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Effect of Lactic Acid Bacteria on D- and L-Lactic Acid Contents of *Kimchi*

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Abstract The D-form of lactic acid is frequently detected in fermented foods, and an excessive dietary intake of D-lactic acid may induce metabolic stress in both infants and patients. This work was carried out to determine the prevailing microorganisms relevant to the accumulation of D-lactic acid in *kimchi*. *Leuconostoc* (*Leuc.*) *mesenteroides* and *Leuc. citreum* primarily synthesized D-lactate with a small quantity of L-form. *Leuc. gelidum* and *Leuc. inhae* evidenced patterns similar to this. *Lactobacillus* (*Lb.*) *plantarum* and *Lb. brevis* were shown to convert glucose into a balanced mixture of D-/L-lactic acid, whereas *Lb. casei* principally synthesized L-lactic acid and a very small quantity of D-lactic acid. When *kimchi* was incubated at 8 or 22°C, D-lactic acid was over-produced than L-form. *Leuconostoc* was determined as the primary producer between the initial to mid-phase of fermentation and *Lb. plantarum* or *Lb. brevis* seemed to boost D-lactic acid content during later stage of acid accumulation.

Keywords: D-lactic acid, *kimchi*, acidosis, lactic acid bacteria, leuconostocs

Introduction

D- and L-Lactic acids are optical isomers, which are generated and metabolized into pyruvate by the enzyme lactate dehydrogenase (LDH). The predominant form of lactic acid normally present in fruits, human blood, and other vertebrates is L-lactic acid, which is derived from pyruvate by L-LDH. D-Lactic acid is converted primarily by D-LDH from pyruvate, and infrequently by racemase from L-lactic acid. The presence of D-lactic acid in fermented foods and the dietary ingestion of lactic acid bacteria result in no adverse effects in the vast majority of the adult population. Nevertheless, it has been reported that after the consumption of certain foods, D-lactic acid accumulates within the blood of patients that suffer from short-bowel syndrome or intestinal failure, culminating in D-lactic acidosis and encephalopathy (1-4). Furthermore, newborn infants may not be able completely to metabolize the D-lactic acid ingested or generated internally by intestinal microorganisms, due to liver immaturity (3, 5). Thus, food products containing these ingredients are not recommended for infants and young children up to 3 years of age (6).

Humans ingest D-lactic acid via the consumption of milk or fermented vegetables, including pickles, sauerkraut, *kimchi*, and yogurt, all of which harbor both D- and L-lactic acids. D- and L-Lactic acid contents were analyzed in those foods (7-9). Otherwise, it was suggested that the colonizing intestinal lactobacilli producing D-lactic acid were the main factor in the pathogenesis (10, 11). *Kimchi* is a Korean traditional fermented dish, with a sour, hot, salty, and characteristically carbonic taste, which is the result of lactic acid fermentation in the vegetables. In our previous study (9), we determined that significant quantities of D-lactic acid existed in all types of *kimchi* tested, regardless of the producers and raw materials used in its formulation. Considering the substantial amount of *kimchi* consumed in Korea alone (total 500 million USD in Korea in 2004), the possible effects of D-lactic acid intake via *kimchi* should not be overlooked.

In lactic acid bacteria (LAB), the presence of one type of LDH or another varies among species. In *Lactobacillus* (*Lb.*) *plantarum*, genes for an L-LDH and a D-LDH have been characterized and mutants have been generated (12). With regard to the LAB inherent to *kimchi*, an understanding of their roles in the accumulation of D- and L-lactic acid is clearly necessary. Thus, in this study, we have determined the profiles of D-/L-lactic acid synthesis of the primary LAB contained in *kimchi*, and investigated their D-lactic acid production levels during *kimchi* fermentation, in an effort to determine the prevailing microorganisms relevant to the accumulation of D-lactic acid in *kimchi*.

