PCR-DGGE analysis of population dynamics of lactic acid bacteria in kimchi by addition of bacteriocins

Miran Jeong, Jinseon Kim, Eunji Choi, Joon-soo Kim, and Wang June Kim*

Department of Food Science and Biotechnology, Dongguk University, Goyang 10326, Gyeonggi, Korea

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*Corresponding Author
Tel/Fax: +82-31-961-5144
E-mail: wjkim@dongguk.edu

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Abstract The effects of two crude bacteriocins (DF01 and K10) on lactic acid bacteria (LAB) communities and pH during kimchi fermentation were analyzed by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). Crude LAB bacteriocins, prepared by ammonium sulfate precipitation, were added at 5 AU/mL, and kimchi was incubated at 20°C for 7 days. The pH and titratable acidity of the kimchies were determined daily, and the amplified 16S rRNA products were analyzed by PCR-DGGE. The common and main LAB were *Weissella* spp., *Leuconostoc* spp., and *Lactobacillus* spp. from both control and bacteriocin-treated samples. Among them, *W. koreensis*, *W. confusa*, and *Lb. sakei* were the predominant microorganisms throughout the fermentation period. Some obligate and facultatively heterofermentative LAB were detected from the bacteriocin-treated samples. The pH of the kimchi samples treated with each bacteriocin was higher (ca. 0.8 unit) than that of the control at day 4 and 5.

Keywords: PCR-DGGE, kimchi, lactic acid bacteria, bacteriocin

Introduction

Kimchi is a generic term used to denote a group of lactic fermented vegetables in Korea and its health benefits are globally recognized (1). The microfloral changes that take place during kimchi fermentation have been extensively studied (2). According to the literature, kimchi fermentation is initiated by heterofermentative *Leuconostoc* spp. Being heterofermentative in nature, they produce less lactic acid than homofermentative lactic acid bacteria (LAB), but produce CO₂, ethanol, and acetic acid, which contribute to the desirable taste and flavor of kimchi. However, as fermentation continues, depending upon the temperature, the pH gradually decreases, less-aciduric *Leuconostoc* spp. fade away, and in the final stage, major over acid-producing LAB, such as *Lactobacillus plantarum* and *Lb. sakei*, become the predominant flora and kimchi loses its acceptable quality owing to low pH (3). In order to preserve kimchi, refrigeration is the most effective and commonly used method. The addition of a preservative can be an alternative solution. However, chemically synthesized preservatives should be carefully considered as they can have some side effects on human consumption. An appropriate solution could be the application of biocontrols or biopreservation. The use of a bacteriocinogenic food-grade LAB starter originated from lactic acid fermented foods or addition of their bacteriocins could be attractive methods for kimchi biopreservation (4,5). Nisin is undoubtedly the most extensively studied bacteriocin, and two studies on the effect

of nisin in kimchi fermentation have been conducted (6,7).

The use of bacteriocin-producing LAB as a kimchi starter has also been studied. Moon *et al.* (8) tried a bacteriocinogenic *Enterococcus* sp. starter in kimchi fermentation. The *in situ* production of bacteriocin in kimchi was effective, and the shelf life (the period at which the pH is maintained at 4.2, the most desirable pH for consumption) was extended by approximately 14 days. Chang and Chang (9) also reported that the shelf life of kimchi was extended by a bacteriocin-producing *Leuconostoc citreum* starter. However, none of the abovementioned studies investigated the population dynamic change of various LAB in kimchi fermentation upon the addition of bacteriocin.

Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) focusing on the V3 region has been used as an effective tool to analyze microbial diversity and identify microbial communities in numerous different areas. In comparison with the culture-dependent method, PCR-DGGE reflects the whole microbial community in the sample, because DNA is directly extracted from the samples and analysis is faster (10). Recently, PCR-DGGE was applied to study the population dynamics of LAB in different types of kimchi at different fermentation conditions (11–13). However, none of the reports has stated the effect of bacteriocin on population dynamics as well as pH change in kimchi fermentation at 20°C.

In this study, kimchies were fermented at 20°C to simulate extreme environments where refrigerators are not available, and thereafter fermentation has been accelerated. The population dynamics of LAB

in kimchi after addition of different bacteriocins were analyzed by PCR-DGGE. In addition, pH and titratable acidity were measured to see if bacteriocin had an inhibitory effect against pH reduction during fermentation.

