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Isolation, Characterization, and Control of *Pseudomonas kribbensis* and *Pantoea vagans* that cause Soft-rot Disease Isolated from Chinese Cabbages

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ABSTRACT - The bacterial soft-rot disease is one of the most critical diseases in vegetables such as Chinese cabbage. The researchers isolated two bacteria (*Pseudomonas kribbensis* and *Pantoea vagans*) from diseased tissue samples of Chinese cabbages and confirmed them as being the strains that cause soft-rot disease. Lactic-acid bacteria (LAB), were screened and used to control soft-rot disease bacteria. The researchers tested the treatments with hypochlorous acid water (HAW) and LAB supernatant to control soft-rot disease bacteria. The tests confirmed that treatments with the HAW (over 120 ppm) or LAB (*Lactobacillus plantarum* PL203) culture supernatants (0.5 mL) completely controlled both *P. kribbensis* and *P. vagans.*

Key words: Soft-rot disease, *Pseudomonas kribbensis*, *Pantoea vagans*, Hypochlorous acid water, Antimicrobial activity

Bacterial soft-rots diseases are caused by several soil-borne bacteria of but most commonly by gram-negative bacteria such as *Pectobacterium* sp., *Erwinia* sp., *Pseudomonas* sp., and *Pantoea* sp. It is a dangerous disease of plants such as fruits, flowers, and vegetables, such as Chinese cabbage, potato, and tomato found worldwide[1-4]. The disease occurs under high temperature and high humidity conditions. It is one of the diseases that are very difficult to control. Soft-rot pathogens survive in infected organ groups in storage and in the field, in debris, on roots or other parts of host plants, in ponds and streams used for water irrigation, occasionally in the soil, and several insects. When soft-rot disease bacteria enter wounds of plant, they feed at first on the liquids released by the broken cells on the wound surface of plant tissues. They produce pectolytic enzymes that break down the pectic substances of the plant tissues[5,6].

Chinese cabbage is one of the most important vegetables as the main ingredient for Kimchi, a traditional fermented food in Korea. It is generally harvested from the summer to winter seasons. However, Chinese cabbage harvested during the summer is difficult to store for longer than a month, because of high temperatures and wet weather[7,8]. Especially, it is a raw material whose price fluctuates depending on the conditions of the crop. For example, during the rainy season in July and August, the prices of Chinese cabbages may rise significantly because of supply and demand instability. Therefore, for the industrial production of Kimchi, a stable supply of Chinese cabbage is necessary throughout the year. In order to supply enough Chinese cabbage, much research, such as the development of storage technology for Chinese cabbage, is needed. Recently, antibiotics have been the main ingredient in the control pharmaceuticals for Chinese cabbage soft-rot diseases; these may have caused antibiotic resistance in the Chinese cabbages that we eat as food[9]. Therefore, it is necessary to study the control methods using effective materials.

In Korea, research on control methods for soft-rot disease of Chinese cabbage by functional oils has been partially reported; however, studies on the control of bacteria that cause soft-rot disease are still insufficient. Microbiological control is one of the most effective methods for the control of plant disease, including soft-rot disease. Previous studies revealed some bacteria, such as *Bacillus, Actinomycete*, and *Serratia*, which may be useful as microbiological control against plant pathogens[3,10-14]. Antimicrobial substances derived from lactic acid bacteria (LAB) such as bacteriocin have a number of positive properties such as high thermal

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stability and activity in wide pH range for various applications. They are colorless, odorless and tasteless, so they are good for use in the food industry[15]. Despite the long time use of antimicrobial substances of LAB, there have been no reports on the resistant bacteria.

Hypochlorous acid water (HAW) is a typical chlorine-based disinfectant water produced by peroxidation of chloride ions. It showed a strong and broad spectrum of antimicrobial activity against microorganisms, such as bacteria and fungi; with an available chlorine concentration, it has the advantage of being less corrosive for equipment, less irritating for the human body, and having less impact on the environment[16-18]. However, in the presence of organic matter, its antimicrobial activity is known to decrease. Especially, the HAW is unstable, especially when exposed to sunlight and contact with air, so it cannot remain after sterilization even when used on vegetables such as Chinese cabbages[19]. Actually, in kimchi manufacturing, during washing of ingredients such as cabbage, the HAW is mostly removed. Hypochlorous acid has been approved by the U.S. FDA for use as disinfectant, food additive, or sanitizer in the food industry. In Korea, HAW is used to wash fruits and vegetables, and the maximum concentration is 200 ppm[20-22].

