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Analysis of microbial diversity in Shenqu with different fermentation times by PCR-DGGE

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ABSTRACT

Shenqu is a fermented product that is widely used in traditional Chinese medicine (TCM) to treat indigestion; however, the microbial strains in the fermentation process are still unknown. The aim of this study was to investigate microbial diversity in Shenqu using different fermentation time periods. DGGE (polymerase chain reaction-denaturing gradient gel electrophoresis) profiles indicated that a strain of *Pediococcus acidilactici* (band 9) is the predominant bacteria during fermentation and that the predominant fungi were uncultured *Rhizopus*, *Aspergillus oryzae*, and *Rhizopus oryzae*. In addition, pathogenic bacteria, such as *Enterobacter cloacae*, *Klebsiella oxytoca*, *Erwinia billingiae*, and *Pantoea vagan* were detected in Shenqu. DGGE analysis showed that bacterial and fungal diversity declined over the course of fermentation. This determination of the predominant bacterial and fungal strains responsible for fermentation may contribute to further Shenqu research, such as optimization of the fermentation process.

Introduction

Shenqu, also known as Liushenqu, is commonly used in Chinese medicine clinics to protect the stomach and spleen and stimulates appetite and digestion. Current research efforts have revealed that some digestive enzymes (amylase enzymes, protease enzymes, glucoamylase), vitamins and other substances play a main role in stimulating appetite and digestion.[1] Resistance theory is the earliest work to mention Shenqu. Shenqu is traditionally processed as follows: wheat bran, flour, ricebean powder (*Vigna umbellata* [Thunb.] Ohwi and Ohashi), and bitter apricot seed powder (*Prunus mandshurica* [Maxim.] Koehne) are blended in a particular ratio. Various Chinese medicine decoctions are then added, including *Polygonum pubescens* (Blume), *Xanthium sibiricum* (Patr.), and *Artemisia annua* (L.). The mixture is then kneaded and divided into bricks, which are put into a mold. Finally, the bricks are covered with adhesive-bonded cloth and placed in a box at constant temperature and humidity. After a few days of fermentation, the product is cut into small lumps and dried at a low temperature. The quality of the resulting Shenqu can vary due to differences in the amount of the mixed bacteria and fungi that are present during fermentation. It is worth noting that the fungus *Aspergillus flavus* produces aflatoxin,

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a carcinogen, during fermentation. This is one of the reasons why Shenqu is not included in the Chinese Pharmacopeia. However, the current theoretical support endorses Shenqu for stimulating appetite and digestion.a better understanding of the microbes involved in Shenqu fermentation may lead to improved methods of fermentation.

There are two main types of methods for assessing bacterial diversity, traditional culture-dependent methods and culture-independent methods. Thus far, studies on the microbial diversity of Shenqu have been mainly based on traditional culture-dependent methods,[2–4] such as PCR-SSCP (single strand conformation polymorphism)[5] and DGGE.[6] PCR-DGGE (polymerase chain reaction-denaturing gradient gel electrophoresis) is a culture-independent method designed to analyze the genetic diversity in a sample. It overcomes the disadvantages of culture-dependent methods,[7] making it a common tool for molecular biological investigations into microbial communities. PCR-DGGE has been used widely to analyze microbial community structure across different fields, such as food microbiology, oral microbiology, soil microorganisms, environmental microbiology, and other areas.[8–11] In this study, we used culture-independent PCR-DGGE and TA cloning to determine the microbial diversity of Shenqu across different fermentation periods. The aim of this study was to investigate eubacteria microbial diversity during fermentation and identify several dominant fermentation bacteria and fungus.

Materials and methods

Shenqu sample collection

Shenqu fermentation parameters were based on our previous study and response surface methodology.[12] Raw materials were crushed in a grinder. Fourteen grams of *Polygonum pubescens* (Blume), *Xanthium sibiricum* (Patr.) and *Artemisia annua* (L.) were mixed with water and decocted for 1 h at 32 °C and 75% relative humidity and then mixed with 60 g of flour, 140 g of wheat bran, 8 g of bitter apricot, and 5.2 g of ricebean. Eight samples were processed and designated as 1–8 for fermentation for varying lengths of time, representing days 1–8, respectively. Each Shenqu sample, of approximately 100 g, was collected during days 1–8. All samples were collected in a sterile environment, transferred to sterile polyethylene bags and stored at −70 °C until they were analyzed.

