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Microbial Diversity during Fermentation of Sweet Paste, a Chinese Traditional Seasoning, Using PCR-Denaturing Gradient Gel Electrophoresis

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The aim of this study was to elucidate the changes in the microbial community and biochemical properties of a traditional sweet paste during fermentation. PCR-denaturing gradient gel electrophoresis (DGGE) analysis showed that *Aspergillus oryzae* was the predominant species in the koji (the fungal mixture), and the majority of the fungi isolated belonged to two *Zygosaccharomyces* species in the mash. The bacterial DGGE profiles revealed the presence of *Bacillus subtilis* during fermentation, and *Lactobacillus acidipiscis*, *Lactobacillus pubuzihii*, *Lactobacillus* sp., *Staphylococcus kloosi,* and several uncultured bacteria were also detected in the mash after 14 days of main fermentation. Additionally, during main fermentation, amino-type nitrogen and total acid increased gradually to a maximum of 6.77 ± 0.25 g/kg and 19.10 ± 0.58 g/kg (30 days) respectively, and the concentration of reducing sugar increased to 337.41 ± 3.99 g/kg (7 days). The 180-day fermented sweet paste contained 261.46 ± 19.49 g/kg reducing sugar and its pH value remained at around 4.65. This study has used the PCR-DGGE technique to demonstrate the microbial community (including bacteria and fungi) in sweet paste and provides useful information (biochemical properties) about the assessment of the quality of sweet paste throughout fermentation.

Keywords: Sweet paste, PCR-DGGE, microbial diversity, fermentation

Introduction

Sweet paste, a traditional seasoning produced through the fermentation of flour by fungi and bacteria, has been consumed for centuries as a flavoring ingredient and as the most important condiment of Beijing roast duck in China. Owing to its unique and delicious sweet taste with a slight and particular caramel flavor, sweet paste has also been used as a cooking sauce for dishes such as diced pork fillet or as a dip for green Chinese onion and noodles [1]. In the first phase of its manufacturing process, steamed flour is mixed with the spores of *Aspergillus oryzae*, followed by cultivating the mixture known as the "koji" for approximately 2 days. In the second phase, the koji is mixed with an appropriate amount of brine to form another mixture known as the "mash" [2]. After about 6 months of aging, a red-brown flour sauce called "sweet paste" is produced. A total of 84 volatile compounds have been identified from the Chinese fermented flour paste (a kind of sweet paste) using simultaneous distillation and extraction and gas chromatography-mass spectrometry [1].

The sauce quality is affected by the microflora involved, the fermentation process employed, and the basic ingredients used [3, 4]. Compared with culture-dependent methods, the culture-independent approach is based on the diversity of 16S rDNA and 18S rDNA genes, which not only facilitates the detection of microbes that are difficult to culture, but also can obtain an overview of the microbial community

structure [5, 6]. Among the various culture-independent methods, PCR-denaturing gradient gel electrophoresis (DGGE) is one of the most widely used methods for analyzing microbial diversity. Recently, PCR-DGGE has been used to investigate the microbial communities involved in many foods or seasonings, such as soy sauce [7], kimchi (Korean fermented cabbage) [8], doenjang (Korean fermented soybean paste) [9], soybean paste [10], daqu (Chinese liquor fermentation starter) [11], wine, and vinegar [12, 13]. It has been shown that molds, yeasts, and bacteria, including the genus *Bacillus* and lactic acid bacteria (LAB), are the dominant microorganisms and play important roles during fermentation [14–16]. For example, LAB such as *Leuconostoc mesenteroides*, *Tetragenococcus halophilus*, and *Enterococcus faecium* were the predominant bacterial species, and *Mucor plumbeus*, *A. oryzae*, and *Debaryomyces hansenii* were the most common fungal species in the doenjang samples [9].

However, little information is available about the microbial diversity in sweet paste using culture-independent methods. The aim of the present study was to investigate the microbial diversity of a traditional Chinese sweet paste using PCR-DGGE and analyze the changes in its biochemical properties during fermentation.

