differences in microbial diversity during paocai fermentation. It is generally believed that, with fixed raw material and environmental conditions (Seo et al., 2010), the initial flora structure of paocai should be the same. The microorganisms in paocai fermentation have been reported in the past and include *Enterobacter*, *Staphylococcus*, *Pseudomonas*, etc. (H.-W. Lee et al., 2017; Park et al., 2012). Some microorganisms are uncultured, resulting in a differential bacterial composition for culture-dependent and culture-independent methods. From the analysis of the microbial flora structure

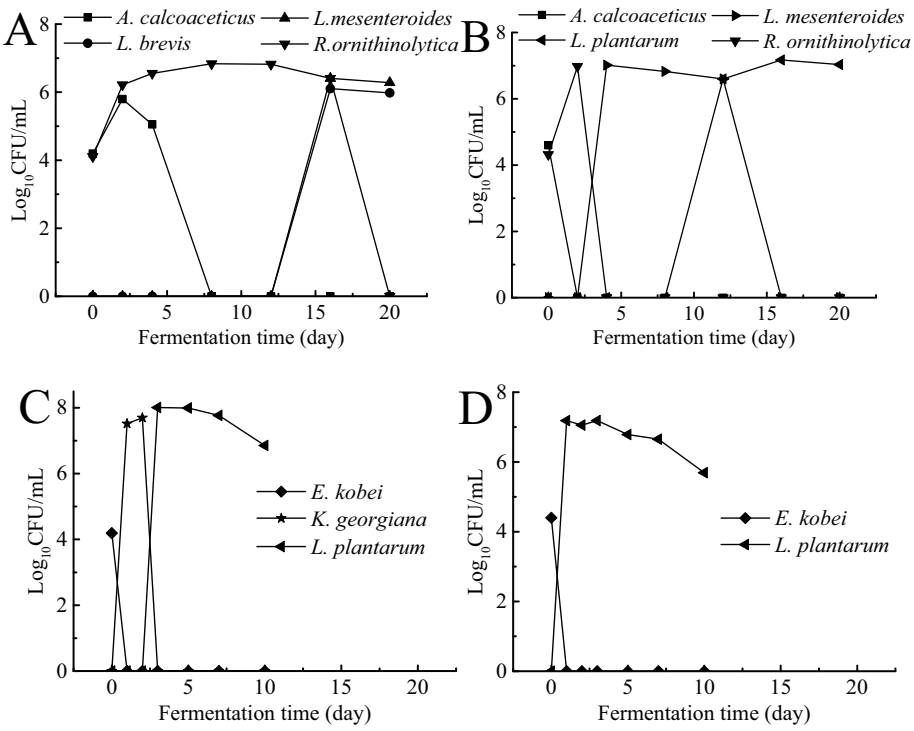

Fig. 4. Analysis of the number of bacteria in fermented paocai at different temperatures by culture-dependent methods. Trace of the number of identified bacteria through the record of similar bacteria in the previous period. (A) 10 °C, (B) 15 °C, (C) 25 °C, (D) 35 °C.

in the culture-independent method, it is known that in the initial fermentation, there is an abundance of > 1% of *Lactobacillus*, unclassified_*Enterobacteriaceae*, *Lactococcus*, *Acinetobacter*, *Leuconostoc*, *Weissella*, *Raoultella*, unclassified_*Lactobacillales*, *Bacillus*, *Erwinia*, and *Pseudomonas*. However, the culture-dependent method detects initial fermentation microorganisms such as *Acinetobacter*, *Enterobacter*, and *Raoultella*, including *A. calcoaceticus*, *E. cloacae* and *R. ornithinolytica*. As the fermentation progresses, the micro ecological environment changes; some microbial flora that are not suitable for the paocai system are gradually eliminated, and a phenomenon of "self-purification" appears in the fermentation process, resulting in a significant decline in bacterial diversity. This observation is consistent with the fermentation process of many fermented vegetables (Jeong et al., 2013c; Jeong et al., 2013b; Xiong et al., 2012). Under medium high-temperature fermentation conditions, the changes in acidity and other conditions of the fermentation system are more intense, and this accelerates the evolution of microbial diversity. However, high-temperature fermented paocai is susceptible to bacterial infection, which may lead to increased diversity in later stages.

