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Real-Time PCR Monitoring of *Lactobacillus sake*, *Lactobacillus plantarum*, and *Lactobacillus paraplantarum* during *Kimchi* Fermentation

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Abstract Semi-quantitative monitoring of *Lactobacillus sake* and *Lactobacillus plantarum*, major and minor microorganisms in *kimchi*, respectively, and *Lactobacillus paraplantarum*, recently shown to be present in *kimchi*, was carried out by real-time polymerase chain reaction (PCR). Changes in the 3 species during *kimchi* fermentation were monitored by the threshold cycle (C_T) of real-time PCR. As fermentation proceeded at 15°C, the number of *L. sake* increased dramatically compared to those of *L. plantarum* and *L. paraplantarum*. During fermentation at 4°C, the growth rates of the 3 species decreased, but the proportions of *L. plantarum* and *L. paraplantarum* in the microbial ecosystem were almost constant. Considering the C_T values of the first samples and the change in the C_T value, the number of *L. sake* is no doubt greater than those of *L. plantarum* and *L. paraplantarum* in the *kimchi* ecosystem. *L. sake* seems to be one of the major microorganisms involved in *kimchi* fermentation, but there is insufficient evidence to suggest that *L. plantarum* is the primary acidifying bacterium.

Keywords: *kimchi, Lactobacillus sake, Lactobacillus plantarum, Lactobacillus paraplantarum*, real-time PCR

Introduction

Kimchi is one group of traditionally fermented vegetables originating from Korea that are produced by mixed fermentation, usually involving naturally occurring lactic acid bacteria (LAB). The microorganisms in *kimchi* were first studied in Korea in 1939, but were not actively investigated until the study of Mheen and Kwon (1). They identified the major LAB in *kimchi* as *Leuconostoc mesenteroides*, *Lactobacillus brevis*, *Lactobacillus plantarum*, *Pediococcus cerevisiae*, and *Streptococcus faecalis* on the basis of morphology, fermentation type, dextran formation, and acid production. They showed that the population density of *L. mesenteroides* peaked during the main ripening period of *kimchi* and then declined as the pH decreased, whereas that of lactobacilli, tolerant to low pH, continued to increase until the last stage of fermentation. In particular, *L. plantarum* became predominant when *L. mesenteroides* began to diminish during fermentation. From these observations they inferred that *L. mesenteroides* is the primary microorganism of *kimchi* fermentation while *L. plantarum* is the main acidifying bacterium. Subsequent reports (2-4) have supported the results of Mheen and Kwon (1), and consequently *L. mesenteroides* and *L. plantarum* are now generally considered to be responsible for the good taste and overripening of *kimchi*, respectively.

Since 2000, molecular techniques have been applied to detect and identify LAB in *kimchi*, and results using such methods have differed from those obtained by conventional methods. Using polymerase chain reaction (PCR) with a strain-specific primer, sodium dodecyl sulfate polyacryamide gel electrophoresos (SDS-PAGE) of the whole-cell proteins, and 16S rRNA gene sequence analysis of LAB from the *kimchi* in the middle stage of fermentation at 20°C, Kim *et al.* (5) suggested that *Lactobacillus sake* outnumbered *L. mesenteroides*. The study of diversity and change in microbial communities during *kimchi* fermentation at 4°C by denaturing gradient gel electrophoresis (DDGE) analysis revealed species of leuconostocs and lactobacilli, and recently identified *Weissella koreensis* (6). Based on this analysis, *L. sake*, *Leuconostoc gelidum*, and *W. koreensis* are considered as dominant strains throughout fermentation, but *L. plantarum* and *L. brevis*, considered the main acidifying microorganisms during *kimchi* fermentation, were not detected. A low population density of *L. plantarum* was also implied by species-specific PCR amplification during *kimchi* fermentation at 15°C (7). The discrepancies between results obtained with molecular and conventional methods are due mainly to the similar nutritional and growth requirements of LAB which make it difficult to distinguish species using conventional methods based solely on physiological and phenotypic characteristics (8-10). These difficulties are also related to changes in taxonomic criteria due to the advent of molecular analyses.

The illumination of microbial communities in *kimchi* during fermentation is an important goal for food microbiologists, not only to understand the precise mechanism of *kimchi* ripening, but also to control the ripening process for quality-controlled *kimchi* production in industry.

