Title

Difructose anhydride III promotes iron absorption in the rat large intestine.

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Running title

Difructose Anhydride III & iron absorption

Abstract (250 words)

Objectives: We examined the intestinal segment responsible for and the mechanisms involved in the nondigestible disaccharide difructose anhydride (DFA) III-mediated enhancement of iron absorption.

Methods: Net iron and calcium absorption (%) were observed in male SD rats fed an AIN93G-based diet with or without DFAIII (30 g/kg diet) for 2 weeks after cecectomy or laparotomy (Exp.1). The absorption rates of calcium and iron from ligated jejunal loops (Exp.2) or ligated cecal sacs (Exp.3) in anesthetized rats fed a control or DFA diet were observed with or without the addition of DFAIII to the instilled mucosal fluid. Levels of the major iron transporter DMT-1 mRNA per 28S rRNA were evaluated by real-time PCR in the intestinal mucosa.

Results: Net absorption (%) of iron and calcium was higher in the DFA group than in the DFA-free group in laparotomized rats. The enhancement of iron absorption was almost and calcium absorption was partly abolished by cecectomy. The absorption rate of calcium, but not iron, from the jejunal loops was increased by the addition of DFAIII (100 mmol/L). Iron absorption was clearly higher in the ligated cecal sacs of the DFAIII-fed group than in the DFA-free group with or without the mucosal addition of DFAIII. DMT-1 mRNA level per 28S rRNA was maintained in the cecal mucosa through large increases in cecal size due to DFAIII feeding.

Conclusion: DFAIII-induced increases in iron absorption are the result of increased cecal iron absorptive capacity through expansion of the cecal mucosa maintaining DMT-1 mRNA expression.

Key words: Iron absorption; Difructose anhydride III; Small intestine, Cecum; Divalent cation transporter-1

Iron intake is relatively low in young females, and iron deficiency can result in anemia, with even a marginal deficiency also know to impair learning performance [1,2]. Enhancement of the iron absorption rate is effective for prevention of iron deficiency. We have demonstrated that a nondigestible disaccharide, difructose anhydride III (DFAIII), promotes calcium absorption in rats [3] and humans [4], and also promotes iron absorption in rats [5-7]. DFAIII has been manufactured and used as a food supplement, and this disaccharide is slowly fermented in human large intestine [8]. Nondigestible oligosaccharides generally produce some gastrointestinal symptoms when ingested in large doses in humans; however, the abdominal symptoms associated with DFAIII were comparable to those of palatinose [9].

The mechanisms for the promotion of calcium absorption by DFAIII feeding have been reported [10,11]. However, there is no information on the mechanisms for the DFAIII-mediated increase in iron absorption. The disaccharide promotes calcium absorption in both the small and large intestines [3,12]. The microbial fermentation products of nondigestible saccharides are responsible for the promotive effects observed in the latter segment [13-17].

The present study examined whether cecal fermentation of DFAIII is responsible for the enhancement of iron absorption by using cecectomized (CX) rats, and examined the direct effects of DFAIII on both the small and large intestinal iron absorption using the ligated jejunum and the cecum of anesthetized rats. We also examined adaptive changes in iron absorptive activity or capacity of the ligated cecum and the mucosal mRNA levels of divalent metal transporter-1 (DMT-1), the intestinal brush border membrane iron transporter, as adaptive increases in calcium absorption have been observed in the cecum of rats fed DFAIII [12,18].

Materials and Methods

Animals and diets

Male Sprague-Dawley rats (Japan SLC, Hamamatsu, Japan), weighing about 130-180 g were used in all experiments, and were housed individually in stainless-steel cages with mesh bottoms. The cages were placed in a room with controlled temperature (22-24°C), relative humidity (40-60%) and lighting (lights on: 0800-2000 h). Rats were given a semi-purified stock diet (shown in Table 1) for 7 days for acclimation, and then divided into several groups using the randomized block design based on body weight for four separate experiments. Rats were allowed free access to all diets and deionized water

during the acclimation and test diet period. Body weight and food intake were measured every day. All spilled feed was carefully collected and weighed. Food intake for the entire period was corrected by subtraction of the spillage.

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

Balance study for iron and calcium absorption using sham and cecectomized rats

In experiment 1, the rats were divided into two groups, with one group of rats undergoing cecectomy (CX) and the other group undergoing laparotomy (sham). Both operations were performed under the same anesthetic procedure (Nembutal: sodium pentobarbital 40 mg/kg of body weight; Abbott Laboratories, North Chicago, IL). All animals had free access to deionized water and the stock diet for a 6-day recovery period. After postoperative recovery, sham and CX rats were randomly assigned to two subgroups of 8-9 rats, and were fed a control or 3% DFA III diet (30 g DFAIII/kg diet) shown in Table 1 for 2 weeks. Di-D-fructose-1, 2′: 2, 3′-anhydride is a disaccharide consisting of two fructose residues with two glycoside linkages, and was kindly provided from Nippon Beet Sugar MFG Co. LTD (Obihiro, Japan).

Feces were collected from day 11 to day 14 to evaluate the absorption rates of calcium and iron. On the last day of the experiment, all rats were killed by withdrawal of the aortic blood under pentobarbital anesthesia (Nembutal: sodium pentobarbital 50 mg/kg of body weight; Abbott Laboratories). The liver was collected after a saline perfusion from the portal vein. The cecum was removed together with its contents, weighed, frozen immediately with liquid nitrogen, and stored at – 40°C until pH and organic acid analyses. The cecal contents were collected by cutting open the frozen cecal wall and washing it with saline. The content weight was evaluated as the difference in weight between the cecum with and without contents.

