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Original article

Effects of kestose on gut mucosal immunity in an atopic dermatitis mouse model

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ABSTRACT

Background: Atopic dermatitis (AD) is recently increasing among populations, but the underlying mechanisms remain controversial. Interactions between the gut microbiota and mucosal immunity are considered to be a crucial etiology. Fructooligosaccharide (FOS), prebiotics have been reported as activators of the gut microbiota.

Objective: The aim of this study was to investigate the effects of kestose, the smallest FOS and FOS on atopic dermatitis in mice.

Methods: An AD mouse model was developed by (ovalbumin) epidermal sensitization using BALB/c mice. Kestose (1%, 5%, and 10%) or FOS (5%, positive control) was orally administered throughout the study.

Results: In comparison with the values observed for the control AD mice, transepidermal water loss (TEWL), clinical score, and skin inflammation on histopathology were significantly decreased by the oral administration of kestose. Total IgE, thymic stromal lymphopoietin (TSLP) in skin, and IL-4 were also suppressed by this administration. In addition, the population of CD4⁺Foxp3⁺ cells in mesenteric lymph nodes (MLNs) and acetate concentrations in feces were significantly increased by kestose treatment.

Conclusions: These findings suggest that kestose activates the gut immune system to induce the tolerance against allergic skin inflammations in AD.

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1. Introduction

Atopic dermatitis has markedly increased in western countries and has been investigated with respect to the relationship between environmental factors and the human immune system [1,2]. Allergic diseases could be driven by in response to environmental or food antigens [2]; however, the mechanisms are not clearly understood. Recently, one of the more interesting studies investigating intestinal microbiota reported that commensal

bacteria and their metabolites interact with the development of the immune system.

Food antigens also affect gut mucosal immune responses, which could be used to modulate gut microbiota. Prebiotics are defined as nondigestive food ingredients that stimulate the growth and activity of beneficial bacteria in the intestines, thereby improving host health [3]. Recently, many prebiotic studies have used short-chain fructooligosaccharides (FOS; degree of polymerization, 2–9) and long-chain inulin (degree of polymerization, 10–60) [4].

Some studies have shown that kestose, the smallest short-chain FOS (glucose–fructose–fructose) significantly stimulated the growth of several species of *Bifidobacterium* [5–7]. *Bifidobacterium*, one of the probiotics, is a predominant component of the intestinal microbiota in infants and is believed to provide beneficial effects pertaining to the prevention of AD [1,4,8]. The growth of

Abbreviations: AD, atopic dermatitis; TEWL, transepidermal water loss; TSLP, thymic stromal lymphopoietin; FOS, fructooligosaccharide; SCFA, short-chain fatty acids; OVA, ovalbumin; MLNs, mesenteric lymph nodes.

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Faecalibacterium prausnitzii was also stimulated by kestose in the intestinal tract, thereby improving AD symptoms in infants [4]. However, the clinical effects and mechanisms of kestose are still not clearly understood.

We previously demonstrated the effects of probiotics on AD and allergic march mouse models [9,10]. In addition to the established conclusions, these studies indicated a potential of an increase in probiotics in the intestinal environment having beneficial effects on AD and suggested that the mechanism eliciting systemic regulation of allergic inflammation was related to regulatory T cells (Tregs). In the present study, we investigated the effects of kestose on an AD mouse model to identify the mechanism responsible for the interaction between the intestinal microbiota and mucosal immunity.

2. Materials and methods

2.1. Mice

Female BALB/c mice weighing 16–20 g (4-week old) were purchased from Orient Bio (Orient Bio Inc., Seongnam, Korea) and treated in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Asan Medical Center and Ulsan University College of Medicine.

