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Diversity of Lactic Acid Bacteria in the Korean Traditional Fermented Beverage Shindari, Determined Using a Culture-dependent Method

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Abstract: The fermented food Shindari is a low-alcohol drink that is indigenous to Jeju island, South Korea. In this study, the diversity of lactic acid bacteria (LAB) in Shindari was determined using a culture-dependent method. LAB were cultivated from Shindari samples using two different LAB culture media. Twenty-seven strains were randomly selected and identified by 16S rRNA gene sequence analysis. The identified LAB strains comprised 6 species within the *Enterococcus*, *Lactobacillus* and *Pediococcus* genera. Five of the species, namely *Enterococcus faecium*, *Lactobacillus fermentum*, *L. plantarum*, *Pediococcus pentosaceus* and *P. acidilactici* were isolated from MRS medium, while 1 species, *L. pentosus,* was isolated from Rogosa medium. Most of the isolated strains were identified as members of the genus *Lactobacillus* (78%). This study provides basic microbiological information on the diversity of LAB and provides insight into the ecological roles of LAB in Shindari.

Keywords: lactic acid bacteria, indigenous fermented food, Shindari, culture-dependent method

The lactic acid bacteria (LAB) are acid-tolerant, low-G+C content, non-spore-forming, rod- or cocci-shaped Gram-positive microbes that primarily produce lactic acid as the major metabolic end-product of carbohydrate fermentation (Makarova *et al.*, 2006). LAB hold a "generally recognized as safe (GRAS)" status, as they contribute to the normal healthy microflora of human mucosal surfaces and are frequently found in industrially important foods. Historically, LAB have been used for food fermentation, as acidification inhibits the growth of pathogenic microorganisms that can cause damage to human health. Furthermore, the metabolic products of LAB (lactic acid or lactate residue) contribute to the organoleptic and textural profile of a food item. Many LAB strains are used for the production of yogurt, cheese, sauerkraut, pickles, beer, wine, cider, and other fermented foods, as well as Korean traditional fermented foods, such as kimchi, doenjang (soybean paste), and jeotgal made of shrimp or shellfish (Cho *et al.*, 2006, Nam *et al.*, 2012a, Nam *et al.*, 2012b, Roh *et al.*, 2010b, Steinkraus, 1983). In many traditional salting processes used for food preservation, vegetables or seafood are submerged in brine, and salt-tolerant LAB are able to grow by feeding on the natural sugars present in the food. The resulting combination of salt and LAB prevents food spoilage by inhibiting the growth of bacteria and fungi (Caplice and Fitzgerald, 1999). The LAB genera belonged to the order Lactobacillales include *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, and *Streptococcus*, and the more peripheral *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Sporolactobacillus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*.

Shindari, a low-alcohol fermented food indigenous to Jeju island, South Korea, is made via the fermentation of gelatinized rice or barley and the addition of *nuruk*, which is major ingredient of Korean traditional drinks (Kim *et al.*, 1999). In the past, when cooked rice quickly turned sour as a result of the summer heat, fermentation

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of the rice would generate nutritious alcoholic drinks, such as Shindari. It might be possible to enhance the flavor of Shindari by altering the LAB population used in the fermentation process; however, this has not yet been attempted. The traditional production of Shindari was regionally limited to Jeju island; therefore, it is expected that Shindari possesses a unique LAB population. For this reason, in this study, we sought to discover the diversity of LAB in Shindari samples collected from Jeju island using a culture-dependent method.

