Food Sci. Biotechnol. Vol. 16, No. 6, pp. 994 ~ 998 (2007)

Food Science and Biotechnology

Identification of Lactic Acid Bacteria Involved in Traditional Korean Rice Wine Fermentation

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Abstract Changes in microflora, pH, reducing sugar content, lactic acid content, and ethanol content during Korean rice wine fermentation were investigated. Typical quality characteristics of Korean rice wine fermentation including pH, reducing sugar content, lactic acid content, and ethanol content were evaluated. While a fungus was not detected in our Korean rice wine mash, yeast was found to be present at fairly high quantities ($1.44\text{-}4.76 \times 10^8$ CFU/mL) throughout the fermentation period. It is assumed that lactic acid bacteria (LAB) had effects on the variations of fragrance and flavor for traditional Korean rice wine. The main LAB during the Korean rice wine fermentation was determined and identified as a Gram-positive, straight rod-shaped cell. Genotypic identification of the isolated strain by amplification of its 16S rRNA sequence revealed that the isolated strain was most closely related to *Lactobacillus plantarum* (99%) strains without any other comparable *Lactobacillus* strains. Therefore, we designated the major LAB identified from traditional Korean rice wine fermentation as *L. plantarum* RW.

Keywords: 16S rRNA, lactic acid bacteria, *Lactobacillus plantarum*, *nuruk*, traditional Korean rice wine

Introduction

Korean rice wine is an alcoholic beverage brewed by the yeast *Saccharomyces cerevisiae*, which is similar to grape wine but made from rice. The history of Korean rice wine is extensive, dating back to the period of Koguryeo (approximately 3rd century A.D.) when Chinese literature recorded the manners and customs of consuming Korean rice wine. Until the early 20th century, the culture affiliated with Korean rice wine flourished as did the types of Korean rice wine. The popularity of Korean rice wine, however, dramatically decreased due to liquor laws enacted during the period of Japanese occupancy (1-3). Today, the consumption and demand for various traditional Korean rice wines has increased dramatically due to the development of various high quality traditional Korean wines and modernization of the techniques necessary to make Korean rice wine (4).

Different from the grape wine-making process in which yeast converts natural sugars of the grapes into alcohol and carbon dioxide that bubble and then dissipate, the brewing process for Korean rice wine involves the use of a mold known as *nuruk*, which saccharifies the rice starch during fermentation. *Nuruk* is a starter culture made from whole wheat flour/grits (3, 5). The *nuruk* is moistened to make cake-shaped products through pressure and natural fermentation, allowing the growth of many natural types of molds, bacteria, and yeasts. Saccharification is required because rice does not have the abundance of mono- and oligosaccharides that are present in grape products. For complete brewing, a 3-step procedure is involved, which includes the liquefaction of starch by α-amylase, the saccharification of the liquefied starch by glucoamylase, and the production of alcohol with glucose (1). The brewing process of Korean rice wine is demonstrated in Fig. 1. During the brewing process of Korean rice wine, various enzymes derived from mold act on the rice. While amylolytic enzymes convert starch to fermentable sugar, proteolytic enzymes break down proteins into small peptides, and more than 50 other enzymes responsible for the flavor and taste of Korean rice wine make various organic acids and related metabolic compounds (1, 5, 6). Yeast (*S. cerevisiae*) primarily converts sugar to alcohol and carbon dioxide. The enzymes also produce various compounds like esters, alcohols, acids, and other chemical substances that impact variations in fragrance and flavor of rice wine (7).

Nuruk is considered to contain various types of microorganisms such as fungi, yeast, and lactic acid bacteria (LAB) (1). As fungi, *Absidia* sp., *Rhizopus* sp., *Mucor* sp., and *Aspergillus* sp. were found in the traditional Korean *nuruk* (8). Other than fungi, *S. cerevisiae, Bacillus subtilis,* and LAB were also known to be present in traditional Korean *nuruk* (9). Although fungi and yeast were known to be involved in the conversion of starch to fermentable sugar and conversion of this fermentable sugar to alcohol and carbon dioxide, the contributions of other microorganisms in Korean rice wine fermentation have not been studied in detail.

