Oral Intake of *Lactobacillus helveticus*-Fermented Milk Whey Decreased Transepidermal Water Loss and Prevented the Onset of Sodium Dodecylsulfate-Induced Dermatitis in Mice

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We investigated the effects of oral intake of *Lactobacillus helveticus*-fermented milk whey on the intact and sodium dodecylsulfate (SDS)-exposed skin of Hos:HR-1 hairless mice. The mice were allowed to drink 10% *L. helveticus*-fermented milk whey in distilled water *ad libitum* for 5 weeks. SDS solution was topically applied to the dorsal skin at 4 weeks, leading to the development of dermatitis. The skin moisture content, transepidermal water loss, and sizes of the dermatitis areas were periodically measured. Compared with oral intake of water alone, oral intake of water containing *L. helveticus*-fermented milk whey for 4 weeks significantly lowered transepidermal water loss from intact skin, significantly reduced in size the areas of early SDS-induced dermatitis, and ameliorated both the SDS-induced decrease in moisture content and the increase in transepidermal water loss. These results suggest that oral intake of *L. helveticus*-fermented milk whey might be effective in promoting the epidermal barrier function and in preventing the onset of dermatitis.

Key words: *Lactobacillus helveticus*; fermented milk whey; skin; dermatitis

In many parts of the world, fermented milk has been consumed for centuries as a valuable food. Many studies have demonstrated several beneficial effects of fermented milk on health, including improvement of the gastrointestinal condition, antitumor effects, and antihypertensive effects.¹⁻³ With respect to the skin, fermented milk has been used in the clinical treatment of wounds and burns,¹ and is now being used widely as a cosmetic material. Recently, we reported that *Lactobacillus helveticus*-fermented milk whey (LHMW) promoted the differentiation of cultured normal human epidermal keratinocytes and increased the expression of profilaggrin,⁴ which is further proteolytically processed and used in the generation of a natural moisturizing factor (NMF) believed to be crucial to epidermal hydration and flexibility.⁵ However, little has been reported regarding the in vivo effects of ingestion or topical application of fermented milk on skin homeostasis, or on the in vitro effects of fermented milk components on cells constituting the skin. Hence we decided to further investigate the effects of fermented milk on skin condition. In this study, we investigated the effects of oral intake of LHMW on the fundamental properties of mouse skin, including skin moisture content and barrier function, and we also examined the ability of this product to prevent the onset of dermatitis induced by SDS exposure. Further, we compared the efficacy of LHMW with that of other materials generally regarded as beneficial to the skin, viz., collagen peptides, konjac ceramide, hyaluronic acid, and ascorbic acid, the efficacies of which have hitherto not been evaluated in a single study.

Materials and Methods

Preparation of fermented milk whey and artificially acidified milk whey. Fermented milk was prepared as reported previously.⁴ In brief, reconstituted pasteurized 9% wt/wt skim milk (Yotsuba Milk Products, Sapporo, Japan) was fermented with the *Lactobacillus helveticus* CM4 strain selected from a starter culture of Calpis sour milk (Calpis, Tokyo) at 37 C for 24 h. Artificially acidified milk was obtained by the addition of L- lactic acid (Wako Pure Chemical Industries, Osaka, Japan) to reconstituted pasteurized 9% wt/wt skim milk to a final concentration of 2.29% wt/wt, to create the same acidity as that of the *L. helveticus*-fermented milk. These samples were separated into the whey fraction by centrifugation at 12,000 × g for 20 min, and were named *L. helveticus*-fermented milk whey (LHMW) and artificially acidified milk whey (AAMW).

Animals. Male Hos:HR-1 hairless mice (7 weeks old) were obtained from Sankyo Labo Service (Tokyo), and examinations were performed after they were housed for 7 d. The animals were equally divided into 4 or 6 groups according to their weight, maintained at 4 mice/cage, and housed in a room under controlled temperature (24 ± 1 C), humidity (55 ± 5%), and light (light on 08:00–20:00h). In all experiments, diet (CRF-1; Oriental Yeast, Tokyo) and water were freely available, and intake and body weight were measured every d. The procedures for the animal experiments were conducted in accordance with the “Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain” (Notice no. 88, Ministry of the Environment, Government of Japan).

