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Effects of Epilactose on Calcium Absorption and Serum Lipid Metabolism in Rats

MEGUMI NISHIMUKAI,*,†,‡ JUN WATANABE,† HIDENORI TAGUCHI,† TAKESHI SENOURA,† SHIGEKI HAMADA,‡ HIROKAZU MATSUI,‡ TAKESHI YAMAMOTO,§ JUN WASAKI,† HIROSHI HARA,‡ AND SUSUMU ITO†

Creative Research Initiative 'Sousei', Hokkaido University, N21W10 Kita-ku, Sapporo 001-0021, Japan, Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, N9W9 Kita-ku, Sapporo 060-8589, Japan, and Nihon Shokuhin Kako Co. Ltd., 30 Tajima, Fuji, Shizuoka 417-8530, Japan

Epilactose (4-*O*-β-galactopyranosyl-D-mannnose) is a rare disaccharide in cow milk that can be synthesized from lactose by the cellobiose 2-epimerase of *Ruminococcus albus*. In this study, we examined the biological activities of epilactose using male Wistar-ST rats. The apparent rates of calcium and magnesium absorption of rats fed epilactose and fructooligosaccharide diets were greater than those fed control and lactose diets, accompanied by greater weight gain of the cecal wall and higher levels of short-chain fatty acids and other organic acids. Epilactose also increased the calcium absorption in everted small intestinal sacs. In addition, the levels of plasma total cholesterol and nonhigh-density lipoprotein cholesterol were lower in epilactose-fed rats. These results indicate that epilactose promotes calcium absorption in the small intestine and possibly lowers the risk of arteriosclerosis. Cecal microbes may efficiently utilize epilactose and contribute to these biological activities.

KEYWORDS: Epilactose; calcium absorption; plasma cholesterol

INTRODUCTION

Nondigestible saccharides such as dietary fibers and oligosaccharides are known to exhibit various health-promoting biological activities (*1*−*3*). Intake of some mineral like calcium and iron is lower than the dietary reference intake in Japan; therefore, it is necessary to supply the deficiency (*4*). Some nondigestible saccharides promote mineral absorption, and the large intestine is involved in this beneficial effect (*5, 6*). Nondigestible saccharides are often fermented by intestinal microorganisms, generating short-chain fatty acids (SCFAs). SCFA formation in the large intestine has been proposed to be partly responsible for the increase in calcium absorption (*7*−*9*). Fructooligosaccharides (FOS), which are nondigestible oligosaccharides, are the most popular and well-known oligosaccharides that are highly fermentable and that increase calcium absorption. In addition, several studies have indicated that nondigestible saccharides improve lipid metabolism (*10*−*13*).

Epilactose (4-*O*-β-galactopyranosyl-D-mannnose) is a rare disaccharide (*14*−*16*). A considerable amount of epilactose is known to be formed from cow milk by heat and alkali treatments (*14, 16*). Recently, we found that cellobiose 2-epimerase (EC 5.1.3.11) from the ruminal strain *Ruminococccus albus* NE1 converts lactose to epilactose, and we showed that epilactose highly resisted the rat intestinal enzyme *in vitro* (*17*). Also, lactose, which is the original saccharide to produce epilactose, is known to increase calcium absorption. These observations suggest that epilactose increases calcium absorption. However, the biological activities of this unusual sugar have not yet been examined to date.

The aims of this study were to examine the effects of feeding epilactose on mineral absorption and plasma lipid levels in rats compared with those of feeding FOS and lactose.

MATERIALS AND METHODS

Diets and Animals. Epilactose was synthesized from lactose by using a recombinant cellobiose 2-epimerase from *R. albus*, as described by Ito et al. (*16*). The reaction mixture was applied to a sugar SP0810 column (Shodex, Tokyo, Japan), and the fractions containing epilactose were collected and lyophilized. The epilactose preparation contained 90% epilactose and 10% lactose. Male Wistar-ST rats (4-week-old; Japan SLC, Hamamatsu, Japan) were housed in individual stainless-steel cages in a room with controlled temperature (22 ± 2), relative humidity (40% to 60%), and a 12-h light/12-h dark cycle (lights on from 8:00 a.m. to 8:00 p.m.). They were allowed free access to a semipurified stock diet (sucrose-based, AIN-93G formula) and tap water for 5 days for an acclimation piriod. Then the rats were divided into four groups ($n = 6$), and each group was fed the control, epilactose, lactose, or fructooligosaccharide (FOS; Meioligo P; Meiji Foodmateria,

* Corresponding author.
† Creative Research Initiative 'Sousei', Hokkaido University.
‡ Graduate School of Agriculture, Hokkaido University.
§ Nihon Shokuhin Kako Co. Ltd.