Materials and Methods

Preparation of crude bacteriocin Bacteriocin-producing *Lb. brevis* DF01 (14) and *Pediococcus acidilactici* K10 (15,16) strains were previously isolated from fermented flat fish and kimchi, respectively, using *Lb. plantarum* as an indicator.

Each strain was inoculated (2%, v/v) in 300 mL of de Man, Rogosa and Sharpe (MRS) broth (Difco, Spark, MD, USA) and incubated at 37°C for 24 h. The cells were harvested by centrifugation (16,600×g, 15 min at 4°C, 1736R; LaboGene, Seoul, Korea) and the supernatants were filtered through a 0.22-μm pore size cellulose acetate filter (Toyo Roshi Co., Ltd., Tokyo, Japan). While stirred at 4°C, ammonium sulfate (Samchun Pure Chemical Co., Ltd., Pyeongtaek, Korea) was added to reach 50% saturation. The saturated solution was centrifuged (16,600×g, 15 min at 4°C) and the precipitate was dissolved in 5 mL of distilled and deionized water (ddH₂O). The solution was dialyzed against 2 L ddH₂O using a dialysis sac filter (benzoylated cellulose, 1,200 molecular weight cut-off, Sigma-Aldrich, St. Louis, MO, USA) for 24 h at 4°C. The dialysate was refiltered through the same syringe membrane filter and stored at 4°C before use. The arbitrary units (AU/mL) of each crude bacteriocin were determined by two-fold dilution method against *Lb. plantarum* NCDO 955 indicator (17).

Preparation of kimchi and fermentation Kimchi cabbages were purchased from a local supermarket in Seoul. Three kimchies (control, DF01 treated, and K10 treated) were prepared with a modified method as reported previously and each kimchi sample was prepared in triplicate (15). Crude bacteriocin solution was added separately to obtain a final concentration of 5 AU/g kimchi, and all the samples were incubated at 20°C for 7 days.

Culture and isolation of genomic DNA Four reference strains (*Lb. plantarum* NCDO 955, *Leu. mesenteroides* ATCC 10830, *Lb. brevis* DF01, and *Ped. acidilactici* K10) were used as DGGE references. They were incubated in 10 mL of MRS broth for 24 h at 37°C. The cells were pelleted by centrifugation (16,600×g, 15 min, 4°C), washed twice with 10 mL of sterile 0.1% peptone water (Difco), and the cell suspension was diluted with sterile 0.1% peptone water to a final inoculum level of 9 log CFU/mL.

Genomic DNAs of reference strains were isolated using a genomic DNA extraction kit (MiniBEST Genomic DNA kit for bacteria, TaKaRa, Seoul, Korea). The genomic DNAs were electrophoresed on 1% agarose gel and confirmed by gel documentation system (Bio-Rad Laboratories, Hercules, CA, USA).

pH, lactic acid, and remaining bacteriocin activity Every day, 70 g of kimchi was taken from each sample (DF01, K10, and control). The sample was homogenized (51BL32; Waring Commercial, Torrington, CT, USA) and 20 g of juice was used for DNA extraction, and remainder was used for pH, titratable acidity, and bacteriocin activity analyses. For the analysis of bacteriocin activity, kimchi juice was neutralized (pH 7.5) and filtered through a 0.22-μm pore size cellulose acetate filter (Toyo Roshi Co., Ltd.). Fifty μL of kimchi juice was loaded on *Lb. plantarum* NCDO 955 indicator lawn.

The kimchi juice from each sample was obtained by filtering the sample through a sterilized cheesecloth, and the pH was measured using a pH meter (ORION 3 STAR; Thermo Fisher Scientific, Waltham, MA, USA). Titratable acidity was determined by titration of the filtrate of the obtained samples at pH 8.3 with 0.1 N NaOH using a pH meter, and the ratio of lactic acid was converted with the used volume of NaOH. Remaining bacteriocin activity was tested by the agar well diffusion assay. Kimchi juice (100 μL) from each sample was placed in the wells (9.5 mm diameter) of the agar plate that was pre-inoculated with the indicator strains (10⁷ CFU/mL) to allow diffusion at 4°C for 12 h before incubation at 37°C. The remaining bacteriocin activity against indicator was indicated by formation of a clear zone around the well. Each sample was tested in triplicate.

