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Perilla Oil, An Omega-3 Unsaturated Fatty Acid-Rich Oil, Enhances Diversity of Gut Microbiota and May Relieve Constipation in Sedentary Healthy Female: A Nonrandomized Placebo-Controlled Pilot Study

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Abstract: This study shows the effect of omega-3 unsaturated fatty acids via perilla oil intake on gut microbiota and constipation. Eight sedentary females participated in a nonrandomized placebo-controlled pilot study consisting of eight-week perilla oil (OIL) and placebo (PLA) intervention phases. There was a 10-month washout period between phases. All participants received 9 g of perilla oil-containing jelly in the OIL phase, and a placebo jelly in the PLA phase. Gut microbiota, α -diversity, and constipation scores were measured pre- and post-intervention in both phases. The α -diversity, an important indicator of gut microbiota diversity, was significantly increased post-intervention (4.5 ± 0.1) compared to pre-intervention (3.8 ± 0.3) in the OIL only ($p = 0.021$). Notably, the level of α -diversity was maintained even after the washout period of 10 months. Butyrate-producing bacteria, Lachnospiraceae (%), did not change in the OIL but were significantly reduced post-intervention (15.1 ± 4.8) compared to pre-intervention (20.1 ± 7.0) in the PLA ($p = 0.040$). In addition, the constipation scores were significantly or tended to be reduced during the OIL phase only ($p < 0.05$, $p < 0.1$). In conclusion, an eight-week perilla oil supplementation may enhance and establish the diversity of gut microbiota, which may relieve constipation.



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Keywords: microbiota; α -diversity; omega-3 unsaturated fatty acids; constipation score

1. Introduction

The gut microbiota is a collection of bacteria that live in inside the gut. It is closely related to the metabolism of the whole-body [1,2], and enhancement of the gut microbiota may lead to health promotion. In particular, short-chain fatty acids (SCFAs) are gut bacteria-derived metabolites involved in systemic metabolic regulation. SCFAs produced in the gut microbiota are transported through the blood and react with SCFA receptors, such as the G protein-coupled receptor, which are distributed in tissues throughout the body [3]. Stimulation by the receptors then promotes physiological responses [3]. For example, in skeletal muscle, SCFAs enhance energy metabolism by modulating insulin sensitivity and mitochondrial activity [4,5].

One of the major SCFAs is butyrate, and there are several other types of SCFAs such as acetate and propionate. In particular, butyrate is an important modulator of energy metabolism in skeletal muscle [6]. Gao Z et al. determined that butyrate intervention increases energy expenditure by enhancing mitochondrial function in an animal experiment [6]. Butyrate is produced by butyrate-producing gut bacteria, such as Lachnospiraceae, Bacteroidaceae, and Clostridiaceae. Therefore, increasing the abundance rate of butyrate-producing gut bacteria may contribute to the improvement of energy metabolism.

The gut microbiota is influenced by several lifestyle factors. In particular, dietary habits play a key role in establishing its status [7]. Gut bacterial diversity is a major indicator for assessing the gut microbiota, and there is a positive correlation between a habitual

healthy diet and the gut microbiota diversity [8]. Therefore, dietary interventions aimed at increasing the bacterial diversity and the abundance of butyrate-producing bacteria will be effective for health promotion. It is well known that dietary fiber intake increases butyrate-producing bacteria and gut diversity. Recently, Manor O. et al. reported that gut microbiota diversity is positively associated with blood levels of omega-3 unsaturated fatty acids as well as consumption of fruits and vegetables, which provide dietary fiber [8]. Furthermore, the omega-3 unsaturated fatty acids increase the SCFA butyrate through an increase in Lachnospiraceae [9,10], which plays an important role in energy metabolism via activating cell receptors [11,12].

Previously, we investigated the effect of omega-3 unsaturated fatty acids intake through perilla oil on the gut health in female athletes [13]. Perilla oil is a seed oil rich in omega-3 unsaturated fatty acids, a traditional Japanese food and familiar to the Japanese. In addition, the oil has antioxidant, anti-inflammatory effects [14,15] and is useful as an energy source. Even vegans or people from regions unfamiliar with fish diets who have difficulty consuming fish oil can easily get the omega-3 unsaturated fatty acids they need with just a spoonful of perilla oil. Therefore, we chose it as a research food. As an important result, 9 g/day of perilla oil intake increased the abundance of butyrate-producing bacteria and relieved constipation. Furthermore, no increase in body weight and body fat was observed despite 81 kcal of perilla oil per day. This suggests that a daily intake of 9 g perilla oil improves the gut environment without causing weight gain or fat accumulation. Although previous studies have shown that omega-3 unsaturated fatty acids have multiple health benefits, such as lowering blood pressure [16] and reducing the risk of disease or death [17–19], we focused on the gut microbiota. However, the baseline gut microbiota differs between athletes and healthy individuals [20]. Therefore, it was necessary to investigate the effect of perilla oil intervention in healthy females for health promotion.

