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Small Intestinal Goblet Cell Proliferation Induced by Ingestion of Soluble and Insoluble Dietary Fiber Is Characterized by An Increase in Sialylated Mucins in Rats[1-3]

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[8] Abbreviations used: BF, beet fiber; BrdU, 5'-bromo-deoxyuridine; CH, corn husk; GG, guar gum; HID/AB, high-iron diamine/alcian blue; KM, konjac mannan; PAS, periodic acid Schiff; PS, psyllium; PSF, polystyrene foam; WB, wheat bran.

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Abstract

The study aimed to examine the effects of insoluble and soluble fibers on mucin sialylation and sulfation in the small intestine. First, diets containing soluble (konjac mannan, psyllium or guar gum (50 g/kg)) or insoluble (polystyrene foam, wheat bran or cornhusk (80 g/kg)) fiber were fed to rats for 13 d. The fiber-fed groups had more goblet cells in the ileum than the fiber-free control. High-iron diamine/alcian blue staining showed more sialylated mucin-producing cells in the fiber-fed groups than in the control, while sulfated mucin-producing cells were fewer (insoluble fibers) or unchanged (soluble fibers). Second, feeding of konjac mannan (50 g/kg) and beet fiber (80 g/kg) diets for 7 d had a higher ileum *Siat4C* expression than the control, but *Gal3ST2* and *Gal3ST4* expression was comparable. Luminal mucin content correlated with sialic acid ($r = 0.96$, $P < 0.001$) or sulfate ($r = 0.62$, $P < 0.01$), but the slope of the sialic acid-derived equation was greater than that of the sulfate-derived equation, indicating preferred increase in sialylated mucins. Third, rats were fed the control diet for 10 d under antibiotic treatment. Analysis of the luminal mucin showed that sialylated mucins were more vulnerable to bacterial degradation than sulfated mucins. Finally, a study of bromo-deoxyuridine incorporation in rats fed a beet fiber diet indicated that goblet cell proliferation accompanied by increased sialylated mucin appeared to be related to accelerated ileal epithelial cell migration. We conclude that intestinal goblet cell responses to insoluble and soluble fibers are characterized by increases in sialylated mucin production.

Introduction

The absorptive surface of the intestine is covered by a layer of mucins that are synthesized and secreted by specialized goblet cells. Mucins are heavily glycosylated molecules that consist of threonine/serine rich-polypeptide backbones and *O*-linked oligosaccharide side chains (1). These mucus gels present a barrier that prevents potential pathogens and antigens from gaining access to the underlying epithelium and also serve as binding sites for immunoglobulins, particularly for secretory IgA (2). Mucin oligosaccharide chains are often terminated with sialic acid or sulfated sugar, which account for their polyanionic nature and visco-elastic properties (3). Because the presence of high levels of sulfate in a mucin decreases its susceptibility to bacterial glycosidases and limits the rate and extent of degradation, it has been proposed that reduced mucin sulfation might be closely correlated with the increase in bacterial translocation in murine models of gut disease (4) and the exacerbation of colitis in humans (5).

Consumption of dietary fiber appears to enhance the total capacity for mucin secretion in the small intestinal lumen, although the stimulatory effect on mucin secretion depends on the quantity, as well as the quality of dietary fiber ingested (6-9). Our previous studies showed that small intestinal mucins were secreted in proportion to the settling volume in water (a numerical representation of bulk-forming properties) of water-insoluble dietary fibers (7) or the viscosity of water-soluble dietary fibers (9). The stimulatory effects of both soluble and insoluble fibers on mucin secretion appear to be linked to epithelial cell turnover and the

51 subsequent increase in goblet cell number (9, 10). However, there appears
52 to have been little investigation of changes in the patterns of small
53 intestinal mucin sulfation and sialylation in response to different types of
54 dietary fiber.
55
56 Cassidy et al. (11) conducted histochemical analyses using high-iron
57 diamine/alcian blue (HID/AB) staining to differentiate HID^+ sulfomucin
58 and AB^+ sialomucin-containing goblet cells in the rat intestine. They
59 reported that, compared to a fiber-free control, feeding of both gel-forming
60 and bulking insoluble fibers, at a dietary level of 5-10% of the diet, tended
61 to produce an increase in the percentage of HID^+ goblet cells in the
62 terminal ileum. Piel et al. also suggested that a diet containing 10%
63 carboxymethylcellulose predominantly increased sulfomucin-containing
64 goblet cells in the ileum of pigs (12). However, in the course of our studies
65 of the mucin secretory effects of dietary fiber, we noticed that, compared
66 with rats fed a fiber-free semi-purified diet, those fed a non-purified diet
67 had an increased number of total goblet cells in the ileum as well as in the
68 jejunum. This increase could be accounted for by an increased number of
69 AB^+ goblet cells, accompanied by a corresponding decrease in the number
70 of HID^+ goblet cells. This non-purified diet contains a number of fiber
71 types and has a total dietary fiber value of more than 16%, which is
72 generally regarded as a high bulk diet (13, 14). These observations are just
73 the opposite of the previous findings that indicated that bulky fiber
74 ingestion increases the number of HID^+ goblet cells (11). This finding
75 prompted us to examine whether insoluble (bulky) and soluble (viscous)

fibers with a capacity for induction of goblet cell proliferation share common characteristics in terms of their influence on the pattern of sulfation and sialylation of the oligosaccharide chains of small intestinal mucins.

For this purpose, we fed several fibers with a bulky or viscous nature to rats, and then histochemical analyses by HID/AB staining, measurements of the sulfate and sialic acid content of in the small intestinal mucins, and gene expression analyses of the sialyltransferase *Siat4C* and the sulfotransferases *Gal3ST2* and *Gal3ST4* were conducted in the ileum tissue. We also examined differences in the susceptibility of sialylated and sulfated mucins to bacterial mucinase by measurements of the sialic acid and the sulfate content of small intestinal mucins in rats with or without antibiotic treatment. Finally, the effects of a non-purified diet on the epithelial cells in the ileum that were observed in our preliminary study were re-evaluated and compared with those of a semi-purified diet including a bulky fiber.

Methods

Materials. Konjac mannan (KM, a copolymer of glucose and mannose (1:1.6) joined through β-1,4-glucosidic linkages) was provided by Shimizu Chemical Co. (PROPOL A, 1000-2000 kDa; Hiroshima, Japan). Guar gum (GG, Sunfiber) and psyllium (PS) were provided by Taiyo Kagaku (Mie, Japan) and Bizen Chemicals (Okayama, Japan), respectively. Polystyrene foam (PSF), with an experimentally determined expansion ratio (7) of 54.9,

was provided by JSP Co., Ltd. (Tokyo, Japan). PSF was powdered using a Wiley mill with an adjusted mesh size of 30-100. Wheat bran (WB, 30-70 mesh), beet fiber (BF, 30-70 mesh) and corn husk (CH, 30-70 mesh) were gifts from Nisshin Seifun Group Inc. (Saitama, Japan), Nippon Beet Sugar Manufacturing Co., Ltd. (Obihiro, Japan) and Nihon Shokuhin Kako Co. Ltd. (Shizuoka, Japan), respectively. WB preparation was washed in boiling water to remove starch, and was then washed repeatedly with 99% ethanol and dried. Dietary fiber contents (dry matter basis), as determined by the Prosky method (15), were: KM (95%), GG (81.5%), PS (90%), WB (77%), BF (78%) and CH (89%), respectively. The viscosity of a 1.0% solution of each soluble dietary fiber, defined as the area under the viscosity curve described by Dikeman et al. (16), was 599 Pa (KM), 165 Pa (GG), and 67.6 Pa (PS), respectively (9). The settling volume in water (7) of each insoluble dietary fiber was 10 mL/g (PSF), 9.0 mL/g (BF), 9.0 mL/g (WB) and 5.0 mL/g (CH), respectively.

