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Oligosaccharide Promotes Bioavailability of a Water-Soluble Flavonoid Glycoside, αG-Rutin, in Rats

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This study examined the effects of a nondigestible saccharide, difructose anhydride (DFA) III, and fructooligosaccharides (FOS) on the intestinal absorption and metabolism of αG-rutin, a quercetin glycoside in rats during a 2 week feeding period with diets containing 1% αG-rutin with or without 1.5 or 3% DFAIII and FOS. Blood concentrations and urinary excretion of quercetin derivatives were largely and dose-dependently increased during the test period with feeding DFA III and FOS. The amounts of quercetin derivatives in the cecal contents and feces were also much higher in both saccharide groups than in the control group. The degradation rate of aglycone, estimated by differences between ingestion and sum of fecal and urinary excretion, were suppressed in the both saccharide groups. Cecal fermentation was dose-dependently modified by the oligosaccharides. It was concluded that suppression of degrading quercetin aglycone in the large intestine has a major role for increasing αG-rutin bioavailability by DFA III and FOS feedings.

KEYWORDS: Flavonoid; difructose anhydride III; fructooligosaccharides; metabolism; absorption

INTRODUCTION

Flavonoids are polyphenolic compounds mainly found in fruits and vegetables (*1*). These compounds act as antioxidants in foods and the body (*2*). Quercetin is a well-known natural flavonoid rich in onions, green tea, and sophora (*3*). Epidemiologic studies have suggested that a high flavonoid intake reduces the risk of cardiovascular disease in subjects (*4*). Quercetin is usually present in glycosylated forms, mainly as *β*-glucosides, in plant foods (*3, 5*). The nature of the glycosylation probably influences absorptive efficiency of quercetin aglycone (*6*). Quercetin-3-*O*-glucoside (isoquercitrin) and rutin (quercetin-3-*O*-glucosyl-rhamnose) are major naturally occurring quercetin glycosides.

For absorption of quercetin-3-*O*-glucoside, hydrolysis of the glucose moiety by mucosal lactase phlorizin hydrolase (LPH) is suggested to be a major step in intestinal absorption (*5*). Contribution of a glucose transporter (SGLT1) has been also suggested for absorption of this quercetin glucoside in the isolated rat intestinal mucosa (*7*); however, a recent study showed no contribution of SGLT1 in quercetin-3-*O*-glucoside transport (*8*). Another abundant quercetin glycoside, rutin, is not cleaved by LPH (*9, 10*) but partly hydrolyzed the sugar moieties by the colonic microflora (*9, 10*). The released quercetin aglycone from rutin is absorbed from the large intestine (*11*).

Recently, a new quercetin glycoside, αG-rutin (**Figure 1**), has been developed and manufactured commercially. This glycoside is a very water-soluble *β*-glucose adduct of rutin. Our previous studies showed that αG-rutin is transported to the serosal side as the intact form in the isolated rat intestinal mucosa (*12*), and the transported αG-rutin is increased linearly with increases in mucosal αG-rutin concentrations, which suggests that αG-rutin is absorbed via a simple diffusion pathway, most likely via the paracellular pathway in the epithelium of the intestinal mucosa (*12*).

Efficiencies of intestinal absorption of flavonoids are usually low, and little is known about enhancement of soluble flavonoid absorption in an in vivo study (*13*). We previously demonstrated that a nondigestible saccharide, difructose anhydride (DFA) III, promotes intact αG-rutin absorption with a single bolus administration by using portal and duodenal cannulated rats and ligated jejunal and ileal loops (in situ) and also in isolated mucosa of rat intestines (in vitro) (*15*). This disaccharide is evidenced as an enhancer of the paracellular transport in the small intestine (*14*). Furthermore, DFA III is fermentable in the large intestine (*16*), and it has been suggested that the bacterial fermentation of DFA III affects the bioavailability of flavonoid glycosides (*17*). It has been also suggested that fructooligosaccharides (FOS), which are the most popular nondigestible oligosaccharides and well-established to be readily

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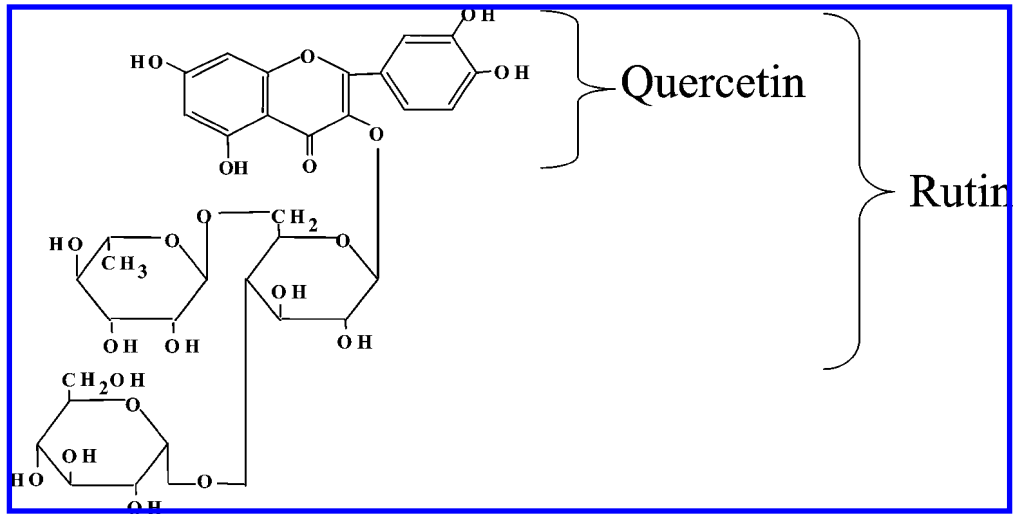

Figure 1. Chemical structure of αG-rutin.

fermentable (*18*), increased isoflavone glycoside absorption with promotion of intestinal fermentation (*19*).

