Food Sci. Biotechnol. 25(1): 173-178 (2016)
DOI 10.1007/s10068-016-0026-6

Food Science
and
Biotechnology

Isolation and Characterization of Lactic Acid Bacteria from Fresh Chinese Traditional Rice Wines Using Denaturing Gradient Gel Electrophoresis

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Received June 1, 2015
Revised October 23, 2015
Accepted November 10, 2015
Published online February 29, 2016

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pISSN 1226-7708
eISSN 2092-6456

Abstract Lactic acid bacteria (LAB) are a prevalent bacterial group in rice wine maturation that contributes to flavor, texture, and nutritive value. To better understand LAB diversity in rice wines, 6 rice wine varieties from different regions in China were investigated using denaturing gradient gel electrophoresis (DGGE). *Lactobacillus plantarum*, *L. namurensis*, and *Pediococcus acidilactici* were identified using DGGE. Forty nine isolates were screened using a culture-dependent method. Prominent taxa were identified as *Enterococcus* sp., *Lactobacillus delbrueckii*, *L. rhamnosus*, *L. plantarum,* and *Pediococcus acidilactici*. Isolates were grouped and used for fermentation of rice wines. Greater numbers of species involved in fermentation lead to better sensory attributes of wine. DGGE analysis combined with a culture-dependent method can be a tool for investigation of the bacterial compositions of fermented rice wines.

Keywords: lactic acid bacteria, traditional rice wine, isolation, characterization

Introduction

Chinese traditional rice wine is a popular fermented alcoholic beverage in China. Rice wine has a long history of 9,000 years, with an origin approximately the same time that barley beer and grape wine were being made in the Middle East (1). Traditional rice wine is known as makgeolli in Korea, and zhuojiu or laozao in China. However, traditional rices wine from Korea and China differ in starters, raw materials, alcohol contents, color, and flavor. Chinese rice wine is fermented from high-quality glutinous rice, wheat, and Chinese koji, which is a kind of mold. The structure and bacterial community varies and contributes to the flavor of Chinese rice wine. Fresh Chinese traditional rice wine is different from Chinese Yellow wine and Shaoxing rice wine. The whole fermentation process takes a few days or weeks and does not include a distillation process. Fresh Chinese traditional rice wine, some of which even contains a rice residue, has a delicate acidic fragrance and a sweet-tart taste, with high protein, low fat, and low alcohol contents (2-5). Awareness of the health benefits of rice wine has been raised, resulting in an increased appetite for wine for treatment of chronic atrophic gastritis in speeding up blood circulation, improving high density lipoprotein (HDL) levels in patients with hyperlipidemia and atherosclerosis, and helping to recover from fatigue (6,7).

Wine made from rice undergoes microbial interactions throughout the entire fermentation process. During fermentation of rice wine, mold, yeast, and lactic acid bacteria (LAB) strengthen nutritional values, enrich food specialties, degrade anti-nutritional factors, and increase amounts of essential substances, such as vitamins and anti-oxidants (8-13).

A few studies have been conducted to investigate microbial diversity in alcoholic fermented beverages. *Pediococcus pentosaceus*, *P. acidilactici*, *Bacillus* sp., *Lactobacillus plantarum,* and *L. brevis* have been identified in Wuyi Hong Qu glutinous rice wine (14). *Janthinobacterium lividum*, *L. plantarum*, *Weissella soli*, *Leuconostoc mesenteroides*, *L. brevis*, *P. pentosaceus*, *L. lactis* subsp. *lactis*, and *P. acidilactici* have been isolated from Fuzhou Hong Qu glutinous rice wine. *P. pentosaceus*, *L. plantarum*, *L. brevis*, *Weissella confusa*, and *Weissella paramesenteroides* have been identified in Vietnamese starters, known as banh men (15). However, bacterial dynamics in Chinese traditional rice wines have rarely been reported (14).