Care of animals. The study (No. 22-18) was approved by the Animal Use Committee of Shizuoka University, and animals were maintained in accordance with the guidelines of Shizuoka University for the care and use of laboratory animals. Male rats of the Wistar strain (purchased from Shizuoka Laboratory Animal Center, Hamamatsu, Japan) were housed in individual wire screen-bottomed, stainless steel cages in a temperature (23 ± 2 °C) and lighting (lights on from 08:00-20:00) controlled room. For adaptation, rats were fed a control diet for at least 5 d. This diet (7) was formulated from 250 g/kg of casein, 652.5 g/kg of cornstarch and 50 g/kg of corn oil. The remainder of the diet consisted of vitamins including

choline (12.5 g/kg) and minerals (35 g/kg). Subsequently, rats were allocated to groups on the basis of body weight to give a similar mean body weight and allowed free access to experimental diets and water. Each dietary fiber was added at the expense of an equal amount of cornstarch in the diet. Accordingly, dietary starch levels differed in diets and were 572.5 g/kg (insoluble fiber-added diet) or 602.5 g/kg (soluble fiber-added diet). Body weight and food intake were recorded every morning before replenishing the diet. In the present series of experiments, dietary inclusions of soluble and insoluble dietary fibers were set at 50 g/kg and 80 g/kg diet, based on the settling volume and the viscosity of the respective dietary fibers, to ensure a sufficient quantity of fiber for induction of goblet cell proliferation.

Expt. 1. Forty-two rats weighing 120-140 g (age, 6 weeks) were allocated to seven groups of 6 rats each and were allowed free access to the control diet or to a diet containing 50 g of KM, PS or GG/kg diet, or 80 g of PSF, WB or CH/kg diet for 13 d. Diets were withdrawn overnight and rats were then killed by decapitation and the small intestine was excised. Luminal contents were collected by flushing with 15 mL of ice-cold phosphate buffer saline (pH 7.4) containing 0.02 mol sodium azide/L and the same volume of air. The contents were freeze-dried and stored for luminal mucin analysis. For histological evaluation, the upper half of the small intestine except the duodenum was defined as the jejunum, and the lower half was defined as the ileum. The mid-portions of ileum segments (approx. 5 cm) and the terminal ileum (approx. 5 cm in length, cut at a distance of 2 cm

151 from the ileo-cecal valve) were removed, opened longitudinally, placed in
152 10% buffered formalin and used for tissue examination.
153 ***Expt. 2.*** Thirty-six rats weighing 127-147 g (age, 6 weeks) were allocated
154 to three groups of 12 rats each and were fed the control diet or a diet
155 containing 50 g of KM or 80 g of BF/kg diet for 7 d. Then, each dietary
156 group was further divided into two equal groups. One group was killed by
157 decapitation without prior food deprivation. A part of the ileal segment
158 (approximately 5 cm) was opened longitudinally, and the mucosa was
159 scrapped with a glass slide and used for total RNA isolation. The other
160 group of rats was fasted overnight and killed by decapitation. Luminal
161 mucin sampling procedures and intestinal tissue collection were as
162 described for *Expt. 1*.
163 ***Expt. 3.*** Twelve rats weighing 130-156 g (age, 6 weeks) were allocated to
164 two groups of 6 rats each and were allowed free access to the control diet
165 with or without antibiotics (benzyl penicillin potassium, 50kU/L; neomycin
166 sulfate, 2000 mg/L; cefoperazone sodium, 500 mg/L; WAKO Chemicals,
167 Tokyo, Japan) in the drinking water for 10 d. The rats were then killed by
168 decapitation, and the small intestinal contents and the cecal contents were
169 gathered. The small intestinal mucin sampling procedure was as described
170 for *Expt. 1*. The cecal contents were used for the measurement of organic
171 acids (17) and for bacterial culture.
172 ***Expt. 4.*** To re-evaluate our preliminary study, twelve rats weighing
173 133-158 g (age, 6 weeks) were allocated to three groups of 4 rats each and
174 were allowed free access to the control diet, to a diet containing 80 g of
175 BF/kg diet, or to a non-purified diet (MF-2, Oriental Yeast, Tokyo, Japan) for

10 d. For examination of epithelial cell migration, 5′-bromo-deoxyuridine (BrdU) (50 mg/kg bw) was injected intraperitoneally into the rats on d 9 (10:00–11:00). At 24 h after administration, without feed-deprivation, the rats were killed by decapitation, and the mid-portions (approx. 5 cm) of the ileum were removed and treated as described for *Expt. 1*.

Mucin analysis. The mucin fraction was isolated by the method of Lien et al. (18), with some modification, as described previously (7) and was dissolved in 5.0 mL of distilled water for analyses. After an appropriate dilution of the mucin fraction, *O*-linked oligosaccharide chains were measured as described previously (7). Standard solutions of *N*-acetylgalactosamine (Sigma-Aldrich, St. Louis, MO, USA) were used to calculate the amount of oligosaccharide chains liberated from mucins during the procedure.

Sialic acid determination. Part of the mucin fraction (0.1 mL) was hydrolyzed with 50 mmol sulfuric acid/L for 60 min at 100 °C, and sialic acid was determined by a previously described method (19). N-acetylneuraminic acid was used as a standard.

Sulfate determination. An appropriate volume of the mucin fraction (approximately 20 μg protein) was completely dried, re-suspended in 200 μL of 4 mol/L HCl, and hydrolyzed at 100 °C in a heating block for precisely 4 h. Determination of sulfate in the mucin fraction was performed basically using the method of Harrison and Packer (20). Solutions of 0.79, 1.59, 3.18, 6.38 and 12.8 mmol sulfate/L (Multi-anion standard solution-1, Wako Pure Chemicals, Tokyo, Japan) were used as standards.

Histochemical analyses. Six 5-µm-thick cross-sections were prepared from paraffin-embedded samples of each tissue for each staining. Five complete villi (entire crypt/villus axis) per section were selected, and villus length and the numbers of epithelial cells and goblet cells per villus (left side) were determined. Two observers (blind to treatments) independently analyzed each section by light microscopy using an Olympus BH2 instrument fitted with a micrometer eyepiece. Goblet cells were stained with periodic acid Schiff (PAS) and counter-stained with hematoxylin. HID/AB staining was performed using the method of Spicer (21) with a slight modification. Briefly, de-paraffinized and rehydrated sections were immersed in HID solution (240 mg of N, N-dimethyl-*m*-phenylenediamine dihydrochloride (Sigma-Aldrich, St. Louis, MO, USA), 40 mg of N, N-dimethyl-*p*-phenylenediamine hydrochloride (Wako Pure Chemicals, Osaka, Japan) and 4.2 mL of 40% ferric chloride (Wako Pure Chemicals) in 100 mL of distilled water for 21 h. After washing with running tap water for 5 min, the sections were immersed in 1% alcian blue in 3% acetic acid (pH 2.5) for 1 h. The sections were then rinsed with distilled water, dehydrated with increasing concentration of ethanol, cleared with xylene and mounted with mounting media (MP500, Matsunami Glass, Osaka, Japan). This HID/AB technique stains sulfomucin black/brown and stains sialomucin blue. Both types of reaction, i.e., HID^+ and AB^+ (2-4 counts/villus, left side) were observed in a small population of goblet cells. We ascribed these cells to one type of reaction, depending on the predominant tone.