DNA extraction

Five grams of each Shenqu sample were suspended in 50 mL of phosphate buffered saline (PBS, 0.1 mol/L, pH 8.0) and shaken for 10 min. The mixed suspension was centrifuged at 10,000 × *g* for 10 min and washed three times using the same PBS buffer. Total genomic DNA was extracted from the pellets using a ONE-4-ALL Genomic DNA Mini-Preps Kit (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions. The samples were ground using liquid nitrogen and lysis buffer, then rapidly thawed in a water-bath at 65 °C for an hour. The samples were shaken every 10 min during lysis. The crude DNA was electrophoretically analyzed on 1.2% (w/v) agarose gels; samples were then kept in a clean 0.5-mL microcentrifuge tube and stored at −20 °C.

PCR amplification

All primers used in this study are listed in Table 1. General bacterial 16S rRNA gene primers 338F and 518R were used to assess bacterial diversity. A touch-down PCR technique was employed in order to increase sensitivity. The thermal cycling conditions were as follows: 5 min denaturation at 95 °C; 5 cycles of 30 s at 94 °C, 30 s at 62 °C (with each cycle reduced by 2 °C), and 90 s at 72 °C; 25 cycles of 30 s at 94 °C, 30 s at 50 °C, and 90 s at 72 °C; and final extension for 10 min at 72 °C. A GC clamp (5′-CGC CCG CCG CGC GCG GCG GGCGGG GCG GGG GCA CGG GGG G-3′) was attached to the 5′ end of primer 338F for the DGGE analysis.

A nested PCR technique was employed in order to increase sensitivity. PCR amplification of general bacterial 18S rRNA was performed using universal gene primers NS1 and FR1 in the first step, followed by nested PCR using NS1 and GC-Fung. The thermal cycling conditions were as follows: 5 min denaturation at 95 °C; 30 cycles of 30 s at 94 °C, 30 s at 50 °C, and 90 s at 72 °C; and final extension for 10 min at 72 °C. PCR products from the first step were diluted with 10 times the amount of ddH_2O and served as the template for the second round of nested PCR.

DGGE analysis

The PCR products of bacteria and fungi were analyzed using DGGE and the D-code Universal Mutation Detection System (Bio-rad, USA). For assessing bacterial diversity, 10%

Table 1 – Primers used in this study.

Targets	Primers	Sequence (5′–3′)	References
Bacteria	338F[a]	CCTACGGGAGGCAGCAG	Muyzer G[13]
	518R	ATTACCGCGGCTGCTGG	Muyzer G[13]
Fungi			
First PCR round	NS1	GTAGTCATATGCTTGTCTC	Vainio EJ[14]
	FR1	AICCA TCA ATC GGT AIT	Vainio EJ[14]
Second PCR round	NS1	GTAGTCATATGCTTGTCTC	May LA[15]
	Fung[b]	ATTCCCCGTTACCCGTTG	May LA[15]

F, forward primer; R, reverse primer.

[a] Primer with a 41-bp GC clamp (CGCCCGGGGCGCGCCCCGGGGCGGGGCGGGGGCGCGGGGGG).

[b] Primer with a 40-bp GC clamp (CGCCGGCCGCGCCCCGCGCCGGCCCGCCGCCCCGCCCC).

of the polyacrylamide gradient (acrylamide:bisacrylamide, 37.5:1) was used. The optimal separation was achieved by a 40–70% denaturant gradient. For assessing fungal diversity, 8% polyacrylamide and 25–40% denaturant gradient were used. Electrophoresis was then performed for 1 h at 60 V and 15 h at 100 V (60 °C). After electrophoresis, gels were stained with SYBR Green I (Molecular Probes, BBI, Candia) for 30 min. The gels were observed, and photographs were taken using a KETA G series Image System (Wletch, USA).

