Original Article

High poly-γ-glutamic acid-containing natto improves lipid metabolism and alters intestinal microbiota in mice fed a high-fat diet

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Several beneficial effects of poly-γ-glutamic acid (γ-PGA) have been reported. To test whether natto, a fermented soy food rich in γ-PGA, can improve intestinal microbiota content and lipid metabolism in a high-fat diet, we compared the intestinal microbiota content, plasma, liver, and fecal contents, and changes in gene expression in the livers and large intestines of a group of mice fed a high-fat diet supplemented with cooked soybeans (SC group) and a group fed a high-fat diet supplemented with natto (NA group) for 42 days; high-fat diet-fed mice were used as a control (Con group). Hepatic lipid levels were significantly lower, the fecal bile acid and lipid levels were significantly greater, and the *Bacteroidetes*/*Firmicutes* ratio was significantly higher in the SC and NA groups as compared to Con group. Additionally, plasma glucose and triglyceride levels, the expression of liver fatty acid synthase, and the relative abundance of *Lactobacillaceae* was significantly higher in the NA group than in the Con group. Although both natto and cooked soybeans impacted the metabolic response to a high-fat diet, the addition of natto had a greater effect on glucose and lipid metabolism. γ-PGA may play an important role in natto functionality.

Key Words: **poly-γ-glutamic acid, lipid metabolism, microbiota composition, *Firmicutes*/*Bacteroidetes*, high-fat diet**

Natto, a fermented soy food that is commonly consumed in Japan, is produced when boiled soybeans are fermented by *Bacillus subtilis* subsp. natto.[1] However, there are significant differences in some ingredients between soybeans and natto. For example, menaquinone-7 (MK-7), a form of vitamin K_2, is rich in fermented soybean products such as natto;[2] however, soybeans contain low levels of MK-7. Nattokinase (NK), an enzyme expressed by *B. subtilis* subsp. natto during the fermentation of soybeans to produce natto that is commonly used to treat cardiovascular diseases.[3]

Several beneficial effects of soybeans and soybean-derived food such as natto have been reported. Glucose homeostasis is improved and the progression of diabetes can be delayed in db/db mice fed soy.[4] It has also been reported that a soybean β-conglycinin diet induces β-oxidation, downregulates fatty acid synthase, and suppresses serum triglyceride (TG) levels in normal and genetically obese mice.[5] Recently, much attention has been paid to fermented soy products.[6,7] In an experiment involving 12 healthy male volunteers, a natto meal significantly suppressed the rise in blood glucose levels compared to volunteers fed the control meal.[8] In the Japanese Population-Based Osteoporosis (JPOS) Cohort Study, natto intake potentially contributed to avoiding postmenopausal bone loss due to the effects of the MK-7 or bioavailable isoflavones abundantly contained in this food product.[9] Additionally, two weeks of consuming a breakfast of natto and viscous vegetables improved insulin sensitivity, serum lipid, and oxidative stress in overweight subjects with impaired glucose tolerance.[6] Based on these reports, natto is regarded as an important functional food.

Poly-γ-glutamic acid (γ-PGA) is a major component of natto. γ-PGA is a polymer composed of D-glutamic and L-glutamic acids. The main component of natto that contributes to its sticky quality is γ-PGA. Several prebiotic effects of γ-PGA have also been reported.[10] For example, the abundance of *Lactobacillales* increases after oral administration of γ-PGA in mice.[10] In a human study, meals containing low-γ-PGA or high-γ-PGA natto were administered to men ($n = 29$) and postmenopausal women ($n = 7$); the incremental area under the curve for blood glucose within 45 min was significantly lower in subjects administered a meal containing high-γ-PGA natto than in those administered a meal containing low-γ-PGA natto.[11] Considering these reports, γ-PGA can be considered one of the major components of natto's functional properties.

In this study, we hypothesized that natto rich in γ-PGA would improve lipid metabolism and alter the intestinal microbiota of mice fed a high-fat diet. To test this assumption, we compared the intestinal microbiota, hepatic lipids, plasma lipids, and blood glucose levels between a group of mice fed a high-fat diet plus cooked soybeans (SC) and a group fed a high-fat diet plus natto (NA), which is high in γ-PGA while using high-fat diet-fed mice as a control group. In this study, we demonstrate that supplementing a diet with food rich in γ-PGA can alter intestinal microbiota content and impact lipid and glucose metabolism.

Materials and Methods

Dietary materials. The natto used in the animal study was made from natto produced by fermentation using γ-PGA-high producing *Bacillus subtilis* natto. Natto with a high γ-PGA content of 2.3% was produced by Takanofoods Co., Ltd. (Ibaraki, Japan). The γ-PGA content of natto was measured using previously described methods.[12] The Natto was freeze-dried and crushed in a mill. The milled natto was sieved through a 24-mesh sieve. Boiled soybeans were obtained from Takanofoods Co., Ltd., which were also freeze-dried and crushed in a mill and then sieved through a 24-mesh sieve.

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Table 1. Composition of the experimental diet

Ingredient (g/kg diet)	Con diet	SC diet	NA diet
Corn starch	362.479	333.529	331.429
Casein	200	139.4	136.7
α-Cornstarch	132	132	132
Sucrose	100	100	100
Soy bean oil	30	30	30
Lard	75	37.8	46.35
Cellulose	50	26.75	23
Mineral mix (AIN-93G-Mix)	35	35	35
Vitamin mix (AIN-93-Mix)	10	10	10
L-Cystine	3	3	3
Choline bitartrate	2.5	2.5	2.5
Tert-butylhydroquinone	0.021	0.021	0.021
Natto powder (high γ-PGA)	—	—	150
Boiled soy bean powder	—	150	—

Animal experiments. Male Jcl:ICR mice (6 weeks old) were purchased from CLEA Japan, Inc. (Tokyo, Japan). All mice were specific pathogen-free (SPF) and randomly subdivided into three groups of seven animals each. The mice were housed individually in suspended stainless steel cages with wire mesh bottoms in a room maintained at 24 ± 0.5°C under a relative humidity of 65% with 12-h periods of light and darkness. The mice were fed the regular maintenance AIN-93G diet for 7 days. After 7 days, their diet was replaced with a 15% high γ-PGA natto and high-fat diet (NA group), a 15% boiled soybeans and high-fat diet (SC group), or a control high-fat diet for 42 days. Freeze-dried natto and boiled soybeans were analyzed by the Japan Food Research Laboratories according to the manual of analytical methods for standard tables of food composition in Japan. The nutritional components of natto with a high γ-PGA content were as follows: moisture, 2.2%; protein, 42.2%; lipids, 19.1%; ash, 6.0%; sugar, 12.5%; and dietary fiber, 18.0%. The nutritional components of the boiled soybeans were as follows: moisture, 3.3%; protein, 40.4%; lipids, 24.8%; ash, 5.6%; sugar, 10.4%; and dietary fiber, 15.5%. Based on this information, we adjusted the composition of the NA, SC, and control diets (Con) so that each had similar protein, lipid, and carbohydrate contents. The adjusted diet composition was 20% protein, 10.5% fat, 5% dietary fiber, and 60.4% carbohydrate. The diet composition (g/kg diet) is presented in Table 1. Feces were collected 3–5 days before dissection. Feces were dried in a lyophilizer (FD-1000; Tokyo Rikakikai Co., Ltd., Tokyo, Japan) for 24 h, and the trap cooling temperature was −45°C. The mice were fasted for 4 h prior to dissection. The mice were anesthetized by isoflurane inhalation and euthanized by exsanguination. Blood from the heart was collected in heparinized tubes during anesthesia. The plasma was separated from whole blood by centrifugation and stored at −80°C until the later analysis of plasma lipids. Liver, visceral fat, and cecal contents were collected. Visceral fat weight was determined by summing the perirenal fat weight, mesenteric fat weight, and periadrenal fat weight. Livers were stored at −80°C until liver lipid analysis. Cecal contents were stored at −80°C for later DNA extraction. All procedures involving mice in this study were approved by the Animal Care Committee of the Food Research Institute (Tsukuba, Japan) (H31-024) in accordance with the "Guidelines for Animal Care and Experimentation" of the Food Research Institute, National Agriculture and Food Research Organization (Tsukuba, Japan).

