Nondigestible Saccharides Suppress the Bacterial Degradation of Quercetin Aglycone in the Large Intestine and Enhance the Bioavailability of Quercetin Glucoside in Rats

Noriko Matsukawa,[†] Megumi Matsumoto,[‡] Aki Shinoki,[†] Masahito Hagio,[†] Ryo Inoue,[§] and Hiroshi Hara*,[†]

[†]Laboratory of Nutritional Biochemistry, Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Sapporo, Hokkaido 060-8589, Japan, [‡]Meiji Dairies Research Chair, Creative Research Initiative Sousei (CRIS), Hokkaido University, Sapporo, Hokkaido, 001-0021, Japan, and [§]Laboratory of Animal Science, Kyoto Prefectural University, Shimogamo, Kyoto 606-8522, Japan

Contribution of intestinal bacterial degradation of quercetin aglycone to the promotive effects of fructooligosaccharides and di-D-fructose anhydride III (DFAIII) on quercetin-3-O-β-glucoside (Q3G) bioavailability was examined. Male Sprague–Dawley rats were fed 0.68% Q3G diets with or without 1.5% or 3% oligosaccharides for 2 weeks. Blood levels and urinary excretion of quercetin and methylquercetin conjugates, measured by methanol extraction and LC-MS analyses, were dose-dependently and adaptively increased by the oligosaccharide supplementation with increasing cecal fermentation (Experiment 1). Degradation of Q3G and quercetin aglycone by cecal bacteria in oligosaccharide-fed rats was much lower than that in the control rats using an anaerobic culture system (Experiment 2). Using the ligated intestinal sacs of anesthetized rats, we found that the cecum possessed high absorptive capacity for quercetin derivatives (Experiment 3). These results demonstrate that feeding of the oligosaccharides strongly suppresses the bacterial degradation of quercetin aglycone in the cecum, thus largely contributing to the increased bioavailability of Q3G.

KEYWORDS: Fructooligosaccharide; difructose anhydride III; quercetin glycoside; cecal bacteria; bioavailability; rats

INTRODUCTION

Flavonoids are polyphenolic compounds naturally occurring in various plants (*1, 2*). Quercetin glycosides are the most abundant flavonoids in fruits and vegetables, such as onions and apples, and have been suggested to function as anticarcinogenic, antidiabetic, and antiatherogenic agents (*3−5*). However, the absorptive rates of quercetin glycosides vary depending on their sugar moieties or food sources (*6−8*), and the beneficial effects depend on the bioavailability of the flavonoid. More than one mechanism for the intestinal absorption of quercetin glycosides, especially quercetin monoglucosides, has been identified. The first mechanism proceeds through absorption of the flavonoid as quercetin aglycone after the removal of sugar moieties by a β-glucosidase in the brush border membrane, lactase-phlorizin hydrolase (LPH), especially for quercetin-3-O-glucoside (*9, 10*). Another proposed mechanism suggests that the flavonoid is absorbed as a glucoside via an intestinal brush border glucose transporter, sodium-dependent glucose transporter 1 (SGLT-1) (*11*); however, this mechanism is still controversial (*12*). These absorptive mechanisms are considered to function in the small intestine. In humans, the bioavailability of quercetin glucoside is also largely different among food matrixes (*7, 13*), and absorptive

advancement between quercetin aglycone and its glucoside is still controversial (*14*).

Though the absorptive rates vary between several reports, the bioavailability of these flavonoids is generally accepted to be relatively low as mentioned above, which means that unabsorbed quercetin or quercetin glycosides in the proximal intestine flow into the large intestine. Absorption of quercetin or its glycosides in the large intestine is mostly unknown. Rutin, a quercetin rutinoside, is resistant to LPH but is hydrolyzed by bacterial β-glycosidase and is assumed to be absorbed from the large intestine (*15*). However, intestinal bacteria not only hydrolyze sugar moieties but also breakdown quercetin aglycone (*16, 17*). This metabolism of flavonoids by the commensal bacteria in the large intestine probably influences flavonoid bioavailability. Short-chain fructooligosaccharides (FOS) are well-known, nondigestible and fermentable sugars that induce marked changes in the intestinal bacterial populations (*18*). Feeding of a diet containing isoflavone glycoside with FOS enhanced the increases in blood isoflavone concentration, which suggests that the bacterial metabolism of the isoflavone glycoside affects flavonoid bioavailability (*19*). The authors have proposed that removal of the sugar moieties promotes the absorption of isoflavone glycoside.

We previously showed that the absorption of a water-soluble quercetin glycoside, αG-rutin, is promoted by feeding a nondigestible disaccharide, di-D-fructose anhydride III (**Figure 1**, DFAIII), as a consequence of the restoration of quercetin

*Corresponding author. Kita-9, Nishi-9, Kitaku, Sapporo, Hokkaido 060-8589, Japan. Tel: +81-11-706-2504. Fax: +81-11-706-3352. E-mail: hara@chem.agr.hokudai.ac.jp.

