ORIGINAL

Effects of fermented brown rice on the intestinal environments in healthy adult

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Abstract : Purpose : The aim of this study is to investigate the prebiotic effects of brown rice fermented by *Aspergillus oryzae* (FBRA) on the intestinal environment *in vitro* and in healthy adults. Methods : Fresh fecal slurries from six healthy adults were incubated with FBRA to confirm prebiotic potentials of FBRA. Another thirty-six healthy adults were randomly allocated to 2 groups for the clinical study. Subjects consumed 21.0 g/day of either FBRA or control food for 2 weeks, followed by a 12-week intermission and then 2-week ingestion *vice versa*. Main outcome measures were bifidobacterial numbers and organic acid concentration in feces. Sub outcome measures were fecal microbiota, fecal environments and bowel function. Results : Incubation of fecal slurries with FBRA *in vitro* resulted in increased organic acids with individual-specific patterns. Bifidobacterial numbers were increased during incubation. In the clinical study, all participants safely completed this study. FBRA had little effect on fecal number of bifidobacteria, concentrations of organic acids or putrefactive metabolites, fecal pH, or fecal microbiota. Conclusion : FBRA has the potentials as a prebiotic, however, we could not detect its effects on the intestinal environment *in vivo*. The results in a clinical study indicated that FBRA could be safely used for healthy adults. J. Med. Invest. 58 : 235-245, August, 2011

Keywords **:** *dietary fiber, fermented brown rice and rice bran, intestinal microbiota, organic acid, randomized controlled trial*

INTRODUCTION

In Japan, increased consumption of European/American food and rising stress levels has elevated the incidence of intestinal disorders (1). Along with the growing consumer awareness of intestinal health and enteric bacteria, some types of beneficial bacteria contained in yogurts, fermented milk, and other fermented foods have been recognized as probiotic (2). Some non-digestible dietary fibers, such as inulin or fructooligosaccharides, have been shown to increase levels of beneficial bacteria for human health such as bifidobacteria and lactobacilli, resulting in reduced intestinal pH, improved intestinal environments, and suppressed intestinal disorders (3, 4). Non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number

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of bacterial species already resident in the colon are defined as prebiotic (4).

Brown rice fermented by *Aspergillus oryzae* (FBRA) is a processed food prepared by fermenting brown rice and rice bran with *A. oryzae*. It has previously been reported that FBRA has a preventive effect on azoxymethane-induced colon carcinogenesis and dextran sulfate sodium-induced acute colitis in rats (5, 6). FBRA contains a large proportion of fiber, and dietary administration of 10% FBRA to rats increased viable cell numbers of lactobacilli in feces (7). Beta-glucan and arabinoxylan, the main constituents in dietary fiber in FBRA, have been reported to stimulate growth of bifidobacteria and lactobacilli strains *in vitro* (8). Moreover, FBRA has been eaten for over 30 years in Japan by many people, as well as consumed in clinical trials, and no serious side effects have been reported (9, 10).

In the present study, we first examined the prebiotic effects of FBRA in *in vitro* incubation with feces. We then conducted a placebo-controlled, double-blinded, crossover study to examine the effects of FBRA on the intestinal environments in human adults. As ingestion of prebiotics and probiotics in adults is thought to prevent a range of diseases (4, 11, 12), we investigated the effects of FBRA in adults with no obvious illnesses, even though the effects of prebiotics and/or probiotics have been mostly examined in patient groups, elderly, or infants. The main end points of the study were increased levels of bifidobacteria and organic acids in feces. Organic acids were produced by intestinal bacteria in the large intestine, decrease intestinal pH, thereby suppressing the growth of pathogenic bacteria and increase in putrefactive metabolites, and have growth-promoting and differentiation activities on the colonic epithelium (13-16). In addition, organic acids have been noted to have immunomodulatory effects on colonic inflammation (16, 17). The effects of FBRA on fecal microbiota, fecal environments including pH, and putrefactive components, and bowel function were also investigated as sub end points.

MATERIALS AND METHODS

Chemicals, media and bacterial strains

Blood liver agar (BL), Rogosa SL agar, Trypticase soy blood agar (TS) and desoxycholate hydrogen sulphide lactose agar (DHL) were obtained from Nissui Pharmaceuticals Co., Ltd. (Tokyo, Japan). *Bifidobacterium longum* strain JCM1217 was obtained from the Japan Collection of Microorganisms, RIKEN Bioresource Center. Chemicals, unless otherwise stated, were reagent grade or higher and obtained from Sigma Chemical Co. (St. Louis, MO, USA) or Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan).

In vitro incubation of FBRA in human fecal slurries

Fresh feces from 6 adult volunteers were suspended in 9 volumes of 0.1M $NaHCO_3/CO_2$ buffer (pH 6.8) and incubated anaerobically with 10% FBRA for 0, 6, and 24 hr. Aliquots of each sample were centrifuged, and the supernatants used to measure organic acid content. Fecal DNA in the pellets were extracted and used to examine changes in microbiota by T-RFLP analysis and bifidobacteria-specific real-time PCR.

