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Two weeks feeding of difructose anhydride III enhances calcium absorptive activity with epithelial cell proliferation in isolated rat cecal mucosa

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Running title: Difructose Anhydride III and Ca absorption in the cecum

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ABSTRACT

OBJECTIVE: Ingestion of Difructose Anhydride (DFA) III enhances Ca absorption in rats. The aim of the present study was to determine the mechanism involved in increased Ca transport by DFA III ingestion. The short-term and long-term effects of DFAIII feeding on Ca transport were determined using isolated epithelium from the small and large intestine in rats.

METHODS : Male Sprague-Dawley rats were fed an 8% cellulose or 5% cellulose plus 3% DFAIII diet for 14 days. Net epithelial Ca transport in the small intestine, cecum and colon were compared between the two diet groups using an Ussing chamber. The contents and the epithelial tissues in the cecum were analyzed.

RESULTS: There were no differences in basal and luminal DFAIII-induced Ca transport in the isolated small intestinal and colonic mucosa between the two diet groups. Basal and lamina DFAIII-induced Ca transport in the cecum in the DFAIII fed group was higher than that in control group. A decrease in pH, and increases in Ca pools, short-chain fatty acids (SCFAs) or organic acids in the cecal contents and in the depth and number of cells in crypts in the cecal tissue were observed.

CONCLUSIONS: The increase in Ca transport involved two mechanisms, the presence of DFAIII in the small intestine directly affected the epithelial tissue and caused to increase Ca absorption as a short-term effect, and the degradation of DFAIII by microbial fermentation produced SCFAs and subsequently enhanced Ca absorption in the large intestine as a long-term effect, respectively.

KEY WORDS: Ca absorption, indigestible saccharide, Ussing chamber, intestine, rats

INTRODUCTION

Difructose anhydride (DFA) III (Fig. 1), a newly developed indigestible saccharide, consists of two fructose residues and is prepared from inulin with *Arthrobactor* sp. H65-7 fructotransferase (1). A procedure was established for the mass production of DFAIII using a bacterial enzyme (1) and the products of DFAIII have already been sold in Japan. Ingestion of DFAIII (2) and its isomer, DFAIV (3), increases Ca absorption in male rats as demonstrated in in vivo balance studies. The appearance Ca absorption was significantly higher in rats fed DFAIII than in rats wed well-known indigestible oligosaccharides such as raffinose or fructooligosaccharides (2). Feeding DFAIII has restored Ca absorption in female rats after ovariectomy, which induces ovarian hormone deficiency followed by a reduction in Ca absorption (4). DFAIII increases the Ca content, and the breaking force and mineral density of bone in exercised rats (5). Thus, the ingestion of DFAIII might play a beneficial role in the absorption and retention of Ca in the body.

In in vitro experiments, the application of DFA III or DFA IV in the mucosal medium was found to stimulate Ca absorption in everted sacs of the rat small intestine (2, 3) or colon (4). The application of DFAIII and DFAIV in the luminal medium was also found to increase net Ca absorption in the isolated epithelium of the small and large intestine in rats using the Ussing chamber technique (6). These findings suggest that DFAIII flowing into the lumen directly affects the epithelium of the small and large intestine and promptly enhances Ca transport. In our recent report, Ca transport in the isolated intestinal epithelium was increased by luminal DFAIII application and this increase in Ca absorption occurred via the paracellular route, which is regulated by tight junctions (TJs) (7).

In the above-mentioned in vivo balance studies using rats (2,4), DFAIII was repeatedly ingested for at least 2-4 weeks and the small and/or large intestinal mucosa

were continuously exposed to this sugar throughout the feeding period. The transport and absorption system of the intestinal mucosa might have adapted to the continuous stimulation of DFAIII in vivo, since the ingestion of DFAIII has been reported to increase the weight of the cecal wall in rats (2, 4). In addition, feeding DFAIII is reported to improve microbial fermentation and produce short-chain fatty acids (SCFAs) or other organic acids in the cecum in rats (2, 4).

In our latest report, ingestion of a 3%DFAIII diet for 14 d enhanced Ca absorption via intercellular passage in the isolated cecal epithelium in rats (8). This increase in Ca transport was not occurred in the small intestinal or colonic mucosa isolated from the same rats fed 3%DFAIII diet (8). In the cecum, which is thought to be a main portion of microbial fermentation, adaptive changes in its function should be induced by DFAIII ingestion for an extended period.

Thus, the aim of the present study was to clarify the mechanism for increase in Ca absorption through the small and large intestine in rats fed DFAIII for 2 weeks. As DFAIII itself directly stimulates Ca absorption from the intestine, Ca transport in the isolated epithelial tissue of the small and large intestine was compared with or without applying DFAIII into the mucosal medium using Ussing chamber system (9). In addition, since DFA III is known to be fermented in the cecum in rats, differences in morphological changes in the cecal epithelium, and pH, Ca pools and SCFA concentrations in the cecal contents were compared between in rats fed control diet and in rats fed DFAIII diet.

MATERIALS AND METHODS

This study was approved by the Hokkaido University Animal Committee and the animals were maintained in accordance with the Hokkaido University guidelines for the care and use of laboratory animals.

Animals and diets

Sixteen male Sprague-Dawley rats (4 wk old; Japan SLC, Shizuoka, Japan) were housed in individual stainless-steel metabolic cages with wire-mesh bottoms. The cages were placed in a room with controlled temperature (22-24ºC), relative humidity (40-60%) and lighting (light 0800-2000 h). The animals were fed the stock diet shown in Table 1 for an acclimation period of 14 days and then assigned to two groups based on body weight. Compositions of the stock (control) and test diets are shown in Table 1. DFA III was kindly provided by Nippon Beet Sugar MGF., Ltd. (Obihiro, Japan). One group (control group) was fed the control diet, while the other group (DFAIII group) was fed the test diet containing 30g of DFAIII per kilogram of diet. Rats were fed the assigned test diets for 14 days. All animals were allowed free access to deionized water throughout the test period. Body weight and food intake were measured every day.

