Dietary Eicosapentaenoic Acid and Docosahexaenoic Acid Ethyl Esters Influence the Gut Microbiota and Bacterial Metabolites in Rats

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Abstract: Dietary fish oil containing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) has been reported to affect the diversity and composition of gut microbiota and bacterial metabolites. However, few reports have focused on the effects of EPA and DHA on gut microbiota diversity and bacterial metabolites. This study evaluated the effects of dietary EPA-ethyl ester (EE) and DHA-EE on steroid metabolism, gut microbiota, and bacterial metabolites in Wistar rats. Male rats were fed the experimental diets containing 5% (w/w) soybean oil-EE (SOY diet), EPA-EE (EPA diet), and DHA-EE (DHA diet) for four weeks. The lipid contents in the serum and liver, mRNA expression levels in the liver, and the diversity, composition, and metabolites of the gut microbiota were evaluated. The EPA and DHA diets decreased serum and liver cholesterol contents compared to the SOY diet. In addition, there were no significant changes in gene expression levels related to steroid metabolism in the liver between the EPA and DHA groups. Rats fed the DHA diet had lower microbiota diversity indices, such as Simpson and Shannon indices, than rats fed the SOY and EPA diets. In addition, rats fed EPA and DHA had significant differences in the relative abundance of microbiota at the genus level, such as *Phascolarctobacterium*, *Turicibacter*, and *Eubacterium*. Therefore, it was concluded that EPA and DHA have different effects on the diversity and composition of gut microbiota under the experimental conditions employed herein.

Key words: eicosapentaenoic acid, docosahexaenoic acid, microbiota, cholesterol, rat

1 Introduction

Fish oil is a rich source of *n*-3 polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and has health-promoting functions in humans. For example, *n*-3PUFA intake has proven beneficial against cardiovascular diseases¹, hyperlipidemia², Alzheimer's disease³, inflammation⁴, and cancer⁵. Accordingly, various organizations recommend the intake of *n*-3PUFA, especially EPA and DHA⁶.

Most prior studies investigated the health-promoting effects of dietary *n*-3PUFA employed commercially available fish oil, which is a mixture of EPA and DHA. However, the molecular structures and physiological functions of EPA and DHA are different. EPA (C20:5*n*-3) has a shorter carbon chain (20 vs. 22) and fewer double bonds (5 vs. 6) per molecule than DHA (C22:6*n*-3). DHA has a greater influence on membrane fluidity and the activity of membrane

proteins and ion channels⁷. EPA and DHA are sources of bioactive metabolites with different properties, such as eicosanoids and docosanoids⁸. In addition, DHA is preferentially retained over EPA, and EPA is more β -oxidized than DHA⁹. Thus, the differences in the physiological functions of EPA and DHA are becoming clear.

The gut microbiota is closely related to the health of the human host¹⁰. The change in gut microbiota composition has been implicated in the development of metabolic diseases, including type-2 diabetes and obesity¹¹. Recent studies have reported that dietary fish oil alters the composition and metabolites of gut microbiota. For example, *n*-3PUFA alters the abundance of beneficial gut bacteria, particularly the *Akkermansia* genus, in mice¹² and attenuate the increase in the Firmicutes/Bacteroidetes ratio observed in mice fed a high-fat diet¹³. Our previous study showed that the consumption of menhaden oil (EPA 14.6

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mol% and DHA 9.9 mol%) and tuna oil (EPA 7.3 mol% and DHA 24.3 mol%) with different EPA and DHA ratios had different effects on the composition of gut microbiota in normal rats¹⁴⁾. Therefore, EPA and DHA may have different effects on the diversity and composition of gut microbiota as the effects on gut microbiota were different when rats were fed the oils with different EPA and DHA ratios. To the best of our knowledge, the different effects of EPA and DHA on gut microbiota and bacterial metabolites have not been reported. This study sought to determine whether EPA-ethyl ester (EE) and DHA-EE have different effects on gene expression related to steroid metabolism, gut microbiota, and bacterial metabolites in normal rats.

