Cecal Environment and TBARS Level in Mice Fed Corn Oil, Beef Tallow and Menhaden Fish Oil

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Summary To examine the dietary effects of three lipids from different sources on intestinal conditions, diets containing 10% corn oil (CO), 1% corn oil + 9% beef tallow (BT) or 1% corn oil + 9% menhaden fish oil (MO) were administered to male mice for 4 wk. Levels of total cholesterol (TC), non-esterified fatty acids (NEFA) and glucose in plasma were lower in mice fed on MO than in mice fed on CO or BT. The plasma phospholipid (PL) level was lower for mice fed MO than in mice fed BT. Levels of the thiobarbituric acid-reactive substances (TBARS), soluble saccharides and soluble proteins in the cecum were significantly higher in mice fed on MO than in mice fed on CO or BT. The number of fecal bacteroidaceae was lower in mice fed on MO than in mice fed on BT. On the other hand, the number of fecal bifidobacteria was greater in mice fed on MO than in mice fed on CO. These results suggest the possibility that menhaden oil affects lower intestinal microflora through the increase of not only oxide products but also saccharides and proteins in the intestine.

Key Words fish oil, cecal TBARS, fecal microflora, mice, menhaden

The microflora and their fermented products in the lower (large) intestine are very important to the host for the prevention of deterioration, cancer and infection (1, 2). It is known that foodstuffs such as dietary fiber, resistant starch and protein undigested in the small intestine affect the microflora and conditions of the large intestine in humans (1–3).

Various effects of dietary fish oils on human and animals, for example, the hypocholesterolemic and glucose tolerance effect, have been reported (4–6). These effects are thought to be dependent on their high concentration of (n-3) polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA) and eicosapentaenoic acids (EPA). On the other hand, the antimicrobial activities of many fatty acids in vitro are known (7). Kabara and Vrable (8, 9) suggested that the immune activity of mammal milk is related to the activity of its lipase, which induces fatty acids. Morishita (10) indicated that the antimicrobial activity of lipids on intestinal bacteria is different for varying kinds of lipids in vitro.

According to these reports, about not only microbiological but also nutritional effects, different lipids are considered to have different effects on intestinal microflora. However, the changes of nutrients, such as saccharides and proteins, by fish oil or other lipid intake have not been cleared. In this study, to clarify the dietary effects of fish oil and other lipids on intestinal microflora and conditions, we examined fecal microflora and products fermented by microflora in the cecum in male ICR mice fed diets containing 10% corn oil (CO), 1% corn oil + 9% beef tallow (BT) or 1% corn oil + 9% menhaden fish oil (MO) for 4 wk. In addition, cecal thiobarbituric acid-reactive substances (TBARS), soluble saccharides and soluble proteins were also determined.

MATERIALS AND METHODS

Diets. Composition of the test diets are shown in Table 1. The test diets were stored at −20°C. The long-chain fatty acid (LCFA) composition, acid value (AV), peroxide value (POV) and TBARS of the lipids are shown in Table 2. AV, POV and TBARS were measured by standard methods (11, 12). TBARS was expressed in terms of malondialdehyde. To analyze the LCFA composition, 0.1 g of the lipid was hydrolyzed in 100 mL of 1 mol/L KOH/methanol at 100°C for 2 h and 0.1 mL of the hydrolyzed lipid solution was neutralized by 1 mL of 0.1 mol/L HCl. Then the LCFA, in 100 μL of the neutralized solution, was labeled with 2-nitrophenylhydrazine by a test kit (S-FA-CR-O1, YMC, Kyoto) and analyzed by HPLC (column, YMC-Pack FA; eluent, CH₃CN:H₂O=85:5 (pH 4.8); flow rate, 1.0 mL/min; detection, 400 nm; column temperature, 35°C).

Animal care. Male ICR mice (CLEA Japan, Tokyo) weighing initially 13.0–15.0 g were used after acclimating them to laboratory conditions (temperature, 20–25°C; relative humidity range, 50–60%). Five or six mice were housed in a wire bottom cage and fed CO. After six days, the mice were redivided at random into three groups and fed CO (n=5), BT (n=6) or MO (n=6) for 28 d. Fresh diets and water were supplied every day.

