Safety Evaluation of the Excessive Intake of Ceramide-Containing Acetic Acid Bacteria - A Randomized, Double-Blind, Placebo-Controlled Study Over a 4-week Period

Yoshihiro Tsuchiya^{1*}, Masayasu Ban¹, Mikiya Kishi¹, and Takahiro Ono²

¹ Central Research Institute, Mizkan Holdings Co., Ltd., 2-6 Nakamura-Cho, Handa-Shi, Aichi 475-8585, JAPAN

² Ueno Asagao Clinic, 2-7-5 Higashiueno, Taitou-Ku, Tokyo 110-0015, JAPAN

Abstract: Ceramide plays an important role in maintaining the skin barrier function. Aging and atopic dermatitis are known to reduce the levels of ceramide. Application of exogenous ceramide to the skin can restore the barrier function. In recent years, the effect of oral intake of ceramide has been demonstrated to improve the skin barrier function, and it has been marketed as a food supplement. Therefore, it is important to provide information on the safety of unintentional overdose of ceramide. This randomized, double-blind, placebo-controlled study was conducted in 30 healthy adults, aged between 20 and 60 years of age (both female and male). The subjects consumed either dietary supplement, comprising 1197 mg of acetic acid bacteria containing 9.06 mg of ceramide, or placebo for four consecutive weeks. Safety was evaluated based on physical measurements, blood test, urinalysis, adverse events, and side effects. The results showed several significant differences in physical measures and blood tests between the two groups. However, these differences were considered to be unrelated to the intake of the ceramide-containing acetic acid bacteria or placebo. Thus, no adverse effects or clinically concerning changes in physical, blood, and urine parameters were observed due to the excessive intake of the ceramide-containing acetic acid bacteria in the present study.

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Key words: ceramide-containing acetic acid bacteria, safety evaluation, excessive intake

1 Introduction

The skin barrier function not only prevents the evaporation of water from inside the stratum corneum, but also plays an important role in protecting the living body from external stressors such as ultraviolet rays, infiltration of chemical substances, and microbial infection. The barrier function of the epidermis is strongly related to the intercellular lipid layer structure of the stratum corneum. Intercellular lipids are mainly composed of ceramide, cholesterol, and free fatty acids, and among these components, ceramide is the most important molecule for maintaining skin barrier homeostasis¹⁾. Reports indicating decreased levels of ceramide²⁾, increased transepidermal water loss (TEWL), and decreased water content in the stratum corneum of skin lesions in patients with atopic dermatitis all support this theory³⁾. Ceramide levels are also known to

decrease in the stratum corneum of aged skin²⁾.

Supplementing ceramide to the skin contributes in maintaining or improving the skin barrier function. It has been reported that the application of ceramide-containing cream improved the destruction of the skin barrier, induced by tape stripping or sodium lauryl sulfate treatment⁴⁾. Oral intake of plant-derived ceramides has also been reported to reduce transepidermal water loss (TEWL) in mice^{5, 6)}, in healthy adults⁶⁾, and even in patients with atopic dermatitis⁷⁾.

Ceramides are known to exist mainly in plants and animals, but they have also been found in a very limited number of species of Gram-negative bacteria^{8, 9)}. Acetic acid bacteria is a general term for Gram-negative aerobic bacteria that oxidize and ferment ethanol to produce acetic acid. These bacteria are also used in the production of

*Correspondence to: Yoshihiro Tsuchiya, Central Research Institute, Mizkan Holdings Co., Ltd., 2-6 Nakamura-Cho, Handa-Shi, Aichi 475-8585, JAPAN

E-mail: yoshihiro_tsuchiya@mizkan.co.jp

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brewed vinegar, Caspian Sea yogurt, and Nata de coco¹⁰⁾. Acetic acid bacteria are able to tolerate osmotic stress and low pH caused by the acetic acid, produced by the bacteria themselves, and it has been reported that the molecule that confers this resistance is ceramide accumulated in the cells¹¹⁾. *Acetobacter malorum* NCI 1683 (S24), derived from fermented milk, is known to accumulate a large amount of ceramide¹²⁾, and the oral intake of this bacteria has been reported to improve the skin barrier function¹³⁾. The ceramide used in this study refers to the dihydroceramide produced by hydroxylation of the sphingosine base, unless otherwise stated. In detail, the ceramide molecule is 2-hydroxypalmitoyl-sphinganine (dihydroceramide), which has no double bond, and its chemical formula is $C_{34}H_{69}NO_4$.

There has been progress in the development of cosmetics and foods to improve skin function, and these products are currently marketed as food supplements. Although previous human clinical trials have not yet identified any problematic events, in terms of safety, for ceramide-containing acetic acid bacteria¹³⁾, food supplements pose the risk of unintentional overdose because these products are easy to take. Thus, it is important to evaluate safety of food supplements with ceramide-containing acetic acid bacteria, in case of overdose. Therefore, we conducted a randomized, double-blind, placebo-controlled, parallel-group comparison study in healthy adults, aged 20 to 60 years, to evaluate the safety of excessive intake of ceramide-containing acetic acid bacteria. The dose used as an excessive intake was 1197 mg (per day as acetic acid bacteria, 9.06 mg as ceramide), which was more than five times the recommended daily intake, as specified in the precautions stipulated during preparation of the application for food for specified health uses, and safety of this dose has been confirmed in animal experiments (In-house examinations: data not shown).

2 Experimental

2.1 Study implementation system

This study was reviewed and approved by the Ethics Review Committee of the Oriental Occupational Health Association Tokyo Branch, Oriental Ueno Medical Center (approval date: December 6, 2018), which comprised of a third party not involved in the study, to protect the human rights and safety of the subjects and to ensure the reliability of the test data. The study was then implemented from January to February 2019, based on the study protocol of the Ueno Asagao Clinic (Investigator: Takahiro Ono) (UMIN study ID: UMIN000035481). This study complied with ethical principles and was conducted in accordance with the tenets of the Declaration of Helsinki (amended at the 2013 WMA Fortaleza General Assembly [Brazil]) and the Ethical Guidelines for Medical and Health Research Involving

Human Subjects (Notice from the Ministry of Education, Culture, Sports, Science and Technology and Ministry of Health, Labour and Welfare).

