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Changes in physicochemical property and lactic acid bacterial community during *kimchi* fermentation at different temperatures

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Abstract This study aimed to investigate the change in physicochemical properties and lactic acid bacterial communities during *kimchi* fermentation at different temperatures (8, 15, and 25 °C) using two molecular genetics approaches, multiplex polymerase chain reaction and 16S rRNA gene sequencing. The pH during fermentation at 8, 15, and 25 °C decreased from 6.17 on the initial fermentation day to 3.92, 3.79, and 3.48 after 54, 30, and 24 days of fermentation, respectively, while the acidity increased from 0.24% to 1.12, 1.35, and 1.54%, respectively. In particular, the levels of lactic acid increased from 3.74 g/L on the initial day (day 0) to 14.43, 20.60, and 27.69 g/L during the fermentation after 24, 18, and 12 days at 8, 15, and 25 °C, respectively, after that the lactic acid concentrations decreased slowly. The predominance of lactic acid bacteria (LAB) in the fermented *kimchi* was dependent on fermentation stage and temperature: *Lactobacillus sakei* appeared during the initial stage and *Leuconsotoc mesenteroides* was observed during the optimum-ripening stage at 8, 15, and 25 °C. *Lac. sakei* and *Lactobacillus plantarum* grew rapidly in *kimchi* produced at 8, 15, and 25 °C. In addition, *Weissella koreensis* first appeared at days 12, 9, and 6 at 8, 15, and 25 °C of fermentation, respectively. This result suggests that LAB population dynamics are rather sensitive to environmental conditions, such as pH, acidity, salinity, temperature, and chemical factors including free sugar and organic acids.

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Keywords Fermentation temperatures · *Kimchi* · Lactic acid bacteria · Microbial diversity · Multiplex-polymerase chain reaction · Organic acids

Introduction

Kimchi is a popular side dish in Korea. During the last three decades, many genera and species of microorganisms found in *kimchi* have been isolated and reported. The microorganisms in *kimchi* were actively investigated, for the first time, in a study by Mheen and Kwon [1]. Reportedly, the microorganisms involved in *kimchi* fermentation included approximately 200 species of bacteria and several yeasts [2]. In fact, the major microorganisms responsible for *kimchi* fermentation are lactic acid bacteria (LAB). Previously, the LAB that were isolated and identified from fermented *kimchi* included: *Leuconsotoc citreum*, *Leuconsotoc gasicomitatum*, *Leuconsotoc mesenteroides*, *Lactobacillus brevis*, *Lactobacillus curvatus*, *Lactobacillus plantarum*, *Lactobacillus sakei*, *Lactococcus lactis*, *Pediococcus pentosaceus*, and *Weissella Korenesis* etc. [3-10].

The common quality indices of *kimchi* are pH and acidity, which are affected by a number of factors during fermentation [1,5]. The important factors that affect *kimchi* fermentation are microorganisms, temperature, oxygen level, pH, salt concentration, fermentable carbohydrates, and other available nutrients or any inhibitory compounds in the raw materials used [4]. However, the key factor that affects and controls the fermentation of Chinese cabbage *kimchi*, has been reported to be fermentation temperature [11]. In fact, the characters of *kimchi* originate from the action of LAB during fermentation.

Molecular ecological studies have received increasing attention for exploring the microbial diversity in *kimchi* including polymerase chain reaction (PCR) with a strain-specific primer [5], sodium dodecyl sulfate polyacrylamide gel electrophoresis [12], PCR-denaturing gradient gel electrophoresis (DGGE) [9], genome-

probing microarray [13], and 16S rRNA gene sequence [6-8,14]. In particular, a multiplex PCR method allows the simultaneous amplification of more than one target sequence in a single PCR reaction, which saves considerable time and effort and decreases the number of reactions that need to be performed to assess the possible presence of microorganisms in the food [5,15,16].

Increasing our knowledge regarding the microbial communities in *kimchi* during fermentation is an important goal to food microbiologists, not only to understand the precise mechanism of *kimchi* fermentation but also to control the fermentation process for quality-controlled production of *kimchi* in industry. Also, several researchers have reported correlations between the microbial community and fermentation environment in *kimchi* ecosystem [17-19]. Previously, we designed and reported species-specific primers for specific detection and identification of each LAB species [5] during *kimchi* fermentation. In this study, we tried to investigate that the characteristics of *kimchi* fermentation through changes in the physicochemical properties (including pH, acidity, reducing sugar, and salinity), organic acid contents, and LAB community during *kimchi* fermentation at different fermentation temperature of 8, 15, and 25 °C.

Materials and Methods

Bacterial strains and growth media

Reference LAB were collected from the Korean Type Culture Collection, Korean Culture Center of Microorganisms, and Korean Agricultural Culture Collection [5]. The LAB were grown overnight at 30 °C in Lactobacilli MRS broth (MRS, Difco, Becton Dickinson Co., Sparks, MD, USA). *Escherichia coli* DH5α and recombinant *Esc. coli* cells were cultured in Luria-Bertani broth (Difco, Becton Dickinson Co.) media containing the appropriate antibiotics (ampicillin, 50 μg/mL) at 37 °C.

Preparation of *kimchi*

Kimchi (3 kg) was obtained from Jonggajib (DaeSang FNF Co., Geochang-gun, Gyeongnam-do, Korea) in Geochang-gun, Korea. A *kimchi* sample (1 kg) was placed in a glass jar with a cap and fermented at either 8±2, 15±1, and 25±1 °C.

pH and acidity

This procedure was adapted as previously described by Cho et al. [5]. *Kimchi* samples were blended and the pH was measured with a pH meter (MP220, pH meter, UK). To estimate the acidity, 20 mL of *kimchi* filtrate was titrated with 0.1 N NaOH at pH 8.2±0.2. The pH and acidity measurements were performed in triplicate. The acidity was calculated as follows: Acidity (%, as lactic acid) =0.009×mL of 0.1 N NaOH×F×100/ Sample (mL), F: factor of 0.1 N NaOH.