RNA isolation and quantitative real-time PCR. Total RNA was isolated

using the Takara RNAiso reagent (Takara Bio, Tokyo, Japan) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed using the Takara Prime Script RT reagent (Takara Bio) at 37 °C for 15 min. The synthesized cDNA was amplified by PCR using a LightCycler System (Roche Applied Science, Tokyo, Japan). The primer pairs and protocols for PCR of *Muc2*, *Muc3* (22), *Siat4c*, *Gal3ST4* (23) and 18S rRNA (24) have been reported. 18S rRNA was used as an endogenous reference gene. PCR reactions were carried out in a total volume of 20 μL containing 400 nmol/L each of gene-specific primers, cDNA, and SYBRPremix Extaq II (Takara Bio). To confirm amplification specificity, the PCR products from each primer pair were subjected to a melting curve analysis and subsequent agarose gel electrophoresis. Gene expression was quantified using the comparative C_T method (25), and the data were expressed relative to the control group. In the present study, C_T (threshold of cycles) values of the 18S rRNA gene among the dietary treatments were 9.1 ± 0.1 (control), 9.2 ± 0.1 (BF) and 9.2 ± 0.1 (KM), and there were no differences between the groups.

Bacterial culture. After the rats were killed, the cecal contents were immediately removed, weighed and then placed in grinding tubes containing anaerobic phosphate buffer. The cecal contents were homogenized under oxygen-free carbon dioxide gas (26). Bacteriological procedures and media were essentially the same as the method described previously (26, 27).

BrdU staining. Six 5-μm-thick cross-sections of intestinal tissue per animal were collected on aminopropyltriethoxysilane-coated slides. After

de-paraffinization and re-hydration, the sections were immersed in preheated 10 mmol/L citrate buffer (pH 6.0) and heated at 100 °C for 20 min for antigen retrieval. BrdU staining was then performed as described previously (9). BrdU-positive cells were subsequently counted in the same manner as for goblet cell staining.

Statistical analyses. Data were analyzed by one-way ANOVA and significant differences among means were identified by the Tukey-Kramer test. The results were expressed as means ± SEM and a 5% level of probability was considered a significant difference for all analyses. When variances were not homogenous by the Bartlett test, data were logarithmically transformed. When variances were not homogenous even after logarithmic transformation, the data were presented as medians with range and were then analyzed by Kruskal-Wallis ANOVA followed by Kolmogotov-Smirnov two-sample tests. For *Expts. 3* and *4*, differences were analyzed by Student's *t*-test. Regression analysis was used to examine the relationship between *O*-linked oligosaccharide chains (as mucin) and sialic acid or sulfated sugar in the mucin fractions. If a significant correlation was observed, the sialic acid or sulfate content as a response variable was predicted from the contents of *O*-linked oligosaccharide chains as a function of regressor variables by each regression line. When the intercept was not zero, the mean slopes were compared by analysis of covariance with *O*-linked oligosaccharide chains as a covariate. All calculations were done using the JMP8 software (SAS Institute).

Results

Expt. 1. Rats fed KM and GG diets had lower food intake than those fed the control, PSF, WB and CH diets (**Table 1**). Food intakes in the viscous fiber-fed groups were also lower than in the bulky fiber-fed groups. Body weight gain in rats fed the GG diet was lower than in those fed the control and the other fiber- added diets, except the KM diet. The total amount of *O*-linked oligosaccharide chains (measured as mucin) in the small intestinal contents was significantly greater in KM, PS, PSF, and WB groups than in the control. The difference between KM and GG groups was also significant. However, neither the sialic acid nor sulfate content of the mucin fraction differed significantly among the dietary groups. Linear regression analyses showed a significant correlation between the mucin content and sialic acid or sulfate content, but the slope of the sialic acid-derived equation was significantly greater than that of the sulfate-derived equation indicating that fiber ingestion predominantly increased sialylated mucins (**Fig. 1, A, B**). In the mid ileum, villus heights in rats fed viscous fiber diets were significantly greater than in those fed bulky fiber diets. The difference between the control and PS groups was also significant. All of the fiber-fed groups had a higher number of PAS^+ goblet cells than the control group with the PS and PSF groups showing significantly more cells than in the GG and CH groups. These increases were accounted for by an increase in the number of AB^+ goblet cells in the epithelial cells. On the other hand, only the PSF, WB, CH and GG groups had fewer HID^+ goblet cells than the control group (**Table 1**, **Supplemental Fig. 1**). In the terminal ileum, villus heights in rats fed the PS diet were significantly greater than in those fed the control, PSF, WB and CH

diets. The fiber-fed groups, except CH group, had a higher number of PAS^+ goblet cells than the control group. The PAS^+ goblet cells in the PS and PSF groups were also higher than in the CH group. The AB^+ and HID^+ goblet cells in the GG, PSF and WB groups were higher and lower than in the control group (**Table 1** and **Supplemental Fig. 2**).

Expt. 2. In the 7 d-feeding study of KM and BF, the KM group had lower food intake and body weight gain than the control and the BF groups (**Table 2**). The amount of *O*-linked oligosaccharide chains (mucins) in the small intestinal contents was greater in the KM and BF groups than in the control group. In the mucin fraction, the contents of sulfate and sialic acid were greater in the BF group than in the control group. There was a significant correlation between the mucin content and the sialic acid or sulfate content, but the slope of the sialic acid-derived equation was significantly greater than that of the sulfate-derived equation, indicating that fiber ingestion predominantly increased sialylated mucins (**Fig. 1, C, D**). In the ileum tissue, the number of PAS^+ goblet cells was significantly increased in the KM and BF groups compared to the control group. These increases were accounted for by an increase in the number of AB^+ goblet cells, while a lower number of HID^+ goblet cells were observed in the BF group compared with the control group (**Table 2** and **Supplemental Fig. 3**). *Muc2* gene expression was slightly but significantly greater in the BF group compared to the other groups. *Siat4C* expression was 6 to 10 times higher in the KM and BF groups than in the control, whereas the gene expression of *Gal3ST2* and *Gal3ST4* did not differ among the groups (**Table 2**).

326

327 ***Expt. 3.*** Food intake for 10 d was significantly lower in rats with
328 antibiotic treatment (117 ± 3 g) than in those without antibiotic treatment
329 (144 ± 4 g), but the body weight gain did not differ between rats with (55 ±
330 2 g) or without antibiotic treatment (52 ± 2 g) due to a huge increase in the
331 weight of cecal contents in rats treated with antibiotics (15.9 ± 0.9 g vs.
332 control as 1.9 ± 0.1 g). The concentration of cecal organic acids (the sum of
333 acetate, propionate, butyrate, lactate and succinate) was 58.7 ± 2.3 μmol/g
334 content in rats without antibiotic treatment, but only negligible amounts of
335 total organic acids were detected in rats with antibiotic treatment (< 0.3
336 μmol/g). The total number of anaerobes, lactobacillus and clostridia in the
337 cecal contents was significantly reduced in rats with antibiotic treatment
338 (4.0 ± 0.5, 0.4 ± 0.3, and 2.7 ± 0.9 log of cfu/cecum respectively) compared
339 with the number in rats without antibiotic treatment (13.5 ± 0.4, 12.7 ± 1.6,
340 and 11.2 ± 0.2 log of cfu/cecum respectively). The amount of *O*-linked
341 oligosaccharide chains and sialic acid in the small intestinal contents
342 increased in rats with antibiotic treatment by 500% compared with the
343 amount in rats without antibiotic treatment, while the amount of sulfate
344 increased by 200% with antibiotic treatment (**Fig. 2**).

