

A competitive quantitative polymerase chain reaction method for characterizing the population dynamics during kimchi fermentation

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Abstract The aim of this study was to develop a competitive quantitative-PCR (CQ-PCR) method for rapid analysis of the population dynamics of lactic acid bacteria (LAB) in kimchi. For this, whole chromosome sequences of *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, and *Lb. brevis* were compared and species-specific PCR primers targeting dextranucrase, 16S rRNA, and surface layer protein D (SlpD) genes, respectively, were constructed. The tested strains were quantified both in medium and kimchi by CQ-PCR and the results were compared with the data obtained using a conventional plate-counting method. As a result, the three species were successfully detected and quantified by the indicated primer sets. Our results show that the CQ-PCR method targeting species-specific genes is suitable for rapid estimation of LAB population to be used in the food fermentation industry.

Keywords *Leuconostoc mesenteroides* · *Lactobacillus plantarum* · *Lactobacillus brevis* · Kimchi · CQ-PCR

Introduction

Kimchi, a fermented vegetable product, is made of cabbage and is mixed with different spices including garlic,

ginger, green onion, chives, fish source, salt and hot red pepper [1, 15]. In recent years, kimchi has become a globally popular food because of its taste as well as its health-promoting effects [6, 9, 17]. The microbial diversity analysis of kimchi revealed that *Leuconostoc mesenteroides* and *Lactobacillus plantarum* were the predominant species [7, 12]. Since 2004, *L. mesenteroides* DRC has been used as a starter culture in the Korean kimchi industry for achieving higher organoleptic effects. Previous studies reported that *Leuconostoc* spp. were dominant from the initial to the middle stages of the kimchi fermentation and the population gradually decreased when the pH of kimchi decreased below 4.0. Populations of homo-fermentative *Lb. plantarum* and *Lb. brevis* having strong tolerance to acidic pH continuously increased to the final fermentation stage [3, 14]. Therefore, after mixing all ingredients, kimchi is incubated at a low temperature (10 °C) for 1–3 days until the various LAB achieve uniform and exponential growth, so that the ripened product with a consistent flavor should be delivered to the consumers. Producers need a rapid method for quantifying LAB populations during the kimchi fermentation so that the optimal incubation time for high quality product can be determined.

PCR-based methods are frequently used to detect changes in microbial populations. Microorganisms can be monitored by DNA fingerprinting techniques such as PCR-DGGE, quantitative PCR, and metagenomic analysis via next generation sequencing [8, 13, 16]. Although they are potential tools to obtain information of microbial populations, the operations usually take long time (≥ 24 h) due to the multiple steps of genetic manipulations and thus they are not suitable for the use in fermentation industries. Meanwhile, competitive quantitative-PCR (CQ-PCR) may be an alternative approach; it has been used for the quantification of cellular DNA and RNA as well as viral and

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bacterial communities [11, 19, 22]. In competitive PCR, a known amount of DNA fragment (competitor) is added to the sample (this competitor must contain sequences for the primers that are used to amplify the target sequence). When the target DNA and competitor are amplified together, both templates compete for the same set of the primers and due to this competition the ratio of the amounts of the two amplified products reflects the ratio of the amounts of the target DNA and competitor [5, 21]. Since the initial amount of the competitor is known, the amount of the target DNA can then be estimated from the $T:C$ ratio (T = amount of PCR amplified product from the target DNA; C = amount of PCR amplified product from the competitor). (Further information can be obtained in subsection 2.3 of the “Materials and methods” section and Fig. 2 cited in the Results section).

Therefore, in this study a CQ-PCR method was developed to monitor the population changes of LAB during kimchi fermentation. Dongchimi-kimchi was selected among several types of kimchi, due primarily to the fact that this type of kimchi is produced via liquid-type fermentations; this allowed significantly improved simplicity and accuracy with regard to control and analysis. And, *L. mesenteroides*, *Lb. plantarum*, and *Lb. brevis* were chosen as dominant LAB in kimchi and their whole genome sequences were analyzed to discover species-specific gene sequences. Based on the results, we constructed species-specific primers and CQ-PCR results were compared with the data obtained using a conventional plate-counting method.