The main purpose of this study was to investigate the effects of daily perilla oil intake in healthy females on the gut microbiota, constipation, and biomarkers. Furthermore, the second aim was to investigate whether the effect of perilla oil was achieved only in the intervention phase or continued after the intervention. In other words, we aimed to clarify the long-term effects of perilla oil intake on the gut microbiota.

2. Materials and Methods

2.1. Participants

Eleven sedentary healthy females were recruited, but three of them did not meet the inclusion criteria based on the results of the questionnaire. Finally, eight females (age: 36.6 ± 1.6 years, height: 161.3 ± 1.7 cm, body weight: 57.5 ± 2.7 kg) participated. All participants had no exercise habits and performed sedentary work in the same workplace every weekday. The number of subjects finally enrolled in the study was limited because of the COVID-19 pandemic, but all eight completed the study. None of the participants were using supplements or medications, had a history of chronic disease, or were smokers. The entire study, including recruitment, data collection, and follow-up, was conducted at the Nippon Sport Science University from October 2019 to December 2020.

Body composition was measured using bioelectrical impedance analysis (InBody730, InBody Co., Ltd., Seoul, Republic of Korea), and the physical characteristics of the participants are shown in Table 2.

2.2. Experiment Design

This study was designed as a placebo-controlled pilot study consisting of 8-week perilla oil intervention (OIL) and placebo intervention (PLA) phases. There was a washout period of 10 months between the OIL and PLA phases. Since the gut microbiota could be affected by seasonal changes [21], we eliminated external environmental factors such as ultraviolet levels and temperature as much as possible by intervening during the same month in both phases. Participants were given 3 g of perilla oil-containing jelly three times a day (9 g/day perilla oil) in the OIL phase, and a placebo jelly the same number of times

(0 g/day perilla oil) in the PLA phase. The placebo jelly had the same shape and taste as the perilla oil jelly and contained no fat. The jellies were lemon-flavored, which masked the natural nutty taste of the perilla oil. Participants were blinded to their phase and assigned a subject number. They were instructed to maintain their diet and lifestyle during the intervention phases.

We assessed physical characteristics, gut microbiota, and urinary biochemical indices (8-hydroxydeoxyguanosine:8-OHdG, indoxyl sulphate) pre- and post-intervention in both phases. In addition, data on constipation score and subjective condition were collected pre-intervention and every week thereafter in the OIL and PLA phases using the visual analogue scale (VAS) method [22] (Figure 1).

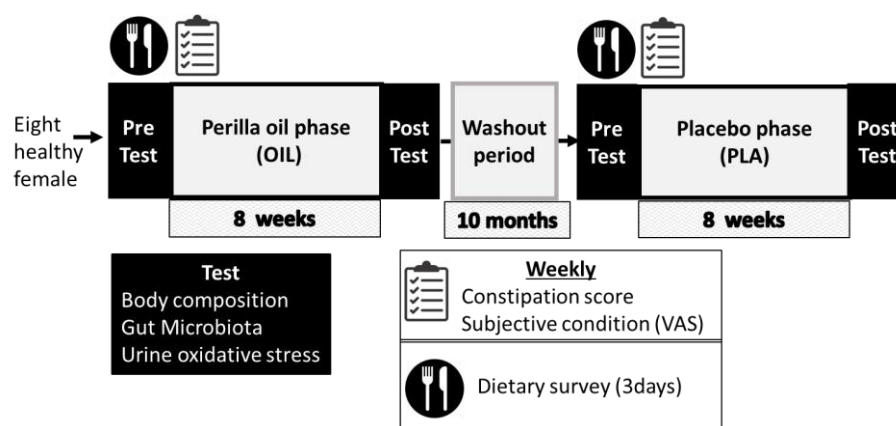


Figure 1. Experiment design.

2.3. Gut Microbiota

Bacterial DNA from feces was collected to test the gut microbiota. Fecal samples were collected at home using a kit and transported at room temperature within 24 h to a company (SheepMedical Co., Ltd. Tokyo, Japan) for analysis. Samples were prepared for gut microbiota detection using a next-generation sequencer, and approximately 300 bases, including the V1 to V2 variable region, were analyzed as previously described [23]. The following polymerase chain reaction (PCR) conditions were used: 10 s at 98 °C, 10 s at 55 °C, and 5 s at 72 °C for 20 cycles. The α -diversity and bacteria were then identified. These evaluations were performed using a software (Ekuseru-Toukei 2015, Social Survey Research Information Co., Ltd., Tokyo, Japan), and the results of α -diversity were shown using the Shannon index. In this study, all data on the major changes at the phylum, family, and genus levels were analyzed.

2.4. Constipation Score

A constipation scoring system [24] was used pre-intervention and every week thereafter to assess constipation status during the OIL and PLA phases. Constipation status was scored from 0 to 30, with 0 indicating normal constipation and 30 indicating severe constipation.

