

Oral intake of heat-killed cells of *Lactococcus lactis* strain H61 promotes skin health in women

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Abstract

We conducted a double-blind, placebo-controlled trial to evaluate the effect of heat-killed cells of *Lactococcus lactis* strain H61 on various skin properties of Japanese women. Volunteers (age 31–62 years) were randomly assigned to receive test food with or without 60 mg of heat-killed strain H61 (fifteen women in each group; H61 and control groups, respectively) daily for 8 weeks. Results were analysed for three age categories (30s, 40s and 50–60s). Compared with that at week 0, skin hydration at the inner forearm at weeks 4 and 8 decreased in all volunteers (except those in their 50–60s) because of the environmental change from autumn to winter. The oldest H61 group maintained skin hydration at the inner forearm throughout the study. Skin elasticity and melanin content in the cheek decreased and sebum content increased throughout the test period due to seasonal environmental change, regardless of age or H61 treatment. Self-evaluation scores for apparent hair follicles and dryness of the throat at week 8 were higher in the overall H61 group than in the combined placebo group. The 30s H61 group noted marked improvements in self-surveyed skin elasticity at week 8 compared with at week 0 and with the placebo group at week 8. The results of the present study indicate that oral intake of heat-killed cells of *L. lactis* strain H61 can improve some skin properties and body characteristics in women. This strain would probably be useful in increasing the quality of life in an ageing population.

Key words: Lactic acid bacteria: Skin: Human studies

Many countries are faced with the challenge of maintaining good health and a high quality of life in an ageing population that is living longer than ever before. In this context, the probiotic properties of lactic acid bacteria have garnered particular interest. Probiotics have been defined by the Food and Agriculture Organization–World Health Organization as ‘live microorganisms which, when administered in adequate amounts, confer a health benefit to the host’. For example, probiotic lactic acid bacteria can reduce serum lipid content⁽¹⁾, improve the imbalances of intestinal microbiota⁽²⁾ and exert immunomodulatory activity⁽³⁾ in human subjects. Lactic acid bacteria probably have additional probiotic properties as well; our previous study showed that oral administration of heat-killed *Lactococcus lactis* subsp. *cremoris* strain H61 to aged senescence-accelerated mice was associated with a decreased incidence of skin ulcers, reduced bone density loss and reduced hair loss compared with those of controls that did not receive strain H61⁽⁴⁾. Strain H61 has already been shown to be empirically safe through its use in manufactured dairy

products in Japan. To fully explore the anti-ageing potential of strain H61, further investigations in human trials are required.

Various endogenous and environmental factors, including ageing, exposure to sunlight, chemicals and mechanical damage^(5,6), are known to affect various properties of the skin. In addition, the consumption of various foods is thought to alter the condition of skin. For example, several nutrients including cocoa flavanol⁽⁷⁾ and lycopene⁽⁸⁾ have been shown to be beneficial in maintaining or improving skin characteristics. In addition, increased intake of vitamin C and linoleic acid and decreased consumption of fats and carbohydrates are associated with an improved appearance of ageing skin⁽⁹⁾.

In the present study, we investigated the effects of oral supplementation with heat-killed cells of *L. lactis* strain H61 on various properties of human skin. When presented as heat-killed or live cells or in fermented milk, strain H61 improved the condition of the skin of senescence-accelerated mice⁽⁴⁾. We used heat-killed cells for this study because they are easier to

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handle than are viable bacteria. In addition, we analysed the effect of age on the results by dividing the subjects into three age categories (30s, 40s and 50–60s).

Materials and methods

Subjects and study design

A total of thirty healthy women (age 31–62 years) from our Institute were enrolled in this study. The study was performed between October 2010 and December 2010 as a randomised, double-blind placebo-controlled application test, in two groups, consisting of fifteen women each. Exclusion criteria were pregnancy, breast-feeding and those who routinely used medical products for various diseases including metabolic, cardiovascular and hepatic diseases. Volunteers were allocated to three age-range categories: 30s (35.1 (SE 1.08) years), 40s (44.7 (SE 0.86) years) and 50–60s (54.5 (SE 1.61) years). Age distribution was comparable between the control and treatment populations.

