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Application of Quantitative Real-Time PCR for Enumeration of Total Bacterial, Archaeal, and Yeast Populations in Kimchi

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Kimchi is a Korean traditional fermented food made of brined vegetables, with a variety of spices. Various microorganisms are associated with the kimchi fermentation process. This study was undertaken in order to apply quantitative real-time PCR targeting the 16S and 26S rRNA genes for the investigation of dynamics of bacterial, archaeal, and yeast communities during fermentation of various types of kimchi. Although the total bacterial and archaeal rRNA gene copy numbers increased during kimchi fermentation, the number of yeasts was not significantly altered. In 1 ng of bulk DNA, the mean number of rRNA gene copies for all strains of bacteria was 5.45×10^6 which was 360 and 50 times greater than those for archaea and yeast, respectively. The total gene copy number for each group of microorganisms differed among the different types of kimchi, although the relative ratios among them were similar. The common dominance of bacteria in the whole microbial communities of various types of kimchi suggests that bacteria play a principal role in the kimchi fermentation process.

***Keywords*:** quantitative PCR, kimchi, bacteria, archaea, yeast

Food fermentation is a natural process that influences food taste and preservation (Ross *et al*., 2002). Kimchi is a traditional fermented food made of various vegetables such as Chinese cabbage, radish, and spring onions with spices (red pepper, garlic, ginger, and etc) in Korea. Many researchers demonstrated the microbial communities of kimchi and their role in fermentation (Kim and Chun, 2005; Nan *et al*., 2005; Li *et al*., 2006). Among the whole bacterial community, the diversity and dynamics of lactic acid bacteria were revealed through genome-probing microarray and 16S rRNA gene analysis during kimchi fermentation (Bae *et al*., 2005; Kim and Chun, 2005). Yeasts have been isolated from ripened kimchi at pH 4, and they can inhabit hypersaline environments (3% NaCl concentration, w/v) (Mheen and Kwon, 1984; Oh and Han, 2003; Butinar *et al*., 2005). Halophilic archaea have been isolated from jeotgal, which is a salted seafood in Korea and generally used as an ingredient of kimchi (Roh *et al*., 2007), and was known to inhabit kimchi. Halophilic archaea could habit kimchi because the sodium chloride concentration averages 3% (w/v).

The dynamics among microbial communities such as bacteria, archaea, and yeast during kimchi fermentation was observed using culture-independent denaturing gradient gel electrophoresis (DGGE) (Chang *et al*., 2008). Although the DGGE technique provides a sectional diagram for the microbial structure and can be used for qualitative analysis of microbial communities, it is not a quantitative method (Suzuki and Giovannoni, 1996; Kanagawa, 2003). Quantitative real-time PCR (qPCR) can be used to amplify and simultaneously quantify a targeted DNA by employing a PCR-based technique that enables one to quantify the number of gene copies or relative number of gene copies in a complex DNA sample (Furet *et al*., 2004). The amplified gene copy number from bulk DNA reflects the relative abundance of the microorganisms in the community. This technique can be applied to quantitatively analyze microbial composition in natural environments and has been used to monitor microbial dynamics in fermented foods such as sausages and milk (Furet *et al*., 2004; Martin *et al*., 2006). In this study, we used a culture-independent qPCR technique in order to monitor and compare the dynamics of microbial communities including bacteria, archaea, and yeast involved in kimchi fermentation.

Materials and Methods

Kimchi sampling

Kimchi (designated P kimchi) was obtained from a commercial factory at the point of production and immediately stored at 4°C until the time of sampling. The microbial dynamics and pH changes of P kimchi were monitored at 2- to 3-day intervals during fermentation. The measurement of pH was determined by a pH meter (Corning, USA). To study how microbial populations varied with the type of kimchi, seven different kinds of kimchi (K1, K2, K3, K4, K5, K6, and K8) and a mixture of spices (K7) were analyzed 11 days after preparation. K1, K4, and K5 were Chinese cabbage kimchi, K2 and K6 were young radish kimchi, K3 was pony-tail radish kimchi, K7 contained a spice from the K5 kimchi, and K8 was a radish water kimchi. The kimchi samples were centrifuged at 7,000 rpm