2.5. Urinary Biochemical Index

For the analysis of urinary biochemical indices, participants collected their first morning urine at home. 8-OHdG is a biomarker of oxidative DNA damage [25], and indoxyl sulphate is a gut-derived uremic toxin mainly produced by tryptophan-containing foods [26]. These biochemical indices were outsourced for analysis (Healthcare Systems Co., Ltd., Aichi, Japan).

2.6. Perilla Oil Supplementation and Dietary Assessment

Perilla oil jelly (Gifu Prefectural Research Institute for Agricultural Technology in Hilly and Mountainous Areas) was provided for the OIL phase and the participants consumed one jelly after breakfast, lunch, and dinner, a total of three times. The nutritional components per jelly of perilla oil are shown in Table 1. The jellies contained perilla oil, fructose dextrose liquid sugar, lemon juice, sugar, citric acid, and gelling agent, and were individually wrapped in film.

Table 1. Nutritional components of perilla and placebo jelly.

Energy and Nutrients		Perilla Oil Jelly (20 g/One Portion)	Placebo Jelly (20 g/One Portion)
Energy (kJ) ((kcal))		9 (38)	3 (12)
Protein (g)		0	0
Fat (g)		3	0
Omega-3	α -linolenic acid	1.9	0
Omega-6	Linoleic acid	0.5	0
Omega-9	Oleic acid	0.4	0
Others		0.3	0
Carbohydrate (g)		2.8	3.3
Salt (g)		0.1	0.1

Others: Palmitic acid, Stearic acid, etc.

A dietary assessment was conducted to calculate the nutrient intakes in the OIL and PLA phases. Participants' diets were recorded for 3 consecutive days using a food diary and camera in both phases. The diet was then reviewed by a dietitian for follow-up and nutrient intakes were estimated using software (NEW HEALTHY ver. IV, Tokyo Shoseki Co., Ltd., Tokyo, Japan).

2.7. Subjective Condition

The degrees of subjective conditions regarding fatigue, sleep quality, appetite, and psychological distress were measured using the VAS method. The degree of a subjective condition was indicated on a 100 mm horizontal line. The left side (0 mm) indicated 'feeling bad', while the right side (100 mm) indicated 'feeling good'.

2.8. Statistical Analysis

All data are presented as mean $\pm$ standard error (SE). The Shapiro–Wilk test was used to measure the normality of the variables. Differences within the phases of physical characteristics were tested using the paired *t* test. For parameters without normality, non-parametric statistical analysis, Wilcoxon signed-rank test, or Friedman's test was performed for the gut microbiota, constipation score, urinary biochemical index, nutrient intake, and subjective condition. Cohen's *d* was calculated to measure the effect size. Statistical analysis was performed using SPSS ver.25 (IBM Japan Inc., Tokyo, Japan), and statistical significance was set at *p*-value < 0.05.

3. Results

3.1. Characteristics of the Participants

There were no significant differences in body weight (kg) and body mass index (BMI; kg/m²) between pre- and post-intervention in both phases. However, body fat (%) was increased only in the PLA phase (Table 2). All participants showed excellent adherence to the intervention, with no dropouts. Mean habitual sleep hours (h:min) in the PLA and OIL phases were 7:41 $\pm$ 0:33 and 7:19 $\pm$ 0:28, respectively, with no difference between groups.

Table 2. Physical characteristics pre- and post- intervention in the OIL and PLA phases.

Physical Characteristics	OIL			PLA		
	Pre	Post	Change	Pre	Post	Change
Body weight (kg)	57.5 ± 2.7	58.0 ± 2.6	0.5 ± 0.3	58.3 ± 3.0	58.9 ± 2.9	0.6 ± 0.3
BMI (kg/m ²)	22.1 ± 0.8	22.3 ± 0.7	0.2 ± 0.1	22.4 ± 1.0	22.6 ± 0.9	0.2 ± 0.1
Body fat (%)	28.8 ± 6.1	28.9 ± 6.3	0.1 ± 0.3	29.8 ± 2.4	30.6 ± 2.3	0.8 ± 0.3 *

Data are presented as the mean ± standard error, $n = 8$. * < 0.05 vs. Pre, OIL: perilla oil intake phase, PLA: placebo intervention, Pre: pre-intervention, Post: post-intervention, BMI; body mass index.