Molecular culture-independent approaches based on 16S rDNA gene diversity have been proven to be powerful tools for development of a more complete inventory of the microbial diversity of wine. Denaturing gradient gel electrophoresis (DGGE) is one of widely applied and economical molecular methods for study of microbial diversity (16). PCR-DGGE can be used for analysis of complicated

environmental bacterial diversity, such as porcine gastrointestinal microbiota, rumen, and soil bacterial communities (17-19), and also bacterial diversity in fermented foods (20-22).

In this study, DGGE was used with specific primers for analysis of LAB in Chinese rice wines. Combination of DGGE analysis with traditional isolation methods allowed successful monitoring of LAB diversity in Chinese rice wines.

Materials and Methods

Wine Six fresh Chinese traditional rice wine varieties were obtained from different Chinese provinces. Five varieties were home-made by local farmers. The rice wines were produced from glutinous rice, and Chinese koji was used as starter. Varieties NA1, NA2, and NA3 were from Ningan City, Heilongjiang province and the MDJ1 and MDJ2 varieties originated from Mudanjiang City, Heilongjiang province. Another variety was a commercial wine (FS1) from Fushun City, Liaoning province. All wines were obtained in liquid form.

DNA extraction DNA extraction was performed following the method of Iacumin *et al*. (23) with modification. Briefly, 0.5 mL of rice wine was placed in a 2 mL sterilized centrifuge tube with an extraction solution (100 mM Tris HCl at pH 8.0, 100 mM EDTA, 100 mM sodium phosphate buffer solution at pH 8.0, 1.5 M NaCl, 1% CTAB, and 1% PVPP) and mixed. Cells were disrupted by placing the mixture into liquid-nitrogen for 3-5 min. DNA was extracted at 60°C until the solution was thawed in 30 min. The freezing-thawing step was repeated 3x. Then, 20 μL of 20 mg/mL protease K (Sigma-Aldrich, St. Louis, MO, USA) was added, followed by 50 mg/mL, lysozyme (Sigma-Aldrich), and 100 μL of 10% SDS (Sigma-Aldrich). The mixture was swirled by vortexing for 5 min and allowed to stand at 60°C for 2 h, then centrifuged at 6,000×*g* for 10 min using a refrigerated centrifuge (Sigma 3-30k; Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The supernatant was treated with 2 mL 2% Triton X-100 and the same amount of phenol:chloroform:isoamyl alcohol (25:24:1) was added. After swirling by vortexing for 2 min, 200 μL of TE (10 mM Tris, 1 mM EDTA, pH 7.6) was added and centrifuged (Sigma Laborzentrifugen GmbH) at 16,000×*g* and 4°C for 10 min. The supernatant was collected and combined with a 2.5x volume of absolute ethanol. DNA was precipitated using centrifugation (Sigma Laborzentrifugen GmbH) at 16,000×*g* and 4°C for 10 min. Pellets were washed with 70% ethanol and dried at 25°C. 50 μL of TE containing 2 U of RNase was added to tubes for 30 min for digestion of RNA at 37°C. DNA samples were checked for purity using PCR in a Thermal Cycler (2720; Applied Biosystems, Foster City, CA, USA).

PCR amplification and primers The variable region (V3) of the 16S rDNA bacterial gene was amplified using touchdown PCR (24). This procedure was performed using primers 338f-gc, 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGAGGCAGCAG-3' and 518r, 5'-ATTACCGCGGCTGCTGG-3' (25). The PCR procedure was performed in a 2720 Thermal Cycler (Applied Biosystems) and the 50 μL mixture contained 1 μL of DNA template, 0.2 μmol of each primer, 0.25 mmol of dNTPs, 5 μL of 10× PCR buffer, 2.5 mmol of $MgCl_2$, and 2.5 U of Taq DNA polymerase. Touchdown PCR included initial denaturing at 94°C for 5 min, followed by 20 cycles at 94°C for 1 min, annealing at 65-55°C for 50 s, and extension at 72°C for 1 min. The initial temperature was decreased by 1°C per second until the last cycle, with final extension at 72°C for 10 min. Amplification products were detected using 2% agarose gel.

