

Monitoring of the bacterial and fungal biodiversity and dynamics during *Massa Medicata Fermentata* fermentation

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Abstract The microbial community dynamics play an important role during *Massa Medicata Fermentata* (MMF) fermentation. In this study, bacterial and fungal communities were investigated based on the culture-dependent method and polymerase chain reaction-denaturing gradient gel electrophoresis analysis. Meanwhile the dynamic changes of digestive enzyme activities were also examined. Plating results showed that MMF fermentation comprised two stages: pre-fermentation stage (0–4 days) was dominated by bacterial community and post-fermentation stage (5–9 days) was dominated by fungal community. The amount of bacteria reached the highest copy number 1.2×10^{10} CFU/g at day 2, but the fungi counts reached 6.3×10^5 CFU/g at day 9. A total of 170 isolates were closely related to genera *Enterobacter*, *Klebsiella*, *Acinetobacter*, *Pseudomonas*, *Mucor*, *Saccharomyces*, *Rhodotorula*, and *Amylomyces*. DGGE analysis showed a clear reduction of bacterial and fungal diversity during fermentation, and the dominant microbes belonged to genera *Enterobacter*, *Pediococcus*, *Pseudomonas*, *Mucor*, and *Saccharomyces*. Digestive enzyme assay showed filter paper activity; the activities of amylase, carboxymethyl cellulase, and lipase reached a peak at day 4; and the protease activity constantly increased until the end of the fermentation. In this study, we carried out a detailed

and comprehensive analysis of microbial communities as well as four digestive enzymes' activities during MMF fermentation process. The monitoring of bacterial and fungal biodiversity and dynamics during MMF fermentation has significant potential for controlling the fermentation process.

Keywords *Massa Medicata Fermentata* fermentation · PCR-DGGE · Digestive enzyme · Bacterial community · Fungal community

Introduction

Massa Medicata Fermentata (MMF) is also known as Liu Shenqu; it is one of the most widely used traditional fermented Chinese medicines and is also one of the earliest medicines recorded in Chinese Pharmacopoeia. In the clinic, it is mainly useful for indigestion, vomiting, diarrhea, and other symptoms caused by poor diet (Tao 2000). The fermentation of MMF is a spontaneous process, whose composition consists of five traditional Chinese medicinal materials (red bean, bitter almond, *Artemisia annua*, *Xanthium sibiricum*, and *Polygonum hydropiper*), flour, and wheat bran. Different microbes coexisting in this fermentation process can produce a variety of digestive enzymes, which can decompose higher molecular compounds and transform them into some new smaller functional substances that are beneficial to be absorbed by the human body such as glycosides, essential oils, vitamins, small peptides, and so on (Gao and Jia 2002). The biotransformation can greatly enhance the efficacy and reduce some side effects of the medicine (Wu et al. 2013). At present, it has become a hot topic in medicinal microbe fermentation and transformation of traditional Chinese medicine (Wu et al. 2013). Thus, it is necessary to investigate the digestive enzymes as well as the microbial diversity during MMF fermentation process.

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So far, the digestive enzyme researches were mainly about amylase and protease (Wang et al. 2012). The microbial researches are mainly based on conventional culture-dependent methods (Gao and Jia 2002; Hu et al. 2004). And these approaches consist of isolating and enumerating microbial groups by growing them on different selective media and identifying the isolates by using phenotypic and molecular techniques. Although plate counting can provide quantitative data, the conventional culture-dependent methods are time consuming because of long culture periods. Also, it is known that only a small proportion of microorganisms can be cultured, and this often results in an incomplete representation of the true microbial diversity present (Hugenholtz et al. 1998). In recent years, culture-independent methods which are based on molecular biology techniques allowed for a rapid analysis of the microorganism diversity and dynamics during fermentation without culturing, such as polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), restricted fragment length polymorphisms, and single-strand conformation polymorphism. PCR-DGGE analysis was widely used to analyze the microflora in various traditional fermented products, such as Chinese liquor Daqu (Wang et al. 2011), Japanese shochu (Endo and Okada 2005), Portuguese fermented sausage (Albano et al. 2008), Italian cheese (Fontana et al. 2010), and so on.

Plenty of data have been published regarding microbes, and digestive enzyme activities were mainly about commercial MMF (Zhang et al. 2012a). However, there is little information about the dynamic changes of bacterial and fungal communities and four digestive enzymes' (amylase, protease, cellulase, and lipase) activities during MMF fermentation process. In this study, we sought to fill these gaps by exploring the microbiota, combining both PCR-DGGE technology and cultivation-dependent methods as well as monitoring the activity dynamics of the four digestive enzymes during MMF fermentation process. To our knowledge, this is the first report about the microbiota of MMF during fermentation process using PCR-DGGE analysis. The dynamics monitoring of the microbiota and digestive enzymes' activities during MMF fermentation has significant potential for controlling MMF fermentation.

