J Appl Biol Chem (2019) 62(4), 441–446
https://doi.org/10.3839/jabc.2019.060

Online ISSN 2234-7941
Print ISSN 1976-0442

Article: Food Science

Origin of lactic acid bacteria in *mulkimchi* fermentation

Chung Eun Hwang[1] · Md. Azizul Haque[2] · Su Young Hong[3] · Su Cheol Kim[1] · Kye Man Cho[1]

Received: 5 September 2019 / Accepted: 22 November 2019 / Published Online: 31 December 2019

Abstract The assortment of endophytic lactic acid bacteria (LAB) in *kimchi* derives from its raw vegetables, which include Chinese cabbage, radish, welsh onion, onion, garlic, red pepper, and ginger. These vegetables were examined during *mulkimchi* fermentation using gene-specific multiplex polymerase chain reaction and 16S ribosomal RNA sequence analysis. Sixteen species from five LAB genera (*Leuconostoc*, *Lactobacillus*, *Lactococcus*, *Pediococcus*, and *Weissella*) appeared in the raw *kimchi* materials. Interestingly, nine LAB species were identified in *mulkimchi* on fermentation day 0 as follows: *Leuconostoc carnosum*, *Leuconostoc citreum*, *Leuconostoc gelidum*, *Leuconostoc inhae*, *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, *Lactobacillus sakei*, *Lactococcus lactis*, and *Weissella confusa*. Seven additional LAB species were present in *mulkimchi* at fermentation day 9 as follows: *Leuconostoc gasicomitatum*, *Leuconostoc kimchii*, *Lactobacillus brevis*, *Lactobacillus curvatus*, *Lactobacillus pentosus*, *Pediococcus pentosaceus*, and *Weissella koreensis*. These species corresponded completely with the LAB in *kimchi* vegetables. *Wei. confusa* was the predominant LAB during early fermentation (pH 6.20 to 4.98 and acidity 0.20 to 0.64%), while *Lac. sakei*, *Lac. plantarum*, and *Wei. koreensis* became dominant later in fermentation (pH 4.98 to 3.88 and acidity 0.64 to 1.26%). These results collectively demonstrate that the LAB involved in *mulkimchi* fermentation originates from the raw vegetables examined.

Keywords Endophytic · *Kimchi*-vegetables · Lactic acid bacteria · *Mulkimchi* · Multiplex-PCR · Origin

Kye Man Cho (✉)
E-mail: kmcho@gntech.ac.kr

[1]Department of Food Science, Gyeongnam National University of Science and Technology, Jinju 52725, Republic of Korea

[2]Department of Biochemistry & Molecular Biology, Hajee Mohammad Danesh Science & Technology University, Dinajpur 5200, Bangladesh

[3]Food Science Research Institute, SBT Business Division, Kolmar BNH Inc., Sejong 30003, Republic of Korea

Introduction

Kimchi is a Korean traditional fermented vegetable that is typically composed of the following components: Chinese cabbage, radish, green onion, red pepper powder, garlic, ginger, and fermented sea food. Because failures in the fermentation of *kimchi* occur often, efforts are being made to control the fermentation process. Specifically, the main bacterial species that are actively involved in the raw materials (vegetables) and in the fermentation process need to be identified. Most of the lactic acid bacteria (LAB) was previously isolated and identified from fermented *kimchi* and includes *Leuconostoc mesenteroides, Leuconostoc citreum, Leuconostoc gasicomitatum, Lactobacillus brevis, Lactobacillus curvtaus, Lactobacillus plantarum, Lactobacillus sakei, Lactococcus lactis, Pediococcus pentosaceus,* and *Weissella koreensis* [1-5]. A soup-based Korean food known as *mulkimchi* is prepared from fermented *kimchi* and fresh red peppers, which can be acidified and softened more rapidly by LAB than can other *kimchi*.