Materials and Methods

Strains and growth conditions The type strains used in this study were *Leuconostoc* (*Leuc.*) *mesenteroides* KCTC 3719, *Leuc. gelidum* KCTC 3527, *Leuc. inhae* KCTC 3774, *Pediococcus* (*Ped.*) *pentosaceus* KCCM 11729, and *Enterococcus* (*Ent.*) *faecalis* KCCM 11902. The other strains utilized were *Leuc. citreum*, *Lb. plantarum*, *Lb. brevis*, *Lb. sake*, and these strains were isolated from *kimchi* and identified via 16s rRNA analysis in our research group. For the analyses of D-/L-lactic acid synthesis, type cultures were inoculated in MRS

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fermentation medium (Difco Laboratories, Detroit, MI, USA) for 24 hr at optimum temperature under static conditions. The gene sequences involved in the D- and L-lactate fermentation of LAB in *kimchi* were obtained using the GeneBank genomic database, and were analyzed with regard to their possible roles in the accumulation of D- and L-lactic acid content.

The growth of lactic acid bacteria in *kimchi* was expressed in colony-forming units (CFU) per mL of liquid. In order to determine the viable cell count of the leuconostocs, we utilized NLS agar medium containing novobiocin and vancomycin (13), and for the total LAB cell counts, we utilized Lactobacilli MRS medium (Difco).

Fermentation of *kimchi* (*Dongchimi-kimchi*) A whole radish (80 g) was first cut into 4 pieces. After washing and peeling the outer layer, the pieces were mixed with salt (40 g) in a plastic jar, and incubated for 12 hr at 20°C, until the pieces became soft. The salted radish and extract solution were then mixed with crushed garlic (10 g), ginger (3 g), and chopped green onions (20 g). The jar was then filled with 4 L of drinking water and sealed tightly with a plastic lid. The fermentation temperature was maintained for 14 days at 8 and 22°C. The liquid samples were then decanted, centrifuged at 10,000×g, and analyzed immediately after filtration using a 0.45 μm pore size filter.

HPLC quantitation The analysis method of Okubo *et al.* (14) was employed in the quantitation of D- and L-lactic acids with some modifications. The normal phase HPLC system consisted of a YOUNG-LIN M930 Solvent Delivery pump (Younglin, Seoul, Korea) with a YOUNG-LIN M720 Absorbance Detector (Yonglin) and a 8.0×50 mm Shodex ORpac CRX853 column with a CRX-G column (Showa Denco, Tokyo, Japan). Lithium salts of racemic D- and L-lactic acids were provided by Sigma (St. Louis, MO, USA), and stock standard solutions were constructed at 20.0 mM in distilled water.

The mobile phase was 1.0 mM $CuSO_4$ in H_2O. The flow rate of the mobile phase was 1 mL/min, and the temperature of the column was maintained at room temperature. The analytes were monitored at 250 nm using a UV spectrophotometric detector. The concentration of each analyte was determined from the ratio of its peak area against that of the working standard solution.

On the basis of the chromatograms of D- and L-lactic acids for standards and *kimchi*, the retention times of D- and L-lactic acid were determined to be 11.43 and 8.86 min, respectively. When standard plots were constructed using standard solution, linear lines were obtained between 0.63 and 10.00 mM for D- and L-lactic acids.

