Profiling of Dynamic Changes of the Microbial Environment in the Semen Sojae Praeparatum Fermentation Process

Liyuan Wang1, Jing He2, Haizhen Zhu3, Weihua Xie4, Kai Long4, Mingshen Su3 and Xiaomei Xie3,*

1School of Basic Medical Science, Jiangxi University of Traditional Chinese Medicine, Nanchang 330004, China
2Institute Translational Medicine, Nanchang University, Nanchang 330031, China
3Key Laboratory of Modern Preparation of Traditional Chinese Medicine, Ministry of Education, Jiangxi University of Traditional Chinese Medicine, Nanchang 330004, China
4School of Pharmacy, Jiangxi University of Traditional Chinese Medicine, Nanchang 330004, China

*Corresponding author e-mail: jxxm1964@sina.com

Abstract. As a traditional fermented food in China, Semen sojae praeparatum (SSP) is used as both medicine and food. SSP is a kind of solid state fermentation production which be fermented of black soybean, mulberry leaves and artemisia carvifolia, and the unique flavor and function of SSP is determined by the microbial environment in the fermentation process. To track changes in dynamic microbial communities during fermentation process, traditional culture and denaturing gradient gel electrophoresis (DGGE) were used to detect the number and species of dominant microorganisms in the fermentation. Our study showed that Bacillus subtilis, Klebsiella oxytoca and Lactobacillus concavus were dominant species in the SSP fermentation. During the first 6 days of fermentation process, which we called ‘Yellow Cladding’ stage, Bacteria were very high in species diversity and Aspergillus species were the major specimens in the positive fungal. We found that after the 6th day Lactobacillus and Cryptococcus act as key species in the secondary fermentation also. When the material were determined, the microbial population of the fermented environment would decide the unique flavor, function, and the medicinal values, too.

1. Introduction

Semen Sojae Praeparatum (SSP), an ancient fermented food also known as ‘Dan Douche’ [1] in China, is processed with black soybean, mulberry leaf and artemisia carvifolia as the principal raw material. Either as medicinal works of past dynasties or Pharmacopoeia of People's Republic of China record, ‘Yellow Cladding’ and secondary fermentation must be important in typical SSP production process [2]. The microbial environment of the fermentation determined the unique flavor, function and edible safety of SSP. Therefore, a great contribution to special characters of anaerobic culture may produce some special organic acids, enzymes and aromatic substances [3, 4].
Some microbiology studies about SSP fermentation reported. For example, expression products of *Bacillus subtilis* in SSP fermentation was clear, TOSHIO HARA et al found that *Bacillus* play an important role in the procession [1]. Zhang tried to find out how Aspergillus works in black bean fermentation, also [5]. These studies indicates that it would be meaningful to study in what roles the microorganisms play in the fermentation process.

Our study would try to describe the microbial diversity in SSP processing. Amplificon of 16S rDNA and 18S rDNA of the dominant microbial population was analyzed with DGGE. The sequences and the GeneBank data would be measured to describe the microbial changes. The dynamic change of microbial environment in the process would be described for further study of the fermented mechanism of SSP processing.

2. Material and method

2.1. Materials and reagents

Black soybean, mulberry leaves and artemisia carvifolia cultivars produced in the summer of 2014 in Nanchang, Jiangxi, China were used for SSP preparation. Restriction enzymes and T4 DNA ligase were purchased from TaKaRa. Taq and pfu DNA polymerases were purchased from BioStar and TIANGEN Biotech. All other reagents were the purest ones commercially available.