The aims of this study were to determine whether feedings of DFA III and FOS increase the bioavailability of αG-rutin in a rat balance study and to examine the involvement in the paracellular transport in the small intestine and bacterial fermentation in the large intestine for αG-rutin bioavailability. We measured periodical changes in concentrations of αG-rutin and its metabolites in blood as well as the excretion of metabolites in urine and feces during 2 weeks of feeding of test diets including αG-rutin and DFA III or FOS. Possible metabolites of αG-rutin in the intestinal lumen are rutin, quercetin-3-*O*-glucoside (isoquercitrin), and quercetin aglycone, which are hydrolysis products of αG-rutin by LPH (intestinal *β*-glycosidase) and bacterial glycosidases. After absorption, some of the αG-rutin metabolites are converted to methylated quercetin (isorhamnetin and tamarixetin). The nonmethylated and these methylated quercetins may be conjugated with glucuronic and sulfuric acids as glucuronide and sulfate in the intestinal and hepatic cells. These possible metabolites were measured by LC-MS analyses.

MATERIALS AND METHODS

Chemicals. αG-rutin was kindly donated by Toyo Sugar Refining Co., Ltd. (Tokyo, Japan). Quercetin and rutin were obtained from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan). All other reagents and chemicals were of the highest grade commercially available. Difructose anhydride III (DFA III; di-D-fructofuranosyl 1,2′:2,3′-dianhydride), a disaccharide comprising two fructose residues with two glycoside linkages, was provided by Nippon Beet Sugar Mfg. Ltd. (Obihiro, Japan).

Animals and Diets. Male Sprague−Dawley rats (6 weeks old; Japan Clea, Tokyo, Japan) weighing about 150 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 9:00 a.m.−9:00 p.m.). The rats had free access to deionized water and a semipurified diet based on the AIN93G formulation (basal diet) for an acclimation period of 7 days. The test diets [basal, 1% αG-rutin (control), 1% αG-rutin with 1.5 or 3% DFA III, and 1% αG-rutin with 1.5 or 3% FOS] shown in **Table 1** were given at 6:00 p.m. and removed at 9:00 a.m. every day. This study was approved by the Hokkaido University Animal Committee, and the animals were maintained in accordance with the Hokkaido University guideline for the care and use of laboratory animals.

Study Design. Acclimated rats were divided into six groups of six rats and then given one of the test diets mentioned above for 2 weeks. Body weight and food intake were measured daily. The day when the basal diet was replaced with the test diets was assumed to be day 0. Feces were collected from day 4 to day 7 (week 1) or from day 11 to day 14 (week 2) after feeding of the test diets. Urine was collected for 24 h on days 6 and 13 and diluted to a final volume of 40 mL. On the last day, the rats were anesthetized and then killed after collection of the aortic blood. Tail blood was also sampled at 10:00 a.m. and 5:00 p.m. on days 1 and 7 to evaluate periodical changes in concentrations of blood quercetin metabolites.

Plasma and Urine Treatment. Plasma (100 μL) was separated from the aortic and tail blood by centrifugation; the plasma and diluted urine

Table 1. Composition (Grams per Kilogram of Diet) of the Experimental Diets

compound	basal	αG-rutin (control)	αG-rutin + 1.5% NDS	αG-rutin + 3% NDS
casein	250.0	250.0	250.0	250.0
mineral mixture[a]	35.0	35.0	35.0	35.0
vitamin mixture[a]	10.0	10.0	10.0	10.0
choline chloride	2.5	2.5	2.5	2.5
soybean oil	50.0	50.0	50.0	50.0
cellulose	50.0	50.0	50.0	50.0
sucrose	602.5	592.5	577.5	562.5
NDS[b]			15.0	30.0
αG-rutin		10.0	10.0	10.0

[a] The mineral mixture and the vitamin mixture were prepared according to the AIN93G formulation. [b] NDS, nondigestible saccharides, difructose anhydride III or fructooligosaccharides.

