



# Skin Enhancement Efficacy of Supplements Containing Four Amino Acids in Healthy Japanese Women: A Randomized Double-blind Placebo-controlled Study

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## ● Abstract

**Objective:** The objective of this research was to investigate the effectiveness of daily ingestion of a supplement containing RHIZOPUS/RICE FERMENT FD POWDER, which features Glutamine acid, Aspartic acid, Proline, and Arginine, on the condition of the human skin.

**Methods:** A randomized, placebo-controlled, double-blind study was conducted to verify the skin condition. In this study we measured wrinkles, the amount of surface spots (visible spots and hidden ones), the amount of melanin, the whiteness of the complexion, the stratum corneum water content and the trans-epidermal water loss (TEWL), three kinds of elasticity and also carried out the subjective reporting in form of questionnaire. To evaluate the safety of the test foods, adverse events were collected by means of a written questionnaire during the study.

**Results:** From all of 155 applicants, 108 were eliminated according to not meeting inclusion criteria. The 47 subjects were randomly assigned to intervention groups and made a start with ingestion. Among 47 subjects, 2 were withdrawn due to personal reason. Data obtained with 45 subjects (Test; 22, Placebo; 23) was used for the analysis of efficacy. After the 12-week ingestion the test group showed a significant difference in “Wrinkle grade”, “The whiteness of the complexion”, “The stratum corneum water content”, “Melanin”, and “ElasticityR2” compared to the Placebo. On the other hand, the tendency was not seen in “Spot” or “The trans-epidermal water loss”. As for the subjective evaluation, we observed a significant difference in 7 out of 20 items. No adverse effects were observed after the ingestion of the test product.

**Conclusion:** We found out that the 12-week ingestion of the test product containing RHIZOPUS/RICE FERMENT FD POWDER, which has a plenty of amino acids such as glutamine acid, aspartic acid, proline, and arginine, contributed to the improvement of the skin condition such as elasticity, tightness or moisture. In addition, no safety-related matter was observed during the 12-week test period.

**Key Words:** RHIZOPUS/RICE FERMENT FD POWDER, amino acid, skin condition, elasticity, wrinkle grade

## 1. INTRODUCTION

In modern Japanese society, the words “skin care” is attracting much interest from people, irrespective of age and sex. Society is flooded with many products or information about skin care, and the category of these items or information varies from cosmetics to supplements, massage technique and dietary treatment. Recently, many people are inflicted with emotional or physical harm caused by the lack of exercise, bad eating habits or stress, and as a result they suffer from skin problems such as rough skin, pale skin or lack of tension. In addition, substantially more people start to have the skin problems by the external factors such as photo-aging caused by the increase of ultraviolet<sup>1)</sup> or dry air triggered by the heavy usage of air-conditioning<sup>2)</sup>. The skin

condition is greatly influenced by the type of nutrition consumed. Although there exists some individual variation, people with an unbalanced diet tend to have rough skin. The recent dietary habit in Japan is shifting to European-style, which is said to have problems such as excessive intake of fat or lack of minerals<sup>3)</sup>. In terms of the relationship between the skin condition and the food consumed, it is reported that the specific food ingredients such as collagen peptide<sup>4)</sup>, elastin<sup>5)</sup> and placenta<sup>6)</sup> exercise a positive effect on the skin condition.

In Japan, the fermentation technique with the use of microorganisms (such as the production of Miso or soy sauce) has been cultivated since ancient times. Fermentation applies to the changing process of the original food ingredients to another type with the help of the development of microorganisms, and, at the same time, a process which is beneficial to humans. Therefore the fermentation often produces ingredients of higher nutrition than the original. Rice has been used for fermentation technique for many years since it has been consumed from ancient times in Japan, and many

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fermented foods that derived from rice have been invented and developed so far. The representative example of the rice-derived fermented foods is sake (rice wine). It is known that sake has more nutrition such as amino acids than the rice used as raw material<sup>7)</sup>. Rice ferment extract is produced from rice (which has a wealth of carbohydrate), and by applying a high fermentation technique to it. Therefore it is well known that the rice ferment extract contains plenty of vitamins, amino acids and carbohydrate, and it helps to keep skin enriched, moisturized high in tension. Also, the amino acids contained in the rice ferment extract are reported to have a high function of moisture by the skin absorption<sup>8)</sup>. RHIZOPUS/RICE FERMENT FD POWDER is a powder produced by a filtration of extract which has a seed of *Oryza sativa* as its substrate and undergoes a process of fermentation via *Rhizopus*.

Although rice ferment extract is used in many cosmetics and is renowned for its function, there are few studies which focus on the efficacy of rice ferment extract to the skin condition when it is ingested orally. Therefore in this study, we conducted a randomized, placebo-controlled, double-blind study to verify the efficacy of the food containing RHIZOPUS/RICE FERMENT FD POWDER, which is rich in amino acids, for the skin condition.

## 2. METHODS

### 2.1. Trial design

A randomized, placebo-controlled, double-blind study was conducted with the aid of a fund from RISOU Co., Ltd (Tokyo) at JACTA (Tokyo). The study period was 12 weeks, from March 26<sup>th</sup> to June 19<sup>th</sup>, 2016. This study was conducted in accordance with the ethical principles of the declaration of Helsinki. The study protocol was approved by the Institutional Review Board of Pharmaceutical Law Wisdoms (Tokyo). Written informed consent was obtained from all subjects.