Published on Web 10/06/2009

Article

J. Agric. Food Chem., Vol. 57, No. 20, 2009 **9463**

aglycone in the gastrointestinal tract (*20*). DFAIII is a unique nondigestible disaccharide, which is found as a strong enhancer of Ca absorption (*21*), and is a slowly fermentable saccharide. Also, DFAIII is fermented by different intestinal bacteria from those for FOS, for example, *Bifidobacterium* favorably utilizes FOS, but does not utilize DFAIII (*22*). We have proposed that suppression of the bacterial degradation of aglycone in the intestine rather than the removal of sugars from the glycoside is involved in the increase in the bioavailability of αG-rutin resulting from the feeding of the nondigestible saccharide.

The aim of the present study was to provide evidence that the feeding of nondigestible oligosaccharides actually suppresses the degradation of quercetin aglycone by the large intestinal bacteria and that this suppression contributes to the promotion of quercetin glycoside availability by three separate experiments of rats. First, we examined the absorption of an abundant and naturally occurring quercetin glycoside, quercetin 3-*O*-β-glucoside (isoquercitrin, Q3G), after feeding of test diets including Q3G with FOS or DFAIII in rats. Next, we evaluated the degradation of Q3G and quercetin derivatives (quercetin aglycone and methylquercetin) by the cecal bacteria in rats fed FOS or DFAIII, and finally, we evaluated the absorptive activity of the cecum for quercetin derivatives in the cecal lumen.

MATERIALS AND METHODS

Chemicals. Quercetin-3-*O*-β-D-glucoside was kindly donated by San-Ei Gen F.F.I., Inc. (Osaka, Japan). Quercetin and tamarixetin (monomethylquercetin) 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 (DFAIII; di-D-fructofuranosyl 1,2′/2,3′ dianhydride), a disaccharide comprising two fructose residues with two glycoside linkages, was provided by Fancl Co. (Yokohama, Japan). Fructooligosaccharide (FOS, Meioligo-Pt, Meiji Seika Kaisha, Ltd., Tokyo, Japan) is a mixture of 42% 1-kestose, 46% nystose, and 9% 1*F*-β-fructofuranosyl nystose.

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 09:00−21:00 h). The rats had free access to deionized water and a semipurified diet based on the AIN93G formulation for an acclimation period of 7 days. The test diets were given at 18:00 and removed at 9:00 everyday. 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.

Experiment 1: Changes in Absorption and Metabolism by Prolonged Feeding of Quercetin Glucoside Together with Nondigestible Oligosaccharides. Acclimated rats were divided into six groups of eight rats and given one of the test diets (flavonoid-free basal diet, oligosaccharide-free control diet containing 0.68% Q3G, and diets containing 0.68% Q3G with 1.5 or 3% FOS or DFAIII) shown in **Table 1** for 2 weeks. The level of Q3G used in the present study was adjusted to the same aglycone level of the test diet including a water-soluble αG-rutin used in our previous study (*20*). Body weight and food intake were measured every day. The day switched to the test diets was assumed to be day 0. Tail blood was sampled at 10:00 on day 1, and at 10:00 and 17:00 on days 7 and 13, respectively. Feces were collected from day 11 to day 14. Urine was collected for 24 h on day 13 under normal feeding conditions and diluted to a final volume of 100 mL. On the last day, the rats were anesthetized and killed by withdrawal of aortic blood, then the cecum with their contents were collected.

Experiment 2: Comparison of Quercetin Degradation Ability of Cecal Microbiota in Rats Fed FOS or DFA. To get cecal bacteria adapted to oligosaccharides, acclimated rats were divided into three groups and were fed the control, 3% FOS, or 3% DFAIII diet for 2 weeks. We observed in vitro degradation of Q3G (*n* = 7 per group) or of quercetin aglycone and methylquercetin (*n* = 6 per group) by using the
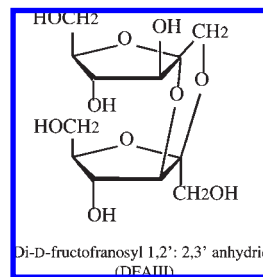

Figure 1. Chemical structure of difructose anhydride III.

Table 1. Composition of the Experimental Diets

	g/kg diet			
	basal	control	1.5% FOS or DFAIII	3% FOS or DFAIII
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	595.7	580.7	565.7
FOS or DFAIII[b]			15.0	30.0
quercetin-3-*O*-D-glucoside		6.8	6.8	6.8

[a] The mineral mixture and the vitamin mixture were prepared according to the AIN93G formulation. [b] DFAIII, di-D-fructose anhydride III; FOS, fructooligosaccharides.

cecal contents including the adapted bacteria. On the last day, the cecum was removed, and cecal contents were immediately collected. The cecal contents (1 g as wet weight) were diluted to 10 mL with anaerobic phosphate buffer (50 mmol/L NaH_2PO_4, 50 mmol/L Na_2HPO_4, 0.5 g/L L-cysteine, 3 g/L Na_2CO_3, and 1 mg/L resazurin sodium salt, pH 6.5), and Q3G, quercetin, or tamarixetin (the final concentration, 1 mmol/L in 0.1% DMSO) was added and cultured at 37 °C for 120 min filled with CO_2. The concentrations of quercetin derivatives were measured by LC/MS analysis.