Salinity and reducing sugar

Kimchi samples were blended and after filtered to collect the fluid portion and the salinity was measured with a salt meter (Atago Co., Tokyo, Japan). Reducing sugar in *kimchi* filtrate was measured with the Dinitro-salicylate method [20].

Organic acids

High performance liquid chromatography (HPLC) was performed in order to determine the organic acids present during fermentation of *kimchi*. A 5 mL sample of the culture was collected and centrifuged. One milliliter of supernatant was filtered through a 0.45-μm Minipore PVDF filter (Schleicher & Schuell, GmbH, Dassel, Germany) for HPLC analysis. Injection volume was 10 μL of the sample. The analysis of organic acids was carried out using HPLC (Perkin-Elmer 200 series, Perkin-Elmer Co., Norwalk, CT, USA) with a TSKgel ODS-100V column (4.6×250 mm, 5 μm, Tosoh Corp., Tokyo, Japan). The 0.1% phosphoric acid (H_3PO_4) was eluted with a flow rate of 1 mL/min at 40 °C. The various organic acids were measured at 210 nm using a UV detector (Perkin-Elmer UV 200 series, Perkin-Elmer Corp.).

Total LAB cell numbers and isolation of LAB from *kimchi*

One mL of each blended *kimchi* sample was diluted in 9 mL of sterile 0.85% physiological saline. Aliquots of 1 mL were serially diluted tenfold using the 0.85% physiological saline, and 100 μL samples were spread on MRS agar plates and incubated at 30 °C for 48 h. Ninety-six colonies were randomly selected from the total viable LAB cells on MRS agar plates. The number of cells in each specific LAB isolate was calculated as previously described by Cho et al. [5]. Each specific LAB was calculated as follows: Each of specific LAB viable cells (log CFU/mL)= (detection of each of specific LAB colonies by multiplex PCR or 16S rRNA gene sequencing÷isolated 96 colonies)×total LAB viable cells on MRSA.

Extraction of genomic DNA from isolated LAB

Genomic DNA was extracted by a method described in total DNA extraction G-spin™ Genomic DNA Extraction Kit (iNtRON Biotechnology, Suwon, Korea), or by the method of boiling and vortexing bacterial pellets for 10 min at 80 °C. The extracted DNA was used as a template for the multiplex PCR.

Primer, multiplex PCR reaction, and agarose gel electrophoresis

Ten species-specific primers were designed for the identification of *Leu. carnosum, Leu. mesenteroides, Lac. brevis*, *Lac. plantarum*, *Lac. pentosus*, *Lac. sakei*, *Lac. lactis*, *Ped. pentosaceus*, *Wei. confusa*, and *Wei. korenesis*, and multiplex PCR was performed as previously described [5]. PCR of 16S rDNA genes were amplified and these sequences were analyzed as previously described [21,22].

16S rRNA PCR, transformation, and sequence analysis

LAB isolates not identified by multiplex PCR were subjected to further identification via 16S rRNA gene sequencing. The PCR primers used to amplify 16S rRNA gene fragments were the universal primers (Forward, 5-CGGAGAGTTTPATCCTPG-3; reverse, 5-TACGGCTACCTTPTTAGCGAC-3). PCR of 16S rDNA genes were amplified and these sequences were analyzed as previously described [21].

Results

Change in pH and acidity during *kimchi* fermentation at different temperatures

The changes in pH and acidity in the *kimchi* during fermentation at 8, 15, and 25 °C are shown in Fig. 1. In the case of *kimchi* fermentation at 8 °C, sharp decreases in pH were observed during the first 15 days; thereafter, the pH values were moderately decreased from 15 to 21 days, while the pH values were negligibly increased from 21 to 54 days. Overall, the pH decreased from 6.17 (0 day of fermentation) to 4.16 after 54 days of fermentation. On the other hand, the acidity in the *kimchi* during fermentation gradually increased for 24 days; thereafter, a negligible increase was observed after 54 days. In total, the acidity increased from 0.24 to 1.14% in the *kimchi* after 54 days of fermentation (Fig. 1A). The pH was dramatically decreased within 9 days, and negligibly decreased thereafter until 30 days of fermentation at 15 °C. Overall, the initial pH decreased from 6.17 to 3.92 after 30 days of fermentation. However, the acidity sharply increased until day 15 and negligibly increased until 30 days of fermentation. In total, the initial (day 0) acidity of 0.24 was increased to 1.35% at the end of fermentation (30 days) (Fig. 1B). Similarly, for *kimchi* fermentation that proceeded at 25 °C, the pH sharply decreased until 6 days, slowly decreased from 6 to 9 days and remained almost unchanged until 24 days of fermentation. The pH decreased from 6.17 (0 day) to 3.48 at the end of fermentation (24 days). In contrast, the acidity was markedly increased until 6 days and slowly increased from 6 to 9 days, then negligibly increased until 24 days. Overall, the acidity increased from 0.24 to 1.54% in *kimchi* after 24 days of fermentation (Fig. 1C).

Change in salinity and reducing sugar during *kimchi* fermentation at different temperatures

The reduction of salinity and reducing sugar during fermentation of *kimchi* at 8, 15, and 25 °C is described in Fig. 2. In the case of fermentation at 8 °C, the salinity (3.3%) remained constant until 15 days of fermentation; thereafter, it decreased slowly from 3.3 to 2.7% after 54 days of fermentation. Similar to salinity, the reducing sugar concentration (43.07 g/L) remained almost unchanged until 15 days of fermentation, and rapidly decreased thereafter to 15.25 g/L at the end of fermentation (Fig. 2A). The salinity gradually decreased from 3.3 to 2.6% at the end of