The allocation of the test product to the subjects was carried out by the person in charge of allocation. The allocation list was sealed and strictly controlled in a safe deposit box of JACTA until the end of the study.

### 2.2. Subject

Healthy subjects participated in the present study. All of the subjects in this study were public volunteers who had enrolled in the monitor bank of CROee Inc. (Tokyo), recruited from February through March, 2016.

#### 2.2.1. Inclusion criteria

- (1) Females aged between 35 and 59 years.
- (2) Females with wrinkle problems (evaluated between grades 1 and 3 upon the guideline for evaluation of anti-wrinkle products by a task-force committee for evaluation of anti-aging function<sup>9)</sup>
- (3) Non-smoker

#### 2.2.2. Exclusion criteria

- (1) Individuals diagnosed with photosensitive dermatosis;
- (2) Individuals with skin lesions at the test site (atopic

**Table 1** Nutritional content of the test samples per 4 capsules

Item	RS	Placebo
Energy	7.74 kcal	5.91 kcal
Protein	0.02 g	0.00 g
Lipid	0.06 g	0.02 g
Carbohydrate	1.77 g	1.43 g
Salt equivalent	0.00 g	0.00 g

dermatitis, hives, inflammation, eczema, injury, acne spots, knots, speckles, or their traces);

(3) Individuals on medication, including herbal medicines that may affect the test (adrenal cortex hormone, collagen, hyaluronic acid, etc.);

(4) Individuals with a pollen allergy or may be allergic to the test materials (collagen peptide, gelatin, dextrin, flavor, etc.);

(5) Individuals who are pregnant, nursing, or likely to become pregnant during the trial;

(6) Individuals judged to be unsuitable to participate in the trial by the principle investigator.

### 2.3. Randomization

From all of 155 applicants, 108 were eliminated according to the wrinkle grade upon the guideline for evaluation of anti-wrinkle products. The inclusion criteria was judged by the principle investigator. All subjects were sequentially allocated to Group A (n=24) and Group B (n=23) using a random number table. In the process of subject assignment, background factors such as age was taken into consideration to avoid biased distribution. Subjects in Group A ingested the placebo and subjects in Group B ingested the test sample for 12 weeks.

### 2.4. Description of test foods and blinding

The active material of the test product is RHIZOPUS/RICE FERMENT FD POWDER, which features special 4 amino acids; Glutamine acid, Aspartic acid, Proline, and Arginine. The test product "Repair Supple" ("RS") was prepared by RISOU Co., Ltd. The amount of daily intake was 4 capsules (1 capsule weighs 495 mg, therefore 4 capsules weigh 1,980 mg). The Placebo does not include RHIZOPUS/RICE FERMENT FD POWDER. Both capsules were indistinguishable in shape, color, or taste, and were managed by an identification symbol. All involved were blinded. **Table 1** shows the nutritional contents of the samples.

### 2.5. Experimental procedures

#### 2.5.1. Experimental protocol

Subjects consumed 4 capsules of the supplement with hot or cold water every day for 12 weeks. Subjects were instructed as follows: to take the assigned foods as indicated; to maintain their usual lifestyles and habits; to avoid excessive amounts of food, drink, or alcohol; to avoid direct sunlight; to avoid excessive exercise; to maintain a daily record of that included the intake of test foods (or not), the status of facial skin and physical

**Table 2** Schedule for the study

Item \ Term	Screening	Pretrial test	Test period		
			4 w	8 w	12 w
Informed consent	●				
Selection and/or allocation	●				
Wrinkles	●	●	●	●	●
Surface spots		●	●	●	●
Melanin		●	●	●	●
Whiteness of the complexion		●	●	●	●
Stratum corneum water content		●	●	●	●
TEWL		●	●	●	●
Elasticity		●	●	●	●
Subjective reporting		●			●
Ingestion of test foods			↔		
Log			↔		

● : Implementation

↔ : Daily practice during the test period

condition during the test period; and to send the diary to the study coordinator.

### 2.5.2. Outcome

According to the schedule shown in **Table 2**, we measured parameters on efficacy and safety. These assessments were conducted upon pre-intervention and post-intervention.

Wrinkles, the amount of surface spots (visible spots and hidden ones), the amount of melanin, the whiteness of the complexion, the stratum corneum water content, the trans-epidermal water loss (TEWL), and the elasticity of the skin was measured to evaluate the skin condition as the primary outcome.

The subjects stayed in the environmental laboratory (temperature;  $22 \pm 2$  °C, humidity;  $50 \pm 10\%$ ) for twenty minutes before starting the trial.

