

## Article

# Influence of Oral Administration of Lactic Acid Bacteria Metabolites on Skin Barrier Function and Water Content in a Murine Model of Atopic Dermatitis

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**Abstract:** The effects of orally administered lactic acid bacteria metabolites on the skin were studied using an atopic dermatitis-like murine model created by feeding mice with HR-AD. The lactic acid bacteria metabolites were obtained by inoculating 35 strains of 16 species of lactic acid bacteria into soy milk and culturing them. The atopic dermatitis-like murine model was created by feeding HR-1 mice HR-AD for 40 days. The skin condition of mice that were fed HR-AD worsened compared with normal mice, i.e., reduced water content in the stratum corneum, increased transepidermal water loss (TEWL), reduced ceramide AP content in the stratum corneum, and increased epidermis thickness. When mice that had been fed the HR-AD diet was administered a raw liquid of lactic acid bacteria metabolites orally, the measured values related to water content in the stratum corneum, TEWL, ceramide AP content in the stratum corneum, and epidermis thickness improved. To find out the active components for these effects, filtrate and residue from the raw liquid of lactic acid bacteria metabolites and lipid components extracted from the raw liquid were examined at the same time. Results showed that the water-soluble components or residue after filtration did not demonstrate effects but the raw liquid and the lipid fraction did. These findings suggest that lactic acid bacteria metabolites improve skin injury in an atopic dermatitis-like murine model.

**Keywords:** oral administration; lactic acid bacteria metabolites; skin barrier function; water content; atopic dermatitis

## 1. Introduction

One hundred trillion enteric bacteria inhabit the human intestines, among which lactic acid bacteria are known to exist. Lactic acid bacteria metabolize sugar to produce lactic acid bacteria metabolites. If orally ingested lactic acid bacteria reach the intestines, it is believed that orally ingested lactic acid bacteria which reach the intestines have difficulty proliferating or colonizing the intestines because they are different from indigenous intestinal bacteria (intestinal flora). Ingested lactic acid bacteria are excreted from the body quickly. Therefore, it is hard for ingested lactic acid bacteria to exert their effects. Lactic acid bacteria metabolites are a fermentation product of lactic acid bacteria, and their effectiveness as biogenics has been reported in recent years [1]. It has been reported that biogenics activate immune function in the intestines and this effect is not mediated by intestinal flora, and that biogenics decrease reactive oxygen [2]. These lactic acid bacteria metabolites are present in foods such as cheese, yogurt, pickles, etc., and are therefore present in our everyday diet. An increase in interest in the field has led to lactic acid bacteria metabolites becoming a subject of research. The importance of food components that work directly or via intestinal flora on biological regulation, host defense, disease prevention, recovery, aging control, etc.—examples of which are immunostimulation, cholesterol-lowering effect, blood pressure-

lowering effect, regulatory effect on intestinal function, antitumor effect, antithrombotic effect, and hematopoiesis—has been reported by Mitsuoka et al. [3-6].

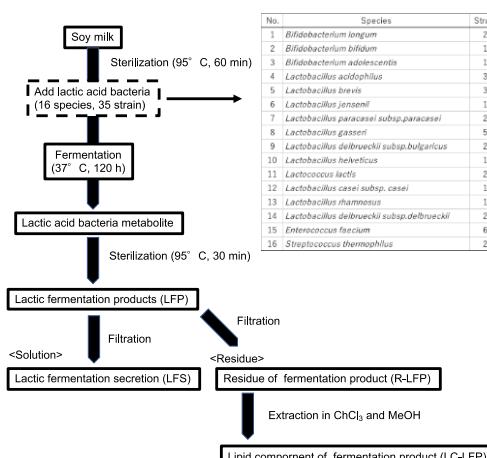
The incidence of atopic dermatitis is increasing. Dry skin, skin inflammation, and pruritus are the main symptoms [7]. These symptoms are often accompanied by the overproduction of IgE [8]. The pathogenic mechanisms of atopic dermatitis are complex, and it has been reported that there is less ceramide in the stratum corneum of atopic dermatitis patients compared with the stratum corneum of normal subjects [9-11]. Filaggrin gene mutations have also been reported to be a cause of atopic dermatitis [12, 13]. However, the fundamental causes or treatments have not been found yet and are being assiduously studied by researchers across the globe. Atopic dermatitis models using NC/Nga mice [14] and hairless mice fed with HR-AD have been reported [15,16]. Decreased water content in the stratum corneum, acanthosis, and changes in the content and composition of ceramide in the skin were noted in NC mice that developed dermatitis [14] while decreased water content in the stratum corneum, increased number of scratches, acanthosis, increased levels of total IgE in the blood, etc. were observed in hairless mice that were fed HR-AD [17].