Materials and Methods

Sampling Procedures

Koji and mash samples were collected from a sweet paste factory (Jiatai Co., Ltd., China). Approximately 1,000 kg of steamed wheat flour (40°C–45°C, 45%–50% of moisture content) was inoculated with *A. oryzae* (10^{8-9} spores per kg materials, premixed with 25 kg dry wheat flour) and then incubated at 30°C–35°C for 43 h. Subsequently, the koji was mixed in a fermentation tank with brine to obtain a mash with a final concentration of 7.5% salt and 55% moisture content. The samples were fermented from September 2014 to February 2015, with an average monthly temperature of 23.7°C, 19.1°C, 13.0°C, 8.0°C, 8.4°C, and 9.8°C in September, October, November, December, January, and February, respectively. Solid koji samples from the koji making and mash samples from the outdoor aging were collected separately at 0, 7, 14, 21, 30, 60, 90, 120, 150, and 180 days in sterile bottles and transported to the laboratory at room temperature within 30 min.

DNA Extraction

DNA was extracted as previously described with some modifications [17–19]. Briefly, 10 g of the koji or mash samples was suspended in 15 ml of sterile saline (0.75%) and shaken for 1 min. The mixture was centrifuged at 6,000 ×*g* for 10 min at 4°C to pellet the cells, followed by washing three times with sterile saline, supplementing the cells with 50 µl of (20 mg/ml) proteinase K solution, and shaking the mixture at 37°C for 30 min at 130 rpm. Subsequently, 150 µl of 20% SDS was added, and the mixture was incubated at 65°C for 2 h and shaken smoothly at an interval of 15 min. After centrifugation at 6,000 ×*g* for 10 min at 4°C, the supernatant was collected and mixed with an equal amount of phenol-chloroform-isoamyl alcohol (25:24:1 (v/v)), followed by another centrifugation and mixing the supernatant with 0.6 volume of chloroform. After centrifugation at 12,000 ×*g* for 10 min at 4°C, the DNA was washed with 70% ethanol, and the pellet was then dried and dissolved in 100 µl of sterile water and stored at –20°C for further use.

PCR-DGGE Analysis

For the DGGE analysis of fungal communities, the fungal 18S rDNA was amplified by PCR using the primers GCFung (5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCATTCCCCGTTACCCGTTG-3′) and NS1 (5′-GTAGTCATATGCTTGTCTC-3′) [20]. PCR amplification was performed using a Thermal Cycler PCR apparatus (Bio-Rad, USA) under the following conditions: pre-incubation at 94°C for 4 min; 30 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min; and a final extension step of 72°C for 4 min.

For the DGGE analysis of bacterial communities, the universal bacterial PCR primers F-968-GC (5′-AACGCGAAGAACCTTACCGCCCGGGGCGCGCCCCGGGCGGCCCGGGGGCACCGGGGG-3′) and R-1401(5′-CGGTGTGTACAAGACCC-3′) were used to amplify the 16S rDNA V6–V8 region [21]. Each PCR mixture (50 µl) consisted of 1 µl of DNA template (approximately 10 ng), 5 µl of 10× Taq buffer, 2 µl of dNTP (2.5 mM of each; Takara, China), 25 pmol of each primer, and 0.5 U of Taq DNA polymerase (Takara). PCR amplification was performed using a Thermal Cycler PCR apparatus (Bio-Rad) as follows: pre-incubation at 94°C for 7 min; 30 cycles of 94°C for 30 sec, 56°C for 20 sec, and 68°C for 30 sec; and a final extension step of 68°C for 7 min.

The PCR products were analyzed by DGGE using a DCode apparatus (Bio-Rad). For the fungal DGGE analysis, electrophoresis experiments were performed using 8% gradient polyacrylamide gels containing a 20–40% denaturing gradient, with a 100% denaturant corresponding to 7 M urea and 40% formamide [22]. For the bacterial samples, gradient polyacrylamide gels ranging from 48% to 55% were produced. Electrophoresis was performed at 80–85 V and 60°C for 12–16 h. The gels were silver stained for 15 min after electrophoresis [23], and then the gel image was photographed. The selected bands in the gels were excised using a clean scalpel blade under UV illumination. Each slice was placed in an Eppendorf tube, and the DNA was eluted with 30 µl of sterile water by incubation at 4°C overnight. The extracted DNA was re-amplified using the same primers as described above. The re-amplified PCR products were purified with a PCR purification kit (Omega, USA) according to the manufacturer's instructions. Subsequently, the re-amplified PCR products were run on another DGGE gel along with the PCR products of the original samples to

confirm the presence of the bands at the same positions. Finally, the PCR products were sent for sequencing (Bio Sune Biotechnology Co., Ltd., China).