2 Experimental Procedures

2.1 Materials

Soybean oil-EE (SOY-EE) was prepared from soybean oil (Merck KGaA, Darmstadt, Germany) using an NaOH-ethanol solution. EPA-EE and DHA-EE (99%; w/w) were provided by Bizen Chemical Co., Ltd. (Akaiwa, Japan). The components of the experimental diet were purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan) and Fujifilm Wako Pure Chemical Co. (Osaka, Japan). All other reagents were obtained from Merck KGaA, Fujifilm Wako Pure Chemical Co., and Nacalai Tesque, Inc. (Kyoto, Japan).

2.2 Preparation of the experimental diets

The control diet (SOY) was prepared using a modified semi-purified AIN-93G formulation¹⁵⁾ containing 10% (w/w) soybean oil and 5% (w/w) SOY-EE. The experimental diets contained 10% (w/w) soybean oil and 5% EPA-EE and DHA-EE (EPA and DHA diets, respectively). The detailed diet composition is shown in Table S1.

2.3 Analysis of fatty acid composition

The fatty acids (FA) compositions of the experimental oils (SOY-EE, EPA-EE, and DHA-EE) and diets (SOY, EPA, and DHA) were determined using a gas chromatography (GC) system (GC-2014; Shimadzu Co., Kyoto, Japan) with an Omegawax[®] capillary GC column (cat. no. 24136; Merck KGaA) after the methylation of FA with boron-trifluoride-methanol as described in our previous report¹⁶⁾. Tables 1 and S2 show the FA compositions of the experimental diets and oils, respectively.

2.4 Animal experiment

Animal experiments were conducted according to the Guide for the Care and Use of Experimental Animals issued by the Prime Minister's Office of Japan and reviewed and approved by the Animal Ethics Committee of Kansai University (approval no. 1704).

Four-week-old male Wistar rats obtained from Japan

Table 1 Composition of FA in the experimental diets.

	Diets		
	SOY	EPA	DHA
	mol%		
C16:0	11.5	7.5	7.5
C18:0	3.8	2.4	2.4
C18:1 <i>n</i> -9	21.8	14.0	14.0
C18:2 <i>n</i> -6	53.6	36.3	36.3
C18:3 <i>n</i> -6	0.6	0.3	0.3
C18:3 <i>n</i> -3	6.5	4.4	4.4
C20:5 <i>n</i> -3 (EPA)		33.2	0.3
C22:6 <i>n</i> -3 (DHA)			33.0
Others	2.3	1.8	1.7

DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; SOY, soybean oil

SLC, Inc. (Hamamatsu, Japan) were housed in individual cages and housed at 21–23°C with a 12-hour light-dark cycle (lights on: 08:00–20:00), and allowed free access to tap water and the diets. Following an acclimatization period of seven days, rats were divided into three dietary groups (SOY, EPA, and DHA), each consisting of eight rats with similar mean body weights (BW). Food consumption and BW were recorded every two days. Before dissection, the feces were collected from each rat daily for two days and stored at –35°C. After four weeks of diet administration, rats that had not fasted were killed under isoflurane (Fujifilm Wako Pure Chemical Co.) anesthesia from 9:00 to 11:00. Blood samples were collected without anticoagulant use, and serum was obtained by centrifugation at 4°C, 1,500 × *g* for 20 min. Rat liver, cecum, and abdominal white adipose tissue (WAT) from the epididymis, mesentery, and perinephria were quickly removed, weighed, rinsed with cold saline, frozen in liquid nitrogen, and stored at –80°C until further analyses. Aliquots of liver were stored in RNA-Later Storage Solution (Merck KGaA) for quantitative real-time PCR.

2.5 Analysis of the lipid parameters in serum and liver

Serum biochemical parameters (triacylglycerol [TAG], phospholipids [PL], total cholesterol, high-density lipoprotein [HDL]-cholesterol, non-HDL-cholesterol, and non-esterified fatty acids [NEFA]) were measured using an Olympus AU5431 automatic analyzer (Olympus Co., Tokyo, Japan) by a commercial service (Japan Medical Laboratory, Osaka, Japan). Blood glucose levels were measured via the tail vein at the time of dissection using a handheld glucometer (Glutest Neo alpha; Sanwa Kagaku Kenkyusho Co., Ltd., Nagoya, Japan).

