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A Species-Specific qPCR Method for Enumeration of *Lactobacillus sanfranciscensis*, *Lactobacillus brevis*, and *Lactobacillus curvatus* During Cocultivation in Sourdough

Hyun-Wook Baek[1] · Seul-Ah Kim[2] · Won-Ki Min[3] · Shin Dal Kang[4] · Sangmin Shim[4] · Nam Soo Han[2] · Jin-Ho Seo[1,4]

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Abstract

Lactobacillus sanfranciscensis, *Lactobacillus brevis*, and *Lactobacillus curvatus* are frequently isolated from cereal-based fermented foods and are the dominant lactic acid bacteria in Korean sourdough. Detection of the individual species and their enumeration during fermentation are of great importance for maintaining the quality of bakery products. Here, we developed a species-specific quantitative polymerase chain reaction (qPCR) method to monitor the population of these three bacterial species during cocultivation in sourdough. Target genes, including those encoding nucleoside hydrolase, hypothetical protein, and glyoxalase, were selected by MegaBLAST for the detection of *L. sanfranciscensis*, *L. brevis*, and *L. curvatus*, respectively. The specificities of PCR primer sets were verified with qPCR; constant cycle threshold (Ct) values were obtained from mixed genomic DNAs (gDNAs) of target and non-target strains. The qPCR results were unaffected by the sourdough matrix. The cell numbers derived from qPCR were 10–65% higher than those obtained using a conventional plate-counting method. The qPCR standard curves for the three *Lactobacillus* species were established, and their populations were successfully enumerated during sourdough propagation. This method would enable quantification of three *Lactobacillus* species during cocultivation in sourdough and provide useful information on microbial commensalism that is essential to obtain high-quality bread.

Keywords Sourdough · Real-time quantitative polymerase chain reaction · *Lactobacillus sanfranciscensis* · *Lactobacillus brevis* · *Lactobacillus curvatus*

Introduction

Sourdough has been used in bread production worldwide due to the improved rheological, organoleptic, and nutritional properties compared to the products made by straight dough processes (Hansen and Schieberle 2005; Pétel et al. 2017). The difference between two bakery products is based on metabolic compounds produced by lactic acid bacteria (LAB) at dough stage (Gänzle and Ripari 2016). The beneficial contributions during sourdough fermentation from LAB include production of exopolysaccharides that improved volume, texture, and shelf life (Lin and Gänzle 2014) and formation of anti-microbial and volatile compounds (Choi et al. 2012; Leroy and De Vuyst 2004). Traditional sourdoughs have geographical and cultural identities all over the world because of their natural ingredients as well as fermentation methodologies, and thus these differences result in LAB microflora in sourdough (Dertli et al. 2016). In our previous study, we identified *Lactobacillus sanfranciscensis*, *L. curvatus*, and *L. brevis* as prevalent species in Korean sourdough that represented 56, 27, and 9% of the total isolates, respectively (Lee et al. 2015). They are also frequently detected in other types of sourdough originated from various substrates (De Vuyst et al. 2014; Liu et al. 2018). Growth of *Lactobacillus* spp. may positively or negatively affect to each other due to their metabolites such as organic acids and bacteriocins and their

Hyun-Wook Baek and Seul-Ah Kim contributed equally to this work.

✉ Nam Soo Han
namsoo@cbnu.ac.kr

[1] Department of Agricultural Biotechnology, Center for Food and Bioconvergence, Seoul National University, Seoul 08826, Republic of Korea

[2] Brain Korea 21 Center for Bio-Resource Development, Division of Animal, Horticultural, and Food Sciences, Chungbuk National University, Cheongju 28644, Republic of Korea

[3] Department of Food Science and Development, Kyungil University, Gyeongbuk 38428, Republic of Korea

[4] Research Institute of Food and Biotechnology, SPC Group, Seoul 08826, Republic of Korea

enzymatic activities including proteases and amylases (Zhang et al. 2019a). Nowadays, fermentation industries often employ defined-starter cultures consisted of couple of strains to produce high-quality products (Bintsis 2018; Özcan et al. 2020). As the same in the bakery industry, maintenance of the microbial ecosystem in the starter culture could improve and maintain the quality of the products, and thus their population changes during fermentation should be analyzed and monitored at species level (Gaglio et al. 2020). However, there are no methods to specifically and simultaneously quantify these species in sourdough.

Compositions of complex microflora in sourdough result in difficulties in enumeration of particular species by conventional plate counting (Moser et al. 2017; Yazar and Tavman 2012). To guarantee quality of the sourdough, controlling and monitoring microbiota for reproducibility throughout the fermentation progress has been considered necessary (Lin and Gänzle 2014; Park et al. 2019). For this, culture-independent methods like real-time quantitative polymerase chain reaction (qPCR) have been developed for enumerating individual species from complex DNA sample by quantifying the number of copies for target gene (Kim et al. 2015). This qPCR method can be potential alternative of conventional plate counting due to its rapidness and sensitivity for the enumeration of certain bacteria (He et al. 2017). However, the qPCR has often used universal primers targeting 16S rRNA-encoding gene, and it has difficulty in discrimination in species levels of *Lactobacillus* genus due to their similarities in nucleotide sequences (Pontonio et al. 2017; You and Kim 2020; Kim et al. 2020, b). In addition, monitoring population changes of individual species in the food samples fermented by multiple *Lactobacillus* species is much more difficult. Besides, the current metagenomic method based on 16S rRNA gene amplicon sequencing often does not reveal microbial diversity past the genus level and exhibits only moderately accurate profile (Hillmann et al. 2018). Therefore, a reliable and rapid method is needed to monitor dynamics of multiple *Lactobacillus* species during sourdough fermentation (You and Kim 2020).