Materials and methods

LAB strains and culture condition

Lb. plantarum KCCM 11322 and *L. mesenteroides* KCCM 11324 were obtained from the Korean Culture Center of Microorganism (KCCM, Seoul, Korea) and *Lb. brevis* K9 was donated by Prof. Hong-hui Han at Inha University. Culture stocks of *Lb. plantarum*, *L. mesenteroides*, and *Lb. brevis* K9 stored at -80°C were thawed and incubated in de Man, Rogosa, and Sharpe (MRS) broth (Difco, Detroit, MI, USA) at pH 6.5 with shaking at 250 rpm. Batch fermentations of mixed cultures were carried out in 500 mL baffled flasks containing 100 mL of MRS broth at 30°C with shaking at 250 rpm. The initial pH of the MRS broth was adjusted to pH 6.5 with 2 N NaOH.

Sample preparation from fermented kimchi for PCR

The DNA templates for CQ-PCR were prepared as described below [18]. A watery radish kimchi, so-called dongchimi

was prepared by mixing radish(80 g), garlic(10 g), scallion(20 g) in 4 L of salty water (3 % NaCl) and it stored at 10°C for 19 days for fermentation. Kimchi juice samples (5 mL) taken periodically from fermented kimchi were spun down at $10,000\times g$ at 4°C for 30 min and the supernatant was discarded carefully. The pellet was washed three times with phosphate-buffered saline (PBS) buffer at pH 7.4 and then resuspended with 1 mL distilled water (DW). They were subsequently diluted with DW and the diluted samples were used as a template for CQ-PCR. CQ-PCR analysis was carried out at regular intervals to monitor the changes in the population of the three LAB species during fermentation at 10°C which is classic storage temperature of kimchi.

Species-specific primers design

To design species-specific primers for three LAB strains, we used a sequence alignments program VISTA (<http://genome.lbl.gov/vista/index.shtml>) using chromosome sequences of *L. mesenteroides* subsp. *mesenteroides* ATCC 8293 (http://www.ncbi.nlm.nih.gov/genome/1078?genome_assembly_id=170424), *Lb. plantarum* subsp. *plantarum* ST-III (http://www.ncbi.nlm.nih.gov/genome/1108?genome_assembly_id=170519) and *Lb. brevis* ATCC 367 (http://www.ncbi.nlm.nih.gov/genome/1110?genome_assembly_id=170542). On the basis of these comparative genomic sequence output, target genes which had no significant similarity in nucleotide sequence among other LAB were selected for PCR targets. These species-specific primers were synthesized by Bioneer Inc. (Daejun, Korea).

Competitors' construction

The competitor of each target gene was used as an internal standard template during the PCR amplification process between the templates and products. DNA competitors were prepared according to the manufacturer's instructions for the Competitive DNA Construction Kit (Takara, Kusatsu, Japan). The sequences at both 5'-ends were the same for primers amplifying the target templates of the three microorganisms in mixed cultures and kimchi samples. The different sizes of CQ-PCR amplified targets and internal sequences were distinguishable in agarose gel. The sense and antisense primers for the construction of the DNA competitor were prepared by flanking the 3'-termini of each sense and antisense primers of the three target templates with the sequence for amplification of the template DNA.

Multiplex PCR

As preliminary experiments prior to multiplex PCR, each strain was individually cultured and was subjected to PCR

using three primer set combined to check non-specific amplification and to determine optimum condition. Multiplex PCR amplification was performed in 20 μL of reaction mixture containing 5 μL of the diluted sample ($\sim 10^3$ CFU/mL of the strains) as PCR template, 0.5 μM each primer, 0.25 mM deoxynucleoside triphosphates (dNTPs), 2.5 U *Taq* DNA polymerase and 1 μL of competitor diluted with UltraPure™ Glycogen solution. The amplification program was as follows: 1 cycle of pre-denaturation for 5 min at 94 °C; 30 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; 1 cycle of final extension at 72 °C for 7 min. For multiplex PCR, amplification reactions were performed in a 50 μL reaction mixture containing 5 μL sample of each strain culture as PCR template, 0.5 μM each primer, and 0.25 mM dNTPs. Various concentrations of Mg^{2+} and HotStar *Taq* DNA polymerase (Qiagen, Hilden, Germany) were used to determine the optimal reaction concentrations.

