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Bacterial Community Monitoring of Commercial Kimchi Produced in Korea and China with Evidence of Bacilli Spore Formation during Fermentation

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Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis was adopted to explore rapid differentiation in the diversity and dynamics of bacteria in kimchi made in Korea and China for future application in kimchi origin discrimination. T-RFLP analysis supported the reproducible and rapid detection of major lactic acid bacteria known to be involved in kimchi fermentation. The taxonomic resolution level of this T-RFLP analysis was between the species and genus level, but was not specific enough for the detection of a bacterium found only in one origin, either Korea or China. The bacterial community structure successions in kimchi samples from Korea and China analyzed by T-RFLP analysis occurred with a similar pattern. *Bacillus* spp. which were not detected in the early microbial studies of kimchi were constantly detected until the late fermentation stage of kimchi in our T-RFLP analysis and their existence was proved by culture-based identification. Additionally, sporulation of *Bacillus* spp. during kimchi fermentation was discovered.

Keywords: Kimchi, bacterial community, T-RFLP, *Bacillus*, spore formation

Introduction

Kimchi is the generic term given to a group of fermented vegetable foods in Korea. Kimchi is classified according to major ingredients such as Chinese cabbage, radish, cucumber, and other vegetables. Its fermentation depends on the indigenous microflora on these raw materials. Kimchi has traditionally been made at home, but has begun to be produced industrially as the increased demand by institutional food services follows the rapid growth of industry in the 1970's. In addition, the demand for commercial kimchi is increasing due to economic growth, changes in family structure, the development of the food processing industry and the increasing number of women working outside the home. At the 1988 Seoul Olympics, kimchi was introduced to many foreign countries and became a global export. Since the registration of kimchi on Codex Alimentarius in 2001, its status has been elevated to a global food, and its market continues to expand worldwide [4].

Currently, Korean imports of price-competitive kimchi are sharply increasing due to the expansion of agricultural imports and the increased demand of the domestic food service industry. According to a survey by the Korea International Trade Association (http://www.kita.net) and the Korea Agro-Fisheries Trade Corp. (http://www.at.or.kr), domestic kimchi demand in 2008 was estimated to be about 1,640,000 tons. Imported kimchi accounted for about 220,000 tons, mostly from China. As imports of the low-priced Chinese kimchi increase, the kimchi industry in Korea is greatly impacted, affecting production, supply and price of raw materials. Some distributors intentionally mislabel the imported Chinese kimchi as domestically-made kimchi which harms the Korean kimchi industry.

According to the report by the Korea Food and Drug

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Administration (KFDA, http://www.kfda.go.kr/) from 2005 to July, 2009, 178 cases of imported kimchi were judged to be unsuitable for import due to sanitation violations. Of those cases, 89 had foreign substances, 36 violated import declarations, 32 used prohibited food additives, 18 violated the food additive usage standards, 2 were considered decomposed or spoiled and 1 case violated the standards for preservation and distribution. It is feared that the distribution of low-quality and falsely marked kimchi may lead to a decline in the reliability of commercial kimchi, cause distrust between producers and customers, and decrease the competitiveness of Korean-made kimchi.

In order to solve these problems, to promote the establishment of healthy distribution orders and to provide reliable information about commercial kimchi to customers, the Ministry for Food, Agriculture, Forestry and Fisheries of Korea (http://www.mifaff.go.kr) has implemented a mark-of-origin regulation for kimchi. This regulation affected restaurants whose sizes were larger than 100 m^2 from December 28, 2008, and extends to all restaurants since August 11, 2010. However, due to the absence of scientific methods that can distinguish the origin of kimchi, the authenticity of geographical origins is not verifiable. According to the survey on kimchi R&D research papers [8], studies on kimchi have been conducted to develop manufacturing techniques and processes for quality assurance, preservation, and packaging. Additionally, studies on the ingredients, the microorganisms involved in fermentation, and the health functions of kimchi have been conducted. No study has been performed to assert the differences of domestic kimchi against the imported kimchi in spite of the rapid growth of Chinese imports.

Our laboratory employed T-RFLP (terminal restriction fragment length polymorphism), a popular culture-independent microbial community analysis, to determine its suitability as a rapid differentiation method to discriminate between the bacteria found in kimchi made in Korea and China.

