Specific Quantitative Detection of *Leuconostoc* spp. During Food Fermentation by Real-time PCR Using SYBR Green I

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ABSTRACT

In our study, a SYBR green-based real-time PCR assay was used to detect quantitatively leuconostoc spp. that appear in the fermented milk and vegetables from the early stages of fermentation based on specific-amplification of dextransucrase gene as a target. We examined genomic DNAs extracted from 33 references strains including 13 leuconostocs and 20 non-leuconostocs and from 4 sauerkraut samples fermented at 10 and 17 °C for 10 days. Specific PCR products of *Leuconostoc* spp. were confirmed by the results of melting curve analysis and agarose gel electrophoresis. Quantification was linear over at least 4 log units using both serial dilutions of purified genomic DNA and cell suspensions from *Leuconostoc mesenteroides* B-512F. In addition, it was able to determine bacterial changes by addition of starter and sugar during sauerkraut fermentation. This result is available to specifically determine a real-time PCR method to monitor the quantitative detection of *Leuconostoc* spp. of the bacterial population in mixed culture.

Key words : Sauerkraut, Leuconostocs, SYBR green, real-time PCR

INTRODUCTION

Sauerkraut is a traditional German vegetable food, but it can also be found as a dish in the Dutch, Estonian and other Northern European and Central European cuisines. It also is a prominent feature of cuisines from most of the cold regions of Europe, and it is eaten in many parts of Northeast China, Northern China, the USA, Chile, and Canada. Sauerkraut to use finely shredded cabbage has been fermented by several lactic acid bacteria, including mainly *Leuconostoc mesenteroides, Lactobacillus brevis, Lactobacillus plantarum,* and *Pediococcus pentosaceus.* Recently, *Leu. fallax* strains were isolated from brine samples during sauerkraut fermentation [Barrangou et al., 2002]. The sauerkraut fermentation process has three phases according to the occurrence of bacteria as the follows : 1) anaerobic bacteria such as *Klebsiella* and *Enterobacter* lead the early fermentation 2) *Leuconostoc mesenteroides* and other *Leuconostoc* spp. take dominance to start acid production such as lactic acid ; 3) various *Lactobacillus* species including *Lb. brevis* and*Lb. plantarum* ferment remaining sugars, further lowering the pH.

Leuconostoc spp. belong phylogenetically to

hetero-fermentative lactic acid bacterial group and consist the major bacterial population in fermented food such as sauerkraut and kimchi during initial or middle fermentation [Um et al., 2006 Seo et al., 2007]. This genus is currently consists of eight species, namely *Leu. mesenteroides*, *Leu. lactis*, *Leu.gelidum*, *Leu. carnosum*, *Leu. citreum*, *Leu. kimchii*, *Leu. pseudomesenteroides*, *Leu. fallax* and *Leu. argentinum* [Holzapfel & Schillinger, 1992 Dicks et al., 1993, 1995 Kim et al., 2000]. During fermentation stages, these strains contribute to the production of various constituents such as lactic acid, acetic acid, and mannitol. Especially, dextransucrase (EC 2.4.1.5) excreted by the genus *Leuconostoc*transfers the glucose moiety of sucrose to form dextran and also contributes to synthesis of functional isomaltooligosaccharides such as panose after addition of sucrose and maltose during kimchi fermentation [Robyt, 1995].

Culture-dependent method such as plate counting to use cultivatable media is still used to monitor the quantitative assay of LAB populations occurred during fermentation in various foods and some commercial identification systems are currently used for an easy species- identification. But, these methods can be time-consuming and labor intensive to examine completely. Moreover, in some cases, the results of phenotypic analysis are strongly dependant on the physiological state of the cells [Renouf et al., 2006].

In order to increase the rapidity of the response, many researchers are used the primers and the PCR were adapted for real-time PCR using a fluorescent double stranded DNA intercalating dyes such as SYBR Green I. This method can be useful for identification of different organisms by DNA melting curve analysis. Based on the PCR melt curves, the specific melting temperature (*Tm*) could be attributed to each species [Martin et al., 2006].

