学位論文

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Journal of Pediatric Surgery 54 (2019) 2514–2519

Contents lists available at ScienceDirect

Journal of Pediatric Surgery

journal homepage: www.elsevier.com/locate/jpedsurg

Partially hydrolyzed guar gum alleviates small intestinal mucosal damage after massive small bowel resection along with changes in the intestinal microbiota

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ARTICLE INFO

Article history:
Received 7 August 2019
Accepted 24 August 2019

Key words:
Partially hydrolyzed guar gum
Dietary fiber
Short bowel syndrome
Intestinal failure
Dysbosis
Short chain fatty acids

ABSTRACT

Purpose: Short bowel syndrome is associated with intestinal mucosal inflammation and microbial dysbiosis, leading to intractable complications. Partially hydrolyzed guar gum (PHGG) has trophic and anti-inflammatory effects on the intestine. We investigated whether PHGG ameliorates small intestinal mucosal damage and alters the intestinal microbiota using a rat small bowel resection (SBR) model.
Methods: Sprague Dawley rats were divided into sham operation (Sham), Sham/PHGG, SBR, and SBR/PHGG groups. On day 21, all rats were euthanized. To assess small intestinal mucosal damage, the degeneration rate was morphometrically evaluated and immunohistochemically examined using anti-CD45 antibodies. Analyses of fecal microbiota using 16S rRNA and short-chain fatty acid production were also performed.
Results: The mucosal degeneration rate was significantly higher in the SBR group than in the Sham or SBR/PHGG groups. The number of CD45-positive cells was significantly higher in the SBR group than in the Sham, Sham/PHGG, or SBR/PHGG groups. The relative abundance of family *Lachnospiraceae* was significantly higher in the SBR/PHGG group than in the SBR group.
Conclusions: PHGG administration alleviated small intestinal mucosal damage which could be associated with modulation of the intestinal microbiota.

Although long-term parenteral nutrition is often required in children with short bowel syndrome (SBS) to enhance growth and development, it causes intestinal mucosal atrophy. Intestinal epithelial cells play an important role in preventing invasion of pathogens via an intercellular physical barrier consisting of the tight junction, a thick mucus layer on the cell surface, and secreted antimicrobial peptides in the mucus [1].

Reduced diversity of intestinal microbiota and bacterial overgrowth seen in SBS patients may be caused not only by failure of the barrier function but also by several factors, including compensatory dilatation of the intestinal tract, impaired intestinal motility, and increased levels of nonabsorbing substances within the intestinal tract [2]. Furthermore, the dysbiosis of microbiota can be related to intestinal failure-associated liver disease (IFALD) and catheter-related blood stream infections (CRBSI), which are intractable complications in SBS patients [2]. Accordingly, it is likely that prevention of the dysbiosis of microbiota and alleviation of the damage of the intestinal mucosa lead to the prevention of IFALD and CRBSI in SBS patients.

Partially hydrolyzed guar gum (PHGG), which is a water-soluble dietary fiber, has various physiological activities, including modulation of the intestinal microbiota [3,4], effective production of short-chain fatty acids (SCFAs) [4], and anti-inflammatory effects in colitis [4]. However, the effects of PHGG on the intestinal mucosa and intestinal microbiota in SBS patients remain unknown. Therefore, in this study, we investigated whether PHGG ameliorates small intestinal mucosal damage and alters the intestinal microbiota using a rat SBS model.

1. Methods

1.1. Animals

Eight-week-old male Sprague Dawley rats weighing 280–320 g were purchased from Charles River Laboratories Japan, Inc. (Yokohama,

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https://doi.org/10.1016/j.jpedsurg.2019.08.048
0022-3468/

Japan). The animals were individually housed in cages with ad libitum access to the liquid rat diet F2LCW (Oriental Yeast Co., Ltd., Tokyo, Japan) and water and were acclimatized to their environment for 7 days before the experiments. All experimental procedures were approved by the Kagawa University Animal Ethics Committee (Approval number: 17633-1).

1.2. Study design

Twenty rats were assigned to one of the following four treatment groups: sham operation (bowel transection with reanastomosis) group (Sham group; $n = 5$); Sham plus PHGG group (Sham/PHGG group; $n = 4$); 70% small bowel resection group (SBR group; $n = 5$); and SBR plus PHGG group (SBR/PHGG group; $n = 6$). PHGG (Sunfiber®, containing 0.85 g fiber/g) was purchased from Taiyo Kagaku (Tokyo, Japan). The rats in the PHGG groups were fed 10% PHGG diet from the first postoperative day. On day 21, all rats were euthanized, the small intestines were harvested for histological analysis, and fecal contents were collected for analysis of SCFAs. SCFA levels in the ileum and ileal microbiome were examined in selected rats.

