High-throughput sequencing method to study the dynamic changes of microbial communities in second-generation fermented lettuce

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Abstract: Pickle has been loved by consumers since ancient times because of its unique taste, strong flavor, simple process and long shelf life. Complex microbial succession activities promote the fermentation process of pickle. In this paper, high-throughput sequencing methods were used to study the bacterial microbial dynamics of the second-round fermentation lettuce artificially inoculated with Lactobacillus. The results showed that as the fermentation progressed, at the bacterial genus level, Lactobacillus are the dominant genus in the fermentation process. In the late fermentation stage, the dynamic changes of microorganisms tend to be stable. These results provide ideas and new strategies for the development of pickle products using lettuce as the main vegetable raw material.

1. Introduction
China has a long history of fermented vegetables, which can be traced back to thousand years ago.[1] In addition to being popular with people due to its unique flavor, it is also rich in many nutrients, such as vitamins, amino acids, etc.[2] In South Korea, local chili powder, seafood, fruits, etc. are added to fermented vegetables to produce the world-renowned Korean pickle.[3] Due to different regions, the types of fermented vegetables are also different. Common ones include Sichuan pickle, Northeast sauerkraut, Korean pickle, Japanese pickle, German sauerkraut, and American pickled pickled cucumbers.

Fermented vegetables refer to the products in which salt, auxiliary materials, water, etc. are added to the vegetables for sealed preservation and fermentation. During the fermentation process, microorganisms are used to decompose the nutrients and their products in the vegetables to improve the flavor of the vegetables.[4] The main microorganisms involved in the fermentation process are lactic acid bacteria, yeasts, acetic acid bacteria, molds, etc. [5]

The fermentation process of kimchi is highly characterized to the succession of complex microorganisms, thereby forming various vitamins, amino acids and other nutrients, forming a special flavor. In recent years, in order to improve the quality of fermented vegetables, many scholars have conducted research on the changes of microorganisms during the fermentation process, but there are few fermented vegetable products using lettuce as the main raw material.

In recent years, it was generally tended to inoculate pickle with suitable fermentation strains at the
beginning of fermentation to improve the sensory properties and shelf life. Inoculation of brine from fermented vegetables and putting in new raw materials for secondary or multiple fermentation is the initial artificial inoculation and fermentation method. An-jun Chen \[^6\] found that multiple inoculation of fermented vegetables with brine can speed up the fermentation process and shorten the fermentation cycle. The aim of this study was to investigate the dynamic changes of microbial communities in the second-generation fermented lettuce through denatured gradient gel electrophoresis (DGGE) and High-throughput sequencing, which provides the possibility for fermented vegetable products using lettuce as the main raw material.

2. Materials and methods

2.1 Bacterial culture conditions
Preparation of strain activation and cultivation methods refer to Chen’s. \[^7\] In detail, the LV02(*Lactiplantibacillus plantarum* V02 MH885507) and LV73(*Lentilactobacillus diolivorans* V73 MH885506) were respectively incubated in MRS broth at 37 ℃ for 24 h to 8 log CFU/ml, then activate twice with 5% inoculum.

2.2 Preparation of pickle and sampling
Lettuces were purchased from the local supermarket in Beijing University of Agriculture, Beijing, China. The preparation of pickle was performed as follows:
Fresh lettuces, parsley and carrots were cut, the mass ratio is 8:1:1, mixed and placed in a jar. Add 3% salt to the pepper water. The bacterial liquid was inoculated into fermented vegetables at a concentration ratio of LV02:LV73=1:1, and fermented for 7 days as a round of fermented lettuce samples. The brine of the first round of fermented lettuce was inoculated into the new round of fermented lettuce at a 5% inoculation amount.