Liver total lipids were extracted using the method described by Bligh and Dyer¹⁷⁾. Liver TAG content in liver

total lipids dissolved in 2-propanol was determined using the Triglyceride-E-Test (Fujifilm Wako Pure Chemical Co.). The liver PL content was measured using phosphorus analyses¹⁸. Liver cholesterol content was determined using GC (GC-2014; Shimadzu Co.) with a DB-5 capillary GC column (cat. no. 122-5532; Agilent Technologies, Santa Clara, CA, USA) under the conditions described by Kaneda *et al.*¹⁹. Liver FA content was evaluated after methylation on a GC system as described above¹⁶, using tridecanoic acid (C13:0) as an internal standard.

2.6 Analysis of steroid composition in feces

The collected feces were weighed and ground using a conventional mill. Fecal neutral steroids (cholesterol and coprostanol) contents were analyzed by GC, applying the method shown above¹⁹. Fecal bile acids (BA) content was measured using a total bile acid test kit (Fujifilm Wako Pure Chemical Co.), following the manufacturer's protocol. Fecal total steroids content was calculated by adding fecal neutral steroids content to BA content.

2.7 Analysis of bile acids and short-chain fatty acids (SCFA) composition in cecal content

Cecum containing cecal content was suspended in cold saline. Thereafter, the cecum was removed and weighed. The BA content in cecal content was analyzed using a total bile acid test kit (Fujifilm Wako Pure Chemical Co.). The composition of SCFA in cecal contents was analyzed using GC, as described in our previous study¹⁴.

2.8 RNA isolation and real-time quantitative reverse transcription PCR

Livers that had been stored in RNA-Later Storage Solution were homogenized using a bead beater-type homogenizer (MicroSmash MS-100R; TOMY SEIKO Co., Ltd., Tokyo, Japan). Total RNA was isolated and purified using TRIzol[®] reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's protocol. The content and purity of total RNA were determined by measuring the absorbance at wavelengths of 260 and 280 nm by UV spectroscopy (UV-1800; Shimadzu Co.) using Hellma[®] TrayCell[™] (Hellma Analytics, Müllheim, Germany). cDNA was synthesized using the GoScript[™] Reverse Transcription System (Promega Co., Madison, WI, USA). The mRNA expression levels were measured using a Thermal Cycler Dice[®] Real Time System (Takara Bio Inc., Kusatsu, Japan) and GoTaq[®] qPCR Master Mix (Promega Co.). The primers were designed using Primer3Plus (<http://primer3plus.com/>); the primer sequences are listed in **Table S3**. Gene expression levels were standardized to the expression level of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and expressed as the fold-change in gene expression relative to that of the SOY group (set at 100).

2.9 16S rRNA amplicon sequence and bioinformatics

Fecal genomic DNA was extracted using a NucleoSpin[®] DNA Stool Kit (Takara Bio Inc.) according to the manufacturer's protocol. 16S rRNA amplicon sequence analysis was performed using a next-generation sequencing (NGS) system (Ion PGM[™], Thermo Fisher Scientific Inc.), as described in our previous report²⁰.

The raw sequence data were analyzed using Ion Reporter Software with the workflow of Metagenomics 16S w1.1 v5.14 (Thermo Fisher Scientific Inc.). Alpha diversity indices (Chao-1, Observed_species, Simpson, and Shannon), bacterial identification, and sequence numbers obtained at the phylum and genus levels for each sample were acquired from Ion Reporter Software based on the QIIME (v1.8.0), which used a threshold of 97% sequence identity. Beta diversity was estimated and visualized using principal component analysis (PCA) based on the relative abundance of all detected bacteria at the phylum and genus levels. Linear discriminant analysis effect size (LEfSe)²¹ was performed to identify high-dimensional biomarkers and to characterize the differences among the groups at the genus or higher taxonomy levels using Galaxy (<http://huttenhower.sph.harvard.edu/galaxy/>).

2.10 Statistical analysis

Data are presented as mean values and standard errors of the mean (SEM). Differences between multiple groups were evaluated using analysis of variance (ANOVA) and Tukey's multiple comparison test. Statistical significance and tendency were set as $p < 0.05$ and $0.05 \leq p < 0.10$, respectively. The relationships between the relative abundance of bacteria and fecal coprostanol content were evaluated using the Pearson's correlation coefficient test. Statistical analyses were performed on a personal computer (iMac, Apple Inc., Cupertino, CA, USA) with the macOS Mojave 10.14.6 operating system and GraphPad Prism7 (ver. 7.0d, GraphPad Software, San Diego, CA, USA).