Table 2. Initial and Final Body Weights and Total Food Intakes in Rats Fed the Test Diets for 2 Weeks[a]

	initial body wt (g)	final body wt (g)	food intake (g/day)
basal	198 ± 3	303 ± 4 a	20.8 ± 0.5 a
αG-rutin (control)	198 ± 2	302 ± 6 a	20.5 ± 0.6ab
αG-rutin + 1.5% FOS	198 ± 2	301 ± 6 a	19.3 ± 0.4bc
αG-rutin + 3% FOS	198 ± 2	294 ± 5ab	18.2 ± 0.3 c
αG-rutin + 1.5% DFA III	198 ± 2	300 ± 4 a	19.7 ± 0.4ab
αG-rutin + 3% DFA III	198 ± 2	285 ± 6 b	18.2 ± 0.6 c
one-way ANOVA *P* value	0.999	0.037	0.001
two-way ANOVA *P* value			
saccharide	0.987	0.042	0.497
level	0.981	0.347	0.005
saccharide × level	0.975	0.450	0.715

[a] Values are means ± SEM for six rats. Values in a column with a different letter are significantly different by Duncan's test ($P < 0.05$). One-way ANOVA was performed for all groups. Means not sharing a common letter differ significantly, $P < 0.05$. Two-way ANOVA was performed for four oligosaccharide-fed groups without the basal and control groups (saccharide and level).

Table 3. Cecal Wall, Cecal Contents, pH, and Organic Acid Pools in the Cecal Contents of Rats Fed the Experimental Diets for 2 Weeks[a]

	cecal wall (g/100 g of body wt)	cecal contents (g/100 g of body wt)	pH of cecal contents	total organic acids (μmol/cecal contents)
basal	0.75 ± 0.05a	1.93 ± 0.16 c	7.65 ± 0.08a	81.1 ± 9.6 d
αG-rutin (control)	0.91 ± 0.05a	3.69 ± 0.42 b	7.02 ± 0.11b	132.4 ± 14.2cd
αG-rutin + 1.5% FOS	1.19 ± 0.05c	4.79 ± 0.26ab	6.77 ± 0.11b	233.3 ± 18.8 b
αG-rutin + 3% FOS	1.56 ± 0.06a	6.01 ± 0.74 a	6.28 ± 0.12c	345.6 ± 50.0 a
αG-rutin + 1.5% DFA III	1.07 ± 0.05c	3.87 ± 0.39 b	6.72 ± 0.12b	148.0 ± 18.5cd
αG-rutin + 3% DFA III	1.38 ± 0.05b	6.08 ± 0.71 a	6.27 ± 0.06c	178.4 ± 31.7bc
one-way ANOVA *P* value	<0.001	<0.001	<0.001	<0.001
two-way ANOVA *P* value				
saccharide	0.009	0.466	0.787	<0.001
level	<0.001	0.006	<0.001	0.038
saccharide × level	0.581	0.391	0.835	0.218

[a] Values are means ± SEM for six rats. Values in a column with a different letter are significantly different by Duncan's text ($P < 0.05$). Total organic acids: sum of succinic, lactic, acetic, propionic, and *n*-butyric acids. One-way ANOVA was performed for all groups. Means not sharing a common letter differ significantly, $P < 0.05$. Two-way ANOVA was performed for four oligosaccharide-fed groups without the Basal and control group (saccharide and level).

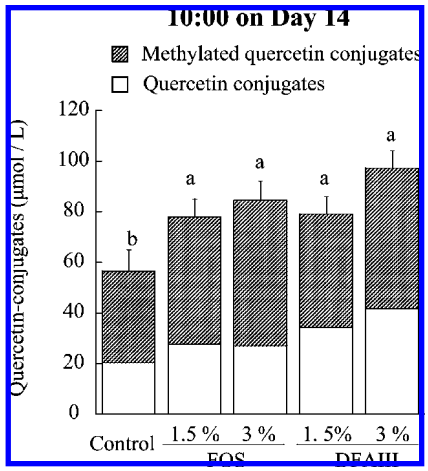

Figure 2. Plasma concentration of quercetin and monomethylated quercetin (sum of isorhamnetin and tamarixetin) conjugates in the abdominal aortic blood of rats fed the control (only αG-rutin), 1.5 or 3% FOS, and 1.5 or 3% DFA III diets at 10:00 a.m. on day 14. Values are means ± SEM, $n = 6$. Error bars refer to sum of metabolites. *P* values by one-way ANOVA were 0.028. Means not sharing a common letter differ significantly, $P < 0.05$. *P* values estimated by two-way ANOVA for the four nondigestible saccharide groups were 0.024 for saccharide, 0.452 for level, and 0.371 for saccharide × level.

samples (100 μL) were acidified (to pH 4.9) with 10 μL of acetic acid (0.58 mol/L) and then treated with 10 μL of *Helix pomatia* extract (Sigma G0876, 5106 U/L, β-glucuronidase and 25105 U/L sulfatase) for 30 min at 37 °C to measure conjugated forms with glucuronic acid and sulfuric acid as quercetin aglycone and methylated forms of quercetin, isorhamnetin, and tamarixetin. The reaction mixture was then added to 100 μL of MeOH and heated at 100 °C for 1 min, and the supernatant was collected by a centrifugation for 3 min at 9000*g*. This extraction procedure was repeated three times without heating. The combined supernatant was applied to an Oasis HLB cartridge (Waters Co. Ltd., Milford, MA), and the eluent (100% MeOH) was dried and dissolved in 100 μL of 50% MeOH solution (sample solution). We confirmed that conjugates were not degraded by the extraction procedure, including heating. Recovery rates of standard αG-rutin, rutin, quercetin, and methylated quercetin (isorhamnetin and tamarixetin) added to the plasma and feces were over 90% with the same sample treatment.