2.2. Development of AD and administration of kestose in mice

In the AD model, OVA sensitization was performed as previously described with few modifications [11]. In brief, to develop an AD mouse model using skin sensitization, 6-week-old female mice were anesthetized and their backs were shaved. Skin injury was induced by repeated tape stripping, after which 100 µg of OVA (ovalbumin, grade V; Sigma, St Louis, MO) in 100 µL of PBS or PBS only was applied on a sterile patch and attached to the tape-stripped area with an adhesive tape. Treatment was repeated once during the 1-week sensitization period, after which a 2-week resting period was allowed, and then a similar sensitization treatment was again performed. The mice were euthanized and samples were collected 24 h after the last treatment. Samyang Genex Co. (Seoul, Korea) provided FOS (95% FOS powder) and kestose (79.1% kestose syrup). The mice received kestose (1%, 5%, or 10%/mouse/day, respectively) orally for 2 weeks before primary sensitization until the end of the study. FOS (5%/mouse/day) was administered as a positive control for kestose (Fig. 1).

2.3. Estimation of clinical signs and skin barrier function in the AD mouse model

Dorsum lesions were scored for erythema, scaling, and excoriation after each sensitization using a 0–3 scoring system, where 0 = no lesion, 1 = mild lesion, 2 = moderate lesion, and 3 = severe lesion [9]. The same investigator performed all scoring evaluations throughout the study. Three investigators

randomized performed scoring evaluations throughout the study, then calculate the average.

To determine whether epidermal permeability barrier function was altered due to OVA-induced AD, we measured transepidermal water loss (TEWL) using a vapometer (SWL-3; Delfin Technologies Ltd., Kuopio, Finland). We assessed the baseline TEWL at the beginning of the experiment, and then performed one TEWL assessment after each sensitization.

The dorsal skins of experimental mice were removed on the final day of the schedule and fixed in 10% phosphate-buffered formalin before being embedded in paraffin for histopathological evaluation. Serial paraffin sections (4.5-mm thick) were stained with hematoxylin and eosin (H&E) for evaluation of skin inflammatory cell infiltration.

2.4. Quantitation of serum immunoglobulin (Ig) levels

Serum samples were obtained from blood taken during exsanguinations of the mice after sensitization treatments and stored –80 °C until analyzed. Total serum IgE levels were detected using the Mouse IgE ELISA kit (eBioscience, San Diego, CA, USA). Determinations were performed in duplicate. The optical density was measured at 450 nm.

2.5. Real-time PCR

To measure the expression of IL-4 and TSLP in mice skin, RNA was extracted from the dorsal skin of experimental mice using the RNeasy kit (Qiagen, Valencia, CA, USA). Real-time PCR was performed using the TaqMan method on an ABI 7900 system (Applied Biosystems, Piscataway, NJ, USA). Each signal was normalized against the levels of GAPDH mRNA in the same sample.

2.6. Flow cytometry

Mouse Tregs were collected from the mesenteric lymph nodes (MLNs) and skin lymph nodes. Mouse Treg-staining kits (using FITC-labeled anti-CD4, APC-labeled anti-Foxp3, and respective isotype controls; eBioscience, San Diego, CA) were used to analyze CD4 and Foxp3 expression in accordance with the manufacturer's directions. Lymphocytes were gated based upon expression of CD4 to identify Foxp3 expression in the total CD4⁺ fractions. Staining was analyzed by flow cytometry using FACS Calibur with CellQuest software (BD Biosciences, Mountain View, CA).

2.7. Measurement of short-chain fatty acids (SCFA) from feces

For measurement of SCFAs, 100 mg of mice feces were homogenized with 4 volumes of deionized water. Then supernatants were collected and an internal standard solution (100 µL of 10 mg/mL propionic acid-2,2-d₂, Sigma-Aldrich Co Ltd, Yongin, Korea), 20 µL of 0.6 N HCl, and 2 mL diethyl ether were added. The upper layer was collected, and the step was repeated as necessary.

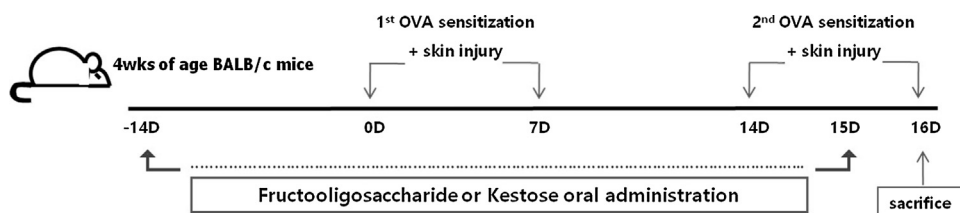


Fig. 1. The present study design. BALB/c mice were given a total of three 1-week epicutaneous exposures to OVA, with 2-week intervals between exposures. Kestose was orally administered to mice for 2 weeks before primary sensitization until the end of the study. Fructooligosaccharide was also administered as a positive control.

