

Preventive effects of enzyme-treated rice fiber in a restraint stress-induced irritable bowel syndrome model

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Abstract. Irritable bowel syndrome (IBS) is a common health issue that is characterized by abdominal pain, abnormal bowel movements, altered visceral perception, and abnormal metabolism of 5-hydroxy triptamine (serotonin; 5HT). The use of prebiotics or probiotics treatment for IBS has become increasingly important as an adjunct to pharmaceutical options. The aim of this study was to determine the efficacy of enzyme-treated rice fiber (ERF) on an IBS model. We obtained a new prebiotic from defatted rice bran that was developed as an insoluble dietary fiber through amylase and hemicellulase treatment followed by removal of the soluble fraction. Containing ~70% hemicellulose, ERF is utilized by *Lactobacilli* and subsequently converted to butyrate using *Eubacterium limosum*. We employed a restraint stress IBS model which involved the continuous application of stress for 4 h per day for 3 days. Polycarbophil Ca (PC) (500 mg/kg body weight) was used as a positive control and ERF was added to the diet at 4% in diet. During restraint stress, ERF significantly attenuated urgent fecal excretion, colonic mucosal 5HT secretion, and hyperalgesia compared with the control. ERF also significantly increased cecal butyrate production as well as total organic acid content. PC was only effective in regard to preventing increases in 5HT levels. Furthermore, there were no significant levels of pro-inflammatory markers CINC-1 and TNF- α among the groups. Although more detailed studies are needed, the ERF prebiotic demonstrated potency in attenuating major symptoms of IBS.

Introduction

Irritable bowel syndrome (IBS) is a common health disorder that is characterized by abdominal pain, gas excretion and abnormal bowel movements (1). Clinically, IBS patients are divided into three sub-types, namely, diarrhea- and constipation-dominant and alternating types. Depending on the criteria used to define IBS, its prevalence in the general population is 5-11% in developing countries (1,2). Although the overall mechanism of IBS is still unclear, disturbed gastrointestinal motility, altered visceral perception, metabolism of 5-hydroxy triptamine (serotonin; 5HT), and various psychological factors are regarded as important mechanisms that interact with each other in the development of the condition (1,3). Because IBS is not a fatal disease, the most important treatment criteria are ensuring patient safety and improving their quality of life. According to a recent review concerning IBS, there are 3 basic treatment options, including dietary treatment (which aims to increase dietary fiber), psychological treatment, and pharmacological treatment using anti-spasmodics, anti-depressants, serotonin receptor agonists/antagonists and anti-diarrhea agents (loperamine or polycarbophil-calcium) (1,2).

Post-infectious low-grade mucosal inflammation is thought to be one of several possible causes of IBS in a specific subset of patients (4-7). IBS has a heterogeneous clinical presentation that includes abnormal bowel movements and abdominal pain, which are also symptoms of inflammatory bowel disease (IBD) (8-10). In addition, Schoepfer *et al* reported that antibodies against flagellin, the primary structural component of bacterial flagellae, were significantly more frequent in IBS patients than in control patients (7). Furthermore, levels of human β defensin 2, which is considered to play a pivotal role in the mucosal innate immune response, were higher in ulcerative colitis and IBS patients than in control subjects (5). In regard to the relationship between IBS and inflammation, Ohman *et al* reported that, in comparison to the control subjects, IBS patients showed a significant increase in the frequency of peripheral blood CD4⁺ and CD8⁺ T cells expressing the gut homing integrin β 7 as well as an increase

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in lamina propria CD8⁺ T cells in the ascending colon (6). In consideration of previous findings, the current methods of treating IBD, especially dietary modification, could also be appropriate for the treatment of IBS (11-14).

Although it is clear that IBD and IBS differ in regard to pathophysiology (in particular the lack of abnormal endoscopic findings in IBS patients), probiotics such as VSL#3 and prebiotics such as bran have shown efficacy in IBD treatment as well as in the clinical or preclinical treatment of IBS (11,13,15). The microbiota of the gastrointestinal tract form a complex ecosystem, and the use of probiotics and antibiotics has been reported to change the diversity and quantity of microbiota in IBS patients (11,15,16). Spiller noted that, in comparison to control patients, the microbiota in IBS patients displayed abnormalities related to malfermentation in the lower intestine and a change in transit time (17). While the details are still not completely clear, it has been reported that probiotics change the microbiota in IBS patients and improve mucosal immune, motor and barrier functions. These effects subsequently result in positive changes in fermentation and visceral hypersensitivity. However, in the case of probiotic therapy, the administered probiotics are excreted in the feces within several days after the termination of administration (18,19). Prebiotics have also been reported to alleviate IBS symptoms mainly through the modulation of the microbiota and the increase of short chain fatty acid (SCFA) production (20). Although prebiotics generally must be present in relatively high dose volumes to show efficacy in IBS patients, prebiotics have been reported to selectively increase beneficial endogenous microbiota without the administration of exogenous microbiota (probiotics).

Enzyme treated rice fiber (ERF) is a newly developed prebiotic which contains hemicellulose-rich dietary fiber. In this study, we evaluated the potency of ERF in the treatment of IBS. As mentioned above, maintaining the microbiota in the GI tract in a good condition by means of prebiotics is considered to be one of the best methods of treating IBS. In this study, we initially examined the utilization of ERF by microbiota as well as the production of SCFAs, especially butyrate (which has anti-inflammatory effects with respect to mucosa), *in vitro*. We thereafter determined the efficacy of ERF in a restraint stress model.

