Appl Microbiol Biotechnol (2014) 98:4701–4709
DOI 10.1007/s00253-014-5535-8

ENVIRONMENTAL BIOTECHNOLOGY

Caecal environment of rats fed far East Asian-modelled diets

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Received: 15 November 2013 / Revised: 8 January 2014 / Accepted: 9 January 2014 / Published online: 16 February 2014

Abstract To clarify the effect of type of foods on the intestinal environment, Far East Asian- (FEA; rich in rice starch, soy protein and soy oil) and Far East Asian marine- (FEAM; rich in rice starch, fish meal, fish oil and brown alga) modelled diets and sucrose, casein and beef tallow-rich (SCB) diet were prepared. After the 2-week administration of diets in rats, caecal organic acids and putrefactive compounds (ammonia, indole, phenol and H_2S, which are regarded as putative risk factors for tumours) were determined. The caecal microbiota was also analyzed using denaturing gradient gel electrophoresis and pyrosequencing with bar-coded primers targeting the bacterial 16S rRNA gene. Levels of *n*-butyrate, acetate, indole and phenol were high in rats fed FEA. On the other hand, H_2S was clearly suppressed by both FEA and FEAM comparing with SCB. These results suggest that FEAM is preferable to FEA for optimal intestinal environment and host health. Both microbial analyses showed that the diversity of microbiota in the FEAM group was lower than in the other diet groups. Ratio of *Firmicutes*, *Bacteroidetes* and *Proteobacteria* in the SCB group was about 5:4:1. *Firmicutes*, particularly *Lachnospiraceae*, was promoted by FEA and FEAM.

Keywords Far East Asian diets · Intestinal environment · Intestinal microbiota · Rat · Brown algae · Pyrosequencing

Introduction

The adult human intestine contains 10^{13} to 10^{14} bacteria, involving at least 500 different species or strains, which make up the gut microbiota (Dethlefsen et al. 2008). While up to ten different bacterial phyla typically comprise the microbiota, the three phyla *Firmicutes*, *Bacteroidetes* and *Actinobacteria* predominate (Simrén et al. 2013). The intestinal microbiota plays an important role in host health that can be seen as twofold, with both beneficial and harmful effects on the host. Beneficial effects include prevention of pathogen colonization and stimulation of immune responses (Chung et al. 2012; Feng et al. 2011), assistance in digestion, absorption and synthesis of nutrients (Willing and Van Kessel 2010), and aging (Biagi et al. 2011). Harmful effects include the production of intestinal putrefactive compounds, such as ammonia, H_2S, amines, phenols and indoles; these putrefactive compounds are regarded as putative carcinogens and toxins (Davila et al. 2013; Kuda et al. 2005). The intestinal microbiota depends on various factors, such as aging, stress, climate, infectants, disease, drugs and diet (Biagi et al. 2011). Moreover, diet composition is dependent on geographic location and culture. Such differences can also affect the intestinal microbiota (De-Filippo et al. 2010).

Food materials, food consumption and cuisine vary not only by climate but also by culture, region and society. Diets of Far East Asian areas, such as Korea, Japan and East coasts of China, are characterized by the consumption of rice, vegetables and marine products, including various fishes and algae (Kuda and Ikemori 2009; Ogawa et al. 2002). Additionally, various processed soy products, such as *miso*, *shoyu*, *natto* and *tofu*, are utilized in the cuisines of these countries. There are reports of food-associated beneficial effects, such as hypolipidemic, antihypertensive and/or antioxidant effects of fish oil and canned fish products (Aguilera et al. 2004; Biagi et al. 2011; Kuda and Yano 2009), as well as antioxidant, antibacterial, immune promoting and anti-inflammatory activities of edible algae and soybean products (Kim et al. 2008; Kuda et al. 2007; Kuda et al. 2012a; Kuda et al. 2012b).

In our previous studies, soluble polysaccharides (dietary fibres) of brown algae, laminaran and alginic acid, showed inhibitory effects on intestinal putrefactive compounds with

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organic acid fermentation (An et al. 2013a; Kuda et al. 2005). On the other hand, although *n*-3 polyunsaturated fatty acids improved hypocholesterolemia in rats, an increase in intestinal oxidation, evaluated as thiobarbituric acid reactive substances (TBARS), was observed (Kuda et al. 2000). Furthermore, compared with milk-casein, the intake of soy protein and fish meal increased intestinal putrefactive compound levels (An et al. 2014). Therefore, an analysis of the combined effects of various food materials on fermentation by the intestinal microbiota and the generation of putrefactive compounds is anticipated. It was reported that *Lactobacillus*, *Bifidobacterium*, *Eubacterium*, *Clostridium* and *Enterococcus* (Gram positive) in the rat caecum were impaired by a European-modelled diet compared with a Japanese-modelled diet (Morishita 1990). However, this determination was made using only culture-dependent methods.

