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Comparison of Bacterial Community Changes in Fermenting *Kimchi* at Two Different Temperatures Using a Denaturing Gradient Gel Electrophoresis Analysis

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A polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) technique followed by sequencing of the 16S rDNA fragments eluted from the bands of interest on denaturing gradient gels was used to monitor changes in the bacterial microflora of two commercial *kimchi*, salted cabbage, and ingredient mix samples during 30 days of fermentation at 4°C and 10°C. *Leuconostoc* (*Lc.*) was the dominant lactic acid bacteria (LAB) over *Lactobacillus* (*Lb.*) species at 4°C. *Weissella confusa* was detected in the ingredient mix and also in *kimchi* samples throughout fermentation in both samples at 4°C and 10°C. *Lc. gelidum* was detected as the dominant LAB at 4°C in both samples. The temperature affected the LAB profile of *kimchi* by varing the pH, which was primarily caused by the temperature-dependent competition among different LAB species in *kimchi*. At 4°C, the sample variations in pH and titratable acidity were more conspicuous owing to the delayed growth of LAB. Temperature affected only initial decreases in pH and initial increases in viable cell counts, but affected both the initial increases and final values of titratable acidity. The initial microflora in the *kimchi* sample was probably determined by the microflora of the ingredient mix, not by that of the salted cabbage. The microbial distributions in the samples used in this study resembled across the different *kimchi* samples and the different fermentation temperatures as the numbers of LAB increased and titratable acidity decreased.

Key words: Bacterial community, PCR-DGGE, *kimchi*, lactic acid bacteria

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Kimchi is a traditional Korean fermented condiment that is made with vegetables such as Chinese cabbage and minor ingredients such as garlic, green onion, and red pepper powder. The majority of *kimchi* fermentation is natural and, thus, wild microorganisms in the vegetables and ingredients compete with each other under the presence of salt at the beginning of fermentation.

The wild microorganisms initially present in the major and minor ingredients include lactic acid bacteria (LAB), aerobic bacteria, and yeasts [4, 21]. Changes in microbial communities during fermentation are continuous and are closely related to salt concentration and fermentation temperature [21, 25, 30]. During *kimchi* fermentation, LAB is primarily responsible for supressing the growth of aerobic bacteria through acid production and is creating the typical flavor profile of *kimchi* by producing acids, ethanol, mannitol, and CO_2 [14]. Tolerances to differing levels of acids are also distinct for different LAB groups. During the earlier stage of *kimchi* fermentation, the pH is relatively high and the LAB profile is diverse. However, in later stages, the pH is typically lower than 4.0 [21] and the LAB profile becomes simple and occupied primarily by *Lactobacillus plantarum*, which is possibly due to the extinction of less acid-tolerant LAB such as the *Leuconostoc* species. *Leuconostoc* (*Lc.*), *Weissella* (*W.*), *Lactobacillus* (*Lb.*), and *Pediococcus* (*P.*) species are major LAB in *kimchi* that have been consistently studied [10, 24]. *Weissella* and *Leuconostoc* species are also the dominant LAB in green onion and garlic [9, 14], which are basic minor ingredients in *kimchi*. *Lb. plantarum* in *kimchi* is not typically desired because it causes a soft texture and extreme sourness [19, 21, 29]. Yeasts also grow in over-ripened *kimchi* at a pH lower than 4.0, but the quality of *kimchi* at this stage is generally not acceptable owing to its extreme sourness and off-flavors [19, 21].

Temperature affects the quality and shelf-life of *kimchi* by impacting the growth of LAB and the relative growth rate of each LAB group. Mheen and Kwon [21] compared the sensory quality of *kimchi* at low (5°C and 14°C) and high (20°C and 30°C) fermentation temperatures and reported that it was better at low temperatures. They also found that the acidity remained at an acceptable level after 30 days when the *kimchi* was fermented at 10°C, but not at 15°C and 20°C [21]. Chung *et al.* [6] estimated the shelf-life of *kimchi* at four different fermentation temperatures and reported that the estimated shelf-life at 4°C was approximately 10 times longer than that at 28°C. The lag time and the generation time of growth curve for each LAB group at a given fermentation condition are important for determining the relative growth of each LAB group. For example, So and Lee [29] reported that the lag time and generation time of *Lb. plantarum* were longer than those of *Lc. paramesenteroides* at 10°C, but were shorter at 30°C. They also found that a greater number of LAB viable cells were observed at 10°C and 20°C compared with higher temperatures [29]. According to these results, fermentation temperature clearly affects the initial competition among LAB groups and, consequently, affects the overall changes in microflora and quality of *kimchi*.