Experiment 3: Absorptive Activities of the Ligated Cecal and Ileal Segments for Luminal Quercetin Derivatives in Anesthetized Rats. The absorptive activity of the cecum for Q3G derivatives was compared with that of the small intestine. To prepare the Q3G derivatives in the cecal contents, six acclimated rats were fed a 1% Q3G diet without oligosaccharides for 5 days, then the cecal contents were removed after euthanizing the rats under pentobarbital anesthesia. The cecal contents were combined, diluted with four volumes of saline, and homogenized using a Teflon homogenizer. The supernatant was collected after centrifugation (1,580*g* for 20 min), which included 363 μmol/L of an unconjugated form of methylquercetin (isorhamnetin and tamarixetin), and the content of quercetin aglycone was negligible.

Twelve acclimated rats fed the flavonoid-free basal diet were divided into two groups and placed on a warm plate under ketamine−xylazine anesthesia after fasting for 18 h. The ligated whole cecum or 10-cm ileal segment (at 3 cm proximal to the ileocecal junction) was prepared through an abdominal midline incision (3−4 cm). The ligated cecal or ileal segment was washed out with warmed saline, then instilled with 2 or 1 mL supernatant of the cecal contents containing methylquercetin (363 nmol/mL), respectively. Blood was collected from the cecal vein or the mesenteric vein in the ligated ileal segment at 45 min after instillation. The rats were euthanized by withdrawal of aortic blood, the cecal or ileal segments were removed together with their contents, and the luminal solution was collected. The surface area of the cecal or ileal segment was measured using a digital camera and Image-J software. To collect all unabsorbed quercetin derivatives, the mucosa of the intestinal segments were also removed with a slide glass, combined with the luminal solution, and homogenized.

Sample Treatment for the Measurement of Quercetin Derivatives. The plasma (100 μL) of the tail blood (Experiment 1) or mesenteric blood (Experiment 3) was acidified (to pH 4.9) with 10 μL of acetic acid (0.58 mol/L) and treated with (total flavonoids) or without

Table 2. Initial and Final Body Weights and Daily Food Intake in Rats Fed a Flavonoid-Free Basal Diet or Test Diets Containing Q3G with or without 1.5% or 3% FOS or DFAIII for 2 Weeks[a]

	initial body weight (g)	final body weight (g)	food intake (g/day)
basal (flavonoid-free)	236 ± 3	355 ± 7 a	24.6 ± 0.7 a
control	236 ± 3	357 ± 8 a	24.3 ± 0.5 ab
1.5% FOS	237 ± 3	343 ± 5 ab	22.1 ± 0.7 ab
1.5% DFAIII	237 ± 3	341 ± 4 ab	22.6 ± 0.5 ab
3% FOS	237 ± 3	338 ± 5 b	21.6 ± 0.5 b
3% DFAIII	237 ± 3	335 ± 4 b	21.0 ± 0.8 b
1-way ANOVA *P* value			
	0.999	0.043	0.001
2-way ANOVA *P* value			
saccharide	0.987	0.601	0.107
level	0.995	0.235	0.911
saccharide × level	0.991	0.917	0.373

[a] Values are the means ± SEM for eight rats. Values not sharing a common letter are significantly different (*P* < 0.05). One-way ANOVA was performed for all groups, and 2-way ANOVA was performed for the four oligosaccharide groups.

Table 3. Cecal Wall, Cecal Contents, and pH and Organic Acid Pools in the Cecal Contents of Rats Fed a Flavonoid-Free Basal Diet or Test Diets Containing Q3G with or without 1.5% or 3% FOS or DFAIII for 2 Weeks[a]

	cecal wall (g/100 g body weight)	cecal contents (g/100 g body weight)	pH of cecal contents	total organic acids (μmol)
basal (flavonoid-free)	0.24 ± 0.02 c	0.55 ± 0.02 c	7.65 ± 0.05 a	80 ± 7 b
control	0.30 ± 0.02 c	1.04 ± 0.05 b	7.02 ± 0.05 b	138 ± 11 b
1.5% FOS	0.41 ± 0.02 ab	1.39 ± 0.09 a	6.77 ± 0.04 b	244 ± 14 a
1.5% DFAIII	0.38 ± 0.03 b	1.34 ± 0.12 a	6.72 ± 0.01 b	153 ± 11 b
3% FOS	0.50 ± 0.02 a	1.57 ± 0.11 a	6.78 ± 0.02 b	324 ± 36 a
3% DFAIII	0.49 ± 0.02 a	1.73 ± 0.12 a	6.22 ± 0.04 c	306 ± 27 a
1-way ANOVA *P* value				
	<0.001	<0.001	<0.001	<0.001
2-way ANOVA *P* value				
saccharide	0.302	0.596	<0.001	0.032
level	0.003	0.014	<0.001	<0.001
saccharide × level	0.506	0.361	<0.001	0.141

[a] Values are the means ± SEM for eight rats. Values not sharing a common letter are significantly different (*P* < 0.05). Total organic acids: sum of succinic, lactic, acetic, propionic, and *n*-butyric acids. One-way ANOVA was performed for all groups, and 2-way ANOVA was performed for the four oligosaccharide groups.