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Table 1. List of PCR primer pairs used in this study

	Designation	Sequence (5'→3')
Bacteria-specific primers	bac1055YF	ATGGYTGTCGTCAGCT
	bac1392R	ACGGGCGGTGTGTAC
Archaea-specific primers	arch349F	GYGCASCAGKCGMGAAW
	arch806R	GGACTACVSGGGTATCTAAT
Yeast-specific primers	YEASTF	GAGTCGAGTTGTTTGGGAATGC
	YEASTR	TCTCTTTCCAAAGTTCTTTTCATCTTT

for 30 min and pelleted cells were used for the DNA extraction. DNAs were extracted using glass bead-beating method as previously described (Yeates *et al*., 1998). Extracted DNA samples were purified using an UltraClean Microbial DNA Isolation kit (Mo Bio Laboratories, USA).

Quantitative real-time PCR conditions (qPCR)

In order to quantify the total number of bacteria, bacterial standard curves were generated by plotting the threshold cycle (Ct) versus the concentration of purified PCR product obtained by amplification of the rRNA genes from the genomic DNA of *Weissella confusa* (KCTC 3499). This DNA was diluted serially from 17.8 ng/μl to 17.8×10^{-4} ng/μl. PCR primer pairs used to amplify the rRNA genes of each group of microorganisms used in this study are listed in Table 1. The qPCR amplifications were performed using the universal primer pair bac1055YF/bac1392R (Ritalahti *et al*., 2006). For enumeration of total archaea, standard curves were also created by plotting threshold cycle (Ct) versus concentration of purified PCR product from amplification of the 16S rRNA genes of *Haloterrigena thermotolerans* (DSM 11552); the sample concentration was serially diluted from 0.4 ng/μl to 0.4×10^{-4} ng/μl. The qPCR amplifications were performed using the universal primer pair arch349F/arch806R (Takai and Horikoshi, 2000). For quantification of total yeast, PCR products from the amplification of the D1/D2 region of the 26S rRNA gene of *Saccharomyces cerevisiae* (KCTC 7445) were used as the standard control. Standard curves were created by plotting threshold cycle (Ct) versus concentration, with the DNA serially diluted from 2.0 ng/μl to 2.0×10^{-5} ng/μl. Amplification for qPCR was performed using the universal yeast primer pairs YEASTF/YEASTR (Hierro *et al*., 2006). The initial DNA concentration was quantified using a spectrophotometer (Nanodrop Technologies, USA), and the DyNAmo™ HS SYBR® Green qPCR kit (FINNZYMES, Korea) was used for qPCR analysis (DNA Engine OPTICON™2 System, MJ Research, Korea). The qPCR reactions (20 μl) contained 1 μl template DNA, 10 pmol of each primer, 7 μl nuclease-free water, and 10 μl of 2× master mix (containing the hot start version of a modified *Tbr* DNA polymerase, SYBR Green I, optimized PCR buffer, 5 mM $MgCl_2$, and dNTP mix including dUTP). The following qPCR conditions were used: initial denaturation, 15 min at 95°C; followed by 60 cycles of 94°C for 20 sec, 58°C for 30 sec, and 72°C for 45 sec; and a final extension of 5 min at 72°C. PCR amplification was followed by melting curve analysis with the temperature decreased from 95 to 65°C at a rate of 0.2°C/sec, with continuous monitoring of the decline in fluorescence.

qPCR of kimchi samples and calibration

After determination of the qPCR conditions required for quantitative analysis of total bacteria, archaea, and yeast in kimchi, we tested the purified bulk DNA from eight kimchi samples. The gene copy number for the bacterial genomic DNA was calibrated as described previously (Ritalahti *et al*., 2006). We assumed that the genome size of the *Weissella* species was 2 Mb and that the 16S rRNA gene copy number was eight (Chelo *et al*., 2004). In order to calibrate gene copy number for a known amount of PCR product, we used the following equation: gene copy number = {[(0.978× 10^9)×DNA content (pg)] / PCR product size (bp)} (Dolezel *et al*., 2003). The sizes of the PCR products from archaea and yeast were 1,500 and 600 bp, respectively.