LAB are often fastidious on artificial plate media even though they grow actively in fermenting food substrates. The polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) of 16S rRNA followed by 16S rDNA sequencing analysis has been used as an effective culture-independent identification method to detect key LAB in fermented food samples [12, 13], since it implies direct DNA extraction from samples instead of cultivating the fastidious LAB and has been known as a useful tool to study the microbial diversity in biological samples [22]. Lee *et al*. [17] analyzed *kimchi* microflora at two different fermentation temperatures (10°C and 20°C) by PCR-DGGE. However, at fermentation temperatures higher than 10°C, changes in microflora may be too fast to examine specifically, based on reports by Ku *et al*. [16] and Shin *et al*. [27]. Ku *et al*. [16] found that the pH of *kimchi* reached 4.2 in about 2 days at 15°C and in only 1 day at 25°C. Shin *et al*. [27] also reported that the pH of *kimchi* reached to within 3 days at 15°C and 2 days at 25°C. The range of temperature for relatively fast *kimchi* fermentation is 10–25°C and the fermentation is considered relatively slow below 10°C in reports [6, 16, 17, 21, 29]. Slow fermentation is often required for mass production of *kimchi*, unless *kimchi* is stored at or slightly lower than 0°C after the fermentation and until it is consumed. Maintaining the storage temperature to 0°C would not be easy and would be expensive for *kimchi* manufacturers. Comparative information on microflora in *kimchi* under the low and high fermentation temperatures is thus important, but the difference in the microflora in this specific range of fermentation temperatures has not been analyzed by a culture-independent identification method.

The purpose of this study was to evaluate the effect of relatively low and high temperatures on bacterial microflora in fermenting *kimchi* and *kimchi* ingredients. To examine these bacterial community changes closely, 4°C and 10°C were chosen as fermentation temperatures based on previous reports [6, 16, 21, 27, 29]. Salted Chinese cabbage and a minor ingredient mix were collected from two commercial *kimchi* producers to track the microbial transition in between the commercial *kimchi* materials and *kimchi*. *Kimchi* was prepared by combining salted cabbage and minor ingredient mix in the laboratory. Microflora was analyzed using a culture-independent method and was monitored during fermentation. It was also compared with the initial microflora in *kimchi* materials. Changes in pH, titratable acidity, and viable cell numbers in fermenting *kimchi* were also monitored for better comprehension of bacterial community changes.

Materials and Methods

Samples

Salted cabbage and *kimchi* ingredient mix were packed with ice gel pouches and were provided by the *kimchi* companies H and W on the same day of production in June 2012. All ingredients were used immediately for *kimchi* preparation. The salted cabbage (1.5 kg) was cut and combined with the ingredient mix (1 kg) thoroughly and was fermented at 4°C and 10°C in 3-liter-plastic containers. Salt concentrations for the cabbage were 2% for sample H and 1.5% for sample W. The contents of the ingredient mix from the two companies were dycon, red pepper powder, red pepper, garlic, green onion, ginger, onion, salted shrimp, fish sauce, and sugar. The percentage of each component of the ingredient mix was available only for dycon (54.1% for sample H and 36.3% for sample W). During the 30 days of fermentation, samples were taken every 3 days and were analyzed with control (day 0) samples, salted cabbage, and ingredient mix.

Measurement of pH, Titratable Acidity, and Viable Cell Counts

For pH and titratable acidity measurements, each sample (50 g) was ground using a blender (HM-1300G; Hibell, Hwaseong, Gyeonggi, Korea) and was filtered through two layers of gauze. The pH of 30 ml of filtrate was measured in triplicate using a pH meter (Orion Star Series; Thermo Scientific, Beverly, MA, USA). For titratable acidity, 1 ml of filtrate was diluted with 50 ml of deionized water and was titrated with 0.1 N NaOH until the pH reached 8.3 in triplicate. The milliliter of 0.1 N NaOH was converted into the percentage of lactic acid (%, v/v). For viable cell counts, each sample (10 g) was diluted with 90 ml of sterilized water and was put into Stomacher® filter bags (BA6141/STR; Seward, Worthing West, Sussex, England). Samples were homogenized for 30 s at 230 rpm using the Stomacher® (Circulator stomacher 400, Seward). After 30 min of settling, 100 µl of the supernatant was inoculated on a de Man Rogosa Sharpe (MRS) plate (Difco, Becton & Dickinson, Sparks, MD, USA) and a plate count agar (PCA) plate (Difco) after

serial dilution. The MRS plates were placed into an air-tight container with Anaeropack (Mitsubishi Gas Chemical, Tokyo, Japan) and both plates were incubated for 36 h at 30°C before counting colonies. This was done in duplicate.

PCR-DGGE and Sequence Analysis of 16S rDNA

The PCR-DGGE method used has been previously described [13]. The same diluted and homogenized sample that was used for viable cell count was used for DNA extraction. After homogenization, samples were filtered through two layers of cheese cloth and were centrifuged at 14,000 ×*g* for 15 min at 4°C. The pellet was washed twice with sterile water and then subjected to DNA extraction using the Genomic DNA Prep kit (SolGent, Daejeon, Korea). The yield and quality of DNA were analyzed electrophoretically on 1% (w/v) agarose gel. Extracted DNA was then used as a template for PCR to amplify the 16S rRNA gene.

