Daily consumption of fermented soymilk helps to improve facial wrinkles in healthy postmenopausal women in a randomized, parallel-group, open-label trial

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Submission Date: January 4th, 2018, Acceptance Date: February 25th, 2018, Publication Date: February 28th, 2018

Citation: Kano M., Haga K., Miyazaki K, Ishikawa F., Daily consumption of fermented soymilk helps to improve facial wrinkles in healthy postmenopausal women in a randomized, parallel-group, open-label trial, Functional Foods in Health and Disease 2018; 8(2):107-121. https://doi.org/10.31989/ffhd.v8i2.412

ABSTRACT

Background: Soymilk fermented by lactobacilli and/or bifidobacteria is attracting attention due to the excellent bioavailability of its isoflavones. This study aims to investigate the effects of fermented soymilk containing high amounts of isoflavone aglycones on facial wrinkles and urinary isoflavones in postmenopausal women in a randomized, parallel-group, open-label trial.

Methods: Healthy Japanese women were randomly divided into active (n = 44, mean age 56.3 ± 0.5) or control (n = 44, mean age 56.1 ± 0.5) groups, who consumed or did not consume a bottle of soymilk fermented by Bifidobacterium breve strain Yakult and Lactobacillus mali for 8 weeks. Maximum depth of wrinkles around the crow’s feet area and other wrinkle parameters were evaluated as primary and secondary endpoints respectively at weeks 0, 4, and 8 during the consumption period. Urinary isoflavone levels were determined by liquid chromatography-mass spectrometry.

Results: The active group demonstrated significant improvements in the maximum depth (p=0.015) and average depth (p=0.04) of wrinkles, and significantly elevated urinary isoflavones (daidzein, genistein, and glycitein; each p < 0.001) compared with the control during the consumption period. No serious adverse effects were recorded.
**Conclusion:** These findings suggest that fermented soymilk taken daily may improve facial wrinkles and elevate urinary isoflavones in healthy postmenopausal women.

**Key words:** postmenopausal women; isoflavone; fermented soymilk; phytoestrogen; facial wrinkle

**BACKGROUND**

Soy is widely used in traditional Japanese foods and is a good source of nutrients because of a well-balanced combination of protein, lipids, and carbohydrates. Soy-containing foods are heavily consumed in Japan and China but not in Western countries. The amount of soy consumed has an inverse association with the incidence of cardiovascular disease, osteoporosis, breast cancer, prostate cancer, and menopausal symptoms [1–3]. Unsurprisingly, soy products have recently attracted much attention for their potential health benefits. Isoflavones are believed to be one of the active compounds in soy products that leads to these effects, and isoflavone aglycones, namely phytoestrogens, mimic the structure of female sex hormones and demonstrate estrogenic and anti-estrogenic activities. Accordingly, isoflavones help to prevent and/or treat estrogen-dependent diseases such as breast cancer, prostate cancer, cardiovascular disease, and osteoporosis, in addition to menopausal symptoms [4–10].

Signs of skin aging, such as increased wrinkles, decreased elasticity, and increased dryness are a serious problem for women. It is arguably important for adult women around the world to maintain the appearance of youthful skin, with no signs of skin aging, in order to optimize their quality of life. Estrogen deficiency due to menopause not only promotes skin aging and thinning with less collagen, but also reduces the skin’s defense against oxidative stress. In fact, the skin ages remarkably at areas exposed to sunlight, which contains ultraviolet rays. Conversely, estrogen is known to delay skin aging by preserving the moisture and elasticity of the skin in postmenopausal women [11].

Various in vitro, animal, and human studies have demonstrated the effectiveness of isoflavones in the treatment of skin conditions [12–20]. However, many studies have been based on topical applications and not oral administration of soy products and soy isoflavone supplements. Furthermore, few studies have investigated the effects on age-related skin changes in postmenopausal women. Additionally, it is important to examine the bioavailability of isoflavones together with their effectiveness in a clinical trial. Our previous studies have demonstrated that a single intake of fermented soy milk containing a higher amount of isoflavone aglycones leads to excellent isoflavone bioavailability in healthy adults [21], while isoflavone aglycones can be delivered to the skin in sufficient amounts via the circulation in hairless mice after daily administration of fermented soymilk [22].