Results and Discussion

D-/L- Lactic acid profiles of LAB in *kimchi* Strains of LAB isolated from *kimchi* include the following: *Leuc. mesenteroides, Leuc. citreum, Leuc. gelidum, Leuc. inhae, Lb. plantarum, Lb. fermentum, Lb. brevis, Lb. casei, Ped. pentosaceus,* and *Ent. faecalis* (15-17). They were inoculated in MRS medium and cultured for 24 hr at optimum pH. After converting glucose into lactic acid, the D-/L-lactic acid contents in these samples were analyzed via HPLC, and some of the generated chromatograms are shown in Fig. 1. As is shown in the figure, *Leuc. mesenteroides* and *Lb. plantarum* produced both D- and L-lactic acids in different ratios, and they also secreted acetic acid to some degree. The D-/L-lactic contents, and their ratio in all microflora tested in this experiment are shown in Table 1. All tested leuconostocs synthesized D-lactic acid as a primary form (58-91 mM) as compared to the L-form (17-20 mM) and the ratio of D-/L-lactic acid was approximately 5:1 (Table 1). However, the lactobacilli evidenced two distinct spectrums; *Lb. plantarum, Lb. brevis,* and *Lb. fermentum* generated comparable quantities of D-/L-forms, but *Lb. casei* generated mainly the L-form. The total quantities of lactic acids (D+L) fermented by the lactobacilli were between 120-200 mM, values that were superior to those of the leuconostocs (77-108 mM). This was attributed to their different lactic acid fermentation patterns; specifically, the hetero-type for the leuconostocs vs. the homo-type for the lactobacilli. *Ped. pentosaceus* and *Ent. faecalis* also synthesized D-lactic acids at 43 vs. 0 mM and L-lactic acids at 114 vs. 31 mM, respectively. When the levels of D-lactic acid contents regardless of L-form were compared as shown in Table 1, the most abundant D-lactic acid producers in *kimchi* were found to be *Leuconostoc* spp., *Lb. plantarum,* and *Lb. brevis.*

D-/L-Lactic acid contents during *kimchi* fermentation Using the information obtained in the above

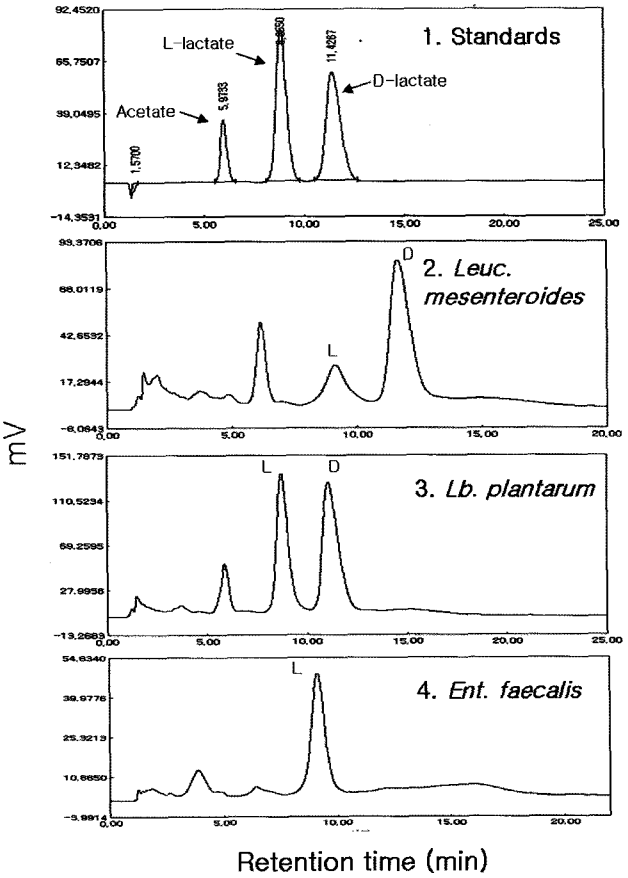

Fig. 1. Chromatograms of D-/L-lactic acid in LAB culture broth.

Table 1. D-/L-Lactic acid contents of LAB culture broth

Strains	Optical density (550 nm)	D-Lactic acid (mM)	L-Lactic acid (mM)	Total lactic acids (D+L) (mM)	Ratio (D:L)
Leuconostoc mesenteroides	2.336	75.28	19.68	94.96	80:20
Leuc. citreum	2.372	85.00	16.87	101.87	83:17
Leuc. gelidum	2.564	91.00	17.29	108.29	84:16
Leuc. inhae	1.016	57.91	19.03	76.94	75:25
Lactobacillus plantarum	4.784	114.13	81.74	195.87	58:42
Lb. brevis	4.688	115.47	84.77	200.24	58:42
Lb. fermentum	2.856	48.59	72.07	120.66	40:60
Lb. casei	6.376	8.20	185.36	193.56	4:96
Pediococcus pentosaceus	4.938	43.30	114.22	157.52	28:72
Enterococcus faecalis	0.716	ND[1)]	30.78	30.78	0:100