In this study, we isolated and identified the soft-rot disease bacteria from the soft-rot diseased Chinese cabbage samples during storage of Chinese cabbage and investigated the basic characteristics of these bacteria. Finally, two bacteria and one fungus were isolated as causes of soft-rot disease; we identified them as *Pseudomonas kribbensis*, *Pantoea vagans* and *Penicillium oxalicum*. In order to find a method to control these soft-rot disease bacteria, the control effect was verified by using HAW and natural antimicrobial substances produced by LAB (*Lactobacillus plantarum*). We intend these results to be used as the data related to the development of Chinese cabbage storage technology in the future. There are no research data on soft-rot disease by *P. kribbensis* and *P. vagans* in previous studies.

Materials and Methods

Isolation and identification of soft-rot disease bacteria

Soft-rot disease bacteria were isolated from Chinese cabbages (Haenam and Cheongju cities, Korea) that had been stored for 12-15 weeks in a kimchi manufacturing factory. We cut fresh and diseased tissue samples of Chinese cabbage into small pieces (about 10 cm × 10 cm). The cut samples were homogenized with 9 mL of diluted saline (3M Diluent) and were plated onto Plate Count Agar (PCA; DB, Franklin Lakes, NJ, USA), Nutrient agar (NA; Oxoid Ltd, Hampshire, UK) for aerobic bacteria and Dichloran-Rose Bengal-Chloramphenicol Agar (DRBC agar; DB, Franklin Lakes, NJ, USA) for yeast and fungi. The PCA and NA plates were incubated at 35°C for 24-48 h, and DRBC media were incubated at 25°C for 72-96 h. After incubation, we selected various colonies according to the characteristics of the colonies, such as shape, color, and size.

The soft-rot disease bacteria were selected from the soft-rot diseased tissue samples using Dong's modified method[23]. Normal Chinese cabbage was cut into sizes of about 3.0 cm × 3.0 cm, washed once with sterile distilled water, and used for control test of soft-rot disease bacteria using HAW and supernatants of LAB. We dispensed 0.5 mL of the isolated bacterial culture (soft-rot disease bacteria, about 5.0×10^5 cfu/g of cabbage) onto the Chinese cabbage; The samples were incubated at 25°C for 3-6 days to confirm the outbreak of soft-rot disease.

The isolated strains were identified by 16s ribosomal RNA (16s rRNA) gene sequencing by polymerase chain reaction (PCR). We did PCR using the primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The conditions of PCR were 40 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min. We confirmed the 1400 base pairs of PCR-amplified products by agarose gel electrophoresis and identified nucleotide sequences at Macrogen Inc. (Seoul, Korea). We used the nucleotide BLAST programs to identify homologous 16S rRNA gene sequences in the database (http://www.ncbi.nlm.nih.gov/BLAST).

Growth properties of soft-rot disease bacteria

P. kribbensis and *P. vagans* were grown in nutrient broth (NB) for 24 h at 35°C with shaking and inoculated into 100 mL of fresh NB with different growth conditions of initial pH of broth (2.0–9.0) and temperature(5-45°C). The bacterial cultures were incubated for 96 h and the OD_{600} values (Absorbances) were measured during cultivation using INFINITE 200 PRO microplate reader (TECAN; Grödig, Austria)

Isolation and screening of antimicrobial activity LAB against soft-rot disease bacteria

We isolated antibacterial LAB strains from various fermented foods (various type of kimchi, cheese), fruits, and vegetables. The samples were homogenized with sterile distilled water and spread onto de Man, Rogosa, and Sharpe (MRS; DB, Franklin Lakes, NJ, USA) agar medium. After incubation for 48 h at 30°C, the various colonies were collected as LAB and used. The LAB strains were cultured in MRS broth for 24 h at 30°C (the total LAB counts were $3.00 - 7.40 \times 10^9$ cfu/mL in MRS broth at 24 h), and the cultures were centrifuged at 8,000 rpm for 20 min at 4°C.

The supernatants were filtrated using a 0.22-μm syringe filter (Pall Corporation, Fajardo, Puerto Rico) and neutralized by adding 10 N NaOH solution. The supernatants of LAB were used in this test.