Clinical study participants

Participants were recruited on complying with the protocol approved by the Tokushima University Hospital Ethics Committee on Clinical Research. Exclusion criteria were presence of food allergy, serious illness, and periodic taking of antibiotic drugs or agents known to influence bowel condition. After the significance and protocol of the study had been explained, informed consent was obtained from all participants. Thirty-six adults (14 male and 22 female, aged 22-67, Table 1) participated in the study. This study was conducted according to the Helsinki Declaration of 1975, as revised in 2000.

Table 1 Profile of participants

	FBRA	NF	age
Participant	18	18	22-67
male	8	6	22-45
female	10	12	22-67

Test foods

Test foods used in this study were provided by Genmaikoso Co., Ltd. (Sapporo, Japan). The main components of FBRA and control food (non functional food, NF) are shown in Table 2. FBRA was made from brown rice and rice bran fermented with *A. oryzae*. NF was made from roasted flour and cornstarch (85 : 15, W/W). Both foods were granulated and 3.5 g amounts packed individually.

Table 2 Composition of test food

		FBRA		NF	
		/100 g	/21 g	/100 g	/21 g
Energy	(kcal)	421.0	88.4	376.0	79.0
Protein	(g)	16.3	3.42	8.2	1.72
Lipid	(g)	24.7	5.19	1.0	0.21
Glycol	(g)	21.3	4.47	82.6	17.35
Dietary fiber	(g)	24.1	5.06	2.2	0.42
soluble fiber	(g)	3.8	0.80	1.1	0.23
insoluble fiber	(g)	20.4	4.28	1.0	0.21

Study Design

We conducted a placebo-controlled, double-blinded crossover study. Subjects were instructed not to change their normal daily activities such as dietary and exercise habits. The study period was 16 weeks. Participants were randomly divided into two groups by drawing lottery for group according to written ID. Group A ingested FBRA for two weeks prior to NF ingestion and group B ingested *vise versa*. We set a 12-week intermission period between the ingestion periods to wash out the influence of the former test food. Participants took 21 g per day of the test food (7.0 g after every meal) and fresh feces were collected before and at the end of each ingestion period. Feces were kept cold and anaerobic until sent to our laboratory and processed. In this clinical trial, administrators were Kataoka Ph.D for enrollment, Arimochi Ph.D for allocation and blinding, and Sei MD.Ph.D (Institute of Health Biosciences, the University of Tokushima Graduate School) for the data fixation and supervision of safety and ethics.

Dietary habits and bowel-function questionnaire

Dietary habits were evaluated by using Excel-Eiyoukun ver.4 software and Food Frequency Questionnaire Based on Food Groups ver.2 software (FQQg, Office of Nutrition Data Base, Shikoku University, Tokushima, Japan). The frequency of evacuation, shape, and amount of stools, as well as any abdominal discomfort or pain were self-reported using a bowel-function questionnaire. Participants filled in the questionnaires before and after each ingestion period.

Fecal microbiota analysis by culture methods

Viable cell numbers were analyzed according to the procedure of Mitsuoka *et al.* (18) with slight modification as described by Kataoka *et al.* (7). BL agar and Rogosa SL agar were used to count anaerobic bacteria and bifidobacteria. TS agar and DHL agar were used for counting aerobes, *Enterobacteriaceae*, and Gram-positive cocci. Results were calculated as $\log_{10}$ of colony forming unit (CFU) per gram (wet weight) of the initial material. Total numbers of fecal bacteria were counted by microscopy after Gram stain.

Fecal microbiota analysis by Terminal-Restriction Fragment Length Polymorphism (T-RFLP)

Fecal and bacterial DNA were isolated according to the method of Morita *et al.* (19) and T-RFLP analysis of fecal microbiota performed as described by Sakamoto *et al.* (20) Briefly, 16S ribosomal DNA from samples were amplified with PCR Thermal Cycler Dice® Standard (Takara, Otsu, Japan) and digested with either *Hha*I or *Msp*I (Takara). Length of the terminal-restriction fragments (T-RFs) was determined using an ABI Prism 310 Genetic analyzer, GeneScan® Analysis Software 3.7.1 (Applied Biosystems, Japan). GeneScan™ 1200 LIZ (Applied Biosystems) was used as a size standard. T-RFs patterns were analyzed by BioNumerics™ ver.5.01 software (Applied Maths, Sint-Martens-Latem, Belgium) to construct dendrograms. Distances between samples were represented graphically by constructing a dendrogram based on Dice coefficients of the T-RFLP profiles. The Ward method was used for establishing the dendrogram type. Microbiota Profiler software (INFOCOM CORPORATION, Tokyo, Japan) was used to find intestinal bacteria with T-RFs that increased or decreased in response to FBRA ingestion.