[1)]ND; not detected.

experiments, we attempted to determine the relationship between microbial population changes and the D-/L-lactic acid contents observed during *kimchi* fermentation (Fig. 2 and 3). *Dongchimi-kimchi* was selected among several types of *kimchi,* due primarily to the fact that this type of *kimchi* is produced via liquid-type fermentation; this allowed for significantly improved simplicity and accuracy with regard to control and analysis. As is shown in Fig. 2, when the *dongchimi-kimchi* was prepared and incubated at 8°C, the viable total LAB cell counts gradually increased to 1×10^9 during 9 days of fermentation, and decreased slightly thereafter. The quantity of leuconostoc strains followed the same pattern, with total cell counts reaching a level of 1×10^8, but evidencing a precipitous drop after 9 days. This was attributed to the sensitivity of *Leuconostoc* spp. to acidic medium conditions, and their growth was inhibited at pH values below 4.0. However, other LAB represented by lactobacilli were resistant to these conditions (18). The levels of D-/L-lactic acid increased gradually with the growth of LAB cells for 6 days, and D-lactic acid content increased till 11th day, reaching a level of 10.5 mM, at which the concentration of L-lactic acid was 6 mM. When the *dongchimi-kimchi* was incubated at higher temperatures (22°C) as is shown in Fig. 3, the viable cell counts of the total LAB and leuconostoc strains rapidly increased for 3 days, reaching levels of 1×10^9 and 3×10^8, respectively, with a slow decline occurring thereafter. The level of D-lactic acid (14 mM) exceeded

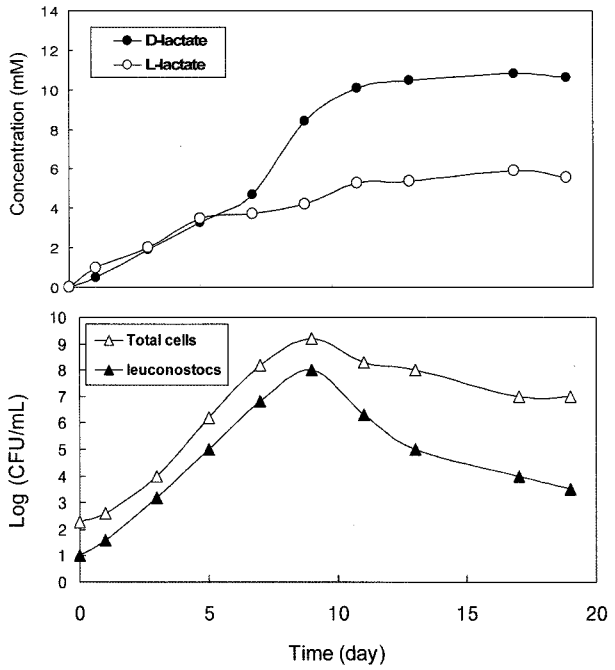

Fig. 2. Changes in lactic acid concentration during *kimchi* fermentation at 8°C.

