J. Microbiol. Biotechnol. (2018), 28(7), 1112–1121
https://doi.org/10.4014/jmb.1803.03034

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Identification of Lactic Acid Bacteria in Galchi- and Myeolchi-Jeotgal by 16S rRNA Gene Sequencing, MALDI-TOF Mass Spectrometry, and PCR-DGGE S

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Received: March 27, 2018
Revised: May 30, 2018
Accepted: June 4, 2018

First published online
June 6, 2018

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Supplementary data for this paper are available on-line only at http://jmb.or.kr.

pISSN 1017-7825, eISSN 1738-8872

Jeotgal is a Korean traditional fermented seafood with a high concentration of salt. In this study, we isolated lactic acid bacteria (LAB) from galchi (*Trichiurus lepturus*, hairtail) and myeolchi (*Engraulis japonicas*, anchovy) jeotgal on MRS agar and MRS agar containing 5% NaCl (MRS agar+5% NaCl), and identified them by using 16S rRNA gene sequencing and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) as culture-dependent methods. We also performed polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) as a culture-independent method to identify bacterial communities. Five samples of galchi-jeotgal and seven samples of myeolchi-jeotgal were collected from different regions in Korea. A total of 327 and 395 colonies were isolated from the galchi- and myeolchi-jeotgal samples, respectively. 16S rRNA gene sequencing and MALDI-TOF MS revealed that the genus *Pediococcus* was predominant on MRS agar, and *Tetragenococcus halophilus* on MRS agar+5% NaCl. PCR-DGGE revealed that *T. halophilus*, *Tetragenococcus muriaticus*, and *Lactobacillus sakei* were predominant in both types of jeotgal. *T. halophilus* was detected in all samples. Even though the same species were identified by both culture-dependent and -independent methods, many species identified by the culture-dependent methods were not in the bacterial list identified by the culture-independent methods. The distribution of bacteria in galchi-jeotgal was more diverse than in myeolchi-jeotgal. The diverse LAB in galchi- and myeolchi-jeotgals can be further studied as candidates for starter cultures to produce fermented foods.

Keywords: Lactic acid bacteria, galchi-jeotgal, myeolchi-jeotgal, 16S rRNA gene sequencing, MALDI-TOF MS, PCR-DGGE

Introduction

Jeotgal or jeot is traditional fermented and salted seafood widely consumed in regions that have rice-based diets, such as Korea, Japan, and Southeast Asia. This food product is manufactured by adding salt to various types of seafood to prevent spoilage and has a distinctive flavor as a result of enzymatic reactions among the ingredients. The various types of Korean traditional fermented and salted seafood, such as jeotgal, aekjeot (fish sauce), and sikhae (fermented fish with grains) are distinguished by the manufacturing process. In Korea, jeotgal is made using diverse raw materials, including whole meat, intestines, or eggs of fish and shellfish. There are approximately 150 different kinds of jeotgal in Korea that differ by main ingredient and/or processing method [1]. Jeotgal is also made in other countries; it is known as pla-ra and pa-daek in Thailand and Laos [2], and nuoc-man in Vietnam [3]. Jeotgal is made by adding salt to a final concentration of a 20–30% to the raw material and allowing the mixture to ferment for 2–3 months [1]. Jeotgal is used as an important condiment in kimchi or for seasoning Korean-style stews to enhance flavor and taste. Additionally, the bacterial communities in jeotgal are thought to improve appetite

and digestion [1].

In general, most bacteria in jeotgal originate from the seafood used as the main ingredient or the environment of that seafood. Owing to the high salt concentration of jeotgal, halophilic or halotolerant bacteria can survive and participate in the fermentation, whereas the growth of harmful bacteria is decreased [1]. Examples of bacterial genera present in jeotgal include *Halobacterium* and *Halomonas* in addition to *Bacillus*, *Brevibacterium*, *Flavobacterium*, *Micrococcus*, *Pediococcus*, *Pseudomonas*, and *Staphylococcus* [1]. The bacterial communities vary according to the ingredients and fermentation period. There has been extensive research into the bacteria present in jeotgal because they contribute to its fermentation and ripening. However, few studies have reported the lactic acid bacteria (LAB) communities present in jeotgal and their functions in the fermentation process [4]. This is mostly because LAB cannot survive in the high salt environment of jeotgal. In this study, we identified LAB communities present in different jeotgal samples using agars containing salt.

Tools to investigate bacterial community composition can be categorized as culture-dependent or -independent. Culture-dependent methods rely on the application of molecular techniques such as 16S rRNA gene sequencing and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to identify isolates from cultured media [5–7]. 16S rRNA gene sequencing is one of the most commonly used techniques for bacterial identification [4, 8]. However, this technique is time-consuming because genes from extracted DNA need to be amplified by the polymerase chain reaction (PCR) and be purified for sequencing. In contrast, MALDI-TOF MS is an effective and accurate identification technique that can be performed in a short time period using only a single colony, and a large number of isolates can be analyzed simultaneously [9, 10]. This quick and accurate technique has previously been used to characterize bacterial communities in diverse fermented foods from various countries, including fermented meat nem chua from Vietnam [11] and vegetable dua muoi and ca muoi [10], including mukeunji, a long-term aged kimchi [12], as well as yogurt and probiotics [13]. Nevertheless, few studies have used MALDI-TOF MS analysis to identify bacterial communities in jeotgal.

