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# Diversity Analysis of Lactic Acid Bacteria in *Takju*, Korean Rice Wine

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**To investigate lactic acid bacterial population in Korean traditional rice wines, biotyping was performed using cell morphology and whole-cell protein pattern analysis by SDS-PAGE, and then the isolates were identified by 16S rRNA sequencing analysis. Based on the morphological characteristics, 103 LAB isolates were detected in wine samples, characterized by whole-cell protein pattern analysis, and they were then divided into 18 patterns. By 16S rRNA gene sequencing, the isolates were identified as *Lactobacillus paracasei*, *Lb. arizonensis*, *Lb. plantarum*, *Lb. harbinensis*, *Lb. parabuchneri*, *Lb. brevis*, and *Lb. hilgardii* when listed by their frequency of occurrence. It was found that the difference in bacterial diversity between rice and grape wines depends on the raw materials, especially the composition of starch and glucose.**

**Keywords:** *Takju*, lactic acid bacteria, SDS-PAGE, 16S rRNA

Korean traditional rice wines and liquors have long been brewed by classical ways using *nuruk* or *koji*, made from rice and/or flour, yeasts, and medicinal plants. Before dilution, rice wines contain 15–18% alcohol (in the case of *takju*, above 6% [23]) and they are kept at acidic pH 3.4–4.5 as a result of the production of organic acids such as citric and tartaric acids that are the highest contents in *takju* by lactic acid bacteria (LAB) [13, 14]. These LAB contribute to produce lactic acid and acetoin, imparting a sour taste and a pleasant flavor, thus having a significant impact on food quality parameters such as taste and texture [1]. In addition, acidity is used to indicate the quality of tartness or sharpness to the taste. In general, the titratable acidity of *takju*, expressed as % lactic acid, is 0.5%; however, it was below 1.0%, according to Kim [7]. Therefore, an adequate acidity is important to improving the taste and maintaining wine quality.

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Winemaking can be summarized as the biotransformation of substrates into wine, which is performed principally by yeast, such as the genus *Saccharomyces*, during the primary or alcoholic fermentation [15]. Secondary malolactic fermentation (MLF) is a biodeacidification that is encouraged by LAB [16, 25]. MLF usually occurs either spontaneously or after inoculation with selected bacteria after alcoholic fermentation [18]. Rojo-Bezares *et al.* [21] introduced the four genera of LAB (*Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Oenococcus*) that usually can be found in grape must and wines. Specifically, during wine fermentation, *Oenococcus oeni* is a well-known species that contributes to the malolactic fermentation, improving wine stability and quality [3, 25]. Therefore, it is important to understand their existence and the biochemical reactions in spontaneous fermented rice wines without addition of LAB.

Until now, various fermented alcoholic beverages have been studied regarding the LAB diversity and their roles in grape wines or Japanese rice wines [5, 25], however, little study has been carried out on LAB in Korean rice wines (*yakju* or *takju*), except for a brief report by Shin and Cho in 1970 [24]. They confirmed the existence of only *Leuconostoc mesenteroides* and *Lactobacillus casei* in *takju* mash. Considering the effects of sour taste and flavor influenced by LAB on the quality of Korean rice wines, the bacterial population of LAB involved in the malolactic fermentation should be elucidated. For this purpose, we investigated the distribution of the LAB occurring naturally in a Korean traditional rice wine, *takju*, by using biochemical and genotypic methods such as cell morphology, whole-cell protein patterns, and 16S rRNA gene sequencing analyses.

To obtain samples, fifteen rice wines were purchased from local markets in Cheongju (Korea). Products with no heat treatment for sterilization were chosen, and some of them were brands distributed to the whole country and some were local brands. The pHs of the rice wines were determined as previously described [22] and were in the range of 3.4 to 3.9. The titratable acidities were also shown in the values of 0.89 to 1.0. All samples had an alcohol content of 6.0% to 7.5%. These values were similar to the previous studies