Sampling of the intestinal contents and tissue preparation

On the last day of the experiment, the rats were anesthetized with pentobarbital sodium (30 mg/kg). The entire length of the small intestine from the ligament of Treitz to the ileocecal junction, the entire cecal sac, and the entire length of the colon were quickly removed. The contents of the small intestine, cecum and colon were collected, weighed and placed into test tubes. The intestinal contents were stored at –80 º C until future analysis. The outside and inside surface of the isolated intestine was washed with ice-cold (4 ºC) saline (154 mmol/L NaCl), and the wet weight of each specimen was measured. Segments of the small intestine (a 6-cm section from the center), cecum (whole sac) and colon (a 6-cm section from the center) were quickly isolated,

and each specimen was then cut open along the mesenteric border to produce a flat sheet, and rinsed with ice-cold HEPES buffer solution (HBS). The HBS used in this study consisted of 125 mmol/L NaCl, 4 mmol/L KCl, 6 mmol/L L-glutamine, 30 mmol/L 2-[4-(2-hydoroxymethyl)-1-piperazyl] ethanesulfonic acid (HEPES), and 1.25 or 10 mmol/L $CaCl_2 \cdot 2H_2O$, and it was gassed with 100% O_2 to maintain a constant pH of 7.4. HBS containing 1.25 mmol/L Ca was used as the bathing solution for the serosal component during the experiment and as a stabilizing solution for both the mucosal and the serosal sides between experiments. The serosa and muscle layers were removed, and stripped preparations, consisting of the mucosa and the submucosa, were mounted onto Ussing chambers (Diffusion chamber system, Corning Costar Co., Cambridge, UK) that exposed a circular area of the epithelium of 0.64 cm^2. The serosal and mucosal sides of the segments were bathed in 1 mL of HBS containing 1.25 mmol/L Ca and continuously exposed to 100% O_2 gas. After a 30-min stabilization period, the medium on both sides of the tissue was removed by aspiration and 1 mL portions of the appropriate solutions were added to the mucosal and serosal sides in the chamber system.

Saccharide and permeable marker

DFAIII is soluble in water at 100 mmol/L. In our preliminary evaluation using the same chamber system, a maximal Ca transport response was obtained with 100mmol/L DFAIII on the luminal side (6,7). Lucifer Yellow CH dilithium salt (LY: FW 457.2, Sigma Chemical Co., St. Louis, MO) was used as the paracellular permeable marker (10) at a concentration of 21.8 μmol/L in the 10 mmol/L Ca-HBS mucosal medium. The pH of 10 mmol/L Ca plus LY-HBS containing any of these sugars at 0 (control) or 100 mmol/L was found to remain constant at 7.4.

Experimental procedure

Fresh HBS containing 1.25 mmol/L Ca was applied to the serosal bath and 10 mmol/L Ca-HBS with or without 100 mmol/L DFAIII was applied to the mucosal bath in the experiments using the small intestine, cecum and colon preparations. After a 30-min incubation period, the serosal solution was transferred to a polyethylene test tube.

Morphological index of cecal epithelium

The cecal preparation for morphological observation was obtained by cutting a 6 mm wide strip of tissue along the circumference of the cross section of the cecum at 3/4 of the distance between the ileocecal valve to the end of the cecum. The strip was fixed in a 3.3 mmol/L buffered formaldehyde solution for future histological analysis. The fixed 6-mm cecal segments were cut into 7-μm cross-sections on a glass slide using cryostat, and stained with hematoxylin and eosin. Only 10 well-oriented crypts, in which the base, lumen and the top of the crypt could all be seen, were selected per segment of the cecum. The consistency of the crypts was obtained by positioning the micrometer along the luminal surface and counting the number of complete crypts per 1 mm. The depth of the crypts from the top to the base was measured in μm, and the number of cells in the crypt was counted (cells/crypt) using an optical microscope.
The electrical portrait of the whole cross section of the cecal tissue was obtained and analyzed by computer system. The outside circumference along serosa and the inside circumference along the top of the crypts in the cecal section, which is expected to indicate the inner area of the lumen, was measured using soft ware program (Scino Image, Scino corptation, USA). The two parameters are expected to indicate the outer and inner area of the cecum, respectively.

Bacterial enumeration in cecal content

The population of anaerobic bacteria in the cecal contents was compared in rats fed the control and DFAIII diets. About 0.2 g (wet weight) of cecal content from one rat fed the control or DFAIII diet was immediately transferred into 1.8 ml of diluting solution containing (g/l): KH_2PO_4, 4.5; Na_2HPO_4, 6.0; L-cystein-HCl, 0.5; tween 80, 0.5; and Bacto Agar (DIFCO, USA), 1.0. The samples were kept under anaerobic conditions using Anaero Pack (Mitsubishi Gas Chem. Co. Inc. Japan) during transportation to our laboratory and were then spread on GAM agar plates containing (g/l): peptone, 10.0; soy peptone, 3.0; protease peptone, 10.0; hydrolyzed serum powder, 13.5; yeast extract, 5.0; meat extract, 2.2; liver extract, 1.2; glucose, 3.0; KH_2PO_4, 2.5; NaCl, 3.0; soluble starch, 5.0; L-cystein-HCl, 0.3; thioglycolate-sodium salt, 0.3; and agar, 20.0. The pH of medium was adjusted to 7.1 before autoclaving. The bacterial population was counted after 2 days incubation at 37°C in an anaerobic chamber (Coy Laboratory Products, Inc. USA) and expressed as colony forming units (CFUs).