Evaluation of cholesterol, TG, glucose, HDL-cholesterol, and NEFA contents in plasma. Cholesterol, TG, glucose, HDL-cholesterol, and NEFA levels were analyzed using kits purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Total plasma cholesterol concentrations were evaluated using a cholesterol oxidase-based cholesterol E-test kit. Plasma TG concentrations were determined using a TG E-test kit, based on the glycerol-3-phosphate oxidase method. Plasma glucose concentrations were quantified using a glucose CII-test kit containing glucose oxidase and peroxidase. Plasma HDL-cholesterol levels were evaluated using a cholesterol oxidase-based HDL-cholesterol E-test kit. Plasma NEFA concentrations were evaluated using the NEFA C-test kit involving acyl-coenzyme A (CoA) synthase, acyl-CoA oxidase, and peroxidase.

Evaluation of lipid, TG, and cholesterol contents in liver. Liver lipids were extracted using the Bligh and Dyer method.[13] Extracted liver lipids were dissolved in 2-propanol containing 10% Triton X-100. Liver cholesterol and TG concentrations were evaluated using methods and kits similar to those used to determine plasma cholesterol and TG levels.

Evaluation of fecal weight and fecal lipid extraction. Feces were collected for 2 days, both 4 days before dissection and 2 days before dissection. Feces were dried in a lyophilizer (FD-1000; Tokyo Rikakikai Co. Ltd.) for 24 h; the trap cooling temperature was maintained at −45°C. Lyophilized feces were then weighed and pulverized in a food mill (TML17; TESCOM Co. Ltd., Tokyo, Japan) for 30 s. Fecal lipids were extracted from fecal powder using the Bligh and Dyer method.[13]

Evaluation of fecal bile acid content. Lyophilized fecal samples were weighed and pulverized before evaluating their bile acid content. The concentration of bile acid in feces was measured using a previously described method.[14] Lyophilized fecal samples collected for 2 days during the 4 days before dissection and 2 days before dissection (50 mg) were suspended in a glass test tube containing 99.5% ethanol (2.5 ml), vortexed for 30 s, incubated for 1 h at 65°C, and centrifuged at 3,000 rpm for 10 min at 4°C. Supernatants were then transferred to a glass test-tube. An equivalent volume (2.5 ml) of 99.5% ethanol was added to the sediment and the procedure was repeated. The supernatants from both extractions were pooled in the same glass test tube and dried at 65°C using N_2 gas. After drying, 90% ethanol (0.5 ml) was added to the residue and the mixture was vortexed for 30 s. Total bile acid concentrations were evaluated using a total bile acid test (FUJIFILM Wako Pure Chemical Corporation), according to the manufacturer's instructions.

Quantitative RT-PCR analysis. Total RNA was extracted from the large intestine and liver samples using an RNeasy Mini Kit (Qiagen KK, Tokyo, Japan), according to the manufacturer's instructions. Total RNA (1 µg) was reverse-transcribed into cDNA using SuperScript II reverse transcriptase and random primer oligonucleotides (Takara Bio, Shiga, Japan) in a 20 µl final volume. Quantitative RT-PCR was performed using a Real-Time QPCR System QuantStudio (Thermo Fisher Scientific Inc., Waltham, MA) in a total volume of 20 µl in the presence of cDNA (1 µl solution) plus 19 µl of the reaction mix [0.4 µl each of 10 µM primer and 10 µl of 2× KAPA SYBER FAST qPCR Master Mix Universal (Kapa Biosystems Inc., Wilmington, MA)]. Qunatitative PCR (qPCR) was carried out under the following conditions: one cycle for 3 min at 95°C, 40 cycles for 3 s at 95°C, and 30 s at 62°C for the expression analysis of *Fxr* (liver), *Fasn* (liver), *Ho-1* (liver), *Il-4* (large intestine), *Il-10* (large intestine), and *Tnf-α* (large intestine); one cycle for 3 min at 95°C, 40 cycles for 3 s at 95°C, and 50 s at 62°C for the expression analysis of *Srebp-1c* (liver), *Pepck* (liver), *Gck* (liver), *G6pase* (liver), and *Chrebp* (liver); one cycle for 3 min at 95°C, 40 cycles for 5 s at 95°C, 20 s at 62°C, and 25 s at 72°C for the expression analysis of *Il-6* (large intestine); and one cycle for 3 min at 95°C, 40 cycles for 5 s at 95°C, 20 s at 62°C, and 30 s at 72°C for the expression analysis of *Ifn-γ* (large intestine). We used primer sets for *Fasn*, *Fxr*, *G6pase*, *Gck*, *Ho-1*, *Pepck*, *Srebp-1c*, and *Chrebp* for the qPCR analysis of gene expression

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Table 2. Plasma lipids, glucose and general observations

	Chol (mg/dl)	TG (mg/dl)	NEFA (mEq/L)	HDL-Chol (mg/dl)	Glucose (mg/dl)	Final body weight (g)	Visceral fat (g/100 g BW)	Liver weight (g/100 g BW)	Cecal contents (g/100 g BW)	Food consumption (g/day)
Con	175.0 ± 10.0	108.5 ± 28.5[a]	0.99 ± 0.13	69.3 ± 3.4	305.3 ± 20.0[a]	44.9 ± 1.2	6.51 ± 0.39	4.78 ± 0.13	0.43 ± 0.05[a]	4.55 ± 0.09
SC	152.5 ± 12.1	75.0 ± 9.9[a]	0.92 ± 0.08	65.7 ± 4.5	268.7 ± 13.5[a]	44.2 ± 0.6	6.12 ± 0.40	4.40 ± 0.23	0.71 ± 0.06[b]	4.61 ± 0.08
NA	163.0 ± 11.6	53.4 ± 4.7[b]	0.87 ± 0.09	68.5 ± 3.3	216.6 ± 18.4[b]	43.0 ± 0.9	5.25 ± 0.61	4.27 ± 0.20	0.63 ± 0.08[a,b]	4.53 ± 0.08

Significant differences ($p<0.05$) between different superscript. Values are expressed as mean ± SE ($n = 7$).

in the liver, and primer sets for *Il-4*, *Il-6*, *Il-10*, *Ifn-γ*, and *Tnf-α* for the qPCR analysis of gene expression in the large intestine. Primer sequences are shown in Supplemental Table 1*.

The relative amount of each transcript of *Fasn*, *Fxr*, *G6pase*, *Gck*, *Ho-1*, *Il-4*, *Il-10*, *Pepck*, *Srebp-1c*, *Tnf-α*, and *Chrebp* was normalized to the amount of *Gapdh* in the same cDNA. The relative amount of each transcript of *Il-6* and *Ifn-γ* was normalized to the amount of *β-actin* in the same cDNA. To ensure the specificity of this PCR analysis, a melting curve analysis was conducted after amplification. The melting curves were obtained by heating at temperatures from 60°C to 95°C with continuous fluorescence monitoring.

Microbiota analysis. Cecal DNA was prepared using previously described methods.[15] Equivalent amounts of cecal DNA (10 ng each) were used as templates, and the V3 and V4 regions of 16S rRNA genes were amplified using the 341F (5'-CCTACGGGNGGCWGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') primers,[16,17] which were linked to the Illumina overhang adapter sequences. An additional PCR cycle was performed to add barcodes to each sample. Amplicons were pooled in equal amounts and paired-end (2 × 300 bp) sequencing was performed using the MiSeq System (Illumina Inc., San Diego, CA) and MiSeq Reagent Kit ver. 3 (Illumina Inc.) following quantification. QIIME2 2019.4.0 (https://qiime2.org) was used to analyze the sequences in demultiplexed format. Denoising of merged paired-end reads was performed using the DADA2 error model.[18] Sequence variants that originated from chloroplasts and mitochondria were removed from further analyses, and the Greengene database was used for each representative sequence to annotate the taxonomic information.[19,20] Finally, α- and β-diversities were analyzed by rarefying the feature table at a consistent sample depth of 57,000.