Materials and methods

Preparation of ERF and its chemical composition. Defatted rice bran (1 kg) was suspended in 4 l of hot water (80°C) and 2.0 g of heat-resistant amylase (Sumizyme A10; Shin Nihon Chemical Co., Ltd. Aichi, Japan) was subsequently added. The suspension was maintained at 80°C for 60 min in order to remove the residual starch fraction. After incubation, the insoluble fraction was isolated using a #200 meshed sieve (aperture size, 75 µm) and transformed into a suspension with a volume of 4 l and a pH of 5.0 via the addition of lactic acid and distilled water. The re-suspended fraction was then hydrolyzed using 2.0 g of hemicellulase (Sumizyme NX; Shin Nihon Chemical) and 2.0 g of protease (Sumizyme LPL; Shin Nihon Chemical) at 50°C for 12 h.

Thereafter, the fraction was heated at 80°C for 20 min to halt enzyme activity and the insoluble fraction was isolated

Table I. Chemical composition of rice bran and enzyme-treated rice fiber (ERF).

	Rice bran	ERF
Protein	17.6	14.9
Lipids	3.5	12.9
Dietary Fiber ^a	22.8	74.5
Cellulose ^b	(30.2%)	(32.8%)
Hemi-cellulose ^b	(48.2%)	(42.8%)
Lignin ^b	(21.5%)	(24.4%)
Carbohydrate ^c	33.0	
Ash	11.6	2.1
Water	11.5	3.6

^aData obtained by method of Southgate *et al.*; ^bData within parentheses indicate % of dietary fiber; ^cCarbohydrate data were calculated by the formula, 100 - protein - lipids - dietary fiber - ash. All values are in g/100g.

using a #200 meshed sieve. This insoluble fraction was subsequently sterilized and dried in hot air to obtain the ERF. The recovery was ~20%. The chemical composition of ERF is shown in Table I. ERF is a heterogeneous mixture of dietary fiber and protein. It contains ~70% hemicellulose rich dietary fiber by weight. The physiological function of dietary fiber seems to have a relation with its physical properties. The settling volume (SV) in water of dietary fiber is a convenient physical parameter of dietary fiber and contributes to the bulk-forming activity (21). The settling volume of ERF in water was determined to be 30.1 ml, a value which has been reported to be important in assessing the bulking effect in the lower intestinal tract (22). The settling volume of ERF is considerably higher than those of other insoluble fibers and rice bran, which is the raw material of ERF (Table II).

Utilization of ERF by microbiota. The utilization of ERF by intestinal microbiota and the accompanying production of organic acids were investigated using 7 representative anaerobic human intestinal bacterial strains, including 2 *bifidobacterium* strains (*B. breve*; JCM 1192 and *B. longum*; JCM 1217) and 2 *lactobacilli* strains (*L. acidophilus*; JCM 1132 and *L. casei*; JCM 1134) as probiotics, 2 *bacteroides* strains (*B. distasonis*; JCM 5825 and *B. ovatus*; JCM 5824) as opportunistic microbiota, and the butyrate producing microbiota, *Eubacterium limosum* (*E. limosum*; JCM 6421). The composition of the culture medium (peptone-yeast medium; PY medium) and incubation conditions were the same as those reported previously (23,24). Briefly, test tubes containing 15 ml of PY medium were inoculated with 300 µl of pre-incubated steady state bacteria (3x10⁷ CFU) and subsequently incubated anaerobically for 48 h at 37°C. An AnaeroPack (Mitsubishi Gas Chemical Co., Tokyo, Japan) apparatus was used to create anaerobic conditions. ERF was added to the PY medium (sole carbon source) at a rate of 0.5% (PY-ERF medium). After incubation, the pH of each bacterial culture was measured with a pH meter and the concentrations of organic acids (acetate, propionate, butyrate, iso-butyrate, succinate, lactate) were determined by HPLC as described

Table II. Settling volume of insoluble dietary fiber.

Fiber source	Settling volume (ml)
Cellulose	3.5 ^a
Corn husk	5.0 ^a
Beet fiber	7.0 ^a
Wheat bran	8.0 ^a
ERF	30.1
Rice bran	4.5

^aData from Tanabe *et al* (21).

below. The utilization of ERF by each bacterial strain was estimated from the increase in organic acid production in the PY-ERF broth. Cell growth was not evaluated from the optical density of the PY-ERF broth because ERF is insoluble and therefore rendered the broth opaque. However, our previous study showed a strong relationship between an increase in SCFA production and an increase in the growth of probiotics (24).

In addition, 6 of the above-mentioned strains (excluding *E. limosum*) were used to investigate the mechanism of bacterial butyrate production. Samples were prepared by inoculating 15 ml of PY-ERF medium with 300 μ l of pre-incubated *E. limosum* and one other strain. These samples were incubated anaerobically for 48 h at 37°C. After incubation, the organic acid content of the PY-ERF broth was determined by HPLC (23). The organic acids were separated using a Shim-pack SPR-H 250L (Shimadzu Co. Ltd., Kyoto, Japan). The mobile phase was 4 mM of p-Toluene sulfonic acid and an electrical conductivity detector was utilized (Shimadzu CDD-6A) (25).