Collected feces and livers were freeze-dried and milled to a fine powder, and the powdered feces and liver were dry-ashed at a temperature linearly elevated to 550℃ for 6 hr, and then maintained at 550℃ for 18 hr using an electric furnace (EYELA, TMF-3200, Tokyo Rikakikai, Tokyo, Japan). The ashed samples were treated with 5.49 mol/l HCl at 200℃ for 30 minutes and were dissolved in 0.82 mol/l HCl. Iron and calcium concentrations in the ashed sample solutions were measured by atomic absorption spectrometry (Z-5310, Hitachi, Tokyo, Japan) after appropriate dilution.

Net absorption of iron or calcium was calculated using the following formula:

Net Fe or Ca absorption (%) = 100×(total Fe or Ca intake−fecal Fe or Ca excretion) / Fe or Ca intake.

The cecal contents were diluted with 4 volumes of deionized water and homogenized using a Teflon homogenizer. The pH of these homogenates was measured with a semiconducting electrode (ISFET pH sensor 0010-15C, HORIBA, Kyoto, Japan) as the pH of cecal contents. Soluble iron concentrations in the homogenate were determined using an assay kit (Fe C-test and UIBC-test, Wako Pure Chemical Industries), and ferrous iron concentrations were evaluated using the same kit without the use of a reducing agent. Concentrations of organic acids (acetic, propionic, butyric, succinic and lactic acids) in the homogenate of the cecal contents were measured after sample preparation by the procedure described previously [19, 20] using a HPLC system (LC-10ADvp, Shimadzu Seisakusyo, Kyoto, Japan) equipped with two Shim-pack SCR-102H columns (8 mm i.d. × 30 cm long, Shimadzu Seisakusyo) and an electroconductibility detector (CDD-6A, Shimadzu Seisakusyo).

Iron and calcium absorption rates in ligated jejunal loops (Exp.2) and ligated cecal sacs (Exp. 3)

In experiment 2, a 15-cm ligated jejunal loop was prepared in each acclimated rat under pentobarbital anesthesia. Briefly, the upper jejunum was drawn out from a small abdominal midline incision, the jejunal lumen was washed out with saline, and the segment was ligated 3 cm distal to the ligament of Treitz (the proximal end of the loop). Three ml of the control or test solution was then instilled into the loop by syringe with a blunted needle from the distal end of the segment, and the segment was ligated, prior to removal of the needle, at 15 cm distal to the proximal end. The ligated loop was returned to the abdominal cavity, and the abdominal incision was closed with a clip. The anesthetized rats were placed on a warm plate to maintain body temperature. The rats were killed 20 min after the instillation. The ligated loop was immediately removed, and the mucosal fluid was collected for the measurement of iron and calcium concentrations.

The mucosal solution instilled into the jejunal loops contained 10 mmol $CaCl_2$, 0.5 mmol $FeCl_2$, 10 mmol ascorbic acid, 106 mmol NaCl, 6 mmol L-glutamine, 30 mmol MOPS (pH 6.5) and 0.025 mmol [^{3}H] 1,2-polyethylene glycol 4000 (18.5 MBq / g, NEN Research Products, Boston, MA) / L. DFA III (100 mmol / L) was replaced in part with NaCl in the control solution to adjust osmolarity.

In experiment 3 using the ligated cecal sacs, acclimated rats were given a control or 3% DFAIII diet for 2 weeks. On the last day of the feeding period, a ligated cecal sac was prepared in each rat from both the control and DFAIII diet groups through an abdominal midline incision (about 3 cm) under pentobarbital anesthesia. Briefly, the proximal and distal ends of the cecum were ligated, and a small cut was made at the distal end of the cecum. The lumen was gently washed out with warmed saline and the experiment was initiated by the instillation of the mucosal medium containing short-chain fatty acids with or without DFAIII. The volume of instilled medium injected into the cecal sacs was 2 ml for the control rats and 4 ml for DFA-fed rats as determined from the wet weight of the cecal contents in each diet group (Table 3). The anesthetized rats were warmed on the plate, and were killed 20 min after the instillation. The ligated cecal sacs were immediately removed, and the mucosal fluid was collected. The ligated cecal sacs after fluid collection were weighed and the surface area of the mucosa was estimated using a digital camera and image analysis (ImageJ, NIH).

The mucosal solution used for the ligated cecal sacs was the same as that for the ligated jejunum except that it contained 84 mmol of sodium salts of short-chain fatty acids (SCFA, 48 mmol acetate, 24 mmol propionate and 12 mmol butyrate/ L) instead of L-glutamine as energy sources. DFA III (80 mmol /L) and SCFAs were replaced in part with NaCl in the control medium for the ligated cecal sacs to adjust osmolarity.

Calcium and iron concentrations in the mucosal fluids collected from the jejunal and cecal lumen were measured by atomic absorption spectrophotometry (Z-5310, Hitachi) after treatment with perchloric acid (final 0.5 mole/L). Radioactivity of [^{3}H] 1,2-polyethylene in the mucosal fluid collected from both the ligated jejunum and cecum was measured using a liquid scintillation system (LSC-5100, Aloka, Tokyo, Japan) with a scintillation cocktail containing 32 mmol DPO and 1 mmol dimethyl-POPOP / L of toluene: ethylene glycol monoethyl ether (1:1). Concentrations of short-chain fatty acids were also measured in the collected fluid from the cecal sacs by HPLC systems described above. All reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan) or Sigma Chemicals Co. (St. Louis, MO).

Absorption rates in the *in situ* experiments were expressed as nmol/cm segment • min for the jejunal loops or as nmol/cecum • min for the cecal sacs using values of iron or calcium absorption (%). Iron or calcium absorption (%) by the ligated jejunal loops and the cecal sacs were calculated using the following equation:

Absorption (%) = 100 - [([^{3}H] 1,2-polyethylene / iron or calcium) in the collected mucosal fluid] / [([^{3}H] 1,2-polyethylene / iron or calcium) in the instilled solution] x 100.

