Research Article **Open Access**

Heat-killed *Lactiplantibacillus plantarum* N1487-7 induces IL-10 production and enhances skin barrier function

Eriko Uehara[1]*, Hideki Hokazono[1]

[1]Research & Development Laboratory, Sanwa Shurui Co., Ltd., 2231-1 Yamamoto, Usa, Oita 879-0495, Japan

***Corresponding author:** Eriko Uehara, Ph.D., Research & Development Laboratory, Sanwa Shurui Co., Ltd., 2231-1 Yamamoto, Usa, Oita 879-0495, Japan

Submission Date: October 18th, 2024, **Acceptance Date**: November 19th, 2024, **Publication Date**: November 22nd, 2024

Please cite this article as: Uehara E., Hokazono H. Heat-killed Lactiplantibacillus plantarum N1487-7 induces IL-10 production and enhances skin barrier function. *Functional Foods in Health and Disease* 2024; 14(11): 856-870. DOI: https://doi.org/10.31989/ffhd.v14i11.1483

ABSTRACT

Background: Interleukin (IL)-10, an anti-inflammatory cytokine, suppresses the release of inflammatory cytokines, such as IL-6, and inhibits the decline in skin barrier function. Mice orally administered test substances by gavage are subjected to physical stress, which results in the secretion of stress hormones. Stress hormones cause inflammation and reduce skin barrier function.

Objective: The effects of heat-killed *Lactiplantibacillus* (*Lpb.*) *plantarum* N1487-7 (N1487-7-HK) on IL-10 production in Caco-2 cells and macrophages, and skin barrier function in physically stressed mice was investigated.

Methods: *Lpb. plantarum* N1487-7 was cultured in fermented barley extract medium at 30 °C for 22 h with shaking (100 rpm). The washed cells were killed by heating at 110 °C for 10 minutes. The cells were then dried in a centrifugal evaporator and ground to obtain the cell powder. The powder was administered to Caco-2 cells and macrophages, and *IL-10* mRNA and IL-10 protein expressions were measured by real-time quantitative polymerase chain reaction and Western blotting, respectively. N1487-7-HK cells or distilled water were orally administered daily by gavage to Hos:HR-1 mice for 5 weeks (the N1487-7-HK group or the control group). During weeks 3 and 5 only, dextran sulfate sodium (DSS) was mixed into the drinking water of these groups to induce mild colitis. The normal group was not orally

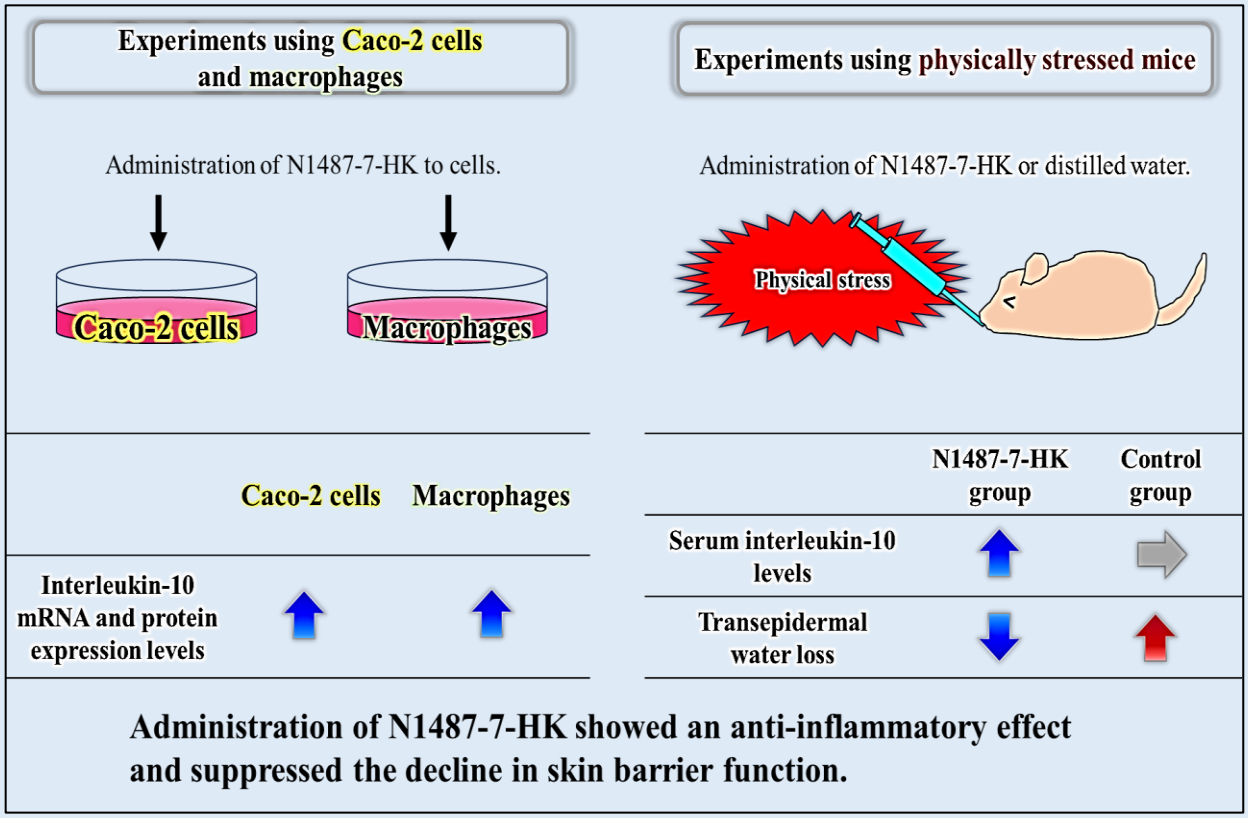
administered anything by gavage and was not given DSS. Serum IL-10 and the trans epidermal water loss (TEWL) of mice were measured.

Results: N1487-7-HK promoted the expression of *IL-10* mRNA and IL-10 protein in both Caco-2 cells and macrophages. In mice subjected to physical stress, serum IL-10 levels in the N1487-7-HK group were significantly increased compared to the control group. The transepidermal water loss (TEWL) in the control group was notably higher than that in the normal group during Weeks 1 and 2. In contrast, the N1487-7-HK group showed no significant difference in TEWL compared to the normal group.

The N1487-7-HK group showed a significantly lower TEWL at Week 4 compared to Week 0.

Conclusions: N1487-7-HK promoted IL-10 production from intestinal epithelial cells and macrophages. Intake of N1487-7-HK inhibited the decline in skin barrier function in mice subjected to physical stress.

Keywords: *Lactiplantibacillus plantarum*, Interleukin (IL)-10, Caco-2, macrophages, skin barrier



Graphical Abstract: Heat-killed Lactiplantibacillus plantarum N1487-7 induces IL-10 production and enhances skin barrier function

INTRODUCTION

In the early twentieth century, dermatologists Stokes and Pillsbury were among the first to propose that the gut, skin, and brain are interconnected [1, 2]. When the intestinal barrier function is weakened, foreign substances such as toxins and pathogens enter the bloodstream. To eliminate these foreign substances, T helper 2 immune cells release inflammatory cytokines such as interleukin (IL)-6, which suppress the release of the anti-inflammatory cytokine IL-10. It has been reported that particulate matter worsens atopic dermatitis, increases IL-6, and reduces the expression of the skin barrier protein filaggrin [3–5]. Atopic dermatitis is a frequently recurring chronic inflammatory skin condition marked by elevated transepidermal water loss (TEWL) and reduced skin hydration levels [6].