CQ-PCR

CQ-PCR was performed with kimchi samples to verify the application of the primers to analyze the three LAB species and concomitantly, to evaluate if it is ready to be used for this purpose. CQ-PCR was performed with serially diluted competitor DNA in an UltraPure™ Glycogen solution (10 $\mu\text{g}/\text{mL}$: Life Technologies, Carlsbad, CA, USA) at concentrations sufficient to validate the predicted size and competing ratios. The conditions for CQ-PCR were set up as follows: 1 cycle of pre-denaturation for 5 min at 94 °C; 36 cycles for *Lb. brevis* K9 and 28 cycles for *L. mesenteroides* and *Lb. plantarum* consisting of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s; and 1 cycle of final extension at 72 °C for 30 s. PCR amplification was performed in a total 20 μL reaction mixture containing 5 μL of PCR template, 0.5 μM each primer, 0.25 mM dNTPs, 2.5 U *Taq* DNA polymerase, and 1 μL of competitor. The amplification density of PCR products was analyzed with a densitometer (Bio-Rad, Richmond, CA, USA). All regression and data expression analyses were carried out using Sigmaplot 2000 (Systat Software Inc., San Jose, CA, USA). From the results of competitive

PCR, the initial amount of target DNA template could be estimated from the ratio of *T* to *C* (*T* = amount of PCR amplified target template; *C* = amount of PCR amplified competitor). By determining the amount of competitor that gave a *T/C* ratio of 1 for each sample, the relative amounts of target DNA could be obtained. In this method, the amplification efficiency of the target template and of the competitor was assumed to be equal.

Plate-counting for LAB quantification in kimchi and metabolite analysis

For the determination of cell numbers, a conventional plate-counting method was used. An aliquot of the individual culture broth of three strains was taken at the indicated time intervals, diluted serially with a saline solution and plated on the MRS medium supplemented with 0.002 % bromophenol blue. The number of colony-forming units per milliliter (CFU/mL) was determined following incubation at 30 °C. To quantify the concentrations of glucose and lactic acid, the culture broth was filtered through a 0.45 μm filter paper (Sigma Aldrich, St. Louis, MO, USA), and the filtrate was analyzed by high-performance liquid chromatography (HPLC: Agilent, Walnut Creek, CA, USA) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) and a refractive index detector. An H_2SO_4 solution (0.01 N) was used as the eluent at a flow rate of 0.6 mL/min.

Results

Primers' design and competitors' construction

Genes encoding dextransucrase of *L. mesenteroides*, 16S rRNA of *Lb. plantarum*, and SlpD of *Lb. brevis* K9 were chosen for target DNA and the primers were designed based on their sequences (Table 1). To confirm multiplex PCR amplification of the genes, the three constructed primer sets were tested with a mixed cell culture of the strains. As shown in Fig. 1, two bands were obtained from each reaction; the length of the PCR products was 603 bp for *L. mesenteroides*, 410 bp for *Lb. plantarum*, and 506 bp

Table 1 Strain-specific primers of the three LAB species used in this study

Target	Primer	Sequence 5' → 3'	Coding gene	Reference
<i>Leuconostoc mesenteroides</i>	mes (Forward)	GTAGATGCTGTTGATAACGTT	Dextransucrase	[10]
	mes (Reverse)	TTGCCATGTATTGAACCATC		
<i>Lactobacillus plantarum</i>	pla (Forward)	TGATTGGTGCTTGCATCATG	16S rRNA	This study
	pla (Reverse)	TGAACAGTTACTCTCAGATA		
<i>Lactobacillus brevis</i> K9	bre (Forward)	CATACTTATCAACTACGACT	Surface layer protein D	This study
	bre (Reverse)	ACATCCTTCTTGGCACCCCTTA		

for *Lb. brevis* K9, while those for the amplified competitors were 500, 300, and 400 bp, respectively. The optimal multiplex PCR conditions were 3 mM MgCl₂ and 2.5 U Taq DNA polymerase per 20 μL reaction volume.