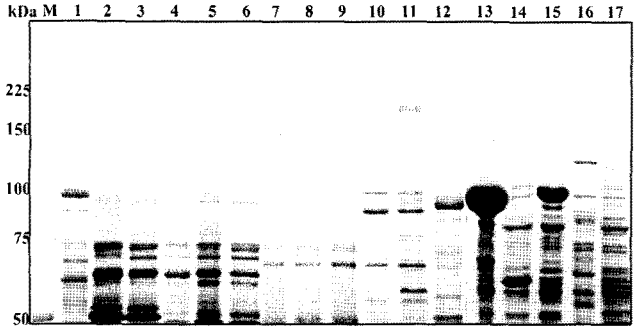


**Fig. 1.** Whole-cell protein patterns of the representative isolates from rice wines by SDS-PAGE analysis.
Lanes: M, molecular size markers (Promega, U.S.A.); 1, *Ln. pseudomesenteroides* KCTC3652; 2–3, *Lb. paracasei* isolates; 4, *Lb. casei* KCTC3109; 5–6, *Lb. casei* isolates; 7, *Lb. plantarum* KCTC3104; 8–9, *Lb. plantarum* isolates; 10–11, *Lb. arizonensis* isolates; 12, *Lb. fermentum* isolate; 13–15, *Lb. harbinensis* isolates; 16, *Lb. parabuchneri* isolate; 17, *Lb. harbinensis* KCTC13106. * *Lb. paracasei*, *Lb. arizonensis*, and *Lb. parabuchneri* were identified by 16S rRNA sequencing analysis.

[7, 23], and we did not consider that these values significantly affected the diversity of LAB among the samples.

For screening of LAB isolates, the rice wine samples were mixed in physiological saline [0.85% (w/v) NaCl; Junsei Chem., Japan] and serially diluted ($10^{-0, -2, -4, \text{and } -6}$). Diluents were spread onto five selective media; lactobacilli MRS (Difco, Franklin Lakes, NJ, U.S.A.) for lactobacilli, modified MRS-tomato juice medium containing 20% (v/v) tomato juice (MT; pH 4.8) for oenococci, BL (Difco) containing 5% (v/v) sheep blood for total lactic acid bacteria, phenylethyl alcohol containing 2% (w/v) sucrose (PES; Difco) for leuconostocs, and Enterococcosel (EN; Difco) agars for enterococci. After incubation at 37°C for 3–4 days under the anaerobic conditions using a commercial anaerobic gas pack (BD Gas-Pak EZ Anaerobic Indicator System, U.S.A.), a total of 251 colonies of typical LAB formed on each selective media were transferred onto BL agar, subcultured three times, and stored at −80°C before use. Reference strains used in this study are shown in Fig. 1. Based on the phenotypic characteristics of colony and cell morphologies, the isolates were observed using a light microscope with magnification of 1,000 times after Gram-staining [17]. The 251 isolates were distinguished between Gram-positive and -negative bacteria, and then the morphological characteristics of each isolate were recorded in terms of cell shape and size [8]. These isolates were classified into three microbial groups consisting of 103 isolates of LAB (41%), which are characterized by the properties of nonsporing, catalase-negative, rod, and cocci, 97 of yeasts (38.7%), and 51 of Gram-negative bacteria (20.3%) (*data not shown*).

SDS-PAGE has frequently been used for successful LAB classification and identification in various samples, based on whole-cell protein profiling [19, 20]. Therefore, SDS-PAGE analysis of whole-cell protein profiles was carried out according to the method described by Lee *et al.* [12]. The bacterial cells were mixed with 2 × sample dye buffer [8] and then treated for 2 min with an Ultrasonic Processor (Sonics and Materials Inc., Newtown, CT, U.S.A.).

**Table 1.** Species identification of the lactic acid bacterial isolates from rice wines using 16S rDNA sequencing.

| Patterns by SDS-PAGE | Isolates | Species-identification | Identity (%) | GenBank accession No. | No. of the isolates |
|---|---|---|---|---|---|
| 1 | T7-10 | *Lactobacillus paracasei* | 99.9 | AB368901 | 24 |
| 2 | T11-9 | *Lactobacillus paracasei* | 100 | AB368902 | 25 |
| 3 | T4-3 | *Lactobacillus paracasei* | 99.9 | AB368900 | 4 |
| 4 | T2-2 | *Lactobacillus paracasei* | 99.4 | AB368899 | 1 |
| 5 | T4-5 | *Lactobacillus plantarum* | 99.8 | AB368903 | 6 |
| 6 | T6-7 | *Lactobacillus arizonensis* | 99.9 | AB368908 | 6 |
| 7 | T5-10 | *Lactobacillus arizonensis* | 99.8 | AB368907 | 7 |
| 8 | T10-14 | *Lactobacillus arizonensis* | 99.7 | AB368909 | 12 |
| 9 | T1-16 | *Lactobacillus arizonensis* | 99.9 | AB368906 | 1 |
| 10 | T14-8 | *Lactobacillus arizonensis* | 99.8 | AB368910 | 4 |
| 11 | T10-3 | *Lactobacillus brevis* | 99.4 | AB368912 | 1 |
| 12 | T8-7 | *Lactobacillus plantarum* | 99.8 | AB368904 | 1 |
| 13 | T12-7 | *Lactobacillus parabuchneri* | 100 | AB368913 | 2 |
| 14 | T3-10 | *Lactobacillus arizonensis* | 99.9 | AB368905 | 1 |
| 15 | T3-12 | *Lactobacillus hilgardii* | 99.6 | AB368911 | 1 |
| 16 | T9-8 | *Lactobacillus harbinensis* | 98.2 | AB368915 | 4 |
| 17 | T9-11 | *Lactobacillus parabuchneri* | 99.4 | AB368914 | 2 |
| 18 | T12-11 | *Lactobacillus harbinensis* | 99.7 | AB358916 | 1 |
| Total | | | | | 103 |