Supplementation with a probiotic formulation consisting of *Bifidobacterium* (*B.*) *lactis* Bi-07, *B. lactis* HN019, *Lactobacillus acidophilus* NCFM, *Lacticaseibacillus* (*L.*) *rhamnosus* HN001 and *L. paracasei* Lpc-37 twice a day for 12 weeks significantly reduced TEWL in subjects at high risk for alopecia and metabolic syndrome [7]. In studies investigating the relationship between intestinal barrier function and skin barrier function, dextran sulfate sodium (DSS) is administered to experimental animals to induce colitis and then the condition of the skin is examined [8].

IL-10 functions as an important anti-inflammatory cytokine, acting as a negative regulator of immune responses. This cytokine is synthesized by several immune cell types, including regulatory T cells, regulatory B cells, anti-inflammatory macrophages (M2), and dendritic cells. [9] Furthermore, IL-10 is also produced by intestinal epithelial cells, where it plays a key role in preserving intestinal balance and homeostasis [10].

Lactiplantibacillus (Lpb.) plantarum is utilized in traditional fermented foods worldwide and is generally regarded as safe (GRAS) with qualified presumption of safety (QPS) status. In a whey protein-induced allergic rat model, administration with both live and heat-killed *Lpb. plantarum* DPUL-F232 administration was effective in promoting IL-10 secretion by regulatory T cells and restoring intestinal barrier function, and allergic symptoms were significantly alleviated [11]. Heat-killed *Lpb. plantarum* OLL2712 highly induced IL-10 production in mouse-derived dendritic cells and peritoneal macrophages [12]. Heat-killed *Lpb. plantarum* YIT 0132 induced IL-10 in peritoneal macrophages and was shown to suppress Japanese cedar pollinosis symptoms [13]. Probiotic extracts of multiple strains, including *Lpb. plantarum* EMRO 009, can be used as anti-inflammatory agents for the treatment of chronic diseases [14]. Fermented fruit juices containing *Lpb. plantarum* and *Ligilactobacillus salivarius* showed antibacterial activity against *Salmonella Typhi* DMST 22842 and increased antioxidant activity [15]. Lactobacillus probiotics, which increase the amount and variety of gut bacteria, may contribute to weight management by regulating immune function and improving metabolic rate. [16] We isolated *Lpb. plantarum* N1487-7 (N1487-7) from rice bran pickles, a traditional Japanese fermented food. In a bacterial reverse mutation test, heat-killed N1487-7 (N1487-7-HK) showed no gene mutation-inducing ability under the test conditions. In an acute oral toxicity test using mice, the 50% lethal dose value was estimated to be greater than 2000 mg/kg body weight for both males and females. In other words, a minimum level of safety was confirmed both in vitro and in vivo. The Functional Food Center (www.functionalfoodscenter.net/about-us.html) has defined “functional foods” as follows: Natural or processed foods that contain biologically-active compounds, which, in defined, effective, non-toxic amounts, provide a clinically proven and documented health benefit utilizing specific biomarkers, to promote optimal health and reduce the risk of chronic/viral diseases and enable management of symptoms [17–19].

As mentioned above, *Lpb. plantarum* has been reported to promote the production of IL-10 in dendritic cells and peritoneal macrophages. Therefore, N1487-7-HK may promote the production of IL-10 and prevent the deterioration of intestinal barrier function, thereby maintaining skin barrier function. Biomarkers of skin barrier function include cellular and serum IL-10 levels, and TEWL. We therefore investigated the effect of N1487-7-HK, on IL-10 production in human intestinal epithelial cells and macrophages. Furthermore, we investigated the effects of N1487-7-HK on serum IL-10 levels and skin barrier function in a DSS-induced colitis model of hairless (Hos:HR-1) mice.

MATERIALS AND METHODS

Bacterial strain, culture conditions, and cell preparation: *Lpb. plantarum* N1487-7 was isolated from rice bran pickles and identified based on 16S rRNA gene (16S rDNA) sequence analysis, morphological observation, physiological and biochemical tests, and recA gene analysis (SIID48544 report, TechnoSuruga Laboratory Co., Ltd., Shizuoka, Japan).

N1487-7 was cultured in fermented barley extract (Sanwa Shurui Co., Ltd., Oita, Japan) medium (Brix 4.0) supplemented with 6% hydrated crystalline glucose (Sunus Co., Ltd., Kagoshima, Japan) for 22 h at 30 °C and pH5.5 [20]. The cells were washed twice with tap water and centrifuged at 20,000 × g in a continuous centrifuge, after which they were sterilized in an autoclave at 110 °C for 10 minutes. The cells were freeze-dried and ground in a mixer. Bacterial cell death was confirmed by plating the suspensions onto Difco™ Lactobacilli MRS Agar (Becton, Dickinson and Company, Bergen, NJ, USA) and incubating for 18 h at 30 °C.

Culture of intestinal epithelial cells and macrophages: Caco-2 cells (KAC Co., Ltd., Kyoto, Japan), derived from human colon tissue, were utilized in this study. These cells were plated at a density of 2 × 10^5 cells/cm² in 24-well plates, each containing 1 mL of Dulbecco's modified Eagle's medium (low glucose D6046, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 1% penicillin-streptomycin-glutamine (100×, Thermo Fisher Scientific, Waltham, MA, USA). The cells were incubated at 37 °C in a 5% CO_2 atmosphere for 24 h. Once they reached confluence, the medium was replaced with DMEM containing 1% FBS and 1% penicillin-streptomycin (test medium). N1487-7-HK cells, mixed with phosphate-buffered saline (PBS), were then added to the test medium to achieve final concentrations of 10 and 100 µg/mL. The PBS was added in a way that kept its volume at 10% or less of the total test medium to maintain appropriate concentrations of other components. Recombinant human interferon-gamma (IFN-γ, AF-300-02, Pepro Tech Inc., Cranbury, NJ, USA), dissolved in ultrapure water, was introduced to the test medium at a concentration of 50 ng per well to induce an inflammatory response. The medium containing N1487-7-HK cells and IFN-γ was refreshed every 24 h. After 2 d of this regimen, cells were harvested for mRNA analysis, and after 6 d, they were used for Western blotting.

Additionally, 28SC-ES cells (JCRB1647, National Institutes of Biomedical Innovation, Health and Nutrition, Osaka, Japan), an established human macrophage-like cell line, were employed. These cells were seeded at a density of 8 × 10^4 cells/cm² in 24-well plates with 1 mL of RPMI medium (Sigma-Aldrich), supplemented with 1% FBS and 1% penicillin-streptomycin-glutamine (100×, Thermo Fisher Scientific). N1487-7-HK cells were introduced to the wells at concentrations of 10 and 100 µg/mL without the addition of IFN-γ. Cells were cultured at 37 °C and 5% CO_2 for 24 h. After this incubation, they were used for mRNA analysis. The medium containing N1487-7-HK cells was replaced daily, and after 4 d of this treatment, cells were collected for Western blot analysis.

Real-time quantitative polymerase chain reaction (PCR): Total RNA was isolated from the cells using the TRIzol™ Plus RNA Purification Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. For reverse transcription, SuperScript IV VILO Master Mix and ezDNase Enzyme (Thermo Fisher Scientific) were used with random primers. The resulting cDNA was then amplified by PCR with specific primers for the target genes. mRNA levels were quantified using real-time PCR on a LightCycler® 96 System (Roche, Basel, Switzerland). The PCR program consisted of an initial 10-minute denaturation at 95°C, followed by 45 cycles of 95°C for 10 seconds, 60°C for 10 seconds, and 72°C for 10 seconds. The relative expression of mRNA was normalized to GAPDH levels using the comparative threshold cycle method.

The sets of primers used in this study were as follows: *GAPDH* forward, 5'-GGTGAAGGTCGGAGTCAACGGA-3'; *GAPDH* reverse, 5'-GAGGGATCTCGCTCCTGGAAGA-3', and *IL-10* forward, 5'-GGTGAAGGTCGGAGTCAACGGA-3'; *IL-10* reverse, 5'-GAGGGATCTCGCTCCTGGAAGA-3'.