The bacterial community structure evolution during fermentation at different temperatures was different, and the results of the culture-dependent and culture-independent methods were consistent. *L. lactis* started paocai fermentation. In the middle-stage of paocai fermentation, *Weissella*, *L. mesenteroides*, and *L. brevis* were present as major populations. Members of the genus *L. plantarum* appeared as one of the dominant populations at late stage fermentation. Interestingly, the higher the temperature is, the earlier the emergence of *L. plantarum*.

The microbial community structure during different temperature paocai fermentation showed that *L. plantarum*, *L. brevis*, *L. lactis*, *L. mesenteroides*, *A. calcoaceticus*, and *R. ornithinolytica* were the dominant bacteria. It is well known that biological characteristics of microbes inhabiting fermented foods could determine the microbiota structure. On the one hand, in terms of the temperature tolerance of the flora, these genera are medium- and low-temperature bacteria, and the optimum growth and fermentation temperature range is 20–40 °C (Schleifer, 2009). In particular, *L. plantarum*, *L. brevis*, *L. lactis*, and *L. mesenteroides* are associated with the acid production of paocai and can be grown at lower temperatures (Lee et al., 2006; Lee et al., 2005). In combination with TTA, this also explains why higher temperature paocai fermentation produces acid more quickly. On the other hand, as fermentation progresses, the pH reduction also affects the dominant flora. For example, *Lactobacillus* predominates (10 °C after 12 days) or is the dominant genus (15 °C after 8 days) in the low-temperature group of paocai, which has a significant acid tolerance compared with that of other groups. *L. mesenteroides*, *L. lactis*, and other strains are weaker than *L. plantarum* (Herreros et al., 2005) and disappear after fermentation. In addition, it has also been reported that lactic acid bacteria have products such as metabolic bacteriocins that can antagonize other gram-negative microorganisms such as *Escherichia coli* (Todorov and Dicks, 2006; Yang et al., 2012) and may also be one of the factors for the decline of *Enterobacteriaceae* abundance in the middle and late stages of fermentation. From the perspective of fermentation characteristics, it can be seen that low-temperature is more conducive to the growth of heterofermentative lactic acid bacteria to produce acetic acid, ethanol, and other substances, and 10 °C is more conducive to the promotion of paocai flavor (Xiao et al., 2018). From the perspective of microorganisms, the ripening of paocai is related to the growth of *L. plantarum*. *Leuconostoc*, *Weissella*, and other lactic acid bacteria produce less acid, but the facultatively heterofermentative *L. plantarum* produces a large amount of lactic acid, accelerating the ripening of paocai (Jeong et al., 2013b). The artificial regulation of paocai should be combined with environmental factors and lactic acid bacteria characteristics. In the initial stage, low medium-temperature fermentation is carried out, and *L. brevis*, *L. mesenteroides*, and *L. lactis* are added to strengthen the aroma. In the middle of fermentation, *L. plantarum* is added to rapidly produce acid and shorten the fermentation time.

In the past, a large number of research results have shown that the dominant microbial community structures in the processes of starting fermentation, that lead to fermentation and termination of fermentation are not the same. For example, when Pederson studied core cabbage, he noted that *Leuconostoc mesophila* started paocai fermentation (Pederson, 1930). Jeong et al. (2013a) used 454-pyrosequencing

