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In Vitro: Antimicrobial Effect of *Lactobacillus salivarius* on *Staphylococcus pseudintermedius*

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Abstract : *Lactobacillus* spp. are the bacteria most commonly used as probiotics and it has been proven that they inhibit pathogenic bacterial growth and improve skin repair in humans. This study was conducted to investigate the growth inhibitory effect of *Lactobacillus* on *Staphylococcus pseudintermedius*, the most commonly isolated pathogen in canine pyoderma, and whether *Lactobacillus* could inhibit the adhesion capability of *S. pseudintermedius* to canine corneocytes. For this study, *L. salivarius* and *S. pseudintermedius* were isolated from healthy beagle fecal samples and the skin surface of dogs with skin infection, respectively. *S. pseudintermedius* was co-cultured with *L. salivarius* to assess the inhibitory effect. For the adhesion assay, corneocytes were collected from healthy beagle ventral abdominal skin. Both bacterial species attached to corneocytes and were assessed in number. As a result, *L. salivarius* significantly inhibited the growth of *S. pseudintermedius* in the culture medium. Moreover, *L. salivarius* reduced attachment of *S. pseudintermedius* in the adhesion assay. These results suggest that *L. salivarius* has an inhibitory effect on *S. pseudintermedius* and may be effectively used in the topical therapy of canine skin infections.

Key words **:** dog, *Lactobacillus salivarius*, probiotics, *Staphylococcus pseudintermedius*.

Introduction

Probiotics are defined as live microorganisms contained in food that provide a benefit to the health of the host (6). Among several bacterial strains, *Lactobacillus* are the most commonly used probiotics in foods and oral supplements for human health and disease control (7,10).

Lactobacillus show antimicrobial ability through several pathways. First, *Lactobacillus* produce organic acid and bacteriocin, which have antimicrobial ability (12). Second, another main property of *Lactobacillus* is inhibition of other bacteria's adhesion. Adhesion proteins such as fibronectine and fibrinogen play a major role in binding at adhesion sites when *Staphylococcus* initiate skin infection in the presence of atopic dermatitis (5). *Lactobacillus* have characteristics such as autoaggregation and hydrophobicity associated with binding at the cell surface, which may help inhibit staphylococcal infection (14). Biofilm formation is another mechanism of antibacterial and immunomodulatory effects of *Lactobacillus* (8).

Canine pyoderma is one of the most common infectious skin diseases in dogs, occurring with or without atopic dermatitis. *S. pseudintermedius* is the pathogen isolated in most of these cases (1,18). Adhesion to the skin is an important factor in establishing of infection and likely represents the initiation of bacterial colonization (1). Atopic skin is more susceptible than normal skin to adhesion by *S. pseudintermedius* (11). Thus, we hypothesized that inhibition of *S. pseudintermedius* could be helpful to prevent the canine pyoderma especially on atopic dogs.

In this study, we evaluated the antimicrobial ability of *L. salivarius* by growth and adhesion inhibitory effect on *S. pseudintermedius*.

Materials and Methods

Staphylococcus isolates

Four isolates of *S. pseudintermedius* were used, obtained from the skin surface of dogs with pyoderma. The samples were cultured aerobically on sheep blood agar (Asan Pharmaceutical, Hwasung, Korea) and grown at 37°C for 24 h. Each sample was subcultured in tryptic soy broth (TSB, Becton Dickinson and Company, Frankline Lakes, NJ) at 37°C for 24 h.

The polymerase chain reaction (PCR) band method was performed for identification of organisms. The pure PCR product of the 16S rRNA gene was obtained and sequenced, and then subjected to a search with the Basic Local Alignment Search Tool of the National Center for Biotechnology Information database bacterial DNA nucleotide sequences for identification.

Lactic acid bacteria isolation and identification

Fecal samples from three healthy beagles were collected aseptically using cotton swabs and then cultured in De Man Rogosa-Sharpe (MRS) broth (Becton Dickinson Company) at 37°C for 48 h anaerobically for enrichment of *Lactobacillus*. These cultures were inoculated on MRS agar (Becton Dickinson Company) in the same condition, and a single col-

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ony on solid medium was subcultured for preparation of bacterial identification.