Western blotting: For Western blot analysis, all materials were sourced from Thermo Fisher Scientific unless noted otherwise.

The cell supernatants were removed, and proteins were extracted using M-PER™ Mammalian Protein Extraction Reagent, supplemented with a mixture of protein and phosphatase inhibitors. Protein concentration was measured by the Bradford assay, ensuring 20 µg of protein was present in each well. The samples were then combined with Bolt™ LDL Sample Buffer and Reducing Agent, heated at 70°C for 10 minutes. Protein samples were loaded onto Bolt™ 4–12% Bis-Tris mini gels and separated by electrophoresis at 200 V for 22 minutes using Bolt™ MES SDS running buffer. Following electrophoresis, proteins were transferred to a PVDF membrane using the iBlot2 system. The iBind Western System was used for antigen-antibody reactions targeting GAPDH and IL-10 proteins. Monoclonal antibodies for GAPDH (60004-1-lg) and IL-10 (60269-1-lg) from Proteintech Group Inc. were employed as primary antibodies, with goat anti-mouse IgG (H + L)-HRP as the secondary antibody, according to the manufacturer's recommended concentrations. After membrane washing with deionized water, the membrane was incubated with SuperSignal West Dura reagents for 5 minutes. Imaging was conducted using the ChemiDoc XRS+ system, and band intensity was analyzed with Image Lab™ 5.0 software from Bio-Rad. Protein expression levels were normalized to GAPDH.

Animal experiment: To avoid any possible interference from sex, 20 female Hos:HR-1 (hairless) mice (6 weeks old) were obtained from Hoshino Laboratory Animals, Inc. (Ibaraki, Japan). Experiments were conducted at Japan SLC, Inc. (Shizuoka, Japan). The animal experimental procedures were approved by the Committee on the Ethics of Animal Experiments of Japan SLC, Inc. (approval number: BT20039). All animal experiments were performed in compliance with all relevant ethical regulations. The experiment was conducted in a breeding room within a barrier facility under the following conditions, air conditioning: all fresh air, temperature: 23 °C, humidity: 50%, lighting duration: 12 h (7:00–19:00). The experiment schedule is shown in Figure 1. After 6 d of preliminary breeding, mice were randomly divided into three groups of 6 mice each. The experimental groups consisted of the normal group, the control group, and the N1487-7-HK group. The control group was orally administered 100 µL /mouse of distilled water, and the N1487-7-HK group was orally administered 0.8 mg/mouse of N1487-7-HK cells suspended in 100 µL distilled water once a day for 35 d (5 weeks). Oral administration was performed by gavage. The normal group was not subjected to oral

administration of anything via gavage. In the control and N1487-7-HK groups, mice were given free access to drinking water containing 1.5% DSS for 7 d each from Week 2 to Week 3 and from Week 4 to Week 5 to induce mild colitis. The general condition of mice was observed every day. Body weight was measured every week using an electronic balance (MP-3000, Chyo Balance Corp, Kyoto, Japan). The TEWL of mice was measured using a TEWL measuring device (VAPO SCAN AS-VT100RS, Asahi Techno Lab Co., Ltd., Yokohama, Japan) every week. The rate of change in the measured TEWL relative to pre-dose Week 0 was calculated. Approximately 1 h before the evaluation, mice were housed individually in cages with a floor covering. The condition of the feces was evaluated using the disease activity index (DAI) and was scored weekly according to the following criteria: score 0, normal (moderately dry, shape maintained); score 1, shape maintained but slightly loose; score 2, loose (sticking to paper); score 3, loose (loose shape); and score 4, diarrhea. Although not scored, the presence or absence of occult blood in the feces was also observed. On the day after the final administration, whole blood was collected from the posterior vena cava under isoflurane inhalation anesthesia. The collected blood was centrifuged to separate serum, and frozen and stored at -20°C. The large intestine was removed and the length from the colon to the anus was measured as the large intestine length.

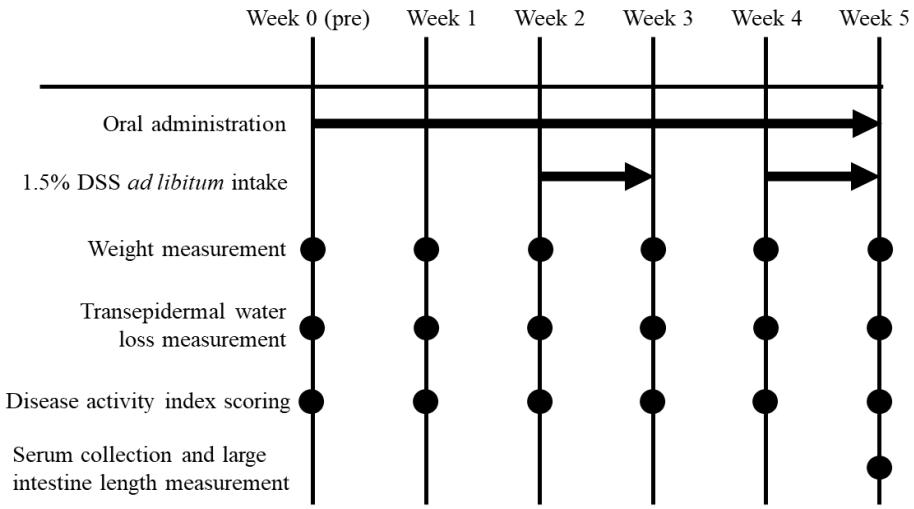


Figure 1. Animal experiment schedule.

ELISA for serum IL-10: A solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) for mouse IL-10 (Mouse IL-10 ELISA Kit KE10008) was obtained from Proteintech Group Inc. IL-10 quantification in thawed serum samples was conducted following the manufacturer's guidelines. The color intensity, which correlates with the IL-10 protein concentration, was measured at 450 nm, with a correction wavelength set to 630 nm using a spectrophotometric plate reader, using sample diluents as blanks. A four-parameter best-fit standard curve was generated using reference solutions with known IL-10 concentrations, allowing the calculation of serum sample concentrations (pg/mL) based on this standard curve. The concentration values of diluted samples were then adjusted by multiplying with the dilution factor.

Statistical analysis: All cell experiment results are expressed as the mean ± standard deviation from three independent trials. The Shapiro-Wilk test assessed the distribution of the variables, while the Bartlett test evaluated variance equality. For datasets with normal

distributions and equal variances, the Tukey-Kramer test was utilized to determine significant differences. In cases that did not meet these criteria, the Steel-Dwass test was employed. A *p*-value of less than 0.05 was deemed statistically significant. All analyses were conducted using R v4.2.1 (R Development Core Team, Vienna, Austria). [21] In the animal experiments, trans epidermal water loss (TEWL), the rate of change in TEWL, disease activity index (DAI) score, and serum IL-10 levels were reported as the mean ± standard deviation for each group. Statistical analyses were conducted using paired t-tests to compare Week 0 with each subsequent week within each group. For intergroup comparisons each week, Tukey's multiple comparison test was applied. A significance level of 5% ($p < 0.05$) or lower was considered statistically significant.

Deviations from the animal experiment protocol: No deviations occurred during the experiment that could have affected the results.

RESULTS

Effects of N1487-7-HK on IL-10 mRNA and protein expression in Caco-2 cells: Caco-2 cells were exposed to 10 and 100 μg/mL of N1487-7-HK. The relative expression of *IL-10* mRNA in Caco-2 cells stimulated with IFN-γ was measured using real-time PCR, normalized to the *GAPDH* mRNA (Figure 2A). The results showed that at 100 μg/mL N1487-7-HK, *IL-10* mRNA expression was significantly higher than in the IFN-γ(+) control group ($p < 0.05$) and was equivalent to the IFN-γ(–) control group.