Materials and methods

The fermentation of MMF and sample collection

Under laboratory conditions, the MMF fermentation was performed. Each raw material was firstly crushed by a grinder; then 100 g of *A. annua*, 100 g of *P. hydropiper*, and 100 g of *X. sibiricum* were mixed with the appropriate water, boiled for 1 h, and finally mixed with 500 g of flour, 20 g of red bean, 20 g of bitter almond, and 1,000 g of wheat bran. The mixture

was shaped in small boxes, then incubated at around 30–37 °C and 70–80 % of the humidity for about 9 days. The samples were collected every 24 h for measurement of digestive enzymes' activities and analysis of microflora.

Digestive enzyme assay

Five-gram samples were suspended in 12.5 mL of the appropriate buffer and mildly agitated at 4 °C overnight. The samples were centrifuged at 10,000×g at 4 °C for 30 min, and the supernatant was collected for the digestive enzyme assay. All tests were carried out in triplicate.

Amylase activity was assayed by the dinitrosalicylic acid (DNS) method of Miller (1959) with slight modifications, using 1 % starch solution in 0.1 M phosphate buffer (pH 6.5) containing 17 mM NaCl as a substrate. The substrate solution (350 µL) and 50 µL of enzyme solution were incubated at 37 °C for 15 min; then 50 µL of 2 M NaOH was added to stop the reaction. Fifty microliters of the DNS reagent was added to the reaction solution and incubated in a boiling water bath for 5 min; absorbance values at 540 nm were measured. Blanks contained phosphate buffer instead of a digestive enzyme solution. One unit of amylase activity (U/g MMF) was defined as the amount of enzyme required for the liberation of 1 µmol of maltose per minute under the described conditions.

Protease activity was determined according to Sigma's non-specific protease activity assay using 0.65 % (w/v) casein solution in 50 mM potassium phosphate buffer (pH 7.0) as a substrate. Of the enzyme solution, 0.5 mL was mixed with 2.5 mL of casein solution and incubated at 37 °C for 10 min, and then 2.5 mL of trichloroacetic acid reagent was added and incubated at 37 °C for 30 min to stop the reaction. The samples were centrifuged at 12,000×g for 5 min. One milliliter of supernatant was mixed with 2.5 mL of 500 mM Na₂CO₃, and 0.5 mL of Folin's reagent was added immediately. The mixture was incubated at 37 °C for 30 min. Absorbance at 660 nm was measured. Blanks contained phosphate buffer instead of digestive enzyme solution. One unit of protease activity (U/g MMF) was defined as the amount of enzyme required for the liberation of 1 µg of tyrosine per minute under the described conditions.

Cellulase activities were determined according to the method described by Ghose (1987) using carboxymethyl cellulose sodium and Whatman no. 1 filter paper in 50 mM citrate buffer (pH 4.8) as substrates, respectively. The reaction mixture consisting of 500 µL of substrate and 250 µL of enzyme solution was incubated at 50 °C for 1 h. Then 250 µL of reaction mixture with 500 µL of DNS reagent was mixed and boiled for 5 min. Absorbance at 540 nm was measured. Blanks contained citrate buffer instead of digestive enzyme solution. One unit of cellulase activity (U/g MMF) was

defined as the amount of enzyme required for the liberation of 1 μmol of glucose per minute under the described conditions.

We assayed lipase activity by the method of Kordel et al. (1991) with slight modifications. The substrate solution was prepared by adding one part of solution A (16.5 mM *p*-nitrophenyl palmitate in isopropanol) into nine parts of solution B (0.4 % Triton X-100 and 0.1 % gum arabic in 50 mM Tris–HCl buffer (pH 8.0)) by stirring until fully dissolved. Fifty microliters of enzyme solution was added to 450 μL of substrate solution and incubated at 37 °C for 30 min. Absorbance was read at 405 nm. Blanks contained Tris–HCl buffer instead of digestive enzyme solution. One unit of lipase activity (U/g MMF) was defined as the amount of enzyme required for the liberation of 1 μmol of *p*-nitrophenyl per minute under the described conditions.

Enumeration and isolation of microorganisms

One-gram sample was homogenized with 9 mL of 0.85 % (*w/v*) sterile physiological saline; after serial tenfold dilutions, 100 μL of the appropriate aliquot was spread on Luria-Bertani (LB) agar plates and Martin agar plates in triplicate. LB agar plates were used for enumeration of bacterium and incubated at 37 °C for 24 h. Martin agar plates supplemented with 3,4,5,6-tetrachlorofluorescein (1.5 mg/mL) were used for counting of fungi and incubated at 30 °C for 48–72 h. After incubation, plates with 30–300 colonies were enumerated and recorded as colony-forming unit per gram of MMF samples (CFU/g). For isolation of microorganisms, LB medium, Czapek's medium, and Malt extract medium were used for isolation of bacteria, mold, and yeast, respectively.

Extraction of total genomic DNA

The total genomic DNA of MMF samples made by our lab was extracted by the method previously described (Zhou et al. 1996) with some modifications. The pellet of crude nucleic acids was resuspended in sterile Tris–EDTA (TE) buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), after which it was stored at –40 °C. The DNA from the pure isolates was extracted using Rapid Bacterial Genomic DNA Isolation Kit (Sangon, Shanghai, China) and Rapid Fungi Genomic DNA Isolation Kit (Sangon, Shanghai, China), respectively.