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Table 1. Composition of Diets

component	stock diet	test diet			
		control	epilactose	lactose	FOS
			g/kg		
casein[a]	200	200	200	200	200
sucrose	649.486	644.486	599.486	599.486	599.486
epilactose			50		
lactose		5		50	5
FOS					45
corn oil	50	50	50	50	50
cellulose[b]	50	50	50	50	50
AIN-93G MX	35	35	35	35	35
AIN-93 VXM	10	10	10	10	10
L-cystine	3	3	3	3	3
choline bitartrate	2.5	2.5	2.5	2.5	2.5
tert-butylhydroquinone	0.014	0.014	0.014	0.014	0.014

[a] Casein (ALACID; New Zealand Dairy Board, Wellington, New Zealand). [b] Cellulose (Ceolus PH102; Asahi Chemical Industry, Tokyo, Japan). Prepared according to the AIN-93G formulation.

Tokyo, Japan) diet (**Table 1**) with freely available deionized water for 15 days. To adjust the lactose concentrations of the control and epilactose diets, 5 g/kg of lactose was substituted for sucrose in the control diet.

Body weight and food intake were measured every day. Blood was collected in a fed state (9 a.m.) and after fasting for 10 h (7 p.m.) by venipuncture of the tail vein using a heparinized capillary tube on days 0, 7, and 14 of feeding the test diets to measure total cholesterol and triglyceride (TG) concentrations. Feces were collected from day 13 to 15 of feeding the experimental diets to evaluate apparent mineral absorption. At the end of the experiment, rats were anesthetized by sodium pentobarbital (Nembutal, 40 mg/kg of body weight; Abbott, Chicago, IL) for collection of aortic blood with a heparinized syringe. The blood was centrifuged (1300*g* for 10 min at 4 °C) to obtain the plasma. The rats were killed, and the liver and kidneys were removed and weighed. The cecum with its contents was removed and weighed. The contents were collected and stored at −40 °C until subsequent analyses.

Four consecutive segments (3 cm each) were cut from the jejunum (12 cm in length after Treitz ligament) and ileum (12 cm before a point 5 cm from the ileocecal valve) from rats of both the control and epilactose groups. The intestinal segments were everted with a blunt plastic rod and ligated with surgical silk at one end. The other end of each sac was ligated after the instillation of 0.8 mL of serosal fluid (30 mmol/L Tris-HCl buffer (pH 7.4), 125 mmol/L NaCl, 4 mmol/L KCl, 10 mmol/L glucose, and 1.25 mmol/L $CaCl_2$), and the sacs were incubated at 37 °C in plastic tubes containing 30 mL of mucosal fluid (30 mmol/L Tris-HCl buffer (pH 7.4), 125 mmol/L NaCl, 4 mmol/L KCl, 10 mmol/L glucose, and 10 mmol/L $CaCl_2$ with 0, 50, or 100 mmol/L epilactose). Both fluids were bubbled with 95% O_2 and 5% CO_2, and warmed to 37 °C before use. After 30-min incubation at 37 °C, the calcium concentrations of the artificial serosal fluid in the everted sacs were measured using a Calcium-C Test kit (Wako Pure Chemical, Osaka, Japan).