(unconjugated flavonoids) 10 μL of *Helix pomatia* extract (Sigma G0876, 5,106 U/L, β-glucuronidase and 25,105 U/L sulfatase) for 30 min at 37 °C. To extract quercetin derivatives, the reaction mixture was added to 100 μL of methanol, heated at 100 °C for 1 min, and centrifuged. The procedure was repeated 3 times. The combined supernatant was applied to a C18 cartridge (Oasis HLB, Waters Co. LTD, Milford, MA, USA). After washing with 1 mL of water, the eluent with methanol was dried and dissolved in 100 μL of 50% methanol (sample solution). Diluted urine (100 μL, Experiment 1) and the homogenized luminal solution containing the mucosa (100 μL, Experiment 3) was treated in the same manner as that used for the plasma.

Freeze-dried feces (Experiment 1) were milled, and the powdered feces (0.5 g) were added to 5 mL of 80% methanol solution, sonicated for 20 s (42W, TAITEC Co., Ultra Homogenizer, Saitama, Japan), heated at 60 °C for 60 min, and centrifuged for 15 min at 3,000*g*. The extraction process was repeated twice without heating. The combined supernatant was applied to a C18 cartridge as performed for the plasma. The cecal contents (100 μL, Experiment 1) and the cultured media (100 μL, Experiment 2) were also prepared in the same manner as that used for the plasma. These samples were not treated with the β-glucuronidase/sulfatase mixture as no conjugate peaks were detected in the UV spectra at 360 nm in the HPLC analyses described below. We confirmed that conjugates were not degraded by the extraction procedure.

Quantification of Quercetin Derivatives by LC/Mass Spectrometry (MS). Quercetin and its metabolites were identified and quantified by a LC/MS system using an electric spray ionization (ESI) interface (Acquity UPLC, Waters Co. Ltd., Milford, MA). The temperature of the capillary heater and the vaporization heater was 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 2,000 and in selected ion monitoring (SIM) mode (m/z) +303 for quercetin, (m/z) +317 for monomethylquercetin (isorhamnetin and tamarixetin), and (m/z) +465 for Q3G. The UPLC system was fitted with a 1.7-μm C18 column (ACQUITY UPLC BEH, 2.1 × 100 mm, Waters Co. Ltd.) set at 40 °C. Solvents A (water/methanol/formic acid, 70:30:0.1) and B (methanol/formic acid, 100:0.1) were run at a flow rate of 0.3 mL/min using a linear gradient from 20% up to 35% solvent B for 2 min and from 35% up to 80% solvent B for 2 min, then reduced linearly back to 20% solvent B for the next 1 min and subsequently maintained at the initial condition for 1 min. Injection volume was 10 μL. Concentrations of Q3G, monomethylquercetin (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 monomethylquercetin concentrations after the enzymatic treatment.

Organic Acid Analyses in the Cecal Contents. Organic acids in diluted cecal contents with 4 volumes of water were measured using a method previously reported (*23*) using crotonic acid (4.17 mmol/L) as an internal standard. The samples were analyzed by HPLC equipped 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). Succinic, lactic, acetic, propionic, and butyric acids were quantified, and the sum of these acids is presented as organic acid in **Table 3**.

Statistics. Statistical analyses were performed by one-way ANOVA. The differences among treatment groups were analyzed with the Tukey−Kramer's multiple range test and were considered significant at *P* < 0.05. Statistical analyses were also performed by two-way ANOVA for the four oligosaccharide-fed groups, excluding the basal and control groups, to analyze the effects of FOS and DFA (saccharide) as well as the effects of oligosaccharide levels in diets (level, Experiment 1).

RESULTS

Bioavailability of Q3G after Prolonged Feeding of FOS and DFAIII. Final body weight and food intake were not affected by the feeding of Q3G or Q3G with 1.5% FOS and DFAIII; however, these parameters were slightly but significantly lower in the 3% FOS and DFAIII groups compared to those of the basal group (**Table 2**). Wet weight of the cecal wall and contents were greater in all oligosaccharide groups than in the control and basal groups (**Table 3**). Total organic acid pool in the cecal contents similarly changed with these cecal parameters except for the 1.5% DFAIII group in which the value was lower than that in the 1.5% FOS group. Values of pH in the cecal contents changed reciprocally with the other cecal parameters, with the value in the 3% DFAIII group being the lowest among all of the groups. Oligosaccharide levels were found to affect all of these cecal parameters according to the results of 2-way ANOVA among the four oligosaccharide groups.