L. salivarius identification was conducted by 16S rRNA gene sequencing using the PCR band method, as described above for Staphylococcal identification.

Bacterial competition assay

The densities of both bacterial cultures were adjusted spectrophotometrically to include bacteria required at a certain number. The same number of bacteria was adjusted by measuring the optical density at 600 nm and 540 nm for *S. pseudintermedius* and *L. salivarius* (9,16).

Aliquots of overnight cultures of *L. salivarius* and *S. pseudintermedius* were inoculated into 10 ml of fresh broth (using the same volume of TSB and MRS broth at 5 ml). *S. pseudintermedius* with TSB and *L. salivarius* with MRS broth were used as controls. The tested broth was divided into three groups.

- Group A: The control group with *S. pseudintermedius* only
- Group B: The control group with *L. salivarius* only
- Group C: The group in which *S. pseudintermedius* and *L. salivarius* were co-cultured.

After 24 h at 37°C under aerobic conditions, the bacterial number of each sample was assessed with the serial dilution plate count method after 24 h of culture, using Muller-Hinton agar (Asan pharmaceutical) and MRS agar for *S. pseudintermedius* and *L. salivarius.*

Collection of corneocytes

Three intact male beagles were used as donors of corneocytes. The dogs had no signs of disorders in the skin or other organs. Ventral abdominal skin was used as the sampling site because it was previously that a constant number of cells may be collected from this region. The hair of the ventral abdomen was removed with a clipper. To remove the skin debris and remnant hair, four layers of adhesive tape (Scotch Tape; 3M, Maplewood, MI) were serially pressed down.

Corneocytes were collected using standard D-Squame® discs (CuDerm Corporation, Dallas, TX). The adhesive side was applied to the skin 10 times with the same force each time. The same investigator collected samples in a standard manner to avoid variability. The adherence assay for all samples was conducted in 1 day. Before performing the adherence assay, the non-adhesive end of the D-Squame disc was attached to a slide glass with adhesive tape.

Adherence assay

For the bacterial competition assay described above, the colony numbers of *S. pseudintermedius* and *L. salivarius* were adjusted for optical densities to reach the same value. Aliquots of bacterial suspensions containing 3.75×10^7 of each bacterial species were applied onto the collected corneocytes on the D-Squame discs and incubated in a humid environment using a stainless tray and wet gauze for 90 min at 37°C. Three groups were tested for the adherence assay.

- Group A: The control group with D-Squame discs on which only *S. pseudintermedius* was applied.
- Group B: The control group with D-Squame discs on which only *L. salivarius* was applied.
- Group C: The control group with D-Squame discs on which *S. pseudintermedius* and *L. salivarius* were applied simultaneously.

After incubation, the slides were flushed with phosphate buffered saline to wash off the non-adherent bacteria and stained with crystal violet (Difco™, USA) for 30 s. The number of adherent bacteria was counted in each slide from 10 random high-powered fields (1000x magnification) in areas of 3.14×10^{-1} cm^2 and the mean value of each counting was recorded. A triple-blind study was conducted for each slide's examination. Corneocytes that were severely folded or attached to foreign materials were excluded from counting.

Statistical analysis

All statistical tests were conducted with commercial statistical software (SigmaPlot for windows version 12.0, Systat Software, Inc., Richmond, CA). To analyze the percent of inhibited bacterial growth and adhesion, repeated-measures analysis of variance was performed. A P value of < 0.05 was considered to indicate statistically significant differences.

Results

Bacterial competition assay

Competition assays showed significant reduction in the growth of *S. pseudintermedius* during 24 h coculture with *L. salivarius* compared to that in axenic cultures ($P < 0.05$). For the four *S. pseudintermedius* isolates, the inhibition rate of *S. pseudintermedius* in the presence of *L. salivarius* was 99.95%.