Feces and Cecal Contents Treatment. Freeze-dried feces were milled, and the powdered feces (0.5 g) were added to 5 mL of 80% MeOH solution and sonicated for 20 s (42W, ULTRAS homogenizer, Taitec Co. Ltd., Nagoya, Japan). The reaction mixture was heated at 60 °C for 60 min and then centrifuged for 15 min at 3000*g*. The precipitate was added to 2.5 mL of 80% MeOH solution, and the extraction procedure was repeated twice without heating. Cecal contents were diluted to 40 mL. Cecal samples (100 μL) were added to 100 μL of MeOH and heated at 100 °C for 1 min, and the supernatant was collected by a centrifugation for 3 min at 9000*g*. This extraction procedure was repeated three times without heating. The combined supernatant of the feces or cecal contents was subjected to the same procedure as described above for plasma using Oasis HLB cartridges. The feces and cecal contents were not treated with β-glucuronidase and sulfatase mixture because no conjugate peaks were detected in the UV spectra at 360 nm in the HPLC analyses described below.

Organic acids in the cecal contents were measured using a method previously reported (*17*). Briefly, cecal contents were diluted with 4 volumes of deionized water and homogenized using a Teflon homogenizer (Iuchi-Seleido, Osaka, Japan). The homogenate was mixed with NaOH solution (final concentration = 20.8 mmol/L) containing crotonic acid (4.17 mmol/L). The mixture was then centrifuged and passed through a filter disk with a pore size of 0.2 μm. Filtered samples were analyzed by HPLC constructed with a solvent delivery system (SLC-10 AVP; Shimadzu, Co. Kyoto, Japan), a double ion-exchange column (Shim-Pack SCR-102 h, 8 × 300 mm; Shimadzu), and an electroconductivity detector (CDD-6A; Shimadzu).

LC-MS Analysis. αG-rutin and its metabolites were identified and quantified with a HPLC/mass spectrometry system using an electric spray ionization (ESI) interface (ZQ 2000, Waters Co. Ltd.). The temperatures of the capillary heater and the vaporization heater were maintained at 100 and 300 °C, respectively. The flow rate of the sheath gas (nitrogen) was 70 arb. LC-ESI/MS was carried out in scan mode from m/z +50 to 2000 and in selected ion monitoring (SIM) mode at m/z +303 for quercetin, m/z +317 for monomethylated quercetin (isorhamnetin and tamarixetin), m/z +611 for rutin, and m/z +773 for αG-rutin. The HPLC system was fitted with a 5 μm C_{18} Waters Puresil column (150 × 4.6 mm^2, Waters Co. Ltd.), and the temperature was maintained with the column oven set at 40 °C. Solvent A (water/methanol/trifluoroacetic acid, 70:30:0.1) and B (methanol/trifluoroacetic acid, 100:0.1) were run at a flow rate of 1 mL/min using a linear gradient from 10 to 30% solvent B for 20 min, then reduced linearly back to 10% solvent B for the next 5 min, and then maintained at the initial condition. αG-rutin metabolites were also monitored by absorbance at 360 nm.

Calculation and Statistics. Concentrations of αG-rutin, rutin, monomethylated quercetin (isorhamnetin and tamarixetin), and quercetin were calculated from the peak area of each mass spectrum and calibration curves of each standard compound. The concentrations of conjugated derivatives in the plasma and urine were estimated as quercetin or monomethylated quercetin (isorhamnetin and tamarixetin) concentrations after the enzymatic treatment. Statistical analyses were performed by one-way ANOVA. The differences among treatment groups were analyzed with Duncan's multiple-range test and were considered to be significant at $P < 0.05$. Statistical analyses were also performed by two-way ANOVA for four oligosaccharide-fed groups without the control group to analyze effects of FOS and DFA (saccharide) and the effects of oligosaccharide levels (level). Two-way ANOVA was applied to **Tables 2** and **3** and **Figures 2**−**6**. The statistics