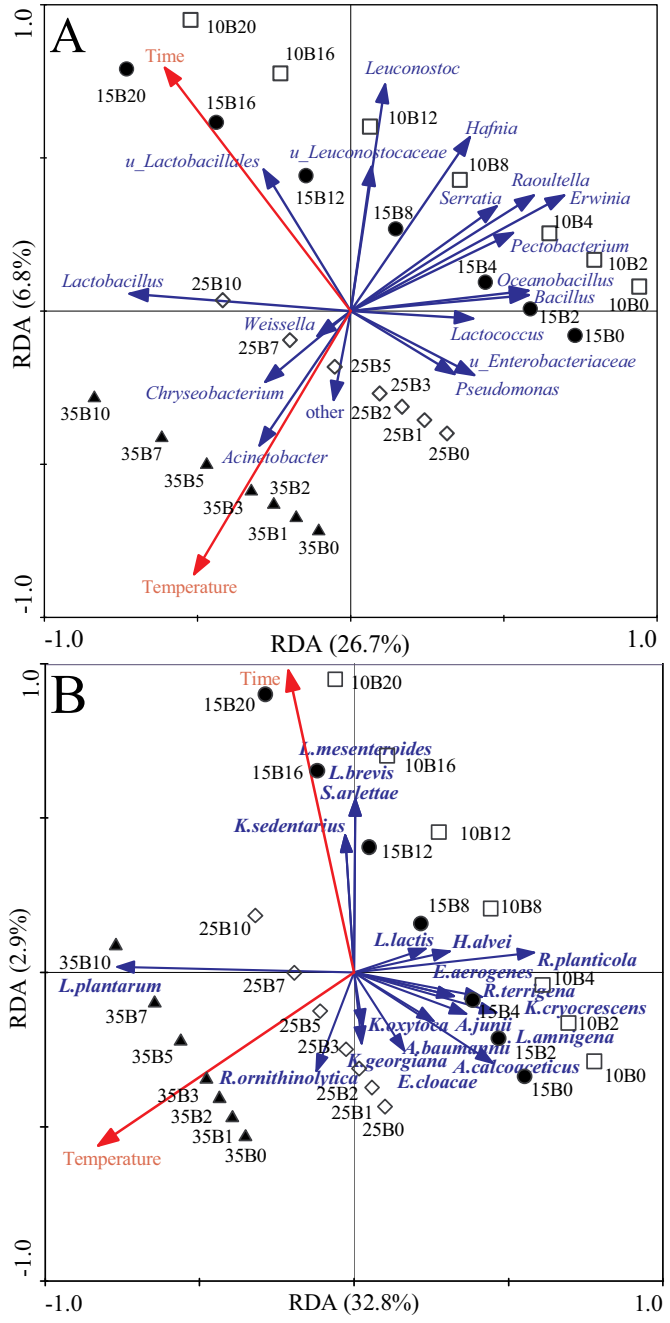

Fig. 5. Redundancy analysis (RDA) based on microbial community. (A) culture-independent methods analysis genus level, (B) culture-dependent methods species analysis level.

technology to study the initial stages of radish fermentation with *Leuconostoc*, *Lactobacillus*, *Pseudomonas*, *Pantoea*, and *Weissella*, of which *Leuconostoc* was the dominant genus in the first 3 days. M. Lee et al. (2017) used 454-pyrosequencing technology to indicate that Chinese-made Chinese cabbage was dominated by *Weissella*, *Lactobacillus*, and *Proteobacteria* in the early stage of fermentation, and that *Lactococcus*, *Weissella*, and *Lactobacillus* were the dominant bacteria at the end of fermentation. Xiong et al. (2012) studied the natural fermentation of paocai by a culture-dependent method. The initial stage was found to be dominated by *Enterococcus faecalis* and *L. lactis* subsp. *lactis*. *L. mesenteroides* subsp. *mesenteroides* was the dominant bacterium in the middle stage, and *L. plantarum* and *Lactobacillus casei* were the dominant bacterium in the later stage. Jung et al. (2011) used macro sequencing to study *Leuconostoc* in the early stage of kimchi fermentation, followed by rapid growth of *Lactobacillus* and *Weissella* in the middle stage. *Leuconostoc*, *Lactobacillus*, and *Weissella* were dominant in the main fermentation stages.