The wrinkles found around the corner of the left and right eye were graded by the study conductor through optical observation of the skin with photos taken by a professional photographer, according to the guideline for evaluation of anti-wrinkle products by a task-force committee for the evaluation of anti-aging function<sup>9</sup>. The average grade of the two eyes was then calculated per subject. The amount of two kinds of surface spots on the face was measured by VISIA<sup>®</sup> Evolution (CANFIELD Imaging Systems, Inc.)<sup>10</sup>. The amount of melanin was measured by Mexameter<sup>®</sup> MX18 (Courage & Khazaka electronic GmbH)<sup>11,12</sup>, and for the whiteness of the complexion, lightness (L\*) was measured by a spectrophotometer, NF555 (Nippon Denshoku Industries Co., Ltd)<sup>13</sup>. For the stratum corneum water content, the TEWL, and the skin elasticity, Corneometer<sup>®</sup> CM825, Tewameter<sup>®</sup> TM300, and Cutometer<sup>®</sup> MPA580 (Courage & Khazaka electronic GmbH, Germany) were used respectively<sup>14-16</sup>. For the amount of surface spots, the

amount of melanin, the whiteness of the complexion, stratum corneum water content, TEWL, and the skin elasticity, both sides of the face were measured. The average value between both sides was then calculated per subject. Regarding the skin elasticity, 3 kinds of indexes were measured; R2 (Recovery ratio of the skin length), R5 (Ratio of elasticity part when elongated and during constriction), and R7 (Ratio of elasticity part during constriction)<sup>17,18</sup>.

Further, subjective reporting of the skin was observed by a questionnaire as the primary outcome. The questionnaire covered 20 items (**Appendix 1**). Responses to each question were rated on an ordinal scale of 0 to 9, with higher scores indicating a better result.

To evaluate the safety of the test foods, adverse events were collected by means of a written questionnaire during the study.

### 2.6. Data analysis

A full analysis set was adopted in the present study and no sample size design was used. All statistics were expressed as mean  $\pm$  standard deviation (SD).

For the wrinkle's grade, changes from baseline in the same group were assessed using Wilcoxon signed-rank test. Mann-Whitney U test was used for intergroup comparisons of changes from the baseline. With respect to the amount of the two kinds of surface spots, the amount of melanin, the lightness (L\*), stratum corneum water content, TEWL, the three kinds of elasticity, scores of subjective reporting, and blood biochemical and urine analysis, changes from baseline in the same group were assessed using the paired t-test. Student's t-test was used for intergroup comparisons of changes from baseline. Student's t-test was used to compare subject backgrounds between groups.

Multiplicity according to the occasions was not adjusted.