1.3. The surgical procedure and maintenance methods

The animals were fasted overnight. For surgery, the animals were anesthetized with sevoflurane. A 70% SBR operation was performed in SBR-operated rats as follows: The intestinal tract of interest was resected, leaving 15 cm of the ileum above the ileocecal valve and 15 cm of the jejunum below the ligament of Treitz. Then, the remaining intestinal stumps in the body were anastomosed. Bowel anastomosis was completed using interrupted 6-0 polydioxanone sutures (Ethicon Inc., Cincinnati, OH, USA). The abdominal incision was closed with 3-0 polyglycolic sutures (Ethicon Inc.). In the Sham group, the ileum was transected at 15 cm above the ileocecal valve and was reanastomosed. All animals received ampicillin/sulbactam (30 mg/kg, subcutaneously; Meiji Seika Pharma Co., Ltd., Tokyo, Japan) preoperatively to prevent postoperative infection and buprenorphine (0.01 mg/kg, subcutaneously; Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) for analgesia. The animals were allowed ad libitum access to water immediately after surgery. From day 1 to day 5 postoperatively, the animals were fed a liquid rat diet ad libitum. The rats in the PHGG groups were fed 10% PHGG diet from the first postoperative day. From day 6, the liquid diet was changed to a normal rat diet in powder form: MF (Oriental Yeast Co., Ltd., Tokyo, Japan) ad libitum. The rats in the PHGG group were fed a 10% PHGG diet. Glucose (Fuso Pharmaceutical Industries, Ltd., Osaka, Japan) was added to diets of the rats in the non-PHGG-fed groups to obtain the same number of calories as the rats in the PHGG-fed groups. On day 21, after an overnight fast, all rats were anesthetized by sevoflurane inhalation. The animals were euthanized by exsanguination.

1.4. Histopathological analysis

Samples for histopathological analysis were harvested from the small intestine of the anastomotic site (3 cm around the anastomotic site). They were fixed in neutral buffered formalin for 48 h and embedded in paraffin. Paraffin sections were cut at 4-μm thickness and stained with hematoxylin and eosin. Ten areas composed of well-oriented villi/crypt units were chosen in each sample for analysis. On the basis of the results, the degeneration rate in the small intestinal mucosa around the anastomotic site was evaluated as follows (Fig. 1): The morphometric analysis in the small intestinal mucosa was performed by measuring (A) the length from the muscularis mucosae to the top and (B) the length from the muscularis mucosae to the place where the epithelial cells remained clean. The degeneration rate was defined as $(1 - B/A) \times 100$.

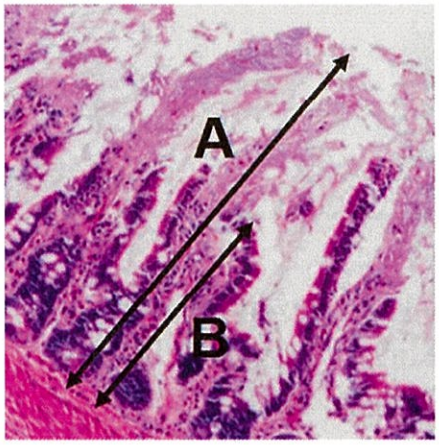

Fig. 1. The definition of degeneration rate of the small intestinal mucosa. The length from the muscularis mucosae to the top is defined as A, while the length from the muscularis mucosae to the place where epithelial cells remained clean is defined as B. The degeneration rate of the small intestinal mucosa is defined as $(1 - B/A) \times 100$ (%).

1.5. Immunohistochemical procedure

To assess the infiltration of inflammatory cells, immunohistochemical examination using anti-CD45 (also known as leukocyte common antigen) antibodies (Abcam ab10558, Cambridge, UK) was conducted. The sections were treated with 3% H_2O_2 for 10 min; they were then heated at 95 °C in 0.01 mol/l citrate buffer (pH 6.0) for 20 min, blocked with bovine serum albumin for 20 min, and incubated in the presence of the anti-CD45 antibody (diluted 1:1000) at room temperature for 1 h. The sections were incubated with a secondary antibody (Nichirei, Tokyo, Japan) for 30 min. Color was developed with 3,30-diaminobenzidine tetrahydrochloride and hydrogen peroxide (Nichirei) at room temperature for 10 min. The sections were counterstained with hematoxylin for several seconds. The number of CD45-positive cells per 10 villi/crypt units was counted.