Fermentation by LAB can reduce the microbial contamination and inhibit the growth of foodborne pathogens in fermented foods [6]. Several studies have reported the role of LAB in different phases of *kimchi* fermentation [4,7,8]. Previously, Cagno et al. [9] emphasized the role of LAB involvement in the spontaneous fermentation of fruits and vegetables. However, the origin of the fermenting LAB in *kimchi* remains unclear [10-12].

We previously designed sets of special multiplex PCR primers for the rapid detection of LAB during *baechukimchi* fermentation [3]. In this study, we identified the endophytic LAB species of raw

kimchi materials (vegetables), including Chinese cabbage, radish, welsh onion, onion, garlic, red pepper, and ginger as well as the LAB species of *mulkimchi* prepared from all of these vegetable mixtures, using multiplex polymerase chain reaction (PCR) and 16S ribosomal RNA (rRNA) sequence analysis.

Materials and methods

Bacterial strains and growth medium

The LAB were grown overnight at 30 °C in Lactobacilli MRS broth/agar (MRSB/MRSA, Difco, Becton Dickinson Co., Sparks, MD, USA). *Escherichia coli* DH5α and recombinant *E. coli* cells were cultured in LB (Becton Dickinson Co.) media containing the appropriate antibiotics (ampicillin, 50 μg/mL) at 37 °C.

Preparation of raw *kimchi* materials and *mulkimchi*

Seven raw materials, including Chinese cabbage (CC), young radish (YR), welsh onion (WOn), onion (On), garlic (Ga), red pepper (RPe), and ginger (Gi), were obtained from Gyeongnam Agricultural Research and Extension Services in the Jinju area of Korea and transferred to a sterile bag for same-day analysis. The root and leaf surfaces of CC, YR, WOn, On, Ga, RPe, and Gi were disinfected with 1% sodium hypochlorite for 10 min. The external portion of the roots (approximately 0.5 cm from the margin) was removed with a sterile blade, and the root tissue was triturated in a sterile porcelain mortar in sterile 10 mM phosphate buffer (pH 7.2) [13]. One hundred microliters of samples were spread-plated on MRS agar and incubated at 30 °C for 48 h. All raw materials from *kimchi* sources were washed with tap water and sliced to prepare *mulkimchi* as previously described [14]. Prepared *mulkimchi* samples were placed in a capped 1,000 mL glass jar (1,800 mL) and fermented at 25 °C for 6 h and then at 8 °C for 30 days. One hundred grams [Solutions of *mulkimchi* : solids of *mulkimchi* =4 (80 g):1 (20 g)] of each *mulkimchi* sample (was collected at 0, 3, 6, 9, 12, 18, 24, and 30 days of fermentation.

pH and acidity

The pH and acidity of each blended *mulkimchi* sample was measured according to Cho et al. [14]. Briefly, one hundred grams of each *mulkimchi* sample was ground with a brand blender and after each blended sample was filtered to collect the fluid portion and the pH was measured with a pH meter (MP 220 pH meter, London, UK). To estimate the acidity, 20 mL of *mulkimchi* filtrate was titrated with 0.1 N NaOH at pH 8.2±0.2.

Total viable LAB cells and isolation of LAB from *mulkimchi* samples

The total LAB cell numbers in the *mulkimchi* samples were determined on MRS agar plates, and colony counts were carried out according to Cho et al. [14]. In short, 1 mL of each blended *mulkimchi* sample was diluted in 9 mL of sterile 0.85% NaCl water. Aliquots of 1 mL were serially diluted ten-fold using the buffer and 100 μL samples were spread-plated on MRS and incubated at 28 °C for 48 h.