Analyses

The Ca concentrations in the serosal medium were measured by a colorimetric method using a commercial kit (Calcium C-Test, Wako Chemical Co., Osaka, Japan). Intra- and inter-assay coefficients of variation for Ca determination were below 2% (stock standard sample) and 9% (same sample at 5 assays). The net transepithelial passage of Ca was expressed as nmol Ca transferred per min per square cm of surface area. The LY in the serosal solution was determined fluorometrically at 430nm for excitation and 540nm for emission (FP-550, JAS.Co., Tokyo, Japan) after appropriate dilution of the solution with purified water. The contents of the small intestine, cecum or colon were diluted 4 vol of deionized water and homogenized with a

Teflon homogenizer. The pH of the intestinal homogenate was measured with a semi-conducting electrode (ISFET pH sensor 0010-15C, HORIBA, Ltd., Kyoto, Japan) as intestinal contents. Amount of total Ca in the homogenates were determined by atomic absorption spectrometry (Polarized Zeeman Atomic Absorption Spectrophotometor, A-5310, HITACHI, Tokyo, Japan) after wet-ashing with an acid mixture. Soluble Ca in the supernatant obtained after centrifugation (30000g for 20 min at 4 ºC) of the homogenate was determined by the above-mentioned atomic absorption sectrophotometry after deproteinization with 9 mol/L of perchoric acid. Concentrations of short-chain fatty acids (SCFAs) and organic acids (acetic, propionic, butyric, succinic and lactic acids) in the homogenates of the cecal and colonic contents were measured according to the method of Hoshi et al. (11) using high-performance liquid chromatography (LC-10 ADvp SHIMAZU, Kyoto, Japan) equipped with two Shima-packSCR-102H columns (8mm inner diameter and 30 cm long; SHIMAZU, Kyoto, Japan) and an electroconductibility detector (CDD-6A, SHMAZU).

Statistical analyses

All results are expressed as means ± SEM (n=8). Statistical analyses were performed by unpaired t-test for comparison of values between the two diet groups. For comparison of the two diet groups (control and DFAIII diets, D), luminal application of DFAIII in the mucosal medium in vitro (with or without 100mmol/L DFAIII in the mucosal chamber, L), and interaction of (D) x (L), analyses were performed by two-way ANOVA following Duncan's multiple range test. A difference with $P<0.05$ was taken to be statistically significant. Regression analysis was used for analysis of the relationship between two factors.

RESULTS

There were no significant differences in initial and final body weights, body weight gain or food intake over the experimental period between groups fed the control and DFAIII diets (Table 2). Similarly, there were no differences in the wet weights of the small intestine or colon, or in their contents between the two diet groups (Table 3). The wet weights of the cecum ($P < 0.005$) and cecal contents ($P < 0.001$) in rats fed the DFAIII diet were, however, significantly greater than those in rats fed the control diet. The pH in the contents of the small intestine was similar between the two diet groups, while the pH in cecal and colonic contents were lower ($P < 0.001$) in rats fed the DFAIII diets than in rats fed the control diets.

Regarding the morphological indices of the cecal epithelium (Table 4), the outside ($P < 0.05$) and luminal circumference ($P < 0.01$), and the ratio of luminal: outside circumference ($P <0.005$) of the cecal tissue preparations from rats fed the DFAIII diet were greater than those in rats fed the control diet. The depth of crypt ($P < 0.001$) and the number of cells per crypt ($P < 0.005$) in the DFAIII groups were significantly greater than those in the control diet groups. There were no significant differences in the width of epithelial cells in the crypts or the number of crypts per 1 mm of cecal epithelial circumference between the two diet groups.

There were no significant differences in total and soluble Ca pools, soluble Ca concentration, or the ratio of soluble Ca pool in the contents of the small intestine and the colon between the two diet groups (Table 5). The total ($P < 0.001$) and soluble Ca pools ($P < 0.001$), and soluble Ca concentration ($P < 0.05$) in the cecal contents of rats fed the DFAIII diets were higher than those in the rats fed the control diets. There were no significant differences in the percentage of the soluble Ca pool to total Ca pool in the cecal contents between the two diet groups.

There were no differences in basal (without luminal DFAIII application) net Ca absorption or LY permeability in the small intestinal mucosa between rats fed the

control diet and rats fed DFAIII diet (Fig.2AB). Application of 100 mmol/L DFAIII in the mucosal medium enhanced net Ca transport and LY passage in the small intestinal preparations in both diet groups ($P < 0.05$). However, there were no differences in the increase in net Ca absorption or LY permeability induced by luminal application of DFAIII between the two diet groups.

For the large intestinal epithelial preparations, luminal application of DFAIII affected the net Ca absorption and LY permeability in the cecum (Fig. 3AB). Basal Ca transport in the cecal epithelium in rats fed the DFAIII diets was higher than that in rats fed the control diets. There were no differences in basal LY permeability between rats fed the control and the DFAIII diets. Application of DFAIII in the mucosal medium enhanced net Ca transport and LY passage in the cecum of both diet groups ($P < 0.05$). The degree of the increase in net Ca absorption and LY passage were greater in the rats fed the DFAIII diet than in rats fed the control diet ($P < 0.05$).

There were no differences in basal net Ca absorption or LY permeability in the colonic mucosa between rats fed the control diet and rats fed DFAIII diet (Fig. 4AB). Application of 100 mmol/L DFAIII in the mucosal medium enhanced net Ca transport and LY passage in the colonic preparations in both diet groups ($P < 0.05$). However, there were no differences in the increase in net Ca absorption or LY permeability induced by luminal application of DFAIII between two the diet groups.

Regression analysis for the relation between the net Ca absorption and LY permeation was evaluated for the three portions of the intestine, respectively (Fig. 2-4C). Statistically significant ($P < 0.001$) positive linear relations were observed between net Ca absorption and LY passage in the small intestine, cecum and colon isolated from the rats fed the control and the DFAIII diets.

The amount of acetic and propionic acid, total SCFA (sum of acetic, propionic, n-butyric, iso-butyric, n-valeric and iso-valeric acids), succinic and lactic acid in the cecal contents were higher ($P < 0.001$, 0.005 or 0.01) in rats fed the DFAIII diets than in

rats fed the control diets (Table 6). However, there were no differences in the amount of main (acetic, propionic and butyric acid) and total SCFA, and lactic acid in the cecal contents between the control and test diet groups (Table 7). Anaerobic bacterial population in the cecal contents in one rat fed the control or DFAIII diet was 2.8 X 10^9 (control) and 2.0 X 10^{10} CFU (DFAIII), respectively.

