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Buffering Effects of Calcium Salts in *Kimchi*: Lowering Acidity, Elevating Lactic Acid Bacterial Population and Dextransucrase Activity

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This study investigates the buffering effects of calcium salts in *kimchi* on the total acidity, microbial population, and dextransucrase activity. Calcium chloride or calcium carbonate was added to *dongchimi-kimchi*, a watery radish *kimchi*, and the effects on various biochemical attributes were analyzed. The addition of 0.1% calcium chloride produced a milder decrease in the pH after 24 days of incubation, which allowed the lactic acid bacteria to survive longer than in the control. In particular, the heterofermentative *Leuconostoc* genus population was 10-fold higher than that in the control. When sucrose and maltose were also added along with the calcium salts, the dextransucrase activity in the *kimchi* was elevated and a higher concentration of isomaltooligosaccharides was synthesized when compared with the control. Calcium chloride was determined as a better activator compound of dextransucrase than calcium carbonate, probably because of its higher solubility. Therefore, the results of this study confirm the ability of the proposed approach to modulate the *kimchi* fermentation process and possibly enhance the quality of *kimchi* based on the addition of dietary calcium salts.

Keywords: Dextransucrase, *kimchi*, *Leuconostoc*, calcium carbonate, calcium chloride, isomaltooligosaccharide

Leuconostoc species are heterofermentative lactic acid bacteria (LAB) and important bacterial populations in *kimchi* or sauerkraut from the initial to the middle stages of fermentation [7, 14]. During these stages, these bacteria produce various constituents, such as lactic acid, acetic acid, alcohol, CO_2, and mannitol, all of which contribute to the flavor of the fermented foods. For this reason, in 2005, a leading *kimchi* producer in Korea began to use *L. mesenteroides* as a starter culture for taste improvement and quality control. However, the microorganisms belonging to the *Leuconostoc* genus are more acid-labile than other LAB and their growth is inhibited by the lactic acid excreted during *kimchi* fermentation below pH 4.0 [15]. In addition, the acid content influences the taste of *kimchi*; a total acidity of 0.6–0.8% is recognized to give the best taste, whereas acidity above this range produces a strong acid taste and lowers the quality of the *kimchi* [12]. The concentration of lactic acid in *kimchi* can be reduced by the addition of calcium salts to neutralize the lactate into calcium salt (*i.e.*, calcium lactate).

The dextransucrase (E.C. 2.4.1.5) secreted by *Leuconostoc* species transfers the glucose moiety of sucrose to form dextran-like glucan, and catalyzes the transfer of glucose from sucrose (donor) to other sugars (acceptors) by linking an α-(1→6)-glucosyl bond [20, 22]. When the acceptor is a monosaccharide or disaccharide, a series of oligosaccharide acceptor products is usually produced, and maltose has been shown to be the best acceptor molecule in experiments using *L. mesenteroides* NRRL B-512F [2]. Therefore, using this reaction, the current authors already proposed a synbiotic oligosaccharide synthesis method for *kimchi* and fermented milk [5, 6, 8]. In the *kimchi* manufacturing process, the simple addition of sucrose and maltose to the ingredients has achieved a high conversion yield of isomaltooligosaccharides (IMOs) *via* the reaction of the dextransucrase excreted by the inherent *Leuconostoc* bacteria.

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Miller and Robyt [16] also reported that calcium ions are an activator of dextransucrase based on increasing the V_{max} and decreasing the K_m for sucrose.

Accordingly, this study used the addition of calcium salt to *kimchi* for two goals: first, to decrease the lactic acid content through the formation of calcium salts to reduce the acid stress against leuconostocs and thereby maintain the population of beneficial microflora; and second, to activate the dextransucrase excreted from leuconostocs to synthesize more IMOs in *kimchi*. For this purpose, calcium chloride and calcium carbonate were selected as the calcium salts, since they are soluble in water and acceptable for dietary use. After adding the salts to the *kimchi* preparation, the microbial cell counts, including the LAB and leuconostocs, were monitored, and the dextransucrase activities with the amounts of IMOs synthesized were assayed during the whole period of *kimchi* fermentation.

Materials and Methods

Materials

The dextranase, sucrose, and standard chemicals were all purchased from Sigma Inc. (St. Louis, MO, U.S.A.), and the maltose was from Duksan Pharmaceuticals (Yongin, Korea). The lactobacilli MRS broth was from Difco (Detroit, MI, U.S.A.), and cubic plastic jars with sealing lids were used as the fermentation vessel. The radishes, red peppers, green onions, and other constituents were purchased from a local grocery store.