For the preparation of test food including cells of *L. lactis* subsp. *cremoris* H61, constituted broth (0.5 % meat extract, 0.5 % yeast extract, 1 % sodium succinate, 1 % sodium chloride and 1 % glucose) was inoculated to the level of 1 % (v/v) with strain H61 (MAFF 400007) and cultured for 1 d at 35°C. Bacterial cells were harvested and washed twice with 0.85 % NaCl and then resuspended in the same solution. Heat-killed cells were prepared by treatment at 121°C for 15 min, followed by centrifugation and lyophilisation. Daily doses totalled 2.0 g and comprised vehicle (potato starch, lactose and citrate) with or without 60 mg of lyophilised bacterial cells.

The duration of the study was 8 weeks, with assessment of skin parameters at weeks 0, 4 and 8. This study was conducted according to the guidelines in the Declaration of Helsinki, and all procedures involving human subjects were approved by the local medical ethics committee at our Institute. All test subjects received detailed information listing all parameters relevant to the study, and they submitted a written declaration of consent for participation in the study.

Evaluation of skin properties

Hydration of the skin of the right and left inner forearms was measured, and hydration and other parameters of the right and left lateral angles of the cheeks were assessed in the study volunteers. Before measurements, each subject washed her cheeks once with soap in tap water and sat quietly for 20 min to equilibrate to the conditions in the testing room (22 ± 2 °C; relative humidity, 50 ± 5 %). Two symmetrical regions of the cheeks were evaluated. Skin hydration was measured by using a corneometer (CM 825, Courage & Khazaka Electronics)^(10,11). Malar skin elasticity was measured by an absorption method (Cutometer SEM575, Courage & Khazaka Electronics)^(11,12). In brief, a 2-mm region of skin was aspirated at 300 mbar for 2 s and then released. The ratio of immediate retraction to total distension was recorded. The melanin and sebum contents in the cheeks were measured by using a Mexameter (Courage & Khazaka Electronics)⁽¹³⁾ and

a Sebumeter SM810 (Courage & Khazaka Electronics)⁽¹¹⁾, respectively. Skin hydration and melanin content were measured three times at each location and time point. Skin elasticity was measured four times at each site and time point. Sebum content was measured once per time point. The skin parameters of each subject were measured before intake and, based on those values, the subjects were randomly assigned to either group.

Self-evaluation

The self-evaluation questionnaire comprised two sections to assess perceptions of skin-specific attributes (section I) and indicators of general health (section II) at weeks 4 and 8 as compared with week 0, according to their impressions. Section I addressed skin properties of lustre, elasticity, darkness, spots, sagging, apparent hair follicles, crevices, wrinkles (forehead, mouth and eyes), dryness, stickiness, freedom from blemishes, redness, ability to retain cosmetics, the number of pimples, size of pimples and the presence of dark circles around eyes. Section II included swelling, oversensitivity to cold, paleness of skin, dizziness, stiff neck, headache, energy level, irritation, constipation, diarrhoea, lustre of hair, appetite, dryness of the throat, sweatiness and ease of falling asleep. Subjects evaluated these conditions by responding to questionnaire items by using a five-point scale, in which scores ranged from –2 (the characteristic was markedly worse than previously) to 0 (no change in the characteristic) to +2 (the characteristic was markedly better than previously) for each item.

Statistics

Data are expressed as least-square means. For all parameters in skin properties and all time points (weeks 0, 4 and 8), descriptive statistics were calculated and were analysed by using a general linear model procedure (SAS version 9.1; SAS Institute). Least-square means were compared by applying the Tukey–Kramer test (PDIF option of the general linear model procedure). In addition, the interaction between age, treatment and week was tested. Subgroup analyses were performed only when the interaction was significant ($P < 0.05$) or tended towards significance ($0.05 < P < 0.1$). Self-evaluation of skin and body conditions in each subject was analysed by using non-parametric tests (NPAR1WAY procedure of SAS). The effects of treatment, week and age on all parameters were analysed by using the Tukey–Kramer test.