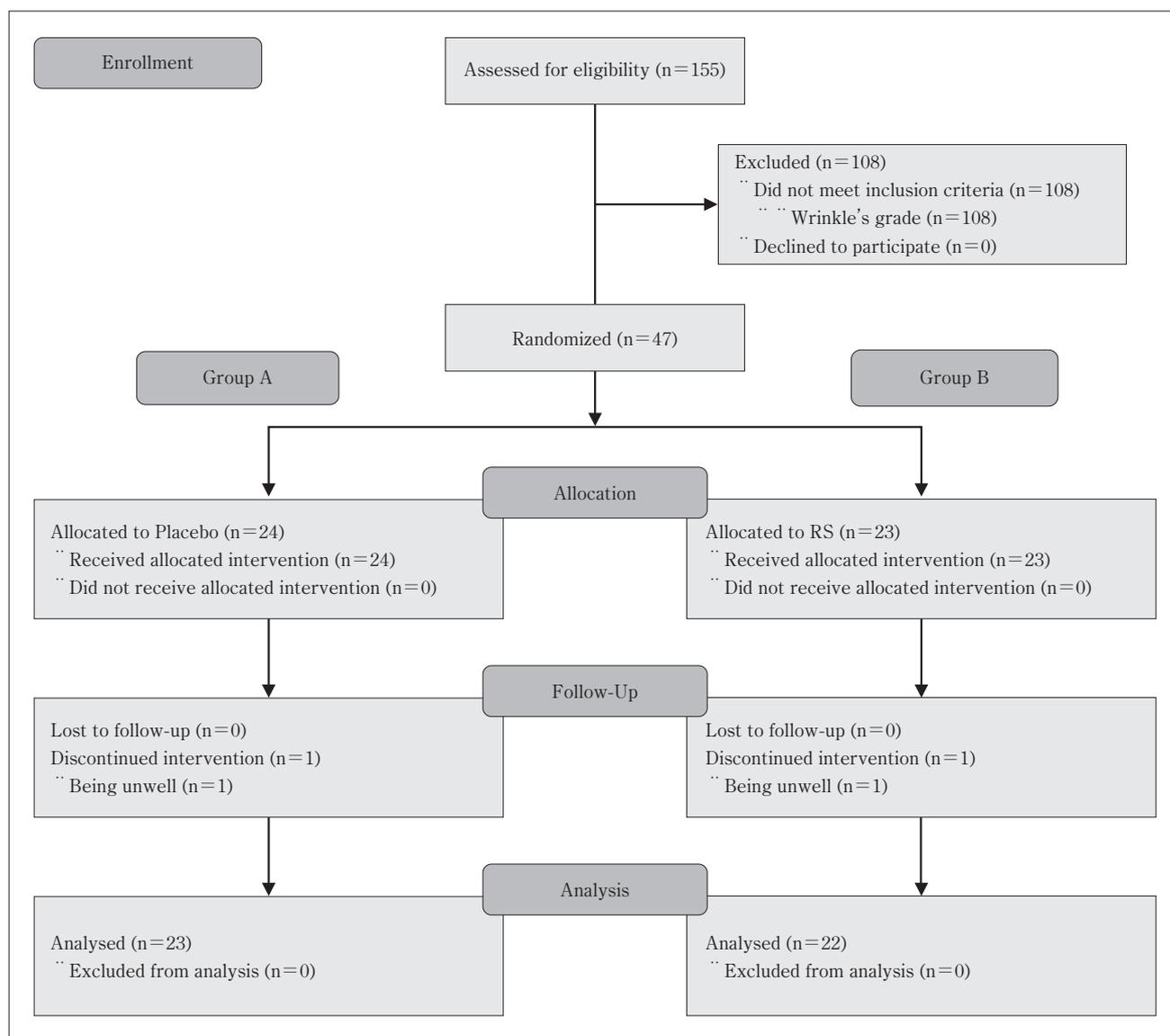


Figure 1 Flow diagram of subject disposition

Table 3 Subject demographics

Item	Unit	RS	Placebo
Subjects	numbers	22	23
Age*	years	45.5 ± 5.1	45.4 ± 4.9

mean ± SD

\*No significant difference

Any subjects with missing values were eliminated from the analysis. Statistical analyses were performed using Statcel 4 (Yanai, 2015). The results were considered significant at a <5% level in the two-sided test.

### 3. RESULTS

#### 3.1. Participant demographics

The 47 subjects were randomly assigned to intervention groups and made a start with ingestion. 2 were

withdrawn (unwell), and 45 subjects completed the study. Thus, data obtained from 45 subjects (RS; 22, Placebo; 23) were used for efficacy analysis (Figure 1). There were no significant differences in the age between groups (Table 3).

#### 3.2. Wrinkle grade<sup>9)</sup>

The results of the wrinkle grade are shown in Table 4. The average score for wrinkles at the corner of the right and left were significantly lower in RS compared with

baseline data. Further, a significant difference of changes was observed between the two groups after 4, 8, and 12 weeks of ingestion.

### 3.3. Two kinds of surface spots<sup>10)</sup>

The average amount of visible spots and hidden ones on both sides of the face are shown in Table 4. In RS, values of both visible and hidden spots increased significantly at week 8 and 12.

### 3.4. Melanin<sup>11)12)</sup>

The average amount of melanin on both sides of the face are shown in Table 4. Whilst in both groups, melanin increased significantly at week 4, 8, and 12, significant differences between the two groups were found in  $\Delta$  0-4w,  $\Delta$  0-8w, and  $\Delta$  0-12w.

### 3.5. The whiteness of the complexion (L\*)<sup>13)</sup>

For the whiteness of the complexion, Table 4 represents the amount of L\* on both sides of the face. At week 8, L\* decreased significantly in Placebo. Moreover  $\Delta$  0-8w, and  $\Delta$  0-12w showed a significant difference between the two groups.

### 3.6. The stratum corneum water content<sup>14)15)</sup>

Table 4 illustrates the average value of stratum corneum water content on both sides of the face. Values of stratum corneum water content was statistically significant in RS at week 8 and 12. Furthermore,  $\Delta$  0-8, and  $\Delta$  0-12w show a significant difference between the two groups.

### 3.7. The trans-epidermal water loss (TEWL)<sup>16)</sup>

Table 4 shows the average value of TEWL on both sides of the face. Both groups resulted in a significant decrease at week 4, 8, and 12 compared to the baseline, however no significant difference was detected in the between-group comparison of changes from pretrial value.

### 3.8. The three kinds of elasticity<sup>17)18)</sup>

The results of the three kinds of elasticity are shown in Table 4. At week 12 of R2, elasticity increased significantly in RS. R2 also differed significantly in the comparison of  $\Delta$  0-12w between the groups.

### 3.9. Subjective skin condition assessments

The results of subjective skin condition assessments are shown in **Table 5**. Moisture, Softness, Skin texture, Foundation adhesion, Skin condition, Crow's feet, and Eye bag's sagginess were significantly different after 12 weeks of between-group comparison of changes from pretrial value.

### 3.10. Safety

No adverse effects associated with the test product were observed in the course of the reporting.

## 4. DISCUSSION

We conducted a randomized, placebo-controlled, double-blind study in order to examine how the food containing RHIZOPUS/RICE FERMENT FD POWDER affects the skin condition. As the primary outcome, the RS showed a significant difference in "Wrinkle grade", "The whiteness of the complexion", "The stratum corneum water content", "Melanin", and "Elasticity R2" after 12-week

ingestion, compared to the Placebo. Also, as for the subjective reporting in form of a questionnaire, the between-group comparison of changes from pretrial value showed a significant difference in the items of "Moisture", "Softness", "Skin texture", "Foundation adhesion", "Skin condition", "Crow's feet", and "Eye bag's sagginess" after ingestion of 12 weeks. On the other hand, the improvement tendency was not observed in "Spot" or "The trans-epidermal water loss". In addition, as the secondary outcome, no adverse effects associated with the test product were observed in the course of the reporting, and the safety of ingesting the test product was suggested.

### Main Findings

This study showed the significant improvement in the items related to moisture, wrinkle or elasticity, in both test categories of the objective assessments (measurements) and the subjective assessments (questionnaire).

