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Title:

Ingestion of Difructose Anhydride III Partially Restores Calcium Absorption
Impaired by Vitamin D and Estrogen Deficiency in Rats

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Column Title:

DFAIII prevents Ca malabsorption in rats

Abbreviations:

DFAIII, difructose anhydride III; GAPDH, glyceraldehydes-3-phosphate
dehydrogenase; IGF-1, insulin-like growth factor 1; OVX, ovariectomy;
RT-PCR, reverse transcription-polymerase chain reaction; SCFA, short-chain
fatty acid; VD, vitamin D; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃.

Summary

Background Vitamin D (VD) and estrogen deficiencies impair Ca absorption and bone mineralization, and the relevance of the interaction between these factors has not been fully understood. **Aim of the study** The aim of the present study was to clarify the effects of a nondigestible saccharide, difructose anhydride III (DFAIII), on the interaction of VD and estrogen deficiencies involved in Ca malabsorption by assessing changes in intestinal Ca absorption and bone mineralization by feeding of DFAIII in rats with VD or estrogen deficiency or with a combined loss of VD and estrogen. **Methods** Three-wk-old female Sprague-Dawley rats were divided into four groups: two groups were ovariectomized (OVX) and two were laparotomized (sham). One group each of OVX and sham rats were fed an AIN93G-based normal diet, and the other groups were fed a VD-deficient diet for 8-wk. Rats from the four groups were divided into two subgroups and fed the normal or VD-deficient diet with or without DFAIII for next 4-wk. **Results** VD-deficiency decreased Ca absorption and bone mineralization with reductions in duodenal calbindin-D9k mRNA and serum Ca levels. There were no additional reductions in these parameters in the OVX. The reductions in Ca absorption and femoral Ca were restored partially or fully by DFAIII. Recovery of Ca absorption rate by DFAIII was greater in the OVX than in the sham showing an interaction between OVX and VD-deficiency in, at least, the DFAIII-fed groups. The cecal pH was lower and the level of short-chain fatty acids in the cecal contents was higher in all the DFAIII groups than those in the control groups. **Conclusions** VD deficiency impaired Ca absorption and bone mineralization, and feeding DFAIII partially restored Ca malabsorption and fully recovered bone Ca in VD-deficient rats. No additional reductions in these parameters with a combination of VD deficiency and OVX were noted: however, interactions were found between these factors in the DFAIII-induced increase in Ca absorption.

Key words: DFAIII, calcium absorption, vitamin D deficiency, ovariectomy, rats

Introduction

1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] is recognized as playing a role in transcellular calcium (Ca) absorption and maintenance of Ca homeostasis [1-4]. The active transcellular process involving vitamin D (VD) requires three groups of Ca transport proteins, Ca transport protein type 1, calbindin-D9k (the intracellular Ca transporter) and Ca ATPase [1, 2]. Several reports show that VD increases the level of calbindin D9k in cells [1, 2] and that the calbindin-D9k mRNA level is decreased in the duodenum of VD receptor-knockout mice and VD-deficient animals [1-4]. Moreover, a previous study demonstrated that 1,25(OH)₂D₃ also increases paracellular Ca flux through changes in the chemical structure of tight junctions [5], which is another important pathway for intestinal Ca absorption. Therefore, VD deficiency may decrease Ca absorption through both transcellular and paracellular pathways.

Estrogen also influences active intestinal Ca absorption. The presence of estrogen receptors in rat duodenal cells was confirmed [6, 7] and estrogen may have a direct physiological role via these estrogen receptors in regulating intestinal Ca absorption [8, 9]. It has been reported that estrogen deficiency reduced duodenal Ca transport protein type 1 mRNA expression [3]. However, some reports showed that estrogen deficiency decreases VD receptor and calbindin D9k mRNA levels in the intestinal mucosa, and these reductions are recovered by estrogen treatment [10, 11]. These results suggest that down regulation of the VD receptor contributes to the Ca malabsorption in estrogen deficiency.