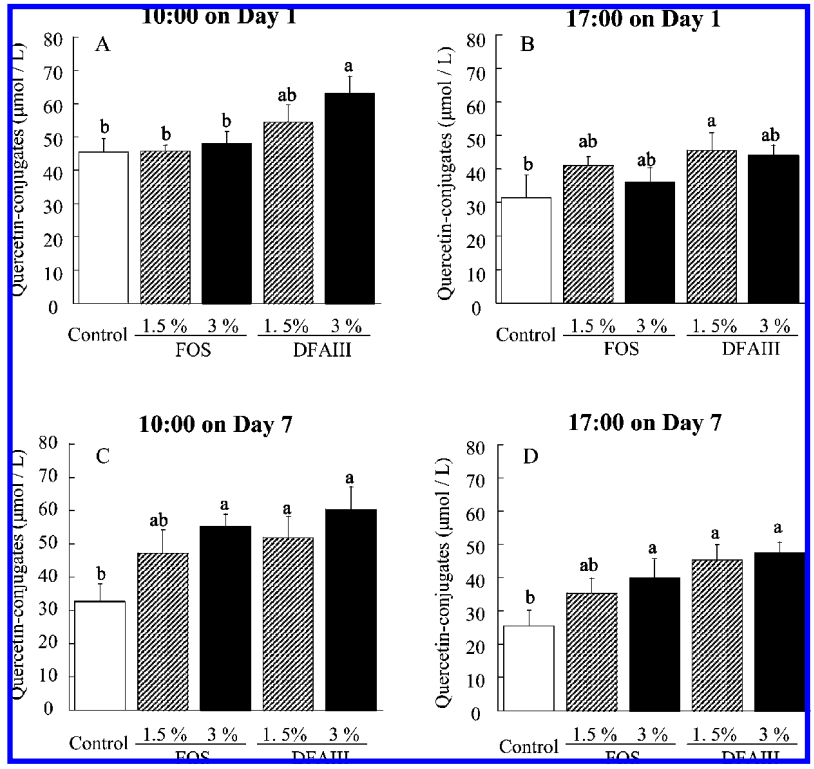

Figure 3. Plasma concentration of quercetin conjugates in the tail blood of rats fed the control (only αG-rutin), 1.5 or 3% FOS, and 1.5 or 3% DFA III diets at 10:00 a.m. (**A**), and 5:00 p.m. (**B**) on day 1 (1 day after start on the test diets) and at 10:00 a.m. (**C**) and 5:00 p.m. (**D**) on day 7 after the start of feeding the test diets. Values are means ± SEM, $n = 6$. P values by one-way ANOVA were 0.029 (10:00 a.m. on day 1), 0.039 (5:00 p.m. on day 1), 0.029 (10:00 a.m. on day 7), and 0.020 (5:00 p.m. on day 7). Means not sharing a common letter differ significantly, $P < 0.05$. P values estimated by two-way ANOVA for the four nondigestible saccharide groups were 0.008 for saccharide, 0.191 for level, and 0.448 for saccharide × level (10:00 a.m. on day 1) and 0.137 for saccharide, 0.440 for level, and 0.688 for saccharide × level (5:00 p.m. on day 1); 0.037 for saccharide, 0.197 for level, and 0.711 for saccharide × level (10:00 a.m. on day 7); and 0.038 for saccharide, 0.686 for level, and 0.934 for saccharide × level (5:00 p.m. on day 7).

Table 4. Estimation of Ingested and Excreted Quercetin Aglycone into the Urine (Day 13−14) and Feces (Day 11−14) per Day and Amounts of Aglycone Degradation per Day[a]

	ingested total aglycone (μmol/day)	excreted total aglycone into the urine and feces (μmol/day)	degraded aglycone (μmol/day)	rate of degraded aglycone (%)
αG-rutin (control)	265	34.6	230	87.2
αG-rutin + 1.5% FOS	250	79.7	170	68.1
αG-rutin + 3% FOS	235	105	131	55.7
αG-rutin + 1.5% DFA III	258	69.2	189	73.4
αG-rutin + 3% DFA III	245	120	124	50.9

[a] Degraded aglycone (μmol/day) = (ingested aglycone) − (sum of total aglycone excreted into urine and feces). These data were estimated as amounts of aglycone per day. Rate of aglycone degradation (%) = degraded aglycone/ingested total aglycone × 100.

processing was done only for the sum of metabolites presented in **Figures 2**, **4**, and **6**.

RESULTS

Body Weight, Food Intake, Cecal Weight, Cecal pH, and Organic Acid Pools. Final body weight was slightly but significantly lower in the 3% DFA III group than in the basal and control groups (**Table 2**). Food intake in the 1.5 and 3% FOS groups was lower without suppression of body weight gain compared with that in the basal group, and that was also lower in the 3% DFA III group than in the basal and control (only αG-rutin) group. Food intake was also lower in the 1.5% oligosaccharide groups than in the 3% oligosaccharides groups according to the result of two-way ANOVA ($P = 0.005$).

The cecal wall and content weights in rats fed αG-rutin (the control group) were greater than those in the basal group, and feeding of both saccharide diets further increased the cecal wall and contents in dose-dependent manners (**Table 3**). Weights of the cecal contents were 3-fold higher in the 3% FOS and DFA III groups compared with that in the basal group. The cecal pH was lower in the 3% DFA III and FOS groups compared with the basal and control groups. These changes by both saccharides were dose-dependent. Weights of the cecal contents were higher and the cecal pH was lower in the 3% oligosaccharide groups than in the 1.5% oligosaccharide groups according to the results of two-way ANOVA ($P = 0.006$ for weights, 0.038 for pH, **Table 3**). Total organic acids pools, which are the sum of succinic, lactic, acetic, propionic, and *n*-butyric acid, were also increased in the 3% DFA and 1.5 and 3% FOS groups compared with the basal and control groups, but the changes in the organic acid pools with DFA feeding were less than those with FOS feeding. Changes in the individual organic acids are not shown, but increased organic acid in the 1.5% FOS group was acetic acid, whereas increasing lactic acid mainly contributes to the