3.2. Gut Microbiota

All data were analyzed at the family and genus levels, and the abundance (%) of Bacteroidaceae, Lachnospiraceae, Bifidobacteriaceae, and Clostridiaceae at the family level and Bacteroides, Faecalibacterium, Eubacterium, Lactobacillus, Prevotella, and Streptococcus at the genus level were detected, including the main changes (Table 3). The diversity of bacteria species was indicated by the α -diversity (Shannon index). The α -diversity was significantly increased post-intervention (4.5 ± 0.1) compared to pre-intervention (3.8 ± 0.3) in the OIL phase ($p = 0.017$, $d = 0.92$). The α -diversity in the PLA phase was changed from 4.5 ± 0.4 to 4.39 ± 0.15 without significant difference. The change (%) was significantly higher in the OIL phase ($p = 0.036$, $d = 1.20$). The most remarkable point was that the increase in α -diversity was maintained even during the washout period of 10 months. Regarding bacterial changes at the family level, butyrate-producing bacteria, Lachnospiraceae (%), were not changed in the OIL phase. In contrast, the bacteria were significantly decreased post-intervention (15.1 ± 1.7) compared to pre-intervention (20.1 ± 2.5) in the PLA phase ($p = 0.036$, $d = 0.73$). The change tended to be lower in the PLA phase ($p = 0.093$, $d = 1.43$). Furthermore, a tendency of Lachnospiraceae to increase was observed after the washout period ($p = 0.050$, $d = 0.55$) (Figure 2). In addition, Clostridiaceae, which are also butyrate-producing bacteria, tended to be increased post-intervention (2.4 ± 0.5) compared to pre-intervention (0.9 ± 0.3) only in the OIL phase ($p = 0.065$, $d = 1.96$). Other bacteria detected at the phylum, family, and genus level did not change pre- and post-intervention in either phase, except for Bifidobacteriaceae.

Table 3. Gut microbiota pre- and post- intervention in the OIL and PLA phases.

Level	Bacteria	OIL		PLA	
		Pre	Post	Pre	Post
Family	Bacteroidaceae	30.4 ± 7.3	22.7 ± 5.6	23.1 ± 3.7	18.8 ± 3.7
	Lachnospiraceae	12.8 ± 1.8	16.7 ± 2.2	20.1 ± 2.5	15.1 ± 1.7 *
	Bifidobacteriaceae	13.7 ± 6.6	10.2 ± 3.6	2.5 ± 1.1	6.3 ± 1.9 *
	Clostridiaceae	0.9 ± 0.3	2.4 ± 0.5 §	2.7 ± 0.9	4.3 ± 1.3
Genus	Bacteroides	29.9 ± 7.4	21.5 ± 5.5	22.7 ± 3.7	18.3 ± 3.6
	Faecalibacterium	17.6 ± 3.3	16.0 ± 2.8	14.4 ± 3.6	21.3 ± 5.0
	Eubacterium	4.7 ± 0.9	6.6 ± 1.9	7.3 ± 2.0	7.0 ± 2.3
	Lactobacillus	0.4 ± 0.1	0.7 ± 0.4	1.0 ± 0.3	0.7 ± 0.2
	Prevotella	0.2 ± 0.1	1.3 ± 1.2	4.2 ± 4.0	0.04 ± 0.04
	Streptococcus	0.9 ± 0.6	2.0 ± 0.9	2.9 ± 0.9	3.3 ± 2.4

Data are presented as the mean ± standard error, $n = 8$. * < 0.05 vs. Pre, § < 0.1 vs. Pre, OIL: perilla oil intake phase, PLA: placebo intervention, Pre: pre-intervention, Post: post-intervention.

3.3. Constipation Score

Constipation scores were significantly or tended to be decreased at two weeks ($p = 0.035$), three weeks ($p = 0.020$), four weeks ($p = 0.095$), five weeks ($p = 0.054$), and post ($p = 0.052$) compared to pre in the OIL phase and tended to be decreased during the OIL phase ($p = 0.072$). However, there was no change in the PLA phase. In addition, the

decrease in constipation score during the OIL phase was reversed after a 10-week washout period (Figure 3).