Experimental design (Experiment 1). Thirty-two mice were divided into four groups (eight mice per group). Over a period of 5 weeks, the mice were given ad libitum access to various solutions. The first group was given 10% vol/vol LHMW diluted with distilled water (DW), the
second group was given 10% vol/vol AAMW diluted with DW, and the remaining two groups were given DW alone. As summarized in Fig. 1, we measured the skin moisture content and transepidermal water loss (TEWL), which is index of epidermal barrier function,7–9) every week to evaluate the effects of LHMW and AAMW intake on intact skin.

At 4 weeks after sample intake, to irrigate the skin and induce dermatitis, 500 μl of 10% wt/vol SDS solution diluted with 70% vol/vol ethanol was applied topically to the dorsal skin of the mice using a pasteurized soft cotton pad (2 cm × 2 cm) (Toyokagaku, Shiga, Japan) for 30 min per d. SDS-treatments were repeated for 4 consecutive d., and the mice were maintained for a further 4 d. The mice were housed in separate cages to prevent them from scratching the lesions of other mice after the first SDS treatment. As a control for the intact skin, one of the groups given DW alone was maintained without SDS exposure (the non-irritated group). In order to evaluate the preventive effects of LHMW and AAMW on SDS-induced dermatitis, we made daily measurements of skin moisture content, TEWL and skin lesion size.

Experimental design (Experiment 2). As shown in Fig. 1, 48 mice were divided into six groups (eight mice per group). Over a 5-week period, the mice were given ad libitum access to various solutions. The first group was given DW alone and the second group given 10% vol/vol LHMW diluted with DW. The remaining 4 groups (collagen peptides, konjac ceramide, hyaluronic acid, and ascorbic acid) were given solutions of collagen peptides (9.33 mg/ml DW; Nippi, Tokyo), konjac ceramide (0.14 mg/ml DW; Unitika, Osaka, Japan), hyaluronic acid (0.22 mg/ml DW; QP, Tokyo), or L(+)-ascorbic acid (0.93 mg/ml DW; Wako Pure Chemical Industries, Osaka, Japan) respectively. Konjac ceramide contains ceramide at a concentration of 4 mg/g. With the exception of LHMW, the concentrations that were set made it possible for the mice to ingest 10 times the effective daily dose when taken in 5 ml of distilled water.

Experimental Flow.

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-breeding</td>
<td>Pre-breeding</td>
</tr>
<tr>
<td>Fed with DW alone, LHMW, or AAMW</td>
<td>Fed with DW alone, LHMW, collagen peptides, konjac ceramide, hyaluronic acid, or ascorbic acid</td>
</tr>
</tbody>
</table>

At 4 weeks after sample intake, the dorsal skin of the mice was treated with SDS solution in the same way as in exp. 1. Skin moisture content and TEWL were measured at 4 weeks after the start of intake and at 7 d after SDS exposure. At 7 d after SDS exposure, the SDS-induced skin lesion area was also measured.

Measurement of skin moisture content and TEWL. Skin moisture content and TEWL were measured on the dorsal skin of each mouse. To measure the skin moisture content, a Moisture Checker MY-707S (Scalar, Tokyo) was used, and each measurement was performed 3 times. TEWL was measured using a Tewameter TM300 (Courage+-Khazaka Electronic, Cologne, Germany) and recorded twice during a 10-s period when TEWL readings had stabilized, approximately 20 to 30 s after the probe was placed on the skin. All measurements were conducted under the same conditions as for the housing of the animals.

Measurement of dermatitis. Dorsal areas with dermatitis induced by SDS exposure were photographed using a digital camera (Fine Pix 2500Z, Fujifilm, Tokyo) and the images were captured using a CCD camera (CCD-IRIS DXC-108 MODEL, Sony, Tokyo). Using an image measurement program (SCD-150, Scalar), percentages of the skin areas, showing skin erythema, epidermal hyperplasia, or desquamation of the stratum corneum in the irritated areas were calculated.

Statistical analysis. Statistical significance among the groups was determined by multiple comparisons using a two-way ANOVA, followed by Tukey’s HSD test.