2.2 Study participants

The study details were explained to the subjects at recruitment, and a preliminary examination (SCR: Screening) was conducted for 68 subjects who were deemed suitable as test subjects. The selection criteria for this study were (1) men and women, aged between 20 and 60 years (inclusive) at the time of consent for participation in the study, (2) healthy individuals with no chronic physical disease, including skin disease, (3) individuals who had received sufficient explanation about the purpose and content of this study, had the ability to consent, had agreed to participate voluntarily with full understanding of the content of the study, and were able to consent to participate in this study in writing, (4) individuals who were able to visit the clinic on designated examination days, and able to undergo examination, and (5) individuals deemed to be suitable for participation in the study by the principal investigator. The exclusion criteria for this study were (1) individuals currently receiving drug treatment for some kind of disease, (2) individuals with a medical history or current mental disease, sleep disorder, hypertension, diabetes, dyslipidemia, and/or serious disease, (3) individuals with a habit of taking medication to treat disease in the past month (excluding single dose medication for headache, menstrual pain, common cold, etc.), (4) individuals with serious medical history or current condition of the liver, kidneys, heart, lungs or blood, (5) individuals with serious medical history or comorbidity of the digestive system, (6) individuals with body mass index (BMI) $< 18.5 \text{ kg/m}^2$ or $\geq 30 \text{ kg/m}^2$, (7) individuals with a systolic blood pressure of $\geq 140 \text{ mmHg}$, a diastolic blood pressure of $\geq 90 \text{ mmHg}$, and/or a pulse rate of $\geq 100 \text{ bpm}$, (8) individuals who had donated more than 200 mL of blood within the last month or more than 400 mL of blood within the last 3 months, (9) individuals who felt unwell after previous blood collection, (10) individuals who may develop allergy symptoms to the test food, or who may develop serious allergy symptoms to other foods or drugs, (11) individuals who regularly took food supplements for specified health uses, foods with functional claims, and/or health foods (however, this did not apply to individuals who were able to stop taking the foods in question for the duration of the study after consent was obtained), (12) individuals whose mean daily alcohol consumption normally exceeded 60 g/day, (13) individuals whose daily life may change during the study period (going on extended holidays, etc.), (14) women who were pregnant, breastfeeding, or may be pregnant, (15) individuals who were currently participating in another human clinical trial, or who participated in another human clinical trial less than 3 months ago, (16) individuals or

their family members who worked at companies that develop, manufacture or sell health food, foods with functional claims and/or cosmetics, and (17) other individuals who were deemed unsuitable for participation in this study by the principal investigator. Based on the selection criteria, 30 subjects were selected for participation in this study.

As the basis for setting the number of subjects considered the importance of incorporating a broad age range of the population expected to take the test food, as the purpose of this study was to ascertain the safety of the test food. Therefore, it was considered necessary to incorporate equal ratio of male and female subjects, across a wide age range (aged 20-60 years). Considering withdrawals and the short study period (4 weeks), the number of subjects was set to 15 per group.

2.3 Selection, randomization, and blinding

The subjects' gender, age, and BMI at preliminary examination (SCR) were used as stratification factors, and after randomly assigning the subjects to two groups, using the stratified block randomization method, it was confirmed that there was no significant difference between the allocation groups.

Two types of food supplements were investigated: test food (food containing acetic acid bacteria) and placebo food (food not containing acetic acid bacteria). The study contractor ensured that each type of test food could not be identified based on their appearance, by printing the relevant identification codes on the test food and delivering it to the study outsource provider. The identification codes were sealed in an envelope and sent to the person-in-

charge of allocation, who confirmed that each test food sent from the study contractor could not be distinguished by its appearance and/or smell. Next, the person-in-charge of allocation replaced the test food (food containing acetic acid bacteria) and placebo food (food not containing acetic acid bacteria) codes with another test product control code that was not easy identifiable, thereby blinding the study. The products were then handed over to the test product manager. The test item control codes were tightly sealed in an envelope together with the correspondence table and kept by the person-in-charge of allocation, until the study was unblinded.

2.4 Test food

Dried *A. malorum* NCI 1683 acetic acid bacteria strain and *A. malorum* NCI 1683 acetic acid bacteria strain-derived ceramides were used as the test food.

The ingredients and nutritional compositions of the test food and placebo food used in the study are shown in **Tables 1a** and **1b**. Hard gelatin capsules, containing dried *A. malorum* NCI 1683 acetic acid bacteria strain 1197 mg containing 9.06 mg of dihydroceramides as mentioned in the introduction. Since the industrial know-how regarding the optimal fermentation of *A. malorum* NCI 1683 (S24) found from fermented milk was included, the culture conditions, preparations for the bacteria were not described. In briefly, the ceramide was prepared by solvent extraction using chloroform/methanol. 7.5 mL of chloroform and 7.5 mL of methanol were added to 80 mg of lyophilized bacterial cells. The mixture was extracted effectively under sonication. 6 mL of distilled water was added to chloroform/methanol mixture to extract effectively under sonication.

Table 1a All components of test food (per 9 capsules/day).

Component	Test Food (Food containing acetic acid bacteria)	Placebo Food (Food not containing acetic acid bacteria)
Acetic acid bacteria	1197 mg (contains 9.07 mg of ceramide)	0 mg
Dextrin	243 mg	1440 mg

Table 1b Nutritional component of test food (per 9 capsules).