In the present study, we developed a species-specific real-time quantitative polymerase chain reaction (qPCR) method for *L. sanfranciscensis*, *L. brevis*, and *L. curvatus* to monitor their population during cocultivation in sourdough. To target the species-specific genes of *L. brevis* and *L. curvatus*, we searched the unique nucleotide sequences present in the corresponding genomes using the Mega Basic Local Alignment Search Tool (MegaBLAST) analysis of genome sequences of LAB. Based on the results, we selected the genes for a glyoxalase and hypothetical protein as targets for the species-specific detection of *L. brevis* and *L. curvatus*, respectively. For *L. sanfranciscensis*, we used our previously developed species-specific primer set targeting the nucleoside hydrolase gene of the bacterium (Lee et al. 2015). The specificities of the PCR primer sets were verified by colony PCR and qPCR against different species of LAB. The standard curves for qPCR of the three *Lactobacillus* species were successfully established and applied to monitor the changes in their populations during sourdough propagation.

Materials and Methods

LAB and Culture Conditions

Table 1 lists the microorganisms used in this study. *L. sanfranciscensis*, *L. brevis*, and *L. curvatus* were isolated from the Korean sourdough inoculated with traditional *nuruk*, a preculture of natural microorganisms on a disc prepared from cooked rice and wheat bran. The culture stocks stored at −80 °C were thawed and incubated in MRS medium (Difco, MI, USA) at 30 °C with shaking at 250 rpm under anaerobic conditions. Batch fermentations of mixed cultures were carried out in 500-mL baffled flasks containing 100 mL of MRS broth at 30 °C and 250 rpm. The initial pH of the MRS medium was adjusted to pH 6.5 using 2 N sodium hydroxide (NaOH).

Design of Strain-Specific qPCR Primer Sets

The complete genome sequence data of *L. brevis* KB290 and *L. curvatus* CRL705 were retrieved from National Center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov/genome/). To select each qPCR target, their unknown, hypothetical, and unannotated genes from each genome sequence were screened in priority, and genetic uniqueness was examined with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). As per the comparative genomics, qPCR target genes that lacked any significant similarity in nucleotide sequence among other LAB were selected, and the final target nucleotide sequences were chosen after repetitive assessment for specificity. Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to confirm the specificities of primers toward qPCR amplicon. All primer sets were synthesized by Bioneer Co. (Daejeon, Korea).

Genomic DNA (gDNA) Preparation

For the extraction of gDNA from pure cultures in MRS media, the cells were cultivated until the density reached 10^8-10^9 cells. Cells were centrifuged at 13,000 × *g* for 2 min and the supernatant was discarded. To extract total DNA from sourdough samples, 10 g of sourdough was dissolved in a sterile 0.85% sodium chloride (NaCl) solution and the final volume was made up to 100 mL. After complete resuspension, 50 mL of the suspension was centrifuged at 200 × *g* for 5 min to remove large particles, and the cells were harvested by centrifugation for 15 min at 13,000 × *g*. Cell pellets were washed

Table 1 List of microorganisms used in this study

Species	Strain designation	Source
Lactobacillus sanfranciscensis	ATCC 27651	San Francisco sourdough
L. sanfranciscensis	101	* *Nuruk*
L. brevis	DSM 6235	Spoiled beer
L. brevis	111	*Nuruk*
L. curvatus	ATCC 25601	Milk
L. curvatus	114	*Nuruk*
L. plantarum	ATCC 8014	N.I.
L. paracasei	ATCC 25302	Milk products
L. sakei subsp. *sakei*	ATCC 31063	Pickled cabbage
L. casei	ATCC 393	Cheese
Lactococcus lactis subsp. *lactis*	ATCC 19435	N.I.
Pediococcus pentosaceus	ATCC 33314	Sake mash
Leuconostoc mesenteroides subsp. *mesenteroides*	ATCC 9135	N.I.
L. crustorum	KACC 16344	Artisan wheat sourdough
Le. citreum	ATCC 49370	Honeydew of rye ear
L. paralimentarius	JCM 10415	Japanese sourdough
L. buchneri	ATCC 4005	Tomato pulp

ATCC American Type Culture Collection, Rockville, Maryland, USA; *DSM* Deutsche Sammlung für Mikroorganismen und Zelkulturen, Braunschweig, Germany; *KACC* Korean Agricultural Culture Collection, Suwon, Korea; *JCM* Japan Collection of Microorganisms, Tsukuba, Japan; *N.I.* Not Informed. * *Nuruk* is a preculture to ferment Korean rice wine or sourdough

twice with 1 mL of sterile phosphate-buffered saline (PBS: 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in 1 L at pH 7.4) and the chromosomal DNA was prepared using the genomic DNA prep kit (SolGent, Seoul, Korea). The quantity and purity of gDNA were measured with NanoVue Plus (GE Healthcare Bio-Sciences Co., Piscataway, NJ, USA).

Conditions for PCR and qPCR

PCR was carried out in a 20 μL reaction mixture containing 10 pM primer set, 100 ng of extracted gDNA, 8 μL of double distilled water, and 10 μL of the AccuPower™ PCR PreMix (Bioneer Co., Daejeon, Korea). GeneAmp PCR System 2400 (Applied Biosystems, Foster city, CA, USA) was used, and the amplification program was as follows: denaturation step at 94 °C for 5 min; 40 cycles of denaturation at 94 °C for 1 min, annealing at 40 °C for 1 min, and extension at 72 °C for 1 min; and a final extension step at 72 °C for 10 min (Venturi et al. 2012). DNA electrophoresis was carried out for 1 h at 90 V using Vari-Gel™ Horizontal Mini System (Denville Scientific, MA, US) filled with Tris-acetate-ethylenediaminetetraacetic acid (EDTA) buffer (TAE: 40 mM Trizma base, 20 mM acetic acid, and 1 mM EDTA at pH 8.3).