Isolation of genomic DNA in kimchi and PCR-DGGE analysis The bead beating method (18) was modified and used to extract genomic DNA from kimchi samples. Briefly, 20 g of each kimchi sample was mixed with 180 mL of 0.85% NaCl solution. From them, 2 mL was taken and centrifuged (16,600×g, 2 min), and the supernatant was discarded. The cell resuspension solution (20 mL; Bioneer Co., Ltd., Daejeon, Korea) and sterile glass beads (0.1 mm, Next Advance, Averill Park, NY, USA) were mixed with the cell pellet and centrifuged (16,600×g, 5 min). DNA extraction was performed using a *PowerPrep*TM DNA extraction kit from Food and Feed (Kogen Biotech, Seoul, Korea) following the procedures described by the manufacturer.

Extracted genomic DNA from each kimchi sample and reference strains were amplified using a thermal cycler (T100; Bio-Rad Laboratories). Primers gc338F, 5'-CGCCCGCCGCGCGGCGGGCGGGGCGGGGCGA CGGGGGGACTCCTACGGGAGGCAGCAG-3' that contained GC clamp, and 518R, 5'-ATTACCGCGGCTGCTGG-3', were used to amplify the V3 region of the bacterial 16S rRNA gene (1). The confirmation primers 338F, 5'-ACTCCTACGGGAGGCAGCAG-3', and 518R were used. The PCR mixture (final volume, 50 μL) included 3 μL of the template DNA, each primer at a concentration of 0.1 μM, each deoxynucleoside triphosphate (dNTP) at a concentration of 10 mM, 5 μL of the 10× *Taq* reaction buffer, which is mixed with 25 mM MgCl₂, and 1.0 U of *Taq* polymerase (Solgent, Daejeon, Korea). After pre-denaturation at 95°C for 5 min, 45 cycles of PCR were performed; furthermore, denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 2 min were conducted. The cycling was concluded with a final extension step at 72°C for 10 min. The PCR

products were electrophoresed on 1% agarose gel and confirmed by the gel documentation system before implementation of the DGGE analysis.

PCR amplicons were analyzed by the DCode Universal Mutation Detection System (Bio-Rad Laboratories). The PCR products were applied to 8% polyacrylamide (37.5:1, acrylamide:bisacrylamide, 40% (w/v) solution) gels containing 30% to 60% urea and formamide in 1x TAE buffer (40 mM Tris base; 20 mM glacial acetic acid; 1 mM EDTA, pH 8.0; TaKaRa). The gels were electrophoresed at 20 V for 30 min and at 60 V for 16 h and stained with ethidium bromide (10 mg/mL) for 1 h. The gel image was captured by the gel documentation system (19).

DGGE fragment sequencing The major bands of interest were excised with a sterile blade, placed in 1.5 mL Eppendorf tubes, and incubated overnight at 4°C in Tris-EDTA buffer (pH 8.0) to allow DNA diffusion out of the polyacrylamide matrix, and the DNA was recovered. For sequencing, the eluted DNA from the DGGE band was re-amplified using the same primer pairs but without the GC clamp using the condition described above. The PCR products for sequencing were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) following the procedure described by the manufacturer. The samples were analyzed by a 3730xl DNA analyzer. Searches in GenBank with BLAST were performed to determine the closest known relatives of the partial ribosomal DNA sequences obtained (20).

Results and Discussion

pH, acidity, and remaining bacteriocin The pHs of the kimchies decreased during fermentation. The pHs of the control and bacteriocin-treated samples were slightly increased for the first 2 days and then rapidly decreased. Both the bacteriocin-treated samples showed higher pH than that of the control. In particular, at day 4, the pH was 0.8 pH units higher than that of the control (Fig. 1). The acidity of the kimchi samples showed a similar pattern (Fig. 1). Bacteriocin activity was found in the DF01 and K10 treated kimchies up to 3 days; however, no activity was observed in the control kimchi.