Sequencing of DGGE bands

Representative bands were excised from gels with a sterile blade. The gel pieces were ground using tissue-grinding pestles (Sangon, Shanghai, China) and then incubated overnight at 4 °C in TE buffer (pH 8.0). The DNA solution with TE was then amplified with primers with no GC clamp. Purified PCR products were ligated into a pUCm-T vector and then transformed into Trans5α Chemically Competent Cells (Transgen Biotech, Beijing, China). Individual white colonies were amplified with PCR using the primers M13-47[16] and M13-48 (Sangon, Shanghai, China). Samples were then sent to a sequencing company for sequencing (Sangon, Shanghai, China). The resulting gene sequences were aligned with those in a Gen Bank with the Blast program to identify the closest known relatives.

Statistical analyses

Quantity One software (Bio-rad, USA) was used to analyze the DGGE profiles and perform cluster analysis. Statistical analysis of the data sets was performed using MATLAB 2013a software (Mathworks, USA). The Shannon–Wiener index was determined by the relative intensity of bands.

Results

Bacterial and fungal community diversity

The DGGE profile for the bacterial community of fermenting Shenqu is shown in Fig. 1. Notably, the bacterial community differed over the course of fermentation, while the fungal community did not differ. Diversity indices of microbes in Shenqu were calculated based on the DGGE profile. The bacterial diversity indices over 8 days of fermentation were as follows: day 1, 21 bands, Shannon–Wiener index 2.38; day 2, 23 bands, index 2.56; day 3, 13 bands, index 2.07; day 4, 13 bands, index 2.05; day 5, 18 bands, index 2.19; day 6, 18 bands, index 2.15; day 7, 19 bands, index 2.35; and day 8, 7 bands, index 1.52. The fungal diversity indices over the 8 days were as follows: day 1, 8 bands, Shannon–Wiener index 1.69; day 2, 10 bands, Shannon–Wiener index 1.92; day 3, 4 bands, Shannon–Wiener index 1.36; day 4, 8 bands, Shannon–Wiener index 1.77; day 5, 7 bands, Shannon–Wiener index 1.77; day 6, 7 bands, Shannon–Wiener index 1.35; day 7, 7 bands, Shannon–Wiener index 1.59; and day 8, 7 bands, Shannon–Wiener index 1.71. The species richness varied over the eight samples, and most bands were observed in the sample from day 2 (Fig. 1A and B). The sample from day 2 also had the highest Shannon–Wiener indices (2.56 and 1.92) of the PCR-DGGE profiles.

Bacterial and fungal diversity after varying durations of fermentation

The sequencing of bacterial DGGE bands highlighted the presence of various bacterial strains, including *Enterobacter cloacae* (band 1, 100% identity to NCBI accession KM408606), *Klebsiella oxytoca* (bands 2 and 10, 100% identity to KM408607 and KM408615), *Erwinia billingiae* (bands 3 and 11, 100% identity

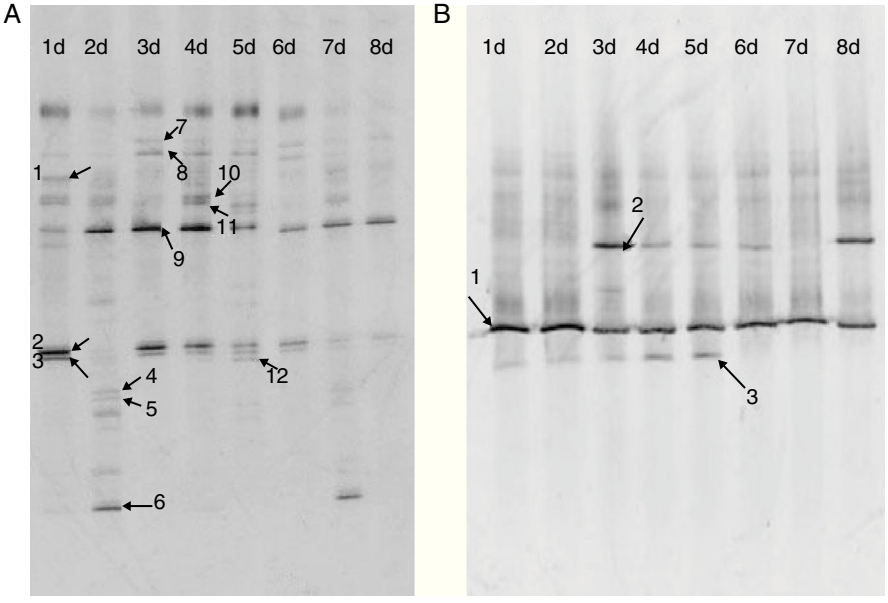

Fig. 1 – Touchdown PCR-DGGE and nested PCR-DGGE profile of bacterial community diversity of Shenqu from the 16s rDNA and 18s rDNA obtained from Shenqu after varying durations of fermentation. Lanes 1–8d refer to samples derived from the 1st to the 8th day of fermentation, respectively. (A) A 40–70% denaturing gradient was used. (B) A 25–40% denaturing gradient was used.