The objective of the present study is to analyze the changes in microflora, pH, reducing sugar content, lactic acid content, and ethanol content during Korean rice wine fermentation. The involvement of LAB during Korean rice wine fermentation was investigated and furthermore, the main LAB during the Korean rice wine fermentation was selected and identified.

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Received June 1, 2007; accepted June 27, 2007

Materials and Methods

Korean rice wine making procedure A flowchart describing the Korean rice wine making procedure is outlined in Fig. 1. The first brew (mother brew) was initiated by soaking 2.0 kg of polished white rice for 12 hr, draining the rice, and then autoclaving for 40 min at 105°C. Autoclaved rice was then cooled at room temperature. *Nuruk* used in this study was purchased from Korea Enzyme Co., Ltd. (Hwasung, Korea). Water (*Saemul*, Sunchang, Korea) was added to ground *nuruk* powder (40 g) to form the starter culture *nuruk*. At the same time, dried yeast (1.4 g, Fermipan Instant Yeast Red, DSM Bakery Ingredient, Dordrecht, Netherlands) was dissolved into water. In a traditional Korean soy jar, the starter culture *nuruk* and the dissolved yeast culture were mixed together and water was added up to 3 L. The first brew was finished by adding the autoclaved rice to the jar. After incubating 2 days at 25°C, twice the volume of all ingredients, except the yeast, were added to the first brew. This second brew was incubated for 5 days at 25°C, leading to the completion of the Korean rice wine making procedure (6, 10).

The enumeration of yeast, fungi, and lactic acid bacteria One mL of the brewing sample taken from the fermentation broth was mixed with 9 mL sterilized water and appropriate serial dilutions were made to count various microorganisms including fungi, yeast, lactic acid bacteria, and other bacteria cells. For yeast counts, Rose Bengal agar (Difico Lab., Detroit, MI, USA) with 100 ppm of chloramphenicol was used. For lactic acid bacteria counts, MRS agar (Difco Lab.) was used (11, 12). All plates were incubated at 30°C for 24 to 48 hr, and the number of microorganisms was counted by colony forming units (CFU).

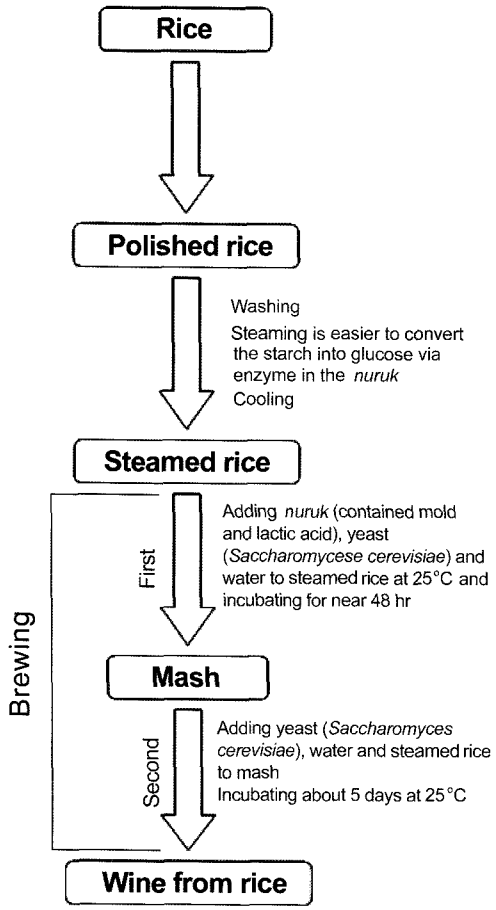

Fig. 1. Flow diagram of the steps used to make Korean rice wine.