Extraction of total DNA from LAB

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

Primers, multiplex PCR reaction, and agarose gel electrophoresis

Ten species-specific primers were designed for the PCR application of *Leu. carnosum, Leu. mesenteroides, Lac. brevis*, *Lac. plantarum*, *Lac. pentosus*, *Lac. sakei*, *Lac. lactis*, *Ped. pentosaceus*, *Wei. confusa*, and *Wei. koreensis*, and multiplex PCR was performed as previously described [3]. The PCR reaction mixture (50 μL) consisted of 5 μL of template DNA, 2 μL each of mixture of ten primer sets (20 pmol, Bioneer Co., Daejeon, Korea), 5 μL of reaction buffer with 2.5 mM $MgCl_2$, 5 μL of 2.5 mM deoxynucleoside triphophate, 1 μL of Super-Therm DNA polymerase (5.0 unit, JMR, Side Cup, Kent, UK), and 30 μL of sterile water. Multiplex PCR was carried out through 40 cycles following a preheat step at 95 °C for 5 min. Each cycle designed of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min, and extension at 72 °C for 2 min. The final step was followed by a last extension at 72 °C for 10 min. The PCR products were analyzed by agarose gel electrophoresis as described by Cho et al. [14]. Additionally, the unidentified LAB confirmed by 16S rRNA gene sequencing.

16S rRNA PCR and sequence analysis

LAB isolates that remained unidentified by multiplex PCR were subjected to further analysis via 16S rRNA gene sequencing. To amplify the 16S rRNA gene fragments, the following universal PCR primers were used: Forward, 5'-CGGAGAGTTTPATCC TPG-3'; reverse, 5'-TACGGCTACCTTPTTAGCGAC-3'. The 16S rRNA genes were amplified by PCR, and these sequences were analyzed as previously described [15].

Results and Discussion

Distribution of endophytic LAB from raw *kimchi* materials

This study was initiated to explore the endophytic LAB communities in raw *kimchi* materials (vegetables) and during *mulkimchi* fermentation periods using our previously developed multiplex PCR [3] and 16S rRNA sequence methods. When multiple target-bacteria were included in a reaction mixture containing ten primer-sets, corresponding amplicons of different sizes were observed. Each species-specific primer set produced a single PCR product with an expected product size that corresponded to the

species specificity of the primer set [3]. Instead of 16S rRNA, the multiplex PCR primer sets contained 10 different gene sequences, which successfully identified the LAB in the raw vegetables and in the *mulkimchi* samples examined in this study. Additionally, the 16S rRNA gene sequence primers identified LAB that were otherwise not identifiable using the gene-specific multiplex PCR assay.

The distribution of LAB was assessed in samples obtained from CC, Ra, WOn, On, Ga, RPe, and Gi plants. Total LAB cell numbers varied from 1.24 to 2.53 log cfu/g of the raw materials. A total of 515 colonies were isolated from the CC, Ra, WOn, On, Ga, RPe, and Gi roots and leaves. The CC root sample consisted of 92 isolates representing 12 species, which included *Leu. carnosum*, *Leu. citreum*, *Leu. gelidum*, *Leu. inhae*, *Leu. mesenteroides*, *Lac. brevis*, *Lac. plantarum*, *Lac. sakei*, *Lac. lactis*, *Ped. pentosaceus*, *Wei. confusa*, and *Wei. korenesis*. The CC leaf sample consisted of 88 isolates representing 10 species that corresponded to the CC root sample, not including *Leu. inhae*, *Lac. brevis*, and *Ped. pentosaceus*. Additionally, it consisted of one unique isolate-*Leu. kimchii*. The Ra root sample consisted of 45 isolates representing 13 species that corresponded to the CC root sample, not including *Leu. carnosum* and *Leu. gelidum*. However, the Ra leaf sample provided 40 isolates representing 9 species that corresponded to the CC root sample, not including *Leu. gelidum*, *Leu. inhae*, *Lac. brevis*, *Leu. kimchii*, and *Wei. koreensis*. Additionally, it consisted of one unique isolate-*Lac. curvatus*. The LAB from the Ga, Gi, On, RPe, and WOn plants consisted of 48, 44, 52, 50, and 56 isolates representing 8, 8, 9, 9, and 9 species, respectively. All plants consisted of *Leu. citreum*, *Leu. mesenteroides*, *Lac. plantarum*, *Lac. sakei*, and *Weu. confusa*. Importantly, *Lac. lactis* and *Wei. koreensis* were represented in all plant samples except Ga and Won, respectively. The distribution of *Wei. confusa* was dominant in all plant samples, estimated at 33.3-51.8% (Table 1).