The relation between net Ca absorption without luminal DFAIII application in chamber system (basal Ca transport) and some factors in the cecum or the cecal contents in rats fed control and DFAIII diets were evaluated by regression analysis (Table. 8). There was positive correlation between the basal Ca transport and the weight of the cecal wall, luminal circumference of cecal section, the ratio of lumen: outside length of section, the depth of crypt, or the number of cells per crypt height. There was a negative correlation between the Ca absorption rate in and the pH of the cecal contents. The Ca absorption in the cecal epithelium was correlated with the concentration of acetic, propionic or iso-butyric acids, total SCFA, succinic, and lactic acid in the cecal contents. There were no significant relations between the wet weight of cecal contents and Ca transport in cecal epithelium.

DISCUSSION

In the present study, ingestion of DFAIII for 14 days did not affect growth, food intake, or weight of the small intestine or colon in rats. The DFAIII feeding also have no affect on net absorption of Ca and permeability of LY in the isolated epithelium of the small intestine or colon. Repeated ingestion of DFAIII did increase in cecal weight, and net Ca transport and LY permeation in the isolated cecal epithelium. Positive linear regressions were obtained between net Ca absorption and LY permeation in the small intestine, cecum and colon ($P < 0.001$). LY is reported to act as a marker of paracellular passage, in which is regulated by TJs (10). Thus, the increase in Ca

transport and LY passage with repeated feeding of DFAIII is thought to occurr via the paracellular route in the cecal tissue. These changes induced by the feeding of DFAIII are similar to those in our latest report (8). The repeated ingestion of DFAIII affects the ability of the epithelial tissue in the cecum in rats.

Morphological indices of the cecal mucosa, such as the luminal and outer circumference of the cross section, depth of crypts, and the cell population of crypts, were also increased with DFAIII feeding. The increase in the weight of the cecal wall was due to an increase in the population of epithelial cells and their supporting tissue such as submucosal tissue and smooth muscle layer. At the same time, a decrease in pH and increases in acetic, propionic, total SCFA, succinic, and lactic acid concentrations were observed in the cecal contents. The feeding of DFAIII obviously affects the microbial environment and enhances the fermentation process in the cecum.

Although repeated feeding of DFAIII affects Ca transport activity and LY permeation in the cecum, this effect may be also induced by changes in the paracellular route via TJs in the epithelial tissue itself. An adaptation to enhance Ca transport ability might, therefore, occur in the epithelial tissue of the cecum after feeding of DFAIII for extended periods. Positive linear relations were obtained between the basal Ca transport and the depth of crypt, the number of cells per crypt and the intra-luminal circumference. Thus, the absorptive area in the cecal tissue was substantially increased and these morphological changes may be important factors in the increase in the efficeince of Ca transport in rats fed DFAIII diet for 14 days.

Ingestion of indigestible saccharides, such as sugar alcohols (12,13), oligosaccharides (14,15) and polysaccharides (16,17), has also been reported to increase Ca absorption in rats during balance studies in vivo. The effect of DFAIII ingestion on Ca absorption has also been reported in rats in vivo (2,3). To date, there are two aspects of Ca absorption known to be improved by indigestible saccharides; the direct effect of the sugar itself in the small and/or large intestine and the indirect effect of the

SCFAs or organic acids produced from indigestible sugars by microbial fermentation in the large intestine.

Intraluminal infusion of sorbitol increases Ca absorption in the small intestine of rats (18). Using a tracer technique with ^{45}Ca, maltitol was found to stimulate Ca absorption in rats (19). In in vitro experiments using everted sacs or isolated preparations, maltitol (20), and polydextrose (21) were also seen to stimulate Ca absorption in the small intestine of rats. Various indigestible sugars were also found to increase net Ca absorption in the isolated epithelium of small and large intestine in rats using an Ussing chamber technique in vitro (6). Using everted sacs, DFA III in the mucosal medium has been found to stimulate Ca transport from the mucosal side to the serosal side in the rat jejunum, ileum (2) and colon (4). Our preliminary experiment using the same chamber system also revealed that luminal application of DFAIII increased Ca absorption in isolated epithelial tissue of the small and large intestine in rats (6,7). The present study also revealed that luminal application of DFAIII directly affected the epithelium itself, and increased Ca transport in the small and large intestine in rats.

Improved fermentation of resistant carbohydrates is also thought to be a factor in promoting transepithelial Ca transport in the large intestine (22,23). SCFAs and other organic acids (lactic acid, succinic acid, etc.) produced through microbial fermentation decrease the pH of the cecal contents and thereby convert insoluble Ca to a soluble ionic form (22,24). Indeed, in the present study, a decrease in the pH, and increases in the soluble Ca pools and the soluble Ca concentrations were observed in the cecal contents in rats fed the DFAIII diets.

In balance studies in vivo, a positive correlation exists between apparent Ca absorption and total SCFA, acetic, propionic and butyric acid concentrations in the cecum of rats fed oligosaccharides (25) or soluble soybean fiber (26). The infusion of SCFAs into the colon or rectum in known to stimulate Ca absorption in humans in vivo

(27,28). Luminal perfusion of SCFAs also enhances Ca absorption in the colon in rats (29). In our previous study using the same chamber technique, luminal application of SCFA enhanced net Ca transport from the mucosal side to the serosal side in isolated rat epithelial tissue in vitro (30). These in vivo and in vitro experiments indicate that SCFAs in the lumen promptly stimulate Ca transport in the epithelial tissue of the large intestine. However, in this experiment, the isolated cecal epithelial tissue was not exposed to SCFAs in the chamber system. Thus, changes in Ca transport activity, especially in the basal conditions, must have already occurred before isolation from the body.