345

346 ***Expt. 4.*** Food intake and body weight gain did not differ between rats fed
347 the control and BF diets. The villus height and the number of epithelial
348 cells in the ileum tissue also did not differ between the two groups (**Table**
349 **3**). The number of PAS^+ goblet cells in the ileum was higher in rats fed the
350 BF diet than in those fed the control diet (**Table 3 and Supplemental Fig.**

4). The number of AB^+ and HID^+ goblet cells was higher and lower respectively in rats fed the BF diet than in rats fed the control diet. The position of the uppermost BrdU-labeled cell from the bottom of the villus was significantly higher in rats fed the BF diet than those fed the control diet. In terms of goblet cell variability and BrdU-labeled cells, the non-purified diet gave similar results to the BF diet (**Table 3 and Supplemental Fig. 4**).

Discussion

In accordance with our previous studies (7-9), an increase in small intestinal mucin content was consistently observed after bulky or viscous fiber ingestion (*Expt. 1*). The amount of small intestinal mucins in these fiber-fed groups increased in proportion to the number of PAS^+ goblet cells in the mid-ileum ($r = 0.80$, $P < 0.05$) as well as in the terminal ileum ($r = 0.72$, $P = 0.07$). Under the conditions of our HID/AB staining assay of the mid-ileum, it appeared that bulky fiber ingestion stimulated an increase in the number of AB^+ goblet cells with a concomitant decrease in the number of HID^+ goblet cells, while viscous fiber ingestion resulted in an increased number of AB^+ goblet cells with a constant number of HID^+ goblet cells. These results remained essentially similar in the terminal ileum, except that there were no differences in the total (PAS^+) goblet cell numbers in rats on the CH diet compared with the control. These findings are totally different from those of Cassidy et al. (11), who reported that chronic ingestion (4 weeks) of viscous and bulky fibers by rats led to an alteration in the

intestinal goblet cell population from predominantly AB^+ to predominantly HID^+ goblet cells. The reason for this discrepancy is unclear, but could be explained by the differences in the duration of the feeding studies. Thus there may be a chronic effect of dietary fiber on changes in the pattern of sulfation and sialylation of intestinal mucins. Another possible explanation may be that HID/AB stained goblet cells were categorized differently in the two studies. When this technique is used, a small population of goblet cells demonstrates both types of reaction. We ascribed theses cells to one type of reaction, depending on which tone was predominant, whereas Cassidy et al. designated these cells as "mixed" (11).

Mucins have been categorized as neutral mucins, sialomucins or sulfomucins on the basis of the density and types of acidic groups present in their oligosaccharide side chains. However, as indicated by Robertson and Wright (28), the intensity of HID/AB staining does not necessary correlate with actual biochemical measurements of mucin sulfate levels and, furthermore, the quantity of sulfate needed to qualify a mucin for categorization as a sulfomucin is unclear. In the present study, therefore, we biochemically measured both sialic acid and sulfate content in the small intestinal mucin fractions in rats fed a bulky or a viscous fiber diet. There was a significant correlation between the mucin content and the sialic acid or the sulfate content. However, the slope of the sialic acid-derived equation was significantly greater than that of the sulfate-derived equation (*Expts. 1* and *2*). These results indicate that fiber ingestion increases mucin sialylated oligosaccharides rather than sulfated oligosaccharides.

Studies on the transcription levels of the sialyltransferase *Siat4C* and the sulfotransferases *Gal3ST2* and *Gal3ST4* genes provided further support for the predominant increase in sialylated mucins following fiber ingestion. Both bulky WB and viscous KM ingestion for 7 d strongly up-regulated the gene expression of *Siat4C* (by 6-10 fold) compared with the fiber-free control, while the expression levels of *Gal3ST2* and *Gal3ST4*, which are the major mucin sulfotransferases in the intestine (23, 29), were comparable among the dietary groups (*Expt. 2*). Thus, the results of gene expression analyses are in accordance with those of the histochemical analyses of the ileum. On the other hand, the relative amounts of sialic acid and sulfate in the small intestinal mucin fractions do not necessary reflect expression of the related genes or the ileum histochemistry (*Expts. 1* and *2*). This result may be partly due to the differences in susceptibility of sialomucin and sulfomucin in the small intestinal fluid to bacterial degradation.

Enteric bacteria possess both sialidases and glycosulfatases that are essential for mucin degradation (30), but the optimum pH of the glycosulfatases (pH 5.0) is much lower than that of the sialidases (pH 7.8) (31). Therefore, in small intestinal fluid of neutral pH, bacterial degradation of sulfomucin might be considerably less than that of sialomucin. Indeed, in the presence of antibiotics, not only the amount of *O*-linked oligosaccharide chains but also the amount of both sialic acid and sulfate in the small intestinal contents was significantly greater than that in the absence of antibiotics. However, the increased in sialic acid (500%) was much greater than that in sulfate (200%)

426 in antibiotic treated rats (**Fig. 2**). These findings suggest that there might have
427 been a greater underestimation of the sialic acid contents measured in *Expt. 1*
428 and *2* than of the sulfate contents due to differences in their susceptibility to
429 bacterial degradation. This possibility may partly explain the disparity
430 between the sialic acid and sulfate content of luminal mucins and the results
431 of gene expression or ileum histochemical analyses. Accordingly, at least as
432 far as the findings of histochemical analyses, biochemical measurements and
433 gene expression are concerned, it is plausible to conclude that the ingestion
434 of bulky and viscous fibers predominantly increases sialylated mucins
435 rather than sulfated mucins in the rat small intestine.
436
437 Decreased mucin sulfation has been proposed to lead to enhanced mucin
438 degradation and penetration of the secreted mucus barrier by microbes,
439 thereby giving increased access to the epithelial cell surface (32). Dawson et
440 al. reported that *NaS1* sulfate transporter null (*Nas1*$^{-/-}$) mice, which display
441 increased urinary sulfate excretion and hyposulfatemia (33), show reduced
442 intestinal sulfomucin content, enhanced susceptibility to toxin-induced colitis,
443 and an impaired intestinal barrier to bacterial translocation (4). In this regard,
444 the predominant increase in sialylated mucin by dietary fiber ingestion might
445 be considered less beneficial for mucosal physiology. However, it has been
446 repeatedly observed in animal experiments that the incidence of bacterial
447 translocation is low in rats fed a high fiber diet or in rats fed a non-purified
448 diet compared with those fed a highly defined elemental diet or those treated
449 with total parenteral nutrition (34-36). As we also observed in rats fed a
450 fiber-added diet or a non-purified diet (**Table 3 and Supplemental Fig. 4**), a

marked increase in the total number of goblet cells is linked to the accelerated epithelial cell migration (23, 37, 38). Possibly, an increased capacity for mucin secretion accompanied by accelerated epithelial cell turnover may effectively function as an intestinal barrier, irrespective of sialylation/sulfation of mucins, at least under normal conditions. However, further studies are needed to clarify the physiological relevance of such an alteration in the pattern of sulfation and sialylation of small intestinal mucins.