In this study, semi-quantitative monitoring of *L. sake* and *L. plantarum*, recently established as major and minor microorganisms in *kimchi*, respectively, was performed using real-time PCR. Monitoring of *Lactobacillus paraplantarum*, recently shown to be present in *kimchi* (11, 12), was also performed to compare its levels with those of *L. plantarum* and *L. sake*.

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Materials and Methods

Bacterial strains and growth conditions The reference strains used in this study were purchased from the Korean Collections for Type Cultures and grown at 30°C in MRS broth (Difco, Detroit, MI, USA) under facultative-anaerobic conditions.

PCR primers Oligonucleotide primers were synthesized by Takara (Sungnam, Korea); their sequences are presented in Table 1. The specific primer sets for *L. plantarum* (16/Lpl2) and *L. paraplantarum* (paraF/paraR) designed for the specific detection of each strain in *kimchi* were described in our previous study (11). For the specific detection of *L. sake*, *recA* fragment sequences of *L. plantarum* (AJ286119), *Lactobacillus pentosus* (AJ286118), *L. paraplantarum* (AJ286120), *Lactobacillus fermentum* (AJ579534), and *L. sake* were aligned, and *L. sake*-specific regions were selected. The partial *recA* sequence of *L. sake* subsp. *sake* KCTC 3603 (ATCC 15521) was amplified by PCR with the degenerate primers reported by Dewat *et al.* (13) and determined in this study (DQ 307756). All sequences are available from GenBank, and were aligned by CLUSTAL W software.

PCR amplification The *L. sake*-specific PCR product was amplified in a UNOII Thermocycler (Biometra, Germany). The PCR reaction mixture consisted of template DNA, each primer at a concentration of 0.5 μM, 1 U of *Taq* polymerase (Takara), 2.5 mM $MgCl_2$, 100 mM dNTPs, and 1× PCR buffer supplied by the manufacturer. Total DNA was extracted from LAB with a Dneasy Tissue Kit (Qiagen, Germany). Samples were preheated for 5 min at 95°C, and then amplified by 30 cycles of 1 min at 94°C, 1 min at 62°C, and 1 min at 72°C. For the specific detection of *L. plantarum* and *L. paraplantarum*, the annealing temperatures of PCRs were 56°C. The annealing temperature for *L. sake recA* fragment amplification was 63°C. PCR products were separated by electrophoresis in a 2% agarose gel and stained with ethidium bromide. The expected sizes of PCR products were 231 bp for *L. sake*, 223 bp for *L. plantarum*, and 154 bp for *L. paraplantarum*.

DNA sample preparation from *kimchi* *Baechukimchi*, the most common type of *kimchi*, was purchased from Poonggi, a manufacturer in Suwon, Korea. Just after the preparation of a *kimchi* sample at the factory, the lot was divided and evenly distributed into 300-mL airtight containers. Fermentation was allowed to proceed at 4 and 15°C. Sampling was started after 1 day, and the samples that were fermented at 15°C were collected every other day. The samples that were fermented at 4°C were collected every week. The juice of facultative anaerobically fermented *kimchi* in airtight containers was filtered through sterilized gauze and stored at -20°C until DNA extraction. Total DNA from the filtrates was isolated with a Dneasy Tissue Kit (Qiagen), suspended in TE buffer (pH 8.0), and quantitatively evaluated using spectrophotometry (Shimadzu, Japan).

Real-time PCR quantification Real-time PCR was performed with FastStart DNA Master Hybridization Probes (Roche Diagnostics, Mannheim, Germany) in a LightCycler real-time PCR system (Roche Diagnostics). The 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA) labeled double-dye oligonucleotide probes for fluorescence emission were synthesized by Takara. The sequences of probes for each species were complimentary to the target genes and specific to each species (Table 1). For real-time PCR, each 25 μL reaction mixture contained template DNA, 3 mM $MgCl_2$, 2.5 μL of the master mix, a 500 nM concentration of each primer, and 200 nM of each probe. The cycle parameters for *L. sake* monitoring consisted of a 10-min denaturation at 95°C followed by 40 cycles of 95°C for 15 sec, 5 sec-annealing at 62°C, and 15 sec-extension at 72°C. For the monitoring of *L. plantarum* and *L. paraplantarum*, the annealing temperatures were set at 56°C. The amplification