Molecular identification of isolates

The primers used in this study are shown in Table 1. The primers 27F (Nakai et al. 2012) and 1401R (Yong et al. 2011) were used to amplify the full length of bacterial 16S rDNA. The primers NS1 and Fung (May et al. 2001) were used to amplify the fungal 18S rDNA gene. The PCR fragments were ligated with pMD18-T vector (Takara, China), subsequently

sequenced by Beijing Huada gene company (Beijing, China), and were identified using BLAST search.

PCR-DGGE analysis

PCR amplification of 16S and 18S rDNA sequences

The microbial composition of MMF was investigated by amplifying partial sequences of ribosome encoding genes from the total DNA extracts. For bacteria, the V6–V8 region of the 16S rRNA gene of bacterial groups was amplified using the primers 968F-GC and 1401R (Yong et al. 2011). Amplifications were carried out in a final volume of 50 μL , containing 5 μL of 10 \times PCR buffer (Mg^{2+} plus), 4 μL dNTP mixture (2.5 mM each), 0.4 μM of each primer, 1.25 units of *rTaq* polymerase (Takara, Dalian, China), and 1 μL of template DNA. The reactions were carried out as follows: initial denaturation at 94 °C for 4 min, 30 cycles of denaturation at 94 °C for 40 s, annealing at 55 °C for 40 s, and extension at 72 °C for 1 min; and a final extension at 72 °C for 10 min. For fungi, the approximately 350-bp fragment of the 18S rRNA gene was amplified using the primers NS1 and GCFung (May et al. 2001). The amplification program was 94 °C for 4 min; 30 cycles of denaturation at 94 °C for 40 s, annealing at 51 °C for 50 s, and extension at 72 °C for 50 s; and a final extension step at 72 °C for 10 min. The sizes and quantities of the PCR products were determined using 2 % agarose gel electrophoresis.

Analysis of PCR products by DGGE

The DGGE was performed with a DGGE-2401 system (CBS Scientific Company) as described previously (Muyzer and Smalla 1998). Electrophoresis was performed in a 0.75-mm polyacrylamide gel (8 % (*w/v*) acrylamide/bisacrylamide 37.5:1) in 1 \times TAE buffer (40 mM Tris base, 20 mM glacial acetic acid, 1 mM EDTA 0.5 M, pH 8.0) at 60 °C. In the case of bacteria, gel electrophoresis was carried out for 17 h at 80 V on a 8 % acrylamide/bisacrylamide (37.5:1) gel with a 45–55 % denaturant gradient (100 % denaturant corresponding to 7 M urea and 40 % (*w/v*) formamide), increasing in the electrophoretic run direction. In the case of fungi, gel electrophoresis was carried out for 17 h at 80 V on 8 % acrylamide/bisacrylamide (37.5:1) gel with a 25–35 % denaturant gradient. After electrophoresis, DGGE gels were stained with SYBR Green I. Dominant DGGE bands were excised using sterile blades and washed with sterile distilled water thrice in microtubes, and the DNA was extracted in 50 μL of TE buffer overnight at 4 °C. One microliter of eluted DNA was reamplified as described above. The amplified fragments were sequenced by Beijing Huada gene company (Beijing, China).

Table 1 The primers used for PCR in this study

Primer	Target	Sequence (5'–3')	Reference
27F	Bacteria	AGAGTTTGATCCTGGCTCA	Nakai et al. 2012
1401R	Bacteria	GCGTGTGTACAAGACCC	Yong et al. 2011
968F-GC	Bacteria	<u>CGCCCGCCGCGCGCGGGC</u> <u>GGGGCGGGGCACGG GGGG</u> AACGCGAAGAACCTTAC	Yong et al. 2011
NS1	Fungi	GTAGTCATATGCTTGTCTC	May et al. 2001
GCFung	Fungi	<u>CGCCCGCCGCGCCCCGCCCC</u> <u>GGCCCGCCGCCCCCGCCCC</u> ATTCCCGTTACCCGTTG	May et al. 2001

The GC clamp is underlined

Sequencing and phylogenetic analysis

Obtained sequences were compared to the GenBank database with the BLAST program. The phylogenetic tree was constructed using the neighbor-joining method by the software MEGA 5.0 with 1,000 bootstraps. Band intensities were calculated using the software BIO-1D, and the relative band intensities were calculated by dividing the peak area of a band by the sum of the peak areas of all bands in a lane.

Nucleotide sequence accession numbers

The sequences reported in this study were deposited in GenBank. The sequences obtained from DGGE are under accession numbers KF411361 to KF411383, and those of the isolates are under accession numbers KF411346 to KF411360.