Skin consists of three layers: epidermis, dermis and subcutaneous tissue. The epidermis has four layers of stratum corneum, granular layer, stratum spinosum and basal layer, while in the dermis, collagen fiber and elastic fiber are complexly intertwined and the area between these two fibers is filled with mucopolysaccharide and fibroblasts<sup>19)</sup>. The Skin aging is caused by two factors: intrinsic aging and extrinsic aging<sup>20)</sup>. The increasing age is categorized as the intrinsic aging, and because of the increasing age, the whole epidermis becomes thinner and the stratum spinosum adversely becomes thicker. These changes cause the deterioration of moisture retention and the acceleration of drying of the skin<sup>21)</sup>. They also degrade the functions such as sebum secretion or sweat glands, and the amount of the trans-epidermal water loss increases<sup>22)</sup>. Furthermore, they cause the decrease of the amount of ceramide or amino acid in the dermis<sup>23)</sup>. The extrinsic aging, on the other hand, is triggered by the drying or oxidizing. Also, ultraviolet is a great enemy to the skin; once the skin is exposed to ultraviolet, the aging of skin dramatically becomes advanced. UVB (290 ~ 320 nm) mainly stimulates the epidermal keratinocyte and promotes the production of cytokine. The cytokine stimulates the pigment cells to help the melanin production, or work on the fibroblasts of dermis and severs collagen or elastic fiber. UVA (320 ~ 400 nm) directly stimulates the fibroblasts of dermis to promote wrinkle formation<sup>21)</sup>. In addition, the exposure of the horny cell layer to ultraviolet leads to the decrease of intercellular lipid or NMF (Natural Moisturizing Factor). This decrease triggers the loss of moisture retention function of the layer, the increase of moisture evaporation and eventually the loss of moisture level of the layer<sup>24)</sup>. Based on the above aging phenomena, the skin is involved with several problems such as wrinkles, spots, sagging, porous skin and/or dullness.

RHIZOPUS/RICE FERMENT FD POWDER contained

Table 4 Results of test analyses

Item (unit)	Time points	Scores/ Values <sup>1)</sup>		P-value <sup>2)</sup>
		RS (n=22)	Placebo (n=23)	
Wrinkles (scores)	Baseline	2.63 ± 0.51	2.14 ± 0.48	<0.001 <sup>##</sup>
	4-week	2.22 ± 0.37**	2.15 ± 0.48	
	Δ 0-4 w	-0.41 ± 0.26	0.01 ± 0.05	
	8-week	2.18 ± 0.41**	2.15 ± 0.48	
	Δ 0-8 w	-0.44 ± 0.28	0.01 ± 0.05	
	Δ 0-12 w	-0.50 ± 0.28	0.01 ± 0.05	
Visible spots (numbers)	Baseline	102.5 ± 25.1	103.3 ± 30.4	0.205
	4-week	100.4 ± 26.3	105.8 ± 31.9	
	Δ 0-4 w	-2.1 ± 13.8	2.4 ± 9.3	
	8-week	108.1 ± 28.0*	106.2 ± 31.4	
	Δ 0-8 w	5.5 ± 12.4	2.8 ± 10.4	
	Δ 0-12 w	5.4 ± 10.2	4.2 ± 10.6	
Hidden spots (numbers)	Baseline	302.3 ± 35.0	302.1 ± 39.6	0.702
	4-week	309.3 ± 29.8	306.8 ± 45.7	
	Δ 0-4 w	7.1 ± 20.6	4.7 ± 20.8	
	8-week	312.8 ± 30.8*	302.1 ± 56.3	
	Δ 0-8 w	10.6 ± 21.7	0.0 ± 28.5	
	Δ 0-12 w	13.3 ± 20.1	4.2 ± 24.6	
Melanin (index)	Baseline	126.2 ± 32.0	123.2 ± 27.8	<0.001 <sup>##</sup>
	4-week	152.5 ± 29.5**	173.0 ± 29.7**	
	Δ 0-4 w	26.3 ± 18.3	49.7 ± 15.7	
	8-week	161.6 ± 27.0**	176.5 ± 32.0**	
	Δ 0-8 w	35.4 ± 17.7	53.2 ± 17.2	
	Δ 0-12 w	39.1 ± 20.7	67.6 ± 29.0	
L* (index)	Baseline	62.80 ± 3.04	62.32 ± 2.85	0.546
	4-week	61.34 ± 6.59	61.77 ± 2.36	
	Δ 0-4 w	-1.45 ± 6.43	-0.55 ± 3.04	
	8-week	62.59 ± 2.00	60.06 ± 2.35**	
	Δ 0-8 w	-0.21 ± 2.16	-2.26 ± 3.65	
	Δ 0-12 w	0.68 ± 2.21	-1.01 ± 2.86	
Stratum corneum water content (index)	Baseline	56.14 ± 15.78	44.76 ± 12.09	0.391
	4-week	50.26 ± 13.35 †	34.45 ± 15.55*	
	Δ 0-4 w	-5.89 ± 14.80	-10.32 ± 19.12	
	8-week	64.89 ± 12.22**	39.32 ± 12.11	
	Δ 0-8 w	8.75 ± 13.11	-5.44 ± 15.58	
	Δ 0-12 w	24.50 ± 12.60	-5.38 ± 21.97	
TEWL (g/hm <sup>2</sup> )	Baseline	17.13 ± 4.63	18.21 ± 7.09	0.561
	4-week	14.48 ± 4.04**	14.97 ± 6.58**	
	Δ 0-4 w	-2.65 ± 2.95	-3.24 ± 3.76	
	8-week	13.57 ± 4.92*	14.37 ± 6.04**	
	Δ 0-8 w	-3.57 ± 6.05	-3.85 ± 4.47	
	Δ 0-12 w	-5.23 ± 7.21	-5.01 ± 7.63	
Elasticity (R2) (ratio)	Baseline	0.683 ± 0.083	0.575 ± 0.046	0.089 †
	4-week	0.634 ± 0.074**	0.551 ± 0.052*	
	Δ 0-4 w	-0.049 ± 0.052	-0.023 ± 0.045	
	8-week	0.645 ± 0.070	0.537 ± 0.053**	
	Δ 0-8 w	-0.038 ± 0.106	-0.038 ± 0.049	
	Δ 0-12 w	0.745 ± 0.085*	0.569 ± 0.056	
Elasticity (R5) (ratio)	Baseline	0.450 ± 0.062	0.352 ± 0.045	0.948
	4-week	0.402 ± 0.052**	0.305 ± 0.048**	
	Δ 0-4 w	-0.048 ± 0.039	-0.047 ± 0.039	
	8-week	0.398 ± 0.069**	0.319 ± 0.054**	
	Δ 0-8 w	-0.051 ± 0.066	-0.033 ± 0.053	
	Δ 0-12 w	0.457 ± 0.083	0.334 ± 0.058	
Elasticity (R7) (ratio)	Baseline	0.312 ± 0.052	0.247 ± 0.034	0.620
	4-week	0.297 ± 0.039 †	0.227 ± 0.036**	
	Δ 0-4 w	-0.015 ± 0.037	-0.020 ± 0.026	
	8-week	0.293 ± 0.054 †	0.231 ± 0.038*	
	Δ 0-8 w	-0.019 ± 0.044	-0.016 ± 0.029	
	Δ 0-12 w	0.332 ± 0.064 †	0.242 ± 0.041	