Animals and diets. Thirty 5-week-old male SD rats were purchased from Charles River Japan (Kanagawa, Japan). The rats were housed individually in cages in a room maintained at a temperature of 20-25°C and a relative humidity of 40-60% with a 12-h lighting cycle (08:00-20:00) (26). They were allowed free access to food and drinking water. The experiment was approved by the Kirin Holdings Ethics Committee for Animal Experimentation. The 30 rats were initially fed laboratory chow for 1 week as an acclimatization period before the experiments began. We subsequently divided the rats into 3 groups (n=10/group) - a control diet group, an ERF diet group, and a polycarbophil Ca (PC) diet group. In addition to anti-spasmodics and anti-cholinergic agents, PC is often used as a treatment for IBS in Japan (27). PC was chosen as a positive control in this study because it has a similar efficacy as dietary fiber due to its bulk-forming character. PC has no serotonergic or cholinergic effects.

The rats were fed the respective diets *ad libitum* for 14 days and were subjected to restraint stress during the last 3 days of this period. PC was added to the diet at ~500 mg/kg body weight (28) and ERF was added at rate of 4%. As in our previous studies, the total volumes of protein and dietary fiber in the experimental diets were adjusted to 14.6 and 3.0%, respectively. The compositions of the three diets are shown in Table III.

Table III. Composition of respective diets.

	Control	ERF ^a	PC ^b
Casein	146.0	140.4	146.0
Vitamin mix ^c	10.0	10.0	10.0
Mineral mix ^c	35.0	35.0	35.0
Choline chloride	2.0	2.0	2.0
Cellulose	30.0	0.0	24.0
ERF		40.0	
PC			6.0
Corn oil	50.0	50.0	50.0
Corn starch	727.0	722.6	727.0

^aEnzyme-treated rice fiber (protein, 14.9%; dietary fiber, 74.5%);

^bPolycarbophil Ca (Colonel; Astellas Pharma Inc., Tokyo, Japan);

^cAccording to AIN 93G formula, protein and dietary fiber contents in three diets were adjusted to the same value using casein and cellulose, respectively. All values are g/kg.

Restraint stress model. After 11 days of pre-feeding with the respective experimental diets, the rats in each group were divided into two subgroups: stress-positive (n=5) and stress-negative (n=5). In accordance with the restraint stress model described by Miyata *et al*, the stress-positive rats were placed in individual wire-meshed narrow compartments (KN-468, Natsume Seisakusho Co. Ltd., Tokyo, Japan) in an animal room under the same conditions as described above for 4 h (09:30-13:30) per day for 3 consecutive days. The stress-negative rats in the respective groups were kept in their cages in an animal room without being subjected to restraint (29).

Behavioral responses to colorectal distention. The pain threshold to colorectal distention (CRD) was determined using a Barostat system (Distender IIR, Starmedical, Tokyo, Japan), Protocol PlusTM apparatus (Starmedical), and a custom made balloon catheter (RTBR3, Starmedical). The rats were placed in a rat holder for drawing blood (KN-328, Natsume Seisakusho). In a modification of the method of Ren *et al*, the balloon catheter was gently inserted into the distal colon with the distal tip 1.5 cm from the anal verge and secured to the base of the tail with surgical adhesive tape (30). The balloon was then distended with air to exert a pressure of 5 mmHg for 1 min and the baseline colonic contraction was confirmed. After this baseline evaluation, the rats were allowed to rest and their baseline of CRD reached a steady state (Fig. 4A). Thereafter, CRD was performed in a stepwise fashion. This process involved 30 sec of distention followed by 20 sec of rest from 10 to 52 mmHg with 3 mmHg increases in pressure (31,32). As per the method of Al-Chaer *et al*, behavioral responses to CRD were assessed in all 6 groups by measuring the abdominal withdrawal reflex (AWR) using a semi-quantitative score under a blinded evaluation (31). We determined an AWR score of 3, a level featuring strong contraction of the abdominal muscles and lifting of the abdomen off the platform, as the threshold of visceral hypersensitivity (31). During acclimatization periods, we measured the pain threshold to CRD once to inure the animals to CRD measurement.

Fecal output. During the restraint stress period, fecal samples were collected from all groups. The number of fecal samples was counted and the dry weight was measured after lyophilization. An increase in fecal weight has been reported to be a major IBS symptom in the restraint model utilized in this study (29). No diarrhea was observed in any of the 6 groups. We determined the fecal output during restraint stress for 4 h and the total fecal output for 24 h including the stress treatment period.

Colonic mucosal 5HT, CINC-1 and TNF- α content. After removing half of the longitudinal distal colon, the colonic mucosa were centrifuged in a potter homogenizer with deadeacyltrimethylammonium bromide buffer (33). The prepared mucosal homogenate was used for cytokine-induced neutrophil chemoattractant (CINC)-1 and 5HT determinations using commercially available ELISA kits for rats (CINC; GRO/CINC-1, Rat, RPN2730, GE Healthcare UK Ltd., 5HT; Serotonin EIA, BA10-0900, Rocky Mountain Diagnostics Inc., USA, TNF- α ; Rat TNF ELISA set BD Biosciences Pharmingen, USA). The protein content was determined using a commercially available kit (BCA protein assay kit, Pierce Biotechnology, Rockford, IL, USA).