We modified the agar-well diffusion method to detect antimicrobial activities of supernatants isolated from LAB strains[24]. We added 1.0 mL of the indicator strain culture to 10 mL of 0.7% tryptic soy agar medium (TSA; Merck, Darmstadt, Germany). The indicator mixtures were poured on top of the TSA medium (1.5% agar), which was completely solidified. We then prepared 6-8 mm diameter wells and loaded cell-free supernatants from isolated LAB in the wells (100 μL/well). After a 24-h incubation at 35°C, we measured clear zones (inhibition zone). In this test, two soft-rot disease bacteria (*P. kribbensis* and *P. vagans*, the strains isolated in this study) and various pathogen indicator strains (*Listeria monocytogenes* NCCP14714, *Escherichia coli* O157:H7 NCCP14541, *Staphylococcus aureus* NCCP14780, *Salmonella enteritidis* NCCP12243, *Bacillus cereus* NCCP16296; The strains were distributed in the Korean Disease and Prevention Agency and used for experiments). As a result, various antibacterial active LAB were isolated, and one of them, *L. plantarum* PL203 strain, was used for this test.

The natural antimicrobial substance produced by LAB was tested for resistance against heat treatments (80°C, 90°C, 100°C for 30 min and 121°C for 15 min). The 5 mL of supernatants of *L. plantarum* PL203 was injected into a 10 mL glass test tube, treated at each temperature and time conditions, and examined using the agar-well diffusion method mentioned above.

Control of soft-rot disease bacteria using HAW and LAB supernatant

To control the soft-rot disease bacteria of Chinese cabbages, we did the test using HAW treatment and supernatant of *L. plantarum* PL203, as in the way used for inoculating the cabbage samples to find the bacteria of soft rot disease. Fresh Chinese cabbage was cut into 3.0 cm × 3.0 cm pieces, which were washed once with sterile distilled water and used in this test. We dispensed 0.5 mL of the soft-rot bacteria (about 5.0×10^5 cfu/g of cabbage) onto the Chinese cabbages, stored them at room temperature for 30 min, and inoculated each of them with antimicrobial *L. plantarum* PL203's supernatants (0.5 mL) and HAW (80, 120, and 200 ppm).

The inoculated sample were incubated at 25°C for 5 days to confirm the occurrence of soft-rot disease. The quantitative reduction levels of soft-rot disease bacteria were confirmed by microbial analysis of each sample. We used the culture supernatant of *L. plantarum* PL203 after calibrating it to pH 7.0 with 10 N NaOH.

Results and Discussion

Isolation and identification of soft-rot disease bacteria

As shown in Fig. 1, the stem tissue of soft-rot disease Chinese cabbages turned brown and softened. We did microbial analysis and isolated bacteria from the soft-rot disease tissue and fresh tissue samples, from which we found that the aerobic bacteria in the soft-rot disease samples (2.50×10^8 cfu/g) were about 100-fold higher those in the fresh tissue samples (3.10×10^6 cfu/g). The fungi were also detected at about 100-fold higher in the soft-rot disease samples (1.20×10^6 cfu/g) than those in the fresh tissue samples (1.00×10^4 cfu/g). Twenty bacteria and five fungi isolated based on their shape, size, and color from soft-rot disease samples, respectively. When they were inoculated and cultured in fresh Chinese cabbage samples, it was confirmed that the two strains of bacteria and one of fungi directly caused the soft-rot disease of Chinese cabbages, which began to appear at 48 hours and 72 h after inoculation of the selected bacteria and fungus, respectively. The two strains of soft-rot disease bacteria were identified as *P. kribbensis* and *P. vagans* and the fungus was identified as *Penicillium oxalicum* (The results of the fungus' data were not showed in this study). The bacteria associated with cabbage soft-rot disease were mostly *Pectobacterium* sp. and *Erwinia* sp.[2,24], but *P. kribbensis* and *P. vagans* did not have any research data related to soft-rot disease of Chinese cabbage. In addition, we confirmed that the soft-rot disease bacteria were originated from soil. When the microbial analysis of the soil samples of the Chinese cabbage production area in Haenam and Cheongju in Korea was done, the same microbes such as *P. kribbensis* and *P. vagans* were detected. In other previously papers, *P. kribbensis* and *P. vagans* were shown to be species that have been mostly isolated from natural resources such as soil[25,26]. *P. kribbensis* was confirmed to have white colonies and be aerobic, gram-negative, rod-shaped, non-spore forming, motile, and catalase positive[25]. *P. vagans* was also gram-negative, short rod-shaped, motile, non-spore forming, oxidase-negative and facultatively anaerobic, with beige to yellow colonies[27].