Scores and Values are expressed as the mean ± SD.

1) † p &lt; 0.1, \* p &lt; 0.05, \*\* p &lt; 0.01 against baseline.

2) ‡ p &lt; 0.1, # p &lt; 0.05, ## p &lt; 0.01 between-group difference in change from baseline.

Table 5 Results of questionnaire analyses

Item	Time points	Scores <sup>1)</sup>		P-value <sup>2)</sup>
		RS (n=22)	Placebo (n=19)	
1 Moisture	Baseline	3.7 ± 1.3	4.5 ± 1.3	<0.001 <sup>##</sup>
	12-week	5.9 ± 1.5 <sup>**</sup>	4.6 ± 1.1	
	Δ 0-12 w	2.2 ± 1.7	0.1 ± 0.9	
2 Refreshing feeling	Baseline	4.3 ± 1.0	4.3 ± 1.2	0.840
	12-week	4.8 ± 1.3 <sup>*</sup>	4.7 ± 1.5 <sup>†</sup>	
	Δ 0-12 w	0.5 ± 0.9	0.4 ± 1.2	
3 Greasiness	Baseline	4.3 ± 1.4	4.6 ± 1.1	0.698
	12-week	4.8 ± 1.3	5.2 ± 1.3 <sup>*</sup>	
	Δ 0-12 w	0.5 ± 1.3	0.6 ± 1.3	
4 Softness	Baseline	4.0 ± 1.2	4.6 ± 1.2	<0.001 <sup>##</sup>
	12-week	5.7 ± 1.4 <sup>**</sup>	4.5 ± 1.2	
	Δ 0-12 w	1.8 ± 1.2	0.0 ± 0.7	
5 Skin elasticity	Baseline	3.5 ± 1.3	3.4 ± 1.5	0.072 <sup>‡</sup>
	12-week	5.5 ± 1.3 <sup>**</sup>	4.6 ± 1.4 <sup>**</sup>	
	Δ 0-12 w	2.0 ± 1.5	1.2 ± 1.2	
6 Skin texture	Baseline	3.4 ± 1.6	3.1 ± 1.9	0.041 <sup>#</sup>
	12-week	5.2 ± 1.6 <sup>**</sup>	4.0 ± 1.9 <sup>**</sup>	
	Δ 0-12 w	1.9 ± 1.6	0.9 ± 1.4	
7 Sagginess	Baseline	2.9 ± 1.4	2.6 ± 1.8	0.111
	12-week	4.8 ± 1.4 <sup>**</sup>	3.8 ± 1.7 <sup>**</sup>	
	Δ 0-12 w	1.9 ± 1.7	1.2 ± 1.2	
8 Pore distinctiveness	Baseline	3.3 ± 1.7	2.9 ± 1.9	0.165
	12-week	4.7 ± 1.8 <sup>**</sup>	3.7 ± 1.8 <sup>**</sup>	
	Δ 0-12 w	1.3 ± 1.5	0.8 ± 1.2	
9 Facial wrinkle	Baseline	3.0 ± 1.3	2.9 ± 1.5	0.291
	12-week	4.8 ± 1.8 <sup>**</sup>	4.2 ± 1.6 <sup>**</sup>	
	Δ 0-12 w	1.8 ± 1.6	1.3 ± 1.5	
10 Surface spots	Baseline	2.6 ± 1.5	2.7 ± 1.9	0.054 <sup>‡</sup>
	12-week	4.5 ± 1.7 <sup>**</sup>	3.7 ± 1.7 <sup>**</sup>	
	Δ 0-12 w	1.9 ± 1.7	1.0 ± 1.4	
11 Foundation adhesion	Baseline	3.3 ± 1.2	3.8 ± 1.3	<0.001 <sup>##</sup>
	12-week	5.3 ± 1.7 <sup>**</sup>	4.0 ± 1.3 <sup>†</sup>	
	Δ 0-12 w	2.0 ± 1.4	0.2 ± 0.5	
12 Make up degradation	Baseline	3.7 ± 1.3	3.8 ± 1.3	0.481
	12-week	5.1 ± 1.7 <sup>**</sup>	4.8 ± 1.5 <sup>**</sup>	
	Δ 0-12 w	1.4 ± 1.3	1.1 ± 1.5	
13 Skin condition	Baseline	3.6 ± 1.2	3.7 ± 1.3	0.022 <sup>#</sup>
	12-week	5.8 ± 1.8 <sup>**</sup>	4.7 ± 1.5 <sup>**</sup>	
	Δ 0-12 w	2.2 ± 1.7	1.0 ± 1.5	
14 Dark circle	Baseline	3.4 ± 1.3	2.8 ± 1.6	0.606
	12-week	4.8 ± 1.3 <sup>**</sup>	3.9 ± 1.9 <sup>**</sup>	
	Δ 0-12 w	1.3 ± 1.0	1.1 ± 1.6	
15 Dullness	Baseline	3.2 ± 1.4	2.7 ± 2.1	0.612
	12-week	4.9 ± 1.5 <sup>**</sup>	4.1 ± 1.9 <sup>**</sup>	
	Δ 0-12 w	1.7 ± 1.5	1.4 ± 1.5	
16 Skin luster	Baseline	3.5 ± 1.3	3.2 ± 2.0	0.312
	12-week	4.8 ± 1.6 <sup>**</sup>	4.8 ± 1.6 <sup>**</sup>	
	Δ 0-12 w	1.2 ± 1.2	1.6 ± 1.3	
17 Acne/ Pimple	Baseline	4.4 ± 1.2	4.3 ± 2.0	0.581
	12-week	5.5 ± 1.4 <sup>**</sup>	5.1 ± 1.6 <sup>†</sup>	
	Δ 0-12 w	1.0 ± 1.3	0.8 ± 1.8	
18 Redness	Baseline	3.7 ± 1.1	4.0 ± 1.7	0.419
	12-week	4.5 ± 1.1	4.5 ± 1.3	
	Δ 0-12 w	0.7 ± 1.0	0.5 ± 1.2	
19 Crow's feet	Baseline	3.2 ± 1.3	3.8 ± 1.6	0.012 <sup>#</sup>
	12-week	4.8 ± 1.9 <sup>**</sup>	4.4 ± 1.4 <sup>*</sup>	
	Δ 0-12 w	1.6 ± 1.5	0.6 ± 1.0	
20 Eye bag's sagginess	Baseline	3.5 ± 1.2	3.5 ± 2.0	<0.001 <sup>##</sup>
	12-week	4.7 ± 1.7 <sup>**</sup>	3.7 ± 1.8	
	Δ 0-12 w	1.2 ± 1.2	0.1 ± 0.6	