1.6. Analysis of SCFAs

SCFAs (acetate, butyrate, and propionate) in the supernatants of sample suspensions were derivatized with 2-nitrophenylhydrazine using a YMC-Pack FA kit (YMC Co. Ltd.) and were extracted with hexane and diethyl ether. Standards of acetate, butyrate, 2-ethyl-butyrate (2-EA), and propionate were obtained from Wako Chemical Co Ltd. 2-EA was used as the internal standard. The high performance liquid chromatography system consisted of a Waters e2695 separation module (0–29 MPa) and a Waters-UV detector connected in series. Chromatographic records were obtained using Gold Nouveau software, version 1.7 (Waters). Chromatographic separations were performed on YMC-FA-250 × 6 mm. Columns were purchased from YMC Co. Ltd. Injections (15 ml of sample) were performed using an autoinjector. Analysis was performed by continuous elution with acetonitrile: methanol:deionized water 30:16:54 (v/v/v) with the pH adjusted to 4.5 using 0.01 N HCl. The flow rate was constantly set to 0.5 ml/min. The monitored UV wavelength was 400 nm. SCFAs were monitored with UV detection.

1.7. Analysis of fecal microbiota

Ileal samples were suspended in PBS (pH 7.4) to 0.1 g/ml, centrifuged, and washed once with Tris-EDTA buffer (TE, pH 8.0). After centrifugation, the pellets were frozen at −80 °C until further use. DNA extraction was performed according to the method reported by Morita et al. [5]. Sequencing libraries were prepared by amplifying the V3-V4 region of the 16S rDNA. After initial amplification, a second PCR was

performed to attach Illumina adaptors as well as barcodes that allowed multiplexing (24 samples). Amplifications were performed in 25-μl reactions containing 2.5 μl of the diluted template, 12.5 μl of 2× KAPA HiFi HotStart Ready Mix, and 5 μl each of primers. Thermal cycling consisted of an initial denaturation step (3 min at 95 °C), followed by 25 cycles of denaturation (30 s at 95 °C), annealing (30 s at 55 °C) and 30 s extension at 72 °C. Final extension consisted of 5 min at 72 °C. Amplicons were purified using AMPure XP beads (Beckman Coulter). Sequencing was performed on an Illumina MiSeq platform (MiSeq Reagent Kit ver. 3, 600 cycles) according to the manufacturer's specifications to generate paired-end reads of 300 bases in each direction. Primer sequences were trimmed away, and paired-end reads were merged using fastq-join [6] with default parameters and processed with QIIME 1.8.0 pipeline. After chimera check by Usearch, 20,000 Illumina reads per sample (average quality score above 20) were randomly selected for further analysis. Using the UCLUST [7] algorithm built into the QIIME pipeline, sequences were clustered at 97% identity against the Greengenes reference database. Using the QIIME pipeline, weighted UniFrac distances were produced and used for the investigation of beta diversity by plotting PCA coordinates.

1.8. Statistical analysis

Data are presented as mean ± standard error. Statistical significance was evaluated using two-way analysis of variance followed by the Tukey–Kramer or Steel–Dwass tests (EZR, Saitama Medical Center, Jichi Medical University, Saitama, Japan). Relative abundances in the ileal microbiome between groups (SBR group and SBR/PHGG group) were compared using the t-test (StatFlex ver.6, Artec Co., Ltd., Tokyo). A p-value of less than 0.05 was considered statistically significant.

2. Results

2.1. Morphometric analysis of the small intestinal mucosa

Fig. 2A shows the representative histological images of the small intestinal mucosa. The tip of the small intestinal epithelium underwent complete coagulative necrosis or was partially injured. These findings were most notably observed in the SBR group. The degeneration rate in the SBR group was significantly higher than that in the Sham group (Fig. 2B). However, the degeneration rate was significantly improved by PHGG administration. Fig. 3A shows representative immunohistochemical images in the small intestinal mucosa using the anti-CD45 antibody. The number of CD45-positive cells was significantly higher in the SBR group than in the Sham and Sham/PHGG groups. However, the number was significantly lower in the SBR/PHGG group than in the SBR group (Fig. 3B).

