

Profiling of dynamic changes in the microbial community during the soy sauce fermentation process

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Abstract Soy sauce is a traditional condiment manufactured by natural inoculation and mixed culture fermentation. As is well known, it is the microbial community that plays an important role in the formation of its flavors. However, to date, its dynamic changes during the long period of fermentation process are still unclear, intensively constraining the improvement and control of the soy sauce quality. In this work, we revealed the dynamic changes of the microbial community by combining a cultured dependent method and a cultured independent method of polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis. Results indicated that the two methods verified and complemented each other in profiling microbial community, and that significant dynamics of the microbial community existed during the fermentation process, especially the strong inhibition of the growth of most of the microbes when entering into the mash stage from the koji stage. In the analysis of bacterial community, *Staphylococcus* and *Bacillus* were found to be the

dominant bacteria and detected in the whole fermentation process. *Kurthia* and *Klebsiella* began to appear in the koji stage and then fade away in the early stage of the mash fermentation. In the analysis of fungal community, *Aspergillus sojae* and *Zygosaccharomyces rouxii* were found to be the dominant fungi in the koji and mash fermentation, respectively. It was clearly shown that when *A. sojae* decreased and disappeared in the middle stage of the mash fermentation, *Z. rouxii* appeared and increased at the meantime. *Aspergillus parasiticus*, *Trichosporon ovoides* and *Trichosporon asahii* also appeared in the koji and the early period of the mash fermentation and disappeared thereafter. Similar to *Z. rouxii*, *Millerozyma farinosa* and *Peronospora farinosa* were also found spontaneously which appeared in the mid-late period of the mash fermentation. The principal component analysis suggested that the microbial community underwent significant changes in the early period of the fermentation and, thereafter, tended to the stabilization in the mid-late periods. This study gave us important clues to understand the fermentation process and can serve as a foundation for improving the quality of soy sauce in the future.

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Introduction

Soy sauce is a traditional fermented condiment and popular worldwide because of its special flavor. Its brewing costs a long time, more than half a year, and involves a well-established two-step fermentation process, short-term koji fermentation, and followed long-term mash fermentation brought by adding of brine. Koji fermentation is to provide a specific fermented cereal containing molds, yeasts, and bacteria (Wu et al. 2010), while mash fermentation is to increase the flavors of soy sauce and produce the final products. The flavor of soy sauce is

attributed to many kinds of microbial metabolites such as alcohols, aldehydes, esters, ketones, organic acids, furanones, and miscellaneous compounds (Leejeerajumnean et al. 2001; Suezawa et al. 2006). Obviously, the formation process of the flavor is closely related to the growth of the microbes in the fermentation process. Soy sauce is produced by natural inoculation and mixed-culture fermentation. Thus, it is the microbial community, not a single microbe, which functions in the formation of the flavor of soy sauce. However, to date, the changes of microbial communities during the whole fermentation process are still unclear, which conferred difficulty in improving and controlling the quality of soy sauce.

In previous studies, microbes existing in soy sauce fermentation were usually identified by cultured-dependent method with different selective medium (Ito and Dou 1994; Tanasupawat et al. 2002). The cultivation method is time consuming, tedious, and cannot cover the non-cultivable microbes by current isolation and culture methods. Thus, an increasing number of cultured independent methods for overcoming the drawbacks of the conventional method are being developed. They use the total DNA of microbes without the need of culturing and perform microbial identification through sequencing of phylogenetic markers. Bacteria are identified by the sequence of 16S rDNA (Whiteley et al. 2012; Xie et al. 2011), and fungi are identified by the sequence of 18S rDNA (or 25S rDNA–28S rDNA) (Fell et al. 2000). These methods include the denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis, terminal restriction fragment length polymorphism, 454 pyrosequencing, random amplification of polymorphic DNA and multiplex PCR coupled with rDNA sequencing (Akopyanz et al. 1992; Marsh 1999; Muyzer and Smalla 1998). Recently, the method of PCR-DGGE has been widely used to study microbial communities present in milk, vinegar, shochu, and cocoa bean (Cocolin et al. 2002; De Vero et al. 2006; Endo and Okada 2005; Garcia-Armisen et al. 2010; Mamlouk et al. 2011). We hypothesized that the PCR-DGGE method could reveal more and new information about the microbes present in the soy sauce fermentation process.

In the present study, the diversity and distribution of microbial populations in soy sauce fermentation process were investigated by both culture-dependent method and culture-independent method. In addition of using selective mediums, we also used PCR-DGGE to analyze the microbial communities in soy sauce fermentation by identifying the V3 regions of 16S rDNA (for bacteria) and D1 regions of 26S rDNA (for fungi). The dynamic changes in microbial communities were examined by principal component analysis (PCA). The determination of the relationship of microbial community changes with the fermentation progression will strengthen the understanding of microbial community in soy sauce fermentation process and help to improve the production of high-quality soy sauce.

Materials and methods

Soy sauce fermentation

Soy sauce koji and mash samples were collected from a factory. The soy sauce was fermented according to a method modified from that of Rölöng in this factory (Rölöng et al. 1996). Briefly, the defatted yellow soybean was boiled and then cooled to room temperature. Wheat was roasted and then cooled to room temperature, followed by the roasted wheat, which was grinded by the grinder. A mixture of steamed defatted yellow soybean and ground wheat (v/v, 5.5:4.5) was inoculated with *Aspergillus* and kept at 35 °C for 42 h, resulting in a formation of soy sauce koji. Soy sauce koji were then mixed with 2.5 times volume of salted water (18~20 °Be, salt concentration approximately 20 %) in a fermentation tank for mash fermentation, which was held at room temperature (approximately 30 °C) for more than half a year and finally resulted in a formation of ripened soy sauce mash. This is the general practice employed in most of factories.