Results

Body weight change and daily intake of water and food

At each weekly time point, there were no significant differences among the groups in terms of body weight (Table 2). Similarly, there were no significant differences among the groups with respect to daily intake of food. However, in exp. 1, the daily intake of water containing LHMW in the LHMW group was lower than the intake of water alone in the control group (Table 3).

Effects of the LHMW on intact skin

To determine the effects of oral intake of LHMW on intact skin, we evaluated the changes in skin moisture content and TEWL during the 4-week sample intake period. There were no significant differences among the groups in terms of skin moisture content at 4 weeks; however, the value for the LHMW group was higher than that for the control group at 1 and 3 weeks (p < 0.05, Fig. 2A). When the TEWL of the LHMW group was compared with that of the control group, TEWL was lower at 2 weeks (p < 0.05), and this continued until the end of the 4-week intake period (Fig. 2B). At the 3-week time point, the TEWL of the LHMW group was lower than that of the AAMW group (p < 0.05). In the AAMW group, the TEWL was also lower than in the control group at 4 weeks (p < 0.05), but the values were not significantly different from the control group until the third week (Fig. 2B).

Inhibitory effects of LHMW on the development of SDS-induced dermatitis

The size of the skin lesion, which was calculated as the total area showing erythema, epidermal hyperplasia, or desquamation of the stratum corneum, increased depending on the frequency of SDS exposure up to 4 d, and the extent of the dermatitis area was maintained until the end of the study. In this state, skin moisture content gradually decreased and TEWL rapidly increased with barrier disruption. In all the SDS-treated groups, the size of the skin lesion and TEWL significantly increased and the
skin moisture content decreased after day 2, as compared with the non-irritated group ($p < 0.05$, Fig. 3).

The skin lesions in the LHMW group were smaller than in the control group at day 1 ($p < 0.05$, Fig. 3A). After day 2, there was no significant difference in the size of the skin lesions between the LHMW group and the control group. However, at each time point, the lesions in the LHMW group were on average 20% smaller than those in the control group. Further, the SDS-induced reduction in skin moisture content was ameliorated in the LHMW group after day 4 ($p < 0.05$), and recovery from the increase in TEWL after day 5 ($p < 0.05$) was also promoted as compared to the control and the AAMW group (Fig. 3B, C). There was no significant difference between the AAMW group and control group in the three parameters measured (Fig. 3).

**Comparison of the efficacy of LHMW with other materials**

The ability of LHMW to ameliorate skin function was compared with other materials generally regarded as substances with beneficial skin care effects: collagen peptides, konjac ceramide, hyaluronic acid, and ascorbic acid. The comparisons were performed at 2 time points before and after the SDS exposure period following the 4-week sample intake. At 4 weeks after the start of intake, there was no significant difference in skin moisture content or TEWL between the groups (Fig. 4). At 7 days after the start of skin irritation, there was no significant difference among the groups with respect to the size of SDS-induced skin lesion and TEWL, but the SDS-induced reductions in skin moisture content in the LHMW and collagen peptides groups were smaller than those in the control group ($p < 0.05$, Fig. 5).

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**Table 2. Body Weight Changes during the Experiments**

<table>
<thead>
<tr>
<th>Group</th>
<th>0 wks</th>
<th>1 wk</th>
<th>2 wks</th>
<th>3 wks</th>
<th>4 wks</th>
<th>5 wks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-irritated</td>
<td>26.5 ± 1.0</td>
<td>27.3 ± 1.5</td>
<td>28.4 ± 1.7</td>
<td>28.6 ± 1.4</td>
<td>29.1 ± 1.5</td>
<td>29.3 ± 1.9</td>
</tr>
<tr>
<td>Control</td>
<td>26.6 ± 1.2</td>
<td>26.7 ± 1.6</td>
<td>27.8 ± 2.1</td>
<td>29.1 ± 1.6</td>
<td>29.8 ± 1.8</td>
<td>30.6 ± 1.6</td>
</tr>
<tr>
<td>LHMW</td>
<td>26.6 ± 1.0</td>
<td>26.9 ± 1.1</td>
<td>27.0 ± 1.2</td>
<td>28.5 ± 1.0</td>
<td>28.9 ± 1.2</td>
<td>29.0 ± 1.2</td>
</tr>
<tr>
<td>AAMW</td>
<td>26.2 ± 0.7</td>
<td>26.5 ± 1.2</td>
<td>26.8 ± 1.2</td>
<td>28.3 ± 1.3</td>
<td>28.7 ± 1.4</td>
<td>28.9 ± 1.3</td>
</tr>
</tbody>
</table>