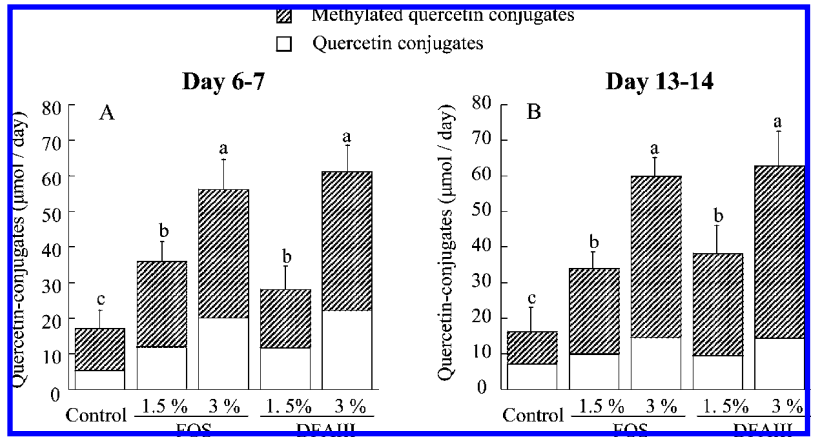

Figure 4. Urinary excretion of quercetin and monomethylated quercetin (sum of isorhamnetin and tamarixetin) conjugates in rats fed the control (only αG-rutin), 1.5 or 3% FOS, and 1.5 or 3% DFA III diets on day 6−7 (**A**) and day 13−14 (**B**) after the start of feeding the test diets. Values are means ± SEM, $n = 6$. Error bars refer to the sum of metabolites. P values by one-way ANOVA were 0.002 (day 6−7) and <0.001 (day 13−14). Means not sharing a common letter differ significantly, $P < 0.05$. P values estimated by two-way ANOVA for the four nondigestible saccharide groups were 0.302 for saccharide, <0.001 for level, and 0.146 for saccharide × level (day 6−7); and 0.042 for saccharide, <0.001 for level, and 0.614 for saccharide × level (day 13−14).

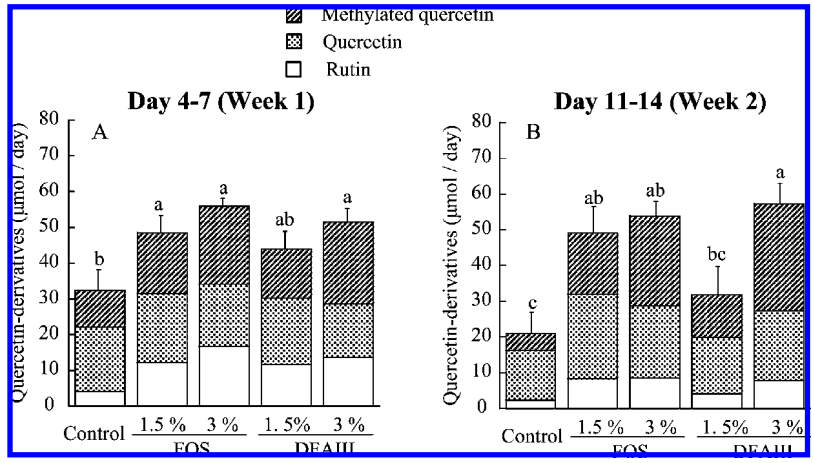

Figure 5. Fecal excretion of quercetin derivatives, rutin, quercetin, and monomethylated quercetin (isorhamnetin and tamarixetin) in rats fed the control (only αG-rutin), 1.5 or 3% FOS, and 1.5 or 3% DFA III diets at week 1 (**A**) and week 2 (**B**) after the start of feeding the test diets. Values are means ± SEM, $n = 6$. Error bars refer to the sum of metabolites. P values by one-way ANOVA were 0.042 (week 1) and 0.010 (week 2). Means not sharing a common letter differ significantly, $P < 0.05$. P values estimated by two-way ANOVA for the four nondigestible saccharide groups were 0.473 for saccharide, 0.162 for level, and 0.764 for saccharide × level (week 1); and 0.087 for saccharide, 0.557 for level, 0.151 for saccharide × level (week 2).

expansion of total organic acids in the 3% FOS group. The higher organic acid pool in the 3% DFA group depends on increases in acetic and lactic acid pools.

Changes in αG-Rutin Metabolites in the Blood Plasma, Urine, Feces, and Cecal Contents. Quercetin and monomethylated quercetin (sum of isorhamnetin and tamarixetin) glucuronide/sulfate were presented as metabolites in the plasma and urine because nonconjugated forms of quercetin and monomethylated quercetin were not detectable in the plasma and urine. Glycosylated quercetin and dimethylquercetin were also not detectable. In the feces and cecal contents, rutin and nonconjugated forms of quercetin and monomethylated quercetin (isorhamnetin and tamarixetin) could be detected. Other glycosides and conjugated forms of quercetin were not detectable. Intact αG-rutin was not detectable in any samples.