The collected solutions were completely dried under vacuum. Then, 100 μ L of 58 mg/mL pentafluorobenzyl bromide in acetonitrile and 20 μ L N, N-Diisopropylethylamine were added to the dried matter. The mixtures were vortexed well and incubated at room temperature for 20 min. After the derivatization reaction, the sample solutions were dried under vacuum, and reconstituted with 100 μ L acetonitrile.

SCFAs were analyzed with GC–MS (7890A/5975A (Agilent)) and HP-5 MS 30 m $\times$ 250 μ m $\times$ 0.25 μ m column (Agilent 19091S-433). He (99.999%) was used as a carrier gas. The oven program was set at an initial temperature of 50 $^{\circ}$ C and was then increased up to 100 $^{\circ}$ C at 10 $^{\circ}$ C/min increments and held for 3 min. Then the temperature was increased to 130 $^{\circ}$ C at 2.5 $^{\circ}$ C/min increments. Finally, the temperature was increased to 230 $^{\circ}$ C at 30 $^{\circ}$ C/min increments and held for 9 min. A 6-min solvent delay and SIM mode were applied. The extracted ion chromatogram corresponding to specific SCFAs was used for quantification.

2.8. Statistical analysis

Statistical significant differences were calculated (GraphPad Prism 5.0 San Diego, CA) using one-way analysis of variance (ANOVA; Tukey's multiple comparison test). A *p* value of <0.05 was considered to indicate statistical significance.

3. Results

3.1. Kestose significantly suppressed skin inflammation and improved TEWL and clinical scores in the AD mouse model

Histopathological evaluation revealed that skin inflammatory cell infiltrations were significantly reduced by treatments with kestose and FOS as compared to those by treatment with OVA only (Fig. 2a). In addition, TEWL and clinical scores were significantly suppressed by the administration of 5% and 10% kestose treatments compared to those by the administration of OVA only (Fig. 2b, c); however, there were no significantly different effects on these parameters between the two kestose concentrations.

These findings indicate that kestose could suppress skin inflammation, thereby improving skin barrier function and clinical symptoms at appropriate concentrations in the AD mouse model.

3.2. Kestose significantly suppressed systemic immune response and skin Th2 cytokines in the AD mouse model

Serum IgE levels were significantly suppressed by kestose as well as FOS (Fig. 3a). Kestose (1%, 5%, 10%) and FOS (5%) administration compared with only OVA administration also suppressed skin Th2-related cytokines, TSLP, and IL-4 (Fig. 3b,