The 16S rRNA gene was amplified using 16S universal primers (Bionics Co., Ltd., Seoul, Korea) by PCR. The sequence of the forward primer (27F) was 5'-AGAGTTTGATCCTGGCTCAG-3' and of the reverse primer (1492R) was 5'-GGCTACCTTGTTACGACTT-3'. PCR (PC808; Astec, Fukuoka, Japan) was repeated for 30 cycles with conditions of pre-denaturing for 5 min at 95°C, annealing for 1 min at 45°C, and extension for 2 min at 72°C. The final extension was done for 10 min at 72°C and 0.1 μl of Taq polymerase (5 U/μl; Takara Biotechnology, Otsu, Shiga, Japan) was used. The PCR products were purified using a QIA quick PCR purification kit (QIAGEN, Valencia, CA, USA) and were used as templates for a nested PCR targeting the V3 region of the 16S rRNA gene. The DNA fragment suitable for denaturing gradient gel electrophoresis (DGGE) was obtained using DGGE primers of GC-338f and GC-518r. The sequence of the forward primer (GC-338f) was 5'-CGC CCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGAC TCCTACGGGAGGCAGCAG-3' and of the reverse primer (GC-518r) was 5'-ATTACCGCGGCTGCTGG-3'. PCR amplification was done in a final volume of 25 μl containing 5 μl of template, 2.5 μl of 10× PCR buffer, 2 μl of dNTP mixture (2.5 mM each), 0.1 μl of *Taq* polymerase (5 U/μl; Takara Biotechnology, Otsu, Shiga, Japan), and a final concentration of 0.4 pM of each primer. Reactions were performed in a Mastercycler (Eppendorf, Hamburg, Germany). PCR products were directly applied onto 8% (w/v) polyacrylamide gels in a running buffer containing 1× TAE [20 mM Tris, 10 mM acetate, 0.5 mM EDTA (pH 8.0)] and a denaturing gradient of 20% to 50% urea and formamide. Gels were run on a Dcode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA) for 30 min at 40 V and for 15.5 h at 60 V. Gels were stained with ethidium bromide for 30 min and the gel images were captured using a digital camera (COOLPIX 4300; Nikon, Japan) attached to a transilluminator (SL-20 Image Visualizer; Seoul, Korea).

Bands of interest were excised from the gel using a sterile blade and incubated overnight at 4°C in ultrafiltered water to allow for DNA diffusion out from the polyacrylamide matrix. The solution was then used directly for further amplifications. Excised bands were reamplified using the DGGE primers of GC-338f and GC-518r and re-run on denaturing gradient gels to confirm their identity and to improve the purity prior to sequencing. For sequencing, the eluted DNA was amplified using 27F and 1492R primers. PCR products for sequencing were purified using the QIAquick PCR purification kit (QIAGEN, Valencia, CA, USA). The samples were analyzed with an automated DNA sequencer (Jenotech, Daejeon, Korea). Searches in GenBank with BLAST [1] were performed to determine the closest known relatives of the partial rDNA sequences obtained.

Cluster analysis of PCR-DGGE patterns of the 16S V3 rRNA gene was performed with the numerical taxonomic system using a multivariate statistics program (NTSYSpc, ver. 2.02j; Exeter Software, Setauket, NY, USA) [28]. For each sample, numerical data were constructed by expressing each DGGE band that represents a particular microorganism as being present (value 1) or not present (value 0). Jaccard's similarity coefficient was estimated using NTSYS pc software with the numerical data converted from the DGGE band patterns. Jaccard's coefficients are defined as a/(a+b+c), where a is the number of positive matches (*i.e.*, bands common to two samples), and b and c refer to the number of bands present only in each sample, respectively [31]. The unweighted pair group method using arithmetic averages (UPGMA) clustering was performed using the SHAN option of NTSYSpc, and a dendrogram representing the relationship among samples was derived from the TREE option.

RESULTS AND DISCUSSION

Changes in pH, Titratable Acidity, and Viable Cell Count

Changes in pH and titratable acidity are shown in Fig. 1. As predicted, the decrease in pH and increase in titratable acidity were slow at 4°C (Fig. 1). Variations in both pH and titratable acidity changes between the two different samples were small at 10°C but large at 4°C (Fig. 1). At 10°C, it took about 6 days for sample H and about 9 days for sample W to reach a pH of 4.2, which was reported as the optimum pH of *kimchi* with the highest sensory quality [21]. However, it took about 18 days for sample H and about 27 days for sample W to reach a pH of 4.2 at 4°C (Fig. 1). The titratable acidities of samples H and W were

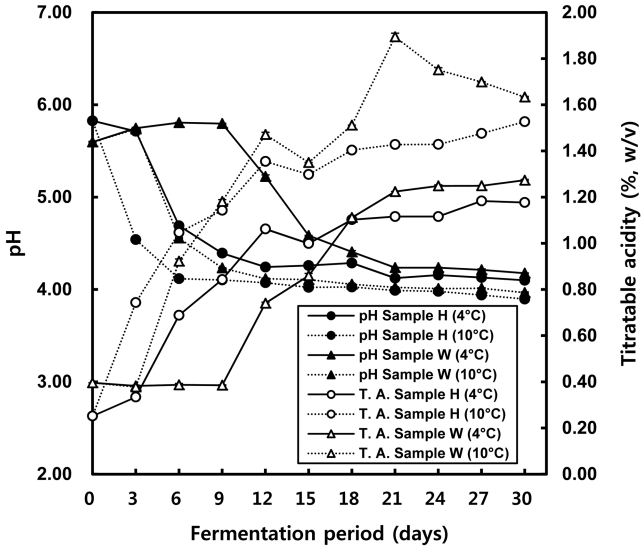

Fig. 1. Changes in pH and titratable acidity in H and W *kimchi* samples during fermentation at 4°C and 10°C.