Table 1. Sequences of PCR primers and double-dye probes

Primer	Description[1)]	Oligonucleotide sequence (5′ → 3′)[2)]	Specificity	Reference
16	16S rRNA gene, 3′ end, F	GCTGGATCACCTCCTTTC	16S rRNA gene	(17)
LeuF	Internal fragment of *recA*, F	TTHATYGANGCYGARCAYGC	Degenerate primer for *recA*	(13)
LeuR	Internal fragment of *recA*, R	CCWCCWGKWGTYGTYTCNGG	Degenerate primer for *recA*	(13)
Lpl2	16S/23S spacer region of *L. plantarum* DNA, R	CATGAGGTATTCAACTTATG	*L. plantarum*	(11)
paraF	*recA* sequence of *L. paraplantarum* DNA, F	TAGTGGTGCCGTTGATATTTTG	*L. paraplantarum*	(11)
paraR	*recA* sequence of *L. paraplantarum* DNA, R	AGTCTTGTTCAACGTTCCGG	*L. paraplantarum*	(11)
skF	*recA* sequence of *L. sake* DNA, F	CTAAGTATGCAACAGCACTTG	*L. sake*	This study
skR3	*recA* sequence of *L. sake* DNA, R	CGTCCCTGATAATTTACGTAA	*L. sake*	This study
Lpl	16S/23S spacer region of *L. plantarum* DNA, P	CGGAAACCTACACATTCTTCGAAACTTTGT	*L. plantarum*	This study
Lpara	*recA* sequence of *L. paraplantarum* DNA, P	CAAGCTCGACTCATGTCACAGGCATTACGA	*L. paraplantarum*	This study
Lsk	*recA* sequence of *L. sake* DNA, P	CTTGGTATCAAGTGGGGCCGTTGAT	*L. sake*	This study

[1)]F, R, and P, forward primer, reverse primer, and double-dye oligonucleotide probe, respectively.
[2)]Degenerate primer code: H is mixture of A, T, and C; K is mixture of G and T; N is mixture of A, T, G, and C; R is mixture of A and G; W is mixture of A and T; Y is mixture of C and T.

of PCR fragments from each target gene was detected by monitoring the increase in fluorescence from the dye-labeled probe. The threshold cycle (C_T) is defined as the PCR cycle where a significant increase in fluorescence was first observed (according to the LightCycler owner's manual version 1.2). This value is normalized against background fluorescence.

Results and Discussion

Specificity of PCR The phenotypic and genotypic diversity within *Lactobacillus curvatus* and *L. sake*, as well as the close relatedness of these two species, have been revealed by many studies. Two sub-groups within each species have been detected by genotypic analyses of randomly amplified polymorphic DNA (RAPD)-PCR, DNA-DNA hybridization, and protein fingerprinting, however it is still difficult to rapidly distinguish these 2 strains by specific PCR amplification (14, 15). Therefore, we designed *L. sake*-specific PCR primers to differentiate *L. sake* from closely related lactobacilli at the species level. The 16S rRNA sequences from *L. curvatus*, *Lactobacillus graminis*, and *L. sake* are closely related (16), and the 16/23S rRNA spacer regions in these strains also show high sequence similarity (17).

Based on our successful detection of *L. pentosus* and *L. paraplantarum* by *recA*-specific PCR primers in previous studies, *L. sake*-specific PCR primers were designed based on the *recA* sequence. The expected size of the PCR product was 231 bp and the skF/skR3 primer pair generated a unique DNA fragment of the expected size without producing PCR products from nontarget species (Fig. 1). Consequently, the primer set can be used for the specific detection of *L. sake* in *kimchi* without detecting closely related *L. curvatus* and *L. graminis*. The identity of the amplicon was confirmed by sequencing. The specificity of PCR primer sets for the detection of *L. plantarum* and *L. paraplantarum* was demonstrated in our previous study (11).

Monitoring of *L. sake*, *L. plantarum*, and *L. paraplantarum* during *kimchi* fermentation by real-time PCR Different amounts of DNA were tested for real-time PCR amplification, and finally, 100 and 150 ng concentrations of template per reaction were used for the monitoring of *L. sake*, *L. plantarum* and *L. paraplantarum* in *kimchi* fermentation at 15 and 4°C, respectively. The change in the levels of each species during *kimchi* fermentation was monitored using the C_T value from real-time PCR.