Results

Digestive enzyme assay

We investigated the changes of four digestive enzymes during MMF fermentation process (Fig. 1a). The four digestive enzymes included amylase, cellulase, lipase, and protease. With the exception of protease, the activities of the three other digestive enzymes showed a similar trend. The filter paper activity (FPA) and the activities of amylase, carboxymethyl cellulase (CMCase), and lipase significantly increased during the first 4 days and reached a peak at day 4 (up to 2.2, 304.7, 3.6, and 117.2 U/g, respectively), indicating that some microorganisms rapidly increased in the early fermentation stage and produced a large number of enzymes. Afterwards, the amylase activity slightly decreased (249 U/g) until the end of the fermentation process. Unlike amylase, FPA, CMCase activity, and lipase activity significantly decreased from day 4 to day 5, then slightly decreased (up to 0.9, 1.8, and 43.2 U/g, respectively) until the end of the fermentation. We supposed that the environment was not suitable for the growth of some microorganisms or antagonism between various microorganisms

inhibited the metabolism of some microorganisms, thus resulting in a decline of enzyme activities. However, the protease activity exhibited a different trend compared with the abovementioned enzymes. Its activity slowly increased in the first 4 days of fermentation and, subsequently, exponentially

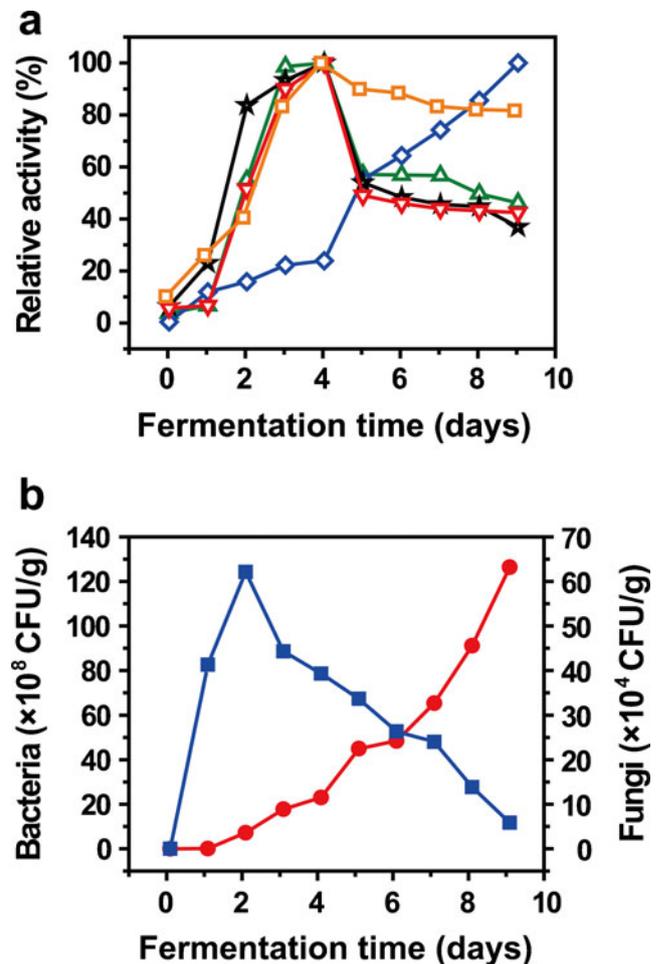


Fig. 1 Changes of the relative activities of digestive enzymes (a) and bacteria and fungi cell counts (b) during MMF fermentation process. Orange open square, amylase; black open pentagon, lipase; green open upper triangular, CMCase; red open lower triangular, FPA; blue open diamond, protease; blue filled square, total bacteria; red filled circle, total fungi

increased up to 777.9 U/g until the end of fermentation. The protease activity and total fungi counts showed a similar trend (Fig. 1b), indicating that the protease that we measured was mainly produced by fungi during the fermentation process.

Cultivable microbial counting during MMF fermentation process

The statistical analysis results of cultivable microbial dynamic changes were illustrated in Fig. 1b. The quantity of bacteria and fungi showed different trends during the fermentation process. Bacteria numbers significantly increased for the first 2 days, reached a peak (1.2×10^{10} CFU/g) at day 2, and then decreased. Fungi counts increased slowly for the first 4 days, then rapidly increased and obtained the maximal counts (6.3×10^5 CFU/g) at the end of the fermentation.

DGGE analysis of bacteria

Bacterial DGGE patterns and the dynamic changes of the relative intensities of DGGE bands are shown in Fig. 2. A total of 14 bands were detected (Fig. 2a). A clear reduction of

