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

Hee Yul Lee¹ · Md. Azizul Haque¹² · Kye Man Cho¹

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 fermentation day to 14.43, 20.60, and 27.69 g/L during the particular, the levels of lactic acid increased from 3.74 g/L 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 fermentation day 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 Leuconostoc 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.

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: Leuconostoc citreum, Leuconostoc gasicomitatum, Leuconostoc mesenteroides, Lactococcus 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 (H3PO4) 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 rRNA genes were amplified and these sequences were analyzed as previously described [21,22].
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
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, and 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. gascomitatum, 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. koreensis).

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), respectively; 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...
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. gascomitatum* first appeared at 12 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. 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, aceton, 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\(^d\) 0.32±0.02 0.31±0.02 0.08±0.00 nd nd nd nd nd nd |
|              | Leu. citreum\(^d\) 0.71±0.04 0.47±0.02 0.44±0.02 0.28±0.01 nd nd nd nd nd nd |
|              | Leu. gasicomitatum\(^d\) 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. mesenteroides\(^d\) 0.38±0.02 2.88±0.14 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\(^d\) nd nd nd 0.31±0.02 0.09±0.00 nd nd nd nd nd nd |
|              | Lac. curvatus\(^d\) nd nd nd nd nd nd 0.28±0.01 nd nd nd nd |
|              | Lac. pentosus\(^d\) nd nd nd nd nd 0.14±0.01 nd nd nd nd nd |
|              | Lac. plantarum\(^d\) 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\(^d\) 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 |
| *Lactococcus* | Lac. lactis\(^d\) 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\(^d\) 0.16±0.01 nd nd nd 0.09±0.00 nd nd nd nd nd nd |
| *Weissella* | Wei. confusa\(^d\) 0.65±0.03 0.31±0.02 0.44±0.02 nd 0.09±0.00 nd nd nd nd nd |
|              | Wei. koreensis\(^d\) nd nd 0.08±0.00 0.64±0.03 1.16±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\(^d\) 0.32±0.02 0.57±0.03 0.36±0.02 0.36±0.02 nd nd nd nd nd nd |
|              | Leu. citreum\(^d\) 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\(^d\) 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. mesenteroides\(^d\) 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\(^d\) nd 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\(^d\) nd nd nd nd nd nd nd nd nd nd |
|              | Lac. pentosus\(^d\) 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\(^d\) 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\(^d\) 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 |
| *Lactococcus* | Lac. lactis\(^d\) 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\(^d\) 0.16±0.01 0.21±0.01 0.18±0.01 nd nd nd nd 0.12±0.01 nd |
| *Weissella* | Wei. confusa\(^d\) 0.65±0.03 0.44±0.02 nd 0.24±0.01 nd nd nd nd nd nd |
|              | Wei. koreensis\(^d\) 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 |
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

### Table 1 Continued

| Temperature Genus/Species | Specifica and total 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   |
| Leu. citreum‡             | 0.71±0.04 0.54±0.03 0.56±0.03 0.25±0.01 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 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 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   |
| Lactococcus               |                                                         |
| 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 nd   |
| Weissella                 |                                                         |
| Wei. confusa†             | 0.65±0.03 0.62±0.03 0.22±0.01 nd nd nd nd nd nd nd nd   |
| Wei. koreensis†           | nd 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
§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
|||
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 (5.34, 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 CO₂, 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 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 Leuconsotoc 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 Leuconsotoc, 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. gascomitatum, 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 account 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].
ripening when kimchi was fermented at 8, 15, and 25 °C and Lact. sakei and Lact. 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.

Acknowledgment This work was supported by Gyeongnam National University of Science and Technology Grant (2020 year), Republic of Korea.