Component	Test Food (Food containing acetic acid bacteria)	Placebo Food (Food not containing acetic acid bacteria)
Calories	5259 cal	1656 cal
Protein	1003 mg	0 mg
Fats	91 mg	0 mg
Carbohydrates	290 mg	1499 mg
Sodium chloride equivalent	36 ng	14 ng

15 mL of chloroform and 7.5 mL of methanol were added and mixed by convective vortex, and centrifuged at 3000 rpm for 10 minutes to remove the upper layer and insoluble bacterial cells. 20 mL of distilled water was added and mixed by convective vortex, and centrifuged at 3000 rpm for 10 minutes to remove the upper layer and insoluble bacterial cells. The lower layer was transferred to a glass tube and dried using a rotary evaporator at 3000 rpm and 42°C. The dried residue highly including the ceramide was reconstituted by chloroform: methanol (2:1) solution and subjected to quantification analysis.

The absorbance could not be measured, because there is no double bond. Therefore the ceramide molecule was quantified using a combination of an evaporative light scattering detector (ELSD) and HPLC using a separation column Shim-Pack HRC-SIL (4.6 × 250 mm; Shimadzu Corporation, Japan).

The placebo capsules contained dextrin only. It was confirmed that that test food could not be identified based on its appearance or smell.

The test food was taken as 9 capsules per day. All the capsules were taken once a day with water or lukewarm water.

In the nutritional components shown in **Table 1b**, calories were calculated assuming that protein was 4 kcal/g, fat was 9 kcal/g, and carbohydrate was 4 kcal/g. Protein was measured by the combustion method, lipid by the acid decomposition method, and sodium by the atomic absorption spectrophotometry. Carbohydrates were calculated by subtracting protein, fat, ash and water content from the total weight. The ash content was measured by the direct ashing method. Sodium chloride equivalent was calculated as sodium equivalent (g/100 g) = sodium content in food (mg/100 g) × 2.54/1,000.

2.5 Study design

This was a placebo-controlled, randomized, double-blind, parallel-group study. The main endpoints were the presence or absence of side effects, and the number and incidence of side effects. Secondary endpoints included the number and incidence of adverse events, blood pressure (systolic/diastolic)/pulse, body weight/body fat percentage/BMI, hematology tests, biochemical examination of blood, and general urinalysis.

During the study period, the subjects were instructed to avoid an irregular lifestyle (lack of sleep, excessive drinking and eating, etc.). They were instructed to refrain from drinking excessive alcohol (500 mL of beer per day: within the equivalent of 20 g based on alcohol conversion). They were also prohibited from suddenly changing the amount of alcohol consumption, such as suddenly stopping alcohol. The subjects were instructed to maintain the same quantity and quality of diet, exercise, and sleep as before the start of this study. In particular, excessive exercise and lack of sleep on the day before the tests was prohibited. The use

of pharmaceuticals (including external preparations), quasi-drugs, and herbal medicines was generally prohibited. If pharmaceuticals were used or the use was unavoidable, the subjects were asked to contact the consultation counter and to record the name of the product used, the manufacturer, and the reason for use in a diary. Intake of food for specified health uses, foods with functional claims, and/or health foods was prohibited. When intake was unavoidable, the name of the product, composition, and the amount taken were recorded in the diary. There were no particular restrictions on smoking during the study period. However, the subject was asked to maintain their usual smoking habits (frequency, brand, etc.) as much as possible, and sudden changes in smoking, such as suddenly stopping smoking, were prohibited. Blood collection other than those performed in this study and blood donation were prohibited from the time of becoming a subject to the end of the study (blood collection for health checkups etc. was allowed). The subjects were instructed to complete the diary every day and to submit it on the specified day. They were asked to visit the hospital on the designated examination day and obliged to follow the precautions at the time of the examination. Participation in other human clinical trials was prohibited during the trial period. Other matters that may affect the test results were also prohibited. Consumption of alcohol was prohibited from the day before the test until completion of the test on the test day. The subjects fasted for 12 hours before the scheduled blood sampling time until the completion of the test. However, the minimum required amount of water was allowed.

All procedures followed were in accordance with the Ethics Committee of the Oriental Ueno Detection Center, General Incorporated Association Oriental Occupational Health Association Tokyo Branch (Tokyo, Japan; Study ID: HR-2018-MKH03) and with the Helsinki Declaration of 1964 and later versions.

2.6 Test items

Measurements were taken for blood pressure (systolic/diastolic)/pulse, height, weight/body fat ratio/BMI. Hematology tests measured white blood count (WBC), red blood count (RBC), hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT), and white blood cell profile. Biochemical examination of blood measured total protein (TP), albumin quantification (ALB), urea nitrogen (UN), creatinine (CRE), uric acid (UA), aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ -glutamyl transpeptidase (γ -GT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatine kinase (CK), CRP quantification, total cholesterol (T-cho), triglycerides (TG), LDL cholesterol quantification (LDL-C), HDL-cholesterol (HDL-C), total bilirubin (T-BIL), sodium (Na), potassium (K),

chloride (Cl), calcium (Ca), iron (Fe), glucose (GLU), and HbA1c. General urinalysis measured urobilinogen, occult blood reaction, bilirubin, ketone body qualitative measurement, glucose qualitative measurement, protein, pH, and specific gravity.

2.7 Statistical analysis

Statistical analysis was performed using appropriate statistical analysis software, such as SAS (SAS 9.4) or SPSS (Statistics 25), and the significance level for all tests was 5% on both sides and the trends at 10% level. Calculated data shown in the table and graphs, was presented as the mean $\pm$ standard deviation.

Fisher's exact test was used for intergroup comparison of the number and incidence of side effects, and the number and incidence of adverse events. The paired t-test was used for intergroup comparison of blood pressure (systolic/diastolic)/pulse, weight/body fat ratio/BMI, hematology tests, and biochemical examination of blood. The Wilcoxon signed rank test was used for semi-quantitative urinalysis. The unpaired t-test was used for intergroup comparison.

3 Results

3.1 Patient disposition

This study conducted a preliminary examination on 68 people, amongst which 30 were selected as subjects for this study based on the selection criteria. One subject could not attend the clinic for the examination before starting the intake of the test food due to contracting influenza, hence the subject withdrew from the study. Therefore, the study was initiated with 29 subjects: test food group: $n = 15$ (men: $n = 7$, women: $n = 8$) and placebo food group: $n = 14$ (men: $n = 7$, women: $n = 7$). The study was completed with one subject withdrawing during the study due to personal circumstances. Subject background (intent to treat analysis set) included age of the test food group which was 34.1 ± 11.8 years (men: 35.3 ± 14.1 years, women: 33.1 ± 10.3 years). The mean age of the subjects in the placebo food group was 35.1 ± 8.6 years (men: 34.9 ± 7.6 years, women: 35.4 ± 10.0 years) (Fig. 1, Table 2).