2.3 DNA extraction and PCR amplification
Microbial community genomic DNA was extracted from pickle samples using the FastDNA SPIN for soil kit (MP Biomedicals, Solon, USA) according to manufacturer’s instructions. The DNA extract was checked on 1% agarose gel, and DNA concentration and purity were determined with NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA). The hypervariable region V3-V4 of the bacterial 16S rRNA gene were amplified with primer pairs 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R(5'-GGACTACHVGGGTWTCTAAT-3') by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). The PCR amplification of 16S rRNA gene was performed as follows: initial denaturation at 95 ℃ for 3 min, followed by 27 cycles of denaturing at 95 ℃ for 30 s, annealing at 55 ℃ for 30 s and extension at 72 ℃ for 45 s, and single extension at 72 ℃ for 10 min, and end at 10 ℃. The PCR mixtures contain 5 × *TransStart* FastPfu buffer 4 μL, 2.5 mM dNTPs 2 μL, forward primer (5 μM) 0.8 μL, reverse primer (5 μM) 0.8 μL, *TransStart* FastPfu DNA Polymerase 0.4 μL, template DNA 10 ng, and finally ddH2O up to 20 μL. PCR reactions were performed in triplicate. The PCR product was extracted from 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to manufacturer’s instructions and quantified using Quantus™ Fluorometer (Promega, USA).

2.4 Illumina MiSeq sequencing
Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq PE300 platform/NovoSeq PE250 platform (Illumina, San Diego,USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database.

2.5 Processing of sequencing data
The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered by fastp version 0.20.0
and merged by FLASH version 1.2.7 with the following criteria: (i) the 300 bp reads were truncated at any site receiving an average quality score of <20 over a 50 bp sliding window, and the truncated reads shorter than 50 bp were discarded, reads containing ambiguous characters were also discarded; (ii) only overlapping sequences longer than 10 bp were assembled according to their overlapped sequence. The maximum mismatch ratio of overlap region is 0.2. Reads that could not be assembled were discarded; (iii) Samples were distinguished according to the barcode and primers, and the sequence direction was adjusted, exact barcode matching, 2 nucleotide mismatch in primer matching.

Operational taxonomic units (OTUs) with 97% similarity cutoff were clustered using UPARSE version 7.1, and chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analyzed by RDP Classifier version 2.2 against the 16S rRNA database (eg. Silva v138) using confidence threshold of 0.7.

3. Result

3.1 alpha diversity and Bar diagram during pickle fermentation

| Sample | OTUs | Shannon | Simpson | Ace | Chao | Coverage | Sobs |
|--------|------|---------|---------|-----|------|----------|------|
| D0     | 82   | 1.7216  | 0.2518  | 94.5021 | 71.9206 | 0.9996 | 56   |
| D1     | 88   | 2.0771  | 0.1626  | 95.2458 | 81.4762 | 0.9996 | 60.6667 |
| D2     | 88   | 1.8471  | 0.1989  | 90.0037 | 92.0333 | 0.9995 | 63.3333 |
| D3     | 102  | 2.1043  | 0.1659  | 113.8643 | 89.1255 | 0.9996 | 74.6667 |
| D4     | 95   | 1.8697  | 0.2044  | 163.1540 | 127.8846 | 0.9994 | 79   |
| D5     | 99   | 1.6442  | 0.2951  | 111.7399 | 91.0278 | 0.9996 | 70.6667 |
| D6     | 51   | 1.3598  | 0.4155  | 61.9144 | 59.9643 | 0.9998 | 42.3333 |
| D7     | 74   | 1.5508  | 0.3809  | 101.9141 | 83.6329 | 0.9996 | 61.6667 |

The OTU coverage rate exceeds 99% (Table 1), indicating that the sampling is reasonable and can reflect the richness and diversity of the microbial community in the sample. The Shannon Index maintained a relatively high level in the initial stage of fermentation, and then slowly decreased. This indicates that the microbial diversity in pickle was gradually decreasing. Chao and ACE were indicators to measure the grade of species, further indicating that the sample was the most abundant on the 4th day of fermentation.