2.2. SSP processing and sampling

SSP was made in our laboratory according to the traditional process [2]. 100 grams of mulberry leaf was boiled with 1 kilograms ddH2O twice, and 100 grams of artemisia carvifolia was boiled with 0.8 kilograms ddH2O twice also. The filtrate was both collected to soak black soybean for 6 hours, then the soybeans was hold in 140°C for 1.5 hours (sampling as the original sample). The soybeans was covered with the residues of boiled mulberry leaf and artemisia carvifolia, keep 30°C and 70% humidity for 6 days. We called this procession ‘Yellow Cladding’, and sampled once a day. Then washed away the dregs of the fermentation products, kept the mixture fermenting at 30°C and 70% humidity in an airtight container for 15 days. The mixture need airing and sampling every three days at this stage which we called secondary fermentation. After totally 21 days’ fermentation, the mixture will be drying after 10 minutes steamed, and the SSP product were prepared. All the samples would be storage at -60°C for following analysis.

2.3. Microbiological culture and isolation with selective mediums

Every mixed sample (1 g) was transferred to test tube and homogenized with 9 mL sterile water. Gradient dilution coating method was used to for Microbiological isolation. Proper concentration samples was coating on brain heart infusion broth (BHIB) medium, peptone-yeast extract-glucose medium [6] and potato dextrose agar (PDA) medium, and the latter two medium supplemented with streptomycin sulfate (40 μg per mL) as necessary. These coating mediums were cultivated at 37°C at 55°C for 24–48 hour. All counts were repeated triplicate, and the results were reported as the means±SD (n=3).

2.4. BIOLOG assays.

Enrichment cultured the microorganisms from each samples for 24–48 hour, and prepared suspension. With spectrophotometer detected, 95% bacterial suspension, 47% yeast suspension and 75% mould suspension were prepared. Each suspension of bacterial cells in saline was used to inoculate BIOLOG GENIII plates (100 μL per well), which were then incubated at 33°C for 22 h. Yeasts and molds were inoculated on BIOLOG GENIII YT or FF plates (100 μL per well), then incubated at room temperature for 72 hour or 120 h. Keep microstation host readings each 24 hour until the microorganisms were identified.
2.5. DGGE gel and analysis
Amplicons of 16S and 18S rDNA were used for sequence-separation by denaturing gradient gel electrophoresis (DGGE) according to the method described by Fujiwara, K. [7]. DGGE was performed on 8% polyacrylamide gels containing acrylamide, bisacrylamide, formamide, and a gradient of 40-60% of urea for bacteria and 20%-40% of urea for fungus, and the TAE buffer was used as the electrophoresis buffer in a Bio-Rad DGGE system (Bio-Rad, Hercules, CA, USA).

DGGE of PCR amplicons were performed according to the method of Nicolaisen and Ramsing [8]. The obtained DGGE patterns were subsequently normalised and analysed using Quantity One® (version 4.5.0, Bio-Rad Laboratories). During this processing, different band was identified, similarity of the cluster and relative quantitative were analyzed.

2.6. Total DNA extraction, PCR amplification and sequencing
5g sample was soaked in 45 mL sterilized normal saline, after shaking for 30 min, then suspension matters were collected. Wash the collection sterile phosphate buffered saline (pH 7.3) three times before cell broken. Then DNA was isolated according to the method of Donskey [9]. After phenol-chloroform extraction, DNA was precipitated with ethanol and suspended in 30 μL of ultrapure water.

Table 1. PCR primers used in this study.

| Target organism | Primer* | Sequence |
|-----------------|---------|----------|
| Bacteria        | GC-341(F) | 5'-CGCCCGCCGCCCCGCCCCCCCCCCCCCCCCCTACGGGAGGCAGCAG-3' |
|                 | 341(F)   | 5'-CTACGGGAGGCAGCAG-3' |
|                 | R518(R)  | 5'-GTATTACCGCGGCTGCTGG-3' |
| Fungi           | NS1(F)   | 5'-GTAGTCATATGCTTGTCTC-3' |
|                 | GC-Fung(R) | 5'-CGCCCGCCGCCCCGCCCCCCCCCCCCCCCCATCCCGGAGGCAGCAGCAG-3' |
|                 | Fung(R)  | 5'-ATTCCCCGTTACCCCGTG-3' |