1.05 and 0.92 at 10°C, but were 0.69 and 0.39 at 4°C on day 6 (Fig. 1). Thus, at low temperatures, the pH and acidity of fermenting *kimchi* may be more dependent on the initial physicochemical status of the *kimchi* ingredients because of the initial slow growth of LAB. Fermenting, distributing, and storing *kimchi* at 4°C can extend its shelf-life as long as the initial aerobic bacterial growth is under control. Previous study has reported an optimum titratable acidity of *kimchi* as 0.6%, which is lactic acid with a pH of 4.2 [21]. *Kimchi* quality is generally unacceptable when the pH is lower than 4.0 and titratable acidity is 1.5–2.0% [20]. In the present study, pH values at 4°C and 10°C were similar on day 30 (Fig. 1), but the titratable acidities at 10°C were at unacceptable levels compared with those at 4°C. Overall, the temperature affected the initial pH decrease more than the final pH in *kimchi,* and the titratable acidity was affected by the temperature at both the initial increase and final values.

The viable cell count on MRS and PCA plate media are shown in Fig. 2. There were temperature and sample differences in time elapsed to reach the maximum viable cell counts. Sample W required more time than sample H, and both samples at 4°C required more time than at 10°C to reach maximum viable cell counts (Fig. 2). There were no differences in maximum viable cell counts between samples and between temperatures in this study despite the difference in initial viable cell counts between samples H and W (Fig. 2). The viable cell counts in the salted cabbage and ingredient mix for sample H were similar to each other (about 10^6 CFU/ml, data not shown). In contrast, the viable cell counts in the salted cabbage for sample W were much lower (10^{2-4} CFU/ml, data not shown) than those in the ingredient mix (10^{6-7} CFU/ml, data not shown). Because of this imbalance in viable cell counts for sample W, the microflora in this sample would be expected to rely on the microflora from the ingredient mix rather than that of the salted cabbage. The times required to reach maximum viable cell counts on MRS and PCA plates were similar (Fig. 2).

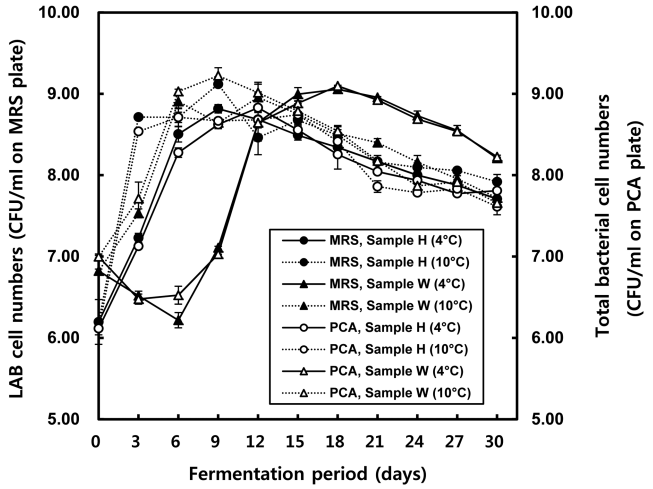

Fig. 2. Changes in LAB and total bacterial cell counts (Log CFU/ml) in H and W *kimchi* samples during fermentation at 4°C and 10°C.

Changes in Microbial Community in Fermenting *Kimchi* at 4°C and 10°C

The PCR-DGGE patterns of 16S rDNA are shown in Fig. 3A and 3B and the results for sequencing of the 16S rDNA fragments are listed in Table 1. Among the 22 different bands analyzed on the denaturing gradient gels, 17 were identified as LAB. These LAB included 8 *Lactobacillus*, 5 *Leuconostoc*, 2 *Weissella*, 1 *Lactococcus* (*L.*), and 1 *Streptococcus* (*S.*) species (Table 1). Such LAB strains, except for *Streptococcus* and *Lb. spicheri*, previously have been either isolated or detected in *kimchi* samples based on their 16S rRNA sequences [5, 10, 15, 17]. Some