**Table 3. Daily Intake of Water and Food during the Experiments**

**Experiment 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Daily intake of water and food</th>
<th>Water (g)</th>
<th>Food (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-irritated</td>
<td>Daily intake of water and food</td>
<td>5.6 ± 0.5</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td>Control</td>
<td>6.2 ± 1.4</td>
<td>4.1 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>LHMW</td>
<td>5.1 ± 1.7</td>
<td>4.0 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>AAMW</td>
<td>5.6 ± 2.1</td>
<td>4.1 ± 0.6</td>
<td></td>
</tr>
</tbody>
</table>

**Experiment 2**

<table>
<thead>
<tr>
<th>Group</th>
<th>Daily intake of water and food</th>
<th>Water (g)</th>
<th>Food (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.5 ± 1.3</td>
<td>4.5 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>LHMW</td>
<td>5.9 ± 1.5</td>
<td>4.4 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>Collagen peptides</td>
<td>6.7 ± 1.8</td>
<td>4.4 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>Konjac ceramide</td>
<td>6.8 ± 1.7</td>
<td>4.3 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>6.8 ± 1.7</td>
<td>4.3 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>6.4 ± 1.6</td>
<td>4.5 ± 0.8</td>
<td></td>
</tr>
</tbody>
</table>

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Fig. 2. Effects of Fermented Milk Whey Intake on Intact Skin.

The values of skin moisture content (A) and TEWL (B) were expressed as means ± SEM. Mice were provided with DW (○), 10% LHMW (●), or 10% AAMW (○). * $p < 0.05$ indicates a significant difference from the control group. † $p < 0.05$ indicates a significant difference between the LHMW and the AAMW group.
Discussion

We have reported that LHMW promoted the differentiation of cultured normal human epidermal keratinocytes and increased expression of profilaggrin at the mRNA level.4) Further, *L. helveticus* CM4 strain showed very potent protease activity and efficiently produced several milk protein-derived biologically active peptides during fermentation. 6) Hence we expected that oral administration of LHMW, including a large amount of peptides, might have beneficial effects on the skin. In this study, we attempted to examine this hypothesis in intact and detergent-irritated skin.

We observed that oral intake of LHMW significantly decreased TEWL in intact skin (p < 0.05) and significantly promoted recovery from the increase in TEWL (p < 0.05) after induction of dermatitis by SDS exposure (Figs. 2B, 3C). In view of the fact that TEWL has been reported to be an indicator of skin barrier function, and given that individuals with dry skin exhibit higher TEWL values than those with skin of normal appearance,5–9) intake of LHMW might be beneficial in strengthening the physical properties of skin. Consistently with this assumption, we also observed that intake of LHMW ameliorated the SDS-induced decrease in skin moisture content (p < 0.05), which reflects elevated transpiration (Fig. 3B). Moreover, the intake of LHMW tended to reduce the size of the SDS-induced skin lesions as compared with the control group (Fig. 3A).

SDS exposure is a standard method used in many experiments to induce dermatitis, but a standardized protocol has yet to be established.10–12) Our procedure, a modified version of the method reported by Kawano,13) induced a decrease in skin moisture content, to less than 50% of the level of native skin, and this state persisted for 4 d after the cessation of SDS exposure. Since the differences among the evaluated parameters between the sample group and the control group were found to be significant, our procedure is considered to be appropriate for evaluation of the efficacy of skin care regimens.

