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Bowel movement improvement by Mulukhiyah (*Corchorus olitorius*)-containing food (AOTSUBU®) consumption: A randomized, double-blind, placebo-controlled, parallel- group comparison trial

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ABSTRACT

Background: Mulukhiya (*Corchorus olitorius*) richly contains dietary fiber and is suggested to improve bowel movement.

Objective: This study aimed to investigate the effects of mulukhiya-derived dietary fiber (MDF) on the intestinal environment in healthy Japanese adult subjects.

Methods: This randomized, double-blinded, placebo-controlled study enrolled 22 healthy Japanese adult subjects who typically defecate three to five times per week and do not consume enough dietary fiber. All subjects were randomly allocated into the MDF group (4 men and 7 women; 45.1 ± 11.4 years) or the placebo group (3 men and 8 women; 41.6 ± 9.5 years) by using a computerized random number generator. Each subject was administered with assigned 30 tablets (active [77-mg dietary fiber] or placebo) daily for two weeks. We asked the subjects to record their defecation condition in a bowel movement diary from 1 week before the start of test food consumption to the day before two weeks after the start of the test-food consumption (three weeks in total). Then, we evaluated the items in the bowel movement diary such as the occupancy rate of enteric, organic acids in feces, and subjective symptoms related to constipation.

Results: At one and two weeks after the start of the test-food consumption, the MDF group exhibited a significant increase in stool days, stool frequency, and stool volume compared with the placebo group ($P < 0.05$). Regarding the occupancy rate of enteric bacteria, *Prevotella* ($P = 0.025$) and *Clostridium* cluster IV ($P = 0.045$) were significantly increased in the MDF group compared with those in the placebo group at 2 weeks after the start of

the test-food consumption. As for organic acids in feces, n-butyric acid was significantly higher in the MDF group than in the placebo group at 2 weeks after the start of the test-food consumption ($P = 0.037$). Furthermore, no safety concerns were noted.

Conclusions: The consumption of MDF-containing food for 2 weeks resulted in the increase of stool frequency, stool volume, useful enteric bacteria, and organic acids in feces in healthy Japanese adult subjects.

Clinical trial registration number: UMIN-CTR: UMIN000035613.

Keywords: Mulukhiya, enterobacterial flora, dietary fiber, stool frequency, organic acid levels

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BACKGROUND

As food processing and lifestyle improve, people's intake of food rich in carbohydrates, fats, free sugars, and salts are increasing; however, dietary fiber intake is decreasing worldwide[1]. The joint expert consultation report of the World Health Organization and the Food and Agriculture Organization of the United Nations recommends the consumption of >25 g/day of total dietary fiber (>20 g/day of non-starch polysaccharides) through the intake of vegetables, fruits, and wholegrain foods[2]. In Japan, "Dietary Reference Intakes for Japanese (2015)" determined the dietary goals (DGs) of dietary fiber as follows: ≥ 20 g/day for male aged 18–69 years, ≥ 18 g/day for female aged 18–69 years, ≥ 19 g/day for male aged ≥ 70 years, and ≥ 17 g/day for female aged ≥ 70 years[3]. However, according to the results of "The National Health and Nutrition Survey in Japan, 2016," the median of daily dietary fiber intake in Japanese adult people is approximately 14.6 g/day[4]; hence, Japanese people do not achieve the DGs of dietary fiber. Therefore, the revised DGs of dietary fiber will be announced in "Dietary Reference Intakes for Japanese (2020)." Based on a comprehensive analysis of epidemiological studies, the reference value for calculating the revised DGs of dietary fiber (19.3 g/day) is generated according to the median of daily dietary

fiber intake in Japanese adult people (14.6 g/day) and the adequate intake for dietary fiber (24 g/day) in the US–Canada dietary reference intakes[5]. The revised DGs of dietary fiber will be set as follows: ≥ 21 g/day for males aged 18–64 years, ≥ 18 g/day for females aged 18–64 years, ≥ 20 g/day for males aged ≥ 65 years, and ≥ 17 g/day for females aged ≥ 65 years[5].

A negative correlation was found between dietary fiber intake and the development or mortality of lifestyle diseases, such as stroke[6,7] and type 2 diabetes mellitus[8,9]. In addition, dietary fiber intake influences bowel habits[10]. In an epidemiological study, dietary fiber intake negatively correlated with stool frequency and constipation prevalence[11]. In addition, constipation is reported to be associated with carcinogenesis through several mechanisms, caused by the increasing transit time of stool in the gut, the circulating toxic metabolites from the microbial cells in gut microbiota, etc.[12]. Therefore, constipation is an important health problem. Mulukhiya (*Corchorus olitorius*) is a leafy vegetable of Sparmaniaceae [13], which is the main component of the test food in this study. It is widely produced in tropical regions of Asia, America, and Africa, and the leaves are used for food and herbal medicine [13].

Mulukhiya richly contains polysaccharide (a kind of water-soluble dietary fiber)[14] and suggested to have an effect of increasing fecal water content in a clinical trial[15]. Water-soluble dietary fiber can increase the water holding capacity of the gastrointestinal lumen contents[16]; as fecal moisture increases, the defecation amount also increases [17]. Therefore, the consumption of mulukhiya-derived dietary fiber (MDF) may contribute to achieving the DGs of dietary fiber, and it can be an effective approach for improvement of bowel movements. However, studies investigating the effects of MDF on bowel movements remain unavailable. Hence, this study aims to investigate the effect of increasing the daily dietary fiber intake through MDF consumption on bowel movements and intestinal environment.

METHODS

Study design: The study was conducted as a randomized, double-blind, placebo-controlled, parallel-group comparison trial. The ethics committee of the Takara Clinic, Medical Corporation Seishinkai approved the study protocols on January 7, 2019 (approval ID: 1901-1811-AT01-01-TC), and the protocol was registered at the University Hospital Medical Information Network Clinical Trials Registry (UMIN000035613). This study, which was conducted from January 22, 2019, to April 21, 2019, was performed in accordance with the principles of the Declaration of Helsinki (2013), the ethical guidelines for medical and health research involving human subjects in Japan, and broader medical ethics.