3 Results

3.1 Growth parameters, organ weights, and serum biochemical parameters

Table 2 shows the growth parameters, organ weights, and serum biochemical parameters. There were no significant differences in growth parameters, including initial BW, final BW, BW gain, and food intake among the groups. The relative epididymal WAT (eWAT) weight in the DHA group was significantly lower than that in the SOY group. Other relative organ weights, including liver, cecum, mesentery WAT, and perirenal WAT, were not significantly different among the groups. The EPA and DHA groups had significantly decreased serum TAG, PL, total cholesterol, HDL-

Table 2 Growth parameters, organ weights, and serum biochemical parameters of the three groups.

	Groups		
	SOY	EPA	DHA
Growth parameters			
Initial BW (g)	80.6 ± 2.3	81.0 ± 1.6	81.1 ± 2.1
Final BW (g)	295.7 ± 4.9	295.3 ± 5.2	303.7 ± 4.8
BW gain (g/day)	7.68 ± 0.16	7.65 ± 0.16	7.95 ± 0.14
Food intake (g/day)	15.1 ± 0.3	14.9 ± 0.3	14.5 ± 0.1
Organ weights (g/100 g BW)			
Liver	4.38 ± 0.04	4.41 ± 0.11	4.67 ± 0.09
Cecum	0.98 ± 0.08	0.88 ± 0.11	0.89 ± 0.03
Epididymal WAT	1.39 ± 0.07 ^b	1.24 ± 0.03 ^{ab}	1.17 ± 0.03 ^a
Mesentery WAT	1.47 ± 0.12	1.35 ± 0.13	1.37 ± 0.08
Perirenal WAT	1.28 ± 0.07	1.43 ± 0.07	1.35 ± 0.03
Serum biochemical parameters			
TAG (mg/dL)	74.6 ± 9.0 ^b	36.5 ± 9.6 ^a	38.8 ± 4.7 ^a
PL (mg/dL)	149 ± 5 ^c	91 ± 4 ^a	105 ± 2 ^b
Total cholesterol (mg/dL)	83.0 ± 2.0 ^b	49.9 ± 2.6 ^a	56.6 ± 1.7 ^a
HDL-cholesterol (mg/dL)	57.1 ± 1.6 ^c	34.8 ± 1.8 ^a	44.4 ± 1.2 ^b
Non-HDL-cholesterol (mg/dL)	25.9 ± 0.8 ^b	15.9 ± 1.1 ^a	12.3 ± 1.3 ^a
NEFA (Eq/L)	0.45 ± 0.05	0.38 ± 0.01	0.38 ± 0.02
Blood glucose (mg/dL)	158 ± 3	159 ± 8	159 ± 5

Data are presented as mean ± SEM (n = 8). Values in the same row that do not share a common superscript are significantly different at $p < 0.05$, using Tukey's multiple comparison test.

BW, body weight; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HDL, high-density lipoprotein; NEFA, non-esterified fatty acids; PL, phospholipids; SEM, standard error of the mean; SOY, soybean oil; TAG, triacylglycerol; WAT, white adipose tissue.

cholesterol, and non-HDL-cholesterol contents compared with the SOY group. Moreover, the EPA diet significantly reduced the serum PL and HDL-cholesterol contents compared with the DHA diet. There were no significant differences in serum NEFA and blood glucose contents among the groups.

3.2 Liver lipid composition and gene expression

Figure 1 shows the liver TAG, cholesterol, PL, and FA contents, and mRNA expression levels related to steroid metabolism. The EPA and DHA diets significantly decreased liver TAG and cholesterol contents compared to the SOY diet (Figs. 1A and 1B). Liver PL content did not significantly change among the groups (Fig. 1C). The EPA and DHA groups had significantly lower liver C14:0, C16:0, C18:1*n*-9, C18:1*n*-7, C18:2*n*-6, C18:3*n*-6, C18:3*n*-3, and C20:4*n*-6 contents than the SOY group (Fig. 1D). Moreover, the liver C20:5*n*-3 (EPA) content in rats fed EPA-EE was