Real Time PCR for bifidobacteria

As bifidobacteria were difficult to detect precisely by T-RFLP conditions used in this study (21, 22), changes in bifidobacterial numbers in response to test food was examined by real time PCR according to Matsuki *et al* (23). SYBR® Premix Ex Taq™ II (Takara) and an ABI Prism 7000 were used according to the manufacturers' instructions. The amplification program used was : preheating at 95°C for 30 sec ; 40 cycles of denaturation at 95°C for 5 sec, annealing at 57°C for 5 sec, and extension at 72°C for 31 sec ; followed by a final dissociation protocol to verify specific amplification. As a copy number standard, pCRBL2 plasmid DNA was used at 10^2-10^8 copies. pCRBL2 consisted of the pCR2-TOPO vector (Invitrogen, Tokyo, Japan) that harbored 16S

ribosomal DNA of *B. longum* strain JCM 1217. The genetic recombination experiments performed in this study were approved by the University of Tokushima.

Measurement of fecal concentration of organic acid

Portions (about 0.2 g each) of homogenized stool were suspended in 1 ml water and centrifuged at 4°C at 10 krpm for 10 min. Supernatants were stored at -80°C until gas chromatograph analysis.

Organic acid concentrations in the fecal extracts were measured using a gas chromatograph instrument (GC-7AG, Shimadzu, Kyoto, Japan) and a GLC glass column (3 mm × 2 m) packed with 10% Reoplex 400 on Chromosorb W (80-100 mesh, AW). The column temperature was 130°C and the temperature of the injection port and flame ionization detector was 210°C. Nitrogen was used as the carrier gas at a flow rate of 50 ml/min. Mixtures of volatile fatty acids (VFAs) (acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, isocapuronic acid, capuronic acid, 10 mM each) and non-volatile fatty acids (NVFAs) (lactic acid, succinic acid, 10 mM each) were used as external standards. Fecal extract (0.5 ml) and standard VFAs (0.5 ml) were acidified with 5 µl 50% sulfuric acid, extracted with an equal volume of diethylether, and 1 µl of the ether layer injected into the gas chromatograph. VFAs in the fecal extracts were removed by two more extractions with diethylether. The remaining water layer (100 µl) and NVFA standard solution (100 µl) were esterified by heating at 100°C for 5 min in the presence of an equal volume of boron trifluoride methanol complex (Nacalai Tesque, Inc., Kyoto, Japan). NVFA methylesters were extracted with 100 µl chloroform and injected into the gas chromatograph.

Fecal pH and water content

Stool samples (0.1~0.2 g) were homogenized in 1 ml of water. Fecal pH was measured with a pH meter (Sartorius B021610 007). Water content of feces was calculated as the weight difference before and after freeze-drying a portion of the feces (EYELA FDU-1000).

Measurement of fecal putrefactive metabolites

Fecal levels of indole were measured as described by Shioiri *et al.* (24) Briefly, stools (approximately 0.1 g) were homogenized with 9 volumes of 0.1 mM Phosphate buffer (PB) and centrifuged. The supernatant was appropriately diluted with PB. 0.2 ml of diluted sample was mixed with 1 ml of coloring solution (*p*-dimethyl aminobenzaldehyde in a sulfuric acid/alcohol mixture) and kept at room temperature for 20 min. The absorbance at 570 nm was measured using UV-VIS spectrophotometer (Shimadzu UV-1200). As a control, 0 to 0.3 mM indole solution was prepared just before use. Ammonia concentration was measured using Ammonia Test Wako (Wako Pure Chemical Industries Co. Ltd., Osaka, Japan).

Statistical analysis

Mann-Whitney U test and repeated measure ANOVA using Microsoft® Excel 2007 and Statcel2 add-in software (OMS publishing, Tokorozawa, Japan) was used for the statistical analysis. A probability of less than 0.05 was taken to indicate statistical significance.

RESULTS

Organic acid production in human fecal slurries with FBRA

In vitro experiments were performed using fresh feces from 6 healthy adults. Acetate was commonly produced as the major metabolite, and lactate was also a major metabolite except for slurry C (Fig. 1). Although the pattern of accumulated organic acids was different for each sample, substantial amounts of propionate, butyrate, and succinate were detected in most samples. Concentrations of total organic acids were increased to about 300 µmol/g slurry by incubation of feces with FBRA (Fig. 1). Incubation of FBRA alone or fecal slurries alone resulted in no increase of organic acids (data not shown).