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

Analyses. Freeze-dried powdered feces (wet weight, 1.5 g) and the experimental diets (1 g) were incinerated at temperatures increasing linearly to 550 °C for 6 h and then maintained at 550 °C for 18 h in an electric furnace (EYELA TMF-3200; Tokyo Rikakikai, Tokyo, Japan). The ash samples were treated with 5.49 mol/L HCl at 200 °C for 30 min and dissolved in 0.82 mol/L HCl. For the determination of calcium and magnesium, $LaCl_3$ (final concentration 7.2 mmol/L) was added to ash solutions to overcome the interferance of phosphate ion. After suitable dilution, the calcium, magnesium, zinc, and iron concentrations of the solutions were measured by atomic absorption spectrometry (AA-6400F; Shimadzu, Kyoto, Japan).

The cecal contents were suspended in four volumes of deionized water and homogenized by a Teflon homogenizer. The pH of the homogenates, which was taken as the pH of the cecal contents, was measured with a semiconducting electrode (ISFET pH sensor 0010-15C; Horiba, Kyoto, Japan). Concentrations of organic acids (acetic, propionic, butyric, succinic, and lactic acids) in the homogenate were measured by high-performance liquid chromatography (LC-10ADvp; Shimadzu) equipped with two Shim-pack SCR-102H columns (8 mm × 30 cm; Shimadzu) and an electroconductibility detector (CDD-6A; Shimadzu) as described previously (*18*). The amounts of total calcium, magnesium, and zinc in the homogenate were determined by the atomic absorption spectrometric method described above after dry-ashing. Soluble mineral in the supernatant obtained upon centrifugation (30,000*g* for 20 min at 4 °C) of the homogenate was determined by atomic absorption spectrometry after deproteinizing with 0.5 mol/L of perchloric acid.

Total cholesterol and high-density lipoprotein (HDL) cholesterol concentrations in the plasma were quantified enzymatically (Cholesterol-E Test Wako and HDL-cholesterol Test Wako; Wako Pure Chemical). The concentration of low-density lipoprotein (LDL) + very low-density lipoprotein (VLDL) cholesterol was calculated by subtracting HDL from total cholesterol. Plasma triglyceride (TG) and phospholipid (PL) concentrations were quantified using TG-EN and PL-EN kits, respectively (Kainos Laboratories, Tokyo, Japan). The calcium concentration of the artificial serosal fluid in the everted sacs was measured using a Ca C-Test kit (Wako Pure Chemical).

Calculation and Statistical Analyses. Apparent mineral absorption (%), solubility of mineral in cecal contents, and absorption speed (mmol/h) by everted sacs were calculated using the following equations, respectively: apparent mineral absorption rate (%) = [(total mineral intake − mineral excretion in feces)/total mineral intake] × 100, solubility of mineral in cecal contents (%) = [(mineral dissolved in liquid part of cecal contents)/(total mineral of cecal contents)] × 100, and calcium absorption speed by everted sacs (nmol/h/cm of sacs) = (calcium amount in everted sacs before incubation − calcium amount in everted sacs after 0.5-h incubation)/0.5 h/length of everted sac.

Values are shown as the means with SEM. The results were analyzed by one-way (**Table**s **2** and **3**, and **Figures 1** and **4**), two-way (**Figure 2**), or repeated measure ANOVA (**Figure 3**), and the significance of differences between groups was evaluated by the Tukey−Kramer posthoc test. Data analysis was performed with StatView for Macintosh (version 5.0, SAS Institute Inc., Cary, NC). Differences with $P < 0.05$ were taken to be statistically significant, and values not sharing a common lowercase letter are significantly different.

RESULTS

Body weight gain and food intake were not different among the four test groups of rats fed control, epilactose, lactose, and FOS diets (**Table 2**), and liver and kidney weights relative to body weight did not differ among the groups (data not shown). Weights of the cecal wall and cecal contents were greater in rats fed epilactose and FOS diets than those in rats fed control and lactose diets. The pH values of the cecal contents were lower in rats fed epilactose and FOS diets than those in rats fed control and lactose diets (**Table 2**). The apparent rates of calcium and magnesium absorption were higher in the epilactose-fed group than in the control- and lactose-fed groups, and that of zinc absorption was higher in the epilactose-fed group than in the control-fed group (**Figure 1**). The rate of iron absorption was not very different among the test groups (data not shown). Calcium solubility in the cecum was higher in rats fed epilactose (32.6 ± 1.2% for epilactose, 26.5 ± 6.9% for FOS) than in rats fed lactose and control diets (18.8 ± 2.4% for lactose, 13.9 ± 1.1% for control, $P = 0.017$). Magnesium solubility in the cecum was higher in rats fed epilactose (79.2 ± 3.4% for epilactose, 64.3 ± 7.7% for FOS) than in rats fed lactose and the control diet (53.5 ± 5.4% for lactose, 59.9 ± 2.7% for control, $P = 0.018$). Zinc solubility in the cecum was higher in rats fed lactose and the control diet (13.0 ± 1.5% for control,