Bacterial Strain and Enzyme

The dextransucrase was obtained using *Leuconostoc citreum* KACC 91035, which is a psychrotrophic strain secreting highly active dextransucrase across a broad temperature range [6]. The strain was cultivated in a sucrose broth (500 ml) under aerobic conditions for 48 h at 28°C. The S-medium was composed of 24.7 g sucrose, 4.2 g peptone, 4.2 g yeast extract, 20 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 2H_2O$, 0.1 g NaCl, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $MnSO_4 \cdot H_2O$, and 0.13 g $CaCl_2 \cdot 2H_2O$ per liter of distilled water [11], where 0.5–1 mg/ml Tween 80 was added for enzyme stabilization. To determine the effect of the calcium ions on the dextransucrase, the enzyme was purified using the method described by Miller *et al.* [15, 16]. The cells were separated from the supernatant by centrifugation at 10,000 rpm for 10 min at 4°C. To digest the dextran, lyophilized dextranase was added to the culture supernatant, which was then dialyzed overnight against 0.02 M sodium acetate (pH 5.2), 0.05 M NaCl, in regenerated cellulose dialysis membrane tubing (3.5 kDa molecular mass cutoff; Spectra/Por, Spectrum Laboratories Inc., CA, U.S.A.) at 4°C with two changes of buffer. The dialyzed solution was concentrated by dehydration with polyethylene glycol 6000 at 4°C overnight. The dextransucrase was further purified by DEAE-cellulose column chromatography equilibrated with 0.02 M sodium acetate (pH 5.2) and 0.05 M NaCl with a 0.2 M NaCl linear gradient in 0.02 M imidazole-HCl (pH 6.7). The purified enzyme fractions were then filtered through a 0.22-μm filter and stored at −70°C until use. The dextransucrase activities were measured by assaying the changes in the fructose concentration [19] after modification [21] using dinitrosalicylic acid (DNS) methods [17] in a 20 mM Na-acetate buffer solution (pH 5.2) containing 100 mM sucrose, 1 mM $CaCl_2$, and 0.02% NaN_3. One unit of dextransucrase was defined as the amount of enzyme used to produce 1 μmole of fructose per minute at 25°C.

Kimchi Preparation and Fermentation

Dongchimi-kimchi is a popular watery radish *kimchi*. The whole radish (800 g) was washed, the outer layer peeled off and cut into small pieces. The pieces were then mixed with salt (40 g) in a plastic jar and incubated at 20°C for 6 h until they became soft. Next, the salted radish and extract solution were mixed with crushed garlic (10 g), ginger (3 g), and chopped green onions (20 g). To synthesis IMOs, sucrose and maltose were added to make a final concentration of 1% (w/v). The jar was then filled with 4 l of drinking water and tightly sealed with a plastic lid. [3]. The *dongchimi* samples with added sucrose and maltose [each 1% (v/v)] were grouped as follows: A, control without calcium salt; B, *dongchimi* with 0.1% calcium chloride; and C, *dongchimi* with 0.1% calcium carbonate. The fermentation temperature was kept at 20°C for 2 days after reaching the maximum level of *Leuconostoc* sp. growth and dextransucrase activity. Thereafter, the temperature was dropped to 4°C to reduce the bacterial growth and sugar consumption. *Kimchi* samples (10 ml liquid) were harvested periodically to analyze the microbiological and physicochemical changes during fermentation.

Microbiological Analysis

The viable bacteria were counted using MRS and phenylethanol agars (Difco, U.S.A.) with 2% sucrose (PES [15]). Each sample was serially diluted with 0.85% (w/v) physiological saline. The total number of LAB was determined by spread-plating onto the MRS agar and incubating at 28°C for 48 h [10], and the *Leuconostoc* genus population was counted by spread-plating onto the PES agar after incubation at 20°C for 48 h [13].

Chemical Analysis

The pH of the *dongchimi* samples was measured using a pH meter (IQ 240, I.Q. Scientific Inc., U.S.A.) and the titratable acidity was determined by titrating with 0.1 N NaOH to an end point of pH 8.3 [1]. The percentage of lactic acid in the sample was calculated by multiplying the volume of the NaOH solution (ml). For a quantitative analysis of the sugars, 1 ml of each sample was loaded onto a Merck K5 TLC plate and developed three times with acetonitrile/distilled water [85:15 (v/v)]. The separated sugars were then detected by dipping the plate in ethanol containing 0.5% (w/v) α-naphthol and 5% (v/v) sulfuric acid, followed by heating at 110°C for 5 min. The final sugar analysis was performed using the Sigmagel program (Sigma Inc., U.S.A.) [6].