It is well known that most of the intestinal microbiota is non-cultureable using general medium. Therefore, since the 1990s, the microbiota has been analyzed using a variety of molecular techniques, such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP), because these methods are not culture-based (Kibe et al. 2005). However, these methods can detect only tens of mainly microbes, though there are so many species. Recently, pyrosequencing of 16S rRNA genes has been used for the analysis of complex microbiota in various environments, including the animal intestine (An et al. 2013a; An et al. 2014; Van den Bogert et al. 2011). This method can evaluate thousands to tens of thousands of reads of genes from a sample in one run.

In this study, to clarify the effects of the types of foods on the intestinal environment and host health, caecal organic acids and putrefactive compounds in rats fed the sucrose, casein and beef tallow-rich diet, Far East Asian-modelled diet (rich in rice starch, soybean protein and soy oil) and Far East Asian marine modelled diet (rich in rice starch, fish protein and fish oil) were analysed. Furthermore, the caecal microbiota was analyzed using DGGE and amplicon pyrosequencing with bar-coded primers targeting the bacterial 16S rRNA gene.

Materials and methods

Animal and diets

The animal experiment was performed in compliance with the guidelines for the proper conduct of animal experiments and related activities in academic research institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan, and approved by the animal experiment committee of Tokyo University of Marine Science and Technology (approval no. 2012-10).

As shown in Table 1, the three diets were prepared as sucrose, wheat starch, milk-casein and beef tallow-rich (SCB) diet, Far East Asian-modelled (FEA; rich in rice starch, soybean protein and soy oil) diet and Far East Asian marine-modelled (FEAM; rich in rice starch, fish protein and fish oil) diet; diets in this study were based on the report of Murata et al. (2002); however, diets had some changes comparing with diets of the report. In the report, the SCB diet was regarded as European-modelled diet. Rice starch, soy protein, soy oil and high dietary fibre were found in FEA. FEAM contained fish meal, fish oil and *wakame* (*Undaria pinnatifida*).

Four-week-old male Wistar rats were housed separately in metal wire cages and allowed free access to water and food. After acclimation with AIN-76 based diet for 7 days, the animals were divided into three groups (n=6) and given the SCB, FEA or FEAM for 14 days. Faecal frequency was a number of faeces and faeces of rats were counted in every morning. Then, rats were anesthetized with diethyl ether and exsanguinated from the abdominal aorta. The caecum were excised and weighed. A portion of the caecal content was used for direct total cell counts using the Gram stain method (Miriam and Buenviaje 1989). The remaining caecal content was stored at −80 °C until used in subsequent experiments.

Table 1 Composition of the test diets used in this study (g/100 g)

		AIN-76	Test diets		
			SCB	FEA	FEAM
Carbohydrates	Sucrose	50.0	36.0	5.0	2.9
	Corn starch	15.0			
	Wheat starch		30.0		
	Rice starch			60.3	57.7
	Cellulose	5.0	2.0	6.9	
	Undaria pinatifida [a]				4.9
Protein	Milk casein	20.0	20.0		
	Soy protein			7.9	3.9
	Fish meal			7.9	11.8
Lipid	Corn oil	5.0			
	Beef tallow		7.0		
	Soy oil			5.0	2.0
	Fish oil			2.0	4.9
Micronutrients	Mixed minerals (AIN-76)	1.0	1.0	1.0	1.0
	Mixed vitamins (AIN-76)	3.5	3.5	3.5	3.5
	DL-methionine	0.3	0.3	0.3	0.3
	Choline bitartrate	0.2	0.2	0.2	0.2

[a] Wakame: rich in insoluble and soluble dietary fibers

Blood lipid and glucose levels

Blood samples were placed in tubes with heparin and centrifuged at 2,190×*g* for 10 min to collect plasma. Levels of plasma total-cholesterol, triglyceride, phospholipid, non-esterified fatty acid and glucose were determined using commercial test kits (T-Cho E Test Wako, TG E Test Wako, PL C Test Wako, NEFA C Test Wako and Glucose CII Test Wako; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and a grating microplate reader (SH-1000 Lab; Corona Electric, Ibaraki, Japan).