In our study, the objective was developed for the rapid quantification of the genus *Leuconsotoc*, which is the key microorganisms in sauerkraut fermentation, by real-time PCR analysis using partial sequence of the dextransucrase gene. In addition, we monitored bacterial changes by the addition of starter and sugars for the development of novel fermentation process of sauerkraut.

MATERIALS and METHODS

Bacterial strains and growth conditions

All LAB strains were grown on MRS agar (BD/Difco Laboratories, Detroit, MI, USA) at 37 °C for 2 days except for *Oenococcus oeni* on tomato juice agar (pH 4.8) at 37 °C for 3 days under the anaerobic conditions. They include 13 leuconostocs and 20 non-leuconostoc strains and are listed in Table 1. Reference strains were supplied by the Korean Culture Centre of Microorganisms (KCCM), Korean Collection for Type Cultures (KCTC), and Korean Agricultural Culture Collection (KACC).

Preparation of sauerkraut samples

Eight groups of sauerkraut kimchi consisting of approximately 5 kg of cabbage were sampled for this study. Each fermentation was carried out with approximately 2.5 % NaCl (final equilibrated concentration) using shredded cabbage. This sauerkraut sample without sugar and starter strain was referred to as the control. The other groups were prepared by adding sugars of 1% sucrose (w/w) and 1% maltose (w/w) to the control (B), by adding simultaneously sugars and *Leuconostoc mesenteroides* subsp. *mesenteroides* KCTC

Table 1. Reference strains used in this study.

Group	Species	Srains	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	-
	Lactococcus 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 mesentreroides subsp. *cremoris*	KCTC 3529	+
	Leuconostoc mesentreroides	NRRL B-512F	+
	Leuconostoc mesentreroides subsp. *dextranicum*	KCTC 3530	+
	Leuconostoc mesentreroides subsp. *mesenteroides*	KCTC 3100	+
	Leuconostoc citreum	KCTC3526	+
	Leuconostoc citreum HJ-P4	KACC 91035	+
	Leuconostoc mesentreroides	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

3100 as starter(C), and by adding starter without sugars (D). The compositions of starter and sugars added in each sauerkraut sample are summarized in Table 2. The samples were manually put in the glass tanks, covered with flexible vinyl sheeting, and weighted down with heavy stoneon top of the sheeting. The fermentation was controlled for 10 days at 10 and 17 °C, which are the temperatures in the commercial fermentations. Each sample (10 ml) was obtained for 6 microbial and biochemical analysis with a 0.5-cm-diameter, perforated thin

Table 2. Composition of sauerkraut kimchi used in this study.

Composition	Sauerkraut kimchi[a]			
	A	B	C	D
Sliced cabbage (5 kg)	O	O	O	O
Sucrose : Maltose (1% of each)		O	O	
Leuconostoc mesenteroides subsp. *mesenteroides* KCTC3100			O	O

[a] All kimchi were added to 2.5% (W/W) of sodium chloride.

plastic tube that was sealed at the bottom of the fermentation tanks and was placed in 50 ml sterile plastic tubes. All samples were maintain a temperature at below 4 °C and processed immediately.

DNA Extraction and PCR Amplification

Four ml of fermented sauerkraut samples and culture broth grown in MRS broth was centrifuged for 10 min at 12,000 x g and 4°C. The supernatant was carefully discarded, and the pellets were resuspended in 0.85% (w/v) NaCl solution. DNA extraction from the precipitants of sauerkraut samples and the reference strainsgrown on MRS plates, was performed using Genomic DNA Prep kit for bacteria (SolGent Co. Ltd., Daejeon, Korea) according to the manufacturer's instruction. The PCR amplification of the total DNA from each sample and 33 reference strains including 20 non-leuconostocs and 13 leuconostocs was performed using the dextransucrase as a target gene for quantitative analyses of the genus *Leuconsotoc* [Koh et al., 2002]. The primers used in this study were followed: mesF, 5′-GTAGATGCTGTTGATAACGTT-3′ and mesR, 5′-TTGCCATGTATTGACCATCA-3′ [Koh et al., 2002]. PCR amplification was performed with a 2.5 U of *Taq* DNA polymerase (Takara Co. Ltd., Japan), 2.5 pmol of each primer, 0.25mM of each dNTPs, and 1 μl of genomic DNA template, in a final volume of 25 μl. After pre-denaturation at 94 °C for 5min, 30 cycles of polymerase chain reactions were performed at 94°C for 1at 58 °C for 1min, and at 72 °C for 1The cycling was concluded with a final extension step of 10at 72 °C. The PCR products were confirmed by electrophoresis in 1% agarose gel before they were applied to real-time PCR analyses [Koh et al., 2002 Martin et al., 2006 Elizaquível et al., 2008].