*F, forward primer. R, reverse primer

PCR was performed using the Taq DNA polymerase kit (TIANGEN biotech, China) in PCR system. The primers were listed in Table 1. Touchdown PCR were used for the bacterial DNA amplification with following program. The PCR reaction (50 μL) used 25 μL of 2*PCR Mix, 1 μL of primers (5 pmol), 2 μL of 10-fold diluted DNA template (approximately 0.5 ng) and lastly distilled water. The samples for bacterial total DNA were amplified in PCR system, pre-denaturation at 94 ℃ for 5 min, with 20 cycles of 94 ℃ for 45 s, the annealing temperature was decreased by 0.5 ℃ every cycle from 65 ℃ in the first cycle to 56 ℃ during the final cycle, and 72 ℃ for 60 s in a Biosci PCR system, then the samples were amplified with 10 cycles of 94 ℃ for 45 s, 55 ℃ for 30 s, and 72 ℃ for 60 s before the last extension at 72 ℃ for 10 min. The PCR reaction (50 μL) for fungal DNA used 25 μL of 2*PCR Mix, 1 μL of primers (5 pmol), 4 μL of 10-fold diluted DNA template (approximately 0.5 ng) and lastly distilled water. The samples were amplified in a Biosci PCR system, pre-denaturation at 94 ℃ for 5 min, with 30 cycles of 94 ℃ for 30 s, 58 ℃ for 45 s, and 72 ℃ for 60 s in a Biosci PCR system before the last extension at 72 ℃ for 10 min. Primers used in this study were listed in Table 1. Aliquots of 5 μL were analyzed by electrophoresis on an agarose gel (1%) to check the size and amounts of the amplicons.

PCR was performed using the pfu DNA polymerase kit (Biostar, Canada) in PCR system with the eluted DNA as template, used the primer without GC clamp (Table 1). The amplified DNA fragments were attached to pEASY-T3TM Cloning Vector to send to BGI (The Beijing Genomics Institute) to get commercial sequencing.

3. Conclusion

3.1. Dominant microbes in SSP fermentation
In the ‘Yellow Cladding’ stage (0–6 d), bacteria, molds and yeasts were significantly increased during the first 4 days of fermentation. The number of bacterial colonies reached 1*10^{10.6} CFU per mL,
compared with the previous $1 \times 10^{16}$ CFU per mL at the beginning (Fig.1). The number of mould and yeast colonies reached $1 \times 10^{9.8}$ CFU per mL and $1 \times 10^{8.8}$ CFU per mL, composed none of them before. In the first 5 days of fermentation, the number of microorganisms increased gently. Then the number of microorganisms decreased in the second fermentation (7~21 d) stage. Between the ninth day and the twenty first day of this stage, the number decreased steadily, and the decreasing became significantly after the ninth day. At the end of the secondary fermentation, the number of bacterial colonies was $1 \times 10^{3.2}$ CFU per mL, the number of bacterial colonies was $1 \times 10^{4.0}$ CFU per mL, and no mould be found.

![Figure 1. Changes in the amount of microorganisms in SSP fermentation.](image)

The bacteria play an important role in the whole process, especially in the ‘yellow cladding’ stage. But it decreased in the secondary fermentation stage. The yeast would be the most important microbes during the late of the SSP fermentation. Though molds were not found from the SSP products, they may worked in some way because the number of molds increased in the ‘yellow cladding’ stage and decreased in the secondary fermentation.