Materials and Methods

Bacterial strains and growth conditions

The type strains of *Lactobacillus plantarum, Lactobacillus sakei, Leuconostoc mesenteroides, Weissella cibaria, Weissella confusa*, and *Weissella soli* used for references in this study were purchased from the Korean Collection for Type Culture (KCTC). Reference strain *Weissella koreensis* KK0101 was isolated from kimchi and identified phylogenetically by 16S ribosomal RNA gene (rDNA) sequence analysis. Lactic acid bacteria (LAB) were grown at 30°C in MRS broth (Difco, USA) under facultative-anaerobic conditions and agar was added at 1.6% (w/v) concentration when needed.

Kimchi samples and DNA extraction

Commercial *Baechukimchi*, the most common type of kimchi, manufactured in Korea and China was purchased in Korea. Seven Korean kimchi samples were purchased at local supermarkets in Gyeonggi area and the same numbers of Chinese kimchi samples produced by different companies in China were purchased at the wholesale market in Anyang, Korea. Sample times were days 0, 3, and 30. Samples were stored at 10°C between samplings. Each sample was filtered through sterilized gauze to collect the fluid portion. pH was measured with a pH meter. Cells in the filtrate were concentrated by centrifugation. Total DNA was extracted with a Bacterial Genomic DNA Miniprep Kit (Axygen, USA). The extracted DNA was quantitatively evaluated using spectrophotometry and stored at −20°C.

PCR for T-RFLP and restriction digest

The conserved eubacterial forward primer 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and the reverse primer 16R (5'-TGA CGG GCG GTG TGT ACA AG-3'), valid for the analysis of lactic acid bacterial community in kimchi, were used for the amplification of 16S rDNA [14]. Primer 27F was labeled with 6-FAM (6-carboxyfluorescein, Takara, Japan). For PCR, each 100 ml of reaction mixture contained 100 ng template DNA, each primer at a concentration of 20 pmol, 0.25 mM dNTPs, and 1 U of *Taq* polymerase (Roche, Germany). The reaction mixtures were amplified in a T3000 Thermocycler (Biometra, Germany) using the following program: 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 58°C for 1 min, 72°C for 1 min, and a final extension period of 72°C for 5 min. The expected length of amplified product of approximately 1.5 kb was confirmed by electrophoresis on 0.8% agarose gel and the fragment was purified with a Gel & PCR purification system (SolGent, Korea). Purified PCR products were digested with 10 U of *Alu*I, *Hae*III, *Mse*I, and *Msp*I (New England Biolabs, USA) for 5 h at 37°C.

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T-RFLP analysis

The lengths for terminal-restriction fragments (T-RFs) were analyzed by electrophoresis on a model ABI PRISM 3100 Genetic Analyser (Applied Biosystems, USA) in GeneScan mode. Fragment sizes were estimated by the Local Southern method in Peak Scanner Software v1.0 (Applied Biosystems). GS-500 and GS-1000 ROX (Applied Biosystems) were used as the internal standards. T-RFLP analysis for each sample was repeated three times independently and the proportions of each T-RF were calculated as the mean percentages of the total T-RF area on the graph. Compiled data were exported to Excel and relative peak areas were calculated. Peaks with an area below 1% of total were considered as background noise and with a size less than 50 bp were excluded from further analysis. Peaks that were not present in the triplicate analyses were considered as PCR artifacts. T-RFLP profiles were aligned by identifying and grouping homologous fragments and normalized by calculating relative abundances of each T-RF from peak area. Combining data from each restriction enzyme, the normalized T-RFLP profiles were compared. Dominant T-RFs were assigned to bacterial species using the virtual digest tool at the MiCA III website (http://mica.ibest.uidaho.edu/). Restriction analysis of the available sequences in the RDP-II and GenBank databases was performed by Webcutter 2.0, an on-line tool for restriction mapping nucleotide sequences (http://bio.lundberg.gu.se/cutter2/).

Enumeration and confirmation of spore forming bacteria in kimchi

To prove the existence of bacilli until the late stage of kimchi, a freshly made kimchi sample was purchased at a local supermarket in Gyeonggi area and fermented at 10°C. Sampling began at date of purchase and continued until day 31. The filtrate from kimchi was spread on tryptic soy agar (TSA, Difco) which is generally used for the enumeration and isolation of *Bacillus* spp. After 24 h-incubation at 30°C, cells were enumerated. Endospore-forming bacteria were enumerated on TSA plates after heat treatment of the filtrate at 73°C for 30 sec. Twenty different types of colonies were collected from agar plates based on differences in their morphology and growth characteristics, then purified by successive transfer on TSA. The identity of the isolates was confirmed by 16S rDNA sequence analysis.