The qPCR was carried out in a final volume of 25 μL in a reaction mixture comprising 12.5 μL of SYBR® Premix Ex Taq (Takara Bio. Inc., Shiga, Japan), 8.5 μL of sterile distilled water, 2 μL of template DNA (< 500 ng), and 0.4 μM of each primer. CFX Manager™ real-time thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used, and annealing temperatures ranging from 58 to 63 °C were tested to determine optimum qPCR conditions yielding the lowest Ct value, a high final fluorescence value, and a melting curve with a single peak. All samples were analyzed in duplicates. Thermal cycling, fluorescence data collection, and data analysis were carried out using CFX Manager™ Software v 3.0 (Bio-Rad Laboratories). The Ct value was defined as the PCR cycle at which the fluorescence signal exceeded the background level.

Enumeration of Three LAB by qPCR

For enumeration of *L. sanfranciscensis*, *L. brevis*, and *L. curvatus* in sourdough samples, their Ct values were interpolated on a linear regression model generated from the gDNA extracted from reference strain cultures in MRS media. Serial dilutions ranging from 10^3 to 10^9 copies/μL of each gDNA were prepared, and standard curves were obtained by plotting the Ct values against the target gene copy number/μL via qPCR. Each standard curve was derived from qPCR data in triplicates. The reliability criteria were determined by the square

of correlation coefficient (R^2) and amplification efficiency. R^2 value is indicative of how well the data fit to the first-order equation, suggestive of the agreement between actual Ct values in triplicates and the linear range of the assay. Efficiency of qPCR (E) was calculated as follows: $E = 10^{(-1/b)} - 1$, where b is the slope of the linear fit. Quantification limit was defined as the lowest concentration of gDNA at which linearity was maintained. Intra-assay repeatability was evaluated on triplicates of one of the gDNA samples analyzed in a single qPCR experiment. The coefficient of variation (CV) based on Ct values was obtained at different concentrations of gDNA not only in the same qPCR run but also in three independent qPCR runs, estimating intra-assay and inter-assay reproducibility. Copy numbers (or cell numbers) were calculated for all standards using the following formula: Number of copies/μL = $(6.022 \times 10^{23} \times$ gDNA concentrations)/number of bases pairs $\times$ 660 Da, where 6.022×10^{23} is Avogadro's number (molecules/mole) and 660 Da is the average weight of a single base pair (Dhanasekaran et al. 2010).

Sourdough Fermentation and Propagation

L. sanfranciscensis, *L. brevis*, and *L. curvatus* were precultured in MRS medium at 30 °C and 250 rpm under anaerobic conditions. Cells were recovered by centrifugation at 10,000 × *g* for 2 min and suspended in sterile 0.85% NaCl. To prepare 500 g of sourdough, wheat flour (225 g), rye (25 g), tap water (250 mL), and a mixture (5 mL) of three microbial suspensions were continuously mixed for 5 min on a mixer (Chopin & Co., Boulogne, Seine, France). Sourdoughs were fermented at 23 °C for 5 h and stored at 4 °C for 19 h in the back-slopping process during the 7 days of long propagation.

Statistical Analysis

Statistical analysis of the qPCR results was performed using SPSS 12.0 (SPSS Inc, Chicago, IL, USA). Data were considered statistically significant when $p < 0.05$.

Results

Species-Specific Primers

To develop species-specific primer sets for LAB, we retrieved the complete genome sequence data and screened candidate unique genes in their genomes. The genes that showed high identity or similarity with other species in nucleotide sequences were excluded by consecutively running MegaBLAST. The selection criteria were optimized for both highly similar sequences (MegaBLAST) and more dissimilar sequences (discontinuous MegaBLAST). Several candidate genes were used to match and finally the hypothetical gene (LVISKB_0189) of *L. brevis* and glyoxalase gene (EHE85023.1) of *L. curvatus* were selected as targets for qPCR. The corresponding primer sets to obtain final qPCR amplicons were designed for the amplification of each PCR amplicon below 170 bp, as primers producing around 150 bp-sized amplicons are ideal for real-time qPCR (Dhanasekaran et al. 2010). As shown in Fig. 1, the two primer sets BrevF/BrevR and CurvF/CurvR originating from the hypothetical gene of *L. brevis* and glyoxalase gene of *L. curvatus*, respectively, showed no sequence match with any other sequences uploaded in NCBI database. In addition, these primer sequences were commonly present in the gDNAs of 15 strains of *L. brevis* (Fig. 2a) and 12 strains of *L. curvatus* (Fig. 2b), respectively. The resulting primer sets are listed in Table 2. In addition, we used our previously developed species-specific primer set for *L. sanfranciscensis* targeting the nucleoside hydrolase gene of the bacterium (Lee et al. 2015).

Assessment of Primer Specificity Using PCR Amplification

The specificity of each primer set was assessed with PCR using gDNAs prepared from pure cultures of seven different LAB such as *L. sanfranciscensis*, *L. brevis*, *L. curvatus*, *L. plantarum*, *L. buchneri*, *L. sakei*, and *Pediococcus pentosaceus*. The references of LAB were selected such that they were mainly detected in sourdough or *nuruk*. As shown in Fig. 3, single specific bands of expected sizes were obtained from each PCR reaction; the length of PCR products was 129 bp for *L. sanfranciscensis*, 162 bp for *L. brevis*, and 120 bp for *L. curvatus*, while we failed to detect any nonspecific amplification with gDNA samples of other bacteria. The results of DNA sequencing of PCR amplicons sub-cloned into the TA cloning vector also proved that each primer set amplified the right nucleotide regions located at the intended loci in their genomes (data not shown). Thus, the primer sets could be used to selectively detect the gDNA of *L. sanfranciscensis*, *L. brevis*, or *L. curvatus*.