The absorption rates of SCFAs from the ligated cecal sacs were similarly evaluated.

Changes in iron transporter, DMT-1 mRNA, levels in each intestinal segment in rats fed DFAIII

In experiment 4, acclimated rats were allowed free access to a control diet or DFAIII diet for 3 days or 7 days. Rats were killed by withdrawal of aortic blood under pentobarbital anesthesia. For measurement of intestinal DMT-1 mRNA levels, the mucosa of the duodenum, jejunum (15 cm distal to the Treitz ligaments), ileum (5 cm proximal to the ileocecal valve) and cecum were lightly scraped from each intestinal segment using a glass slide. The cecum and its contents were collected, and their wet weight and pH were measured as in experiment 1.

The mucosa collected for the evaluation of DMT-1 (NM_013173) [21] mRNA levels were immediately homogenized in Isogen (RNA extraction mixture, Nippon Gene, Tokyo, Japan) using a Polytron homogenizer (KINEMATICA, Amlehnhalde, Switzerland), and extracted RNA was estimated by absorbance at 260 nm. Relative DMT-1 mRNA levels (referenced by 28S rRNA) were quantified by reverse transcription (RT) followed by the real-time polymerase chain reaction method (LightCycler^{TM}, Roche Diagnostics, Tokyo, Japan). Complementary DNAs (cDNAs) were produced from 600 ng of intestinal total RNA using random hexamers and oligo (dT) primers (Takara Bio Inc., Ohtsu, Japan). The real-time PCR was performed in a final volume of 10 μL containing 180 ng cDNA, 1 μL of reaction mixture containing LightCycler-FastStart DNA master SYBR Green I (Roche Diagnostics), and 3.3 pmol of the specific upper and lower primers. The PCR conditions were 10 min at 95°C followed by 45 cycles of 10 s at 95°C, 5 s at 54°C and 3 s at 72°C for DMT-1, and by 45 cycles of 10 s at 95°C, 14 s at 63°C and 14 s at 72°C for 28S rRNA. The primers for DMT-1 were 5’-GTGGCTAATGGTGGAGTTGG-3’ (upper) and 5’-GGCAGACAGGAGATTGATGG-3’ (lower), and those for 28S rRNA were 5’-GCTCGCTTGATCTTGATTTTCAGT-3’ (upper) and 5’-AAACCCAGCTCACGTTCCCTATTA-3’ (lower). Message levels of DMT-1 were normalized to those of 28S rRNA within the sample, and are expressed as arbitrary units.

Statistical analyses

All values are expressed as means ± the standard error of the mean. Statistical analyses were performed by one-way ANOVA, two-way ANOVA followed by Tukey's post-hoc test (one-way ANOVA, Table 5 and Fig. 4; two-way ANOVA, Tables 2 and 4, Figs. 1 and 3) or Student's *t*-test (Table 3, Fig. 2). A difference with a *P* value < 0·05 was considered significant. These statistical analyses were performed using the general linear models procedure of the Statistical Analysis Systems program (SAS Institute Inc., Cary, NC).

Results

Contribution of the cecum to the effects of DFAIII on iron and calcium absorption in a balance study using CX rats (Exp. 1)

The results of two-way ANOVA showed that cecectomy influenced body weight gains without affecting food intake (Table 2), with the body weight gains in CX rats found to be less than those in sham rats. Liver iron concentration in the DFAIII group was clearly higher than that in the control group in sham rats but not in CX rats.

Iron absorption (%) in sham rats was 50% higher in the DFAIII group than in the control diet group, but there were no significant differences between the diet groups in the CX rats (Fig. 1). The results of two-way ANOVA indicate that there is an interaction between the CX operation and Diet for iron absorption, but not for calcium absorption. Calcium absorption rates were influenced by DFAIII feeding, with higher rates observed in both sham and CX rats fed a DFAIII diet than in rats fed a control diet. However, the difference between the control and DFAIII diet groups was reduced by CX operation.

Wet weight of the cecal wall was greater in the DFA group than in the control group (Table 3). With regard to cecal contents, the pH value was lower and total SCFA pool was higher in the DFA-fed rats than in the control rats. The levels of all evaluated organic acids, except for butyric acid, were much higher in the DFA group than in the control group.

Effects of DFAIII on iron and calcium absorption in the ligated jejunum and cecum of anesthetized rats (Exp. 2 and 3)

In the ligated jejunal segments of anesthetized rats fed a control diet, the rate of calcium absorption per cm segment was higher from the instilled mucosal fluid containing DFAIII than from the DFA-free fluid; however, the rate of iron absorption was not changed by the addition of DFAIII to the mucosal medium instilled into the ligated jejunal lumen (Fig. 2).

Rates of iron and calcium absorption by ligated whole cecal sacs in rats fed a DFAIII diet were greater than those by the ligated sacs in rats fed a control diet, according to the results of two-way ANOVA (*P* values for Diet were both < 0.001, Fig. 3). An interaction was observed between Diet (DFA feeding) and Medium (addition of DFA to the mucosal fluid) for iron absorption, but not for calcium absorption. The rate of iron absorption from the mucosal fluid containing DFAIII by the cecal sacs in rats fed a DFAIII diet was more than 2-fold higher than that from the fluid without DFAIII by the sacs in the control rats. The former condition simulates the cecum of DFA-fed rats and the latter simulates the cecum of the control rats.

Absorption rate of acetic, propionic and butyric acids included in the mucosal fluid by the whole cecal sacs in rats fed a DFAIII diet were higher than those in rats fed a control diet, according to the results of two-way ANOVA (Table 4). The rates of absorption of the three short-chain fatty acids were increased with the addition of DFAIII to the mucosal fluid.