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Table 1. Composition of the diets.

| Compositions | Diet | CO | BT | MO |
|--------------|------|----|----|----|
|              | g/100 g |     |     |     |
| Casein       |        | 20.0 | 20.0 | 20.0 |
| DL-Methionine|        | 0.3  | 0.3  | 0.3  |
| Sucrose      |        | 45.0 | 45.0 | 45.0 |
| Corn starch  |        | 15.0 | 15.0 | 15.0 |
| Cellulose    |        | 5.0  | 5.0  | 5.0  |
| Mixed minerals<sup>1</sup> | | 3.5  | 3.5  | 3.5  |
| Mixed vitamins<sup>2</sup> | | 1.0  | 1.0  | 1.0  |
| Choline bitartrate | | 0.2  | 0.2  | 0.2  |
| Corn oil<sup>3</sup> | | 10.0 | 1.0  | 1.0  |
| Beef tallow<sup>3</sup> | | 0.0  | 9.0  | 0.0  |
| Menhaden fish oil<sup>3</sup> | | 0.0  | 0.0  | 9.0  |

<sup>1</sup> Based on AIN-93.
<sup>2</sup> Based on AIN-76.
<sup>3</sup> Corn oil, beef tallow and menhaden fish oil were purchased from Ajinomoto, Co. Inc., Tokyo, Wako Pure Chemical Ind. Ltd., Osaka, and Sigma Chemical Co., St. Louis, respectively.

Table 2. Fatty acid composition, acid value, peroxide value and TBA-reactive substances (TBARS) of the lipids used in the diets.

| Lipid Source | Corn oil | Beef tallow | Menhaden fish oil |
|--------------|----------|-------------|-------------------|
| Fatty acid (area %)<sup>1</sup> |          |             |                   |
| 16:0         | 12.2     | 26.4        | 13.9              |
| 18:0         | &lt;1     | 12.1        | 2.3               |
| 16:1         | &lt;1     | 5.5         | 21.9              |
| 18:1         | 26.8     | 39.7        | 7.8               |
| 18:2         | 61.0     | 7.5         | &lt;1              |
| 18:3         | &lt;1     | —           | 1.5               |
| 20:5         | —        | —           | 9.5               |
| 22:6         | —        | —           | 20.7              |
| Others       | &lt;1     | &lt;9       | &lt;23             |
| Acid value   | 0.18     | 0.15        | 0.83              |
| Peroxide value (μEq/g) | 0.87 | 1.73        | 13.73             |
| TBARS<sup>2</sup> (μmol/g) | 0.04 | 0.17        | 2.13              |

<sup>1</sup> Determined with HPLC using an analysis kit (S-FA-CR-01, YMC, Kyoto). Only the major fatty acids are listed.
<sup>2</sup> Expressed in terms of malondialdehyde.
<sup>3</sup> —: not detected.

Fresh feces of the mice were collected for microflora analysis on the last two days. At the end of the experiment, blood was collected from the abdominal aorta and vein under diethyl ether anesthesia. The cecum, spleen, liver and kidneys were removed, and then weighed. The plasma, cecal contents and liver were stored at −20°C until their chemical analyses.

Analysis of fecal microflora. Fecal microflora were analyzed using the method and media of Mitsuoka et al. (13). A fecal sample (0.1 g) was suspended in 9.9 mL of anaerobic diluent “A,” then a decimal dilution series from 10⁻² to 10⁻⁸ was prepared. From the appropriate dilution, 0.05 mL aliquots were inoculated with three non-selective agar media (EG, BL, Trypticase soy blood (TS)) and four selective agar media (BS for bifidobacteria), LBS (for lactobacilli), TATAC (for streptococci), and DHL (for enterobacteriaceae). Anaerobic agar plates (EG, BL, BS) were incubated at 37°C in jars containing reduced steel wool under CO₂ gas replacement for 72 h. Aerobic agar plates were incubated for 72 (TATAC) and 24 h (TS, DHL) at 37°C. LBS agar plates were incubated at 37°C in jars without reduced steel wool under CO₂ gas replacement for 72 h. After incubation, each plate was examined for microbial colonies. Microbial groups were identified based on using Gram staining, colonial and cellular morphologies, spore formation and aerobic growth. The number of bacteria was expressed as log₁₀ of the number of microbes per gram found in wet feces.