Some conclusions are consistent with this study, but there are also reports of significant differences. Fig. 2 showed that *Lactococcus* was found in all the samples (at 0 days) of the different temperature groups, which grew rapidly as starter bacteria in the early fermentation stages. This observation indicates that the initial bacteria in the raw material can act as a fermentation starter and may strongly influence the flora structure at the initial fermentation stage of paocai. The flora associated with the beginning and termination of fermentation of paocai is affected by temperature. Fermentation in low medium-temperature paocai is initiated by *Lactococcus*, while fermentation at a high temperature is initiated by *Lactococcus*, *Weissella*, and *Lactobacillus*. The ripening of low-temperature paocai is due to *L. brevis* and *L. mesenteroides*, while the ripening of medium-high-temperature paocai is due to *L. plantarum*. These results are inconsistent with the conventionally reported fermentation by *L. mesenteroides*, and the termination of fermentation by *L. plantarum*. The differences are mainly due to factors such as production process, production area, and processing conditions, and the results are difficult to compare. For the first time, this study used culture-dependent and culture-independent methods to demonstrate that changes in temperature can lead to the evolution of different dominant flora, which in turn form paocai products of different flavors. In addition, the culture-dependent and culture-independent results are basically the same, showing that paocai is a simple fermentation system. This system is the same system utilized for most fermented foods, such as cheese and kefir milk. Most of the strains are cultivable (Garrote et al., 2002; Ozturkoglu Budak et al., 2016), which also lays the foundations for regulating the fermentation of paocai.

It is worth noting that a large number of *Enterobacteriaceae* microorganisms were found in all temperature groups of paocai, and *Enterobacteriaceae* microorganisms are important indicators of the hygienic environment. In the paocai fermentation process, especially in the early stage of fermentation, the number and variety of *Enterobacteriaceae* were high, including *Enterobacter*, *Klebsiella*, *Kluyvera*, *Lelliottia*, and *Raoultella* (Table 1). More species and a higher quantity of *Enterobacteriaceae* were found in the low-temperature paocai. Although it is generally believed that acidic fermented foods, especially fermented vegetable products, are microbiologically safe (Liu et al., 2011; Nout, 1994), problems with food-borne pathogenic bacteria in acidic foods have been reported in recent years (Cho et al., 2014; Inatsu et al., 2004), which deserves our close attention. In addition, during the experiment, we also found that some samples of the 35 °C paocai group showed some spoilage and odor during late fermentation (10th day). The abundance of *Acinetobacter*, a class of spoilage microorganisms, significantly increases during this process, and such microorganisms are commonly reported in other spoiled foods such as milk, shrimp, and fish products (Li et al., 2018; Ribeiro Junior et al., 2018; Zhu et al., 2018). This microbe may be a major cause of paocai spoilage and we are currently researching it. At the same time, this result shows that there are certain health and safety hazards in natural fermentation, and that it is necessary to regulate the fermentation of paocai.

5. Conclusion

In this paper, based on culture-independent and culture-dependent methods combined with multivariate statistics, the effects of temperature on bacterial community structure during paocai fermentation were systematically studied. In a certain temperature range, increasing the fermentation temperature is beneficial for rapid acid production and shortening the paocai maturity period, whereas low-temperature paocai fermentation is beneficial for maintaining the number of lactic acid bacteria in the fermentation process. The difference in temperature led to microbial diversity in the paocai fermentation process. There was

more diversity in the start-up fermentation stage, which gradually decreased with the progression of fermentation. *Lactobacillus* was the dominant bacterial genus in the late fermentation stage. This study found that the results of culture-dependent and culture-independent methods are consistent, indicating that the dominant bacteria in the paocai fermentation process were mostly cultured, which provides conditions for the artificial control of paocai. However, more detailed studies are needed to better understand the relationships of quality and paocai microbial communities with fermentation temperature.

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Declaration of competing interest

The authors state no conflict of interest.