At present, the mechanism by which bulky and viscous fiber ingestion increases sialylation of mucins in the small intestine remains unclear. However, Specian and Oliver showed that immature goblet cells in the small intestine produce neutral mucins that contain less sialic acid, but, as they mature and migrate to the villus tip the mucins become increasingly sialylated (39). This observation may suggest that accelerated epithelial turnover may be linked with an increase in sialylated mucin production. Besides dietary fiber, administration of medium-chain triglycerides (38) or silver nanoparticles (40) also increases the level of sialylated mucin and is accompanied by goblet cell proliferation and accelerated epithelial turnover.

In conclusion, goblet cell responses to the ingestion of insoluble (bulky) and soluble (viscous) fibers are characterized by a predominant increase in sialylated mucin of the rat small intestine.

Acknowledgments

T.M. designed the research and wrote the manuscript; S.H., N.T., A.M., and H.K. conducted the research, and K.S. and S.A. analyzed the data. All authors read and approved the final manuscript.

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Figure legends

Fig. 1 Correlations between the amount of O-linked oligosaccharide chains and the amount of sialic acid or of sulfate in rats fed the control diet, or a diet containing 50 g of KM, PS or GG/kg diet, or a diet containing 80 g of PSF, WB or CH/kg diet for 13 d (*Expts. 1*: panels A, B), or in rats fed the control diet, or a diet containing 50 g of KM/kg diet or 80 g of BF/kg diet for 7 d (*Expt*. 2; panels C, D).

Fig. 2 The amount of O-linked oligosaccharide chains, sulfate and sialic acid in the mucin fraction of the small intestinal contents of rats fed the control diet with or without antibiotics for 10 d (*Expt. 3*).
Each column and bar indicates the mean ± SE (n = 6).
*$P < 0.05$ compared with the corresponding value for rats with no antibiotic treatment by Student's *t* test.

Table 1 Food intake, body weight gain, amount of *O*-linked oligosaccharide chains, sulfate and sialic acid in the small intestinal mucin fraction, and histological variables in the small intestinal tissue in rats fed the control diet, a diet containing 50 g of KM, PS or GG/kg diet, or a diet containing 80 g of PSF, WB or CH/kg diet for 13 d (***Expt. 1***)[1]

Groups	Control	5% KM[2]	5% PS[3]	5% GG[4]	8% PSF[5]	8% WB[6]	8% CH[7]
Food intake, *g/13 d*	179 ± 5^{ab}	150 ± 4^{cd}	171 ± 5^{bc}	140 ± 3^{d}	210 ± 7^{a}	208 ± 6^{a}	197 ± 5^{a}
Body weight gain, *g/13 d*	66 ± 3^{b}	59 ± 2^{bc}	68 ± 4^{ab}	49 ± 3^{c}	69 ± 2^{ab}	79 ± 4^{c}	70 ± 1^{ab}
Intestinal contents							
O-linked oligosaccharide chains, *μmol/intestine*	0.9 ± 0.9^{c}	2.3 ± 0.3^{a}	1.9 ± 0.2^{ab}	1.1 ± 0.1^{bc}	1.8 ± 0.1^{ab}	2.0 ± 0.2^{ab}	1.6 ± 0.2^{abc}
Sulfate, *μmol/intestine*	0.59 ± 0.12	0.91 ± 0.09	0.88 ± 0.08	0.70 ± 0.03	0.81 ± 0.05	1.03 ± 0.12	0.75 ± 0.14
Sialic acid, *μmol/intestine*	0.51 ± 0.07	0.97 ± 0.15	0.90 ± 0.17	0.61 ± 0.13	0.89 ± 0.12	0.88 ± 0.08	0.75 ± 0.10
Mid-ileum							
Villus height, *μm*	321 ± 6^{bcd}	352 ± 8^{ab}	354 ± 8^{a}	341 ± 9^{abc}	303 ± 5^{d}	312 ± 7^{cd}	307 ± 6^{d}
PAS^{+} cells, *n/villus*	10.4 ± 0.4^{c}	15.0 ± 0.4^{ab}	16.2 ± 0.3^{a}	13.6 ± 0.5^{b}	15.7 ± 0.6^{a}	14.7 ± 0.5^{ab}	13.3 ± 0.5^{b}
HID^{+} cells, *n/villus*	6.6 ± 0.3^{a}	6.4 ± 0.4^{ab}	6.6 ± 0.2^{a}	4.9 ± 0.5^{bc}	3.7 ± 0.3^{cd}	3.3 ± 0.4^{d}	4.4 ± 0.3^{cd}
AB^{+} cells, *n/villus*	2.4 ± 0.2^{c}	6.7 ± 0.4^{b}	7.6 ± 0.2^{b}	8.0 ± 0.7^{b}	10.5 ± 0.5^{a}	10.6 ± 0.5^{a}	6.1 ± 0.2^{b}
Terminal ileum							
Villus height, *μm*	227 ± 7^{bc}	254 ± 8^{ab}	264 ± 4^{a}	246 ± 5^{abc}	226 ± 12^{bc}	219 ± 10^{c}	220 ± 5^{c}
PAS^{+} cells, *n/villus*	9.9 ± 0.3^{c}	13.0 ± 0.2^{ab}	13.9 ± 0.7^{a}	12.4 ± 0.3^{ab}	14.0 ± 0.9^{a}	12.8 ± 0.4^{ab}	11.4 ± 0.3^{bc}
HID^{+} cells, *n/villus*	8.2 ± 0.7^{ab}	6.7 ± 0.5^{bc}	8.6 ± 0.4^{ab}	5.6 ± 0.2^{cd}	4.3 ± 0.6^{d}	5.1 ± 0.2^{cd}	9.7 ± 0.7^{a}
AB^{+} cells, *n/villus*	3.4 ± 0.9^{cd}	6.2 ± 0.1^{bc}	5.2 ± 0.4^{bcd}	7.0 ± 0.4^{ab}	9.2 ± 1.2^{a}	7.6 ± 0.3^{ab}	3.1 ± 0.2^{d}

[1] Data are mean ± SE, $n = 6$. Means in a row with superscripts without a common letter differ ($P < 0.05$).

[2] Diet containing 50 g of konjac mannan/kg diet.

[3] Diet containing 50 g of psyllium/kg diet.

[4] Diet containing 50 g of guar gum/kg diet.

[5] Diet containing 80 g of polystyrene foam/kg diet.

[6] Diet containing 80 g of wheat bran/kg diet.

[7] Diet containing 80 g of corn husk/kg diet.

Table 2 Food intake, body weight gain and amount of *O*-linked oligosaccharide chains, sulfate and sialic acid in the small intestinal mucin fraction, histological variables and mucin-related gene expression in the ileum tissue of rats fed the control diet or a diet containing 50 g of KM/kg diet or 80 g of BF/kg diet for 7 d (***Expt. 2***) [1]