Real-time PCR quantification

Real-time PCR was performed in a 20 μl reaction mixture volume containing 10 μl of 2× iQ SYBR green supermix (Bio-Rad Laboratories, Hercules, CA), 0.125 μM of each primer, and 2 μl of sample template to dilute 10-fold with RNase-free sterile water. Amplification was performed using the iQ5 cycler detection system (Bio-Rad Laboratories, Hercules, CA) using the following program: (i) an initial denaturing step at 95°C for 5 min; (ii) 40 cycles, with 1 cycle consisting of denaturation at 95 °C for 20 s, annealing at 55°C for 20 s, and extension at 72 °C for 20 s. A melting curve was analyzed at 60 °C for 10 s with an increase of 0.5 °C per 10 s. The threshold cycles (Ct) were calculated using the Optical interface iQ5 software and the Ct values over 40 were considered negative. Besides, a negative (no-template) control was used to test for false-positive results or contamination. The production of non-specific amplicon or primer dimers was confirmed by formation of a single melting peak in a melting curve analysis using the iCycler iQ5 software. The PCR products were confirmed the presence of non-specific products or primer dimmers by gel electrophoresis on 1.0% agarose gels stained with ethidium bromide.

Standard curves for quantification assays

Standard curves were calculated for quantification purposes using 10-fold serial dilutions of a 24 h culture of two strains, *Leu. mesenteroides* NRRL B-512F and *Leu. citreum* KACC91035 in sterile saline solution (0.85% NaCl), covering the range from 1.6 to 1.8 x 10^9CFU/mL (determined by plate count on MRS). Four milliliter from each dilution was subjected to DNA extraction using the Genomic

DNA Prep kit (SolGent, Korea). Standard curves based on purified DNA and calibrated cell suspensions were each calculated using the same cell or DNA extraction batch. Averaged Ctvalues (from triplicates) and SD were calculated for each dilution.

RESULTS and DISCUSSION

Specificity of PCR

16S rRNA gene-based identification of bacteria has been widely used as a molecular base [Sacchi et al, 2002]. However, some strains can be occurred non-specific amplification to unknown region because these contain multiple rRNA operons with slightly different 16S rRNA gene sequences. In this study, we considered appropriate to quantify the leuconostocs related to transglycosylation reaction of dextransucase secreted by leuconostocs sauerkraut fermentation at the genus.

The PCR amplification was performed using the primers (mesF and mesR) designed from the dextransucrase gene to detect *Leuconsotoc* spp. Various reference strains belongs to non-leuconostocs were compared and the results are shown in Table 1 and Fig. 1. As a result, 20 non-leuconostocs strains were not amplified and 13 leuconostocs were amplified by mesF and mesR primers. The size of PCR amplicon was 600 bp for partial sequences of dextransucrase gene. Although these primers were designed to dextransucrase gene of *Leu. mesenteroides* as previously described by Koh et al. [2002], we could successfully detect the only leuconostoc group using mesF and mesR PCR primers. Consequently, these primers can be used for the specific detection and quantification of the leuconostocs in various fermented foods without detection of non-leuconostocs.

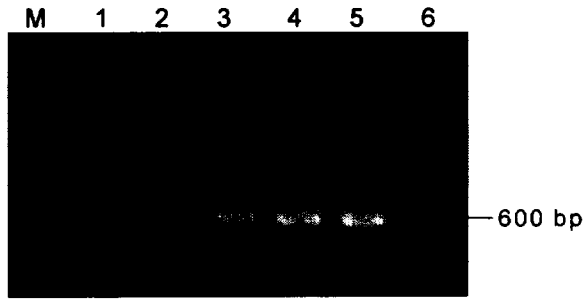

Fig. 1. PCR amplification of the gene encoding the dextransucrase using mesF and mesR primer pair. Lanes : M, 1 kb DNA size marker ; 1, *Oenococcus oeni* KCTC 3072 ; 2, *Lactobacillus fermentum* KCTC 3112 ; 3, *Leu. mesenteroides* B-512F ; 4, *Leu. citreum* KACC91035 ; 5, *Leu. mesenteroides* subsp. *mesenteroides* KCTC 3100; 6, *Weissella paramesenterides* KACC 10213.