As the dominant microbes isolated by Biolog technology shown in Table 2, the species of dominant bacteria, molds and yeasts at the ‘yellow cladding’ stage became much richer than before. Bacillus Subtilis was the dominant strain of the fermentation. The dominant molds were Aspergillus sp., and the specie of yeast changed greatly at the different stage of fermentation.

| Time  | Bacteria                          | Mycetes                   | Yeasts                      |
|-------|-----------------------------------|---------------------------|-----------------------------|
| 0 day | Bacillus subtilis                 | none                      | none                        |
| 3 day | Bacillus subtilis, Bacillus       | Aspergillus parasiticus   | Debaryomyces Hansenii C     |
|       | amyloliquefaciens, Staphylococcus | Aspergillus flavus        |                             |
|       | sciuri                            |                            |                             |
|       | Acinetobacter radioresistens      |                            |                             |
|       | Enterococcus casseliflavus        |                            |                             |
| 6 day | Bacillus subtilis                 | Aspergillus parasiticus   | Rhodotorula acheniorum      |
|       | Staphylococcus sciuri             |                            | Trichosporon beigeli A      |
|       | Acinetobacter radioresistens      |                            | Debaryomyces Hansenii C     |
|       | Enterococcus casseliflavus        |                            |                             |
| 9 day | Bacillus subtilis, Escherichia    | Aspergillus niger          | Rhodotorula acheniorum      |
|       | hermannii, Klebsiella oxytoea     |                            |                             |
| 21 day| Bacillus subtilis, Klebsiella     | none                      | Cryptococcus marinus        |
|       | oxytoea                           |                            | Cryptococcus laurentii      |

### 3.2. DGGE result of bacteria

To detect the fluctuation of bacteria, molds and yeasts community during the fermentation process, the DGGE was carried out with different primers. The bacterial DGGE pattern showed that though microbial number reached maximum since the 5th day (Fig.1), its bacterial composition was obviously
different from the other samples. The amplicon of 16S rDNA bacterial were detected using DGGE. The result was shown in Fig.2a, the bands represent bacterial species and the brightness of the bands represents the number of bacteria. Analyzed the result of DGGE with Quantity One®, the bands in Fig.2b represents the number of bacterial species. Abundant bacterial florae in the semen sojae praeparatum fermentation process that 29 bacteria were detected. On the day 3 and the day 6, the number of bands increased from 15 to 17. At the stage of secondary fermentation, the number of bands decreased, it were 14 bands on day 9, 9 bands on day 15 and 7 bands at last.

**Figure 2.** The result of bacterial DGGE. Figure 2a left while Figure 2b right. **Figure 3.** The result of fungal DGGE. Figure 3a left while Figure 3b right.

Not only the number of bands changed in different lanes, but also the position of the bands were different, the number and species of bacteria increased gradually at the ‘yellow cladding’ stage of the SSP fermentation, and they decreased at the stage of secondary fermentation. The DGGE result showed the same trend as the result of cultured and Biolog before, it would reflect that the microbial community of bacteria in semen sojae praeparatum fermentation changed dynamically.

### 3.3. DGGE result of fungi

The amplicon of 18S rDNA PCR were detected using DGGE technology. As shown in the Fig.3a, the bands represent the same as before. Analyzed the result of DGGE with Quantity One®, the bands in Fig.3b represents the number of fungal species. Abundant fungal florae in the semen sojae praeparatum fermentation process that 19 fungi were detected. On day 1, there was no fungi was detected. Then bands were gradually increasing from day 3 to day 9. There were 7 bands on the day 3, 9 bands on day 6, and 12 bands on day 9. The bands decreased that there was only 7 bands on day 15, and 4 bands at last. All bands of day 15 and day 21 were not obvious that they were all weak.

Not only the number of bands changed in different lanes, but also the position of the bands were different, the number and species of fungi increased gradually at the ‘yellow cladding’ stage of the SSP fermentation, and they decreased at the stage of secondary fermentation. On the day 9, six news strains of fungi were found in the samples while most of other strain were undetected. The DGGE result showed the same trend as the result of cultured and Biolog before, it would reflect that the microbial community of fungi in semen sojae praeparatum fermentation changed dynamically, and most of them disappeared at last.

### 3.4. Strains identification results

22 representative strains of bacteria were isolated from DGGE gel. They belonged to four phylum of bacteria, and 54.5% of these strains was Proteobacteria as the most dominant phylum. The following
phylum was Firmicutes which accounted for 36.4%, while Bacteroidetes and Actinobacteria accounted for 4.5% both.  