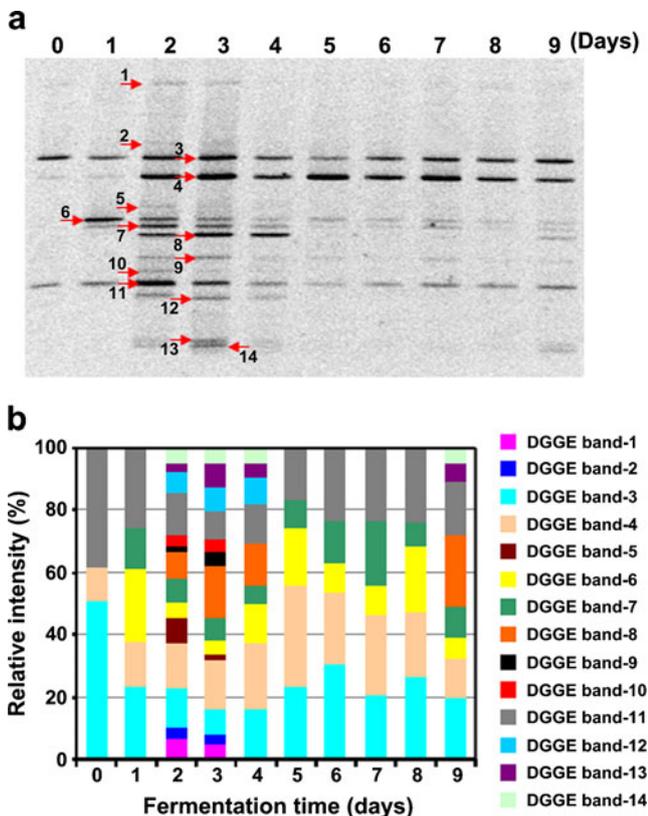


Fig. 2 PCR-DGGE profile (a) of the bacterial community and relative intensity (b) based on the band intensities of bacterial DGGE community profile from MMF samples during fermentation (bands that were excised for sequence analysis are *numbered*)

bacterial diversity was shown from day 4 and remained stable in the late period of the fermentation. The bands 3, 4, and 11 could be detected throughout the fermentation process and were dominant bands during all MMF fermentation process with highest relative intensity (Fig. 2b). The results indicated that these three bacteria were involved in all MMF fermentation process and were the dominant bacteria during the fermentation process. Some new bands appeared at day 2 and day 3, then disappeared at day 5, and the results showed a similar trend with total bacteria counts, which indicated that the intensity of DGGE bands can reflect the changes of microbial community but were also restricted by total viable counts.

DGGE analysis of fungi

Fungal DGGE profile and relative intensity dynamics of DGGE bands are shown in Fig. 3. Figure 3a showed that the fungi were simple. A total of nine bands were detected. The bands (1 and 6) had the highest relative intensity (Fig. 3b), which can indicate that they were the dominant fungi during fermentation.

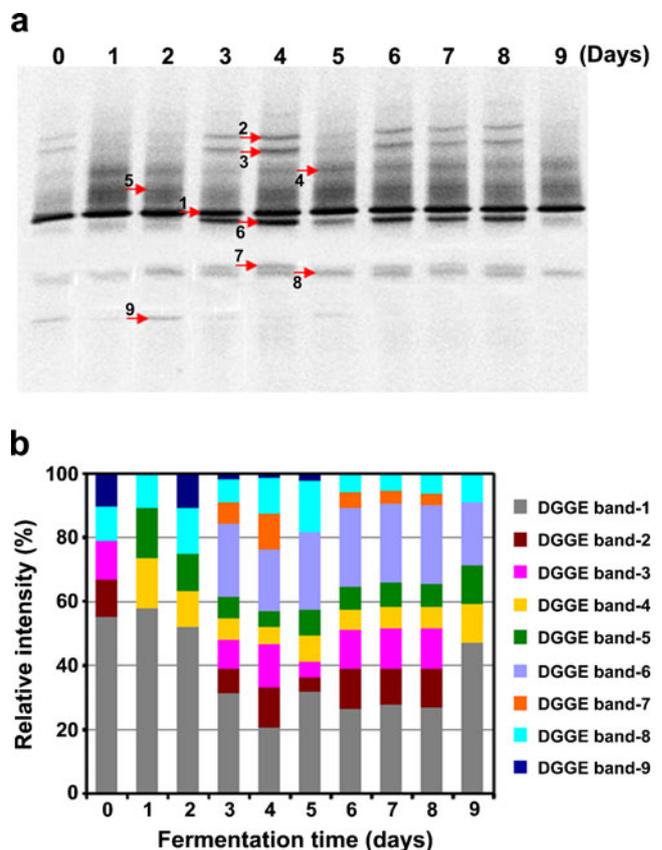


Fig. 3 PCR-DGGE profile (a) of the fungal community and relative intensity (b) based on the band intensities of fungal DGGE community profile from MMF samples during fermentation (bands that were excised for sequence analysis are *numbered*)

Isolation and molecular identification of pure cultures

One hundred seventy pure cultures were isolated from the ten samples collected during MMF fermentation process. The bacteria included 91 strains and the fungi included 79 strains. Sequences obtained from isolates were compared to the GenBank database with the BLAST program. The 170 isolates were identified to 15 species (Table 2). Eight of them were bacteria, five of them were molds, and two of them were yeasts. The results indicated that the bacteria that existed during MMF fermentation process were closely related to the genera *Enterobacter*, *Klebsiella*, *Pseudomonas*, and *Acinetobacter*, spanning the families *Enterobacteriaceae*, *Moraxellaceae*, and *Pseudomonadaceae*. The molds were affiliated with the genera *Amylomyces* and *Mucor*, belonging to family *Mucoraceae*. The yeasts were close to the genera *Saccharomyces* and *Rhodotorula*, spanning the families *Saccharomycetaceae* and *Cryptococcaceae*. These results were partly coincidental with those obtained from DGGE analysis.