Surface area and wet weight of the cecal wall in the DFA group (11.2 ± 0.29 cm^2, 0.657 ± 0.013 g/100 g body weight) were both 2-fold greater than those in the control group (5.56 ± 0.17 cm^2, 0.336 ± 0.009 g/100 g body weight). No significant differences were observed in body weight gain or food intake between the control and DFA groups.

Changes in mRNA levels of divalent cation transporter (DMT)-1 in the intestine by feeding with DFAIII

There were no significant differences in the levels of DMT-1 mRNA normalized by 28S rRNA in either the jejunal or cecal mucosa on day 3 or day 7 between the control and DFAIII diet groups (Fig. 4). Average values of the mucosal mRNA levels of DMT-1 normalized by 28S rRNA for 30 rats (sum of 5 groups) were 12.9 (*P* = 0.881) for the duodenum, 12.8 (*P* = 0.555) for the jejunum, 1.52 (*P* = 0.504) for the ileum and 0.85 (*P* = 0.287) for the cecum.

Weights of the cecal wall and contents in the DFAIII group were higher than those in the control group on both day 3 and day 7 (Table 5). The cecal wall weight in the DFAIII group was greater on day 7 than on day 3. The pH value of the cecal contents was lower in the DFAIII group than in the control group on both day 3 and 7. Soluble total iron concentration in the cecal contents of rats fed a control or DFAIII diet for 7 days was 196 ± 40 μmol/L and 209 ± 39 μmol/L, respectively, and the cecal ferrous iron concentration

of rats fed a control or DFAIII diet for 7 days was 21.9 ± 6.7 μmol/L and 33.9 ± 4.1 μmol/L , respectively. There were no differences in these iron concentrations between the diet groups. Body weight gain and food intake were not influenced by feeding with the test diets.

Discussion

Results of the rat balance study showed that feeding with DFAIII increased net iron and calcium absorption. Cecectomy largely reduced the promotive effect of DFAIII on iron absorption; however, the effect on calcium absorption was restored to a greater degree than that on iron absorption in CX rats. The results of two-way ANOVA showed a significant interaction between CX and DFA for iron absorption, which suggests that the effects of DFAIII feeding on iron absorption differ between sham and CX rats. These results indicate that the enhancement of iron absorption by DFAIII largely depends on the cecum, which may in turn be dependent on the cecal fermentation of DFAIII. The enhancement of calcium absorption by DFAIII was also reduced, but remained relatively constant after CX, suggesting that DFA promotes calcium absorption in intestinal segments other than the cecum; i.e., in the small intestine. It has been shown that DFAIII increases calcium absorption through the paracellular transport pathway in the small intestine [22,23].

The addition of DFAIII to the luminal fluid stimulated calcium absorption in the ligated jejunum of rats fed a control diet, which indicates that adaptive changes are not necessary to increase calcium absorption in the small intestine. This finding agrees with the results of previous *in vitro* studies using everted intestinal sacs [3] and stripped intestinal mucosa [8]. In contrast, iron absorption by the ligated small intestine was not affected by the addition of DFAIII to the luminal fluid, which agrees with the results of an *in vivo* balance study using CX rats. This result suggests that the contribution of the small intestine to the enhancement of iron absorption is very small. These results also suggest that iron is not absorbed via the paracellular transport pathway in the small intestine.

An experiment using the ligated cecum was performed in rats fed a control or DFAIII diet for 2 weeks to examine the adaptive responses to DFAIII feeding as DFAIII has been known to be fermented by cecal bacteria [24]. The addition of DFAIII to the luminal fluid of the ligated cecum in the control rats did not produce any effect on the absorption rate of either iron or calcium. In contrast, the absorption rates of both minerals were clearly increased in the cecal sacs of the DFA-fed rats compared with those of the

control rats, according to the results of 2-way ANOVA (*P* values for Diet are both <0.001). These findings reveal that the feeding of DFAIII adaptively increased the absorptive activities or capacities for iron and calcium in the cecum. In the case of iron absorption, there was a 3-fold increase in the cecal sacs of rats fed the DFA diet compared with those fed the control diet when DFAIII was added to the luminal fluid in the ligated cecum. These results suggest that the increase in absorption activity or capacity in the cecum is associated with the promotion of iron absorption by feeding DFAIII in the balance study. The mucosal surface area and weight of the cecal wall were doubled by feeding DFAIII. We previously showed increases in the crypt depth and cell number/crypt in rats fed DFAIII, and these changes are highly correlated to the increase in calcium absorption in the stripped cecal mucosa [25]. This mucosal enlargement with increased epithelial cell proliferation may be involved in the increase in the cecal capacity for iron absorption in DFA-fed rats. The present study shows increases in cecal pools of short-chain fatty acids, which are known to stimulate epithelial cell proliferation [26]. We also found that absorption of short-chain fatty acids (acetic, propionic and butyric acids) included in the instilled luminal fluid was increased by the addition of DFAIII to the fluid in the ligated cecum. Iron absorption by the ligated cecum of DFA-fed rats also tended to be increased with the addition of DFA. The enhancement of cecal iron absorption might be associated with the increases in SCFA absorption stimulated by the mucosal application of DFAIII.

Divalent metal transporter-1 is well known as a major iron transporter in the small intestinal brush border membrane [27], and the small intestine is known to play a major role in iron absorption. We found DMT-1 mRNA to be expressed in the cecal mucosa. This finding suggests that DMT-1 is involved in cecal iron absorption as well as in that occurring in the small intestine. The cecal level of DMT-1 mRNA was one-fifteenth that of the levels found in the duodenal and jejunal mucosa. However, the transit speed of the cecal contents is much slower than that of the upper small intestine, which reveals that iron absorption in the cecum may contribute significantly to the total iron absorption of the entire intestinal tract. The soluble ferrous iron concentration in the cecal contents was 20-30 μmol/L, as described in the Results section, and the level of ferrous iron has been shown to be much higher than the *Kt* value for DMT-1; that is, 1–2 μmol/L [28]. Relative levels of DMT-1 mRNA in the cecal mucosa and in the jejunum were not changed by feeding with DFAIII; however, these values are normalized against 28S rRNA. We showed both the mucosal surface area and the weight of the cecal mucosa

to be increased. These results indicate that DMT-1 mRNA in the whole cecum is increased with expansion of the cecum induced by DFAIII feeding, and the increase in the iron transporter per whole cecum probably contributes to the enhancement of iron absorption in the cecum of the DFA-fed rats.