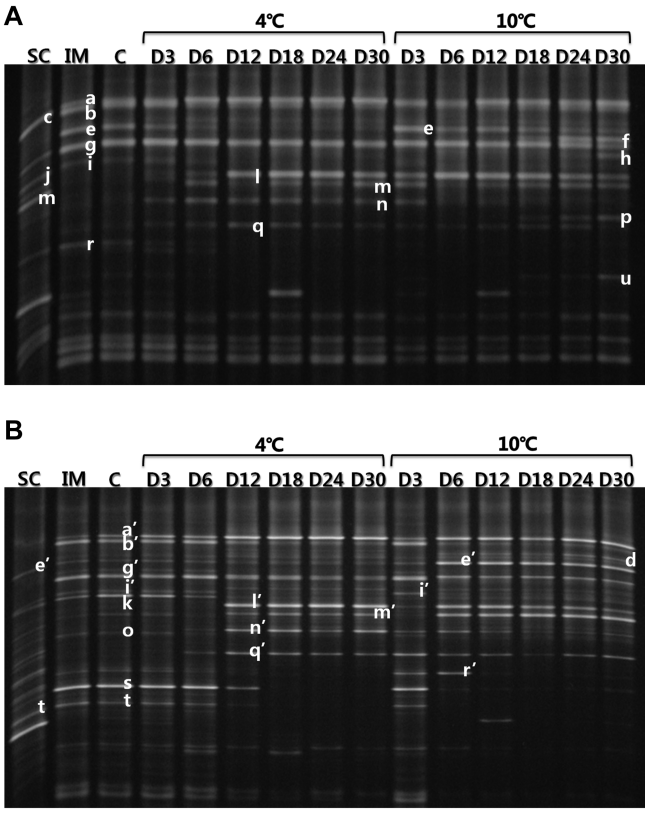

Fig. 3. PCR-DGGE patterns of 16S V3 rRNA gene sequences in sample H and W.
(**A**) PCR-DGGE patterns of 16S V3 rRNA gene sequences in sample H during fermentation at 4°C and 10°C using a 338f-GC clamp and 518r primer pair (SC, salted cabbage; IM, ingredient mix; C, day 0 control sample; D3–D30, fermentation period in days). Sequencing results of the excised fragments indicated as lowercase letters are listed in Table 1. (**B**) PCR-DGGE patterns of 16S V3 rRNA gene sequences in sample W during fermentation at 4°C and 10°C using a 338f-GC clamp and 518r primer pair (SC, salted cabbage; IM, ingredient mix; C, day 0 control sample; D3–D30, fermentation period in days). Sequencing results of the excised fragments indicated as lowercase letters are listed in Table 1.

Table 1. Identification of the bacteria in *kimchi* samples by sequencing the 16S V3 rDNA fragments excised from PCR-DGGE.

Bands[a]	Species identification	Homology (%)	Accession no.
a, a'	*W. confusa*	94	JX041938
b, b'	Uncultured bacterium	93	JQ986605
c	*Lb. pentosus*	95	AB548883
d	*W. koreensis*	95	CP002899
e, e'	*Lc. citreum*	97	JQ712017
f	*Lb. parabrevis*	95	FJ609222
g, g'	Uncultured bacterium	95	HQ905766
h	*Lb. parabrevis*	98	AB626068
i, i'	*Lc. mesenteroides*	97	GU458344
j	*Lb. brevis*	91	JX046150
k	*Lc. lactis*	97	JQ754450
l	*Lb. sakei*	97	DQ412704
m, m'	*Lb. curvatus*	95	AB600200
n, n'	*Lc. gelidum*	92	JF756111
o	Uncultured bacterium	92	GU513213
p	*Lb. plantarum*	91	JN671595
q, q'	*Lc. carnosum*	97	JF756282
r, r'	*L. lactis*	95	GU428033
s	*S. salivarius*	93	AM157451
t, t'	*B. subtilis*	90	JX035948
u	*Lb. spicheri*	92	AB626069

[a]Band designation refers to Fig. 3A and 3B.

of the LAB also have been found in garlic or green onion samples [9, 11]. The microflora in sample H included 4 *Leuconostoc* and 2 *Lactobacillus* species at 4°C, but 3 *Leuconostoc* and 6 *Lactobacillus* species at 10°C (Fig. 3A). In sample W, which showed a slower fermentation (Fig. 1 and 2), there were no differences in microflora at the two different temperatures. However, there were more *Leuconostoc* than *Lactobacillus* species in sample W at both temperatures (Fig. 3B). This suggests that *Leuconostoc* is likely dominant over *Lactobacillus* in a slow fermenting *kimchi*. In general, LAB were slightly more diverse at 10°C but the difference was not significant (Fig. 3A and 3B).

Lc. carnosum (band q in Fig. 3A and Table 1) and *Lc. gelidum* (band n in Fig. 3A and Table 1) were two major strains at 4°C in sample H. *Lc. lactis* (band k in Fig. 3B and Table 1) and *Lc. gelidum* (band n' in Fig. 3B and Table 1) were two major strains at 4°C in sample W. *Streptococcus* (band s in Fig. 3B) was also dominant in sample W at 4°C, but it may be that this strain was decreasing along with the pH decrease rather than being distinct in samples at 4°C because it also appeared in the D3 sample at 10°C (Fig. 3B and Table 1). *Lc. citreum* (band e in Fig. 3A and e' in Fig. 3B) was a distinct strain at 10°C for sample W and C-D12 samples of H at 10°C. *Lb. sakei* (band l in Fig. 3A and Table 1) was a distinct strain in sample H at 10°C and in the later stage samples of H at 4°C, which was under the decreased pH condition.