Quercetin and methylquercetin (isorhamnetin and tamarixetin) totally existed in conjugated forms, and no Q3G was detected in the blood plasma and urine. No quercetin derivatives were detected in the blood or urine in rats fed the flavonoid-free diet (basal group). Blood concentrations of methylquercetin conjugates were higher than those of quercetin conjugates. There was no difference in the sum of these concentrations at 10:00 on day 1 among the groups, but the values in the 3% FOS and DFAIII groups were higher than that in the control group at 10:00 on day 7 (**Figure 2**). On day 13, FOS and DFAIII were dose-dependently

Article

*J. Agric. Food Chem.,* Vol. 57, No. 20, 2009 **9465**
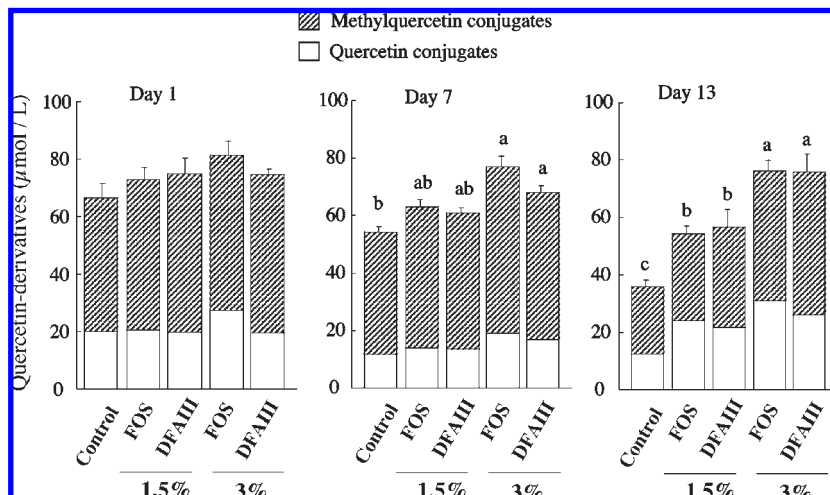
Figure 2. Plasma concentration of quercetin and monomethylquercetin (sum of isorhamnetin and tamarixetin) conjugates in the tail blood of rats fed the control (only Q3G), FOS, or DFAIII diets at 10:00 on days 1, 7, and 13. Values are the means ± SEM, n = 8. Error bars refer to the sum of metabolites. P values by one-way ANOVA were <0.001. Means not sharing a common letter differ significantly; P < 0.05. According to the results of 2-way ANOVA, saccharide (P = 0.014), the level (P < 0.001) on day 7, and the level (P < 0.001) on day 13 affected the concentrations.
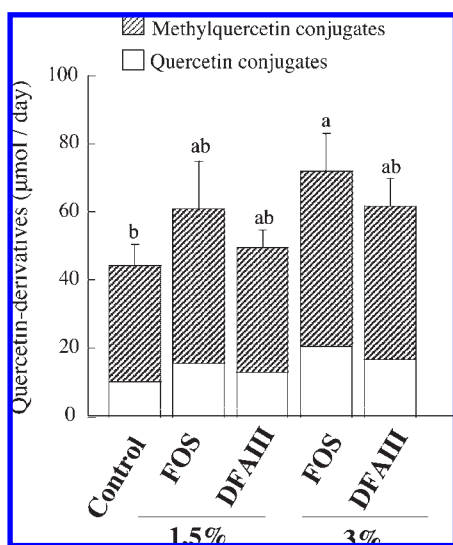
Figure 3. Urinary excretion of quercetin and monomethylquercetin (sum of isorhamnetin and tamarixetin) conjugates in rats fed the control (only Q3G), FOS, or DFAIII diets on days 13 and 14 after the start of feeding the test diets. Values are the means ± SEM, n = 8. Error bars refer to the sum of the metabolites. P values by one-way ANOVA were 0.020. Means not sharing a common letter differ significantly; P < 0.05. According to the results of 2-way ANOVA, saccharide (P = 0.033) affected the excretion.

and significantly increased in the blood flavonoid concentrations. The values for the 3% groups were more than 2-fold higher than the value for the control group but were similar between FOS and DFAIII groups. The concentrations and variations among groups were similar at 17:00 to those at 10:00 on day 7 and 13 (data not shown).