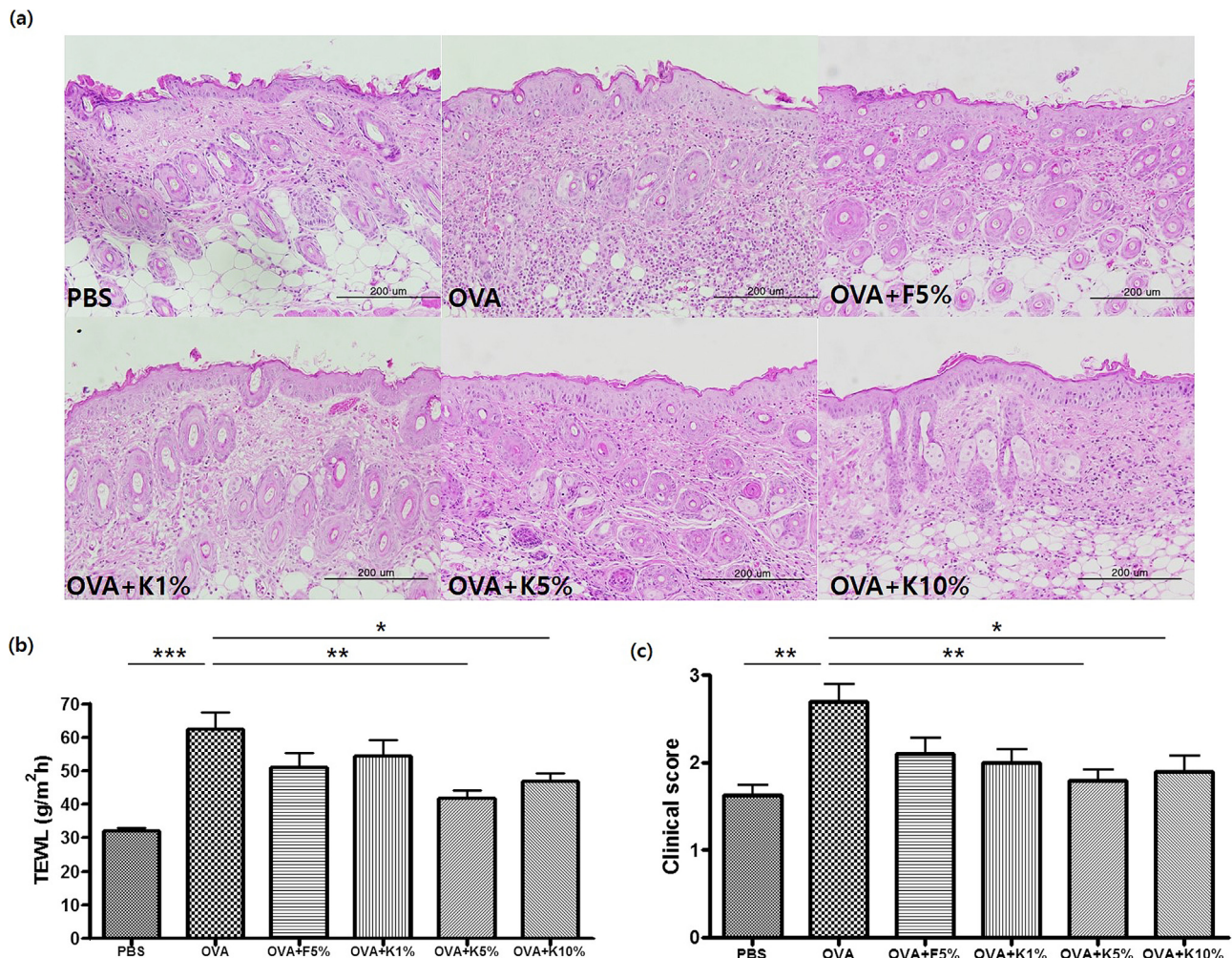


Fig. 2. Effects of kestose on skin histology and the epidermal permeability barrier in the AD mouse model. (a) Hematoxylin and eosin (H&E) staining, (b) transepidermal water loss (TEWL), and (c) clinical score (**P* < 0.05, ***P* < 0.01, ****P* < 0.001). OVA: ovalbumin; F: Fructooligosaccharide; K: kestose.