Several reports have indicated that ingestion of oligosaccharides and fermentable dietary fibers increase Ca absorption in rats [12-15]. Difructose anhydride III (DFAIII) is a newly manufactured nondigestible saccharide prepared from inulin with *Arthrobacter* sp. H65-7 inulin fructotransferase (Inulinase II; EC 2. 4. 1. 93). Recent studies show that DFAIII promotes Ca absorption in *in vivo*, *in situ* and *in vitro* experiments [16-21]. The proposed mechanisms for the promotion of Ca absorption are that intact DFAIII stimulates paracellular Ca absorption in the small intestine [16, 17, 19], and DFAIII increases the large

intestinal Ca absorption with enhancement of bacterial fermentation [20]. Our previous study demonstrated that feeding DFAIII restored the ovariectomy-induced reduction in Ca absorption and led to increases in femoral Ca content [22]. However, it is still not known whether DFAIII ingestion recovers impairment of Ca absorption associated with VD-deficiency or whether DFAIII has an effect on the interactive actions of VD and estrogen deficiencies in Ca absorption. DFAIII promotes paracellular Ca transport and VD has a role in the paracellular transport process as mentioned above. It is not clear whether DFAIII promotes Ca absorption under when both transcellular and paracellular pathways are down-regulated.

The aim of the present study was to clarify the effects of DFAIII on the interaction of VD and estrogen deficiencies involved in postmenopausal Ca malabsorption by assessing changes in intestinal Ca absorption and bone mineralization by feeding of DFAIII in rats with VD or estrogen deficiency or with a combined loss of VD and estrogen.

Materials and methods

Animals and diets.

Three-week-old female Sprague-Dawley rats (Japan Clea, Tokyo, Japan) weighing about 50 g were housed in individual stainless-steel cages with wire-mesh bottoms. The cages were placed in a room with controlled temperature (22-24°C), relative humidity (40-60%) and lighting (lights on 8:00-20:00 h). The rats had free access to water and the semipurified basal diets (Table 1) based on AIN93G formulation [23] for an acclimation period of 4 days. The final dietary content of Ca, magnesium (Mg) and phosphate (P) were 3000 mg Ca/kg diet, 507 mg Mg/kg diet and 3000 mg P/kg diet. The recommended dietary amount is same except for Ca. The recommended amount of Ca is 5000 mg Ca/kg diet.

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

Experimental design.

At 3-week-old, rats were divided into four groups, two groups underwent

94 bilateral ovariectomy (OVX) and the other two groups underwent bilateral
 95 laparotomy (sham). One group each of sham and OVX rats were fed an
 96 AIN93G-based normal diet, and rats of the other groups were fed a diet from
 97 which VD was excluded (VD-deficient) for 8 weeks (Table 1). Fluorescent lights
 98 were covered with sheets to cut ultraviolet radiation to inhibit light-synthesis VD.
 99 Food intake of rat in each group was adjusted to the average intake of the group
 100 with the lowest value in each day (pair-feeding). All rats were given free access to
 101 deionized water. At 11-week-old, the four groups of rats were divided into two
 102 subgroups of 8 rats, and fed a normal or VD-deficient diet with or without DFAIII
 103 (15g/kg diet) as shown in Table 1 for 4 weeks (the control or DFAIII group),
 104 respectively.

105 The body weight and food intake were measured every day. Feces were
 106 collected during the last 4 days of the test period. On the last day, the rats were
 107 anesthetized (Nembutal: sodium pentobarbital, 50 mg/kg body weight, Abbott
 108 Laboratories, North Chicago, IL, U.S.A.), and then killed after aortic blood was
 109 taken. Blood was centrifuged (1,300 g for 10 min at 4°C) to obtain the serum. The
 110 proximal duodenum (0-5 cm distal to the pylorus) was removed, washed in cold
 111 saline (0.9% NaCl solution), slit lengthwise, and the mucosa collected onto a glass
 112 microscope slide. The mucosa was quickly frozen with liquid nitrogen for analysis
 113 of calbindin mRNA. The uterus was removed from each rat and weighed to
 114 confirm the success of the ovariectomy. The cecum was removed with its contents
 115 and weighed. The contents were collected and stored at -40°C until subsequent
 116 analyses. The femur was removed from each rat, carefully cleaned of adherent
 117 tissue, and freeze-dried for measurement of mineral contents.