Sample collection

The samples of koji were sampled at the end of the koji fermentation period. The samples of mash were collected at different time points of the mash fermentation: 9, 30, 66, 112, 140, 174, 202, and 227-d mash. For each time point, five parallel samples were sampled from parallel batches of soy sauce fermentation. All the five samples from the same time point were mixed into one sample before the followed microbe identification by the cultivation method and the culture-independent method of PCR-DGGE.

Cultivation method for microbe profiling with selective mediums

Every mixed sample (1 g) was transferred to test tube and homogenized with 9 mL sterile water at high speed twice. Appropriate serial dilutions were prepared by the same diluent. One-milliliter portions of the diluted suspensions were mixed with molten (45 °C) medium and poured in duplicate plates. Total mesophilic aerobic bacteria (TMAB) were measured using pour plates of Plate Count Agar at 37 °C for 28 h. The counting of bacterial spores were performed via pasteurized samples (80 °C, 10 min) and then cultured on Plate Count Agar at 30 °C for 48–72 h. Lactic acid bacteria (LAB) were counted on de Man, Rogosa and Sharpe Agar medium (MRS Agar) with 0.1 % natamycin at 30 °C for 72 h. To confirm the presence of presumptive LAB, Gram staining and catalase reactions were carried out. *Enterobacteriaceae* were enumerated on violet-red bile glucose agar (VRBGA) at 30 °C for 36 h. To confirm the presence of presumptive colonies of *Enterobacteriaceae*, oxidase- and glucose-fermentation tests

were carried out. Yeasts and molds (fungus) were enumerated in pour plates of Rose Bengal Chloramphenicol Agar (RBCA) with 100 mg/L chloramphenicol at 30 °C for 4–5 days. All media were sterilized at 121 °C for 15 min. All counts were repeated triplicate, and the results were reported as the means \pm SD ($n=3$).

Total DNA extraction

Total DNA was extracted from each mixed sample according to the method of Ni (Ni et al. 2010) with some modifications. About 0.2 g of sample was ground in liquid nitrogen until a powdery form was achieved. The powder was suspended in 1 mL of extraction buffer (100 mM Tris–HCl and 100 mM EDTA, pH 8.0), and 100 μ L of 10 % SDS was added. The mixture was incubated at 65 °C for 1 h with mixing and softly overturning every 20 min. After centrifuging the mixture at 10,000 \times g for 10 min at 4 °C, the aqueous phase was removed, placed in a fresh 2-mL tube and extracted with an equal volume of 25:24 phenol/chloroform at 10,000 \times g for 5 min at 4 °C. The aqueous phase was again removed, placed in a fresh 2-mL tube and extracted with an equal volume of chloroform at 10,000 \times g for 5 min at 4 °C. The aqueous phase (0.6 mL) was added with half its volume of 7.5 M ammonium acetate (pH 7.5) and an equal volume of isopropanol. Subsequently, the aqueous phase was incubated at –20 °C for 1 h. The DNA precipitates were collected by centrifugation at 10,000 rpm for 10 min, washed with 200 μ L of 70 % ethanol, dried for 5 min in air, and left to resuspend in 100 μ L of TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8.0).

PCR amplification of V3 regions of 16S rDNA and D1 regions of 26S rDNA

V3 regions of 16S rDNA was amplified in a thermal cycler using the 518R/338f-GC primer set (518R: 5'-ATT ACC GCG GCT GCT GG-3'; 338 F: 5'-ACT CCT ACG GGA GGC AGC AG-3'; 338 F-GC clamp with 5'-GCC GCC CGC CGC GCG CGG CGG GCG GGG CGG GGG CAC GGG GGG-3' attached to the 5' end of 338 F). Touchdown (TD)-PCR was performed to amplify V3 regions of 16S rDNA. PCR was performed as follows: initial denaturation at 94 °C for 4 min; followed by a touchdown step (20 cycles) with denaturation at 94 °C for 45 s, annealing at temperatures lowered from 55 to 45 °C at 0.5 °C intervals every cycle for 45 s; and elongation at 72 °C for 20 s. Ten additional cycles of annealing at 45 °C followed, and a final extension at 72 °C for 10 min ended the amplification reaction to eliminate artificial bands (Janse et al. 2004).

D1 regions of 26S rDNA was amplified in a thermal cycler using the NL1-GC and LS2 primer set (NL1: 5'-GCC ATA TCA ATA AGC GGA GGA AAA G-3'; LS2 : 5'-ATT CCC AAA CAA CTC GACT C-3'; NL-1-GC clamp with 5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA

CGG GGG-3' attached to the 5' end of NL-1). TD-PCR was performed to amplify D1 region of 26S rDNA. PCR was performed as follows: initial denaturation at 94 °C for 4 min; followed by a touchdown step (20 cycles) with denaturation at 94 °C for 45 s, annealing at temperatures lowered from 62 to 52 °C at 0.5 °C intervals every cycle for 45 s; and elongation at 72 °C for 20 s. Ten additional cycles of annealing at 52 °C followed, and a final extension at 72 °C for 10 min ended the amplification reaction (Cocolin et al. 2002). The PCR products were analyzed on 2 % (*w/v*) agarose gels (Euroclone) using a GeneRuler DNA Ladder Mix (Fermentas International, Burlington, Canada) as a molecular weight standard. The gels were run at 6 V/cm (constant voltage) in 1 \times TAE and stained with 0.5 μ g/mL ethidium bromide.

