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Synergistic Effects of Bacteriocin-Producing *Pediococcus acidilactici* K10 and Organic Acids on Inhibiting *Escherichia coli* O157:H7 and Applications in Ground Beef

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Abstract When used in combination with organic acids, *Pediococcus acidilactici* K10 or its bacteriocin was effective in inhibiting *Escherichia coli* O157:H7 *in vitro* and *in situ*. *P. acidilactici* K10, a strain of bacteriocin-producing lactic acid bacteria (LAB), was previously isolated from kimchi in our laboratory, and the molecular weight of its bacteriocin was estimated to be around 4,500 Da by SDS-PAGE. Initially, *P. acidilactici* K10 and its bacteriocin could not inhibit *E. coli* O157:H7, when used alone. However, when they were used together with organic acids such as acetic, lactic, and succinic acids, they greatly inhibited *E. coli* O157:H7 *in vitro*. Based on these *in vitro* results, a real sample test with ground beef was conducted at 4°C with acetic acid (0.25%) or lactic acid (0.35%) alone, and then in combination with *P. acidilactici* K10 (10^5 CFU/g of sample). Combined treatment of *P. acidilactici* K10 with lactic acid showed the most inhibitory effect: a 2.8-$\log_{10}$-unit reduction of *E. coli* O157:H7 in ground beef during storage at 4°C. This result suggests that the combination of bacteriocin-producing *P. acidilactici* K10 and organic acids has great potential as a food biopreservative by inhibiting the growth of *E. coli* O157:H7.

Key words: Bacteriocin, *Pediococcus acidilactici*, organic acids, *Escherichia coli* O157:H7, ground beef, biopreservative, synergistic effect

Enterohemorrhagic *E. coli* (EHEC) causes human diseases, such as bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome [13, 17, 32]. *E. coli* O157:H7 is a representative EHEC and most of its infections are associated with consumption of contaminated ground beef, milk, water, and apple juice products that have been improperly handled, stored, or cooked [2, 8, 9]. Previous studies showed that *E. coli* O157:H7 survives at pH 3.4 and at both refrigeration and freeze storage temperatures, and that it is moderately salt tolerant [4, 11, 24, 25]. These studies indicate that *E. coli* O157:H7 has the potential to survive harsh conditions, including low pH, low water activity, and refrigerated storage.

In order to inhibit foodborne pathogens including *E. coli* O157:H7, various physical, chemical, and biological factors have been applied. Furthermore, hurdle technology, a combination of these methods, has been recently used as a multi-target preservation tool [14, 16, 23, 34]. For instance, a combination of competitive microflora, pH, salt, temperature, and bacteriocin has been shown to reduce *E. coli* O157:H7 significantly [5, 10, 12].

Bacteriocin-producing bacteria and their bacteriocins have been the subject of intensive study in the area of biopreservatives, because they increase the shelf-life of food [15, 26, 30]. Kimchi (Korean fermented vegetables) and jeot-gal (fermented fish foods) are among the sources of bacteriocin-producing LAB [18, 19, 22]. Major classes of bacteriocins produced by LAB are those bacteriocins that inhibit the organisms closely related to LAB and those bacteriocins that inhibit a broad spectrum of Gram-positive bacteria associated with food spoilage and foodborne illnesses [7, 27, 36]. However, few Gram-negative bacteria are sensitive to LAB bacteriocins, because their outer membrane acts as a barrier and protects them from bacteriocins [35]. To expand bacteriocin usage as a biopreservative for controlling Gram-negative pathogens, Cutter and Siragusa [6] used nisin in combination with chelating agents and found that nisin was effective in reducing *E. coli* O157:H7 and *Salmonella typhimurium in vitro* [5]. However, they reported later that such reductions of >4 $\log_{10}$ CFU/cm^2 *in vitro* were not observed *in situ*.