In the aortic blood plasma, concentrations of quercetin and monomethylated quercetin (isorhamnetin and tamarixetin) glucuronide/sulfate were increased with feeding of FOS and DFA III at both 1.5 and 3% levels on day 14 at 10:00 a.m. (**Figure 2**). Values of summed quercetin and monomethylated quercetin (isorhamnetin and tamarixetin) glucuronide/sulfate were higher in the 1.5 and 3% levels of both nondigestible saccharide groups than in the control group. The concentrations were greater in the DFA III groups than in the FOS groups according to the results of two-way ANOVA ($P = 0.024$, **Figure 3**).

The tail blood analyses showed that concentrations of quercetin glucuronide/sulfate were dose-dependently increased with DFA III feeding, but not FOS feeding, on day 1 at 10:00 a.m. (after an overnight feeding of αG-rutin diet). These changes were, however, less marked at 5:00 p.m. (in the postabsorption state) compared to those at 10:00 a.m. with lowering of the concentration in the control group (**Figure 3A**,**B**). On day 7, conjugate concentrations in the 3% FOS group were also higher than those in the control group. The results of two-way ANOVA for the four groups fed oligosaccharides showed that the concentrations of quercetin conjugates at 10:00 a.m. on day 1 (**A**) and day 7 (**C**), and at 5:00 p.m. on day 7 (**D**) were influenced by kind of oligosaccharide (saccharide, $P < 0.05$). We did not examine the methylated form in the tail blood because of the limited availability of tail blood samples.

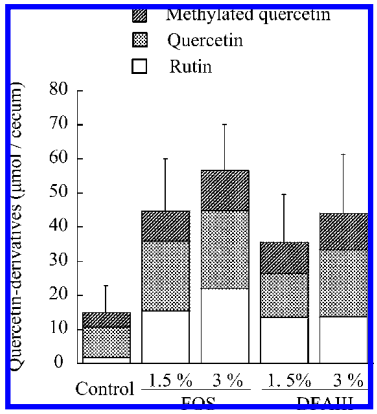

Figure 6. Cecal pools of quercetin derivatives, rutin, quercetin, and monomethylated quercetin (isorhamnetin and tamarixetin) of rats fed the control (only αG-rutin), 1.5 or 3% FOS, and 1.5 or 3% DFA III diets 2 weeks after the start of feeding the test diets. Values are means ± SEM, $n = 6$. Error bars refer to the sum of metabolites. *P* values by one-way ANOVA were 0.162. *P* values estimated by two-way ANOVA for four nondigestible saccharide groups were 0.934 for saccharide, 0.850 for level, and 0.989 for saccharide × level.

In the urine collected for 24 h on days 6−7 and 13−14, conjugated forms (glucuronide/sulfate) of quercetin and monomethylated quercetin (isorhamnetin and tamarixetin), but no nonconjugated form, were detected. The urinary excretion of quercetin and monomethylated quercetin glucuronide/sulfate was increased with the feeding of both nondigestible saccharides compared with that in the control group in both periods. As the results of two-way ANOVA show, there were clear influences in oligosaccharide level on the conjugate excretion ($P < 0.001$ on days 6−7 and 13−14). The increases in conjugates were similar between the DFA and FOS groups. Fluctuations between quercetin and monomethylated quercetin (isorhamnetin and tamarixetin) glucuronide/sulfate were very similar within each group on both periods (**Figure 4**).

In feces, all quercetin derivatives, rutin, nonconjugated forms of quercetin, and monomethylated quercetin (isorhamnetin and tamarixetin), were increased in the nondigestible saccharide groups compared with the control group in both weeks 1 and 2 (**Figure 5**). The sum of the three quercetin derivatives was significantly higher in the 1.5 and 3% FOS groups and the 3% DFA III group than in the control group in each period. The changes in quercetin derivatives with feeding nondigestible saccharide diets were similar between weeks 1 and 2. Kinds of oligosaccharide (saccharide) and feeding levels (level) did not influence the fecal excretion of quercetin metabolite according to the results of two-way ANOVA. In the cecal content, changes in the total amounts of the quercetin derivatives among the groups were very similar to those in the fecal excretion in week 2 without significant differences (**Figure 6**).

DISCUSSION

In this study, we examined whether prolonged feedings of nondigestible disaccharides, DFA III and FOS, affect αG-rutin absorption and metabolism. On day 14 after feeding of test diets, quercetin conjugate concentrations in the aortic blood were increased by feeding of FOS and DFA III diets. Moreover, the urinary excretion of quercetin conjugates was also increased with the feedings of both nondigestible saccharides, which showed clear dose dependency for diet levels of oligosaccharides. In addition, total organic acid pools were higher and the cecal pH was lower in both oligosaccharide groups than in the control groups, and these changes were dependent on the level of oligosaccharides. These results strongly suggest that increased fermentation in the large intestine enhanced αG-rutin availability by oligosaccharides.

Overnight feeding of αG-rutin together with DFA III, but not FOS (10:00 a.m., day 1), increased quercetin conjugate concentrations in the tail blood compared with that in the control group. This result suggests that DFA III, not FOS, enhances absorption of αG-rutin in the early stage of feeding. We previously demonstrated that αG-rutin is absorbed via the paracellular pathway by detection of intact αG-rutin in the portal blood (*20*). The increase in blood of quercetin conjugates just after overnight feeding of DFA III suggests that DFA III promotes absorption of αG-rutin via the paracellular pathway in the small intestines, which may not be associated with bacterial fermentation in the large intestine. In the present study, quercetin conjugates, but not αG-rutin and aglycone, were detected in the tail and aortic blood, whereas intact αG-rutin was detected in the portal vein in our previous study, as mentioned above. It is speculated that the absorbed αG-rutin in the portal blood may be readily converted to conjugated forms in the liver.