Therefore, we hypothesized that the daily consumption of soy foods containing high amounts of isoflavone aglycones prevents or improves skin aging in postmenopausal women due to phytoestrogenic activities. To test this hypothesis, a preliminary randomized, parallel-group, open-label trial was conducted to examine the effects of daily consumption of fermented soymilk containing high amounts of isoflavone aglycones on facial wrinkle parameters and urinary isoflavone levels in healthy postmenopausal Japanese women.
METHODS

Chemicals
The chemicals were sourced as the following: isoflavones (daidzein, genistein, and glycine) from Fujikko Co., Ltd. (Kobe, Japan), β-glucuronidase/sulfatase from Sigma-Aldrich Corp. (St. Louis, MO), and equol from Extrasynthèse (Lyon, France). O-Desmethylangolensin (O-DMA) was synthesized from 1,3-dimethoxybenzene and 4-methoxyphenyl chloride [23]. All other reagents and chemicals were commercially available products of extra-pure grade.

Subjects
Healthy postmenopausal women with amenorrhea lasting more than 1 year, and ranging in age from 50 to 65 years old were recruited for this trial. The exclusion criteria were the following: 1) taking or applying hormone drugs or bone metabolism improving agents; 2) habitual ingestion of functional foods that might affect female hormones; 3) habitual ingestion of functional foods (tablets, supplements, beverages, etc.) containing isoflavones or soymilk, or using cosmetics containing isoflavones; 4) dietary intake of isoflavones providing isoflavone aglycones of over 22 mg/day (average daily intake of isoflavones in postmenopausal women) from soy foods (natto, tofu, and soymilk); 5) receiving medical treatment; 6) history of serious liver, kidney, heart, lung, or gut disease; 7) irregular eating habits and meal contents; 8) excessive alcohol consumption (drinking more than 6 days/week or drinking 60 g/day alcohol equivalent amount); 9) possibility of changes to daily habits, such as meal contents, or use of cosmetics during the examination period; and 10) being deemed ineligible for this study by the physician-in-charge.

The trial was conducted according to the guidelines laid down in the Declaration of Helsinki. All procedures were approved by the Clinical Ethics Committee of the Sapporo Dermatology Clinic. Written, informed consent was obtained from all participants after a precise explanation of the purpose and potential risks of the study. According to the previous report [17], 88 subjects were recruited into the trial. The 88 subjects who satisfied the criteria were randomly assigned to the active (n = 44) or control (n = 44) groups, and matched for the following items evaluated during the pre-consumption period: age, height, weight, body mass index, systolic blood pressure, and diastolic blood pressure.

Study design
This randomized, parallel-group, open-label clinical trial was conducted from February to May in 2007 at the Kita Jusanjo Internal Medicine Dermatology Clinic, Hokkaido, Japan. The trial was performed by outsourcing to TTC Co. Ltd. (Tokyo, Japan), a contract research organization (CRO).

Test beverage
As described previously [21], soymilk (Shikoku Kakoki Co., Ltd., Tokushima, Japan) was fermented with Bifidobacterium breve strain Yakult (YIT 4065) and Lactobacillus mali (YIT 0243) obtained from the Culture Collection Research Laboratory of Yakult Central Institute at 37 °C for 21 hours to produce fermented soymilk, in which a portion of the isoflavone glycosides were converted to their aglycones. Table 1 shows the isoflavone composition of the fermented soymilk.
Table 1. Isoflavone composition in fermented soymilk beverage.

<table>
<thead>
<tr>
<th>Isoflavones</th>
<th>mg/bottle (125 mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daidzin</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>Genistin</td>
<td>0.21 ± 0.02</td>
</tr>
<tr>
<td>Glycitin</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>Malonyl daidzin</td>
<td>0.63 ± 0.07</td>
</tr>
<tr>
<td>Malonyl genistin</td>
<td>0.85 ± 0.05</td>
</tr>
<tr>
<td>Malonyl glycitin</td>
<td>0.54 ± 0.14</td>
</tr>
<tr>
<td>Daidzein</td>
<td>5.40 ± 0.52</td>
</tr>
<tr>
<td>Genistein</td>
<td>7.87 ± 0.51</td>
</tr>
<tr>
<td>Glycitein</td>
<td>1.27 ± 0.26</td>
</tr>
<tr>
<td>Total</td>
<td>17.12 ± 0.80</td>
</tr>
<tr>
<td>Aglycone ratio %</td>
<td>91.34 ± 0.80</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± standard error of the mean (n = 4). Isoflavone composition was determined by high-performance liquid chromatography, as described in a previous report [21].