Results

Mechanical analysis of skin parameters in women after treatment with strain H61

Table 1 indicates the mean measurements associated with various skin parameters in thirty women, as compared according to H61 intake, age and week of analysis. H61 treatment alone had no effect on any skin parameter (Table 1). Age had a significant ($P = 0.0162$) effect only on melanin content in the cheek, which was lower in women in their 30s compared



Table 1. Effect of heat-killed cells of *Lactococcus lactis* strain H61 on skin parameters of women (Least-squares means)

	Inner forearm: hydration (AU)	Cheek			
		Hydration (AU)	Elasticity	Melanin (MI)	Sebum ($\mu\text{g}/\text{cm}^2$)
Treatment					
Control	40.5	57.5	0.317	167	46.2
H61	42.8	59.0	0.323	173	50.6
<i>P</i>	NS	NS	NS	0.0162	NS
Age (years)					
30s	38.9	59.6	0.330	141 ^b	47.5
40s	41.2	56.8	0.325	188 ^a	40.5
50–60s	44.7	58.3	0.306	181 ^{a,b}	57.1
<i>P</i>	NS	NS	NS	0.0162	NS
Week					
0	45.1 ^a	57.9	0.330 ^a	188 ^a	15.0 ^b
4	40.1 ^b	58.7	0.322 ^a	168 ^b	54.5 ^a
8	39.6 ^b	58.1	0.308 ^b	154 ^c	75.7 ^a
<i>P</i>	< 0.0001	NS	0.0014	< 0.0001	< 0.0001
Treatment × age: <i>P</i>	NS	NS	NS	NS	NS
Treatment × week: <i>P</i>	NS	NS	NS	NS	NS
Age × week: <i>P</i>	NS	NS	0.0149	NS	NS
Treatment × week × age: <i>P</i>	0.0851	NS	NS	NS	NS

AU, arbitrary units; MI, melanin index.

^{a,b,c} Mean values within a category (treatment, age and week) within a column with unlike superscript letters were significantly different ($P < 0.05$).

with the other two age groups (Table 1). Week (seasonal change) had a significant ($P < 0.001$) effect on all measured parameters except cheek hydration (Table 1). Skin hydration of the inner forearm ($P < 0.0001$), cheek elasticity ($P = 0.0014$) and melanin content ($P < 0.0001$) decreased, whereas sebum content in cheeks ($P < 0.0001$) increased in response to environmental changes, such as temperature and UV exposure, from autumn to winter.

Neither interaction between treatment and age nor interaction between treatment and week had an effect on any parameters of the inner forearm or cheek. A significant ($P = 0.0149$) interaction between age and week was obtained for cheek elasticity, in that women in their 30s had higher elasticity at week 0 than at week 8 (Fig. 1). Skin elasticity in cheeks was significantly ($P < 0.05$) higher in the youngest group than in the oldest women.

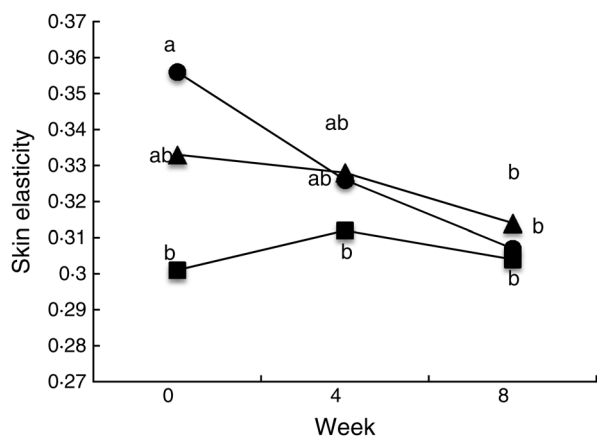


Fig. 1. Skin elasticity of cheeks of women in three age categories (30s, ●; 40s, ▲; 50–60s, ■) who received a placebo or heat-killed cells of *Lactococcus lactis* strain H61. Values are means (eight to twelve subjects). ^{a,b} Values with unlike letters were significantly different ($P < 0.05$).