Statistical analysis. Data are expressed as the mean ± SE. All data were analyzed in Sigma Plot 11 (Systat Software, Inc., San Jose, CA) using a one-way analysis of variance (one-way ANOVA); When an ANOVA p value was significant, Tukey's test was performed. $P<0.05$ was considered to be statistically significant. We also used Spearman Rank Order Correlation and Pearson Product Moment Correlation for the correlation analysis of the data.

Results

General observations between the Con, SC, and NA groups. No significant differences were observed among the three dietary groups in terms of final body weight, visceral fat, and food consumption (Table 2). The liver weight (g/100 g BW) were not significantly different among three group (Table 2). The cecal content of the SC group was significantly greater than that of the Con group (Table 2).

Plasma lipid and glucose concentrations. Plasma total cholesterol, TG, non-esterified fatty acids (NEFA), high-density lipoprotein (HDL)-cholesterol, and glucose concentrations are shown in Table 2. Plasma TG levels were significantly lower in the NA group than in the Con group. Plasma TG levels tended to be lower in the SC group than in the Con group, although these differences were not statistically significant. Plasma glucose levels were significantly lower in the NA group than in the Con group. Plasma glucose levels also tended to be lower in the SC group than in the Con group, although these differences were not statistically significant. No significant differences were observed in NEFA or HDL cholesterol levels among the three groups.

Hepatic lipid levels are altered in the SC and NA groups. Table 3 shows lipid, TG, and cholesterol levels in the liver. Hepatic lipid levels were significantly lower in the SC and NA groups than those in the Con group. Liver TG levels were also significantly lower in the SC and NA groups than those in the Con group. The mean liver TG levels in the NA group were the lowest among the three groups and were significantly different when compared with those in the Con group. Finally, liver cholesterol levels were significantly lower in the SC and NA groups than those in the Con group ($p<0.05$).

Fecal bile acid amounts are altered in the SC and NA groups. The amount of bile acid excreted in the feces is shown in Fig. 1. The amount of bile acid excreted in the feces was significantly greater both in the SC (1.10 ± 0.09 μmol/day) and NA (1.13 ± 0.11 μmol/day) groups than in the Con group (0.64 ± 0.10 μmol/day) ($p<0.05$).

Table 3. Hepatic lipids

	Hepatic total lipids (mg)	TG (mg)	Chol (mg)
Con	199.3 ± 16.6[a]	141.6 ± 13.1[a]	12.4 ± 1.5[a]
SC	119.1 ± 7.5[b]	65.7 ± 8.0[b]	6.6 ± 1.1[b]
NA	109.3 ± 6.3[b]	55.8 ± 5.4[b]	5.5 ± 0.9[b]

Significant differences ($p<0.05$) between different superscript. Values are expressed as mean ± SE ($n = 7$).

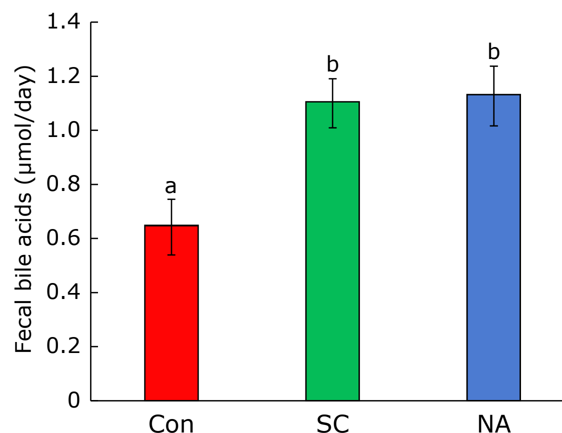

Fig. 1. Amounts of bile acid excreted in the feces of mice in the control (Con), a high-fat diet plus soybeans (SC), and a high-fat diet plus natto (NA) groups (g/day). Values are presented as mean ± SE (n = 7). Significant differences ($p<0.05$) are indicated by superscript.

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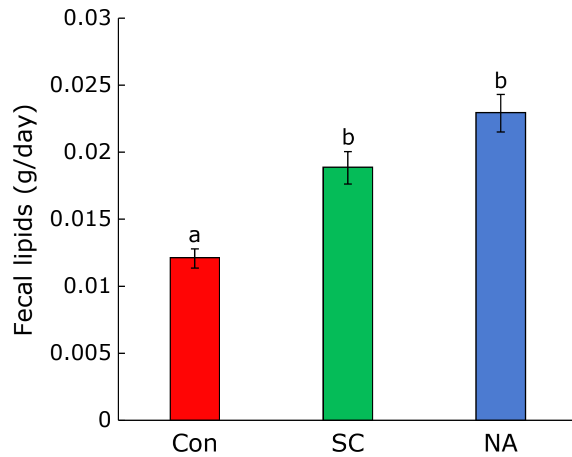

Fig. 2. Amounts of fecal lipids in the Con, SC, and NA groups (g/day). Values are expressed as mean ± SE ($n = 7$). Significant differences ($p<0.05$) are indicated by superscript.

Fecal lipid amounts are altered in the SC and NA groups. The amount of lipids in freeze-dried feces was significantly greater in the SC (0.019 ± 0.001 g/day) and NA (0.023 ± 0.001 g/day) groups than in the Con group (0.012 ± 0.001 g/day) (Fig. 2). The mean fecal lipid excretion amount was highest in the NA group.

Changes in gene expression are observed in the SC and NA groups. The results of the gene expression analysis in the liver using quantitative real-time (RT)-PCR are shown in Fig. 3. The NA group showed a significantly lower liver expression level of fatty acid synthase (*Fasn*) than the Con group (Fig. 3D), but there was no significant difference between the SC and Con groups. The NA and SC groups showed significantly lower liver expression levels of sterol regulatory element binding protein-1c (*Srebp-1c*) than the Con group (Fig. 3E). The NA group showed a significantly higher liver expression level of heme oxygenase-1 (*Ho-1*) than the Con group (Fig. 3G). There were no significant differences in the expression of glucokinase (*Gck*) (Fig. 3A), glucose 6-phosphatase (*G6pase*) (Fig. 3B), phosphoenolpyruvate carboxykinase (*Pepck*) (Fig. 3C), farnesoid x receptor (*Fxr*) (Fig. 3F), or carbohydrate-responsive element-binding protein (*Chrebp*) (Fig. 3H) in the liver among the three groups.

Gene expression results in the large intestine are shown in Fig. 4. No significant differences were observed in the expression of interleukin 6 (*Il-6*) (Fig. 4A), tumor necrosis factor-alpha (*Tnf-α*) (Fig. 4B), or interferon-gamma (*Ifn-γ*) (Fig. 4C) in the large intestine among the three groups. The expressions of interleukin 4 (*Il-4*) (Fig. 4D) and interleukin 10 (*Il-10*) (Fig. 4E) in the large intestine tended to be higher in the NA group than in the SC and Con groups. The expression ratio of *Ifn-γ* to *Il-4* (Fig. 4F) was significantly lower in the SC and NA groups than in the Con group.