**Procedures**

Subjects were randomly allocated to either the active or control group during the 4-week pre-consumption period. During the 8-week consumption period, only subjects in the active group consumed one bottle (125 mL) of fermented soymilk daily before breakfast. The 4-week post-consumption period was set up to analyze the effects of stopping daily consumption. Subjects in both groups were asked to limit the daily amount of soybean foods they consumed and eat smaller than usual amounts of other foods which affect the condition of the skin, to refrain from exercise, excessive alcohol consumption, and to maintain their daily routines during the trial. During the trial, subjects recorded information about their consumption of the test beverage, physical condition, subjective symptoms, food intake, exercise quantity, alcohol consumption, smoking, cosmetic use, and medicine intake in a diary. Subjects visited the clinic in the first week of the pre-consumption period, in weeks 0, 4, and 8 during the consumption period, and in week 12 at the end of the post-consumption period. Skin measurements, clinical findings, physical examinations, clinical examinations, and responses to questionnaires were recorded. Subjects were instructed not to eat or drink after 10 PM on the day before the visit. For 1 week before the day of each visit, skin condition, bowel movements (frequency, hardness, and strain), exercise habits, soybean food intake, and meal contents were recorded in a diary. The day before the visit, each subject self-collected a 24-hour urine sample using a urine-collecting system (Urinemate P, Sumitomo Bakelite Co., Ltd, Tokyo, Japan).

**Outcomes**

As a primary endpoint of the trial, the maximum depth of wrinkles around the crow’s feet area of the eye was evaluated. As secondary endpoint criteria, other wrinkle parameters (maximum width, average depth, wrinkle area rate, and wrinkle volume) and urinary isoflavone levels were evaluated.
Analysis of wrinkle parameters

Silicone rubber replicas of facial wrinkles were collected using a silicone rubber, Silflo® (Flexico Developments Ltd., London, UK), under standardized conditions (temperature 20 ± 1 °C, humidity 45% ± 5%) by Exam Co, Ltd. (Sapporo, Japan). Silicone rubber replicas were stored until the analysis. To quantitatively analyze the facial wrinkles, two-dimensional image analysis of silicone rubber replicas [23–25] and reflective three-dimensional skin analysis software (ASA-03R, Asahi Biomed Ltd., Kanagawa, Japan) [26, 27] were employed. The data was calculated using a standard scale to correct the values. These wrinkle assessment methods (replica method and two-dimensional image analysis method) are based on the guidelines prepared by the Japanese Cosmetic Science Society [28]. Wrinkle parameters, such as the maximum depth of wrinkles, maximum width of wrinkles, average depth of wrinkles, wrinkle area rate, and wrinkle volume around the crow’s feet area of the eye were determined. The analysis was outsourced to Inforward, Inc. (Tokyo, Japan) to avoid bias.

Analysis of isoflavones in urine

Isoflavones in urine were quantified by liquid chromatography–mass spectrometry (LC-MS) as described previously [20]. Briefly, 50 μL of serum or urine was mixed with 50 μL of acetate buffer (0.2 mol/L, pH 5.0) containing 100 units of β-glucuronidase. The mixture was incubated for 15 h at 37 °C to release the aglycone forms of isoflavones from the glucuronide and sulfate conjugates. Methanol (400 μL) was added to the mixture and mixed by vortex and sonication, and centrifuged at 5,000 x g for 5 min at 4 °C. The supernatant fluid was filtered through an Ultrafree-MC 0.45-μm filter unit (Merck Millipore, Darmstadt, Germany). A portion was subjected to LC-MS. The analysis was outsourced to SRL, Inc. (Tokyo, Japan) to avoid bias.

Statistical analyses

All data are expressed as the mean ± standard error of the mean (SEM). All statistical analyses were performed using the SAS software (SAS Institute Japan, Tokyo, Japan). Analysis of covariance (ANCOVA) was used for intergroup comparisons of each outcome. The covariates included in this ANCOVA model were the baseline values of outcome (week 0) and those at the time points. The interactions between groups and time points were also evaluated in the ANCOVA model. Paired t-tests were used for intragroup comparisons. Two-tailed P values of less than 0.05 were considered statistically significant. Considering multiplicity, a Bonferroni correction was carried out for the intragroup comparisons.

