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Alteration of intestinal flora by the intake of enzymatic degradation products of adlay (*Coix lachryma-jobi* L. var. *ma-yuen* Stapf) with improvement of skin condition

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ABSTRACT

Adlay has been used as a traditional Chinese medicine and nutrient for its beneficial effects on bowel movements and skin care. This study examined the effect of enzymatic degradation product of adlay, "Super Hatomugi" (SPH) on human skin and the intestinal flora in a randomized, double-blind placebo-controlled study. The subjects were divided into three groups: 500 mg SPH, 1000 mg SPH, and placebo, taken daily for 4 weeks. Hematological and skin condition examinations as well as an analysis of intestinal flora were performed 2 weeks before and 10 weeks after the start of the SPH intake. Skin condition was improved by SPH intake as revealed by a reduction in the number of nucleated epidermal cells. In addition, an increase in the fecal population of *Bacteroidetes* followed the SPH intake. These results show the possibility that SPH improves the skin condition and changes the proportions of intestinal flora.

1. Introduction

Skin aging is a complex biological process influenced by a combination of endogenous and exogenous factors (Debacq-Chainiaux, Leduc, Verbeke, & Toussaint, 2012). A variety of bioactive components derived from plants such as vitamins, amino acids and polyphenols have been reported to support the health and beauty of skin (Martorana et al., 2013; Schagen, Zampeli, Makrantonaki, & Zouboulis, 2012), and thus foods and supplements derived from these plants have been of particular interest in terms of health promotion.

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Adlay (*Coix lachryma-jobi* L. var. *ma-yuen* Stapf) is an annual crop cultivated mainly in India, China and Japan (Hu, Zhao, Liang, Qiu, & Chen, 2007). Adlay seeds have been used widely as a traditional Chinese medicine for the treatment of edema, rheumatism, and neuralgia.

Adlay was found to have various pharmacological effects. For example, rats fed adlay oil showed significant decreases in serum insulin and leptin levels (Huang, Chiang, Yao, & Chiang, 2005). In rats with hyperlipidemia, adlay seed oil intake reduced the volume of abdominal fat tissue and the level of low-density lipoprotein concentration, and increased the total antioxidant capacity (Yu, Gao, Zeng, & Liu, 2011). These reports suggest that adlay may help ameliorate metabolic syndrome, a cluster of conditions that is characterized by abdominal obesity and hyperlipidemia. In addition, other research groups have revealed that dehulled adlay reduced the risk of colorectal carcinogenesis through the modulation of COX-2 expression in a rat model (Hung & Chang, 2003; Shih, Chiang, & Kuo, 2004).

The consumption of adlay has also been thought to prevent or improve various skin diseases such as dry skin, warts and atopic dermatitis. Adlay's therapeutic effects for skin disorders are speculated to be based on the recovery of skin barriers by an increase in ceramide (Huang, Chung, Kuo, Lin, & Chiang, 2009). It is also speculated that adlay exerts anti-allergic effects by producing a shift in the Th1/Th2 balance from Th2 to Th1 dominance (Hsu, Lin, Lin, Kuo, & Chiang, 2003).

Several studies have indicated that the intestinal flora play an important role in obesity and cancer, as well as inflammatory bowel disease (IBD) (Bajzer & Seeley, 2006; Bien, Palagani, & Bozko, 2013; Jobin, 2013). It is now empirically recognized that an individual's skin condition can reflect the environment of his or her intestinal flora (Bisgaard et al., 2011); however, the influence of the intestinal flora on the skin's condition has not been well investigated. Concerning the change of intestinal flora in association with adlay intake, rats fed adlay showed a significant change in the population of lactic acid bacteria in faeces (Chiang, Cheng, Chiang, & Chung, 2000). Experimental data regarding the relationship between the proportion of intestinal flora and the skin's condition in humans could provide valuable information about the skin's condition in light of the presence of microbiota.