Participants: The target subjects were healthy Japanese aged ≥ 20 years whose stool frequency was three to five times per week and who reportedly did not consume enough fiber in their usual dietary pattern. The

exclusion criteria were as follows: (a) subjects who are undergoing medical treatment or have a medical history of malignant tumor, heart failure, or myocardial infarction; (b) subjects who have a pacemaker or an implantable cardioverter defibrillator; (c) subjects who are undergoing treatment for any of the following chronic diseases: arrhythmia, liver disease, kidney disease, cerebrovascular disease, rheumatism, diabetes mellitus, hyperlipidemia, hypertension, or any other chronic diseases; (d) subjects who take "Foods for Specified Health Uses," "Foods with Functional Claims," or other functional food/beverage daily; (e) subjects who are currently taking medicines (including herbal medicines) and supplements; (f) subjects who regularly use anticoagulants, such as warfarin; (g) subjects who are allergic to medicines and/or the test-food-related products; (h) subjects who are pregnant, breastfeeding, and planning to become pregnant; (i) subjects who have been enrolled in other clinical trials within the last 3 months before the agreement to participate in this trial or plan to participate in another trial during this trial; and (j) subjects who are judged as ineligible to participate in the study by the physician. All subjects were enrolled through the website (<https://www.go106.jp/>) operated by ORTHOMEDICO Inc. (Tokyo, Japan). The study protocols were comprehensively explained to all subjects at the office of ORTHOMEDICO Inc. Furthermore, all subjects signed an informed consent prior to their participation in the study. No sponsors or members of the funding companies participated in the study. The tests were conducted at Takara Clinic (Medical Corporation Seishinkai, Tokyo, Japan).

Sample size: The sample size was determined to be 20 subjects within the maximum utility of the budget. In addition, one extra subject was included to each group

(11 subjects per group) in case one will withdraw from the study.

Selection, randomization, and blinding: Among the 30 subjects who provided an informed consent, 22 were determined eligible by the physician and had relatively less defecation frequencies at a week before screening (Scr; before consumption). Test foods were provided by the sponsor of the contract research organization. After the test foods were confirmed to be indistinguishable, a person in charge of shipping, who is a member of the contract research organization, gave the code of the test foods to an allocation controller who was not directly involved in the studies. The subjects were randomly allocated to either the MDF group or the placebo group (n = 11 each) according to the stratified randomized allocation of stool frequency, intestinal flora, age, and sex. The allocation ratio was 1:1. Such allocation was performed by the allocation controller using StatLight #11 version 2.10 (Yukms Co., Ltd., Kanagawa, Japan), which is a computerized random number generator, according to the provided codes. The allocation table with the coded test foods was

provided only to the person in charge of the shipping, who sent the test foods to each subject according to the table. The allocation controller locked the allocation table until the key opening day. The sponsors; principal investigator; sub-investigators; entire contract research organization staff (i.e., the director of the trial, the director of trial conduction, the person in charge of monitoring, the director and staff of statistical analysis, and the person in charge of shipping); medical institution staff; institutional review board members; contract laboratory; and others who were related to this study were not aware of the group assignments and were not involved in the allocation

Intervention: The composition and nutrition facts of the test foods in each tablet are shown in Table 1. We asked all subjects to take 30 tablets of either active (MDF-containing tablet) or placebo every day. The length of the intervention period was 2 weeks. The ethics committee declared that both tablets were identical in color, odor, and flavor.

Table 1. Contents of test tablets (per one tablet)

Test Food	Form	Component	Amount
Tablet containing mulukhiya	Tablet	Powdered mulukhiya*	200 mg
		Potato starch	156 mg
Placebo tablet	Tablet	Powdered tea	20 mg
		Sodium, Iron, and Chlorophyllin	20 mg
		Calcium stearate	4 mg

*77 mg of dietary fiber

OUTCOMES

The study schedule is shown in Table 2.

(1) Primary outcome: Stool frequency per week

Stool frequency per week was evaluated by counting

the frequency of defecation recorded in a bowel movement diary. Then, we examined the stool frequency per week. A week before Scr to the day before Scr and a week before the start of test-food consumption (-1w) to the day before 2 weeks after the start of the test-food consumption (2w) (3 weeks in total), the subjects recorded the existence and condition of defecation in the bowel movement diary by themselves.

(2) Secondary outcomes: Stool days per week, stool volume, stool shape, stool smell, and exhilarating feeling.

We examined the subjects' stool days per week, stool volume, stool shape, stool smell, and exhilarating feeling. Stool days per week were evaluated by counting the frequency of defecation recorded in the bowel movement diary. The stool volume was evaluated by the subjects themselves by visually converting the whole size of their stool into the number of standard size film case No. 7 (volume, 20 mL; diameter, 26 mm; height, 57.0 mm; Roppon-Ashi Entomological Books, Tokyo, Japan). Stool shape was classified as a seven-point grading scale, and subjects were selected from the following seven items: 1, separate hard lumps resembling nuts; 2, sausage shaped but lumpy; 3, sausage or snake shaped but with cracks on its surface; 4, sausage or snake shaped but smooth and soft; 5, soft blots with clear cut edges; 6, fluffy pieces with ragged edges, a mushy stool; and 7, watery, no solid pieces. Moreover, the smell of stool was classified as a five-point grading scale, grading scale, and subjects were selected from the following five items: 1, the smell is quite tight; 2, the smell is a bit tight; 3, the smell is usual; 4, the smell is a bit weak; and 5, the smell is quite weakened. Meanwhile, exhilarating feeling was classified as a three-point grading scale, and subjects were selected from the following three items: 1, clear feeling; 2, usual feeling; 3, relaxed feeling.

(3) Secondary outcomes: Occupancy rate of enteric bacteria.

We examined the occupancy rate of enteric bacteria such as *Bifidobacterium*, *Lactobacillales*, *Bacteroides*, *Prevotella*, *Clostridium* cluster IV, *Clostridium* cluster IX, *Clostridium* cluster XI, *Clostridium* subcluster XIVa, and *Clostridium* cluster XVIII. We evaluated the intestinal environment by using the terminal restriction fragment length polymorphism (T-RFLP) [18]. The subjects extracted and submitted the first feces sample within 2 days before and after each assessment point (the day before Scr and 2w). If they could not submit it personally, they sent it via post mail. The occupancy rate of each enteric bacteria was determined by T-RFLP[18] at Techno Suruga Laboratory Co.,Ltd. (Shizuoka, Japan). First, the amplification products were digested with restriction enzymes and terminal restriction fragments (T-RFs) of DNA were generated. Then, T-RF lengths were determined with ABI PRISM-3130xl DNA Sequencer (Applied Biosystems; California, USA) and each kind of enteric bacteria was detected from fragment length.