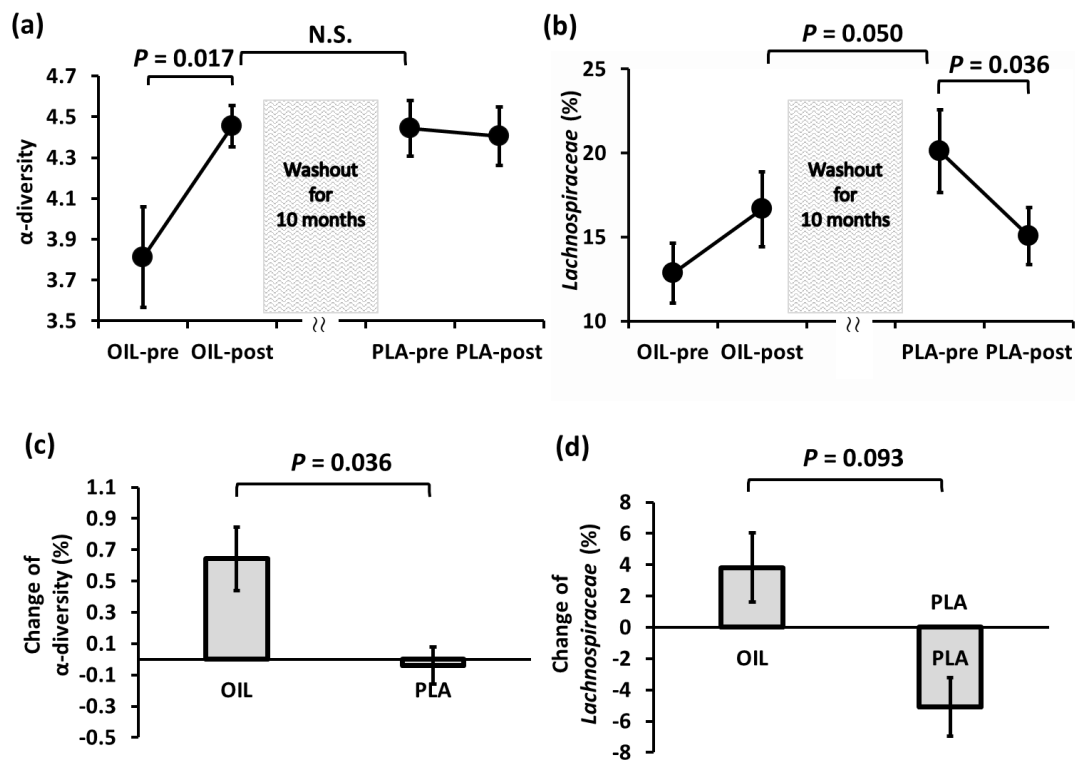


Figure 2. Changes in the gut microbiota pre- and post-intervention at the OIL and PLA phases. (a,b) Comparison of the gut microbiota within and between the phases. (c,d) Changes of the gut microbiota within and between the phases. Data are presented as the mean $\pm$ standard error, $n = 8$. OIL: perilla oil intake phase, PLA: placebo intervention, Pre: pre-intervention, Post: post-intervention, N.S.: not significant.

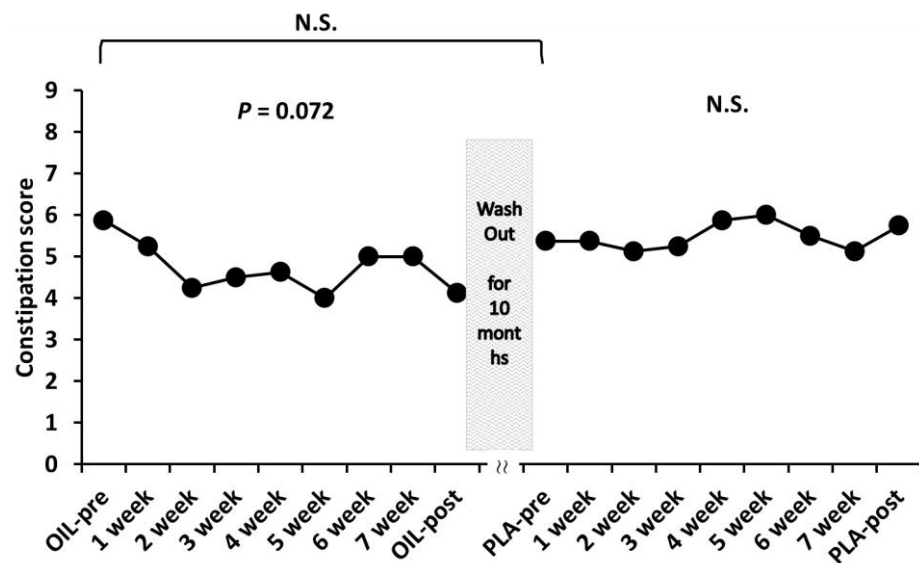


Figure 3. Changes in the constipation score at the OIL and PLA phases. Data are presented as the mean $\pm$ standard error, $n = 8$. OIL: perilla oil intake phase, PLA: placebo intervention, Pre: pre-intervention, Post: post-intervention, N.S.: not significant. Score 0 indicates no constipation and a higher score indicates severe constipation.

3.4. Urinary Biochemical Index

8-OHdG (ng/mg Cr), a biomarker of oxidative damage to DNA, did not change between pre- and post-intervention in the OIL phase. However, it was significantly increased post-intervention (5.8 ± 1.2) compared to pre-intervention (3.0 ± 0.5) in the PLA phase ($p = 0.028$, $d = 1.85$). Indoxyl sulphate ($\mu\text{g/mg Cr}$), which is an indicator of the deterioration of the intestinal environment, also did not change between pre- and post-intervention in the OIL phase and tended to increase post-intervention (59.5 ± 3.1) compared to pre-intervention (45.9 ± 6.0) in the PLA phase ($p = 0.066$, $d = 0.80$) (Figure 4).