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In particular, we have been very much interested in f ıding out whether the bacteriocin-producing microorganism i olated from kimchi, its bacteriocin, and organic acids c ɔuld effectively inhibit *E. coli* O157:H7 *in situ* as well as *i vitro*. Therefore, the present study was undertaken to e ɛamine the effects of *Pediococcus acidilactici* K10, its b acteriocin, and organic acids on the growth inhibition of *l . coli* O157:H7, using artificial media, and then this p ·ocess was repeated using ground beef, which is a p ɔtential food carrier of *E. coli* O157:H7, at a refrigerated t ·mperature of 4°C.

] MATERIALS AND METHODS

! ·trains and Culture Conditions

. '. coli O157:H7 ATCC 43894 and *Lactobacillus plantarum* . ATCC 14917 were purchased from American Type Culture ('ollection (ATCC, Rockville, MD, U.S.A.). Bacteriocin-] roducing *P. acidilactici* K10 was previously isolated from] imchi in our laboratory [20]. *E. coli* O157:H7 was cultured : erobically in tryptic soy broth (TSB, Merck, Darmstadt, ' ermany) at 37°C for 18 h. For the selective enumeration ' f *E. coli* O157:H7, CT-SMAC Agar (Merck, Darmstadt, ' ermany) was used. *P. acidilactici* K10 and *Lb. plantarum* ATCC 14917 were cultured aerobically using MRS broth Merck, Darmstadt, Germany) at 37°C for 18 h. For the numeration of *P. acidilactici* K10, ROGOSA Agar (Merck,)armstadt, Germany) was used.

Chemicals

Lactic acid (mixture of D and L isomers), acetic acid, uccinic acid, citric acid, boric acid, tartaric acid, and ɔropionic acid were purchased from Sigma (St. Louis, J.S.A.). Stock solutions of organic acids (1 M) were ɔrepared in distilled de-ionized water (DDW) and diluted o 10-50 mM for the experiments.

Food Sample

Ground beef was purchased from a local market (Bundang, Korea) and stored at -20°C until used.

Confirmation of Bacteriocin Production from *P. acidilactici* K10

Bacteriocin production by *P. acidilactici* K10 was confirmed with an agar diffusion assay using *Lb. plantarum* ATCC 14917 as an indicator strain [31]. Two microliters of the overnight culture of *P. acidilactici* K10 were spotted on tryptic soy agar (TSA) and MRS plates, and the plates were then incubated at 37°C for 12 h. *Lb. plantarum* ATCC 14917 in 5 ml of MRS with 0.7% agar (MRS top agar) was overlaid to the level of 10^7 cells/ml on the plate. After incubation, inhibition zones were checked.

Preparation of Crude Bacteriocin

The overnight culture of *P. acidilactici* K10 was centrifuged for 30 min at 10,000 rpm and 4°C, and the supernatant was filtered through a 0.2 μm-pore size membrane filter (Sartorius, Goettingen, Germany). While stirred at 4°C, ammonium sulfate was added to the filtrate to give 50% saturation. The saturated solution was centrifuged for 30 min at 10,000 rpm and 4°C, and the precipitate was recovered. The precipitate was reconstituted in distilled water (DW) and dialyzed in Spectra/Por membrane (MWCO 1,000, Spectrum Medical Industries, Houston, U.S.A.) against DW for 24 h at 4°C. After dialysis, the crude bacteriocin was freeze-dried and stored at -20°C until used [21, 29].

The bacteriocin activity was expressed as AU (arbitrary unit) per ml. To determine the bacteriocin activity of the original preparation, the reciprocal of the highest dilution that gave a definite zone of inhibition of over 1 mm was multiplied by a conversion factor (e.g. 200 if 5 μl is used) [31].