Identification of the isolates by 16S rDNA sequence analysis

Colony isolate genomic DNA was extracted using a Bacterial Genomic DNA Miniprep Kit (Axygen). Amplification of 16S rDNA was performed with universal primers, 27F and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') using a T3000 Thermocycler (Biometra). The PCR mixture consisted of the template DNA, 0.5 mM of each primer, 1 U of *Taq* polymerase (Roche), 100 mM dNTPs, and 2.5 mM $MgCl_2$. Samples were preheated for 5 min at 95°C and then amplified using 30 cycles of 1 min at 95°C, 1 min at 65°C, and 1 min at 72°C. The PCR products were purified using a Gel and PCR purification system (SolGent) and sequenced using a custom service provided by SolGent. The 16S rDNA sequence similarities were searched using the BLAST programs in the National Center for Biotechnology Information database and EzTaxon server 2.1 [3]. The taxonomic identities of the isolates were determined by 16S rDNA sequence analysis.

Results

T-RFLP profiles of Korean kimchi and Chinese kimchi

T-RFLP analysis was applied to 14 kimchi samples manufactured in Korea and China to evaluate its potential as a culture-independent assay to define the difference of bacteria in two origins of fermented vegetables (Table 1). After PCR, restriction enzymes *Alu*I, *Hae*III, *Mse*I, and *Msp*I were applied to cut the amplified 16S rDNA to see if there were differences between T-RF profiles of Korean and Chinese kimchi samples. Each restriction enzyme produced different lengths and numbers of T-RFs, but the numbers of major T-RFs produced by each enzyme were almost identical, regardless of kimchi origins (Fig. 1). Specific T-RFs were only detected from one sample, but the T-RFs were not kimchi origin specific. We could not discern a specific T-RF which is consistent with either Korean or Chinese kimchi.

Analysis of phylotypes (OTUs, operational taxonomic units) detected by T-RFLP

We detected 3-5 (median 4) T-RFs per community DNA preparation with *Alu*I, 8-10 (median 9) with *Hae*III, 5-7 (median 6) with *Mse*I, and 5-6 (median 5.5) with *Msp*I (data not shown). Among the four enzymes, *Hae*III and *Mse*I pro-

Table 1. The pHs of kimchi samples manufactured in Korea and China used in this study.

Kimchi origin	Sample number[a]	Fermentation time (day)[b]		
		0	3	30
Korea	1	4.4	4.2	3.9
	2	4.7	4.4	4.0
	3	5.7	4.9	4.1
	4	5.8	4.9	3.7
	5	6.2	5.5	3.9
	6	5.2	4.7	3.6
	7	6.0	4.8	4.1
China	1	4.8	4.5	4.0
	2	5.4	4.8	4.1
	3	5.9	4.9	4.1
	4	5.4	4.8	4.2
	5	4.8	4.6	4.5
	6	6.2	4.6	4.1
	7	5.6	4.5	4.1

[a]Kimchi samples were discretionally numbered.
[b]Commercial kimchi samples were fermented at 10°C; Day 0 = date of purchase.

duced more T-RFs than the other two enzymes. The major T-RFs obtained by *Hae*III corresponded to the predicted T-RFs: 236, 266, 310, 323, 326, 333, and 340 bp by *in silico* analysis (Fig. 1). Band lengths obtained with *Mse*I also corresponded to the predicted T-RFs: 75, 183, 200, 228, 458, 598, 604, and 616 bp. By combining T-RFs produced by *Hae*III and *Mse*I, the existence of a maximum of 16 species of LAB was predicted together with bacilli (Table 2). The additional use of *Alu*I and *Msp*I bands did not increase the numbers of identified bacteria. The T-RFLP analysis with reference strains produced the expected T-RFs without producing unrestricted and pseudo T-RFs. The maximum T-RF length difference with the predicted size was 4 bp (data not shown). The primers and two enzymes used in this study could detect and discriminate the dominant LAB found in kimchi: *Leuconostoc* spp., *Lactobacillus* spp., *Weissella* spp., *Enterococcus* spp., *Pediococcus* spp., and *Bacillus* spp. The assay was not able to identify minorities existing in the early stage of kimchi such as species in the genera, *Pseudomonas* and *Serratia* [9]. The taxonomic res-