significantly higher than that in rats fed SOY-EE and DHA-EE. The DHA diet significantly decreased the liver C16:1*n*-7 content and increased the liver C22:6*n*-3 (DHA) content compared to the SOY diet. The EPA and DHA groups had significantly lower liver cytochrome P450 family 7 subfamily A member 1 (*Cyp7a1*) levels and higher liver nuclear receptor subfamily 0 group B member 2 (*Nr0b2*) levels than the SOY group (Fig. 1E). The liver acetyl-CoA acetyltransferase 1 (*Acat1*) level in the DHA group was significantly higher, and tended to be higher in the EPA group than in the SOY group ($p = 0.07$).

3.3 Steroid composition in feces and cecal content

Figure 2 shows the steroid composition in the feces and cecal content. The EPA and DHA groups had significantly lower fecal total steroids and BA excretions than the SOY group (Fig. 2A). There were no significant changes in fecal neutral steroids and their constituents (coprostanol and

Fig. 1 Liver lipid content, FA contents, and relative mRNA expression levels of steroid metabolism-related proteins (A) Liver TAG content. (B) Liver cholesterol content. (C) Liver PL content. (D) Liver FA contents. (E) Liver relative mRNA expression levels of steroid metabolism-related proteins.

Data are presented as mean $\pm$ SEM (n = 8). Different letters indicate significant difference at $p < 0.05$, using Tukey's multiple comparison test.

The mRNA expression levels were determined and normalized using the *Gapdh* expression level. The levels are expressed relative to those determined using the livers of rats in the SOY group (set at 100).

DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; PL, phospholipids; SEM, standard error of the mean; SOY, soybean oil; TAG, triacylglycerol. The abbreviation names of the genes are listed in Table S3.

cholesterol) excretions among the groups (Figs. 2A and 2B). The cecal content weight did not change among the groups (data not shown). The amount of BA in the cecal content was not significantly different among the groups (Fig. 2C). Moreover, no significant differences in the amount of SCFA in cecal content were observed among the groups (Fig. S1).

3.4 Diversity and composition of fecal microbiota

As fecal microbiota composition has been reported to

largely reflect that of the large intestine²²⁾, we evaluated the fecal microbiota using 16S rRNA amplicon sequencing in this study. There was no significant difference in the number of valid reads among the SOY, EPA, and DHA groups after the processing of reading length and read abundance filtering using Ion Reporter ($187,241 \pm 11,270$, $183,534 \pm 10,118$, and $174,421 \pm 10,482$, respectively). Figure 3 shows the results of alpha and beta diversity indices and the relative abundance of microbiota in feces. The rarefaction curves of Chao-1 and Observed_species

Fig. 2 BA and neutral steroids compositions in feces and cecal content (A) Fecal total steroids, neutral steroids, and BA excretions. (B) Fecal cholesterol and coprostanol excretions. (C) Amount of BA in the cecal content. Data are presented as mean $\pm$ SEM ($n = 8$). Different letters indicate significant difference at $p < 0.05$, using Tukey's multiple comparison test.

'Total steroids' represents the sum of neutral steroids and BA.

BA, bile acids; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; SEM, standard error of the mean; SOY, soybean oil.

indices in the EPA and DHA groups were lower than those in the SOY group (Figs. 3A and 3B). Moreover, the DHA group was lower in the rarefaction curves of the Simpson and Shannon indices than in the SOY and EPA groups (Figs. 3C and 3D). The PCA results (Figs. 3F, 3G, 3I, and 3J) revealed the primary component contribution rates at the phylum and genus levels. PC1 accounted for 38.1% and 23.2% of the variance for the phylum and genus levels, respectively, while PC2 accounted for 27.6% and 14.8%, and PC3 accounted for 19.9% and 12.7%, respectively. As shown in Fig. 3J, large distances were observed between the EPA and DHA groups. Further, the EPA group had a significantly lower relative abundance of *Bifidobacterium* than the SOY diet (Fig. 3P). Compared with the EPA group, the DHA group had significantly lower relative abundances of *Phascolarctobacterium* and *Turicibacter* and higher relative abundance of [*Eubacterium*] (Figs. 3N, 3Q, and 3R). In the DHA group, the relative abundance of *Clostridium* and *Parabacteroides* tended to be higher and lower, respectively, than those in the EPA group (Figs. 3M and 3O).