Although the distribution of viable cells in each sample can be determined and isolates acquired through culture-dependent methods, bacteria present at very low levels or dead bacteria cannot be cultured [14]. These hard-to-culture microorganisms can be detected effectively by culture-independent methods [15]. One conventional culture-independent method is polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). Genomic DNA extracted directly from each sample is separated by the GC content of the DNA, with different bacteria showing specific band patterns in the gradient gel after electrophoresis. This method has been used to detect diverse bacterial communities grown in culture as well as organisms that cannot be cultured. PCR-DGGE has been used in several studies to investigate microorganisms in various fermented foods, including jeotgal [2, 15–18]. Among the many kinds of jeotgals made using various seafoods, galchi (hairtail)- and myeolchi (anchovy)-jeotgal are common kimchi ingredients that are widely consumed in Korea.

Our aim in this study was to investigate the bacterial communities, especially LAB, present in galchi- and myeolchi-jeotgals using diverse identification methods. First, the bacterial communities from various jeotgal samples purchased from different regions of South Korea were identified using 16S rRNA gene sequencing and MALDI-TOF MS of isolates grown on agar plates with or without salt as culture-dependent methods. Additionally, the bacterial communities were analyzed using PCR-DGGE as a culture-independent method.

Materials and Methods

Jeotgal Sample Preparation and Measurement of pH and Salinity

Five samples of galchi-jeotgal were collected from five markets (Se-1, Se-2, Se-3, So-1, and Y-1) in three regions (Seoul, Sokcho, and Yeosu) and seven samples of myeolchi-jeotgal were purchased from seven markets (B-1, B-2, B-3, Se-1, Se-2, Se-3, and Y-1) in three regions (Busan, Seoul, and Yeosu) in Korea. To measure the pH and salinity of each sample, 1 g of sample and 10 ml of sterilized water were mixed and centrifuged at 16,200 ×*g* for 10 min. The pH of the collected supernatant was then measured using an Orion star a211 pH meter (Thermo Fisher Scientific, USA) and the salinity was measured using a PAL-03S refractometer (ATAGO, Japan).

Isolation of Lactic Acid Bacteria

Ten grams of each sample was mixed with 90 ml of sterile water in a Stomacher filter bag (Seward Limited, UK) and homogenized using a Stomacher 400 (Seward Limited, UK) for 30 sec at 230 rpm. After serial dilution of the homogenized samples with phosphate-buffered saline, 100 µl of highly diluted sample was spread onto MRS agar (Acumedia, USA) and MRS agar containing 5% NaCl (MRS agar+5% NaCl), and incubated anaerobically for 72 h at 30°C to culture LAB. Single colonies that differed in morphological characteristics were picked from each plate and subcultured in MRS broth (Acumedia, USA) and MRS broth

containing 5% NaCl (MRS broth+5% NaCl) for 24 h at 30°C. Enriched bacterial isolates in both broths were mixed with 80% glycerol at the ratio of 7:3 and stored at −80°C.

Comparison of Whole Cell Protein Patterns through SDS-PAGE Analysis

Each isolate was cultured in MRS broth or MRS broth+5% NaCl for 24 h at 30°C and centrifuged at 16,200 ×*g*, 4°C for 15 min. The collected pellet of each isolate was washed twice with sterile water and mixed with 50 mM (pH 8.0) Tris-HCl buffer. Glass beads (425–600 μm diameter; Sigma, USA) were added to the suspended solution, which was then vortexed for 8 min. The same amount of 2× buffer was added and heated for 5 min in an ALB64 heat block (FINEPCR, Korea). After cooling to room temperature, the supernatant obtained through centrifugation for 5 min was subjected to gel electrophoresis. Samples (5 μl of whole cell protein extract) were electrophoresed on a 30% acrylamide gel run at 100 V for 90 min. After gel electrophoresis, whole cell protein patterns were determined by staining the gel with staining buffer (1.25 g/l Coomassie brilliant blue, 500 ml/l methanol, 900 ml/l acetic acid, and 400 ml/l H_2O) for 1 h, followed by destaining with destaining buffer (100 ml/l acetic acid, 300 ml/l methanol, and 600 ml/l H_2O) for 3 h. Whole cell protein patterns were analyzed after scanning with a high resolution scanner (Perfection V700 photoscanner; Epson, USA). Each isolate was classified into different groups according to pattern similarities. Representative isolates were selected from each group for bacterial identification.

Identification by 16S rRNA Gene Sequencing

The 16S rRNA gene of representative isolates from each group was sequenced. Genomic DNA extracted using the G-spin Genomic DNA Extraction kit (Intronbio, Korea) was used as the template to amplify the 16S rRNA gene by PCR with 16S universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGCTACCTTGTTAGCGACTT-3′). After purifying the amplicons using the QIAquick PCR purification kit (Qiagen, Germany), the amplicons were sequenced using an automated DNA sequencer (Applied Biosystems, USA). The 16S rRNA gene sequences were then analyzed by BLAST searches of the NCBI database.