Urinary excretion levels of quercetin and methylquercetin conjugates were also greater in the 3% FOS group and tended to be greater in the other oligosaccharide groups than in the control group (**Figure 3**). Quercetin derivatives in the feces collected on the last 4 days, all of which were excreted in unconjugated forms, were mostly methylquercetin with only low levels of quercetin aglycone present. The fecal excretion of the quercetin derivatives was doubled in the 3% oligosaccharide groups (**Figure 4**). The cecal contents included only unconjugated
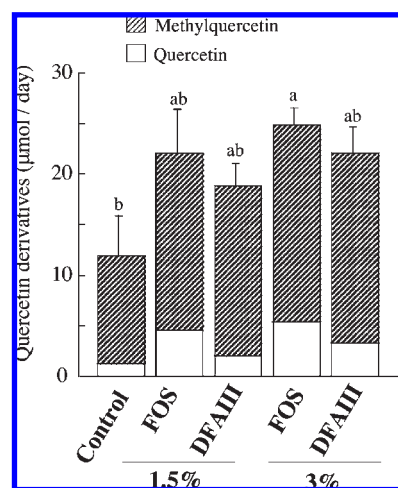
Figure 4. Fecal excretion of quercetin derivatives, quercetin and mono-methylquercetin (isorhamnetin and tamarixetin), in rats fed the control (only Q3G), FOS, or DFAIII diets in week 2 after the start of feeding the test diets. Values are the means ± SEM, n = 8. Error bars refer to the sum of the metabolites. P values by one-way ANOVA were 0.013. Means not sharing a common letter differ significantly; P < 0.05.

forms, and the increases in the cecal levels of these quercetin derivatives were dose-dependent (**Figure 5**). The amount of quercetin aglycone degraded, which was evaluated by subtracting the sum of aglycone in the quercetin derivatives excreted into the feces and urine from the total ingested aglycone in Q3G (**Table 4**), was clearly and dose-dependently lower in rats fed FOS and DFAIII diets than in the control rats. The remaining quercetin aglycone is a percentage of restored aglycone against ingested aglycone composed in the Q3G molecule, and the value in the 3% FOS group was twice that observed in the control group.

Q3G, Quercetin, and Methylquercetin Degradation by the Cecal Contents in Rats Fed the Test Diets. Q3G was completely degraded by incubation with the cecal contents from the control rats for 120 min, but the level of degradation was much lower for rats fed FOS or DFAIII. Quercetin glucoside was restored by more than 80% in the DFA group and 60% in the FOS group (**Figure 6A**). Free quercetin aglycone levels were increased during the incubation of Q3G with the cecal contents, and the increase in
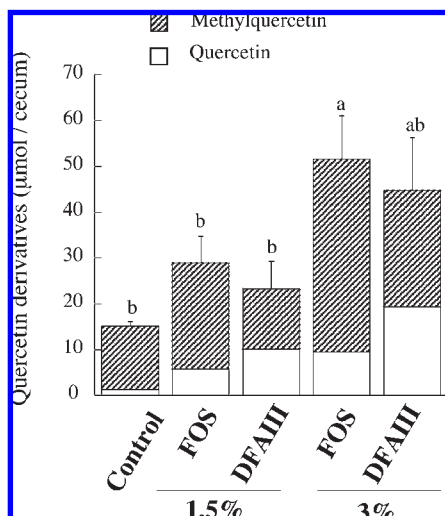
Figure 5. Cecal pools of quercetin and monomethylated quercetin (isorhamnetin and tamarixetin) in rats fed the control (only Q3G), FOS, or DFAIII diets 2 weeks after the start of feeding the test diets. Values are the means ± SEM, $n = 8$. Error bars refer to the sum of the metabolites. P-values by one-way ANOVA were 0.007. Means not sharing a common letter differ significantly; $P < 0.05$. According to the results of 2-way ANOVA, level ($P = 0.015$) affected the cecal pools.

Table 4. Amount of Quercetin Aglycone Degraded per Day and Rate (%) of Remaining Aglycone in Rats Fed Test Diets Containing Q3G with or without 1.5% or 3% FOS or DFAIII for 2 Weeks[a]

|	ingested aglycone (μmol/day)	excreted aglycone (μmol/day)	degraded aglycone (μmol/day)	remaining aglycone (%)
control	335 ± 7 a	55 ± 5 b	280 ± 4 a	16 ± 1 c
1.5% FOS	305 ± 10 ab	76 ± 12 ab	217 ± 15 bc	28 ± 4 ab
1.5% DFAIII	312 ± 7 ab	68 ± 4 ab	245 ± 7 b	22 ± 1 bc
3% FOS	299 ± 7 b	95 ± 10 a	205 ± 7 c	32 ± 3 a
3% DFAIII	289 ± 11 b	78 ± 3 ab	218 ± 8 bc	26 ± 1 ab
1-way ANOVA P value	0.007	0.018	<0.001	<0.001
2-way ANOVA P value				
saccharide	0.910	0.136	0.058	0.025
level	0.106	0.092	0.044	0.106
saccharide × level	0.374	0.610	0.464	0.805

[a] Values are the means ± SEM for eight rats. Values not sharing a common letter are significantly different ($P < 0.05$). One-way ANOVA was performed for all groups, and 2-way ANOVA was performed for the four oligosaccharide groups. Excreted aglycone (μmol/day) = sum of total aglycone excreted into urine and feces as free, methylated, and conjugated forms. Degraded aglycone (μmol/day) = ingested aglycone − excreted aglycone. Remaining aglycone (%) = 100 − (degraded aglycone/ingested aglycone × 100).

aglycone shows a reciprocal change against the decrease in Q3G among the diet groups (**Figure 6B**). The remaining rate of total aglycone (the sum of Q3G and quercetin) in the culture media was reduced to 70% in the control group; however, the level of aglycone in the FOS and DFAIII groups was almost completely restored (**Figure 6C**).