Vegetables are naturally contaminated by eukaryotic (molds and yeasts) and prokaryotic (bacteria) organisms. The microbial community sizes and the relative species proportions on the vegetables vary in response to temperature variations, rainfall, insect- and mold-induced physical damage, and the use of insecticides and fungicides. Also, the microbial diversity of

Table 1 Distribution of endophytic lactic acid bacteria (ELAB) in each of the Chinese cabbage leaf and root, radish leave and root, welsh onion, onion, garlic, red pepper, and ginger samples

Genus Species	Distribution of ELAB in raw *kimchi* materials (%)*								
	Chinese cabbage		Young radish		Garlic [48]	Ginger [44]	Onion [52]	Red Pepper [50]	Welsh Onion [56]
	Leaf [88]†	Root [92]	Leaf [40]	Root [45]					
Leuconostoc									
Le. carnosum[1]	7.1	2.2	2.5					2.0	1.8
Le. citreum[2]	6.8	7.8	7.5	7.5	8.3	11.4	3.8	6.0	7.1
Le. gasicomitatum[2]				1.1		2.3			
Le. gelidum[2]	7.1	5.5			3.8				3.6
Le. inhae[2]	0	2.1		1.1			1.9		
Le. kimchii[2]	4.8			2.2				2.0	
Le. mesenteroides[1]	7.1	10.6	17.5	13.3	12.6	6.8	7.7	6.0	5.4
Lactobacillus									
Lb. brevis[1]		5.2		2.2	2.2		1.9		
Lb. curvatus[2]			2.5						1.8
Lb. pentosus[1]				2.2					
Lb. plantarum[1]	4.8	3.4	2.5	4.5	10.4	4.5	9.7	8.0	7.1
Lb. sakei[1]	7.1	8.3	5.0	4.5	14.6	9.1	7.7	10.0	8.9
Lactococcus									
Lc. lactis[1]	12.3	10.3	10.0	8.9		13.6	13.5	16.0	12.5
Pediococcus									
Pe. pentosaceus[1]		2.1	2.5	2.2					
Weissella									
We. confusa[1]	38.1	40.3	50.0	50.0	33.3	45.5	51.9	46.0	51.8
We. koreensis[1]	4.8	2.2		2.5	2.2	6.8	1.9	4.0	

[1]The specific LAB was identified by multiplex PCR method
[2]The specific LAB was identified by 16S rRNA sequencing analysis
*Percentage of microcosmic each of in raw kimchi materials
†Numbers in square brackets give the total number of the corresponding isolates in the samples

Table 2 Change of lactic acid bacteria (LAB) during *kimchi* fermentation at 8 °C for 30 days

Genus Species	Distribution of LAB in *kimchi* fermentation day (%)*							
	0 [96]†	3 [96]	6 [96]	9 [96]	12 [96]	18 [96]	24 [96]	30 [96]
Leuconostoc								
Le. carnosum[1]	2.1	3.1	2.1		2.1			
Le. citreum[2]	12.4	15.6	13.5	8.3	6.3	6.3	2.1	
Le. gasicomitatum[2]			3.1	8.3	12.6	16.7	17.7	5.2
Le. gelidum[2]	5.2	4.2			1.0			
Le. inhae[2]	2.1	1.0		2.1	2.1	2.1		
Le. kimchii[2]		1.0	2.1	2.1	3.1		2.1	
Le. mesenteroides[1]	4.2	8.3	12.6	14.6	9.4	8.3	2.1	
Lactobacillus								
Lb. brevis[1]				3.1	1.0			
Lb. curvatus[2]			1.0		1.0			
Lb. pentosus[1]				1.0	2.1	3.1		
Lb. plantarum[1]	3.1	4.2	5.2	7.3	8.3	5.2	10.4	12.6
Lb. sakei[1]	6.3	7.3	10.4	12.6	16.7	19.8	25.0	29.0
Lactococcus								
Lc. lactis[1]	9.4	9.4	6.3	2.1		2.1		
Pediococcus								
Pe. pentosaceus[1]				1.0				
Weissella								
We. confusa[1]	55.2	45.9	39.5	29.2	11.4	8.3	6.3	10.4
We. koreensis[1]			4.2	8.3	22.9	28.1	34.3	42.8