Colonic mucosal 5HT positive cells. Half of the longitudinal distal colon was removed and fixed in 4% neutral buffered paraformaldehyde. After fixation, the specimens were embedded in paraffin, and sectioned at 5- μ m width. Each section was deparaffinized, hydrated, and immersed in 0.01% hydrogen peroxide in tris-HCl buffer at a pH of 7.4 for 10 min to inhibit endogenous peroxidase activity. The sections were washed three times with Tris buffer and treated with 1% bovine serum albumin for 30 min to block the non-specific binding sites. The specimens were incubated overnight with 5HT antibody (Progen Biotechnik GmbH, Heidelberg, Germany). Sections were incubated for 30 min with biotinylated swine anti-rabbit IgG or biotinylated anti-mouse IgG diluted 1:200. They were subsequently incubated with the avidin-biotin-peroxidase complex diluted 1:100 for 30 min. Development of the section was performed in 50 ml Tris buffer containing 10 ml of 30% H₂O₂ and 25 mg diaminobenzidine hydrochloride (DAB) (Dako A/S, Glostrup, Denmark) (34). The number of 5HT-positive cells [including enterochromaffin (EC) and mast cells] was counted in 5 different areas and the average number was calculated.

Organic acid analysis on cecal contents. The organic acid content of the cecal contents of the rats fed with the each of the respective diets was determined and modified according to the details in our previous study (25). Briefly, 1.0 ml of Milli-Q water was added to 0.2 g of cecal contents and the mixture was incubated at 4°C for 30 min. After centrifuging at 12,000 rpm at 4°C for 10 min, the obtained supernatant was continuously filtered using a 0.22 μ m filter and the organic acid content was determined by HPLC as described above.

Statistical analysis. All animal experimental data are presented as the mean \pm SE. Comparison among groups was performed using one-way ANOVA, and Dunnett's test or the Student-Newman-Keuls test was subsequently applied. Differences

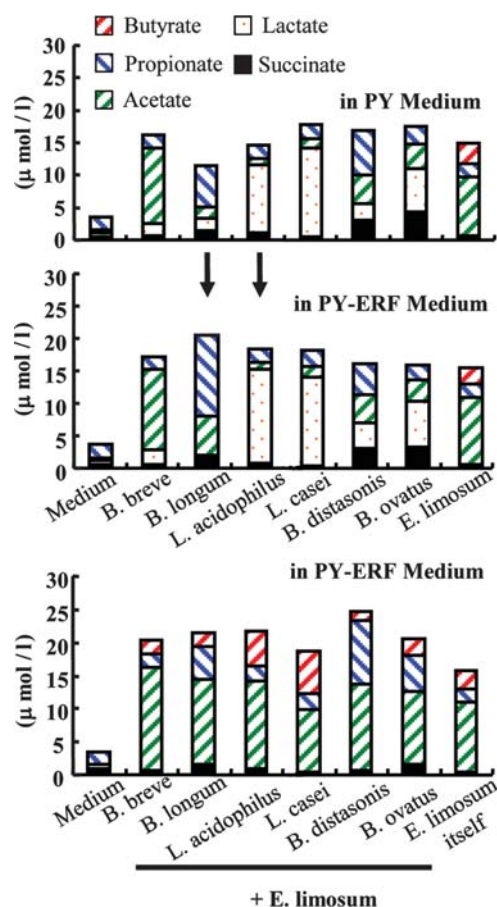


Figure 1. Change in organic acid production profiles of representative intestinal microbiota. PY medium, peptone yeast medium and PY-ERF medium, PY medium containing 0.5% ERF (enzyme-treated rice fiber). We used seven representative microbiota, namely, 2 *bifidobacterium* strains (*B. breve*; JCM 1192 and *B. longum*; JCM 1217) and 2 *lactobacilli* strains (*L. acidophilus*; JCM 1132 and *L. casei*; JCM 1134) as probiotics, 2 *bacteroides* strains (*B. distasonis*; JCM 5825 and *B. ovatus*; JCM 5824) as opportunistic microbiota, and the butyrate producing microbiota, *Eubacterium limosum* (*E. limosum*; JCM 6421). Total organic acid production was greater for *B. longum* and *L. acidophilus* in the PY-ERF medium than in the PY medium. *B. breve*, *L. casei*, the 2 *bacteroides* strains, and *E. limosum* could not directly utilize ERF because no major increases in organic acid production were observed in the PY-ERF medium as compared with the PY medium. Data are the means of triplet samples. In regard to *L. acidophilus* and *L. casei*, the butyrate content was greater than that for *E. limosum* alone. For both *lactobacillus* strains, there was a dramatic decrease in lactate production and a marked increase in acetate and butyrate production. In the case of the 2 *bacteroides* strains, there was an increase in the total organic acid content and a decrease in the succinate content, although there was no increase in butyrate content, as compared with *E. limosum* alone. Data shown are the means of triplet samples.

between means were considered significant at a level of $P < 0.05$.