Changes in the LAB profile during fermentation are shown in Table 2. Viable cell counts for sample W at 4°C were low, expecially on MRS until day 9 (Fig. 2). *Streptococcus* and *Bacillus* were detected in sample W at 4°C only prior to the increase of LAB viable cell counts (Table 2). The pH of *kimchi* at the time of disappearance of these two strains was about 4.5 (Fig. 1). *Lc. citreum* was detected in samples H and W only at 10°C (Table 2). It is likey then that 10°C is more suitable for *Lc. citreum* to grow. A previous report indicates that the doubling times for *Lc. citreum* are 51.5 h at 4°C and 4.8 h at 10°C [7]. In our study, it was the dominant LAB in sample W for a longer period of time than in sample H at 10°C, possibly due to the delayed pH decrease in sample W (Table 2 and Fig. 1). In a report by Park *et al*. [23], *Lc. citreum* was detected as the dominant LAB in *kimchi* with a pH higher than 4.7, which is a likely explanation for the results of the present study.

Lc. mesenteroides existed in the ingredient mix and fermenting samples for H and W only until the pH reached to 4.5–4.7 (Table 2 and Fig. 1). With the faster pH decrease at 10°C, the extinction of the *Lc. mesenteroides* was faster. The disappearance of this strain is thought to be due to the low pH that had been caused by a higher temperature, since there were few differences in the growth of *Lc. mesenteroides* at 4°C and 10°C in a previous report [7]. This strain has been reported to be a dominant LAB at the early stage of *kimchi* fermentation that inhibits the growth of aerobic bacteria by lowering the pH of *kimchi*, and the delayed the propagation of *Lactobacillus* when it was inoculated as a starter culture in *kimchi* [8]. It does not exist in *kimchi* at a pH of 4.2 or lower in a report by Park *et al*. [23]. In Fig. 1, the timing of the pH that was closest to the lowest value was similar to the point of the initial decrease in viable cell count in this study (Fig. 2). In other words, when the pH was close to 4.0, some of the LAB, especially the *Leuconostoc* species, might not have been able to survive as the dominant LAB. *Lc. citreum* and *Lc. mesenteroides* have been found in garlic and green onion [9, 11] and were also detected in the salted cabbage and ingredient mix in this study.

In the present study, *Lc. carnosum* was also present until the later stage of *kimchi* fermentation (Table 2). Previous studies on *Lc. carnosum* have evaluated the spoilage of vacuum-packaged meat products [2] or chill-stored meats [26]. It has been inconsistantely isolated from *kimchi* samples, but its role in *kimchi* fermentation is not yet clear [5]. *Lc. gelidum* has been found as a dominant LAB in *kimchi* samples at low temperatures [5, 17, 23]. This strain has also been found as an undesirable contaminant in modified atmosphere packaged raw meat under increased pH [3]. In our study, it was particularly dominant in sample W with a pH of 4.2 or higher at 4°C (Table 2 and Fig. 1). *Lc. lactis* was also detected in the salted cabbage, ingredient

Table 2. Changes in bacteria profile[a] in H and W *kimchi* samples during fermentation at 4°C and 10°C.

LAB strains identified	Sample H														Sample W													
				Fermentation temperatures														Fermentation temperatures										
				4°C						10°C								4°C						10°C				
	Salted cabbage	Ingredient mix	Control sample[b]	Fermentation period (days)						Fermentation period (days)						Salted cabbage	Ingredient mix	Control sample[b]	Fermentation period (days)						Fermentation period (days)				
				3	6	12	18	24	30	3	6	12	18	24	30				3	6	12	18	24	30	3	6	12	18	24	30
W. confusa	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Uncultured bacterium	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	+	-	-	-	-	-
Lb. pentosus	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
W. koreensis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Lc. citreum	-	+	+	+	-	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	+	+	+	+	+
Lb. parabrevis[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Uncultured bacterium	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lb. parabrevis[d]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lc. mesenteroides	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	+	-	-	-	-	-
Lb. brevis	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lc. lactis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
Lc. sakei	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	-	+	+	+	+	+
Lb. curvatus	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	-	+	+	+	+	+
Lc. gelidum	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-
Uncultured bacterium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
Lb. plantarum	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lc. carnosum	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
L. lactis	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
S. salivarius	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	+	-	-	-	-	-
B. subtilis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	+	-	-	-	-	-
Lb. spicheri	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a]+, detected; -, not detected.
[b]Day 0 sample. [c]Band f in Fig. 3A. [d]Band h in Fig. 3A.

mix, and samples at an earlier stage of fermentation in sample W (Table 2).