118 **Analytical methods.**

119 Total RNA in the duodenal mucosa was isolated using ISOGEN (Nippon
 120 Gene, Tokyo, Japan) according to manufacturer's suggested procedure, and the
 121 concentration of RNA was determined by the absorbance at 260 nm. Reverse
 122 transcription-polymerase chain reaction (RT-PCR) was performed to detect
 123 calbindin D9k and glyceraldehydes-3-phosphate dehydrogenase (GAPDH)
 124 mRNA. Total RNA (10 µg) was subjected to cDNA synthesis using reverse

transcriptase and a random primer. The cDNA was amplified in a PCR master mix (Promega, Madison, WI, U.S.A) with specific primers. Primers and annealing temperature were: calbindin D9k forward AAGAGCATTTTTC AAAAATA: reverse GTCTCAGAATTTGCTTTATT: annealing temperature = 42°C: GAPDH forward TCCACCACCCTGTTGCTGTAG: reverse GACCACAGTCCATGACATCACT: annealing temperature = 54°C. Twenty cycles for calbindin D9k and twenty-nine cycles for GAPDH were employed for semi-quantification. Ten microliters of PCR products were fractionated on a 1.5% agarose gel containing ethidium bromide and bands were visualized and quantified under UV light.

Freeze-dried feces were milled, and the powdered feces were wet-ashed with an acid mixture (16 mol/L HNO₃: 9 mol/L HClO₄ = 3:1) without drying. The amounts of Ca and Mg in the right femurs were measured after the samples had been wet-ashed in the same way as the feces. Ca and Mg concentrations in those solutions were measured by atomic absorption spectrophotometry (AA-6400F; Shimadzu Corporation, Kyoto, Japan) after appropriate dilution with 0.1 mol/L HCl. P was determined in the femoral solutions by the molybdovanadate method [24]. Serum Ca concentration was assayed with a commercial kit (Calcium-C test; Waco Pure Chemical Industries, Osaka, Japan).

The cecal contents were homogenized with nine volumes of deionized water. The pH values of these homogenates were measured with a semiconducting electrode (ISFET pH sensor 0010-15C, Horiba, Ltd., Kyoto, Japan). The organic acids in the cecal contents were measured by high-performance liquid chromatography (Organic Acid Analysis System, Shimadzu Corporation, Kyoto, Japan) as previously described [25].

Calculations and statistical analyses.

Ca absorption was calculated by the following equation:

$$\text{Ca absorption rate (\%)} = 100 \times (\text{Ca intake} - \text{Ca excretion in feces}) / \text{Ca intake}.$$

Values shown represent the means $\pm$ SEM. Statistical analyses were performed by three-way ANOVA (vitamin D deficiency $\times$ operation $\times$ diet). The significance of inter-group differences was evaluated by Duncan's multiple-range test [26] ($P <$

0.05). If the variance was unequal, log transformations of the data were performed before ANOVA. All statistical analyses were done using SPSS for Windows, Version 11.0 J (SPSS, Chicago, IL, U.S.A).

Results

The results of the 3-way ANOVA showed that final body weight, body weight gain and uterine weight were affected by OVX. There was an interaction between VD deficiency and OVX for final body weight (Table 2). The post hoc test showed that the final body weights of the VD-deficient rats were lower than those of the VD-normal rats (normal diet groups) in sham rats, but not in OVX rats.

Serum Ca concentration (Table 3) and calbindin D9k mRNA level (Fig. 1) were affected by VD deficiency according to the results of 3-way ANOVA. Neither operation nor diet influenced serum Ca concentration and calbindin D9k mRNA levels.

The ANOVA results show that VD deficiency and diet influenced Ca absorption rates (Fig. 2). Moreover, there were interactions between VD deficiency and operation, and between operation and diet. The Ca absorption rates were much lower in VD-deficient rats than in normal rats in the sham-control groups and also in the OVX-control groups by the results of post hoc test. OVX reduced Ca absorption in VD-normal rats, but the reduction was smaller than that in VD-deficient rats. OVX did not further reduce the absorption in VD-deficient rats. The absorption rate of Ca was higher in the DFAIII groups than in the control groups in both sham and OVX rats fed a normal or VD-deficient diet except for in the normal-sham rats. In the VD-deficient rat groups, Ca absorption rate in OVX rats fed the DFAIII diet was higher than in sham rats fed the DFAIII diet.