Analysis of pH, total acid, alcohol, and reducing sugar One-hundred g of brewing sample taken from the fermentation broth was distilled and its alcohol content was measured by a Gay-Lussac meter (7, 8, 13). After measuring pH with a pH meter (Corning, New York, NY, USA), the sample was titrated with 0.1 N NaOH solution until the pH reached 7.0 in order to determine the titratable acidity of the sample (7, 8, 13). Reducing sugar content of the sample was determined via the 3,5-dinitro salicylic acid (DNS) method (14).

16S rRNA sequencing Polymerase chain reaction (PCR) was performed with genomic DNA purified from *Lactobacillus plantarum* RW as a template, a pair of 16S rRNA-specific universal primers (16S-F, 5'-GAGTTTGA TCCTGGCTCAG-3' and 16S-R, 5'-AGAAAGGAGGT GATCCAGCC-3'), and *Taq* DNA polymerase (Takara Korea, Seoul, Korea). The amplification procedures consisted of initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and an additional extension at 72°C for 5 min in the final cycle (15, 16). The PCR-generated fragment was purified with the QIAEX II gel extraction kit (Qiagen, Hilden, Germany) and the nucleotide sequence of the PCR-generated gene was determined with the BigDye Terminator Cycle Sequencing Kit with ABI377 PRISM (Perkin Elmer Corp., Lombard, IL, USA). The 16S rRNA sequence was confirmed and analyzed using the BLAST search program (NCBI, Bethesda, MD, USA).

Results and Discussion

Biochemical changes during Korean rice wine brewing While the pH of the Korean rice wine brewing broth (mash) decreased from 4.8 to 3.6 by the second day of fermentation, the total acid content of mash increased from 0.1 to 0.27%, and was maintained between 0.3-0.4%. This result is similar to observations by Han *et al.* (17). When mash qualities of *makgeoli (takju)* prepared by many different sources of *nuruk* were investigated, they found that pH dramatically decreased from the starting pH of 4.85-5.50 to 2.92-3.45 on the second day of fermentation. It was also observed that the pH of the mash had slightly increased to 3.30-3.96 by the fourth day of fermentation and this pH was then maintained throughout the brewing period. Surprisingly, the pH of the mash decreased somewhat from 3.6 to 3.4 on the first day of the second brew (4 days following initialization of fermentation) and then increased slightly during fermentation as shown in Fig. 2. This result implies that microorganisms such as LAB in the *nuruk* may produce various organic acids

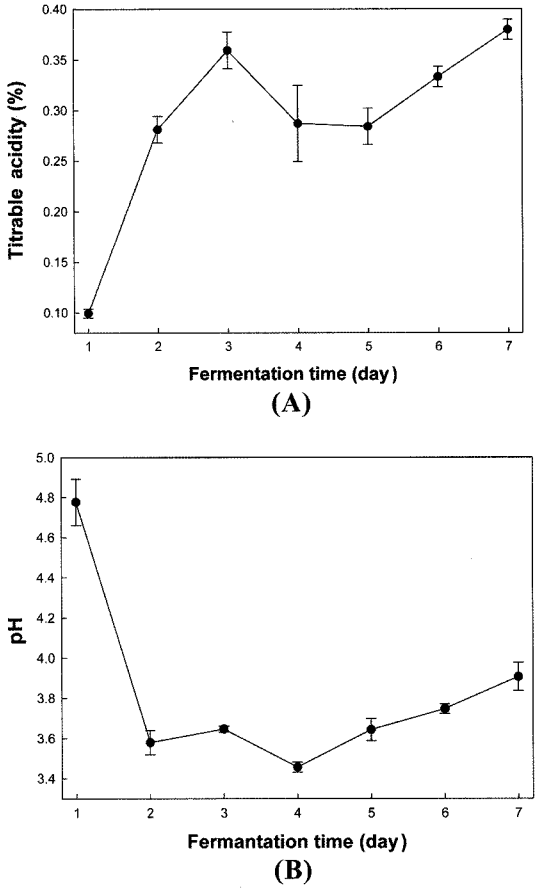

Fig. 2. Change of titratable acidity (A) and pH (B) during fermentation of Korean rice wine. Each point was obtained by triplicate determinations.