[1]The specific LAB was identified by multiplex PCR method
[2]The specific LAB was identified by 16S rRNA sequencing analysis
*Percentage of microcosmic each of in the periods of mulkimchi fermentation
†Numbers in square brackets give the total number of the corresponding isolates in the samples

vegetables can vary not only with raw materials, but also with environmental factors, such as growing area, soil, climate, season and harvest time etc. [13,15]. Vegetable-associated microorganisms may follow the different phases of flour preparation and are found in products made with flour [16]. *Leuconostoc*, *Lactobacillus*, *Pediococcus* and *Weissella* strains have frequently been observed in plant materials and fermented vegetables, particularly in the early phase of *kimchi* fermentation, and their populations throughout the fermentation period have been studied [1,2,4,5,17].

Change of LAB communities during the fermentation of *mulkimchi*

To explore these data further, samples of *mulkimchi* were examined during fermentation (0 to 30 days) to characterize its LAB population using a similar experimental approach (Table 2). *Leu. carnosum*, *Leu. citreum*, *Leu. gasicomitatum*, *Leu. gelidum*, *Leu. inhae*, *Leu. kimchi*, *Leu. mesenteroides*, *Lac. brevis*, *Lac. curvatus*, *Lac. pentosus*, *Lac. plantarum*, *Lac. sakei*, *Lac. lactis*, *Ped. pentosaceus*, *Wei. confusa*, and *Wei. Koreensis* were prevalent throughout the fermentation process. At fermentation day 0, the *mulkimchi* sample consisted of 9 species that included *Leu. carnosum*, *Leu. citreum*, *Leu. gelidum*, *Leu. inhae*, *Leu. mesenteroides*, *Lac. plantarum*, *Lac. sakei*, *Lac. lactis*, and *Wei. confusa*, which corresponded to the isolates represented in the raw vegetables. Importantly, the number of endophytic LAB species gradually increased up to fermentation day 12 (14 species, not including *Lac. lactis*, *Pde. pentosaceus*) and markedly decreased thereafter (5 species) until fermentation day 30. The distribution of endophytic LAB was dynamic during the fermentation periods examined. Specifically, the number of *Wei. confusa* isolates reached a maximum of 55.2% on day 0, dramatically decreased to 11.4% by day 12, and fluctuated thereafter to 10.4% by day 30. Conversely, the viable *Lac. sakei* cell numbers gradually increased from 6.3% (0 days) to 29.0% (30 days) by the end of fermentation. Interestingly, the *Lactobacillus* sp. (including *Lac. brevis*, *Lac. curvatus*, and *Lac. pentosus*) appeared with *Lac. plantarum* and *Lac. sakei* at fermentation day 12, while all *Lactobacillus* sp. (not including *Lac. curvatus*) appeared at fermentation day 9. The existence of the maximum number of *Lactobacillus* sp. on fermentation days 9 through 12 may increase the production of lactic acid in *mulkimchi*. On fermentation day 6, an increase in viable *Wei. koreensis* cell numbers was measured, reaching a maximum value of 42.8% by the 30th day. However, the number of LAB species, particularly *Leu. carnosum*, *Leu. gasicomitatum*,