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References

Ahmadsah, L.S., Min, S.G., Han, S.K., Hong, Y., Kim, H.Y., 2015. Effect of low salt concentrations on microbial changes during kimchi fermentation monitored by PCR-DGGE and their sensory acceptance. J. Microbiol. Biotechnol. 25, 2049–2057.
Cheigh, H.S., Park, K.Y., 1994. Biochemical, microbiological, and nutritional aspects of kimchi (Korean fermented vegetable products). Crit. Rev. Food Sci. Nutr. 34, 175–203.
Cho, J.-I., Joo, I.-S., Park, K.-S., Han, M.-K., Son, N.-R., Jeong, S.-J., Heo, J., Kim, Y.-J., Oh, M.-H., Kim, S.-H., Lee, S.-H., 2014. Characterization of pathogenic Escherichia coli strains linked to an outbreak associated with kimchi consumption in South Korea, 2012. Food Sci. Biotechnol. 23, 209–214.
Di Cagno, R., Minervini, G., Sgarbi, E., Lazzi, C., Bernini, V., Neviani, E., Gobbetti, M., 2010. Comparison of phenotypic (Biolog System) and genotypic (random amplified polymorphic DNA-polymerase chain reaction, RAPD-PCR, and amplified fragment length polymorphism, AFLP) methods for typing Lactobacillus plantarum isolates from raw vegetables and fruits. Int. J. Food Microbiol. 143, 246–253.
García, F.C., Bestion, E., Warfield, R., Yvon-Durocher, G., 2018. Changes in temperature alter the relationship between biodiversity and ecosystem functioning. Proc. Natl. Acad. Sci. 115, 10989–10994.
Garrote, G.L., Abraham, A.G., De Antoni, G.L., 2002. Chemical and microbiological characterisation of kefir grains. J. Dairy Res. 68, 639–652.
Hamady, M., Walker, J.J., Harris, J.K., Gold, N.J., Knight, R., 2008. Error-correcting barcoded primers for pyrosequencing hundreds of samples in multiplex. Nat. Methods 5, 235.
Haruta, S., Kondo, M., Nakamura, K., Aiba, H., Ueno, S., Ishii, M., Igarashi, Y., 2002. Microbial community changes during organic solid waste treatment analyzed by double gradient-denaturing gradient gel electrophoresis and fluorescence in situ hybridization. Appl. Microbiol. Biotechnol. 60, 224–231.
Herreros, M.A., Sandoval, H., González, L., Castro, J.M., Fresno, J.M., Tornadijo, M.E., 2005. Antimicrobial activity and antibiotic resistance of lactic acid bacteria isolated from Armada cheese (a Spanish goats' milk cheese). Food Microbiol. 22, 455–459.
Holzapfel, W., 1997. Use of starter cultures in fermentation on a household scale. Food Control 8, 241–258.
Hong, Y., Yang, H.-S., Chang, H.-C., Kim, H., 2013. Comparison of bacterial community changes in fermenting kimchi at two different temperatures using a denaturing gradient gel electrophoresis analysis. J. Microbiol. Biotechnol. 23, 76–84.
Inatsu, Y., Bari, M.L., Kawasaki, S., Isshiki, K., 2004. Survival of Escherichia coli O157:H7, Salmonella Enteritidis, Staphylococcus aureus, and Listeria monocytogenes in Kimchi. J. Food Prot. 67, 1497–1500.
Jeong, S.H., Jung, J.Y., Lee, S.H., Jin, H.M., Jeon, C.O., 2013a. Microbial succession and metabolite changes during fermentation of dongchimi, traditional Korean watery kimchi. Int. J. Food Microbiol. 164, 46–53.
Jeong, S.H., Lee, S.H., Jung, J.Y., Choi, E.J., Jeon, C.O., 2013b. Microbial succession and metabolite changes during long-term storage of Kimchi. J. Food Sci. 78, M763–M769.
Jeong, S.H., Lee, H.J., Jung, J.Y., Lee, S.H., Seo, H.Y., Park, W.S., Jeon, C.O., 2013c. Effects of red pepper powder on microbial communities and metabolites during kimchi fermentation. Int. J. Food Microbiol. 160, 252–259.
Jung, J.Y., Lee, S.H., Kim, J.M., Park, M.S., Bae, J.-W., Hahn, Y., Madsen, E.L., Jeon, C.O., 2011. Metagenomic analysis of kimchi, a traditional Korean fermented food. Appl. Environ. Microbiol. 77, 2264–2274.