Groups	Control	5% KM[2]	8% BF[3]
Food intake, *g/7 d*	102 ± 2[a]	83 ± 1[b]	95 ± 2[a]
Body weight gain, *g/7 d*	39 ± 1[a]	32 ± 1[b]	36 ± 1[a]
Intestinal contents			
O-linked oligosaccharide chains, *μmol/intestine*	1.1 ± 0.2[b]	1.9 ± 0.2[a]	2.1 ± 0.3[a]
Sulfate, *μmol/intestine*	0.73 ± 0.06[b]	0.94 ± 0.08[ab]	1.05 ± 0.06[a]
Sialic acid, *μmol/intestine*	0.56 ± 0.09[b]	1.01 ± 0.13[ab]	1.32 ± 0.20[a]
Ileum tissue			
Villus height, *μm*	335 ± 4[b]	365 ± 4[a]	325 ± 4[b]
PAS^+ cells, *n/villus*	11.4 ± 0.2[b]	15.6 ± 0.3[a]	15.1 ± 0.3[a]
HID^+ cells, *n/villus* [4]	6.2 (5.8 – 7.0)	6.6 (3.7 – 8.6)	1.1 (0.5 – 1.8)*†
AB^+ cells, *n/villus* [4]	3.3 (2.6 – 3.5)	7.1 (4.8 – 9.8)*	11.4 (10.7 – 12.7)*†
Gene expression			
Muc2	1.0 ± 0.1[b]	1.7 ± 0.1[a]	1.3 ± 0.1[ab]
Siat4c	1.0 ± 0.1[b]	10.3 ± 2.2[a]	7.5 ± 3.7[a]
Gal3ST2	1.0 ± 0.6	0.7 ± 0.5	0.2 ± 0.0
Gal3ST4	1.0 ± 0.1	0.8 ± 0.0	0.8 ± 0.1

[1] Data are mean ± SE or median (range), *n* = 6 or 12 (food intake, body weight gain). Means in a row with superscripts without a

common letter differ ($P < 0.05$).

[2] Diet containing 50 g of konjac mannan/kg diet.

[3] Diet containing 80 g of beet fiber/kg diet.

[4] The effects of dietary treatment were examined by Kruskal-Wallis one-way ANOVA, followed by Kolmogotov-Smirnov two-sample tests. *$P < 0.05$ vs. control.Different from control, $P < 0.05$. †Different from 5% KM, $P < 0.05$.

Table 3 Food intake, body weight gain, histological variables and incorporation of BrdU into epithelial cells in the ileum tissues of rats fed the control diet, a diet containing 80 g of BF/kg diet, or a non-purified diet (as a reference) for 10 d (***Expt. 4***) [1]

Groups	Control	8% BF[2]	Non-purified diet
Food intake, *g/10 d*	150 ± 12	134 ± 3	168 ± 8
Body weight gain, *g/10 d*	47 ± 6	44 ± 3	44 ± 5
Ileum tissue			
Villus height, *μm*	364 (303 – 392)	337 (324 – 339)	335 (330 – 340)
Total epithelial cells, *n/villus*	79 ± 3	71 ± 5	68 ± 1
PAS^+ cells, *n/villus*	10.2 ± 0.2	13.6 ± 0.3*	15.3 ± 0.4
HID^+ cells, *n/villus*	5.3 ± 0.3	1.3 ± 0.2*	0.5 ± 0.1
AB^+ cells, *n/villus*	2.8 ± 0.3	10.2 ± 0.2*	12.9 ± 0.8
Position of upper-most BrdU-labeled cell [3]	23.2 ± 1.3	32.9 ± 3.0*	42.7 ± 2.5

[1] Data are mean ± SE or median (range), $n = 4$. * Different from control ($P < 0.05$) when analyzed by Student's *t*-test.

[2] Diet containing 80 g of beet fiber/kg diet.

[3] Values indicate the highest position of BrdU-labeled cells from the bottom of the villus at 24 h after BrdU injection.

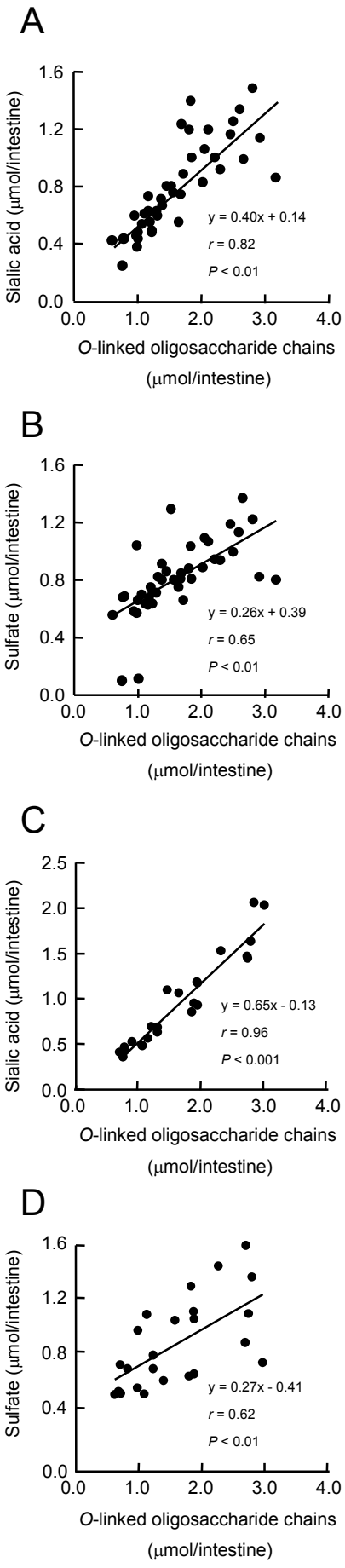

A
Sialic acid (μmol/intestine)
y = 0.40x + 0.14
r = 0.82
P < 0.01
O-linked oligosaccharide chains
(μmol/intestine)
B
Sulfate (μmol/intestine)
y = 0.26x + 0.39
r = 0.65
P < 0.01
O-linked oligosaccharide chains
(μmol/intestine)
C
Sialic acid (μmol/intestine)
y = 0.65x - 0.13
r = 0.96
P < 0.001
O-linked oligosaccharide chains
(μmol/intestine)
D
Sulfate (μmol/intestine)
y = 0.27x - 0.41
r = 0.62
P < 0.01
O-linked oligosaccharide chains
(μmol/intestine)