DGGE analysis DGGE analysis was performed using a Decode Universal Mutation Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA). 20 μL PCR products were loaded on 8% (w/v) polyacrylamide gels with a denaturing gradient of 30 to 60% urea and formamide, and a polyacrylamide to bis-acrylamide ratio of 37.5:1. Electrophoresis was performed at a constant 100 V for 5 to 7 h at 60°C with 1×TAE was used as a buffer. Gels were stained using Gelred following manufacturer instructions and photographed using a gel documentation system (Universal Hood, Bio-Rad Laboratories Inc.).

DGGE fragments were removed using a sterile scalpel by autoclaving and placed in a 2 mL sterile tube. Then 20 μL of TE was added and the tube was stored at 4°C in refrigerator overnight for elution of DNA from DGGE fragments. 2 μL of eluted DNA from each DGGE band was re-amplified under the same PCR conditions as used for amplification of the LAB variable region (V3) described above. Amplified PCR products were sequenced at the Sangon Biotech Co., Ltd. in Shanghai, China.

LAB isolation Ten serial decimal dilutions of each wine sample were obtained using a sterile saline buffer (0.85% NaCl). LAB were isolated using four reformative selective media (MRS, M17, LBS, and TYC) under aerobic and anaerobic conditions to isolate as many LAB as possible. The isolated LAB colonies were cultivated in biochemical incubator at 37°C for 24 h, then placed on MRS and M17 media and stored in refrigerator at 4°C for further study.

Identification LAB were identified based on 16S rDNA sequencing using primer 1,5'-AGAGTTTGATCCTGGCTCAG-3' and primer 2,5'-ACG GTTACCTTGTTACGACTT-3'.

Rice wine fermentation Rice wine fermentation was performed following the method of Kim *et al*. (26) with modification. Raw rice materials were steamed by rice cooker, then 0.4% saccharogenic enzyme (100,000 U/g) (Sigma-Aldrich) was added for hydrolyzation of starch to glucose. *Saccharomyces cerevisiae* (2%, v/w), sterile water (20%, v/w), and LAB isolates (2%, v/w) were added, followed by incubation in biochemical incubator at 30°C for 48 h (Fig. 1). Rice wine fermentation was performed by using 5 LAB groups as shown in Table 1. All fermentations were performed in triplicate.

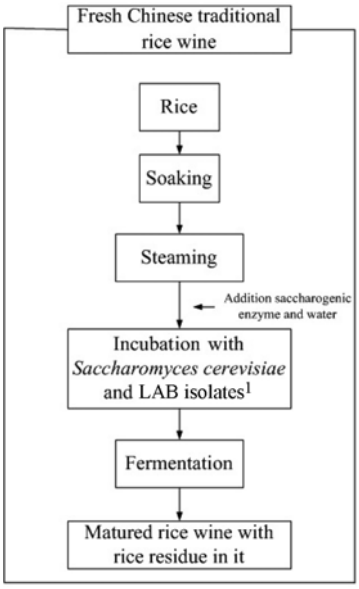

Fig. 1. Processing of fresh Chinese traditional rice wine.

Table 1. LAB isolate combinations for rice wine fermentation

Group	Species[1)]
1	*Lactobacillus plantarum*
2	*L. plantarum*; *L. delbrueckii*
3	*L. plantarum*; *L. delbrueckii*; *Pediococcus acidilactici*
4	*L. plantarum*; *L. delbrueckii*; *Pediococcus acidilactici*; *L. rhamnosus*
5	*L. plantarum*; *L. delbrueckii*; *Pediococcus acidilactici*; *L. rhamnosus*; *Enterococcus faecium*

[1)]*L. plantarum*, *L. delbrueckii*, *Pediococcus acidilactici*, *L. rhamnosus* and *Enterococcus faecium* were isolated using a culture-dependent method. Five groups were identified based on bacterial frequency of occurrence.

Sensory analysis A consumer taste panel was used for comparison of sensory attributes of rice wines fermented by using 5 different LAB groups. Fifty untrained volunteers familiar with rice wine were selected for the sensory panel. Panelists performed evaluations in separate sensory booths using numerically identified, blind-coded cups of different rice wines for overall appearance, color, flavor, and taste on a 4 point Hedonic scale with 4 being extremely good and 0 being extremely poor.