RESULTS

Subjects

In this trial, a total of 88 subjects who satisfied the criteria were registered, and 44 subjects were randomly assigned to either the active group or the control group. None of the subjects were withdrawn or dropped out during the trial.

One subject in the control group, who had not menstruated for more than 1 year, showed extremely high levels of serum estradiol (182.8 pg/mL) compared with the postmenopausal baseline (18.0 pg/mL). Furthermore, progesterone, LH, and SFH levels were also significantly higher than those of the other subjects. Therefore, this subject was excluded from all analyses.
because the doctor responsible deemed that her gonadal function remained sufficient and she was not postmenopausal. As a result, 43 and 44 subjects in the control group and the active group, respectively, were included in the per protocol set analysis. Moreover, it was impossible to precisely analyze the silicone rubber replicas collected from four of the subjects (2 subjects in each group), and accordingly they were also excluded. Finally, 41 and 42 subjects in the control and active groups respectively were included in the analysis of facial wrinkles.

The background characteristics of the subjects are shown in Table 2. There were no significant differences among any of the items between the groups during the pre-consumption period. Additionally, the rate of consumption of the test beverage was good at 95% or more during the consumption period.

**Table 2. Background characteristics of the subjects.**

<table>
<thead>
<tr>
<th>Items</th>
<th>Control (n = 43)</th>
<th>Active (n = 44)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>56.1 ± 0.5</td>
<td>56.3 ± 0.5</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>154.6 ± 0.8</td>
<td>154.9 ± 0.7</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>56.7 ± 1.3</td>
<td>54.2 ± 1.2</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.8 ± 0.6</td>
<td>22.6 ± 0.5</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>124.4 ± 2.6</td>
<td>124.8 ± 2.6</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>80.4 ± 1.5</td>
<td>81.1 ± 1.6</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± standard error of the mean. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure.

**Facial wrinkles**

Table 3 depicts the changes in facial wrinkle parameters in both groups during the trial. Figure 1 shows a typical replica photograph of a facial wrinkle. Group effects among the wrinkle parameters were statistically significant or trended for maximum depth, average depth, and volume ratio (P=0.015, 0.041 and 0.069, respectively). Furthermore, there were significant time effects on maximum depth, average depth, and volume ratio (P=0.009, 0.021 and 0.027, respectively). However, there were no significant interaction effects.

With-group comparisons between week 0 and each subsequent time point (week 4 and week 8) were carried out by paired *t*-tests. The active group had significantly decreased maximum depth and average depth at week 8 compared with week 0 (both P=0.0013). In contrast, the control group demonstrated no significant changes in any of the parameters during the trial.
Table 3. Changes in facial wrinkle parameters in the control and active groups during the trial.

<table>
<thead>
<tr>
<th>Wrinkle parameters</th>
<th>Group</th>
<th>Consumption period</th>
<th>Post observation period</th>
<th>P value†</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Baseline/Week 0</td>
<td>Week 4</td>
<td>Week 8</td>
<td>Week 12</td>
</tr>
<tr>
<td>Maximum depth (μm)</td>
<td>Control</td>
<td>617.5 ± 12.8</td>
<td>632.9 ± 15.5</td>
<td>620.4 ± 13.0</td>
<td>0.015</td>
</tr>
<tr>
<td></td>
<td>Active</td>
<td>608.5 ± 9.1</td>
<td>615.4 ± 9.5</td>
<td>580.3 ± 8.9</td>
<td>0.009</td>
</tr>
<tr>
<td>Maximum width (μm)</td>
<td>Control</td>
<td>783.1 ± 13.6</td>
<td>786.7 ± 18.1</td>
<td>788.6 ± 17.6</td>
<td>0.104</td>
</tr>
<tr>
<td></td>
<td>Active</td>
<td>782.8 ± 15.7</td>
<td>784.3 ± 16.2</td>
<td>752.5 ± 15.9</td>
<td>0.191</td>
</tr>
<tr>
<td>Average depth (μm)</td>
<td>Control</td>
<td>218.4 ± 4.8</td>
<td>221.4 ± 5.9</td>
<td>219.2 ± 5.5</td>
<td>0.041</td>
</tr>
<tr>
<td></td>
<td>Active</td>
<td>217.6 ± 4.4</td>
<td>219.1 ± 4.1</td>
<td>210.1 ± 4.1</td>
<td>0.021</td>
</tr>
<tr>
<td>Area ratio (μm²/mm²/100)</td>
<td>Control</td>
<td>1.99 ± 0.06</td>
<td>1.96 ± 0.07</td>
<td>1.95 ± 0.06</td>
<td>0.320</td>
</tr>
<tr>
<td></td>
<td>Active</td>
<td>2.02 ± 0.06</td>
<td>1.97 ± 0.06</td>
<td>1.94 ± 0.07</td>
<td>0.505</td>
</tr>
<tr>
<td>Volume ratio (μm³/mm³/100)</td>
<td>Control</td>
<td>496.3 ± 22.7</td>
<td>499.8 ± 26.2</td>
<td>485.0 ± 23.6</td>
<td>0.069</td>
</tr>
<tr>
<td></td>
<td>Active</td>
<td>502.3 ± 21.7</td>
<td>494.8 ± 22.4</td>
<td>462.0 ± 22.0</td>
<td>0.027</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± standard error of the mean. † Upper = between group, lower = time point (ANCOVA).
Figure 1. Typical skin replicas of the (A) control group and (B) active group. Each image represents 11 mm x 11 mm.