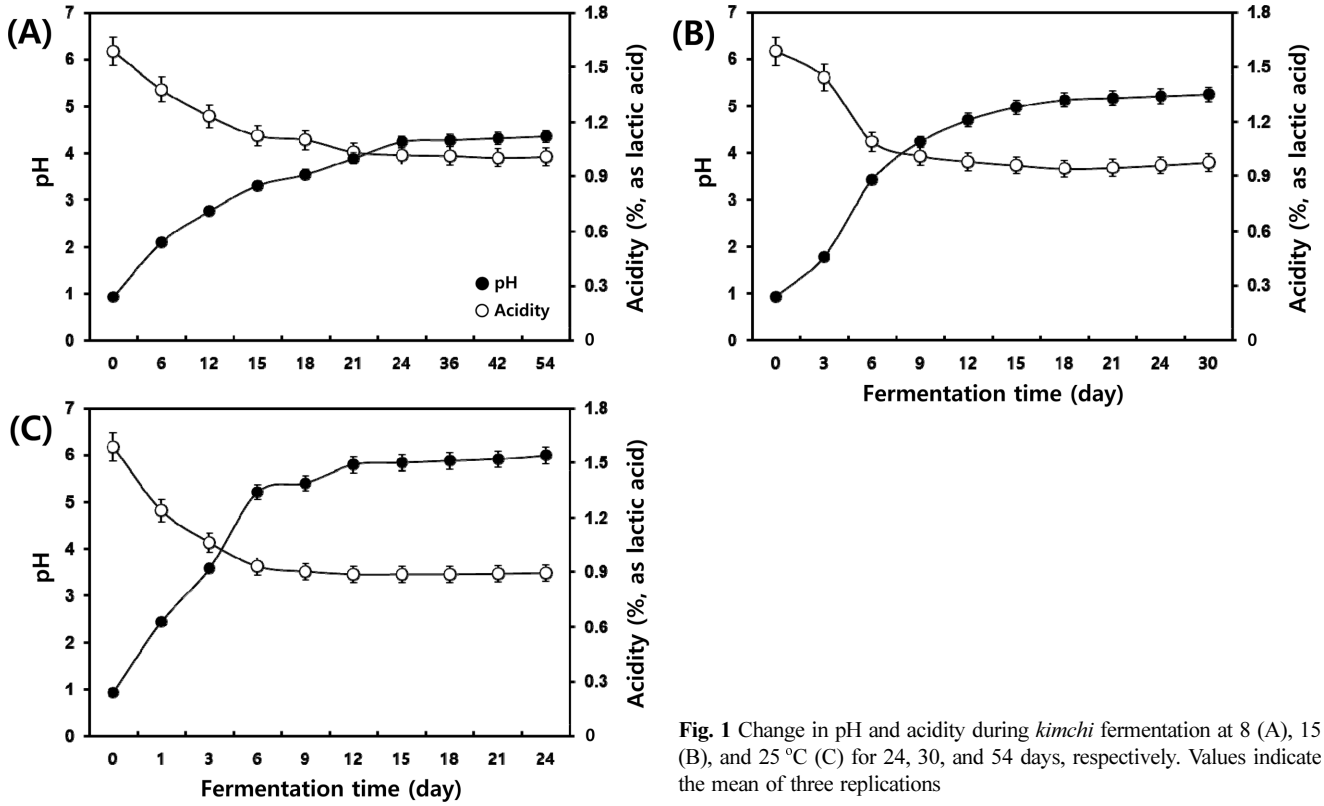

Fig. 1 Change in pH and acidity during *kimchi* fermentation at 8 (A), 15 (B), and 25 °C (C) for 24, 30, and 54 days, respectively. Values indicate the mean of three replications