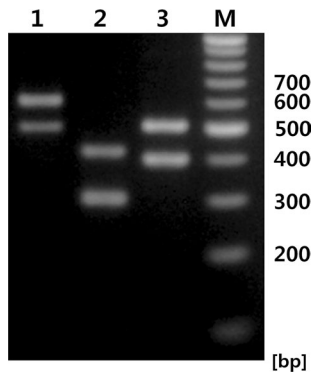


Fig. 1 Agarose gel electrophoresis of CQ-PCR-amplified target and competitor DNAs. Lane 1 amplification products of competitor (500 bp) and target DNA (Dextranucrase) of *L. mesenteroides* (603 bp); lane 2 amplification products of competitor (300 bp) and target DNA (16 s rRNA) of *Lb. plantarum* (410 bp); lane 3 amplification products of competitor (400 bp) and target DNA (slpD) of *Lb. brevis* (506 bp); lane M DNA marker; (bp), nucleotide base pair

Standard curves

The PCR products for the three strains and the serially diluted competitors at known concentrations were obtained (Fig. 2). When the concentration of the competitor decreased (Fig. 2, bottom panel), the signal densities of the 500-, 300-, and 400-bp bands also decreased, and the densities of the 603-, 410-, and 506-bp target bands increased. This result indicates that the target DNAs and the competitor DNAs competed for the same primers. As shown in the upper panel of Fig. 2, standard curves were obtained for an appropriate range of CFU/mL based on the competitive PCR results. Competitors were appropriately diluted to obtain the same signal density as those for target bands (known values of CFU/mL). The equations obtained by the least square method demonstrate relatively good correlation between the copy number of the competitor (y-axis) and CFU/mL (x-axis).

Monitoring of cell growth by CQ-PCR during mixed culture

To ensure a proper PCR amplification signal in a mixed culture, the CQ-PCR method was used during the mixed

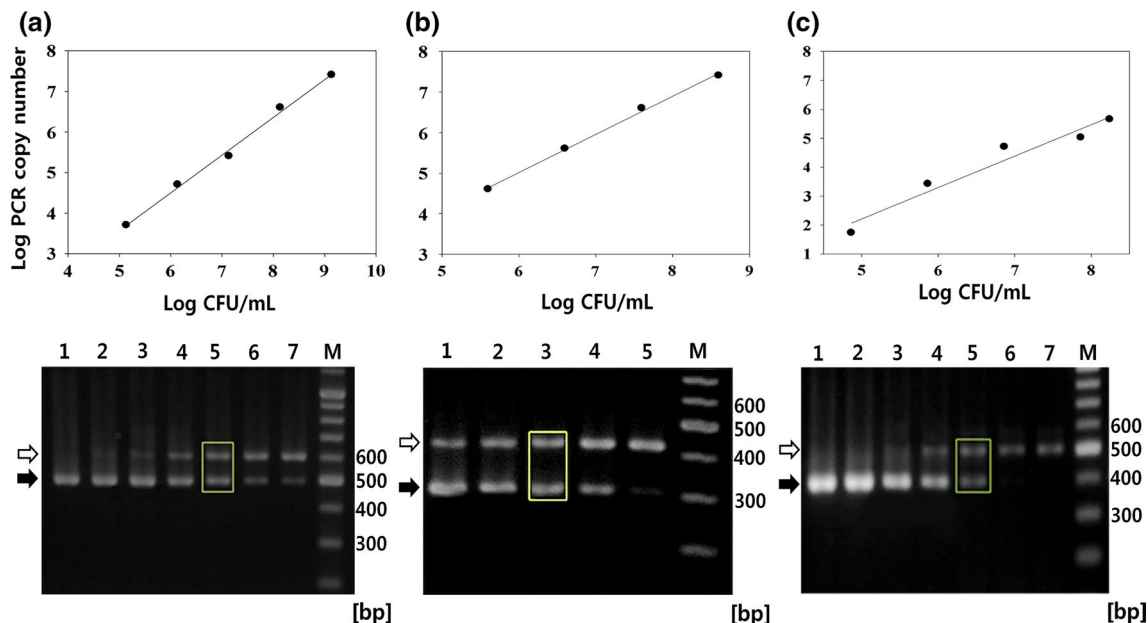


Fig. 2 Correlation between colony-forming units and copy numbers of *L. mesenteroides* (a), *Lb. plantarum* (b), and *Lb. brevis* (c) Agarose gel electrophoresis of CQ-PCR after serial dilution of DNA (lower panels), visualized by EtBr staining. The copy numbers of the competitor were 10^8 , 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , and 10^2 for *L. mesenteroides* (lane 1–7), 7×10^8 , 7×10^7 , 7×10^6 , 7×10^5 and 7×10^4 for *Lb. plantarum* (lane 1–5), and 4×10^7 , 4×10^6 , 4×10^5 , 4×10^4 , 4×10^3 , 4×10^2 and 4×10 for *Lb. brevis* (lane 1–7). Black and white arrows indicate competitor and target PCR amplicons, respec-