Ohta et al. showed that feeding of fructooligosaccharides increased iron absorption in iron-deficient rats [29]. Feeding of fructooligosaccharides also improves anemia associated with iron malabsorption in gastrectomised rats [30]. These reports also suggested that the cecal and colonic fermentation of these oligosaccharides is responsible for the beneficial effect observed in the present study. Kim et al. demonstrated that a high methoxy, low molecular weight pectin enhanced iron absorption in rats, and suggested that the maintenance of iron solubility in the small intestine by negatively charged pectin is associated with the enhancement of iron absorption [31]. However, this may not be in the case for DFAIII as this disaccharide has no electrical charge to bind the iron or calcium ions. In fact, the results from CX rats indicate that DFAIII has little or no effect on iron absorption in the small intestine.

Conclusion

Changes in cecal tissues with the increased microbial fermentation were largely responsible for the enhancement of iron absorption induced by DFAIII feeding, and an increase in the level of iron transporter in the whole cecum with the mucosal expansion may be involved in these promotive effects.

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Figure legends

Fig. 1 Iron and calcium absorption (%) as evaluated by a balance study using sham-operated and cecectomized rats fed a control or DFAIII diet for 2 weeks. Values are mean ± SEM (n= 7-9), and means not sharing a superscript letter differ significantly according to the post-hoc test ($P < 0.05$). *P* values of 2-way ANOVA were 0.781, 0.016 and 0.041 for CX, Diet and CX x Diet for iron absorption, respectively, and 0.100, 0.001 and 0.237 for CX, Diet and CX x Diet for calcium absorption, respectively.

Fig. 2 Iron and calcium absorption rates from instilled mucosal medium with or without DFAIII by ligated jejunal loops in anesthetized rats fed a control diet. Values are mean ± SEM (n=8). The asterisk represents a significant difference between groups ($P < 0.05$).

Fig. 3 Iron and calcium absorption rates from instilled mucosal medium with or without DFAIII by ligated cecal sacs in anesthetized rats fed a control or DFAIII diet for 2 weeks. Values are mean ± SEM (n=8). Means not sharing a superscript letter differ significantly according to the post-hoc test ($P < 0.05$). *P* values of 2-way ANOVA were < 0.001, 0.196 and 0.015 for Diet, Medium and Diet x Medium in iron absorption, respectively, and < 0.001, 0.212 and 0.186 for Diet, Medium and Diet x Medium in calcium absorption, respectively.

Fig. 4 Divalent metal transporter-1 (DMT-1) mRNA levels in the jejunal and cecal mucosa of rats fed a control or DFAIII diet for 3 and 7 days. Values are mean ± SEM (n=6).

Table 1 Composition of stock and test diets

	Stock diet & Control diet (g/kg diet)	DFAIII diet (g/kg diet)
Casein *	200	200
Dextrin †	400	400
Sucrose	200	170
Soybean oil	70	70
Mineral mixture ‡	35	35
Vitamin mixture ‡	10	10
Choline bitartrate	2.5	2.5
L-Cystine	3.0	3.0
Tert-butylhydroquinone	0.014	0.014
Crystallized cellulose §	80	80
DFAII I‖	0	30

* ALACID (New Zealand Dairy Board, Wellington, New Zealand).

† TK-16 (Matsutani Chemical Industry, Hyogo, Japan).

‡ AIN-93G formulation. Iron and calcium were present in the 35 mg/kg diet as ferric citrate and 5,000 mg/kg diet as calcium carbonate, respectively.

§ Avicel PH102 (Asahi Chemical Industry, Tokyo, Japan).

‖ Difructose Anhydride III (Nippon Beet Sugar Manufacturing, Tokyo, Japan).

Table 2 Changes in body weight, food intake and liver iron concentration in sham and cecectomized rats fed a diet with or without DFAIII (Exp. 1)

	Body weight gain (g/day)	Food intake (g/day)	Liver Fe (μg/g dry liver)
Sham rats			
Control diet	7.15 ± 0.23	23.6 ± 0.43 [a]	1.99 ± 0.23 [b]
DFAIII diet	6.68 ± 0.34	20.7 ± 0.36 [b]	3.00 ± 1.00 [a]
Cecectomized rats			
Control diet	6.55 ± 0.31	23.4 ± 0.50 [a]	2.25 ± 0.35 [b]
DFAIII diet	5.86 ± 0.42	23.3 ± 0.50 [a]	2.16 ± 0.22 [b]
ANOVA *P* values			
Cecectomy (C)	0.044	0.160	0.255
Diet (D)	0.095	0.074	0.071
C x D	0.759	0.025	0.034

Values are mean ± SEM (n = 7-9). Means not sharing a superscript letter differ significantly according to the post-hoc test ($P < 0.05$).

Table 3 Changes in several parameters for cecal fermentation in sham rats fed DFAIII for 2 weeks (Exp.1)

	Control diet	DFAIII diet
Weight of cecal contents (g)	2.59 ± 0.18	4.50 ± 0.53*
pH of cecal contents	7.55 ± 0.10	5.99 ± 0.04*
Organic acid pools (μmol/cecum)		
Total SCFAs	167 ± 9	255 ± 36*
Acetic acid	117 ± 8	182 ± 26*
Propionic acid	19.3 ± 2.0	44.6 ± 9.6*
Butyric acid	30.6 ± 1.7	28.0 ± 5.7
Lactic acid	2.5 ± 0.9	11.4 ± 2.2*
Succinic acid	56.0 ± 15.0	143 ± 33*

Values are mean ± SEM (n = 7-9). Asterisks represent significant differences between diet groups according to Student *t*-test ($P < 0.05$).