Microbiota alteration in human fecal slurries with FBRA

T-RFLP analysis of fecal slurries showed that the initial microbiota patterns were different between the slurries, and continued to vary following incubation with FBRA (Fig. 2). In fecal slurry A (Fig. 2A), *Bacteroides* and clostridia were dominant, with the percentages of *Bacteroides* and *Clostridium* subcluster XIVa increasing after FBRA incubation. In fecal slurry B (Fig. 2B), in which lactobacilli was the main species, addition of FBRA increased the percentage of lactobacilli further (Fig. 2B). Although the area of the 352-357 bp peak in Fig. 2B also increased, a matching species except uncultured bacteria could not be found using the Microbiota

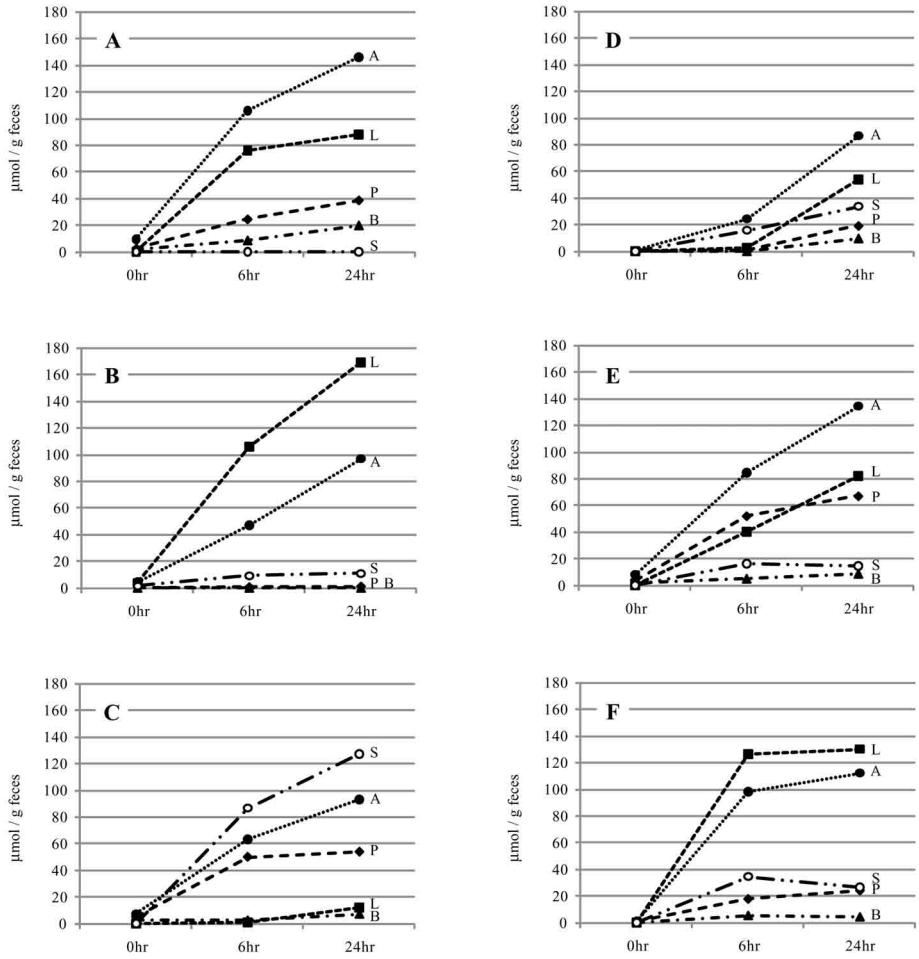

Fig. 1 Production of organic acids after *in vitro* incubation of fecal slurries with FBRA.
Fecal slurries from 6 adults were anaerobically incubated with 10% FBRA and aliquots removed at 0, 6, 24 h to determine the concentrations of organic acids (panel A-F). Organic acid concentrations were measured using a gas chromatograph instrument. VFAs and NVFAs were used as external standards as described in Materials and Methods. A, acetate ; P, propionate ; B, butyrate ; L, lactate ; S, succinate.