Growth properties of soft-rot disease bacteria

We examined the growth of two strains at different temperatures and initial pH conditions. As shown in Fig. 2 (A), *P. vagans* strains grew rapidly at 25-45°C and reached the highest absorbance values (OD_{600} = 1.3-1.5) in 12 h. The strain did not grow at 5°C, and at 15°C, it grew at reduced rates. On the other hand, *P. kribbensis* strains grew rapidly at 15°C and reached the highest absorbance values (OD_{600} = 1.1) in 12 h, but it did not grew at 45°C (Fig. 2, B). That *P.*

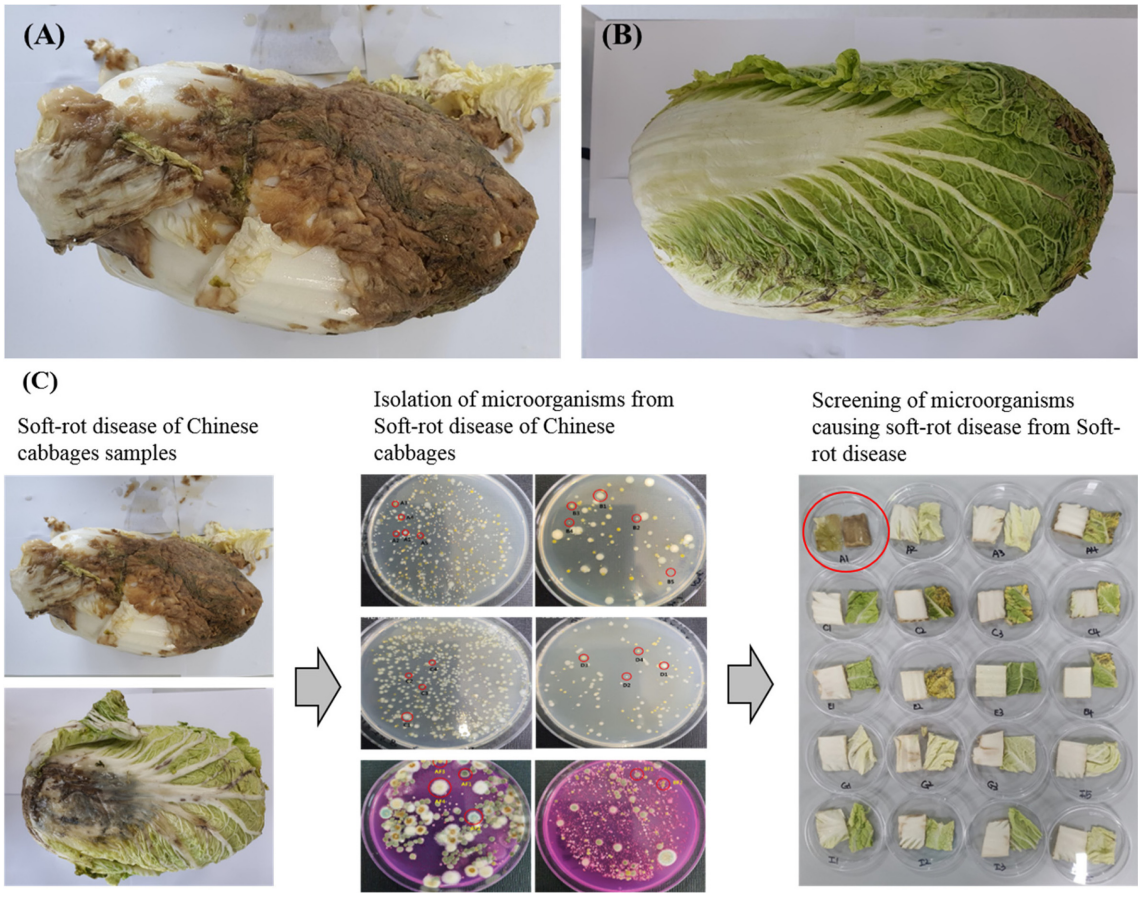

Fig. 1. Symptoms of soft-rot disease of Chinese cabbages at 95% of humidity after 12-15 weeks of storage period. (A) The stem tissue of soft-rot disease (brown-color and softness). (B) Normal tissue of Chinese cabbages after 12-15 weeks of storage times. (C) Screening of bacteria causing soft-rot disease from soft-rot disease of Chinese cabbages.