The results of 3-way ANOVA demonstrated that diet and OVX operation, but not VD deficiency influenced femoral Ca content, and there was a clear interaction between VD deficiency and DFAIII diet (Table 4). Femoral Ca content was lower in VD-deficient rats than in normal rats in the sham-control and OVX-control groups, and was higher in the DFAIII groups than in the control groups in VD-deficient sham and OVX rats. The results of ANOVA also showed

that diet and OVX operation influenced femoral Mg content. The Mg content was higher in the DFAIII group than in the control group only in the VD-deficient sham rats. Femoral P contents were influenced by VD deficiency and OVX operation, but not by DFAIII.

Cecal wall weight was affected by VD deficiency, OVX, and DFAIII diet (Table 5). Cecal pH value and the pool of total SCFA in the cecal content were affected by the DFAIII diet. VD deficiency and OVX operation showed a clear interaction with regard to total SCFA pools.

Discussion

In the present study, we demonstrated that both VD and estrogen deficiencies impaired Ca absorption in rats: however, the reduction in absorption was much greater in the VD-deficient rats. No combined effect of VD and estrogen deficiencies was found (Fig. 2). These results suggest that VD has a crucial role in both transcellular and paracellular Ca transport. It has been reported that VD receptor-knockout impairs Ca absorption and decreases calbindin D9k mRNA levels in mice [3, 4, 27]. In the present study, we showed that the duodenal calbindin D9k mRNA level was clearly decreased in all VD-deficient rat groups without any inter-group differences among the VD-deficient rats (Fig 1). These results confirm the induction of VD deficiency by our experimental conditions.

Estrogen deficiency with OVX impaired Ca absorption without any changes in calbindin D9K mRNA level (Fig. 1 & 2). Liel *et al* reported that duodenal calbindin D9k mRNA was decreased by OVX [10]. Another report showed that estrogen receptor- α knockout mice displayed a significant reduction in duodenal Ca transport protein type 1 mRNA expression [3]. We used a semiquantitative method to estimate duodenal calbindin D9k mRNA levels because we measured this mRNA level to confirm VD deficiency. A more quantitative method would likely show that OVX slightly but not significantly reduces calbindin D9k mRNA.

Ingestion of DFAIII was effective as a treatment for Ca malabsorption associated with estrogen deficiency, and was partly restored Ca malabsorption associated with VD deficiency (Fig. 2). Previously, we reported that DFAIII

promotes Ca absorption both in the small and large intestines [16-22]. In the large intestine, ingestion of DFAIII increases the SCFA pool and decreases the pH value, which may be associated with the increase in Ca absorption by DFAIII [20]. The active intracellular transport pathway may contribute to the enhanced absorption because there is a potential active Ca transport system in the large intestine [28] and DFAIII has a very limited effect of on the paracellular Ca transport in the cecum (unpublished results). Our current study showed a decrease in cecal pH and a tendency toward an increased SCFA pool in the DFAIII groups (Table 5), which agrees with the previous study and shows stimulation of cecal fermentation. These findings suggest that DFAIII enhances Ca absorption in the large intestine via an active transport pathway.

In the small intestine, DFAIII promotes Ca absorption via the paracellular transport pathway through tight junctions [16, 17, 19]. Ingestion of DFAIII did not fully restore Ca malabsorption in the VD-deficient group (Fig. 2). VD plays a role in paracellular Ca transport [5], and VD deficiency may impair paracellular absorption. It is possible that the promotive effect of DFAIII on paracellular Ca transport is weak under VD-deficient conditions, which would explain the incomplete recovery of VD deficiency-induced Ca malabsorption with DFAIII feeding.

In the case of VD-deficient rats fed DFAIII, Ca absorption rate was higher in OVX rats than in sham rats (Fig. 2), which was closely associated with the changes in bone Ca content (Table 4). Previous studies demonstrated that Ca homeostasis is also regulated by insulin-like growth factor 1 (IGF-1), and that OVX elevates serum and duodenal levels of IGF-1 [29-31]. On the other hand, VD receptor-knockout reduces serum IGF-1 levels and inhibits normal growth in mice [32]. The current study showed the lowest final body weight in VD-deficient sham rats with the same food intakes as in the other groups (15.2 g/d, $P = 0.994$, $n = 64$), and that OVX improved the low body weight in VD-deficient rats (Table 2). This suggests that the lower level of IGF-1 is partly associated with the lower Ca absorption in VD-deficient animals, and the increase in IGF-1 level due to OVX is involved in the higher Ca absorption and higher body weight in OVX rats under

249 VD-deficient conditions. This unexpected increase in Ca absorption by OVX is
250 true only in the DFAIII groups. Some factors limiting Ca absorption may exist in
251 the control diet groups: for example, low solubilization of the cecal Ca. Further
252 studies will be necessary to determine the role of IGF-1 levels on Ca absorption
253 and growth in VD- and estrogen-deficient rats.