3.2 Adverse drug reactions and adverse events

There were no side effects thought to be caused by the test food in any of the subjects.

Adverse events occurred in both the groups: test food group ($n = 3$: 4 events: elevated CK, knee burn, pharyngitis, influenza A), and placebo food group ($n = 2$: 2 events: common cold (cough, pharyngitis, nasal discharge), boil (left foot)) (Tables 3a and 3b).

Fig. 1 Consolidated standards of reporting trial diagram: Enrollment, random assignment, and follow-up of subjects.

Table 2 Patient demographics (ITT analysis set).

Item	(Unit)	Test Food Group			Placebo Food Group		
		Overall	Men	Women	Overall	Men	Women
Number of subjects	(people)	15	7	8	14	7	7
Age	(years)	34.1 ± 11.8	35.3 ± 14.1	33.1 ± 10.3	35.1 ± 8.6	34.9 ± 7.6	35.4 ± 10.0
Height	(cm)	163.53 ± 7.08	167.80 ± 6.22	159.80 ± 5.73	164.91 ± 8.96	171.33 ± 6.85	158.50 ± 5.57
Weight	(kg)	61.72 ± 10.21	67.09 ± 11.09	57.03 ± 7.01	61.64 ± 9.85	65.53 ± 10.04	57.74 ± 8.59
BMI	(kg/m ²)	23.03 ± 3.16	23.81 ± 3.75	22.34 ± 2.59	22.62 ± 2.72	22.27 ± 2.51	22.97 ± 3.07

Mean ± standard deviation

Table 3a List of adverse events: Test Food Group.

Subject No.	Age	Sex	Symptoms and findings	Grade	Seriousness	Causal relationship
P5512	26	M	Elevated CK	Mild	Not serious	Probably not related
K23142	38	F	Knee burn	Moderate	Not serious	Not related
K23142	38	F	Pharyngitis	Mild	Not serious	Not related
P4131	46	F	Influenza A	Mild	Not serious	Not related

Table 3b List of adverse events: Placebo Food Group.

Subject No.	Age	Sex	Adverse event symptoms and findings	Grade	Severity	Causal relationship
K21550	46	M	Common cold (cough, pharyngitis, nasal discharge)	Mild	Not serious	Not related
P5536	32	M	Boil (left foot)	Mild	Not serious	Not related

Table 4 Changes in blood pressure and pulse.

Item (Unit)	Ingested food	Before intake		Two weeks after intake	Four weeks after intake
		Test Food Group: n = 15 Placebo Food Group: n = 14		Test Food Group: n = 14 Placebo Food Group: n = 14	Test Food Group: n = 14 Placebo Food Group: n = 14
Systolic blood pressure (mmHg)	Test Food Group	110.1 ± 6.8	#	106.7 ± 9.9	104.3 ± 6.1**
	Placebo Food Group	101.7 ± 11.7		111.3 ± 13.0**	108.7 ± 9.3*
Diastolic blood pressure (mmHg)	Test Food Group	69.7 ± 6.4	#	57.9 ± 10.5***	67.1 ± 6.4
	Placebo Food Group	63.9 ± 8.5		60.1 ± 8.7	68.6 ± 9.2**
Pulse (bpm)	Test Food Group	68.7 ± 9.2		67.4 ± 11.9	71.0 ± 12.6
	Placebo Food Group	73.4 ± 10.5		74.3 ± 9.8	70.4 ± 7.3

Mean ± standard deviation

* A paired t-test was used for comparison with Before intake. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ # An unpaired t-test was used for intergroup comparison at each time point. : # $p < 0.05$

3.3 Blood pressure (systolic/diastolic), pulse

For blood pressure and pulse, the systolic blood pressure significantly decreased in the test food group at four weeks after starting the intake compared to that before the intake (Table 4). Conversely, in the placebo food group, systolic blood pressure significantly increased at two weeks and four weeks after starting the intake compared to that

before the intake. Similar result was seen in the amount of change (Δ value), and especially with the amount of change between groups, the test food group was significantly lower than the placebo food group at two weeks and four weeks after starting the intake. The diastolic blood pressure significantly decreased in the test food group at two weeks after starting the intake compared to that

before the intake. In the placebo food group, diastolic blood pressure significantly increased at four weeks after starting the intake compared with before the intake, while a decreasing tendency was noted at two weeks after starting the intake. In terms of the amount of change, the test food group had a significant decrease at two weeks after starting the intake compared to that before the intake. While the placebo food group had a significant increase at four weeks after starting the intake compared to that before the intake, and a decreasing tendency was noted at two weeks after starting the intake. The test food group had a significantly lower amount of change than the placebo food group at two weeks and four weeks after starting the intake, compared to that in the placebo food group. There was no significant variation in either the measured value or the amount of change in the pulse throughout the study period, in both the test food and placebo food groups.

3.4 Body weight, body fat ratio, BMI

Amongst weight, body fat ratio, and BMI, both the measured value and the amount of change were significantly decreased for body fat ratio, only in the test food group at 4 weeks after the intake compared to that before the intake ($p=0.0475$). No significant change was observed for weight and BMI throughout the study period in both measured values and amount of change for both the groups (data not shown).