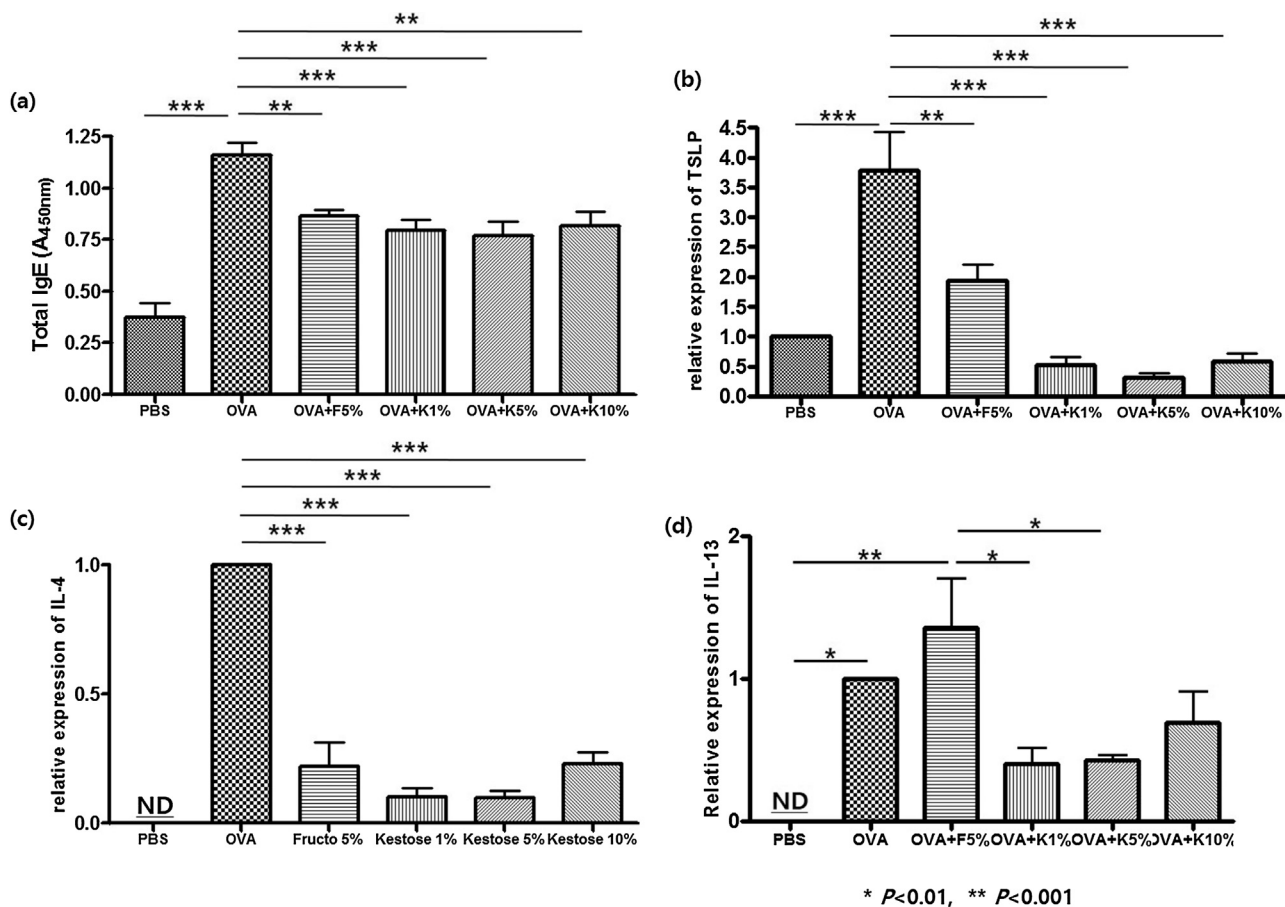


Fig. 3. Effects of kestose on IgE serum levels and Th2-related cytokine in the AD mouse model. (a) Serum levels of total IgE were assessed by ELISA. Skin mRNA expression levels of Th2-related cytokines (b. TSLP; c. IL-4) were assessed by real-time PCR (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). OVA: ovalbumin; F: Fructooligosaccharide; K: kestose.

c). All concentrations of kestose worked beneficially, and there were no differences in efficacy among concentrations.

These findings indicate that allergic systemic response and skin Th2-related inflammation could be suppressed by kestose in the AD mouse model.

3.3. Kestose significantly increased mesenteric CD4⁺Foxp3⁺Treg cells in the AD mouse model

The population of CD4⁺Foxp3⁺Treg cells was significantly increased in MLNs and skin lymph nodes by kestose (1%, 10%)