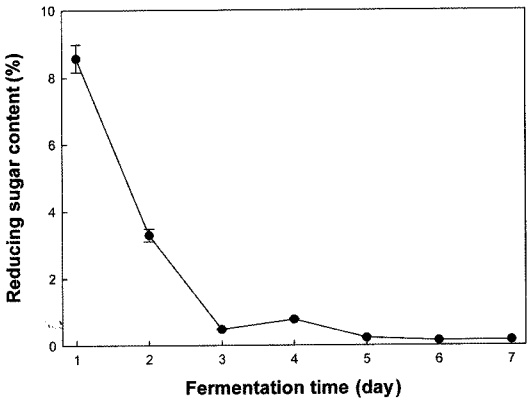

Fig. 3. Changes in reducing sugar during fermentation of Korean rice wine. Each point was obtained by triplicate determinations.

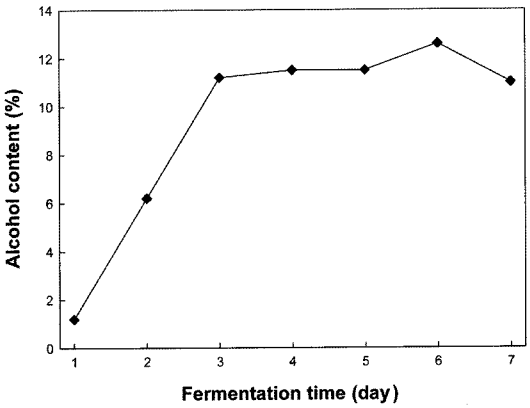

Fig. 4. Changes in alcohol content during fermentation of Korean rice wine. Each point was obtained by triplicate determinations.

during the early stages of fermentation. Reducing sugar and alcohol content in the mash showed patterns typically observed in traditional rice wine fermentation (Fig. 3 and 4). The alcohol content at the initialization of fermentation was 1.0% and increased significantly to 11% by the third day of fermentation. This alcohol content was then maintained for the duration of the fermentation period.

Microbial changes during Korean rice wine brewing

While a fungus was not detected in our Korean rice wine mash, yeast was found to be present at fairly high quantities (1.44 - 4.76 × 10^8 CFU/mL) throughout the fermentation time period (Table 1). Previously, there were different reports regarding the fungal population in Korean rice wine mashes (8, 11). Comparing the presence of microflora in traditional Korean rice wine (*samhaeju*) versus industrial rice wine (*cheongju*), fungus was not detected in industrial rice wine for the duration of the fermentation period, but some fungus grew in the early stages of the *samhaeju* fermentation and then continuously decreased in the late stages of fermentation (11). The major differences between these two processes were brewing temperatures and the addition of pure yeast culture. In the traditional fermentation process, yeast was naturally inoculated by *nuruk* and continuously increased in number at low temperatures over time. In the *cheongju* fermentation process, however, a high number of pure yeast cultures were inoculated, suggesting that the culture may inhibit the growth of many fungal strains included in *nuruk*. Similar results were found in the traditional *Andong-soju* fermentation (9).