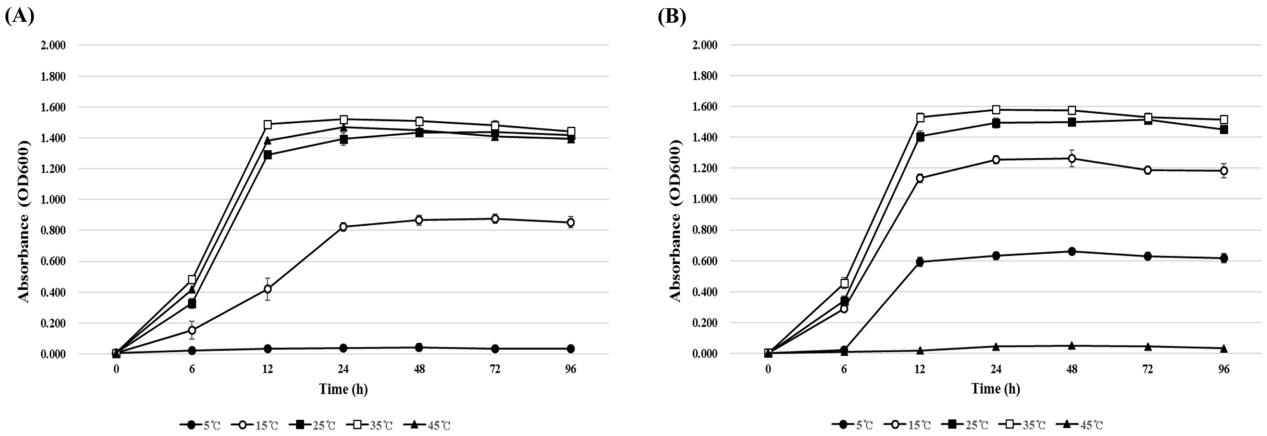

Fig. 2. Growth of soft rot disease bacteria at different temperatures for 96 h. (A) *P. vagans* (B) *P. kribbensis* ●, 5°C; ○, 15°C; ■, 25°C; □, 35°C; ▲, 45°C.

kribbensis can grow at lower temperatures such as 5°C, related to the general storage of Chinese cabbage at a low temperature, so the degradation of cabbage quality is possible.

As shown in Fig. 3, *P. kribbensis* can grow at initial pH 6.0-10.0 conditions, and *P. vagans* can grow at an initial pH of 4.0-10.0 at 35°C. *P. kribbensis* grew rapidly at initial pH of 6.0-9.0 and reached the highest absorbance values (OD_{600}

= 1.3-1.5) (It did not grow under conditions of pH 3.0 and 4.0, and it is not shown in the figure). *P. vagans* grew rapidly at initial pH of 5.0-9.0 and reached the highest absorbance values (OD_{600} = 1.3-1.5). However the strains did not grow at initial pH of 4.0 or 11.0. These results suggest that treatment with materials of pH 4.0 or less may help control the soft-rot disease bacteria.

