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# Incidence and Control of Coliform Bacteria in the Manufacturing of Commercial Kimchi

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As consumption of kimchi has increased, factories have begun to produce this traditional Korean fermented vegetable dish on a large scale. Following the rise in manufacturing, the hygienic conditions under which commercial kimchi is being made have become an issue. We isolated 17 coliform bacteria from commercial kimchi that had not been fully fermented. These bacteria were partially identified as one of seven different species from three genera by 16S rDNA sequence analysis as follows: *Enterobacter intermedius*, *Ent. cloacae*, *Ent. amnigenus*, *Klebsiella terrigena*, *K. ornithinolytica*, *K. oxytoca*, and *Hafnia alvei*. *Lactobacillus paraplantarum* KNUC25 has been isolated from over-fermented Chinese cabbage kimchi and its antimicrobial activity reported in the literature. In our study, the KNUC25 strain showed antibacterial activity against isolated coliform bacteria and some pathogenic coliform bacteria through spot-on-the-lawn tests and viable cell tests. Through development and use of a cell-free supernatant of *L. paraplantarum* KNUC25, we effectively controlled coliform bacteria in commercial kimchi.

**Key words:** Antibacterial LAB, coliform bacteria, kimchi

## Introduction

Kimchi is a traditional Korean vegetable food fermented by lactic acid bacteria (LAB). As consumption of kimchi has increased nationally and internationally due to its beneficial effects, large-scale factory production of kimchi has increased. Unfortunately, hygienic problems can occur in the manufacture of kimchi because of increasing shelf life without introducing early fermentation, and the presence of a variety of coliform bacteria from *Escherichia coli* to *Salmonella* has been reported in some commercial types [24].

Coliform bacteria are regarded as belonging to any of the genera *Escherichia*, *Citrobacter*, *Enterobacter*, and *Klebsiella* [20]. Most coliform bacteria are present in large numbers in the intestinal flora of humans and other warm-blooded animals, but they also exist in fecal waste. Accordingly, the detection of higher concentrations of coliform bacteria is used as an index of fecal contamination through cross contamination or inadequate food treatment [20, 22]. Control of coliform bacteria in kimchi manufacturing as a concept of the GRAS (generally recognized as safe) system is essential.

Recently, to protect foods from harmful microorganisms and/or to improve the preservation of food, a variety of research on bacteriocin or bacteriocin-like substances produced by LAB has been reported [4, 5, 13, 15, 19, 25, 27, 28]. It has also been reported that an isolate of over-fermented Chinese cabbage kimchi, *Lactobacillus paraplantarum* C7, produced a bacteriocin that could inhibit some Gram-positive bacteria [15, 16].

In the present study, we particularly focused on the antibacterial activity of *L. paraplantarum* KNUC25 against various coliform bacteria and its potential as a food preservative to control coliform bacteria in the manufacturing of commercial kimchi.

## Materials and Methods

### Bacterial strains and culture conditions

Pathogenic coliform bacterial strains such as *E. coli* ATCC25922, *Citrobacter freundii* ATCC6750, *Klebsiella pneumoniae* ATCC13883, *Enterobacter cloacae* ATCC13047, *Ent. aerogenes* ATCC29751, and *Erwinia carotovara*

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ATCC15390 were used as indicator strains. *Salmonella enteritidis* ATCC13076 and *Shigella sonnei* ATCC25931, which are coliform bacteria that cause food poisoning, were also used as indicator strains. *L. paraplantarum* KNUC25, which is a lactic acid bacterium isolated from over-fermented Chinese cabbage kimchi, was used to evaluate antibacterial activity against coliform bacteria.

All coliform bacteria were grown in Luria-Bertani (LB) broth at 37°C. *L. paraplantarum* KNUC25 was grown on MRS agar (de Man, Rogosa, and Sharpe, Merck, Germany) [6, 14, 17] supplemented with 0.5% calcium carbonate [28] or in MRS broth at 25°C. All of the strains were stored at -75°C with glycerol 15% (v/v).