254 We showed decreases in femoral Ca contents and serum Ca concentration
255 in VD-deficient rats compared with VD-normal rats (Table 3 & 4). And feeding
256 DFAIII fully recovered bone Ca content in VD-deficient rats, but did not affect
257 hypocalcemia. It has been reported that the primary cause for hypocalcemia in
258 VD deficiency is reduction in the capacity of osteoclastic resorption [33]. Under
259 the suppression of the bone resorption with VD deficiency, increase in bone
260 formation with increasing calcium absorption by feeding DFAIII may prevent
261 the reduction of bone calcium, but not hypocalcemia. Higher body weight may
262 be a factor for bone recovery in the OVX rats: however, this was not true in the
263 OVX-control group under VD-deficient conditions. In this group, no recovery in
264 bone strength was noted in rats with higher body weight. Some factors
265 influenced by DFAIII ingestion other than Ca absorption may also affect bone
266 metabolism. Both VD and estrogen deficiencies are well known to be involved in
267 osteoporosis [9, 10, 34, 35], and estrogen replacement therapy as well as VD
268 treatment has been shown to be effective in preventing bone loss [34-37]. An
269 adequate supply of Ca with improvement in Ca absorption by DFAIII may
270 effectively prevent bone loss.

271 In conclusion, VD deficiency impaired Ca absorption and bone
272 mineralization, and feeding DFAIII partially restored Ca malabsorption and fully
273 recovered bone Ca in VD-deficient rats. No additional reductions in these
274 parameters with a combination of VD deficiency and OVX were noted: however,
275 interactions were found between these factors in the DFAIII-induced increase in
276 Ca absorption. The large intestine may be partly involved in the beneficial effects
277 of DFAIII.

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Table 1 Composition of basal and test diets¹

Ingredient	Normal diet		Vitamin D-deficient diet	
	Control	DFAIII	Control	DFAIII
	g/kg			
Casein ²	250	250	-	-
Vitamin-free casein ³	-	-	250	250
Corn oil ⁴	50	50	50	50
Mineral mixture ⁵	35	35	35	35
Calcium carbonate ⁶	7.5	7.5	7.5	7.5
Vitamin mixture ⁷	10	10	-	-
Vitamin D-free Vitamin mixture ⁷	-	-	10	10
Choline bitartrate ⁸	2.5	2.5	2.5	2.5
Cellulose ⁹	50	35	50	35
DFAIII ¹⁰	-	15	-	15
Sucrose ¹¹	595	595	595	595

¹ The basal diet was same as normal-control diet; ² ALACID; New Zealand Dairy Board, Wellington, New Zealand; ³ Waco Pure Chemical Industries, Osaka, Japan; ⁴ Ajinomoto Co. INC., Tokyo, Japan; ⁵ AIN-93G mixture except for Ca. It provided (mg/kg diet): Ca 3000, P 1561, K 3600, S 300, Na 1019, Cl 1571, Mg 507, Fe 35.0, Zn 30.0, Mn 10.0, Cu 6.0, I 0.2, Mo 0.15, Se 0.15, Si 5.0, Cr 1.0, F 1.0, Ni 0.5, B 0.5, Li 0.1, V 0.1. Waco Pure Chemical Industries, Osaka, Japan; ⁶ Waco Pure Chemical Industries, Osaka, Japan; ⁷ AIN-93 mixture with or without vitamin D. It provided (U/kg diet): Nicotinic acid 30 mg, Pantothenate 15 mg, Pyridoxine 6 mg, Thiamin 5 mg, Riboflavin 6 mg, Folic acid 2 mg, vitamin K 750 µg, D-Biotin 200 µg, vitamin B₁₂ 25 µg, vitamin A 4000 IU, vitamin D₃ 1000 IU, vitamin E 75 IU. Waco Pure Chemical Industries, Osaka, Japan; ⁸ Waco Pure Chemical Industries, Osaka, Japan; ⁹ AVICEL; Asahi Chemical Industry Co. Ltd., Tokyo, Japan; ¹⁰ Nippon Beet Sugar Mfg. Co. Ltd., Obihiro, Japan; ¹¹ Nippon Beet Sugar Mfg. Co. Ltd., Obihiro, Japan