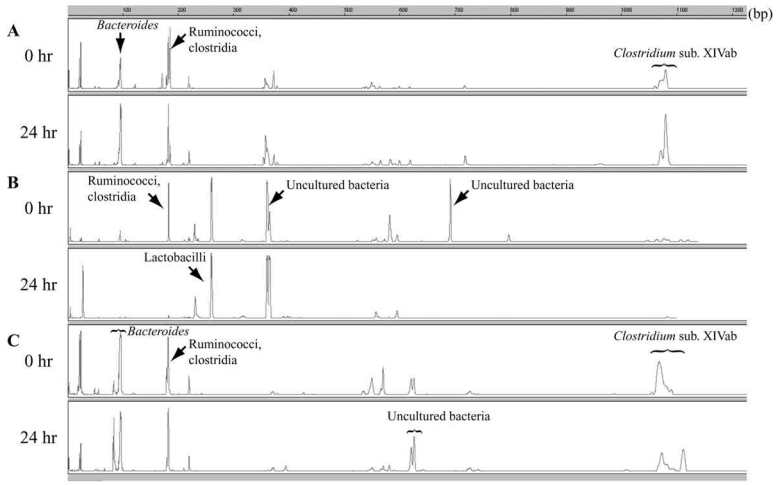

Fig. 2 T-RFLP analysis of microbiota in fecal slurries before and after incubation with FBRA.
Fecal slurries prepared from 6 adults were anaerobically incubated with 10% FBRA and aliquots removed at 0 and 24 h for T-RFLP analysis, as described in Materials and Methods. Representative T-RFLP patterns after *Hha*I digestion in 3 fecal slurries are shown. These data correspond to panels A, B, and C in Fig. 1, respectively. The peak area at 95-98 bp was mainly derived from *Bacteroides* ; that at 185-190 bp was ruminococci and clostridia ; that at 253-256 bp was lactobacilli ; and that at 1070-1080 bp was *Clostridium* subcluster XIVa. The peak area at 352-357, 620-623, and 697-698 bp were deu to uncultured bacteria. These species described above were designated using Microbiota Profiler and the combined data of *Hha*I and *Msp*I digestion.

Profiler. Increased bifidobacterial numbers following *in vitro* incubation with FBRA was confirmed by real time PCR as shown in Fig. 3. Bifidobacteria increased in 4 out of 6 fecal slurries (B, D, E, and F in Fig. 3). In fecal slurry C, *Bacteroides* was increased and remained most dominant during incubation with FBRA (Fig. 2C). Levels of bifidobacteria in sample C were below the detection limit (< 100 copies/10 pg fecal DNA) before and after incubation.

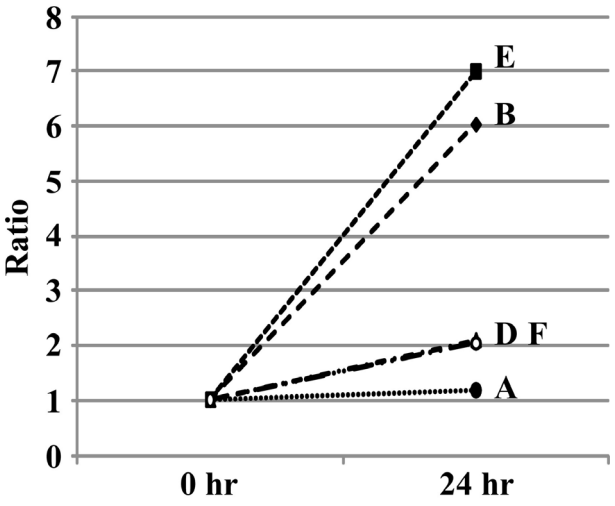

Fig. 3 The increasing ratio of bifidobacteria after *in vitro* incubation of fecal slurries with FBRA.
Fecal slurries from 6 adults were incubated with 10% FBRA and aliquots removed at 0 and 24 h to quantify bifidobacteria by real time PCR. A copy number standard pCRBL2, harboring the 16S ribosomal DNA of *B. longum* strain JCM 1217, was used to calculate the copy number of the bifidobacterial 16S ribosomal RNA gene. Values from one subject, corresponding to panel C in Fig. 1 and Fig. 2, are not shown as they fell below the detection limit (< 100 copies/10 pg fecal DNA).

Participant flow, recruitment, and adverse events

Participant flow is shown in Fig. 4. The recruitment period was from 28th Oct. 2005 to 14th Apr. 2006 and the intervention period was from 7th Nov. 2005 to 16th Oct. 2006. Participants were randomly divided into two groups and took test diets as described in Materials and Methods. The male to female ratio and age composition were similar between the two groups. All participants completed the trial without reporting any adverse events. One male participant in group A was excluded from statistical analysis because he consumed antibiotics for 3 days during the first ingestion period.

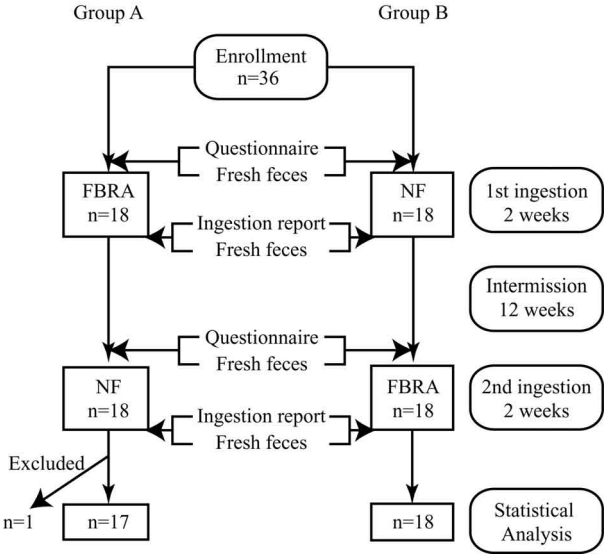

Fig. 4 Clinical study design and participant flow.
Participants were randomly divided into 2 groups. Intervention was for 16 weeks : first ingestion (2 weeks), intermission (12 weeks), and second ingestion (2 weeks). The intervention was completed with no adverse events. One subject in group A was excluded from analysis because of antibiotic intake during the intervention period. As questionnaires at the end of second ingestion were not collected from all participants, analyses were performed on first ingestion period data only.