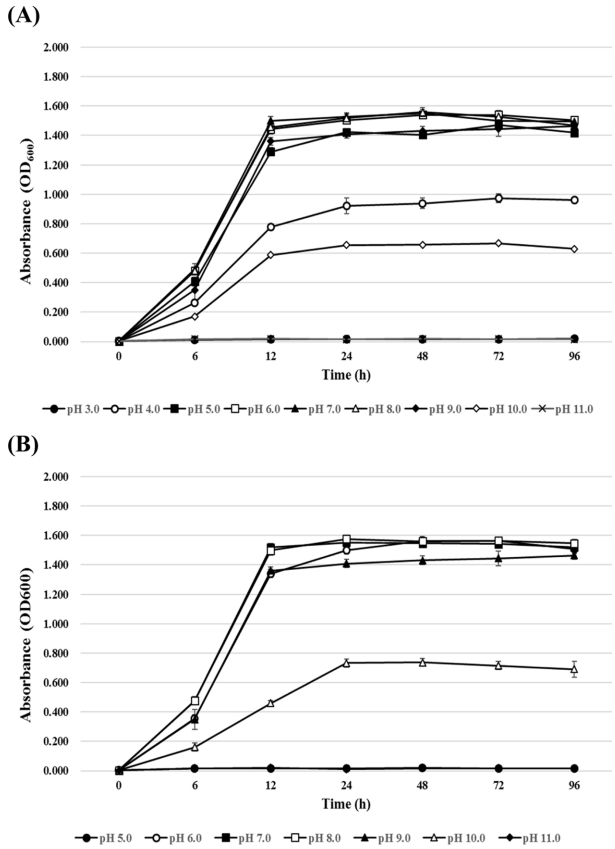

Fig. 3. Growth of soft rot disease bacteria at initial pH conditions at 35°C during 96 hours: (A) *P. vagans* (B) *P. kribbensis* ●, pH 5.0; ○, pH 6.0; ■, pH 7.0; □, pH 8.0; ▲, pH 9.0; △, pH 10.0; ◆, pH 11.0.

Selection of antimicrobial activity LAB strains against soft-rot disease bacteria

We screened 754 LAB strains that were isolated from various fermented foods and agricultural raw materials for antimicrobial activity against several pathogens. Eight LAB strains showed antibacterial activity; among them, PBio203, isolated from *Angelica keiskei*, showed antibacterial activity against soft-rot disease bacteria (Fig. 4). *Angelica keiskei* is a hardy perennial, belonging to the family *Umbelliferae*, and is called 'Myeong Il Yeob' in Korea. *Angelica keiskei* has traditionally been used as a tonic, diuretic, and mild cathartic. It is mainly distributed in Asian countries such as Korea and Japan, and it is used as a raw material of green vegetable juice for daily healthy food[28,29].

In addition, the strain had antimicrobial activity against *Bacillus cereus* and *Listeria monocytogenes* (data not shown). The strain was identified as *L. plantarum* by 16S rRNA gene sequencing and was tested as a method for control of soft-rot disease bacteria in this study. There have been many studies on bacteria that have antibacterial activity against gram-negative bacteria and pathogenic bacteria. Wang et al.[4] reported that *L. plantarum* isolated from Tibetan yaks could inhibit the growth of *Escherichia coli* and *Staphylococcus aureus*. *Lactobacillus plantarum* BY showed antibacterial activity against soft-rot disease bacteria such as *Pectobacterium* sp. It significantly reduced soft-rot disease in onion, tomato, and potato[11].

Controls of soft-rot disease bacteria using HAW and antimicrobial substances

We tested treatment of HAW and antimicrobial activity of LAB (*L. plantarum* PL203) supernatant to control soft-rot disease bacteria such as *P. kribbensis* and *P. vagans*. The tests confirmed that the HAW treatment completely

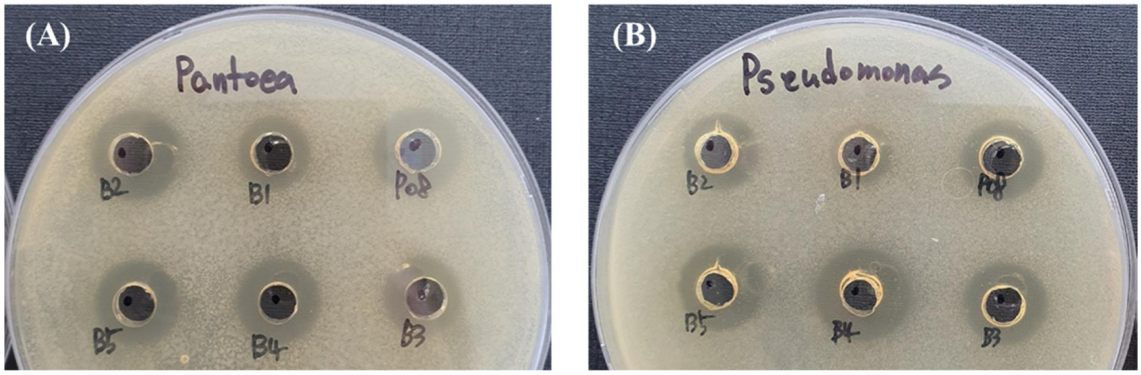

Fig. 4. Selection of antimicrobial activity LAB strains against soft-rot disease bacteria. The B4 labeled strain is the *Lactobacillus plantarum* PL203 strain used in this study. Clear zones were formed around supernatant of LAB strains on a BHI agar medium containing soft-rot disease pathogens, *P. vagans* (A) and *P. kribbensis* (B).