Measurement of chemicals of the plasma, liver and cecal content. Levels of triacylglycerides (TG), total cholesterol (TC), non-esterified fatty acids (NEFA), phospholipids (PL), glucose and albumin in plasma were determined by using commercially available kits (Triglyceride G-test Wako, Cholesterol E-test Wako, NEFA C-test Wako, PL Test Wako, Glu B-test Wako and Alb Test Wako, Wako Pure Chemical, Osaka). Liver lipids (TC, NEFA and PL) were determined after being prepared by the method of Kates and Volcani (14). Cecal pH was determined by a glass-electrode (6636 type, Horiba, Kyoto) with 20 volumes (v/w) of deionized water. Cecal NEFA and protein were determined by test kits (DC Protein Assay, Bio-Rad, Hercules, CA). Cecal volatile basic nitrogens (VBN) were determined by Conway’s micro-diffusion method (15) after centrifugation at 2,220×g for 10 min with 9 volumes (v/w) of 10% trichloroacetic acid (TCA). TBARS in the TCA solution was determined as described above (12). Cecal water-soluble saccharides were determined by the phenol-sulfuric acid method (16) after centrifugation at 2,220×g for 10 min with 9 volumes (v/w) of deionized water. Organic acids (lactic acid, acetic acid, propionic acid and n-butyric acid) in the water solution were determined by a fatty acid analysis kit (S-FA-CR-01) with HPLC in accordance to a previous report (17).

Statistical analysis. Data were expressed as the mean±SD. One-way ANOVA was used to determine any significant differences (p<0.05) between means. When significant differences were found, Duncan’s multiple-range test was used to determine the exact nature of the difference.

RESULTS

Body and organ weight

The body and organ weights are summarized in Table 3. There were not significant differences among the three groups in body weight gain and the wet weight of kidneys, spleen and cecum per body weight. The liver weight was significantly greater for mice fed CO than for mice fed BT and MO.

Plasma lipids, glucose and albumin levels and liver lipids

Table 4 summarizes the results of plasma lipid, glu-
Table 3. Body and organ weight of mice fed diets containing corn oil, beef tallow or menhaden oil.1

| Diet | CO       | BT       | MO       | ANOVA2 |
|------|----------|----------|----------|--------|
| Initial body weight (%) | 22.3±0.6 | 22.2±0.5 | 21.4±1.1 | NS     |
| Body weight gain (g/28 d) | 16.6±2.3 | 17.5±2.5 | 18.3±3.4 | NS     |
| Organ weight (g/100 g body weight) |          |          |          |        |
| Liver | 6.13±0.35a | 5.23±0.55b | 5.10±0.35b | **     |
| Kidneys | 1.63±0.31 | 1.52±0.18 | 1.75±0.22 | NS     |
| Spleen | 0.33±0.04 | 0.30±0.05 | 0.32±0.04 | NS     |
| Cecum | 0.51±0.12 | 0.55±0.09 | 0.58±0.07 | NS     |

1 The mice (male, 4 wk old) were fed diets containing 10% corn oil (CO, n=5), 1% corn oil +9% beef tallow (BT, n=6) or 1% corn oil +9% menhaden oil (MO, n=6) for 28 d. Values are means±SD.

ab Means within a diet group not sharing the indicated letter superscripts are significantly different at p<0.05 as determined by one-way ANOVA followed by Duncan’s multiple range test.

2 Significant diet effects: ** p<0.01; NS, not significant at p>0.05.

cose and albumin levels and liver lipid levels. The levels of plasma TC and NEFA in mice fed MO were significantly lower than those of mice fed other diets. Furthermore, the PL level was lower in mice fed MO than in mice fed BT. The glucose level of MO was 20 and 34% lower than that of mice fed CO or BT, respectively. No significant effect was shown in the levels of plasma TG and albumin. The level of TC was lower in mice fed MO than in mice fed BT. The level of NEFA in mice fed MO was 49 and 35% lower than that of mice fed CO or BT, respectively. The level of PL was lower in mice fed MO than in mice fed BT.