The relative expression of IL-10 protein in Caco-2 cells stimulated with IFN-γ was measured using western blotting, normalized to the GAPDH protein (Figure 2B). The results showed that at 100 μg/mL N1487-7-HK, IL-10 protein expression was significantly higher than in the IFN-γ(+) control group ($p < 0.05$) and the IFN-γ(–) control group ($p < 0.05$).

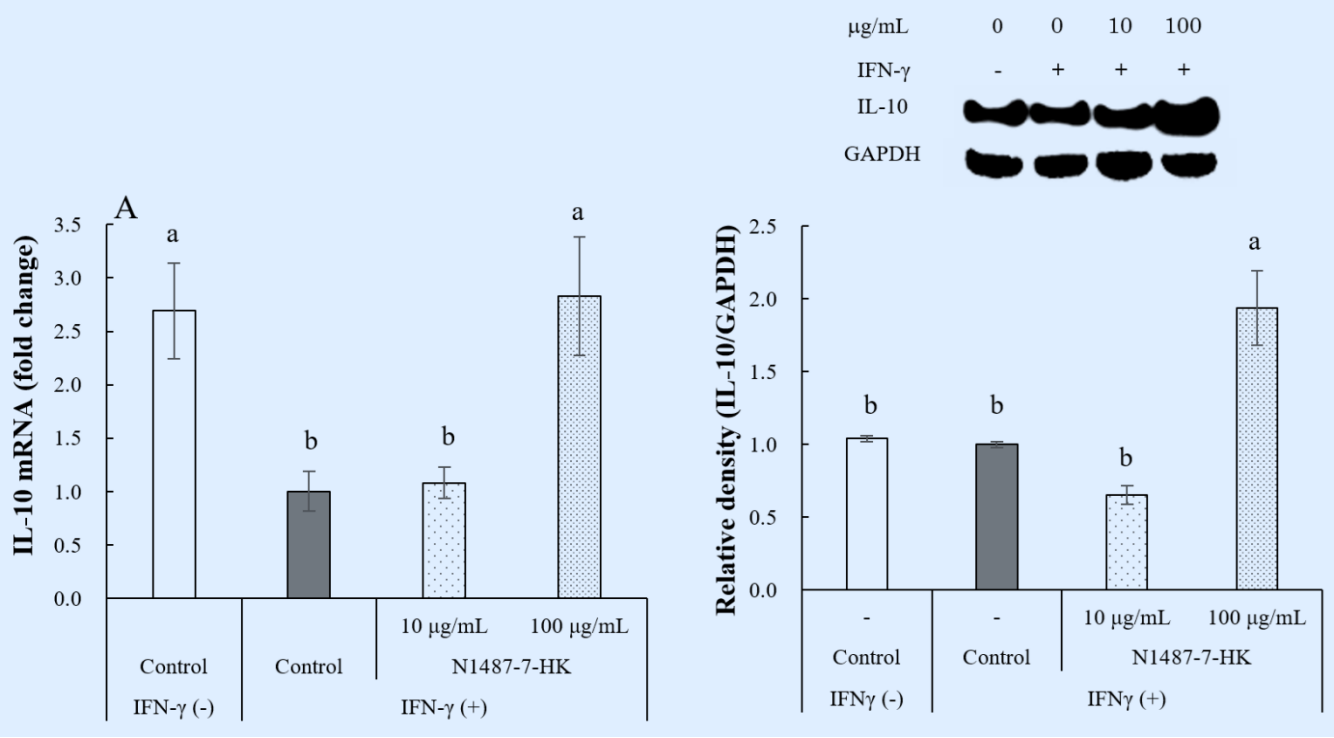


Figure 2. Effects of heat-killed *Lpb. plantarum* N1487-7 (N1487-7-HK) cells on *IL-10* mRNA (A) and IL-10 protein (B) expression in Caco-2 cells stimulated with IFN-γ.

Cells stimulated with IFN-γ were incubated for 2 d with 10 and 100 μg/mL of N1487-7-HK, with the medium refreshed every 24 h. The relative levels of *IL-10* transcripts were assessed using real-time PCR, with normalization to the *GAPDH* gene (A). In a separate experiment, IFN-γ-stimulated cells were maintained for 6 d under the same conditions, and the medium was again changed every 24 h. For this longer duration, IL-10 expression was evaluated via Western blotting, normalized to GAPDH protein levels (B). Each data point reflects the mean ± standard deviation from three independent experiments, with different letters (a or b) above the bars indicating statistically significant differences ($p < 0.05$).

Effects of N1487-7-HK on IL-10 mRNA and protein expression in 28SC-ES cells: 28SC-ES cells were exposed to 10 and 100 μg/mL of N1487-7-HK. The relative expression of *IL-10* mRNA in 28SC-ES cells was measured using real-time PCR, normalized to the *GAPDH* mRNA (Figure 3A). Concentrations greater than 10 μg/mL N1487-7-HK significantly promoted *IL-10* mRNA expression ($p < 0.05$).

The relative expression of IL-10 protein in 28SC-ES cells was measured using western blotting, normalized to the GAPDH protein (Figure 3B). Concentrations greater than 10 μg/mL N1487-7-HK significantly promoted IL-10 protein expression ($p < 0.05$).

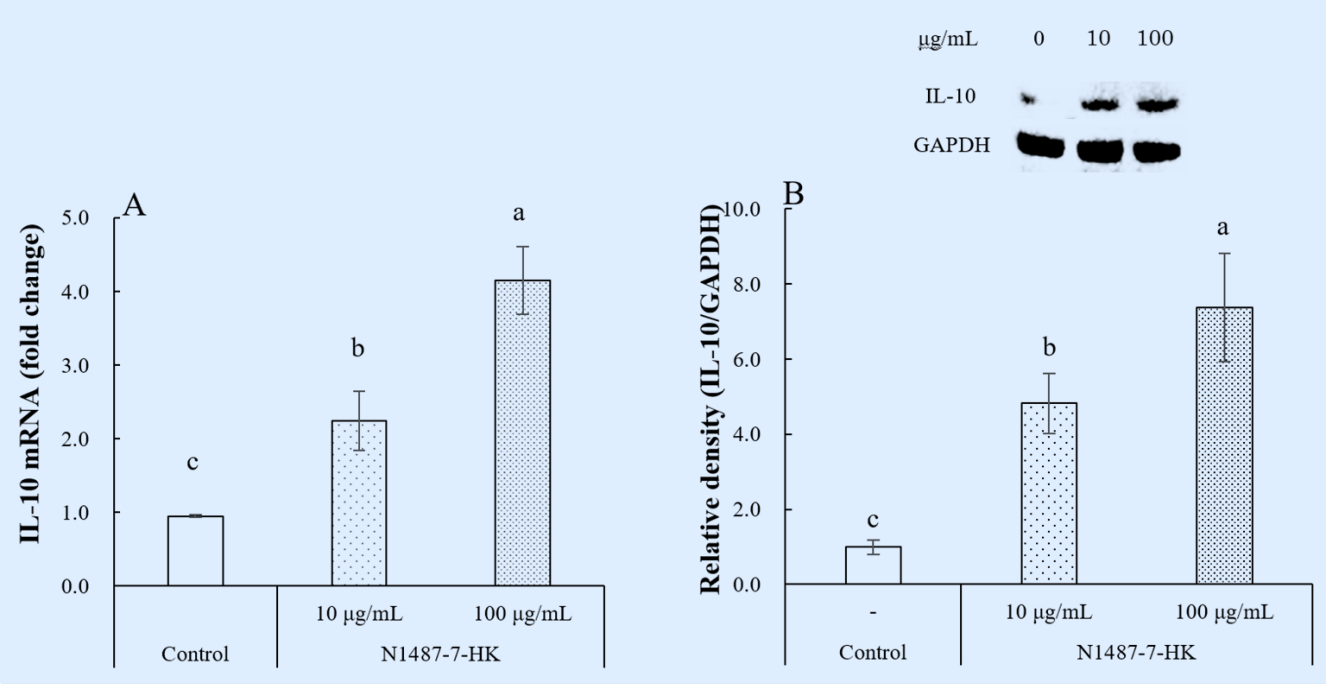


Figure 3. Effects of heat-killed *Lpb*. *plantarum* N1487-7 (N1487-7-HK) cells on *IL-10* mRNA (A) and IL-10 protein (B) expression in 28SC-ES cells.