2.2. Effect of PHGG on SCFAs production

Fig. 4A shows the production of several SCFAs in the ileum. Although there were no significant differences, the production of SCFAs was greater in the SBR/PHGG group than in the SBR group. Fig. 4B shows the production of several SCFAs in the colon. The production of all SCFAs was greater in the SBR/PHGG group than in the SBR group, but there were no significant differences. In particular, the production of acetate in the colon tended to increase with PHGG administration (Sham, Sham/PHGG, SBR, and SBR/PHGG groups: 36.4 ± 5.5, 39.3 ± 9.8, 37.3 ± 4.7, and 64.5 ± 10.2 μmol/g, respectively; $p = 0.085$).

2.3. Alteration of the ileal microbiome in SBR after PHGG administration

The ileal microbiome was examined to address the mechanism of the anti-inflammatory effect observed in the small intestinal mucosa. PCoA analysis showed that the ileal microbiome in the SBR group was more variable than those in the Sham and SBR/PHGG groups (Fig. 5). Furthermore, the ileal microbiome in the SBR/PHGG group was more similar to that in the Sham group than to that in the SBR group. As shown in Fig. 6, the relative abundance of family *Lachnospiraceae* was significantly greater in the SBR/PHGG group than in the SBR group ($p = 0.045$).

3. Discussion

Our study revealed that PHGG administration alleviated small intestinal mucosal damage and inhibited the infiltration of leukocytes, which could be associated with modulation of the intestinal microbiota.

The findings indicating the degeneration of the small intestinal mucosa around the anastomotic site were rarely observed in sites other than the small intestine. This may occur for two reasons. First, there is the possibility that temporary ischemia and decrease in blood flow occurred only in the anastomotic site, followed by degenerative changes. Second, inflammation occurring in the vicinity of the anastomotic site may induce degenerative changes, because intestinal mucosal inflammation often occurs after SBR [8]. The mucosal inflammatory response may be related to reduced microbial diversity [8]. Therefore, it is likely that the combination of ischemia and inflammation strongly occurs in animals in the SBR model, thereby inducing degenerative changes.

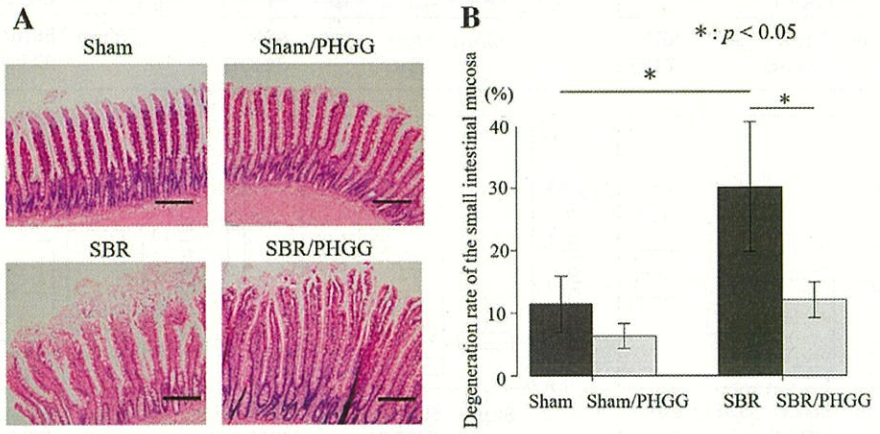

Fig. 2. Intestinal morphometric analysis using histological images. (A) Representative histological images of animals in the Sham, Sham/PHGG, SBR, and SBR/PHGG groups. Scale bars = 200 μm. (B) The degeneration rates of the small intestinal mucosa in the four groups are shown. The degeneration rate in the SBR group was significantly higher than that in the Sham group, while that in the SBR group was also significantly higher than that in the SBR/PHGG group.