Cecal microbiome composition is altered in the SC and NA groups. The α-diversity results demonstrating microbiome diversity in the cecum are shown in Table 4. Faith pd, a phylogenetic metric, in the SC and NA groups was significantly greater than that in the Con group. The observed Operational Taxonomic Units (OTUs) from the NA group were significantly greater than that in the Con group. No significant differences were observed in the Shannon index among the three dietary groups. The results of the β-diversity (Unweighted UniFrac) analysis are shown in Fig. 5. The Con group was distinguishable from the NA and SC groups in the Unweighted UniFrac analysis. Analysis of the intestinal microbiota at the phylum and family levels revealed significant differences in the composition of microbiota among the three dietary groups (Table 5). At the phylum level, the relative abundance of *Firmicutes* was significantly lower in the NA and SC groups than in the Con group. The relative abundance of *Actinobacteria* was significantly higher in the NA group than in the Con group. The relative abundance of *Bacteroidetes* was significantly higher in the SC group than in the Con group. The *Bacteroidetes*/*Firmicutes* ratio was significantly higher in the NA (0.11 ± 0.02) and SC (0.13 ± 0.03) groups than in the Con group (0.04 ± 0.01). The composition of microbiota at the family level was then analyzed. Relative abundances of *Turicibacteraceae*, *Streptococcaceae*, *Clostridiaceae*, and *Peptococcaceae* were significantly lower in the NA group

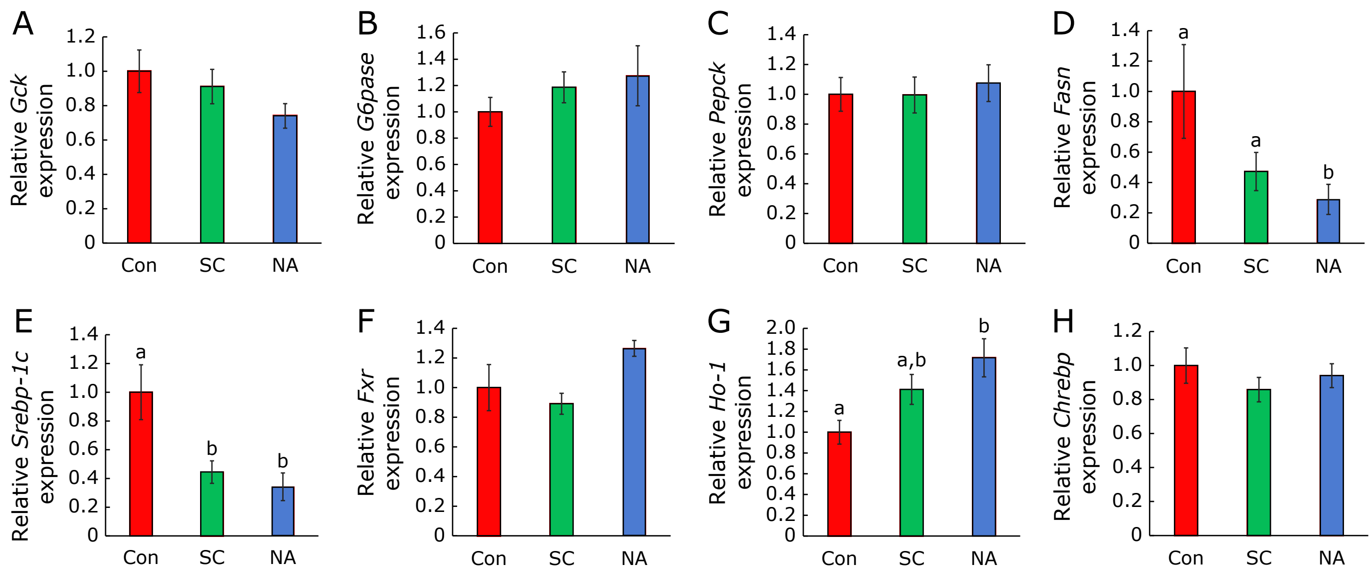

Fig. 3. mRNA expression levels in the liver, as determined by quantitative real-time (RT)-PCR, of (A) glucokinase (*Gck*), (B) glucose 6-phosphatase (*G6pase*), (C) phosphoenolpyruvate carboxykinase (*Pepck*), (D) fatty acid synthase (*Fasn*), (E) sterol regulatory element binding protein-1c (*Srebp-1c*), (F) farnesoid x receptor (*Fxr*), (G) heme oxygenase-1 (*Ho-1*), and (H) carbohydrate-responsive element-binding protein (*Chrebp*) in the Con, SC, and NA groups. Significant differences ($p<0.05$) are indicated by superscript.

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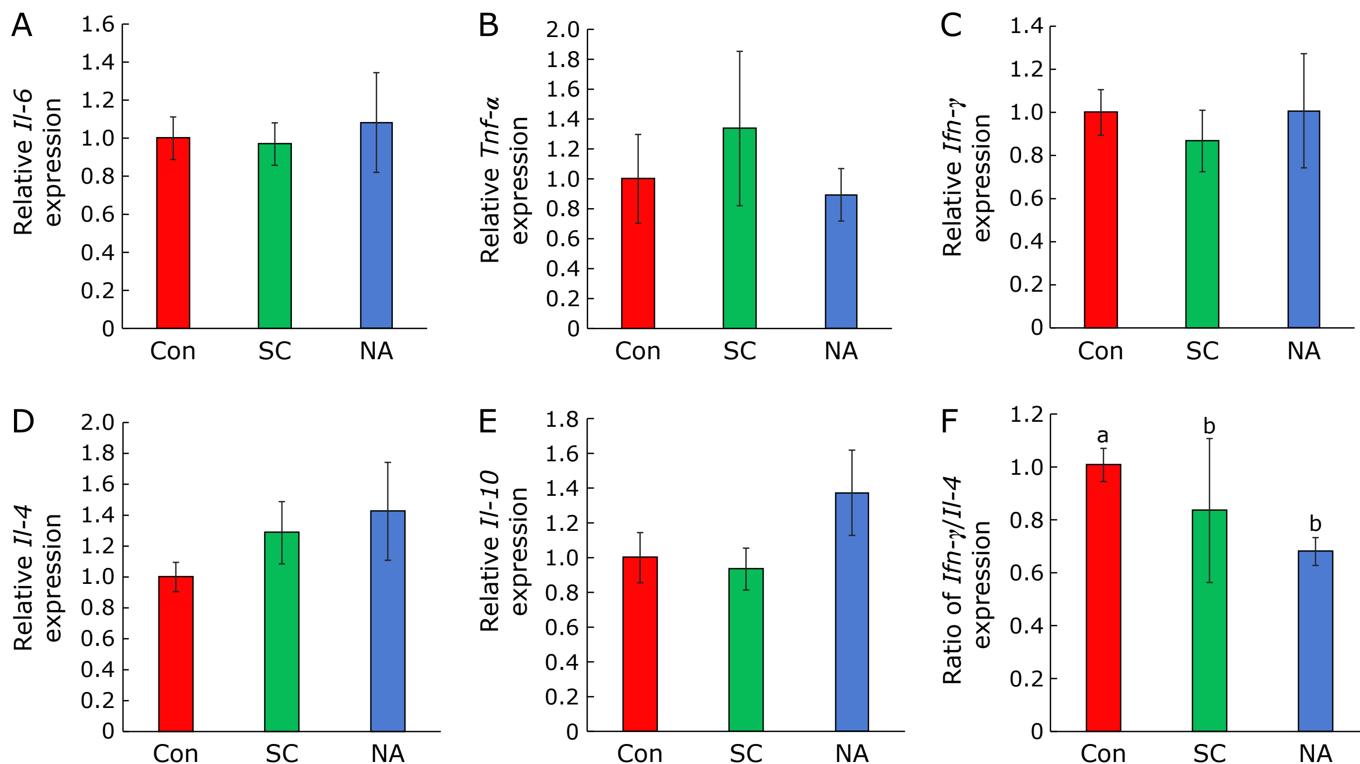

Fig. 4. mRNA expression levels in the large intestine, as determined by quantitative RT-PCR, of (A) interleukin 6 (*Il-6*), (B) tumor necrosis factor-alpha (*Tnf-α*), (C) interferon-gamma (*Ifn-γ*), (D) interleukin 4 (*Il-4*), and (E) interleukin 10 (*Il-10*) in the Con, SC, and NA groups. (F) The expression ratio of *Ifn-γ* to *Il-4* (i.e., expression of *Inf-γ*/expression of *Il-4*) among the three groups. Significant differences (p<0.05) are indicated by superscript.