To investigate the effects of EPA and DHA on the key types of fecal microbiota in rats, we used LEfSe analysis to identify the phylotypes with the greatest differences among the groups (Fig. 4). At the phylum level, the Actinobacteria phylotype was more abundant in the SOY group. In contrast, the Negativicutes phylotype at the class level and Coriobacteriales phylotype at the order level were more abundant in the EPA and DHA groups, respectively.

Figure S2 shows the results of the correlation analysis between fecal coprostanol content and the relative abundance of *Clostridium* at the genus level. A positive correlation was found between fecal coprostanol content and

the relative abundance of *Clostridium* ($R = 0.53$).

4 Discussion

The EPA and DHA diets increased liver EPA and DHA contents, respectively, compared with the SOY diet; these alterations reflected the FA composition of these diets (Table 1 and Fig. 1D). In addition, the EPA and DHA diets caused decreased liver TAG content compared to the SOY diet (Fig. 1A). Because of the decrease in liver TAG content in the EPA and DHA groups, the C16:0, C18:1, and C18:2n-6 contents in the liver were also decreased. The TAG-lowering effects of EPA and DHA are well known²³. EPA and DHA suppress FA synthesis through sterol regulatory element-binding protein-1c expression²⁴, and enhance FA β -oxidation through the activation of peroxisome proliferator-activated receptor- α ²⁵. Previous study suggested that EPA and DHA intake decreased WAT weight by inducing uncoupling protein-1 expression via the sympathetic nervous system²⁶. As previously reported²⁶, relative eWAT weights were lower in the DHA group and tended to be lower in the EPA group ($p = 0.07$) than in the SOY group.

The EPA and DHA diets decreased the serum cholesterol-related parameters (total cholesterol, HDL-cholesterol, and non-HDL cholesterol) and liver cholesterol content compared with the SOY diet (Table 1 and Fig. 1B). Previous studies reported that fish oil decreased the plasma total cholesterol level²⁷ and decreased intestinal cholesterol absorption by downregulating niemann-pick C1-like 1, a cholesterol transporter²⁸. Moreover, the EPA group had significantly lower serum HDL-cholesterol content and

Fig. 3 The diversity and composition of fecal microbiota

(A)–(D) Bacterial rarefaction curves based on Chao-1, Observed species, Simpson, and Shannon indices at the genus level, respectively. (E) Relative abundance of fecal microbiota at the phylum level. (F) and (G) PCA of the bacterial compositions at the phylum level. (H) Relative abundance of fecal microbiota at the genus level. (I) and (J) PCA of the bacterial compositions at the genus level. (K)–(R) Relative abundance of *Bacteroides*, *Lactobacillus*, *Clostridium*, *Phascolarctobacterium*, *Parabacteroides*, *Bifidobacterium*, *Turicibacter*, and *[Eubacterium]*, respectively.

Data are presented as mean $\pm$ SEM ($n = 8$). Different letters indicate significant difference at $p < 0.05$, using Tukey's multiple comparison test.

Square brackets [] around a genus indicate that the name awaits appropriate action by the research community to be transferred to another genus. Graphs (E) and (H) are sorted from the higher relative abundance in SOY group, and with less than 0.5% of the relative abundance summarized as Others.

DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PCA, principal component analysis; SEM, standard error of the mean; SOY, soybean oil.

Fig. 4 Comparisons of fecal microbiota using the LEfSe. Results (A) and cladogram (B) derived using the LEfSe method, indicating the phylogenetic distribution of microbiota associated with the experimental groups. LDA scores (log₁₀) > 2 and $p < 0.05$ are listed.

DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size; SOY, soybean oil.

tended to have lower serum total cholesterol content ($p = 0.09$) than the DHA group. These findings were supported by a previous study where the EPA diet was found to be more effective at lowering serum total cholesterol content than the DHA diet²⁹. In a human study, the increased HDL-cholesterol level caused by DHA intake compared to EPA intake has been suggested to involve the inhibition of cholesteryl ester transfer protein (CETP) activity by DHA³⁰. However, rodents, such as rats and mice, are naturally deficient in CETP. In this study, the mechanism by which EPA intake lowers serum HDL-cholesterol content more than DHA intake was not determined.