Identification by MALDI-TOF MS

A single colony of each isolate was spotted directly on a target plate (Bruker Daltonics GmbH, Germany) overlaid with 1 μl of 70% formic acid and air-dried. All spots were overlaid with a matrix solution (Bruker Daltonics GmbH, Germany) of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid. Each spot was measured using a microflex LT mass spectrometer (Bruker Daltonics GmbH, Germany). Parameters were as follows: ion source 1, 20.0 kV; ion source 2, 18.2 kV; lens, 6.0 kV; initial laser power; 25%; and maximal laser power; 35%. Mass spectra were analyzed using Flexcontrol 3.0 software and the MALDI Biotyper database.

Analysis of Bacterial Communities by PCR-DGGE

Ten grams of each sample was mixed with 90 ml of sterile water in a Stomacher filter bag and homogenized using a Stomacher 400 at 230 rpm for 30 sec. The homogenized samples were filtered through four layers of cheesecloth and centrifuged at 4,250 ×*g*, at 4°C for 15 min. The collected pellets were washed twice with sterile water, and genomic DNA was extracted using the P&C Bacterial Genomic DNA Extraction kit (Biosolution, Korea). The yield and quality of extracted DNA were analyzed by electrophoresis using a 1% (w/v) agarose gel. Extracted DNA was used as the template for initial PCR targeting the 16S rRNA gene, using the primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGCTACCTTGTTAGCGACTT-3′). This amplicon was used as a template for nested PCR to amplify the V3 region of the 16S rRNA gene, using the DGGE primers GC338F (5′-GCCCGC CGCGCGCGGCGGGCGGCGGGGGCACGGGGGGACTCCTAC GGGAGGCAGCAG-3′) and 518R (5′-ATTACCGCGGCTGCTGG-3′). For DGGE analysis, the nested PCR product was applied directly to a denaturing gradient gel containing 20–50% urea (USB, USA)–formamide (Sigma, USA), and electrophoresis was carried out using the DCode system (Bio-Rad, USA) with samples electrophoresed for 40 min at 40 V and then 15 h at 60 V. During electrophoresis, genomic DNA of each sample was separated into diverse bacterial DNAs with different GC contents. The separated DNA was visualized by staining the gel with ethidium bromide for 1 h and imaged using the Quantum ST5 gel documentation system (Vilber Lourmat, Germany). Bands of interest on the gel were excised using sterile blades, washed twice with sterilized water, and incubated overnight at 4°C. During the overnight incubation, bacterial DNA diffused out from the gel. The eluate was then used as a template for re-amplification using the primers GC 338F and 518R. To confirm the presence of a single band and improve the purity, the amplicon was re-run on a DGGE gel. After obtaining a single band on the gel, the eluted DNA was used for amplification of the V3 region of 16S rRNA using the same primer pairs without a GC clamp for sequencing. The PCR products were purified using a QIAquick PCR purification kit and sequenced using an automated DNA sequencer. Partial 16S rRNA gene sequences were compared with the NCBI database using BLAST and closest known relatives were determined.

Results and Discussion

pH and Salinity of Galchi- and Myeolchi-Jeotgals

The jeotgal samples used in this study were collected from four different regions in Korea. Five galchi-jeotgal samples were obtained from Seoul, Sokcho, and Yeosu, and seven myeolchi-jeotgal samples were obtained from Busan, Seoul, and Yeosu. As shown in Table 1, the pH ranges of the galchi- and myeolchi-jeotgal samples were 5.83–6.62 and 5.65–6.17, respectively, and the salinity range was 30–48% and 26–40%, respectively.

Table 1. Sample information and chemical characterization of the galchi- and myeolchi-jeotgal samples used in this study.

Sample (fish material)	Region[a]	Market[b]	pH	Salinity (%)
Galchi-jeotgal	Seoul	Se-1	5.83	32
(Hairtail)	Seoul	Se-2	5.89	33
	Seoul	Se-3	5.98	48
	Sokcho	So-1	6.62	30
	Yeosu	Y-1	6.17	37
Myeolchi-jeotgal	Busan	B-1	5.89	33
(Anchovy)	Busan	B-2	6.16	30
	Busan	B-3	6.17	26
	Seoul	Se-1	5.65	40
	Seoul	Se-2	6.15	34
	Seoul	Se-3	5.98	36
	Yeosu	Y-1	5.66	36

[a]Samples were purchased from four different regions of Korea: Busan, Seoul, Sokcho, and Yeosu.

[b]Markets in each region are abbreviated as follows: Busan: B; Seoul: Se; Sokcho: So; and Yeosu: Y.

Grouping of Jeotgal Isolates by SDS-PAGE Whole Cell Protein Patterns

A total of 327 isolates, comprising 139 and 188 colonies from the galchi-jeotgal samples, were collected on MRS agar and MRS agar+5% NaCl, respectively, whereas 395 colonies, comprising 170 and 225 isolates from the myeolchi-jeotgal samples, were isolated on MRS agar and MRS agar+5% NaCl agar, respectively. These isolates were subjected to SDS-PAGE to observe whole cell protein patterns. Galchi-jeotgal isolates grown on MRS agar and MRS agar+5% NaCl could be differentiated into 8 and 7 groups, respectively, whereas myeolchi-jeotgal isolates grown on both agars could be differentiated into 8 and 9 groups, respectively, based on these whole cell protein patterns (Fig. S1).