Table 2 Final body weight, food intake and uterine weight of sham and ovariectomized (OVX) rats on a normal or vitamin D-deficient diet with or without difructose anhydride III (DFAIII) for 4 weeks¹

	Final body weight	Body weight gain	Uterine weight
	g	g/day	mg/100g body wt
Normal			
Sham-control	252 ± 5.3 bc	1.1 ± 0.08 ab	254 ± 28.2 a
Sham-DFAIII	242 ± 6.6 cd	0.7 ± 0.14 b	301 ± 25.3 a
OVX-control	267 ± 3.7 a	1.1 ± 0.10 ab	21.5 ± 2.6 b
OVX-DFAIII	263 ± 3.1 ab	0.9 ± 0.07 ab	20.2 ± 1.7 b
Vitamin D deficiency			
Sham-control	238 ± 4.3 d	0.9 ± 0.11 ab	268 ± 27.1 a
Sham-DFAIII	235 ± 3.9 d	0.8 ± 0.13 ab	301 ± 38.6 a
OVX-control	270 ± 3.8 a	1.3 ± 0.09 a	17.5 ± 0.6 b
OVX-DFAIII	268 ± 5.5 a	1.1 ± 0.12 ab	21.3 ± 1.9 b
<i>P</i> -values			
Vitamin D (V)	0.324	0.358	0.842
Operation (O)	0.001	0.020	0.001
DFAIII (D)	0.123	0.003	0.176
V × O	0.027	0.156	0.767
V × D	0.434	0.357	0.873
O × D	0.630	0.526	0.202
V × O × D	0.617	0.342	0.743

¹ Values are means ± SEM, *n* = 8. Values in a column not sharing a common letter differ, *P* < 0.05.

Table 3 Serum calcium concentration in sham and ovariectomized (OVX) rats on a normal or vitamin D-deficient diet with or without Diffructose anhydride III (DFAIII) for 4 wk¹

Serum calcium concentration	
	mmol/L
Normal	
Sham-control	2.54 ± 0.079 a
Sham-DFAIII	2.49 ± 0.064 a
OVX-control	2.49 ± 0.062 a
OVX-DFAIII	2.39 ± 0.065 a
Vitamin D deficiency	
Sham-control	2.02 ± 0.145 b
Sham-DFAIII	1.99 ± 0.031 b
OVX-control	1.83 ± 0.089 b
OVX-DFAIII	1.95 ± 0.059 b
<i>P</i> -values	
Vitamin D (V)	0.001
Operation (O)	0.110
DFAIII (D)	0.767
V × O	0.767
V × D	0.308
O × D	0.666
V × O × D	0.435

¹ Values are means ± SEM, *n* = 8. Values in a column not sharing a common letter differ, *P* < 0.05.

Table 4 Femoral mineral contents of sham and ovariectomized (OVX) rats on a normal or vitamin D-deficient diet with or without Diffructose anhydride III (DFAIII) for 4 wk¹

	Calcium	Magnesium	Phosphate
	mmol/femur	μmol/femur	mmol/femur
Normal			
Sham-control	1.57 ± 0.012 bc	156 ± 2.4 bc	1.50 ± 0.068 abcd
Sham-DFAIII	1.58 ± 0.004 bc	157 ± 1.0 ab	1.46 ± 0.043 bcd
OVX-control	1.60 ± 0.005 b	162 ± 0.7 a	1.67 ± 0.052 a
OVX-DFAIII	1.59 ± 0.003 b	162 ± 0.5 a	1.64 ± 0.089 ab
Vitamin D deficiency			
Sham-control	1.50 ± 0.031 d	153 ± 1.9 c	1.42 ± 0.065 cd
Sham-DFAIII	1.60 ± 0.009 b	157 ± 1.7 ab	1.35 ± 0.036 d
OVX-control	1.53 ± 0.020 cd	158 ± 0.8 ab	1.52 ± 0.052 abcd
OVX-DFAIII	1.66 ± 0.019 a	162 ± 1.8 a	1.55 ± 0.048 abc
<i>P</i> -values			
Vitamin D (V)	0.199	0.092	0.013
Operation (O)	0.010	0.001	0.001
DFAIII (D)	0.001	0.040	0.515
V × O	0.200	0.867	0.637
V × D	0.001	0.134	0.826
O × D	0.728	0.487	0.492
V × O × D	0.371	0.719	0.591