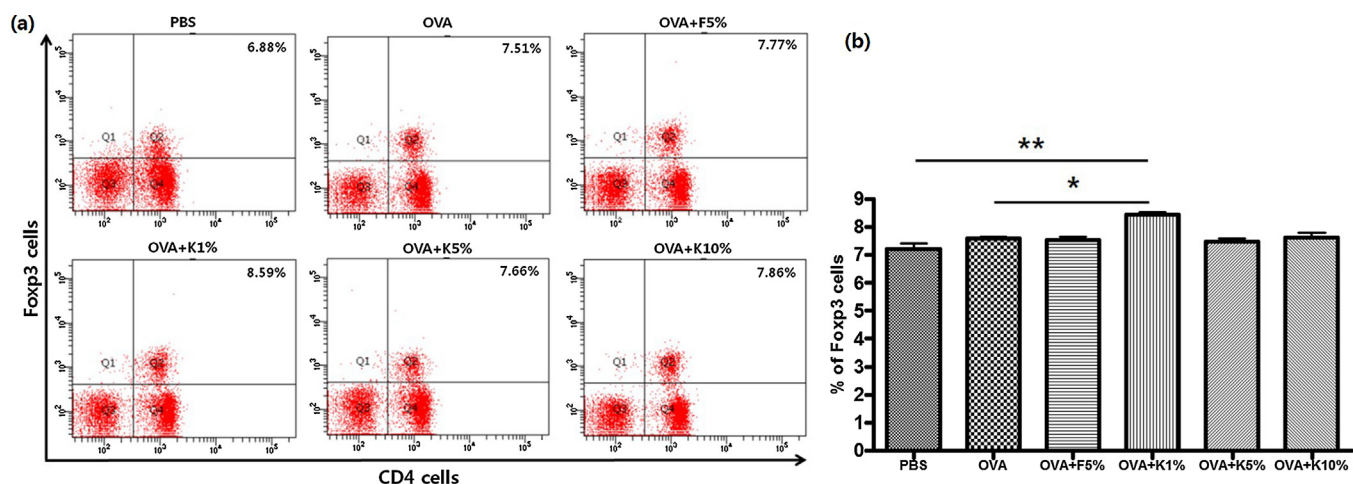


Fig. 4. Effects of kestose on CD4⁺Foxp3⁺ cells of the small intestine in the AD mouse model. The frequency of CD4⁺Foxp3⁺ cell were assessed by flow cytometry (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). OVA: ovalbumin; F: Fructooligosaccharide; K: kestose.

and FOS treatments, compared to those with only OVA treatment (Fig. 4, Suppl. Fig. 1). There was a significant difference observed in cell populations between 1% and 5% kestose concentrations (Fig. 4b). This finding suggests that kestose could elicit the regulation of systemic allergic immune response in mice by increasing CD4⁺Foxp3⁺Treg cells in the small intestinal mucosa. In addition, the ability could not correlate with concentration of kestose in the AD mouse model.

3.4. Kestose significantly increased acetate concentrations in feces in the AD mouse model

Concentrations of SCFA in feces showed increasing tendencies in the 1% kestose treatment compared with those in only OVA treatment (Fig. 5). Particularly for acetate concentrations, there was a significant difference between the 1% kestose treatment and the OVA treatment ($P < 0.05$, Fig. 5a).

This finding suggests that kestose stimulated intestinal microbiota to generate SCFAs, particularly acetate, in the AD mouse model.

4. Discussion

The present study showed that kestose as prebiotics improved clinical signs and Th2- related skin inflammation with systemic allergic suppression in an AD mouse model. The associated mechanism may be connected with increasing SCFA concentrations in the intestinal microbiota that modulate CD4⁺Foxp3⁺Treg cells in MLNs. This is the first study described the potential use of kestose as an alternative food ingredient for AD management using AD mouse model.

FOS is a well-commercialized and researched prebiotic, and several beneficial properties have been reported [12]. Daily intake of FOS increases levels of fecal bifidobacteria, which are associated

with an increase in specific immune response in infants [13], and modulates cytokine secretion in human peripheral blood monocytes [14]. FOS comprises different ratios of 1-kestose, nystose, and fructofructanynystose, which have 1–3 fructose monomers linked with sucrose via β -2,1 glycosidic bonds [15]. The key components of prebiotic activity have not been well investigated.

Previous studies showed that kestose stimulated the growth of beneficial microbes (lactic acid bacteria) *in vitro* and in human clinical data [4–7]. It has also been reported from clinical studies that kestose exerts a beneficial effect on clinical symptoms in infants with AD [7]. These studies indicated that kestose could stimulate the growth of beneficial intestinal microbes that control the host's intestinal immune system; however, there is not enough evidence currently for choosing kestose over other prebiotics as a supportive healthy food ingredient.