Dietary habits, test food consumption, and bowel-function questionnaires

Three participants (two in Group A and one in Group B) were excluded from dietary habits analysis because of inadequate response of questionnaires. Survey of dietary habits revealed no differences in dietary fiber intakes between Groups A and B (Table. 3). Intake of the test foods was checked by self-reports and the number of returned test food portions. All subjects consumed the intended doses of test food, although some missed doses were taken within a day or as an added intervention day with 21.0 g as the maximum daily dose. Samples obtained in the first ingestion period only were used for statistical analysis, because ingestion reports, questionnaires about bowel condition, and questionnaires to check blinding were not collected from all subjects after the latter period. Also, it appeared that the bowel conditions at the start of the second ingestion period were inconsistent with those of the first ingestion period in some subjects. For the bowel-function questionnaires, no significant differences in frequency of evacuation, fecal amount, shape, and water content of feces before and after test diet ingestion were observed for both Groups A and B.

Table 3 Daily intake of dietary fiber before intervention

	Group A (n=15/17)		Group B (n=17/18)		p-value
	median	IQR	median	IQR	
Total fiber (g)	10.8	9.1 - 14.3	12.2	8.0 - 14.0	0.955
Soluble fiber (g)	2.6	2.2 - 3.3	2.6	1.7 - 3.5	0.895
Insoluble fiber (g)	7.4	6.0 - 10.1	8.5	5.6 - 9.5	0.777

Total fiber amount was summed up of soluble and insoluble fiber amount. IQR : Interquartile range. Mann-Whitney U-test was used.

Effect of FBRA ingestion on the fecal concentration of bacterial metabolites

The concentrations of total and individual organic acids did not significantly increase after FBRA ingestion and were not significantly different between Groups A and B, as shown in Table 4. Fecal pH was not significantly altered. Concentration of ammonia and indole in feces were not affected significantly between each group.

Analysis of fecal bacteria by culture and Real time PCR methods

Total numbers of fecal bacteria counted by microscopy after Gram-staining was not significantly different between Groups A and B, nor between before and after experimental diets (Table 5). Viable cell numbers of bifidobacteria and *Enterobacteriaceae* in feces were not significantly altered following test diet ingestion in both Groups A and B. Real time

Table 4 Concentration of metabolites and pH in feces before and after first ingestion of test diet

	Group A (FBRA)				Group B (NF)				p Value
	Before		After		Before		After		
	median	IQR	median	IQR	median	IQR	median	IQR	
Fecal pH	7.21	6.74 - 7.51	7.13	6.69 - 7.20	6.87	6.48 - 7.18	7.22	6.49 - 7.61	0.631
Total organic acid	86.97	71.73 - 102.73	85.75	80.48 - 96.41	100.04	82.58 - 107.87	90.74	81.94 - 107.27	0.460
Acetate	40.31	32.89 - 47.66	41.71	38.25 - 48.61	50.04	35.24 - 54.17	42.55	35.52 - 50.35	0.893
Propionate	10.98	6.95 - 17.71	11.66	9.30 - 13.98	15.47	11.95 - 18.99	15.61	12.56 - 18.91	0.138
Butyrate	6.81	4.32 - 9.62	8.71	5.93 - 10.57	9.92	7.66 - 11.85	9.41	7.54 - 11.55	0.200
Lactate	19.46	17.80 - 21.05	20.59	17.83 - 22.22	19.86	17.40 - 20.95	18.34	15.69 - 22.28	0.793
Ammonia	475	283 - 641	394	199 - 644	412	276 - 463	345	301 - 529	0.569
Indole	1.04	0.33 - 1.34	0.99	0.54 - 1.67	0.78	0.60 - 0.89	1.37	0.95 - 1.87	0.542

Total organic acid concentration was summed up of each measured concentration including isobutyrate, isovalerate valerate, isocapuronate, and capuronate and succinic acid. Values are μmol/g feces (wet weight) in organic acid and indole, μg/g feces (wet weight) in ammonia. IQR : Interquartile range. Values less than detection limit are not included. Repeated measure ANOVA was used to compare between each group.

Table 5 Analysis of fecal bacteria in subjects before and after first ingestion period

	Group A (FBRA)				Group B (NF)				p Value
	Before		After		Before		After		
	median	IQR	median	IQR	median	IQR	median	IQR	
Total bacteria	11.40	11.20 - 11.63	11.50	11.28 - 11.60	11.55	11.30 - 11.78	11.55	11.40 - 11.60	0.530
Culture methods									
Bifidobacteria	9.60	9.35 - 9.95	9.60	9.20 - 9.95	9.75	9.50 - 10.03	9.65	9.23 - 10.00	0.216
Enterobacteriaceae	7.40	6.50 - 7.90	6.90	5.20 - 8.10	7.40	6.15 - 7.60	7.10	6.73 - 7.75	0.663
aerobic G(+)C	6.70	5.60 - 7.30	7.00	6.53 - 7.53	6.65	6.33 - 8.08	7.30	6.48 - 7.98	0.350

Total bacteriawere counted undera microscope after Gram-staining.Valuesare $\log_{10}$CFU/gfeces in culture method. Valuesless thandetection limit are not included. Repeated measure ANOVA was used to compare between each group. IQR, Interquartile range ; G(+)C, gram positive cocci.