¹ Values are means ± SEM, *n* = 8. Values in a column not sharing a common letter differ, *P* < 0.05.

Table 5 Cecal wall weight, pH value, and short-chain fatty acid (SCFA) pool in the cecal contents of sham and ovariectomized (OVX) rats on a normal or vitamin D-deficient diet with or without Diffructose anhydride III (DFAIII) for 4 wk¹

	Cecal wall weight	pH	Total SCFA ²
	g/kg body wt		μmol/cecum
Normal			
Sham-control	1.75 ± 0.155 cd	7.11 ± 0.123 a	45.8 ± 8.22 bc
Sham-DFAIII	2.30 ± 0.100 c	6.67 ± 0.081 b	78.0 ± 13.9 ab
OVX-control	2.16 ± 0.137 c	7.02 ± 0.092 a	27.2 ± 4.07 c
OVX-DFAIII	3.74 ± 0.369 a	6.98 ± 0.087 a	53.3 ± 6.79 abc
Vitamin D deficiency			
Sham-control	1.94 ± 0.164 cd	7.16 ± 0.053 a	44.2 ± 7.44 bc
Sham-DFAIII	2.15 ± 0.144 c	6.78 ± 0.084 b	56.9 ± 8.18 abc
OVX-control	1.59 ± 0.069 d	7.06 ± 0.087 a	61.3 ± 10.9 abc
OVX-DFAIII	2.96 ± 0.108 b	6.77 ± 0.059 b	84.8 ± 19.4 a
<i>P</i> -values			
Vitamin D (V)	0.012	0.350	0.169
Operation (O)	0.001	0.774	0.958
DFAIII (D)	0.001	0.001	0.003
V × O	0.008	0.566	0.006
V × D	0.282	0.903	0.474
O × D	0.001	0.263	0.876
V × O × D	0.794	0.467	0.582

¹ Values are means ± SEM, *n* = 8. Values in a column not sharing a common letter differ, *P* < 0.05.

² Total SCFA: sum of acetic, propionic and butyric acids in the cecal content.

Fig. 1

Fig. 2

Fig. 1

Reverse transcription-polymerase chain reaction (RT-PCR) of duodenal calbindin D9k mRNA levels and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) in sham and ovariectomized (OVX) rats on a normal or vitamin D-deficient diet with or without DFAIII. Gel data of calbindin D9k mRNA were recorded using a NIH-imaging System. Normal diet sham-control (A): 221 ± 14.4 , sham-DFAIII (B): 215 ± 12.2 , OVX-control (C): 206 ± 11.8 , OVX-DFAIII (D): 211 ± 12.8 , vitamin D-deficient diet sham-control (E): 140 ± 18.5 , sham-DFAIII (F): 141 ± 18.3 , OVX-control (G): 143 ± 15.9 , and OVX-DFAIII (H): 141 ± 18.0 . Values are means $\pm$ SEM, $n = 8$. *P*-values estimated by three-way ANOVA were <0.001 for vitamin D-deficiency (V), 0.712 for operation (O), 0.946 for DFAIII (D), 0.602 for $V \times O$, 0.995 for $V \times D$, 0.865 for $O \times D$ and 0.755 for $V \times O \times D$.

Fig. 2

Ca absorption rate in sham and ovariectomized (OVX) rats on a normal or vitamin D-deficient diet with or without DFAIII. Values are means $\pm$ SEM, $n = 8$. *P*-values estimated by three-way ANOVA were <0.001 for vitamin D-deficiency (V), 0.864 for operation (O), <0.001 for DFAIII (D), <0.001 for $V \times O$, 0.108 for $V \times D$, 0.024 for $O \times D$ and 0.862 for $V \times O \times D$. Means not sharing a common letter differ, $P < 0.05$.