DGGE analysis

DGGE was performed for bacterial communities with a Bio-Rad DCode system (Bio-Rad, USA) using 8 % polyacrylamide gel with 30 to 60 % denaturing concentrations in 1 \times TAE buffer as previously described (Muyzer et al. 1993). Electrophoresis was performed at 200 V and 60 °C for 4 h. Meanwhile, DGGE was performed for fungal communities using a Bio-Rad DCode system (Bio-Rad, USA) using 8 % polyacrylamide gel with 30 to 50 % denaturing concentrations in 1 \times TAE buffer. Electrophoresis was performed at 200 V and 60 °C for 4.5 h. The gel was stained for 15 min with SYBR green after electrophoresis and visualized with a UV illumination using a Gel Documentation System (Bio-Rad Laboratories-Segrate, Milan, Italy).

Amplification of excised DGGE bands and sequencing

Bands of interest were excised from the DGGE gel using a sterile scalpel. DGGE bands were collected using a PAGE Purification Recovery Kit (Biomed, China) following the manufacturer's instructions. PCR amplification was performed using 338 F and 518R primers for bacterial community and using NL1 and LS2 primer for fungal community. Then, the PCR products were sent to BGI for sequencing with an Illumina HiSeq 2000 Automated Sequencer (BGI, Beijing, China). The obtained V3 regions of 16S rDNA sequences were compared with sequence in the Ribosomal Database Project (RDP) to determine the nearest matches. The obtained D1 regions of 26S rDNA sequences were compared with sequences in the National Center for Biotechnology Information (NCBI) database to determine the nearest matches.

Calculation of diversity indices, cluster analysis, and statistical analysis

The microbial complexity of different fermented mash samples was expressed by various indices of biodiversity

calculated from the DGGE patterns, namely, the margale index of species richness dMa and the Shannon–Weaver index of general diversity H . These parameters were determined based on the number and relative intensity of bands of each DGGE profile. The software Quantity One was used to convert individual DGGE lanes to densitometric profiles. Cluster analysis of the band patterns was performed with the unweighted-pair group method using average linkages. The band intensity corresponded to peak height in the densitometric curves. PCA was performed by PAST based on the number and relative intensity of the bands of each DGGE profile (Oguntoyinbo et al. 2011).

Nucleotide sequence accession number

The nucleotide sequences of V3 regions of 16S rDNA and D1 regions of 26S rDNA have been deposited with GenBank under the accession number KC778587–KC778621.

Results

Total count of representative bacteria and fungi by cultured method

The count of the microbe present in soy sauce fermentation measured by cultivating on selective media was shown in Fig. 1. The total count of mesophilic aerobic bacteria (TMAB, Plate Count Agar medium) was $2.3 \times 10^{10} \pm 4 \times 10^9$ CFU/g at the end of the koji fermentation, then dropped to $3.4 \times 10^6 \pm 7 \times 10^5$ CFU/g at the ninth day of the followed mash fermentation, decreased further to $8.8 \times 10^4 \pm 4 \times 10^3$ CFU/g at

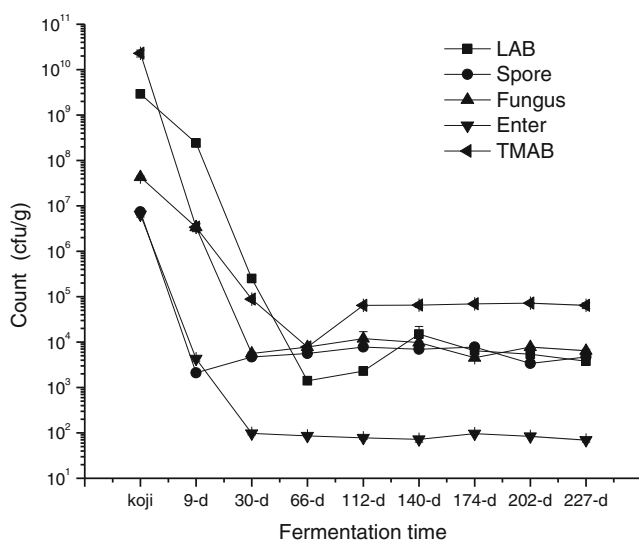


Fig. 1 Enumeration of different fermentation time during soy sauce fermentation process (LAB, lactic acid bacteria; Spore, bacterial spores; Enter, *Enterobacteriaceae*; TMAB, total mesophilic aerobic bacteria)