**Isolation and identification of coliform bacteria**

Coliform bacterial strains used as indicator microorganisms were isolated from commercial kimchi without fermentation bought at market, by using Petrifilm™ *E. coli* Count Plates (3M Microbiology Products, St. Paul, MN, USA) [3] that contained the following ingredients: yeast extract, 9.6 g; pancreatic digest of gelatin, 20.9 g; bile salt No.3, 1.6 g; peptic digest of animal tissue, 1.6 g; lactose, 21.4 g; sodium chloride, 5.3 g; crystal violet, 2 mg; neutral red, 0.1 g; guar gum (cold water soluble gelling agent), 65.7 g; and 2, 3, 5-triphenyltetrazolim chloride, 0.11 g. The commercial kimchi, which was stored at 5°C to repress fermentation, was used to isolate coliform bacteria after grinding and filtration under sterile conditions. The ground kimchi was diluted and inoculated onto Petrifilm™ *E. coli* Count Plates and then incubated for 24 h at 35°C. Using these plates, coliform bacteria appear as red colonies with gas bubbles, but *E. coli* specifically appear as a blue colony with gas bubbles. Seventeen coliform bacteria, which appeared as red colonies with bubbles on the Petrifilm™, were selected. They were partially identified by 16S rDNA sequence analysis. Primers for the 16S rDNA sequencing analysis were GF1 (5'-TAACACATGCAAGTCGAACG-3') and GR1 (5'-GGTGTGACGGGCGGTGTGTACAAG-3') [7]. Each polymerase chain reaction (PCR) mixture contained 30 ng of template DNA and primers (each at a concentration of 10 pM) in the PCR premix (2.5-U *Taq* DNA polymerase, 250-μM dNTPs, 10-mM Tris-HCl [pH 9.0], 40-mM KCl, 1.5-mM $MgCl_2$ stabilizer, and tracking dye; Bioneer, Daejeon, Korea). The thermocycling conditions consisted of an initial denaturation step at 94°C for 3 min, 35 amplifying cycles of 30 sec at 94°C, 30 sec at 60°C, 45 sec at 72°C, and a final extension step at 72°C for 15 min. After the PCR amplification, approximately 1.4-kb DNA fragments were observed on 1% (w/v) agarose gel. The amplified fragments were purified from the agarose gel using AccuPrep Gel Purification Kit (Bioneer) and then sequenced. The resulting nucleotide sequences were deposited in the National Center for Biotechnology Information (NCBI) GenBank (Table 1) and were compared with known sequences from the NCBI database using the basic local alignment search tool (BLAST) algorithm [2].

**Preparation of the cell-free supernatant**

The strain KNUC25 was cultured in MRS broth at 25°C for 24 h and centrifuged at 10 000 rpm at 4°C for 30 min. The supernatant was then sterilized by filtration through a 0.22-μm pore-size filter (Millipore Corp., Billerica, MA, USA). The cell-free supernatant of KNUC25 was frozen at -75°C for more than 24 h and then lyophilized. The cell-free supernatant was then dissolved in 1× PBS and used to investigate antibacterial activity against various coliform indicator strains.

**Antibacterial activity test**

The cell-free supernatant of KNUC25 was screened for antibacterial activity with the spot-on-the-lawn test [6, 14, 19]. The coliform indicator strains were grown in 5-ml LB

**Table 1. Coliform bacterial strains isolated from kimchi.**

| Strain | Homologous microorganism (% identity)[a] | GenBank accession no. |
|---|---|---|
| KNUC173 | *Enterobacter intermedius* (99%) | EF474081 |
| KNUC174 | *Ent. intermedius* (99%) | EF474082 |
| KNUC175 | *Ent. intermedius* (99%) | EF474083 |
| KNUC176 | *Ent. cloacae* (99%) | EF474084 |
| KNUC177 | *Ent. intermedius* (99%) | EF474085 |
| KNUC178 | *Ent. intermedius* (99%) | EF474086 |
| KNUC179 | *Ent. intermedius* (99%) | EF474087 |
| KNUC180 | *Klebsiella ornithinolytica* (99%) | EF474088 |
| KNUC181 | *K. oxytoca* (99%) | EF474089 |
| KNUC182 | *Ent. intermedius* (99%) | EF474090 |
| KNUC183 | *Ent. amnigenus* (99%) | EF474091 |
| KNUC184 | *Hafnia alvei* (99%) | EF474092 |
| KNUC185 | *Ent. intermedius* (99%) | EF474093 |
| KNUC186 | *Ent. intermedius* (99%) | EF474094 |
| KNUC187 | *K. ornithinolytica* (100%) | EF474095 |
| KNUC188 | *K. ornithinolytica* (100%) | EF474096 |
| KNUC189 | *K. terrigena* (100%) | EF488764 |

[a] The multiple alignments for 16S rRNA gene full sequences of isolated strains were performed by the BLAST search.

broth until the optical density at 600 nm ($OD_{600}$) reached between 0.3-0.4; the cultures were then spread onto an LB agar plate. The 10 μL of 30-fold concentrated cell-free supernatant was spotted onto lawns of the coliform indicator strain cultures and incubated at 37°C for at least 4 h. Antibacterial activity was determined by the presence of a clear zone (2 mm or more) of growth inhibition of the indicator strain around the spot.