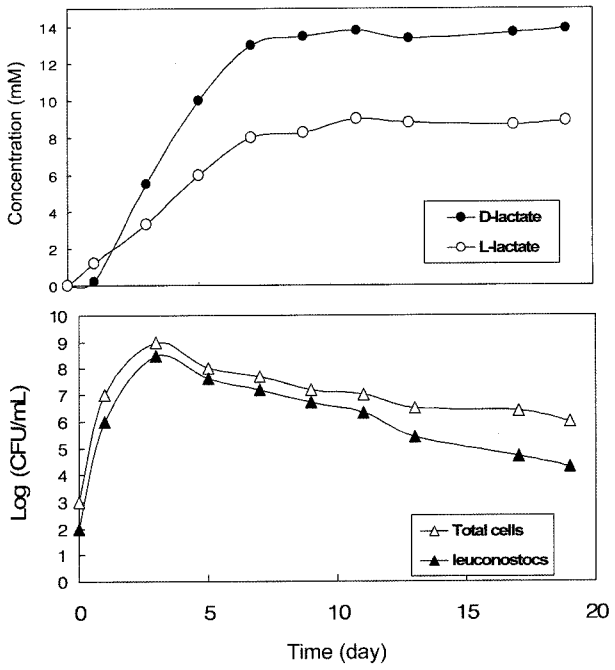

Fig. 3. Changes in lactic acid concentration during *kimchi* fermentation at 22°C.

that of L-lactic acid (8 mM) throughout the entire experimental period.

The classical identification of bacterial isolates from *kimchi* revealed that *Leuc. mesenteroides* and *Lb. plantarum* were the predominant species in *kimchi* (15, 19). *Leuconostoc* spp., a hetero-fermentative type bacterial species, has been demonstrated to be the major bacterial population in *kimchi* from the initial to the middle stage of fermentation, and *Lb. plantarum*, a homo-fermentative type bacterial species, becomes the dominant species afterward, due to its acid-tolerance in the middle stage (20). *Ped. pentosaceus* and *Ent. faecalis* were detected on rare occasions in *kimchi* (15), and their role in D-lactic acid synthesis was generally regarded as negligible. Accordingly, *Leuconostoc* spp., which overproduce D-lactate, have been noticed to lead D-lactic acid synthesis during the initial stages of fermentation, and *Lactobacillus* spp., also other D-lactate producers, are considered to increase D-lactate levels during the stationary phase of cell growth. Particularly *Lb. plantarum* is regarded as the key strain among many lactobacilli species along with the period, since it is the most acid tolerant. This strain was determined to be a profound producer of D-lactate in this study.

Lactate, or 2-hydroxypropanoate, was discovered in 1780 by a Swedish chemist, Scheele, who initially isolated it from sour milk (21). Lactate is the simplest of the hydroxycarboxylic acids, and exists as 2 stereoisomers, or enantiomers, as the consequence of its asymmetric C2 atom. Lactate has a p*K* of 3.86, and dissociates freely at physiological pH, yielding a lactate ion:lactic acid ratio of 3,000:1. Normal serum lactate concentration is 1-2 mM, and is considered to be entirely L-lactate, as the lactate generated in mammalian cells is nearly all in this form, with the exception of the D-lactate which is formed in nanomolar concentrations via the methylglyoxal pathway. Exogenous sources of D- and L-lactate include fermented foods based on milk or vegetables, as well as microbial fermentation within the colon. Initially, D-lactic acidosis has been thought to be the result of the over-ingestion of L-lactic acid through meals, or of overproduction by intestinal bacteria (4). Recently, mammals (including humans) have been shown to harbor the D-LDH gene, and appear to metabolize D-lactate via the secretion of D-LDH within the liver (22). This finding indicates that the development of D-lactic acidosis requires an impaired ability to metabolize D-lactic acid, in addition to excessive intake. Nonetheless, although healthy adults tend to exhibit a sufficient ability for D-lactic acid metabolization after intake, excessive dietary absorption may induce metabolic stress in infants and patients. In particular, intake of D-lactic acid with *kimchi* can not be overlooked considering its size of consumption in Korea. In this study, the profiles of D-/L-lactic acid synthesis of the principal LAB in *kimchi* were analyzed, and the relationship between D-/L-lactic acid contents and microbial populations was investigated during fermentation. When the *kimchi* was incubated at 8 or 22°C, far more D-lactic acid was produced than the L-form. The results of this experiment also showed that the leuconostocs and *Lb. plantarum, Lb. brevis* stood out as the primary D-lactic acid-generating strains during *kimchi* fermentation.