We compared the effects of LHMW with those of other materials generally regarded as beneficial for the skin and which have been widely used in foods, drinks, or cosmetics. Kikuchi reported that daily intake of a drink containing collagen peptides (10,000 mg/d) and ascorbic acid (1,000 mg/d) for 6 weeks increased skin elasticity and reduced wrinkling of the skin.14) Kajimoto et al. found that daily intake of hyaluronic acid (240 mg/d) for 6 weeks increased skin moisture content and improved the smoothness of human skin.15) Mukai indicated that daily intake of ceramide (600 µg/d, equivalent to the 150 mg/d as konjac ceramide used in
this study) for 4 weeks also improved surface water content and reduced itching in human skin.\(^{16}\) The daily intake of 10% LHMW and mouse final body weight in exp. 1 were 5.1 ml/d and 29.0 g respectively. The dose of 5.1 ml/d is equivalent to 1,055 ml of undiluted LHMW/d in a 60-kg human, and we assume that this dose is approximately 10 times higher than that consumed in actual food and drink. In exp. 2, taking into account the above consideration, we set the dose is approximately 10 times higher than that of the estimated effective dose for mice based on a body weight conversion (Table 1).

We observed that the ability of LHMW to ameliorate skin damage after SDS exposure was not less than that of the materials we compared with it in this study. However, the doses of these materials were determined from human effective doses, because there was no comparable mouse study. Therefore, a question remains as to whether these selected doses were appropriate (Fig. 5), and further studies are necessary to compare strictly the efficacies of these materials.

Recently, we conducted human trials over an 8-week intake period and confirmed that drinking a beverage containing 90 g of \(L.\) helveticus CM4-fermented milk ameliorated stratum corneum barrier function.\(^{17}\) When this human effective dose was compared with the mouse effective dose of LHMW, the method of calculation used in this study was found to be valid, although we have not examined the minimal effective dose of LHMW in mice.

Though we do not have a precise understanding of the detailed mechanisms and the effective substances of the LHMW, we speculate that components in intact milk whey are not responsible for most of our observations, since no amelioration of the parameters aggravated by SDS exposure were observed in the AAMW group (Fig. 3). AAMW contains the same amount of lactic acid as LHMW, known as a component of NMF,\(^{18}\) suggesting that intake of lactic acid too may not be involved. As shown in Table 4, the amounts of fundamental nutrients in LHMW were lower than those in AAMW. This decrease in nutrient content is to be attributed to consumption by \(L.\) helveticus CM4 during fermentation, and suggests that factors other than the fundamental nutrients in LHMW contributed to our findings.

In addition, it is noteworthy that even though the volume of the daily intake of water containing LHMW was lower than that of water alone (Table 3), the moisture content of intact and SDS-exposed skin in the LHMW group was ameliorated to a greater extent than in the control group (Figs. 2A, 3B). Fermentation increases the contents of various milk protein-derived peptides, which are produced by proteolytic hydrolysis of casein, and \(L.\) helveticus has strong proteolytic activity and produces peptides efficiently.\(^{19-21}\) Hence we suppose that one or more biologically active peptides generated by the \(L.\) helveticus CM4-fermentation were responsible for our results. In this study, we did not examine the \textit{in vivo} expression of filaggrin; furthermore, the relationship between the LHMW effects observed in this study and those observed in a previous \textit{in vitro} study\(^{22}\) remain unclear. In order to clarify the mechanisms and the effective substances involved, further investigation is required.

In conclusion, oral intake of LHMW lowered TEWL in mouse intact skin, and ameliorated the reduction in moisture content and increase in TEWL when skin lesions developed due to SDS exposure. Therefore, intake of \(L.\) helveticus CM4-fermented milk or its whey fraction, LHMW, might be an effective approach to strengthening epidermal barrier function and preventing the development of dermatitis.

### Table 4. Nutrient Contents of LHMW and AAMW

<table>
<thead>
<tr>
<th>Nutrient content</th>
<th>LHMW</th>
<th>AAMW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal/100g)</td>
<td>16</td>
<td>28</td>
</tr>
<tr>
<td>Water (g/100g)</td>
<td>95.4</td>
<td>92.3</td>
</tr>
<tr>
<td>Protein (g/100g)</td>
<td>0.4</td>
<td>1.0</td>
</tr>
<tr>
<td>Fat (g/100g)</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Carbohydrates (g/100g)</td>
<td>3.5</td>
<td>5.9</td>
</tr>
</tbody>
</table>

Values are expressed as contents in the undiluted solutions.
References