Three samples of homemade Shindari were obtained in Jeju island, South Korea. The samples were spread onto MRS (Difco^{TM} Lactobacilli MRS Agar; BD) and Rogosa (Difco^{TM} Rogosa SL Agar; BD) plates. The MRS medium contained ($g \cdot L^{-1}$): protease peptone no. 3 (10.0), beef extract (10.0), yeast extract (5.0), dextrose (20.0), polysorbate 80 (1.0), ammonium citrate (2.0), sodium acetate (5.0), $MgSO_4 \cdot 7H_2O$ (0.1), $MnSO_4 \cdot 4H_2O$ (0.05), K_2HPO_4 (2.0), and agar (15.0). The Rogosa medium contained ($g \cdot l^{-1}$): tryptone (10.0), yeast extract (5.0), dextrose (10.0), arabinose (5.0), saccharose (5.0), sodium acetate (15.0), ammonium citrate (2.0), KH_2PO_4 (6.0), $MgSO_4 \cdot 7H_2O$ (0.57), $MnSO_4 \cdot 4H_2O$ (0.12), $FeSO_4 \cdot 7H_2O$ (0.03), polysorbate 80 (1.0), and agar (15.0). The plates were incubated at 25ºC for 2 wk and the colonies were successively restreaked to obtain pure cultures.

Twenty-seven LAB colonies from the MRS and Rogosa plates were randomly selected and used for further analysis. The 16S rRNA genes of the selected strains were amplified by PCR using the AccuPower PCR PreMix (Bioneer) and the universal 16S ribosomal RNA gene primer set, 27F (5-AGAGTTTGATCMTGGCTCAG-3) and 1492R (5-GGTTACCTTGTTACGACTT-3). The amplified 16S rRNA genes were sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) together with an automated DNA analyzer system (PRISM 3730XL DNA analyzer, Applied Biosystems), as described previously (Roh *et al.*, 2008). The sequence fragments of the 16S rRNA gene were assembled using the SeqMan software program (DNASTAR). Comparisons of the 16S rRNA gene sequences were performed using the EzTaxon-e server (http://

Table 1. Bacterial strains identified in this study based on the 16S rRNA gene sequences using the EzTaxon-e database

No.	Strain	Medium	Taxon*	Identity (%)
1	MRS-S1-1	MRS	*Lactobacillus fermentum* CECT 562^T	100.0
2	MRS-S1-2	MRS	*Enterococcus faecium* ATCC 19434^T	99.9
3	MRS-S1-3	MRS	*Lactobacillus plantarum* subsp. *plantarum* ATCC 14917^T	99.5
4	MRS-S1-4	MRS	*Lactobacillus plantarum* subsp. *plantarum* ATCC 14917^T	99.9
5	MRS-S1-5	MRS	*Lactobacillus plantarum* subsp. *plantarum* ATCC 14917^T	99.9
6	MRS-S1-6	MRS	*Lactobacillus fermentum* CECT 562^T	99.9
7	MRS-S1-7	MRS	*Lactobacillus plantarum* subsp. *plantarum* ATCC 14917^T	100.0
8	MRS-S1-8	MRS	*Lactobacillus fermentum* CECT 562^T	99.9
9	MRS-S1-9	MRS	*Lactobacillus fermentum* CECT 562^T	99.9
10	MRS-S1-10	MRS	*Lactobacillus fermentum* CECT 562^T	100.0
11	MRS-S2-3	MRS	*Lactobacillus fermentum* CECT 562^T	99.8
12	MRS-S2-4	MRS	*Lactobacillus fermentum* CECT 562^T	99.9
13	MRS-S2-8	MRS	*Lactobacillus fermentum* CECT 562^T	99.9
14	MRS-S3-1	MRS	*Pediococcus pentosaceus* DSM 20336^T	99.9
15	MRS-S3-3	MRS	*Lactobacillus fermentum* CECT 562^T	99.9
16	MRS-S3-5	MRS	*Lactobacillus fermentum* CECT 562^T	99.9
17	MRS-S3-6	MRS	*Pediococcus acidilactici* DSM 20284^T	99.8
18	MRS-S3-7	MRS	*Pediococcus acidilactici* DSM 20284^T	99.2
19	MRS-S3-9	MRS	*Lactobacillus fermentum* CECT 562^T	99.9
20	MRS-S3-10	MRS	*Lactobacillus fermentum* CECT 562^T	99.9
21	MRS-S3-11	MRS	*Lactobacillus plantarum* subsp. *plantarum* ATCC 14917^T	99.9
22	MRS-S3-12	MRS	*Pediococcus pentosaceus* DSM 20336^T	99.9
23	MRS-S3-13	MRS	*Pediococcus pentosaceus* DSM 20336^T	99.9
24	Rogosa-S1-5	Rogosa	*Lactobacillus pentosus* JCM 1558^T	100.0
25	Rogosa-S1-6	Rogosa	*Lactobacillus pentosus* JCM 1558^T	100.0
26	Rogosa-S1-9	Rogosa	*Lactobacillus pentosus* JCM 1558^T	100.0
27	Rogosa-S2-5	Rogosa	*Lactobacillus pentosus* JCM 1558^T	100.0