Cells were incubated for 1 d with 10 and 100 μg/mL of N1487-7-HK. The expression levels of *IL-10* transcripts were measured using real-time PCR, with results normalized to the *GAPDH* gene (A). In a separate experiment, cells were maintained for 4 d with the same concentrations of N1487-7-HK, with the medium replaced every 24 h. The IL-10 expression was assessed via Western blotting, normalized to the GAPDH protein levels (B). Each data point represents the mean ± standard deviation from three independent trials. Statistically significant differences are indicated by different letters (a, b, or c) above the bars ($p < 0.05$).

Effects of N1487-7-HK on TEWL and serum IL-10 levels in colitis-model hairless mice: Hairless mice were orally administered with distilled water or N1487-7-HK. They were given free access to drinking water containing 1.5% DSS from Week 2 to Week 3 and from Week 4 to Week 5.

General condition and body weight: Body weight increased evenly in all groups, and no significant differences were observed between groups (data not shown). No weight loss was observed after administration of 0.8 mg/mouse/day N1487-7-HK (35 d). No weight loss was observed after DSS intake in both control and N1487-7-HK groups. No mice died and no adverse events were observed after oral administration of N1487-7-HK.

TEWL: The results of TEWL measurements are shown in Figure 4. At Weeks 1 and 2, the control group showed significantly higher values than the normal group. On the other hand, the N1487-7-HK group showed no significant difference from the normal group. In addition, the N1487-7-HK group showed a significantly lower value at Week 4 compared to Week 0 (Figure 4).

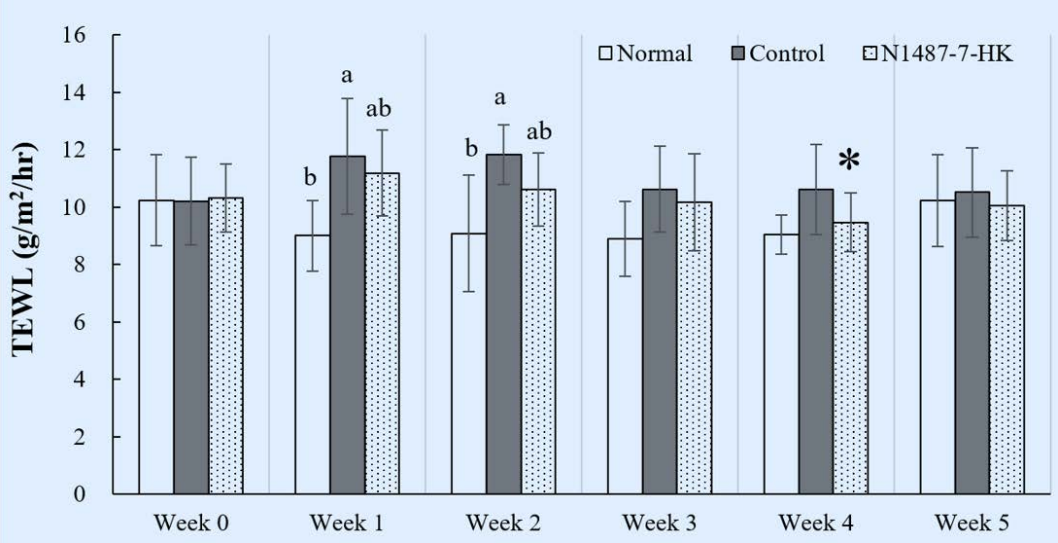


Figure 4. The effect of heat-killed *Lpb. plantarum* N1487-7 (N1487-7-HK) on transepidermal water loss (TEWL) in Hos:HR-1 mice.

Hairless mice were divided into three groups; N1487-7-HK and distilled water were orally administered daily to the N1487-7-HK and control groups, respectively. The normal group was not exposed to oral administration stress. For a period of 7 d each from Week 2 to Week 3 and from Week 4 to Week 5, the N1487-7-HK and control groups were given free access to drinking water containing 1.5% DSS to induce mild colitis. Each data point represents the mean ± standard deviation (n=6). TEWL was measured every week for 5 weeks using a TEWL meter. Different letters (a, b) above the columns indicate significant differences between the groups ($p < 0.05$). An asterisk indicates a significant difference vs. Week 0 ($p < 0.05$).

The rate of change in TEWL from Week 0 at each time point is shown in Figure 5. At Week 2, the control

group showed a significantly higher value than the normal group. On the other hand, the N1487-7-HK group showed no significant difference from the normal group. During the period from Week 3 to 5, N1487-7-HK reduced TEWL compared to before administration, although there was no significant difference between or within the groups.

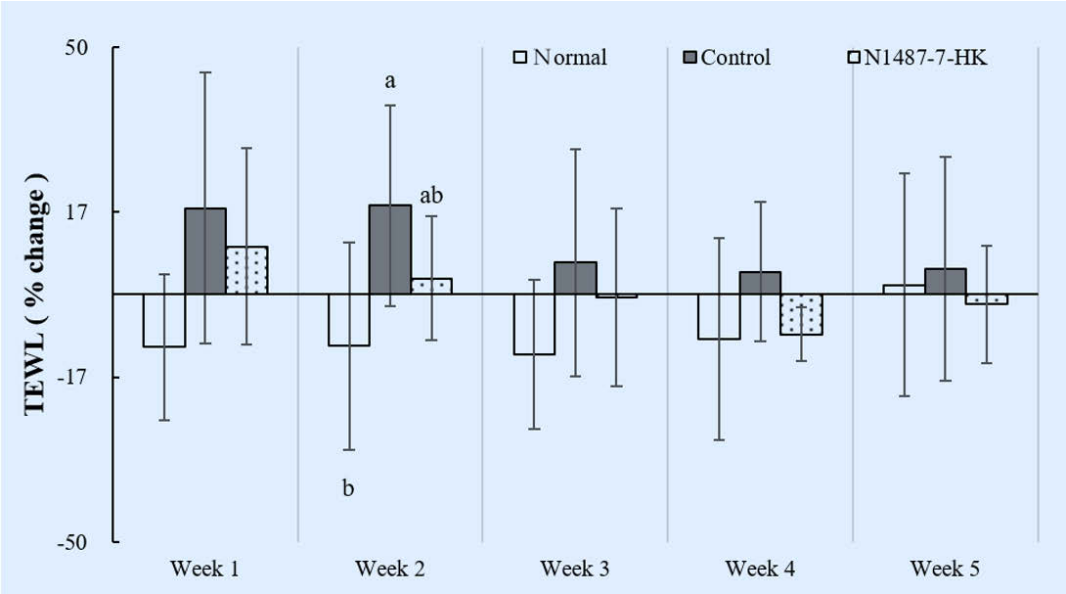


Figure 5. The effect of heat-killed *Lpb. plantarum* N1487-7 (N1487-7-HK) on the rate of change in transepidermal water loss (TEWL) in Hos:HR-1 mice.