Caecal chemical compounds

Caecal contents were diluted with four volumes of distilled water, and levels of organic acids and putrefactive compounds were determined. Organic acids (lactic acid, acetic acid, propionic acid and *n*-butyric acid) were determined using high-pressure liquid chromatography (HPLC) according to a previous report (Kuda et al. 2010). Briefly, 0.2 ml of the caecal suspension was acidified with 0.05 ml of 1 mol/l sulphuric acid and centrifuged at 15,000×*g* for 3 min at 4 °C. After centrifugation, the supernatant was passed through a 0.45-μm-pore-size filter and injected into the HPLC instrument under the following conditions: column, ICSep ICE-ORH-801 (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan); operating temperature, 35 °C; elution, 0.005 mol/l of H_2SO_4; flow rate, 0.8 ml/min. Eluted compounds were detected by a refractive index detector.

Levels of ammonia, phenol and sulphide compounds were determined using reagent sets for water analysis (nos. 7, 17 and 53, respectively; Kyoritsu Co., Tokyo, Japan) with the grating microplate reader, after dilution with four volumes of distilled water (Kuda et al. 2005). Indole level was measured using Kovac's reagent (Lombard and Dowell 1983).

DNA extraction

Bacterial DNA from each caecal sample was extracted using a NuCleoSpin Tissue (Takara Bio, Shiga, Japan). With DNA extraction, caecal content impurities were removed before being placed on the columns by centrifugation. Purified DNA was dissolved in Tris-EDTA buffer (pH 8) and used as the DNA template in DGGE and pyrosequencing analyses.

DGGE analysis

DGGE analysis was performed as described previously (An et al. 2010; An et al. 2013b). The primer pair was chosen for the amplification of the V3 region (approximately 220 bp) of the 16S rRNA gene: forward primer with GC clamp GC-339f (50-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCT CCTACG GGA GGC AGC AG-30) and reverse primer V3–53r (50-GTATTA CCG CGG CTG CTG G-30). Polyacrylamide gels (8 %, *w*/*v* acrylamide-bis-acrylamide (37.5:1)) in 1x Tris–acetate-EDTA buffer (pH 8), with a denaturing gradient ranging from 30 to 60 % denaturant (100 % denaturation corresponds to 7 mol/l urea and 40 %, *v*/*v* formamide), were electrophoresed at a constant voltage of 200 V at 60 °C for 3 h using the DCode System apparatus (Bio-Rad, Hercules, CA, USA).

Bacterial analysis using pyrosequencing

DNA sequences of caecal contents were amplified individually with primer pairs for the 16S rDNA gene following a previous report (An et al. 2014). The region of the 16S rRNA gene was amplified by PCR using bar-coded primers targeting 27 to 338 bp. The primers contained five-base sample-specific bar code sequences denoted as "X" and common linker (AC) sequences in the 5' end (Turnbaugh et al. 2009). The forward primer was 5'-ctatgcgccttgccagcccgctcag NNNNNNNN AGAGTTTGATCCTGGCTCAG - 3', where the sequence of the A adapter is shown in small letters and N represents an 8-bp bar code that is unique for each sample. The reverse primer was 5'-ctatgcgccttgccagcccgctcag TGCTGCCTCCCG TAGGAGT-3', where the sequence of the A adapter is shown in lowercase letters. PCR amplification was performed in 0.1 ml reaction mixtures composed of 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 50 pmol of each primer, 0.2 mol/l of each deoxynucleoside triphosphate (dNTP), 2.5 U of TaKaRa Ex Taq DNA polymerase (TaKaRa Bio) and 50 ng of template DNA.

PCR was performed using the Genome Sequencer Junior System (Roche, CT, USA). The pooled DNA samples were adapter ligated with beads and amplified by emulsion PCR using a GS Junior Titanium emPCR kit (Lib-A; Roche). Beads were enumerated after emulsion PCR, and the same amount of beads was put in a PicoTiterPlate and sequencing was carried out using Genome Sequencer Junior System. Sequences obtained from the pyrosequencing were analyzed with GS Run Browser in the Genome Sequencer Junior System Software 2.5 (Roche).

Taxonomy-based analyses at the phylum, family and genus levels were performed by assigning taxonomic status to each sequence using Classifier of Ribosomal Database Project (http://rdp.cme.msu.edu/classifier/classifier.jsp). In this study, pyrosequencing data was analyzed at the species level by BLAST analysis through DDBJ (http://blast.ddbj.nig.ac.jp/blast/blastn?lang=ja). Diversity of the microbiota, indicated by operational taxonomy units (OTUs) and the Shannon–Wiener index (SWi), was calculated using FastGroupII (http://fastgroup.sdsu.edu/fg_tools.htm). Data was accepted under the conditions that sequences should be at least 300 bp in length and the Percent Sequence Identity (PSI) was >97 %.

Nucleotide sequence accession number

The sequence obtained from DGGE analysis was in DDBJ under accession numbers AB873092-6. Furthermore, the sequence data of pyrosequencing was in DDBJ under submission accession numbers DRA001220 (Study; DRP001285, Sample; DRS012837-48, Experiment; DRX013029-40, Run; DRR014509-20).