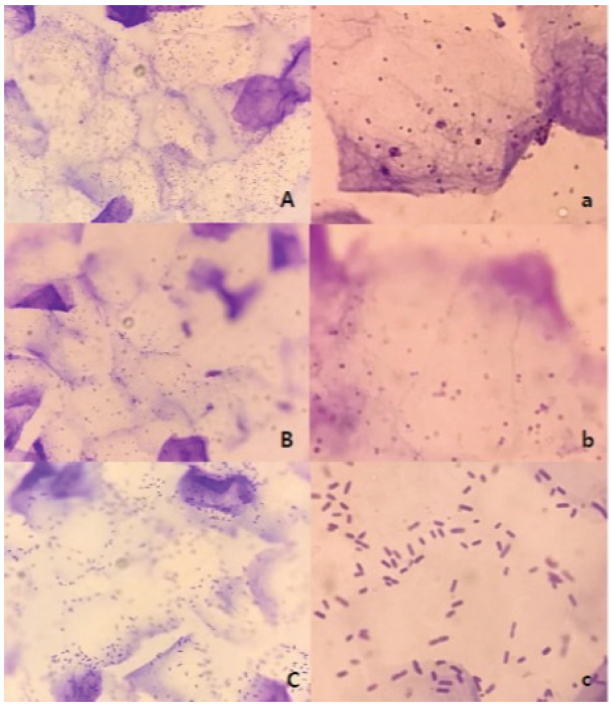

Fig 1. Adhesion of *S. pseudintermedius* (A, a) and *L. salivarius* (B, b) to corneocytes. When *S. pseudintermedius* and *L. salivarius* were attached simultaneously (C, c), adhesion of *S. pseudintermedius* was strongly inhibited by *L. salivarius*. A, B, C; 100 ×, a, b, c; 1000 ×.

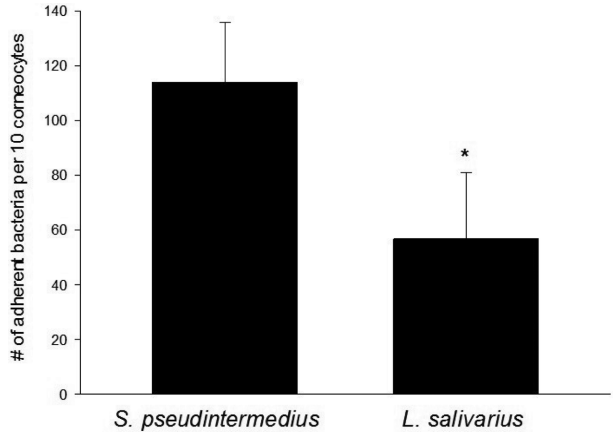

Fig 2. Adhesion of *S. pseudintermedius* and *L. salivarius* to canine corneocytes. Both bacteria showed ability of adherence to corneocytes. The number of adherent bacteria to canine corneocytes per 10 fields was significantly higher for *S. pseudintermedius* than for *L. salivarius* (mean ± SEM, *; $P < 0.01$).

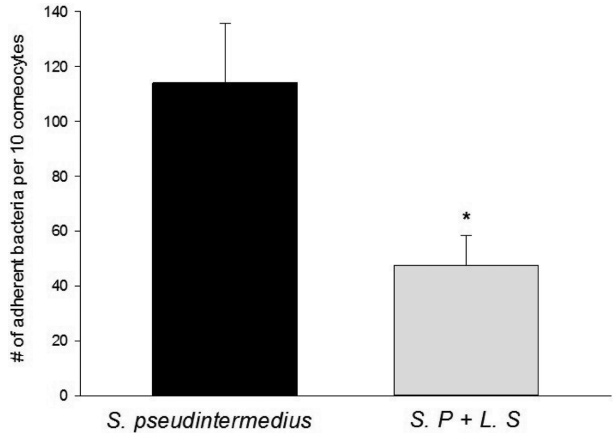

Fig 3. Adhesion of *S. pseudintermedius* to canine corneocytes when applied simultaneously with *L. salivarius*. Compared to the control group with corneocytes in which only *S. pseudintermedius* was attached, adherence of *S. pseudintermedius* was inhibited by 58.51% in the presence of *L. salivarius* (mean ± SEM, *; $P < 0.01$). L; *L. salivarius*, S; *S. pseudintermedius*.

Adherence assay

When the isolates of *S. pseudintermedius* and *L. salivarius* were incubated at a density of 3.75×10^7 each for 90 min, the median number of adherent bacteria to canine corneocytes per 10 fields was significantly higher for *S. pseudintermedius* than for *L. salivarius* ($P < 0.01$, Fig 1 and 2). However, the adherence of *S. pseudintermedius* was inhibited by 58.51% in the presence of *L. salivarius* (Fig 3). The adhesion inhibition rate of *L. salivarius* to corneocytes by *S. pseudintermedius* was 64.85% (Fig 4).