Assessment of Primer Specificity Using qPCR

Primer specificity is especially important when qPCR is associated with SYBR Green, as this intercalating dye is nonspecific and detects all double-stranded DNA fragments. The specificity of each primer set was assessed with qPCR amplification of gDNAs from 12 different bacterial species, mainly covering bacteria that are commonly found in sourdough. Table 3 show that the Ct values ranged from 10.62 to 13.53 under optimal conditions with the amount of DNA present. Non-target DNA showed Ct values over 27, which was different from the value reported for the negative control (blank). In addition, single peaks were observed on the amplicon

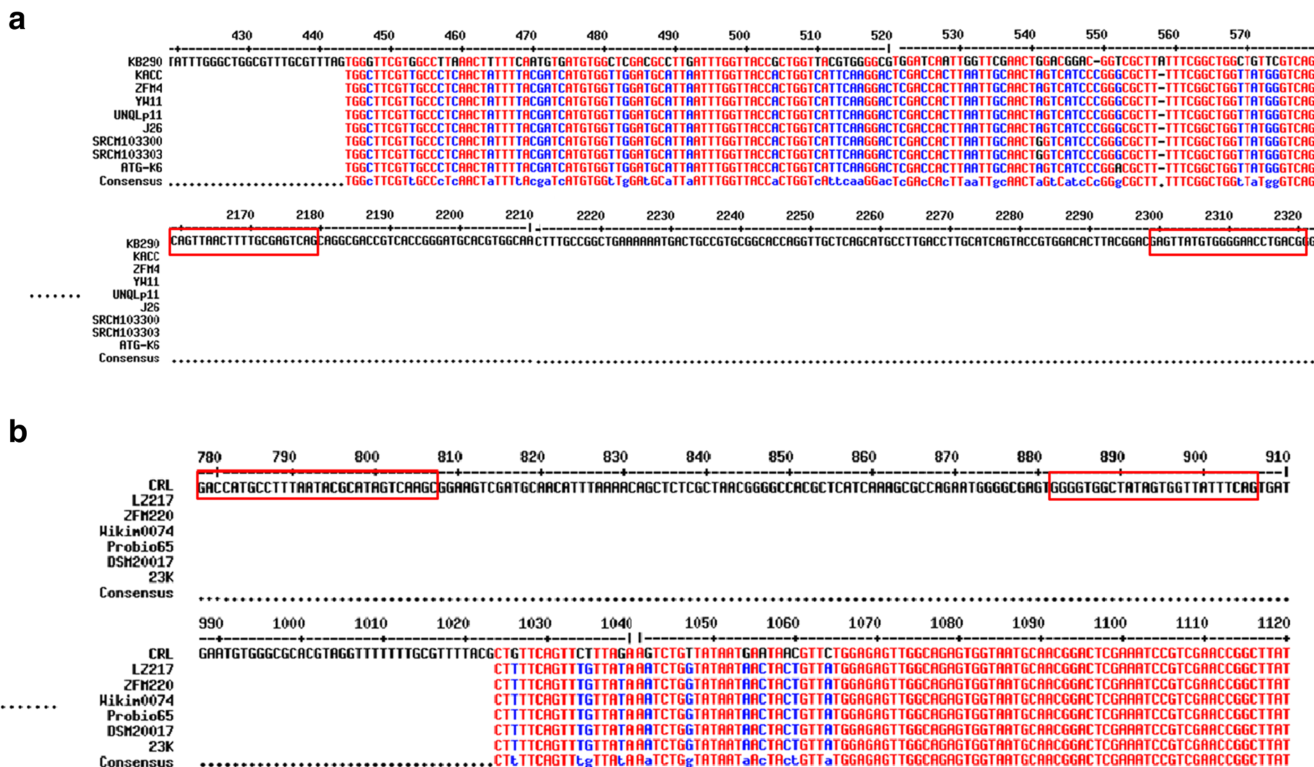

Fig. 1 Alignments of *Lactobacillus brevis* hypothetical protein gene **a** and *L. curvatus* glyoxalase gene **b** with nucleotide sequences from other species that showed sequence similarity based on discontinuous MegaBLAST (NCBI). The alignment was conducted based on MULTALIN (http://multalin.toulouse.inra.fr/multalin/), and intermediate sequences were omitted. Abbreviations: KB290, *L. brevis*; CRL, *L. curvatus* CRL270. Species-specific primers were designed based on the boxes

melting curves for each primer set of *L. sanfranciscensis*, *L. brevis*, and *L. curvatus* at their melting temperatures (Tm) of 84.5, 87, and 78 °C, respectively (data not shown). These observations indicate the specificity of the primer sets against their target DNAs during the amplification reaction of real-time PCR.