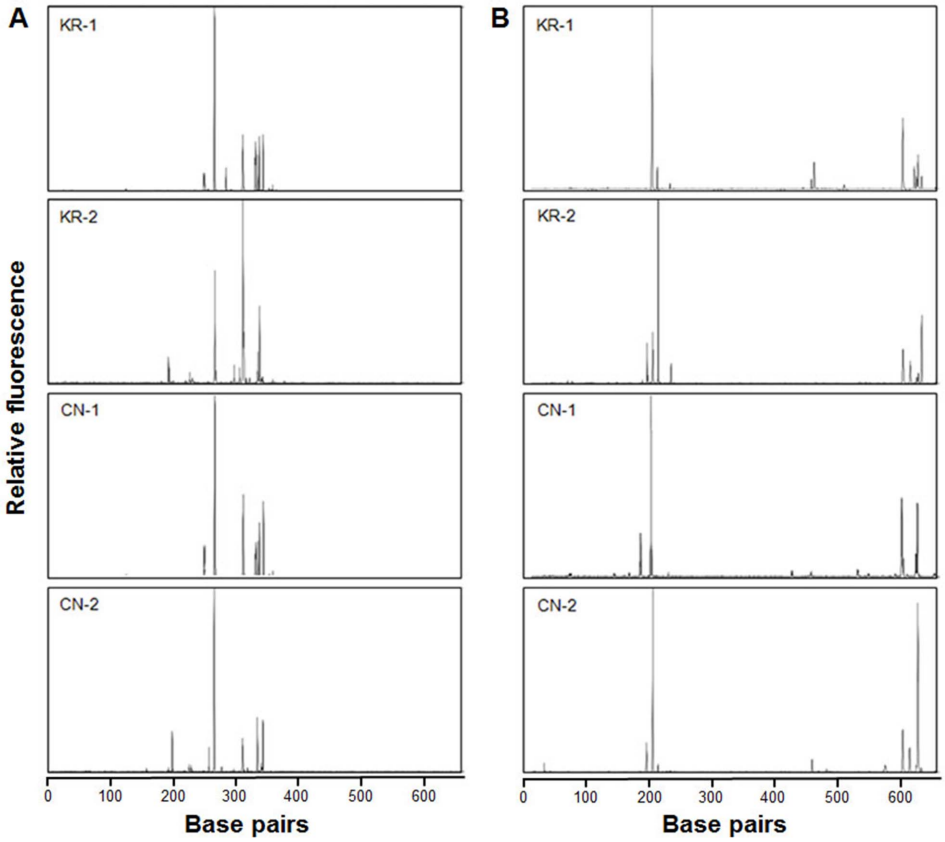

Fig. 1. Representative T-RFLP profiles generated by *Hae*III (A) and *Mse*I (B) digestions of 16S rDNAs amplified from the total DNAs of kimchi manufactured in Korea (KR) and China (CN).

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Table 2. Predicted genus or species in kimchi samples based on the predicted sizes of T-RFs.

Predicted 5'-T-RF size (bp)		Accession number	Predicted genus or species in database	OTU group[a]
*Hae*III	*Mse*I			
236/310	598	-	*Bacillus* spp.	BC
266	200	AF439560	*Leuconostoc inhae* KCTC3774	LM
266	200	CP000414	*Leuconostoc mesenteroides* ATCC8293^T	LM
310	200	AF111948	*Leuconostoc citreum* ATCC49370	LC
310	200	AF173986	*Leuconostoc kimchii* KCTC2386	LC
323	604	AJ301831	*Enterococcus faecalis* LMG7937	Others
326	616	AJ306297	*Lactobacillus paraplantarum* ATCC700211^T	LP
326	616	D79211	*Lactobacillus pentosus* ATCC8041^T	LP
326	616	ACGZ01000098	*Lactobacillus plantarum* ATCC14917^T	LP
333	458	AM113777	*Lactobacillus curvatus* DSM20019	LS
333	458	AM113778	*Lactobacillus graminis* DSM20719	LS
333	458	AM113784	*Lactobacillus sakei* ATCC15521	LS
340	75	AJ305321	*Pediococcus pentosaceus* ATCC33316	Others
340	75	AY028260	*Weissella soli* DSM14420	Others
340	228	AJ295989	*Weissella cibaria* LMG17699^T	WC
340	228	AB023241	*Weissella confusa* JCM1093	WC
340	183	AY035891	*Weissella koreensis* KCTC3621	WK

[a]Mainly detected the T-RFs correspond to more than two closely related OTUs and that of *W. koreensis* are presented as OTU group and minor T-RFs are combined as others.

olution level of T-RFLP analysis was between species and genus.