Recent studies have demonstrated that adlay contains a variety of biologically active compounds including oligosaccharides and polyphenols that may improve bowel functions (Son, Kim, & Lee, 2008) and have an antioxidative effect (Wang, Sun, Yi, Wang, & Ju, 2012), respectively. Unfortunately, adlay extracts lose much of their bioactive compounds through the process of extraction and purification. We thus successfully developed "Super Hatomugi (SPH)," in which whole adlay is crushed and degraded by enzymatic treatment with α-amylase, neutral protease and transglucosidase. We found that SPH contained much higher amounts of free amino acids, polyphenols, and oligosaccharides compared to adlay extracts prepared by conventional methods. To test the efficacy of SPH for human health and skin conditions, we carried out a clinical intervention focusing on the condition of the skin in association with intestinal flora.

2. Methods

2.1. Study subjects

Sixty-eight healthy female volunteers (age 24–47 years) were recruited. None had a history of recent gastrointestinal disorders, pregnancy, significant disease, surgery, severe allergic reaction to food, or current use of any medication. The subjects' body weights and body mass index (BMI) values are listed in Table 1.

The clinical intervention was carried out as a randomized, double-blind placebo-controlled trial. At randomization, the 68 eligible subjects were randomly and blindly assigned to one of three treatment groups: 1000 mg SPH, 500 mg SPH, or Placebo. For 4 weeks, each night before going to bed, the subjects took 1000 mg SPH or 500 mg SPH or placebo (glucose) contained in six capsules. Physical and hematological examinations as well as VAS (Visual Analogue Scale) score measurements were performed at 2 weeks before, baseline (0 week), and 4 weeks after the supplementation started. Measurements of the enteric bacteria profile and skin condition were carried out twice, at 2 weeks before and 4 weeks after the supplementation started.

All subjects provided written informed consent prior to undergoing any study-related tests, and the protocol was approved by the Ethics Committee of Hokkaido Information University. The study protocol was conformed to the Helsinki Declaration.

2.2. Super Hatomugi (SPH)

The SPH was prepared by the use of adlay (*Coix lachryma-jobi* L. var. *ma-yuen* Stapf) harvested in Japan without agricultural chemicals. Adlay seeds with shell were crushed and sterilized at 141–156 °C for 3 min, and then crushed to the particle size of 455 μm or less. The ground product (400 kg) was suspended in water (3600 L) and treated with 25% sodium hydroxide (18 L) for 3 h at 75 °C for gelatinization of starch. After pH adjustment between 5.45 and 5.55, the suspension was treated with α-amylase, SumizymeL® (New Japan Chemical Industry Co. Ltd., Tokyo, Japan) and neutral protease, SumizymeLP® (New Japan Chemical Industry Co. Ltd., Tokyo, Japan) at 50–52 °C. After the enzyme treatment for 3 h, the product was further treated with transglucosidase (Amano

Table 1 – Characteristics of the subjects in the placebo, 500 mg SPH and 1000 mg SPH groups.

Characteristic	Placebo	500 mg SPH	1000 mg SPH
Number of subjects	$n = 22$	$n = 22$	$n = 22$
Age (years)	37.77 ± 6.29	37.91 ± 5.46	38.68 ± 5.27
Body weight (kg)	52.96 ± 7.60	51.65 ± 6.23	51.21 ± 7.70
Height (cm)	158.32 ± 6.71	158.76 ± 6.63	157.81 ± 4.30
Body mass index (kg/m^2)	21.10 ± 2.39	20.54 ± 2.23	20.46 ± 3.45

Values shown are mean ± SD. Statistical analysis was performed by one-way analysis of variance.

Pharmaceutical Co., Ltd., Aichi, Japan) to obtain isomaltooligosaccharide. After enzyme inactivation, the soluble product was concentrated to Brix degrees of 19–22. The final product was prepared by drying and subsequent powdering and encapsulation. Analytical results of the composition of SPH compared to adlay, and coix seed are provided in Table 2. The production and the packing were carried out in HABA Corporation (Tokyo, Japan), under the quality-controlled manufacturing plant in compliance with the Food Sanitation Act (the Ministry of Health, Labor, and Welfare of Japan). The quality and safety of test samples were thoroughly examined by HABA Corporation.