Figure 1. Analysis of flora structure of genes level during the vegetables fermentation
From the genus level (Figure 1), the bacterial community of pickle was significantly different from others. This reflects the characteristics of each fermentation stage. It could be seen that Enterobacter, Pantoea, Pseudomonas and Klebsiella exist in the early stage of fermentation. Pseudomonas, Pantoea, and Enterobacter were the primary bacterial populations on the 0th day of fermentation, which were derived from vegetable raw materials. Research by Miyao et al. [10] had shown that Bacillus and Micrococcus are common on the surface of leafy vegetables, Pseudomonas, Enterobacter and Klebsiella etc. Pseudomonas occupied the largest proportion in D0, about 73.71%, and then dropped sharply to 0.01% in D1, and was hardly observed in the next few days. The surface of the plant was a good habitat for microorganisms. [11] The raw materials of fermented lettuce contained a lot of microorganisms, and the pickle brine contained Lactobacillus and Weissella. Therefore, there were also a small amount of Lactobacillus and Weissella in the early fermentation of lettuce in the mother water. In the early stage of fermentation, Klebsiella and Leuconostoc dominated the fermentation process. As the fermentation progressed, the proportion of Lactobacillus and Weissella increased and dominated the fermentation process. In the process of vegetable fermentation, bacterial genera including Lactobacillus, Leuconostoc, and Lactococcus could play an important role in the glycolysis pathway and fermentation process. [12]

3.2 heat map during pickle fermentation

![Heat map image](image)

Figure. 2. Heat map of clustering during the lettuce fermentation

As shown in the figure 2, cluster analysis was performed on the similarity of species abundance at the genus classification level of 8 samples at different fermentation periods. By analyzing the color gradient and similarity, it can be observed that D0 is significantly different from other samples, D1, D2, D3 are basically close, D4, D5, D6, and D7 are basically close, indicating that as the fermentation matures, the microorganisms between the samples, the differences decrease, and the species are more and more similar. The genera with higher D0 content were Pseudomonas, Pantoea and Enterococcus, which belong to the phylum Proteobacteria. As the fermentation progressed, the genus of the sample bacteria changed greatly. In the early stage of fermentation, the genus of Proteobacteria decreased, Leuconostoc, Klebsiella, Kluyvera, etc. were more abundant, and the acidity decreased at this stage. The Pectobacterium detected in the D4 samples in the mid-fermentation period may be from external pollution. In the late fermentation period, the microbial diversity of the pickle samples was relatively reduced and stabilized, mainly containing Lactobacillus and Weissella belong to Firmicutes.

4. Discussion

In this study, the microbial diversity of the second round of fermented lettuce during fermentation was characterized using Illumina MiSeq sequencing. Lactobacillus and Weissella were observed as the dominant genera during fermentation (Figure 1). These findings were in agreement with our previous studies, and with reports on other fermented vegetables. [7] Lactobacillus can produce a large amount of...
lactic acid, thereby reducing the pH of fermented vegetables, forming an acidic environment, and inhibiting the growth of other acid intolerant microorganisms. 

In this study, the content of many acid intolerant microorganisms such as *Pantoea* and *Kluyvera* decreased in the later stage of fermentation, which is also consistent with the decrease of the shannon index in Table 1.

In this study, the abundance of *Lactobacillus* was increased rapidly at the D4-D7, reached the highest level of 66.92% at D7, during which time the ascending rate of total acid were highest. It gives pickles sour and refreshing taste, which is an important indicator for judging the quality of pickle. Some researchers have demonstrated *Leuconostoc*, *Lactobacillus* and *Weissella* dominate the fermentation.

This is different from the results of this experiment. This experiment found that the abundance of *Leuconostoc* during the fermentation of fermented lettuce Decrease gradually, and contribute little to the fermentation process. The analysis may be due to the inhibition of *Leuconostoc* growth due to low temperature and different raw materials.

5. Conclusions
The microorganism of fermented lettuce changed obviously during the fermentation. With the development of fermentation, *Lactobacillus* and *Weissella* have gradually become the dominant bacteria, and they are the main microorganisms in the fermentation process of lettuce. This was confirmed by high-throughput sequencing of lettuce inoculated with lactic acid bacteria. From the sixth day of fermentation, the microbial changes entered a stable period. It provides enough ideas and reference for the study of fermentation products with lettuce as the main raw material.

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