Interaction between treatment, week and age category on hydration of the inner forearm tended towards significance ($P = 0.0851$; Fig. 2). Skin hydration in the inner forearm was significantly decreased in all subjects in their 30s in the H61 group and 40s in the placebo and H61 groups at week 4 ($P < 0.05$) or week 8 ($P < 0.05$) compared with week 0. In the subjects in their 30s who received the placebo, skin hydration in the inner forearm at weeks 4 and 8 also decreased compared with week 0, but these differences were not significant. In contrast, dehydration in 50–60-year-old subjects given strain H61

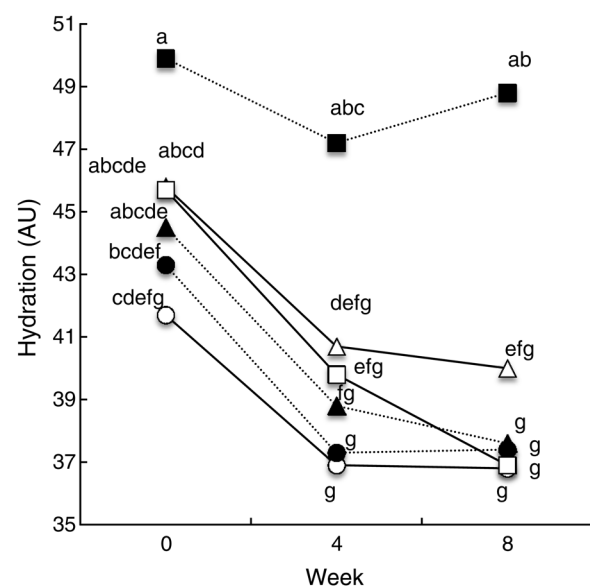


Fig. 2. Skin hydration of the inner forearm of women in three age categories (30s, 40s and 50–60s) who received a placebo or heat-killed cells of *Lactococcus lactis* strain H61. ●, Placebo (30s); ●, H61 (30s); ▲, placebo (40s); ▲, H61 (40s); □, placebo (50–60s); ■, H61 (50–60s); AU, arbitrary units. Values are means (four to six subjects). ^{a–g} Values with unlike letters were significantly different ($P < 0.05$).



Table 2. Effect of heat-killed cells of *Lactococcus lactis* strain H61 on responses to a self-evaluation questionnaire regarding skin and body condition in women (Least-squares means)

	Skin condition			Body condition		
	Elasticity	Lustre	Apparent hair follicles	Swelling	Dizziness	Dryness of the throat
Treatment						
Control	0.056	0.028	0.097	0.067	-0.006	0.028
H61	0.080	0.225	0.219	0.111	0.050	0.160
<i>P</i>	NS	NS	NS	NS	NS	NS
Age (years)						
30s	0.150	0.150	0.100	0.100	-0.100	0.100
40s	0.054	0.125	0.125	0.042	0.042	0.113
50–60s	0.000	0.375	0.250	0.125	0.125	0.063
<i>P</i>	NS	NS	NS	NS	0.0474	NS
Week						
4	0.037	0.111 ^b	0.172	0.067	-0.025	0.052
8	0.098	0.322 ^a	0.144	0.111	0.069	0.132
<i>P</i>	NS	0.0066	NS	NS	NS	NS
Treatment × age: <i>P</i>	0.0984	NS	NS	0.0332	NS	NS
Treatment × week: <i>P</i>	0.0086	NS	0.0302	0.0125	NS	0.0410
Age × week: <i>P</i>	NS	NS	NS	0.0380	NS	NS
Treatment × week × age: <i>P</i>	0.0139	0.0753	NS	NS	NS	NS

^{a,b} Mean values within a category (treatment, age and week) within a column with unlike superscript letters were significantly different ($P < 0.05$).

cells was suppressed ($P < 0.05$) at weeks 4 and 8 of treatment compared with week 0.