Identification of Jeotgal Isolates by 16S rRNA gene sequencing

Based on the whole cell protein patterns, two isolates from each protein pattern group were randomly selected and identified using 16S rRNA gene sequencing. These two isolates were confirmed to be the same species in all cases. Galchi-jeotgal isolates in the eight groups grown on MRS agar were identified as six LAB, whereas those in the seven groups grown on MRS agar+5% NaCl were identified as four LAB and one non-LAB (Table 2). The galchi-jeotgal samples showed a more diverse community of LAB when isolates were grown on MRS agar than when grown on MRS agar+5% NaCl. On MRS agar, *Pediococcus acidilactici* (71 isolates, 51.1%) was predominant, followed by *Pediococcus pentosaceus* (36 isolates, 25.9%), whereas *Tetragenococcus halophilus* (105 isolates, 55.9%) was predominant on MRS agar+5% NaCl. Among all galchi-jeotgal isolates, *P. acidilactici*, *P. pentosaceus*, and *Leuconostoc mesenteroides* were detected in both agars. In contrast, *Enterococcus devriesei*, *Enterococcus faecium*, and *Weissella viridescens* were isolated only on MRS agar, whereas *T. halophilus* and *Staphylococcus epidermidis* were isolated only on MRS agar+5% NaCl. Among the myeolchi-jeotgal isolates that grew on MRS agar, four LAB and one non-LAB were identified from the eight differentiated protein groups (Table 2). A total of six LAB were identified from the nine groups of myeolchi-jeotgal isolates that grew on MRS agar+5% NaCl.

Among the isolates from myeolchi-jeotgal, *P. pentosaceus* (86 isolates, 50.6%) and *T. halophilus* (75 isolates, 33.3%) were predominant on MRS agar and MRS agar+5% NaCl, respectively. In addition, *Lactobacillus sakei*, *L. mesenteroides*, *P. pentosaceus*, and *T. halophilus* were isolated from both agars. *Staphylococcus epidermidis* was isolated only on MRS agar whereas *Lactobacillus curvatus* and *Weissella halotolerans* were isolated only on MRS agar+5% NaCl. The genus *Pediococcus* was isolated from both types of jeotgal and was the predominant isolate on MRS agar.

A previous study demonstrated that some *Pediococcus* species play an important role in fermentation and ripening of fermented foods [4]. Another study also reported that *P. acidilactici* from galchi-jeotgal was the predominant isolate on MRS agar and reduced the amount of histamine, a biogenic amine produced during the fermentation period [19]. The genus *Tetragenococcus* was isolated from both types of jeotgal when isolates were grown on MRS agar+5% NaCl. *T. halophilus* was known as *P. halophilus* in the past, but was reclassified to the genus *Tetragenococcus* on the basis of 16S rRNA gene sequencing [20]. *T. halophilus* is an important starter culture for fish sauce because this species not only increases the flavor and taste of fish sauce by producing various volatile compounds and amino acids as a result of proteolytic activity, but also reduces the amount of histamine during fermentation [19]. Additionally, *T. halophilus* has immune regulatory functions [21]. In a previous study, species in the genus *Tetragenococcus*, namely *T. halophilus* and *T. muriaticus*, were the predominant isolates from fermented seafood including myeolchi [4, 8, 22] and jaridom (*Chromis notatus*) [8]. *Tetragenococcus* has also been found in pla-ra and pa-daek, which are traditionally fermented seafood

Table 2. Isolates identified from galchi- and myeolchi-jeotgal samples by 16S rRNA gene sequencing and MALDI-TOF MS.