Changes in bacterial community structures in Korean and Chinese kimchi during fermentation

We monitored the bacterial community structure changes in Korean and Chinese kimchi during fermentation to see if there were observable differences. Fermentation was allowed to proceed at 10°C to days 3, and 30. A total of 42 samples were classified by 4 stages, based on pH: >5, 5-4.7, 4.7-4.2, and <4.2 (Table 1). The numbers of samples in each stage were 4 to 7. Because the taxonomic resolution level of T-RFLP analysis was not enough to monitor specific bacterial species in either Korean or Chinese kimchi, mainly detected T-RFs corresponded to more than two closely related OTUs and that of *W. koreensis* were presented as OTU group and minor T-RFs are combined as others (Table 2). Mean proportions of OTU groups in each stage were summarized in Table 3. The proportions of each OTU group in each stage of Korean and Chinese kimchi samples were different but the patterns of OTU group alterations were similar (Fig. 2). *Lb. plantarum* group (LP) shared an average of 10.3% and 7.5% total bacterial communities in Korean kimchi and Chinese kimchi, respectively. Their proportions were maintained without much variation during fermentation. *Lb. sakei* group (LS) dramatically increased as the fermentation proceeded and became the most populous OTU group at the end of fermentation in kimchi samples from both Korea and China. *Lc. mesenteroides* group (LM) was predominant at the start of fermentation. They represented *ca.* 27% of the population of kimchi from both origins with pH >5. Their populations in both origins decreased noticeably as the fermentation proceeded. The proportion of the *Lc. citreum* group (LC) was not large as that of *Lc. mesenteroides* group but decreased as fermentation proceeded like the *Lc. mesenteroides* group. The *W. cibaria* group (WC) in Korean kimchi showed most progenitive growth when pH was 4.7-4.2, but *W. koreensis* (WK) was populous when the pH >5.0. However, both *W. cibaria* group and *W. koreensis* in Chinese kimchi proliferated when pH 5.0-4.7. The populations of *Weissella* species in both kimchi were reduced dramatically by the end of fermentation. Considering the community population shift at the genus level, *Weissella* may begin to

Table 3. Mean proportion of OTU groups in Korean and Chinese kimchi at each pH range.

Genus	OTU group	Korean kimchi (pH range)				Chinese kimchi (pH range)			
		>5.0	4.7-5.0	4.2-4.7	<4.2	>5.0	4.7-5.0	4.2-4.7	<4.2
Lactobacillus	LP	11.26	11.29	11.60	6.97	5.56	6.76	8.92	8.69
	LS	5.29	11.09	20.98	51.64	3.26	9.62	23.30	30.18
Leuconostoc	LM	26.57	19.62	13.67	10.09	26.59	24.15	18.20	15.85
	LC	5.41	4.10	2.89	2.40	7.04	5.98	4.62	3.64
Weissella	WC	9.05	12.76	20.60	6.58	8.71	18.05	13.14	9.26
	WK	17.77	13.69	9.07	1.74	11.92	17.35	6.80	2.82
Bacillus	BC	15.47	19.60	14.88	15.56	29.46	11.49	19.30	23.77
	Others	7.09	4.94	3.84	3.20	6.02	4.26	3.30	4.27
	Unidentified[a]	2.11	2.92	2.47	1.82	1.44	2.34	2.42	1.52
Total (%)		100	100	100	100	100	100	100	100

[a]T-RFs were not assigned to bacterial species are presented as unidentified.

