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Quantitative Analysis of *Leuconostoc mesenteroides* and *Lactobacillus plantarum* Populations by a Competitive Polymerase Chain Reaction

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Abstract A multiplex competitive polymerase chain reaction (PCR) method was developed for the rapid identification and quantification of *Leuconostoc mesenteroides* and *Lactobacillus plantarum* populations which are the key microorganisms in kimchi fermentation. The strain-specific primers were designed to selectively amplify the target genes encoding 16S rRNA of *L. plantarum* and dextransucrase of *L. mesenteroides*. There was a linear relationship between the band intensity of PCR products and the number of colony forming units of each model organism. The PCR quantification method was compared with a traditional plate-counting method for the enumeration of the two lactic acid bacteria in a mixed suspension culture and also applied to a real food system, namely, watery kimchi. The population dynamics of the two model organisms in the mixed culture were reliably predictable by the competitive PCR analysis.

Key words: *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, polymerase chain reaction, 16S rRNA, dextransucrase, kimchi

Kimchi is a general term given to a group of fermented vegetables in Korea. Fermentation of kimchi is carried out by various microorganisms, especially lactic acid bacteria such as *Leuconostoc mesenteroides* and *Lactobacillus plantarum* [2, 6, 19]. The number of aerobes increases dramatically in the early stage of kimchi fermentation and then decreases, while the number of anaerobes continues to increase during the middle stage. *L. mesenteroides* actively grows in the early stage of fermentation, producing lactic acid and carbon dioxide which acidify kimchi and create an anaerobic environment, thereby suppressing the growth of other aerobes. *L. plantarum*, *L. brevis*, and *Pediococcus* species actively grow in the middle stage, and acid-tolerant *Lactobacillus* species survive until the late stage, which is classified as the stage when acidifying bacteria causes over-ripening of kimchi [8, 12, 18, 19]. Kimchi fermentation is also markedly affected by salt concentration, temperature, and organic acids produced by lactic acid bacteria [9]. The quality of kimchi, therefore, largely depends on the profile of its microbial populations and their mutual interactions, which need to be more clearly defined.

The qualitative and quantitative methods for assessing microbial communities in a food system have been generally based on the colony-counting method, which involves the incubation of the microorganisms in a selective medium to maintain its suboptimal growth conditions and the number of colonies representing its own peculiar phenotype [10]. However, this method suffers from two major disadvantages. First, only the organisms that have the ability to survive in an artificial medium can be enumerated. In other words, the key organisms present in food materials can be lost during the sample preparation. Second, it takes a significant amount of time to identify and discriminate the strains involved. The current classification methods are based on the physiological properties of microorganisms. They lack the specificity and sensitivity for the accurate and reproducible identification of isolates. To overcome this handicap, the polymerase chain reaction (PCR) method has been suggested for detection of microorganisms in various biological samples [5, 13, 14]. With its extraordinary sensitivity, the PCR method has been employed to detect nucleic acids present in very low quantity [8]. A frequently encountered problem with quantitative PCR application is, however, its poor reproducibility even under the most precisely controlled experimental conditions. The more reliable approaches to quantitative PCR are those based on the competitive co-amplification of reference templates that share the same primer-binding sites with the target molecule [4, 15, 17]. Accordingly, the two templates compete for the primers and subsequent amplification reactions take

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place at the same rate. Predictable and unpredictable variables that affect the amplification reaction exert the same effects on both target and competitor molecules in a competitive PCR, so that the ratio of the amplified products exactly reflects the initial ratio of the targets, rendering the amplification reaction independent of the number of amplification cycles [16, 17].

In this study, a competitive PCR method was developed to specifically detect *L. mesenteroides* and *L. plantarum* in an accurate and reproducible manner and eventually to monitor the population dynamics of these lactic acid bacteria in a mixed culture.