Sample	Concentration of NaCl in MRS agar[a]	16S rRNA gene sequencing (NCBI Accession No.)	Similarity (%)[b]	MALDI-TOF MS	Score value[c]	Ratio of distribution (%)[d]	Market (no. of isolates)
Galchi-jeotgal	0%	*Enterococcus devriesei* (GQ337023.1)	100	*Enterococcus devriesei* DSM 22802T	1.715	10.072	Se-1 (14)
		Enterococcus faecium (CP011828.1)	100	*Enterococcus faecium* 11037	2.379	0.719	Se-2 (1)
		Leuconostoc mesenteroides (KP764082.1)	100	*Leuconostoc mesenteroides* ssp. *mesenteroides* DSM20343T	2.201	10.792	Se-3 (15)
		Pediococcus acidilactici (KT275957.1)	100	*Pediococcus acidilactici* KCTC3101	2.619	51.079	So-1 (21), Y-1 (50)
		Pediococcus pentosaceus (KR010991.1)	100	*Pediococcus pentosaceus* DSM 20206	2.029	15.108	Se-2 (21)
		Pediococcus pentosaceus (KT327865.1)	100	*Pediococcus pentosaceus* DSM 20206	2.319	10.791	So-1 (15)
		Weissella viridescens (LC065637.1)	100	*Weissella viridescens* DSM 20410T	1.878	1.439	Se-2 (2)
			Subtotal			100.000	139
	5%	*Leuconostoc mesenteroides* (KP764082.1)	100	*Leuconostoc mesenteroides* ssp. *mesenteroides* DSM20343T	2.020	7.447	Se-3 (14)
		Pediococcus acidilactici (AP012046.1)	100	*Pediococcus acidilactici* KCTC3101	2.702	13.830	So-1 (26)
		Pediococcus pentosaceus (KR010991.1)	100	*Pediococcus pentosaceus* DSM 20206	1.809	9.043	Se-2 (17)
		Staphylococcus epidermidis (KT427443.1)	100	*Staphylococcus epidermidis* DSM 3269	2.173	13.830	Se-3 (26)
		Tetragenococcus halophilus (AP012046.1)	100	*Tetragenococcus halophilus* ATCC 33315	2.171	13.298	Y-1 (25)
		Tetragenococcus halophilus (KJ699138.1)	100	*Tetragenococcus halophilus* ATCC 33315	2.242	17.552	Se-2 (33)
		Tetragenococcus halophilus (LC071840.1)	100	*Tetragenococcus halophilus* ATCC 33315	2.171	25.000	Se-1 (47)
			Subtotal			100.000	188
			Total				327
Myeolchi-jeotgal	0%	*Lactobacillus sakei* (KJ026631.1)	100	*Lactobacillus sakei* ssp. *carnosus* DSM 15740	2.193	14.706	Se-1 (25)
		Lactobacillus sakei (KT327858.1)	100	*Lactobacillus sakei* DSM 20101	2.208	1.176	B-1 (2)
		Leuconostoc mesenteroides (KP764082.1)	100	*Leuconostoc mesenteroides* ssp. *mesenteroides* DSM20343T	2.16	6.471	Se-3 (11)
		Pediococcus pentosaceus (KP723364.1)	100	*Pediococcus pentosaceus* DSM 20206	2.117	32.353	B-1 (26), B-2 (29)
		Pediococcus pentosaceus (KT757261.1)	100	*Pediococcus pentosaceus* KCTC3507	2.182	18.235	Se-2 (31)
		Staphylococcus epidermidis (KJ571206.1)	100	*Staphylococcus epidermidis* DSM 4851	2.208	11.765	B-3 (20)
		Tetragenococcus halophilus (NR075020.1)	100	*Tetragenococcus halophilus* ATCC 33315	2.021	15.294	Y-1 (26)
			Subtotal			100.000	170
	5%	*Lactobacillus curvatus* (LC063167.1)	100	*Lactobacillus curvatus* DSM 20495	1.375	13.333	Se-2 (30)
		Lactobacillus sakei (KT327858.1)	100	*Lactobacillus sakei* ssp. *carnosus* DSM 15831T	2.238	17.778	B-3 (40)
		Leuconostoc mesenteroides (KP764082.1)	100	*Leuconostoc mesenteroides* ssp. *cremoris* DSM 20346T	2.168	10.222	Se-3 (23)
		Pediococcus pentosaceus (KT327865.1)	100	*Pediococcus pentosaceus* DSM 20206	2.020	12.889	B-2 (29)
		Tetragenococcus halophilus (KC170304.1)	100	*Tetragenococcus halophilus* ATCC 33315	2.021	4.444	Se-1 (10)
		Tetragenococcus halophilus (KJ699143.1)	100	*Tetragenococcus halophilus* ATCC 33315	2.033	5.333	Se-3 (12)
		Tetragenococcus halophilus (KP8452871)	100	*Tetragenococcus halophilus* ATCC 33315	2.348	12.444	Se-2 (28)
		Tetragenococcus halophilus (NR075020.1)	100	*Tetragenococcus halophilus* ATCC 33315	2.110	11.111	Y-1 (25)
		Weissella halotolerans (LC064886.1)	100	*Weissella halotolerans* DSM 20190T	2.004	12.444	B-1 (28)
			Subtotal			100.000	225
			Total				395

[a]Total concentration of NaCl added to MRS agar.

[b]Similarities of the 16S rRNA gene of identified strains to those present in the NCBI sequence database.

[c]Log score values were obtained as follows: ≥2.000: species-level identification; 1.700–1.999: genus-level identification; ≤1.699: not reliably identified.

[d]Ratio of distribution (%) = number of isolates / total number of isolates × 100.

produced in Thailand and Laos [2]. Previous research demonstrated that *Tetragenococcus* could only be cultured in media containing salt [4]. *Tetragenococcus* exhibits maximum growth at high salt concentrations (up to 20%) in the pH range from 5 to 6 [2, 4, 8, 22]. Consequently, some species of *Tetragenococcus* that have strong halophilic characteristics can be important for fermentation in galchi- and myeolchi-jeotgals.