In recent years, the association between intestinal environment and skin condition has been reported [18,19]. As lactic acid bacteria metabolites are presumed to exert various effects by regulating intestinal environment, the author evaluated the effectiveness of orally administered lactic acid bacteria metabolites in mice with atopic dermatitis-like disease induced by HR-AD and compared the water content in the stratum corneum, TEWL, and epidermis thickness in these mice with those of normal mice.

## 2. Materials and Methods

### 2.1. Samples administered

Lactic acid bacteria metabolites were provided by KOEI science laboratory Co. Ltd. (Wako City, Saitama, Japan). Soy milk was heat treated at 95°C for 1 hour. After it was cooled down, it was inoculated with 35 strains of 16 species of lactic acid bacteria and cultured at 37°C for 120 hours in a static culture (Figure 1). After the completion of culture, it was sterilized at 95°C for 30 minutes. The resultant liquid fermented by lactic acid bacteria was called lactic fermentation products (LFP). These lactic fermentation products contain soy milk as the substrate and killed lactic acid bacteria. The LFP were filtrated to obtain lactic fermentation secretion (LFS) and the residue of LFP (R-LFP). LFS is characterized by the absence of soy milk as the substrate and killed lactic acid bacteria. The R-LFP was heated to obtain solid matter. Chloroform and methanol were added onto the solid matter to recover lipids, which was called the lipid components of LFP (LC-LFP). When the weight of the LFP was set at 100%, the weight of the LFS was 80%, R-LFP 18%, and LC-LFP 2%. Each sample was adjusted to the same volume with water.



**Figure 1.** Method of production of the lactic acid bacteria metabolite and the species of microorganism used.

## 2.2. Animals and rearing

Male 6-week-old hairless mice were purchased from Hoshino Laboratory Animals, Inc. (Bando, Ibaraki, Japan) and kept for 1 week, after which the mice were allowed to consume HR-AD (NOSAN Corporation, Yokohama, Kanagawa, Japan) ad libitum. Similarly, a normal mice group was also allowed to consume Labo MR Stock (NOSAN Corporation, Yokohama, Kanagawa, Japan) ad libitum. As for drinking water, the mice were allowed to consume tap water ad libitum. The mice were reared in a room which had a 12-hour light/dark cycle (7 am to 7 pm) and were kept at 20–26 °C. All animal experiments and maintenance were performed under conditions approved by the Animal Research Committee of Josai University (approval date: April 3/2018, approval number: JU18094).

## 2.3. Grouping of animals and administration methods

The atopic dermatitis-like murine model was created by continuously feeding hairless mice with HR-AD for approximately 40 days [15]. Mice were divided into following 5 groups with 6 mice per group according to their weight on the day of experiment and treatment to be administered: a no treatment group (normal group), control group, LFP group, a group to which the filtrate from the LFP (LFS) would be administered, a group to which R-LFP would be administered, and a group to which the lipid components of LFP (LC-LFP) would be administered (Table 1). Each sample was administered orally through a tube at a volume of 0.3 mL per animal for 28 days. Body weight, water content in the stratum corneum, and TEWL were measured. At day 28, animals were sacrificed, and their skin was harvested. Frozen sections were created and stained with hematoxylin and eosin (HE) for light microscopy to observe for acanthosis. The amount of LFS or LC-LFP orally administered to the corresponding group was based on the LFS or LC-LFP that was obtained from LFP. That is, when 0.3 mL of LFP was administered per animal, the amount of LFS or LC-LFP obtained from 0.3 mL of LFP was administered. Ethanol at 10.8%, which was used as a vehicle for these fermented substances, was administered orally to the normal and control groups.