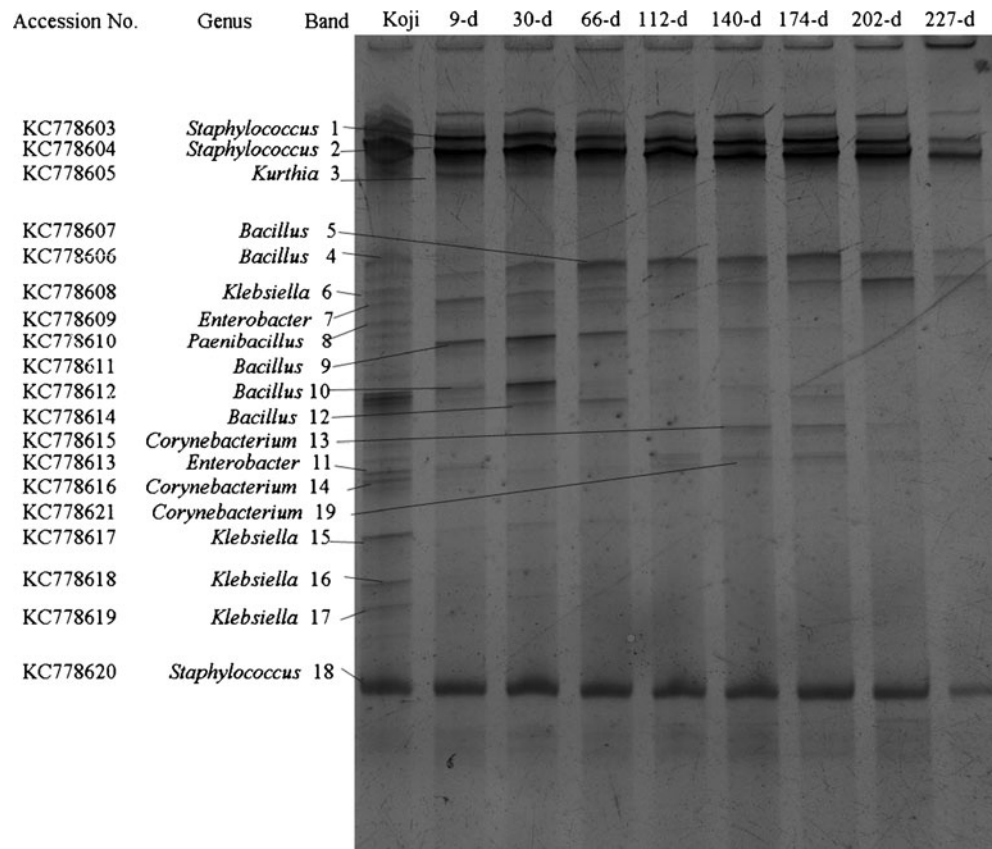
the 30th day, and kept stable thereafter. The LAB (MRSA medium), the *Enterobacteriaceae* (Enter, VRBGA), the mesophilic bacterial spores (Spore, Plate Count Agar), and the yeasts and molds (Fungi, RBCA) counts showed similar trends as TMAB. All their counts tend to be stable in the mid to late periods of the mash fermentation. But their counts were different from each other in the same time point of fermentation. The count of LAB and mesophilic bacterial spores in the koji were $2.9 \times 10^9 \pm 4 \times 10^8$ and $7.4 \times 10^6 \pm 4 \times 10^5$ CFU/g, respectively, with a great difference of three orders. The data showed that the dominant microbe in the koji fermentation were bacteria, especially LAB, and then followed by yeasts and molds. The dominant microbes during the mash fermentation were still the bacteria, although the count of bacteria in the mash was three to five orders less than that in the koji. The result suggested that the growth of most of the microbes were strongly inhibited by the high salty brine when the fermentation entered into the mash period from the koji period.

Profiling of bacterial community using PCR-DGGE

The bacterial communities and diversity in the koji and the mash fermented for different time were investigated by PCR-DGGE. A photograph of the obtained band patterns is shown in Fig. 2. A total of 22 bright bands were selected for amplification, sequencing, and identification by searching the RDP database. As can be seen in Fig. 2, the bands for *Staphylococcus* (Bands 1, 2, and 18), *Bacillus* (Band 5), and *Enterobacter* (Band 7) were detected in the whole process, but most bands were not common among different fermentation time. Several bands, such as Bands 4, 6, 8, 11, 14, 15, 16, and 17 were detected only in the koji and disappeared in the mash. The calculated values for Shannon diversity index (H) suggest that the koji had higher microbial diversity ($H=4.18$) than the mash ($2.19 < H < 2.92$). These results indicate that considerable dynamic changes in the bacterial community existed during the soy sauce fermentation process. Moreover, many abundant bands found corresponded to *Bacillus* and *Staphylococcus*, such as Bands 1, 2, 4, 5, 8, 9, 10, and 12, indicating that they were the dominant bacteria during the soy sauce fermentation.

Different samples were then compared with one another by determining the pairwise similarity coefficient, which ranged from 32.9 to 100. Cluster analysis (Fig. 3) demonstrated that the band pattern of the koji differed from those of other mash samples, and the patterns of the mash fermented from 9 to 66 d also differed from one another. By contrast, the patterns of the mash fermented from 140 to 227 d mash showed high similarities to one another. These results indicated that the bacterial community underwent remarkable changes in the early stage of the mash fermentation, whereas small changes in the late stage.

Fig. 2 DGGE profiles of the bacterial communities in the koji and the mash of different fermentation time. The identified genus of the selected bands and their deposited nucleotide sequence accession number were given at the corresponding position



Profiling of fungal community using PCR-DGGE

Fungal DGGE profiles showed diverse fungal communities in the koji and the mash from different fermentation stages (Fig. 4). A total of 22 DGGE bands were selected for amplification, sequencing, and identification by searching the NCBI database. As can be seen in Fig. 4, the band for *A. sojae* (Band 16) was the dominant band in the koji and then gradually disappeared along with the mash fermentation. The bands for *Aspergillus parasiticus* (Band 12), *Trichosporon ovoides* (Band 7), and *T. asahii* (Band 21) also appeared in the koji

and the mash fermentation before 66 d, and thereafter disappeared in the mid-late period of the mash fermentation. In stark contrast, the band for *Zygosaccharomyces rouxii* (Bands 10 and 11) did not exist in the koji and the mash fermentation before 66 d, but appeared in the mid-late period of the mash fermentation. The bands for *Milleroyzma farinosa* (Band 5) and *Peronospora farinosa* (Band 6) displayed the similar pattern with the band for *Z. rouxii*. In addition, the bands for *C. rugosa* (Band 17) and *Candida* sp. (Band 18) were detected only in the 30-d and 66-d mash, as well as the band for *C. orthopsilosis* (Band 4) only in the 202-d mash and the band for *C. tropicalis* (Band 3) only in the 140-d mash.

