Research Note

Heterofermentative Lactic Acid Bacteria Dominate in Korean Commercial Kimchi

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Abstract Lactic acid bacterial communities in kimchi prepared by representative Korean kimchi companies were investigated using a pyrosequencing method. The pH values of 9 kimchi varieties (K1-K9) ranged from 4.3 to 4.7, and total lactic acid bacterial (LAB) counts ranged from 1.3×10^7 to 1.6×10^9 CFU/g. *Weissella*, *Lactobacillus*, and *Leuconostoc* were the dominant genera accounting for 52, 28, and 20%, respectively, of identified genera. At the species level, *Weissella koreensis* (35%) dominated, followed by *Lactobacillus graminis* (13%), and *Weissella cibaria* (11%). Dominant LAB were all heterofermentative LAB. K1 had the most diverse operational taxonomic units (OTUs), and clustering analysis showed that distributions of microorganisms in K2, K6, K5, and K4 were similar each other. LAB communities in kimchi are diverse and heterofermentative LAB dominate in Korean commercial kimchi.

Keywords: kimchi, lactic acid bacteria, heterofermentative, pyrosequencing

Introduction

Interest in kimchi as a healthy food has increased globally as beneficial health effects have been confirmed. Kimchi has anticancer (1), anti-obesity (2), anti-oxidant (3), anti-aging (4), and anti-diabetic (5) properties, and kimchi contains high levels (10^{7-9} CFU/mL) of lactic acid bacteria (LAB). During the early and middle stages of kimchi fermentation, heterofermentative LAB (*Leuconostoc*) dominate, but with time pH values become acidic and numbers of homofermentative LAB (*Lactobacillus*) increase (6).

The percentage of consumption for home-made kimchi has decreased while the percentage of commercial kimchi has increased (40.0% in 2013) (7). Due to increasing demand and market expansion, some commercial kimchi varieties have been developed using starter inoculation for uniform quality, health functionality, and taste enhancement.

Previous studies have traditionally relied on culture-dependent methods for identification of microorganisms in kimchi. Recently, culture-independent methods have been developed (8-10), and the advent of next-generation sequencing technology (NGS) has enabled identification of microbial communities that were formerly unknown and has eliminated the need for replicate culturing. In this study, 454/Roche pyrosequencing analysis was used for exploration of lactic acid bacterial community dynamics in 9 kimchi varieties prepared by

representative Korean kimchi companies.

Materials and Methods

Kimchi preparation Nine varieties of the most widely consumed *baechu* kimchi made with *baechu* cabbage as a major ingredient by kimchi manufacturing companies in Korea were purchased from several on-line shopping mall sites and from off-line markets in Seoul, Korea in November of 2012. These 9 kimchies are referred to as K1 to K9. K1 (D Co., Hoengseong, Korea), K2 (C Co., Eumseong, Korea), K3 (D Co., Jincheon, Korea), and K4 (P Co., Yeongcheon, Korea) were inoculated with starter (K1: *Leu. mesenteroides*; K2 and K3: *Leu. citreum*; K4: *Lab. plantarum*) at 10^6 CFU/g and the other 5 kimchies (K5 (H Co., Seosan, Korea), K6 (N Co., Andong, Korea), K7 (O Co., Yangpyeong, Korea), K8 (L Co., Iksan, Korea), and K9 (D Co., Paju, Korea)) were naturally fermented. Kimchi samples were kept in plastic pouches for 5 days at 4°C to allow pH values to settle at approximately 4.3.

Measurement of pH and counting of total lactic acid bacteria Kimchi samples were dispensed into 3 polyethylene plastic bags in 1 kg portions for triplicate analyses. Homogenized kimchi soups were filtered through 4 layers of sterile coarse gauze (Daehan Co., Daegu,

Korea) to remove large particles. pH values of homogenized kimchi juice were measured with an M220 pH meter (Corning, Lowell, MA, USA). To obtain bacteria counts in CFU/g, kimchi juice samples were serially diluted 10x with 0.85% sterilized saline, inoculated (100 µL) on Bromocresol Purple (BCP) agar plates, and incubated at 35–37°C for 72±3 h. Numbers of yellow colonies were then counted (11).