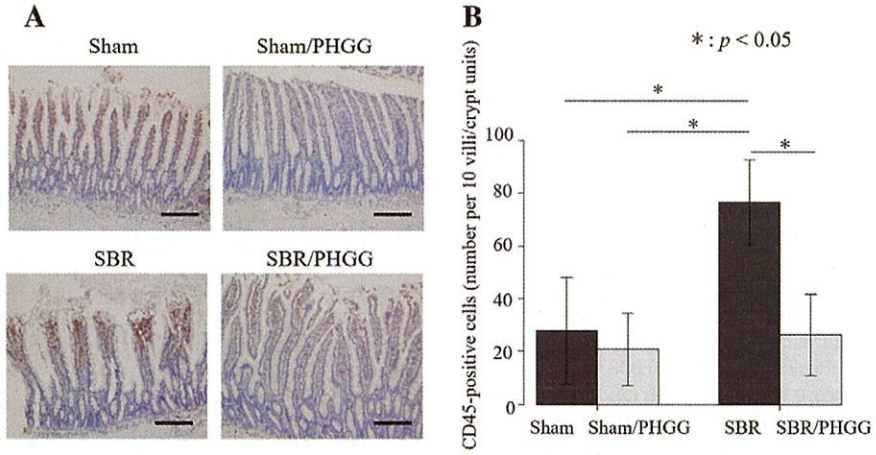

Fig. 3. Intestinal morphometric analysis using immunohistological images. (A) Representative immunohistochemical images using the anti-CD45 antibody at the anastomotic site in the Sham, Sham/PHGG, SBR, and SBR/PHGG groups. CD45-positive cells were primarily observed at the tip of the small intestinal epithelium and were most notably observed in the SBR group. Scale bars = 200 μm. (B) The number of CD45-positive cells was significantly higher in the SBR group than in the Sham and Sham/PHGG groups and that in the SBR group was significantly higher than that in the SBR/PHGG group.

An important result of this study is that the administration of PHGG significantly alleviated the degeneration of the small intestinal mucosa in the SBR group. This repair might be associated with the roles of SCFAs, because SCFAs have a healing effect on gastrointestinal mucosal damage [4,9]. SCFAs promote mucosal cell proliferation in addition to increased epithelial regeneration [9]. In particular, acetate increases colonic blood flow [9], presumably followed by acceleration of collagen maturation. Furthermore, butyrate enhanced histone H3 acetylation in the promoter and conserved noncoding sequence regions of the Foxp3 locus and regulated the differentiation of regulatory T cells with the ability to suppress inflammation [10]. In the present study, the administration of PHGG significantly inhibited the infiltration of leukocytes in the small intestinal mucosa. Although production of acetate and butyrate in the SBR/PHGG group with the ability to inhibit the infiltration of inflammatory cells tended to increase, there were no significant differences in production among animals in the four experimental groups. Accordingly, undetermined factors other than increased production of acetate and butyrate may inhibit the infiltration of inflammatory cells.

We examined the ileal microbiome to address the mechanism of the anti-inflammatory effect observed in the small intestinal mucosa. We found that the ileal microbiome in the SBR group was more variable than those in the Sham and SBR/PHGG groups. Furthermore, the ileal microbiome in the SBR/PHGG group was more similar to that in the Sham group rather than to that in the SBR group. These findings suggest that the alteration of microbiota in the SBR group was ameliorated