Sequence Analysis

Sequence analysis of bacterial DNA and fungal DNA was performed as previously described [24]. The 16S rDNA was amplified using the primers F-968 (5′-CCTACGGGAGGCAGCAG-3′) and R-1401, and the 18S rDNA was amplified using the primers Fung (5′-ATTCCCCGTTACCCGTTG-3′) and NS1. A search of the NCBI database was conducted using the BLAST algorithm to determine the closest relative of the DNA sequence.

Chemical Analysis of Sweet Paste during Fermentation

Total acid and amino-type nitrogen were determined by titration methods with some modifications [25]. Briefly, 5.0 g of each crushed sample was diluted to 100.0 ml. Then, the diluted samples (20.0 ml) were mixed with 60.0 ml of distilled water and titrated to pH 8.2 with 0.05 N NaOH. The volume of consumed NaOH was recorded for determining the total acid content. Subsequently, the samples titrated to pH 8.2 were supplemented with 20.0 ml of formalin solution (40%), and the volume of consumed NaOH was recorded for determining the amino-type nitrogen content. Reducing sugar was measured by the DNS method [26], and its content was determined as previously described [27]. The pH of the samples was directly determined by using a digital pH meter (HM-5 S; TOA Electric Industrial Co. Ltd., Japan). Each analysis was performed in triplicate, and all results are presented as the average of three experiments.

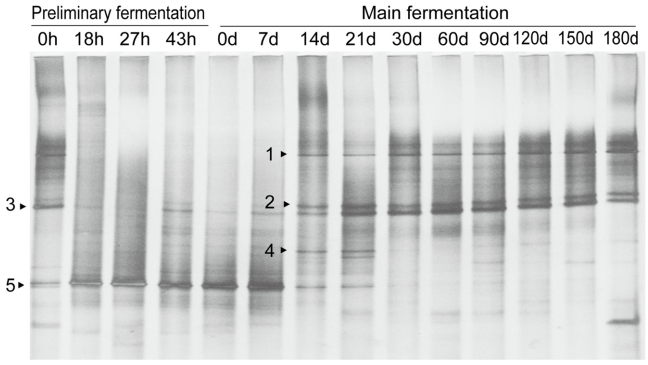

Fig. 1. PCR-DGGE profiles of fungal partial 18 S rDNA gene fragments amplified from the koji and mash.

A 28–40% denaturing gradient gel was used to analyze the PCR products amplified from DNA directly extracted from sweet paste koji and mash. Lanes 0, 18, 27, and 43 h are the preliminary fermentation samples from the koji-making stages for 0, 18, 27, and 43 h; and lanes 0, 7, 14, 21, 30, 60, 90, 120, 150, and 180 d are the main fermentation samples from outdoor aging for 0, 7, 14, 21, 30, 60, 90, 120, 150, and 180 days, respectively. The results of the DNA sequence analysis of the bands are summarized in Table 1.

Results and Discussion

Fungal Composition in Koji and Mash

Fig. 1 shows the DGGE fingerprinting patterns of fungal 18S rDNA of the fermented sweet paste samples. At least 5 bands were identified by PCR-DGGE analysis. A band for the genus *Aspergillus* was detected in the koji fermented for 18 h (Fig. 1, band 5). *Aspergillus oryzae* and *A.tamarii* showed high similarity in DNA sequences, and there is a 99% possibility that band 5 corresponded to *A. oryzae* or *A. tamarii* [28], probably due to *A. oryzae* inoculation in the starting materials. Notably, *A. oryzae* was the dominant microorganism in the preliminary fermentation and the early main fermentation stages. This *A. oryzae* band became weak on day 14 and could not be detected from mash fermented for 30 days, which might be caused by the added brine. The genus *Zygosaccharomyces* was involved in the sweet paste manufacturing process during the main fermentation. Bands of *Z. kombuchaensis* (*Z. lentus* or *Z. bailii*), and *Z. rouxii* (uncultured fungus) were detected by DGGE analysis (Fig. 1, bands 1–3). In addition, the band of *Pichia sorbitophila* (*P. farinose*) was detected from mash fermented for 14 and 21 days (Fig. 1, band 4). The identification results are presented in Table 1.

Fungal species are important for the sauce and stacking fermentation, where they produce amylases and proteases to degrade the complex starch in the raw materials into fermentable sugars, such as glucose and maltose, and

Table 1. Sequencing results of selected DGGE bands from the fungal DGGE fingerprint shown in Fig. 1.