Supplementation of SPH for healthy human volunteers was carried out at doses of 1000 mg/day for 4 weeks. There were no side effects caused by supplementation of SPH. Based on these results, it is considered that SPH is tolerant of humans at the doses of 1000 mg/day for 4 weeks.

2.3. *Preparation of corneocytes*

Skin of 21 randomly selected subjects was tape-stripped for the measurement of nucleated epidermal cells. Cellophane film (Promotool Corp., Tokyo, Japan) was firmly attached to the skin on the subject's cheek and gently pressed by hand over the entire area. The removed films were adhered to slide glasses, and these samples were analyzed using an inverted microscope, IX71 (Olympus, Tokyo, Japan). Nucleated epidermal cell numbers were calculated by viewing five visual fields, and their averages were calculated.

Table 2 – Component of Super Hatomugi (SPH) compared with Coix seed, adlay per 100 g.

Component	SPH	Coix seed	Adlay
Calories (kcal)	300	360	360
Carbohydrate (g)	66.9	72.2	72.2
Protein (g)	10.6	13.3	13.3
Fat (g)	5.5	1.3	1.3
Polyphenol (mg)	441	66	43
Dietary fiber (g)	28.9	1.7	30.6
Total free amino acid (mg)	364	35	24
Arginine	48	6	5
Lysine	14	2	2
Histidine	12	1	2
Phenylalanine	30	–	–
Tyrosine	26	–	–
Leucine	54	–	–
Isoleucine	14	–	–
Methionine	8	–	–
Valine	21	2	–
Alanine	32	5	4
Glycine	6	2	1
Proline	13	4	2
Glutamic acid	34	9	5
Serine	16	1	1
Threonine	11	–	–
Aspartic acid	22	3	2
Tryptophan	3	–	–
Cystine	–	–	–

2.4. *Measure of sebum capacity, transepidermal water loss and moisture*

The sebum secretion of the face was measured using the Sebumeter® SM815 (C-K Electronics, Cologne, Germany), as described (Sator, Schmidt, & Hönigsmann, 2003). In brief, the amount of sebum secretion was recorded at the forehead. To collect sebum, a plastic strip was applied at the site with a constant pressure of 4 N for 30 s. The originally translucent plastic strip becomes transparent when it absorbs sebum. The Sebumeter® calculates the sebum level by measuring the transparency of the strip using a photodetector. All measurements were performed by the same investigator in a room with a constant temperature and constant humidity.

The transepidermal water loss of the upper arm surface and the moisture of the face was measured using the TEWAMETER® TM300 and the CORNEOMETER® CM825 (C–K Electronics, Cologne, Germany) according to the manufacturer's methods.

2.5. *Real-time PCR for analysis of enteric bacteria*

DNA from subjects' fecal samples was extracted using a kit (DNA Extraction kit, Qiagen, Valencia, CA, USA) according to the manufacturer's protocols. Phyla-specific primers were used to quantify 16S rRNA gene copy numbers (rDNA) of Phyla *Bacteroidetes,* Phyla *Firmicutes* and total bacteria. Real-time polymerase chain reaction (PCR) was performed in a 20.0-μL reaction containing 5.0 μL of DNA (<100 ng), 10.0 μL of 2× Light Cycler®480 SYBR Green I Master (Roche Diagnostics, Mannheim, Germany), 3.0 μL of H_2O, and 1.0 μL of 500 nM primers. Amplification was performed on a Light Cycler® 480 qPCR system (Roche Diagnostics, Mannheim, Germany). The total bacteria primers were 5′-ACTCCTACGGGAGGCAGCAGT-3′ and 5′-GTATTACCGCGGCTGCTGGCAC-3′ for forward and reverse primers, respectively. The *Bacteroidetes* primers were forward primer 5′-GGARCATGTGGTTTAATTCGATGAT-3′ and reverse primer 5′-AGCTGACGACAACCATGCAG-3′. The *Firmicutes* primers were forward primer 5′-GGAGYATGTGGTTTAATTCGAAGCA-3′ and reverse primer 5′-AGCTGACGACAACCATGCAG-3′. The percentage of *Bacteroidetes* or *Firmicutes* was calculated as the ratio to the cycle number of total bacteria.