Table 2. Body Weight, Food Intake, Dry Weight of Feces, Cecum Weight, and pH of Cecal Contents[a]

	control	epilactose	lactose	FOS	*P* values
final body weight (g)	212.2 ± 2.0	215.8 ± 4.3	213.8 ± 3.3	215.7 ± 3.7	0.868
body weight gain (g/d)	6.57 ± 0.25	6.87 ± 0.21	6.67 ± 0.14	6.80 ± 0.25	0.783
food intake (g/d)	17.1 ± 0.4	16.9 ± 0.4	17.3 ± 0.2	17.1 ± 0.2	0.928
dry weight of feces (g/4d)	4.49 ± 0.14	4.47 ± 0.19	4.46 ± 0.08	4.92 ± 0.11	0.067
cecal wall weight (g/100 g B.W.)	0.23 ± 0.01 b	0.38 ± 0.02 a	0.24 ± 0.01 b	0.34 ± 0.02 a	<0.001
cecal content weight (g/100 g B.W.)	0.83 ± 0.08 c	2.24 ± 0.09 a	1.03 ± 0.09 c	1.76 ± 0.18 b	<0.001
pH of cecal contents	7.60 ± 0.04 a	6.62 ± 0.18 b	7.52 ± 0.07 a	6.99 ± 0.21 b	<0.001

[a] Each value is the mean ± SEM for 6 rats. Values not sharing a common letter are significantly different.

Table 3. Amount of Organic Acids Produced in Cecum[a]

	control	epilactose	lactose	FOS	*P* values
amount (μmol/ whole cecal contents)					
acetate	88.3 ± 6.9 c	275 ± 19 a	99.1 ± 9.3 c	170 ± 7 b	<0.001
propionate	32.3 ± 3.2 c	127 ± 8 a	41.8 ± 4.6 c	71.6 ± 9.3 b	<0.001
n-butyrate	17.1 ± 1.3 c	46.2 ± 7.7 ab	36.6 ± 4.0 b	81.4 ± 17.7 a	<0.001
total SCFAs	138 ± 11 c	447 ± 31 a	178 ± 16 c	323 ± 22 b	<0.001
succinate	1.52 ± 0.43 b	185 ± 27 a	11.5 ± 5.9 b	22.2 ± 12.9 b	<0.001
lactate	0.742 ± 0.167	44.9 ± 30.6	3.04 ± 1.61	55.9 ± 31.0	0.179
total organic acids	140 ± 11 c	677 ± 49 a	192 ± 18 c	401 ± 60 b	<0.001

[a] Each value is the mean ± SEM for 6 rats. Values not sharing a common letter are significantly different.