Validation of qPCR Assays

Standard curves for the quantification of *L. sanfranciscensis*, *L. brevis*, and *L. curvatus* were obtained from 10-fold serial dilutions of gDNAs of *L. sanfranciscensis* 101, *L. brevis* 111, and *L. curvatus* 114 isolated from *nuruk* (Fig. 4). A good linear correlation was obtained between the Ct values and number of genome copies for all standard curves, and the corresponding R^2 values ranged from 0.995 to 0.999. These correlation coefficients demonstrated the reliability of the qPCR assays over a range of 5 log units. The slope of the standard curve of the gDNA for *L. curvatus* was −3.27, which was close to that of a PCR with an efficiency of approximately 100% (−3.30) (Chandelier et al. 2006). In contrast, the slopes of the gDNAs for *L. sanfranciscensis* and *L. brevis* were less than −3.30, indicative of an efficiency < 100%. The intra- and inter-assay CV values (mean over standard deviation ×100)

Table 2 List of species-specific primers used in this study for quantification of *Lactobacillus sanfranciscensis*, *L. brevis*, and *L. curvatus* by qPCR

Target species	Primer	Sequence (5'- 3')	Target sequences	Amplicon size (bp)	Amplicon Tm (C)	Ref.
Lactobacillus sanfranciscensis	SanfF SanfR	CGGTTTCCGTACTAGCGTTCTTTC ACCCATCATCGCAAGAACTGAC	Nucleoside hydrolase (LSA_RS01825)	129	84–84.5	Lee et al. 2015
L. brevis	BrevF BrevR	CAGTTAACTTTTGCGAGTCAGCAG CGTCAGGTTCCCCACATAACTC	Hypothetical protein (LVISKB_0189)	162	86.5–87	In this study
L. curvatus	CurvF CurvR	GACCCATGCCTTTAATACGC ATAGTCAAGC CTGAAATAACCACTATAGCCACCCC	Glyoxalase (EHE85023.1)	120	78–78.5	In this study.

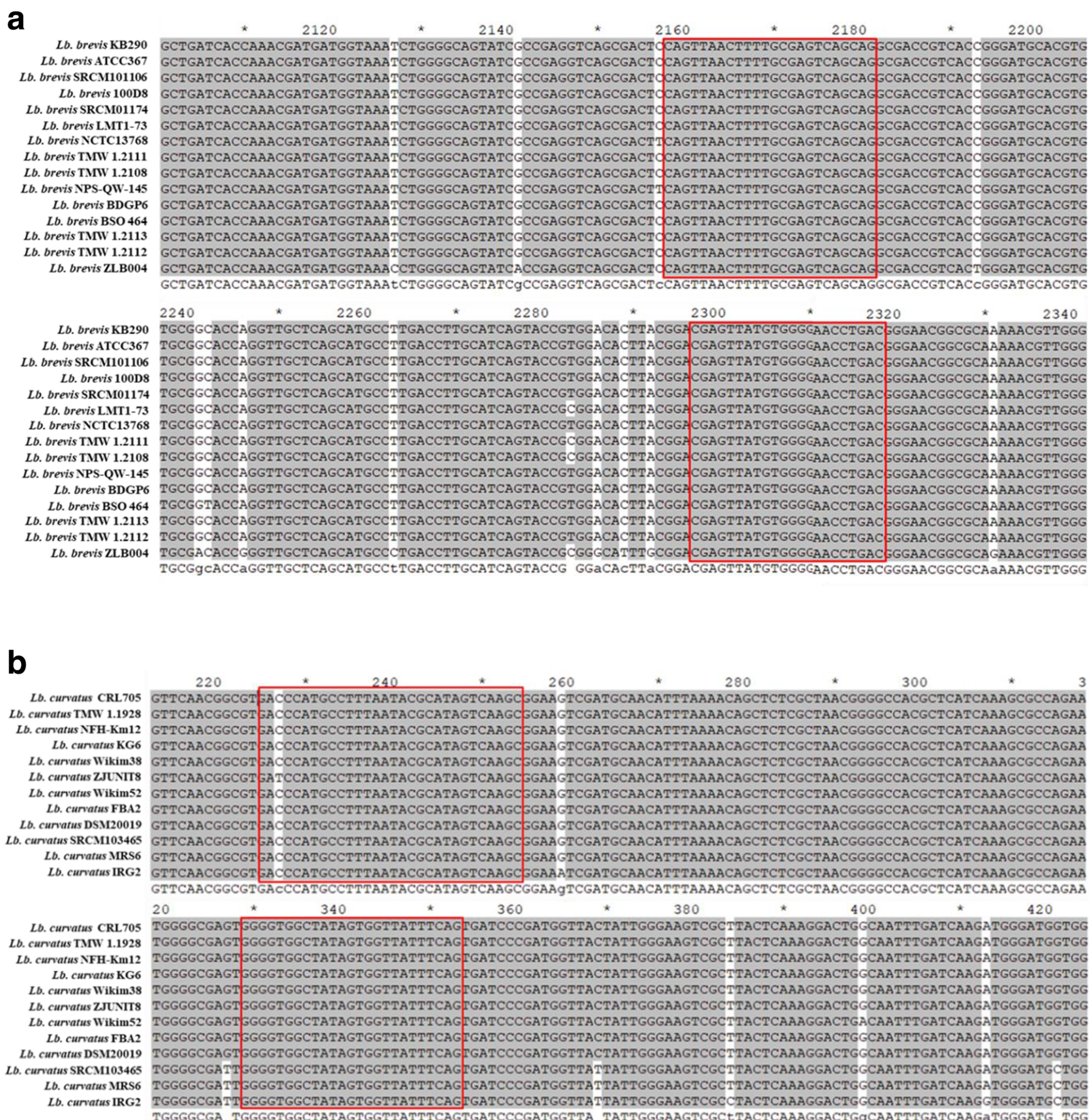

Fig. 2 **a** Homologous region alignment of hypothetical protein gene (LVISKB_0189) from 12 strains of *Lactobacillus brevis* showing sequence differences (white column). The annealing sites for the designed primers are outlined. **b** Homologous region alignment of glyoxalase gene (EHE85023.1) from 12 strains of *L. curvatus* showing sequence differences (white column). The annealing sites for the designed primers are outlined

were measured and the intra-assay CV value was < 1.95% for *L. sanfranciscensis* primer set, < 1.76% for *L. brevis* primer set, and < 1.45% for *L. curvatus* primer set. The inter-assay CV values were slightly higher than the intra-assay CV values.