3.5 Hematology tests

In the hematology tests, no significant change was observed in measured values and amount of change for WBC, MCHC, PLT, and WBC profile, in both the test food group and placebo food group throughout the study period. No significant change was observed in RBC in the test food group throughout the study period. However, in the placebo food group, a significant decline was noted at 2 weeks and 4 weeks after starting the intake, compared to that before the intake. There was no significant difference between the groups in RBC. Similarly, there was no significant variation in the amount of change in the test food group throughout the study period. In contrast, in the placebo food group a significant decline was noted at 2 weeks and 4 weeks after starting the intake, as a result, a statistically significant difference was found between the groups, but no significant change was observed in the test food, in RBC. No significant change was observed in Hb levels in the test food group throughout the study period, while in the placebo food group a significant decline was noted at 2 weeks and 4 weeks after starting the intake when compared to that before the intake. There was no significant difference between the groups. Similarly, while there was no significant variation in the amount of change in the test food group throughout the study period, in the

placebo food group a significant decline was noted at 2 weeks and 4 weeks after starting the intake, when compared to that before the intake, which this resulted in a significant increase in the test food group at 2 weeks after starting intake, and an increasing trend at 4 weeks compared to that in the placebo food group. No significant change was observed in Ht in the test food group throughout the study period. However, in the placebo food group, a significant decline was noted at 2 weeks and 4 weeks after starting the intake, compared to that before the intake. There was no significant difference between the groups. Similarly, there was no significant variation in the amount of change in the test food group throughout the study period, but in the placebo food group a significant decline was noted at 2 weeks and 4 weeks after starting the intake, when compared to that before the intake. This resulted in a significant increase in the test food group at 2 weeks after starting the intake, and an increasing trend at 4 weeks compared to that in the placebo food group. There was a significant decline in the MCV in the test food group at 4 weeks after starting the intake, compared to that before the intake. No significant variation was noted in the placebo food group throughout the study period. The results were similar for the amount of change. A decreasing trend in the MCH levels was noted in the test food group at 4 weeks after starting the intake, compared to that before the intake. No significant variation was noted in the placebo food group throughout the study period. The results were similar for the amount of change. There was a significant decline in basophil count in the test food group at 2 weeks and 4 weeks after starting the intake, compared to that before the intake. No significant variation was noted in the placebo food group throughout the study period. There was no significant difference between the groups. Similarly, there was a significant decline in the amount of change in the test food group at 2 weeks and 4 weeks after starting the intake, compared to that before the intake. No significant variation was noted in the placebo food group throughout the study period. A decreasing trend was noted in the test food group at 2 weeks after starting the intake compared to that in the placebo food group. There was no significant variation in eosinophil count in the test food group throughout the study period. A decreasing trend was noted in the placebo food group at 4 weeks after starting the intake compared to that before the intake; however, there was no difference between the groups. The results were similar for the amount of change. There was a significant increase in neutrophil count in the test food group at 2 weeks after starting the intake compared to that before the intake. There was no significant variation in eosinophil count in the placebo food group throughout the study period. There was an increasing trend in the test food group at 2 weeks and 4 weeks after starting the intake compared to that in the placebo food group. There was no

difference in the amount of change between the groups. There was a significant decrease in the lymphocyte count in the test food group at 2 weeks after starting the intake compared to that before the intake. There was no significant variation in the placebo food group throughout the study period. There was a decreasing trend in the test food group at 2 weeks and 4 weeks after starting the intake compared to that in the placebo food group. The results were similar for the amount of change; however, there was no difference between the groups. There was an increasing trend in monocyte count in the test food group at 2 weeks after starting the intake compared to that before the intake, but there was no significant variation in the placebo food group throughout the study period. There was no difference between the groups. There was an increasing trend in the amount of change in the test food group at 2 weeks after starting the intake compared to that before the intake, but there was no significant variation in the placebo food group throughout the study period. However, there was an increasing trend in test food group at 4 weeks after the intake compared to that in the placebo food group (Table 5).

3.6 Biochemical examination of blood

In the biochemical examination of blood, there were no significant changes in the levels of ALB, UN, CRE, UA, CK, TG, T-BIL, Na, K, Cl, and Fe in either the test food group or the placebo food group throughout the study period. The results were similar for the amount of change. There was a significant decline in TP content in the test food group at 4 weeks after starting the intake compared to that before the intake, and a significant decline in the placebo food group at 2 weeks and 4 weeks after starting the intake. The results were similar for the amount of change. There was no significant variation in AST in the test food group throughout the study period, but a decreasing trend was noted in the placebo food group at 2 weeks after starting the intake compared to that before the intake. The results were similar for the amount of change. There was a decreasing trend in ALT concentration in the test food group at 2 weeks after starting intake compared to before intake. There was no significant variation in the placebo food group throughout the study period. The results were similar for the amount of change. There was a significant decline in γ -GT in the test food group at 2 weeks after starting the intake compared to that before the intake. There was a significant decline in the placebo food group at 2 weeks and 4 weeks after starting the intake compared to that before the intake. The results were similar for the amount of change. There was no significant variation in the ALP in both the test food group and the placebo food group throughout the study period. However, the test food group had an increasing trend in actual measurements compared to that in the placebo food group at 4 weeks

after starting the intake. There was a significant decline in LDH levels in the test food group at 2 weeks after starting the intake compared to that before the intake. There was a significant decline in the placebo food group at 2 weeks and a declining trend at 4 weeks after starting the intake compared to that before the intake. The results were similar for the amount of change. There was a significant decline in T-cho in the test food group at 2 weeks and 4 weeks after starting the intake compared to that before the intake. There was a significant decline in the placebo food group at 4 weeks after starting the intake compared to that before the intake. The results were similar for the amount of change. Analysis of the amount of change revealed a declining trend in the test food group compared to that in the placebo food group at 2 after starting the intake. There was a significant decline in the levels of LDL-C in both the test food group and the placebo food group at 2 weeks and 4 weeks after starting the intake compared to that before the intake. There was a decreasing trend in HDL-C in the test food group at 2 weeks after the starting intake compared to that before the intake, which progressed to a significant decline at 4 weeks. There was no significant variation in the placebo food group throughout the study period. The results were similar for the amount of change. There was a decreasing trend in Ca in the test food group at 2 weeks after starting the intake compared to that before the intake, which progressed to a significant decline at 4 weeks. There was a significant decline in the placebo food group at 2 weeks and 4 weeks after starting the intake compared to that before the intake. The results were similar for the amount of change. There was a significant increase in GLU in the test food group at 2 weeks and 4 weeks after starting the intake compared to that before the intake. There was no significant variation in the placebo food group throughout the study period. The results were similar for the amount of change. Analysis of the amount of change revealed an increasing trend in the test food group compared to that in the placebo food group at 4 weeks after starting the intake. There was a significant increase in HbA1c in both the test food group and the placebo food group at 2 weeks and 4 weeks after starting the intake compared to that before the intake. The results were similar for the amount of change. Analysis of the actual measurements revealed an increasing trend in the test food group compared to that in the placebo food group at 2 weeks and 4 weeks after starting the intake. On the other hand, there was a significant increase in CRP quantification in the test food group compared to that in the placebo food group at 2 weeks after starting the intake. However, no significant variations were seen in either the test food group or the placebo food group throughout the study period (Table 6).