Results and Discussion

The pH and microbial dynamics of P kimchi during the 19-day fermentation period is shown in Fig. 1. For enumeration of the total number of microorganisms in kimchi, standard curves were obtained by qPCR using specific primer sets for the three groups of microorganisms, the serially diluted pure genomes and purified PCR products. Equations for bacterial, archaeal, and yeast standard curves were y= -4.956x + 32.454, y= -9.024x + 60.334, and y= -5.173x + 37.955, respectively (x, $\log_{10}$ DNA concentration in ng/μl; y, cycle threshold). The coefficients (R^2) were 0.983, 0.993, and 0.998, respectively. The gene copy number for total bacteria ranged from 3.31×10^5 to 1.25×10^7 per ng of bulk DNA and these numbers increased 10-fold between P kim-

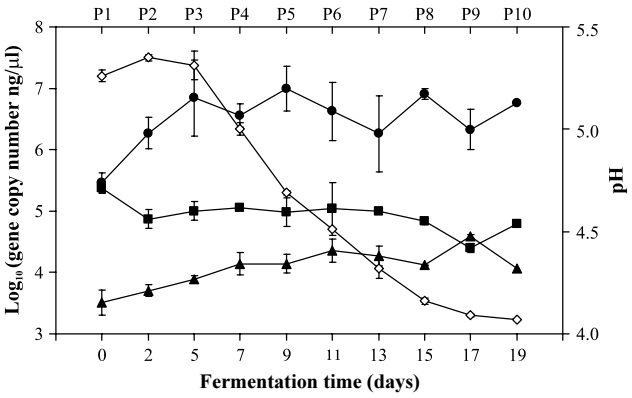

Fig. 1. Shown is the pH change (opened squares) and gene copy numbers for the total bacteria (filled circles), archaea (filled triangles), and yeast (filled squares) during P kimchi fermentation.

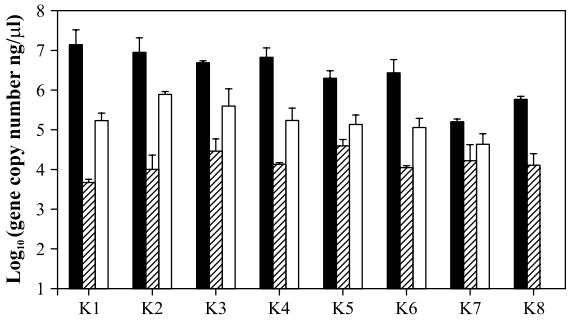

Fig. 2. Shown are the gene copy numbers for the total bacteria (filled bar), archaea (striped bar), and yeasts (open bar) that were derived from quantitative real-time PCR analyses from the seven types of kimchi and one spice after 11 days of fermentation. K1 through K6 plus K8 represent the different types of kimchi. K7 is a spice used in the preparation of K5 kimchi. Standard deviations are shown with error bars.

chi sampling time 1 (P1) and 3 (P3) (see Fig. 1). The gene copy number for total archaea ranged between 3.24×10^3 and 3.87×10^4 per ng bulk DNA and increased gradually during kimchi fermentation. The mean gene copy number for total bacteria was approximately 360-fold greater than that for total archaea. At the initial sampling point P1, the gene copy numbers for bacteria and yeast were similar; however, the gene copy number for bacteria was about 100-fold higher than that for yeast after the peak point of fermentation (P3; see Fig. 1). The gene copy number for total yeast ranged from 2.54×10^4 to 2.43×10^5 per ng of DNA and decreased steadily during fermentation. The mean copy number for yeast was approximately 50-fold lower than the copy number that for bacteria.