Statistical analysis

Data for body, organ and faecal weights, and caecal chemical compounds were expressed as means ± SEM (standard error of the mean). Statistical analysis for the animal experiment was performed using EXCEL Statistic 5.0 (Esumi Co., Tokyo, Japan). One-way ANOVA was used to assess the effects of treatments. Significant differences were accepted at $p<0.05$. When significant variation was found, the Tukey test was used to determine the exact nature of the difference.

Results

Faecal frequency and caecal weights

The initial weight of rats was about 125 g, and the rats gained 82 to 92 g of body weight in the three groups. There were no significant differences in body weight gained between the SCB, FEA and FEAM groups. As shown in Fig. 1a, the faecal frequency of rats fed FEA was 1.8 times higher than rats fed SCB, while that of the FEAM group was 1.6 times higher than the FEA group. Caecal weight (including content) of rats fed FEAM was 1.3 to 1.4 times higher than that of rats fed the other diets.

Blood lipid and glucose levels

Levels of plasma TC, TG, PL, NEFA and Glu in rats fed the test diets are summarized in Table 2. In rats fed SCB, the plasma TC and PL levels were 1.4 and 1.5 times higher, respectively, than the other diet groups. Although there were no significant differences in TG, NEFA and Glu levels among the three groups, TG and NEFA levels showed an increased tendency in rats fed FEAM.

Caecal organic acids and putrefactive compounds

Organic acid contents are shown in Fig. 2a. Lactic and *n*-butyric acid levels (La and Ba in Fig. 2a) and total organic acid were higher in rats fed FEA than in the other groups. Acetic acid (Aa) was high in the FEA and FEAM groups.

As shown in Fig. 2b, caecal indole and phenol contents in the FEA group were higher than in the other groups. Caecal ammonia content was tended to be high in rats fed FEA. On the other hand, caecal H_2S of SCB fed rats was obviously (4 to 16 times) higher than that of rats fed FEA or FEAM.

Caecal microbiota evaluated by DGGE

Direct bacterial counts (using Gram staining) of the caecal content of rats fed SCB, FEA and FEAM were about 10.2 log cells/g, respectively. No differences were observed in the cell counts between the three diet groups.

Diversity in the caecal microbiota was analyzed by DGGE and is shown in Fig. 3. Over 20 bands were visually detected on the DGGE gel for each group. On the lanes representing SCB, many bands were observed in a small region (A in Fig. 3). In the case of FEA, many bands were located in the middle region of the gel (B). Only five of the bands were excised from the gel, since several bands were closely situated.

Although band 1 was clear in rats fed SCB, it could not be identified. Band 4 was clearly detected on the lanes for SCB and was identified as *Barnesiella intestinihominis*; however, the sequence similarity was low (88 %). From the lanes for FEA, bands 2, 3 and 5 were identified. However, the sequence of band 2 showed similarity to the four genera, *Fusobacterium naviforme*, *Lachnospiraceae bacterium*, *Eubacterium xylanophilum* and *Moryella indoligenes*. Band 3 was identified as *Oscillospira guilliermondii* and band 5 was identified

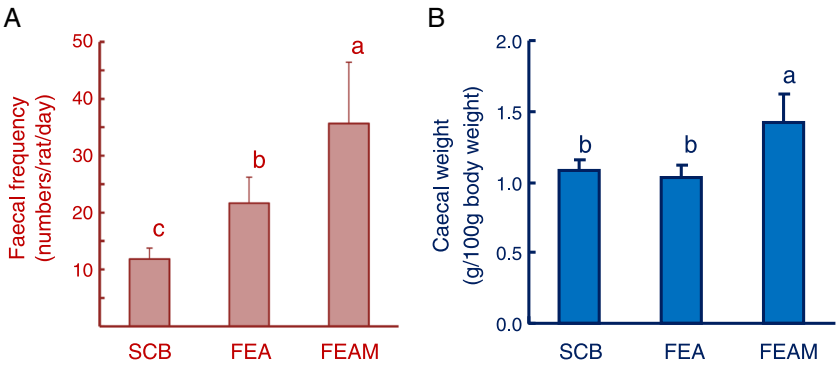

Fig. 1 Faecal frequency (**a**) and caecal weight (**b**) of rats fed sucrose casein beef tallow diet (SCB), Far East Asian-modelled diet (FEA) or Far East Asian marine-modelled diet (FEAM). Values are means ± SEM ($n=6$). *Different letters* indicate significant differences ($p<0.05$) between groups

Table 2 Levels of plasma lipids and glucose in rats fed SCB, FEA and FEAM

	SCB	FEA	FEAM
Cholesterol (mg/100 m)	99.0±4.1[a]	68.5±3.0[b]	68.5±5.3[b]
Triglyceride (mg/100 ml)	143±29	126±16	109±18
Phospholipid (mg/100 ml)	247±12[a]	164±8[b]	166±7[b]
Non-esterified fatty acid (mEq/l)	0.51±0.02[a]	0.48±0.04[a]	0.34±0.02[b]
Glucose (mg/100 ml)	272±23	289±16	319±13

Values are mean and SEM (n=6)

Different letters indicate significant differences (p<0.05) between groups

as *Clostridium difficile*, *Clostridium lituseburense* or *Clostridium bifermentans*.