PCR for bifidobacteria also showed no significant changes in response to the test diets (data not shown).

T-RFLP analysis of human fecal microbiota before and after FBRA ingestion

Effect of FBRA on fecal microbiota was examined by T-RFLP analysis, which focused on the 16S ribosomal DNA. T-RF patterns derived from fecal bacteria before and after FBRA ingestion were quite similar (>70% of similarity index, Fig. 5) in most subjects. In several subjects (12F, 22F, 24F, 25F in Group A, 19N, 31N in Group B, Bold face in Fig. 5), fecal microbiota was comparably altered between before and after ingestion of test foods (<50 % of similarity, Fig. 5).

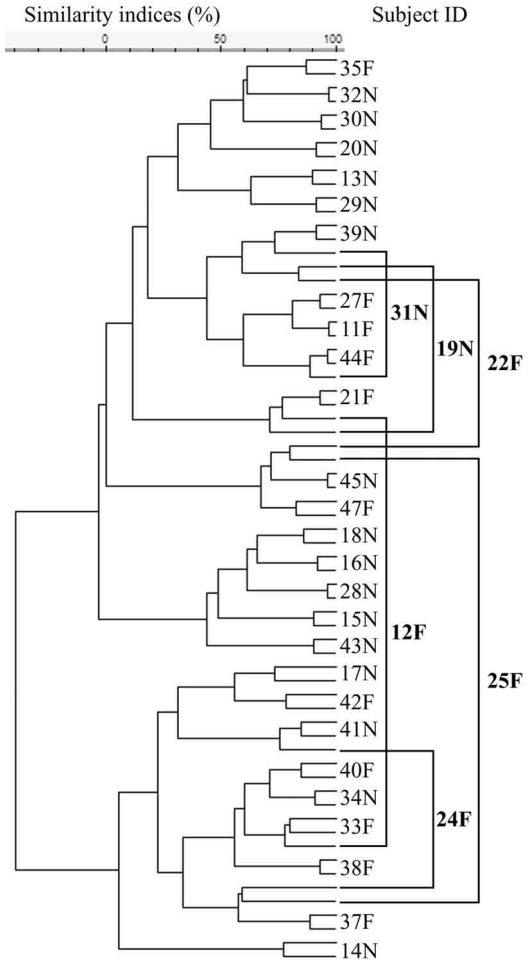

Fig. 5 Cluster analysis of fecal microbiota of subjects before and after first ingestion period.
The dendrogram is based on the T-RFLP patterns derived from *Hha*I and *Msp*I digests. The scale bar represents similarity indices (%). Fecal microbiota of most subjects were not largely changed after intervention. In several subjects (12F, 22F, 24F, 25F in Group A, 19N, 31N in Group B, in bold), T-RFLP patterns after ingestion were comparably different from those before ingestion. F, FBRA ingestion group ; N, Non functional food ingestion group.

DISCUSSION

Some non-digestible dietary fibers have been reported to improve functional constipation by increasing bulk and gas production in the colon (25). They allowed intestinal bifidobacteria and lactobacilli levels to increase, and production of organic acids to enhance including butyrate in the large intestine, leading to decreased intestinal pH that suppresses the overgrowth of pathogenic bacteria and the production of putrefactive components such as indole (26). Organic acids produced by gut-resident bacteria have beneficial aspects for host, including immunomodulatory effects on colonic inflammation (13-16). In addition, butyrate is the major energy source for colonic epithelial cells, and promotes the proliferation and differentiation of colonic cells and inhibits the growth of colonic cancer (16). As FBRA contains a substantial amount of non-digestible dietary fiber, we thought that FBRA could improve the intestinal environment and bowel movement.

The *in vitro* fecal slurry incubation with FBRA showed increased organic acid production and enhanced bifidobacteria, which indicated that FBRA had a potential as a prebiotic. Though the concentration of total organic acid was increased in all samples, the production patterns of organic acids by fecal bacteria differed between individuals (Fig. 1). Coincident with the difference of organic acid productions, T-RFLP analysis showed that fecal microbiota also varied markedly between subjects after incubation with FBRA (Fig. 2), in agreement with previous reports that the effects of prebiotics may depend on the initial condition of the intestinal environments (27). Acetate and lactate were abundant after incubation in fecal slurry B (Fig. 1B), in which bifidobacteria and lactobacilli were dominant. Acetate, lactate and substantial amounts of butyrate were produced in fecal slurry A (Fig. 1A), in which FBRA increased *Bacteroides* and *Clostridium* subcluster XIVa. Succinate was abundant in fecal slurry C (Fig. 1C), in which *Bacteroides* was increased after addition of FBRA but bifidobacteria were extremely low. Intestinal bacteria competitively metabolize carbohydrate to various organic acids via phosphoenolpyruvate and pyruvate. *Bacteroides* can produce succinate from phosphoenolpyruvate and pyruvate, while bifidobacteria produce lactate from pyruvate (28). Therefore, FBRA could be metabolized to organic acids *in vitro*, and the amount and variety of produced organic acids was influenced by

the unique composition of the resident microbiota in each individual.