Sourdough has a highly complex matrix composed of various nutrients and matrix. There is a possibility that the nutrients and matrix may affect the gDNA extraction yield and even interfere with qPCR assay. Therefore, the recovery yield of gDNA purified from the MRS medium or sourdough was compared to determine the effect of sourdough matrix. For this, an equal number of LAB were mixed with the MRS medium or dough, and gDNAs were extracted. The qPCR analysis results showed that there is no significant difference

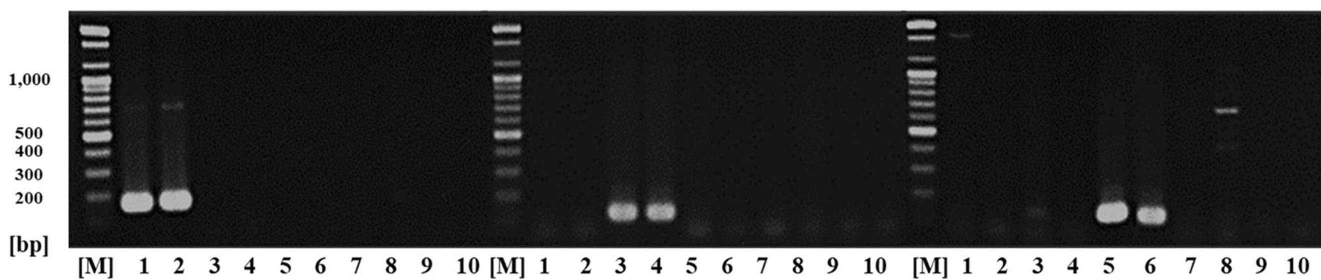

Fig. 3 Species-specific PCR detection. *Lactobacillus brevis* (left), *L. sanfranciscensis* (center), and *L. curvatus* (right) were analyzed with agarose gel electrophoresis of PCR amplicons **a** and real-time PCR amplification. Lane M, DNA marker; lane 1, *L. brevis* 118; lane 2, *L. brevis* 111; lane 3, *L. sanfranciscensis* 117; lane 4, *L. sanfranciscensis* 142; lane 5, *L. curvatus* 104; lane 6, *L. curvatus* 114; lane 7, *L. sakei*; lane 8, *Pediococcus pentosaceus* 224; lane 9, *L. buchneri*; lane 10, *L. plantarum*; [bp], nucleotide base pair

($p < 0.05$) in cell number obtained by qPCR regardless of the strain, revealing no interfering effect of the sourdough matrix on DNA recovery (Fig. 5).

To assess the applicability of qPCR for the direct quantification of *L. sanfranciscensis*, *L. brevis*, and *L. curvatus* in sourdough samples, the cell number of these strains obtained by qPCR was compared with that obtained with the traditional MRS plate counting method. Figure 6 shows that the cell number enumerated by the plate counting method tended to be lower than that observed with the qPCR assay (Fig. 6). In particular, the number of *L. sanfranciscensis* and *L. brevis* determined with qPCR increased by 50 and 65%, respectively, as compared with that evaluated with the plate counting method, while the number of *L. curvatus* was almost constant. The limit of qPCR for cell enumeration is that it may result in false positives or high estimation of cell number, as all gDNAs extracted from cells could be amplified regardless of cell viability (Taylor et al. 2014). Therefore, while the increment is not much, the cell numbers obtained by qPCR method in this study may include both live and dead cells.

Quantification of Three LAB in Sourdough Samples

The qPCR method developed herein using species-specific primer sets was applied to monitor changes in microbial population during back-slopping of sourdough. *L. sanfranciscensis*, *L. brevis*, and *L. curvatus* were inoculated in the dough at different cell densities for sourdough fermentation. For back-slopping, a part of the dough was used as the inoculum for the second fermentation on the following day and so on. During this microbial propagation process of sourdough, any changes in the microbial population were measured with qPCR. As shown in Fig. 7, the number of *L. curvatus* continued to decline during the back-slopping

Table 3 Cycle threshold (Ct) values of three primer sets for *Lactobacillus sanfranciscensis*, *L. brevis*, and *L. curvatus* obtained from quantitative PCR against genomic DNAs of 12 different lactic acid bacteria

Lactic acid bacteria	DNA concentration (ng/μL)	Primers for *L. sanfranciscensis* (Ct)	Primers for *L. brevis* (Ct)	Primers for *L. curvatus* (Ct)
Lactobacillus sakei subsp. *sakei*	54.0	29.31	29.57	30.36
L. paraplantarum	41.5	28.30	27.60	30.28
L. casei	35.0	31.61	30.15	31.90
L. lactis subsp. lactis	35.0	31.91	28.52	29.75
L. curvatus	70.0	27.06	31.29	**13.00**
L. brevis	25.5	27.07	**13.53**	27.76
Pediococcus pentosaceus	42.0	28.00	29.70	29.02
L. plantarum	37.0	28.41	30.83	29.64
L. sanfranciscensis	90.7	**10.62**	33.39	29.80
L. buchneri	22.5	28.17	30.09	27.19
L. paralimentarius	111.0	28.66	31.79	28.56
Leuconostoc mesenteroides	32.0	27.20	29.24	29.60
Negative control: DDW	0	35.94	34.00	37.41