Determination of Molecular Weight of *P. acidilactici* K10 Bacteriocin Followed by Bioassay

To determine the molecular weight of *P. acidilactici* K10 bacteriocin, SDS-PAGE using a Tris-tricine system [33] was performed. In this system, precast 10-20% gradient gel (Bio-Rad, Hercules, CA, U.S.A.) and Mini-Protean III electrophoresis unit (Bio-Rad, Hercules, CA, U.S.A.) were employed. After running gel electrophoresis, the gel was stained with Bio-Safe Coomassie (Bio-Rad, Hercules, CA, U.S.A.).

For the bioassay after SDS-PAGE, the slab gel was washed with DDW to decrease SDS concentration, placed on an MRS plate, and finally overlaid with the indicator strain, *Lb. plantarum* ATCC 14917, which had previously been mixed with 5 ml of MRS top agar. After incubation at 37°C for 12 h, the plate was examined for inhibition zones.

Selection of Effective Organic Acids

Various organic acids were tested for their synergistic inhibitory effects on *E. coli* O157:H7, when used with a competitive microorganism, *P. acidilactici* K10. First, organic acids were added to 5 ml of TSB in which 1% of *E. coli* O157:H7 and *P. acidilactici* K10 were separately inoculated. The final concentration of organic acids in the TSB was 50 mM. After agitation for 12 h at 37°C, the cultures were plated on the selective media (i.e., CT-SMAC agar for *E. coli* O157:H7 and ROGOSA agar for *P. acidilactici* K10) for viable cell counting. Several organic acids showing a good antimicrobial activity against *E. coli* O157:H7 were selected. The organic acids selected were retested in final concentrations of 10, 30, and 50 mM to obtain optimal concentration at which the inhibitory effect on *E. coli* O157:H7 was maximal and *P. acidilactici* K10 was minimally affected. The pHs at the initial and final stages of the

treatments were measured. In order to find out whether the *P. acidilactici* K10 bacteriocin itself could effectively inhibit *E. coli* O157:H7, 50 µl of the bacteriocin (200 AU) and three organic acids, including lactic, acetic, and succinic acids, at the final concentration of 30 mM were applied to the *E. coli* O157:H7 in phosphate buffer.

Organic Acid Analysis During Mixed Culture of *P. acidilactici* K10 and *E. coli* O157:H7

Organic acid analysis during the mixed culture of *P. acidilactici* K10 and *E. coli* O157:H7 was performed. Each culture (10^6 cells/ml) of *E. coli* O157:H7 and *P. acidilactici* K10 was inoculated into 10 ml of MRS broth and TSB, respectively. While cultured at 37°C, 1 ml each volume of the mixed culture from MRS broth and TSB was taken at intervals and filtered through a 0.2 µm membrane filter (Sartorius, Goettingen, Germany). Thirty microliters of the filtrate were injected onto HPLC, equipped with an Aminex HPX-87H ion exclusion column (Bio-Rad, Hercules, CA, U.S.A.). The detector was set at 210 nm, and the solvent, 0.008 N H_2SO_4, ran through the HPLC system with a flow rate of 0.6 ml/min.

Inhibition of *E. coli* O157:H7 in Ground Beef

Among the organic acids tested, lactic acid and acetic acid were chosen and added to the ground beef spiked with *E. coli* O157:H7 in the presence or absence of *P. acidilactici* K10. Five grams of ground beef were put into a falcon tube. To maintain the food properties as intact as possible, *P. acidilactici* K10 (10^5 cells/g sample) and 200 µl of 1 M organic acid (i.e., 0.35% lactic acid or 0.25% acetic acid in the ground beef) were added to the ground beef spiked with *E. coli* O157:H7 (10^5 cells/g sample). The samples were stored at 4°C, and the viable cell number and the pH were periodically measured. *E. coli* O157:H7 and total LAB were enumerated on CT-SMAC agar and ROGOSA agar respectively.