In LAB, both lactate isomers, L-lactate and D-lactate, can be formed via the action of two isomer-specific enzymes. L-LDHs are a group of enzymes that share homology among bacteria, plants and animals, whereas D-lactate is generated by a group of enzymes (D-LDH) that are structurally unrelated to L-LDH showing homology with short-chain fatty acid dehydrogenases (D-2-ketoacid dehydrogenases) (23). In LAB, the presence of one type of LDH or another varies between species. In *Lb. plantarum,* genes for an L-LDH and a D-LDH have been characterized (12). In *Lb. sakei*, the disruption of the single gene (*ldhL*) coding for L-LDH resulted in an abolition of the formation of both lactate isomers, thereby suggesting the presence of racemase activity responsible for the conversion of L-lactate into D-lactate (24).

D-Hydroxyisocaproate dehydrogenase (HicDH) is another enzyme that is responsible for D-lactate synthesis. *Lb. casei* is a lactic acid bacterium relevant to the production of fermented dairy products, and it has been employed for the biosynthesis of lactic acid via the fermentation of a host of substrates including whey ultrafiltrates or fruit juices (25, 26). In this species, there is no gene encoding D-LDH for D-lactate, but this strain was shown to convert glucose into a mixture of D-/L-lactic acid in our experiment, with a 4:96 mM ratio. This result may indicate that some of the L-lactate was converted to D-form by HicDH, as the gene coding for HicDH has been previously detected in this strain (27).

Leuconostoc spp. in *kimchi* generate a variety of constituents, including lactic acid, acetic acid, alcohol, CO_2, mannitol, and dextran, all of which are associated with the taste of *kimchi.* The numbers of these bacteria have been reported to be highest during the initial to middle stages of fermentation. They have been predicted to synthesize only D-lactic acid from glucose, as they harbor a single D-LDH gene, but no L-LDH genes (28, 29). Interestingly, our results indicate that 4 leuconostoc species converted glucose not only into D-lactic acid, but also into L-lactic acid, at a ratio of 5:1 (D:L). In leuconostocs, lactate was formed primarily from pyruvate in the D-form by D-LDH, and citrate has been shown to be another substrate for the production of D-lactic acid (30). Thus, the D-lactate detected in our leuconostoc cultures can be generated both via citrate and glucose metabolism, and exchanges between citrate and lactate favored citrate metabolism. The majority of LAB execute malo-lactic fermentation with the formation of L-lactate and CO_2 from malate, via the action of the malo-lactic enzyme. Recently, two open reading frames encoding for an NAD-dependent malic enzyme were detected within the upstream region of the citrate utilization locus in *Leuc. mesenteroides* (31). Therefore, the production of L-lactic acid by leuconostocs in our experiment might be derived from the enzymes inherent to malate utilization. Further enzymatic and genetic studies will be required to ascertain the veracity of this supposition.

In our study, we elucidated the D-/L-lactate contents in *kimchi* in terms of LAB population. However, in addition to this, we need to investigate another factor that may affect the D-/L-lactate profile. That is the physiological features of microorganisms regarding pH changes. The ratio of the lactate isomers can change at different times in

cultures grown without pH control. *Ped. pentosaceus* and homofermentative lactobacilli evidence high proportions of L-lactic acid early in the growth cycle, and form D-lactic acid in cases in which the medium has a low pH value (28, 32). It has also been reported that the optimal pH of D-LDH changes according to the concentration of pyruvate. However, temperature also affected the D-/L-lactate profile; when the D-lactic acid contents were compared at two different temperature conditions; low temperatures resulted in slightly less D-lactate content, as opposed to what was seen in the case of high temperature. The same trend was reported in the experiment involving fermented milk using *Lb. acidophilus* NCFM (8). Attempts have been made to construct LAB strains in which only the L isomer is produced, which may prove useful for the acquisition of non-racemic lactates via fermentation, suitable both for pharmaceutical biodegradable polymer synthesis and food applications (33-35). For *kimchi*, the construction of *Leuconostoc* spp. with the L-LDH single gene instead of D-LDH gene will allow consumers to overcome the risk of acidosis.

Acknowledgments

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