Scores are expressed as the mean ± SD.

1) † p < 0.1, \* p < 0.05, \*\* p < 0.01 against baseline.

2) ‡ p < 0.1, # p < 0.05, ## p < 0.01 between-group difference in change from baseline.

in the RS has a wealth of amino acids. In particular, it contains plenty of glutamine acids, aspartic acid, proline and arginine. Amino acids constitute the protein that formulates the human organs such as skin, internal organs or blood vessels. Twenty types of amino acids formulating protein become the ingredients for producing collagen, the chief element of the skin. NMF inside the stratum corneum is also composed of amino acids. There are reports that the introduction of amino acids (i.e. iontophoresis) encourages the production of collagen which is damaged as a result of aging<sup>25)</sup>, or the intake of amino acids improves the barrier functions of skin and allow it heal quickly in case of a wound<sup>26)</sup>. The other report illustrates that the ingestion of natural moisturizing amino acids improves dry skin<sup>27)</sup>. Proline and aspartic acid, which are found in large quantities in RHIZOPUS/RICE FERMENT FD POWDER, exist in the dermic layer of skin. They are the major component of collagen which dictates the skin elasticity, and they are also the constituent amino acid of NMF<sup>28)</sup>. Therefore, it is considered that the ingestion of RHIZOPUS/RICE FERMENT FD POWDER promoted the production of collagen and/or NMF. Also, since there is a report that the arginine contained in RHIZOPUS/RICE FERMENT FD POWDER stimulates the production of NO and contributes to blood circulation improvement<sup>29)</sup>, the functions such as increasing overall blood flow and/or alleviating the skin dullness can be expected by the ingestion of the test product. Based upon the discussion above, we can speculate that the ingestion of RHIZOPUS/RICE FERMENT FD POWDER containing amino acids contributed to the promotion of the collagen production on the skin, the promotion of the production of NMF or the blood circulation efficacy, all of which lead to the improvement of skin elasticity or tightness.

Although at the randomization phase prior to the study, we allocated the test subject as equally as possible, the baseline at the beginning of the test showed a significant difference between the RS and Placebo. However, it did not influence the assessment of the test result since the assessment was carried out based on the amount of change. Therefore we can determine that “wrinkle grade” improved significantly compared to the Placebo. R2 (of “Elasticity”) also showed a significant improvement.