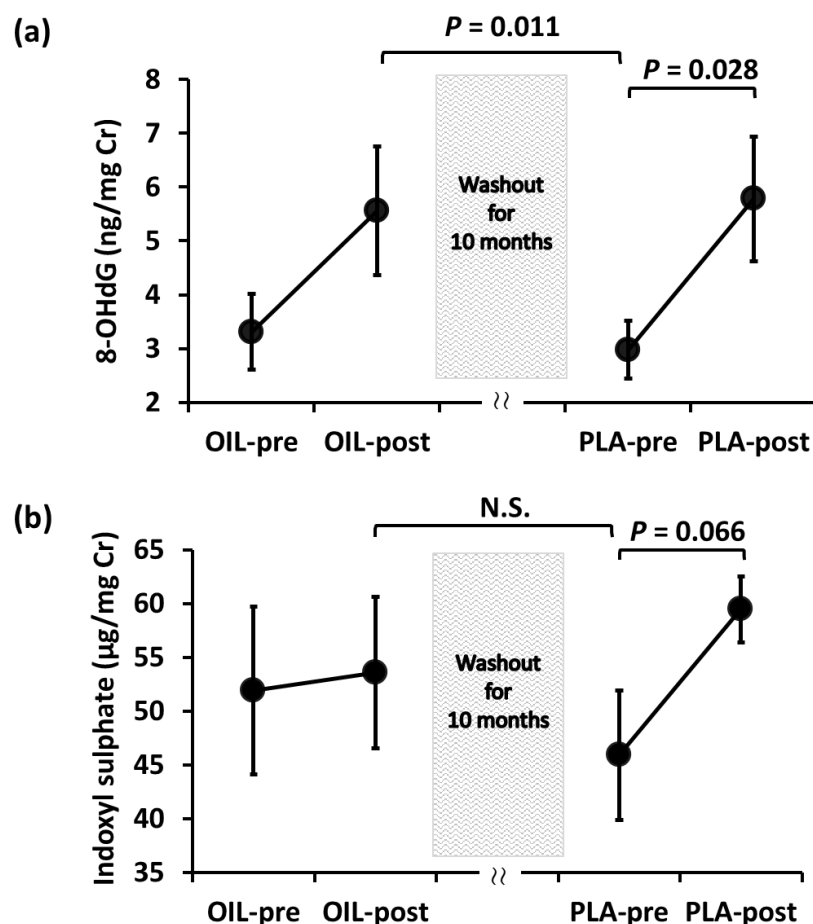


Figure 4. Changes in urinary biochemical index at the OIL and PLA phases. (a) Comparison of 8-OHdG within and between the phases. (b) Comparison of indoxyl sulphate within and between the phases. Data are presented as the mean $\pm$ standard error, $n = 8$. OIL: perilla oil intake phase, PLA: placebo intervention, Pre: pre-intervention, Post: post-intervention.

3.5. Perilla Oil Supplementation and Nutrient Intake

The intake of unsaturated fatty acids, n-3 polyunsaturated fatty acids, and n-6 polyunsaturated fatty acids of the PLA and OIL phases did not differ significantly pre-intervention. Similarly, the intake of total energy and nutrients did not differ significantly between the groups before the intervention (Table 4). Nutrient intakes do not include intakes from perilla oil-containing jelly or placebo jelly. The daily increase in energy intake through consuming the jelly was 114 in the OIL and 12 kcal in the PLA phases. Notably, none of the participants changed their eating habits during the intervention period.

Table 4. Nutrient intake in the OIL and PLA phases.

Energy and Nutrients	OIL	PLA
Energy (kJ/d) ((kcal/d))	428 ± 5.3 (1790 ± 113)	473 ± 5.5 (1979 ± 105)
Protein (g/d)	70.1 ± 5.0	74.5 ± 5.0
Fat (g/d)	67.1 ± 6.3	68.0 ± 4.8
n-3 polyunsaturated fatty acids (g/d)	1.8 ± 0.3	2.7 ± 0.4
n-6 polyunsaturated fatty acids (g/d)	11.5 ± 0.9	11.0 ± 1.3
Carbohydrate (g/d)	204.4 ± 16.3	254.1 ± 25.0
Total dietary fiber (g/d)	13.6 ± 1.3	17.2 ± 2.0
Potassium (mg/d)	2485.4 ± 256.5	2881.0 ± 224.7
Calcium (mg/d)	462.2 ± 41.8	423.0 ± 48.43
Magnesium (mg/d)	264.4 ± 32.1	284.9 ± 27.4
Iron (mg/d)	7.4 ± 0.9	8.3 ± 0.5
Zinc (mg/d)	8.5 ± 0.9	8.3 ± 0.5
Vitamin A (µg/d)	498 ± 67	658 ± 124
Vitamin D (µg/d)	3.8 ± 1.3	6.1 ± 1.0
Vitamin E (mg/d)	6.6 ± 0.7	8.2 ± 0.7
Vitamin B1 (mg/d)	0.9 ± 0.1	1.0 ± 0.1
Vitamin B2 (mg/d)	1.1 ± 0.1	1.2 ± 0.1
Folic acid (µg/d)	272.4 ± 27.1	305.0 ± 30.2
Vitamin C (mg/d)	71.5 ± 6.2	77.5 ± 11.0
Salt (g/d)	9.2 ± 0.5	8.7 ± 0.5

Data are presented as the mean ± standard error, $n = 8$. OIL: perilla oil intake phase, PLA: placebo intervention, Pre: pre-intervention, Post: post-intervention.