Analysis of DGGE fragment and major bands in control kimchi The PCR amplicons were analyzed with 8% polyacrylamide gel using a 30-60% gradient denaturant, and the different size of bands were obtained and compared with the databases (Fig. 2-4).

The DGGE profiles of control kimchi are presented in Fig. 2 and Table 1. *Leu. citreum* (quasi-identical bands "a" and "i") was detected on days 1-5 (early period) and the bands were thick. *Leu. mesenteroides* (band "f") was only detected on day 1 with a thin band. *Leu. paramesenteroides* (band "o") was detected over the whole period. *Lb. brevis* (band "b") was detected on day 3 at its highest intensity and gradually weakened. A thick and distinct *Lb. plantarum* band

Fig. 1. Changes in pH (A) and lactic acid (B) during the storage of kimchi at 20°C. Standard deviation (SD) is presented in the figure.

(band "d") was detected at day 3, but it gradually disappeared. *Lb. sakei* (band "n") and *Weissella koreensis* (band "e") were the most predominant and common throughout the entire fermentation time. Some uncultured bands ("c", "q", and "t") were detected.

Major bands in bacteriocin DF01 treated kimchi The major bands in bacteriocin DF01-treated kimchi are presented in Fig. 3 and Table 1. *Leu. citreum* (quasi-identical bands "a" and "k") was detected for the entire fermentation time, but its bands were thinner than those of the control. Compared to the control, *Leu. mesenteroides* (quasi-identical bands "e" and "l") was detected throughout the fermentation period at very weak intensity, whereas *Leu. paramesenteroides* was not detected. *Lb. brevis* (band "b") was observed during days 3-5 with relatively thicker bands than that of the control. *Lb. plantarum* (band "f") appeared on day 3 and lasted upto day 5, but almost completely disappeared after that. However, its band was thinner than that of the control. *Lb. sakei* (quasi-identical bands "m" and "p") were the predominant bands. *Weissella confusa* (band "c"), which was not detected in the control, was also a predominant component. *Lb. curvatus* (quasi-identical bands "i", "o", and "r") and *Lb. malefermentans* (band "t"), which were absent in the control kimchi, were detected on days 4 and 5 and days 3-6, respectively, and their

Table 1. Identity of bands obtained from DGGE analysis of control kimchi and treated kimchi with bacteriocin DF01 and K10

Band ¹⁾	Control			DF01			K10		
	Closest relative	% Identity ²⁾	Accession No.	Closest relative	% Identity	Accession No.	Closest relative	% Identity	Accession No.
a	<i>Leuconostoc citreum</i>	87	DQ489738	<i>Leu. citreum</i>	95	AF111948	<i>Leu. citreum</i>	84	DQ489737
b	<i>Lactobacillus brevis</i>	99	CP000416	<i>Lb. brevis</i>	99	AF515220	<i>Lb. brevis</i>	98	HM067023
c	Uncultured bacterium	95	AF506711	<i>W. confusa</i>	100	AF506716	Uncultured bacterium	90	AF506711
d	<i>Lb. plantarum</i>	99	FJ455519	Unknown bacterium	-	AF506711	Unknown bacterium	-	EU197817
e	<i>Weissella koreensis</i>	98	AY241937	<i>Leu. mesenteroides</i>	92	CP003101	<i>W. confusa</i>	91	AB023241
f	<i>Leu. mesenteroides</i>	99	AB023242	<i>Lb. plantarum</i>	98	GQ336971	<i>Lb. curvatus</i>	95	FJ751791
g	Unknown bacterium	-	DQ658939	Unknown bacterium	-	EU197817	<i>Lb. plantarum</i>	100	FJ455518
h	<i>Leu. gelidum</i>	95	CP003839	<i>W. confusa</i>	85	AF506717	<i>W. koreensis</i>	100	CP002900
i	<i>Leu. citreum</i>	99	DQ489739	<i>Lb. curvatus</i>	88	AY204894	Uncultured bacterium	91	DQ658939
j	Unknown bacterium	-	GQ180972	<i>Leu. gelidum</i>	90	CP003839	<i>Leu. mesenteroides</i>	94	CP003101
k	<i>Pediococcus acidilactici</i>	99	GQ240304	<i>Leu. citreum</i>	92	AF111949	<i>Leu. gelidum</i>	95	CP003839
l	<i>W. confusa</i>	86	AB023241	<i>Leu. mesenteroides</i>	87	FJ040199	<i>Leu. mesenteroides</i>	96	CP003101
m	<i>W. confusa</i>	90	AF506717	<i>Lb. sakei</i>	100	AY204895	Unknown bacterium	-	GQ180972
n	<i>Lb. sakei</i>	99	AY204896	<i>Leu. pseudomesenteroides</i>	98	AY241941	Uncultured bacterium	90	GU992454
o	<i>Leu. pseudomesenteroides</i>	98	AY241941	<i>Lb. curvatus</i>	99	EF375891	<i>P. acidilactici</i>	88	GQ240304
p	Uncultured bacterium	95	GQ180972	<i>Lb. sakei</i>	98	AY204896	<i>Lb. sakei</i>	100	NC007576
q	Uncultured bacterium	96	GU992454	<i>Leu. pseudomesenteroides</i>	97	AY241941	<i>Lb. pseudomesenteroides</i>	97	AY241941
r	<i>Lb. sakei</i>	95	EU149651	<i>Lb. curvatus</i>	94	AY204891	<i>Lb. curvatus</i>	99	AY204891
s	<i>Lb. sakei</i>	95	AY204895	Uncultured bacterium	90	AY241945	Uncultured bacterium	87	FJ716931
t	Uncultured bacterium	94	FJ716931	<i>Lb. malefermentans</i>	97	AJ575743	<i>Lb. sakei</i>	95	NC007576