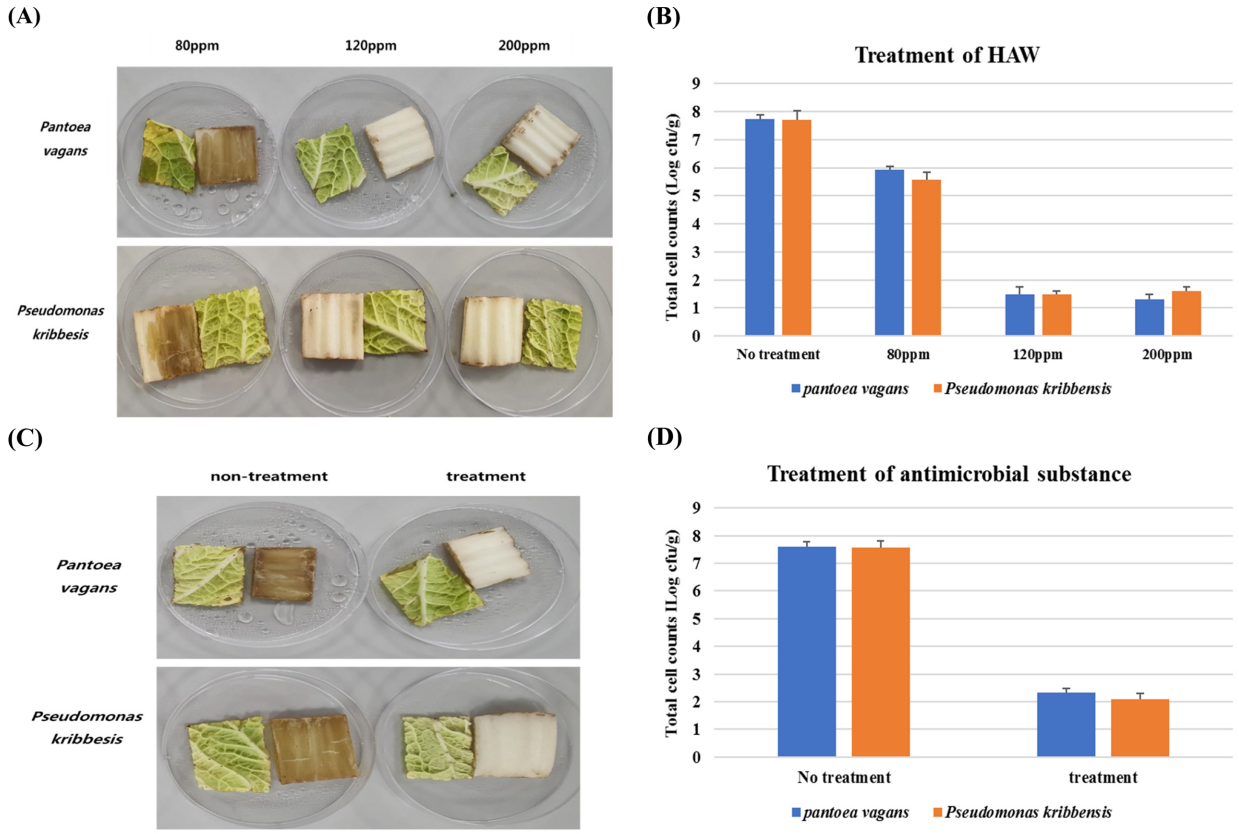

Fig. 5. *In vitro* assay for control methods of soft rot disease. Fresh Chinese cabbage pieces (3.0 cm×3.0 cm) were washed with sterile distilled water. Soft-rot disease bacteria (0.5 mL about 5.0×10^5 cfu/g of Chinese cabbage) onto the Chinese cabbages were stored at room temperature for 30 min and inoculated with HAW and antimicrobial LAB's supernatants. Qualitative (A) and quantitative (B) analysis of HAW treatment (80 - 200 ppm); Qualitative (C) and quantitative (D) analysis of Treatment of 0.5 mL of antimicrobial substances (supernatant) secreted by *Lactobacillus plantarum* PL203.