References
1. Mheen TI, Kwon TW (1984) Effect of temperature and salt concentration on kimchi fermentation. Korean J Food Sci Technol 16: 443–450
2. Kim JM, Song KY, Kim SY, Shin WC, Yoon SS (2004) Effect of eggshell powder on extending the shelf-life of mul kimchi. Food Sci Biotechnol 13: 136–140
3. Chin WS, Breidt F, Fleming HP, Shin WC, Yoon SS (2006) Identifications of predominant bacterial isolates from the fermenting kimchi using ITS-PCR and partial 16S rDNA sequence analyses. J Microbiol Biotechnol 16: 68–76
4. Cho J, Lee D, Yang C, Jeon J, Kim J, Han H (2006) Microbial population dynamics of kimchi, a fermented cabbage product. FEMS Microbiol Lett 257: 262–267
5. Cho KM, Math RK, Islam SM, Lim WJ, Hong SY, Kim JM, Yun MG, Cho JJ, Yun HD (2009) Novel multiplex PCR for the detection of lactic acid bacteria during kimchi fermentation. Mol Cell Probe 23: 90–94
6. Jeong SH, Lee HJ, Jung JY, Lee SH, Seo HY, Park WS, Jeon CO (2013) Effects of red pepper powder on microbial communities and metabolites during kimchi fermentation. Int J Food Microbiol 160: 252–259
7. Jung JY, Lee SH, Jin HM, Hahn Y, Madsen EL, Jeon CO (2013) Metatranscriptomic analysis of lactic acid bacterial gene expression during kimchi fermentation. Int J Food Microbiol 160: 171–179
8. Kim M, Chun J (2005) Bacterial community structure in kimchi, a Korean fermented vegetable food, as revealed by 16S rDNA gene analysis. Int J Food Microbiol 103: 91–96
9. Lee JS, Heo GY, Lee JW, Oh YJ, Park JA, Park YH, Park YR, Ahn JS (2005) Analysis of kimchi microflora using denaturing gradient gel electrophoresis. Int J Food Microbiol 102: 143–150
10. Park H, Shin H, Holzapfel W (2016) Autoinducer-2 properties of kimchi, a fermented cabbage product. Int J Food Microbiol 225: 38–42
11. Park WS, Moon SW, Lee MK, Ahn BH, Koo YJ, Kim KH (1996) Comparison of fermentation characteristics of the main types of Chinese cabbage kimchi. Food Biotechnol 5: 128–135
12. Kim TW, Jung SH, Lee JY, Choi SK, Park SH, Jo JS, Kim HY (2003) Identification of lactic acid bacteria in kimchi using SDS-PAGE profiles of whole cell proteins. J Microbiol Biotechnol 13: 119–124
13. Bae JW, Rhee SK, Park JR, Chung WH, Nam YD, Lee I, Kim H, Park YH (2005) Development and evaluation of genome-probing microarrays for monitoring lactic acid bacteria. Appl Environ Microbiol 71: 8825–8835
14. Kim B, Seo WT, Kim MG, Yun HD, Cho KM (2012) Metagenomic lactic acid bacterial diversity during mul kimchi fermentation based on 16S rRNA sequence. J Korean Soc Appl Biol Chem 55: 787–792
15. Elñifri EO, Ashahi AM, Cooper RJ, Klapper PE (2000) Multiplex PCR: optimization and application in diagnostic virology. Clin Microbiol Rev 13: 559–570
16. Jofré A, Martin B, Garriga M, Hugas M, Pla M, Rodriguez-Lázaro D, Aymerich T (2005) Simultaneous detection of Listeria monocytogenes and Salmonella by multiplex PCR in cooked ham. Food Microbiol 22: 109–115
17. Jung JY, Lee SH, Kim JM, Park MS, Bae JW, Hahn Y, Madsen EL, Jeon CO (2011) Metagenomic analysis of kimchi, a traditional Korean fermented food. Appl Environ Microbiol 77: 2264–2274
18. Lee M, Song JH, Jung MY, Lee SH, Chung JY (2017) Large-scale targeted metagenomic analysis of bacterial ecological changes in 84 kimchi samples during fermentation. Food Microbiol 66: 173–183