Discussion

Lactobacillus are Gram positive, rod-shape microorganism and the most common probiotic (7). Probiotics are live microorganism promoted as having various health benefits (6). They have beneficial effects on health through growth

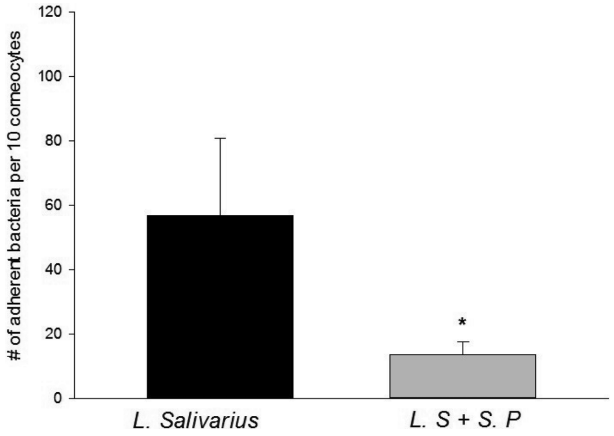

Fig 4. Adhesion of *L. salivarius* to canine corneocytes when applied simultaneously with *S. pseudintermedius*. The inhibition rate of the adhesion of *L. salivarius* to corneocytes by *S. pseudintermedius* was 64.58% (mean ± SEM, *; $P < 0.01$). L; *L. salivarius*, S; *S. pseudintermedius*.

prevention of or resistance to harmful bacteria, improvement of the immune system, and protection against epithelial injury (4,10,13). Among these mechanisms, *Lactobacillus* has antibacterial effects through production of the lactic acid, an organic acid. Lactic acid, which is the most important product of *Lactobacillus* metabolism, is lethal to microorganisms via undissociated molecules that flow through the cell membranes and ultimately change the intracellular pH. This mechanism affects the electron transport system and leads to the microorganism's death. The smaller molecules of lactic acid (90.08 Da) show higher antimicrobial activity than those of citric acid (192.13 Da) and tartaric acid (150.09 Da) (17). One study revealed that *L. johnsonii* has anti-adhesion properties against skin pathogens such as *S. aureus* in humans (2). Some products using this strain can be applied topically to control skin conditions. Thus, we hypothesized that *Lactobacillus* could also be applied to canine skin for the same therapeutic purposes.

L. salivarius is the most frequently isolated lactic acid bacterial species in dogs and has been confirmed to produce lactic acid in high amount (3,15). In previous studies, this strain showed long survival and a marked production rate compared to other *Lactobacillus* spp. (15). In addition, *L. salivarius* showed considerable antimicrobial activities against other bacteria (3). In this study, we conducted bacterial competition and adherence assays to evaluate the antimicrobial effects of *L. salivarius* against *S. pseudintermedius*, the most common bacterial species on the canine skin surface. The results of these assays showed that *L. salivarius* suppressed the growth and adhesion of *S. pseudintermedius*.

Although it was confirmed that *L. salivarius* is capable of attaching to canine corneocytes, there still exists the issue of the resident Lactobacillus on corneocytes. Under aerobic conditions in the skin, *Lactobacillus* cannot proliferate to the same extent with skin pathogens such as *Staphylococcus*. Moreover, as there is also the issue of bacterial colonization of *Lactobacillus*, some prefer topical use of probiotics without cell, produced in cell-free culture medium. To resolve

these issues, powder or topical types of probiotics are used in n human products, such as ointments or shampoo. In addition, a shampoo for pets is recently manufactured used *L. johnsonii* to regulate the pathogenic flora of the cutaneous system of pets.

In conclusion, *L. salivarius* significantly inhibited growth of *S. pseudintermedius*. *Lactobacillus salivarius* can adhere to corneocytes, but *S. pseudintermedius* is more adherent than *L. salivarius.* Adherence of *S. pseudintermedius* was reduced in the presence of *L. salivarius.* Thus, *L. salivarius* can be applied as a topical therapy for canine skin infectious diseases.

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