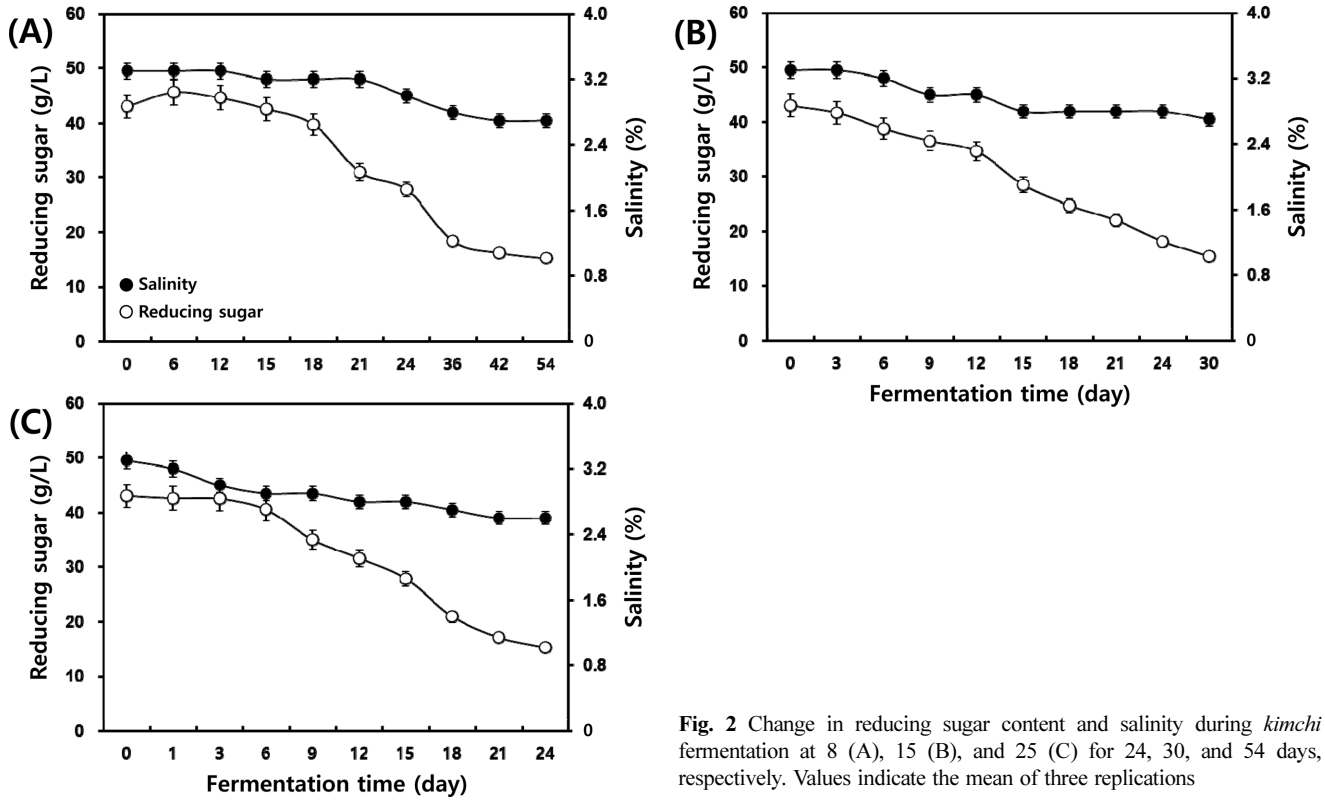

Fig. 2 Change in reducing sugar content and salinity during *kimchi* fermentation at 8 (A), 15 (B), and 25 (C) for 24, 30, and 54 days, respectively. Values indicate the mean of three replications

fermentation (30 days) during *kimchi* fermentation at 15 °C. In addition, the reducing sugar concentration (43.07 g/L) gradually decreased for 15 days, and sharply decreased thereafter to 15.41 g/L at the end of fermentation (Fig. 2B). Similarly, in the case of fermentation at 25 °C, the salinity and reducing sugar concentration decreased from the initial 3.3 and 43.07 to 2.6 and 15.31 g/L at the end of fermentation (24 days) (Fig. 2C).

Change in organic acid contents during *kimchi* fermentation at different temperatures

During *kimchi* fermentation, the total organic acids increased gradually until 12, 21, and 24 days; after that, the total organic acid concentration decreased slowly. In particular, the levels of lactic acid increased rapidly during *kimchi* fermentation. The lactic acid concentration increased from 3.74 at 0 day of fermentation to 10.53, 18.35, 20.5 g/L at the end of fermentation (54, 30, and 24 days) at 8, 15, 25 °C, respectively (Fig. 3). The concentration of succinic acid, acetic acid, maleic acid, and citric acid increased slightly during *kimchi* fermentation at 8 °C, but in the case of oxalic acid concentration unchanged (Fig. 3A). In the case of *kimchi* fermentation at 15 °C, the concentration of succinic acid and acetic acid increased slightly, but the levels of oxalic acid, malic acid, citric acid negligibly increased at the end of fermentation (Fig. 3B). During *kimchi* fermentation at 25 °C, the concentration of succinic acid, oxalic acid, and acetic acid increased slightly, but the values of maleic acid and citric acid a little increased at the end of fermentation (Fig. 3C).

Change in LAB population during *kimchi* fermentation at different temperatures

Changes in specific types of LAB were observed during *kimchi* fermentation using multiplex PCR and 16S rRNA sequence analysis. A sample from each of the 96 colonies was analyzed. The changes in the LAB population during fermentation of *kimchi* at 8, 15, and 25 °C are shown in Table 1. The isolates included five *Leuconostoc* species (*Leu. carnosum*, *Leu. citreum*, *Leu. gasicomitatum*, *Leu. gelidum,* and *Leu. mesenteroides*), five *Lactobacillus* species (*Lac. brevis*, *Lac. curvatus*, *Lac. plantarum, Lac. pentosus,* and *Lac. sakei*), one *Lactococcus* species (*Lac. lactis*), one *Pediococcus* species (*Ped. pentosaceus*), and two *Weissella* species (*Wei. confusa* and *Wei. koreenesis*).

In the case of initial fermentation (0 day), the lactic acid bacterial community included *Leu. carnosum* (0.32 for log CFU/mL), *Leu. citreum* (0.71), *Leu. mesenteroides* (0.38), *Lac. plantarum* (0.16), *Lac. sakei* (1.59), *Lac. lactis* (1.26), *Ped. pentosaceus* (0.16), and *Wei. confusa* (0.65) (Table 1). As fermentation proceeded at 8 °C, the levels of *Leu. carnosum*, *Leu. citreum*, *Lac. lactis*, and *Wei. confusa* decreased until 12 days (0.08), 15 days (0.28), and 18 days (0.09), respectfully; after that, they were not detected during fermentation. *Leu. mesenteroides* population increased greatly until 12 days (3.37), and then decreased. The *Lac. plantarum* concentration increased slowly to a maximum of 1.08 at 18 days, after which it decreased gradually until the end of fermentation (54 days) to 0.31 log CFU/mL. The level of *Lac. sakei* increased greatly until day 36 (7.78), and then decreased