Band No.[a]	Closest relative (NCBI Accession No.)	Identity[b]
1	*Zygosaccharomyces kombuchaensis* (AF339890)	307/307 (100%)
	Zygosaccharomyces lentus (AF339889)	307/307 (100%)
	Zygosaccharomyces bailii (X91083)	307/307 (100%)
2	*Zygosaccharomyces rouxii* (AY227015)	319/319 (100%)
	Zygosaccharomyces rouxii (AY227008)	319/319 (100%)
3	*Zygosaccharomyces rouxii* (CU928181)	316/316 (100%)
	Zygosaccharomyces rouxii (AM943655)	316/316 (100%)
4	*Pichia sorbitophila* (FO082054)	318/318 (100%)
	Pichia farinose (AB054281)	318/318 (100%)
5	*Aspergillus oryzae* (HM536621)	316/316 (99%)
	Aspergillus tamarii (AF516140)	316/316 (99%)

[a]Band numbers correspond to Fig. 1.

[b]Sequencing identity with a sequence in the GenBank database is represented as a percentage (the number of the identical base/total length of the DNA sequence).

protein into amino acids [2, 29, 30]. Previous studies showed that *A. oryzae* not only possessed high saccharolytic and proteolytic enzyme activities but also imparted special flavors to the fermented products [31, 32]. Owing to the low hydrolytic enzyme-producing ability of yeasts [33], filamentous fungi were the main hydrolytic enzyme producers. In this study, after accomplishing its hydrolytic function as a starter strain for sweet paste fermentation, the predominance of *A. oryzae* was taken over by yeasts and LAB during the late stage of fermentation. Among the four identified fungi, *Z. rouxii* was the only one used in the commercial starter cultures for Chinese soybean pastes [10]. As the esters produced by fungal enzymes are responsible for the pleasant flavor and aroma of paste [34], all these fungi could be important for the sweet paste flavor.

Bacterial Composition in Koji and Mash

Fig. 2 shows the DGGE fingerprinting patterns of bacterial 16S rDNA of the fermented sweet paste samples. More than 11 bands were detected by PCR- DGGE analysis. *Bacillus subtilis* and *Bacillus* sp. were detected in the koji (Fig. 2, bands 14 and 16), whereas in the mash, bands were detected for *Lactobacillus acidipiscis* (or *Lactobacillus* sp.), *Lactobacillus pobuzihii*, *Lactobacillus* sp., *Bacillus subtilis*, *Staphylococcus kloosii* (or *Staphylococcus cohnii*), and uncultured *Staphylococcus* and bacterium (Fig. 2). The identification results are presented in Table 2.

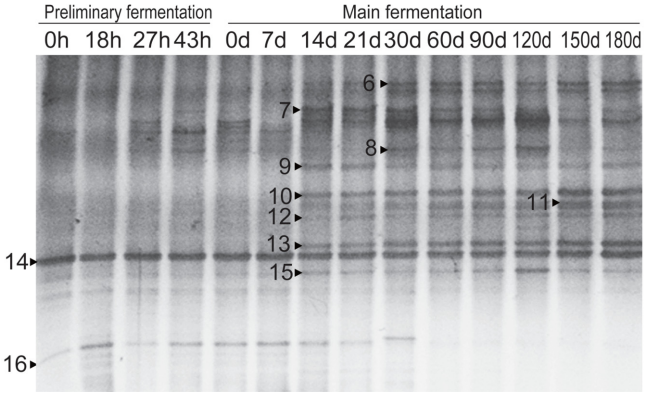

Fig. 2. PCR-DGGE profiles of bacterial V6–V8 16S rDNA gene fragments amplified from the koji and mash.

A 48–55% denaturing gradient gel was used to analyze the PCR products amplified from DNA directly extracted from sweet paste koji and mash. Lanes 0, 18, 27, and 43 are the preliminary fermentation samples from the koji-making stages for 0, 18, 27, and 43 h; and lanes 0, 7, 14, 21, 30, 60, 90, 120, 150, and 180 d are the main fermentation samples from outdoor aging for 0, 7, 14, 21, 30, 60, 90, 120, 150, and 180 days, respectively. The results of the DNA sequence analysis of the bands are summarized in Table 2.