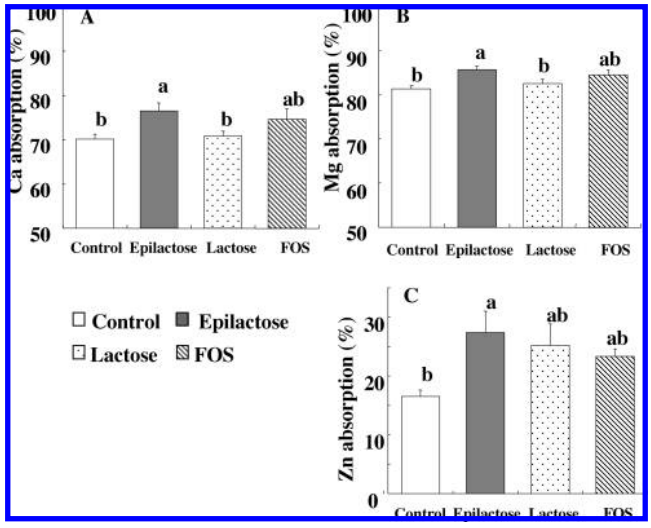

Figure 1. Apparent calcium (**A**), magnesium (**B**), and zinc (**C**) absorption rates in rats after 2 weeks of feeding the control, epilactose, lactose, and FOS diets. Each value is the mean for 6 rats with SEM shown as vertical bars. Values not sharing a common superscript are significantly different ($P < 0.05$). *P* values estimated by one-way ANOVA for calcium, magnesium, and zinc were 0.047, 0.019, and 0.050, respectively.

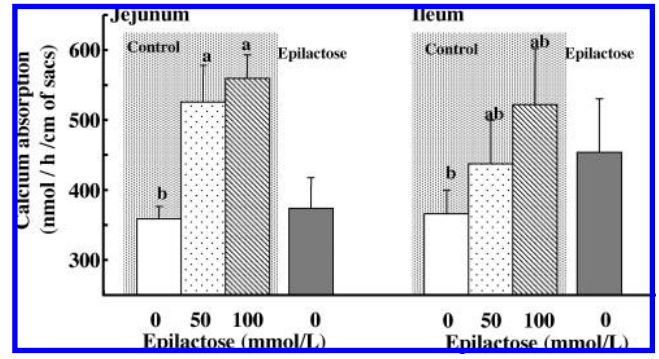

Figure 2. Effect of epilactose on calcium absorption by everted sacs of the jejunum and ileum in rats fed diets with or without epilactose. The everted sacs of the control group were incubated with an artificial serosal fluid containing epilactose (0, 50, or 100 mmol/L). The everted sacs of the epilactose-fed group were incubated with the artificial serosal fluid without epilactose. Each value is the mean for 6 rats with SEM shown as vertical bars. Values not sharing a common superscript are significantly different in the control group ($P < 0.05$). *P* values estimated by two-way ANOVA for the control group were 0.005 for epilactose addition (E), 0.348 for the part of the small intestine (P), and 0.649 for E × P.

12.9 ± 2.0% for lactose) than in rats fed epilactose and the FOS diet (4.9 ± 0.6% for epilactose, 5.6 ± 0.9% for FOS, $P = 0.002$).

The amount of SCFAs (acetic, propionic, and butyric acids) was higher in the epilactose- and FOS-fed groups than in the lactose-fed and control groups. The amounts of acetic acid, propionic acid, total SCFA, and succinic acid were higher in rats fed the epilactose diet than in those fed the FOS diet. The amount of total organic acids (SCFA, succinic acid, and lactic acid) in the cecal contents of the epilactose group was approximately 5 times that of the control group (**Table 3**). The changes in concentrations of organic acids were similar to those in organic acid amounts (data not shown).

Calcium absorption by everted sacs of both the jejunum and ileum isolated from rats fed the control diet was increased by the addition of epilactose (50 or 100 mmol/L) to the mucosal fluid compared with that without epilactose (0 mmol/L) (**Figure 2**). Calcium absorption in jejunal sacs reached a maximum at 50 mmol/L epilactose. In contrast, the absorption in ileal sacs was dose-dependently increased by epilactose, although not significantly (**Figure 2**). Calcium absorption rates from the basal mucosal fluid without epilactose by the jejunal and ileal sacs isolated from rats fed the epilactose diet were similar to absorption by sacs of the control group.

Total plasma cholesterol concentrations in both fed and fasting states tended to be lower in the epilactose-fed group than in the other groups on days 7 and 14, although the differences were not statistically significant (**Figure 3**). Compared with lactose-fed group, the epilactose-fed group was lower in both fed and fasting state on day 7 and in fasting state on day 14. Feeding of epilactose did not have any