MATERIALS AND METHODS

Strains, Culture Conditions, and Analysis

L. plantarum KCCM 11322 and *L. mesenteroides* KCCM 11324 were obtained from the Korean Culture Center of Microorganisms (Seoul, Korea). Frozen cultures of *L. plantarum* and *L. mesenteroides* were thawed and precultured overnight in 5 ml MRS (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) broth [2]. Shake flask fermentations were performed with 100 ml MRS broth at 35°C and 200 rpm. Initial pH was adjusted to 6.5 with 2 N NaOH. The colony forming unit (CFU/ml) was measured using the colony-counting method by properly diluting the culture broth with a sterile saline solution to obtain 200 to 300 colonies on each MRS plate supplemented with 0.002% bromophenol blue dye. The mean CFU was estimated from three independent plates. MRS plates were incubated at 30°C until the diameter of a colony became more than 0.5 mm. Cell growth was also monitored by measuring the optical density of the culture broth at 600 nm.

Polymerase Chain Reaction

Two primer sets were constructed based on the 16S rDNA sequence of *L. plantarum* [11] and the dextransucrase coding gene of *L. mesenteroides* to specifically amplify the respective target gene fragments (Table 1). The amplified fragments of the two target DNAs were 600 bp for the 16S rRNA and 410 bp for the dextransucrase gene.

Template DNAs were prepared by boiling bacterial cell pellets for 10 min, standing 10 min on ice, and then centrifuging for 10 min at 13,000 ×*g* [13]. PCR amplification was performed in a reaction mixture containing 100 ng of template, 25 pmol of each primer, 0.25 mM of each dNTPs, and 2.5 U of *Taq* DNA polymerase (Bioneer Co. Ltd., Korea). The amplification reaction was carried out using the following program: 1 cycle of pre-denaturation for 5 min at 94°C; 26 cycles consisting of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min; final extension at 72°C for 10 min. The PCR products were analyzed by agarose gel (1.2%) electrophoresis and an image analyzer (Bio-Rad, Hercules, CA, U.S.A.). The results obtained by the quantitative PCR amplification were compared with the colony-counting method to examine the time-dependent profiles of the two lactic acid bacteria in a mixed suspension culture.

PCR analysis was also performed on watery kimchi to verify the relative success of the PCR methods for detecting two lactic acid bacteria in fermented kimchi. Watery kimchi was fermented at room temperature (25°C) for 1 day and stored in a refrigerator (5°C) for two weeks. Bacterial cell pellets were harvested from the juice of watery kimchi by centrifugation at 13,000 ×*g* for 15 min, washed twice with 1.0 ml sterile saline solution (0.9%), washed once with sterile water, and then centrifuged at 13,000 ×*g* for 15 min to completely remove food suspensions.

Construction of Competitor as an Internal Standard

Competitor DNA fragments of each target gene used as internal standards were co-amplified with the target DNA to exclude experimental errors which might occur during the reaction process. DNA competitors were prepared, using a Competitive DNA Construction Kit (Takara Co., Tokyo, Japan), that have the sequence at both 5'-ends where the same primers bind to amplify the target DNA. The sense and antisense primers for construction of the DNA competitors were prepared by adding the sequence at the 3'-termini of each sense and antisense primer for amplification of two target DNAs (Table 2). Sizes of the two corresponding amplified competitors were 520 bp for the 16S rRNA gene in *L. plantarum* and 330 bp for the dextransucrase gene in *L. mesenteroides*. In the present study, a quantitative analysis with the competitive PCR method was only performed for measuring the amount of *L. mesenteroides* in a 1-day fermented sample.

Table 1. The sequence of strain-specific primer sets for detection of two lactic acid bacteria used in this study.

Primer sets		Nucleotide sequence (5'-3')	Target	Product size (bp)
Organism	Name			
Lactobacillus plantarum	plaF	TGATTGGTGCTTGCATCATG	16S rRNA	410
	plaR	TGAACAGTTACTCTCAGATA		
Leuconostoc mesenteroides	mesF	GTAGATGCTGTTGATAACGTT	Dextransucrase	600
	mesR	TTGCCATGTATTGACCATCA		

Table 2. The primer sequences for competitor construction.