Table 1 Experimental groups in this study

Experimental group	Oral administration (0.3 ml/day)	Diet
Normal	None	LaboMR Stock (Regular diet)
Control	10.8% ethanol in water	HR-AD
Lactic fermentation product (LFP)	LFP/10.8% ethanol in water	HR-AD
Residue of lactic fermentation product (R-LFP)	R-LFP/10.8% ethanol in water	HR-AD
Lipid component of lactic fermentation product (LC-LFP)	LC-LFP/10.8% ethanol in water	HR-AD
Lactic fermentation secretion (LFS)	LFS/10.8% ethanol in water	HR-AD

## 2.4. Measurement of transepidermal water loss and water content of the stratum corneum

All measurements were performed in triplicate for each mouse, and the mean values were obtained. Water content of the stratum corneum and skin was measured with a Cutometer MPA580

(Courage & Khazaka, Cologne, Germany) and TEWL was assessed using a VAPO SCAN AS-VT100 RS (Asahi Techno Lab. Ltd., Yokohama, Kanagawa, Japan).

## 2.5. Hematoxylin and eosin staining

Cryosections were prepared from tissue samples embedded in optimal cutting temperature (OCT) compound. Skin sections (10  $\mu$ m) were stained with hematoxylin and eosin (HE) and analyzed for structural differences using light microscopy.

## 2.6. Collection of the stratum corneum

A slide glass with one drop of cyanoacrylate adhesive was pressed onto the back of a mouse under anesthesia for 1 minute and it was then peeled off slowly to obtain a sample of the stratum corneum.

## 2.7. Measurement of stratum corneum mass

The slide glass used to collect the stratum corneum was soaked in N, N, dimethylformamide and was sonicated for 15 min. The sonicated solution was passed through a filter that was weighed beforehand. The filter was dried for one week under vacuum. After confirming that the weight of the filter was no longer changing, the weight of the stratum corneum was calculated by subtracting the weight of the filter before use from that of the filter after use.

## 2.8. The extraction of lipid and the quantitation of ceramide (AP)

The slide glass used to collect the stratum corneum was soaked in hexane and ethanol (95:5) and was sonicated at 37 °C for 20 min. After filtering the solution, it was dried under nitrogen gas. Chloroform–methanol (2:1, v/v) was added onto the residue to extract lipids. The extracted sample was separated on a HPTLC plate (Silica Gel 60, Merck, Darmstadt, Germany). The plate was developed twice with chloroform–methanol–acetic acid (190:9:1, v/v) as eluent. Then 10% CuSO<sub>4</sub> and 8% H<sub>3</sub>PO<sub>4</sub> aqueous solution were sprayed onto the plate and the plate was heated at 180 °C for 10 minutes in a plate heater for visualization. Ceramide was quantitated based on the density of the visualized band.