Results

In vitro experiments. All 7 bacterial strains lowered the pH of the PY medium by producing organic acids (data not shown). In the PY-ERF medium, the increase in total organic acid production by *B. longum* and *L. acidophilus* was greater than that in the PY medium, thus indicating that only these 2 strains of bacteria converted ERF to organic acids. However, *E. limosum* was the only strain able to produce butyrate in both

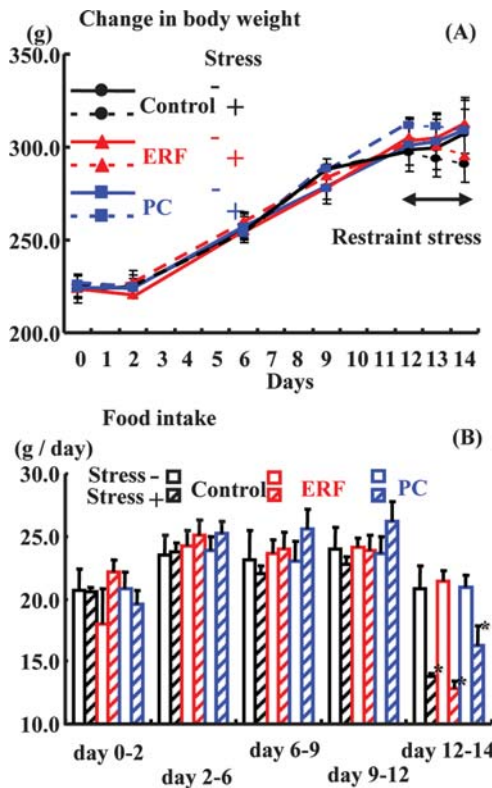


Figure 2. Change in body weight and food intake. Body weight gain and food intake during this experiment are shown. The food intake of the respective groups was significantly decreased by stress treatment in comparison to the food intake of without stress treatment. Data are shown as mean \pm SE. NS, no significant difference between stress-positive and -negative rats in the same dietary group. *Significant difference between stress-positive and -negative rats in the same dietary group ($p < 0.05$).

media. Interestingly, strains from the same genus, namely, the 2 *bifidobacterium* strains, showed different predispositions with respect to ERF utilization in the 2 media, with an increase in organic acid content achieved by only one of them in the PY-ERF medium. *B. breve*, *L. casei*, the 2 *bacteroides* strains, and *E. limosum* did not directly utilize ERF, as evidenced by the fact that no major increases in organic acid production were observed in the PY-ERF medium as compared with the PY medium (Fig. 1). Data are the means of triplicate samples.

The total organic acid contents increased and the organic acid production profiles changed dramatically for all strains incubated together with *E. limosum*. Among the two probiotic strains (*L. acidophilus* and *L. casei*), the butyrate content was greater than that for *E. limosum* alone. Furthermore, there was a dramatic decrease in lactate production and a marked increase in acetate production among both lactobacillus strains. As for the 2 *bacteroides* strains, there was an increase in total organic acid content and a decrease in succinate content, although there was no change in butyrate content, as compared with *E. limosum* alone (Fig. 1).

Animal experiments. Food intake and body weight for the rats were determined 2 or 3 times in a week after the acclimatization period (Fig. 2). In the stress-negative and -positive treatment

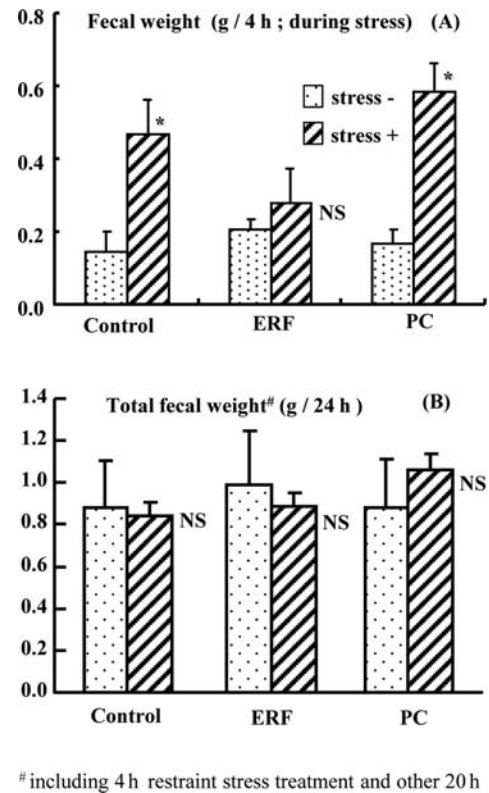


Figure 3. Change in fecal output during restraint stress and daily total output. In restraint stressed rats in the control and PC groups, there was a significant increase in dry fecal dry weight as compared with unstressed rats in the two groups. However, ERF clearly prevented an increase in urgent fecal output in rats under restraint stress. (A) In stressed rats in the control and PC groups, stools were looser than those of rats without stress but no diarrhea was observed in any of the six groups. The total fecal output was not significantly affected by either stress or the 3 diets. (B). Data are shown as the mean \pm SE. NS, no significant difference between stress-positive and -negative rats in the same dietary group. *Significant difference between stress-positive and -negative rats in the same dietary group ($p < 0.05$).

groups, there were no significant differences in body weight among the 6 dietary groups during the experimental period. A significant decrease in food intake was observed in the stress-positive groups as compared with that of the stress-negative group fed the same diets.

Increases in the excreted fecal weight and the frequency of fecal output are reported to be representative symptoms of stress-induced IBS in rats (35). In our experiment, stress-positive rats in the control and PC groups showed significant increases in fecal dry weight as compared with stress-negative rats in these groups. However, the addition of ERF to the diet definitely mitigated any increase in fecal output due to restraint stress (Fig. 3A). In stress-positive rats in the control and PC groups, no diarrhea was observed although the stools were looser than those of the stress-negative rats. In addition, no diarrhea was observed and there was no variation in the appearance of feces between the stress-positive and -negative ERF groups. The total fecal output was not significantly affected by stress or any of the 3 dietary treatments (Fig. 3B). In regard to the ERF treatment, there was no increase in total fecal output or an acute increase in fecal excretion due to restraint stress.