We also directly estimated the degradation of quercetin aglycone and methylquercetin. Seventy percent of quercetin aglycone was degraded by the cecal contents of the control group (remaining rate: 30%), but the remaining rates in both the oligosaccharide groups were around 70%, which indicates that degradation was suppressed by half (from 70% to 30%) with feeding of FOS and DFAIII (**Figure 7A**). The remaining rates of methylquercetin with the cecal bacteria were higher compared with those of quercetin and were more than 80% in the FOS and DFAIII groups and 60% in the control group after 120 min of incubation (**Figure 7B**).

Absorptive Capacity of Luminal Quercetin Derivatives in the Ligated Cecal Sacs. Concentrations of methylquercetin and quercetin conjugates in the mesenteric vein of the cecal sacs in anesthetized rats, which were acclimated with the basal diet, were much higher than those in the ligated ileum segments after instillation of an extract containing quercetin metabolites prepared from the cecal contents of rats fed a 1% Q3G diet (**Figure 8**). The total amount of absorbed quercetin derivatives estimated by subtraction of the remaining quercetin derivatives in the segments (sum of contents and stripped mucosa) from the instilled derivatives was higher in the cecum than in the ileum (**Table 5**). The absorptive rate per mucosal area was higher in the cecal segment than in the ileal segment. These results show that the absorptive capacity of the cecum is comparable to that of the small intestine.

DISCUSSION

We found that the blood levels of quercetin derivatives (quercetin and methylquercetin conjugates) were dose-dependently and adaptively increased by the feeding of a Q3G diet containing 1.5% or 3% FOS and DFAIII compared with those in

rats fed a Q3G diet without oligosaccharides. These increases were accompanied by higher urinary excretions of these quercetin derivatives. This finding indicates the promotion of Q3G bioavailability by FOS and DFAIII. The oligosaccharide levels in the test diet are effective in increasing calcium absorption in rats and are also at the usual and safe levels (7.8 or 15 g/2,000 kcal) for humans. The flavonoid level is rather high but is at a possible intake level as a supplement. Fecal excretion of quercetin derivatives, mainly methylquercetin, was also much higher in the FOS and DFAIII groups than in the control group. The results of the present study were similar to those from our previous study using αG-rutin (20), which is an artificially modified water-soluble quercetin glycoside bearing a very different sugar moiety, suggesting that a common mechanism is involved in the enhancement of quercetin glycoside bioavailability by nondigestible oligosaccharides.

The sum of the urinary and fecal excretion rates of aglycone against the ingested aglycone composed in the Q3G was approximately 30% in the FOS and DFAIII groups and 16.4% in the control group. These values, shown as remaining aglycone in **Table 4**, indicate the restoration rates of quercetin aglycone through the avoidance of degradation in rats after feeding and suggest that these oligosaccharides suppressed the degradation of aglycone in the large intestine because the rats do not possess enzymes necessary for quercetin aglycone degradation but rely on intestinal bacteria for this process (16, 17, 24). We showed that only 16.4% of quercetin aglycone remained in the control group, as mentioned above, and that this result reveals that nearly 85% of quercetin aglycone was degraded by the intestinal bacteria in the control group, which agrees with the previous study showing that a major part of ingested quercetin aglycone degraded and converted to phenolic acids in rat in vivo experiments (25). Usually, the bioavailability of naturally occurring flavonoids such as Q3G is known to be relatively low (13). Our results and previous results demonstrate that the low availability of flavonoids results from the high degradation rates of their aglycone moiety by intestinal bacteria. We found that quercetin conjugate levels in the tail blood of the control group were periodically reduced during the 2-week feeding of the Q3G diet without
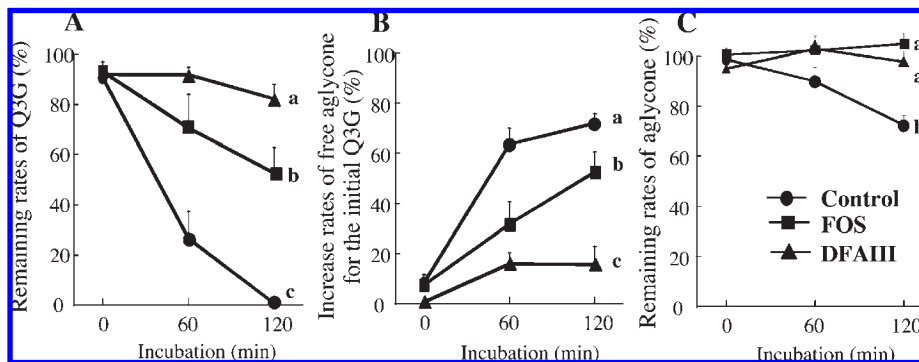
Figure 6. Remaining rates of Q3G for the initial level (**A**), increase in quercetin aglycone against the initial level of Q3G by hydrolysis of Q3G (**B**), and total aglycone (sum of Q3G and quercetin (**C**)) present after incubation with the cecal contents prepared from rats fed the control, DFAIII, or FOS diets for 2 weeks. Q3G was added to each bottle to a final concentration of 1 mmol/L. Values represent the means $\pm$ SEM, n = 7. Means not sharing a common letter differ significantly; P < 0.05.
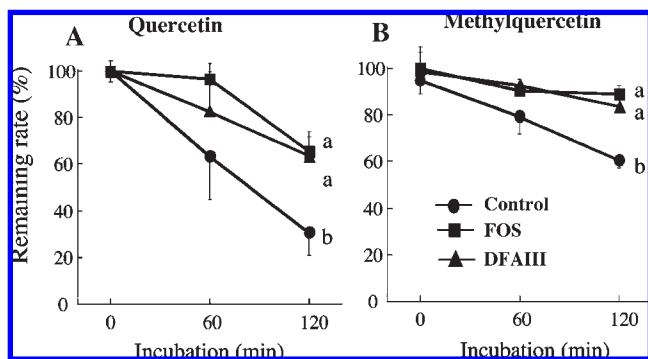
Figure 7. Remaining rates of quercetin (**A**) and methylquercetin (**B**) present after incubation with the cecal contents prepared from rats fed the control, DFAIII, or FOS diets for 2 weeks. Quercetin or methylquercetin (tamarixetin) was added to each bottle to a final concentration of 1 mmol/L. Values represent the means $\pm$ SEM, n = 6. Means not sharing a common letter differ significantly; P < 0.05.
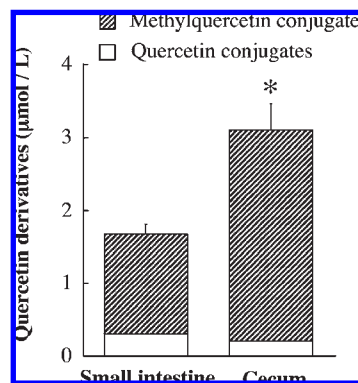