Results and Discussion

Bacteriocin Production by *P. acidilactici* K10

To find out whether *P. acidilactici* K10 produces bacteriocin, a bacteriocin activity test was performed using the agar diffusion method [31] as described in Materials and Methods. As a result, the inhibition zone was clearly shown on both MRS (data not shown) and TSA plates, implying that *P. acidilactici* K10 produced bacteriocin (Fig. 1). Although the definite inhibition zone with *Lb. plantarum* ATCC 14917 was observed, the result of the agar diffusion method with *E. coli* O157:H7 was not satisfactory due to the indistinct inhibition zone (data not shown). It has been reported that Gram-negative bacteria are likely to be resistant to most bacteriocins produced by LAB due to the lack of teichoic acid on their cell envelope [35].

Fig. 1. Antimicrobial activity of *P. acidilactici* K10 on *Lb. plantarum* ATCC 14917.
The arrow indicates an inhibition zone generated by *P. acidilactici* K10.

Determination of Molecular Weight of *P. acidilactici* K10 Bacteriocin and Bioassay

After crude bacteriocin was prepared (refer to Materials and Methods), SDS-PAGE was performed to determine the molecular weight of *P. acidilactici* K10 bacteriocin. As shown in Fig. 2B, the molecular weight of the bacteriocin was estimated to be around 4,500 Da. This result is in good agreement with the result of the earlier paper that reported 4,622 Da, determined by electrospray mass spectrometry [20]. The bioassay result showed an inhibition zone at the band position of 4,500 Da, indicating *P. acidilactici* K10 bacteriocin (Fig. 2A).

Effects of *P. acidilactici* K10 and Organic Acids on the Inhibition of *E. coli* O157:H7

Several organic acids, such as lactic, acetic, citric, boric, tartaric, propionic, and succinic acids, were tested for

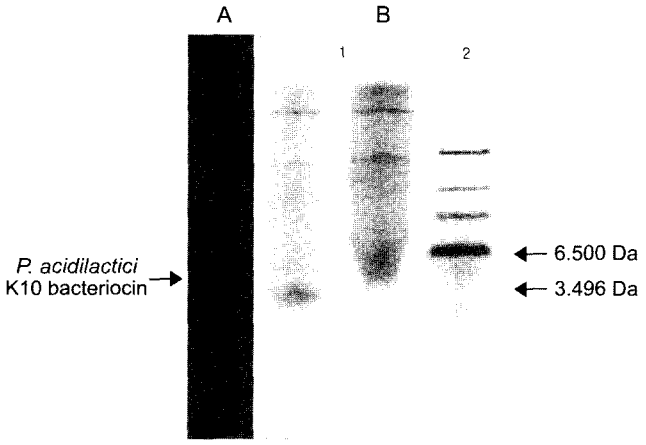

Fig. 2. Tris-tricine SDS-PAGE of *P. acidilactici* K10 bacteriocin.
A, A gel stained for bacteriocin activity; B, A gel stained with Bio-Safe Coomassie; 1, Crude *P. acidilactici* K10 bacteriocin; 2, Polypeptide standards.

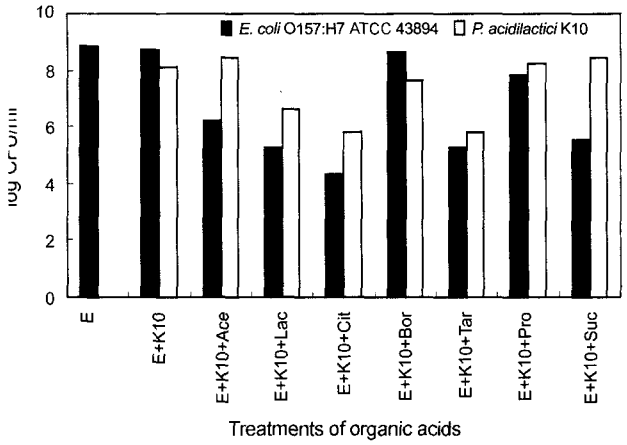

Fig. 3. Synergy effects of organic acids on inhibiting *E. coli* O157:H7, when used with *P. acidilactici* K10.
E, *E. coli* O157:H7; K10, *P. acidilactici* K10; Ace, Acetic acid; Lac, Lactic acid; Cit, Citric acid; Bor, Boric acid; Tar, Tartaric acid; Pro, Propionic acid; Suc, Succinic acid. *E. coli* O157:H7 and *P. acidilactici* K10 were inoculated into 5 ml of TSB with the final concentration of 1%, respectively. The final concentration of organic acid in 5 ml of TSB was 50 mM.