Fig.1

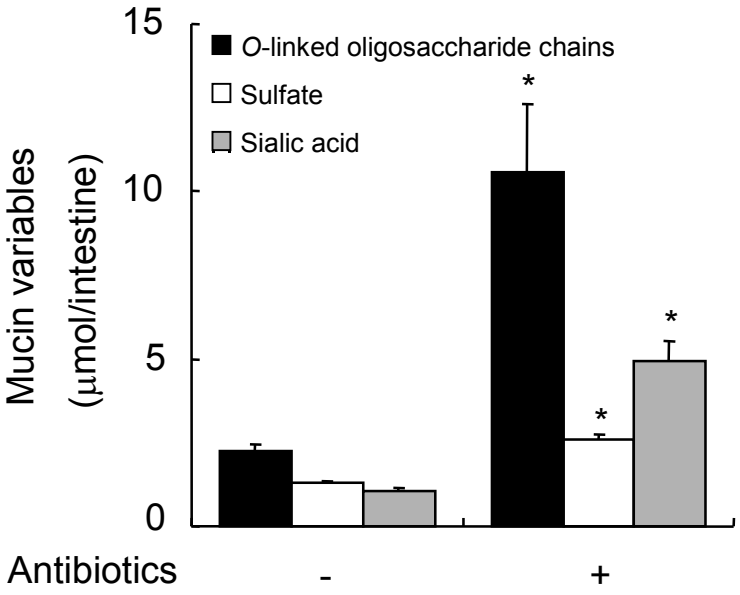
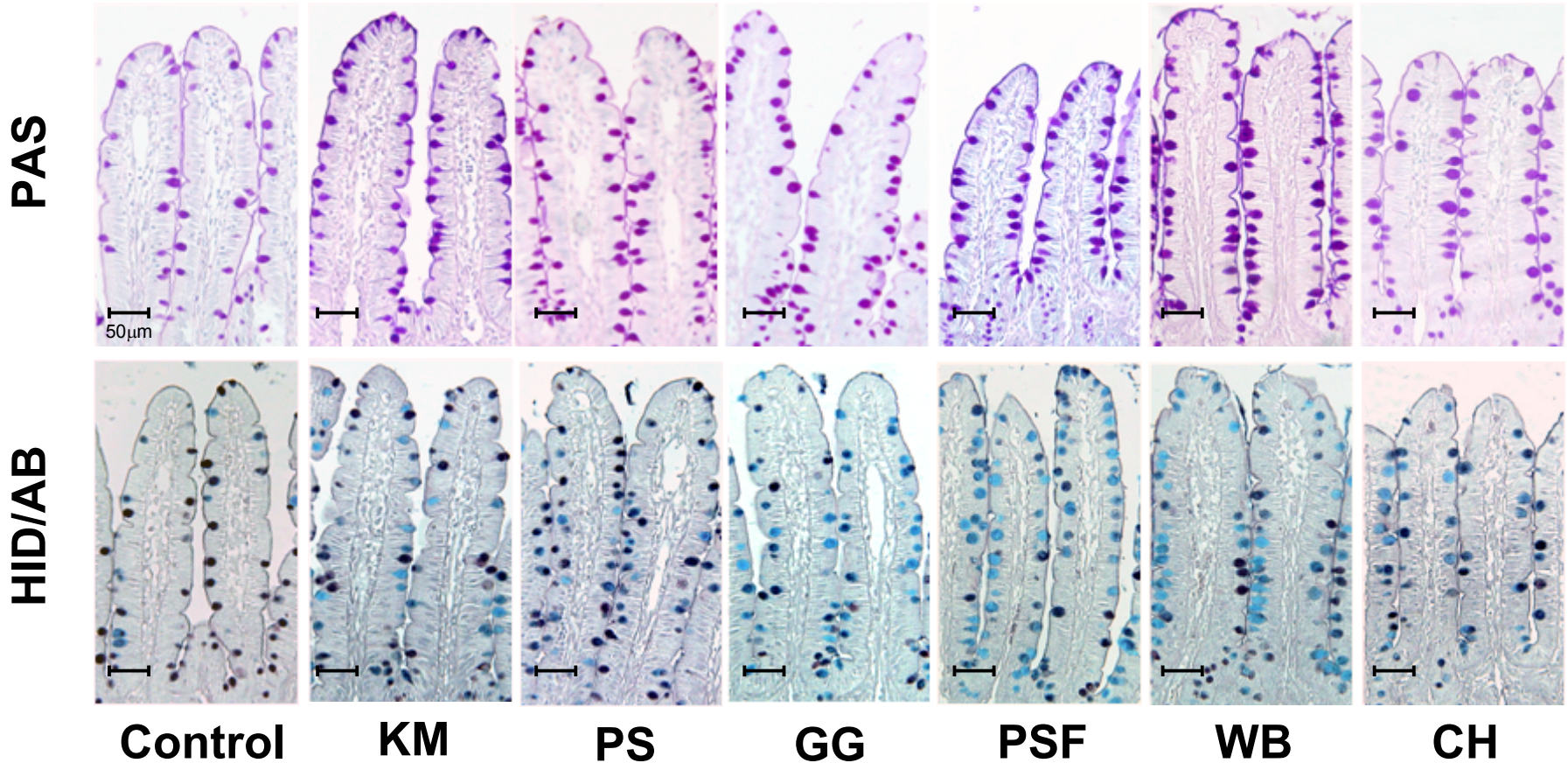

15
10
5
0
Mucin variables
(μmol/intestine)
O-linked oligosaccharide chains
Sulfate
Sialic acid
*
*
*
Antibiotics
-
+