Statistical analysis Data analysis was carried out using SPSS software (ver. 18.0; SPSS, Chicago, IL, USA), Origin Pro 8.0 (Origin Lab, Northampton, MA, USA), Quantity one (442-PC) (Bio-Rad), and MEGA software (ver. 5.10). A one-way analysis of variance (ANOVA) was used for determination of significant differences between means at $p<0.05$. Tukey's test was used to perform multiple comparisons between means. All analyses were performed at least in triplicate ($n=3$). Searches in GenBank (www.ncbi.nlm.nih.gov/genbank/) using BLAST tool were performed to determine the closest known relatives of partial 16S rDNA sequences obtained.

Results and Discussion

LAB diversity determined using a culture-independent method LAB diversity in 6 rice wine varieties were determined by using the DGGE culture-independent method. The variable regions (V3) of 16S rDNA genes were amplified using the F338-gc/R518r primer to obtain 16S rDNA amplicons, which were subjected to DGGE analysis (Fig 2A). Sixteen bands observed in gels were sequenced (Table 2). Bands D and band M were present in gels of all rice wines and exhibited 100% identity with the *Lactobacillus plantarum* sequence. Bands F, I, and K were matched to *L. delbrueckii*. Band J was close to *L. delbrueckii* subsp. *lactis* with an identity of 99%. *L. delbrueckii* was only detected in FS1, MDJ1, and MDJ2 wine samples, and was not found in any of the Ningan wine samples. Band A, which corresponded to *L. namurensis*, was identified in FS1, MDJ1, and NA1 wine samples, and did not detected in MDJ2 and NA2 wine samples. Bands C and G, only detected in FS1, were matched to *L. plantarum* and *L. brevis*, respectively. Band O was close to *Acetobacter*

Table 2. Identification of fragments using 16S rDNA gene sequencing

NO[1)]	Genbank	Species or subspecies	Sequence homology (%)
a	AB626072.1	*L. namurensis* gene for 16S rRNA, partial sequence, strain: NBRC 107158	100%
b	AB601179.1	*L. plantarum* subsp. *plantarum* gene for 16S rRNA, partial sequence, strain: Ni729	97%
c	AB601179.1	*L. plantarum* subsp. *plantarum* gene for 16S rRNA, partial sequence, strain: Ni729	98%
d	JN863682.1	*L. plantarum* strain GB14 16S ribosomal RNA gene, partial sequence	100%
e	GQ267876.1	Uncultured bacterium clone IMAU 79 16S ribosomal RNA gene, partial sequence	95%
f, i, k	JQ801728.1	*L. delbrueckii* strain ZN7a-9 16S ribosomal RNA gene, partial sequence	99%
g	JQ979139.1	*L. brevis* strain YML010 16S ribosomal RNA gene, partial sequence	100%
h	GU429410.1	*L. rhamnosus* clone WWC_C4MKM145 16S ribosomal RNA gene, partial sequence	95%
j	JQ413226.1	*L. delbrueckii* subsp. *lactis* strain LBL 217 16S ribosomal RNA gene, partial sequence	99%
l	AB628985.1	*Pseudomonas* sp. StFRB520 gene for 16S rRNA, partial sequence	99%
m	GQ240304.1	*Pediococcus acidilactici* strain LAB 5 16S ribosomal RNA gene, partial sequence	100%
n	EF669495.1	Uncultured bacterium isolate DGGE band C-8 16S ribosomal RNA gene, partial sequence	98%
o	HQ711333.1	*Acetobacter pasteurianus* strain E4-3 16S ribosomal RNA gene, partial sequence	100%
p	AY527763.1	Uncultured bacterium clone FB46-4 16S ribosomal RNA gene, partial sequence	98%

[1)]Bands were excised from DGGE gel.