DNA extraction, PCR amplification, and pyrosequencing Metagenomic DNA was extracted using a FastDNA Spin Kit (Qbiogen, Carlsbad, CA, USA) from homogenized kimchi samples according to manufacturer instructions. Extracted metagenomic DNA was used for pyrosequencing analysis following the method of Jung *et al.* (9). Briefly, PCR amplification was performed using a C1000 Touch thermal cycler (Bio-Rad, Foster, CA, USA) and barcoded fusion primers (<http://www.ezbiocloud.net/resource/M1001>). A total of 100 ng of template DNA was added to the PCR reaction to achieve a total of 50 µL, which contained Ex Taq buffer, 0.2 mM each dNTP, 0.5 µM each primer, and 2 units Ex Taq (all from Takara, Otsu, Japan). Amplification was conducted using initial denaturation at 95°C for 5 min, followed by 30 amplification cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a final elongation at 72°C for 5 min. PCR products were subjected to electrophoresis and visualized. QIAquick PCR purification kits (Qiagen, Valencia, CA, USA) were used for purification of amplification products. After pooling equal concentrations of purified products, short fragments of non-target products were removed. Product quality and size were measured using a 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA) and a DNA 7500 chip. Mixed amplicons were subjected to emulsion PCR, then deposited on Picotiter plates. Sequencing was carried out at Chunlab, Inc. (Seoul, Korea) using the GS Junior Sequencing system (Roche, Branford, CT, USA) following manufacturer instructions.

Pyrosequencing data analysis Basic pyrosequencing analysis was conducted as described previously (12). The Bellerophon method, which compares BLASTN search results of forward half and reverse half sequences (13), was used for detection of chimeric sequences. Taxonomic classification of each read was compared against the EzTaxon-e database (14), which contains the 16S rRNA gene sequences of type strains with valid published names and representative species

level phenotypes that are present in the GenBank database, with a thorough hierarchical taxonomic classification, following removal of chimeric sequences. Chao1 estimation and Shannon diversity indices at the 3% distance were used for determination of sample richness and diversity. For equalization of sample read sizes, random sub-sampling was performed. Fast UniFrac (15) was used for determination of overall phylogenetic distances between communities using principal coordinate analysis (PCoA). Shared OTUs were obtained based on XOR analysis using the CLcommunity program (Chunlab Inc.) for comparison of OTUs between samples.

Results and Discussion

Kimchi bacterial pyrosequences at the genus and species levels A summary of pyrosequencing results is provided in Table 1. All kimchi varieties were pyrosequenced, and LAB accounted for approximately 100% of bacterial sequences. A total of 78,674 reads were pyrosequenced from 9 kimchi varieties, and 57,316 valid reads (72.9% of total reads) with an average length of ~481 bp were obtained.

The mean pH value of the studied 9 varieties was 4.4, and the average total LAB count was 7.7×10^8 CFU/g (Table 2). LAB counts of starter-inoculated and non-inoculated kimchi varieties were similar and, thus, pH affected total LAB counts more than starter inoculation. Of all genera identified in kimchi samples, *Weissella* at 52.1%, *Lactobacillus* at 27.9%, and *Leuconostoc* at 19.8% dominated (Table 2), and in the 6 kimchi varieties K2, K3, K4, K5, K6, and K8, *Weissella* was dominant. Varieties K1 and K9 contained 45.2 and 54.4% *Lactobacillus* sp., respectively, and K7 contained 98.8% *Leuconostoc* sp. Recent studies (8,10) have also reported that these 3 genera dominate.

In the 9 kimchi varieties studied, abundances at the species level were *Weissella* (*Wei. koreensis*) (34.4%), *Lactobacillus* (*Lab. graminis*) (12.5%), *Wei. cibaria* (11.2%), *Lab. sakei* (9.2%), *Leuconostoc* (*Leu. gelidum*) (8.9%), *Leu. mesenteroides* (3.2%), and others (20.6%) (Table 2). *Wei. koreensis* was most abundant in K2 (59.8%), K3 (86.1%), K4 (43.8%), K5 (39.6%), and K6 (56.2%). *Lab. graminis* was most abundant in K9 (41.0%), and *Wei. cibaria* was most abundant in K8 (85.4%). *Lab. sakei* and *Leu. gelidum* dominated in K1 (23.7%) and K7