Phylogenetic analysis

Bacterial phylogenetic analysis was done based on the sequences of 14 DGGE bands and eight bacterial isolates (Fig. 4). The phylogenetic tree showed that bacteria were grouped into five families. Bands 1–6 and 9, and pure isolates B (1–4), B-8 were classified as *Enterobacteriaceae*. Pure isolate B-7 and band 7

were identified as *Moraxellaceae* and *Lactobacillaceae*, respectively. Bands 8, 10, and 11, together with pure isolates B-5 and B-6 were relatives of *Pseudomonadaceae*. Bands 12, 13, and 14 were detected as *Bacillaceae*. The *Enterobacteriaceae* accounted for 50 % of the total bacteria counts, and *Pseudomonadaceae* and *Bacillaceae* accounted for 21 % of the total bacteria counts, respectively. The results indicated that the family *Enterobacteriaceae* was the predominant bacteria during MMF fermentation process.

Fungal phylogenetic analysis including the sequences of fungal DGGE and fungal isolates was performed to reveal the species and distribution of various fungi (Fig. 5). The fungi were grouped into three families. Bands 2, 3, and 5–7, and pure isolates F (1–5) were classified as *Mucoraceae*. Pure isolate F-7 and band 9 were relatives of *Cryptococcaceae*. Bands 1, 4, and 8, together with pure isolate F-6, were detected as *Saccharomycetaceae*. The results indicated that *Mucoraceae* was the dominant group of fungi and played an important role during MMF fermentation.

Discussion

MMF fermentation is a spontaneous process and involves a succession of changes of microbial community. The study on microbial diversity and dynamic changes during MMF fermentation cannot only be conducive to elucidate the fermentation mechanism and to optimize the fermentation process but also has

Table 2 Alignment of the cultivable bacteria and fungi to its most similar GenBank sequences

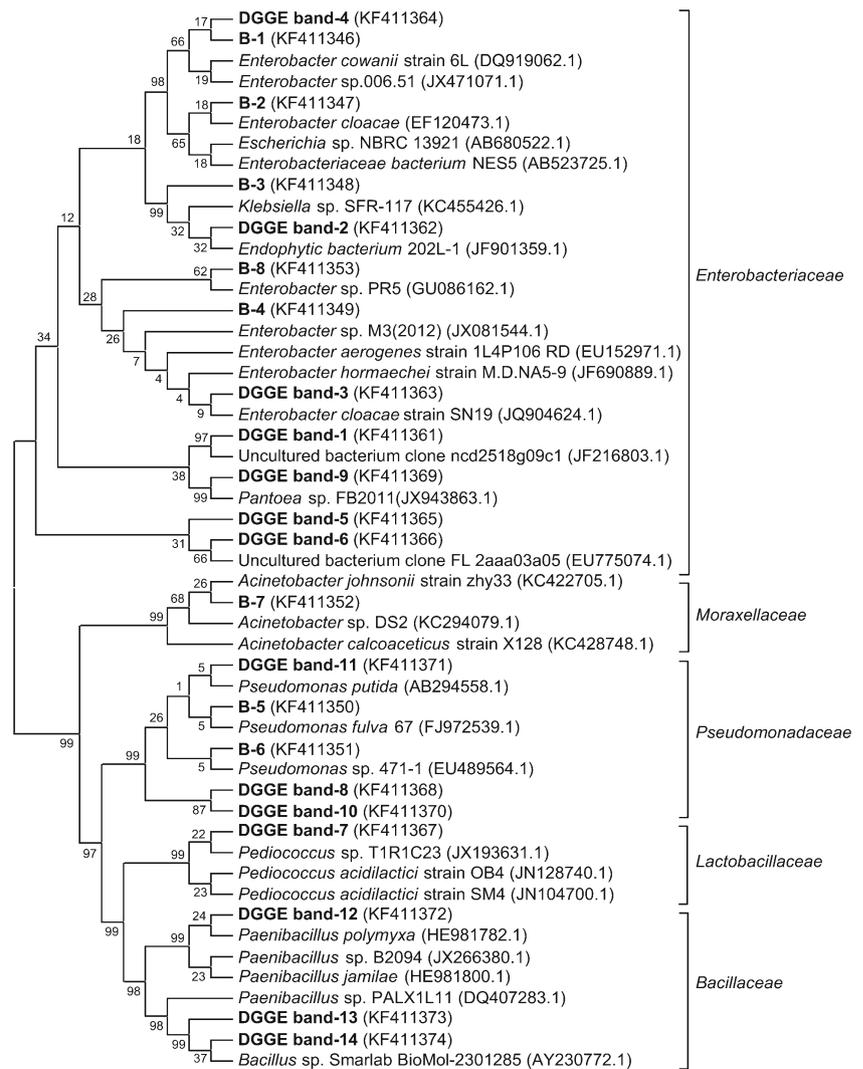
Sequenced strains	Length of 16S or 18S rDNA fragments (bp)	GenBank accession number	Aligned site in GenBank sequence	Similarity rate (%)	Source of the most similar GenBank sequence
B-1	1,397 ^a	DQ919062.1	1-1359	99	<i>Enterobacter cowanii</i> strain 6L
B-2	1,354 ^a	EF120473.1	1-1306	99	<i>Enterobacter cloacae</i>
B-3	1,374 ^a	KC455426.1	9-1321	99	<i>Klebsiella</i> sp. SFR-117
B-4	1,358 ^a	JX081544.1	1-1317	99	<i>Enterobacter</i> sp. M3(2012)
B-5	1,353 ^a	FJ972539.1	4-1313	99	<i>Pseudomonas fulva</i> 67
B-6	1,349 ^a	EU489564.1	1-1304	99	<i>Pseudomonas</i> sp. 471-1
B-7	1,360 ^a	KC422705.1	2-1308	99	<i>Acinetobacter johnsonii</i> strain zhy33
B-8	1,348 ^a	GU086162.1	37-1343	99	<i>Enterobacter</i> sp. PR5
F-1	355 ^b	EU736294.1	1-337	99	<i>Amylomyces rouxii</i> strain FSU761
F-2	355 ^b	HM641689.1	25-377	99	<i>Mucor circinelloides</i> f. <i>circinelloides</i> strain WA0000017591
F-3	355 ^b	AJ271061.1	611-964	99	<i>Mucor racemosus</i>
F-4	355 ^b	JN939019.1	1-322	99	<i>M. circinelloides</i> strain DAOM 225703
F-5	355 ^b	HQ845293.1	1-307	100	<i>M. circinelloides</i> strain NRRL 54372
F-6	349 ^b	GU266277.1	17-365	100	<i>Saccharomyces arboricola</i> strain AS 2.3317
F-7	344 ^b	HQ420261.1	1-347	99	<i>Rhodotorula glutinis</i>