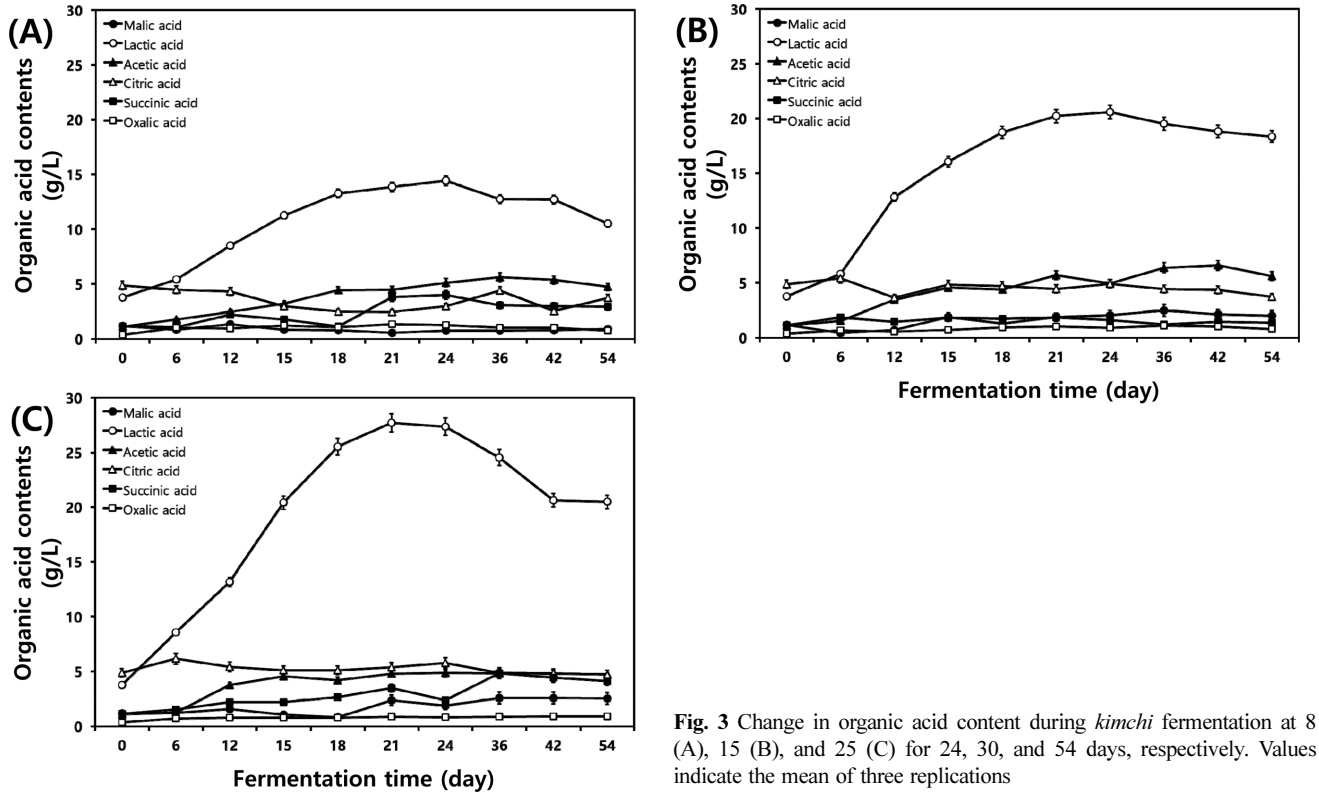

Fig. 3 Change in organic acid content during *kimchi* fermentation at 8 (A), 15 (B), and 25 (C) for 24, 30, and 54 days, respectively. Values indicate the mean of three replications

until 54 days (5.43) (Table 1). In the case of fermentation at 15 °C, the cells of *Leu. carnosum*, *Leu. citreum*, *Lac. lactis*, and *Wei. confusa* decreased until 9 days (0.36), 15 days (0.28), 15 days (0.83), and 9 days (0.24), respectively, and were not detected during fermentation after these time points. The *Leu. mesenteroides* increased until 9 days (3.24), after which they decreased until 24 days (0.26) of fermentation. On the other hand, the *Lac. plantarum* greatly increased until 21 days (6.6), and then decreased. *Lac. sakei* decreased greatly until 3 days (0.93) and increased to a maximum of 3.54 log CFU/mL at 9 days, after which it decreased until 24 days (0.26). On the other hand, *Leu. gasicomitatum* first appeared at 12 days, increased to a maximum of 1.68 at 24 days, and afterwards decreased to 0.73 log CFU/mL at the end of fermentation (Table 1). During fermentation at 25 °C, the numbers of *Leu. carnosum*, *Lac. lactis*, *Wei. confusa* and *Leu. citreum*, decreased until 3 days (0.22, 0.44, and 0.22 log CFU/mL, respectively) and 6 days (0.25) and after that they were not detected during fermentation. However, the *Leu. gasicomitatum* first appeared at 6 days, increased to a maximum of 1.45 log CFU/mL at 9 days, and then decreased at 21 days (0.25). The *Leu. mesenteroides* increased until 1 day (2.57), then decreased until 15 days (0.56) of fermentation and were not detected again during fermentation. On the other hand, the *Lac. plantarum* increased greatly at day 12 (8.63) and later decreased gradually until the end of fermentation (6.31). The *Loc. sakei* population increased to a maximum of 2.35 log CFU/mL at 1 day, after which it decreased until 12 days (0.27) and was not detected again during fermentation. Importantly, *Wei. koreenesis* first appeared at 12 (0.08 log CFU/mL), 9 (0.48), and 6 days (0.5) and then increased markedly until 54 (2.88), 30 (3.86), and 24 days (2.57) of fermentation (Table 1).

Discussion

Without starter cultures, *kimchi* is made through lactic acid fermentation of Chinese cabbage at low temperatures to ensure proper ripening and preservation. Because *kimchi* fermentation is changed from an initial open ecosystem to a later closed ecosystem, each batch of fermented *kimchi* has a different community of bacteria depending on fermentation conditions and ingredients. Lee et al. [17] suggested that the microbial community differed through fermentation conditions (such as salt and sugar concentration, major ingredient, fermentation temperature, and fermentation period), fermentation properties (including pH, acidity, salt and sugar concentration), and different *kimchi* samples (household, commercial, and regional sources). Recently, Lee et al. [19] reviewed that *kimchi* fermentation are carried out by complex microbial metabolisms to produce diverse metabolites including lactate, acetate, CO_2, ethanol, mannitol, amino acids, formate, malate, diacetyl, acetion, and 2,3-butanediol. This study set out to explore the diversity and community dynamics of LAB during *kimchi* fermentation at different temperatures using our previously developed multiplex PCR assay method. To understand

Table 1 Change in the lactic acid bacterial population during *kimchi* fermentation at 8, 15, and 25 °C for 24, 30, and 54 days, respectively