Self-evaluation of skin and body conditions after treatment with strain H61

Table 2 indicates the self-scored skin (elasticity, lustre and apparent hair follicles) and body (swelling, dizziness and dryness of the throat) conditions that yielded significant results concerning the effects of H61 treatment. H61 treatment had improvement effects on all of the items in Table 2, although they were not significant. Improvement of dizziness was significantly ($P = 0.0474$) affected by age, but a significant difference was not observed in age distribution after analysis using the Tukey–Kramer test. Week had a significant ($P = 0.0066$) effect only on the skin parameter of lustre, which was higher at week 8 than week 4.

Treatment and age tended to interact in the elasticity of the cheek ($P = 0.0984$) and significantly ($P = 0.0332$) interacted in

swelling, but Tukey–Kramer analysis failed to reveal any significant difference in these results.

Elasticity of the cheek ($P = 0.0086$), apparent hair follicles in the cheek ($P = 0.0302$), swelling ($P = 0.0125$) and dryness of the throat ($P = 0.0410$) all showed significant interaction of treatment and week (Table 2). At week 8, women who received H61 treatment reported significant ($P < 0.05$) improvement regarding apparent hair follicles in the cheek (Fig. 3(A)) and dryness of the throat (Fig. 3(B)). Tukey–Kramer analysis failed to identify significant differences regarding elasticity in the cheek and swelling in subjects.

Elasticity ($P = 0.0139$; Fig. 4) showed significant interaction between treatment, week and age. Women in the 30s group who received H61 treatment reported a significant ($P < 0.05$) improvement in elasticity at week 8 compared with week 0 and with the placebo group at week 8. Lustre ($P = 0.0753$) also showed a trend toward significance between treatment, week and age, but Tukey–Kramer analysis failed to confirm a significant difference in these results.

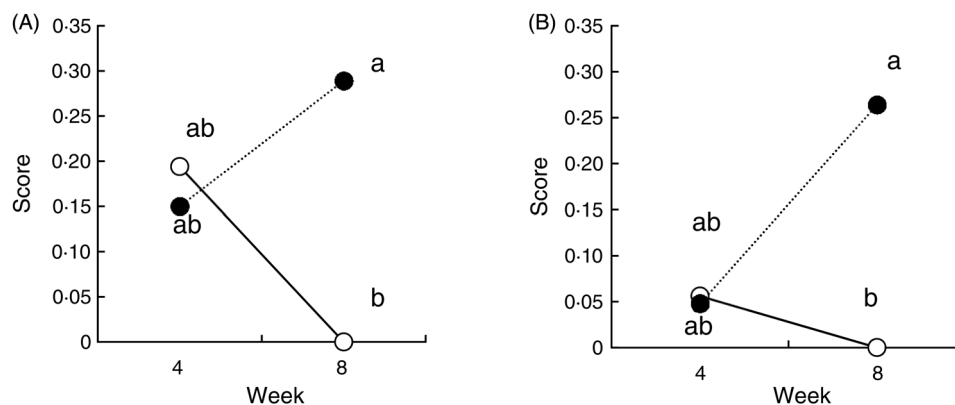


Fig. 3. Self-evaluation of (A) apparent hair follicles and (B) dryness of the throat (score, -2 to +2; relative to week 0) in women (31–62 years) who received a placebo (○) or heat-killed cells of *Lactococcus lactis* strain H61 (●). Values are means (fifteen subjects). ^{a,b} Mean values with unlike letters were significantly different ($P < 0.05$).