Table 5 Changes in hematological parameters.

Item (Unit)	Ingested food	Before intake		Two weeks after intake		Four weeks after intake	
		Test Food Group: n = 15	Placebo Food Group: n = 14	Test Food Group: n = 14	Placebo Food Group: n = 14	Test Food Group: n = 14	Placebo Food Group: n = 14
White blood count (WBC) (μL)	Test Food Group	5513.3 $\pm$ 906.2		6314.3 $\pm$ 2174.3		5657.1 $\pm$ 1122.3	
	Placebo Food Group	4871.4 $\pm$ 1095.7		5107.1 $\pm$ 978.6		5092.9 $\pm$ 816.6	
Red blood count (RBC) ($\times 10^4/\mu\text{L}$)	Test Food Group	464.5 $\pm$ 42.1		464.6 $\pm$ 37.0		460.0 $\pm$ 39.8	
	Placebo Food Group	488.7 $\pm$ 43.5		475.9 $\pm$ 35.5*		473.7 $\pm$ 37.9*	
Hemoglobin (Hb) (g/dL)	Test Food Group	14.09 $\pm$ 1.35		14.10 $\pm$ 1.13		13.87 $\pm$ 1.21	
	Placebo Food Group	14.46 $\pm$ 1.39		14.13 $\pm$ 1.17*		13.96 $\pm$ 1.35**	
Hematocrit (Ht) (%)	Test Food Group	42.61 $\pm$ 3.55		43.13 $\pm$ 3.03		41.91 $\pm$ 3.42	
	Placebo Food Group	44.06 $\pm$ 4.30		43.04 $\pm$ 3.38*		42.24 $\pm$ 3.37**	
Mean corpuscular volume (MCV) (fL)	Test Food Group	91.9 $\pm$ 4.4		93.0 $\pm$ 3.3		91.3 $\pm$ 4.3*	
	Placebo Food Group	90.1 $\pm$ 4.8		90.8 $\pm$ 4.8		89.4 $\pm$ 5.1	
Mean corpuscular hemoglobin (MCH) (pg)	Test Food Group	30.35 $\pm$ 1.67		30.39 $\pm$ 1.61		30.20 $\pm$ 1.65	
	Placebo Food Group	29.65 $\pm$ 1.85		29.71 $\pm$ 1.76		29.50 $\pm$ 1.70	
Mean corpuscular hemoglobin concentration (MCHC) (%)	Test Food Group	33.02 $\pm$ 0.61		32.69 $\pm$ 1.01		33.10 $\pm$ 0.75	
	Placebo Food Group	32.84 $\pm$ 0.97		32.83 $\pm$ 0.87		33.04 $\pm$ 1.13	
Platelet count (PLT) ($\times 10^4/\mu\text{L}$)	Test Food Group	26.94 $\pm$ 4.64		26.82 $\pm$ 3.32		25.39 $\pm$ 4.03	
	Placebo Food Group	26.44 $\pm$ 4.05		27.20 $\pm$ 5.71		25.91 $\pm$ 4.14	
White blood cell profile (%)	Test Food Group	100.0 $\pm$ 0.0		100.0 $\pm$ 0.0		100.0 $\pm$ 0.0	
	Placebo Food Group	100.0 $\pm$ 0.0		100.0 $\pm$ 0.0		100.0 $\pm$ 0.0	
Basophils (%)	Test Food Group	0.69 $\pm$ 0.50		0.45 $\pm$ 0.29*		0.43 $\pm$ 0.27*	
	Placebo Food Group	0.63 $\pm$ 0.36		0.63 $\pm$ 0.33		0.56 $\pm$ 0.35	
Eosinophils (%)	Test Food Group	2.59 $\pm$ 1.65		2.46 $\pm$ 1.61		2.54 $\pm$ 1.86	
	Placebo Food Group	3.31 $\pm$ 2.21		3.01 $\pm$ 1.87		2.90 $\pm$ 1.77	
Neutrophils (%)	Test Food Group	58.74 $\pm$ 8.02		64.29 $\pm$ 9.77*		61.51 $\pm$ 8.81	
	Placebo Food Group	56.01 $\pm$ 8.72		57.93 $\pm$ 7.61		56.16 $\pm$ 5.59	
Lymphocytes (%)	Test Food Group	32.33 $\pm$ 8.38		26.84 $\pm$ 8.94*		29.23 $\pm$ 8.31	
	Placebo Food Group	33.87 $\pm$ 7.23		32.66 $\pm$ 6.95		34.53 $\pm$ 5.70	
Monocytes (%)	Test Food Group	5.65 $\pm$ 1.04		5.96 $\pm$ 1.21		6.29 $\pm$ 1.43	
	Placebo Food Group	6.18 $\pm$ 2.24		5.76 $\pm$ 2.25		5.85 $\pm$ 2.20	

Mean $\pm$ standard deviation* A paired t-test was used for comparison with Before intake. * $p < 0.05$; ** $p < 0.01$

Table 6 Changes in biochemical examination of blood.