Temperature Genus/Species	Specific[a] and total[b] viable LAB cell numbers* (log CFU/mL)									
Temp. 8 °C	Fermentation time (day)									
	0	6	12	15	18	21	24	36	42	54
Leuconostoc										
Leu. carnosum†	0.32±0.02	0.31±0.02	0.08±0.00	nd[c]	nd	nd	nd	nd	nd	nd
Leu. citreum‡	0.71±0.04	0.47±0.02	0.44±0.02	0.28±0.01	nd	nd	nd	nd	nd	nd
Leu. gasicomitatum‡	nd	nd	nd	0.37±0.02	0.48±0.02	0.41±0.02	0.47±0.02	0.26±0.01	nd	nd
Leu. gelidum‡	nd	nd	0.08±0.00	nd	nd	nd	nd	nd	nd	nd
Leu. mesenteroides†	0.38±0.02	**2.88±0.14**[d]	**3.37±0.17**	1.72±0.09	1.16±0.06	1.54±0.08	1.18±0.06	1.28±0.06	1.36±0.07	1.43±0.07
Lactobacillus										
Lac. brevis†	nd	nd	nd	0.28±0.01	nd	0.31±0.02	0.11±0.01	nd	0.37±0.02	0.43±0.02
Lac. curvatus‡	nd	nd	nd	nd	nd	nd	0.11±0.01	nd	nd	nd
Lac. pentosus†	nd	nd	nd	nd	nd	0.10±0.01	nd	nd	nd	nd
Lac. plantarum†	0.16±0.00	0.31±0.02	0.53±0.03	0.56±0.03	1.08±0.05	0.62±0.03	0.94±0.05	0.77±0.04	0.62±0.03	0.31±0.02
Lac. sakei†	**1.59±0.08**	2.50±0.13	3.28±0.16	**4.76±0.24**	**5.21±0.26**	**5.65±0.28**	**6.83±0.34**	**7.78±0.39**	**6.85±0.34**	**5.43±0.27**
Lactoccous										
Lac. lactis†	1.26±0.06	0.71±0.04	0.18±0.01	0.28±0.01	nd	nd	nd	nd	nd	nd
Pediococcus										
Ped. pentosaceus†	0.16±0.01	nd	nd	nd	0.09±0.00	nd	nd	nd	nd	nd
Weissella										
Wei. confusa†	0.65±0.03	0.31±0.02	0.44±0.02	nd	0.09±0.00	nd	nd	nd	nd	nd
Wei. koreensis†	nd	nd	0.08±0.00	0.64±0.03	1.16±0.06	1.23±0.06	1.66±0.08	2.19±0.11	2.73±0.14	2.88±0.14
Total LAB cells	5.23±0.26	7.49±0.37	8.48±0.42	8.89±0.44	9.27±0.46	9.86±0.49	11.30±0.57	12.28±0.61	11.93±0.60	10.48±0.52
Temp. 15 °C	Fermentation time (day)									
	0	3	6	9	12	15	18	21	24	30
Leuconostoc										
Leu. carnosum†	0.32±0.02	0.57±0.03	0.36±0.02	0.36±0.02	nd	nd	nd	nd	nd	nd
Leu. citreum‡	0.71±0.04	0.93±0.05	0.62±0.03	0.60±0.03	0.76±0.04	0.28±0.01	nd	nd	nd	nd
Leu. gasicomitatum‡	nd	nd	nd	nd	0.76±0.04	0.83±0.04	1.66±0.08	1.26±0.06	1.68±0.08	0.73±0.04
Leu. gelidum‡	nd	0.15±0.01	0.18±0.01	nd	nd	nd	nd	nd	nd	nd
Leu. mesenteroides†	0.38±0.02	**2.30±0.12**	**2.67±0.13**	3.24±0.16	3.17±0.16	2.83±0.14	0.98±0.05	0.28±0.01	0.26±0.01	nd
Lactobacillus										
Lac. brevis†	nd	nd	0.36±0.02	nd	0.51±0.03	0.28±0.01	0.56±0.02	nd	0.64±0.03	nd
Lac. curvatus‡	nd	nd	nd	nd	nd	0.28±0.01	nd	nd	nd	nd
Lac. pentosus†	nd	nd	nd	0.24±0.01	0.76±0.01	0.28±0.01	1.26±0.06	0.70±0.04	0	0
Lac. plantarum†	0.16±0.01	0.57±0.03	1.96±0.10	2.78±0.14	**4.32±0.22**	**4.65±0.23**	**5.41±0.27**	**6.60±0.33**	**6.21±0.31**	**6.45±0.32**
Lac. sakei†	**1.59±0.08**	0.93±0.05	1.59±0.08	**3.54±0.18**	1.24±0.06	1.10±0.06	0.70±0.04	1.4±0.07	0.26±0.01	nd
Lactoccous										
Lac. lactis†	1.26±0.06	0.82±0.04	0.62±0.03	0.48±0.02	0.52±0.03	0.83±0.04	nd	nd	nd	nd
Pediococcus										
Ped. pentosaceus†	0.16±0.01	0.21±0.01	0.18±0.01	nd	nd	nd	nd	nd	0.12±0.01	nd
Weissella										
Wei. confusa†	0.65±0.03	0.44±0.02	nd	0.24±0.01	nd	nd	nd	nd	nd	nd
Wei. koreensis†	nd	nd	nd	0.48±0.02	1.24±0.06	1.86±0.09	2.86±0.14	3.20±0.16	3.24±0.16	3.86±0.19
Total LAB cells	5.23±0.26	6.92±0.35	8.54±0.43	11.48±0.57	12.04±0.60	13.22±0.66	13.43±0.67	13.44±0.67	12.41±0.62	11.04±0.55