The calculated values for Shannon diversity index (*H*) suggest that the koji had lower microbial diversity ($H=1.72$) than the mash ($1.94 < H < 3.16$). This is mainly because many bands corresponding to yeast appeared in the mash fermentation. The most abundant band in the koji and the early stage of the mash fermentation were found corresponded to *A. sojae*, while the most abundant band in the mid-late period of the mash fermentation was *Z. rouxii*. Moreover, *A. sojae* presented a decreasing tendency along with the mash fermentation process, while *Z. rouxii* presented an increasing tendency.

Fungal DGGE profiles of different fermentation stages were also compared with one another by determined the pairwise similarity coefficient, which ranged from 19.5

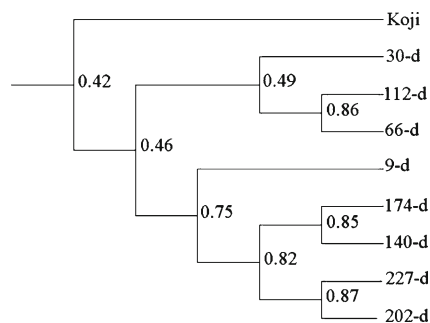
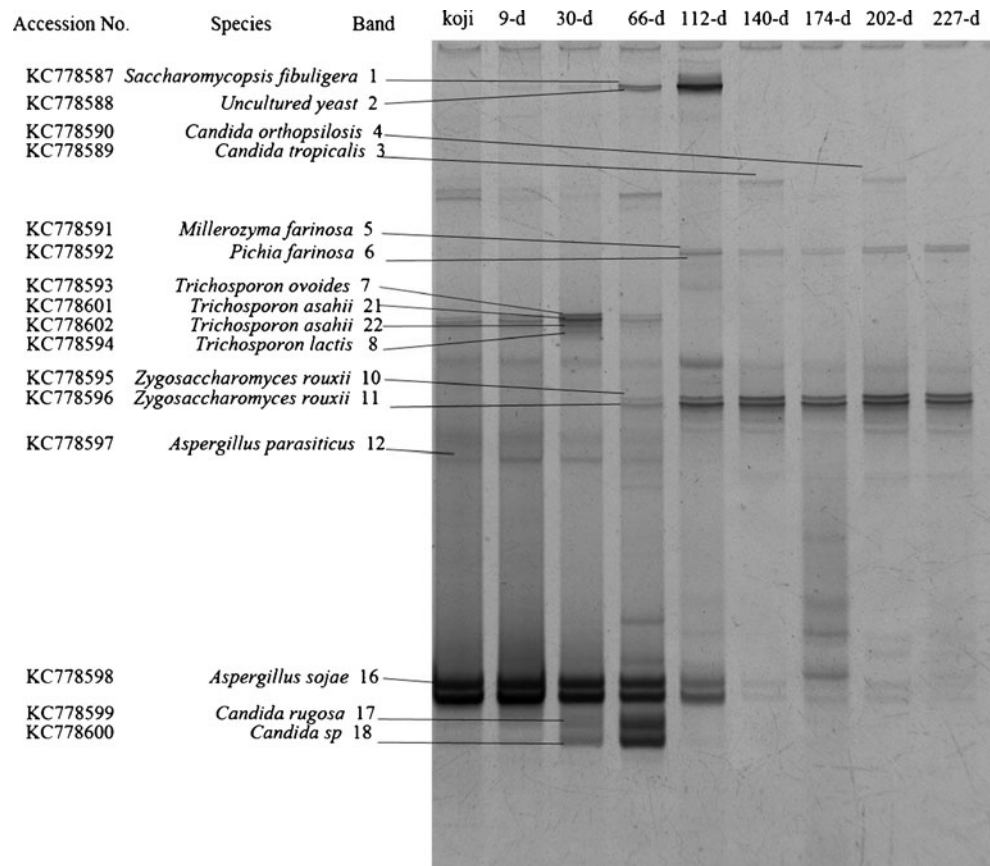


Fig. 3 Cluster analysis of the DGGE band patterns of different fermentation time corresponding to fungal communities with Quantity One software

Fig. 4 DGGE profiles of the fungal communities in the koji and the mash of different fermentation time. The identified species of the selected bands and their deposited nucleotide sequence accession number were given at the corresponding position



100. The results showed that remarkable changes in fungal community existed in the soy sauce fermentation process. Cluster analysis (Fig. 5) indicated that the band pattern of the koji had similarities to the mash of the early stage, and the patterns of the mash fermented from 140 to 227 d were similar to one another. Unlike the drastic change of bacterial community observed between the koji stage and the early stage of the mash fermentation, significant differences in the fungal community were observed in the middle stage of the mash fermentation.

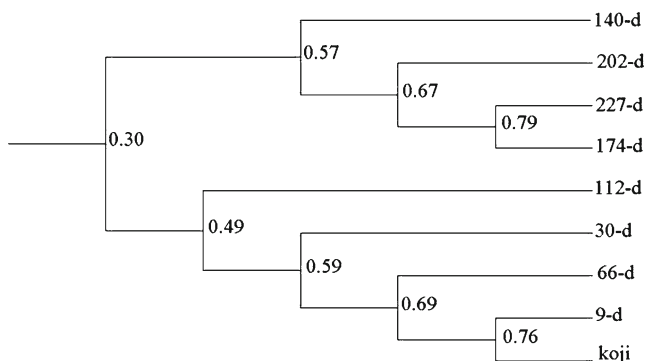


Fig. 5 Cluster analysis of the DGGE band patterns of different fermentation time corresponding to fungal communities with Quantity One software

PCA analysis on microbial communities

A PCA analysis on the bacterial and fungal communities of different fermentation time was carried out to profile their differences. The PCA explained 48.06 (PC1) and 24.14 % (PC2) of the total variance (Fig. 6). The result indicated that the koji and the mash fermented for 9, 30, 66, and 112 d differed significantly with one another in microbial community, while the mash fermented for 140, 174, 202, and 227 d had small differences with one another.