3.6. Subjective Condition

There was no significant change in the subjective fatigue, sleep quality, appetite, and psychological distress each week in either phase (Table 5). The results showed that the physiological conditions of the participants were not different in the two phases.

Table 5. Subjective condition in the OIL and PLA phases.

Subjective Condition	OIL									PLA								
	Pre	1 wk	2 wk	3 wk	4 wk	5 wk	6 wk	7 wk	Post	Pre	1 wk	2 wk	3 wk	4 wk	5 wk	6 wk	7 wk	Post
Fatigue	55.8	54.0	62.0	57.4	56.6	53.8	58.9	58.5	57.8	50.9	47.3	56.5	50.4	47.6	53.5	53.4	57.8	57.9
	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
Sleep quality	6.6	6.3	7.4	8.3	8.4	6.1	8.9	8.0	7.5	9.0	10.3	8.8	9.6	7.5	8.1	7.5	7.1	8.2
	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
Appetite	58.1	61.8	60.9	61.8	65.4	64.9	55.9	59.3	63.4	53.1	55.3	56.4	61.3	56.4	58.5	57.1	65.3	57.5
	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
Psychological distress	9.3	4.8	8.6	8.2	6.5	6.6	9.3	7.3	8.8	8.6	9.3	9.6	7.2	8.4	8.6	7.9	7.8	7.3
	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
	67.6	67.0	68.8	63.0	69.0	65.1	68.6	66.6	65.5	67.0	67.0	68.4	66.3	63.7	60.9	68.7	70.4	68.4
	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
	6.6	6.8	6.9	6.2	8.4	7.6	7.6	6.8	7.3	8.3	8.4	8.7	8.7	8.0	7.6	7.7	7.2	7.8
	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
	56.3	53.9	58.4	56.4	56.1	58.0	60.4	63.5	63.4	64.3	50.3	55.1	54.1	53.1	56.5	64.8	61.1	57.4
	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
	10.1	7.4	8.8	10.3	8.0	8.2	8.9	8.4	8.2	7.4	9.0	8.9	8.3	6.8	7.4	6.4	6.3	7.8
	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±

Data are presented as the mean ± standard error, $n = 8$. OIL: perilla oil intake phase, PLA: placebo intervention, Pre: pre-intervention, Post: post-intervention.

4. Discussion

Our study revealed that omega-3 unsaturated fatty acids intake through 9 g/day perilla oil for eight weeks increased the diversity of gut bacteria. Notably, the diversity was maintained for more than 10 months after the intervention. This result showed that the diversity established by a short-term dietary intervention was not affected by the free eating in a 10-month washout period. It suggested that the diversity of the gut microbiota improved by the perilla oil intervention was maintained at a higher level unless there were significant changes in diet or lifestyle. Previous studies have shown that a Western diet rich in saturated fats decreases total bacteria, whereas a Mediterranean diet rich in unsaturated fatty acids increases them [27]. Therefore, our study suggested that the function of perilla oil, unsaturated fatty acids, is to increase diversity. Future studies need to investigate

whether the change and maintenance of diversity is influenced by lifestyle factors other than perilla oil intake.

We previously investigated 9 g/day perilla oil intake for eight weeks in trained female athletes and showed that the diversity of the gut microbiota was not increased [13]. It is known that exercise habits influence the diversity of gut bacteria and changes in the gut microbiota. In fact, athletes have a higher diversity than the general population [28]. Therefore, differences in the baseline gut microbiota between trained athletes and sedentary healthy females due to exercise habits may result in different responses to gut diversity by dietary intervention. The results of this study suggested that perilla oil supplementation increases α -diversity in sedentary healthy females, who tend to have lower baseline α -diversity than athletes.

Lachnospiraceae are the main butyrate-producing bacteria. The abundance of Lachnospiraceae was maintained in the OIL phase and decreased in the PLA phase. Although the abundance of Lachnospiraceae was not increased in the OIL phase, the perilla oil intervention may have suppressed the degradation of the bacteria. This suggests that the abundance of Lachnospiraceae in the host would be decreased owing to a seasonal fluctuation from the autumn, when this intervention started, to the winter, when it was completed. Bosman ES et al. reported a positive correlation between skin exposure to ultraviolet B radiation and the amount of Lachnospiraceae [21]. Therefore, perilla oil may encourage the maintenance of Lachnospiraceae, which was similar to the results in a previous study [13]. In addition, Clostridiaceae, a butyrate-producing bacteria, also tended to be increased in the OIL phase, suggesting that perilla oil may stimulate a butyrate-producing bacteria. A longer-term intervention is required to increase these butyrate-producing bacteria in sedentary healthy females, which has the potential to increase gut SCFAs and contribute to health promotion. The increase in Bifidobacteriaceae in the PLA phase can be considered a relative change, as other more abundant bacteria at the family level, such as Lachnospiraceae and Bacteroidaceae, decreased.