LAB produced metabolites (lactic acid and bacterial toxins) that not only contribute significantly to the generation of flavor compounds, but also somewhat inhibit the growth of bacteria and pathogenic bacteria [10]. In several fermented foods, LAB are the main producers of lactic acid for ethyl lactate esterified by yeasts [15, 30]. As previously reported, *L. acidipiscis* and *L. pobuzihii* were isolated from fermented fish and pobuzihi (fermented cummingcordia), a traditional food in Taiwan [35, 36]. Interestingly, *L. acidipiscis* (Fig. 2, band 6) and *L. pobuzihii* (Fig. 2, band 7) were also detected from the 14-day and 30-day mash. Another LAB band was identified as the same genus (*e.g.*, *Lactobacillus* sp., Fig. 2, band 13). With recent expansion in the availability of fermentation microbes such as LAB or yeasts, direct addition of selected bacterial and fungal starter cultures to raw materials has been a breakthrough for making fermented foods, achieving a high degree of control over the fermentation process and standardization of the end product [37]. Therefore, we speculate that the screening and scale cultivation of predominant microbes from traditional foods might be a significant research tool for identifying functional fermentation microbes.

Table 2. Sequencing results of selected DGGE bands from the fungal DGGE fingerprint shown in Fig. 2.

Band No.[a]	Closest relative (NCBI Accession No.)	Identity[b]
6	*Lactobacillus acidipiscis* (LN89827)	370/376 (98%)
	Lactobacillus sp. (KM259929)	370/376 (98%)
7	*Lactobacillus pobuzihii* (NR112694)	433/433 (100%)
8	*Staphylococcus kloosii* (KT261093)	376/397 (95%)
	Staphylococcus kloosii (KT261064)	376/397 (95%)
	Staphylococcus cohnii (KT260356)	376/397 (95%)
9	Uncultured *Staphylococcus* (GU385490)	435/437 (99%)
10	Uncultured *Staphylococcus* (GU385490)	433/435 (99%)
11	Uncultured bacterium (KF105630)	389/400 (97%)
12	Uncultured bacterium (KM277709)	417/418 (99%)
13	*Lactobacillus* sp. (HM534774)	360/376 (96%)
14	*Bacillus subtilis* (LN899794)	420/420 (100%)
15	*Staphylococcus kloosii* (KT261093)	435/437 (99%)
	Staphylococcus kloosii (KT261064)	435/437 (99%)
	Staphylococcus cohnii (KT260356)	435/437 (99%)
16	*Bacillus* sp. (KF984466)	436/437 (100%)

[a]Band numbers correspond to Fig. 2.

[b]Sequencing identity with a sequence in the GenBank database is represented as a percentage (the number of the identical base/total length of the DNA sequence).

Bacillus subtilis (band 14) was detected in the mash as well as in the koji (Fig. 2, Table 2), indicating that *B. subtilis* was not affected by conditions like added brine, emerging yeasts, and outdoor aging. *Bacillus subtilis* has been reported to make great contributions to the production of organic acids, amino acids, and lipids, which play an important role in producing more complex flavor components during soy sauce and African locust bean fermentation [38, 39]. Several *Bacillus* species contribute to douchi (Chinese fermented black soybeans) fermentation, which secrete amylases, fungicidal agents, and plasmins to promote douchi aging and benefit human health [39–41].

In addition, the DGGE results did indicate the presence of *Staphylococcus* species. *Staphylococcus kloosii* (or *Staphylococcus cohnii*, Fig. 2, band 8) appeared in the mash during fermentation from days 30 to 120, and uncultured *Staphylococcus* (Fig. 2, band 15) was detected on day 120. *Staphylococcus kloosii* has been observed in soy sauce koji and brine by DGGE analysis [2, 7]. *Staphylococcus kloosii* and *S. gallinarum* were also isolated from miso during its fermentation using the culture-dependent method [42].

Changes in Properties of Sweet Paste during Fermentation

Reducing sugar and amino-type nitrogen of the koji increased gradually and reached 315.13 ± 24.35 g/kg and 7.34 ± 0.25 g/kg, respectively, at the end of preliminary fermentation (Fig. 3A). Despite a decrease with the addition of brine, both of them showed an obvious increase at the early main fermentation stage, and reached the highest content of 337.41 ± 3.99 g/kg (7 days) and 6.77 ± 0.25 g/kg (30 days), respectively, followed by a slight decrease, but with no obvious changes in their biochemical properties. Additionally, both of them maintained 261.46 ± 19.49 g/kg and 6.77 ± 0.12 g/kg, respectively, after 180 days of main fermentation (Fig. 3B). In sweet paste processing, *A. oryzae* mainly provides amylases and proteases for the production of reducing sugar and amino-type nitrogen. The reducing sugar content in the mash began to decrease after 7 days of fermentation, indicating the involvement of yeasts and bacteria in the utilization of sugar, similar to that of liquor fermentation in Maotai-liquor making [13].