The samples were heated, centrifuged at 8,000 ×*g* for 5 min, and analyzed with a 10% polyacrylamide separating gel (Elpis-biotech Co., Daejeon, Korea). A broad range of protein molecular markers (Promega, Madison, WI, U.S.A.), size ranging from 220 to 15 kDa, were used as protein standards. In our results, the 103 LAB isolates were classified into 18 different chemotypes. These groups had protein patterns different from each other, depending on the clearance, thickness, position, and number of the bands. The isolates consisting of different patterns were regarded as different groups (in spite of identification as the same species by 16S rRNA gene analysis, shown in Table 1). In addition, the isolates showing the thickness variance of their major bands, despite of similar protein patterns, were classified as momentary different types. For consistency in whole-cell protein pattern analysis, experiments were repeated three times. The representative protein patterns of several reference strains are shown in Fig. 1. In detail, two isolates (lanes 5 and 6) showed the whole-cell protein patterns similar to lane 4 of the reference strain, *Lb. casei* KCTC3109, and therefore, they were designated as *Lb. casei*. By the same manner, the isolates of lanes 8 and 9 were determined as *Lb. plantarum*, since their protein patterns were matched to that of *Lb. plantarum* KCTC3104. The SDS-PAGE patterns of *Lb. paracasei*, *Lb. arizonensis*, and *Lb. parabuchneri* did not match with those of the stock cultures used, and hence, they were identified by 16S rDNA sequencing.

16S rRNA sequencing was carried out as previously described by Kim and Adachi [8]. The following universal bacterial primer pair was used; 27F (5'-AGAGTTTGATCCTG-GCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') [10]. The 16S rRNA genes were synthesized at SolGent Ltd. (Daejon, Korea). After species identification, the sequences of each isolate were deposited in the DNA databases with accession numbers. As shown in Table 1, the 103 isolates from rice wines were identified as 7 *Lactobacillus* species containing *Lb. paracasei*, *Lb. brevis*, *Lb. plantarum*, *Lb. arizonensis*, *Lb. parabuchneri*, *Lb. hilgadii*, and *Lb. harbinensis*. Most of the isolates showed high homology (above 98.2%) with the reference strains in the GenBank database.

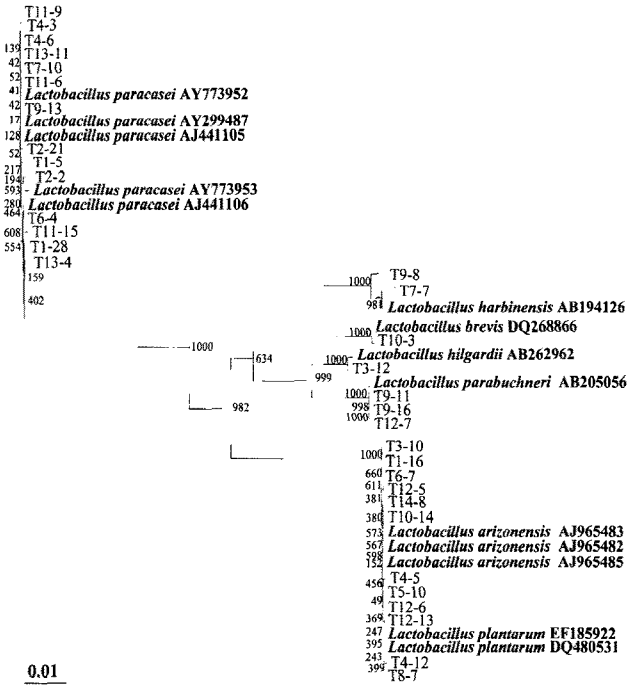


**Fig. 2.** Phylogenetic tree constructed from the neighbor-joining method of 16S rDNA sequences derived by PCR with a universal bacterial primer set (27F and 1492R) among the LAB isolates from rice wines.
The GenBank accession numbers corresponding to each reference strain related to our isolates are indicated in bold. The corresponding bootstrap values (1,000 replications) are shown on each branch.

Phylogenetic tree analysis, showing the relationship of 16S rDNA sequences between the isolates and related reference strains, was performed by using the ClustalW software (Fig. 2). The tree was constructed by a neighbor-joining phylogenetic tree and was estimated by bootstrap analysis for 1,000 replications using the same program [8]. Most isolates were located in the same branches of reference strains obtained from the database in GenBank/EMBL/DDBJ. Notably, we found that the isolates corresponding to *Lb. plantarum* and *Lb. arizonensis* were in the branches linking closely in a same cluster, and this fact revealed that they have very close relationship in 16S rRNA gene sequences.