**Isoflavones**

The average intake of isoflavones per day from foods other than the test beverage ranged from 14 mg to 18 mg (equivalent isoflavone aglycones) in both groups. There was no significant difference between the groups during the trial (Table S1).

Table 4 shows the changes in urinary isoflavone levels in both groups during the trial. Group effects of urinary isoflavones were statistically significant for daidzein, genistein, and glycitein (each p<0.001). However, there were no significant time and interaction effects.

With-group comparisons between week 0 and each subsequent time point (week 4 and week 8) were carried out by paired t-tests. The active group had significant increases in daidzein, genistein, and glycitein at week 4 compared with week 0 (P=0.0188, 0.0073, and 0.0006 respectively) and at week 8 compared with week 0 (P=0.022, 0.0099, and 0.0104 respectively). In contrast, the control group had no significant changes in any of the isoflavones during the trial.

Equol was detected in the urine of 23 and 19 subjects in the control and active groups respectively, at least once during the trial. It was accepted that these subjects were equol producers. However, there were no significant differences in the number of equol producers (chi-square test), and urinary equol and O-DMA levels between the groups.
Table 4. Changes in urinary isoflavone levels in the control and active groups during the trial.

<table>
<thead>
<tr>
<th>Isoflavones</th>
<th>Group</th>
<th>Consumption period</th>
<th>P value†</th>
<th>Post observation period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Baseline/Week 0</td>
<td>Week 4</td>
<td>Week 8</td>
</tr>
<tr>
<td>Daidzein (mg/day)</td>
<td>Control (n=43)</td>
<td>1.98 ± 0.30</td>
<td>1.51 ± 0.20</td>
<td>1.61 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>Active (n=44)</td>
<td>1.53 ± 0.26</td>
<td>2.14 ± 0.17</td>
<td>2.13 ± 0.22</td>
</tr>
<tr>
<td>Genistein (mg/day)</td>
<td>Control (n=43)</td>
<td>1.99 ± 0.33</td>
<td>1.72 ± 0.27</td>
<td>1.56 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>Active (n=44)</td>
<td>1.95 ± 0.37</td>
<td>2.76 ± 0.29</td>
<td>2.96 ± 0.35</td>
</tr>
<tr>
<td>Glycitein (mg/day)</td>
<td>Control (n=43)</td>
<td>0.42 ± 0.08</td>
<td>0.34 ± 0.06</td>
<td>0.34 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>Active (n=44)</td>
<td>0.43 ± 0.07</td>
<td>0.73 ± 0.07</td>
<td>0.69 ± 0.07</td>
</tr>
<tr>
<td>Equol (mg/day)</td>
<td>Control (n=23)</td>
<td>1.57 ± 0.44</td>
<td>1.26 ± 0.32</td>
<td>1.70 ± 0.54</td>
</tr>
<tr>
<td></td>
<td>Active (n=19)</td>
<td>0.65 ± 0.23</td>
<td>1.18 ± 0.41</td>
<td>1.06 ± 0.26</td>
</tr>
<tr>
<td>O-DMA (mg/day)</td>
<td>Control (n=41)</td>
<td>0.72 ± 0.18</td>
<td>0.70 ± 0.22</td>
<td>0.71 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>Active (n=40)</td>
<td>0.90 ± 0.29</td>
<td>1.10 ± 0.27</td>
<td>0.71 ± 0.15</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± standard error of the mean. † Upper = between group, lower = time point (ANCOVA). O-DMA: O-desmethyIangolensin.
Adverse events
No serious adverse events were reported during the trial. However, there were 18 and 12 minor adverse events in the active and control groups respectively, but these were not significantly different between the groups. Only one symptom (constipation) was considered to have a possible association with consumption of the test beverage.