controlled the bacteria at a concentrations of 120 ppm or more. In the non-treatment HAW group, about 10^7 cfu/g levels of total cell counts were detected, but in the groups treated with 120 ppm or more, it was reduced to about 10^1 cfu/g level. The total cell counts of *P. vagans* strain inoculated samples was 5.40×10^7 cfu/g, but it was reduced to 3.00×10^1 cfu/g in the HAW treated samples with 120 ppm, and it was confirmed that there were no symptoms such as soft-rot disease. However, in the 80 ppm treatment samples, although it decreased to 8.40×10^5 cfu/g, it was visually confirmed that soft rot disease still occurred. The results of *P. kribbensis* also showed similar results to *P. vagans*. In the sample treated with 0.5 mL supernatant of the antimicrobial LAB culture, it was also confirmed that both *P. kribbensis* and *P. vagans* were completely controlled (Fig. 5). The total cell counts of *P. vagans* and *P. kribbensis* strains inoculated in samples was 5.70×10^5 cfu/g and 5.32×10^5 cfu/g, respectively. After adding antimicrobial substances and incubating for 5 days, It was confirmed that the sample without antimicrobial substances (supernatant) of LAB increased to a level of $3.82\text{-}4.10\times10^7$ cfu/g, but decreased to a level of $1.20\text{-}2.10\times10^2$ cfu/g in the samples with the addition of antimicrobial substances.

Because many LABs are used for various fermented foods such as Kimchi and yogurt, there have been many studies on improvement of quality and safety of foods. In contrast, the studies on using LAB as a control in agricultural production and storage are relatively limited[30,31]. *Lactobacillus plantarum* is found in various fermented foods and human intestines and is one of the Generally Recognized as Safe (GRAS) LAB, which makes users feel comfortable using antibacterial active substances rather than other agents.

However, if the tested culture supernatant is based on a microbial growth medium, it is difficult to directly use it for Chinese cabbage, because the medium components may grow other microorganisms. Therefore, to use the natural antimicrobial active substances in storage of Chinese cabbages industrially, it is necessary to remove the medium component through purification. However, the costs of purification must also be considered. If an effective method is prepared, it can be applied for storage of Chinese cabbages in companies. In particular, there are few studies

on control using HAW in Chinese cabbage, and there are few cases of industrial application of natural antibacterial activity by LAB.

Currently, we are examining various studies, such as ozone gas, temperature and humidity control systems related to the storage of Chinese cabbage in kimchi manufacture factories for industrial application. One is the use of HAW and natural antimicrobial active substance treatments, which has been confirmed in this study and will also be verified through optimization of conditions, scale-up, and field tests. In addition, although it is not shown in this study, the antimicrobial substance of the LAB strain was concentrated, purified, characteristic study and genomic working are in progress. In the future, these research data will help to Kimchi industry through the stable and useful supply of Chinese cabbages.

Acknowledgment

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국문요약

세균성 무름병은 배추와 같은 채소의 주요 질병중의 하나이며, 특히 김치의 주원료인 배추의 저장에 있어서 발생할 경우, 품질 저하로 인한 경제적 손실을 유발한다. 우리는 무름병이 발생한 배추로부터 원인이 되는 세균들을 분리하였고, 해당 세균이 신선한 배추 조직에 인위적으로 접종하였을 경우, 무름병이 발생하는 것을 확인하였다. 해당 원인균 2종을 동정한 결과, *Pseudomonas kribbensis*와 *Pantoea vagans*로 확인되었다. 이러한 원인 세균을 제어하기 위하여 차아염소산수 처리와 항균활성 유산균의 배양 상등액을 이용하여 제어하는 테스트를 진행하였다. 본 테스트에 사용한 항균활성 유산균은 리스테리아와 같은 병원성 미생물과 *Pseudomonas* 속 계열을 저해하는 *Lactobacillus plantarum* PL203 유산균을 선별하여 사용하였다. 제어 테스트는 정상 배추 조직에 무름병 원인균 2종을 각각 10^5 cfu/g 이 되도록 접종한 후, 차아염소산수를 농도별로 분무하거나 항균활성 유산균 배양상등액을 0.5 mL 분무한 후, 25°C에서 5일간 배양한 결과, 차아염소산수 120 ppm 이상 첨가한 구간과 항균활성 유산균 배양상등액을 처리한 구간에서 대조구와 비교하여 배추 무름 원인세균을 완전하게 제어하는 것을 확인하였다.

Conflict of interests

The authors declare no potential conflict of interest.

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