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Monitoring of *Leuconostoc* Population During Sauerkraut Fermentation by Quantitative Real-Time Polymerase Chain Reaction

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A real-time PCR assay method was established to monitor *Leuconostoc* spp. populations *via* specific amplification of the dextransucrase gene. Quantification of *L. mesenteroides* B-512F using both genomic DNA and cell suspensions yielded a log-linear correlation spanning approximately 5 log units. By using this method, monitoring changes of *Leuconostoc* spp. during sauerkraut fermentation was successfully accomplished with accuracy after inoculation of starter and sugars (sucrose and maltose).

Keywords: Sauerkraut, *Leuconostoc*, real-time PCR, monitoring, dextransucrase

Sauerkraut and *kimchi* fermentation are generally initiated by the natural lactic acid bacteria (LAB) present on cabbage [5]. *Leuconostoc* is the major bacterial genus involved in the early stage of vegetable fermentation, whereas *Lactobacillus plantarum* becomes predominant approximately 5–7 days after the initiation of fermentation [2, 11]. In the genus *Leuconostoc*, various species are known; namely, *L. mesenteroides*, *L. lactis*, *L. gelidum*, *L. carnosum*, *L. citreum*, *L. kimchii*, *L. pseudomesenteroides*, *L. fallax*, and *L. argentinum* [1, 3, 4, 7, 8]. During sauerkraut and *kimchi* fermentation, various end-products, such as lactic acid, acetic acid, and mannitol, are produced. Dextransucrase (E.C. 2.4.1.5), which is secreted by the *Leuconostoc* spp., transfers the glucose moiety of sucrose to form dextran [13]. Usually, a culture-dependent method, which employs the phenyl ethanol agars (Difco, Flanklin Lakes, NJ, U.S.A) with 2% sucrose (PES), is used for selective counting of the *Leuconostoc* spp. in a mixed fermentation [10]. However, the PES medium takes 24–48 h to form a gel, and thus, real-time quantification of *Leuconostoc* spp. by using this method is difficult. Moreover, in some cases, the results of phenotypic analysis are strongly dependant on the physiological state of the cells [12]. On the other hand, real-time polymerase chain reaction (PCR) is a culture-independent method that can detect and quantify PCR amplicons [6]. In this study, we developed a rapid real-time PCR method to quantify *Leuconostoc* spp. using a conserved sequence of the dextransucrase gene as a marker, and monitored the population changes of *Leuconostoc* spp. in sauerkrauts containing various additives such as sugars and starter cultures.

Firstly, to confirm the selective counting of *Leuconostoc* spp. among several LAB, PCR amplification of the dextransucrase gene was performed using the following primers: mesF, 5'-GTAGATGCTGTTGATAACGTT-3', and mesR, 5'-TTGCCATGTATTGACCATCA-3' [9]. As shown in Fig. 1A, amplicons (600 bp) were detected in *L. mesenteroides* B-512F, *L. citreum* KACC 91035, and *L. mesenteroides* subsp. *mesenteroides* KCTC 3100, but not in *Oenococcus oeni* KCTC 3072, *Lactobacillus fermentum* KCTC 3112, and *Weissella paramesenteroides* KACC 10213. When various *Leuconostocs* (n=13) and non-*Leuconostocs* (n=20) reference strains were tested (Table 1), amplicons were detected in the *Leuconostoc* spp. and not in the non-*Leuconostoc* spp. This implies that only the conserved sequences (600 bp) of the dextransucrase gene in the *Leuconostocs* spp. were amplified. Thus, the real-time PCR method may aid in the specific detection and quantification of the *Leuconostocs* spp. To estimate the detection and quantification limits of the real-time PCR assay, 10-fold dilutions of the genomic DNA extracted from *L. mesenteroides* NRRL B-512F and *L. citreum* KACC 91035 grown in MRS broth were analyzed. Fig. 1B