Table 1 Continued

Temperature Genus/Species	Specific[a] and total[b] viable LAB cell numbers* (log CFU/mL)									
Temp. 25 °C	Fermentation time (day)									
	0	1	3	6	9	12	15	18	21	24
Leuconostoc										
Leu. carnosum†	0.32±0.02	0.23±0.01	0.22±0.01	nd	nd	nd	nd	nd	nd	nd
Leu. citreum‡	0.71±0.04	0.54±0.03	0.56±0.03	0.25±0.01	nd	nd	nd	nd	nd	Nd
Leu. gasicomitatum‡	nd	nd	nd	0.88±0.04	1.45±0.07	0.82±0.04	0.7±0.04	0.52±0.03	0.25±0.01	nd
Leu. gelidum‡	nd	0.08±0.00	nd	nd	nd	nd	nd	nd	nd	nd
Leu. mesenteroides†	0.38±0.02	**2.57**±0.13	2.46±0.12	1.98±0.10	0.91±0.05	nd	0.56±0.03	nd	nd	nd
Lactobacillus										
Lac. brevis†	nd	nd	0.44±0.02	nd	0.52±0.03	0.68±0.03	0.70±0.04	0.52±0.03	1.23±0.06	1.54±0.08
Lac. curvatus‡	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Lac. pentosus†	nd	nd	0.12±0.01	0.25±0.01	0.49±0.02	0.68±0.03	0.28±0.01	0.52±0.03	nd	nd
Lac. plantarum†	0.16±0.01	0.39±0.02	**4.0±0.20**	**6.79±0.34**	**7.67±0.38**	**8.63±0.43**	**8.87±0.44**	**7.98±0.40**	**7.74±0.39**	**6.31±0.32**
Lac. sakei†	**1.59±0.08**	2.35±0.12	2.16±0.11	1.36±0.07	0.26±0.01	0.27±0.01	nd	nd	nd	nd
Lactoccous										
Lac. lactis†	1.26±0.06	0.70±0.04	0.44±0.02	nd	nd	0.13±0.01	nd	nd	nd	nd
Pediococcus										
Ped. pentosaceus†	0.16±0.01	nd	nd	nd	nd	nd	nd	nd	nd	nd
Weissella										
Wei. confusa†	0.65±0.03	0.62±0.03	0.22±0.01	nd	nd	nd	nd	nd	nd	nd
Wei. koreensis†	nd	nd	nd	0.50±0.03	1.17±0.06	1.82±0.09	2.29±0.11	2.52±0.13	2.47±0.12	2.57±0.13
Total LAB cells	5.23±0.26	7.48±0.37	10.62±0.53	12.01±0.60	12.47±0.62	13.03±0.65	13.40±0.67	12.06±0.60	11.69±0.58	10.42±0.52

*Values indicate the mean's of three replications (n =3)
†The specific LAB was identified by multiplex PCR method
‡The specific LAB was identified by 16S rRNA sequencing analysis
[a]Each of specific LAB viable cells (log CFU/mL) = (detection of each of specific LAB colonies/isolated 96 colonies) × total LAB viable cells on MRSA
[b]One ml of each blended kimchi was serially diluted. Dilutions were plated onto MRSA and after these incubated at 30 °C for 48 h
[c]nd: not detected
[d]Bold words indicate the predominant during kimchi fermentation

the quality of the *kimchi*, the physicochemical properties, such as pH, acidity, organic acid profile, salinity and reducing sugar concentration, of *kimchi* were investigated. The chemical composition of *kimchi* is different according to the varieties of cabbage and types and amounts of minor ingredients used. In fact, carbohydrates in *kimchi* raw materials are converted by LAB into metabolites such as organic acids, carbon dioxide, ethanol, and aromatic compounds.

Generally, the best quality of *kimchi* can be obtained at pH 4.2 to 4.5 and acidity of 1.5 to 2.0% [1,5]. In this study, *kimchi* fermented at 8 °C had a pH of 4.16 and acidity of 1.14% at the optimum ripening stage that occurred after 54 days of fermentation. However, in the case of fermentation at 15 °C, the optimum pH and acidity appeared after 24 days of fermentation. Similarly, in the case of fermentation at 25 °C, the optimum pH and acidity appeared after 15 days of fermentation. Previously, lactic, acetic, citric, malic, fumaric, succinic, oxalic, tartaric, malonic, maleic, and glycolic acid were identified from *kimchi* samples. Among the organic acids identified, lactic acid and acetic acid are the major acids that are increased by fermentation [9]. In this study, the concentration of lactic acid, as well as other organic acids, was greatly increased at this fermentation time point. In particular, the concentration of lactic acid increased from 3.74 to 10.53, 18.35, and 20.50 g/L at the end of fermentation conducted at 8, 15, and 25 °C, respectively. The results suggested that as the temperature increased, the pH and reducing sugar was greatly decreased, while acidity and total organic acid were markedly increased during the *kimchi* fermentation. Therefore, temperature could be a key factor governing the *kimchi* fermentation. Free sugars play important roles in the taste of *kimchi* because free sugars are not only sweeteners but also serve as carbon sources for LAB to produce various products. Free sugars decreased during heterophic lactic acid fermentation of *kimchi*, while lactate, acetate, and ethanol increased [17].

The predominant LAB species fermented *kimchi* at different stages and temperatures. *Leu. mesenteriodes* was present during the immature stage at 8 °C (2.88, 6, 5.36, 0.54 for log CFU/mL, days, pH, acidity), and 15 °C (2.3, 3, 5.61, 0.46) and during the