(4) Secondary outcomes: Japanese version of constipation assessment scale MT version (CAS-MT)[19–21]

We evaluated the condition of constipation by using the CAS-MT, which included the following eight question items: 1, "Abdominal distention or bloating"; 2, "Change in amount of gas passed rectally"; 3, "Less frequent bowel movements"; 4, "Rectal fullness or pressure"; 5, "Rectal pain with bowel movement"; 6, "Small volume of stool"; 7, "Unable to pass stool"; and 8, "Oozing liquid stool." Subjects answered each question from the following three levels: 0, no problem; 1, some problem; and 2, severe problem. Furthermore, CAS's total score was evaluated as the total score of the eight questions and ranges from 0 = no constipation to 16 = worst possible constipation.

Table 2. Schedule of enrollment, intervention, and assessments

	Screening				Intervention period			
	Examination	One week before screening (-1w [Scr])	Screening (Scr)	Enrollment	Allocation	One week before the start of test-food consumption (-1w)	Start intake (0w)	Two weeks after the start of the test-food consumption (2w)
ENROLLMENT:								
Eligibility screen	●			●				
Informed consent	●							
Allocation					●			
INTERVENTIONS:								
MDF group							↔	
Placebo group							↔	
ASSESSMENTS:								
Primary outcome		↔				↔		
Secondary outcome			●					●
Physical measurements	●		●					●
Physical examination			●					●
Urinalysis			●					●
Hematological and blood biochemical tests			●					●
Dietary survey			●					●
Medical questionnaire			●					●
Daily record							↔	

Closed circles (●) display the execution timing of each items

MDF, mulukhiya-derived dietary fiber

(5) Secondary outcomes: Bristol Stool Scale[22,23]. We used the Bristol Stool Scale to evaluate the subjective symptoms. This scale was classified as a seven-point grading scale, and the subjects were selected from the following seven items: 1, separate hard lumps resembling nuts; 2, sausage shaped but lumpy; 3, sausage or snake shaped but with cracks on its surface; 4, sausage or snake shaped but smooth and soft; 5, soft blots with clear cut edges; 6, fluffy pieces with ragged edges, a mushy stool; and 7, watery, no solid pieces.

(6) Secondary outcomes: Organic acids in feces.

Organic acids in feces were measured by examining succinic acid, lactic acid, formic acid, acetic acid, propionic acid, iso-butyric acid, *n*-butyric acid, iso-valeric acid, and *n*-valeric acid in feces. Subjects extracted and submitted the first feces sample within 2 days before and after each assessment point (the day before Scr and 2w). If they could not submit it personally, they sent it via post mail. Sample of feces (0.1 g) was put in a 2.0-mL-tube with zirconia beads and suspended in MilliQ. Samples were heated at 85°C for 15 minutes, vortexed at 5 m/s for 45 seconds using FastPrep-24 (MP Biomedicals, LLC, California, USA), and centrifuged at $15,350 \times g$ for 10 minutes. The supernatant was filtrated by 0.2- μm filter. Concentrations of organic acid in feces such as formic acid, acetic acid, propionic acid, isobutyrate, butyric acid, valeric acid, isovaleric acid, lactic acid, and succinic acid were measured using an organic acid analysis system (SHIMADZU CORPORATION, Kyoto, Japan): a high-performance liquid chromatography, Prominence;

a detector, CDD-10A; tandemly-arranged two columns, Shim-pack SCR-102H (300 mm \times 8 mm ID); a guard column, Shim-pack SCR-102H (50 mm \times 6 mm ID); a mobile phase, 5-mM *p*-toluenesulfonic acid; and a reaction solution, 5-mM *p*-toluenesulfonic acid, 100- μM EDTA, and 20-mM Bis-Tris. The flow rate and oven temperature were 0.8 mL/min and 45°C, respectively.

(7) Safety evaluation: We conducted physical examination, urinalysis, and hematological and blood biochemical tests.

The subjects' height, weight, BMI, body fat percentage, temperature, systolic and diastolic blood pressures, and pulse rate were measured as the physical examination items. We only measured the height at Scr to calculate the BMI.

Then, we collected urine samples to evaluate the levels of protein, glucose, urobilinogen, bilirubin, ketone bodies, pH, and occult blood. The level of each item was determined by LSI Medience Corporation (Tokyo, Japan) in accordance with the global standard.

Hematological tests were conducted to assess the following: leukocyte count, erythrocyte count, hemoglobin, hematocrit, platelet count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and white blood cell differential count (i.e., percentages of neutrophils, lymphocytes, monocytes, eosinophils, and basophils). For the biochemical tests, we evaluated the following: aspartate aminotransferase, alanine aminotransferase, γ -glutamyl transpeptidase, alkaline phosphatase, lactate dehydrogenase, leucine aminopeptidase, total bilirubin, direct bilirubin, indirect

bilirubin, cholinesterase, total protein, urea nitrogen, creatinine, uric acid, creatine kinase, calcium, serum amylase, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, glycoalbumin, serum iron, sodium, potassium, chloride, inorganic phosphorus, glucose, hemoglobin A1c, and non-specific immunoglobulin E. The level of each time was measured by LSI Medience Corporation.

Furthermore, we asked the subjects to complete a medical questionnaire to determine their health status at each assessment point. We also asked them to report the medication dosage and any changes in their physical condition on a daily basis.