We examined the microbial gene copy number in 1 ng of bulk DNA from seven types of kimchi and one mixed spice (total bacteria, 1.59×10^5 to 1.61×10^7; archaea 4.73×10^3 to 3.98×10^4; yeast, 4.67×10^4 to 7.70×10^5; see Fig. 2). Ratios of the maximum to minimum cell numbers during kimchi fermentation were 7.9, 8.4, and 6.5 for bacteria, archaea, and yeast, respectively. The bacterial gene copy numbers in samples K7 and K8 were lower than those for the bacterial gene copy numbers for the other samples, presumably because the former is a spice and the latter is water kimchi and general fermentation was not able to support comparable bacterial growth in each kimchi sample. While some yeast detected in the DGGE bands had been found in sample K8 in our previous study, the yeast gene copy numbers in sample K8 was not measured in the present study. Therefore, further studies are needed to confirm this method. With the exception of K8 kimchi (water kimchi), the mean gene copy numbers of bacteria, archaea, and yeast ranged from 10^6 to 10^7, 10^3 to 10^4, and 10^4 to 10^5 per ng of bulk DNA, respectively. The total gene copy number for three groups of microorganisms was different for the various types of kimchi; however, similar relative numbers were observed among them.

The analysis of various microorganisms in kimchi during fermentation was investigated by several researchers with improved techniques (Lee *et al*., 1992; Bae *et al*., 2005; Kim and Chun, 2005; Cho *et al*., 2006). Traditional plating methods using selective agar media for the enumeration and detection of microorganisms in food are easy to control but are time-consuming, labor-intensive, and do not detect all microorganisms (Loureiro, 2002). In the case of yeast, at least 3 to 7 days of incubation is needed for detection of colonies (de Boer and Beumer, 1999). The dynamics of lactic acid bacteria in kimchi were confirmed by 16S rRNA analysis and microbial genome-probing microarray (Bae *et al*., 2005; Kim and Chun, 2005). In addition, other investigators monitored archaea and yeast during kimchi fermentation using DGGE analysis (Chang *et al*., 2008). In their study, archaea diversity was changed with decreasing pH but yeast diversity was not. Although microbial diversity from three domains was simultaneously investigated in kimchi, the quantitative relationships among them were not revealed. In this study, we described rapid quantification of total bacteria, archaea, and yeast in kimchi using real-time PCR in order to understand the relationships among them during kimchi fermentation. Although the total number of microorganisms was different for each kind of kimchi, the relative ratios among the microorganisms were similar among the different types of kimchi. A greater number of rRNA gene copies for bacteria in the various types of kimchi implied that bacteria might be able to suppress the growth of other microorganisms in kimchi during fermentation. In the case of yeast, a continuous reduction in the amount of yeast during kimchi fermentation also implies that their growth was restrained through the fermentation process and that innate yeast may not play a significant role in kimchi fermentation. Results from the present study illustrate the dynamics of the bacteria, archaea, and yeast via quantitative analysis during kimchi fermentation and provide a relative comparison among the various kinds of kimchi.

A qPCR assay was successfully applied for the detection and monitoring of fish spoilage bacteria (Reynisson *et al*., 2008). Renard *et al*. (2008) conducted qPCR for detection and quantification of spoilage yeasts in orange juice. Using a qPCR assay, the results can be obtained in 4 to 5 h. Studies on the microbial ecology of foods have frequently employed several techniques such as PCR-DGGE, TGGE (Temperature Gradient Gel Electrophoresis), T-RFLP (Terminal-Restriction Fragment Length Polymorphism), and qPCR; however, these methods are insufficient for analyzing complex and dynamic microbial communities. The qPCR technique allows detection and quantification of several target species or groups. It is possible to analyze and simultaneously monitor comprehensive data by microarray technology. However, it is difficult to monitor specific groups or domains. The combination of qualitative and quantitative methods including PCR-DGGE, qPCR, and microarrays could overcome the limitations of analyzing microbial communities in foods. Improvements in the qPCR assays, however, depend on careful consideration for the selection of microbial genes and primers targeted. In addition, it is difficult to analyze cells found in low numbers in food matrices or microorganisms found with low copy numbers. Finally, we conclude that the qPCR assay can be applied for the simultaneous enumeration and monitoring of several microorganisms in kimchi within a period of a few hours.

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