## 2.9. Measurement of epidermal thickness

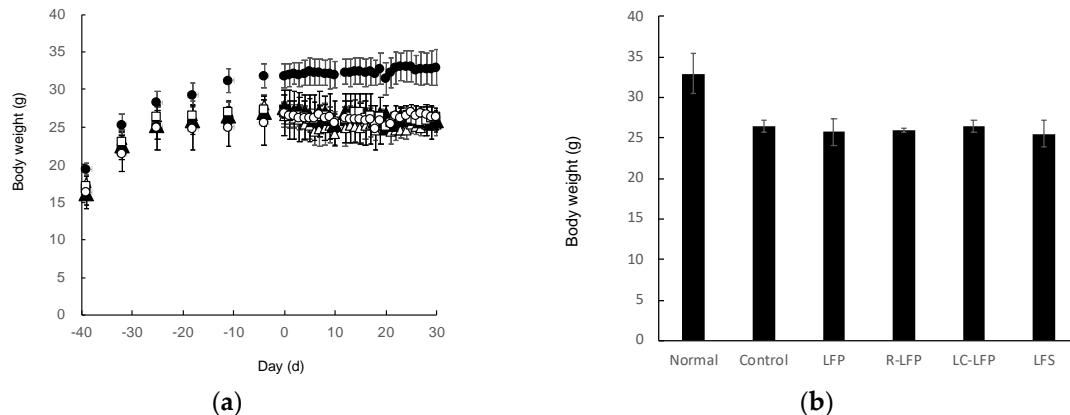
The thickness of the epidermis was measured at the horizontal midpoint of each visual field. Approximately 50 individual measurements were made along the wound margin for each histological section, and mean thickness was evaluated.

## 2.10. Data and statistical analysis

All results are expressed as means  $\pm$  S.D. of four to five experiments. Statistical analysis was performed using Tukey's multiple comparison test (JMP ver. 13, SAS Institute Inc., Cary, NC, USA).

## 3. Results

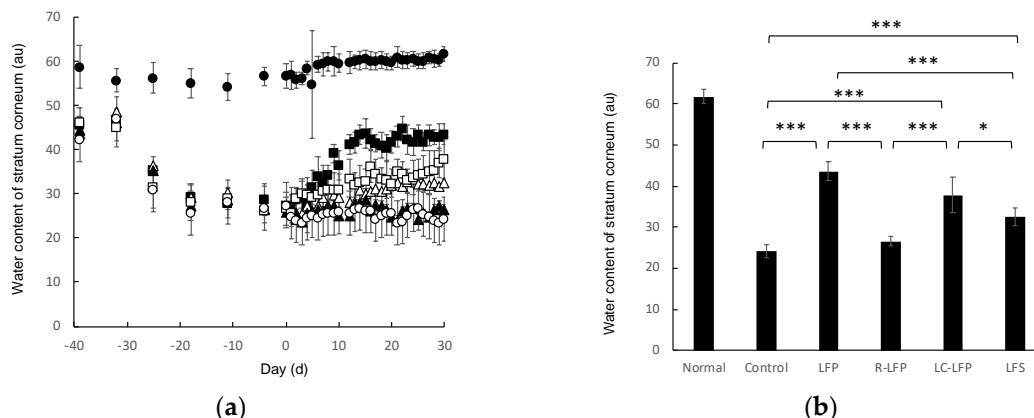
Figure 2 shows the changes in body weight of hairless mice that were fed HR-AD or a regular diet for approximately 40 days. On the final day, the normal group weighed 31.8 g and the groups that were fed HR-AD weighed 27.1 g. Forty days after the start of rearing, oral administration of various samples was initiated, and body weight was measured over time (Figure 2). On the final day, the weight of the normal group that were fed a regular diet was 33.0 g and the weight of the groups that were fed HR-AD were 26.5 g, 25.7 g, 25.5 g, 26.0 g, and 26.4 g in the control group, LFP, LFS, R-LFP, and LC-LFP administration groups, respectively, with no significant differences among them.



**Figure 2.** Body weight changes in HR-AD-fed mice after oral administration of lactic acid bacteria metabolite. Data represent mean and standard deviation ( $n = 4$  to 6).

Symbols: closed circle, normal (regular diet fed, distilled water); open circle, control (HR-AD fed, 10.8% ethanol in water); closed square, lactic fermentation products (HR-AD fed, LFP); closed triangle, residue of LFP (HR-AD fed, LFP); open square, lipid components of LFP (HR-AD fed, LC-LFP); open triangle, lactic fermentation secretion (HR-AD fed, LFS).