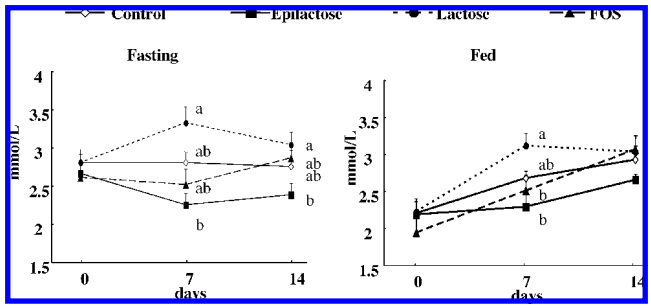

Figure 3. Plasma total cholesterol concentration in tail blood of rats fed the control, epilactose, lactose, or FOS diet on days 0, 7, and 14 (fasting state and fed state). Each value is the mean for 6 rats with SEM shown as vertical bars. Values not sharing a common superscript are significantly different among the four diet groups on the same day ($P < 0.05$). P values estimated by repeated-ANOVA for the fed state were 0.038 for diet (D), < 0.001 for time (T), and 0.005 for D × T. P values for fasted rats were 0.031 for D, 0.878 for T, and 0.017 for D × T.

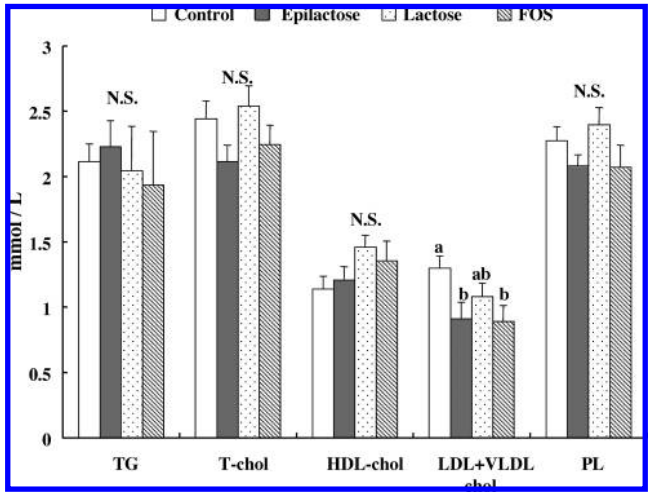

Figure 4. Triglyceride (TG), total cholesterol (T-chol), HDL-cholesterol (HDL-chol), LDL + VLDL cholesterol (LDL + VLDL chol), and phospholipids (PL) in the aorta of rats fed the control, epilactose, lactose, or FOS diet on day 15. Each value is the mean for 6 rats with SEM shown as vertical bars. Values not sharing a common superscript are significantly different ($P < 0.05$). P values estimated by one-way ANOVA for TG, total cholesterol, HDL cholesterol, LDL + VLDL cholesterol, and PL were 0.909, 0.095, 0.124, 0.050, and 0.239, respectively.

significant effect on the plasma TG level (data not shown). Level of LDL + VLDL cholesterol in the aortic blood plasma on day15 (**Figure 4**) was lower in the epilactose group than in the control group, and total cholesterol level tended to be lower in the epilactose group than in the other groups. There were no differences in the TG and PL concentrations between these groups.

DISCUSSION

In the present study, we found that epilactose intake increased calcium absorption in rats. Epilactose increased calcium absorption in the jejunal and ileal sacs of rats when epilactose was added to the mucosal fluid. Two distinct pathways are involved in intestinal calcium absorption, a transcellular pathway and a paracellular pathway (*19*). Transcellular absorption is a saturable, carrier-mediated active transport pathway, whereas paracellular absorption through tight junctions is nonsaturable and diffusive, and requires a gradient of calcium concentrations between the lumen and basolateral sides. Passive absorption in the jejunum and ileum is the major absorptive pathway when calcium intake is adequate or high. In this study, rats were fed adequate calcium (5 g of Ca/kg of diet feeding rate), and therefore, the absorption of calcium by epilactose may proceed mainly via passive transport but not active transport. It has also been shown that difructose anhydride III (DFA III), which is a nondigestible disaccharide, increases calcium absorption by modulation of the tight junction between epithelial cells in everted sacs of the jejunum and ileum and isolated intestinal mucosa in rats (*7, 20*). Furthermore, in a study using Caco-2 cell monolayers, we showed that DFAIII increased net calcium absorption by increasing paracellular transport through the physiologic control of tight junctions without any changes in active calcium transport (*21*). The same mechanism may be involved in the increase in calcium absorption by epilactose intake. We are now conducting further experiments in order to clarify the mechanism of calcium absorption by epilactose. In a previous paper (*17*), we examined the effect of epilactose on the tight junction of Caco-2 cell monolayers and noted an efficient decrease in transepithelial electrical resistance. These results suggest that epilactose increases calcium absorption via the paracellular transport system. Moreover, calcium absorption rates from the mucosal fluid without epilactose did not change in the everted jejunal and ileal sacs isolated from rats fed the epilactose diet when compared with sacs of the control diet-fed group (**Figure 2**). These results indicate that the coexistence of intact epilactose with calcium in the small intestinal lumen enhances calcium absorption but that no adaptive change in the absorptive activity for calcium occurs in the small intestine due to feeding epilactose.