Pyrosequencing analysis of caecal microbiota

In the pyrosequencing analysis, total reads of each sample were 4,462, 4,398 and 4,521 in rats fed SCB, FEA or FEAM, respectively (Table 3). Most of the reads passed the Fast Group II filter: >300 bp in length and 97 % similarity. On the other hand, the number of OTUs and the SWi showed a decreased tendency in rats fed FEAM (OTU 1206; SWi 5.34) compared to rats fed SCB (OTU 1402; SWi 5.73) and JPD (OTU 1462, SWi 5.76).

The microbiotas at the phylum and family levels are shown in Fig. 4. The predominant phyla in the three diet groups were *Firmicutes*, *Bacteroidetes* and *Proteobacteria* (outer circles in Fig. 4). The proportion of *Firmicutes* was the lowest in rats fed SCB, accounting for about 50 % of the genes, and the proportions in the FEA and FEAM groups were 66.8 and 66.1 %, respectively. On the other hand, the proportion of *Bacteroidetes* was the highest in the SCB group (about 41 %). *Proteobacteria* was high in rats fed FEA (8.1 %).

At the family level, *Lachnospiraceae* (l in Fig. 4) was predominant in the three diet groups, though the proportion in each group differed, from 17 % of genes for FEA to 34 % for FEAM. *Ruminococcaceae* (m) was the second most predominant family in the diet groups, accounting for between 15 and 18 % of genes. Of the *Firmicutes*, *Peptostreptococcaceae* (n) was detected in all groups at 1 to 2 %. *Lactobacillaceae* (o) was not detected in the FEA rats. *Bacteroidaceae* (e) was the highest in rats fed SCB (9 %). All *Proteobacteria* genes were identified as *Helicobacteraceae* (h) or *Sutterellaceae* (i).

In this study, a large number of the detected genes could not be classified to the genus level (Fig. 5). At this level, unclassified *Lachnospiraceae* (l) was dominant, though the proportion differed from 14 % for the SCB group to 34 % for the FEAM group. The second most dominant group was unclassified *Bacteroidetes* (a) and unclassified *Ruminococcaceae* (n), with proportions ranging from 10 to 16 %. In the case of FEA rats, unclassified *Bacteroidales* (b) was low (6 %) and unclassified *Firmicutes* (j) was high (13 %). *Bacteroides* (e), *Parasutterella* (i) and *Helicobacter* (h) were high in rats fed SCB.

Discussion

In traditional Japanese cuisine, the main food materials are rice, vegetables, soybean, fishes and algae. However, the intake of animal products, such as pork, beef, chicken and dairy products, as well as sugar and wheat flour has increased recently in Japan (Murata et al. 2002). It is well known that the overconsumption of animal products and sugar is associated with life style-related illnesses, such as hyperlipidemia, hypertension, diabetes, obesity and arteriosclerosis (Poothullil 1993). In this study, levels of plasma TC and PL in the SCB group were the highest among the three diets (Table 2), which agreed with previous studies (Murata et al. 2002). Furthermore, the plasma NEFA level in the FEAM group was lower than the other diet groups. FEAM