Item (Unit)	Ingested food	Before intake		Two weeks after intake		Four weeks after intake	
		Test Food Group: n = 15	Placebo Food Group: n = 14	Test Food Group: n = 14	Placebo Food Group: n = 14	Test Food Group: n = 14	Placebo Food Group: n = 14
Total protein (TP) (g/dL)	Test Food Group Placebo Food Group	7.30 ± 0.30 7.16 ± 0.32		7.19 ± 0.32 7.02 ± 0.30*		7.14 ± 0.34* 6.93 ± 0.38*	
Albumin quantification (ALB) (g/dL)	Test Food Group Placebo Food Group	4.45 ± 0.26 4.49 ± 0.22		4.42 ± 0.29 4.47 ± 0.19		4.41 ± 0.28 4.49 ± 0.22	
Urea nitrogen (UN) (mg/dL)	Test Food Group Placebo Food Group	12.6 ± 4.6 13.5 ± 3.7		11.4 ± 3.7 12.7 ± 3.6		11.8 ± 3.2 13.4 ± 3.6	
Creatinine (CRE) (mg/dL)	Test Food Group Placebo Food Group	0.731 ± 0.148 0.741 ± 0.098		0.718 ± 0.138 0.729 ± 0.097		0.714 ± 0.154 0.736 ± 0.089	
Uric acid (UA) (mg/dL)	Test Food Group Placebo Food Group	4.96 ± 1.28 4.71 ± 1.16		4.84 ± 0.92 4.64 ± 1.24		4.83 ± 1.20 4.64 ± 1.25	
Aspartate aminotransferase (AST) (U/L)	Test Food Group Placebo Food Group	20.9 ± 6.5 18.2 ± 2.5		18.6 ± 4.0 16.9 ± 3.0		21.4 ± 10.2 17.6 ± 4.0	
Alanine aminotransferase (ALT) (U/L)	Test Food Group Placebo Food Group	22.6 ± 14.5 18.6 ± 9.8		17.4 ± 8.6 15.4 ± 4.3		19.2 ± 11.6 16.8 ± 7.9	
γ -glutamyl transpeptidase (γ -GT) (U/L)	Test Food Group Placebo Food Group	20.7 ± 7.7 24.6 ± 20.9		17.6 ± 6.6* 21.1 ± 17.9**		18.9 ± 6.3 21.3 ± 16.6*	
Alkaline phosphatase (ALP) (U/L)	Test Food Group Placebo Food Group	188.6 ± 58.2 163.1 ± 41.2		188.0 ± 63.5 161.4 ± 39.4		189.6 ± 47.4 155.6 ± 48.1	
Lactate dehydrogenase (LDH) (U/L)	Test Food Group Placebo Food Group	171.1 ± 34.6 158.1 ± 19.7		163.1 ± 29.2* 152.4 ± 19.8*		164.7 ± 38.3 152.6 ± 20.5	
Creatine kinase (CK) (U/L)	Test Food Group Placebo Food Group	134.3 ± 115.7 93.2 ± 28.9		106.2 ± 61.5 96.7 ± 26.5		167.7 ± 272.9 94.2 ± 16.9	
CRP quantification (mg/dL)	Test Food Group Placebo Food Group	0.059 ± 0.088 0.040 ± 0.014		0.150 ± 0.192 0.043 ± 0.027	#	0.116 ± 0.240 0.036 ± 0.017	
Total cholesterol (T-cho) (mg/dL)	Test Food Group Placebo Food Group	194.3 ± 27.4 190.8 ± 25.9		185.1 ± 30.4** 187.2 ± 19.3		178.0 ± 29.9** 175.4 ± 25.9**	
Triglycerides (TG) (mg/dL)	Test Food Group Placebo Food Group	68.7 ± 23.6 79.4 ± 38.4		67.7 ± 29.5 77.1 ± 28.1		65.6 ± 22.9 80.2 ± 33.7	
LDL cholesterol quantification (LDL-C) (mg/dL)	Test Food Group Placebo Food Group	110.5 ± 21.9 115.4 ± 26.6		98.5 ± 25.9** 106.6 ± 22.6**		97.8 ± 26.3* 99.6 ± 25.0***	

Table 6 Continued.

Item (Unit)	Ingested food	Before intake		Two weeks after intake		Four weeks after intake	
		Test Food Group: n = 15	Placebo Food Group: n = 14	Test Food Group: n = 14	Placebo Food Group: n = 14	Test Food Group: n = 14	Placebo Food Group: n = 14
HDL-cholesterol (HDL-C) (mg/dL)	Test Food Group Placebo Food Group	71.8 ± 16.1 60.8 ± 13.4		69.6 ± 13.5 60.9 ± 14.4		68.7 ± 14.0* 61.4 ± 14.4	
Total bilirubin (T-BIL) (mg/dL)	Test Food Group Placebo Food Group	0.77 ± 0.35 0.69 ± 0.24		0.76 ± 0.21 0.72 ± 0.29		0.74 ± 0.41 0.71 ± 0.35	
Sodium (Na) (mEq/L)	Test Food Group Placebo Food Group	140.4 ± 2.4 140.2 ± 2.0		139.9 ± 1.7 139.8 ± 1.9		140.0 ± 1.7 140.0 ± 2.0	
Potassium (K) (mEq/L)	Test Food Group Placebo Food Group	4.41 ± 0.75 4.59 ± 0.77		4.32 ± 0.29 4.31 ± 0.38		4.16 ± 0.16 4.26 ± 0.32	
Chloride (Cl) (mEq/L)	Test Food Group Placebo Food Group	104.9 ± 1.8 105.4 ± 1.8		104.4 ± 1.8 104.7 ± 1.5		104.9 ± 1.8 105.6 ± 1.7	
Calcium (Ca) (mg/dL)	Test Food Group Placebo Food Group	9.44 ± 0.27 9.55 ± 0.39		9.31 ± 0.37 9.36 ± 0.35*		9.24 ± 0.36* 9.33 ± 0.32*	
Iron (Fe) (µg/dL)	Test Food Group Placebo Food Group	116.5 ± 46.0 125.3 ± 35.2		103.8 ± 39.8 114.8 ± 53.7		113.6 ± 60.7 106.5 ± 42.1	
Glucose (GLU) (mg/dL)	Test Food Group Placebo Food Group	86.1 ± 8.1 89.0 ± 7.8		88.6 ± 6.9** 90.0 ± 12.0		89.4 ± 5.9** 89.1 ± 10.8	
HbA1c (%)	Test Food Group Placebo Food Group	5.04 ± 0.24 4.91 ± 0.15		5.22 ± 0.22*** 5.09 ± 0.18***		5.30 ± 0.27*** 5.14 ± 0.16***	

Mean ± standard deviation

* Comparison with before intake used a paired t-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ # An unpaired t-test was used for intergroup comparison at each time point. # $p < 0.05$

Table 7 Changes in general urinalysis.