Experimental conditions are described in Figure 4. Each data point represents the mean ± standard deviation (n=6). Data shows the rate of change from Week 0. Different letters (a, b) above or below the columns indicate significant differences between the groups ($p < 0.05$).

DAI score: In all groups except the normal group, most of the animals had a DAI score of 1 or 2, blood on the bedding, and bloody stools on the final day of DSS intake (Weeks 3 and 5). On the final day of DSS non-intake (Week 4), the fecal condition generally improved and was almost the same as before DSS intake. No differences were observed between the groups in either evaluation period (Table 1).

Table 1. Disease activity index (DAI) score.

		Evaluation period (Week)					
	Number	**0**	**1**	**2**	**3**	**4**	**5**
Normal	6	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Control	6	0.00±0.00	0.00±0.00	0.00±0.00	1.17±0.41	0.17±0.41	1.17±0.41
N1487-7-HK	6	0.00±0.00	0.00±0.00	0.00±0.00	1.50±0.55	0.33±0.52	1.17±0.41

Effect of heat-killed *Lpb*. *plantarum* N1487-7 (N1487-7-HK) on DAI score in colitis-model hairless mice. Experimental conditions are described in Figure 4. Fecal scoring is as described in the materials and methods section.

Serum IL-10 levels: The IL-10 concentration in the serum collected on the day after the final administration was measured using an ELISA kit. The N1487-7-HK group showed a significantly higher level (approximately 2-fold greater) than the normal and control groups (Figure 6).

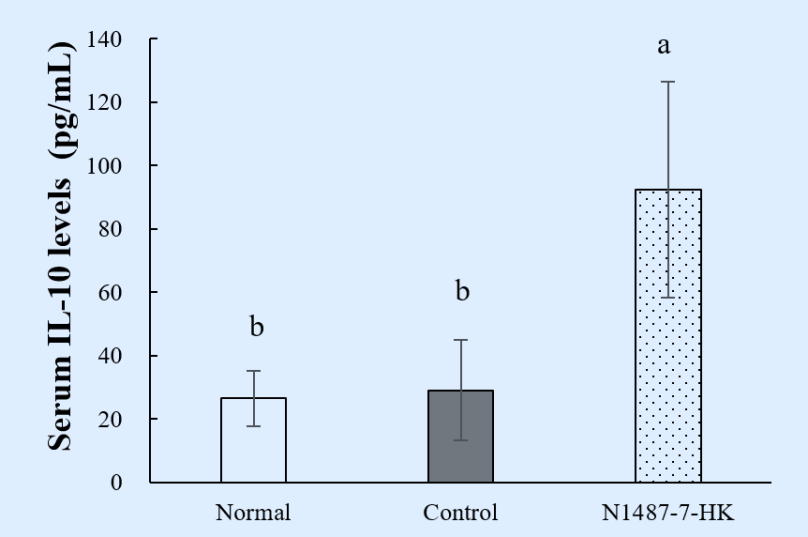


Figure 6. The effect of heat-killed *Lpb*. *plantarum* N1487-7 (N1487-7-HK) on serum IL-10 levels.

Experimental conditions are described in Figure 4. Serum was isolated and assayed for IL-10 concentration using an ELISA kit. Each data point represents the mean ± standard deviation (n=6). Different letters (a, b) above the columns indicate significant differences between the groups ($p < 0.05$).

Large intestine measurement: The large intestine was excised on the day after the final administration, and the length from the colon to the anus was measured as the large intestine length. No significant differences were observed in large intestine length between any of the groups (data not shown).

DISCUSSION

In this study, the treatment with N1487-7-HK resulted in an increase in *IL-10* mRNA and IL-10 protein expressions in intestinal epithelial cells challenged with inflammatory cytokines. However, there was no control of unstimulated cells treated with N1487-7-HK, so the effect of N1487-7-HK on IL-10 expression in the absence of inflammation was unknown. The intestinal mucosa functions as a selective barrier, safeguarding the internal environment from harmful toxins and pathogens. This barrier has a complex, multilayered structure: the outermost layer is the mucus layer, the middle layer consists of intestinal epithelial cells, and the innermost layer is composed of innate and adaptive immune cells [22]. Intestinal epithelial cells can synthesize and release IL-10, playing a critical, intestinal tissue-dependent immune role.[23] It was suggested that N1487-7-HK promotes the production of IL-10 in intestinal epithelial cells during inflammation.

In this study, N1487-7-HK treatment increased the expression of *IL-10* mRNA and IL-10 protein in macrophages. There are two types of macrophages, pro-inflammatory macrophages (M1) and M2, which rapidly and reversibly polarize to one of these types depending on

the environment [24-25]. M1 does not produce or release IL-10, whereas M2 synthesizes and releases IL-10.[26] An open-label study was conducted in healthy male and female aged 40–65, in which they took N1487-7-HK 6 × 10^10 cells/d for 8 weeks (unpublished). Flow cytometry of immune cells showed significantly decreased in the expression of CD80 in macrophages (pre-dose Week 0: 913.18 ± 116.13, Week 8: 839.97 ± 160.79, p = 0.017). Since CD80 is known to be an M1 phenotype [27], intake of N1487-7-HK may polarize M1 to M2. These results suggest that N1487-7-HK polarizes M1 to M2 and promotes IL-10 production by M2.

It has been reported that severe colitis was induced and TEWL was significantly increased in rats given 2.0% DSS water *ad libitum* for the 1st, 18th, and 20th weeks. [28] In another study, female mice (9 weeks old) were given drinking water containing DSS (2.0, 2.5, or 3.0%) for 5 to 10 d. All three groups showed weight loss, increased DAI score due to diarrhea and bloody stools, shortened colon, elevated inflammatory cytokines, and intestinal inflammation. Further, all mice in the 3.0% DSS group died 10 d after DSS administration [29]. Based on the above, in the present study, the DSS concentration was set at a slightly lower level of 1.5%, and the mice were given DSS-treated water *ad libitum* for a period of 7 d each during Weeks 2–3 and Weeks 4–5, with a rest period (recovery period) during Weeks 3–4 to avoid inducing severe colitis. As a result, no mortality was observed, and the body weight of mice increased evenly. The DAI score was 1–2 out of 5, and although some loose stools and bloody stools were observed, the score quickly returned to the lowest score (normal value) of 0 during the DSS intake cessation period. The length of the large intestine was similar in all groups. These results suggest that colitis was not induced under this experimental condition.

In the animal experiment, the control group showed significantly higher TEWL than the normal group at Weeks 1 and 2, which is a non-DSS administration period. On the other hand, the N1487-7-HK group showed higher values than the normal group, but not significantly so. Furthermore, the N1487-7-HK group showed significantly lower TEWL in Week 4, a non-DSS administration period, compared to Week 0. In the N1487-7-HK group, compared to Week 0, from Week 3 onwards, lower TEWL values were observed, although there was no significant difference. In the control group, while no significant difference was observed from Week 3 onwards compared to Week 0, higher values were observed. During the entire study, the normal group was allowed to consume normal food and water *ad libitum*. On the other hand, the control and N1487-7-HK groups were restrained once a day for 35 d, and a gavage was inserted directly into the stomach. Oral gavage administration in rats and mice can lead to considerable stress responses, marked by elevated blood pressure, increased heart rate, and heightened plasma corticosterone levels [30, 31]. In stressful situations, glucocorticoid hormones—corticosterone in rodents and cortisol in humans—are released into the bloodstream [32]. Studies have shown that in mice experiencing psoriasis triggered by sleep deprivation stress, levels of pro-inflammatory cytokines like IL-6 increased significantly, while IL-10 levels decreased notably. Additionally, a positive correlation was observed between corticosterone and IL-6 levels in these stressed mice [33]. In addition, psychological stress increases IL-6 levels [34]. Exposure to ultraviolet B has been shown to significantly increase IL-6 protein expression in hairless mice, which further significantly increased TEWL [35]. Particulate matter significantly increased IL-6 protein expression, which further significantly decreased filaggrin protein expression, skin barrier protein [5]. In humans and mice, reduced filaggrin expression is associated with skin barrier abnormalities and contributes to the development of psoriasis. [36] When heat-killed *L. casei* DKGF7 was