Cholesterol 7 α -hydroxylase, which is the protein for *Cyp7a1*, is the rate-limiting enzyme in the synthesis of BA from cholesterol and plays a critical role in the control of

BA and cholesterol homeostasis³¹. The EPA and DHA groups showed decreased liver *Cyp7a1* expression levels even though the liver cholesterol content was lower than that in the SOY group. Liver *Cyp7a1* expression level is controlled by the negative feedback regulatory pathway of farnesoid X receptor (FXR)/small heterodimer partner (SHP), which is regulated by the enterohepatic circulation of BA³². Therefore, the inhibition of BA reabsorption in the intestine increases liver *Cyp7a1* expression by suppressing the FXR/SHP pathway. In the present study, the amount of BA in the cecal content was not altered among the groups; however, fecal BA excretion was lower in the EPA and DHA groups than in the SOY group (Figs. 2A and 2B). Moreover, the EPA and DHA diets increased the expression levels of liver *Nr0b2*, which is the gene for SHP, compared with the SOY diet (Fig. 1E). Thus, the suppression of liver *Cyp7a1* expression is partly due to the activation of the FXR/SHP pathway by enhancing BA reabsorption in the intestinal tract. On the other hand, the increase in the expression levels of liver *Acat1*, which is related to the esterification of cholesterol, were increased in the DHA group and tended to increase in the EPA group compared with the SOY group (Fig. 1E). Therefore, the evident reduction in liver cholesterol content in rats fed EPA and DHA diets is considered to be a compensatory induction of *Acat1* expression and BA reabsorption in the intestinal tract.

The *n*-3PUFA are thought to affect the gut microbiota mainly through regulation of the type and amount of bacteria in the gut, changes in the levels of inflammatory mediators such as endotoxin and interleukin, and regulation of SCFA concentrations³³. In this study, compared with the SOY group, the EPA and DHA groups had significantly lower the Chao-1 and Observed_species indices, which are used to estimate species richness (abundance-based estimator of species richness) at the genus level (Figs. 3A and 3B). In addition, the Simpson and Shannon indices, which were used to estimate species and evenness (more weight on species evenness) in the DHA group, were lower than those in the SOY and EPA groups (Figs. 3C and 3D). Thus, the EPA group had less species than the SOY group, but the species evenness did not change, while the DHA group had both lower species and species evenness than the SOY group. The *n*-3PUFA could directly modulate the diversity of the gut microbiota. Previous studies have shown that the intake of fish oil affects the diversity of the intestinal microbiota in trout and infants^{34, 35}. In this study, the intake of EPA-EE and DHA-EE reduced the alpha diversity of fecal microbiota, which is different from that found in previous reports^{34, 35}. In this experiment, EPA-EE and DHA-EE (both more than 99% pure) were used as *n*-3PUFA sources; however, previous studies used fish oil. Dietary fish oil containing various FA, besides EPA and DHA, may have beneficial effects on the gut microbiota diversity. Under these experimental conditions, the intake of

n-3PUFA, especially DHA, decreased the alpha diversity of the gut microbiota. The results of PCA, which is used for beta diversity, clearly showed a separation of the EPA group from the DHA group at PC1-PC3 at the genus level (Fig. 3J). The SOY group was located between the EPA and DHA groups at PC1-PC3 at the genus level. These findings suggest that the intake of EPA-EE and DHA-EE had different effects on the fecal microbiota at the genus level.