Discussion

We analyzed the microbial communities involved in the soy sauce manufacturing process by PCR-DGGE and evaluated the microbial communities during this process. The results indicated that dynamic changes of microbial community occurred during soy sauce manufacturing. Previous studies showed that *Bacillus* and *Micrococci* were the dominant bacteria in their soy sauce fermentation (Takazane et al. 1998). However, in our result, *Micrococci* were not detected. Even so, it cannot be judged that *Micrococci* did not exist in our fermentation. The absence of corresponding bands of micrococci in the DGGE gel might be due to other several reasons:

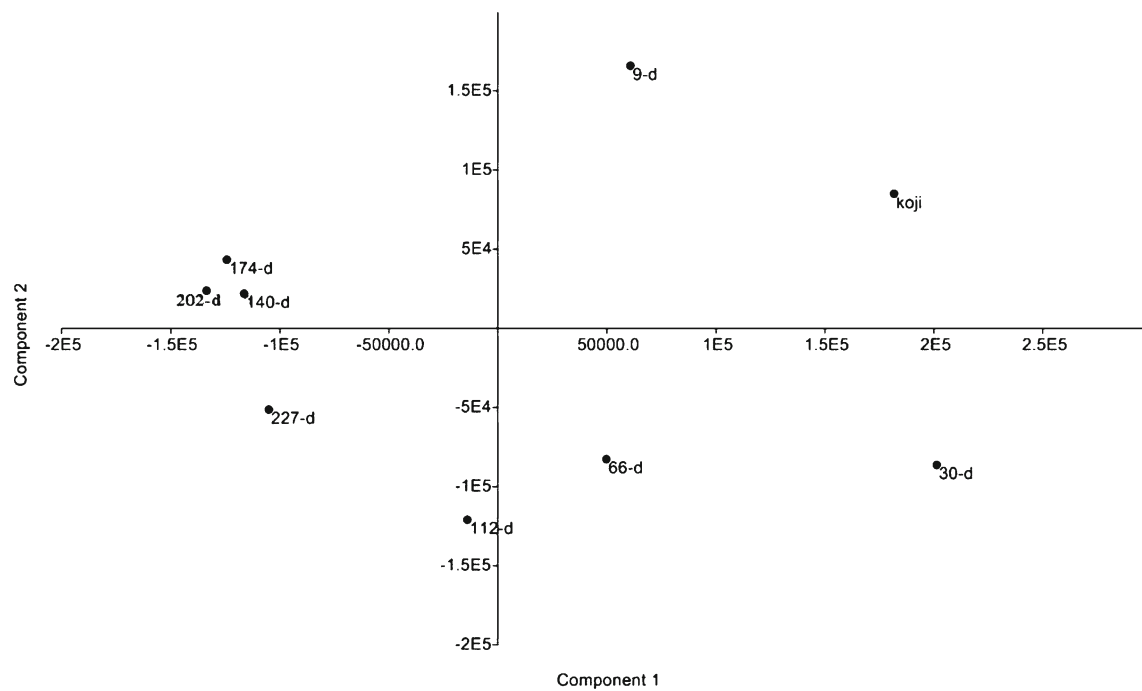


Fig. 6 Principal component analysis of the optical densities produced by microbial activities at different stages of soy sauce fermentation

an insufficient yield of extracted genomic DNA, the unsuitability of the extracted genomic DNA for PCR reactions, and the low species coverage of DGGE method (microbes cannot be observed in DGGE gel when their relative contents are below 1 %).

Lactic acid bacteria were not detected by the DGGE-based method, which was not consistent with the result obtained by the culture-dependent method as well as some previous studies using culture-dependent methods (Uchida 2000; Yan et al. 2013). This indicates the blind spot of the PCR-DGGE technology regarding the species coverage. *Klebsiella* was detected in our study but rarely detected in previous studies. In our result, it began to appear in the koji, then decreased and disappeared in the early stage of the mash fermentation. It is not clear that the effect of uncultured *Klebsiella* on the soy sauce fermentation. Our result also showed that *Kurthia* began to appear in the koji and then fade away in the early stage of the mash fermentation. It was even reported that *Kurthia* functioned in produce protease and volatile fatty acids for the soy sauce fermentation (Goodfellow et al. 1980; Steele et al. 1992).

Staphylococcus was found to be the dominant bacteria during the whole fermentation process of soy sauce in both our study and previous studies. The adding of brine did not restrain the growth of *Staphylococcus* in the mash, indicating the *Staphylococcus* involved in the fermentation of soy sauce was anti-hypertonic. *Staphylococcus* was also reported to be one of the major bacteria involved in other fermentation foods (Guan et al. 2011; Yan et al. 2013) and have a strong ability to survive in the hypertonic environment (Wei et al. 2013). *Staphylococcus* is widely known for the pathogenicity and

considered as food-borne bacterial pathogens, but in fact, most species of the *Staphylococcus* genus are harmless for humans, except for *Staphylococcus aureus*. Instead, it was ever reported that *Staphylococcus* helped to produce volatile fatty acids and contributed to the flavor formation (Wah et al. 2013; Yan et al. 2013).