Table 4. Results of α-diversity

	Faith pd	Observed OTUs	Shannon index
Con	11.4 ± 0.3^{a}	165.4 ± 10.3^{a}	3.6 ± 0.2
SC	12.9 ± 0.4^{b}	187.6 ± 11.1^{a,b}	4.2 ± 0.4
NA	13.1 ± 0.3^{b}	213.1 ± 11.3^{b}	3.8 ± 0.2

Significant differences (p<0.05) between different superscript. Values are expressed as mean ± SE (n = 7).

than in the Con group. In contrast, the relative abundances of *Bacteroidales* family S24-7, *Bacillaceae*, *Coriobacteriaceae*, *Dehalobacteriaceae*, *Mogibacteriaceae*, and *Lactobacillaceae* were significantly greater in the NA group than in the Con group. The relative abundance of *Rikenellaceae* was significantly lower in the NA group than that in the SC group. The relative abundances of *Verrucomicrobiaceae*, *Mogibacteriaceae*, *Eubacteriaceae*, *Dehalobacteriaceae*, *Paraprevotellaceae*, *Bacteroidales* family S24-7, and *Prevotellaceae* were significantly higher in the SC group than in the Con group.

Correlation analysis. We constructed a heat map illustrating the correlation between the relative abundance of different bacteria phyla or families; the amount of liver lipids, cholesterol, and TG; the concentration of plasma cholesterol, TG, glucose, and NEFA; the amount of bile acids and fecal lipids concentration; the amount of visceral fat; the expression of *Fasn*, *Fxr*, *G6pase*, *Gck*, *Ho-1*, *Pepck*, *Srebp-1c*, and *Chrebp* in the liver; and the expression of *Il-4*, *Il-6*, *Il-10*, *Ifn-γ*, and *Tnf-α* in the large intestine between the three dietary groups (Fig. 6). At the phylum level, the abundance of *Bacteroidetes* was negatively correlated with liver lipid, cholesterol, and TG levels, and the expression of *Srebp-1c* and *Fasn*; a positive correlation was

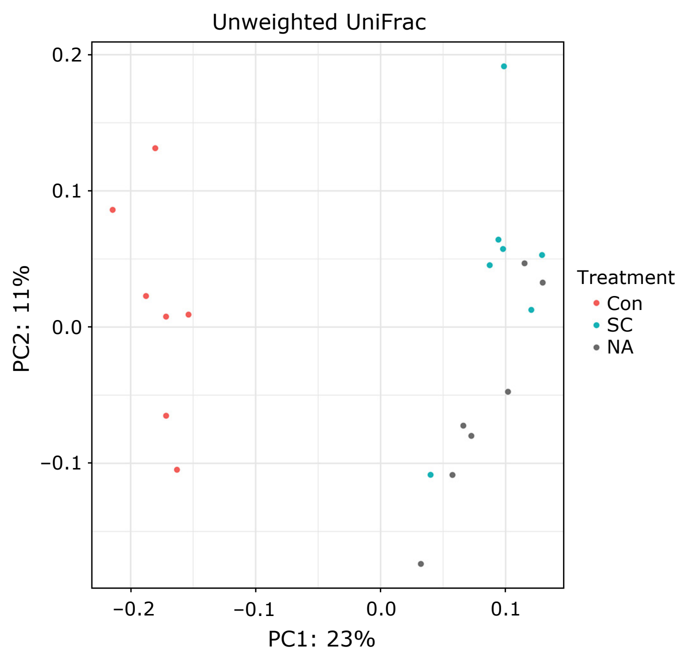

Fig. 5. Comparison of microbial communities in the Con, SC, and NA groups using a β-diversity (Unweighted UniFrac) analysis.

observed with bile acids and fecal lipids. In contrast, *Firmicutes* showed an inverse correlation with *Bacteroidetes*, in which the abundance of *Firmicutes* showed a positive correlation with liver lipid, cholesterol, and TG levels and the expression of *Srebp-1c*

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Table 5. Relative abundance of cecal microbiota of mice in the Con, SC, and NA groups

Phylum/Family	Con	SC	NA
Actinobacteria	3.68 ± 0.89[a]	8.54 ± 2.16[a,b]	10.99 ± 2.35[b]
Nakamurellaceae	0.0005 ± 0.0005	0	0
Bifidobacteriaceae	0.02 ± 0.01	0	0
Coriobacteriaceae	3.66 ± 0.89[a]	8.54 ± 2.16[a,b]	10.99 ± 2.35[b]
Bacteroidetes	3.46 ± 0.90[a]	9.77 ± 1.70[b]	8.02 ± 1.24[a,b]
Bacteroidaceae	0.96 ± 0.34	1.65 ± 0.38	1.28 ± 0.28
Prevotellaceae	0.0006 ± 0.0006[a]	0.19 ± 0.05[b]	0.02 ± 0.01[a,b]
Rikenellaceae	0.26 ± 0.04[a,b]	0.63 ± 0.13[a]	0.27 ± 0.05[b]
Bacteroidales family S24-7	2.07 ± 0.54[a]	6.22 ± 1.07[b]	5.83 ± 0.89[b]
Paraprevotellaceae	0.17 ± 0.07[a]	1.07 ± 0.22[b]	0.61 ± 0.26[a,b]
Firmicutes	91.96 ± 0.67[a]	75.72 ± 2.27[b]	76.38 ± 2.81[b]
Bacillaceae	0.0004 ± 0.0004[a]	0.0025 ± 0.0016[a]	2.23 ± 0.5[b]
Staphylococcaceae	0.0047 ± 0.0026	0.0024 ± 0.0016	0.005 ± 0.004
Enterococcaceae	0.0012 ± 0.0012	0	0.0006 ± 0.0006
Lactobacillaceae	16.65 ± 3.76[a]	25.99 ± 7.47[a,b]	40.26 ± 6.86[b]
Streptococcaceae	0.11 ± 0.02[a]	0.08 ± 0.02[a,b]	0.04 ± 0.01[b]
Turicibacteraceae	11.3 ± 3.36[a]	2.37 ± 1.34[a,b]	0.58 ± 0.36[b]
Christensenellaceae	0.07 ± 0.01[a]	0.02 ± 0.01[b]	0.04 ± 0.01[a,b]
Clostridiaceae	3.81 ± 1.17[a]	0.98 ± 0.67[a,b]	0.01 ± 0.01[b]
Dehalobacteriaceae	0.0093 ± 0.0024[a]	0.11 ± 0.03[b]	0.07 ± 0.02[b]
Eubacteriaceae	0[a]	0.0048 ± 0.0018[b]	0.0017 ± 0.0007[a,b]
Lachnospiraceae	13.64 ± 4.03	7.8 ± 1.5	4.19 ± 0.78
Peptococcaceae	0.0196 ± 0.0032[a]	0.0183 ± 0.0124[b]	0.008 ± 0.002[b]
Peptostreptococcaceae	0.0003 ± 0.0003	0	0
Ruminococcaceae	3.39 ± 0.6	5.21 ± 1.35	3.09 ± 0.41
Veillonellaceae	0	0.0012 ± 0.0012	0
Mogibacteriaceae	0.04 ± 0.01[a]	0.29 ± 0.08[b]	0.16 ± 0.03[b]
Erysipelotrichaceae	37.22 ± 7.78[a]	13.04 ± 5.77[b]	18 ± 5.28[a,b]
Proteobacteria	0.31 ± 0.04	0.53 ± 0.14	0.38 ± 0.05
Alcaligenaceae	0.31 ± 0.04	0.51 ± 0.13	0.33 ± 0.05
Enterobacteriaceae	0.0015 ± 0.0015	0.01 ± 0.01	0.06 ± 0.05
TM7	0.04 ± 0.01	0.12 ± 0.05	0.06 ± 0.03
F16	0.04 ± 0.01	0.12 ± 0.05	0.06 ± 0.03
Verrucomicrobia	0.54 ± 0.28[a]	5.32 ± 1.40[b]	4.17 ± 1.38[a,b]
Verrucomicrobiaceae	0.54 ± 0.28[a]	5.32 ± 1.40[b]	4.17 ± 1.38[a,b]