Unlike fungus, LAB increased dramatically on the third day of fermentation and then severely decreased to low

Table 1. Changes in LAB and yeast during fermentation of Korean rice wine

Microorganisms	Fermentation time (day)						
	1	2	3	4	5	6	7
Lactic acid bacteria (CFU/mL)	2.08×10^8	3.44×10^7	2.30×10^9	3.36×10^7	5.52×10^7	3.02×10^7	1.84×10^8
Yeast (CFU/mL)	4.68×10^8	3.50×10^8	4.76×10^8	3.04×10^8	1.94×10^8	2.36×10^8	1.44×10^8

levels. The combination of enhanced LAB rates with the growth of yeast in the traditional Korean rice wine brewing contributed to lowering of pH, thus leading to the prevention of pathogenic microorganism growth in the fermentation mash. Investigations on the ratio of LAB to total bacteria in the Korean traditional *nuruks* of various regions in Korea revealed that LAB accounted for 61-96% of the total microorganisms (1). It was noted that both the traditional *nuruks* and the commercial *nuruk* we used in this experiment included fairly high populations of LAB, which are partially responsible for the acidic environment and affect the quality of traditional Korean rice wine.

Major LAB in Korean rice wine brewing It is clear that LAB were responsible for the production of various organic acids such as lactic acid, impacting the variations of fragrance and flavor in traditional Korean rice wine (1, 4, 11). Therefore, it is important to identify the distribution of LAB during the fermentation stage. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using whole-cell proteins of LAB was known to be a reliable and fast method for identifying LAB (18).

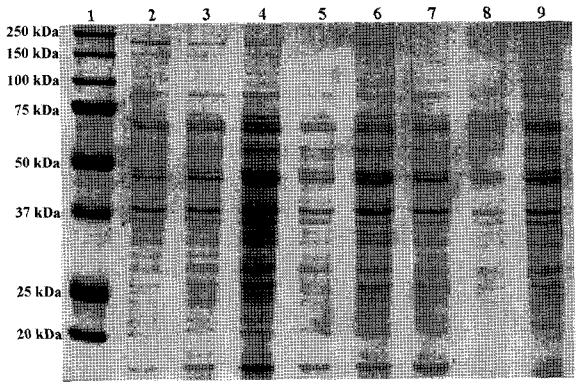

Fig. 5. SDS-PAGE profiles of whole cell proteins of LAB isolated from Korean rice wine mash. Fifty colonies were selected from LAB isolated from Korean rice wine mash 3 days following the start of fermentation. Lane 1 was a molecular size marker. Lanes 2-8 were the whole cell proteins of LAB randomly isolated from the Korean rice wine mash. Lane 9 was *Lactobacillus plantarum* KCTC3104 as a reference strain. Analysis of the patterns for whole cell proteins was performed with the program NTSYS-pc (version 2.02) by using UPGMA (unweighted pair group method using average linkage) cluster analysis.

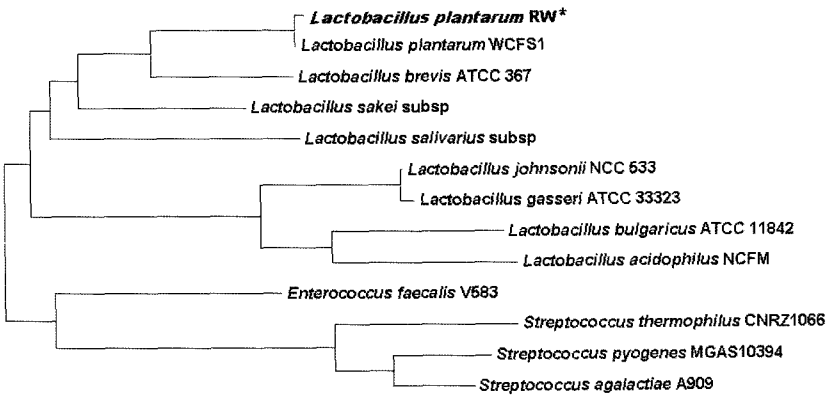

Fig. 6. Phylogenic tree based on 16S rRNA sequence. The isolated *Lactobacillus plantarum* RW were compared with the type strains of some LAB.