Bifidobacteria were increased in 4 out of 6 samples after incubation of fecal slurries with FBRA for 24 h. Rice bran is rich in non-digestible dietary fibers such as beta-glucan and arabinoxylan, and these components are known to be hydrolyzed by *A. oryzae*-mediated fermentation (29). Hydrolysates of these fibers have been reported to be selective substrates for lactobacilli and bifidobacteria (8). Therefore, oligosaccharides produced by *A. oryzae* may contribute to the increased levels of bifidobacteria.

The clinical study was completed without any adverse events, and provided an evidence of safety of FBRA. However, despite the positive findings from the *in vitro* study, the clinical study failed to identify the effects of FBRA on organic acids and bifidobacteria in feces as main end points. Bifidobacteria after FBRA ingestion remained at the same levels as before ingestion. Other bacteria investigated in this study were also unchanged by FBRA ingestion. Likewise, clustering analysis derived from T-RFLP data showed that the fecal microbiota was not affected by FBRA ingestion for 2 weeks (Fig. 5). It has been reported that Japanese adults consume around 14 g of dietary fiber per day (30), while the Ministry of Health, Labour and Welfare, Japan, recommends the intake of 21.0 g dietary fiber per day (31). The United States Department of Agriculture recommend the intake of even more dietary fiber at around 14 g per 1000 kcal, depending on age, gender and energy intake (32). Therefore, we set the FBRA dosage to make up for the shortfall between actual and recommended dietary fiber intake. However, even allowing for the difference of calculating method of dietary fiber, the amount of total dietary fiber did not reach the recommended value (21 g of fiber per day) even in FBRA ingestion group, as the participants had unexpectedly low base intakes of dietary fiber (mean 11.5 g/day). In our previous study (7), we confirmed the prebiotic effects of FBRA in a rat model in which rats ate 1.5 g/day FBRA, which corresponds to 692 g/day in humans on a body weight basis. Therefore, supplementation of dietary fiber with FBRA at the dose used in this study might have been insufficient to modify intestinal microbiota. Further clinical study for adequate ingestion dose for human adults will be necessary.

Reflecting no modifications of fecal microbiota by 2 week ingestion of FBRA (Fig. 5), concentrations of organic acids were also unchanged in the clinical study (Table 4). Even though FBRA could increase total organic acid concentrations in fecal slurries *in vitro*, values were not significantly increased *in vivo*. This result suggested that although FBRA was probably metabolized to organic acids by intestinal bacteria in the human colon, the increased organic acids may have been quickly removed due to their high absorbability in the colon (28). Given the variability in organic acid production patterns observed in *in vitro* experiment and the organic acid absorption in colon, it might be difficult to detect the prebiotic effects from the fecal concentration of organic acids as intestinal environment indicators. Kanauchi *et al.* reported that concentration of fecal butyrate was increased by 4-weeks intake of 30 g per day of germinated barley foodstuff (about 10 g of dietary fiber), while Costabile *et al.* showed that 3-weeks intake of 10 g per day of inulin had no effect on fecal organic acids (33, 34). Considering these reports, the dose of FBRA (21 g, about 5 g per day of dietary fiber) might not be enough to affect on fecal organic acid.

Bowel function and concentrations of ammonia and indole were investigated as sub end points. As FBRA contains substantial amounts of insoluble dietary fiber, we expected FBRA to stimulate bowel peristalsis by increasing the volume of the intestinal contents (26). However, FBRA intake did not significantly affect bowel evacuation quantitatively or qualitatively even in participants with constipation, even though some subjects received around 1.5 times as much dietary fiber as their ordinary diets for 2 weeks. It may be necessary to consume more dietary fiber and/or much longer period to improve constipation. The concentrations of ammonia and indole were also not affected by the test diets. It had been reported that fecal putrefactive components can vary widely in response to daily foods on an intra-individual and inter-individual basis (35). Therefore, the effects of the test foods might have been obscured by the natural variability in ammonia and indole concentrations.

In conclusion, FBRA increased the production of organic acids and bifidobacteria *in vitro*, which supported the use of FBRA as a prebiotic. Clinical study showed the evidence of safety of FBRA. However, as it remains unclear whether FBRA has prebiotic effects on the intestinal environment, further clinical studies for adequate ingestion dose and period of FBRA are necessary.

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