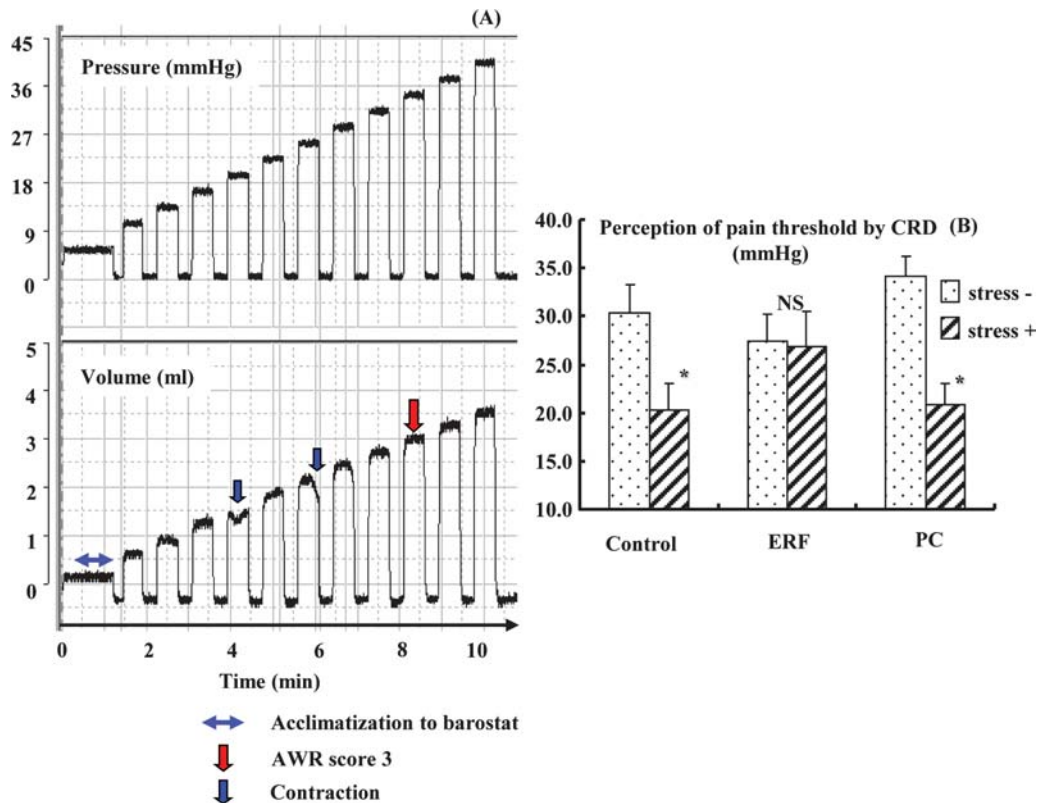


Figure 4. Change in the perception of pain threshold for colorectal distension as affected by the Barostat method. Behavioral responses to CRD were evaluated in all groups by measuring abdominal withdrawal reflex (AWR) scores. A score of 3 was set as the threshold intensity, which was taken as visceral hypersensitivity. A balloon was distended with air to exert a pressure of 5 mmHg for 1 min and the baseline colonic contraction was confirmed. After this baseline evaluation, rats were allowed to calm and their baseline CRD reached a steady state (A). In the control and PC groups, restraint stress significantly decreased the pain threshold. However, ERF prevented any reduction in the pain threshold due to restraint stress (B). Data are shown as the mean \pm SE. NS, no significant difference between stress-positive and -negative rats in the same dietary group. *Significant difference between stress-positive and -negative rats in the same dietary group ($p < 0.05$).

Behavioral responses to CRD were evaluated in all groups by measuring AWR scores, and the CRD threshold intensity was determined as the visceral hypersensitivity (30). The evaluation of the pain threshold with respect to CRD by AWR is superior to measuring the visceromotor reflex using electrodes and wires in the abdominal muscle because it is not necessary to perform surgery to implant electrical detectors with the AWR score-based method. In the control and PC groups, restraint stress significantly decreased the pain threshold. However, ERF prevented the reduction in pain threshold due to restraint stress (Fig. 4B). It was previously reported that the visceromotor response involved a contraction of abdominal and hindlimb musculature, and the distending pressure threshold for the visceromotor response (22.4 ± 0.9 mmHg) was significantly greater than that necessary to evoke a non-nociceptive response (36). In our experiment, the means of the pain threshold levels by CRD in the stress-negative groups were beyond 25 mmHg (36).

Colonic pain signals transmitted to the central nervous system via primary nociceptive afferent neurons are modulated by neurotransmitters (including 5HT). In this study, restraint stress caused a dramatic decrease in the pain threshold with respect to CRD. In the control group, restraint stress produced a significant increase in colonic mucosal 5HT content. However, there were no significant differences in 5HT content between stress-positive and -negative rats in the ERF and PC groups

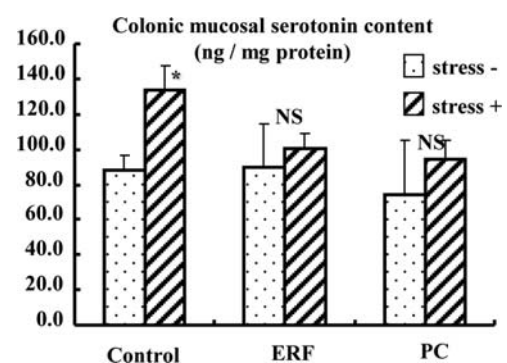


Figure 5. Colonic mucosal serotonin (5HT) content. In the control group, restraint stress produced a significant increase in colonic mucosal 5HT content. However, there were no significant differences in the 5HT content between stress-positive and -negative rats in the ERF and PC groups. Data are shown as the mean \pm SE. NS, no significant difference between stress-positive and -negative rats in the same dietary group. *Significant difference between stress-positive and -negative rats in the same dietary group ($p < 0.05$).