Standard curve and quantification limit of real-time PCR assay using SYBR green

The detection and quantification limit of real-time PCR assay were determined by 10-fold serially diluted genomic DNAs extracted from *Leu. mesenteroides* NRRL B-512F and *Leu. citreum* KACC91035 grown in MRS broth. Two bacterial cells of *Leu. citreum* and *Leu. mesenteroides* calculated ranging 1.6 to 1.8 x 10^9 CFU/ml (determined by plate count on MRS)were indicated Ct values of 12.24±0.37 and 14.27±0.0 (Mean ±SD), respectively.

Quantification was linear over at least 5 log units using both serial dilutions of purified DNA extracted from cell suspensions from *Leu. mesenteroides* B512-F and *Leu. citreum* KACC91035. Standard quantification curves are shown in Fig. 2 and indicate the mean Ct values of three replicates obtained both from 10-fold dilutions of genomic DNAs. They showed a linear correlation between log CFU/ml and Ct with slope and R^2 values (0.997

and 0.998) very similar in two amplifications. The slopes of two linear standard curves, calculated using genomic DNA extracted from cell suspensions as template, were -4.13 and -4.189, respectively. Although both R^2 values were above 0.997 and SYBR green real-time PCR were highly linear, these slopes were not close to the theoretical optimum of -3.32 [Sacchi et al., 2002]. Nevertheless, these assays were successfully able to detect with a minimum threshold of 10^4 CFU/mlfor the accurate quantification of the genus *Leuconostoc*. Accordingly, we used the standard curve obtained from *Leu. mesenteroides*B-512F for quantitative assay of leuconsotocs during sauerkraut fermentation.

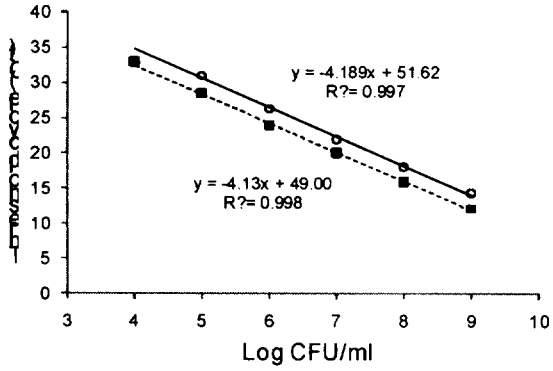

Fig. 2. Standard curves for *Leuconostoc*spp. by SYBR green amplification of the gene encoding the dextransucrase. Two different standard curves were constructed with serially diluted purified DNA from *Leu. mesenteroides* B-512F (■) and *Leu. citreum* HJ-P4 cells (○. The Ct values are the means of three different experiments within triplicates.

Moreover, for SYBR Green based amplicon detection, it is important to analyze a dissociation curve following the real-time PCR. In our study, we confirmed Tm value of the amplicon starts at the point of inflection of the melting curve of each sauerkraut. All leuconostoc strains used in this study indicated *Tm* values ranging from 82 to 82.5℃ The *Tm* values of other strains were not similar to those of leuconosotcs (*data not shown*).

Monitoring of the genus Leuconostoc during sauerkraut fermentation by real-time PCR

To quantifiably monitor leuconostocs in sauerkraut samples at 10 and 17℃, we determined the numerical changes of leuconsotoc occurredin each samples using Ct value from by real-time PCR. The results monitored changes in leuconsotocs were shown in Fig. 3.

As fermentation proceeded at 17℃ the Ct values of leuconostoc increased sharply until 5 days and then maintained a steady state from 7 to 10 days. Especially, control A and sample B withoutstarter were shown the excited growth for 2 and 5 days, respectively. However, samples C and D supplemented with starter were gently grown during sauerkraut fermentation for 10 days.