Jung, J.Y., Lee, S.H., Lee, H.J., Seo, H.Y., Park, W.S., Jeon, C.O., 2012. Effects of Leuconostoc mesenteroides starter cultures on microbial communities and metabolites during kimchi fermentation. Int. J. Food Microbiol. 153, 378–387.
Jung, J.Y., Lee, S.H., Jeon, C.O., 2014. Kimchi microflora: history, current status, and perspectives for industrial kimchi production. Appl. Microbiol. Biotechnol. 98, 2385–2393.
Kim, M., Chun, J., 2005. Bacterial community structure in kimchi, a Korean fermented vegetable food, as revealed by 16S rRNA gene analysis. Int. J. Food Microbiol. 103, 91–96.
Lee, K., Lee, Y., 2010. Effect of Lactobacillus plantarum as a starter on the food quality and microbiota of kimchi. Food Sci. Biotechnol. 19, 641–646.
Lee, J.S., Heo, G.Y., Lee, J.W., Oh, Y.J., Park, J.A., Park, Y.H., Pyun, Y.R., Ahn, J.S., 2005. Analysis of kimchi microflora using denaturing gradient gel electrophoresis. Int. J. Food Microbiol. 102, 143–150.
Lee, D., Han, H., Cho, J., Kim, J., Yang, C., Jeon, J., 2006. Microbial population dynamics of kimchi, a fermented cabbage product. FEMS Microbiol. Lett. 257, 262–267.
Lee, D., Kim, S., Cho, J., Kim, J., 2008. Microbial population dynamics and temperature changes during fermentation of kimjang kimchi. J. Microbiol. 46, 590–593.
Lee, M., Song, J.H., Jung, M.Y., Lee, S.H., Chang, J.Y., 2017a. Large-scale targeted metagenomics analysis of bacterial ecological changes in 88 kimchi samples during fermentation. Food Microbiol. 66, 173–183.
Lee, H.-W., Yoon, S.-R., Kim, S.-J., Lee, H.M., Lee, J.Y., Lee, J.-H., Kim, S.H., Ha, J.-H., 2017b. Identification of microbial communities, with a focus on foodborne pathogens, during kimchi manufacturing process using culture-independent and -dependent analyses. LWT Food Sci. Technol. 81, 153–159.
Li, L., Pan, Z., Shen, Z., 2018. Identification of the dominant microbial species of spoiled crisp grass carp (Ctenopharyngodon idellus C. et V.) and grass carp (Ctenopharyngodon idellus) fillets during cold storage by culture-independent 16S rDNA sequence analysis. J. Food Prot. 81, 84–92.
Liang, H., Chen, H., Zhang, W., Yu, C., Ji, C., Lin, X., 2018. Investigation on microbial diversity of industrial Zhacai paocai during fermentation using high-throughput sequencing and their functional characterization. LWT Food Sci. Technol. 91, 460–466.
Liu, S.-n., Han, Y., Zhou, Z.-j., 2011. Lactic acid bacteria in traditional fermented Chinese foods. Food Res. Int. 44, 643–651.
Liu, A., Li, X., Pu, B., Ao, X., Zhou, K., He, L., Chen, S., Liu, S., 2017. Use of psychrotolerant lactic acid bacteria (Lactobacillus spp. and Leuconostoc spp.) isolated from Chinese traditional paocai for the quality improvement of paocai products. J. Agric. Food Chem. 65, 2580–2587.
Nout, M.J.R., 1994. Fermented foods and food safety. Food Res. Int. 27, 291–298.
Ozturkoglu Budak, S., Figge, M.J., Houbraken, J., de Vries, R.P., 2016. The diversity and evolution of microbiota in traditional Turkish Divle Cave cheese during ripening. Int. Dairy J. 58, 50–53.
Pardali, E., Paramithiotis, S., Papadelli, M., Mataragas, M., Drosinos, E.H., 2017. Lactic acid bacteria population dynamics during spontaneous fermentation of radish (Raphanus sativus L.) roots in brine. World J. Microbiol. Biotechnol. 33, 110.
Park, E.J., Chun, J., Cha, C.J., Park, W.S., Jeon, C.O., Bae, J.W., 2012. Bacterial community analysis during fermentation of ten representative kinds of kimchi with barcoded pyrosequencing. Food Microbiol. 30, 197–204.