to KM408608 and KM408615), *Escherichia hermannii* (band 4, 99% identity to KM408609), *Paenibacillus polymyxa* (band 5, 99% identity to KM408610), *Pantoea vagans* (band 6, 100% identity to KM408611), *Acinetobacter baumannii* (band 7, 100% identity to KM408612), *Desulfotomaculum thermocisternum* (band 8, 100% identity to KM408613), *P. acidilactici* (band 9, 99% identity to KM408614), and *Citrobacter koseri* (band 12, 100% identity to KM408617) (Fig. 1A). Notably, *P. acidilactici* (band 9, 100% identity to KM408614)) was detected throughout the entire fermentation process.

The sequencing of fungal DGGE bands highlighted the presence of three strains: uncultured *Rhizopus* (band 1, 100% identity to NCBI accession KM408618), *Aspergillus oryzae* (band 2, 100% identity to KM408619), and *Rhizopus oryzae* (band 3, 100% identity to KM408620) (Fig. 1B). Again, one species, the uncultured *Rhizopus* (band 1), was detected throughout the entire fermentation process, followed by band 2,3 (*A. oryzae, R. oryzae*).

Discussion

In this study, PCR-DGGE was applied to analyze the microbial community structure of the TCM supplement Shenqu. Shenqu is a natural culture medium containing various nutrients. Conventional culture methods are unable to reflect its full nutritional contents. Therefore, our study adopted the culture-independent method of PCR-DGGE to investigate the bacterial and fungal community structure of Shenqu. The bacterial DGGE fingerprints showed that the *Pediococcus acidilactici* strain (band 9, Fig. 1A) was the predominant bacterial species present during fermentation. Likewise, the predominant fungus during fermentation was uncultured *Rhizopus*, followed by *A. oryzae*, and *R. oryzae*. From Berger's bacterial identification manual and related literature,[17–19] we know that these bacteria can produce amylase, protease enzymes such as glucoamylase, and digestive enzymes. These products are likely to be associated with the appetite stimulating and digestive functions of Shenqu.

The sequencing results showed that the bacterial community included 10 types of pathogenic bacteria, including seven *E. cloacae* strains, *K. oxytoca*,[20] *E. billingiae*, and *P. vagan*.[21] This study confirmed that pathogenic bacteria exist in the traditional Chinese medicine Shenqu. The existence of pathogenic bacteria is likely to affect the quality of various batches of Shenqu compared with batches of Shenqu that have undergone pure bred fermentation[22,6] also investigated the microbial community of Shenqu by PCR-DGGE and found that the dominant microbes belonged to the genera *Enterobacter*, *Pediococcus*, *Pseudomonas*, *Mucor*, and *Saccharomyces*, which are results that are somewhat different from ours. This outcome is probably due to the different proportions of ingredients and fermentation parameters used in the two studies.

In conclusion, the aim of this study was to investigate the microbes of Shenqu over varying durations of fermentation by PCR-DGGE. The results revealed that *P. acidilactici*, *A. oryzae*, and *R. oryzae* were the predominant microbes present. These results may contribute to further study of Shenqu, such as studies focusing on optimizing the fermentation process or pure bred fermentation of Shenqu. Only by purifying the predominant microbes of Shenqu will we be able to examine the microbial biological transformations that occur in Shenqu. Thus, in this study, we suggest that PCR-DGGE should be considered as a preliminary tool for investigating the microbial community structure of Shenqu. Because of technical deficiencies of the PCR-DGGE method, however, some elements of the microbial community may inevitably go undetected. Other new technologies, such as T-RLFP, MLST and high-throughput sequencing, could therefore be adopted for further studies.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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