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shows the real-time PCR amplification plots for the 2 species; a known number of genome equivalents were used as a standard. The mean threshold cycle (Ct) values for *L. mesenteroides* and *L. citreum* were 12.24 (± 0.37) and 14.27 (± 0.0), respectively, when genomic DNA was extracted from 1.6×10^9 and 1.8×10^9 CFU/ml of vegetable cells, respectively. The plots showed a log linear correlation between the *Leuconostoc* genome copy number and the Ct, which spanned 5-log units. The correlation coefficients (R^2) of the standard curves for *L. mesenteroides* and *L. citreum* were 0.997 and 0.998, respectively, and the slopes were −4.13 and −4.19, respectively. The detection limit of the assay for accurate quantification of the *Leuconostoc* spp. was 10^4 CFU/ml. All the *Leuconostoc* strains used in this study indicated Tm values ranging from 82 to 82.5°C.

The population changes of *Leuconostoc* spp. in various sauerkraut samples were monitored using the Ct values obtained *via* real-time PCR. For this experiment, sauerkrauts (5 kg) were prepared using shredded cabbages that were salted with 2.5% NaCl (final equilibrated concentration). Sauerkraut prepared without addition of sugar and starter strain was the control (A). The test group sauerkrauts were prepared by adding sugars and starter strains [14]; 1% sucrose (w/w) and 1% maltose (w/w) (B), 1% sucrose (w/w) and 1% maltose (w/w) and a starter strain (*L. mesenteroides* subsp. *mesenteroides* KCTC 3100) (C), and a starter strain without sugars (D). The first few attempts at direct quantification of *Leuconostoc* in sauerkraut juice failed to yield satisfactory results owing to the sensitivity of the real-time PCR assay to interference in the samples. Therefore, the PCR templates were further purified by improving the efficiency of the bacterial lysis and DNA purification steps as follows. In detail, 4 ml of the samples were centrifuged for 10 min at 12,000 ×*g* at 4°C and the