We proceeded to analyze the effects of EPA and DHA on the relative abundance of bacteria. The EPA diet caused a decrease in the relative abundance of *Bifidobacterium*, which is a probiotic beneficial bacterium, compared with the SOY diet, whereas the DHA diet did not result in a decrease (Fig. 3P). Compared with the EPA diet, the DHA diet caused a decrease in the relative abundance of *Phascolarctobacterium* and *Turicibacter* and tended to reduce the relative abundance of *Parabacteroides* ($p=0.07$). In addition, the DHA group had a higher relative abundance of *Eubacterium* and tended to have a higher relative abundance of *Clostridium* than the EPA group ($p=0.09$). A previous study showed that *n*-3PUFA can also increase the relative abundance of *Bifidobacterium* in a fat-1 transgenic mouse model, which can produce *n*-3PUFA from *n*-6PUFA³⁶. Other studies reported that fish oil intake could prevent gut microbiota dysregulation in mice with alcoholic liver disease³⁷ and increase the relative abundance of lactic acid-producing bacteria (mainly lactobacilli) in the gut of mice fed a high-fat diet³⁸. Dietary fish oil decreased WAT inflammation and increased insulin sensitivity compared with dietary lard in high-fat diet fed mice. The phenotypic differences between the dietary groups (fish oil and lard diets) can be partly attributed to differences in microbiota composition, especially *Akkermansia*³⁹. The increased abundances of several genera, including *Bifidobacterium*, *Roseburia*, and *Lactobacillus* were observed with EPA and DHA interventions in healthy middle-aged volunteers⁴⁰. Thus, most studies have evaluated the gut microbiota of pathological animals fed fish oil, a mixture of EPA and DHA. Although there have been no studies on the effects of EPA and DHA alone on the gut microbiota in rats, we observed for the first time that EPA and DHA had different effects on fecal bacteria, including *Phascolarctobacterium*, *Turicibacter*, and *Eubacterium*, in normal rats.

The metabolites produced by the gut microbiota significantly influence the host's metabolism and health⁴¹. The proportion of SCFA, produced by bacterial fermentation in the gut and exerts several effects on host metabolism and the immune system⁴², in the cecal contents was not altered (Fig. S1). Coprostanol is converted from cholesterol by intestinal bacteria; coprostanol is not detected in tissues and is mainly found in more significant amounts in feces⁴³. Bacteria belonging to *Bifidobacterium*, *Clostridium*, *Eu-*

bacterium, and *Lactobacillus* have been reported to reduce cholesterol to coprostanol in an *in vitro* study^{44–46}. New bacterial phylotypes from Lachnospiraceae and Ruminococcaceae families were associated with coprostanol levels in healthy humans, according to the results of a metagenomic analysis⁴⁷. We analyzed the relationship between the relative abundance of microbiota at the genus level and fecal coprostanol excretion and found a positive correlation between the relative abundance of *Clostridium* and fecal coprostanol excretion (Fig. S2). Therefore, the *Clostridium* genus could be involved in the conversion of cholesterol to coprostanol in this experiment.

Dietary FA is partially metabolized by anaerobic bacteria, such as bifidobacteria and lactobacilli, in the distal intestine, affecting the composition of the gut microbiota⁴⁸. A previous study revealed the presence of conjugated linoleic acids and conjugated linolenic acids, which are bacterial metabolites from PUFA, in the cecum and colon of mice fed sunflower oil or linseed oil⁴⁹. Another study showed that hydroxy FA were mainly generated from PUFA by *Lactobacillus plantarum* and gut bacteria⁵⁰. The specialized pro-resolving lipid mediators in the immune cells synthesized from EPA and DHA may play an essential role in repairing intestinal mucosal epithelial cells⁵¹. Therefore, the difference in diversity and composition of fecal microbiota between the EPA and DHA groups may be partly due to the metabolites of EPA and DHA produced via the actions of bacteria. Further studies are necessary to confirm the bacterial metabolites of EPA and DHA in the intestinal tract.

5 Conclusion

In the present study, we demonstrated the impact of dietary EPA-EE and DHA-EE on steroid metabolism, gut microbiota, and bacterial metabolites in normal rats. Compared with the SOY group, the EPA group had lower species richness while the DHA group had both lower species richness and evenness. In addition, rats fed EPA and DHA had differences in microbiota composition at the genus level, such as *Phascolarctobacterium*, *Turicibacter*, and *Eubacterium*. Overall, our results provide new insights into the differential effects of EPA and DHA on the gut microbiota of normal rats.

Authors Contributions

R.H., A.M., and K.S. performed research, analyzed data, and wrote the original manuscript. T.S., S.K., and T.N., conducted the bioinformatics analysis of gut microbiota and advised in statistical analysis. M.Y. and K.F. supervised the research design and reviewed the article. All authors

read and approved the article.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

Supporting Information

This material is available free of charge via the Internet at doi: 10.5650/jos.ess21189

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