2.6. *Analysis of serum contents of biomarkers*

The subjects fasted overnight, and blood samples were drawn before breakfast on days 0 and 28 for liver function and renal function. The samples were separated immediately by centrifugation (1000×*g*, 10 min) and were stored at −80 °C within 2 weeks until use. Clinical diagnostics tests for alanine aminotransferase, blood urea nitrogen and other biomarkers were carried out using a Roche/Hitachi 912 chemistry analyzer (Roche Diagnostics, Mannheim, Germany).

2.7. *Statistical analysis*

Averages and standard deviations of age and other parameters were calculated for each group. Statistical analyses were performed with the program IBM SPSS Statistic 19 (IBM, Ar-

monk, NY, USA). The Wilcoxon signed-rank test was performed for the data 2 weeks before and 4 weeks after the start of the intake of adlay in each group. The statistical analysis of the value changes of enteric bacteria was carried out by analysis of variance (ANOVA) with the post hoc Games–Howell test.

3. Results

3.1. *Effect of SPH on skin texture*

Sixty-six subjects completed the study; two subjects chose to discontinue due to personal reasons before the supplementation began. There were no significant differences in age, body weight, height, or BMI among the three subject groups (Table 1). First, to determine the effect of SPH on the subjects' skin, we used tape-stripping to measure the number of nucleated epidermal cells and observed the subjects' skin texture. An increase in total nucleated epidermal cells indicates abnormal cell turnover as a results of skin damage. At 4 weeks after the start of the supplementation, we observed that the subjects who ingested SPH at the dose of 1000 mg/day for 4 weeks had significantly decreased numbers of nucleated epidermal cells ($p = 0.018$) (Fig. 1A). In addition, improvement of the structure of skin texture following SPH intake was demonstrated by microscopic analysis (Fig. 1B).

3.2. *Effect of adlay on sebum secretion*

We also examined the effect of SPH on sebum secretion and transepidermal water loss and moisture. Although no significant differences in the rates of skin moisture or transepidermal water loss were observed (data not shown), sebum secretion was increased among the 1000 mg/day SPH subjects, although not significantly so ($p = 0.064$) (Fig. 2).

3.3. *Effect of SPH on intestinal flora*

We analyzed the subjects' skin condition by evaluating the increase of nucleated epidermal cells, an indicator of deterioration of the skin. In parallel, we examined the changes of the subjects' intestinal flora to evaluate the relationship between skin condition and intestinal flora. We analyzed mainly the fecal population of *Bacteroidetes* and *Firmicutes*. The population of *Bacteroidetes* was slightly increased by SPH intake, whereas little change of *Firmicutes* was observed (Fig. 3). Consistent with this result, we found significant increases in *Bacteroidetes* population for all 66 subjects (Fig. 4).

3.4. *Levels of biomarkers of liver and renal functions after SPH intake*

We examined the levels of several biomarkers of liver function and renal function. Parameters for liver function, i.e., AST, ALT, and γ-GTP, and those for renal function, i.e., BUN and creatinine showed minimal changes after the SPH intake, suggesting that SPH intake has no or minimal unfavorable effects on the liver and kidney, even at the dose of 1000 mg/day.

4. Discussion

Many disorders are associated with changes in the composition and metabolism of enteric flora (Guarner, 2006). Functional foods, supplements and vegetables have been well recognized as rich sources of the dietary fiber that exhibit beneficial effects on health promotion through the improvement of the balance of intestinal bacterial flora. It was recently noted that dyspepsia might lead to skin disorders (Roberfroid et al., 2010). In general, however, the correlation between skin condition and intestinal flora is not well investigated.