Weights of the cecal wall and cecal contents in rats fed epilactose and FOS diets were higher than those in rats fed the control and lactose diets. It has been reported that FOS increases the cecal wall weight with increased organic acid concentrations in the cecum (*22*). Organic acids produced from nondigestible saccharides enlarge the large intestine and increase calcium solubility by lowering the pH in the luminal contents; as a result, calcium absorption is increased in the cecum (*22*−*24*). The pH of the cecal contents in rats fed epilactose and FOS diets were lower than those in the other groups. However, calcium solubility in the epilactose-fed group was higher than that in the other groups, and the apparent calcium and magnesium absorption tended to be higher in the epilactose group compared with that in FOS group. The amounts of organic acids in the cecum of the epilactose-fed group were higher than those of the other groups, and epilactose was fermented preferentially to propionic and succinic acids. In particular, the amount of succinic acid in the epilactose group was 6 times that in the FOS group, which may induce higher solubilization of cecal calcium. These results suggest that epilactose intake increases calcium and magnesium absorption in the large intestine by increases in absorptive area and calcium solubility and that the higher solubility may induce higher absorption in the epilactose group than in the FOS group.

Apparent zinc absorption was higher in epilactose-fed rats than in control-fed rats; however, zinc solubility in the cecum was significantly higher in rats fed the control diet than in those fed epilactose. These results suggested that zinc

absorption did not depend on solubility in the cecum and that the small intestine was the major site of zinc absorption.

Unlike previous reports (*25, 26*), the present study showed that lactose intake did not promote the intestinal absorption of calcium. We have no idea why at present, but the amount of lactose added to the diet and the period of lactose intake in the present study might be low compared with those reported in the literature (*27*).

Epilactose intake tended to decrease the plasma cholesterol level (**Figures 3** and **4**). We previously demonstrated that SCFAs lowered the plasma cholesterol level (*12*) and that they inhibited hepatic cholesterol synthesis in rats (*28*). In the present study, epilactose intake elevated the level of SCFAs in the cecal contents (**Table 3**), and SCFAs in cecal contents was negatively correlated with plasma total cholesterol and LDL + VLDL cholesterol levels ($n = 24$, $r = -0.441$, $P = 0.03$ for total cholesterol; $r = -0.457$, $P = 0.023$ for LDL + VLDL cholesterol). Moreover, it has been reported that acetate is involved in the serum cholesterol-lowering effect of oat bran in humans (*29*) and that propionate reduces cholesterol synthesis (*30*). Also, a previous report has shown that feeding sugar beet fiber lowered cholesterol synthesis with coordinated increases in the generation of SCFAs and an increase in fecal steroids excretion (*12*). However, feeding of epilactose did not increase fecal excretion of bile acids (unpublished data). These suggest that the SCFAs generated by microbial fermentation of epilactose contribute to cholesterol synthesis and lower plasma cholesterol level.

In conclusion, epilactose stimulates calcium absorption and lowers plasma total cholesterol in rats. The high ability of cecal microorganisms to ferment epilactose may be related to these biological activities.

ABBREVIATIONS USED

SCFA(s), short-chain fatty acid(s); FOS, fructooligosaccharide; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein; TG, triglyceride(s); PL, phospholipid(s); DFA III, difructose anhydride III.

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