SCFAs are an important factor in the stimulation of epithelial cells and the promotion of cell proliferation in the large intestine. Intraluminal infusion of SCFAs increased the relative weight of the mucosa and submucosa, crypt size, and mitotic index in the cecum in rats (31). Mucosal cell proliferation in the crypts was raised by the addition of SCFAs in vitro using isolated human cecal epithelium obtained through biopsy (32). In the present study, Ca transport activity in the cecal tissue was correlated to the weight of the cecal wall, depth of crypts, and outer circumference of the cecum, as well as with the concentration of some fermentation products (acetic acid, propionic acid, iso-butyric acid, total SCFA, succinic acid and lactic acid) in the cecal contents. Lactic acid stimulated mitosis of the cecal epithelial cell in rats (31). To date, there is no information about the trophic effect of succinic acid on cell proliferation in the gastrointestinal tract.

In balance studies with rats, cecectomy or ingestion of antibiotic drugs completely inhibited the increase in Ca absorption induced by the feeding of fructooligosaccharides or galactooligosaccharides, which are commonly used indigestible saccharides that affect Ca retention in the body (33,34). On the other hand, cecectomy only partially inhibited Ca absorption induced by DFAIII ingestion in rats in vivo (4). These results suggest that the main portion of the intestine for Ca

absorption may vary according to the indigestible saccharide.

In the present study, the increase in Ca absorption induced by repeated feeding of DFAIII may have occurred via two different mechanisms in the small and large intestine in vivo. Firstly, DFAIII flowing into the lumen of the intestine directly affected the epithelial tissue, and promoted Ca absorption. Prompt and direct action of DFAIII mainly occurs in the small intestine and to a minor extent in the large intestine in vivo because DFAIII is thought to be degraded by microbes. Secondly, chronic and indirect action of DFAIII occurred in the large intestine, particularly in the cecum in rats. DFAIII has a prebiotic effect generally induced by ingestion of indigestible saccharides via microbial fermentation. The increased population of epithelial cells and intra-luminal area induced by the increase in SCFA production are important factors in the increased Ca transport in the large intestine. The increase in total and soluble Ca pools, and the stimulatory effect of SCFAs on Ca transport also may contribute the increase in Ca absorption from the epithelial tissue of the large intestine. The paracellular route via TJs is involved in the promotion of diffusional Ca transport in both cases. Thus, in conclusion, DFAIII is thought to increase Ca transport by short-term and/or long-term mechanisms in the small and large intestine, and is thought to benefit Ca absorption and retention in the body.

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TABLE I

COMPOSITION OF CONTROL AND TEST DIETS

Element	Control diet (g)	Test diet (g)
Casein [1]	200	200
Dextrin [2]	400	400
Soybeen oil	70	70
Mineral mixture [3]	35	35
Vitamine mixtture [3]	10	10
Choline bitartate	2.5	2.5
L-cystine	3.0	3.0
Tert- butylhydroquinone	0.014	0.014
Crystallized cellulose [4]	80	50
DFAIII [5]		30
Sucrose	to make 1000 g	

1. Casein (ALACID; New Zealand Daily Bond, Wellington, New Zealand)
2. Dextrin (Pine-dex 4; Matsutani Chemical Industry, Tokyo, Japan)
3. Mineral and vitamin mixture were prepared according to the AIN-93G formulation
4. Crystallized cellulose (AVICEL; Asahi Kasei Ltd. Osaka, Japan)
5. DFAIII (Nippon Beet Sugar MGF., Ltd. Obihiro, Japan)

TABLE II

INITIAL AND FINAL BODY WEIGHT, BODY WEIGHT GAIN, AND FOOD INTAKE OF RATS FED THE CONTROL OR DFAIII DIET FOR 14 DAYS

		Control diet	DFAIII diet	*P*-value
Initial body weight	(g)	213.1±4.9	211.6 ± 6.1	0.848
Final body weight	(g)	307.4 ± 7.4	307.4 ± 9.5	0.997
Body weight gain	(g/d)	6.7 ± 0.3	6.7 ± 0.3	0.815
Food intake	(g/d)	19.2 ± 0.5	18.9 ± 0.5	0.621

Values are mean ± SEM (n = 8). The values of initial and final body weight, body weight gain, and food intake were analyzed by unpaired *t*-test. Asterisk mean significant difference ($P < 0.05$).

TABLE III

WET WEIGHTS OF ISOLATED TISSUES AND THEIR CONTENTS, AND pH IN THE CONTENTS OF THE SMALL INTESTINE, CECUM AND COLON IN RATS FED THE CONTROL OR DFAIII DIET FOR 14 DAYS

	Control diet	DFAIII diet	*P*-value
Isolated tissue (g)			
Small intestine	6.40 ± 0.16	6.92 ± 0.30	0.154
Cecum	1.29 ± 0.08	1.66 ± 0.06*	0.003
Colon	1.03 ± 0.05	1.11 ± 0.05	0.305
Contents (g)			
Small intestine	1.05 ± 0.11	1.05 ± 0.09	0.785
Cecum	2.42 ± 0.20	4.64 ± 0.52*	0.001
Colon	1.10 ± 0.16	0.67 ± 0.15	0.075
pH in contents			
Small intestine	7.86 ± 0.16	7.99 ± 0.09	0.472
Cecum	7.91 ± 0.09	6.97 ± 0.13*	< 0.001
Colon	7.89 ± 0.07	7.47 ± 0.05*	< 0.001

Values are mean ± SEM (n = 8). The values of wet weight of isolated tissues and their contents, and the pH in the contents of the small intestine, cecum and colon were analyzed by unpaired *t*-test. Asterisks mean significant difference ($P < 0.05$).