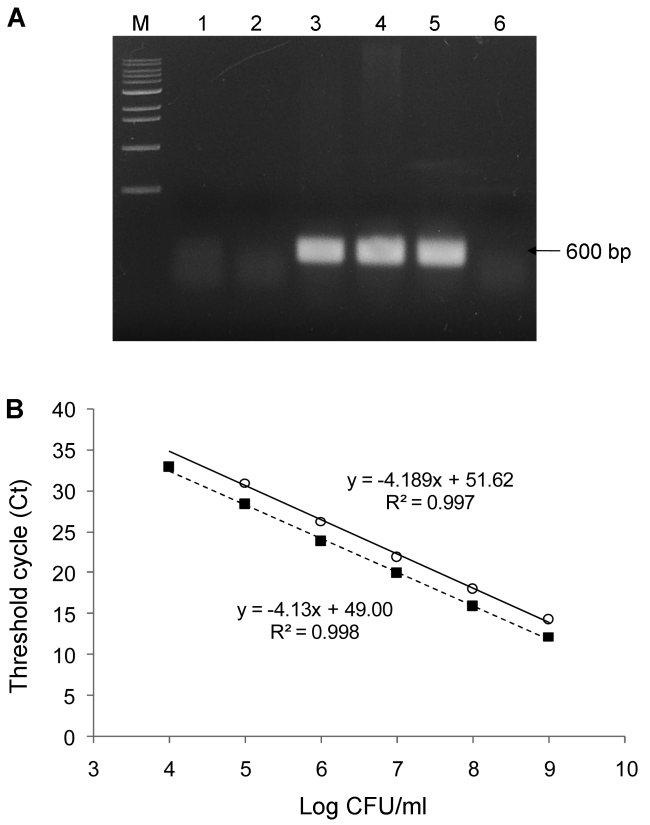

Fig. 1. PCR amplification of the dextransucrase gene.
A. The dextransucrase gene was amplified using the mesF and mesR primer pair and the amplicons were visualized using agarose gel electrophoresis. Lanes: M, 1 kb DNA size marker; 1, *Oenococcus oeni* KCTC 3072; 2, *Lactobacillus fermentum* KCTC 3112; 3, *L. mesenteroides* B-512F; 4, *L. citreum* KACC 91035; 5, *L. mesenteroides* subsp. *mesenteroides* KCTC 3100; 6, *Weissella paramesenteroides* KACC 10213. **B**. Two different standard curves were constructed using serially diluted purified DNA from *L. mesenteroides* B-512F (■) and *L. citreum* KACC 91035 cells (○). Each sample was amplified in triplicate and the mean Ct value was used.

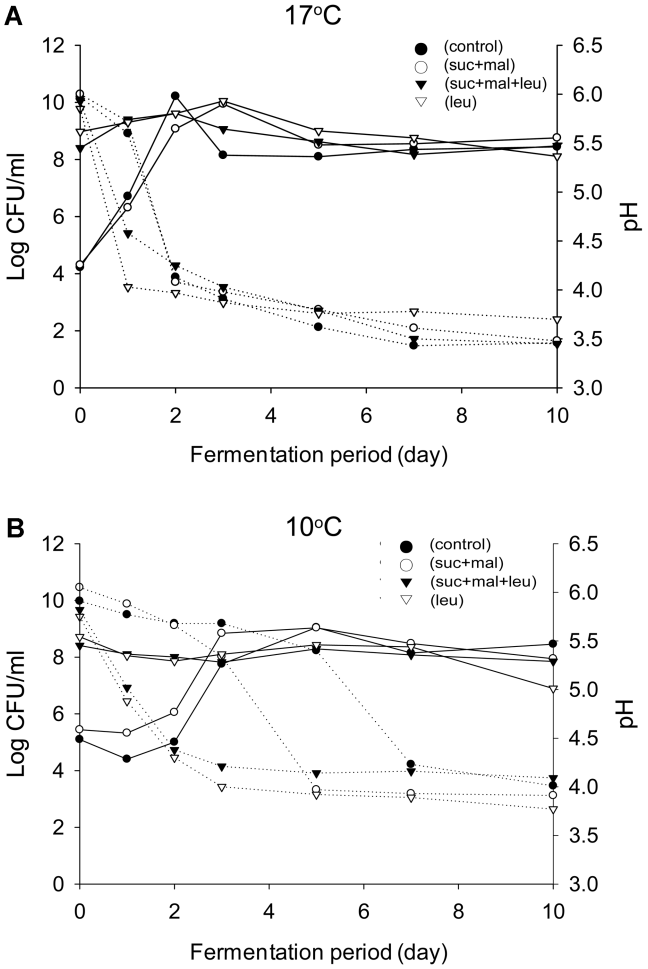

Fig. 2. Changes in the log cell number of *Leuconostoc* (solid line) and pH (dotted line) during sauerkraut fermentation at 17°C (**A**) and 10°C (**B**).
Symbols: ●, control; ○, 1% sucrose and 1% maltose were added to the control; ▼, *L. mesenteroide*s subsp. *mesenteroides* as starter with the sugars (1% sucrose + 1% maltose) was added to the control; ▽, the starter without the sugars was added to the control.