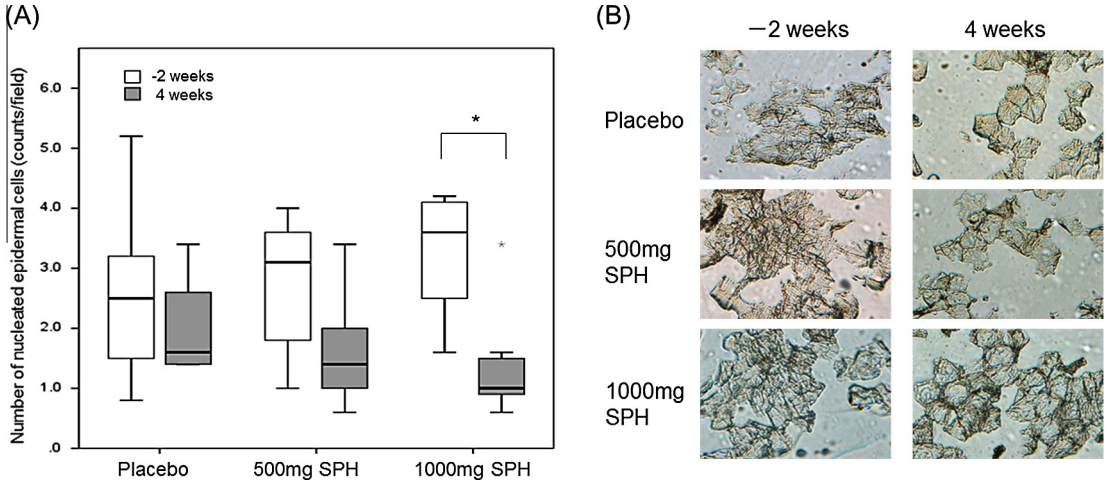

Fig. 1 – Skin condition before and after Super Hatomugi (SPH) intake. (A) Quantitative measurements of nucleated epidermal cells were carried out using tape stripping of subjects who completed a 4-week course of the daily intake of placebo ($n = 8$), 500 mg SPH ($n = 6$), or 1000 mg SPH ($n = 7$). Averages of 5 fields per sample were used. Data for 95th percentiles and medians are shown. $^{*}p < 0.05$ (−2 weeks vs. 4 weeks) by Wilcoxon signed-rank test. (B) The images of corneocytes were captured by an inverted microscope (400×).

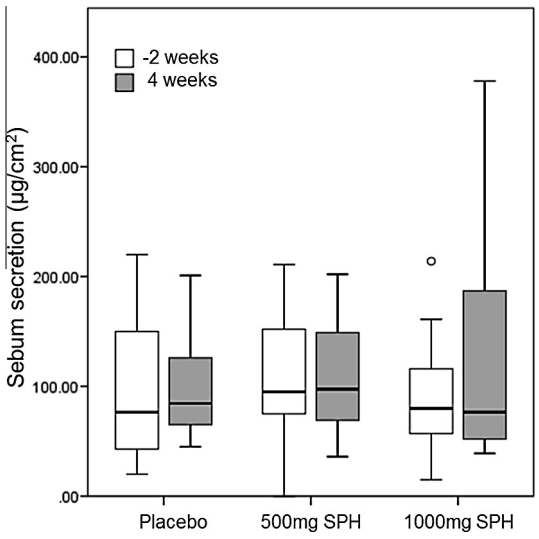

Fig. 2 – The effect of Super Hatomugi (SPH) on sebum secretion after 4 weeks of the ingestion of placebo (*n* = 22), 500 mg SPH (*n* = 22), or 1000 mg SPH (*n* = 22). Data for the 95th percentiles and medians are shown. Statistical analysis was performed by Wilcoxon signed-rank test (−2 weeks vs. 4 weeks).