TABLE IV

MORPHOLOGICAL INDICES OF ISOLATED EPITHELIAL TISSUE IN THE CECUM IN RATS FED THE CONTROL OR DFAIII DIET FOR 14 DAYS

	Control diet	DFAIII diet	*P*-value
Outer circumference (O)(mm)	13.7 ± 1.2	17.5 ± 1.2*	0.040
Inner circumference (I)(mm)	22.1 ± 2.1	36.6 ± 4.1*	0.007
Ratio of (I)/ (O)	1.61 ± 0.07	2.06 ± 0.12*	0.004
Crypt dept (µm)	195.2 ± 4.0	232.2 ± 4.0*	< 0.001
Number of cells/ crypt	32.8 ± 0.5	38.6 ± 1.0*	0.001
Width of one cell (µm)	6.0 ± 0.2	6.1± 0.2	0.776
Number of crypt/(mm)	19.2 ± 0.7	18.1 ± 0.4	0.201

Values are mean ± SEM (n = 8). The values of outer circumference (O), inner circumference (I), ratio of (I)/(O), crypt dept, number of cells / crypt, width of one cell, and number of crypt / mm in the cecal tissue were analyzed by unpaired *t*-test. Asterisks mean significant difference ($P < 0.05$).

TABLE V

TOTAL AND SOLUBLE CA POOL, SOLUBLE CA CONCENTRATION AND THE RATIO OF SOLUBLE CA POOL IN THE CONTENTS OF THE SMALL INTESTINE, CECUM AND COLON IN RATS FED THE CONTROL OR DFAIII DIET FOR 14 DAYS

	Control diet	DFAIII diet	*P*-value
Total Ca pool (µmol in contents)			
Small intestine	114.6 ± 13.1	148.2 ± 15.9	0.125
Cecum	421.2 ± 45.1	862.9 ± 77.6*	< 0.001
Colon	324.0 ± 44.6	272.6 ± 53.8	0.474
Soluble Ca pool (µmol in contents)			
Small intestine	34.9 ± 6.8	39.5 ± 3.9	0.565
Cecum	94.1 ± 8.8	193.6 ± 16.7*	< 0.001
Colon	72.1 ± 9.9	63.5 ± 14.5	0.631
Soluble Ca concentration (µmol/g contents)			
Small intestine	31.8 ± 4.5	36.8 ± 3.0	0.376
Cecum	37.5 ± 3.4	48.7 ± 3.3*	0.031
Colon	58.5 ± 5.1	72.9 ± 5.7	0.081
Soluble Ca pool (%)			
Small intestine	29.1 ± 2.2	27.4 ± 1.7	0.560
Cecum	22.7 ± 0.7	22.5 ± 0.4	0.854
Colon	22.2 ± 0.3	22.6 ± 1.0	0.701

Values are mean ± SEM (n = 8). The values of total and soluble Ca pools, soluble Ca

concentration and the ratio of soluble Ca pool (100 x soluble Ca pool /total Ca pool) of the small intestine, cecum and colon were analyzed by unpaired *t*-test. Asterisks mean significant difference ($P < 0.05$).

TABLE VI

AMOUNT OF EACH AND TOTAL SCFA, AND OTHER ORGANIC ACIDS IN THE CECAL CONTENTS IN RATS FED THE CONTROL OR DFAIII DIET FOR 14 DAYS

	Control diet	DFAIII diet	*P*-value
	(μmol in cecal contents)		
Acetic acid	95.1 ± 12.5	243.8 ± 39.6*	0.003
Propionic acid	28.5 ± 3.6	83.8 ± 11.8*	< 0.001
n-Butyric acid	27.2 ± 3.6	29.1 ± 5.0	0.763
iso-Butyric acid	4.7 ± 2.4	6.4 ± 3.4	0.694
n-Valeric acid	8.7 ± 5.2	5.2 ± 1.1	0.391
iso-Valeric acid	8.8 ± 2.9	5.8 ± 1.6	0.581
Total SCFA	179.2 ± 20.4	481.4 ± 83.0*	0.003
Succinic acid	5.6 ± 1.3	102.1 ± 32.1*	0.009
Lactic acid	0.6 ± 0.3	5.2 ± 1.3*	0.004

Values are mean ± SEM (n = 8). The amount of each and total SCFAs (sum of acetic, propionic, n-butyric, iso-butyric n-valeric and iso-varelic acid) and other organic acids (succinic and lactic acid) in the contents of the cecum were analyzed by unpaired *t*-test. Asterisks mean significant difference ($P < 0.05$).

TABLE VII

AMOUNT OF EACH AND TOTAL SCFA AND OTHER ORGANIC ACIDS IN THE COLONIC CONTENTS IN RATS FED THE CONTROL OR DFAIII DIET FOR 14 DAYS

	Control diet	DFAIII diet	*P*-value
	(μmol in colonic contents)		
Acetic acid	1.49 ± 0.34	1.01 ± 0.24	0.266
Propionic acid	0.48 ± 0.10	0.56 ± 0.22	0.730
n-Butyric acid	0.45 ± 0.09	0.23 ± 0.07	0.091
iso-Butyric acid	0.17 ± 0.09	0.01 ± 0.01	0.082
n-Valeric acid	0.14 ± 0.07	0.15 ± 0.08	0.866
iso-Valeric acid	0.08 ± 0.03	0.05 ± 0.02	0.364
Total SCFA	2.98 ± 0.68	2.01 ± 0.49	0.266
Succinic acid	0.05 ± 0.04	0.80 ± 0.27*	0.017
Lactic acid	0.02 ± 0.01	0.03 ± 0.01	0.386

Values are mean ± SEM (n = 8). The amount of each and total SCFAs (sum of acetic, propionic, n-butyric, iso-butyric n- valeric and iso- varelic acid) and other organic acids (succinic and lactic acid) in the contents of the cecum were analyzed by unpaired *t*-test. Asterisks mean significant difference ($P < 0.05$).

TABLE VIII

CORRELATION BETWEEN NET CALCIUM ABSORPTION OF CECAL EPITHELIUM IN THE ABSENCE OF LUMINAL DFAIII APPLICATION AND CECAL WEIGHT, CECAL CONTENT WEIGHT, CECAL PH, LEVEL OF CECAL SOLUBLE CALCIUM, VARIOUS MORPHOLOGICAL INDICES IN CECAL TISSUE, CONCENTRATIONS OF VARIOUS SCFAS AND ORGANIC ACIDS IN THE CECAL CONTENTS IN RATS FED THE CONTROL AND DFAIII DIETS.