Results and Discussion

Purification of Dextransucrase

To investigate the effects of calcium salts on dextransucrase activity, *L. citreum*, as a representative species of the *Leuconostoc* genus, was cultured in an S-medium and the production of dextransucrase was induced by the addition of sucrose. The dextran polymer produced by dextransucrase

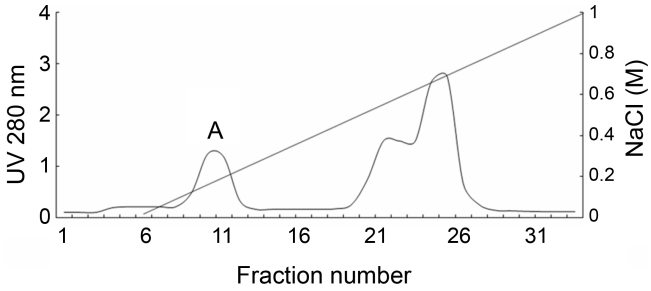

Fig. 1. Dextransucrase purification chromatogram obtained from DEAE-cellulose column chromatography.
Curve absorbance of protein concentration measured at 280 nm; line, concentration of NaCl solution; A, dextransucrase fractions.

when using sucrose as the glucosyl donor usually has an adverse effect on the enzyme purification process owing to the formation of a dextran–enzyme complex. Therefore, dextranase (9 mg, 0.09 IU/mg) was added to break down the polymer selectively and the culture was dialyzed for 24 h. The crude enzyme mixture was then analyzed by DEAE-cellulose column chromatography (Fig. 1) and the fractions (10 and 11) retaining enzyme activity were pooled and concentrated.

Effects of Calcium Salts on Dextransucrase Activity

To determine the effects of calcium salts on dextransucrase, calcium carbonate ($CaCO_3$) and calcium chloride ($CaCl_2$) were used. The salt concentrations used were 0.1% or 0.5% and two different solutions were used for the enzyme reaction; a standard buffer solution (20 mM Na-acetate) for the dextransucrase reaction, and *dongchimi-kimchi* liquid to examine the real effect of calcium salts in *kimchi*. As shown in Fig. 2, the dextransucrase activities increased in both solutions with the addition of $CaCl_2$ or $CaCO_3$, and $CaCl_2$ had a higher activation effect than $CaCO_3$ in both cases. In the sodium acetate buffer, the addition of 0.1% and 0.5% $CaCl_2$ increased the dextransucrase activity by 10% and 60%, respectively, when compared with the control, whereas the addition of $CaCO_3$ increased the dextransucrase activity by 5% and 10%, respectively. When the dextransucrase activities were measured in the real *kimchi* solution, the addition of the calcium salts produced the same results as those obtained with the sodium acetate buffer solution (Fig. 2).

Effects of Calcium Salts on LAB Population

After adding the calcium salts [0.1% (w/v)], the *dongchimi kimchi* was fermented at 20°C for 2 days, then stored at 4°C, and the biochemical changes of the *kimchi* were monitored. As shown in Fig. 3, the initial pH of sample B containing $CaCl_2$ and sample C containing $CaCO_3$ was 7.2 and 6.0, respectively, which was higher than that of the control A (pH 5.2). During the 24-day fermentation period, the pH of the control, sample B, and sample C decreased to