¹⁾Bands as shown in Fig. 2, 3, and 4 were excised from DGGE gel.

²⁾Percentage of identical nucleotides in sequences extracted from DGGE gel and sequences of closest relative found in GenBank.

bands were relatively thick. Other minor bands with weak intensities that appeared only in the DF01-treated sample were *Leu. pseudomesenteroides* (band “n”), unknown (quasi-identical bands “d” and “g”), and uncultured (band “s”) bands, which were absent in the control, were detected with relatively thick bands. In particular, on days 4 and 5, the number was increased.

Major bands in bacteriocin K10 treated kimchi The major bands in bacteriocin K10-treated kimchi are presented in Fig. 4 and Table 1. *Leu. citreum* (band “a”) and *Lb. brevis* (band “b”), which were obvious in the control and DF01-treated samples, were not detected. However, *Leu. gelidum* (band “k”), which was absent from the control, was detected but its intensity was very weak. *Leu. mesenteroides* (quasi-identical bands “j” and “l”) was detected during the entire period at low density. *Lb. plantarum* (band “g”) was detected throughout the period and the maximum intensity was observed on days 4-6. *Weissella confusa* (band “e”), which was not detected in the control, was detected on day 1, lasted to day 5, and its intensity was greatest on days 4-6. *Weissella koreensis* (band “h”) showed a similar pattern to that of *W. confusa*. *Lb. sakei* (quasi-identical bands “p” and “t”) was predominant and common throughout the period. *Lb. curvatus* (quasi-identical bands “f” and “r”) were detected on days 1-4. *Ped. acidilactici* (“o”), which was absent in the control and DF01 sample, was detected. Some uncultured bands (“c”, “i”, “n”, and “s”) and unknown bands (“d” and “m”) were also detected.

This experiment clearly showed that kimchi microflora are complex

and continuously change as fermentation proceeds. However, the microbial communities obtained in this experiment were largely different from the conventional data obtained by culture-dependent method (21-23). The major discrepancies are as follows: First, in this experiment, *W. koreensis*, *W. confusa*, *Lb. sakei*, and *Leu. citreum*, which were not detected in earlier studies, were the most predominant LAB throughout the fermentation period. Thus, these microorganisms seem to play an important role in kimchi fermentation (13). Second, a microfloral shift from less aciduric heterofermentative to aciduric homofermentative LAB was not observed. Third, conventional data showed that *Lb. plantarum* is a major over acid producer and it becomes predominant in the last stage of kimchi fermentation. However, it did not last to the final stage of fermentation; rather, it faded away (Fig. 2). Fourth, *Enterococcus* spp., which has been regarded as one of the dominant genera in the kimchi community, was not detected in this experiment. The culture-dependent method solely relies on the isolation of “randomly selected” LAB colonies that appear on MRS agar by chance and identification of selected strains was mainly performed by rather less accurate physiological or sugar assimilation test (21-23) or for more accuracy, sequencing of 16S rRNA (24-26). However, a selected number of colonies cannot represent all the microflora in kimchi fermentation, and the possibility of neglecting or missing minor flora is still high. Therefore, further studies should be conducted to re-establish and confirm accurate microbial community changes in kimchi fermentation. Many recent researches are strongly supporting this suggestion