**Viable cell test**

A viable cell test was performed by modifying a previously described method [5, 8, 17]. Coliform bacteria were grown in LB broth until the $OD_{600}$ reached between 0.3-0.4. The coliform bacteria culture was then divided into 96-well plates. Into each well, 10 μL of the cell-free supernatant of KNUC25, with or without adjustment of pH, was added, and the OD at 620 nm was measured. After treatment with the cell-free supernatant of KNUC25, the coliform bacteria were cultured at 37°C for 5 h. The pH of the initial cell-free supernatant was pH 4.5 and adjusted to pH 7.0 using 3-M Tris buffer. One percent (v/v) lactic acid was used as control since the level of lactic acid in commercial kimchi after proper fermentation process is equivalent to 1% lactic acid.

**Preparation of the bacterial specimen for scanning electron microscope observation**

The isolates were cultured in 5 mL of LB broth to an $OD_{600}$ of between 0.5 and 0.6 and then harvested by centrifuging at 4°C for 5 min at 8000 rpm. Cell pellets were washed 2-3 times with 0.1-M potassium phosphate buffer (pH 7.2), treated with 2.5% glutaraldehyde, and kept on ice for 90 min. They were subsequently centrifuged at 4°C for 5 min at 8000 rpm, and the supernatants were removed by gentle pipetting. The cell pellets were washed 2-3 times with 0.1-M potassium phosphate buffer (pH 7.2), treated with 1% $OsO_4$ solution, and kept at room temperature for 1 h. The centrifugation and washing steps were then repeated once more. Next, dehydration of the bacterial specimens were carried out sequentially using 50%, 70%, 80%, 90%, 95%, and 100% ethanol. For the last step, bacterial specimens were treated with iso-amyl acetate and kept at room temperature for 1 h. The removal of the supernatant was performed by centrifuging. The bacterial specimens were frozen at -70°C and then lyophilized. After platinum coating (20 mA, 90 s), the morphology of the bacterial specimens were finally observed using the field emission (FE)-scanning electron microscope (SEM) S-4300 (Hitachi, Hitachi City, Japan).

**Measurement of the pH and coliform bacteria count in commercial kimchi**

The pH of the commercial kimchi that was stored at 5°C and 10°C was measured every 3 days for 27 days in the presence/absence of 10 ml of the cell-free supernatant of KNUC25. Kimchi was ground and filtered, and the pH of the kimchi was then determined by pH meter (Radiometer, Lyon, France). Coliform bacteria from the commercial kimchi were enumerated on Petrifilm™ *E. coli* Count Plates after spreading. The spread Petrifilm™ plates were incubated at 35°C for 24 h.

## Results

**Partial identification of coliform bacteria isolated from commercial kimchi**

Coliform bacteria showed a broad diversity of genera and species, whether they belonged to the family *Enterobacteriaceae* or not. The definition of coliform bacteria is based essentially on common biochemical characteristics. They are facultative anaerobic, gram negative, nonspore-forming, and rod-shaped. Some forms ferment lactose with gas and acid formation within 48 h at 35°C or will develop a red colony with a metallic sheen within 24 h at 35°C on endo-type medium containing lactose [22]. It is generally known that the genera to which coliform bacteria belong include *Escherichia*, *Citrobacter*, *Enterobacter*, and *Klebsiella* [20].

The coliform isolates, which were from commercial kimchi that had not undergone the fermentation process, were classified for the most part as one of seven different species from three genera by 16S rDNA sequence analysis as follows: *Enterobacter intermedius*, *Ent. cloacae*, *Ent. amnigenus*, *Klebsiella terrigena*, *K. ornithinolytica*, *K. oxytoca*, and *Hafnia alvei* (Table 1). These coliforms are often recovered from groundwater and soil; therefore, we believed that the isolated coliforms may have come from soil since kimchi vegetables, such as cabbages, green onions, garlic, and ginger, are cultivated in soil.