Primer sets for competitors		Nucleotide sequence (5'-3')	Product size (bp)
Target	Name		
Lactobacillus plantarum	CplaF	TGATTGGTGCTTGCATCATG*-GTACGGTCATCATCTGACAC	330
	CplaR	TGAACAGTTACTCTCAGATA-GGAAGACTCCTGTTATCAAG	
Leuconostoc mesenteroides	CmesF	GTAGATGCTGTTGATAACGTT-GTACGGTCATCATCTGACAC	520
	CmesR	TTGCCATGTATTGACCATCA-CAGGAGAACGAGGATATTGC	

*Each primer has its own strain-specific primer sequences (underlined characters) at 5'-ends to compete with target DNA in quantitative and competitive PCR.

RESULTS AND DISCUSSION

Competitive Polymerase Chain Reaction

PCR amplifications were performed using the primers (mes, pla) designed to specifically detect *L. mesenteroides* and *L. plantarum*. Various amounts of template DNA were used and the results are shown in Fig. 1. The sizes of the PCR product, determined by using the mes primer for amplification of the dextransucrase gene of *L. mesenteroides*, was 600 bp; and 410 bp for the 16S RNA gene of *L. plantarum* using the pla primer [11]. Image analysis and least-square regression indicated that quantitative measurement might be possible within a limited range of template DNA concentration (data not shown), as was reported elsewhere [1, 3, 17]. To obtain more reproducible results of PCR amplification, a competitive PCR method was devised.

The competitor templates were amplified by PCR using the primers listed in Table 2. Sizes of the amplified competitor for *L. mesenteroides* and *L. plantarum* were 520 and 330 bp, respectively. PCR performed in the presence of the competitors also indicated reliable results as illustrated in Fig. 2. While the band intensities of the 520 and 330 bp decreased as the concentrations of competitor were reduced, the band intensities of the amplified target DNA of the 600- and 410-bp sizes increased conversely, suggesting that the target and competitor DNA templates compete with each other for the same primers.

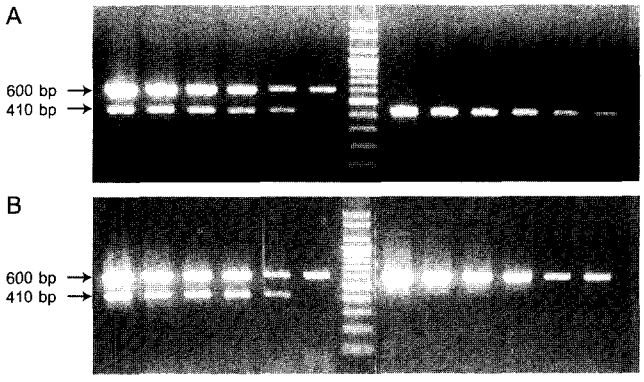

Fig. 1. PCR amplification to detect *L. mesenteroides* (600 bp) and *L. plantarum* (410 bp) using mes and pla primer sets.
Multiplex PCR results (left panel) were compared with those of single PCR (right panel) of *L. plantarum* (A) and *L. mesenteroides* (B). Various concentrations of template DNAs were used for PCR amplification (Lane 1: ×20; 2: ×50; 3: ×100; 4: ×200; 5: ×500; 6: ×800; 7: Marker; 8–13: same dilution factors from lanes 1 to 6).