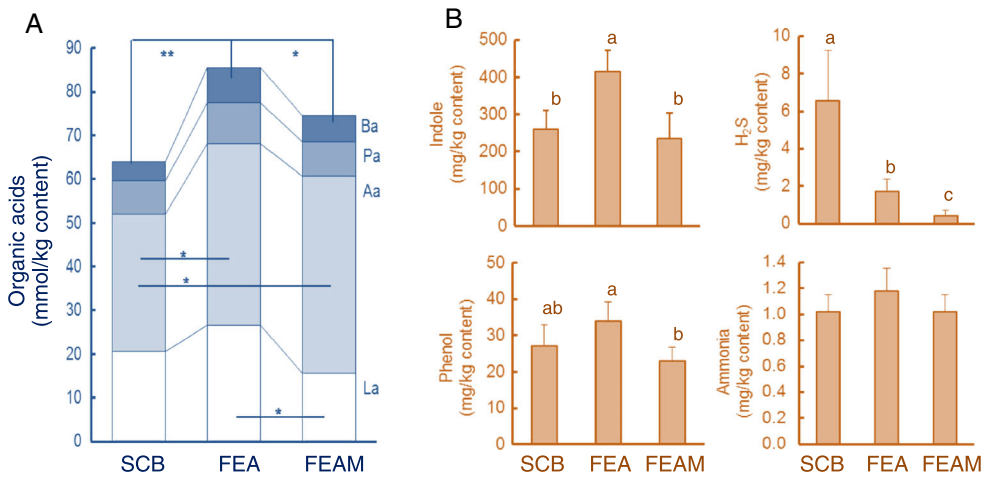

Fig. 2 Caecal organic acid content (**a**) and putrefactive compounds (**b**) in rats fed a model SCB, FEA or FEAM diet. Values are means ± SEM (n=6). **a** Ba, n-butyric acid; Pa, propionic acid; Aa, acetic acid; La, lactic acid. *p<0.05, **p<0.01. **b** *Different letters* indicate significant differences (p<0.05) between groups

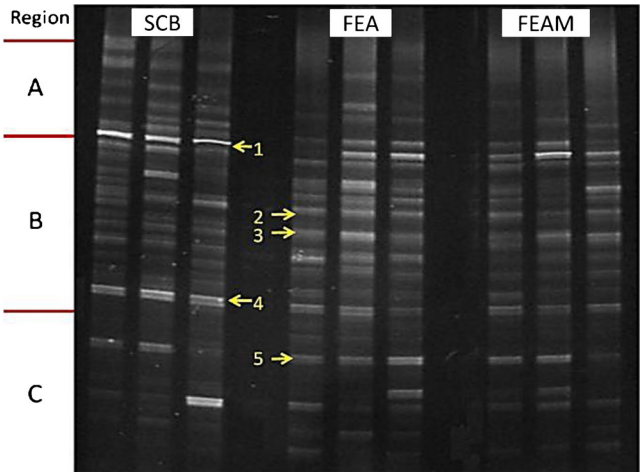

Fig. 3 Representative band patterns from DGGE analysis of the intestinal microbiota of rats fed SCB, FEA or FEAM diet. The main bands were identified as follows: *1*, Not identified; *2*, *Fusobacterium* / Lachnospiraceae bacterium / *Eubacterium xylanophilum* / *Moryella indoligenes* 98 % similarity; *3*, *Oscillospira guilliermondii* 100 % similarity; *4*, *Barnesiella intestinihominis* 88 % similarity; *5*, *Clostridium difficile* / *Cl. bifermentans* 100 % similarity

contained fish oil and brown alga (*wakame*). Fish oil is rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Many studies have indicated that the cholesterol lowering effect of fish oil is related to EPA and DHA (Kuda et al. 2000). Furthermore, it was reported that fish oil and *wakame* showed an ameliorating effect on blood and liver lipid levels (Murata et al. 2002).

Among caecal organic acids (Fig. 2a), *n*-butyric and lactic acids were the highest in the FEA group. FEA was rich in soy protein and fish meal. In our previously study, the AIN-76-based diet containing soy protein or fish meal instead of milk-casein increased caecal *n*-butyric or butyric acids, respectively (An et al. 2014). This suggests that these proteins were degraded and the amino acids fermented, thereby constituting an increased contribution to organic acid levels. However, lactic acid levels were not higher in rats fed FEAM compared to other groups, even though the diet was rich in fish meal. It is thought that the other food materials in the diet, such as fish oil, which was high in FEAM, affected organic acid generation (Kuda et al. 2000).

Table 3 Operational taxonomy units (OTUs) and Shannon–Wiener index of 16S rRNA genes in caecal contents of rats fed the test diets

	SCB	FEA	FEAM
Number of total reads	4,462±57	4,398±154	4,521±184
Number of valid sequences	4,429±49	4,298±125	4,488±191
Number of OTUs	1,402±139	1,462±30	1,280±179
Shannon–Wiener index	5.73±0.41	5.76±0.12	5.34±0.48

Values are mean and SEM

In the previous study, 20 % *w*/*w* soy protein or fish meal increased caecal indole, phenol and H_2S levels (An et al. 2014). In this study, rats fed FEA also showed the highest caecal indole and phenol levels. However, these putrefactive compounds were not high in rats fed FEAM. The inhibitory effect of brown algal dietary fibres, alginate and laminaran, on the generation of caecal putrefactive compounds was reported (An et al. 2013a; Kuda et al. 2005). By extension, it is thought that the production of putrefactive compounds from dietary proteins was suppressed by the dietary fibres contained in FEAM. Additionally, caecal H_2S was suppressed in rats fed FEA or FEAM. Because caecal putrefactive compounds are regarded in the risk factors of cancer and tumours (Davila et al. 2013; Kuda et al. 2005), it is considered FEAM is better in optimal intestinal environment and host health than SEM and FEM.