Table 1. Summary of pyrosequencing

Variety	Total reads	Valid reads	Average length (bp)	OTUs	Chao1	ACE	Shannon	Simpson
K1	11,932	6,704	481	366	520	602	3.88	0.063
K2	10,178	6,864	486	98	140	126	1.92	0.303
K3	3,936	3,261	484	39	47	53	0.82	0.706
K4	6,639	4,351	486	141	172	186	2.73	0.157
K5	9,446	5,860	483	145	204	188	2.62	0.179
K6	4,506	3,334	484	47	65	77	1.87	0.287
K7	13,519	11,995	486	65	91	115	1.63	0.345
K8	13,552	11,587	458	77	158	114	1.10	0.553
K9	4,966	3,360	483	91	139	159	2.42	0.184

Table 2. pH values, LAB colony-forming units (CFU), and identity of bacterial communities in kimchi at the genus and species levels

	K1	K2	K3	K4	K5	K6	K7	K8	K9	Mean
pH	4.3	4.3	4.4	4.3	4.3	4.4	4.6	4.7	4.3	4.4
Total LAB (CFU/g)	9.0×10 ⁸	5.6×10 ⁸	1.2×10 ⁹	1.7×10 ⁹	9.5×10 ⁸	9.6×10 ⁸	1.4×10 ⁷	2.4×10 ⁷	5.9×10 ⁸	7.7×10 ⁸
Genus										
<i>Weissella</i>	14.1	68.6	91	49.9	50.9	60.5	0.6	97.7	36	52.1
<i>Lactobacillus</i>	45.2	26.8	6.3	37.9	40.6	37.8	0	1.9	54.4	27.9
<i>Leuconostoc</i>	40.5	4.4	2.5	12.2	8.5	1.6	98.8	0.3	9.4	19.8
Others	0.2	0.1	0.2	0.1	0	0.1	0.5	0.1	0.1	0.2
Species										
<i>Wei. koreensis</i>	5.4	59.8	86.1	43.8	39.6	56.2	0.6	0.2	18	34.4
<i>Wei. cibaria</i>	0.6	0.1	0.2	0.3	1.5	0.5	0	85.4	12.2	11.2
<i>Wei. confusa</i>	0	0	0	0	0	0	0	3.4	0.3	0.4
other <i>Weissella</i>	24.7	7.3	3	7.6	11.2	3.5	0.6	5.4	8	7.9
<i>Lab. graminis</i>	0.6	15.3	0.2	6.6	15.3	33.7	0	0	41	12.5
<i>Lab. sakei</i>	23.7	7.2	5.5	24.4	15.9	1.3	0	0	5	9.2
other <i>Lactobacillus</i>	9.7	2.8	1.2	3.1	4.4	1.3	0.3	1.9	3	3.1
<i>Leu. gelidum</i>	0.1	0	0	0.8	0.1	0	79.3	0	0	8.9
<i>Leu. mesenteroides</i>	12.5	1.9	0.2	2.6	3.3	1.1	1.6	0.1	5.7	3.2
<i>Leu. inhae</i>	0	0	0	0	0	0	15.5	0	0	1.7
<i>Leu. citreum</i>	0.2	0.9	0.1	5.6	0.6	0	0	0	1.7	1
<i>Leu. gasicomitatum</i>	3.6	0	1.5	0.2	1.1	0	1.4	0	0	0.9
<i>Leu. kimchii</i>	3.7	0	0	0	0.1	0	0	0	0	0.4
other <i>Leuconostoc</i>	14.9	4.5	1.8	4.5	6.8	2.2	0.3	3.5	5	4.8
Others	0.1	0.1	0.2	0.4	0.1	0.2	0.4	0.1	0.2	0.2
Total	99.8	99.9	100	99.9	100	100	100	100	100.1	100

(79.3%), respectively. All LAB strains, except classification as other or etc. (Table 2), were obligate (*Weissella* sp. and *Leuconostoc* sp.) or facultative (*Lab. graminis* and *Lab. sakei*) heterofermentative LAB (16,17) that grow well at low temperatures and in low acidity media to produce lactate, acetate, ethanol, and CO₂, and that produce a better kimchi taste than homofermentative lactic acid producing LAB.