Quantification of *L. mesenteroides* and *L. plantarum* Populations

Application of the competitive PCR method to specifically detect lactic acid bacteria was tested for a real food system, namely watery kimchi. The results of PCR amplification in 1-day and 2-weeks fermented watery kimchi samples are shown in Fig. 3. *L. mesenteroides* was detected only in the 1-day fermented sample (Fig. 3A), while *L. plantarum* was observed only in the 2-weeks sample (Fig. 3B). These results clearly demonstrated that *L. mesenteroides* was a major microorganism in the early stage of watery kimchi fermentation although its population diminishes as the pH decreases during the course of fermentation. In contrast, *L. plantarum* was an organism proliferating in the latter stage

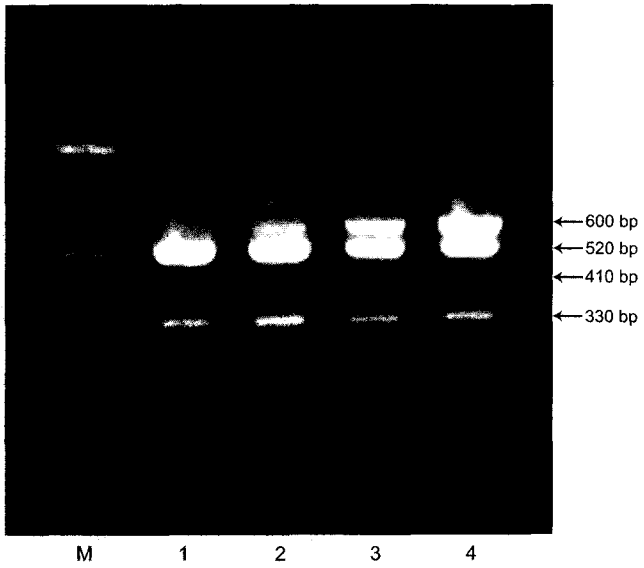

Fig. 2. Multiplex competitive PCR in the presence of competitor.
The sizes of the competitor for *L. mesenteroides* and *L. plantarum* were 520 and 330 bp, respectively (Lane 1: ×1; lane 2: ×10; lane 3: $\times 10^2$; lane 4: $\times 10^3$).

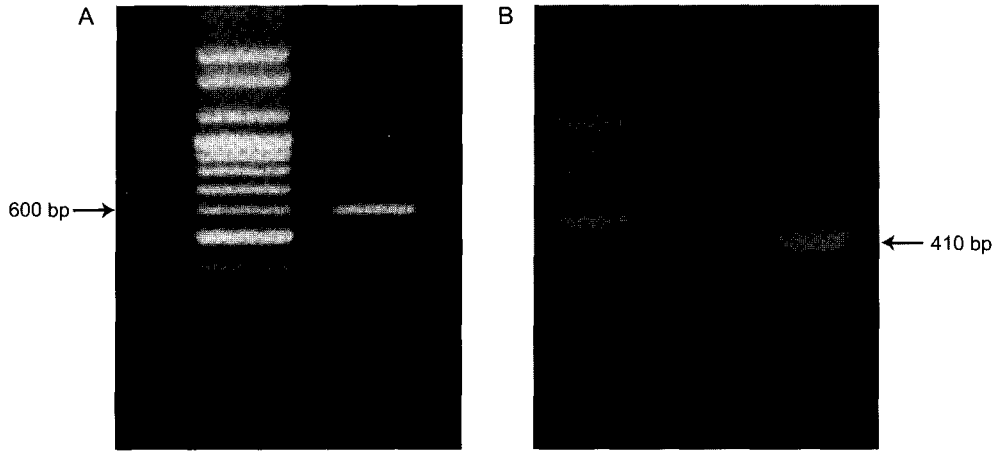

Fig. 3. Specific detection of *L. mesenteroides* and *L. plantarum* in watery kimchi using mes and pla primers.
Detection of *L. mesenteroides* in 1-day fermented watery kimchi (A) and of *L. plantarum* in 2-weeks fermented sample (B).