The results of DGGE analysis revealed that the caecal microbiota diversity of the FEAM group showed a decreased tendency compared to the other diet groups (Fig. 3). This is in agreement with the number of OTUs and the SWi of the pyrosequencing data (Table 3). In the DGGE analysis, the species closely related to *Barnesiella intestinihominis* was detected as one of the dominant components in the SCB group. On the other hand, this species was not detected by pyrosequencing. *Barnesiella* belongs to Bacteroidales and was identified from a human faecal sample using 16S rRNA gene pyrosequencing (Kulagina et al. 2012; Wylie et al. 2012). It is possible that *Barnesiella* was included as unclassified Bacteroidetes in the pyrosequencing result in this study. The DGGE band of *Oscillospira guilliermondii* was clearly visible on the lanes for the FEA group. Though this genus also could not be identified by pyrosequencing, *Oscillospira* belongs to *Ruminococcaceae*. In previous reports, *Bifidobacterium* spp., such as *Bifidobacterium pseudolongum* and *Bifidobacterium animalis*, were detected as main bands on the DGGE gel of caecal contents of rats and hamsters fed AIN-76 or AIN-93 M diets these are rich in sucrose and casein (An et al. 2013b; Martínez et al. 2009). However, a clear DGGE band of *Bifidobacterium* was not detected in this study, even in rats fed SCB. Comparing the AIN-76 and AIN-93 M diets, the main polysaccharide and lipid in SCB were wheat starch and beef tallow instead of corn starch and corn or soybean oil.

According to the pyrosequencing results, the proportion of *Firmicutes* (Gram positives) to *Bacteroidetes* and *Proteobacteria* (Gram negatives) was high in rats fed FEA or FEAM. This agrees with the effect of dietary fibres, such as brown algal polysaccharides (An et al. 2013a) and beet pulp (Middelbos et al. 2010) on intestinal microbiota. On the other hand, there are several reports that indicated that

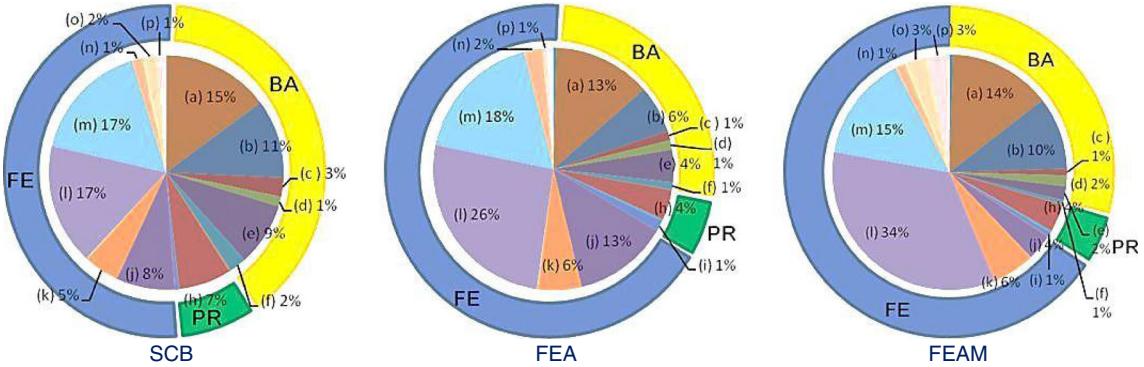

Fig. 4 Characterization of the caecal microbiota of rats fed SCB, FEA or FEAM, using pyrosequencing of bacterial 16S rDNA genes, at the phylum and family levels. The outer circle indicates the phylum percentages. FE is *Firmicutes*, BA is *Bacteroidetes*, and PR is *Proteobacteria*. Additionally, the circle is separated at the family level; (*a*) Unclassified *Bacteroidetes*, (*b*) Unclassified *Bacteroidales*, (*c*) *Rikenellaceae*, (*d*) *Prevotellaceae*, (*e*) *Bacteroidaceae*, (*f*) *Porphyromonadaceae*, (*g*) Unclassified *Proteobacteria*, (*h*) *Helicobacteraceae*, (*i*) *Sutterellaceae*, (*j*) Unclassified *Firmicutes*, (*k*) Unclassified Clostridiales, (*l*) Lachnospiraceae, (*m*) Ruminococcaceae, (*n*) Peptostreptococcaceae, (*o*) *Lactobacillaceae*, (*p*) Unclassified *Selenomonadales*

a high ratio of *Firmicutes* was associated with a high energy intake and obesity (De-Filippo et al. 2010; Ley et al. 2006). Therefore, researchers have proposed that the ratio of *Firmicutes*/*Bacteroidetes* can be used as an index of obesity. However, *Firmicutes* is comprised of many families with various characteristics. Furthermore, although, lactic acid bacteria was detected as predominance in ceacal of rats fed 20 % *w*/*w* fish meal in previous reports (An et al. 2014), it was detected only 3 % in ceacal of rats fed diets containing fish meal. It was indicated that food materials affected each other in the combination diet, not individually.