*CECT, Spanish Type Culture Collection; ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; JCM, Japan Collection of Microorganisms; ^{T}, type strain

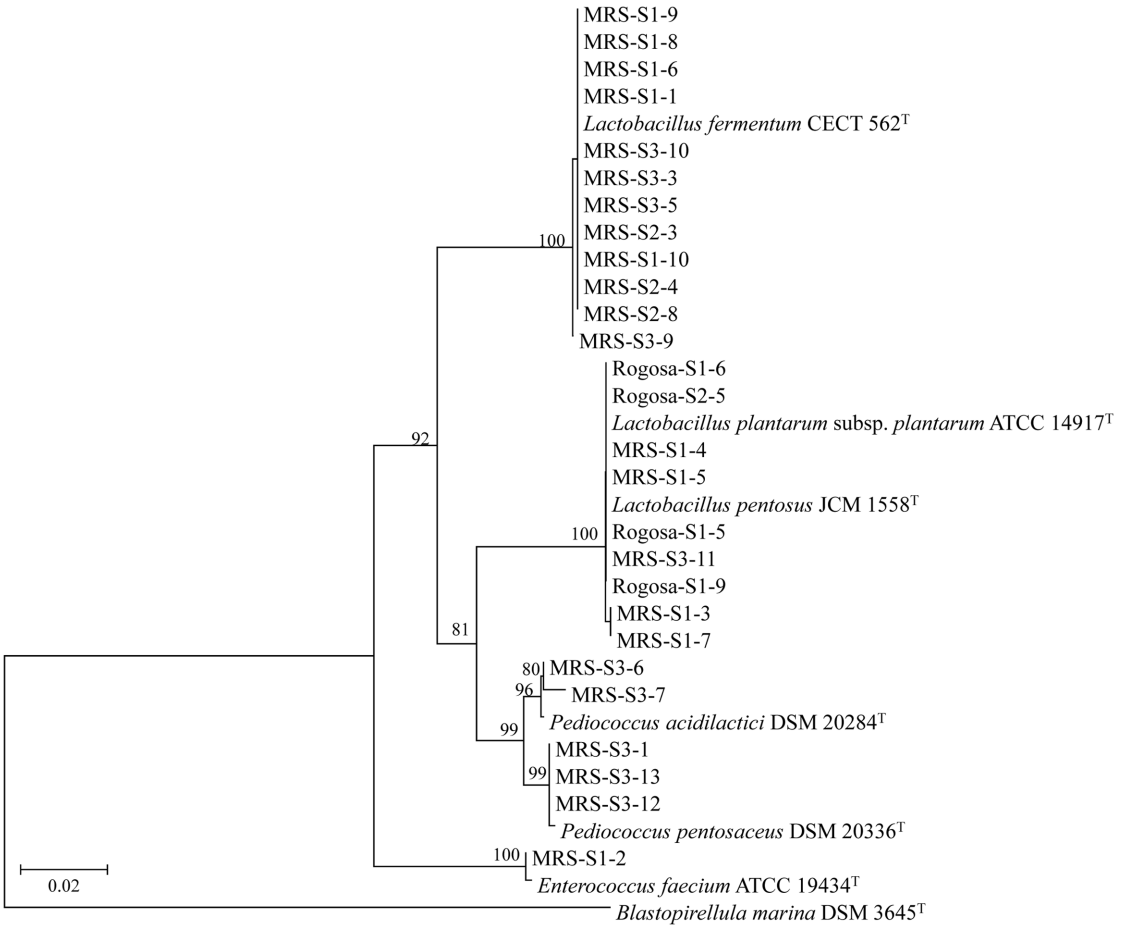

Fig. 1. Neighbor-joining (NJ) phylogenetic tree of the isolates from Shindari samples and phylogenetically closed relatives, based on 16S rRNA gene sequences. The bootstrap values (>70%) calculated using the NJ probabilities are shown at the branching points. *Balstopirellula marina* DSM 3645^T served as the outgroup. Bar, 0.02 accumulated changes per nucleotide.

eztaxon-e.ezbiocloud.net/) (Kim *et al.*, 2012) to identify the nearest related taxa and calculate the pairwise 16S rRNA gene sequence similarities. The 16S rRNA gene sequences of LAB strains and related taxa were aligned using the SILVA Incremental Aligner (Pruesse *et al.*, 2012). A phylogenetic tree was constructed based on the aligned 16S rRNA gene sequences using MEGA5 (Tamura *et al.*, 2011) and the neighbor-joining method (Saitou and Nei, 1987). A bootstrap analysis was performed by obtaining a consensus tree based on 1000 randomly generated trees.

The 16S rRNA gene sequence analysis revealed that the 27 strains comprised 5 species and 1 subspecies within 3 genera, namely *Lactobacillus, Pediococcus*, and *Enterococcus*. All of these genera belong to the class Baclli within the phylum Firmicutes. The genera *Lactobacillus*, *Pediococcus*, and *Enterococcus* are members of the order *Lactobacillales*. Twenty-one of the 27 strains analyzed belong to the genus *Lactobacillus* (78%), while the rest belong to the genera *Pediococcus* (5 strains) and *Enterococcus* (1 strain). The identified *Lactobacillus* spp. were found to be highly similar to *L. fermentum* (99.8~100.0% 16S rRNA gene sequence similarity), *L. plantarum* subsp. *plantarum* (99.5~100.0% similarity) and *L. pentosus* (100.0% similarity). Other LAB strains were closely related to *P. pentosaceus* (99.9% similarity), *P. acidilactici* (99.2~99.8% similarity) and *E. faecium* (99.9% similarity) (Table 1).