(Fig. 5). In addition, the number of 5HT positive cells (primarily EC and mast cells) were similar among the 6 groups (detailed data not shown), and no inflammatory damage was observed (Fig. 6). We also determined the levels of CINC-1 and TNF- α , which are representative inflammatory parameters, but no significant differences were observed (Fig. 7A and B).

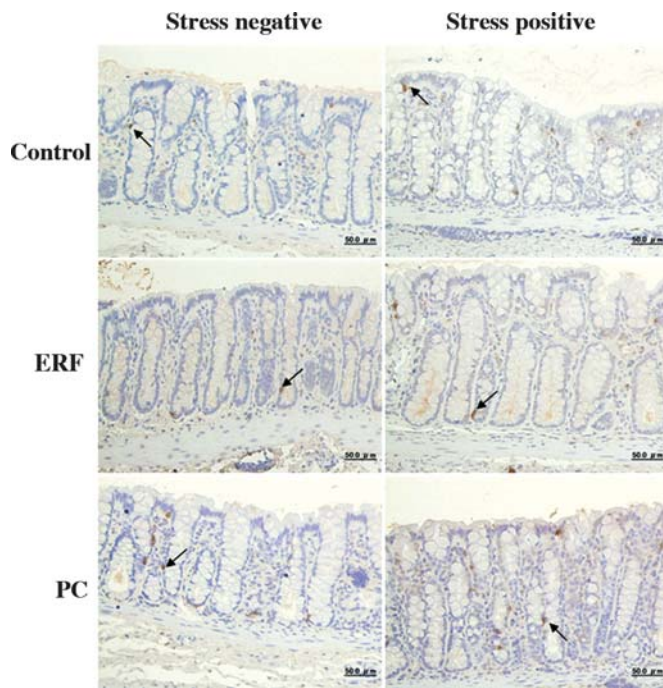


Figure 6. Pathological observation of 5HT-positive cells in colonic mucosa. The arrows show the representative 5HT-positive cells in colonic mucosa (at x200 magnification). In addition, no inflammatory damage was observed among the 6 groups.

Though SCFAs and other cecal organic acids are known to be produced by commensal microbiota from dietary fiber, it is still controversial whether dietary fiber intake is beneficial to IBS patients. Changes in organic acid production are a predictive parameter for the modulation of microbiota (37). In the absence of stress, the butyrate content in the ERF group and the succinate content in the PC group were significantly higher than those in the control rats. In the ERF group, restraint stress significantly decreased acetate and butyrate production, and the butyrate content in the stress-positive ERF group was higher than that of the stress-negative control group (Fig. 8).

Discussion

In order to understand the pathogenesis and treatment of IBS, it is important to examine abnormalities in gastrointestinal sensation, motility, intestinal microbiota, mucosal immunity, and the 5HT pathway (38). In addition, recent reviews have shown that disruption of the intestinal microbiota may contribute to the development and pathogenesis of IBS (1,11,16). ERF was effectively utilized by *L. acidophilus* and *L. casei*, as indicated by the dramatic increase in the total organic acid production in the medium with ERF as compared to the medium without it. Moreover, ERF was effectively converted to butyrate by *E. limosum* with the aid of these 2 strains of *lactobacilli*. *E. limosum* is well known as a butyrate producer in the human GI tract, and among SCFAs, butyrate has the greatest effect on colonic motility (39). In this regard, the present study showed that in the absence of stress, ERF significantly increased the cecal butyrate level as compared with the control and PC groups. ERF also prevented increased fecal output during restraint stress. These findings suggest that modulation of the microbiota by prebiotics maintains

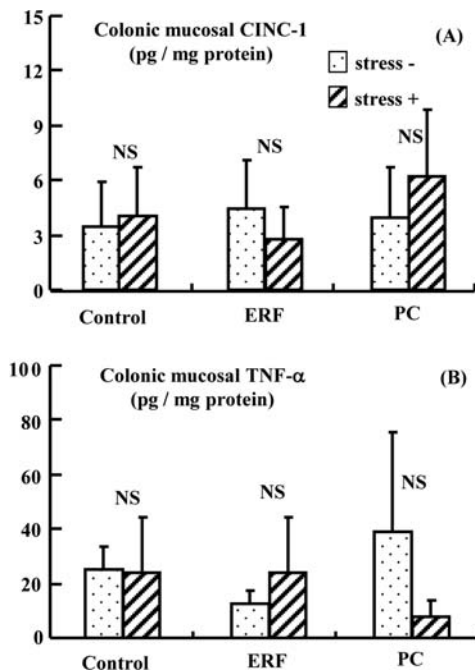


Figure 7. Change in colonic mucosal CINC-1 and TNF- α content. Neither CINC-1 (A) nor TNF- α (B) content showed significant differences among the 6 groups. Data are shown as the mean \pm SE. NS, no significant difference between stress-positive and -negative rats in the same dietary group.