Correlation to net Ca absorption	*R* value	*P* value
Cecal weight	0.531*	0.034
Cecal content weight	0.291	0.273
Cecal content pH	- 0.594*	0.015
Inner circumference	0.482	0.058
Outer circumference	0.621*	0.010
Ratio of inner: outer length	0.544*	0.029
Crypt depth	0.604*	0.013
Number of cells· $crypt^{-1}$	0.485	0.073
Cell width	0.174	0.517
Number of crypt· mm^{-1}	0.023	0.931
Acetic acid	0.579*	0.019
Propionic acid	0.530*	0.035
n-Butyric acid	0.151	0.577
iso-Butyric acid	0.519*	0.039
n-Valeric acid	0.090	0.736
iso-Valeric acid	0.320	0 .227

Total SCFA	0.594*	0.039
Succinic acid	0.681*	0.004
Lactic acid	0.579*	0.005

Relationship between Ca absorption of the cecal epithelium, each cecal factor and the cecal contents were evaluated by regression analysis (n =16). Correlation between Ca absorption (nmol/min· cm) and cecal weight (g), cecal content weight (g), cecal content pH, morphological indices of cecal tissue cross-section, each and total SCFAs, and organic acid concentration (μmol/ g cecal contents) were expressed. Asterisks mean significant correlation ($P < 0.05$).

Legends

FIG. 1. Structural Formula of Di-D-fructosefuranose 1,2´ ; 2,3´ Dianhydride (difructose anhydride III) Produced from Inulin with *Ar-throbacter* sp. H65-7 Inulase II (EC2.4.1.93).

FIG. 2. Effects of luminal application of DFAIII on net Ca absorption and LY permeability, and their relationship in the isolated small intestinal epithelium of rats fed the control or DFAIII diet for 14 days. Values are mean ± SEM (n = 8). The differences in net Ca transport and LY passage induced with (dotted column) or without (open column) the luminal application of 100mmol/L DFAIII in the small intestine isolated from the two diet groups was analyzed by two-way ANOVA (A and B). For net Ca absorption (A), *P* values estimated by two-way ANOVA were = 0.653 for diet (control and DFAIII diet, D), < 0.001 for DFAIII application (with or without DFAIII in luminal medium, L) and =0.391 for (D) x (L). For LY permeability (B panel), *P* values estimated by two-way ANOVA were = 0.889 for (D), < 0.002 for (L) and =0.6175 for (D) x (L). Values not sharing a superscript letter are significantly different according to Duncan's test (P< 0.05). Regression for net Ca transport and LY passage (n = 32) after 30-min incubation is shown for the ileum (C panel).

FIG. 3. Effects of luminal application of DFAIII on net Ca absorption and LY permeability of the isolated cecal epithelium in rats fed the control or DFAIII diet for 14 days. Values are mean ± SEM (n = 8). The differences in net Ca transport and LY passage induced with (dotted column) or without (open column) the luminal application of 100mmol/L DFAIII in the cecum isolated from the two diet groups was analyzed by two-way ANOVA. For net Ca absorption (A), *P* values estimated by two-way ANOVA were < 0.002 for diet (control and DFAIII diet, D), < 0.001 for DFAIII

application (with or without DFAIII in luminal medium, L) and =0.894 for (D) x (L). For LY permeability (B panel), *P* values estimated by two-way ANOVA were = 0.021 for (D), < 0.001 for (L) and =0.307 for (D) x (L). Values not sharing a superscript letter are significantly different according to Duncan's test (P< 0.05). Regression for net Ca transport and LY passage (n = 32) after 30-min incubation is shown for the ileum (C panel).

FIG. 4. Effects of luminal application of DFAIII on net Ca absorption and LY permeability of the isolated colonic epithelium in rats fed the control or DFAIII diet for 14 days. Values are mean ± SEM (n = 8). The differences in net Ca transport and LY passage induced with (dotted column) or without (open column) the luminal application of 100mmol/L DFAIII in the colon isolated from two diet groups was analyzed by two-way ANOVA. For net Ca absorption (A panel), *P* values estimated by two-way ANOVA were = 0.132 for diet (control and DFAIII diet, D), = 0.024 for DFAIII application (with or without DFAIII in luminal medium, L) and =0.682 for (D) x (L). For LY permeability (B panel), *P* values estimated by two-way ANOVA were = 0.239 for (D), < 0.001 for (L) and =0.600 for (D) x (L). Values not sharing a superscript letter are significantly different according to Duncan's test (P< 0.05). Regression for net Ca transport and LY passage (n = 32) after 30-min incubation is shown for the ileum (C panel).