Item	Ingested food	Before intake		Two weeks after intake		Four weeks after intake	
		Test Food Group: n = 15	Placebo Food Group: n = 14	Test Food Group: n = 14	Placebo Food Group: n = 14	Test Food Group: n = 14	Placebo Food Group: n = 14
Urobilinogen	Test Food Group	0.0 ± 0.0		0.0 ± 0.0		0.0 ± 0.0	
	Placebo Food Group	0.0 ± 0.0		0.0 ± 0.0		0.0 ± 0.0	
Occult blood reaction	Test Food Group	0.33 ± 0.90		0.21 ± 0.80		0.43 ± 0.85	
	Placebo Food Group	0.07 ± 0.27		0.14 ± 0.31		0.00 ± 0.00	#
Bilirubin	Test Food Group	0.00 ± 0.00		0.00 ± 0.00		0.00 ± 0.00	
	Placebo Food Group	0.00 ± 0.00		0.00 ± 0.00		0.00 ± 0.00	
Ketone body qualitative measurement	Test Food Group	0.00 ± 0.00		0.00 ± 0.00		0.00 ± 0.00	
	Placebo Food Group	0.00 ± 0.00		0.00 ± 0.00		0.00 ± 0.00	
Glucose qualitative measurement	Test Food Group	0.00 ± 0.00		0.00 ± 0.00		0.00 ± 0.00	
	Placebo Food Group	0.00 ± 0.00		0.00 ± 0.00		0.00 ± 0.00	
Protein	Test Food Group	0.00 ± 0.00		0.00 ± 0.00		0.04 ± 0.13	
	Placebo Food Group	0.07 ± 0.27		0.11 ± 0.29		0.00 ± 0.00	
pH	Test Food Group	6.10 ± 0.66		6.00 ± 0.73		6.04 ± 0.97	
	Placebo Food Group	6.00 ± 0.68		5.96 ± 0.60		5.86 ± 0.69	
Specific gravity	Test Food Group	1.0187 ± 0.0096		1.0174 ± 0.0106		1.0189 ± 0.0089	
	Placebo Food Group	1.0208 ± 0.0076		1.0206 ± 0.0071		1.0194 ± 0.0092	

Mean ± standard deviation

* Mann-Whitney U test was used for intergroup comparison at each time point for urobilinogen, occult blood reaction, bilirubin, ketone body qualitative measurement, glucose qualitative measurement, and protein, while an unpaired t-test was used for pH and specific gravity. : # $p < 0.05$

3.7 General urinalysis

In general urinalysis, there were no significant changes in urobilinogen, bilirubin, ketone body qualitative measurements, glucose qualitative measurements, protein, pH, and specific gravity in either the test food group or placebo food group throughout the study period (Table 7). There was no significant change in occult blood reaction in either the test food group or the placebo food group throughout the study period. In the test food group, occult blood reaction was significantly increased compared to the placebo food group at 4 weeks after starting the intake. However, there was no difference between the groups with respect to the amount of change.

4 Discussion

There were no side effects attributable to the test product observed in any subjects throughout the study period. Adverse events, such as the common cold and influenza, which occurred during the cold and influenza season, as well as other events, disappeared in a short period of time. Moreover, since these adverse events were temporary, the study was continued at the doctor's discretion. No adverse events that may have been attributable to the test product were observed in any of the subjects.

This study also confirmed a significant decrease in blood pressure and body fat ratio due to intake of the test food. However, the scope of decrease in measured values was within the physiological range.

Although there were significant fluctuations in the hematology tests, the fluctuation range was slight and within the physiological range. CRP quantification in biochemical examination of blood confirmed an upward swing in the mean value of the test food group due to the presence of 3 cases, 2 weeks after intake that exceeded the standard value (0.3 mg/dL). The spikes were transient in all cases, and the results were within the normal range when examined 4 weeks later. It was assumed that these spikes may have been due to inflammation associated with some kind of infection because it is not considered to be an effect that occurs with continuous ingestion of the test food. As described above, although significant changes were observed in several parameters in the biochemical examination of blood, all were slight changes, which were considered to be physiological fluctuations within the normal range.

The occult blood reaction test in urinalysis revealed one case with a score of 3 and three cases with a score of 1 in the test food group 4 weeks after intake. All the cases were reported in women. The case with a score of 3 also had a score of 3 before intake (4 weeks before), and the cases with a score of 1 had a score of 2 before intake (4 weeks before), so the involvement of the sexual cycle was suggested. Therefore, it was strongly suspected that menstrual

blood had contaminated the urine. As described above, the intake of the test food is thought to have had no effect on general urinalysis.

5 Conclusion

A safety study on excessive intake of acetic acid bacteria containing ceramide found no clinically significant events for blood pressure (systolic/diastolic)/pulse, weight/body fat ratio/BMI, hematology tests, biochemical examination of blood, general urinalysis, adverse events, and side effects. Therefore, the study confirmed the safety of 4 consecutive weeks' intake of 1197 mg of acetic acid bacteria containing 9.06 mg of ceramide.

Conflict of Interest

This study was financially supported by Mizkan Holdings Co., Ltd., test foods were prepared using equipment owned by Mizkan Holdings Co., Ltd. Study management, sample collection, and data analysis were performed independently, and thus there are no conflicts of interest that would affect the study results.

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