Significant differences ($p<0.05$) are indicated by superscript. Values are expressed as the mean ± SE ($n = 7$).

and *Fasn*; a negative correlation was observed with bile acids and fecal lipids. The ratio of the abundance of *Bacteroidetes*/abundance of *Firmicutes* showed a negative correlation with liver lipid, cholesterol, and TG levels and the expression of *Srebp-1c* and *Fasn*; a positive correlation was observed with bile acids and fecal lipids. At the family level, the abundance of *Coriobacteriaceae* and *Dehalobacteriaceae* was negatively correlated with plasma glucose, liver lipid, liver cholesterol, and liver TG levels and expression of *Srebp-1c* in the liver. A negative correlation was observed between the abundance of both *Prevotellaceae* and *Bacteroidales* family S24-7 and liver lipid, cholesterol, and TG levels; a positive correlation was observed with bile acids and fecal lipids. A positive correlation was observed between the abundance of *Turicibacteraceae* and plasma glucose, liver lipid, liver cholesterol, and liver TG levels, and the expression of *Srebp-1c* and *Fasn*; a negative correlation was observed with bile acids and fecal lipids. A negative correlation was observed between the abundance of *Mogibacteriaceae* and liver lipid, cholesterol, and TG levels, the expression of *Srebp-1c* and *Fasn*, and plasma cholesterol and TG levels; a positive correlation was observed with bile acids and fecal lipids. A positive correlation was observed between the abundance of *Erysipelotrichaceae* and liver lipid, cholesterol, and TG levels; a negative correlation was observed with bile acids and fecal lipids.

Discussion

We compared the intestinal microbiota, liver lipids, plasma lipids, blood glucose levels, fecal lipids concentration, amounts of fecal bile acids, and gene expression levels in the liver and large intestine between three high-fat dietary groups to compare any differences correlated with the addition of soybean or natto.

The abundance of *Bacillaceae* and *Lactobacillaceae* was significantly greater in the NA group than in the Con group. There are few reports on the effects of administration of menaquinone-7 on the intestinal microbiota and on lipid metabolism. It has been reported that administration of *Bacillus*