Statistical analysis: All statistical analyses were two-sided, with a significance level of 5% and without adjustment for multiple comparisons. Data were analyzed using Windows SPSS version 23.0 (IBM Japan, Ltd., Tokyo, Japan). The baseline of stool days per week, stool frequency per week, and stool volume was obtained by summing the values throughout $-1w$, whereas that of stool shape, stool smell, and exhilarating feeling was obtained by calculating the average of the values throughout $-1w$. The changes of stool days per week, stool frequency per week, stool volume, stool shape, stool smell, and exhilarating feeling were determined by subtracting the baselines from the measured values at 1 week after the start of the test-food consumption ($1w$; $\Delta 1w$) and 2w ($\Delta 2w$). The change of secondary outcomes was identified by subtracting the baselines (the measured values at Scr) from the measured values at 2w ($2w-Scr$). The

interaction and main effect of the changes of stool days per week, stool frequency per week, and stool volume recorded in the bowel movement diary were evaluated by two-way repeated measures analysis of variance, which is a repeated-measurement model based on time and group. We also conducted two-way repeated measures analysis of covariance (ANCOVA), with the baseline measurement as a covariate. When two-way repeated measures ANCOVA was used for data analysis, we used the baseline values as covariates and compared the changes at each time point between groups *post hoc*. The occupancy rate of enteric bacteria and organic acids in feces, the measured values and the changes in physical examination, and hematological and blood biochemical tests were expressed as mean and standard deviation. The data at Scr and $2w-Scr$ were analyzed using Student's *t*-test for between-group comparisons. Meanwhile, the data at 2w between groups were analyzed using ANCOVA, with the baseline measurement as a covariate. CAS-MT is shown as median and interquartile range (first and third quartiles), and the data at Scr and 2w between groups were analyzed using Mann-Whitney *U* test. Stool shape, stool smell, exhilarating feeling, and Bristol stool scale recorded in the bowel movement diary and the measured values of urinalysis were set to a code, that is, 1 is defined as true or within the normal range, whereas 0 is defined as false or outside the normal range in each item. Then, such data were expressed as the number of subjects (*n*) and were analyzed between groups by using the chi-square test at Scr and 2w. Subjects who appreciably violated the protocol were excluded from the analysis.

RESULTS

Analysis set: Figure 1 shows the flowchart of this study. The target subjects were healthy Japanese aged ≥ 20 years whose stool frequency was three to five times per week and who reportedly had not enough fiber intake in their usual dietary pattern. We recruited them from January 22, 2019, to February 22, 2019.

Out of 30 subjects, 22 were eligible and were randomly divided into either the MDF group or the

placebo group ($n = 11$ each). All subjects completed this study without violating the protocol, and their consumption rates were $>90\%$. Therefore, 11 subjects (4 men and 7 women; between 27 and 60 years) in the MDF group and 11 subjects (3 men and 8 women; between 26 and 55 years) in the placebo group were included in the safety and validity analyses, on an intention-to-treat dataset. The subjects' background information is shown in Table 3.

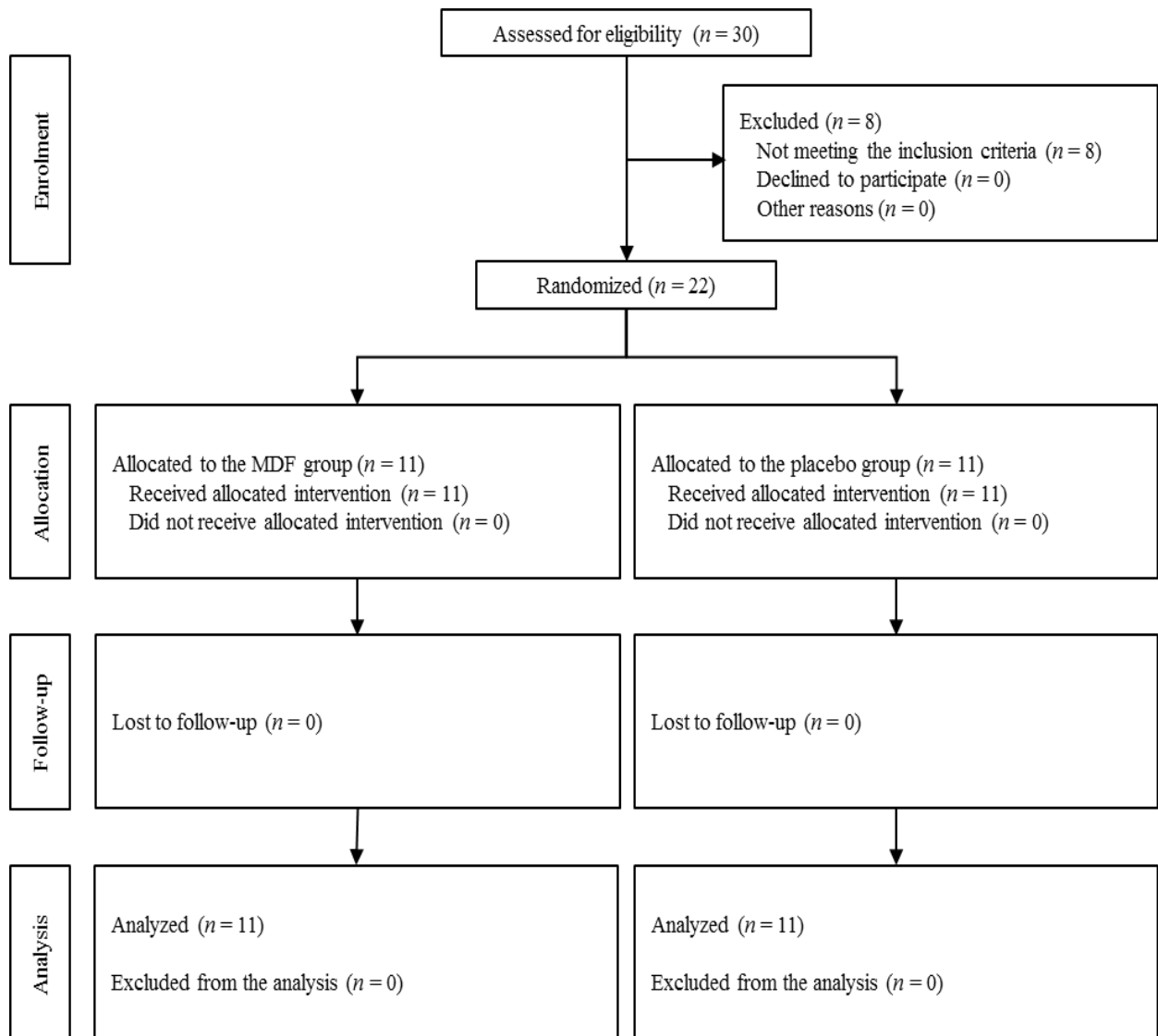


Figure 1. Flowchart of the participants of the study

MDF, mulukhiya-derived dietary fiber

Table 3. Participants' background information

	MDF group (n = 11)	Placebo group (n = 11)	P value ^c
Gender (Male / Female) ^a	4 / 7	3 / 8	1.000
Age (years) ^b	45.1 (11.4)	41.6 (9.5)	0.448
Body height (cm) ^b	159.0 (6.6)	161.8 (6.6)	0.322
non-specific IgE (IU/mL) ^b	139.2 (145.1)	239.6 (403.3)	0.446

a, The data are presented as the number of subjects (n); b, The data are presented as the mean (standard deviation); c, Student's *t*-test; MDF, mulukhiya-derived dietary fiber

Stool days per week, stool frequency per week, and stool volume:

The results of stool days per week, stool frequency per week, and stool volume are shown in Figure 2 and Table 4. The results of any items between the groups at 1w had no significant differences.