The results of the present double-blind and placebo-controlled study demonstrated the potential effects of SPH on the skin condition and the intestinal flora. We observed that SPH improved the subject's skin conditions, possibly through a reduction of nucleated epidermal cells and improvement of skin texture. In particular, an increase of the fecal population of *Bacteroidetes* was clearly associated with the SPH intake. Taken together, these results indicate that SPH could be a beneficial supplement for the improvement of an individual's skin condition, possibly based on a change in the population of intestinal flora.

Adlay has been reported to exhibit an anti-allergic function (Hsu et al., 2003) as well as an antioxidant action (Kuo, Shih, Kuo, & Chiang, 2001), and therefore has been used in China for the treatment of skin diseases (Chen et al., 2011). Oxidative damage by reactive oxygen species (ROS) plays a major role in skin aging (Oresajo, Pillai, Manco, Yatskayer, & McDaniel, 2012; Ristow & Schmeisser, 2011). Antioxidants such as carotenoids, tocopherols, and flavonoids, as well as vitamins, have frequently been referred to as agents capable of promoting skin health and beauty (Schagen et al., 2012). Polyphenols with antioxidative activities were purified and identified from adlay, including three lignan compounds (syringaresinol, 4-ketopinoresinol, and mayuenolide) (Kuo et al., 2002). Our previous study demonstrated that the methanolic and dimethylsulfoxide (DMSO) extraction from SPH had an antioxidant effect, and DMSO extraction reduced the production of tumor necrosis factor-alpha (TNF-α; unpubl. data). As shown in Fig. 1, we observed a reduction in the number of nucleated epidermal cells and an improvement of skin texture following a 4-week SPH intake. We herein confirmed that SPH have more than 10 times amounts of polyphenol compared to adlay (Table 2). Although we did not investigate kinds of polyphenol in SPH, these collective data suggest the possibility that antioxidant action by polyphenols in SPH contributes to the improvement of skin condition.

In addition, we developed SPH to enhance the amount of free amino acid by protease treatment (Table 2). Free amino acids are known to be critical components to collagen product which are helpful for water retention properties of skin (Rawlings & Matts, 2005). Furthermore, it has been demonstrated that free amino acid are important not only as