DISCUSSION
Estrogen deficiency following menopause induces atrophic skin changes and stimulates skin aging, such as an increase in the number of wrinkles, decreased elasticity, and increased dryness [11]. Isoflavone aglycones are phytoestrogens that show estrogenic activity under postmenopausal conditions. Therefore, it is hypothesized that the daily consumption of soy foods containing higher amounts of isoflavone aglycones prevents or improves skin aging in postmenopausal women due to phytoestrogenic activity. To test this hypothesis, a preliminary randomized open-label trial was conducted to examine the effects of the daily consumption of fermented soy milk containing higher amounts of isoflavone aglycones on facial wrinkle parameters and urinary isoflavone levels in healthy, postmenopausal Japanese women.

This study demonstrated that the active group had significantly higher urinary levels of isoflavone aglycones (daidzein, genistein, and glycine) compared with the control group (Table 4) during the consumption period, while the control group maintained constant urinary levels of isoflavone aglycones throughout the trial. The test beverage—fermented soy milk containing higher amounts of isoflavone aglycones—potently enhances the bioavailability of isoflavones in the circulation in healthy adults not only after one dose [21] but also after daily consumption for 8 weeks. It is believed that the subjects in the active group complied with the instructions to consume the test beverage daily, which was demonstrated by the 95% or more consumption rate, and both groups maintained their daily intake of soy foods at a low-constant level to avoid heavy intake, which demonstrated the validity of the present trial.

This study found that the active group experienced significant or trend improvements of wrinkle parameters (maximum depth, average depth, and volume ratio) compared with the control group (Table 3). In contrast, the wrinkle parameters and urinary isoflavone levels remained the same in the control group throughout the trial. Skin wrinkles are promoted not only by aging but also by lifestyle choices, such as sun exposure, loss of body mass via poor nutrition, smoking, poor hydration, change of cosmetics, and a variety of other factors [29]. It is presumed that the subjects in both groups maintained stable lifestyles to minimize the effects on wrinkle parameters during the trial, and that no source of bias affected the subjects in the active group, except for the daily consumption of the test beverage. Our results indicate that the daily consumption of fermented soymilk containing higher levels of isoflavone aglycones for 8 weeks improves facial wrinkles.

Interestingly, the lowest values among the wrinkle parameters were maximum depth and maximum width at week 8 and average depth, area ratio, and volume ratio at weeks 8 and 12 during the trial (Table 3). There were similar changes among urinary isoflavone levels (daidzein, genistein and glycine), maximum depth, and maximum width. These observations suggest that the improvements in maximum depth and maximum width were variable depending on urinary
isoflavone levels, while improvements in average depth, area ratio, and volume ratio were detected after a delay of some weeks from the peak urinary isoflavone levels.

Our previous study demonstrated that isoflavone aglycones can be delivered to the skin in sufficient amounts via the circulation in hairless mice after daily administration of fermented soy milk [22]. The epidermal and dermal layers of human skin both express estrogen alpha and beta receptors [11]. It has been reported that isoflavones stimulate hyaluronic acid synthesis in cultures of human epidermal keratinocytes and in hairless mice [30] as epidermal changes, and promote the proliferation of dermal fibroblasts through estrogen receptor-related signal transduction [31,32] and collagen synthesis in dermal fibroblast cultures [33] as dermal changes, which result in improved skin moisture and elasticity [17,34]. The estrogenic action of isoflavones is thought to be involved in the biological activities that improve facial wrinkles. Additionally, the turnover times of the epidermis and dermis are about 4 weeks or more in humans. This suggests that the improvements in maximum depth and maximum width are based on epidermal changes because they disappeared depending on the urinary isoflavone levels at week 12, which was 4 weeks after stopping the test beverages. Meanwhile, the improvements in average depth, area ratio, and volume ratio may be based not on epidermal changes but on dermal changes because they were independent of the lower urinary isoflavone levels at week 12.