In our result, *A. sojae* and *Z. rouxii* were found to be the dominant fungi in the koji and mash fermentation, respectively. As was well known, both of them are the most important fungi for the fermentation of soy sauce. The key role of *A. sojae* was to produce various hydrolases to hydrolyze proteins, starch, cellulose, and other biopolymers for providing flavor compounds or flavor precursors, while the main role of *Z. rouxii* was to produce important aroma compounds, such as HEMF and HDMF (Wah et al. 2013). Obviously, when *A. sojae* decreased and disappeared between 66 and 112 d, *Z. rouxii* appeared and increased, which may be not a coincidence. It could be speculated that the existence of *A. sojae* restrained the growth of *Z. rouxii*, and the drop of *A. sojae* created the conditions for the appearance of *Z. rouxii*. Except for *Z. rouxii*, *P. farinosa* also appeared and grew in the middle and late period of the mash fermentation. *Pichia* has been known to produce 4-ethylguaicol, which is one of the most important aroma compounds for the special flavor of soy sauce.

Interestingly, some different bands in Fig. 4 were identified as the same fungal species (e.g., Bands 10 and 11 for *Z. rouxii*, and Bands 21 and 22 for *T. asahii*). These sequences were compared through the NCBI website, and the results showed minor differences among them. It could be speculated that these fungal species may have two kinds of rDNAs with

different sequences. The same phenomenon was ever observed in a previous study, which showed that *Z. rouxii* in soy sauce mash was mostly allopolyploid (Gordon and Wolfe 2008). Indeed, the sequences of Bands 10 and 11 matched the 26S rRNA sequence of *Z. rouxii* NRRL Y-229 26S ribosomal RNA gene and the sequence of *Z. rouxii* ZR-1 26S ribosomal RNA gene, respectively.

In this study, both culture-dependent and culture-independent analyses were performed to profile the microbial community changes. The two approaches not only had sufficient overlap in the results of identifying significant microbial groups, but also complemented each other to profile microbial community. For example, *Enterobacter* was detected in the koji by both culture-dependent and culture-independent methods, but in the mash, it was detected only by the culture-dependent method. No band for *Enterobacter* was detected in the culture-independent analysis based on DGGE. These results suggested that it was necessary to combine culture-dependent and culture-independent methods to generate a general view of microbial ecology during soy sauce manufacturing process. As can be seen from both the culture-dependent and culture-independent analyses, the inhibition of the brine on the growth of bacteria was obviously stronger than that on the growth of fungi. In the culture-dependent analysis, the bacteria count fell to 10^4 from 10^{10} CFU/g after adding brine, while the fungal count fell to 10^4 from 10^7 CFU/g. In the DGGE figures of the culture-independent analysis, dramatic changes of the bacterial community were observed after adding brine, while relatively small changes of the fungal community were observed.

The microbial communities detected by PCR-DGGE approach in this work were suitable for PCA analysis. According to the PCA theory, the “principal component” is sufficient to explain the maximum variation in all original data sets (Strauss and Prinsloo 2007). The analysis result suggested that the microbial community underwent significant changes in the early period of the fermentation and thereafter tended to stabilization in the mid-late periods. According to this and the dynamic change of the dominant microbes, the microbial community involved in the whole fermentation process could be divided into three stages: *Aspergillus* stage, transformation stage, and yeast stage. It is the adding of brine that initiated the transformation of the microbe community from the *Aspergillus* stage into the yeast stage, and the transformation in the present fermentation technology cost a long time. This may provide new clues for improving soy sauce fermentation technology. It was speculated that lifting the restraint on the growth of yeast and further adding some yeast could shorten the fermentation period of soy sauce.

Microbe control in the fermentation process is the key of the quality control and stability of the final soy sauce product. Due to the open fermentation environment and the long fermentation period, the microbial community involved in the

soy sauce fermentation showed the characteristics of significant diversity and dynamics. Our study provided a more comprehensive understanding of the dynamic process of the microbial community in soy sauce fermentation by combining culture-dependent and culture-independent methods. We believe it will benefit the optimization and improvement of the fermentation technology of soy sauce in the future research.