Among the *Lactobacillus* species, *Lb. pentosus*, *Lb. brevis*, and *Lb. curvatus* were detected in *kimchi* materials but two of these were not detected in samples at all (Table 2). Among the three strains, only *Lb. curvatus* was detected in samples at the early to mid stages of fermentation, depending on the fermentation rate of each sample, and persisted until the end of fermentation (Table 2). *Lb. curvatus* has been reported to be the dominant culturable LAB in garlic [9]. *Lb. curvatus* and *Lb. sakei* have also been reported to be the dominant culturable LAB in mid to late stages of *kimchi* fermentation [18, 23]. In the present study, *Lb. sakei* showed a similar existence pattern to *Lb. curvatus* (Table 2). Moreover, *Lb. brevis* was not detected in any sample at both temperatures, except for the salted cabbage from sample H, because of the low fermentation temperature. A previous study has found *Lb. brevis* in *kimchi* samples fermented at 20°C, but not at 10°C [17]. In our study, *Lb. plantarum*, *Lb. parabrevis*, and *Lb. spicheri* were detected only in samples H that were fermented at 10°C and when the pH was close to 4.0 (Table 2 and Fig. 1). It may be that *Lactobacillus* species were not detected in sample W because of the relatively slow decreases in pH (Table 2 and Fig. 1). According to the report by Hamasaki *et al*. [7], the growth of *Lb. plantarum* at 4°C is greatly suppressed compared with its growth at 10°C. The different results for sample H at 4°C and 10°C in this study are congruent with results reported by Hamasaki *et al*. [7]. Park *et al*. [23] also analyzed LAB in fermenting *kimchi* at 4°C using PCR-DGGE, and both *Lb. brevis* and *Lb. plantarum* were not detected.

W. confusa was detected in the ingredient mix and all samples, but was not detected in the salted cabbage for both samples H and W (Table 2). This strain seemed to persist at varied temperatures and pH values. More importantly, it has been reported to be a dominant LAB in garlic and green onion [9], which explains the fact that it was detected in the ingredient mix but not in the salted cabbage. These results suggest that the origin of this strain in *kimchi* is likely from the garlic or green onion. There were two bands (band a/a' and g/g') that were consistently detected in all samples (Fig. 3A and 3B). The former was identified as *W. confusa* and the latter was identified as an uncultured bacterium.

UPGMA Clustering of *Kimchi* Samples and Ingredients Based on the PCR-DGGE Band Patterns and the Sequencing Results of the 16S V3 rDNA Gene

Similarities of microbial distributions among samples were checked by deriving a dendrogram (Fig. 4) based on the DGGE patterns (Fig. 3A and 3B) and the identification results (Table 2). *Kimchi* samples at the initial stage of fermentation (HCS, H4D3, WCS, W4D3, W4D6, and W10D3 in Fig. 4), ingredient mixes (HIM and WIM in Fig. 4), and a salted cabbage (WSC in Fig. 4) showed less than 60% of similarity in microflora to other *kimchi* samples at later stages of fermentation. The titratable acidity and viable LAB cell numbers in those *kimchi* samples at the initial stages were below 0.4 and 10^8 CFU/ml, but those values were significantly increased to 0.7–0.9 and over 10^8 CFU/ml in samples at later stages of fermentation (Fig. 1 and 2). Thus, the change of microflora is thought to be closely related to the change of LAB population and titratable acidity. The microflora in *kimchi* samples at the late stage of 10°C fermentation (H10D18–H10D30 in Fig. 4) were separated from others, showing less than 77% similarity, and were probably related to their low pH (< 4.0) and high titratable acidity (> 1.4%) in Fig. 1. The microflora in H *kimchi* samples at mid stage (H10D6, H10D12, and H4D12 in Fig. 4) were more than 94% similar to those in W *kimchi* samples at the late stage (W10D18–W10D30, W4D24, and W4D30 in Fig. 4). This shows that the microflora in the relatively slow-fermenting W sample mostly dictate those in the relatively fast-fermenting S sample once the pH is decreased and the titratable acidity is increased (Fig. 1).

As shown in Fig. 4, the salted cabbage sample from H company (HSC) possessed somewhat different microflora from the ingredient mix H (HIM) and day 0 control sample of H company (HCS), showing less than 60% of similarity. However, the microflora in day 0 control samples (HCS and WCS in Fig. 4) and those in ingredient mix samples (HIM and WIM in Fig. 4) showed higher than 94% of similarity (Fig. 4), which suggests that the initial microflora in *kimchi* sample H should have been determined by the microflora of ingredient mix H (HIM), not by the salted cabbage H (HSC).