Table 2. Levels of 16S rRNA sequence similarity for isolated *Lactobacillus plantarum* RW and type strains of some LAB

	Strain	% Similarity score											
		1	2	3	4	5	6	7	8	9	10	11	12
1	*Lactobacillus plantarum* RW												
2	*Lactobacillus plantarum* WCFS1	98											
3	*Lactobacillus brevis* ATCC 367	92	94										
4	*Lactobacillus sakei* subsp.	91	94	94									
5	*Lactobacillus salivarius* subsp.	90	91	91	91								
6	*Enterococcus faecalis* V583	89	90	89	90	89							
7	*Lactobacillus johnsonii* NCC 533	88	90	90	91	89	88						
8	*Lactobacillus gasseri* ATCC 33323	88	90	90	90	89	88	99					
9	*Lactobacillus bulgaricus* ATCC 11842	86	88	88	89	88	87	92	91				
10	*Streptococcus agalactiae* A909	86	87	87	86	86	90	86	86	85			
11	*Lactobacillus acidophilus* NCFM	85	86	88	89	87	87	92	92	93	85		
12	*Streptococcus pyogenes* MGAS10394	85	87	86	88	86	88	86	86	85	96	85	
13	*Streptococcus thermophilus* CNRZ1066	85	87	86	88	85	88	85	85	85	93	84	94

Therefore, distribution of LAB during the fermentation stage was analyzed with SDS-PAGE for whole-cell proteins. From the colonies observed on the MRS plate, 50 colonies were selected and their whole-cell proteins were analyzed to verify the diversity of LAB within the fermentation mash. During the initial stage of fermentation (first and second days of fermentation), quite a few LAB were observed within the fermentation brew (data not shown). After the third day of the fermentation, however, one major LAB appeared (Fig. 5). This major LAB was isolated and identified to be a Gram-positive, straight rod-shaped cell (data not shown). Genotypic identification of the LAB was performed by amplification of its 16S rRNA sequence. Analysis was conducted using the BLAST search program (NCBI). LAB was most closely related to various *L. plantarum* (99%) strains without any other comparable *Lactobacillus* strains. Figure 6 and Table 2 show phylogenic analysis of 16S rRNA of isolated *L. plantarum*. We have, therefore, designated the major LAB identified within traditional Korean rice wine fermentation as *L. plantarum* RW. When the distribution of LAB and total bacteria in 27 samples of *nuruk* (traditional Korean rice wine starter) were investigated and LAB were isolated from the identified samples, 3 out of 32 were identified as *L. plantarum*. Other LAB was also found: *Leuconostoc mesenteroides* subsp. *mesenteroides* (11 strains), *Pediococcus acidilactici* (7 strains), *L. murinus* (7 strains), and *Enterococcus faecium* (4 strains) (9). When LAB in Korean red wines were made of 'Gerbong', 'Campbell', or a combination of 'Gerbong' and 'Campbell', LAB identified as phylogenetically close to *L. plantarum* and *Lactobacillus pentosus* were isolated (19). On the other hand, Lee and Yu (1) selected three LABs from *nuruk* and identified them as *Lactococcus lactis* subsp. *lactis, Leuconostoc mesenteroides* subsp. *mesenteroides*, and *Pediococcus pentosaceus*. Low water activity of *nuruk* was determined to inhibit the growth of rod-shaped bacteria, resulting in the appearance of cocci-type microorganisms. From these results, we presume that there are many kinds of LABs in *nuruk*. The main observation in our research demonstrated that there is a major type of LAB in Korean rice wine fermentation. This implies that the role of major LAB during fermentation is more important than the distribution of LAB in *nuruk*. The isolated LAB can be employed to make a traditional Korean rice wine in industry. Therefore, it would be extremely interesting to conduct a future study on the biochemical, microbiological, and fermentation analyses of *L. plantarum* RW found in traditional Korean rice wines.

Acknowledgments

This research was supported by the Kyung Hee University Research Fund in 2006 (KHU- 20060543).

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