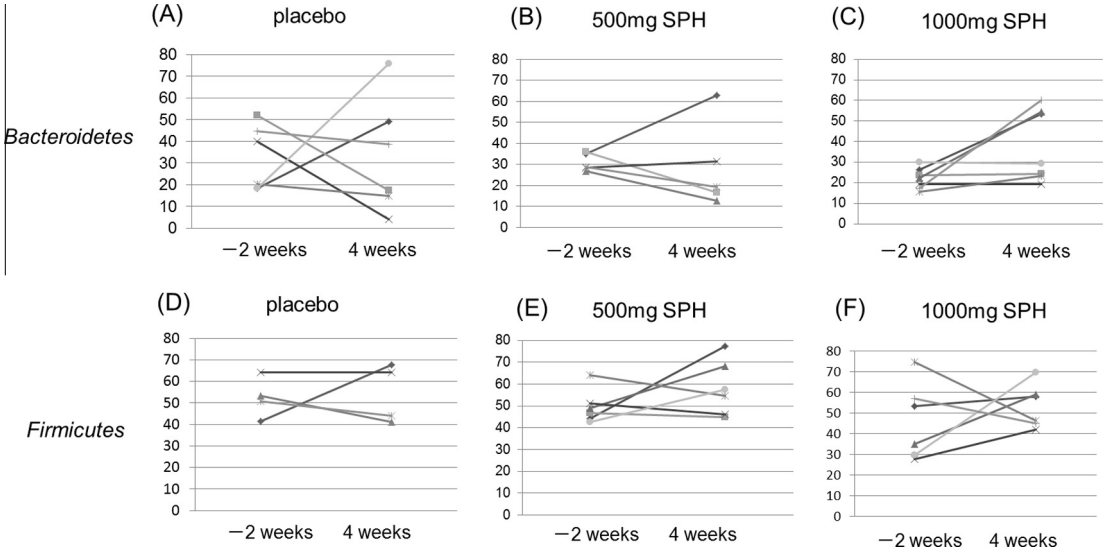

Fig. 3 – Fecal populations of *Bacteroidetes* (A–C) and *Firmicutes* (D–F) compared with total fecal bacteria in the same subjects who underwent tape-stripping for the measurement of the number of nucleated epidermal cells except for a few subjects who showed abnormal bacteria values (i.e., the total ratio of *Bacteroidetes* and *Firmicutes* exceeded 100%). The data are presented as data points for each subject before (−2 weeks) and after (4 weeks) the start of the intake. Placebo group: (A) (*n* = 7), (D) (*n* = 4); 500 mg SPH group: (B) (*n* = 5), (E) (*n* = 6); 1000 mg SPH group: (C) (*n* = 6), (F) (*n* = 6).

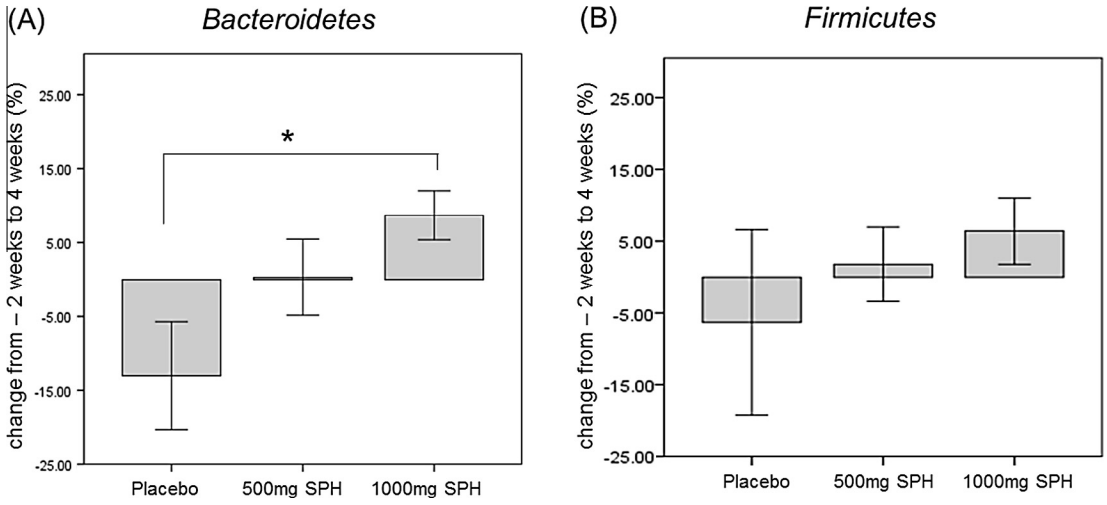

Fig. 4 – Changes in the ratios of fecal *Bacteroidetes* (A) and *Firmicutes* (B) compared with total fecal enteric bacteria after the 4-week supplementation in all subjects except for the few who showed abnormal values (i.e., the total ratio of *Bacteroidetes* and *Firmicutes* exceeded 100%, or the cycle threshold values (Ct) of >25.0). Values are means ± SE. The Games–Howell test was used. *$p < 0.05$. (A) Placebo ($n = 12$), 500 mg SPH ($n = 12$), 1000 mg SPH ($n = 13$). (B) Placebo ($n = 8$), 500 mg SPH ($n = 12$), 1000 mg SPH ($n = 14$).

substrates for various metabolic pathways but also they have a positive effect on signaling pathways (Meijer & Dubbelhuis, 2004). For example, L-threonine regulates meta-stability of embryonic stem cells via PI3K/Akt, MAPKs, and mTOR pathways (Ryu & Han, 2011). These results suggested that free amino acid in SPH relate to the improvement of skin condition as seen in polyphenols.