administered to rats with irritable bowel syndrome induced by restraint stress for four weeks, colonic inflammatory cytokine levels and serum corticosterone levels were reduced in the heat-killed *L. casei* DKGF7-treated group compared to the control [37]. In our mouse experiment, the N1487-7-HK group showed significantly higher serum IL-10 levels than the normal and control groups. These results suggest that, compared to the normal group, the control group experienced a significant increase in TEWL due to the stress of forced oral administration; however, the N1487-7-HK group suppressed the increase in TEWL and the decrease in filaggrin because of the increase in serum IL-10. In other words, N1487-7-HK administration suppressed the decline in skin barrier function caused by stress. However, since there was no control group administered N1487-7-HK without stress, the effect of N1487-7-HK intake on skin barrier function in the absence of inflammation was unknown.

As for the functional components, it is unclear whether all or part of N1487-7-HK acts on intestinal epithelial cells or macrophages to exert the effects. An experiment was performed in which live N1487-7 bacteria were administered to mice under the same conditions as in the present animal experiment, but no significant increase in serum IL-10 levels or reduction in TEWL was observed (unpublished). When bacterial cells are exposed to high temperatures, heat shock proteins are rapidly and transiently synthesized to prevent protein damage [38]. Possible functional components include heat-denatured cell walls and cell membranes, and heat shock proteins that are synthesized instantly and temporarily.

No adverse events occurred during the five-week administration of 0.8 mg/mouse of N1487-7-HK, suggesting a high level of safety.

There have been no reports to date of N1487-7-HK directly acting on intestinal epithelial cells and macrophages to exert an anti-inflammatory effect, or of its ability to inhibit the decline of skin barrier function. In addition, the safety of N1487-7-HK has not been reported, so the findings obtained in this study are highly novel. Based on the findings, a double-blind study was conducted to investigate the effect of N1487-7-HK in improving liver function through its anti-inflammatory effect. An article on this double-blind study will be submitted to this journal soon.

CONCLUSIONS

In this study, N1487-7-HK suppressed the stress-induced TEWL increase by promoting the production of IL-10 from intestinal epithelial cells and macrophages. This suggests that N1487-7-HK intake may improve the skin barrier function through its anti-inflammatory effect. Serum IL-10 level is an important biomarker for anti-inflammatory activity. N1487-7-HK demonstrated high level of safety. These findings suggest that N1487-7-HK is a functional food ingredients /component that can prevent or alleviate diseases caused by inflammation.

Abbreviations: IL: interleukin, TEWL: transepidermal water loss, DSS: dextran sulfate sodium, M2: anti-inflammatory macrophages, *L. plantarum*: *Lactiplantibacillus plantarum*, N1487-7: *Lactiplantibacillus plantarum* N1487-7, N1487-7-HK: heat-killed *Lactiplantibacillus plantarum* N1487-7, IFN-γ: interferon-gamma, real-time PCR: real-time quantitative polymerase chain reaction, Week 0: tests performed before starting oral administration, Week 1–5: test conducted 1–5 weeks after starting oral administration, DAI: disease activity index, xx ± yy: mean value ± standard deviation.

Acknowledgement: The present study was funded by Sanwa Shurui Co., Ltd.

Competing Interests: Uehara and Hokazono are

employees of Sanwa Shurui Co., Ltd. Sanwa Shurui Co., Ltd. concluded an outsourcing contract with Japan SLC Co., Ltd. to outsource the animal experiment. The compensation based on these operating agreements was legitimate business compensation for the implementation of the experiment and does not affect the experiment results.

Author Contributions: Conceptualization: E.U. Data curation: E.U. Formal analysis: E.U. Funding acquisition: E.U. and H.H. Investigation: E.U. Methodology: E.U. Project administration: E.U. and H.H. Resources: E.U. Supervision: H.H. Visualization: E.U. Writing-original draft E.U. Writing-review and editing: H.H.

REFERENCES

1. O'Neill CA, Monteleone G, McLaughlin JT, Paus R: The gut-skin axis in health and disease: a paradigm with therapeutic implications. BioEssays 2016, 38: 1167–1176. DOI: https://doi.org/10.1002/bies.201600008
2. Arck P, Handjiski B, Hagen E, Pincus M, Bruenahl C, Bienenstock J, Paus R: Is there a 'gutbrain-skin axis'? Exp Dermatol 2010, 19: 401–405. DOI: https://doi.org/10.1111/j.1600-0625.2009.01060.x
3. Song H, Yoo Y, Hwang J, Na YC, Kim HS: Faecalibacterium prausnitzii subspecies-level dysbiosis in the human gut microbiome underlying atopic dermatitis. J Allergy Clin Immunol 2016, 137: 852–860. DOI: https://doi.org/10.1016/j.jaci.2015.08.021
4. Mahmud MD, Akter S, Tamanna SK, Mazumder L, Esti IZ, Banerjee S, Akter S, et al.: Impact of gut microbiome on skin health: gut-skin axis observed through the lenses of therapeutics and skin diseases. Gut Microbes 2022, 14: 2096995. DOI: https://doi.org/10.1080/19490976.2022.2096995
5. Roh YJ, Choi YH, Shin SH, Lee M-K, Won YJ, Lee JH, Cho BS, et al.: Adipose tissue-derived exosomes alleviate particulate matter-induce inflammatory response and skin barrier damage in atopic dermatitis-like triple-cell model. PLoS One 2024, 19: e0292050. DOI: https://doi.org/10.1371/journal.pone.0292050
6. Ferrucci S, Romagnuolo M, Maronese CA, Germiniasi F, Tavecchio S, Angileri L, Casazza G, et al.: Skin barrier status during dupilumab treatment in patients with severe atopic dermatitis. Ther Adv Chronic Dis 2021, 12: 20406223211058332. DOI: https://doi.org/10.1177/20406223211058332
7. Yu P, Teng X, Liu I, Li Y, Ni J, Xue S, Wang J: Effect of an oral probiotic formula on scalp and facial skin condition, glucose, and lipid metabolism. Functional Foods in Health and Disease 2022, 12: 394–409. DOI: https://doi.org/10.31989/ffhd.v12i7.944
8. Yokoyama S, Hiramoto K, Koyama M, Ooi K: Impairment of skin barrier function via cholinergic signal transduction in a dextran sulphate sodium-induced colitis mouse model. Exp Dermatol 2015, 24: 779–784. DOI: https://doi.org/10.1111/exd.12775
9. Rutz S, Ouyang W: Regulation of interleukin-10 expression. Adv Exp Med Biol 2016, 941: 89–116. DOI: https://doi.org/10.1007/978-94-024-0921-5_5
10. Eva Latorre, Elena Layunta, Laura Grasa, Julián Pardo, Santiago García, Ana I Alcalde, José E Mesonero: Toll-like receptors 2 and 4 modulate intestinal IL-10 differently in ileum and colon. United European Gastroenterol J 2018, 6: 446–453. DOI: https://doi.org/10.1177/2050640617727180
11. Feng L, Chen G, Guo Z, Yao W, Li X, Mu G, Zhu X: Both live and heat killed Lactiplantibacillus plantarum DPUL-F232 alleviate whey protein-induced food allergy by regulating cellular immunity and repairing the intestinal barrier. Food Funct 2024, 20: 5496– 5509. DOI: https://doi.org/10.1039/d4fo00105b
12. Toshimitsu T, Mochizuki J, Ikegami S, Itou H: Identification of a Lactobacillus plantarum strain that ameliorates chronic inflammation and metabolic disorders in obese and type 2 diabetic mice. J Dairy Sci 2016, 99: 933–946. DOI: https://doi.org/10.3168/jds.2015-9916
13. Kakiyama S, Kubota N, Shida K, Harima-Mizusawa N: Effects of citrus juice fermented with Lactobacillus plantarum YIT 0132 on Japanese cedar pollinosis during probiotic consumption: an open study. Biosci Microbiota Food Health 2020, 39: 137–144. DOI: https://doi.org/10.12938/bmfh.2019-017
14. Nyotohadi D, Kok T: Potential of multi-strain probiotics extract as an anti-inflammatory agent through inhibition of macrophage migration inhibitory factor activity. Functional Foods in Health and Disease 2023, 13: 1–10. DOI: https://doi.org/10.31989/ffhd.v13i1.1033
15. Laosee W, Kantachote D, Chansuwan W, Thongraung C, Sirinupong N: Anti-salmonella potential and antioxidant activity of fermented fruit-based juice by lactic acid bacteria and its biotransformation. Functional Foods in Health and Disease 2021, 11: 368–384. DOI: https://doi.org/10.31989/ffhd.v11i8.813
16. Williams K, Oo T, Martirosyan D: Exploring the effectiveness of