Daidzein is metabolized to dihydrodaidzein, equol, or O-DMA by intestinal bacteria in humans after ingestion [35,36]. Specifically, equol is a very attractive metabolite because it has higher estrogen-like, anti-oxidative, and anti-inflammatory activities than other isoflavones. In fact, equol producers have less menopausal symptoms and lower bone loss than women who do not produce equol after daily intake of isoflavones [37–39], which suggests that equol is involved in the biological activities of isoflavones. However, the ability to metabolize equol differs greatly among individuals. About half of the population of Japan and Korea are equol producers, but in Europe and the United States the rate is much lower [40]. In this study, there were no significant differences among the number of equol producers, urinary equol levels, or O-DMA levels during the trial (Table 4). Furthermore, there were no significant differences in the changes of maximum depth of facial wrinkles between equol producers and non-producers in a sub-group analysis (Figure 2). Our previous study demonstrated that urinary levels of daidzein and genistein are significantly higher after a single intake of fermented soymilk than unfermented soymilk, but the equol levels are similar [21]. These findings indicate that genistein and glycitin rather than equol are involved in improving facial wrinkles, because the active group had higher urinary levels than the control group during the consumption period.

This study also demonstrated that there were no serious adverse events during the trial. There are many arguments surrounding the safety of isoflavones [41–44], because the increased numbers of commercial soy isoflavone products available as supplements imply that it is easier for overdoses to occur. Previous reports have observed that improvements in facial wrinkles via soy isoflavones require oral supplementation for 12 weeks to 6 months [17,45,46]. Long-term consumption of soy isoflavone supplements should be monitored to avoid over-exposure, because they are taken in addition to the many isoflavones consumed in normal diets. In fact, soy foods are staple in the Japanese diet and familiar to Japanese women. However, we believe that
the daily consumption of fermented soymilk for 8 weeks not only improves quality of life in postmenopausal women but also carries no risk of side effects due to excessive intake.

The major limitation of this study was the lack of a placebo control because it was a preliminary randomized, parallel-group, open-label trial. Accordingly, further studies using a double-blind, placebo-controlled design are required to clarify the effect of soymilk fermented by lactic acid bacteria and bifidobacteria on facial wrinkles and urinary isoflavone levels in healthy postmenopausal women. Moreover, because of the complex interactions that exist between probiotics and their hosts, the complete mechanisms of action of fermented soy milk remain unknown. Further studies are needed to establish the evidence.

CONCLUSION
Daily consumption of soymilk fermented by lactic acid bacteria and bifidobacteria may improve facial wrinkles and elevate urinary isoflavones in healthy postmenopausal women.

List of Abbreviations: O-DMA, O-desmethylandolensin

Competing Interests: This clinical trial was performed through outsourcing to TTC Co., Ltd. (Tokyo, Japan) as a CRO with sponsorship and provision of test beverages from Yakult Honsha Co., Ltd. The sponsor provided support in the form of salaries for all authors, but did not have any additional role in the study design, data collection, and interpretation, in addition to the decision to publish or manuscript preparation. All authors declare no potential conflicts of interest with respect to the authorship and publication of this article.

Author Contributions: M.K., K.H., and F.I. conceived and designed the experiments. M.K checked the data. M.K. and K.M. wrote the paper.

Acknowledgments and Funding: We thank all the participants of this trial. We also thank Dr. Osamu Nemoto at the Kita Jusanjo Internal Medicine Dermatology Clinic (Hokkaido, Japan) for performing the physical and clinical examinations, the staff of TTC Co., Ltd. (Tokyo, Japan) for performing the clinical trial management and statistical analyses, the staff of Exam Co., Ltd. (Sapporo, Japan) for collecting the silicone rubber replicas of facial wrinkles, the staff of Inforward, Inc. (Tokyo, Japan) for analyzing the wrinkle parameters, and the staff of SRL, Inc. (Tokyo, Japan) for analyzing the urinary isoflavones. This work was funded by the Yakult Honsha Co., Ltd. The funder provided support in the form of salaries for all authors, but did not have any additional role in the study design, data collection, and interpretation, in addition to the decision to publish or manuscript preparation.

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