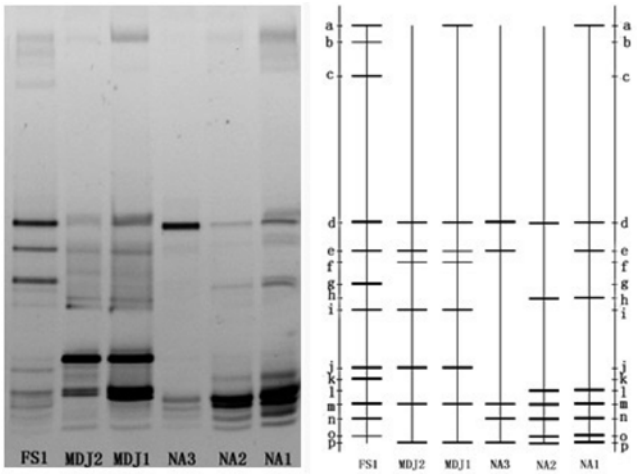

Fig. 2A. DGGE profiles of the 16S rDAN gene of different rice wine samples.

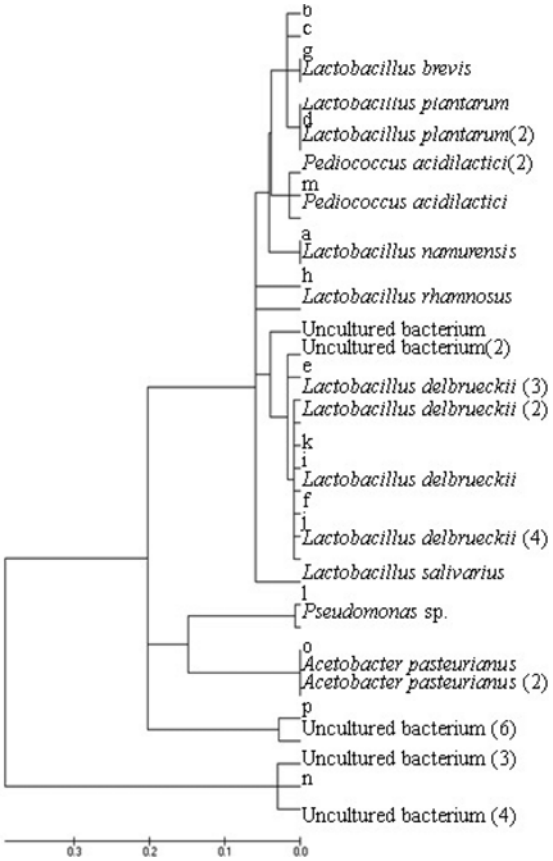

Fig. 2B. Phylogenetic tree of strains detected by DGGE profiles

pasteurianus, which was not present in Mudanjiang rice wine, which might result from the too low concentration. Bands B and L in NA1 and NA2 wine samples were similar to *Pseudomonas* sp., which was often found in fermented foods and beverages (27), but was unexpected in this study because it often causeed food spoilage. Bands N and P were identified as uncultured bacteria.

A phylogenetic tree was built using the neighbor-joining method according to the identified clones and showed that uncultured strains might have been derived from LAB species (Fig 2B). Bands B, E, and H were not species-identified due to low identity levels of 95% to 97%. However, Band H, which corresponded to *L. rhamnosus*, was identified with a higher degree of reliability because this strain was also isolated using the culture-dependent method.

Table 3. Identification of LAB strains using a culture-dependent method

Strains	Genbank	Species	Sequence homology (%)	Isolates count
R-1	HQ853454.1	*L. plantarum*	99%	19
R-2	GU429462.1	*L. rhamnosus*	99%	5
R-3	KC545944.1	*L. delbrueckii*	99%	15
R-4	KF198084.1	*P. acidilactici*	99%	7
R-5	JQ726533.1	Enterococcus faecium	99%	3