pellets were resuspended in 0.85% (w/v) NaCl solution after careful removal of the supernatant. DNA extraction from the precipitates of sauerkraut samples and the reference strains grown on MRS plate was performed using a Genomic DNA Prep Kit (Solgent, Daejeon, South Korea) according to the manufacturer's instructions. Real-time PCR was performed in a 20 μl reaction mixture containing 10 μl of 2 × iQ SYBR green supermix (Bio-Rad Laboratories, Hercules, CA, USA), 0.125 μM of each primer, and 2 μl of the sample template diluted 10-fold with RNase-free sterile water. Amplification was carried out using the iQ5 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) with the following parameters: (i) an initial denaturation step at 95°C for 5 min; and (ii) 40 cycles, consisting of denaturation at 95°C for 20 s, annealing at 55°C for 20 s, and extension at 72°C for 20 s. The melting curve of the PCR products was analyzed at 60°C for 10 s with an increase of 0.5°C per 10 s. The Ct values were calculated using the Optical Interface iQ5 software (Bio-Rad Laboratories); Ct values > 40 were considered negative. In addition, a negative (no-template) control was used to test for false-positive results or contamination. Melting curve analysis yielded a single peak, which indicated the absence of nonspecific amplicon and primer dimers. The quantification results of *Leuconostoc* spp. are shown in Fig. 2. Sauerkraut was fermented at 10°C and 17°C with and without the addition of the *L. mesenteroides* starter culture. During fermentation at 17°C, the Ct values of *Leuconostoc* spp. increased sharply until day 5 and then maintained a steady state from days 7 to 10. For the first 5 days, the changes in *Leuconostoc* spp. of samples A and B were slower during fermentation at 10°C than at 17°C.

Table 1. The reference strains amplified by real-time PCR for amplification of dextransucrase genes.

Group	Species	Strains	Real-time PCR result[a]
Non-leuconostocs	*Enterococcus faecalis*	KCCM 11729	-
	Enterococcus faecium	KCTC 13225	-
	Lactobacillus acidophilus	KCTC 3164	-
	Lactobacillus brevis	KCTC 3498	-
	Lactobacillus casei	KCTC 3109	-
	Lactobacillus coryniformis	KCTC 3159	-
	Lactobacillus curvatus	KCTC 3767	-
	Lactobacillus delbrueckii subsp. *bulgaricus*	KCTC 3635	-
	Lactobacillus delbrueckii subsp. *delbrueckii*	KCTC 1047	-
	Lactobacillus fermentum	KCTC 3112	-
	Lactobacillus harbinensis	KCTC 13106	-
	Lactobacillus plantarum	KCTC 3104	-
	Lactobacillus reuteri	KCTC 3594	-
	Lactobacillus sakei subsp. *sakei*	KCTC 3598	-
	Lactobacillus lactis subsp. *lactis*	KCTC 3769	-
	Oenococcus oeni	KCTC 3072	-
	Pediococcus pentosaceus	KCCM 11902	-
	Streptococcus thermophilus	KCTC 3927	-
	Weissella cibaria	KACC 11845	-
	Weissella paramesenteroides	KACC 10213	-
Leuconostocs	*Leuconostoc lactis*	KCTC 3528	+
	Leuconostoc mesenteroides subsp. *cremoris*	KACC 10213	+
	Leuconostoc mesenteroides	NRRL B-512F	+
	Leuconostoc mesenteroides subsp. *dextranicum*	KCTC 3530	+
	Leuconostoc mesenteroides subsp. *mesenteroides*	KCTC3100	+
	Leuconostoc citreum	KCTC 3526	+
	Leuconostoc citreum HJ-P4	KACC 91035	+
	Leuconostoc mesenteroides	NRRL B-742C	+
	Leuconostoc pseudomesenteroides	KCTC 3652	+
	Leuconostoc fallax	KCTC 3537	+
	Leuconostoc argentinun	KCTC 3773	+
	Leuconostoc inhae	KCTC 3774	+
	Leuconostoc fructosum	KCTC 3544	+

[a]-, Negative real-time PCR results; +, positive real-time PCR results.

Meanwhile, samples C and D supplemented with the *L. mesenteroides* starter strain maintained their bacterial counts for 10 days. The pattern of microbial change obtained using the real-time PCR method was comparable to that obtained using the PES plate count method. Thus, it is suggested that our real-time PCR assay can be used for the rapid detection and quantification of *Leuconostoc* spp. under various conditions of sauerkraut fermentation.

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