A previous study demonstrated that functional constipation is associated with altered butyrate concentrations [29]. In addition, SCFAs were shown to stimulate the mucous membrane of the large intestine to promote intestinal peristalsis [30,31]. Therefore, growing butyrate from omega-3 unsaturated fatty acids may improve bowel function. This study showed that constipation was relieved within eight weeks of perilla oil intake in the OIL phase, whereas the improvement disappeared after 10 months of washout. Therefore, it was suggested that a short period of perilla oil intervention does not completely relieve constipation, and continuous intake would be effective in maintaining relief. Although the mechanism of action remains unclear [32], this study and our previous study [13] suggest that the changes in the microbiota may contribute to the improvement in bowel function. However, it cannot be concluded that gut bacteria influence constipation because the result of this study showed that α -diversity and Lachnospiraceae were maintained after 10 months of washout. In contrast to our study, Brinkworth GD et al. investigated the effect of a high-fat diet rich in saturated fat for eight weeks. They found that dietary intervention decreased fecal volume and frequency, as well as butyrate and SCFA concentrations [33]. These results suggest that changes in gut function and the abundance of butyrate-producing bacteria depend on the type of oil. In the future, it should be clarified whether the relief of constipation is due to the specific function of perilla oil. Several studies have shown that females are more likely to suffer from constipation than males [34,35]. Therefore, finding an effective solution for constipation in females is desirable. This study would suggest the solution for healthy females.

8-OHdG and indoxyl sulphate are indicators of the DNA oxidation or the deterioration of the gut environment, which was suppressed during the OIL phase. It was not clear whether seasonal changes were factors that affected the urinary biochemical index; however, the increase in the urinary biochemical index during the PLA phase may have been influenced by some environmental factors. We also measured subjective indicators of lifestyle and environment, such as fatigue, sleep quality, appetite, and psychological dis-

tress. The results showed that there were no differences between the two phases, suggesting that the interventions could have been carried out under the same conditions.

Finally, body weight and body fat did not change despite the additional energy of 81 kcal/day in the OIL phase. Therefore, daily intake of perilla oil could prevent unexpected weight gain, and perilla oil has the potential to be a daily food that supports the gut microbiota and may relieve constipation. Fat accumulation in the body depends on the type of fatty acid. A previous study indicated that a four-week diet rich in saturated fatty acids increased body weight and body fat mass compared to a diet rich in monounsaturated fatty acids [36]. On the other hand, intervention with omega-3 unsaturated fatty acids such as EPA and DHA did not affect body weight and body composition [37]. Furthermore, it was shown that the intake of α -linolenic acid, a plant-derived omega-3 unsaturated fatty acid, reduces body fat mass. Although our results suggested that 81 kcal/day of perilla oil for eight weeks did not affect body fat accumulation, the oil contains a high amount of α -linolenic acid, and long-term intake might be expected to reduce body fat mass.

Our study showed that the omega-3 unsaturated fatty acid in perilla oil enhanced the gut microbiota and relieved constipation without storing body fat; however, the study has several limitations. First, the sample size is small because this study was conducted during COVID-19. In other words, we could not recruit enough participants to do a randomized crossover design or a randomized controlled trial to observe changes in the gut microbiota 10 weeks after the intervention. Inevitably, we conducted a non-randomized placebo-controlled pilot study with a 10-month washout period. In the future, more reliable study designs should be conducted with larger numbers of subjects. Second, we did not add any oil other than omega-3 unsaturated fatty acids to the placebo group. Therefore, it is not clear whether the effects on the gut microbiota are specific to perilla oil, an oil rich in omega-3 unsaturated fatty acids, or due to oil intake. It is necessary to compare the effects of omega-3 unsaturated fatty acids on the microbiota with several other types of oil. Finally, it was not possible to assess dietary habits throughout the intervention period because of the burden on the subjects. Although the participants did not change their dietary habits during the intervention period, dietary assessment for the entire intervention period, including the washout period, is desirable to clarify the effect of perilla oil intake. In particular, longer-term dietary assessment and dietary control should be conducted in order to conclude whether an eight-week perilla oil intake continuously improves the diversity of the gut microbiota for a year.

In conclusion, this study showed that a daily intake of the omega-3 unsaturated fatty acid-rich perilla oil enhanced the diversity of the gut microbiota and maintained the abundance of butyrate-producing bacteria in sedentary healthy females. In addition, perilla oil may support the relief of constipation related to or independent of these changes in the microbiota. Therefore, daily intake of perilla oil would be beneficial for promoting health through the enhancement of the gut microbiota and relief of constipation.

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