Among the *Firmicutes*, *Lachnospiraceae* was high in rats fed FEA and FEAM. *Lachnospiraceae* belongs to the order *Clostridiales* and includes various genera, such as *Eubacterium*, *Roseburia*, *Oribacterium*, butyric acid-producing bacteria, such as *Butyrivibrio*, *Pseudobutyrivibrio*, as well as *Oscillibacter* (Lee et al. 2013). On the other hand, acetic acid levels were high in FEA and FEAM groups. It is well known that a number of species inhabiting the intestine can produce acetate from dietary fibres. For example, several intestinal acetate-producing bacteria, such as *Ruminococcus flavefacien*, *Bacteroides ovatus* and *Clostridium ramosum*, can degrade cellulose, alginate and/or laminaran (Kuda et al. 1992; Salyers et al. 1977; Varel et al. 1984).

As mentioned above, the FEA group showed high content of caecal indole and phenol with high organic acids, though reduced caecal H_2S was clearly observed. It is thought that the dietary fibres in FEAM promoted excretion of putrefactive compounds from the intestine. Results of this study suggest that FEAM is preferable to FEA. However, the effect of alterations in the intestinal environment and microbiota on host health requires clarification. Further studies on the effects of these diets

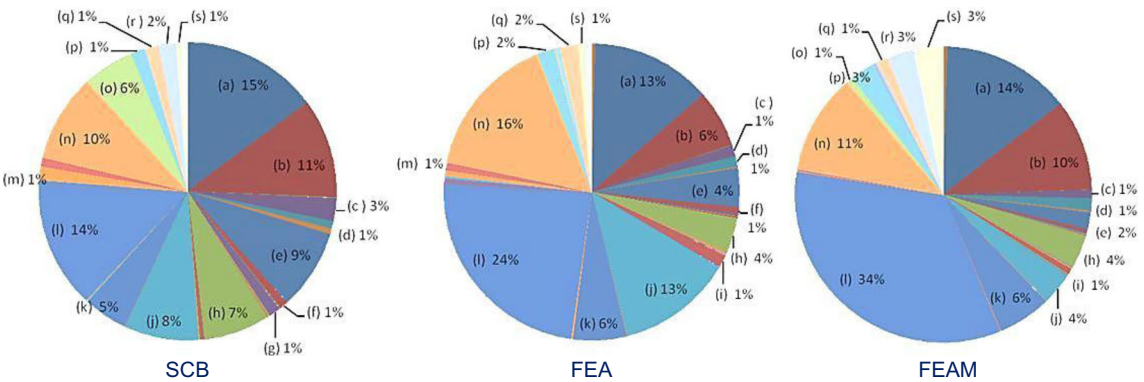

Fig. 5 Characterization of the caecal microbiota of rats fed SCB, FEA or FEAM, using pyrosequencing of bacterial 16S rDNA genes, at the genus level. (*a*) Unclassified *Bacteroidetes*, (*b*) Unclassified *Bacteroidales*, (*c*) *Alistipes*, (*d*) Unclassified *Prevotellaceae*, (*e*) *Bacteroides*, (*f*) Unclassified *Porphyromonadaceae*, (*g*) *Parabacteroides*, (*h*) *Helicobacter*, (*i*) *Parasutterella*, (*j*) Unclassified *Firmicutes*, (*k*) Unclassified Clostridiales, (*l*) Unclassified *Lachnospiraceae*, (*m*) *Clostridium* XlVb, (*n*) Unclassified *Ruminococcaceae*, (*o*) *Ruminococcus*, (*p*) *Oscillibacter*, (*q*) *Clostridium* XI, (*r*) *Lactobacillus*, (*s*) Unclassified *Selenomonadales*

on inflammation bowl disease and food borne pathogen infection are now in progress.

Acknowledgments The present study was supported in part by the Ministry of Education, Science, Sports and Culture, Japan, Grant-in-Aid for Scientific Research (C), #25450300, 2013–2015 and was also supported by a 2013 Grant from Fuji Foundation for Protein Research (Osaka, Japan).

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