Ct values in bold indicate values for target DNA. DDW, double distilled water

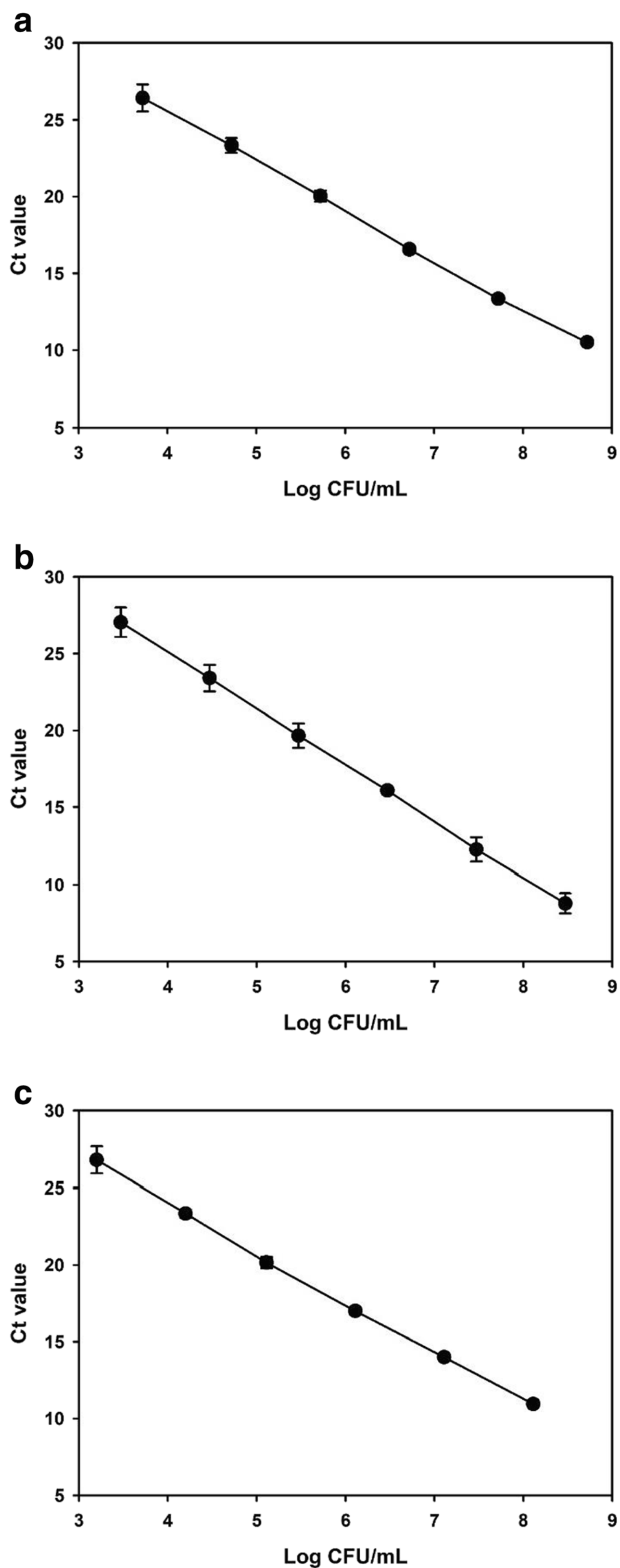

Fig. 4 Standard curves generated from the threshold cycle (Ct) values plotted against the estimated logarithm of each target gene concentration (copies/μL) of *Lactobacillus sanfranciscensis* **a**, *L. brevis* **b**, and *L. curvatus* **c**. Ten-fold serial dilutions of the DNA extracted from *L. sanfranciscensis* 101, *L. brevis* 111, and *L. curvatus* 114 were performed

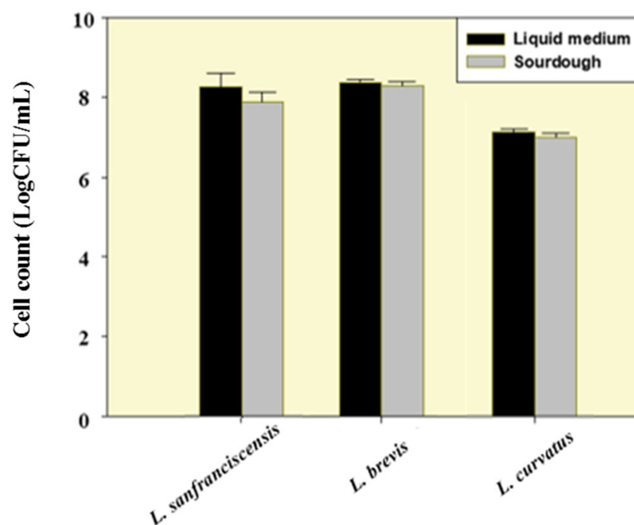

Fig. 5 DNA extraction yield from MRS medium and sourdough samples

process, while that of *L. brevis* was constant. In contrast, *L. sanfranciscensis* population increased and it became the most dominant LAB strain after the seventh cycle. This result reveals the applicability of the qPCR method developed to monitor the population sizes of *L. sanfranciscensis*, *L. brevis*, and *L. curvatus* during their growth in sourdough.

Discussion

Microbiota greatly contributes to the differences in the characteristics of similarly matured products during processing. These dynamics are largely influenced by the interactions between microorganisms (e.g., microbial co-operation and antagonism) and have a marked impact on the survival, growth, and activity of different microbial populations during