lactobacillus probiotics in weight management: A literature review. Functional Food Science 2023, 3: 42–54. DOI: https://www.doi.org/10.31989/ffs.v3i5.1115

17. Martirosyan D, Kanya H, Nadalet C: Can functional foods reduce the risk of disease? Advancement of functional food definition and steps to create functional food products. Functional Foods in Health and Disease 2021, 11: 213–221. DOI: https://doi.org/10.31989/ffhd.v11i5.788
18. Martirosyan D, Lampert T, Lee M: A comprehensive review on the role of food bioactive compounds in functional food science. Functional Food Science 2022, 2: 64–78. DOI: https://www.doi.org/10.31989/ffs.v2i3.906
19. Martirosyan D, Stratton S: Quantum and tempus theories of function food science in practice. Functional Food Science 2023, 3: 55–62. DOI: https://doi.org/10.31989/ffs.v3i5.1122
20. Furuta Y, Maruoka N, Nakamura A, Omori T, Sonomoto K: Novel conversion processes with lactic acid bacteria from shochu kasu into valuable materials. Seibutsu-kogaku 2010, 88: 114–120. (in Japanese)
21. Ihaka R, Gentleman R: R: a language for data analysis and graphics. J Comp Graph Stat 1996, 5: 299–314. DOI: https://www.tandfonline.com/doi/epdf/10.1080/10618600.1996.10474713
22. Yu X, Wang Y, Xu Y, Li X, Zhang J, Su Y, Guo L: Resveratrol attenuates intestinal epithelial barrier dysfunction via Nrf2/HO-1 pathway in dextran sulfate sodium-induced Caco-2 cells. Immun Inflamm Dis 2024, 12: e1193. DOI: https://doi.org/10.1002/iid3.1193
23. Latorre E, Layunta E, Grasa L, Pardo J, García S, Alcalde AI, Mesonero JE: Toll-like receptors 2 and 4 modulate intestinal IL-10 differently in ileum and colon. United European Gastroenterol J 2018, 6: 446–453. DOI: https://doi.org/10.1177/2050640617727180
24. Ma Y, Yang X, Ning K, Guo H: M1/M2 macrophage-targeted nanotechnology and PROTAC for the treatment of atherosclerosis. Life sci 2024, 352: 122811 DOI: https://doi.org/10.1016/j.lfs.2024.122811
25. Lyu Q, Veldhuizen E JA, Ludwig IS, Rutten V PMG, Eden W, Sijts A JAM, Broere F: Characterization of polarization states of canine monocyte derived macrophages. PLoS One 2023, 8: e0292757. DOI: https://doi.org/10.1371/journal.pone.0292757
26. Huang X, Li Y, Fu M, Xin HB: Polarizing macrophages in vitro. Methods Mol Biol 2018, 1784: 119–126. DOI: https://doi.org/10.1007/978-1-4939-7837-3_12
27. Wang Y, Zou Y, Jiang Q, Li W, Chai X, Zhao T, Liu S, et al.: Ox-LDL-induced CD80+ macrophages expand pro-atherosclerotic NKT cells via CD1d in atherosclerotic mice and hyperlipidemic patients. Am J Physiol Cell Physiol 2024, 326: C1563–C1572. DOI: https://doi.org/10.1152/ajpcell.00043.2024
28. Yokoyama S, Hiramoto K, Koyama M, Ooi K: Impaired skin barrier function in mice with colon carcinoma induced by azoxymethane and dextran sodium sulfate. Biol Pharm Bull 2015, 38, 947–950. DOI: https://doi.org/10.1248/bpb.b15-00208
29. Jiang X, Chen X, Dong R, Wang J, Pan Y, Cao Y: Establishment of a mouse model of inflammatory bowel disease using dextran sulfate sodium. Adv Clin Exp Med 2023, 32: 563–573. DOI: https://doi.org/10.17219/acem/156647
30. Brown AP, Dinger N, Levine BS: Stress produced by gavage administration in the rat. Contemp Top Lab Anim Sci 2000, 39: 17–21.
31. Bonnichsen M, Dragsted N, Hansen AK: The welfare impact of gavaging laboratory rats. Animal Welfare 2005, 14: 223–227. DOI: https://doi.org/10.1017/S0962728600029389
32. 32. Pegueros-Maldonado R, Pech-Pool SM, Blancas JJ, Prado-Alcalá RA, Arámburo C, Luna M, Quirarte GL: Inhibition of corticosterone synthesis impairs cued water maze consolidation, but it does not affect the expression of BDNF, CK2 and SGK1 genes in dorsal striatum. Front Behav Neurosci 2024, 18: 1341883. DOI: https://doi.org/10.3389/fnbeh.2024.1341883
33. Hirotsu C, Rydlewski M, Araújo MS, Tufik S, Andersen ML: Sleep loss and cytokines levels in an experimental model of psoriasis. PLoS One 2012, 7: e51183. DOI: https://doi.org/10.1371/journal.pone.0051183
34. Brydon L, Edwards S, Jia H, Mohamed-Ali V, Zachary I, Martin JF, Steptoe A: Psychological stress activates interleukin-1beta gene expression in human mononuclear cells. Brain Behav Immun 2005, 19: 540–546. DOI: https://doi.org/10.1016/j.bbi.2004.12.003
35. Kuo YH, Lin TY, You YJ, Wen KC, Sung PJ, Chiang HM: Antiinflammatory and antiphotodamaging effects of ergostatrien-3β-ol, isolated from antrodia camphorata, on hairless mouse skin. Molecules 2016, 21: 1213. DOI: https://doi.org/10.3390/molecules21091213
36. Zhang Y, Tu C, Wang S, Xiao S: Expression of skin barrier protein filaggrin in skin diseases without atopic dermatitis. J Biosci Med 2018, 6: 101–112. DOI: https://doi.org/10.4236/jbm.2018.61009
37. Seong G, Lee S, Min YW, Jang YS, Kim HS, Kim EJ, Park SY, et al.: Effect of Heat-Killed Lactobacillus casei DKGF7 on a Rat Model of Irritable Bowel Syndrome. Nutrients 2021, 13: 568. DOI: https://doi.org/10.3390/nu13020568
38. Yura T, Nagai H, Mori H: Regulation of the heat-shock response in bacteria. Annu Rev Microbiol 1993, 47: 321–350. DOI:https://doi.org/10.1146/annurev.mi.47.100193.001541