9 representative strains of fungi were isolated from DGGE gel. They belonged to 2 phyla of fungi, Ascomycota and Basidiomycota. 3 order contained, Eurotiales, Tremellales and Polyporales. 4 genus contained, with Aspergillus as the dominant which accounted 55.6%, Trichosporon for the second which accounted 22.2%, Trametes and Cryptococcus accounted 11.1% each. Both Aspergillus oryzae and Trichosporon ovoides increased at the stage of ‘yellow cladding’, and decreased at the stage of secondary fermentation until to none of them were detected at last. Both these two were main strains at the ‘yellow cladding’ stage and disappeared at the following stage, while Cryptococcus randhawii was found only in the end of the whole process. 

Microbial environment is most crucial in the fermentation of SSP processing, with distinctive fermentation methods, fermentation conditions, materials and other factors, a unique complex microbial system play role in the SSP processing. Therefore the dynamic changes and the dominant bacteria are crucial in SSP fermentation. Our following research will try to study how the dominant microorganisms works in SSP processing.

Acknowledgements

This work was supported in part by the National Natural Science Foundation of China (81660664), and the project of the Education Department of Jiangxi Province (GJJ13615, GJJ160858), Health and Family Planning Commission of Jiangxi Province (2015B044, 2016A060) and Jiangxi University of traditional Chinese Medicine (2014BS020)

References

[1] HARA, T., OGATA, S., & UEDA, S. (1993). PLASMID DISTRIBUTION IN γ-POLYGLUTAMATE-PRODUCING BACILLUS STRAINS ISOLATED FROM "DAN-DOUCHI," A "NATTO"-LIKE NON-SALTY FERMENTED SOYBEAN FOOD IN CHINA. The Journal of General and Applied Microbiology, 39 (1), 75-82.
[2] Chinese Pharmacopoeia Commission. (2015). Pharmacopoeia of the People's Republic of China. 1 (2015). China Medical Science Press.
[3] Hussain, A., Bose, S., Wang, J. H., Yadav, M. K., Mahajan, G. B., & Kim, H. (2016). Fermentation, a feasible strategy for enhancing bioactivity of herbal medicines. Food Research International, 81, 1-16.
[4] Tamang, J. P., Watanabe, K., & Holzapfel, W. H. (2016). Review: diversity of microorganisms in global fermented foods and beverages. Frontiers in microbiology, 7.
[5] Zhang, J. H., Tatsumi, E., Fan, J. F., & Li, L. T. (2007). Chemical components of Aspergillus - type Douchi, a Chinese traditional fermented soybean product, change during the fermentation process. International journal of food science & technology, 42 (3), 263-268.
[6] McCroskey, L. M., Hatheway, C. L., Fenicia, L., Pasolini, B., & Aureli, P. (1986). Characterization of an organism that produces type E botulinal toxin but which resembles Clostridium butyricum from the feces of an infant with type E botulism. Journal of clinical microbiology, 23 (1), 201-202.
[7] Fujiwara, K., Yamazaki, M., Abe, H., Nakashima, K., Yakabe, Y., & Otsuka, M., et al. (2009). Effect of bacillus subtilis var. natto fermented soybean on growth performance, microbial activity in the caeca and cytokine gene expression of domestic meat type chickens. Journal of Poultry Science, 46 (46), 116-122.
[8] Nicolaisen, M. H., & Ramsing, N. B. (2002). Denaturing gradient gel electrophoresis (dgge) approaches to study the diversity of ammonia-oxidizing bacteria. Journal of Microbiological Methods, 50 (2), 189-203.
[9] Donskey, C. J., Hujer, A. M., Das, S. M., Pultz, N. J., Bonomo, R. A., & Rice, L. B. (2003). Use of denaturing gradient gel electrophoresis for analysis of the stool microbiota of hospitalized patients. Journal of microbiological methods, 54 (2), 249-256.