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		P-Chol	P-TG	P-Glu	P-NEFA	Visceral fat	Liver lipids	Liver -Cho	Liver -TG	Bile acids	Fecal lipids	*Fxr*	*Srebp -1c*	*Chrebp*	*G6Pase*	*Gck*	*Fasn*	*Pepck*	*Ho-1*	*Il-10*	*Il-6*	*Ifn-γ*	*Tnf-α*	*Il-4*	*Ifn-γ /Il-4*
Phylum	*Actinobacteria*	−0.25	−0.30	−0.39	−0.05	−0.40	−0.80*	−0.62*	−0.80*	0.27	0.58*	0.09	−0.36	−0.11	0.21	−0.17	−0.28	0.05	0.76*	−0.08	−0.17	−0.24	0.00	0.16	−0.55*
	Bacteroidetes	−0.10	−0.01	−0.14	−0.19	−0.04	−0.51*	−0.56*	−0.44*	0.41	0.59*	−0.11	−0.49*	−0.25	0.16	−0.15	−0.27	0.31	0.23	−0.07	−0.04	−0.05	0.01	−0.16	−0.07
	Firmicutes	0.33	0.24	0.36	0.07	0.37	0.80*	0.66*	0.79*	−0.47*	−0.72*	−0.12	0.64*	0.19	−0.18	0.30	0.50*	−0.13	−0.64*	0.09	0.11	0.20	−0.05	−0.06	0.38
	Proteobacteria	−0.23	−0.10	−0.11	−0.17	−0.30	−0.25	−0.31	−0.30	0.27	0.29	−0.03	−0.18	−0.04	0.14	−0.14	−0.19	0.34	0.26	−0.02	0.11	0.19	−0.03	0.18	0.00
	TM7	−0.14	0.05	0.07	−0.09	−0.22	0.05	−0.05	−0.01	0.53*	0.27	−0.16	−0.29	−0.30	−0.06	−0.54*	−0.47*	0.28	−0.11	−0.13	0.08	−0.13	−0.42	0.08	−0.12
	Tenericutes	−0.09	−0.24	−0.15	0.27	0.17	0.03	0.08	−0.04	0.43	0.11	0.24	−0.38	0.02	−0.04	−0.45*	−0.40	0.02	0.16	−0.19	−0.34	−0.37	−0.05	−0.11	−0.29
	Verrucomicrobia	−0.31	−0.11	−0.17	0.23	0.01	−0.46*	−0.33	−0.46*	0.67*	0.53*	0.43	−0.45*	−0.03	0.00	−0.20	−0.43	−0.04	0.30	0.16	−0.03	−0.17	−0.02	0.36	−0.56*
	Bacteroidetes/Firmicutes	−0.11	−0.06	−0.16	−0.17	−0.07	−0.60*	−0.61*	−0.55*	0.42	0.65*	−0.11	−0.54*	−0.23	0.14	−0.15	−0.34	0.31	0.35	−0.05	−0.04	−0.05	0.02	−0.07	−0.16
Family	*Nakamurellaceae*	0.33	0.33	0.37	0.37	0.37	0.33	0.22	0.33	−0.22	−0.30	−0.07	0.33	0.37	0.15	0.37	0.37	0.19	−0.11	0.26	0.11	0.19	−0.22	0.11	0.11
	Bifidobacteriaceae	0.12	0.59*	0.38	0.42	0.35	0.47*	0.37	0.50*	−0.48*	−0.50*	0.00	0.61*	0.51*	0.01	0.53*	0.58*	−0.28	−0.46*	0.11	0.39	0.37	−0.04	0.11	0.39
	Coriobacteriaceae	−0.25	−0.30	−0.39	−0.05	−0.40	−0.80*	−0.62*	−0.80*	0.27	0.58*	0.09	−0.36	−0.11	0.21	−0.17	−0.28	0.05	0.76*	−0.08	−0.17	−0.24	0.00	0.16	−0.55*
	Bacteroidaceae	0.04	0.04	−0.02	−0.11	−0.09	−0.17	−0.16	−0.14	0.12	0.23	−0.12	−0.53*	−0.30	−0.15	−0.31	−0.36	0.25	0.00	−0.27	−0.15	−0.11	−0.02	−0.23	0.09
	Prevotellaceae	−0.20	−0.06	−0.17	−0.02	−0.15	−0.61*	−0.59*	−0.64*	0.55*	0.64*	−0.12	−0.32	−0.32	0.24	0.00	−0.19	0.07	0.42	0.01	0.04	−0.05	−0.09	0.21	−0.46*
	Rikenellaceae	0.01	0.38	0.22	0.17	0.36	0.04	0.10	0.06	0.17	0.00	−0.08	−0.10	−0.18	−0.16	0.08	0.02	0.16	−0.02	−0.15	0.08	0.12	−0.01	0.04	0.08
	Bacteroidales family S24-7	−0.13	−0.17	−0.18	−0.28	−0.08	−0.64*	−0.71*	−0.58*	0.46*	0.75*	−0.07	−0.49*	−0.22	0.30	−0.12	−0.28	0.27	0.36	0.02	−0.04	−0.05	0.12	−0.12	−0.18
	Paraprevotellaceae	−0.09	0.16	−0.20	0.19	−0.17	−0.38	−0.33	−0.45*	0.40	0.45*	0.00	−0.43	−0.26	−0.09	−0.24	−0.41	0.13	0.25	−0.30	0.22	0.08	−0.25	0.15	−0.16
	Bacillaceae	0.13	−0.37	−0.53*	−0.17	−0.45*	−0.55*	−0.58*	−0.56*	0.37	0.60*	0.39	−0.44*	−0.01	0.14	−0.23	−0.44*	0.15	0.32	0.20	0.01	−0.02	0.01	0.13	−0.27
	Staphylococcaceae	0.28	0.10	0.26	0.29	0.31	0.34	0.17	0.32	−0.09	−0.11	0.04	−0.26	0.07	0.00	−0.45*	−0.25	0.43	−0.17	−0.26	−0.28	−0.41	−0.45*	−0.38	0.08
	Enterococcaceae	−0.27	−0.01	−0.26	−0.15	−0.26	0.03	−0.23	−0.02	0.06	0.14	−0.07	0.40	0.32	0.26	0.25	0.22	−0.30	−0.28	0.31	0.48*	0.34	−0.06	0.31	0.12
	Lactobacillaceae	−0.02	−0.56*	−0.47*	−0.13	−0.40	−0.27	−0.29	−0.38	0.34	0.31	0.46*	−0.09	−0.05	0.30	−0.09	−0.14	−0.50*	0.16	0.20	0.04	0.08	0.39	0.19	−0.40
	Streptococcaceae	0.17	0.44*	0.35	0.21	0.32	0.63*	0.36	0.58*	−0.20	−0.37	−0.33	0.23	0.14	−0.18	0.12	0.15	−0.03	−0.85*	−0.10	−0.07	−0.07	−0.28	−0.27	0.46*
	Turicibacteraceae	0.01	0.26	0.45*	−0.29	0.13	0.53*	0.36	0.54*	−0.44*	−0.57*	−0.50*	0.76*	0.17	−0.12	0.44*	0.70*	−0.21	−0.49*	−0.13	0.01	0.24	0.13	−0.27	0.58*
	Christensenellaceae	0.06	−0.15	−0.06	−0.08	0.13	0.37	0.38	0.42	−0.35	−0.40	0.23	−0.07	0.24	−0.36	−0.23	−0.16	0.11	−0.29	−0.05	−0.22	−0.17	−0.04	−0.33	0.34
	Clostridiaceae	−0.04	0.27	0.44*	−0.10	0.19	0.46*	0.40	0.47*	−0.47*	−0.63*	−0.48*	0.65*	0.07	−0.26	0.41	0.62*	−0.01	−0.37	−0.24	−0.04	0.13	−0.03	−0.18	0.35
	Dehalobacteriaceae	−0.15	−0.11	−0.33	0.22	−0.18	−0.51*	−0.37	−0.56*	0.49*	0.55*	0.09	−0.64*	−0.17	−0.20	−0.39	−0.61*	0.21	0.50*	−0.28	−0.05	−0.11	−0.24	0.16	−0.38
	Eubacteriaceae	0.09	−0.14	−0.29	0.01	−0.22	−0.46*	−0.60*	−0.57*	0.30	0.63*	−0.12	−0.20	−0.05	0.28	−0.02	−0.16	−0.27	0.28	−0.19	−0.10	−0.08	−0.04	−0.07	−0.23
	Lachnospiraceae	0.26	0.36	0.45*	−0.13	0.35	0.44*	0.30	0.50*	−0.10	−0.37	−0.23	0.10	−0.09	−0.19	−0.04	0.19	0.39	−0.40	−0.22	−0.08	0.00	−0.08	−0.49*	0.59*
	Peptococcaceae	−0.03	0.27	0.40	−0.02	0.13	0.64*	0.64*	0.70*	−0.22	−0.65*	−0.12	0.17	−0.07	−0.31	−0.11	0.08	0.37	−0.56*	0.07	0.23	0.16	−0.17	0.00	0.42
	Peptostreptococcaceae	0.19	−0.24	0.19	−0.07	−0.04	0.26	0.19	0.22	0.19	−0.33	0.19	0.22	0.30	−0.26	0.00	0.07	0.07	−0.22	0.22	−0.15	0.07	0.04	−0.22	0.30
	Ruminococcaceae	−0.01	0.19	0.10	−0.07	0.04	0.04	−0.11	0.07	0.33	0.11	−0.06	−0.22	0.00	−0.12	−0.26	−0.19	0.38	−0.25	−0.03	0.05	−0.03	−0.25	−0.15	0.25
	Veillonellaceae	0.37	0.30	0.30	0.22	0.26	−0.07	−0.19	−0.07	0.00	0.15	−0.19	0.19	0.07	−0.15	0.15	0.22	−0.33	−0.19	−0.19	−0.33	−0.33	−0.33	−0.04	−0.37
	Mogibacteriaceae	−0.37	−0.43	−0.28	−0.19	−0.35	−0.51*	−0.57*	−0.53*	0.82*	0.76*	0.08	−0.56*	−0.22	0.24	−0.32	−0.51*	0.10	0.37	0.09	−0.10	−0.12	0.05	0.15	−0.43
	Erysipelotrichaceae	0.35	0.33	0.20	0.28	0.34	0.31	0.43	0.38	−0.53*	−0.54*	−0.03	0.28	0.11	−0.27	0.13	0.27	0.27	−0.01	0.04	0.19	0.11	−0.33	0.03	0.20
	Alcaligenaceae	−0.13	0.09	0.08	0.03	−0.10	−0.19	−0.08	−0.18	0.01	0.04	−0.18	−0.32	−0.12	−0.10	−0.22	−0.21	0.44*	0.22	−0.23	−0.12	−0.05	−0.09	−0.05	0.03
	Enterobacteriaceae	−0.39	−0.17	−0.26	−0.36	−0.55*	−0.22	−0.33	−0.27	0.32	0.28	−0.14	0.15	−0.02	0.30	0.16	0.05	−0.07	0.02	0.21	0.38	0.48*	0.27	0.33	0.09
	F16	−0.14	0.05	0.07	−0.09	−0.22	0.05	−0.05	−0.01	0.53*	0.27	−0.16	−0.29	−0.30	−0.06	−0.54*	−0.47*	0.28	−0.11	−0.13	0.08	−0.13	−0.42	0.08	−0.12
	Verrucomicrobiaceae	−0.31	−0.11	−0.17	0.23	0.01	−0.46*	−0.33	−0.46*	0.67*	0.53*	0.43	−0.45*	−0.03	0.00	−0.20	−0.43	−0.04	0.30	0.16	−0.03	−0.17	−0.02	0.36	−0.56*

Fig. 6. A heat map showing the correlation between the relative abundance of different bacteria phyla or families; the amount of liver lipids, cholesterol, and TG; the concentration of plasma cholesterol, TG, glucose, and NEFA; the amount of bile acids and fecal lipids concentration; the amount of visceral fat; the expression of *Fasn*, *Fxr*, *G6pase*, *Gck*, *Ho-1*, *Pepck*, *Srebp-1c*, and *Chrebp* in the liver; and the expression of *Il-4*, *Il-6*, *Il-10*, *Ifn-γ*, and *Tnf-α* in the large intestine between the Con, SC, and NA groups. In the heat map, correlation coefficients closer to −1, +1, and 0 are shown in darker blue, darker red, and no color, respectively; an increasing blue intensity indicates a stronger negative correlation, while an increasing red intensity indicates a stronger positive correlation. *$p<0.05$. See color figure in the on-line version.

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subtilis natto increases *Bacillus* bacteria in the intestinal microbiota but does not affect other intestinal microbiota.[21] On the other hand, there are reports that administration of poly-γ-glutamic acid to humans improves the intestinal microbiota.[22] It has been reported that the abundance of *Lactobacillales* increases by oral administration of γ-PGA in mice.[10] From these reports, it is easy to infer that the poly-γ-glutamic acid contained in the diet of the NA group may have affected the intestinal microbiota. Because natto contains high amounts of γ-PGA, the higher occupation ratio of *Lactobacillaceae* in the NA group may be due to this diet's high γ-PGA content. As the NA group diet contained natto fermented with *B. subtilis*, the higher occupation ratio of the *Bacillaceae* in the NA group may be due to the presence of *B. subtilis*.