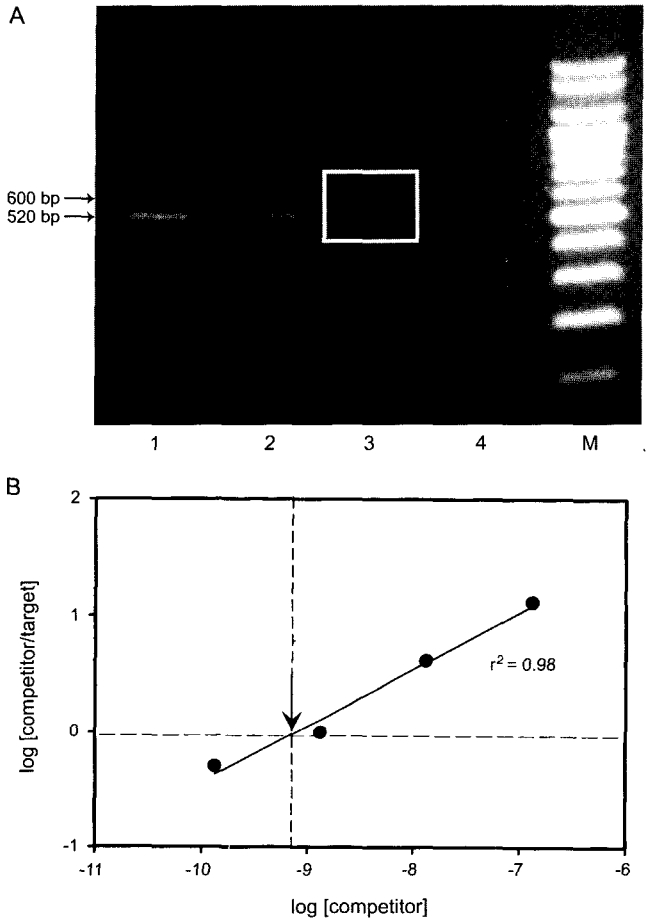

Fig. 4. Competitive multiplex PCR amplification of 1-day fermented watery kimchi (A).
Lane 1: $\times 10^7$; lane 2: $\times 10^8$; lane 3: $\times 10^9$; lane 4: $\times 10^{10}$; M: Marker. The amount of target DNA was estimated from competitor concentration and PCR band intensities (B). Arrow indicates template DNA concentration yielding an identical PCR band intensity identical with that of the competitor (y=0.486x+4.427, X-intercept=−9.1, log [competitor]=−9.1 Amount of target DNA=$1.33\times 10^{-9.1}\times 50=5.2\times 10^{-8}$ [μg/ml]).

of fermentation because of its strong tolerance to lactic acid, as reported elsewhere [19].

To quantitatively enumerate the number of *L. mesenteroides* and *L. plantarum*, linear regression curves were obtained to correlate band intensity of the PCR product with the number of viable cells measured using traditional plate-counting.

Quantitative PCR was applied to the sample of 1-day fermented kimchi. Multiplex PCR results for the target DNA, obtained with competitor template diluted at various factors ($\times 10^7$, $\times 10^8$, $\times 10^9$, $\times 10^{10}$), are illustrated in Fig. 4A and their corresponding band intensities are shown in Fig. 4B. The amount of target DNA (5.2×10^{-8} μg/ml) yielding a PCR band intensity identical with that of the competitor template was estimated by a linear regression analysis of the experimental data, as shown in Fig. 4B.

Populations of *L. mesenteroides* and *L. plantarum* in the mixed suspension culture were measured, using both the quantitative PCR amplification and the traditional plate-counting methods. *L. mesenteroides* grew faster than *L. plantarum* with a high specific growth rate (μ=0.88 h^{-1}) as depicted in Fig. 5. The band intensity of each PCR product correlated well with the growth patterns of *L. mesenteroides* and *L. plantarum* in the mixed suspension culture. Each lane represents the fermentation time (h) from 0 to 20 with the interval being 2 h. The above two quantification methods showed the compatible growth profiles of the two lactic acid bacteria suggesting that this PCR-based method could be applied to selectively estimate the population densities of microflora in a mixed culture.