B-1 to B-8 the strains of the cultivable bacteria, F-1 to F-7 the strains of the cultivable fungi

^a Length of 16S rDNA fragments

^b Length of 18S rDNA fragments

Fig. 4 Phylogenetic tree based on sequences of the bacteria from MMF. *B* isolates of bacteria. The numbers given in the branches are the bootstrap values after 1,000 repetitions



significant potential for controlling the fermentation of MMF. In this study, the culture-dependent method and culture-independent method (PCR-DGGE) were used to analyze bacterial and fungal communities during MMF fermentation process. PCR-DGGE can provide a broad overview within a short time, making it an effective supplement to culturing methods. Although this technique has advantages such as reliability, high throughput, and reproducibility (Muyzer et al. 1993), it still has some drawbacks such as its limited data on the microbes with higher concentration (Ruiz et al. 2010) and not providing accurate quantitative data like plate counting. To obtain more overall profiles of microbial community during MMF fermentation, we also used a culture-dependent method. In this study, we selected three media (LB medium, Czapek's medium, and Malt extract medium) to isolate microorganisms of MMF referring to the results of PCR-DGGE. This can reduce the blindness of directly using traditional microbiological methods and improve targeting.

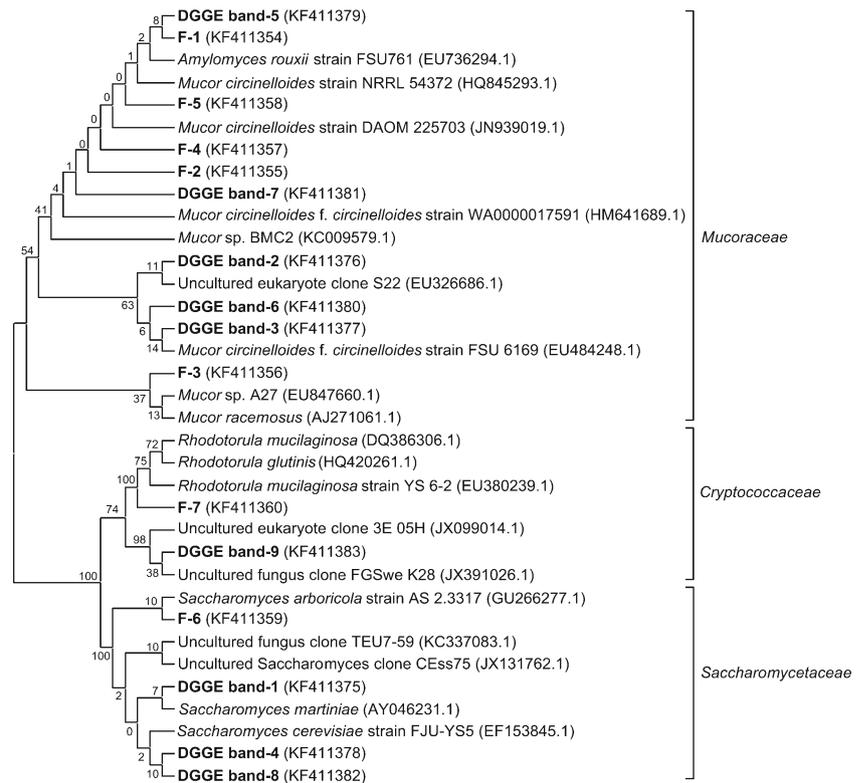
DGGE results showed that the bacteria were more complex than fungi. Fourteen bands were obtained and identified from