The dietary intake of soy food, soy protein, and soy isoflavones is inversely associated with the incidence of type 2 diabetes.[23] Thus, both cooked soybeans in the SC diet and natto in the NA diet seem to be beneficial for carbohydrate metabolism in mice. Plasma glucose levels were significantly lower in the NA group as compared to the Con group, though there were no significant differences in the plasma glucose levels between the SC and Con groups. Chungkookjang paste, which is made from cooked soybeans fermented with *B. licheniformis* and *B. amyloliquefaciens*, is reported to have a higher γ-PGA content and improve glucose metabolism.[7]

In a human study, it was reported that a single administration of high γ-PGA natto diet lowered postprandial blood glucose level of γ-PGA more than a single administration of low γ-PGA natto diet,[11] and in a mouse study, oral administration of starch + γ-PGA significantly lowered blood glucose level than starch alone, suggesting a hypoglycemic effect of γ-PGA.[24] The low blood glucose levels in the NA group are presumably due to the hypoglycemic effect of γ-PGA.

The relative abundances of *Turicibacteraceae*, *Streptococcaceae*, and *Clostridiaceae* were significantly lower in the NA group than in the Con group. However, no significant differences in the relative abundance of these bacteria were observed between the SC and Con groups. Tsumura Suzuki Obese Diabetes (TSOD) mice show typical type 2 diabetes symptoms, and the abundance of *Turicibacter* is significantly greater in obese mice than in non-obese TSNO mice,[25] suggesting the importance of *Turicibacter* in the abnormal metabolism that occurs in type 2 diabetes. It has been reported that the relative abundances of *Clostridiaceae*,[26] *Streptococcaceae*, and *Desulfovibrionaceae* are positively associated with plasma glucose levels in mice.[27] In our results, the relative abundance of these bacterial families was positively correlated with plasma glucose levels. The decreased abundance of these bacteria in the NA group might have contributed to the lower plasma glucose levels in the NA group compared to the Con group.

Steamed soy beans inhibited lipid accumulation in the liver.[28] This report suggests that soybeans may have an effect of lowering liver lipid accumulation. Although the liver lipid-lowering effect of *Bacillus subtilis* natto has been reported, it was reported that there was no serum triglyceride-lowering effect of *Bacillus subtilis* natto.[29] Our results showed that the *Firmicutes*:*Bacteroidetes* ratio was significantly lower in the NA and SC groups than that in the Con group, with significantly lower *Firmicutes* and higher *Bacteroidetes* abundances in the NA and SC groups. *Bacteroidetes* abundance was negatively correlated with hepatic lipid levels and *Srebp-1c* expression, while *Firmicutes* abundance was positively correlated with liver lipid levels and the expression of *Fasn* and *Srebp-1c*. As such, the *Bacteroidetes*:*Firmicutes* ratio was significantly negatively correlated with liver lipid levels and the expression of *Srebp-1c*, suggesting that both *Bacteroidetes* and *Firmicutes* are involved in lipid metabolism. Turnbaugh *et al.*[30] showed that the ingestion of high-fat diets altered the *Firmicutes*:*Bacteroidetes* ratio in the gut microbiota. The increased weight and fat deposition observed in mice were associated with the microbiota composition promoted by their high-fat diet, which contained increased levels of *Firmicutes* and decreased levels of *Bacteroidetes*.[30] Natto in the NA group and cooked soybeans in the SC group possibly contributed to the improved lipid metabolism by altering the intestinal microbiota. Serum lipids were significantly lower in the high-fat diet + γ-PGA-treated mice than in the high-fat diet-treated mice, and liver lipid size was significantly lower in the high-fat diet + γ-PGA-treated mice than in the high-fat diet-treated mice.[31] Fat accumulations and inflammatory degrees within liver and fat cells were also decreased in the γ-PGA containing diet than high fat diet.[32] Mean liver lipid content was lower in the NA group than in the SC group. Plasma TG levels were significantly lower in the NA group than in the Con group. γ-PGA may enhance the hepatic lipid-lowering effect of soybeans.

We found that the abundance of the *Bacteroidales* family S24-7 was also higher in both the NA and SC groups than in the Con group. It has been reported that mice fed a high-fat diet that received estrogen, 17β-Estradiol (E2), showed attenuated weight gain associated with an increased abundance of the *Bacteroidales* family S24-7.[33] In our study, diets for both the SC and NA groups contained processed soybeans containing isoflavones, which have weak estrogenic activity. It is suggested that the isoflavones in the diets of the SC and NA groups may be related to the higher occupancy of the *Bacteroidales* family S24-7 in these groups. Additionally, the abundance of the *Bacteroidales* family S24-7 was negatively correlated with the amount of liver lipids and positively correlated with the amount of bile acid excretion and fecal lipid concentration. It has been reported that bean supplementation in a high-fat obesogenic diet in mice improved the obese phenotype, such as poor intestinal health and adipose inflammation, and that the abundance of *Bacteroidales* family S24-7 in fecal microbiota was significantly greater in mice fed a high-fat diet supplemented with beans than in mice fed only a high-fat diet.[34] Therefore, the *Bacteroidales* family S24-7 may play an important role in the improvement of lipid metabolism in a high-fat diet supplemented with beans.

The fiber content of cooked beans and natto has been reported.[8] Based on this report, the percentages of soluble fiber used in this study were about 1.3% for natto (fermented soybeans) and about 0.7% for cooked soybeans, while the control group's diet did not contain any soluble fiber. Flaxseed dietary fiber contain about 30% dietary fiber and one third of flaxseed dietary fiber are water soluble fiber and flaxseed dietary fiber increased fecal fat.[35] Soluble fiber intake has been reported to enhance fecal bile acid excretion.[36] Rats fed water-soluble chicory extract had significantly greater fecal lipid and bile acid excretions than those fed fiber-free diets.[37] Thus, soluble dietary fiber affects the fecal lipid excretion and fecal bile acid excretion, soluble dietary fiber contained in the SC and NA diet might increase the fecal lipid excretion and fecal bile acid in two groups.

Cheonggukjang is a traditional Korean fermented soybean that contains significant amounts of γ-PGA.[38] Administration of cheonggukjang to mice modulated pro-inflammatory and anti-inflammatory cytokine levels via the suppression of NF-κB and inflammatory mediator signaling pathways.[38] The mRNA levels of anti-inflammatory cytokines (*Il-4* and *Il-10*) in the large intestine of mice were significantly higher in the cheonggukjang group than in the non-administered control group.[38] Our results showed that the mRNA levels of *Il-4* and *Il-10* in the large intestine of the NA group tended to be higher than those in the Con group. Additionally, the ratio of *Ifn-γ*/*Il-4* expression in the large intestine was significantly greater in the Con group than in the NA group Therefore, the γ-PGA contained in the natto provided in the NA group diet might have affected the higher

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mRNA levels of anti-inflammatory cytokines, such as *Il-4* and *Il-10*, in the large intestine of mice.

In summary, there were significant differences in the abundance of the microbiota among the Con, SC, and NA dietary groups analyzed in our work. The abundance of *Bacillaceae* and *Lactobacillaceae* was significantly greater in the NA group than in the Con group, and the relative abundance of *Rikenellaceae* was significantly lower in the NA group than in the SC group. Additionally, liver lipid levels were significantly lower in the SC and NA groups than in the Con group, and plasma glucose and TG levels were significantly lower in the NA group than in the Con group. Finally, expression of *Srebp-1c* in the liver was significantly lower in the NA group than in the Con group. Although both natto and cooked soybeans improved the metabolism induced by a high-fat diet, it can be inferred that natto had a greater impact on carbohydrate and lipid metabolism than cooked soybeans. As such γ-PGA may play an important role in natto functionality.

Author Contributions

Study concept and design, MT; acquisition of data, MT, JW, TNoguchi, and TNishikawa; analysis interpretation of data, MT; drafting of the manuscript, MT and JW; final approval, all authors; study supervision, MT.

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Conflict of Interest

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