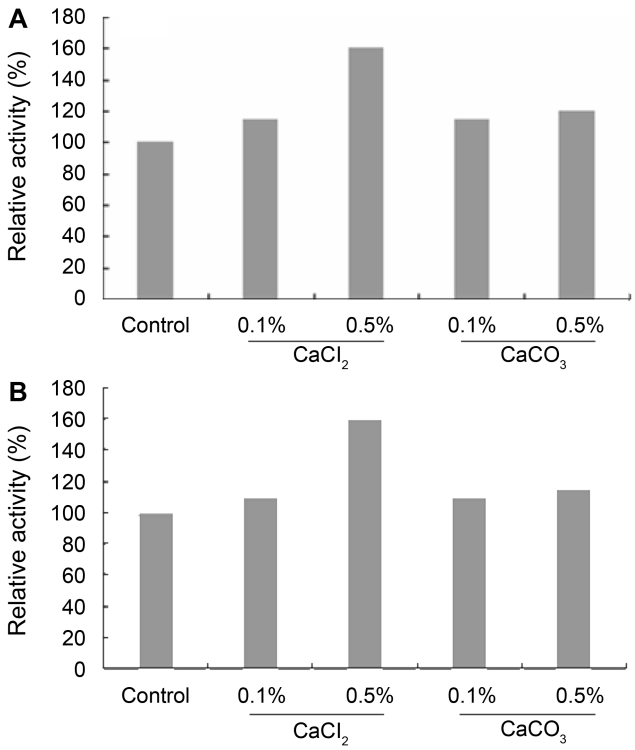

Fig. 2. Comparison of dextransucrase activity in buffer with sodium acetate and acetic acid; pH 5.2 (**A**) and *dongchimi-kimchi* juice (**B**) with addition of calcium salts.
The control indicates the dextransucrase activity without calcium salts.

4.1, 5.2, and 4.4, respectively, indicating that the calcium salts had a neutralizing effect in the acid solution of the *kimchi* from the beginning to the end of the fermentation. Moreover, whereas the total acidity of samples gradually increased during the fermentation period, the rate of increase for the samples with the calcium salts (B and C) was lower than that for the control (A). In particular, sample B with $CaCl_2$ had a lower total acidity value (0.15%) than the control (0.4%) and sample C (0.25%), which was probably because calcium chloride is more easily dissociated than calcium carbonate which is less soluble in water. When the same experiment was conducted with a 0.5% addition of calcium salts, the salts were not dissolved completely and made the *kimchi* turbid.

Fig. 4 shows the changes in the viable counts of LAB and the *Leuconostoc* genus during the spontaneous fermentation of the *dongchimi-kimchi* after the addition of the two calcium salts. The total LAB and leuconostocs counts for the three groups (initial counts; 10^3 CFU/ml) increased at a similar rate for about 7 days, and then decreased until the end of the storage period (24 days). *Leuconostoc* spp. were observed as one of the major isolates among the LAB in the *kimchi* samples. Although no significant changes in the viable bacterial counts were observed between the three groups, the total LAB counts for sample C with 0.1% $CaCO_3$ were much higher (about

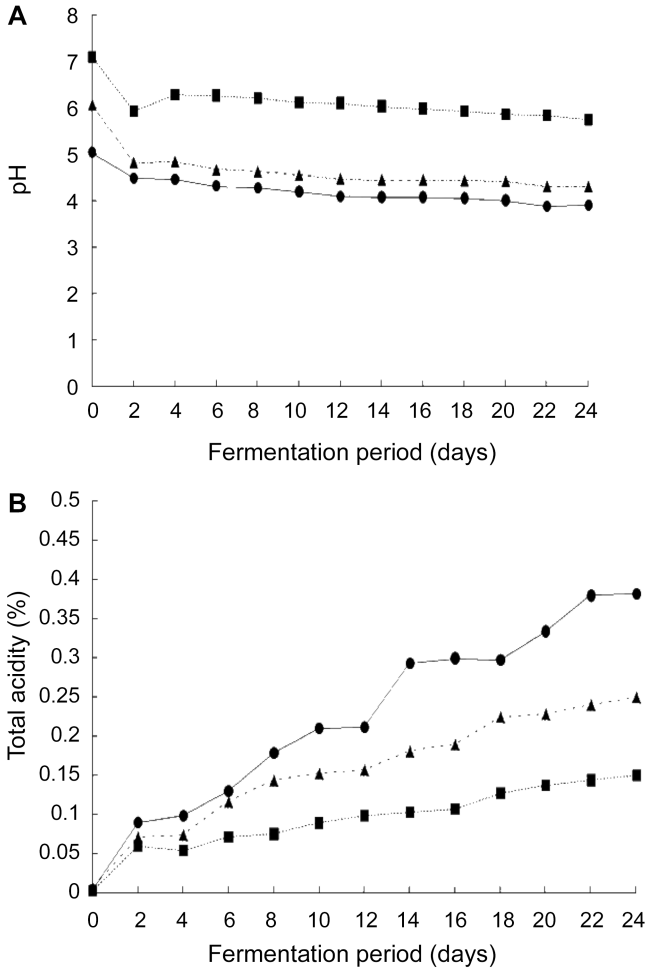

Fig. 3. Changes of pH (**A**) and titratable acidity (**B**) in *dongchimi kimchi* during refrigerated storage for 24 days with addition of calcium salts.
Symbols: ●, Control without calcium salt; ■, Sample with 0.1% (w/v) $CaCl_2$; ▲, Sample with 0.1% (w/v) $CaCO_3$.