Table 4 Changes in absorption rates of short-chain fatty acids in the ligated cecal sacs of anesthetized rats by feeding a control or DFAIII diet for 2 week, or by the instillation of a mucosal medium with and without DFAIII (Exp. 3)

	Acetic acid	Propionic acid (μmol/min・cecum)	Butyric acid
Control diet			
Control medium	159 ± 5.8	89.2 ± 2.89	49.3 ± 1.35
DFAIII-added medium	198 ± 8.2	110 ± 4.1	56.4 ± 2.24
DFAIII diet			
Control medium	259 ± 26.7	149 ± 14.6	84.6 ± 8.01
DFAIII-added medium	337 ± 19.8	189 ± 10.0	99.3 ± 4.92
ANOVA *P* values			
Diet (D)	< 0.001	< 0.001	< 0.001
Medium (M)	< 0.001	< 0.001	0.012
D x M	0.980	0.915	0.956

Values are mean ± SEM (n=8).

Table 5 Periodic changes in cecal parameters in rats fed a control diet or DFAIII diet (Exp. 4)

	Cecal wall (wet g)	Cecal contents (wet g)	Content pH
Day 0	1.00 ± 0.030 [c]	2.11 ± 1.01 [b]	7.23 ± 0.15 [a]
Control Diet			
Day 3	1.00 ± 0.053 [c]	2.34 ± 0.27 [b]	7.47 ± 0.16 [a]
Day 7	0.94 ± 0.021 [c]	2.99 ± 0.23 [b]	7.38 ± 0.07 [a]
DFAIII diet			
Day 3	1.42 ± 0.070 [b]	7.27 ± 0.40 [a]	6.52 ± 0.13 [b]
Day 7	1.97 ± 0.059 [a]	7.83 ± 1.10 [a]	6.32 ± 0.03 [b]
ANOVA *P* values	< 0.001	< 0.001	< 0.001

Values are mean ± SEM (n=8). Means not sharing a superscript letter differ significantly ($P < 0.05$).

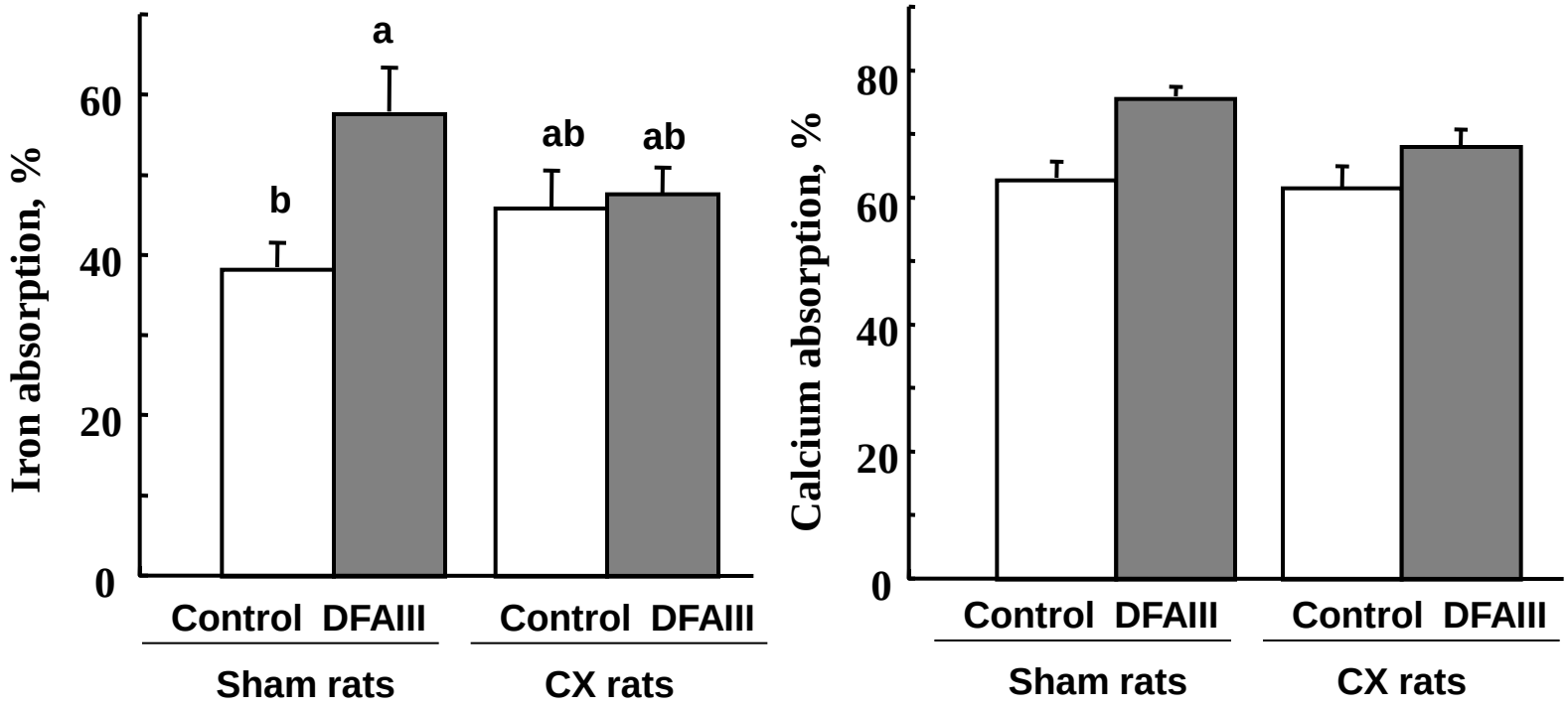
Iron absorption, %
0
20
40
60
a
b
ab
ab
Control
DFAIII
Control
DFAIII
Sham rats
CX rats
Calcium absorption, %
0
20
40
60
80
Control
DFAIII
Control
DFAIII
Sham rats
CX rats