The genus *Hafnia* is one of more than 40 genera that currently comprise the family *Enterobacteriaceae*. Although this genus was described over 50 years ago and has been

recovered from various sources such as mammals, birds, fish, soil, water, and foods, very little is known about these organisms with regard to their characteristics and roles as both human and veterinary pathogens [10, 11]. It has been reported that vegetables do not appear to be a frequent reservoir for these bacteria [10]; nevertheless, they were recovered from the commercial kimchi in this study.

As mentioned above, some harmful bacteria in commercial kimchi have been reported by Shin *et al.* [24]. However, in that study, the authors simply isolated some harmful bacteria from commercial kimchi by using a selection medium and did not identify the isolates in detail. In our study, 17 different coliform bacteria from commercial kimchi were isolated by using a selection medium and also partially identified by 16S rDNA sequence analysis. Therefore, this research may represent the first instance of a molecular biological approach applied to the investigation of bacteria in commercial kimchi. The 16S rDNA sequences of all coliform bacteria were deposited in the NCBI GenBank database with accession numbers as identified in Table 1.

**Inhibition spectrum of the cell-free supernatant of KNUC25**

Previously, *L. paraplantarum* KNUC25 was isolated from over-fermented whole Chinese cabbage kimchi and misidentified as *Leuconostoc carnosum* by 16S rDNA sequencing in 2003 [1]. After an accurate reappraisal in 2005, it was determined that the 16S rDNA of the KNUC25 strain had a 99.2% identity with rDNA from *L. paraplantarum* TKR17B (accession number: AJ878739) [9]. Moreover, comparison of the whole-cell protein patterns in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with other *Lactobacillus* species using the API 50 CHL system assay offered a complementary identification of KNUC25 as *L. paraplantarum* [12]. The 16S rDNA nucleotide sequence of KNUC25 was deposited to the NCBI GenBank (accession number: EF20067).

The strain KNUC25 has shown antibacterial activity against various Gram-positive or Gram-negative indicators [1]. In this study, we focused on the antibacterial activity of KNUC25 against Gram-negative coliform bacteria. The cell-free supernatant showed antibacterial activity against all indicator strains, including the 17 coliform isolates (Table 2). In particular, *S. enteritidis* ATCC13076 was significantly inhibited.

**Table 2. Antibacterial activity spectrum of the cell-free culture supernatant of *L. paraplantarum* KNUC25.**

| Indicator strain | Antibacterial activity[a] |
|---|---|
| Coliform bacteria from kimchi | |
| *Enterobacter intermedius* KNUC173 | ++ |
| *E. intermedius* KNUC174 | +++ |
| *E. intermedius* KNUC175 | ++ |
| *E. intermedius* KNUC177 | +++ |
| *E. intermedius* KNUC178 | ++ |
| *E. intermedius* KNUC179 | ++ |
| *E. intermedius* KNUC182 | +++ |
| *E. intermedius* KNUC185 | ++ |
| *E. intermedius* KNUC186 | ++ |
| *E. cloacae* KNUC176 | +++ |
| *E. amnigenus* KNUC183 | + |
| *K. ornithinolytica* KNUC180 | + |
| *K. ornithinolytica* KNUC187 | ++ |
| *K. ornithinolytica* KNUC188 | + |
| *K. oxytoca* KNUC181 | ++ |
| *K. terrigena* KNUC189 | ++ |
| *Hafnia alvei* KNUC184 | + |
| Pathogenic coliform bacteria | |
| *Escherichia coli* ATCC25922 | ++ |
| *Citrobacter freundii* ATCC6750 | ++ |
| *K. pneumoniae* ATCC13883 | +++ |
| *Ent. cloacae* ATCC13047 | ++ |
| *Ent. aerogenes* ATCC29751 | ++ |
| *Erwinia carotovara* ATCC15390 | ++ |
| Food-poisoning bacteria | |
| *Salmonella enteritidis* ATCC13076 | +++ |
| *Shigella sonnei* ATCC25931 | ++ |

[a] Comparative diameter size (mm) of clear zone: +, 2≤φ<3; ++, 3≤φ<4; +++, 4≤φ<5.