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References

- Akopyanz N, Bukanov NO, Westblom TU, Kresovich S, Berg DE (1992) DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. *Nucleic Acids Res* 20(19):5137–5142
- Cocolin L, Aggio D, Manzano M, Cantoni C, Comi G (2002) An application of PCR-DGGE analysis to profile the yeast populations in raw milk. *Int Dairy J* 12(5):407–411
- De Vero L, Gala E, Gullo M, Solieri L, Landi S, Giudici P (2006) Application of denaturing gradient gel electrophoresis (DGGE) analysis to evaluate acetic acid bacteria in traditional balsamic vinegar. *Food Microbiol* 23(8):809–813
- Endo A, Okada S (2005) Monitoring the lactic acid bacterial diversity during shochu fermentation by PCR-denaturing gradient gel electrophoresis. *J Biosci Bioeng* 99(3):216–221
- Fell JW, Boekhout T, Fonseca A, Scorzetti G, Statzell-Tallman A (2000) Biodiversity and systematics of basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *Int J Syst Evol Micr* 50(3):1351–1371
- Garcia-Armisen T, Papalexandratou Z, Hendryckx H, Camu N, Vrancken G, De Vuyst L, Cornelis P (2010) Diversity of the total bacterial community associated with Ghanaian and Brazilian cocoa bean fermentation samples as revealed by a 16S rRNA gene clone library. *Appl Microbiol Biotechnol* 87(6):2281–2292
- Goodfellow M, Collins M, Minnikin D (1980) Fatty acid and polar lipid composition in the classification of Kurthia. *J Appl Microbiol* 48(2):269–276
- Gordon JL, Wolfe KH (2008) Recent allopolyploid origin of *Zygosaccharomyces rouxii* strain ATCC 42981. *Yeast* 25(6):449–456
- Guan L, Cho KH, Lee J-H (2011) Analysis of the cultivable bacterial community in *jeotgal*, a Korean salted and fermented seafood, and identification of its dominant bacteria. *Food Microbiol* 28(1):101–113
- Ito H, Dou K (1994) Microorganisms of Miso and Soysauce. *Japanese Journal of Food Microbiology* 11(3):151–157
- Janse I, Bok J, Zwart G (2004) A simple remedy against artifactual double bands in denaturing gradient gel electrophoresis. *J Microbiol Meth* 57(2):279–281
- Leejeerajumnean A, Duckham SC, Owens JD, Ames JM (2001) Volatile compounds in *Bacillus*-fermented soybeans. *J Sci Food Agr* 81(5):525–529
- Mamlouk D, Hidalgo C, Torija MJ, Gullo M (2011) Evaluation and optimisation of bacterial genomic DNA extraction for no-culture techniques applied to vinegars. *Food Microbiol* 28(7):1374–1379
- Marsh TL (1999) Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among

- homologous populations of amplification products. *Curr Opin Microbiol* 2(3):323–327
- Muyzer G, Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Anton Leeuw Int J G* 73(1):127–141
- Muyzer G, De Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* 59(3):695–700
- Ni Z, Xu W, Dou W, Xu H, Xu Z (2010) Comparison of total microbial DNA extraction methods from solid-culture of Zhenjiang vinegar. *Acta Microbiologica Sinica* 50(1):119
- Oguntoyinbo FA, Tourlomos P, Gasson MJ, Nabad A (2011) Analysis of bacterial communities of traditional fermented West African cereal foods using culture independent methods. *Int J Food Microbiol* 145(1):205–210
- Röling WFM, Apriyantono A, Van Verseveld HW (1996) Comparison between traditional and industrial soy sauce (kecap) fermentation in Indonesia. *J fermentation and bioengineering* 81(3):275–278
- Steele DB, Fiske MJ, Steele BP, Kelley VC (1992) Production of a low-molecular-weight, alkaline-active, thermostable protease by a novel, spiral-shaped bacterium, *Kurthia spiroforme*, sp. nov. *Enzyme Microb Tech* 14(5):358–360
- Strauss MJ, Prinsloo NM (2007) Real-time principal component analysis of in-line NIR spectroscopic data as applied to heterogeneous catalysis research. *Appl Catal A-Gen* 320:16–23
- Suezawa Y, Kimura I, Inoue M, Gohda N, Suzuki M (2006) Identification and typing of miso and soy sauce fermentation yeasts, *Candida etchellsii* and *C. versatilis*, based on sequence analyses of the D1/D2 domain of the 26S ribosomal RNA gene, and the region of internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2. *Biosci Biotechnol Biochem* 70(2):348–354
- Takazane S, Endo T, Shida O, Tagami H, Takagi H, Kadowaki K (1998) Influence of *Bacillus* species in Shoyu Koji on Shoyu brewing (part 1) isolation and identification of antifungal antibiotics producing bacteria. *Shoyu Kenkyujyo Kenkyu Houkoku* 24:77–82
- Tanasupawat S, Thongsanit J, Okada S, Komagata K (2002) Lactic acid bacteria isolated from soy sauce mash in Thailand. *J Gen Appl Microbiol* 48(4):201–209
- Uchida K (2000) Diversity and ecology of salt tolerant lactic acid bacteria: *Tetragenococcus halophilus* in Soy Sauce fermentation. *Japanese Journal of Lactic Acid Bacteria* 11:60–65
- Wah TT, Walaisri S, Assavanig A, Niamsiri N, Lertsiri S (2013) Co-culturing of *Pichia guilliermondii* enhanced volatile flavor compound formation by *Zygosaccharomyces rouxii* in the model system of Thai soy sauce fermentation. *Int J Food Microbiol* 160(3):282–289
- Wei CL, Chao SH, Tsai WB, Lee PS, Tsau NH, Chen JS, Lai WL, Tu JC, Tsai YC (2013) Analysis of bacterial diversity during the fermentation of inyu, a high-temperature fermented soy sauce, using nested PCR-denaturing gradient gel electrophoresis and the plate count method. *Food Microbiol* 33(2):252–261
- Whiteley AS, Jenkins S, Waite I, Kresoje N, Payne H, Mullan B, Allcock R, O'Donnell A (2012) Microbial 16S rRNA Ion Tag and community metagenome sequencing using the Ion Torrent (PGM) Platform. *J Microbiol Meth* 91(1):80–88
- Wu J, Gullo M, Chen F, Giudici P (2010) Diversity of *Acetobacter pasteurianus* strains isolated from solid-state fermentation of cereal vinegars. *Curr Microbiol* 60(4):280–286
- Xie J, Jiang H, Liu X, Liu X, Zhou J, Qiu G (2011) 16s rDNA based microbial diversity analysis of eleven acid mine drainages obtained from three Chinese copper mines. *J Cent South Univ T* 18(6):1930–1939
- Yan YZ, Qian YL, Ji FD, Chen JY, Han BZ (2013) Microbial composition during Chinese soy sauce koji-making based on culture dependent and independent methods. *Food Microbiol* 34(1):189–195