synergistic effects on the inhibition of *E. coli* O157:H7 under the treatment of *P. acidilactici* K10. Among the organic acids tested, boric and propionic acids showed a little inhibitory effect on *E. coli* O157:H7 at the final concentration of 50 mM (Fig. 3). Therefore, they were excluded from further tests. The rest of the organic acids, including lactic acid, were further tested for their optimal concentrations at which *E. coli* O157:H7 was effectively inhibited with little harm to *P. acidilactici* K10. The pHs at the initial and end of the treatments were measured (Table 1). The optimal concentrations for the organic acids were found to be generally 30 mM (Fig. 4).

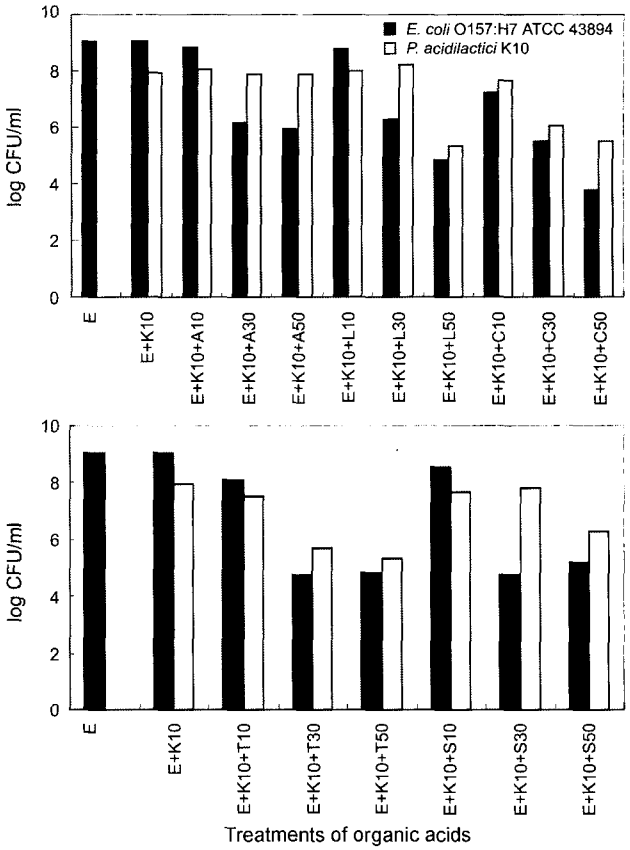

Fig. 4. Synergy effects of organic acids at various concentrations on inhibiting *E. coli* O157:H7 when used with *P. acidilactici* K10.
E, *E. coli* O157:H7; K10, *P. acidilactici* K10; Ace, Acetic acid; Lac, Lactic acid; Cit, Citric acid; Tar, Tartaric acid; Suc, Succinic acid. *E. coli* O157:H7 and *P. acidilactici* K10 were inoculated into 5 ml of TSB each with the final concentration of 1%. The final concentrations of organic acids in 5 ml of TSB were each 10, 30, and 50 mM (i.e. 10, 10 mM; 30, 30 mM; 50, 50 mM).