**Viable cell test**

Both natural cell-free supernatant of KNUC25 (pH 4.5) and cell-free supernatant adjusted to pH 7.0 inhibited the growth of coliform indicator strains (Fig. 1). The antibacterial activity of natural cell-free supernatant was better than 1% lactic acid and was sustained, lasting for several days at room temperature (data not shown). Although the cell-free supernatant adjusted to pH 7.0 showed antibacterial activity, the effect was slight and was maintained for only about 3 h, because antibacterial reaction conditions might not have been optimal at pH 7.0.

**Morphological changes of indicator strains treated with the supernatant of KNUC25**

A SEM was used to observe the morphological changes of coliform indicator strains after treatment with the cell-

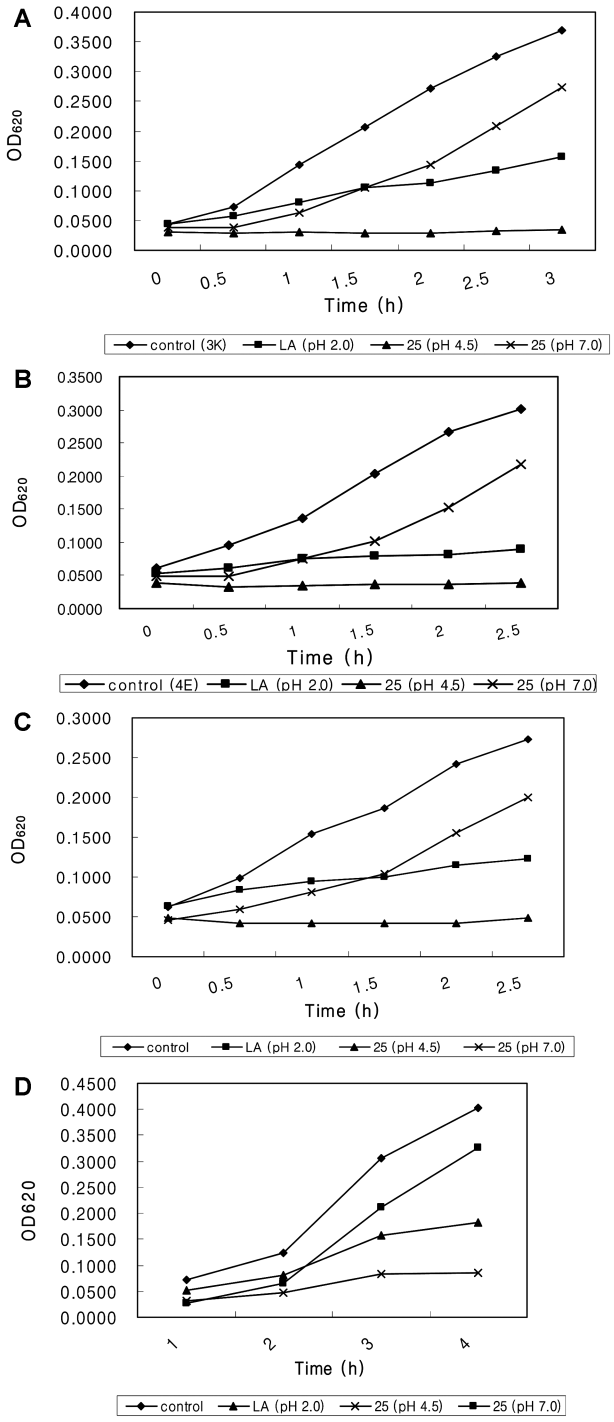


**Fig. 1. Growth inhibition of coliform bacteria by treatment with KNUC25.** Panel **A**, growth inhibition of *K. pneumoniae* ATCC13883; Panel **B**, growth inhibition of *Ent. cloacae* ATCC13047; Panel **C** growth inhibition of coliform bacteria (*Ent. cloacae* KNUC176) isolated from commercial kimchi; Panel **D** growth inhibition of *S. enteritidis* ATCC13076. In each panel, (◆) represents growth of nontreated coliform bacteria; (■) represents coliform bacteria treated with 1% lactic acid (pH 2.0); (▲) represents coliform bacteria treated with the cell-free supernatant of KNUC25 (pH 4.5); (×) represents coliform bacteria treated with the cell-free supernatant of KNUC25 (pH 7.0).

free supernatant of KNUC25. Before treatment, all coliform strains were a typical rod-shape. After treatment with the cell-free supernatant (both at pH 4.5 and pH 7.0) of the KNUC25 strain, the morphologies of coliform strains were severely twisted or burst (data not shown). This result prompted us to theorize that factors other than pH might have an affect on cell morphology change. In particular, the bursting of coliform bacteria cells indicated that these other factors may play roles in the formation of pores on the cell surface of coliform bacteria.