The present study showed that kestose (5% and 10%) significantly improved clinical scores and TEWL compared with FOS in the AD mouse model. In this study, all concentrations of kestose and FOS significantly suppressed Th2-related inflammation in the skin, represented by TSLP and IL-4. All concentrations of kestose also decreased serum IgE levels. There were no differences among groups. These findings suggested that kestose improved clinical symptoms more efficiently than FOS in the AD mouse model. The mechanisms associated with this action were shown to be an increase in CD4⁺Foxp3⁺Treg cells in MLNs as well SCFAs in feces.

We previously reported that a lactic acid microbe, *Lactobacillus rhamnosus*, suppressed skin allergic inflammation and systemic immune response. This was accomplished by modulating the intestinal immune system, as represented by CD4⁺Foxp3⁺Treg cells in MLNs in the AD and allergic march mouse models [9,10]. These studies showed that increasing beneficial intestinal microbes could control the intestinal immune system to block AD allergic reactions. The associated mechanisms from these

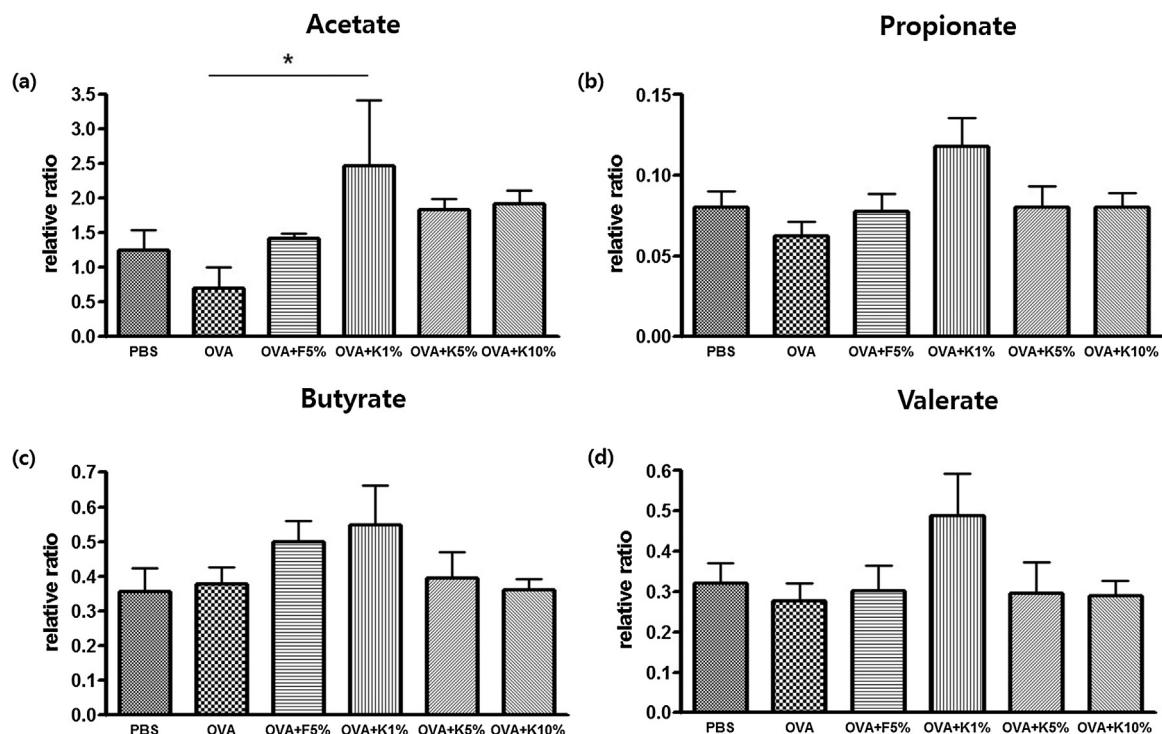


Fig. 5. Effects of kestose on short-chain fatty acids (a–d, SCFA) in feces of the AD mouse model of AD. Representative GC chromatograms of SCFAs were analyzed by the Metaboanalyst software suite (* $P < 0.05$). OVA: ovalbumin; F: Fructooligosaccharide; K: kestose. Relative ratio = Analyte peak area/Intern standard peak area.

studies coincided with those from the current study. Our results suggest that kestose also affects the beneficial intestinal microbiota to modulate allergic immune responses in the AD mouse model. More specifically, the current study showed that kestose increased fecal SCFAs concentration in the AD mouse model.