bacterial DGGE profile, and a total of nine bands were detected for fungi. Phylogenetic analysis indicated that *Enterobacter* sp., *Pediococcus* sp., and *Pseudomonas* sp. were the main representative bacteria during MMF fermentation process; the dominant fungi were *Mucor* sp. and *Saccharomyces* sp..

One hundred seventy isolates were isolated from the ten samples collected during MMF fermentation process. The 170 isolates were identified to 15 species and closely related to the genera *Enterobacter*, *Klebsiella*, *Acinetobacter*, *Pseudomonas*, *Mucor*, *Amylomyces*, *Saccharomyces*, and *Rhodotorula*. The results were partially consistent with those reported by Gao and Jia (2002), who isolated *Bacillus* sp., *Mucor* sp., and *Aspergillus* sp. from MMF.

The culturing results revealed partially coincident results with PCR-DGGE results. The endophytic bacterium, Uncultured bacterium clone, *Pantoea* sp., *Pediococcus* sp., *Paenibacillus* sp., *Bacillus* sp., Uncultured eukaryote clone, and Uncultured fungus clone were only detected by PCR-DGGE analysis; this could be due to the inability of selective media to provide specific growth requirements. The *Klebsiella* sp.,

Fig. 5 Phylogenetic tree based on sequences of the fungi from MMF. *F* isolates of fungi. The numbers given in the branches are the bootstrap values after 1,000 repetitions



Acinetobacter sp., and *Rhodotorula* sp. were only identified by culture-dependent method analysis, owing to the low concentrations of these microorganisms. Generally, only the microbe populations that make up 1 % or more of the total community might be detected by DGGE. The *Enterobacter* sp., *Pseudomonas* sp., *Amylomyces* sp., *Mucor* sp., and *Saccharomyces* sp. were detected by both methods.

Among the bacteria found in MMF, *Enterobacter* sp., *Pseudomonas* sp., and *Paenibacillus* sp. are amylase-producing microorganisms (Kumar and Das 2000; Valente et al. 2006; Rajesh et al. 2013). *Enterobacter* sp. and *Pseudomonas* sp. can also produce lipase (Sinkūniene et al. 2008; Kordel et al. 1991). *Pediococcus* sp. is commonly found in fermented dairy products, fermented vegetables, and meat (Barros et al. 2001) and has emerged as a potential probiotic bacterium. Keuth and Bisping (1994) showed that the bacterium *Klebsiella* can produce Vitamin B₁₂. As we all know, *Mucor* sp. and *Saccharomyces* sp. are important species of the fermentation industry and food processing industry. The *Amylomyces* sp. and *Mucor* sp. can produce numerous extracellular enzymes, such as protease, amylase, cellulase, and lipase (Marcial et al. 2011; Mohapatra et al. 1998; Coca and Dustet 2006; Alves et al. 2005; Mohamed et al. 2011). The *Saccharomyces* sp. can produce protease and amylase (Haq et al. 2002; Moehle et al. 1987). The MMF fermentation process is a biotransformation process, microorganisms play an important role, and they have both individual function and interaction in the process. Microbial growth

and metabolism also can more drastically improve herb efficacy, reduce toxicity, and expand indications than physical or chemical methods and can also improve the extraction, absorption, and utilization rate of the active ingredient (Zhang et al. 2012b). Extracellular enzymes such as cellulase can decompose cell wall of medicinal plants, so that the active ingredient of medicinal plants is fully released, and ultimately enhance herb efficacy (Yang et al. 2005).

On the MMF fermentation mechanism, the results of bacteria and fungi counts of MMF fermentation showed that bacteria was the dominant microbe from day 0 to day 4, and fungi was the dominant microorganism from day 5 to day 9 (Fig. 1b). The DGGE profiles showed the bacteria were active during the first 4 days, and the fungi were active during the last 5 days. These results indicated that MMF fermentation process consisted of two stages, the pre-fermentation stage (0–4 days) dominated by bacteria and the post-fermentation stage (5–9 days) dominated by fungi.

In conclusion, this study provided a more comprehensive knowledge on microbes and digestive enzymes during MMF fermentation. The results showed that *Enterobacter* sp., *Pediococcus* sp., *Pseudomonas* sp., *Saccharomyces* sp., and *Mucor* sp. were dominant microbes during the fermentation process. MMF fermentation included two stages: a pre-fermentation stage dominated by bacteria and a post-fermentation stage dominated by fungi. PCR-DGGE results can be used as a reference for pure culture. However, in order to determine the exact functional microorganisms during

MMF fermentation, more studies in detail should be made further in the future.

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