Fig.1 MINEO H et al

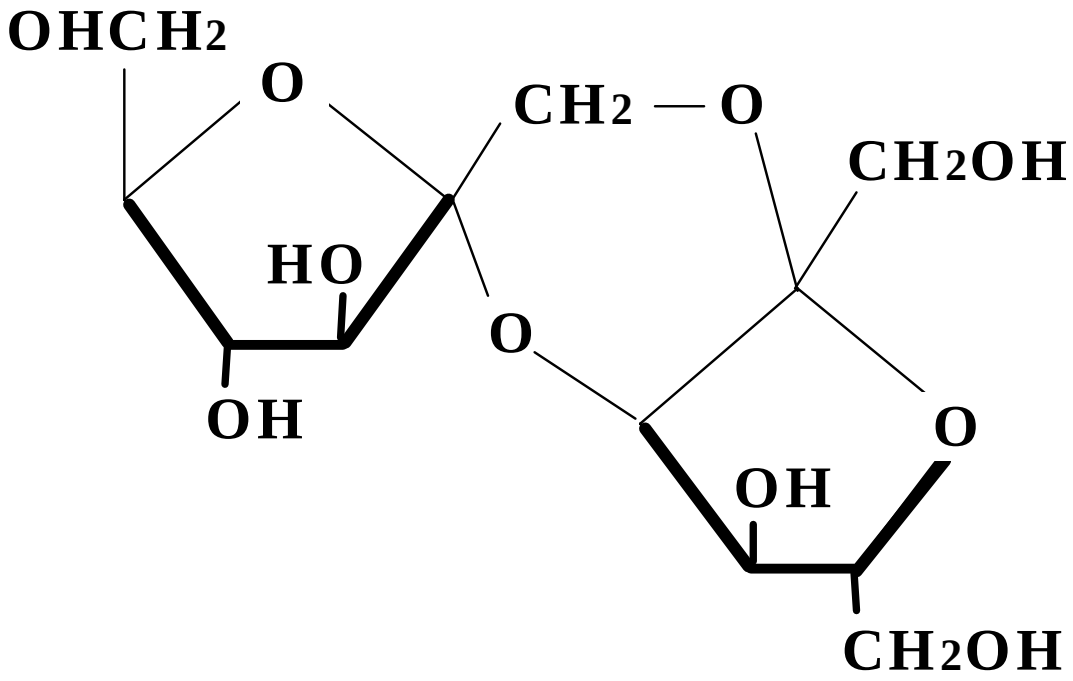


Fig.2 MINEO H et al

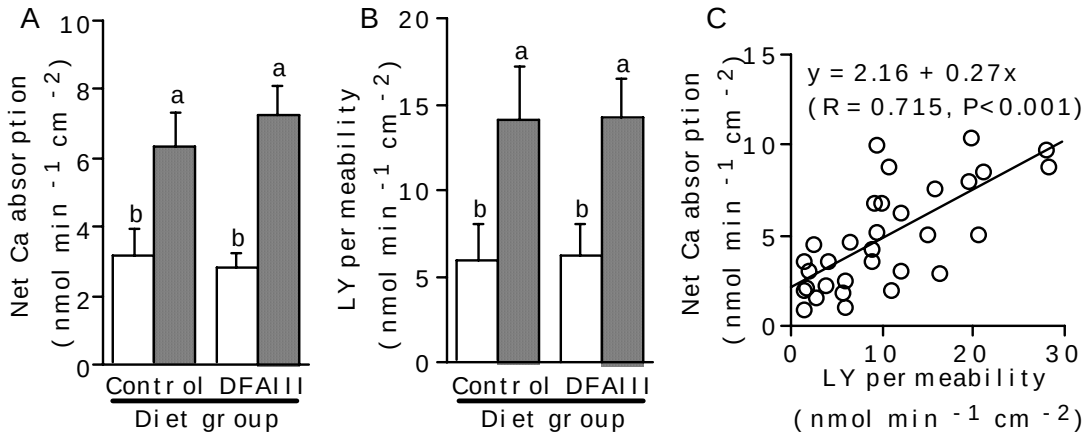


Fig.3 MINEO H et al

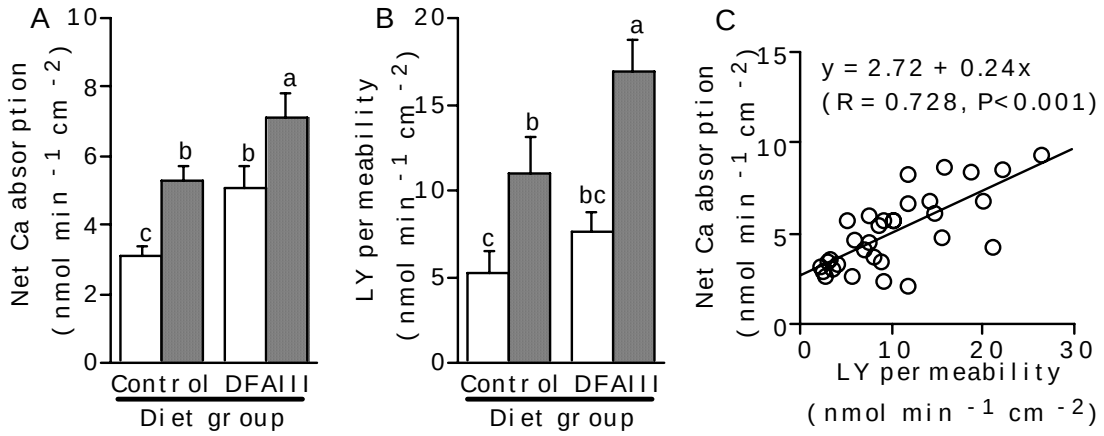
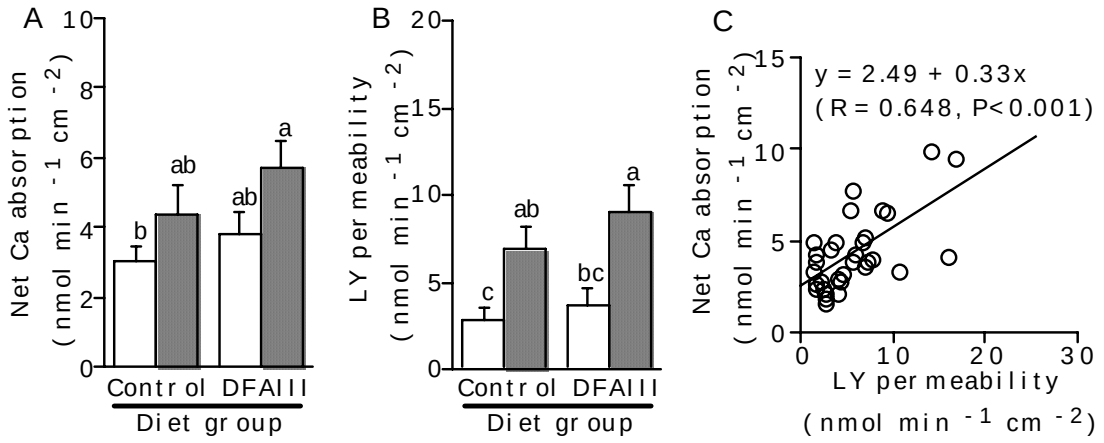


Fig.4 MINEO H et al



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