In conclusion, the key microorganisms *L. mesenteroides* and *L. plantarum* in kimchi fermentation could be specifically detected and enumerated by competitive PCR amplification. The 16S rRNA gene for *L. plantarum* and the dextransucrase gene for *L. mesenteroides* were selected as target DNA for PCR amplification. The competitive PCR methods showed almost the same growth profiles for

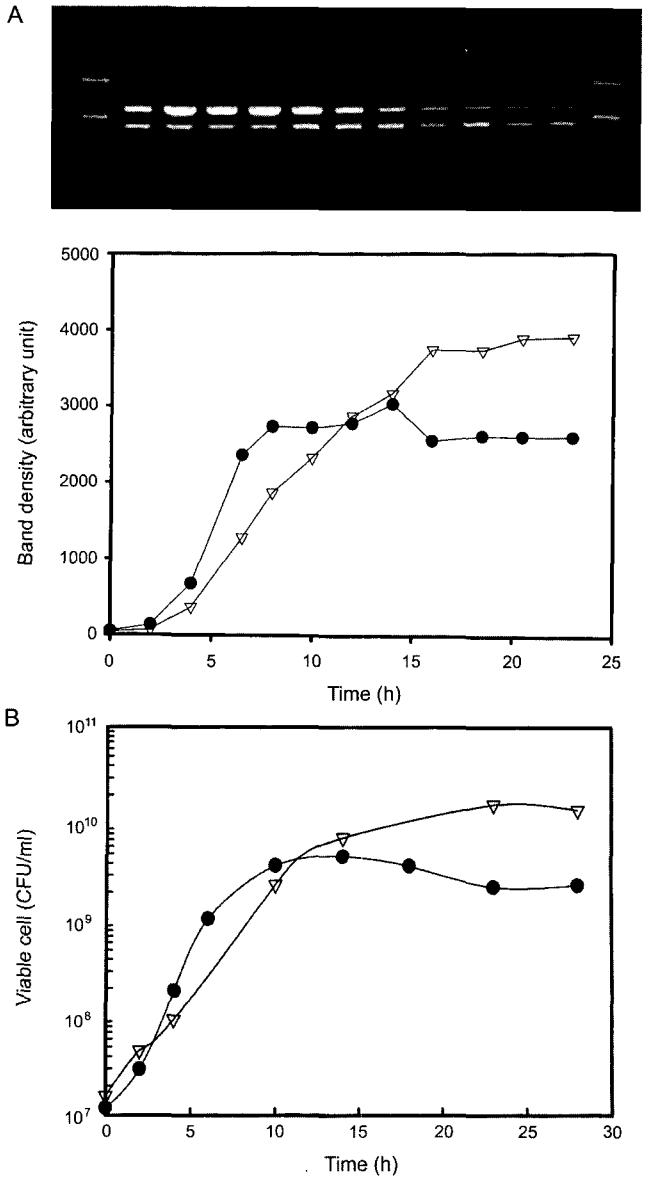

Fig. 5. The comparison of quantitative PCR (A) with the colony-counting method (B) to predict growth profiles of *L. mesenteroides* (●) and *L. plantarum* (▽) in a mixed suspension culture.

the two model organisms, as predicted by the traditional plate-counting method. Since rapid and reliable identification and quantification of a target microorganism is an important issue in a mixed microfloral community, including kimchi fermentation, the method developed in the present study could be well applied to other mixed fermentation systems.