Identification of Jeotgal Isolates by MALDI-TOF MS

Results of bacterial identification based on MALDI-TOF MS are shown in Table 2. Among the 30 representative isolates, 26 (86.7%) were identified to the species level (log score ≥ 2.0), whereas three (10.0%) were identified to the genus level (log score between 2.0 and 1.7). Exceptionally, only a single representative isolate (3.3%) of Se-2, representing *Lactobacillus curvatus* DSM 20495, had an unacceptable score value (1.375) and this was assumed to be caused by the limited reference strains available in the MALDI-TOF MS database [20]. Owing to the lack of *Pediococcus* and *Tetragenococcus* reference strains in the MALDI Biotyper database, we established a local database using three reference strains (*Pediococcus acidilactici* KCTC3101, *Pediococcus pentosaceus* KCTC3507, and *Tetragenococcus halophilus* ATCC 33315). Using this database, most *Pediococcus* and *Tetragenococcus* strains were identified with high score values.

All isolates identified using MALDI-TOF MS were consistent with those identified using 16S rRNA gene sequencing. Based on 16S rRNA gene sequencing, the representative strain of Se-1 isolated from galchi-jeotgal isolates that grew on MRS agar was identified as three species of *Enterococcus* (*E. avium, E. devriesei,* and *E. gilvus*), and the representative strain of B-3 isolated from myeolchi-jeotgal grown on MRS agar was identified as two species of *Staphylococcus* (*S. epidermidis* and *S. captis*). Those isolates were identified as *E. devriesei* and *S. epidermidis* on MALDI-TOF MS. In the bacterial communities present in galchi- and myeolchi-jeotgals identified through MALDI-TOF MS analysis, the genus *Pediococcus* was predominant on MRS agar, whereas the genus *Tetragenococcus* was predominant on MRS agar+5% NaCl. There was no relationship between the distribution of bacterial species and market location.

Identification of Jeotgal Isolates by PCR-DGGE

DGGE banding patterns of the bacterial V3 region of the 16S rRNA gene from each jeotgal sample are shown in Fig. 1, and sequencing results are summarized in Table 3. Bacterial communities in galchi-jeotgal were more diverse than those in myeolchi-jeotgal.

Five LAB, namely *Lb. sakei*, *Leuconostoc citreum, Leuconostoc gelidum, T. halophilus,* and *T. muriaticus,* and three non-LAB, including *Synechococcus* sp. and *Psychrobacter celer*, were identified from galchi-jeotgal. *Streptococcus* sp. was identified only to the genus level, and was therefore not classified as a lactic acid bacterium (Table 3). *T. halophilus* and *T. muriaticus* were present in all galchi-jeotgal samples. *T. halophilus* was represented by nine bands (no. 1, 2, 4, 7, 8, 11, 12, 13, and

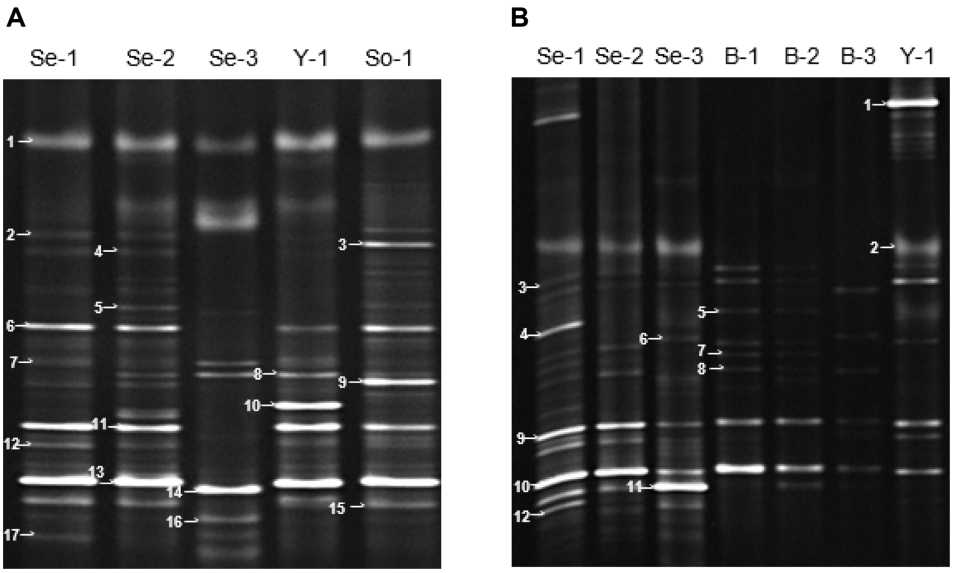

Fig. 1. PCR-DGGE profiles of the bacterial V3 region of the 16S rRNA gene from (**A**) galchi- and (**B**) myeolchi-jeotgal samples.
Sequencing results for excised bands are summarized in Table 3.

Table 3. Isolates present in galchi- and myeolchi-jeotgal samples identified by sequencing of the V3 region of the 16S rRNA gene after DGGE.