PCR-DGGE fingerprinting can be used for identification of microbial diversity in rice wines (24). LAB diversity differed among different rice wines in this study. DGGE analysis showed that LAB microbial diversity in the 3 Ningan wines was similar, as was diversity in rice wines from Mudanjiang. The greatest bacterial diversity was found in sample FS1. LAB species in rice wines appeared to be related to wine origin. Bacteria identified as *Enterococcus* sp. using the culture-dependent method were not identified using DGGE analysis. *Enterococcus faecium* was reportedly identified using a culture-independent method with primers GC341F and 800R (20) and a GYP-agar culture (28). *Enterococcus* sp. strains were not identified using the culture-independent method in this study, probably because primers F338GC and R518 were not suitable for sequencing species of *Enterococcus*, or species of *Enterococcus* was not dominant enough to be detected using DGGE, even though they can grow in an M17 agar culture. Previous studies have demonstrated that PCR-DGGE cannot be used for detection of microbes at concentrations of less than 1% of the total cell population (29,30).

LAB diversity determined based on a culture-dependent method

Culture-dependent method was also used to identify LAB in rice wines and 49 isolates were obtained and identified based on 16S rDNA sequencing (Table 3) and classified into 5 groups (Table 1). Identifed taxa included *L. plantarum*, *L. rhamnosus*, *L. delbrueckii*, *Pediococcus acidilactici*, *Enterococcus* sp., *L. plantarum*, *L. rhamnosus*, *L. delbrueckii* and *P. acidilactici*, which were all also identified using DGGE analysis of the 16S rDNA gene. However, other LAB species identified using DGGE analysis were not isolated using the culture-dependent method, which suffers limits with regards to detection of microbial diversity in rice wine because replication of the natural environment in the laboratory is difficult, including changes in temperature, humidity, and complex microbial interactions. DGGE analysis indicated that *Acetobacter pasteurianus* was common rice wines (Fig 2A), indicating an important effect on rice wine fermentation. However, *A. pasteurianus* was not identified using the culture-dependent method in this study.

Table 4. Sensory evaluation of wines prepared using different LAB isolate groups

Group	Quality attributes				
	Appearance	Color	Flavor	taste	total
1	2.81±0.36[a1)]	2.75±0.29[a]	2.02±0.50[a]	1.92±0.37[a]	9.50±0.79[a]
2	3.08±0.46[b]	2.81±0.41[ab]	2.21±0.39[b]	2.05±0.42[b]	10.16±0.92[b]
3	2.80±0.38[a]	2.78±0.45[ab]	2.79±0.26[c]	2.48±0.17[c]	10.86±0.62[c]
4	3.09±0.44[b]	2.90±0.30[b]	2.82±0.28[c]	2.53±0.22[c]	11.34±0.69[d]
5	3.05±0.53[b]	3.53±0.17[c]	3.28±0.45[d]	3.08±0.40[d]	12.95±1.01[e]

[1)]Different letters indicate significant differences at $p<0.05$.

Sensory analysis Sensory evaluation of 5 sensory attributes of fermented rice wines using different LAB isolate groups is shown in Table 4. Group 5 wines had the highest sensory analysis scores and were the most acceptable wines. The greater number species used to ferment rice wine, the higher sensory analysis score was obtained, probably related to complex species interactions to produce a rich wine flavor.

More research on bacterial species used in rice wine production is imminent. Flavors of rice wines, which are different from different regions, may be related to interactions between LAB. A previous report has indicated that during the later stage of rice wine fermentation, acidity was increased, growth of yeasts was inhibited, production of ethanol stopped, and flavor was established via an esterification reaction (31). Interaction among bacteria may promote strain growth, strengthen strain activity, and increase amounts of secondary metabolites that create a desirable taste. Unfortunately, such interactions can also operate in the opposite manner. Thus, it is important to determine how such interactions operate.

This study revealed that PCR-DGGE combined with a culture-dependent method can ultimately lead to more controlled rice wine fermentation and account for flavor differences. However, limitations in both culture-dependent and culture-independent methods need to be overcome based on further investigation.

Acknowledgments This work was financially supported by the Fund of Harbin application technology research and development projects (No. 2014RFQXJ069).

Disclosure The authors declare no conflict of interest.

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