optimum-ripening stage at 8 °C (3.37, 12, 4.79, 0.71), 15 °C (2.67, 6, 4.24, 0.88), and 25 °C (2.57, 1, 4.82, 0.63). *Lac. sakei* was present during the initial stage (1.59, 0, 6.17, 0.24), the over-ripening stage at 8 °C (5.65, 21, 4.02, 1.0), and 15 °C (3.54, 9, 3.93, 1.09), and the rancid stage at 8 °C (5.43, 54, 3.92, 1.12). *Lac. plantarum* was present during the over-ripening stage at 25 °C (4.0, 3, 4.13, 0.92) and rancid stage at 15 °C (6.45, 30, 3.79, 1.35) and 25 °C (6.31, 24, 3.48, 1.54). These results also suggest that fermentation temperature and acidity are one of the primary determinants of microbial populations in *kimchi* and that complex microbial succession is not crucial for *kimchi* fermentation. Generally, *Leuconsotoc* species typically dominate during the initial fermentation with low acidity at high temperature, while *Weissella* and *Lactobacillus* species have the ability to grow under high acidity and low temperatures, indicating that *kimchi* fermentation temperature and acidity are important determinants of the microbial population [17]. Previously, several studies reported that *Leuconsotoc* species were the major microorganisms at the beginning of *kimchi* fermentation and that they reach their highest population during the optimum-ripening period [5,23]. Cogan and Jordan [24] reviewed that they only obtained energy by fermentation, always producing lactic acid as all other LAB and CO_2, and ethanol or acetate. Therefore, their suggestion that *Lac. plantarum* was responsible for over-ripening of *kimchi* has to be further tested by careful studies at several temperatures. Mheen and Kwon [1] showed that *Lac. plantarum* appeared at 30, 20, and 14 °C, but could not be detected at a lower temperature (5 °C), and similar results were also reported by Lim et al. [25] and Lee et al. [26]. Similarly, Kim et al. [14] reported that *Lac. plantarum* first appeared at the over-ripening stage (36 h, pH 4.03, and acidity 0.88%) and increased during mulkimchi fermentation at 30 °C. The difficulty of discrimination between *Lac. plantarum* and *Lac. pentosus* was recognized in other LAB studies [27,28]. In addition, Lim et al. [25] also noted the difficulty of discrimination between *Lac. plantarum* and *Lac. brevis* by biochemical methods. Additionally, *Lac. sakei* is and acidophilic and/or acid-producing bacterium, which is phylogenetically close to *Lac. plantarum*. Thus, two *Lactobacillus* species, such as *Lac. sakei* and *Lac. plantarum*, proportionally increased with the increase in acidity and lactic acid during *kimchi* fermentation at 8 °C (low temperature), but *Lac. plantarum* only increased during *kimchi* fermentation at 15 and 25 °C (high temperature). Recently, Hwang et al. [22] reported that results similar to those of this study in *mulkimchi* fermentation at 8 °C. Conversely, Chin et al. [3] showed that *Lac. brevis* and *Wei. kimchii/cibaria* are the predominant species in the initial to mid-stage of fermentation (when *kimchi* pH is over 4.0) and *Leu. mesenteroides* is the only predominant species in the early stage of *kimchi* fermentation. Cho et al. [4] reported that *Wei. koreensis*, a psychrophilic bacterium, is probably the dominant species in *kimchi* produced at −1 °C and the predominance of *Leuconostoc* species, including *Leu. citreum*, observed after a short preliminary incubation at 15 °C, results in a delay of the rapid outgrowth of *Wei. koreensis* at −1 °C. Hwang et al. [22] reported that the reduction in pH and increment in acidity and lactic acid concentration were observed, these was gradual increase of *Wei. koreensis* throughout *mulkimchi* fermentation at 8 °C. Additionally, Lee et al. [9] performed microbial fingerprints by a DGGE to investigate the distribution of microorganisms in *kimchi* fermented at 10 to 20 °C, and concluded that *Wei. confusa, Lac. sakei, Leu. citreum*, and *Lac. curvatus* were dominant. Interestingly, *Lac. plantarum*, which has been known as a predominant strain in the later stage of fermented vegetables by culture-independent approaches, was not detected by the culture-independent DGGE analysis. According to one publication [29], *Lac. sakei* and *Leu. mesenteroides* are the most predominant LAB in all types of *kimchi* in the middle stage of fermentation at 20 °C. These results proposed that there could be another important determinant of lactic acid bacterial community in addition to temperature and acidity. Data shown in Figure 1, 2, and 3 strongly indicate that the metabolism of reducing sugar and the production of lactic acid and acetic acid were closely correlated with the growth of *Leuconostoc, Lactobacillus*, and *Weissella*.

We attempted to determine whether our culturing method may have missed a portion of the species that actually existed in the *kimchi* samples, as certain populations may have been unculturable under the experimental conditions. Two pieces of evidence support that predominant species are culturable. First, increases in the total number of viable cells are closely correlated with increases in the levels of fermentation products, including lactic acid and acetic acid. Second, the several reports of microbial diversity in *kimchi* by culture-independent methods determined that the primary bacterial components include: *Leu. citreum, Leu. gasicomitatum, Leu. gelidum, Lac. curvatus, Lac. plantarum, Lac. sakei, Wei. confusa*, and *Wei. koreensis* [5,7-9]. These studies strongly supported our interpretation that the predominant species in *kimchi* samples can, indeed, be cultured on MRS medium. However, these culture-independent analyses are also somewhat limited. As we showed in a previous study, PCR amplification cannot be strictly correlated with the ratio of target DNA to genomic DNA [30]. As a result, some minor population groups may have been missed. Similar to the majority of molecular techniques for the detection, identification, and classification of bacteria [31,32], multiplex PCR commonly targets the 16S rRNA gene, the gene most widely used to infer phylogenetic relationships among bacteria [33]. This gene is sometimes insufficient to distinguish closely related species [34,35]; thus, in order to ensure high specificity of multiplex PCR, other genes need to be taken into accounts for primer designation. The results of multiplex PCR show various types of LAB strains detected in the 154 to 506 bp range [5]. The ideal technique would include DNA sequences that were specific for each species in order to obtain a single band per species and the specific bands would differ in size such that the interpretation of band position would be easy [36].

In conclusion, *Leu. mesenteroides* predominated during optimum-

ripening when *kimchi* was fermented at 8, 15, and 25 °C and *Lac. sakei* and *Lac. plantarum* rapidly grew in *kimchi* produced at 8, 15, and 25 °C. Experiments also demonstrated that population dynamics are rather sensitive to environmental conditions, including fermentation temperature and acidity. Therefore, the microbial population dynamics characterized in this study may be applicable in the use of microbes for the improved control of *kimchi* fermentation and preservation.

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