Fig. 2. PCR-DGGE analysis of partial 16S rRNA gene fragments from control kimchi sample using a gc338f and 518r primer pair. Reference strain composed of *Leu. mesenteroides* ATCC 10836, *P. acidilactici* K10, *Lb. plantarum* NCDO 955, and *Lb. brevis* DF01.

Fig. 3. PCR-DGGE analysis of partial 16S rRNA gene fragments from kimchi sample with bacteriocin DF01 using a gc338f and 518r primer pair. Reference strain composed of *Leu. mesenteroides* ATCC 10836, *P. acidilactici* K10, *Lb. plantarum* NCDO 955, and *Lb. brevis* DF01.

(12,13,27).

This is the first report regarding bacteriocin-altered microbial communities of kimchi. The bacterial communities in the bacteriocin-treated samples were more complex than those in the control. Compared to the control, the *Lb. brevis* band was thicker in the DF01-treated sample (Fig. 4). *Lb. curvatus* (thick bands "o" and "r" in DF01,

Fig. 4. PCR-DGGE analysis of partial 16S rRNA gene fragments from kimchi sample with bacteriocin K10 using a gc338f and 518r primer pair. Reference strain composed of *Leu. mesenteroides* ATCC 10836, *P. acidilactici* K10, *Lb. plantarum* NCDO 955, and *Lb. brevis* DF01.

Fig. 3, and "r" in K10 treated samples, Fig. 4) and *Lb. malefermentans* (thick band "t" in DF01 treated sample, Fig. 3) newly emerged in the bacteriocin-treated samples. These three strains are classified as either Group III obligate heterofermentative or Group II facultatively heterofermentative. Their bands were detected at maximum intensity on day 4 or 5, and during that time, the pH and acidity differences between the control and bacteriocin-treated sample were maximized (ca. 0.8 unit, Fig. 1). Unfortunately, the DGGE profile does not indicate the number of cells; therefore, the intensity of each band is regarded as a criterion for indirect measurement of cell number. In the same period, several miscellaneous bands were detected (Fig. 3 and 4); however, we could not identify them as they were too closely packed and it was rather practically impossible; therefore, we do not know their identity. Perhaps, the addition of bacteriocin affected the ecological niche in such a way that provided preferable conditions for growth of more heterofermentative microbial communities, and this might have delayed the pH reduction.

This is the first report showing that LAB bacteriocin has a positive effect on delaying the pH reduction of kimchi. Ryu *et al.* (7) reported that addition of nisin to prevent over-acidification of kimchi was unsuccessful owing to nisin being inactivated by raw components and the presence of nisin-resistant LAB in kimchi. Choi and Park (6) reported that at the recommended level (100 IU/mL), nisin can be used to preserve mulkimchi (watery kimchi) by inhibiting lactobacilli more effectively than leuconostocs. However, the microbial ecosystems in mul-kimchi and kimchi (in this study) were different and they only focused on two groups of LAB (leuconostocs and lactobacilli); in

other words, not the entire microbial community, and fermentation was conducted at 15°C (a lower temperature than in our experiment). In this experiment, we obtained a 0.8 pH unit difference at 20°C (ambient temperature) by addition of bacteriocin. Considering the high temperature, 0.8 pH units should not be underestimated. For further studies, optimization of bacteriocin treatment, such as dosage, addition time, and use of cocktail bacteriocin, might be helpful to develop LAB bacteriocin as a biopreservative in kimchi and its close relatives such as sauerkraut (28).

Disclosure The authors declare no conflict of interest.

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