tively and yellow boxes show the target densities (concentration) that are the same as the competitors. The point in y-axis was determined where the target density (concentration) was the same as the competitor (upper panels). The equations obtained by the least square method were $y = 0.93x - 1.08$ ($R^2 = 0.99$) for *L. mesenteroides*, $y = 0.94x - 0.63$ ($R^2 = 0.99$) for *Lb. plantarum*, and $y = 1.084x - 3.21$ ($R^2 = 0.95$) for *Lb. brevis* K9, where the y-axis indicates the copy number of the competitor and x-axis indicates CFU/mL. Lane M DNA marker; (bp), nucleotide base pair

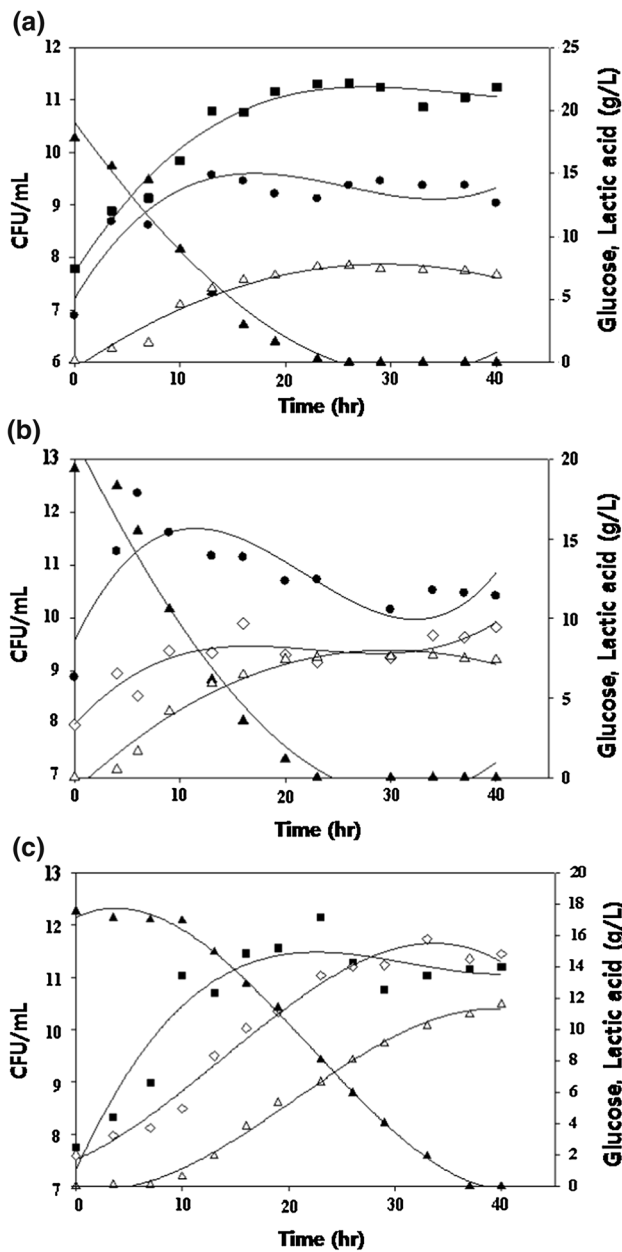


Fig. 3 Mixed fermentation profile of *L. mesenteroides* and *Lb. plantarum* (a), *L. mesenteroides* and *Lb. brevis* (b), and *Lb. plantarum* and *Lb. brevis* (c) monitored by CQ-PCR filled circle *L. mesenteroides*, filled square *Lb. plantarum*, open diamond *Lb. brevis* K9, filled triangle Glucose, open triangle: Lactic acid

cultivation of the strains in three different combinations: *L. mesenteroides* with *Lb. plantarum*, *L. mesenteroides* with *Lb. brevis*, and *Lb. plantarum* with *Lb. brevis*. When *L. mesenteroides* was co-cultured with *Lactobacillus* spp. in MRS broth, *L. mesenteroides* outgrew its partners in the early stage, but the cell numbers of *L. mesenteroides* decreased in the later part of the culture (Fig. 3a, b). The cell numbers of *Lb. plantarum* or *Lb. brevis* increased in the middle and later stages. When *Lb.*