**Table 2.** Frequency of occurrence of the lactic acid bacterial species isolated from the rice wine samples (T1-T15).

| Species identification | Rice wine samples (n=15) | | | | | | | | | | | | | | | No. of the isolates | Frequency of occurrence (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | T1 | T2 | T3 | T4 | T5 | T6 | T7 | T8 | T9 | T10 | T11 | T12 | T13 | T14 | T15 | | |
| *Lactobacillus paracasei* | 6 | 7 | 7 | 2 | | 1 | 2 | 3 | 1 | 2 | 6 | 2 | 4 | 5 | 6 | 54 | 93 |
| *Lactobacillus arizonensis* | 2 | | 2 | 1 | 6 | 3 | 3 | | 2 | 2 | 3 | 2 | 1 | 3 | 1 | 31 | 87 |
| *Lactobacillus plantarum* | | | | 5 | | | | 1 | | | | 1 | | | | 7 | 27 |
| *Lactobacillus harbinensis* | | | | | | | 2 | | 2 | | | 1 | | | | 5 | 20 |
| *Lactobacillus parabuchneri* | | | | | | | | | 3 | | | 1 | | | | 4 | 13 |
| *Lactobacillus brevis* | | | | | | | | | | 1 | | | | | | 1 | 7 |
| *Lactobacillus hilgardii* | | | 1 | | | | | | | | | | | | | 1 | 7 |
| Total | 8 | 7 | 10 | 8 | 6 | 4 | 7 | 4 | 8 | 5 | 9 | 7 | 5 | 8 | 7 | 103 | 100 |

Using 16S rRNA gene sequencing and ribotyping analysis, Kostinek *et al.* [9] suggested that *Lb. arizonensis* is a heterotypic synonym from *Lb. plantarum* owing to their similarity. However, SDS-PAGE analysis of *Lb. arizonensis* isolates revealed that it was distinct from the *Lb. plantarum* isolates and the reference strain with different protein patterns (lanes 7–9 *vs.* lanes 10–12 in Fig. 1). Our data support the suggestion of Swezey *et al.* [26] that *Lb. arizonensis* represents a new species, based on biochemical characteristics. Accordingly, further biochemical analyses are needed to distinguish these species from the *Lb. plantarum* group.

In this study, we found 7 LAB species from the 103 LAB isolates: *Lb. paracasei* (93%), *Lb. arizonensis* (87%), *Lb. plantarum* (27%), *Lb. harbinensis* (20%), *Lb. parabuchneri* (13%), *Lb. brevis* (7%), and *Lb. hilgardii* (7%), when listed by their frequency of occurrence (Table 2). From this result, we recognized that the LAB species isolated most frequently from the Korean traditional rice wines, *takju*, were *Lb. paracasei* and *Lb. arizonensis*. According to the previous reports, *Lb. paracasei* and *Lb. plantarum* were detected from various fermented foods such as *kimchi* and grape wines [2, 11, 25]. Moreover, Endo and Okada [5] reported in 2005 their existence during *shochu* fermentation with *koji* (Japanese distilled spirit). Besides, this *Lb. hilgardii*, *Lb. brevis*, and *Lb. paracasei* were also isolated from the base wine [4]. Although little has been described on *Lb. arizonensis* in fermented foods, which was first isolated from jojoba meal [26], this is the first report on its isolation from Korean rice wine.

Interestingly, although *O. oeni* is well-known as the predominant LAB species during grape and rice wines fermentation [6, 25], it was not detected in our study. The difference of bacterial diversity between rice and grape wines has been suggested to depend on the raw materials, especially the composition of starch and glucose. Despite the general similarity of LAB population between *takju* and other wines, this fact implies that *takju* might follow the malolactic fermentation pathway slightly differently from other wines. Further studies on the biochemical and microbial changes during the fermentation period producing organic acids in rice wine brewing should be carried out. Moreover, in addition to this culture-dependant method, a genetic analysis using PCR technique may make it possible to elucidate more broad microbial diversity including nonculturable bacteria in *takju*.

In conclusion, we demonstrated the differences of the lactic acid bacterial population in Korean rice wine, *takju*, by culture-dependent methods such as morphological, whole-cell protein patterns, and 16S rRNA gene sequencing analyses. *Lb. paracasei* and *Lb. arizonensis* were determined as the predominant species in *takju* except yeasts. The presence and absence of these LAB species could be associated with the wine quality. In the further study, their characteristics of growth and acid conversion during rice wine fermentation should be evaluated for quality improvement and starter development for rice wine.

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