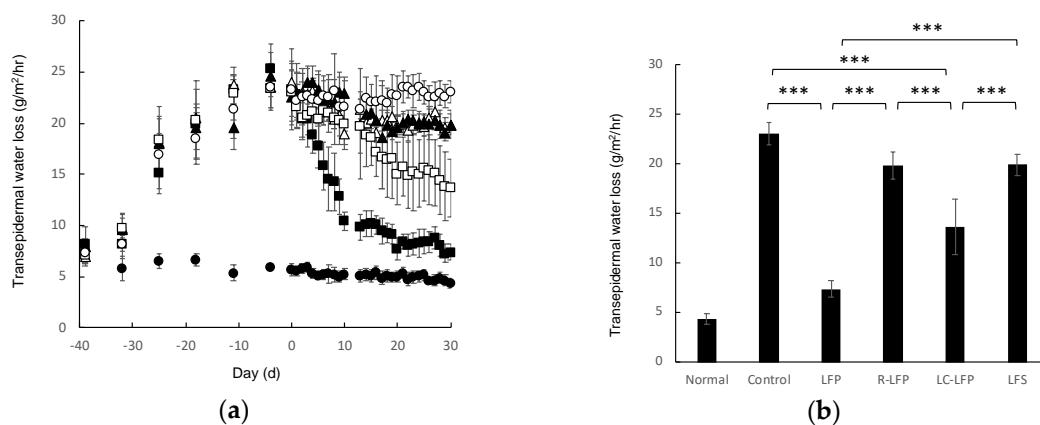
Figure 3 shows changes in the water content of the stratum corneum of hairless mice that were fed HR-AD and were subsequently administered LAB metabolites. The water content in the stratum corneum (arbitrary units, AU) immediately before LAB metabolites were administered was on average 56.6 AU in the normal group and 27.0 AU in hairless mice groups which were fed HR-AD and developed an atopic dermatitis-like condition; the difference between the groups was significant. Among the groups to which LAB metabolites were administered, the water content in the stratum corneum started to increase in the LFP administration group and the LC-LFP administration group from the 10th day after the initiation of oral administration and reached 43.5 and 37.8 AU at 28 days after the initiation of administration, respectively. It was 24.2 AU in the control group and 26.6 AU in the residue administration group, both of which were significantly lower than the values in the LAB metabolite groups. In the lipid administration group, until the 14th day after the initiation of experiment, the water content in the stratum corneum tended to be higher compared with the other groups to which lactic acid bacteria metabolites were orally administered, but the differences were not significant.



**Figure 3.** Water content of the stratum corneum of HR-AD-fed mice after oral administration of lactic acid bacteria metabolite over time (A), and after 40 days (B). Data represent mean and standard deviation ( $n = 4$  to 6). \* $p < 0.05$ , \*\* $p < 0.01$  vs. control group (Dunnett's post-hoc test). Symbols: closed circle, normal (regular diet fed, distilled water); open circle, control (HR-AD fed, 10.8% ethanol in water); closed square, lactic fermentation products (HR-AD fed, LFP);

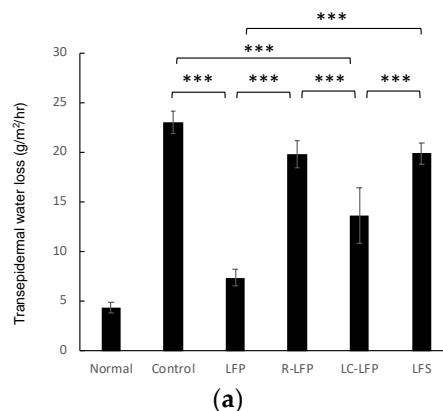
closed triangle, residue of LFP (HR-AD fed, LFP); open square, lipid components of LFP (HR-AD fed, LC-LFP); open triangle, lactic fermentation secretion (HR-AD fed, LFS).