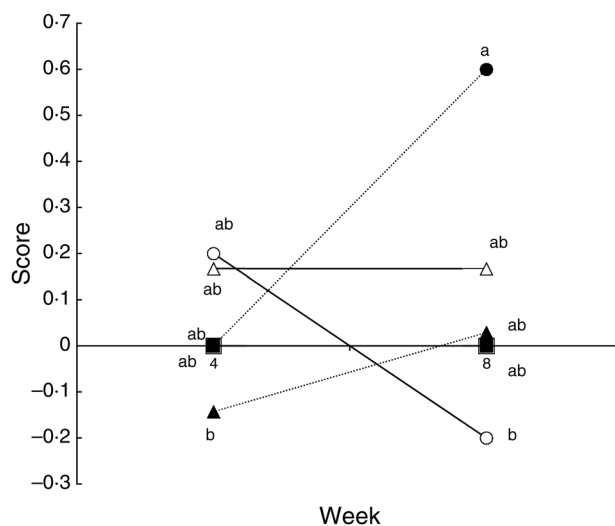


Fig. 4. Self-evaluation of elasticity (score, -2 to $+2$; relative to week 0) of the cheeks of women in three age categories (30s, 40s and 50–60s) who received a placebo or heat-killed cells of *Lactococcus lactis* strain H61. ○, Placebo (30s); ●, H61 (30s); △, placebo (40s); ▲, H61 (40s); □, placebo (50–60s); ■, H61 (50–60s). Values are means (four to six subjects). ^{a,b} Values with unlike letters were significantly different ($P < 0.05$).

Discussion

The present study represents an attempt to modulate the skin parameters of women through the intake of heat-killed cells of *L. lactis* strain H61 and to clarify the effect of this treatment on the skin of women in various age cohorts. Oral intake of 40 mg of soya isoflavone aglycones daily has been reported to improve age-associated attributes of skin (e.g. fine wrinkles and malar skin elasticity) of middle-aged (from late 30s to early 40s) Japanese women⁽¹²⁾. Skin elasticity and the degree of dryness in the cheeks of Japanese women (age 20–39 years) were improved after 4 weeks of consuming yogurt prepared by using *Lactobacillus delbrueckii* subsp. *bulgaricus* 2038 and *Streptococcus thermophilus* 1131⁽¹⁴⁾. However, no previous reports have addressed the effect of heat-killed lactic acid bacterial cells on human skin properties and age distribution. Here, we show that oral intake of heat-killed strain H61 cells was associated with suppression of dehydration due to seasonal change in the inner forearms of women in their 50–60s but not in their 30s and 40s. In response to a self-evaluation questionnaire, subjects in their 30s reported significant improvements in elasticity after treatment with H61 cells, compared with that at week 0, although the results were inconsistent with the data from the mechanical analysis. It is notable that the effect of strain H61 on some skin properties varied according to the age category (30s, 40s and 50–60s). However, the reason for this variation remains unclear as yet.

By what mechanism, then, does strain H61 specifically improve the properties of ageing skin in women? According to responses to the self-evaluation questionnaire, H61 treatment had no effect on general-health items such as constipation, diarrhoea and sleep patterns, which reportedly are associated with skin health⁽¹⁴⁾. Reactive oxygen species, including singlet oxygen and superoxide, are induced in the skin by solar UV radiation and have long been suspected of contributing to the deleterious effects of cutaneous photodamage⁽¹⁵⁾. Reactive oxygen species promote lipid peroxidation, protein oxidation and cross-linking, enzyme inactivation, and DNA

damage⁽¹⁵⁾. Studies using human dermal fibroblasts have suggested that singlet oxygen mediates UVA (320–400 nm)-induced up-regulation of interstitial collagenase in the skin, through the induction of the inflammatory cytokines IL-1 and IL-6⁽¹⁶⁾. Oral administration of antioxidant substances such as vitamin C^(7,17) and carotenoids⁽¹⁸⁾ has been shown to have photoprotective effects on the skin. In our preliminary study, heat-killed cells of strain H61 had an antioxidant effect by scavenging the α,α -diphenyl- β -picrylhydrazyl radical. We are currently investigating potential additional antioxidant effects of heat-killed strain H61, including suppression of oxidative damage of DNA and reactive oxygen species generation in macrophage cells.