Fig. 3C shows changes in the total acid and pH value of koji. The total acid of koji reached the highest content of 15.66 ± 0.73 g/kg at the 27th hour of fermentation, followed by a slight decrease, while its pH value (6.12–6.58) remained almost unchanged. Additionally, the total acid showed a similar change trend to that of amino-type nitrogen during main fermentation, and it reached the highest content

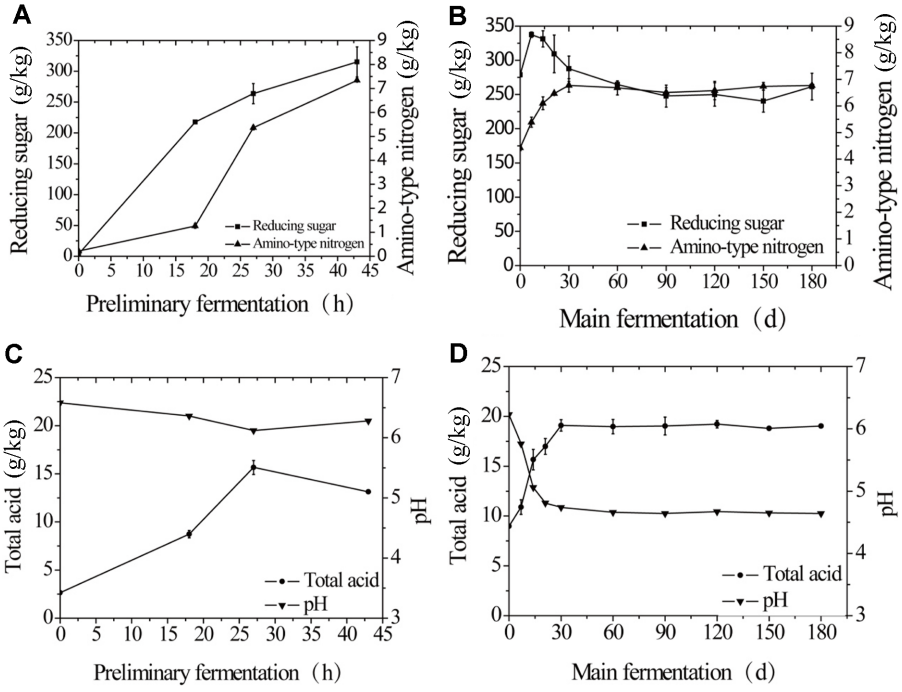

Fig. 3. Changes in chemical properties during sweet paste fermentation.

(**A**) Reducing sugar and amino-type nitrogen of the koji; (**B**) Reducing sugar and amino-type nitrogen of the mash; (**C**) Total acid and pH of the koji; and (**D**) Total acid and pH of the mash. The results are representative of a typical experiment of three different batches of samples. Values are the means ± SD.

of 19.10 ± 0.58 g/kg at day 30, and then remained below 20.00 g/kg. The pH value decreased gradually at the early main fermentation stage and then remained at around 4.65, demonstrating a high correlation to the total acid (Fig. 3D). Furthermore, the total acid of mash samples increased significantly at the early main fermentation stage because of the activity of the fermentation microflora, especially LAB, and its metabolism (acids and bacteriocins), which not only inhibited the growth of pathogens and bacteria with low acid tolerance, but also contributed to the generation of flavor compounds [10, 43].

To the best of our knowledge, this is the first report to reveal the microbial diversity involved in sweet paste production using PCR-DGGE. In the mash, two *Zygosaccharomyces* species were found to be the dominant microbes, and we also detected the presence of *Bacillus subtilis* during fermentation as well as *Lactobacillus acidipiscis*, *Lactobacillus pubuzihii*, *Lactobacillus* sp, *Staphylococcus kloosii*, and several uncultured bacteria in the mash after 14 days of main fermentation. Additionally, the changes of biochemical properties in the fermented sweet paste were also explored. During main fermentation, the reducing sugar, amino-type nitrogen, and total acid increased gradually and reached the highest content of 337.41 ± 3.99 g/kg (7 days), 6.77 ± 0.25 g/kg (30 days), and 19.10 ± 0.58 g/kg (30 days), respectively, with no obvious changes observed in all of these biochemical properties during further fermentation. The overall findings from this study indicate that these microbial populations have close connections with the industrial production of the sweet paste with high quality and safety.

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