In conclusion, the microbial distribution in *kimchi* at the initial stage of fermentation was mainly affected by the microbial distribution of *kimchi* materials and varied by samples and temperatures (Fig. 4). However, once LAB grew over 10^8 CFU/ml (Fig. 2) and dominated the microbial community, the microbial distribution was less varied, even though the rate of changes was different (Fig. 4). The initial microbial distribution in *kimchi*, again, depended more on that in the ingredient mix than that in salted cabbage, as previously stated (Fig. 1 and 4). The dominance of LAB in *kimchi* in this study primarily depended on the variation in pH that was determined by the varied growth rate of LAB under different temperatures. Since pH decreases and acid production are caused mainly by the growth of various LAB that have different tolerance levels for acid, it is possible that temperature affects the initial competition among different LAB species in *kimchi*. Additionally, at a higher temperature (10°C), LAB should have grown actively enough to depress the sample variation in pH and titratable acidity (Fig. 1). However, the sample variation at

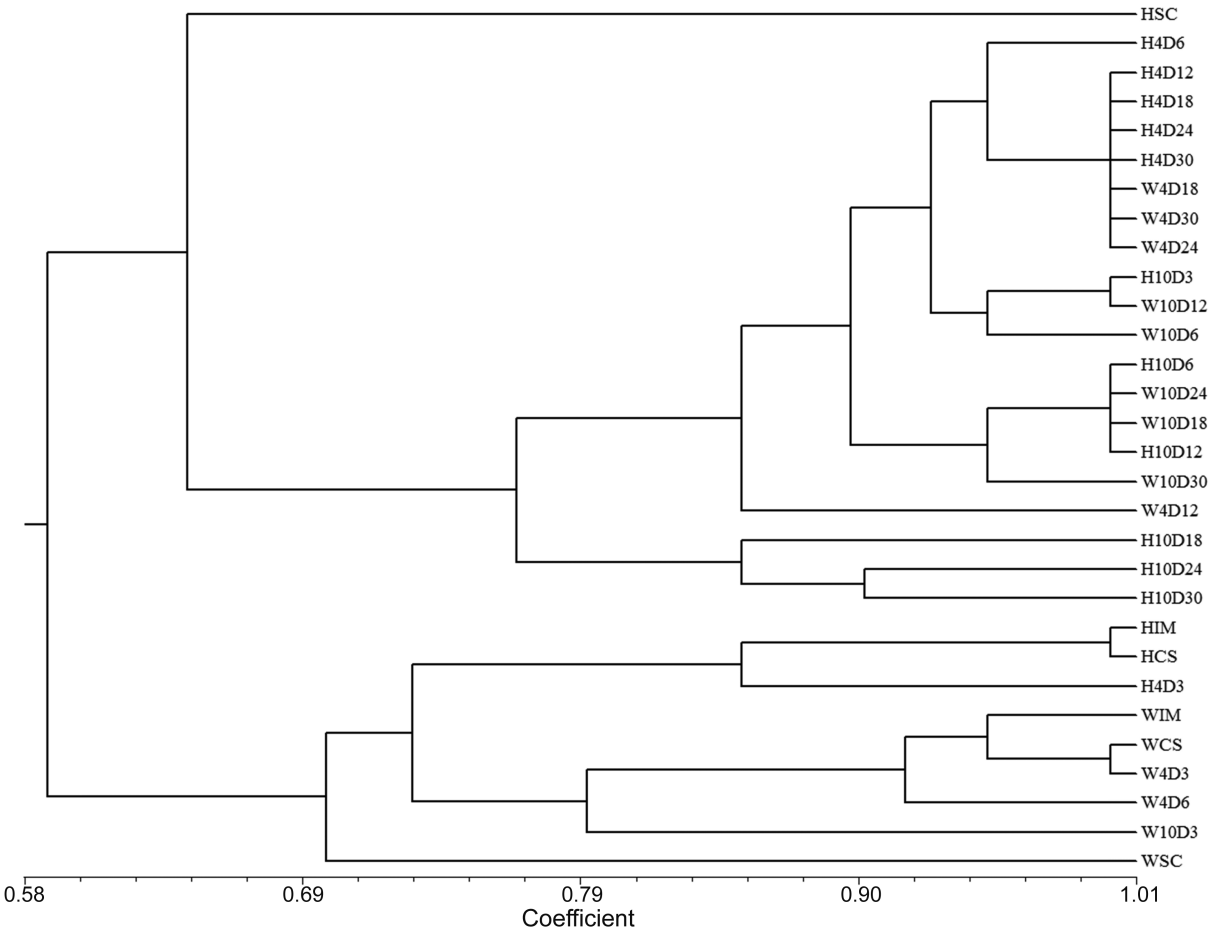

Fig. 4. Dendrogram derived from the UPGMA clustering of correlation coefficients based on the microbial distribution in *kimchi* materials and *kimchi* samples.
The similarity is indicated as a correlation coefficient at the bottom.

4°C was more conspicuous (Fig. 1), possibly due to the delayed growth of LAB at the lower temperature. This delay of initial LAB propagation at low temperature should be counted meaningfully when the *kimchi* fermentation would be done at low temperature, especially with LAB starter cultures. The culture-independent identification method used in this study was an efficient way to monitor the key changes in LAB distribution. The individual change of each LAB species in *kimchi* samples under each given fermentation condition would be able to be investigated through applying more sensitive sequencing techniques, such as pyrosequencing, once the specific role of each LAB group under the specific fermentation condition is revealed and the target LAB are established.

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