The genus *Leuconostoc* appears during the early stage of kimchi fermentation, whereas the genera *Lactobacillus* and *Weissella* appear during the late stage because these taxa are acid-tolerant (6). The 9 kimchi varieties represented the optimum fermentation ripening stage of pH approximately 4.4. K1 was inoculated with *Leu. mesenteroides*, but this species did not dominate with an abundance of only 12.5%. Similarly, starter-inoculated varieties K2, K3, and K4 did not show starter dominance. The abundance of starter strains *Leu. citreum* in K2 and K3, and *Lab. plantarum* in K4, were probably reduced in abundance as pH decreased, and abundances of acid-tolerant *Lactobacillus* and *Weissella* species increased during fermentation. Starter strains in optimally ripened kimchi have been detected (18,19), but the kimchi samples used were prepared at laboratory scales.

Lab. plantarum is representative of the later stage of kimchi fermentation (6). However, *Lab. plantarum* was not detected in quantity in any kimchi sample in this study. In particular, K4 was inoculated with *Lab. plantarum*, but *Wei. koreensis* dominated. Kim

and Chun (8) failed to find *Lab. plantarum* in commercial kimchi, probably attributable to different kimchi ingredients and different analytical and identification methods. Meanwhile, *Lab. graminis* is not known as a dominant LAB in kimchi, but recent study showed that this strain is present in kimchi in the early stage of fermentation when the pyrosequencing method was applied (20).

Studies conducted on the microbiota of kimchi prior to the year 2000 do not mention the genus *Weissella*, which was classified by Collins *et al.* (21), or *Leu. paramesenteroides*. Actually, *Wei. koreensis*, *Wei. cibaria*, and *Wei. confusa* belong to the genus *Weissella*, which belongs to the family *Leuconostocaceae*. Using new culture-independent methods, the genus *Weissella* was confirmed as present at important levels in kimchi (8-10). Several recent studies have reported health functional effects, such as exopolysaccharide production and anti-obesity effects, of *Weissella* species isolated from kimchi (22,23), although more studies are needed.

Comparison of bacterial community structure Rarefaction curves of the 9 kimchi varieties are shown in Fig. 1A. A steep curve gradient indicates diverse operational taxonomic units (OTUs). The gradient of the curve for K1 was steepest, and K1 had the most diverse OTUs with 366. On the other hand, the K3 curve rose gently toward the horizontal and K3 had only 39 OTUs, which was the simplest LAB composition. These tendencies coincided with the diversity of species in each kimchi variety shown in Table 2.

Fig. 1. Diversity of OTUs and similarity of kimchi varieties. Rarefaction curves showing diversity in operational taxonomic units (OTUs), (A) a community tree showing similarities (B) between kimchi varieties.

Beta-diversity analysis based on Fast UniFrac for all varieties for determination of relationships among kimchi types was carried out for construction of an unweighted pair group method with arithmetic mean (UPGMA) dendrogram (Fig. 1B). Clustering analysis revealed that K7 had a more unique taxonomic composition than other varieties. K3 and K8 shared a similar bacterial structure. LAB communities in K2, K4, K5, and K6 were similar. K2 and K3 exhibited different LAB communities although both were *Leu. citreum*-inoculated varieties, suggesting that LAB communities in kimchi are influenced by other factors, such as ingredients, temperature, salt concentration, container type, or other variables (16). Park *et al.* (10) concluded that kimchi microflora are determined largely by pH rather than by ingredients or starter inoculation.

In conclusion, LAB communities were found to be highly diverse in different varieties of kimchi with *Weissella*, *Lactobacillus*, and *Leuconostoc* as the most abundant genera. All bacterial strains detected were heterofermentative LAB, which can grow actively at low temperature. *Weissella* was the dominant genus and *Wei. koreensis* dominated. Furthermore, LAB compositions of commercial kimchi are affected by ingredients and the fermentation environment, and by pH conditions. Detailed studies are needed on the probiotic characteristics and properties of *Weissella*, and more research is needed on use of LAB starters and on efficient methods of inoculation in order to reproducibly prepare healthy and functional kimchi.

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