Cecal contents property

Cecal pH, soluble protein, soluble saccharides, VBN, NEFA, organic acids and TBARS are shown in Table 5. Cecal pH in mice fed MO and BT was significantly lower than that of mice fed CO. The content of soluble protein in mice fed MO was about 1.5 times higher than that of mice fed CO or BT. The content of soluble saccharides in mice fed MO was higher than that of mice fed BT. The content of NEFA in mice fed MO tended to be higher than that of mice fed other diets, though there was no significant difference. Cecal TBARS in mice fed MO was about 8 and 5 times higher than that of mice fed CO or BT, respectively. The major organic acids in the mouse cecum were lactic acid and acetic acid ranging from 10 to 23 and 23 to 34 μmol/g, respectively. Though the lactic acid in mice fed BT tended to be higher than that in mice fed CO, there were no significant differences among the three groups. Cecal propionic acid in mice fed MO was about 1.8 times higher than that of mice fed BT. However, the level was lower than 6 μmol/g.

Fecal Microflora

The results of fecal microflora are shown in Table 6. The number of bacteroides in mice fed BT was 3.2 times greater than that of mice fed MO. On the other
Table 5. Effect of dietary lipids on pH, short-chain fatty acids (SCFAs), lactic acid, volatile basic nitrogen (VBN), non-esterified fatty acid (NEFA), TBARS, soluble protein and soluble saccharides in the cecal content of mice.

| Diet | CO       | BT       | MO       | ANOVA |
|------|----------|----------|----------|-------|
| pH   | 7.83±0.06a | 7.49±0.16b | 7.37±0.13b | **   |
| Organic acids (μmol/g content) | | | | |
| Lactic acid | 10.5±4.0 | 22.9±15.6 | 18.3±4.8 | NS   |
| Acetic acid | 29.9±13.0 | 33.7±15.7 | 23.4±3.8 | NS   |
| Propionic acid | 4.0±1.3b | 3.3±1.6b | 5.9±1.7b | *    |
| n-Butyric acid | 5.4±1.9 | 4.0±1.4 | 3.9±1.3 | NS   |
| VBN (mg/g content) | 1.03±0.33 | 0.91±0.28 | 0.94±0.33 | NS   |
| NEFA (μmol/g content) | 1.5±0.76 | 1.28±0.57 | 2.02±1.43 | NS   |
| TBARS (nmol/g cment) | 100±14a | 139±20a | 789±181b | **   |
| Soluble protein (mg/g content) | 33.2±4.9a | 33.1±4.5a | 48.4±7.2b | **   |
| Soluble saccharide (mg/g content) | 14.2±1.6ab | 12.1±2.7a | 16.4±1.7b | *    |

1 See Table 3.
ab Means within a diet group not sharing the indicated letter superscripts are significantly different at p<0.05 as determined by one-way ANOVA followed by Duncan’s multiple range test.

2 Significant diet effects: * 0.01 < p<0.05; ** p<0.01; NS, not significant at p>0.05.

Table 6. Effect of dietary lipids on fecal microflora in mice.

| Diet | CO       | BT       | MO       | ANOVA |
|------|----------|----------|----------|-------|
| Total viable count\(^1\) | 9.78±0.31 | 9.92±0.37 | 9.58±0.24 | NS   |
| Bacteroidaceae | 9.70±0.38ab | 9.90±0.39a | 9.37±0.23b | *    |
| Bifidobacteriaceae | 8.52±0.29a | 8.75±0.18b | 8.93±0.24b | *    |
| Lactobacilli | 8.22±0.80 | 8.37±0.51 | 8.52±0.58 | NS   |
| Streptococci | 6.78±0.44 | 6.57±0.29 | 6.85±0.48 | NS   |
| Enterobacteraeae | 5.60±0.26 | 5.65±0.81 | 6.81±0.69 | NS   |
| Ratio of bifidobacteria to total viable count (%) | 7.9±7.6° | 8.0±5.6° | 22.0±8.8° | **   |

1 See footnotes of Table 3.
\(^ab\) Means within a diet group not sharing the indicated letter superscripts are significantly different at p<0.05 as determined by one-way ANOVA followed by Duncan’s multiple range test.

2 Significant diet effects: * 0.01 < p<0.05; ** p<0.01; NS, not significant at p>0.05.

3 The counts of colony-forming units on EG agar.

hand, the number of bifidobacteria in mice fed MO was 2.5 times higher than that of mice fed CO. The ratio of fecal bifidobacterial count to total viable count in mice fed MO was higher than that in mice fed CO or BT. The number of enterobacteraeae tended to be higher in mice fed MO than in mice fed other diets.