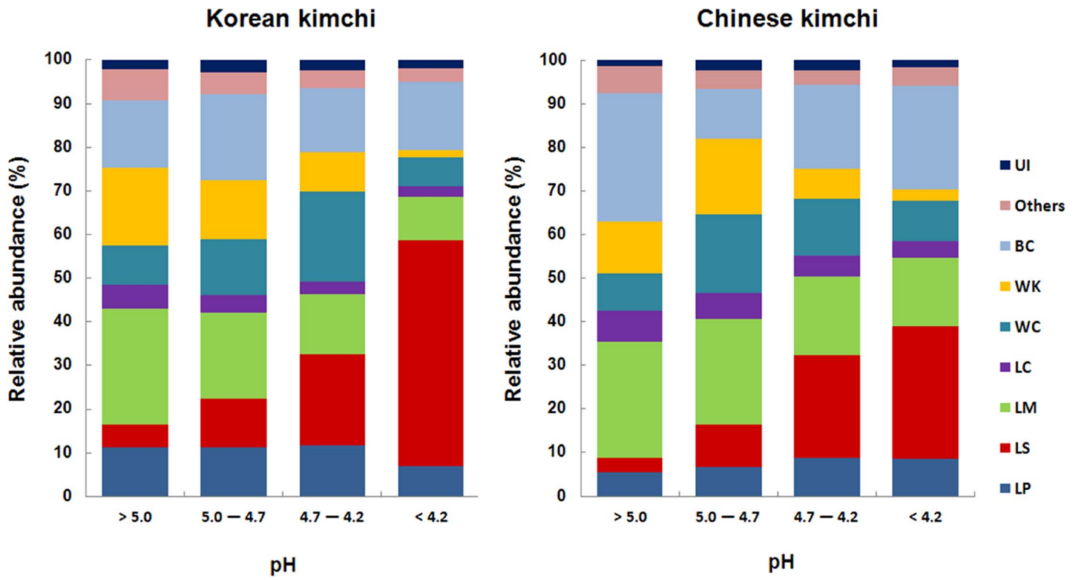

Fig. 2. Changes of bacterial community structures in Korean and Chinese kimchi during fermentation. LP: *Lb. paraplantarum*, *Lb. pentosus*, and *Lb. plantarum*; LS: *Lb. curvatus*, *Lb. graminis*, and *Lb. sakei*; LM: *Lc. inhae* and *Lc. mesenteroides*; LC: *Lc. citreum* and *Lc. kimchii*; WC: *W. cibaria* and *W. confusa*; WK: *W. koreensis*; BC: *Bacillus* spp. Others represent the minor species do not belong to OTU group members. T-RFs were not assigned to bacterial species are presented as unidentified (UI).

proliferate at the start of mid-phase (pH 5.0-4.7) and then begin to slow down its growth at the end of mid-phase growth (pH 4.7-4.2). The population of *Bacillus* spp. in Korean kimchi was almost constant until the late stage of fermentation, but in Chinese kimchi it dramatically decreased when pH was between 5.0-4.7. Later in the fermentation, Chinese kimchi *Bacillus* spp. populations gradually recovered to their original numbers. Greater differences in the production areas and qualities of Chinese kimchi compared to Korean samples might account for differences in *Bacillus* populations. Typically, *Bacillus* cannot proliferate well below pH 4.7 but *Bacillus* species which existed in early stages were constantly detected until the end of fermentation.

Endospore formation of bacilli

Most of the early culture-based microbial studies in kimchi focused on the diversity and transfer of LAB during fermentation [6, 10]. In these studies MRS medium was used. This medium is not optimal for the growth of *Bacillus* spe-

cies. Newer culture-independent bacterial community analyses of kimchi agree with the early culture-based microbial studies concerning the major involvement of LAB in kimchi fermentation [2, 5, 7, 11, 14]. Application of pyrosequencing to several types of kimchi detected *Bacillus* spp. only in the early fermentation stages of a few samples, but the authors neglected to mention their existence [12]. In our previous bacterial community analysis in the initial fermentation stage kimchi samples (above pH 5) using 4 kinds of media, *Bacillus* spp. were the predominant species [9]. Recently, Chang and others [1] reported *Bacillus* spp. as the most populous Gram-positive bacteria in the initial stage of kimchi, maintaining their cell numbers until kimchi fermentation reached around pH 4. We also found through T-RFLP analysis that *Bacillus* spp. were constantly detected until the late fermentation stage of kimchi.

A culture method using TSA was adopted to prove the existence of *Bacillus* spp. until the late stage of kimchi. Viable cells were *ca.* 8.5 log CFU/ml and this population was constantly maintained until day 31 in spite of pH decrease (Fig. 3). When the filtrate was treated with heat at 73°C for

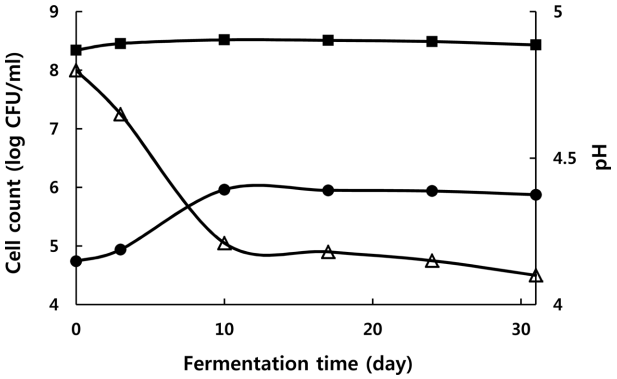

Fig. 3. Monitoring of pHs and viable cell numbers on TSA during kimchi fermentation at 10°C. The filtrate of kimchi sample was used for pH measurement (△) and cell number counting with (●) and without (■) heat treatment.