19. Lee SH, Whon TW, Roh SW, Jeon CO (2020) Unraveling microbial fermentation features in kimchi: from classical to meta-omics approaches. Appl Microbiol Biotechnol 104: 7731–7744
20. Miller GL (1959) Use of dinitrosalicylic acid reagent for the determination of reducing sugar. Anal Chem 31: 426–428
21. Haque MA, Lee JH, Cho KM (2015) Endophytic bacterial diversity in Korean kimchi made of Chinese cabbage leaves and their antimicrobial activity against pathogens. Food Cont 56: 24–33
22. Hwang CE, Haque MA, Hong SY, Kim SC, Cho KM (2019) Origin of lactic acid bacteria in mulkimchi fermentation. J Appl Biol Chem 62: 441–446
23. Choi JY, Jeon Y, Lee JH (2002) Reevaluation of the change of L. plantarum species and Lactobacillus plantarum by PCR during kimchi fermentation. J Microbiol Biotechnol 12: 166–171
24. Cogan TM, Jordan KN (1994) Metabolism of Lactococcus species. J Dairy Sci 77: 2704–2717
25. Lim CT, Park HK, Han HU (1989) Reevaluation of isolation and identification of Gram-positive bacteria in kimchi. Korean J Microbiol 27: 404–414
26. Lee KW, Ko CY, Ha DM (1992) Microbial changes of the lactic acid bacteria during kimchi fermentation and identification of the isolates. Korean J Appl Microbiol Bioeng 20: 102–109
27. Berthier F, Ehrlich SD (1998) Rapid species identification within two groups of closely related lactobacilli using PCR primers that target the 16S/23S rRNA spacer region. FEMS Microbiol Lett 161: 97–106
28. Sliefer KH, Ehmann M, Beinborn C, Brockmann E, Ludwig W, Amann R (1995) Application of molecular methods for the classification and identification of lactic acid bacteria. Int Dairy J 5: 1081–1094
29. Kim TW, Lee JY, Jung SH, Kim YM, Jo JS, Chung DK, Leem HJ, Kim HY (2002) Identification and distribution of dominant lactic acid bacteria in kimchi, a Korean traditional fermented food. J Microbiol Biotechnol 12: 635–642
30. Lee MK, Park WS, Lee BH (2000) Genetic identification of the kimchi strain using PCR-based PevN and 16S rDNA gene sequence. Korean J Food Sci Technol 32: 1331–1335
31. Collins MD, Rodrigues U, Ash C, Aguierre M, Farrow JAE, Martinez-murcia A, Phillips BA, Williams AM, Wallbanks S (1991) Phylogenetic analysis of the genus Lactobacillus and related lactic acid bacteria as determined by reverse-transcribe sequencing of 16S ribosomal-RNA. FEMS Microbiol Lett 77: 5–12
32. Grahn N, Olofsson M, Ellnebo-Svedlund K, Monstein HJ, Jonasson J (2001) Differentiation of predominant bacterial isolates from the fermenting kimchi by recA gene sequence. J Microbiol Biotechnol 11: 441–446
33. Torriani S, Felis GE, Dellaglio F (2001) Differentiation of food-and beverage-associated microorganisms: A review. J Microbiol Meth 44: 1–12
34. Normand P, Poronnet C, Nesme X, Neyra M, Simonet P (1996) ITS analysis of prokaryotes. Molecular Microbiol Ecology Manual. Kluwer Academic Publishers, Dordrecht, 1–12
35. Torriani S, Felis GE, Dellaglio F (2001) Differentiation of Lactobacillus plantarum, L. pentosus, and L. paraplantarum by recA gene sequence analysis and multiplex PCR assay with recA gene-derived primers. Appl Environ Microbiol 67: 3450–3454
36. Settanni L, Corsetti A (2007) The use of multiplex PCR to detect and differentiate food-and beverage-associated microorganisms: A review. J Microbiol Meth 69: 1–22