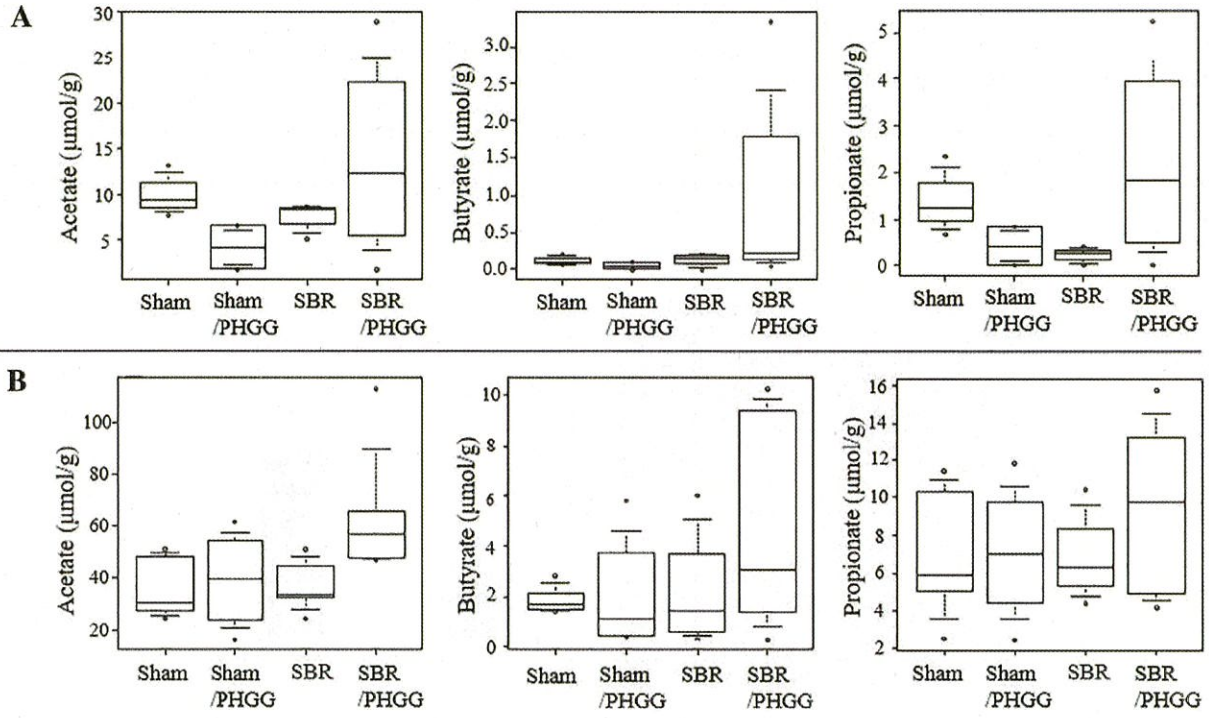

Fig. 4. Production of SCFAs. (A) SCFA levels in the ileum. Although there were no significant differences, production of SCFAs was higher in the SBR/PHGG group than in the SBR group. (B) SCFA levels in the colon. The production of all SCFAs was higher in the SBR/PHGG group than in the SBR group; however, there were no significant differences.

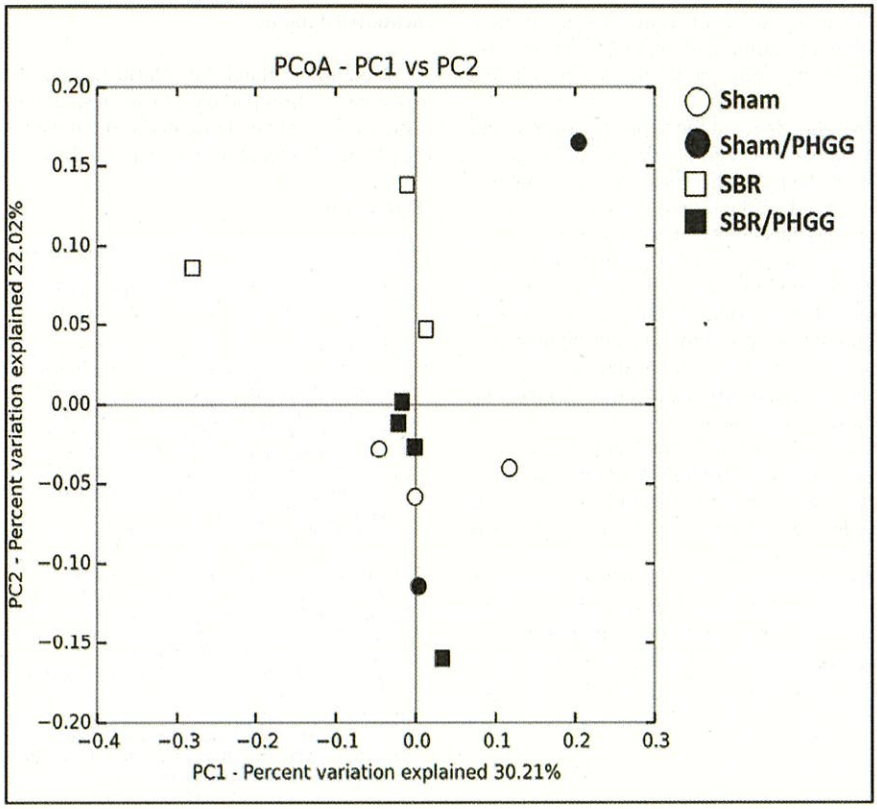

Fig. 5. PCoA plot describing weighted UniFrac distances between ileal contents. Pairwise distances between all samples are projected onto a two-dimensional space where the PCA axis describes the highest degree of variation. Samples that are clustered closely together are therefore considered to share a larger proportion of the phylogenetic tree than samples that have a larger separation.