Figure 4 shows the effect of lactic acid bacteria metabolites on TEWL in mice that were fed HR-AD. In the normal group that was fed a regular diet, TEWL ( $\text{g}/\text{m}^2/\text{h}$ ) was 5.5  $\text{g}/\text{m}^2/\text{h}$  at the start of study and did not demonstrate large variations during the study period. In contrast, TEWL increased immediately after mice were fed HR-AD and reached 24.1  $\text{g}/\text{m}^2/\text{h}$  at the start of study, which was a significant increase. After that, the elevated TEWL was reduced after oral administration of LFP or LC-LFP to 10.4 and 19.9  $\text{g}/\text{m}^2/\text{h}$ , respectively, which showed significant recovery compared with the control group. On the other hand, TEWL in the LFS and the R-LFP administration groups was 18.9 and 22.9  $\text{g}/\text{m}^2/\text{h}$  after the completion of study, respectively, which were not significantly different from that in the control group.



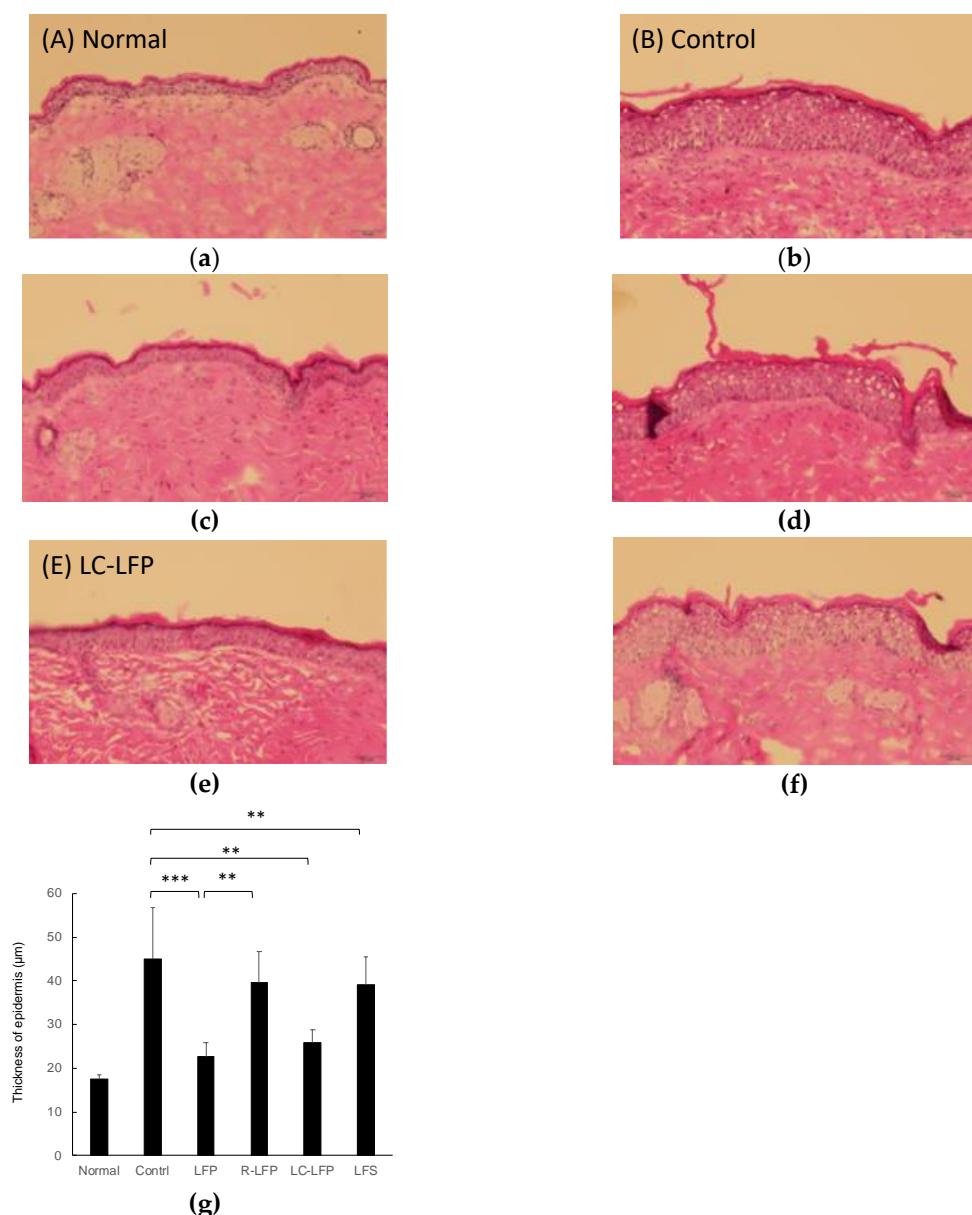
**Figure 4.** Transdermal water loss of HR-AD-fed mice after oral administration of lactic acid bacteria metabolite over time (A), and after 40 days (B). Data represent mean and standard deviation ( $n = 4$  to 6). \* $p < 0.05$ , \*\* $p < 0.01$  vs. control group (Dunnett's post-hoc test). Symbols: closed circle, normal (regular diet fed, distilled water); open circle, control (HR-AD fed, 10.8% ethanol in water); closed square, lactic fermentation products (HR-AD fed, LFP); closed triangle, residue of LFP (HR-AD fed, LFP); open square, lipid components of LFP (HR-AD fed, LC-LFP); open triangle, lactic fermentation secretion (HR-AD fed, LFS).