**Application of *L. paraplantarum* KNUC25 to commercial kimchi**

The culture supernatant of *L. paraplantarum* KNUC25 was added to the kimchi in the manufacturing stage. The change in pH and in the number of coliform bacteria in the kimchi was observed at intervals of 3 days for 27 days. Although there was only a slight difference in the pH of nontreated kimchi and KNUC25-treated kimchi in the early

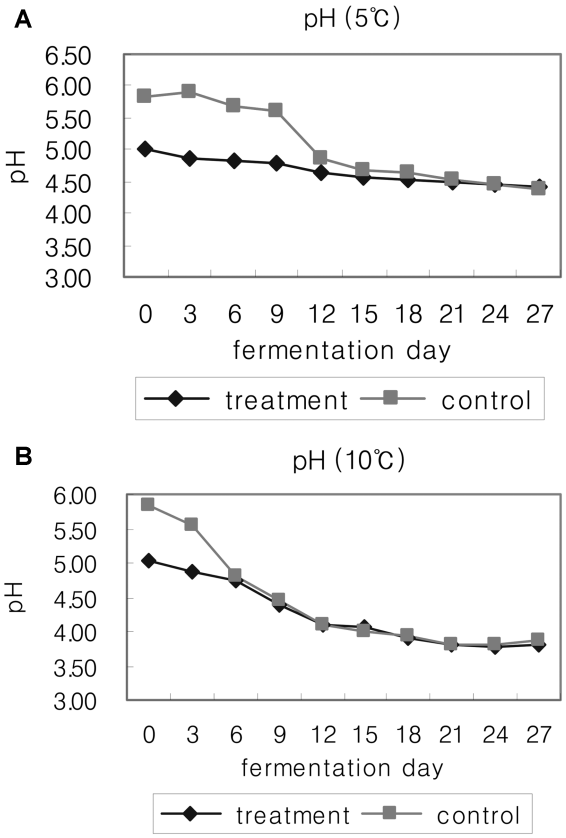


**Fig. 2. Changes in the pH of kimchi during storage at 5°C and 10°C.** Panels **A** and **B** show the changes in the pH of kimchi at 5°C and 10°C, respectively. (◆) represents treatment (commercial kimchi added culture of KNUC 25); (■) representscontrol (commercial kimchi).

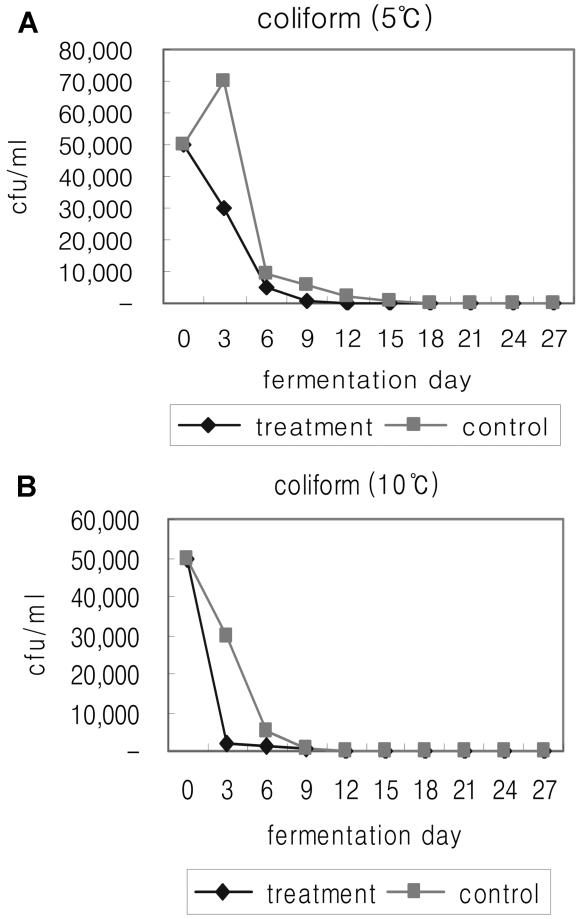


**Fig. 3. Changes in the number of coliforms in kimchi during storage at 5°C or 10°C.** Panels **A** and **B** show the changes in the number of coliforms of kimchi at 5°C and 10°C, respectively. (◆) represents treatment (commercial kimchi added culture of KNUC 25); (■) represents control (commercial kimchi); cfu, colony forming units.

stages of fermentation, the pH values became equal at 9-15 days after treatment and did not affect the taste of the kimchi (Fig. 2). Thus, a change of pH in the kimchi during the manufacturing stage should not cause problems with flavor in the production of commercial kimchi.