Effects of *P. acidilactici* K10 Bacteriocin and Organic Acids on the Inhibition of *E. coli* O157:H7

In addition to the result obtained from the mixed culture above, it was decided to find out whether the *P. acidilactici* K10 bacteriocin itself could effectively inhibit *E. coli* O157:H7. For this test, three organic acids were added with or without the *P. acidilactici* K10 bacteriocin, as described in Materials and Methods. As shown in Fig. 5, when the bacteriocin was added alone, *E. coli* O157:H7 was not inhibited. However, *E. coli* O157:H7 was greatly inhibited under the treatments of organic acids. Even more remarkably, synergistic effects on the inhibition of *E. coli* O157:H7 were observed, when both the bacteriocin and the organic acids were added. This synergistic effect could be explained by the fact that the organic acids first disrupted the outer membrane of *E. coli* O157:H7, thus providing a way for the bacteriocin to attack *E. coli* O157:H7 [1]. In order to verify this possibility, the *E. coli*

Table 1. Initial and final pHs after organic acids treatments.

		Organic acids treatments (mM)																	
		E	EK	EK+Ace			EK+Lac			EK+Cit			EK+Tar			EK+Suc		
				10	30	50	10	30	50	10	30	50	10	30	50	10	30	50
pH	Initial (0 h)	7.0	7.0	6.1	4.9	4.5	6.1	4.5	4.1	5.0	3.8	3.4	5.7	3.7	3.3	5.3	5.3	5.0
	Final (12 h)	6.6	6.8	5.9	4.4	4.2	5.8	4.1	3.9	4.7	3.6	3.2	5.0	3.5	3.1	5.4	4.1	3.8

E, *E. coli* O157:H7; K10, *P. acidilactici* K10; Ace, Acetic acid; Lac, Lactic acid; Cit, Citric acid; Tar, Tartaric acid; Suc, Succinic acid.
E. coli O157:H7 and *P. acidilactici* K10 were separately inoculated into 5 ml of TSB with the final concentration of 1%.

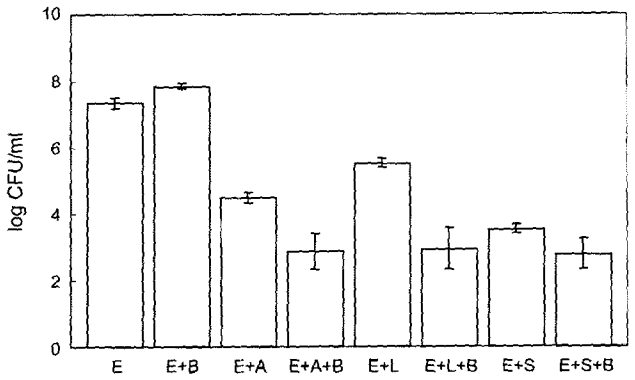

Fig. 5. Synergy effects of *P. acidilactici* K10 bacteriocin and organic acids on inhibiting *E. coli* O157:H7.
E, *E. coli* O157:H7; B, *P. acidilactici* K10 bacteriocin; A, Acetic acid; L, Lactic acid; S, Succinic acid. *E. coli* O157:H7 was inoculated into 5 ml of 0.01 M phosphate buffer (pH 7.0) with the final concentration of 1%. Fifty microliters of the bacteriocin, corresponding to 200 AU, were added. Organic acids were added at the final concentration of 30 mM. Each point is an average of three replicates, and the error bar represents one standard deviation.

O157:H7 culture treated with the bacteriocin was examined by a scanning electron microscope to observe any physical change of cell surface, and UV absorbance of the culture was measured at 260 and 280 nm which indicate an efflux of nucleic acids and proteins [5]. Under the experimental conditions, no indications of membrane disruption and/or release of nucleic acids and proteins could be found. The current study could not find a satisfactory explanation on the mechanism, hence future work to find out the mode of action of the bacteriocin should be followed.