Figure 5 shows ceramide AP content in the stratum corneum at the completion of the study. It was 0.57 mmol/g in the stratum corneum of mice that were fed HR-AD. AP content in the stratum corneum in the LFP, LC-LFP, and LFS administration groups was significantly higher than that in the control group and were 2.25, 2.08, and 1.90 mmol/g stratum corneum, respectively. These values were similar to that in the group that was fed a regular diet (2.29 mmol/g stratum corneum). On the other hand, AP content in the stratum corneum in the R-LFP administration group was 0.78 mmol/g stratum corneum.



**Figure 5.** Ceramide AP content in the stratum corneum of HR-AD-fed mice after oral administration of lactic acid bacteria metabolite for 40 days.

Images of epidermal sections (HE staining) of mice that were fed HR-AD were prepared at 30 days after the start of administration of lactic acid bacteria metabolites and are shown in Figures 6 (A) through (F). When the epidermis of the normal group was used as a reference, the control group that was fed HR-AD and orally administered the solvent had a thicker epidermis. Oral administration of LFP or LC-LFP suppressed acanthosis compared with the control group and the thickness of epidermis was almost similar to that in the normal group. On the other hand, the LFS and the R-LFP administration groups demonstrated acanthosis similar to that observed in the control group. Figure 6 (G) shows the thickness of epidermis. As with the images described above, oral administration of LFP or LC-LFP suppressed acanthosis significantly compared with the control group, while the LFS and the R-LFP administration groups demonstrated acanthosis similar to that observed in the control group.



**Figure 6.** Histological image of HR-AD-fed mice after oral administration of lactic acid bacteria metabolite for 40 days. Groups given LAB metabolite samples were normal (regular diet fed, distilled water), control (HR-AD fed, distilled water), lactic fermentation products (HR-AD fed,

LFP), residue of LFP (HR-AD fed, LFP), lipid components of LFP (HR-AD fed, LC-LFP) and lactic fermentation secretion (HR-AD fed, LFS) groups.

Each skin sample was fixed with 10% formalin in PBS. Then, sections were prepared and stained with hematoxylin and eosin. Scale bar represents 100  $\mu$ m.