One of the health effects of SCFAs is the concomitant reduction of the luminal pH, which alone inhibits pathogenic microorganisms and increases the absorption of some nutrients [16]. One percent kestose treatment concentrations showed an increasing pattern of total SCFA concentrations; however, only increased acetate concentrations due to kestose treatments were statistically significant.

Acetate has been reported as a key player in the ability of bifidobacteria to inhibit enteropathogens [17]. Some studies have reported the effects of SCFAs on intestinal immune function but only addressed butyrate and propionic acid [12,18–20] in terms of promoting colon motility, reducing inflammation, and inhibiting tumor cell progression. In addition, butyrate and propionate have also been reported to induce the differentiation of T-regulatory cells and assist in the control of intestinal inflammation [20]. Based on these studies, increased SCFA concentrations in feces would induce the differentiation of CD4⁺Foxp3⁺Treg cells in MLNs and affect the ability of beneficial microbes to inhibit enteropathogens in the AD mouse model. Interestingly, there were no correlations among the results of clinical data (clinical symptoms and immune responses of the skin) in AD and SCFA concentrations by kestose. On the other hand, there was a partial correlation between CD4⁺Foxp3⁺Treg cells in MLNs and SCFA concentrations by kestose (1%). The results indicated that SCFAs in feces mainly affect mesenteric Treg populations with the appropriate concentration of kestose in the AD mouse model. In addition, clinical signs and immunological changes relating to inflammation of the skin are probably influenced by kestose without a relationship between SCFA concentrations. Further studies are needed.

The current study has not proven that kestose increases the growth of beneficial intestinal microbes in the AD mouse model. More studies are needed to confirm this interaction between kestose and intestinal microbiota growth and composition.

In conclusions, kestose can significantly improve clinical symptoms and Th2- allergic inflammation in the skin by increasing CD4⁺Foxp3⁺Treg cells in MLNs in the AD mouse model. We suggest that the mechanism involves stimulating the growth of beneficial intestinal microbes that generate SCFAs in feces to increase CD4⁺Foxp3⁺Treg cells in MLNs.

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Conflict of interest

The authors have no conflict of interest to declare.

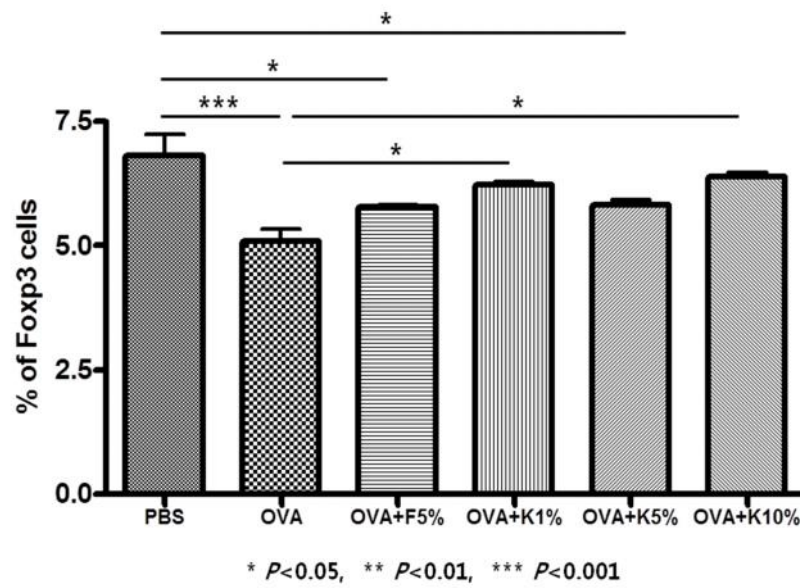
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: [10.1016/j.jdermsci.2017.10.006](https://doi.org/10.1016/j.jdermsci.2017.10.006).

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Supplemenatry Figure



Supplementary Figure 1. Percentage of Foxp3+ cells in draining lymph node from the back skin.