Fig. 1

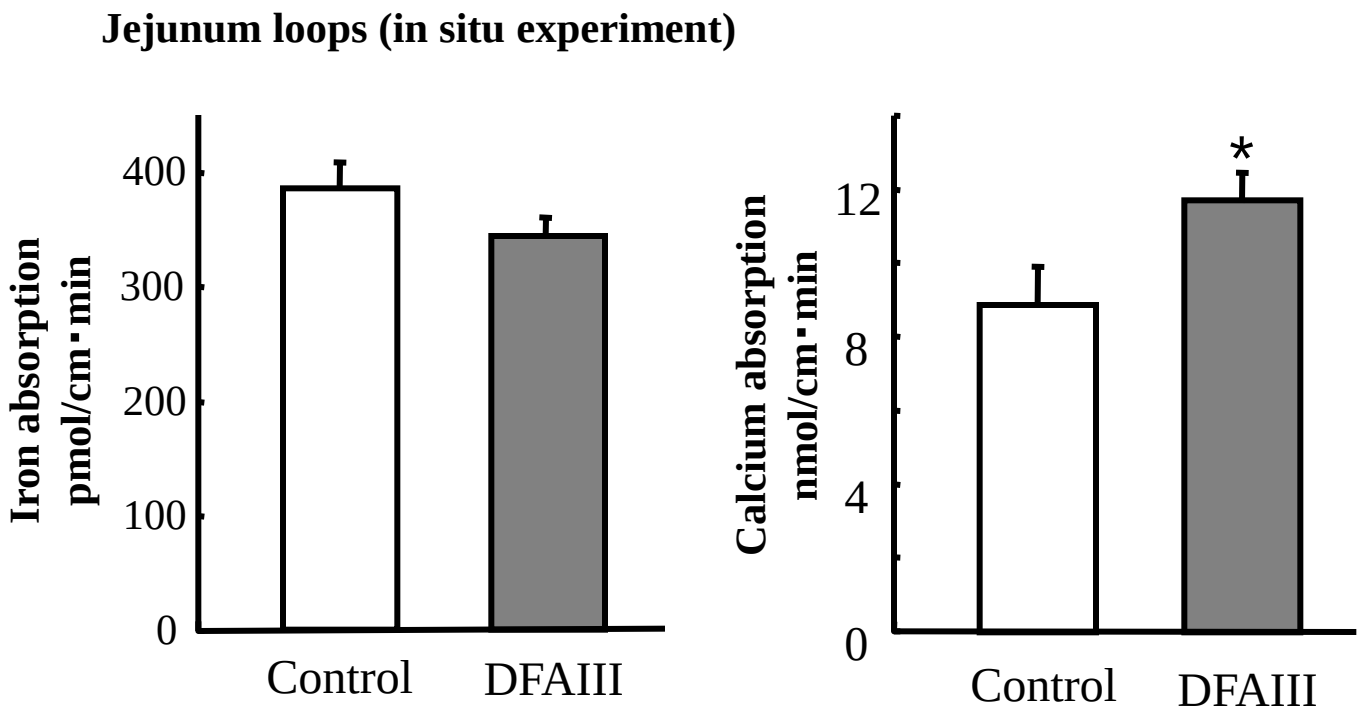
Jejunum loops (in situ experiment)
Iron absorption
pmol/cm·min
400
300
200
100
0
Control
DFAIII
Calcium absorption
nmol/cm·min
12
8
4
0
*
Control
DFAIII

Fig. 2

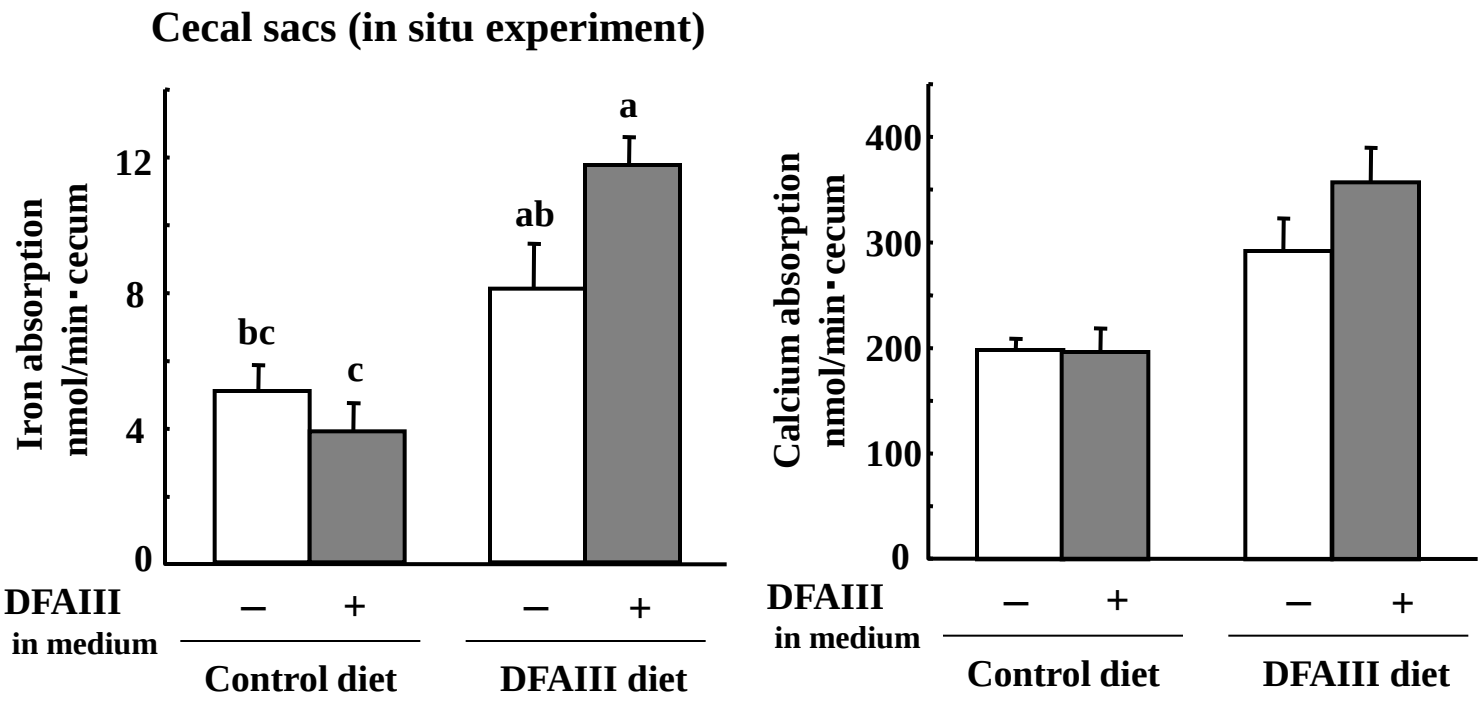
Cecal sacs (in situ experiment)
Iron absorption
nmol/min・cecum
12
8
4
0
bc
c
ab
a
DFAIII
in medium
–
+
–
+
Control diet
DFAIII diet
Calcium absorption
nmol/min・cecum
400
300
200
100
0
DFAIII
in medium
–
+
–
+
Control diet
DFAIII diet