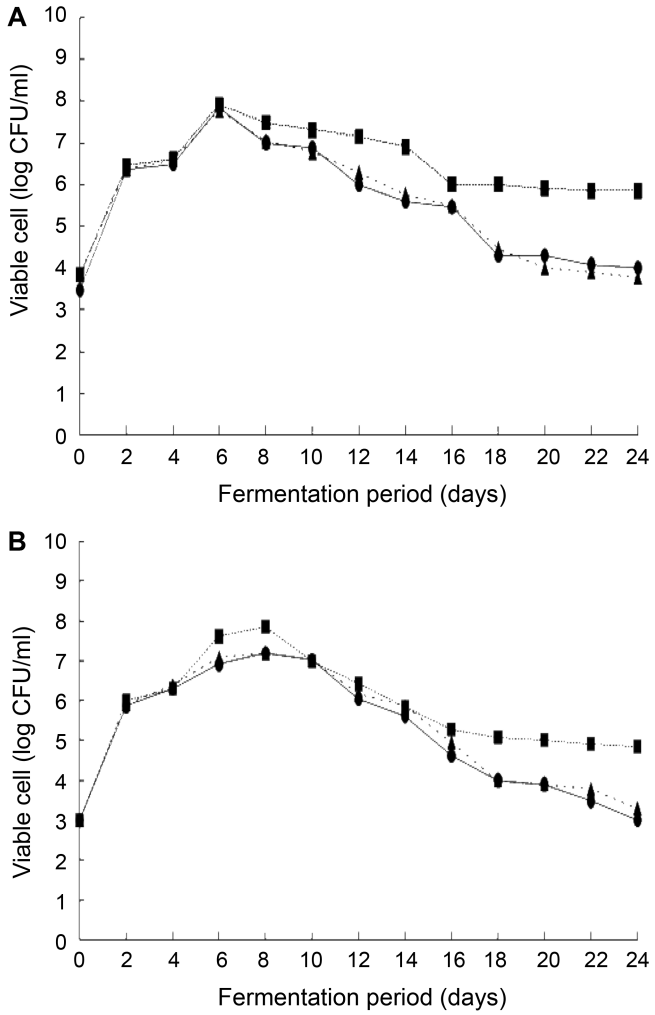

Fig. 4. Changes of total lactic acid bacterial and leuconostoc counts in *dongchimi-kimchi* with addition of calcium salts.
A. Total lactic acid bacteria. **B**. *Leuconostoc* genus. Symbols: ●, Control without calcium salt; ■, Sample with 0.1% (w/v) $CaCl_2$; ▲, Sample with 0.1% (w/v) $CaCO_3$.

10–100 fold) than those for the other two samples (A and B). Furthermore, sample C also exhibited the highest *Leuconostoc* counts until the end of the fermentation period.

Therefore, these results confirmed that calcium chloride, a well-known neutralizing agent [24, 25], extended the survival of LAB, including leuconostocs, under acidic *kimchi* conditions.

Effects of Calcium Salts on IMO Production

The glucosyl transfer reaction of dextransucrase is used for the synthesis of IMOs in *kimchi*. In previous work using the dextransucrase from *L. mesenteroides* NRRL B-512F, an equimolar addition of sucrose and maltose provided the best conditions for a higher production of panose, the major component of IMOs [20]. Thus, 1% sucrose and 1% maltose were added to the *kimchi* with calcium salts during the preparation, and the changes in the sugar concentration in the *kimchi* during the fermentation were then analyzed (Fig. 5). As expected, an acceptor reaction of dextransucrase occurred, where the glucose residue was transferred from sucrose to maltose, resulting in panose as the major product of the acceptor reaction [8]. In all cases, the sucrose (1%) was rapidly consumed within 2 days, whereas only about half of the maltose (0.5%) was used as acceptor molecules. The oligosaccharide concentrations were highest after 4–7 days and these levels were maintained for the remainder of the fermentation at 4°C without any remarkable decomposition. When the concentration of panose was compared among the samples, samples A, B, and C reached their maximum level of 0.50% (on the 14th day), 0.70% (10th), and 0.65% (10th), respectively, and the two samples including the calcium salts maintained a higher concentration of panose throughout the fermentation.