Regarding stool days per week, the simple main effect in the MDF group was significant ($P < 0.001$), and each average at 1w and 2w was significantly higher than that in the placebo group. ($P < 0.001$, $P = 0.002$, respectively; Figure 2a). Furthermore, changes in the stool days per week in the MDF group at $\Delta 1w$ and $\Delta 2w$ were significantly higher than those of the placebo group ($\Delta 1w$, MDF group 1.5 ± 0.9 days/week, placebo group -0.3 ± 0.6 days/week, $P < 0.001$; $\Delta 2w$, MDF group 1.5 ± 0.9 days/week, placebo group 0.0 ± 0.9 days/week, $P < 0.001$; Table 4). The transition of the measured values of stool days per week in the MDF group was as follows: 3.7 ± 0.8 (-1w), 5.3 ± 1.5 (1w), and 5.3 ± 1.3 days/week (2w). In the placebo group, the transition of the measured values of stool days per week was as follows: 4.5 ± 1.1 (-1w), 4.2 ± 1.1 (1w), and 4.5 ± 1.3 days/week (2w).

Regarding stool frequency per week, the simple main effect in the MDF group was significant ($P < 0.001$), and each average time at 1 and 2 weeks after the start of the test-food consumption was significantly higher than that in the placebo group ($P = 0.003$, $P = 0.002$, respectively; Figure 2b). Furthermore, changes in the stool frequency per week in the MDF group at $\Delta 1w$ and $\Delta 2w$ were significantly higher than those in the placebo group ($\Delta 1w$, MDF group: 2.1 ± 1.9 times/week, placebo group: -0.1 ± 0.9 times/week, $P = 0.002$; $\Delta 2w$, MDF group: 2.2 ± 1.8 times/week, placebo group: 0.0 ± 0.9 times/week, $P = 0.002$; Table 4). The transition of the measured values of stool frequency per week in the MDF group was as follows: 3.9 ± 0.7 (-1w), 6.0 ± 2.3 (1w), and 6.1 ± 2.3 times/week (2w). In the placebo group, the transition of the measured values of stool frequency per week was as follows: 4.5 ± 1.1 (-1w), 4.4 ± 1.2 (1w), and 4.5 ± 1.3 times/week (2w).

Regarding stool volume, the simple main effect in the MDF group was significant ($P = 0.002$), and each average amount at 1w and 2w was significantly higher than that in the placebo group ($P = 0.033$, $P = 0.005$,

respectively; Figure 2c). Furthermore, changes in the stool volume in the MDF group at Δ1w and Δ2w were significantly higher than those in the placebo group (Δ1w, MDF group: 6.0 ± 7.0 film canisters/week,

placebo group: -1.1 ± 5.0 film canisters/week, P = 0.012; Δ2w, MDF group: 10.9 ± 9.6 film canisters/week, placebo group:

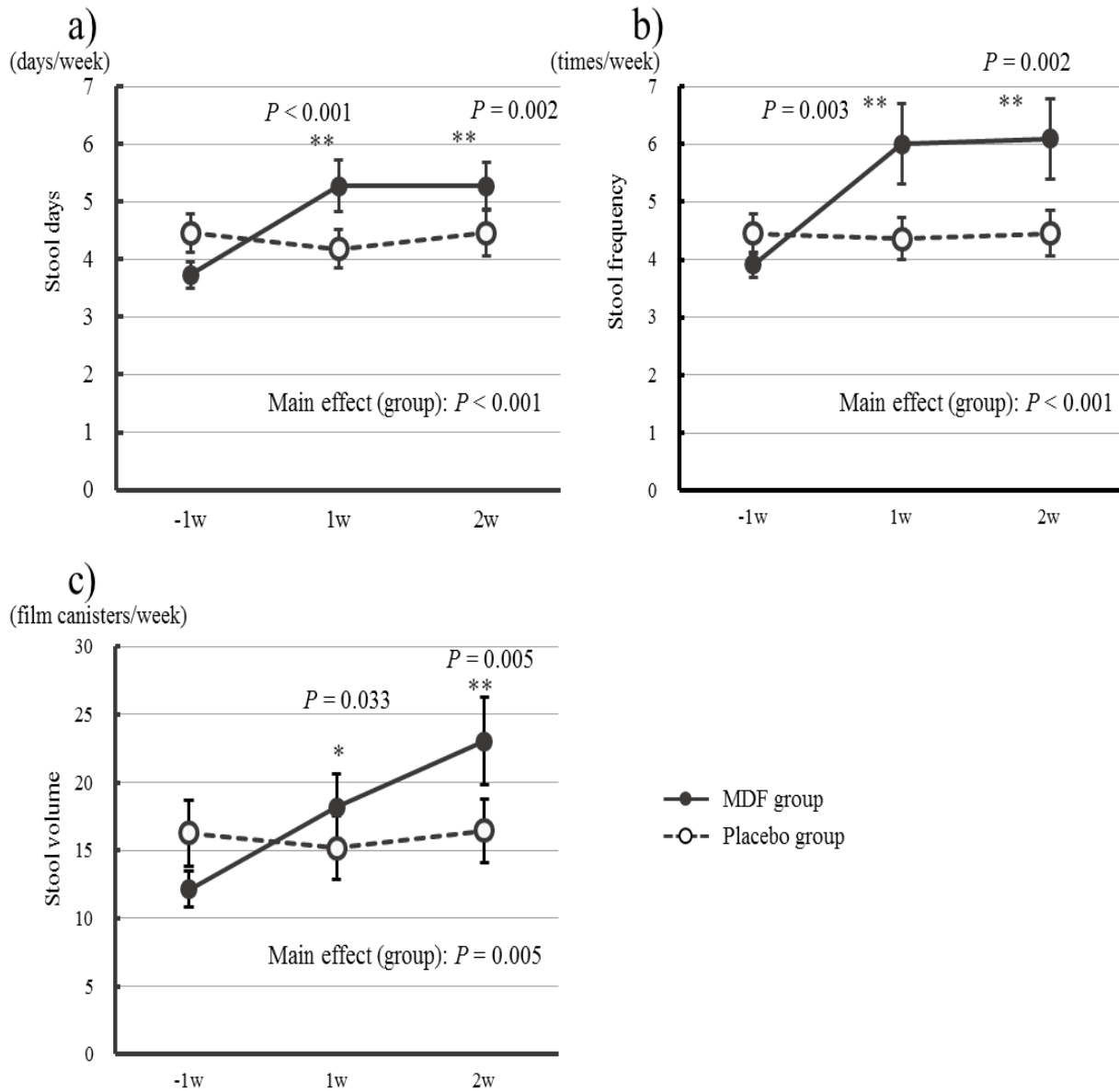


Figure 2. Variation in stool days, stool frequency, and stool volume

a) stool days, b) stool frequency, c) stool volume; Mean ± SE. **: P < 0.01, *: P < 0.05 (vs. Placebo group)

MDF, mulukhiya-derived dietary fiber; -1w, one week before the start of test-food consumption; Scr, screening; 2w, two weeks after the start of the test-food consumption

Table 4. Stool days, frequency, and volume

Item	Unit		-1w		Δ1w (1w-[-1w])		Δ2w (2w-[-1w])		P value				
									-1w ^a	Interaction ^b	Main effect ^b (group)	Post-hoc ^b	
										Δ1w	Δ2w		
Stool days	days/week	MDF group (n = 11)	3.7	(0.8)	1.5	(0.9)	1.5	(0.9)	0.095	0.532	< 0.001*	< 0.001*	< 0.001*
		Placebo group (n = 11)	4.5	(1.1)	-0.3	(0.6)	0.0	(0.9)					
Stool frequency	times/week	MDF group (n = 11)	3.9	(0.7)	2.1	(1.9)	2.2	(1.8)	0.188	1.000	< 0.001*	0.002*	0.002*
		Placebo group (n = 11)	4.5	(1.1)	-0.1	(0.9)	0.0	(0.9)					
Stool volume	film canisters /week	MDF group (n = 11)	12.1	(4.4)	6.0	(7.0)	10.9	(9.6)	0.152	0.206	0.002*	0.012*	0.002*
		Placebo group (n = 11)	16.3	(8.1)	-1.1	(5.0)	0.2	(3.3)					

The data are presented as the mean (standard deviation).

Post-hoc, use the baseline values as covariates and compare the changes at each time point between groups.

a, Student's *t*-test; b, Two-way repeated measures ANOVA

**P* < 0.05 vs. the placebo group.

- 1w, one week before the start of test-food consumption; Δ1w, subtracting the baselines (- 1w) from the measured values at one week after the start of the test-food consumption (1w); Δ2w, subtracting the baselines (- 1w) from the measured values at two weeks after the start of the test-food consumption (2w)

MDF, mulukhiya-derived dietary fiber.

0.2 ± 3.3 film canisters/week, $P = 0.002$; Table 4). The transition of the measured values of stool volume in the MDF group was as follows: 12.1 ± 4.4 (-1w), 18.2 ± 8.1 (1w), and 23.0 ± 10.6 film canisters/week (2w). In the placebo group, the transition of the measured values of stool volume was as follows: 16.3 ± 8.1 (-1w), 15.2 ± 7.7 (1w), and 16.5 ± 7.8 film canisters/week (2w).

Stool shape, stool smell, and exhilarating feeling: The results between the groups had no significant differences (data not shown).

Occupancy rate of enteric bacteria (T-RFLP): The occupancy rates of enteric bacteria are shown in Figure 3. The measured values at Scr were not significantly different. *Prevotella* and *Clostridium* cluster IV at 2w were significantly higher in the MDF group than in the placebo group (*Prevotella*, 12.3% ± 19.0% vs. 5.2% ± 12.9%, $P = 0.025$; *Clostridium* cluster IV, 9.9% ± 6.9% vs. 9.0% ± 7.9%, $P = 0.045$). Furthermore, *Prevotella* and *Clostridium* cluster IV significantly increased from Scr to 2w in the MDF group than in the placebo group (*Prevotella*, 5.6% ± 8.0% vs. -0.1% ± 2.1%, $P = 0.031$; *Clostridium* cluster IV, 3.9% ± 3.9% vs. 0.0% ± 4.4%, $P = 0.038$). *Clostridium* cluster XIVa at 2w was not significantly but marginally higher in the MDF group than in the placebo group (*Clostridium* cluster XIVa, 18.6% ± 5.2% vs. 15.0% ± 8.5%, $P = 0.158$).

Organic acids in feces: The variation in organic acids in feces is presented in Figure 4. The measured values at Scr were not significantly different. *n*-butyric acid at 2w was significantly higher in the MDF group than in the placebo group (0.9 mg/g ± 0.4 mg/g vs. 0.7 mg/g ± 0.4 mg/g, $P = 0.037$).

CAS-MT and Bristol stool scale: The results between the groups had no significant differences (data not shown).

Safety evaluation: Significant between-groups differences were observed with some subjects; however, the mean values remained within the reference ranges, and these were not medically problematic, based on the comprehensive judgment by the physician and the other examination matters (data not shown). Therefore, we observed no medically problematic change in safety evaluation.

DISCUSSION

As mentioned, this study aimed to investigate the effects of 2-week MDF consumption on the bowel movements and intestinal environment of healthy Japanese adult subjects who typically defecate three to five times per week and do not consume enough dietary fiber.

Regarding stool frequency per week, the primary outcome, which is the simple main effect of the MDF group, was significant, and the score of the MDF group was significantly higher than that of the placebo group at 1w and 2w in *post-hoc* analysis. In stool days per week and stool volume per week, the simple main effect of the MDF group was significant, similar to stool frequency per week, and the score of the MDF group was significantly higher than that of the placebo group in *post-hoc* analysis throughout the intervention period. From these results, MDF consumption can increase stool frequency per week and stool volume. In previous studies involving healthy adult subjects, 2 week consumption of mulukhiya tended to increase fecal water content[15]. Furthermore, mulukhiya plentifully contains polysaccharide, which is a kind of water-soluble dietary fiber forming mucus[14]. Mekabu (sporophyll of *Undaria pinnatifrda*), which also richly contains polysaccharide, increases the weight and the water holding capacity of the gastrointestinal lumen contents *in vivo*[16], and it also increases the stool days per week and stool volume in healthy elderly people[24]. Therefore, MDF increases the fecal water content and may increase the stool days per week and stool volume.