#### 4. Discussion

In this article, mice that had been fed HR-AD were orally administered the raw liquid of lactic acid bacteria metabolites, residue after filtration, and extracted lipid components to create an atopic dermatitis model to study their effects on the skin. First, the effect on body weight was confirmed. The mice that were fed HR-AD, the administration of which is believed to create an atopic dermatitis model, lost weight compared with mice that were fed a regular diet. It has been reported that when hairless mice are continuously fed HR-AD and reared, HA-RD causes atopic dermatitis (AD)-like symptoms that are mainly due to a lack of polyunsaturated fatty acids (n-6 PUFAs) and not due to a lack of magnesium [15,16]. A more recent study reported that a lack of polyunsaturated fatty acids and starch causes atopic dermatitis-like symptoms [20]. In the current study, mice reared with this diet with an atopic dermatitis-like condition were used to evaluate the effectiveness of lactic acid bacteria metabolites. Values of water content in the stratum corneum and TEWL were improved in the LFP and LC-LFP groups compared with the normal group (Figure 3 and 4), while in the LFS and the R-LFP groups, the values were almost similar to that in the normal group. It has been reported that ceramide in the stratum corneum tends to decrease in atopic dermatitis patients [9,11]. Ceramide is present intercellularly in the stratum corneum along with cholesterol and fatty acid. Many articles have been published demonstrating that among lipids in the stratum corneum, ceramide is one of the most important for barrier function [21,22]. It is known that water is also present in intercellular lipid in the stratum corneum [23-25]. Based on the results of the present study, it is presumed that decreased lipids in the skin, such as ceramide, in the stratum corneum of animals with atopic dermatitis weakened barrier function, resulting in increased TEWL and decreased water content in the stratum corneum. Therefore, in this study, the ceramide AP content in each group was examined. AP content in the group fed HR-AD without any treatment decreased to approximately 1/4 of that in the normal group (Fig. 5). Ceramide AP content in the stratum corneum tended to be high in mice whose measured values of TEWL and water content in the stratum corneum were good, and these values almost correlated. It has also been reported that when ceramide EOS is concurrently present, ceramide AP contained in the stratum corneum contributes to the stabilization of lamellar structure of the stratum corneum [26]. In the current study, it is possible that lactic acid bacteria metabolites might have had some influence on the barrier function of the stratum corneum through the induction of changes in ceramide AP content in the stratum corneum. A more detailed study to investigate this possibility may be needed in the future. Ceramides other than ceramide AP such as NS, NP and AS, or cholesterol or fatty acid did not demonstrate large variations (data not shown). These may also need to be studied in more detail. The water content in the stratum corneum is greatly influenced by natural moisturizing factors and keratin in the stratum corneum [27]. It is presumed that lactic acid bacteria metabolites influence epidermal differentiation and the production of natural moisturizing factors in the stratum corneum including amino acids, which the author would like to quantitate in the future. The thickness of the epidermis has been reported to increase in mice with an atopic dermatitis-like condition [28]. The epidermis also became thick in mice in this study. It has been reported that in general, acanthosis is caused by the abnormal growth of epidermal cells and in the skin inflammatory response, etc. The possibility that lactic acid bacteria metabolites suppress these causes has been demonstrated, however, the detailed mechanisms of the suppression are not known. Lastly, to characterize effector substances in lactic acid bacteria metabolites, some fractions of the metabolites were examined. Results showed that administration of the water-soluble fraction that had been obtained by filtrating lactic acid bacteria metabolites did not improve TEWL, water content in the stratum corneum, or acanthosis, which suggests that compounds which improve the skin condition of animals with an atopic dermatitis-like condition are contained in fractions other

than the water-soluble fraction of lactic acid bacteria metabolites. Moreover, administration of the residual components, in particular the lipid fraction, exerted effects almost equal to lactic acid bacteria metabolites. Therefore, the effector components are highly likely to be compounds extracted with chloroform or methanol. As a side note, the current study also demonstrated that the residual components are not so potent. It is desirable in the future to identify the effector components by determining their structures. Lactic acid bacteria metabolites may contain unknown compounds that are produced by fermenting soy milk with lactic acid bacteria and which soy milk or lactic acid bacteria themselves do not have. Therefore, future studies are needed to explore these possibilities.

**Author Contributions:** Y.T. designed and performed the experiments. Y.T. analyzed the data, and Y.T. wrote the manuscript.

**Conflicts of Interest:** The authors declare no conflicts of interest.

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