**DISCUSSION**

It is generally considered that the number of intestinal bifidobacteria is suppressed by the intake of a high-lipid and low-dietary fiber diet (18). However, in this study, MO increased the number of fecal bifidobacteria compared with CO and decreased the number of bacteroidaceae compared with BT (Table 6). The ratio of bifidobacteria to total viable count in the feces was higher in mice fed MO than that in either mice fed CO or BT. It is known that peroxide lipids have cytotoxicity induced by free radicals (19). EPA ethyl ester inhibited the growth of Bacteroides thetaiotaomicron, which is one of the dominant bacteria in the large intestine of adult humans (20). On the other hand, linoleic acid has antimicrobial activity against Bifidobacterium rather than against Bacteroides, in vitro (21). The increasing number of bifidobacteria and ratio of bifidobacteria to total bacteria in the intestinal tract is thought to exert a beneficial effect for the host by producing short-chain fatty acids (SCFAs), decreasing putrefactive products, inhibiting the growth of potential pathogens and inducing immune activity (1, 2).

MO and BT lowered the cecal pH compared with CO. The concentration of cecal lactic acid tended to be higher in mice fed BT than that in mice fed CO. It is
known that the mild lowering of intestinal pH is desirable for host health because of the suppression of production and absorption of putrefactive compounds and growth inhibition of bacteria other than lactic acid bacteria (1, 2). The total viable count in feces and the cecal organic acids in mice fed BT tended to be higher than in other test groups. It is reported that dietary tripalmitin, butter and beef tallow suppressed the cecal SCFA content in rats (10, 22, 23), though lactic acid was not discussed in those studies. On the other hand, Morishita (10) indicated that cecal SCFAs in ICR mice were decreased and increased by dietary butter and margarine, respectively. Furthermore, the main fatty acids of butter and margarine are palmitic acid and oleic acid, respectively. Beef tallow contains oleic acid, the same as margarine, suggesting that oleic acid promoted cecal fermentation in the mice fed BT.

Although the cecal pH in mice fed MO was lower than in mice fed CO, there were no differences in the number of fecal viable count and cecal organic acids between the groups. Cecal TBARS was significantly higher in mice fed MO than in the mice fed CO or BT. Furthermore the ratio of TBARS to total lipid in the cecum was much higher than that in the menhaden fish oil used in this test, suggesting that the products generated from menhaden oil by auto-oxidation and intestinal microflora in mice affected the cecal pH.

The results of the plasma and liver lipid and plasma glucose in this study agree with many other reports related to the inhibitory effect of fish oils on the absorption and metabolism of nutrients; for example, the inhibitory effect on cholesterol absorption in rats (24) and the hypoglycemic effect on genetically diabetic (db/db) mice (25). The higher levels of the soluble saccharides and proteins in the cecal contents observed in mice fed MO with lower levels of plasma glucose is very interesting. Commonly, the enzymatic action for poly saccharides (dietary fiber) by intestinal bacteroidaceae is stronger than that of bifidobacteria. However, we have found that the degraded products from dietary fiber by intestinal bacteria (Clostridium ramosum) can promote the growth of bifidobacteria, in vitro (26). Though the proteolytic activity of bacteroidaceae is higher than that of bifidobacteria, a higher number of cecal bifidobacteria and lactobacilli occurred in rats fed a diet containing 22 or 60% (w/w) casein rather than in mice fed a diet containing 5% casein (10). These facts and our results support the notion that the remains and metabolites of nutrients in the intestine increased by MO feeding induced changes to the microflora, such as increasing the ratio of bifidobacteria to total bacteria.

In conclusion, we believe that there are two important causes for the change in intestinal microflora by the intake of fish oils. One of the causes is a difference of antimicrobial effects against bacteroidaceae, bifidobacteria or other bacterial groups. The other cause is the inhibitory effect on nutrient absorption and metabolism from the upper intestine. These nutrients are able to be used by microflora in the lower intestine. These phenomena are considered very important to intestinal conditions and health in mammals. More studies on the cross-effect of lipids and other dietary components on the intestinal microflora and their fermentation are now in progress.

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