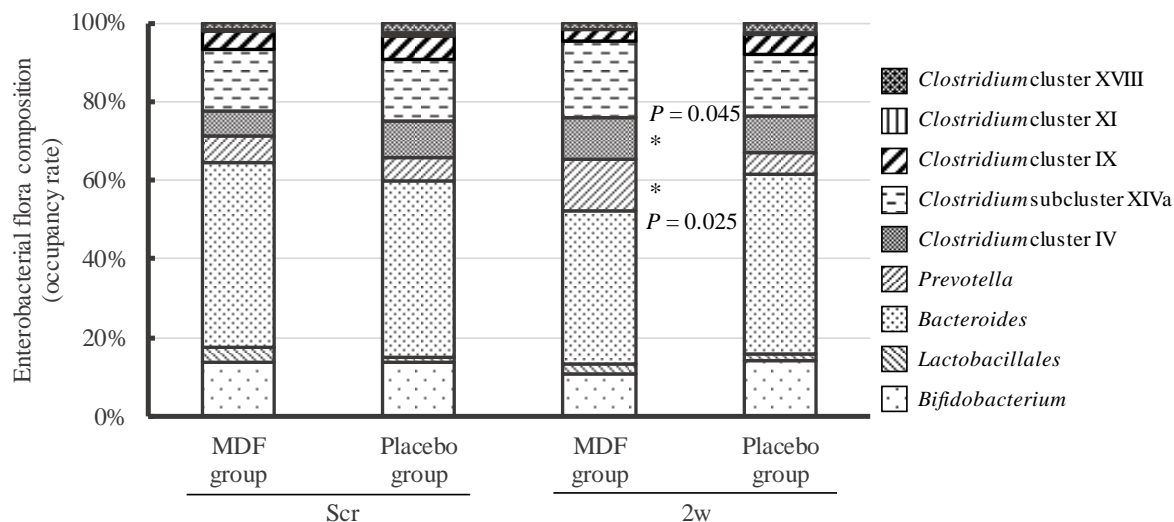


Figure 3. Variation in enterobacterial flora composition

*: $P < 0.05$ (vs. Placebo group)

MDF, mulukhiya-derived dietary fiber; Scr, screening; 2w, two weeks after the start of the test-food consumption

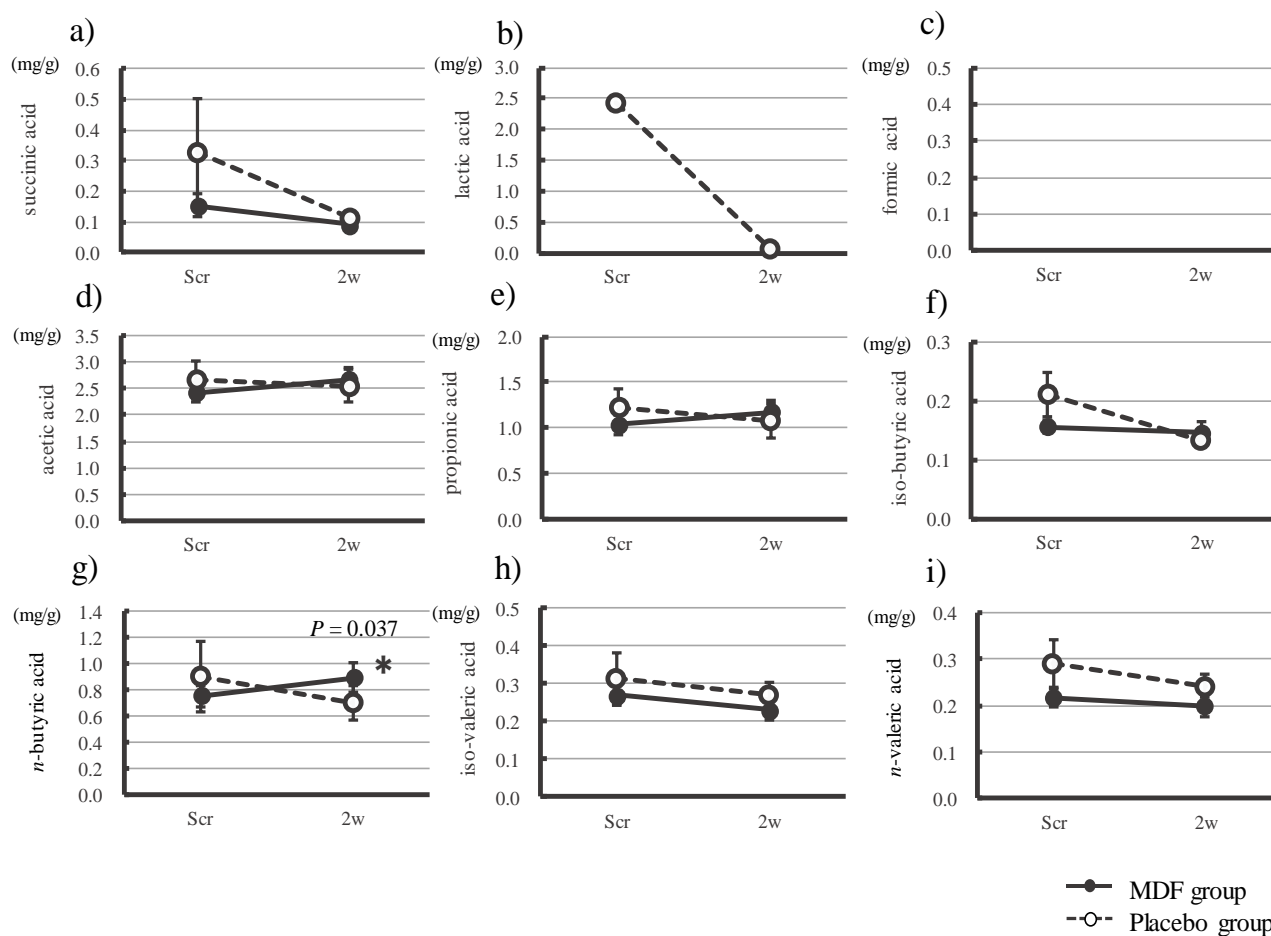


Figure 4. Variation in organic acids in feces

a) succinic acid, b) lactic acid, c) formic acid, d) acetic acid, e) propionic acid, f) iso-butyric acid, g) n-butyric acid, h) iso-valeric acid, i) n-valeric acid