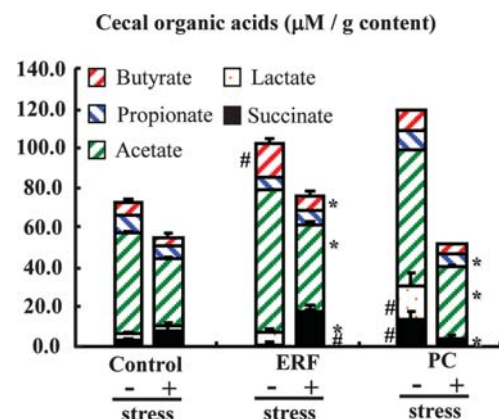


Figure 8. Change in cecal organic acid content. For stress negative rats, the butyrate content in the ERF group and the succinate and lactate contents in the PC group were significantly higher than those in the control group. In the ERF group, restraint stress significantly decreased acetate and butyrate production. In the PC group, the butyrate, acetate and succinate levels were significantly lower in stress-positive rats than in stress-negative rats. Data are expressed as the mean changes \pm SE. *Significant difference between stress-positive and -negative rats in the same dietary group ($p < 0.05$). #Significant difference from the control stress-negative group ($p < 0.05$).

levels of fecal SCFAs and attenuate urgent fecal excretion due to rapid colonic motility under restraint stress. Previous studies reported that PC, a synthesized high-molecular polymer, was insoluble and not absorbable in the GI tract and did not stimulate SCFA production by the fermentation of microbiota (28).

Previous findings suggest that changes in microbiota in IBS models due to probiotics and/or prebiotics are related to improvement in colonic motor function. SCFAs derived from dietary fiber have been reported to potentially prevent the

development of colonic mucosal 5HT receptor hypersensitivity (20,40). Our findings suggest that the relatively high concentration of SCFAs in the colon due to ERF treatment induced hyposensitivity and prevented the enhancement of colonic motility under restraint stress due to suppression of 5HT secretion, as compared with the control and PC groups (20,38,39). However, this effect must be confirmed through more detailed studies. The finding that the total cecal organic acid content in the stress-positive ERF group was similar to that in the stress negative control group suggests that ERF prevents abnormal fermentation under restraint stress and maintains good fermentation in a stress-free control condition. However, we must produce more data in order to evaluate the relationship between 5HT production and SCFA production in further detail. Interestingly, both PC and ERF potentially inhibited colonic mucosal 5HT secretion under restraint stress. It is well known that >90% of 5HT is stored in EC cells. Furthermore, 5HT is thought to be the most important neurotransmitter in visceral nociception and is also reported to induce colonic motility via local nerve networks (41). A previous study reported that 5HT release from EC cells is stimulated by mucosal stroking, microbiota, and SCFAs, and the authors suggested that the presence of mechanical stimuli is the most important factor in 5HT release (38). Owing to their high water-holding capacity and lattice-like physical nature, PC and ERF could attenuate colonic mucosal 5HT release during restraint stress as compared with a control.

Dietary fiber supplementation is generally recommended to manage IBS symptoms. Dietary fiber modulates fecal water content and transit time (when used as a bulking agent), increases mucosal barrier defenses through the activation of microbiota, and alleviates visceral hypersensitivity (17,42). However, there is little convincing evidence to support the use of dietary fiber in IBS for these purposes (43). In the present study, ERF attenuated an increase in fecal output during restraint stress. This effect of ERF is considered in part to be due to an improvement in luminal volume and related motor function (14). ERF has a higher water-holding capacity than other insoluble dietary fibers (22), but the water-holding capacity of PC is more than double that of ERF (44). However, despite its very high water-holding capacity, PC did not prevent an increase in urgent fecal output. Because PC is only partly metabolized by microbiota, it may be necessary for dietary fiber to be fully metabolized by microbiota and converted to SCFAs to modulate bowel movement.

A previous study reported that acute gastroenteritis is one of the highest risk factors for the development of IBS. The authors termed IBS developing after acute gastroenteritis as post-infectious IBS (PI-IBS) and reported that the incidence rate of this condition was 7-33% of total IBS patients (45). Therefore, transient infection seems to play an important role in persistent gut dysfunction by inducing an increase in intestinal permeability and activating the mucosal immune system (32). To examine mucosal inflammation, we targeted the proinflammatory chemokine CINC-1 and the representative proinflammatory cytokine TNF- α . However, their levels were not affected by stress in any of the three dietary groups. Therefore, no inflammation was observed in the three dietary groups with or without moderate stress treatment. Although detailed data are not shown, colonic mucosal damage was not

observed in any of the experimental groups with or without stress treatment by histological observation. In order to clarify this finding, we must carry out more detailed experiments using other types of stress models.

In this study, we used the new prebiotic ERF for the treatment of IBS. The advantage of a prebiotic in comparison to the use of probiotics is that it does not require continuous ingestion of exogenous beneficial microbiota to change the endogenous microbiota population in the host's GI tract (46). ERF was potent in increasing SCFA production and attenuating 5HT, and it significantly prevented urgent fecal excretion and visceral hypersensitivity in the IBS model used in this study. Although ERF demonstrated beneficial effects, more detailed studies will be required in the future to fully evaluate its efficacy in the treatment of IBS.

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