In addition, immune responses are closely associated with skin properties. Exposure to UVB (280–320 nm) can impair specific and non-specific immune responses⁽¹⁹⁾, and UVB-induced immunomodulation contributes to photocarcinogenesis⁽²⁰⁾. In mice, UV exposure reduced the production of T helper type 1 cytokines including IL-2 and interferon- γ and increased the synthesis of the T helper type 2 cytokines IL-4, IL-5 and IL-10⁽²¹⁾. In our previous study⁽⁴⁾, spleen cells from mice fed with strain H61 produced more interferon- γ and IL-12 than did those from control mice that did not receive strain H61, suggesting that administration of strain H61 enhanced T helper type 1 immune responses. Therefore, the immunomodulatory activity of strain H61 in enhancing T helper type 1 responses might underlie its ability to improve various properties of ageing skin. Further investigation is needed to investigate oxidative status and immune responses by analysing serum from subjects supplemented with the cells of strain H61, in order to clarify the mechanism of promoting skin health and the differences in the effect of strain H61 among age categories.

Ageed skin has several typical characteristics, including fine wrinkles, sallowness and loss of elasticity⁽²²⁾. In addition, it has been reported that the secretion of sebum in women reaches a peak during their 20s and declines thereafter⁽²³⁾. Melanin



provides varying degrees of brown coloration at the skin surface, and melanin content is affected by internal and external factors such as age, race and sunlight exposure. Our present data showed that skin hydration, elasticity and sebum content did not differ among age categories, but melanin content in the cheek varied with age. With regard to skin hydration, although skin in the elderly is generally thought to be dry^(24,25), adult women (age 30–48 years) showed higher skin hydration in the cheek and inner forearm than did their children (age 10–14 years)⁽²⁶⁾. In another study⁽²⁷⁾, hydration of normal skin did not differ between young and elderly women. Therefore, data regarding the effect of age on skin hydration are contradictory. The use of instruments that are based on different measurement principles and that assess the skin at different depths and subjects with different ethnic backgrounds may account for the differences reported in the literature.

Seasonal effect is an important factor contributing to skin condition. Skin disorders such as psoriasis and senile xerosis tend to exacerbate in winter. In Japanese women, the hydration state of the stratum corneum on the flexor forearm was significantly lower during winter than during summer⁽²⁸⁾. Our present study showed similar seasonal changes in skin properties: hydration in the forearm and elasticity and melanin content in the cheek were lower during winter than during autumn, whereas sebum content in the cheek was higher in winter. Thermal conditions are apt to affect body temperature regulation and water metabolism in the elderly⁽²⁹⁾, and one's sensitivity to changes in air temperature declines with age⁽³⁰⁾. In the present study, changes in environmental factors such as temperature and humidity from autumn to winter affected skin properties in our subjects. Furthermore, one study⁽²⁸⁾ reported that exposed facial skin became more irritated under the influence of the dry and cold environment of winter than did the forearm. However, the present study showed that seasonal changes were apparent in the hydration of the inner forearm but not the cheek. This difference in hydration may be one of the reasons underlying the apparent effect of strain H61 in the inner forearm in this study. Repeating the examinations used in the present study but during a different season may help to clarify this issue.

In conclusion, we used here a non-invasive mechanical method and self-evaluation survey to show that heat-killed cells of *L. lactis* strain H61 improved skin properties such as skin hydration and apparent hair follicles in middle-aged and aged Japanese women. Although the subjects' impressions should be given much weight, these impressions regarding the effects of oral intake of strain H61 on skin properties (e.g. apparent hair follicles) should be confirmed in future studies by using appropriate measurable instruments.

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wrote the article. R. A. provided technical assistance. K. S. performed the statistical analyses and helped to plan the experimental design. C. S. and K. M. were involved in the conception of the study design and gave final approval of the manuscript. None of the authors had a personal or financial conflict of interest.

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