Table 4. Numbers of the isolated species from a kimchi sample on TSA.

Species	Fermentation time (day)						
	0	3	10	17	24	31	(31)
Bacillus amyloliquefaciens	1						(2)
Bacillus licheniformis		1	1		1	1	(1)
Bacillus methylotrophicus			1	1			(2)
Bacillus safensis							(3)
Bacillus stratosphericus				1			(1)
Bacillus subtilis	2	1		1	1	2	(3)
Bacillus tequilensis			1				(7)
Enterobacter amnigenus			1				
Lactobacillus plantarum	1	1	3	3	4	4	
Lactobacillus pentosus		1	1	1	2	1	
Lactobacillus sakei subsp. *sakei*	3	4	5	3	4	3	
Lactococcus lactis subsp. *lactis*		2	1		2	1	
Leuconostoc citreum	2	2		1		1	
Leuconostoc mesenteroides	2	4	2	2	1	1	
Microbacterium maritypicum	1						
Pseudomonas extremorientalis	1						
Pseudomonas geniculate	1						
Serratia plymuthica	1		1	1	1		
Staphylococcus equorum	1						
Staphylococcus xylosus	1						
Virgibacillus halodenitrificans							(1)
Weissella cibaria	1	2	2	2	1	2	
Weissella confusa						1	
Weissella koreensis	2	2	1	4	3	3	

The sample at day 31 was used with and without heat treatment. The result with heat treatment is shown in parenthesis.

30 sec, cell numbers at the beginning of monitoring (around pH 5) were around 4.7 log CFU/ml. As the pH decreased, cell numbers increased to 6 log CFU/ml and were maintained until the day 31. Bacterial cells which grew on TSA without heat treatment represented the total viable cells, while colonies forming after heat treatment were the endospore-forming cells. This insinuates that the viable bacilli which existed at the beginning of fermentation formed endospores later in the fermentation as the pH decreased.

Twenty different types of colonies which grew on TSA were purified at each sampling day and were identified by 16S rDNA sequence analysis (Table 4). The diversity on TSA at the purchasing day was highest but was reduced to species in the genera, *Bacillus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Weissella* as the fermentation proceeded. When the day 31 sample was heat treated, all isolates were *Bacillus* species except a strain of their relative *Virgibacillus halodentrificans*. Culture method results supported T-RFLP analysis that bacilli were maintained throughout fermentation. In addition to proving the existence of bacilli in kimchi, we established that endospore formation occurred as bacilli progressed through kimchi fermentation.

Discussion

For many years, research on the microflora in foods has relied upon conventional culture-dependent methods, which comprise isolating and culturing microorganisms prior to their identification. Such methods are time-consuming due to the lengthy culture periods and elaborate culture techniques. Moreover, species which occur in low numbers are often out-competed *in vitro* by numerically more abundant microbial species, and some species may not be able to grow *in vitro* on the media or conditions provided in the lab. Therefore, molecular culture-independent approaches have been introduced as powerful tools that provide more complete information on the microbial diversity in specimens.

This study was carried out to explore the possibility of rapid discrimination of kimchi geographic origin by the differences in bacterial community composition. T-RFLP analysis supported reproducible and rapid results but we were not able to detect significant differences between the Korean and Chinese communities or find a bacterium specific to either geographic origin. The taxonomic resolution level of this T-RFLP analysis condition was applicable to a rough view of microbial community composition but not enough to detect a specific species for discrimination within a sample. The LAB assigned to OTU groups in this research all had over 98% 16S rDNA sequence similarity and restriction sites in the same positions (data not shown). Thus, the members of an OTU group will be indistinguishable from each other without multi-locus sequencing which detects single nucleotide polymorphisms.