The fermentation of oligosaccharides enlarges the intestinal mucosa (*21*) and stimulates mucosal blood flow (*22*), which may contribute flavonoid absorption in the large intestine. On day 1, FOS feeding did not increase blood conjugate concentration, which may due to insufficient fermentation. The promotive effects of DFA III on blood levels of quercetin metabolites were small but significantly higher than those of FOS even on days 7 and 14 as shown in the results of two-way ANOVA. DFA III was found to be slowly (*23*, *24*) but extensively fermented after 1 week of feeding DFA III in rats (*25*). DFA III may enhance αG-rutin absorption in the small intestine via the paracellular pathway and also in the large intestine through the promotion of fermentation, which may explain the higher increases in blood flavonoid concentration after feeding of DFA III compared to those after feeding of FOS. The much higher dose-dependent increases in the urinary excretion of quercetin conjugates observed after feeding with both nondigestible saccharides strongly supports the promotion of αG-rutin absorption by these saccharides. The enhanced levels of urinary flavonoid excretion were very similar between the DFA and FOS groups. These results demonstrate that bacterial fermentation has a major role in the promotion of the overall availability of αG-rutin by these oligosaccharides.

The promotion of αG-rutin absorption with feeding of FOS and DFA III was thought to lead to a decrease in the fecal excretion of αG-rutin metabolites. However, fecal excretion of quercetin derivatives was largely increased by feeding both oligosaccharides. In the equilibrium state, the body pool of quercetin derivatives may be constant, and the sum of quercetin aglycone excretion into the feces and urine should be balanced by the total aglycone in ingested αG-rutin if the degradation of aglycone in the body is negligible. It has been reported, however, that quercetin aglycone was degraded by bacteria from the human intestinal tract (*26*, *27*) and by the pig cecal microflora (*28*). We have also found that quercetin aglycone disappeared by 50% after incubation for 150 min at 37 °C in the rat cecal contents in a preliminary experiment (data not shown). In the present study, we estimated bacterial degradation of aglycone by differences between total ingested and total excreted quercetin aglycone in the urine and feces (**Table 4**), which shows that the rate of aglycone degradation was dose-dependently reduced by feeding of both nondigestible saccharides. This finding

suggests that FOS and DFA III increase the bioavailability of αG-rutin by suppressing the degradation of quercetin aglycone by changes in the cecal fermentation. We also observed that concentration of quercetin conjugates decreased on day 7 compared with day 1 in the control group, but not in the FOS and DFA III groups. Possibly, proliferation of quercetin-degrading bacteria or quercetin-degrading enzymes was induced by feeding of αG-rutin every day, which reduced blood levels of quercetin conjugates. Feedings of oligosaccharides may suppress these inductions in the large intestine. In a previous study using an in vitro fermentation system, the addition of FOS allowed the survival of isoflavone aglycone in a fecal bacterial culture (*29*). Tamura et al. also suggested the suppression of intestinal degradation of soybean isoflavones associated with the feeding DFA III in rats (*18*). It is predicted that some kinds of bacteria increased by FOS or DFA III suppress the bacterial proliferation responsible for the degradation of quercetin aglycone. Ingestion of DFA III leads to an increase in bacteria similar to *Ruminococcus productus* (*25*) found in the rat cecum, and FOS selectively increases the number of enterobacteria such as *Bifidobacterium* spp. and *Lactobacillus* spp. (*29*). It should be clarified in future studies which kinds of bacteria are involved in the promotion of αG-rutin bioavailability.

We detected large amounts of conjugated monomethylated quercetin (isorhamnetin and tamarixetin) in the aortic blood and the urine and free monomethylated quercetin in the cecal contents and feces. The existence of monomethylated quercetin in the cecum and feces has not been previously shown. These methylated forms increased together with increases in total quercetin derivatives in the cecal contents and feces after feeding with FOS and DFA III. The methylation of polyphenol compounds occurs in the liver (*30*). The cecal and fecal methylated form may be delivered into the lumen through the bile fluid. Changes in the sum of methylated and nonmethylated quercetin conjugates in the aortic blood on day 14 were very similar to those in the nonmethylated form in the tail blood at 10:00 a.m. on day 7. The increases in tail blood quercetin derivatives may closely reflect changes in total quercetin derivatives.

We conclude that DFA III promotes the availability of αG-rutin by increasing paracellular absorption of the quercetin glycoside in the small intestine; however, suppressing bacterial degradation of quercetin aglycone in the large intestine has a major role in the enhancement of αG-rutin availability by DFA III and also FOS. αG-rutin has already been used as an additive in beverages and food, and the simultaneous ingestion of DFA III and FOS may be an efficient method of eliciting beneficial effects of water-soluble flavonoid glycosides.

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