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References

1. Becker-André, M. and K. Hahlbrock. 1989. Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY). *Nucleic Acids Res.* **17:** 9437–9499.
2. Chang, C. E., S. C. Kim, J. S. So, and H. S. Yun. 2001. Cultivation of *Lactobacillus crispatus* KLB46 isolated from human vagina. *Biotechnol. Bioprocess Eng.* **6:** 128–132.
3. Chagnaud, P., K. Machinis, L.A. Coutte, A. Marecat, and A. Mercenier. 2001. Rapid PCR-based procedure to identify lactic acid bacteria: Application to six common *Lactobacillus* species. *J. Microbiol. Methods* **44:** 139–148.
4. Diviacco, S., P. Norio, L. Zentilin, S. Menzo, and M. Clementi. 1992. A novel procedure for quantitative polymerase chain reaction by coamplification of competitive templates. *Gene* **122:** 313–320.
5. Gerard, M., E. C. de Waal, and A. U. Uitterlinden. 1992. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding 16S rRNA. *Appl. Environ. Microbiol.* **59:** 695–700.
6. Han, N. S., Y. S. Jung, H. J. Eom, Y. H. Koh, J. F. Robyt, and J. H. Seo. 2002. Simultaneous biocatalytic synthesis of panose during lactate fermentation in kimchi. *J. Microbiol. Biotechnol.* **12:** 46–52.
7. Hansen, M. C., T. T. Nielsen, M. Givskov, and S. Molin. 1998. Biased 16S rDNA PCR amplification caused by interference from DNA flanking the template region. *FEMS Microbiol. Ecol.* **26:** 141–149.
8. Henco, K. and M. Heibey. 1990. Quantitative PCR; The determination of template copy numbers by temperature gradient gel electrophoresis (TGGE). *Nucleic Acids Res.* **18:** 6733–6734.
9. Hong, S. I. and W. S. Park. 2000. Application of time-temperature indicators for monitoring kimchi fermentation. *Food Sci. Biotechnol.* **9:** 258–262.
10. Johnsen, K., E. Øivind, C. S. Jacobsen, L. Thirup, and V. Torsvik. 1999. Quantitative selective PCR of 16S ribosomal DNA correlates well with selective agar plating in describing population dynamics' of indigenous *Pseudomonas* spp. in soil hot spots. *Appl. Environ. Microbiol.* **65:** 1786–1789.
11. Kim, T. W., S. G. Min, D. H. Choi, J. S. Jo, and H. Y. Kim. 2000. Rapid identification of *Lactobacillus plantarum* in kimchi using polymerase chain reaction. *J. Microbiol. Biotechnol.* **10:** 881–884.
12. Ko, S. H. and C. Ahn. 2000. Bacteriocin production by *Lactococcus lactis* KCA2386 isolated from white kimchi. *Food Sci. Biotechnol.* **9:** 263–269.
13. Markoulatos, P., N. Siafakas, and M. Moncany. 2002. Multiplex polymerase chain reaction: A practical approach. *J. Clinic. Lab. Anal.* **16:** 47–51.
14. Martin, R. A., M. Mattias, A. Ehrmann, and R. F. Vogel. 2000. Multiplex PCR for the detection of *Lactobacillus pontis* and two related species in a sourdough fermentation. *Appl. Environ. Microbiol.* **66:** 2113–2116.

15. Phillip, H., E. Studer, and J. Lüthy. 1999. Quantitative competitive PCR for the detection of generally modified organisms in food. *Food Control* **10:** 353–358.
16. Polz, M. F. and C. M. Cavanaugh. 1998. Bias in template-to-product ratios in multitemplate PCR. *Appl. Environ. Microbiol.* **64:** 3724–3730.
17. Seibel, P., O. Mell, A. Hannemann, J. Müller-Höcker, and B. Kadenbach. 1991. A method for quantitative analysis of detected mitochondrial DNA by PCR in small tissue sample. *Methods Mol. Cell Biol.* **2:** 147–153.
18. Suzuki, M. T. and S. J. Giovannoni. 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl. Environ. Microbiol.* **62:** 625–630.
19. Yeon, C. J., M. K. Kim, and J. H. Lee. 2002. Re-evaluation of the change of *Leuconostoc* species and *Lactobacillus plantarum* by PCR during kimchi fermentation. *J. Microbiol. Biotechnol.* **12:** 166–171.