Organic Acids Profile During the Mixed Culture of *P. acidilactici* K10 and *E. coli* O157:H7

Organic acids were analyzed during the mixed culture of *P. acidilactici* K10 and *E. coli* O157:H7 in both MRS broth and TSB. When the two microorganisms were cultured in MRS broth, a major organic acid detected was lactic acid (Fig. 6), and the pH of the mixed culture broth finally reached 3.9 after 24 h (data not shown). However, little lactic acid and other organic acids were detected in the TSB culture whose pH was about 6.8 after 24 h (data not shown). The difference between the two media was in the composition. MRS broth was used for *Lactobacilli* and TSB for the common bacteria, including *E. coli*. For this reason, growth of *E. coli* O157:H7 was inhibited in the MRS broth under excessive *P. acidilactici* K10. On the other hand, when *E. coli* O157:H7 was dominant in TSB, it inhibited *P. acidilactici* K10, resulting in little organic acid being fermented in the culture.

Inhibition of *E. coli* O157:H7 in Ground Beef

Lactic acid and acetic acid were chosen for the real sample trial, because they belong to GRAS and exhibited good

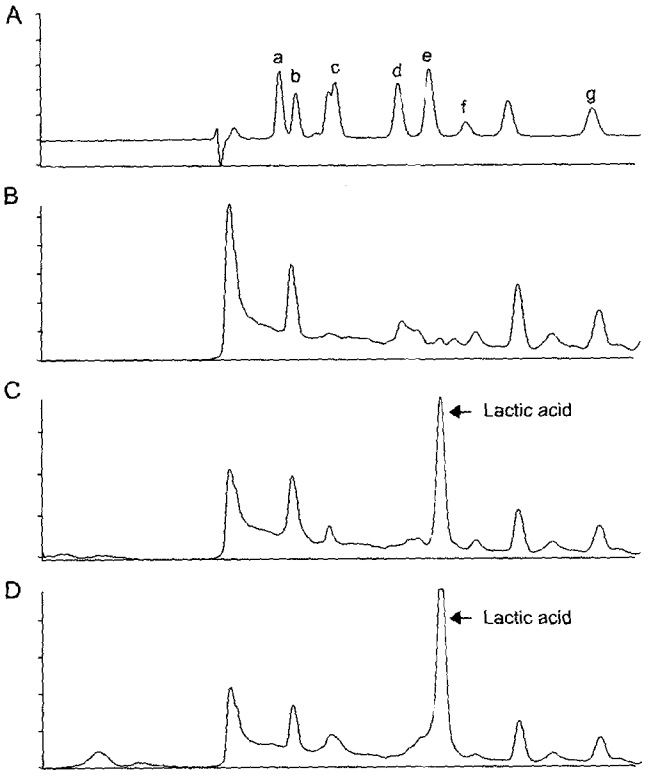

Fig. 6. Organic acid analysis during mixed culture of *E. coli* O157:H7 and *P. acidilactici* K10 in MRS broth at 37°C.
A, standards (a: citric acid, b: tartaric acid, c: malonic acid, d: succinic acid, e: lactic acid, f: fumaric acid, g: acetic acid); B, 0 h-culture; C, 12 h-culture; D, 24 h-culture.

antimicrobial activities on *E. coli* O157:H7 *in vitro*. An *in situ* test with ground beef was performed as described in Materials and Methods. The sample treated with both *P. acidilactici* K10 and lactic acid had the most inhibitory

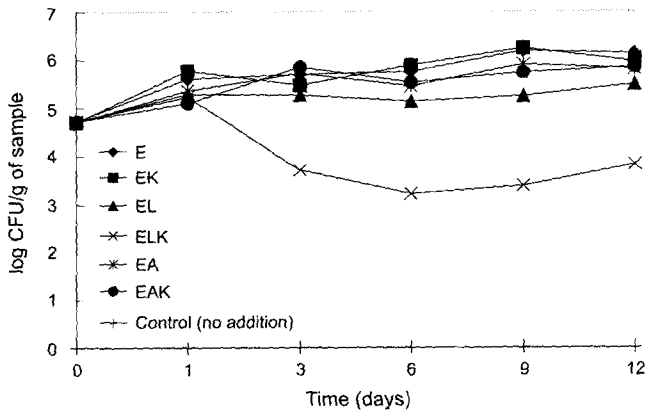

Fig. 7. Changes of viable cell number of *E. coli* O157:H7 in ground beef during storage at 4°C under various treatments.
E, *E. coli* O157:H7; K, *P. acidilactici* K10; L, Lactic acid; A, Acetic acid. The ground beef was spiked with *E. coli* O157:H7 (10^5 CFU/g) prior to the treatments of *P. acidilactici* K10 (10^5 CFU/g) and organic acids. Lactic acid and acetic acid were added at 200 µl of a 1 M solution, respectively.