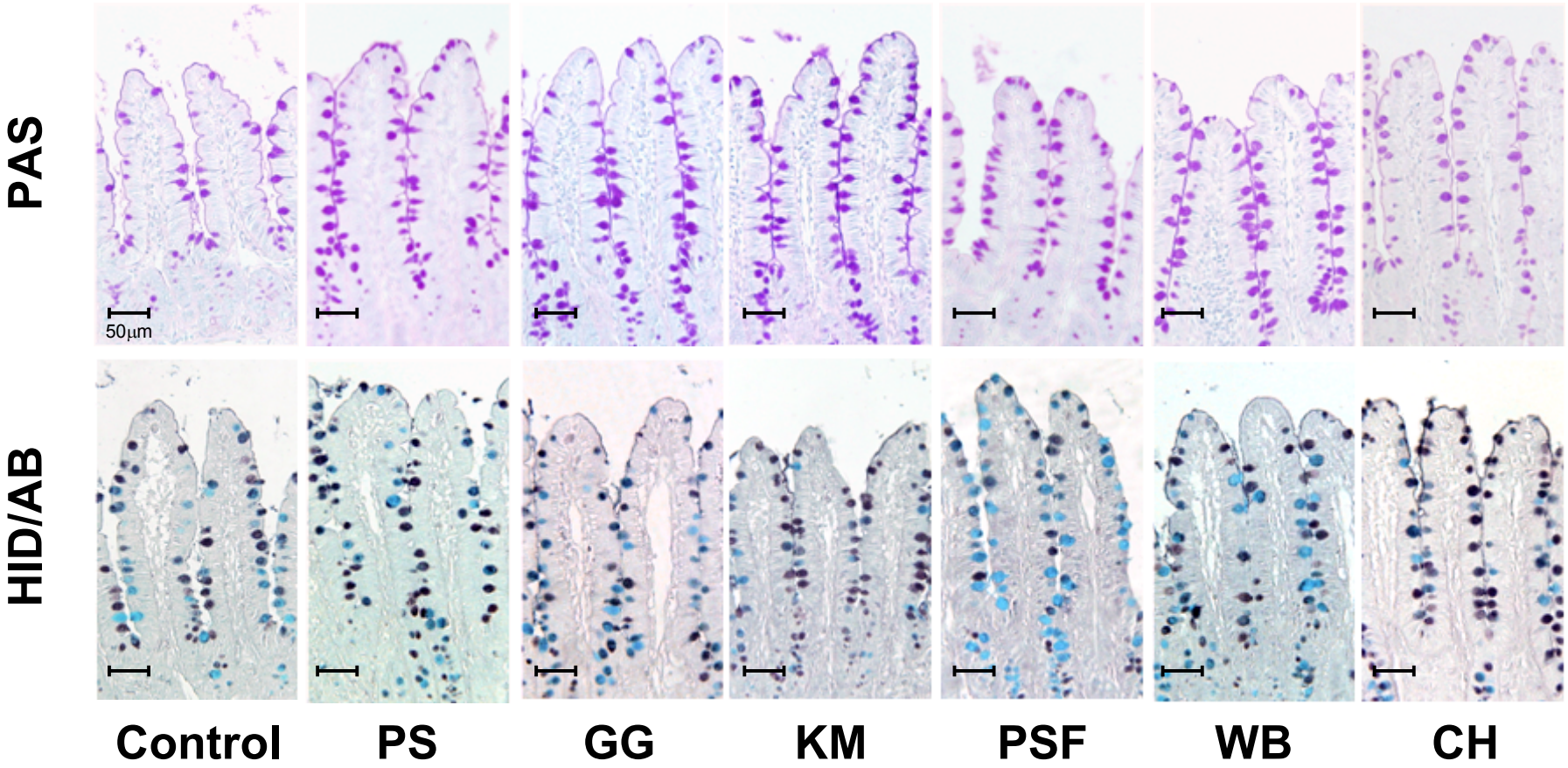
Fig.2

On line supporting material



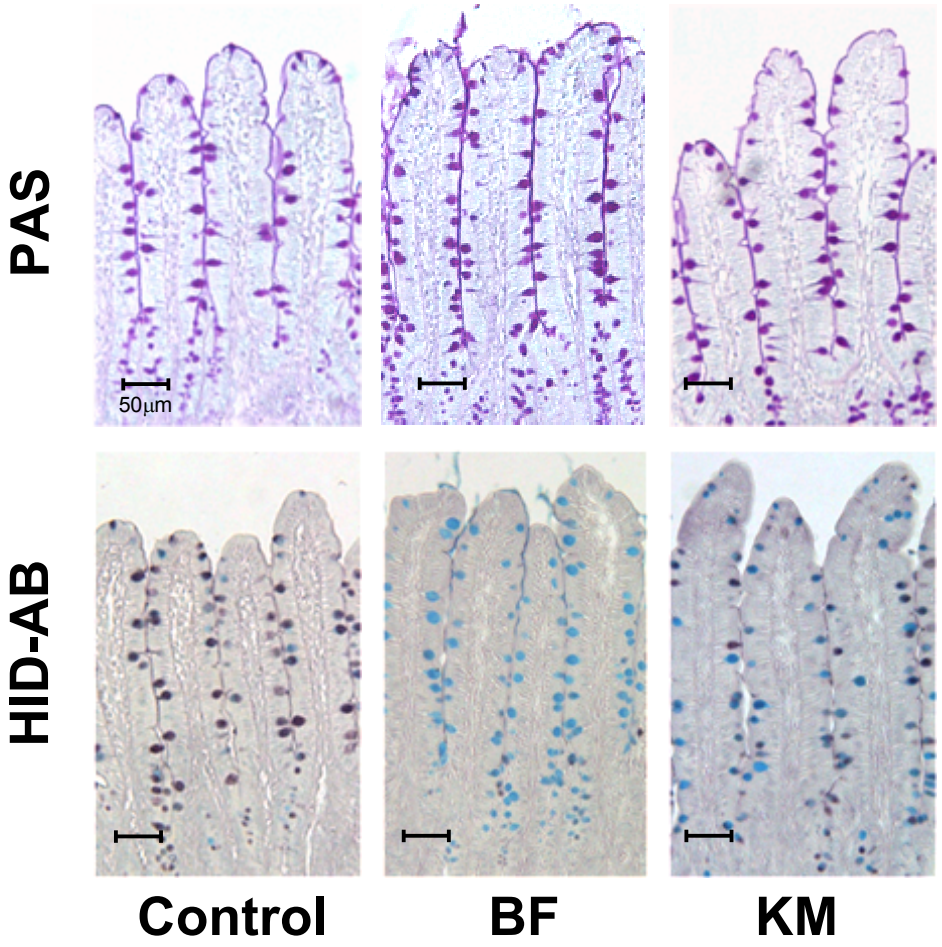
Supplemental Figure 1 PAS and HID/AB staining of the mid-ileum tissue of rats fed the control diet, a diet containing 50 g of KM, PS or GG/kg diet, or a diet containing 80 g of PSF, WB or CH/kg diet for 13 d (*Expt. 1*). Tissues were stained with PAS and HID/AB. Magnification = 200×.

On line supporting material



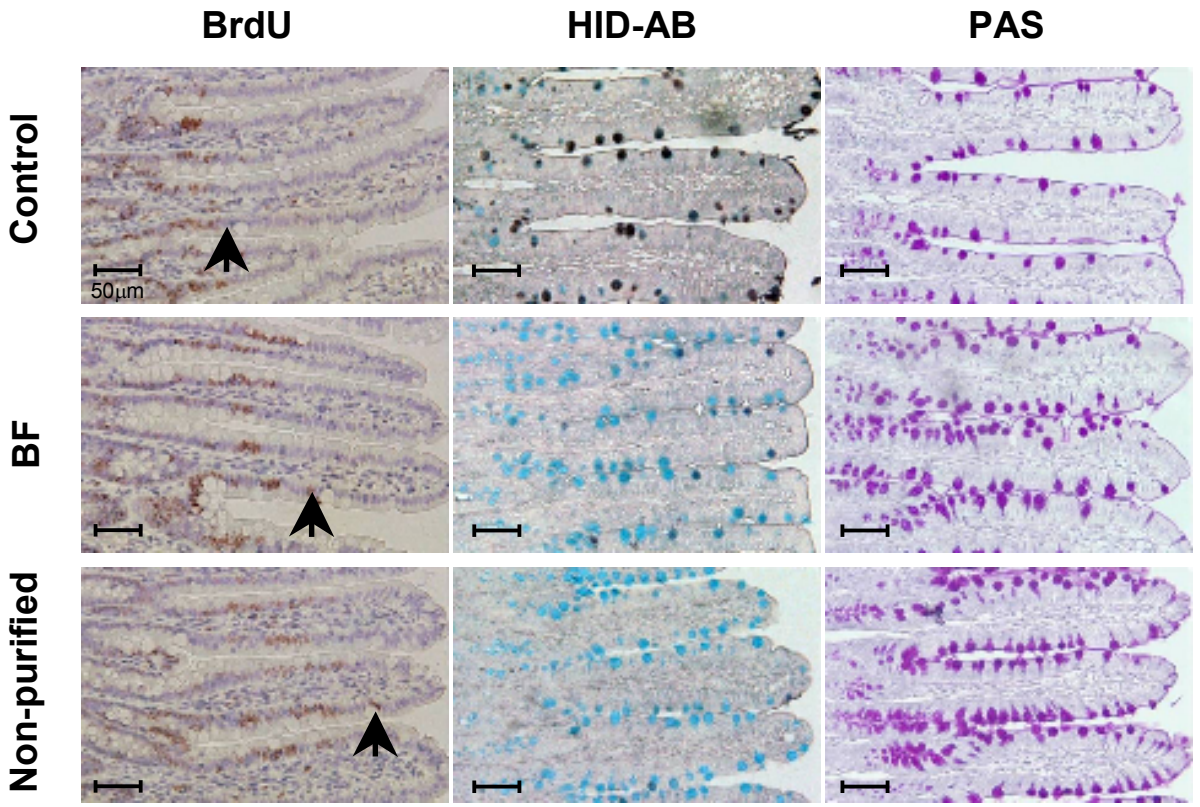
Supplemental Figure 2 PAS and HID/AB staining of the terminal ileum tissue of rats fed the control diet, a diet containing 50 g of KM, PS or GG/kg diet, or a diet containing 80 g of PSF, WB or CH/kg diet for 13 d (*Expt. 1*).
Tissues were stained with PAS and HID/AB. Magnification = 200×.

On line supporting material



Supplemental Figure 3 PAS and HID/AB staining of the mid-ileum tissue of rats fed the control diet, a diet containing 50 g of KM/kg diet, or 80 g of BF/kg diet for 7 d (*Expt. 2*).
Tissues were stained with PAS and HID/AB. Magnification = 200×.

On line supporting material



Supplemental Figure 4 Light micrographs of the mid-ileum tissues of rats fed the control diet, a diet containing 80 g of BF /kg diet, or a non-purified diet (as a reference) for 10 d (*Expt. 4*).
Tissues were stained with PAS or HID/AB or were immuno-stained with anti-BrdU antibody.
Arrows indicate the uppermost of the BrdU-positive cells that migrated on the villi. Magnification = 200×.