Inappropriate amounts of sebum from the glands can lead to several skin disorders, such as acne and seborrhea (Borlu et al., 2012). However, sebum also possesses the beneficial effects of preventing water loss from the skin surface and protecting the skin from infection by bacteria and fungi (Smith & Thiboutot, 2008). In the present study, we found that the sebum capacity was increased by SPH intake. SPH intake also significantly improved skin texture. We suspect that the SPH intake improved the subjects' skin condition by regulating their sebum secretion.

Regarding the change in the population of microbiota due to SPH intake, we observed that *Bacteroidetes* were increased in the 1000 mg/day SPH group, in whom significant improvement of the skin was also observed. *Bacteroidetes* are known as the major population of the microbiota of animals, especially in the gastrointestinal tract (Ishikawa et al., 2013). Members of the *Bacteroidetes* have been known to act as opportunistic pathogens, mostly causing post-operative infections and bacteremia (Murphy, Mörgelin, Cooney, & Frick, 2011). Moreover, *Bacteroidetes* are reported to have the most effective antibiotic resistance of any anaerobic pathogen (Kislak, 1972).

The mechanism underlying the increase in *Bacteroidetes* may be due to the influence of oligosaccharides present in SPH. Dietary oligosaccharides modulate the composition and activity of intestinal microbiota. The intestinal fermentation of oligosaccharides leads to changes in the proportion of intestinal microbiota and the increasing production of short-chain fatty acids (Macfarlane, Steed, & Macfarlane, 2008; Meyer & Stasse-Wolthuis, 2009). *Bacteroidetes* grew, induced α-L-fucosidase activity, and produced abundant lactate or short-chain fatty acid when fed oligosaccharides *in vitro* (Yu et al., 2013). However, most oligosaccharides are converted to monosaccharides and then absorbed from the small intestine. We developed SPH to enhance the amount of oligosaccharides, such as isomaltose and panose, dextrantriose, from 8 to 15 g compared to adlay through a process in which the carbohydrate component of adlay is converted to oligosaccharides by α-amylase and transglucosidase and degradation. Oligosaccharides avoid degradation and absorption in the small intestine, which might lead to an increase in the amount of *Bacteroidetes* in the colon. Moreover, in a preliminary study, we found that SPH enhanced the amount of dietary fibers (28.9/100 g) more than coix seed (1.7/100 g) by the treatment of transglucosidase. It is conceivable that SPH could change the composition of intestinal microbiota through increase of dietary fibers. This needs further investigation.

It was reported that supplementation *with Bifidobacterium* breve, one of the microbiota, prevented UV-induced skin damage through the suppression of the increases in both elastase activity and interleukin (IL)-1β levels in the skin in mouse (Sugimoto et al., 2012). This report supports the idea that enteric flora alterations due to SPH intake could improve the skin's condition.

Consistent with these findings, we speculate that the improvement of skin condition in the present SPH groups may be mediated by the alteration of enteric flora in the colon by oligosaccharides as well as other valuable effect by polyphenols, free amino acid. It is of interest to investigate the mechanism of how SPH affects the population of intestinal flora in association with the improvement of skin conditions.

The scores for abdominal symptoms were also assessed with a Visual Analogue Scale (VAS); however, the subjects' VAS scores were not significantly changed after SPH intake (data not shown). We suspect that the administration period was too short for the effect of fecal properties to be observed.

We are planning to further investigate the effects of long-term intake of SPH on quality of life (QOL).

In summary, the present findings demonstrated the potential efficacy of SPH for the improvement of skin conditions in concert with changes in the microbiota population. However, the underlying molecular mechanisms, including the identification of the functional molecules in SPH, remain to be elucidated. Nonetheless, the results of this clinical trial of SPH broaden our knowledge of the functionality of adlay, particularly SPH, and other functional foods for the improvement of skin conditions and the promotion of health.

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