eff ect against *E. coli* O157:H7. In this sample, a maximum re uction of 2.8-$\log_{10}$-unit occurred. However, in the sample tre ated with only lactic acid, 1-$\log_{10}$-unit reduction occurred. Th e growth of *E. coli* O157:H7 was slightly inhibited in other sa mples treated with acetic acid alone and a combination of *P. acidilactici* K10 and acetic acid (Fig. 7). Unlike the *in vi o* results in which acetic acid showed an inhibitory ef ect on *E. coli* O157:H7 somewhat similar to lactic acid in TSB (Figs. 3, 4, and 5), the inhibitory effect of acetic ac d in the ground beef was only slight. In contrast, lactic ac d still showed good inhibitory effects on both *in vitro* ar l *in situ* tests. The mechanism causing the enhanced in ibition of *E. coli* O157:H7 in the ground beef treated w h a combination of *P. acidilactici* K10 and lactic acid, is nc yet understood. It appeared that organic acids, bacteriocin, or combinations of these factors have different modes of ac ion in the matrix of ground beef. It is reported that re eptors for pediocin AcH are absent in the Gram-ne gative bacteria [3]. This may be one of the reasons why G am-negative bacteria tend to be resistant to bacteriocins. A for the mechanism of action, bacteriocins are known to di sipate proton motive force (pmf), which plays a central rc e in ATP synthesis, active transport, and bacterial motion [2 3]. Bacteriocins also induce leakage of various small ir racellular substances from sensitive cells [28]. An ir eresting report on the mode of action of lactic acid o the Gram-negative bacteria including *E. coli* O157:H7 ir dicates that lactic acid functions as a permeabilizer of the o ter membrane of Gram-negative bacterial and sensitizes th e Gram-negative bacteria to bacteriocins [1]. In this c rrent ground beef study, it may be assumed that lactic a id attacks the outer membrane of *E. coli* O157:H7 to m ake the organism sensitized. The sensitized *E. coli* C 157:H7 is then more susceptible to the lethal activity of b cteriocins that dissipate pmf and induce the leakage of ir tracellular substances.

Bacteriocins from LAB are known to have little effect o i inhibiting Gram-negative bacteria, including *E. coli.* In tl is regard, the findings of the present study agreed with o her studies in that *E. coli* O157:H7 was not inhibited b / bacteriocin-producing *P. acidilactici* K10 in TSB and g ound beef. In an attempt to utilize bacteriocin or bacteriocin-p oducing LAB to control *E. coli* O157:H7, organic acids tl at have synergistic inhibitory effects on *E. coli* O157:H7 v ere screened. Several organic acids presented synergistic i hibitory effects on *E. coli* O157:H7 *in vitro*. Among t ese organic acids, lactic and acetic acids were chosen to a pply to ground beef with or without *P. acidilactici* K10. U nder these conditions, the combination of lactic acid and *l acidilactici* K10 significantly inhibited *E. coli* O157:H7 i the ground beef with a maximum reduction of 2.8-$\log_{10}$-u nit, while any other treatments with acetic acid showed s ight effects. In conclusion, the combination of lactic acid a nd *P. acidilactici* K10 was proved to be a powerful method to inhibit the notorious foodborne pathogen, *E. coli* O157:H7. The results presented herein should be useful when considering the inhibition of foodborne pathogens in foods.

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