Fig. 3

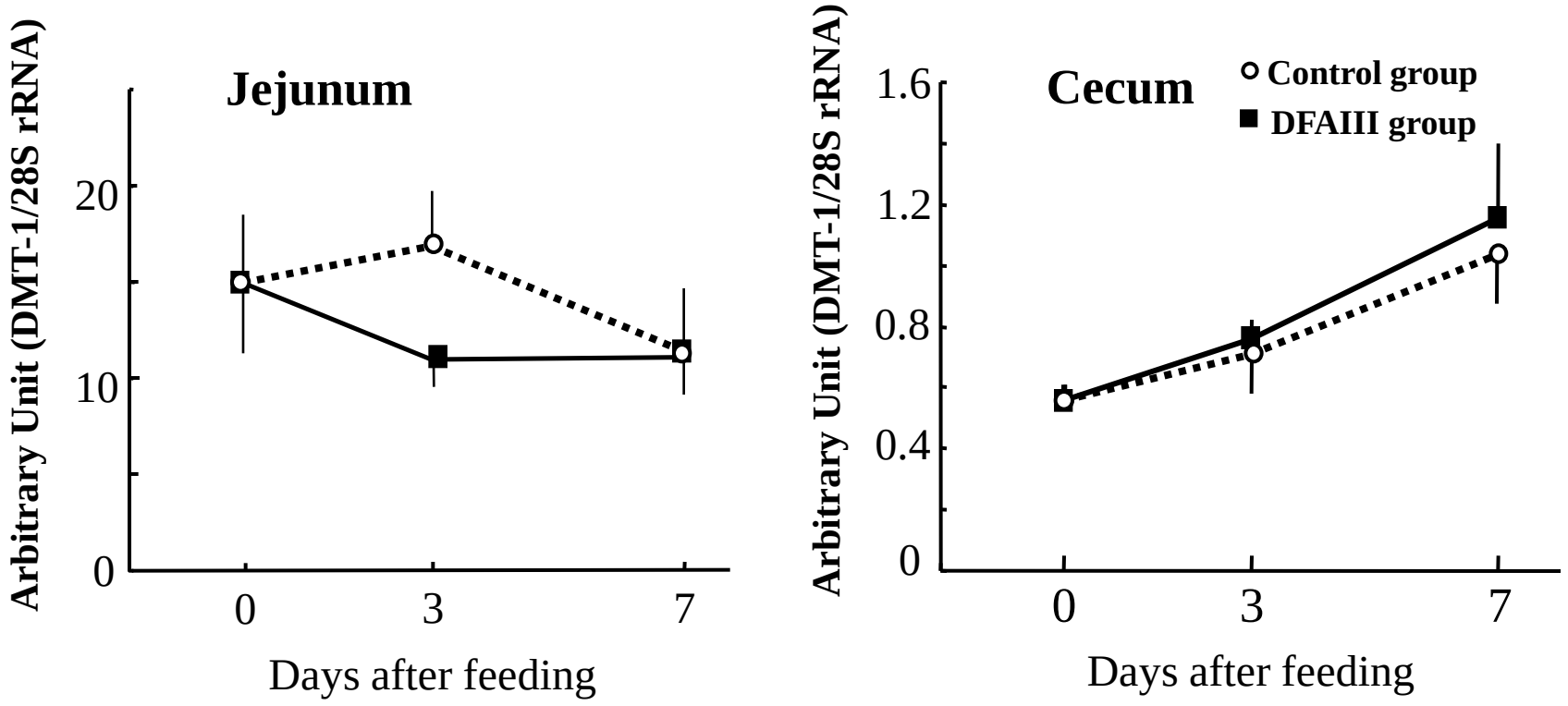
Jejunum
Arbitrary Unit (DMT-1/28S rRNA)
20
10
0
0
3
7
Days after feeding
Cecum
Control group
DFAIII group
Arbitrary Unit (DMT-1/28S rRNA)
1.6
1.2
0.8
0.4
0
0
3
7
Days after feeding

Fig. 4