As fermentation proceeded at 15°C, the C_T value of *L. sake* decreased sharply and then maintained a steady level until day 15 (Fig. 2). However, the values of *L. plantarum* and *L. paraplantarum* decreased gradually until day 15 and then increased slightly. The increases of *L. plantarum* and *L. paraplantarum* coincided with that of *L. sake*. The population decrease of each species after day 15 in the *kimchi* ecosystem insinuates the possibility of a microbial community transition from LAB to other species such as yeast and fungi. The appearance of fungi together with the development of rancidity at the surface of *kimchi* after long-storage is well known, and the population of yeast was reported to increase at late stages of fermentation (1, 18). During fermentation at 4°C, the temperature known to be best for making delicious *kimchi* and inhibiting the growth of lactobacilli involved in acidification, the slower progress of fermentation was confirmed by the decreases in the growth rates of all 3 species. Although the growth rate decreased, the proportion of *L. sake* in *kimchi* increased continuously until the end of the analysis. In the meantime, the C_T values of *L. plantarum* and *L. paraplantarum* remained almost constant.

In previous studies identifying LAB from *kimchi* fermentation under psychrotrophic conditions by conventional methods (1, 3, 4) and DDGE analysis (6), *L. plantarum* was not detected. But in this study, *L. plantarum* was detected from *kimchi* samples fermented at both 4 and 15°C. This means that the proportion of *L. plantarum* in the *kimchi* microbial ecosystem is constant until the end of analysis, but its population is not high enough to influence the flavor of *kimchi* in spite of its increase in number.

Lim *et al.* (3) isolated and identified Gram-positive bacteria from *kimchi* samples fermented at 25, 15, and 5°C and found an increase of *L. plantarum* as the fermentation

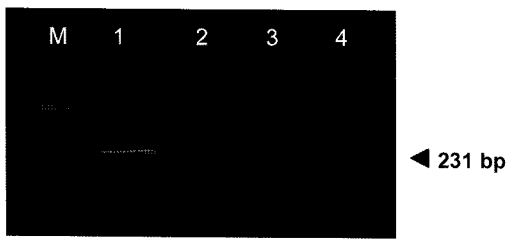

Fig. 1. PCR amplification of genomic DNA from reference strains using skF/skR3 primer pair. Lanes: M, 50-1,000 bp DNA size marker; 1, *L. sake* subsp. *sake* KCTC 3603 (ATCC 15521); 2, *L. casei* KCTC 3109 (ATCC 393); 3, *L. graminis* KCTC 3542 (ATCC 51150); 4, *L. curvatus* subsp. *curvatus* KCTC 3767 (ATCC 25601). All strains are type strains.

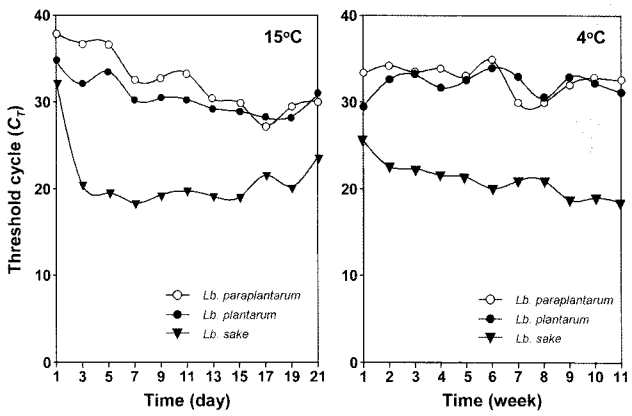

Fig. 2. Change in the threshold cycle value of real-time PCR for the quantification of *L. sake*, *L. plantarum*, and *L. paraplantarum* during *kimchi* fermentation. The amount of template DNA used for real-time PCR was 100 and 150 ng per reaction for *kimchi* samples fermented at 15 and 4°C, respectively.

temperature increased. As the temperature decreased, the number of *L. sake* increased. With regard to the major carbon source requirement, both strains showed minimal differences (19). These facts suggest that the population of both species in *kimchi* may be influenced more by the fermentation temperature than the nutrient requirements.

In this study, the relationship of cell numbers and C_T values was not determined because of the difficulty of determining the criteria for the standardization of experimental conditions. We could not compare the number of each species during fermentation. The change in C_T value reflects the change in each species in the whole microbial community in the sample. Considering the C_T value of the first samples, the number of *L. sake* is no doubt greater than those of *L. plantarum* and *L. paraplantarum*. However, the difference in cell number between *L. plantarum* and *L. paraplantarum* may not be so large. *L. sake* seems to be one of the major microorganisms involved in *kimchi* fermentation at any *kimchi* fermentation temperature, and the microbial population would be expected to have a large proportion of *L. sake* in the *kimchi* microbial ecosystem. *L. plantarum* requires further investigation to be considered as the main acidifying bacterium.

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