During fermentation at 10℃ the changes in leuconostoc of A and B samples were gently increased than those of fermentation proceeded at 17℃ for 5 days. Moreover, samples C and D supplemented with starter were maintained their bacterial counts during sauerkraut fermentation for 10 days.

Fig. 3 indicates the profiles of pH changes for 10 days of fermentation period at 10 and 17 ℃(A, B, C and D, respectively). At 17 ℃ the pH of all samples dropped rapidly, while at 1 0℃ the pH changes in samples A and B without starter were shown the gentle reduction. Generally, the additionof sugar in lactate fermentation is not favored because of the over- acidification of foods. However, in this experiment, sample B added 1% sucrose and 1% maltose was shown the gentle pH

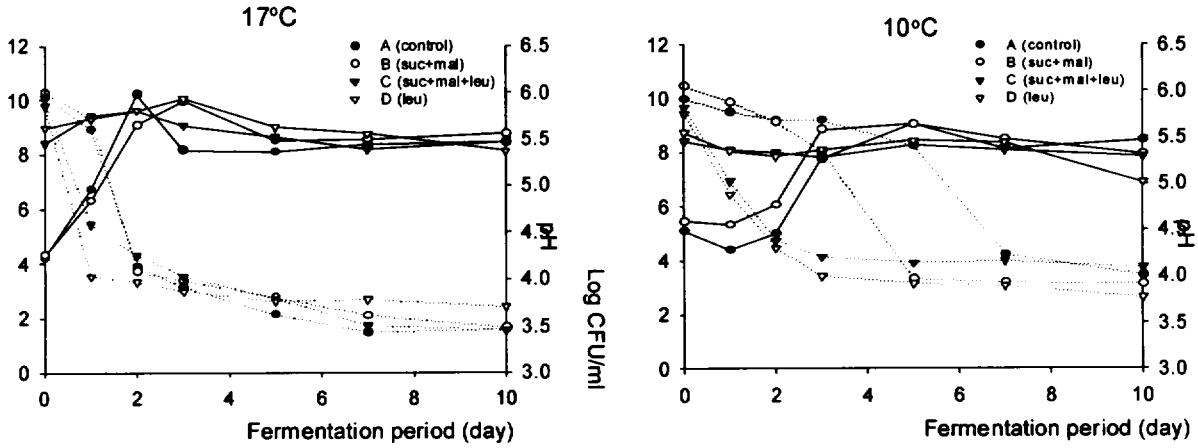

Fig. 3. Changes in the log cell number of leuconostocs (solid line) and pH (dotted line) during sauerkraut fermentation at 10 and 17℃ A, Control; B, 1% sucrose and 1% maltose were added in sample A; C, *Leu. mesenteroides* subsp. *mesenteroides* as starter was added in sample B; D, the starter was added in control A.

decrease when was fermented at 10℃ Accordingly, we describe that the growth of leuconostocs, which is the key lactic acid bacteria in sauerkraut, is able to adjust depending on the addition of the starter and sugar or fermentation temperature. However, several data lead to a lower detection level because of the difficulty in DNA extraction from foods. Nevertheless, we propose that it is better to use specific real-time PCR as a tool to monitor the increase in *Leuconostoc* population in various fermented foods.

In conclusion, the genus *Leuconostoc* was the most predominant strain in all groups with/ without the addition of sugar and starter in sauerkraut [Seo et al., 2007; Plengvidhya et al., 2007]. We developed the rapid quantification of the genus *Leuconsotoc* by real-time PCR analysis using SYBR green I. Moreover, we can predict the enzyme activity in proportion as the cell number of leuconsotoc in foods by using dextransucrase as target gene. Accordingly, real-time PCR assay is useful for rapid detection of undesirable changes in the bacterial population caused by changing qualities of ingredients or false fermentation conditions.

Acknowledgements

This work was supported by the Grape Research funds from Agricultural R&D Promotion Center (ARPC), Ministry of Agriculture and Forestry, Republic of Korea. We were financially supported by BrainKorea21 program and TPC project from KICOS, the Korean Ministry of Education, Science and Technology

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