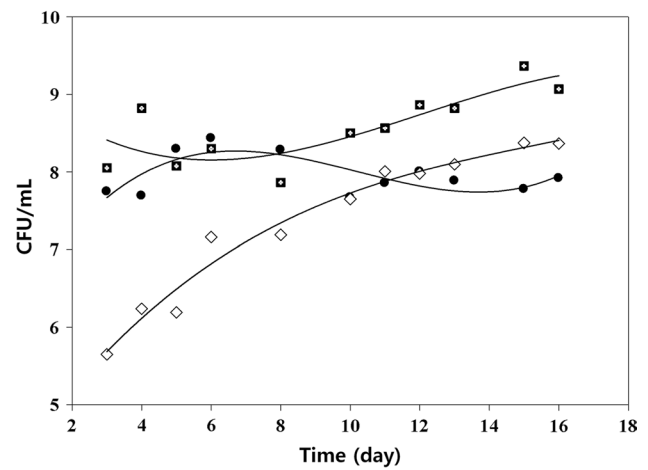


Fig. 4 Application of CQ-PCR during dongchimi-kimchi fermentation. Filled circle *L. mesenteroides*, filled square *Lb. plantarum*, open diamond *Lb. brevis*

plantarum and *Lb. brevis* were co-cultured, the growth rate of *Lb. plantarum* was higher than that of *Lb. brevis* in the early stage of the culture; however, *Lb. brevis* dominated *Lb. plantarum* after 26 h (Fig. 3c). Glucose was rapidly consumed and lactic acid was accumulated in the medium in all three cultures along with cell growth. These results demonstrated that the CQ-PCR method designed in this study can be used to monitor cell growth during dual-starter fermentation in a strain-specific manner.

Monitoring of LAB dynamics in kimchi product by CQ-PCR

To verify the applicability of this method, a kimchi was prepared and incubated at 10 °C for 19 days and the population dynamics of the three LAB species in kimchi was analyzed by CQ-PCR. As shown in Fig. 4, *L. mesenteroides* was the predominant member of the LAB population in the early stage and *Lactobacillus* spp. outgrew the other species in the later stage. This result is consistent with many previous results [10]; *Leuconostoc* spp. dominated from the initial to the middle stages of fermentation (=9 log CFU/mL) but homo-fermentative *Lb. plantarum* and *Lb. brevis* outgrew it in the later stage due to their different pH tolerance.

Discussion

The quantitative methods for the assessment of microbial communities in food systems have been generally based on the colony-counting method, which involves the incubation of microorganisms in a selective medium under

suboptimal growth conditions. However, it is well known that the identification of LAB with conventional methods is complicated and can yield ambiguous results, because similar nutritional conditions for their growth were required in spite of different sub-species. Therefore, the conventional method using selective media is not ideal for monitoring fermentative processes from a quantitative aspect; it takes a relatively long period of time to get results and only the organisms that have the ability to survive in an artificial medium can be enumerated.

As molecular biology techniques have been developed, quantitative PCR methods for detecting 16S rRNA gene sequences [2, 4] or the 16S-23S intergenic spacer regions [20] have been developed using specific PCR primers. However, since these primers are often sensitive to little changes in annealing temperature, multiple gene amplification may occur and it can prevent accurate identification of microbes. Therefore, we designed primers targeting species-specific genes (dextranucrase gene of *L. mesenteroides* and surface layer protein D gene of *Lb. brevis*) after alignment analysis of whole chromosome sequences and it resulted in precise and consistent results compared to 16R rRNA at different ranges of annealing temperatures (data not shown).

The quality of kimchi depends on the composition of the LAB population involved in the fermentation process. Therefore, to control the ripening process for quality-controlled kimchi production, it is important to monitor the proliferation of the microbial community in situ during the fermentation period. The CQ-PCR method developed in this study is determined to be effective for monitoring the important LAB species associated with good-quality kimchi. The primers were specific for three species and CQ-PCR results provided reasonable estimation of their cell growth and, when compared to other recent methods (real time-PCR, PCR-DGGE, and next generation pyrosequencing), the costs for chemicals and equipment of CQ-PCR were not high. Furthermore, a single analysis using CQ-PCR can be carried out within 2 h which is considerably faster than the conventional methods and the procedure is simple to be used in the production processes.

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