However, the succession of major LAB involved in kimchi fermentation was successfully analyzed and corresponded well to previously reported bacteria community studies by culture-independent methods [2, 5, 7, 11]. In our previous research, bacterial diversities in kimchi made in Korea and China were evaluated by culture-dependent methods to evaluate the bacterial community differences between kimchi manufactured at different geographic origins and to find the existence of target bacteria for future use in the confirmation of kimchi origin [9]. In order to maximize the bacterial diversity in kimchi, kimchi samples of initial fermentation stage (above pH 5) were used. Kimchi from China possessed more diverse bacteria than that made in Korea. In spite of higher diversity of bacteria in initial stage Chinese kimchi, its bacterial community structure migration pattern was similar with that of Korean kimchi. High diversities of bacteria in several types of kimchi based on major ingredients and manufacture processes were shown to be converged to the genera *Leuconostoc*, *Weissella*, and *Lactobacillus* by a pyrosequencing [12]. One of the major reasons for the simplification of microbial diversity may be the decrease in pH, hence microbial community succession will be similar regardless of raw materials and geographic origins. Major LAB community succession occurred from heterofermentative *Leuconostoc* spp. to *Weissella* spp. then acid-tolerant homofermentative lactobacilli. This LAB community succession in the genus level was also illuminated in sauerkraut fermentation by DNA fingerprinting method [13]. Even differences in the species involved in kimchi and sauerkraut were found. The migration of LAB involved in cabbage fermentation may occur with the same pattern in the genus level. Therefore LAB community migration will also occur regardless of raw materials. This means T-RFLP analysis cannot be applied to discriminate the geographic origins of kimchi. However, T-RFLP analysis used in this study offers a promising application in the detection and monitoring of LAB and *Bacillus* species involved in food ecosystems, especially in vegetable fermentations.

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Our DNA-based method was successful in the detection of *Bacillus* spp. in kimchi and validated their existence until the late stages of fermentation. *Bacillus* spore formation in late fermentation was illuminated by culture-based methods. As the pH decreases, most of the indigenous bacteria from raw materials will be eliminated by the acids and bacteriocins produced LAB but bacilli will survive by forming endospores. Despite several culture-independent microbial community analyses in kimchi for past ten years in Korea, sporulation of bacilli in kimchi was first illuminated by this study. Although culture methods are labor-intensive and biased, they may still provide a useful means of investigating microbial succession of viable cells at every stage of fermentation and better understanding of the physiological conditions of microorganisms.

The *Bacillus* spores may not influence kimchi quality and safety because spores are the dormant stage of a bacterial life cycle, having ideal structures for dispersal by wind, water, or through the animal gut. However, the existence of *Bacillus* spp. in kimchi may spark controversy over the safety of kimchi. *Bacillus cereus* is considered injurious to human health when present at levels greater than or equal to 4 log CFU/g food, based on FDA standards (http://www.fda.gov). In our previous research, three *B. cereus* strains were detected among totals of 208 *Bacillus* spp. isolated from 22 Chinese kimchi samples but none was detected among 464 *Bacillus* spp. from 26 Korean kimchi samples [9]. The existence of *B. cereus* in Chinese kimchi samples might have originated from imperfect washing of raw materials. The KFDA had compelled the implementation of HACCP system at commercial kimchi manufacturers from December, 2006 to assure the safety of kimchi. Kimchi manufacturers cannot control the existence of *B. cereus* to the zero-tolerance level, but strict HACCP system will render its existence to harmless levels. We believe that thousands of years of kimchi consumption with no safety issues is compelling proof of its wholesomeness.

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국문초록

한국산 및 중국산 김치의 Bacteria 군집 분석 및 발효과정 중 Bacilli 포자 형성 규명

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Bacteria 군집 차이를 이용한 신속한 한국산 및 중국산 김치 원산지 판별 가능성의 검토를 위하여 Terminal Restriction Fragment Length Polymorphism (T-RFLP) 분석법을 적용하였다. T-RFLP 분석은 김치발효에 관여하는 주요 유산균의 빠르고, 재현성 있는 검출에는 효과적이었지만, 종(species) 수준에서의 미생물 확인에는 한계를 가지고 있어 한국산 및 중국산 김치에 특이적으로 존재하는 bacteria의 검출에는 부적합한 것으로 평가되었다. T-RFLP를 적용한 발효과정 중의 한국산 및 중국산 김치에 존재하는 bacteria 군집 천이 분석은 비슷한 양상으로 나타났고, *Bacillus* 속이 발효 후기까지 검출되었다. 또한 *Bacillus* 속은 발효 후기에 포자를 형성하는 것으로 확인되었다.

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