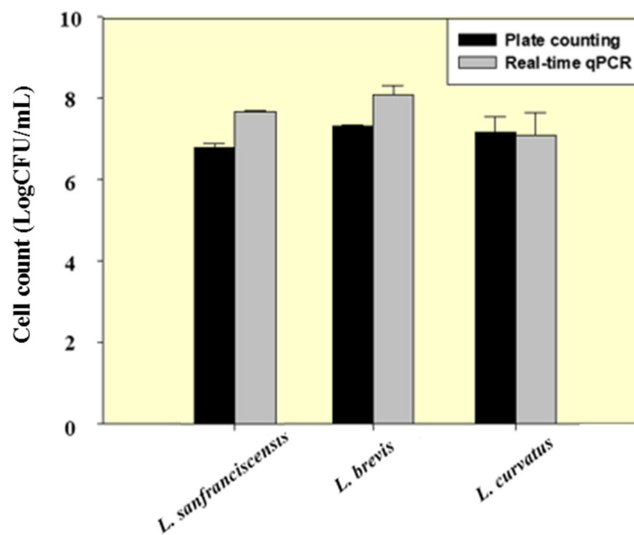

Fig. 6 Comparison of quantification results using qPCR and plate cell counting

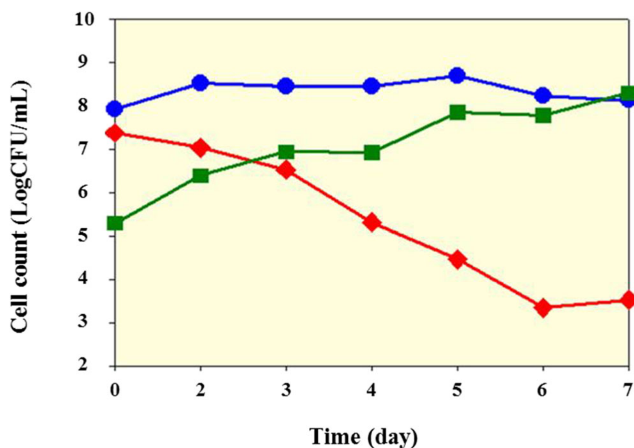

Fig. 7 Persistence of *Lactobacillus sanfranciscensis*, *L. brevis*, and *L. curvatus* starters in sourdough during 7 days of long propagation. Symbols are population sizes of *L. sanfranciscensis* 101 (green square), *L. brevis* 111 (blue circle), and *L. curvatus* 114 (red diamond). Quantification was performed with the qPCR method

processing. It is, therefore, important to reliably quantify physiologically active populations in terms of dynamic changes to understand the role of each microbial population (Achilleos and Berthier 2013). The sourdough has complex microbial composition and the interactions between microorganisms are key to their performance in food habitats (Liu et al. 2018; Sieuwerts et al. 2018). For understanding microbiota of sourdough, studies used molecular fingerprinting techniques: random amplification of polymorphic DNA (RAPD) (Vogelmann and Hertel 2011; Rizzello et al. 2011), rep-PCR (Liu et al. 2016), multiplex PCR (Zhang et al. 2019b), PCR- denaturing gradient gel electrophoresis (PCR-DGGE) (De Vuyst and Vancanneyt 2007; Settanni et al. 2006), and amplified fragment length polymorphism (AFLP) (Fusco et al. 2016). Recently, metagenomics allows high-resolution genomic analysis of unculturable microbes and correlation of the genomes with particular functions in the environment (Menezes et al. 2020). Those methods are easy to identify which microbes exist in sourdough (Meroth et al. 2003), but the accurate quantification of individual species is still challenging (Gao and Li 2018).

To measure the population of microorganism in sourdough, qPCR has been employed using universal primers targeting 16S rRNA-encoding gene (Pontonio et al. 2017). Generally, strains that show > 98% similarity in the 16 S rRNA gene sequence are regarded as the same species (Stackebrandt and Ebers 2006). Therefore, comparison of 16S rRNA gene sequence has limitation in discriminating species level such as *L. acidophilus* group (including *L. acidophilus*, *L. helveticus*, *L. gallinarum*, *L. cripsatus*) (You and Kim 2020) and *L. casei* group (including *L. casei*, *L. paracasei*, *L. rhamnosus*) (Kim et al. 2020b). To overcome this restriction, unique genes of the species other than 16S rRNA gene can be searched and used as target genes for qPCR; glutamate decarboxylase gene for *L. reuteri* in sourdough (Su et al. 2011), phenylalanine-tRNA synthetase gene for *L. helveticus* in cheese, and sometimes a hypothetical protein gene for *L. lactis* in kimchi (Kim et al. 2020a). In this study, we confirmed that genes for nucleoside hydrolase, hypothetical protein, and glyoxalase are unique for *L. sanfranciscensis*, *L. brevis*, and *L. curvatus*, respectively, and those genes can be used as targets for accurate quantification of the species during sourdough fermentation by using qPCR method. This method would enable quantification of three *Lactobacillus* species during cocultivation in sourdough and provide useful information on microbial commensalism that is essential to produce high-quality sourdough.

Conclusions

We developed a species-specific qPCR method for *L. sanfranciscensis*, *L. brevis*, and *L. curvatus* to monitor their growth during sourdough fermentation. The genes encoding nucleoside hydrolase, hypothetical protein, and glyoxalase were selected as targets for the species-specific detection. The specificity of PCR primer sets was verified against different species of LAB and different ratios of target and non-target DNAs. The qPCR methods developed estimated slightly higher number (< 65%) of LAB cell as compared with the traditional plate counting method, probably owing to the detection of both live and dead cells. Considering DNA recovery, no significant interfering effect of sourdough matrix was observed. The standard curves for qPCR of three species were established and successfully applied to monitor their growth during sourdough propagation. Thus, tools that rapidly quantify LAB populations during sourdough fermentation are warranted to facilitate optimization of the entire fermentation process for reproducible organoleptic qualities of products.

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Compliance with Ethical Standards

Conflict of Interests Shin Dal Kang, Sangmin Shim, Deukbuhm Lee, and Jin-Ho Seo are employed at the Research Institute of Food and Biotechnology, SPC Group, that sponsored this research.

Ethical approval This article does not contain any studies with human participants or animals.

Informed consent Not applicable

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