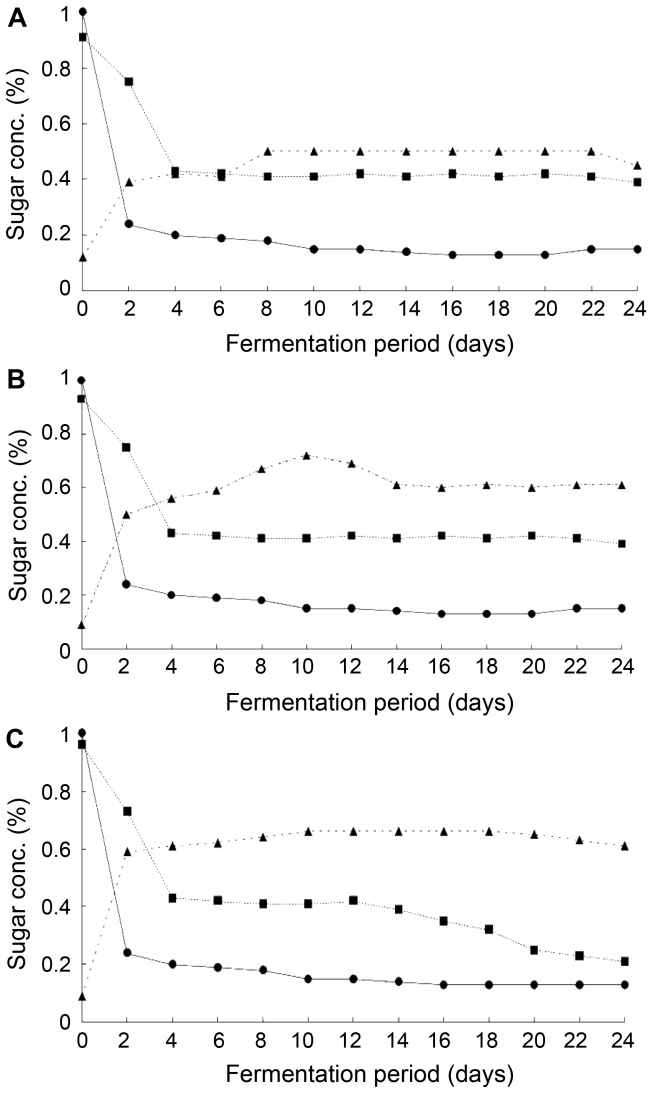

Fig. 5. Profiles of sugar changes in *dongchimi-kimchi* during refrigerated storage for 24 days with addition of calcium salts. **A**. Control without calcium salt; **B**. Sample with 0.1% (w/v) $CaCl_2$; **C**. Sample with 0.1% (w/v) $CaCO_3$. Symbols: ●, sucrose; ■, maltose; ▲, panose.

Therefore, the experimental results indicated that the calcium salts affected the *kimchi* in two ways; first, the calcium salts protected the LAB from acids by neutralization, allowing the LAB population to increase; and second, the leuconostocs were able to increase the amount of dextransucrase owing to the less harsh conditions after neutralization, while the calcium salts activated dextransucrase activity to facilitate the production of more IMOs from sucrose and maltose. Similarly, it has also been reported that calcium carbonate and calcium chloride in the growth medium increase the production of other enzymes, such as amylase [23] and fructosyltransferase [25].

Another interesting aspect of the experiments was the synthesis of calcium lactate. The addition of calcium salts to *kimchi* is known to result in the synthesis of calcium lactate, based on the combination of calcium ions and two lactic acids [4, 18]. As a result of this reaction, the total acidity levels and pH of the *kimchi* did not change significantly in samples B and C (Fig. 2). Calcium lactate is currently used as a representative calcium-fortifying supplement for osteoporosis patients, owing to its higher bioavailability to be absorbed in the intestine rather than free calcium ions [2, 9]. Although assaying the calcium lactate in *kimchi* liquid that contains both free calcium ions and lactic acid is not technically feasible at this moment, it is nonetheless reasonable that the calcium lactate synthesized may provide additional health benefits to the *kimchi* manufacturing process in addition to the increased LAB and synthesis of IMOs.

In conclusion, the proposed method of adding calcium salts during *kimchi* preparation was shown to enable the prolonged growth of LAB and overproduction of beneficial oligosaccharides in *kimchi* by the activation or overproduction of dextransucrase. Therefore, the application of this method will permit the development of new function-added lactate foods.

Acknowledgments

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