Mean \pm SE. *: $P < 0.05$ (vs. Placebo group)

MDF, mulukhiya-derived dietary fiber; Scr, screening; 2w, two weeks after the start of the test-food consumption

As for the occupancy rate of enteric bacteria, the measured values and the changes of the occupancy rates of *Prevotella* and *Clostridium* cluster IV at 2w in the MDF group were significantly higher than those in the placebo group. In a previous study describing the relationships between *Prevotella* and fecal water content or plasticity, the subjects with loose stools had higher occupancy rates of *Prevotella* in their bacterial flora [25]. In the current study, only one subject answered that his/her stool shape was softer than “normal (sausage or snake shaped, smooth and soft)” in the bowel movement diary throughout the intervention period (“soft blots with clear cut edges” at 1w); stool hardness seemed to be relatively high among the subjects. According to the answers for the stool shape in the bowel movement diary, six subjects in the MDF group and nine in the placebo group answered “normal” at 1 week before the start of test-food consumption, with 10 in the MDF group and 7 in the placebo group at 2w. In addition, the stool shape tended to be normalized in the MDF group. Therefore, the normalization of stool shape might be related to the increase of occupancy rates of *Prevotella*. Additionally, *Clostridium* cluster IV is well known as butyrate-producing bacteria[26], and the increase of the occupancy rate of *Clostridium* cluster IV with MDF consumption in this study is consistent with the significant increase of *n*-butyric acid levels in the MDF group than in the placebo group. Enteric bacteria metabolize dietary fiber and generate short-chain fatty acids, such as acetic acid, butyric acid, and propionic acid, and the human body uses them as energy for absorbing water and minerals[27]. Organic acids generated by enteric bacteria increase the peristaltic movement of intestines and mucus secretion at the large-intestine mucosa, allowing the feces to pass more smoothly[28]. Therefore, MDF consumption may promote the peristaltic movement of intestines and

the excretion of feces because of the increasing occupancy rate of *Clostridium* cluster IV and intestinal butyric acid level. Hence, the increase of occupancy rates of *Prevotella* and *Clostridium* cluster IV by MDF consumption improved the bowel movement through the normalization of stool shape and the increase of butyric acid production

The consumption of MDF resulted in the significant increase of stool frequency per week, stool volume per week, useful enteric bacteria, and organic acids in feces. However, this study has three limitations that could be addressed in future research. First, the difference in gut microbiota composition may have caused the variation of the study results. The subjects in this study were mainly in their 30s to 50s. Considering the difference of gut microbiota composition according to the age is reportedly greater than the differences among individuals[29], further investigation should confirm whether MDF can exert the same effects as this study in other generations. Second, items concerning fecal water content were not evaluated in this study. In this study, one of the factors improving bowel movement by MDF consumption might be the increase of the fecal water content. Evaluation of fecal water content and components in the future study is needed to clarify the detailed mechanism of the bowel movement indicating its improvement through MDF consumption. Lastly, intestinal putrefactive products were not evaluated in this study. In the preceding clinical trial, the total amount of fecal putrefactive products and fecal indole levels significantly decreased after mulukhiya consumption[15]. Some enteric bacteria produce harmful putrefactive substances, such as phenol and indole, which directly cause damage to the intestinal tract and sometimes affect the body systemically[30,31]. Further investigation on the effects of MDF consumption on intestinal putrefactive products may reveal

interesting facts.

Finally, regarding physical examination, urinalysis, and blood test, some items showed significant differences between groups; however, the mean values remained within the adequate or reference ranges[32], and these were not medically problematic according to the comprehensive judgment by the physician and the other examination matters. As for the blood test result, the measured value of serum iron at Scr was significantly lower in the MDF group than the placebo group ($78.8 \mu\text{g/dL} \pm 16.2 \mu\text{g/dL}$ vs. $123.0 \mu\text{g/dL} \pm 53.9 \mu\text{g/dL}$, $P = 0.017$) (data not shown). The concentration of iron in the body is related to defecation, and higher level of iron is reported to cause constipation[33]. However, the value in any outcome at Scr or -1w showed no significant difference; in addition, the average of serum iron in each group was within the reference ranges. Therefore, the difference of the measured value of serum iron at Scr has no influence on defecation.

Therefore, the 2-week consumption of MDF was safe under the conditions of this study.

CONCLUSIONS

The effects of the 2-week consumption of MDF on bowel movements and intestinal environment of the healthy Japanese adult subjects who typically defecate three to five times per week and do not consume enough dietary fiber was investigated in this study. Through MDF consumption, the stool frequency per week, stool volume, useful enteric bacteria such as *Prevotella* and *Clostridium* cluster IV, and *n*-butyric acid in feces were increased. Furthermore, MDF consumption was found to be safe under the conditions of this study.

List of Abbreviations: MDF: mulukhiya-derived dietary fiber; ANCOVA: analysis of covariance; DG: dietary goal; T-RFLP: terminal restriction fragment

length polymorphism; CAS-MT: constipation assessment scale MT version; BMI: body mass index; SE: standard error

Declaration of Conflicting Interests: The sponsor of the present study, AOTSUBU CO., LTD., assigned ORTHOMEDICO Inc. to conduct the study. H.N., M.N., and N.O. were affiliated with AOTSUBU CO., LTD., and Y.T. was a member of ORTHOMEDICO Inc. The study was conducted by both AOTSUBU CO., LTD. and ORTHOMEDICO Inc. Furthermore, T.T. (MD) was the principal investigator who monitored all the subjects' conditions.

Authors' Contributions: H.N. designed the study, and wrote the initial draft of the manuscript. T.T. contributed to the collection, interpretation, and analysis of data, and assisted in the preparation of the manuscript. All other authors have contributed to data collection and interpretation, and critically reviewed the manuscript. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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