We found that the amount of coliform bacteria in kimchi treated with the culture of *L. paraplantarum* KNUC25 was remarkably decreased during early stage preserving, when compared with that in untreated kimchi (Fig. 3); in particular, the number of coliform bacteria in kimchi treated with the culture of KNUC25 during storage at 5°C was reduced to 10 colony forming units (cfu)/ml. Therefore, *L. paraplantarum* KNUC25 could be very useful and effective in controlling sanitary conditions during the processing of commercial kimchi.

## Discussion

The natural cell-free supernatant of KNUC25 showed strong and long-lasting antibacterial activity against various coliform bacteria, from pathogenic coliforms to the coliforms isolated from commercial kimchi in this study.

It is generally known that nisin is one of the most effective bacteriocins currently available, and it is the only substance that can be used as a food preservative in more than 50 countries [18, 23]. However, nisin does not control Gram-negative bacteria, yeasts, or molds [21]. Though many kinds of bacteriocins that work effectively on Gram-positive bacteria have been reported, there appear to be no antibacterial materials similar to bacteriocin that work on Gram-negative bacteria [26].

The control of coliform bacteria is very important for sanitary reasons and because coliform bacteria are an index of fecal contamination [22]. By using the KNUC25 cell culture during fermentation, it was possible to decrease the number of coliform bacteria in kimchi during this phase of manufacturing. Thus, kimchi must go through primary fermentation in order to reduce coliform bacteria damage. In the traditional method of preparing kimchi, it is usually stored at a low room temperature after primary fermentation and may serve to control coliform bacteria.

In the manufacturing plant, completing the primary fermentation phase could cause other problems related to the distribution and shelf life of commercial kimchi. If commercial kimchi is distributed after fermentation, the expiration date of the kimchi would be much earlier. However, quite a number of harmful bacteria, including coliform bacteria, could still be in commercial kimchi even after fermentation [24]. Although the coliform bacteria could be removed by mass-producing kimchi with the addition of the KNUC25 cell culture during fermentation, the kimchi might still have serious defects with regard to long-term preservation. However, by adding the cell-free supernatant, these defects could be solved. To keep a special method of long-term kimchi storing for a commercial kimchi company must be very advantageous. Therefore, the antibacterial activity of *L. paraplantarum* KNUC25 against coliform bacteria has very important implications for hygienic control in the manufacturing of kimchi. Furthermore, if the antibacterial components of KNUC25 could be purified, they could be used as effective food preservatives not only for kimchi, but also for other foods

that are mass-produced.

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**국문초록**

## 상업용 김치 생산과정에서 대장균유사세균의 발생과 억제

**이수진 · 한태원 · 김마리 · 설경조 · 박유미 · 진익렬 · 김사열***
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김치 소비가 증가하면서 공장에서 큰 규모로 만들어지게 되었다. 그래서 상업용 김치의 위생 상태는 주 관심사가 되었다. 충분히 발효되지 않은 상업용 김치로부터 17종의 대장균유사세균(coliform bacteria)을 분리하였다. 16S rDNA 분석에 의하여 *Enterobacter intermedius, Ent. cloacae, Ent. amnigenus, Klebsiella terrigena, K. ornithinolytica, K. oxytoca, Hafnia alvei* 등의 3속 7종으로 부분 동정되었다. 한편, 오래된 배추김치로부터 *Lactobacillus paraplantarum* KNUC25라고 부분 동정된 젖산균이 분리되어 그의 항미생물 활성이 보고되었다. 이 균주는 spot-on the lawn test와 세포생존실험을 통하여 본 연구에서 분리한 대장균유사세균과 몇몇 유해한 대장균유사세균에 대하여 항세균 활성을 보여 주었다. 상업용 김치에 있는 대장균유사세균은 *L. paraplantarum* KNUC25균주 배양 상층액의 첨가에 의하여 효과적으로 억제되었다.