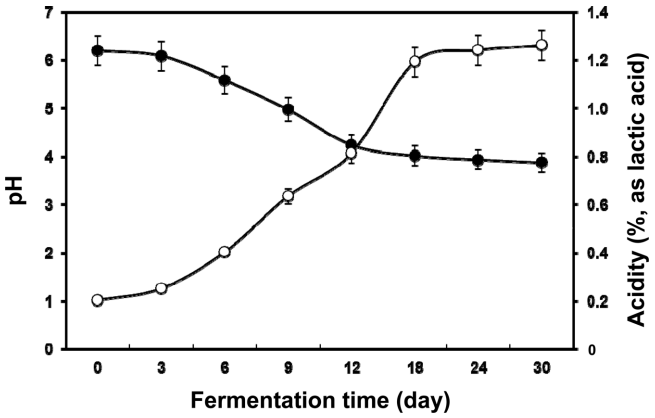

Fig. 1 Change of pH and acidity during *mulkimchi* fermentation at 8 °C for 30 days

Lac. brevis, *Lac. curvatus*, and *Ped. pentosus*, declined gradually after 18 days of fermentation.

A strong decrease in pH was observed (6.15 to 4.20) during the first 2 weeks (0 to12 days) and slowly decreased thereafter to 3.88 at fermentation 30 days. Conversely, the percentage of lactic acid dramatically increased up to 18 days (0.20 to 1.19), and it was slightly increased thereafter to 1.26 at fermentation day 30 (Fig. 1).

The results showed that the LAB identified in the raw materials of the *kimchi* samples also appeared in the *mulkimchi* samples examined on different days of fermentation. Importantly, *Lac. sakei* is an acidophilic and/or acid-producing bacterium, which is phylogenetically close to *Lac. plantarum.* Therefore, viable cells of *Lac. sakei* and *Lac. plantarum* proportionally increased with the increase in acidity during *mulkimchi* fermentation. Moreover, the growth of others LAB were inhibited. A possible explanation is that the lower pH (4.02) or higher acidity (1.19%) inhibited the growth of these LAB during the fermentation period. Therefore, the dynamic community of the LAB was observed throughout the fermentation period, which may have been associated with the total acidity produced by the acidophilic *Lac. plantarum* and *Lac. sakei* bacteria. Importantly, the lactic acid-producing *Lac. sakei* and *Lac. plantarum* bacteria were present throughout the fermentation process, indicating their prominent role in *mulkimchi* fermentation. A similar phenomenon with *Lac. sakei* and *Lac. plantarum* was previously observed during the fermentation of *pogikimchi* and *mulkimchi* [3,17]. Moreover, the proportion of *Leu. mesenteroides* and *Lac. sakei* increased gradually throughout the first week, but their prevalence decreased by the end of leek (vegetable) fermentation [18], which is partially consistent with the current study. Although a reduction in pH was observed, there was a gradual increase of *Wei. koreensis* throughout *mulkimchi* fermentation. This observation is consistent with the previously reported *pogikimchi* fermentation result [3]. These results indicate that the diversity of LAB isolates in raw materials is similar to that of *mulkimchi* during the early stage of fermentation. Therefore, these results were concluded that there is a flow of endophytic LAB into *mulkimchi* fermentation.

In conclusion, the endophytic LAB identified in raw vegetables corresponded to the LAB identified in *mulkimchi* samples examined on different days of fermentation. The predominance of *Wei. confusa* was observed in all raw vegetables examined and throughout the *mulkimchi* fermentation period. Specifically, the acid-producing *Lac. sakei* and *Lac. plantarum* bacteria became predominant as fermentation time and acidity increased during *mulkimchi* fermentation. These results suggest that the LAB involved in *kimchi* fermentation originate from its raw vegetables. Importantly, this study is the first to report the endophytic LAB community in *kimchi* raw vegetables using a multiplex PCR assay.

Acknowledgment This work was supported by Gyeongnam National University of Science and Technology Grant (2018 year), Republic of Korea.

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