Bands (*n*)[a]	Galchi-jeotgal Species (NCBI Accession No.)	Identity[b]	Myeolchi-jeotgal Species (NCBI Accession No.)	Identity[b]
1	*Tetragenococcus halophilus* (KJ699143.1)	100%	*Weissella halotolerans* (LC064886.1)	100%
2	*Tetragenococcus halophilus* (KP845287.1)	98%	*Weissella halotolerans* (LC064886.1)	100%
3	*Leuconostoc citreum* (KU060303.1)	100%	*Tetragenococcus halophilus* (KJ699143.1)	100%
4	*Tetragenococcus halophilus* (LC071840.1)	100%	*Enterococcus* sp. (KJ394442.1)	96%
5	*Psychrobacter celer* (KR051247.1)	98%	*Weissella paramesenteroides* (JN863617.1)	98%
6	*Lactobacillus sakei* (KT626399.1)	99%	*Lactobacillus sakei* (JN851763.1)	100%
7	*Tetragenococcus halophilus* (NR114788.2)	100%	*Tetragenococcus halophilus* (KJ699143.1)	100%
8	*Tetragenococcus halophilus* (LC071840.1)	99%	*Tetragenococcus halophilus* (KJ699143.1)	98%
9	*Leuconostoc gelidum* (LN890331.1)	100%	*Tetragenococcus halophilus* (KJ699143.1)	100%
10	*Streptococcus* sp. (AB371944.1)	98%	*Tetragenococcus halophilus* (NR114788.2)	100%
11	*Tetragenococcus halophilus* (KJ699143.1)	99%	*Tetragenococcus muriaticus* (LC096225.1)	100%
12	*Tetragenococcus halophilus* (EU689055.1)	98%	*Tetragenococcus muriaticus* (LC096225.1)	100%
13	*Tetragenococcus halophilus* (NR114788.2)	100%	-	-
14	*Tetragenococcus halophilus* (KP997167.1)	99%	-	-
15	*Tetragenococcus muriaticus* (KM042034.1)	97%	-	-
16	*Tetragenococcus muriaticus* (KM042034.1)	100%	-	-
17	*Synechococcus* sp. (DQ023295.1)	95%	-	-

[a]Numbers of bands are indicated in Fig. 1.

[b]Percentage identity of the 16S rRNA gene sequence with the most closely related type strain.

14) of a total of 17 bands. In particular, bands no. 11 and 13 were in the same position as bands no. 9 and 10 of myeolchi-jeotgal and were identified as the same strains, respectively. *Lactobacillus sakei* (band no. 6) was detected in all galchi-jeotgal samples, with the exception of jeotgal from Se-3. *L. citreum* (band no. 3) and *L. gelidum* (band no. 9) were found in So-1. *Streptococcus* sp. (band no. 10) was detected from the product from Y-1. As non-LAB present in galchi-jeotgal, *P. celer* (band no. 5) and *Synechococcus* sp. (band no. 17) were detected in samples from markets Se-2 and Se-1, respectively. Comparison of the DGGE banding patterns for the five galchi-jeotgal samples revealed that the major bands were no. 6, 11, and 13, representing *Lb. sakei* and *T. halophilus*.

A total of five LAB (*Lb. sakei*, *T. halophilus*, *T. muriaticus*, *W. halotolerans*, and *W. paramesenteroides*) were identified from the myeolchi-jeotgal samples. *Enterococcus* sp. was identified to the genus level only and was therefore not classified as a lactic acid bacterium (Table 3). *T. halophilus* was detected in all myeolchi-jeotgal samples, and *Lb. sakei* was also detected in all myeolchi-jeotgal samples with the exception of the sample from market B-2. In addition, bands no. 1 and 2 of myeolchi-jeotgal samples from Se-1, Se-2, Se-3, and Y-1 markets were identified as *W. halotolerans*. *Enterococcus* sp. (band no. 4) and *W. paramesenteroides* (band no. 5) were detected only in myeolchi-jeotgal from Se-1 and B-1 markets, respectively. Five bands (no. 3, 7, 8, 9, and 10) that corresponded to *T. halophilus* were predominant. Bands no. 9 and 10 were relatively strong in all samples, indicating that *T. halophilus* was present in these samples at high concentration. Similarly, *T. muriaticus*, represented by bands no. 11 and 12, was also present at high concentration in myeolchi-jeotgal samples from Se-1, Se-2, and Se-3 markets. Myeolchi-jeotgal samples from Se-1 had the most diverse bacterial communities. *Lb. sakei*, *T. halophilus*, and *T. muriaticus* were predominant in both galchi- and myeolchi-jeotgal samples. *Leuconostoc* sp. and *Weissella* sp. were detected specifically in galchi- and myeolchi-jeotgal samples, respectively. According to previous studies, the reason for the difference in microbial communities was reported to be due to the microorganisms present in the raw materials of jeotgal samples [8, 17]. Therefore, galchi- and myeolchi-jeotgals showed the difference of microbial community composition as a result of their raw materials.

Many studies have shown that the bacterial community of kimchi is influenced by fermentation conditions and the

Table 4. Profile of bacteria identified in galchi- and myeolchi-jeotgal samples by culture-dependent and -independent methods.