Park, S.-E., Seo, S.-H., Kim, E.-J., Na, C.-S., Son, H.-S., 2018. Effects of different fermentation temperatures on metabolites of Kimchi. Food Biosci. 23, 100–106.
Pederson, C.S., 1930. Floral changes in the fermentation of sauerkraut. New York (Geneva) Agric. Exp. Sta. Tech. Bull. 168, 1–37.
Ribeiro Junior, J.C., de Oliveira, A.M., Silva, F.G., Tamanini, R., de Oliveira, A.L.M., Beloti, V., 2018. The main spoilage-related psychrotrophic bacteria in refrigerated raw milk. J. Dairy Sci. 101, 75–83.
Schleifer, K.-H., 2009. Phylum XIII. Firmicutes Gibbons and Murray 1978, 5 (Firmacutes [sic] Gibbons and Murray 1978, 5). In: De Vos, P., Garrity, G.M., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.-H., Whitman, W.B. (Eds.), Bergey's Manual® of Systematic Bacteriology: Volume Three. The Firmicutes. Springer New York, New York, NY, pp. 19–1317.
Seo, Y.-H., Jang, J.-H., Moon, K.-D., 2010. Microbial evaluation of minimally processed vegetables and sprouts produced in Seoul, Korea. Food Sci. Biotechnol. 19, 1283–1288.
Todorov, S.D., Dicks, L.M.T., 2006. Screening for bacteriocin-producing lactic acid bacteria from boza, a traditional cereal beverage from Bulgaria: comparison of the bacteriocins. Process Biochem. 41, 11–19.
Wang, Z., Shao, Y., 2018. Effects of microbial diversity on nitrite concentration in pao cai, a naturally fermented cabbage product from China. Food Microbiol. 72, 185–192.
Wouters, D., Grosu-Tudor, S., Zamfir, M., De Vuyst, L., 2013. Bacterial community dynamics, lactic acid bacteria species diversity and metabolite kinetics of traditional Romanian vegetable fermentations. J. Sci. Food Agric. 93, 749–760.
Xiao, Y., Xiong, T., Peng, Z., Liu, C., Huang, T., Yu, H., Xie, M., 2018. Correlation between microbiota and flavours in fermentation of Chinese Sichuan Paocai. Food Res. Int. 114, 123–132.
Xiong, T., Guan, Q., Song, S., Hao, M., Xie, M., 2012. Dynamic changes of lactic acid bacteria flora during Chinese sauerkraut fermentation. Food Control 26, 178–181.
Xiong, T., Peng, F., Liu, Y., Deng, Y., Wang, X., Xie, M., 2014. Fermentation of Chinese sauerkraut in pure culture and binary co-culture with Leuconostoc mesenteroides and Lactobacillus plantarum. LWT Food Sci. Technol. 59, 713–717.
Yan, P.-M., Xue, W.-T., Tan, S.-S., Zhang, H., Chang, X.-H., 2008. Effect of inoculating lactic acid bacteria starter cultures on the nitrite concentration of fermenting Chinese paocai. Food Control 19, 50–55.
Yang, E., Fan, L., Jiang, Y., Doucette, C., Fillmore, S., 2012. Antimicrobial activity of bacteriocin-producing lactic acid bacteria isolated from cheeses and yogurts. AMB Express 2, 48.
Yu, J., Gao, W., Qing, M., Sun, Z., Wang, W., Liu, W., Pan, L., Sun, T., Wang, H., Bai, N., Zhang, H., 2012. Identification and characterization of lactic acid bacteria isolated from traditional pickles in Sichuan, China. J. Gen. Appl. Microbiol. 58, 163–172.
Zhang, Q., Chen, G., Shen, W., Wang, Y., Zhang, W., Chi, Y., 2016. Microbial safety and sensory quality of instant low-salt Chinese paocai. Food Control 59, 575–580.
Zhang, F., Tang, Y., Ren, Y., Yao, K., He, Q., Wan, Y., Chi, Y., 2018. Microbial composition of spoiled industrial-scale Sichuan paocai and characteristics of the microorganisms responsible for paocai spoilage. Int. J. Food Microbiol. 275, 32–38.
Zhu, S., Wu, H., Zhang, C., Jie, J., Liu, Z., Zeng, M., Wang, C., 2018. Spoilage of refrigerated Litopenaeus vannamei: eavesdropping on Acinetobacter acyl-homoserine lactones promotes the spoilage potential of Shewanella baltica. J. Food Sci. Technol. 55, 1903–1912.