Many bacterial strains were originally isolated from fermented food. Members of the genus *Lactobacillus* are particularly well-known LAB, and are present in the normal flora of the vagina and gastrointestinal tract of human. The production of lactic acid makes these environments acidic, which inhibits the growth of some harmful bacteria (Osset *et al.*, 2001; Reid *et al.*, 2009). Interestingly, *L. plantarum* is commonly found in many fermented food products including sauerkraut, pickles, brined olives, kimchi, Nigerian Ogi, sourdough, and other

fermented plant material, and also some cheeses, fermented sausages, and stockfish (Jang and Kim, 2013). *L. fermentum* is also a normal inhabitant of the human intestinal tract, and some strains have been associated with cholesterol metabolism (Mikelsaar and Zilmer, 2009; Pan *et al.*, 2011).

In this study, 27 LAB strains isolated from Shindari samples were analyzed using MRS and Rogosa LAB-selection media. However, culture-dependent methods cannot be used to analyze the whole bacterial population of a given sample. These classical methods must be used in complement with culture-independent methods, such as metagenome or amplicon sequencing using next-generation sequencing technologies (Roh *et al.*, 2010a). The full extent of the population of LAB and other types of bacteria present in Shindari remains to be determined. This study provides basic microbiological information on the diversity of LAB and provides insight into the ecological roles of LAB in Shindari.

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