Sample	Market	Bacterial strains identified by culture-dependent methods (*n*)[a]		Bacterial strains identified by culture-independent methods
		MRS	MRS+5% NaCl	
Galchi-jeotgal	Se-1	*Enterococcus devriesei* (14)	*Tetragenococcus halophilus* (47)	*Lactobacillus sakei* *Leuconostoc gelidum* *Synechococcus* sp. *Tetragenococcus halophilus* *Tetragenococcus muriaticus*
	Se-2	*Enterococcus faecium* (1) *Pediococcus pentosaceus* (21) *Weissella viridescens* (2)	*Pediococcus pentosaceus* (17) *Tetragenococcus halophilus* (33)	*Lactobacillus sakei* *Leuconostoc gelidum* *Psychrobacter celer* *Streptococcus* sp. *Tetragenococcus halophilus* *Tetragenococcus muriaticus*
	Se-3	*Leuconostoc mesenteroides* (15)	*Leuconostoc mesenteroides* (14) *Staphylococcus epidermidis* (26)	*Synechococcus* sp. *Tetragenococcus halophilus* *Tetragenococcus muriaticus*
	So-1	*Pediococcus acidilatici* (21) *Pediococcus pentosaceus* (15)	*Pediococcus acidilatici* (26)	*Lactobacillus sakei* *Leuconostoc citreum* *Leuconostoc gelidum* *Tetragenococcus halophilus* *Tetragenococcus muriaticus*
	Y-1	*Pediococcus acidilatici* (50)	*Tetragenococcus halophilus* (25)	*Lactobacillus sakei* *Streptococcus* sp. *Tetragenococcus halophilus* *Tetragenococcus muriaticus*
Myeolchi-jeotgal	B-1	*Lactobacillus sakei* (2) *Pediococcus pentosaceus* (26)	*Weissella halotolerans* (28)	*Lactobacillus sakei* *Tetragenococcus halophilus* *Weissella paramesenteroides*
	B-2	*Pediococcus pentosaceus* (29)	*Pediococcus pentosaceus* (29)	*Tetragenococcus halophilus* *Tetragenococcus muriaticus*
	B-3	*Staphylococcus epidermidis* (20)	*Lactobacillus sakei* (40)	*Lactobacillus sakei* *Tetragenococcus halophilus*
	Se-1	*Lactobacillus sakei* (25)	*Tetragenococcus halophilus* (10)	*Enterococcus* sp. *Lactobacillus sakei* *Tetragenococcus halophilus* *Tetragenococcus muriaticus* *Weissella halotolerans*
	Se-2	*Pediococcus pentosaceus* (31)	*Lactobacillus curvatus* (30) *Tetragenococcus halophilus* (28)	*Lactobacillus sakei* *Tetragenococcus halophilus* *Tetragenococcus muriaticus* *Weissella halotolerans*
	Se-3	*Leuconostoc mesenteroides* (11)	*Leuconostoc mesenteroides* (23) *Tetragenococcus halophilus* (12)	*Lactobacillus sakei* *Tetragenococcus halophilus* *Tetragenococcus muriaticus* *Weissella halotolerans*
	Y-1	*Tetragenococcus halophilus* (26)	*Tetragenococcus halophilus* (25)	*Lactobacillus sakei* *Tetragenococcus halophilus* *Weissella halotolerans*

[a]Number of identified isolates.

main ingredients, such as garlic, red pepper powder, salted cabbage, and jeotgal [23]. In a previous study, the predominant bacteria in kimchi were reported to be *Leuconostoc*, *Lactobacillus*, and *Weissella* at the genus level [23–27], which we detected in our study as well. Some species of *Weissella* are involved in the early stage of fermentation [23] and *Weissella* species were present mostly in myeolchi-jeotgal in our study. In addition, some species of *Leuconostoc* have been reported to be involved in the late stage of kimchi fermentation [23]; in our study, *Leuconostoc* species were detected mainly in galchi-jeotgal. In particular, *Lb. sakei*, *L. citreum*, and *L. gelidum* were identified in galchi-jeotgal samples based on PCR-DGGE banding patterns. These results are consistent with the bacterial community composition reported for kimchi [25, 27].

In the culture-dependent method, *Pediococcus* species was predominant in MRS agar in two jeotgal samples, and *Tetragenococcus halophilus* was predominant in MRS + 5% NaCl agar. In the culture-independent method, *Lb. sakei* and *T. halophilus* were the dominant species in both jeotgal samples, whereas *T. muriaticus* was the dominant species in only the myeolchi-jeotgal sample. Many species identified by culture-dependent methods were not found in the culture-independent method (Table 4). The reason seems to be due to the presence of low proportions in the sample (<1%) or incomplete DNA extraction or PCR biases [28, 29]. By contrast, the strain shown in the culture-independent method was not isolated because it is not a living strain.

In this study, bacterial communities in galchi- and myeolchi-jeotgal samples were investigated using 16S rRNA gene sequencing, MALDI-TOF MS, and PCR-DGGE. MALDI-TOF MS is an effective tool that can be used to analyze numerous samples in a short time in contrast with 16S rRNA gene sequencing. Additionally, more information about bacterial communities can be acquired by using PCR-DGGE together with a culture-dependent method such as 16S rRNA gene sequencing or MALDI-TOF MS. In our analysis of bacterial communities in galchi- and myeolchi-jeotgal samples, we identified species in the genera *Tetragenococcus*, *Pediococcus*, and *Lactobacillus* as the predominant LAB. The LAB isolated from jeotgal can potentially be used as starter cultures for jeotgal or kimchi.

Acknowledgments

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2017R1A6A3A11031293).

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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