For the assessment of elasticity, we used R2 (Recovery ratio of the skin length), R5 (Ratio of elasticity part when elongated and during constriction), and R7 (Ratio of elasticity part during constriction), and among them R2 and R7 in particular are an important elasticity index of the cutometer. It is reported that R2 and R7 decrease with aging over<sup>30)</sup>; especially, R7 dramatically decreases with advancing of age. As for the test result of R7, the test group showed an improvement tendency compared to the Placebo, and this result indicates tendency that the ingestion of the RS improved the skin elasticity. On the

other hand, it can also be said that the 12-week test period was not sufficient for R7 to show a definite sign of significant difference due to its characteristic of dramatically decreasing with advancing age. As for the test result of R5, it did not even show the improvement tendency. It is unclear that this result was triggered by an insufficient test period (same as that of R7), or it was because the RS is not effective for the improvement of the condition specified in R5. Therefore further study will be needed to clarify the matter. To sum up, it is considered that all test outcomes discussed above were yielded by the increase of collagen (which is forced to decrease by the skin aging) after the ingestion of the RS.

In addition, this study showed an improvement tendency in stratum corneum water content, an index of skin moisture<sup>31)</sup>. This result was possibly produced as a result of the increase of NMF by the ingestion of the RSG. Furthermore, L\* index score which represents the brightness of skin showed a significant improvement compared to Placebo. As for the trans-epidermal water loss (TEWL) which is regarded as an index of skin barrier function<sup>32)</sup>, we could observe significant improvement in the intra-group comparison, whereas the improvement was not seen in the inter-group comparison. Although we cannot determine these results were achieved by ingesting the RS since the same improvement tendency was seen in Placebo, it can be said that the ingestion of the RS somewhat contributed to the improvement of the skin condition by providing some effects such as the improvement of blood circulation and/or skin moisture, and also the improvement of the balance between water and oil on the surface of skin. As for spots or melanin, on the other hand, the study did not show an improvement. Among the amino-acids, cysteine or glycine are reported to have the function of melanin inhibition<sup>33)34)</sup>. However, the RS contains only a small quantity of cysteine, and this fact might affect the test result. The results of subjective reporting (questionnaire) also showed a significant improvement in the items related to moisture or the skin condition. It is considered that these results were based on the objective test outcome discussed above. To sum up, it is speculated that the ingestion of RS contributed to the improvement of the skin condition such as skin elasticity or skin moisture.

#### **Secondary Findings**

In this study, adverse events were collected by means of a written questionnaire during the study, and no abnormal change caused by RS was observed during the ingesting period. During the test period two test subjects discontinued the test. The reasons of discontinuance were personal ones such as being unwell, and had nothing to do with the ingestion of RS. These results indicated the safety of the ingesting the test product for the 12-week test period

#### **General Information**

Skin is a part which primarily catches another's attention,

and therefore affects one's first impression. Also, it is the largest organ in the body overlying the whole body, and plays an important role in the internal metabolism while protecting the vital activity from outward threats. RHIZOPUS/RICE FERMENT FD POWDER is an extract which contains plenty of nutrition such as amino acids. Since the protein is bio-degraded to the form of amino acid (which is easily utilized in the body), the metabolism speed of the extract is fast when ingested. Also, as amino acid is a component material of living organisms, it is safe for ingestion. To sum up, it is contemplated that if we can improve the skin condition by ingesting food which is easily-prepared and safe, it may contribute to not only improving the QOL of women from the standpoint of beauty, but also maintaining the health of the body, such as the prevention or improvement of skin disorders.

### Limitations

In this study we used the food containing RHIZOPUS/RICE FERMENT FD POWDER. The manufacturer of the food confirmed that the category and amount of amino acids contained in RHIZOPUS/RICE FERMENT FD POWDER are stable and without variation from product to product. On the other hand, since it contains quite a lot of amino acids, the outcome of this study was possibly yielded by the complex interactions of the various (not single) amino acids or the admixture contained in the extract. However, the above discussion is merely a speculation since we could not explain rationale for these interactions in this study. Therefore, it is expected that these interactions are studied by way of in vitro or in vivo analysis.

### 5. CONCLUSION

In conclusion, we found out that the 12-week ingestion of RS containing RHIZOPUS/RICE FERMENT FD POWDER, which has plenty of amino acids such as glutamine acid, aspartic acid, proline, and arginine, contributed to the improvement of the skin condition such as elasticity, tightness or moisture. In addition, no safety-related matter was observed during the 12-week test period.

### CONFLICT OF INTEREST

All parts of this study were funded by RISOU Co., Ltd. Katsumi Kurita is the principal. All authors state that the study was conducted in the absence of any other relationships that could be interpreted as a conflict of interest.

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**Appendix 1.** Questionnaire of skin condition

Scale of 0 to 9, with higher scores indicating a better result.

- Question #1: Moisture
  - Question #2: Refreshing feeling
  - Question #3: Tightness
  - Question #4: Softness
  - Question #5: Skin elasticity
  - Question #6: Skin texture
  - Question #7: Sagginess
  - Question #8: Pore distinctiveness
  - Question #9: Facial wrinkle
  - Question #10: Surface spots
  - Question #11: Foundation adhesion
  - Question #12: Make up degradation
  - Question #13: Skin condition
  - Question #14: Dark circle
  - Question #15: Dullness
  - Question #16: Skin luster
  - Question #17: Acne/ Pimple
  - Question #18: Redness
  - Question #19: Crow's feet
  - Question #20: Eye bag's sagginess
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