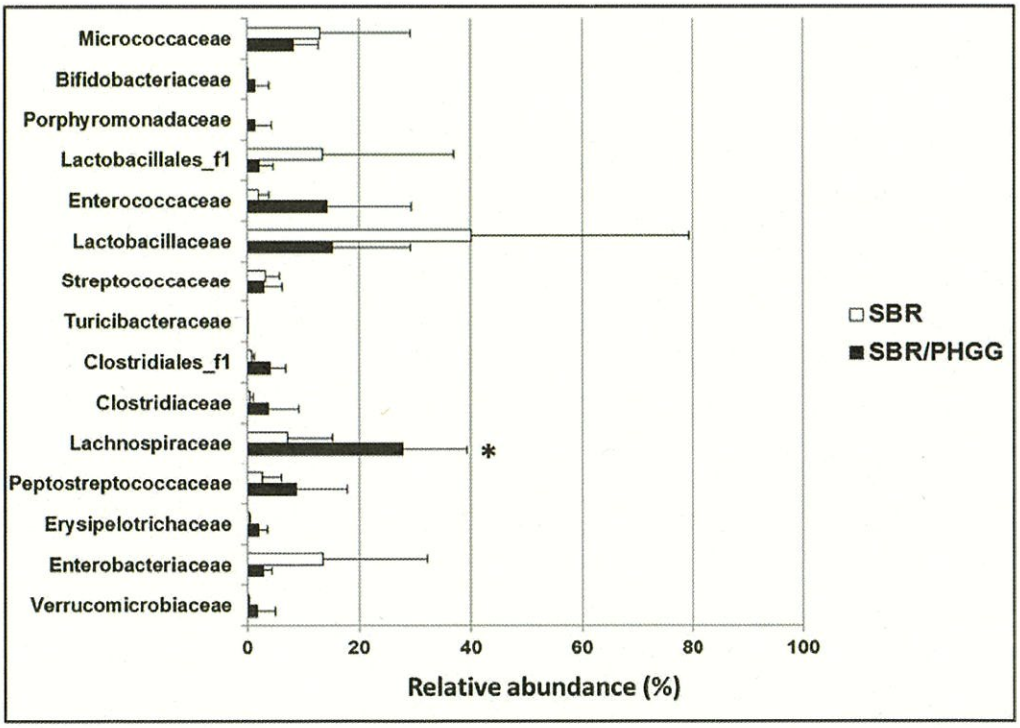

Fig. 6. Comparison of the relative abundance of a microbial family in ileal content between the SBR and SBR/PHGG groups. Bacterial families with relative abundances less than 5.0% in any of the samples were not shown. * $P < 0.05$, vs. SBR.

by the administration of PHGG. The effect of stimulating the growth of health-promoting microbiota by administering PHGG has been reported [3,4], and therefore, such effects should be expected in SBS patients as well.

We found that the relative abundance of the family *Lachnospiraceae* was significantly greater in the SBR/PHGG group than in the SBR group. A previous study showed that the proportion of *Lachnospiraceae* was lower in infants with SBS than in healthy infants [2]. *Lachnospiraceae* is a family of the order of *Clostridiales*. Members of this family are known to produce SCFAs [11], consistent with the increased SCFA production in the SBR/PHGG group in the ileum and colon. Tun et al. reported that the abundance of *Lachnospiraceae* was associated with obesity in infants [12]. Though body weight did not significantly increase after PHGG administration in this study (data not shown), the abundance of *Lachnospiraceae* can contribute to nutrient absorption by the administration of PHGG in SBS patients.

Our study has a limitation. The use of 8-week adult rats might have affected the microbial profile because intestinal microbiota are considerably affected by age. Therefore, further studies using a young SBR rat model may be needed to eliminate the effect of age on the microbial profile.

In conclusion, we found that the administration of PHGG in the SBR model alleviated damage to the small intestinal mucosa, which could be associated with modulation of the intestinal microbiota, especially *Lachnospiraceae*. These findings would be important for preventing complications of SBS patients. Further studies are required to elucidate the mechanism of healing.

Conflict of interest

The authors declare no conflicts of interest in association with this study.

Acknowledgment

The authors thank Ms. Machi Kawauchi for her expert technical assistance in histopathological analysis and Mr. Kazuya Tanaka for his expert technical assistance in the SBR model. This work was supported by JSPS KAKENHI Grant Number JP 16K21204, 16H06276, and 17K10180.

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