Figure 8. Concentration of quercetin and monomethylquercetin in the blood plasma of the cecal vein and mesenteric ileal vein 45 min after instillation. Two or 1 mL of supernatant of the cecal contents from rats fed Q3GM containing 736 nmol or 363 nmol of monomethylquercetin was instilled into the ligated cecal or ileal loop, respectively. Values represent the means $\pm$ SEM, n = 7. *Significant difference (P < 0.05).

Table 5. Absorptive Rate of Luminal Quercetin Derivatives from Rats Fed Q3G by the Ligated Cecum or Ileum of Anesthetized Rats[a]

	cecum	ileum
remaining aglycone (nmol)	406 $\pm$ 40	129 $\pm$ 23
amount of absorption (nmol)	318 $\pm$ 37	226 $\pm$ 23
surface area of the segment (cm^2)	10.9 $\pm$ 1.5	12.5 $\pm$ 2.2
rate of absorption (nmol/cm$^2\cdot$h)	425 $\pm$ 79[b]	251 $\pm$ 30

[a] Quercetin derivatives, containing 736 nmol or 363 nmol of monomethylquercetin prepared from the cecal contents of rats fed Q3G, were instilled into the ligated cecal or small intestinal segments. The luminal solutions in the ligated cecum and ileum were collected 45 min after instillation. Values represent thhe means $\pm$ SEM, n = 7. [b] Significant difference (P < 0.05).

oligosaccharides, which supports the idea that the degradation of luminal Q3G was adaptively increased and the absorption rates of quercetin reduced.

It has been previously suggested that oligosaccharides promote the absorption of isoflavone glycosides by enhancing the removal of the sugar moiety by the intestinal bacteria (*19*). It has been shown that β-glucosidase is derived from *Enterococcus casseliflavus*, an intestinal bacteria, to contribute to the hydrolysis of flavonoid glycosides (*26*). The sugar moiety from isoflavone glycoside was rapidly removed using a culture system with human feces with or without the addition of fructooligosaccharides (*27*). In contrast, we demonstrated that the degradation of Q3G was largely suppressed by the feeding of FOS and DFAIII using cecal contents containing live intestinal bacteria from rats fed the oligosaccharides (**Figure 6A**), which suggests that the effects of oligosaccharides depend on aglycone structure and also suggests that the cecal bacteria adapted to oligosaccharide feedings may preferentially utilize the oligosaccharides rather than the sugar moieties in quercetin glycosides.

We provide evidence that the degradation of quercetin aglycone by cecal bacteria was suppressed by feedings of FOS and DFAIII (**Figure 6C**), which was confirmed by the results of the experiment in which quercetin aglycone and methylquercetin were added to the cultured medium of rat cecal bacteria (**Figure 7**). The feeding of FOS and DFA reduced the degradation activity of cecal bacteria for quercetin aglycone by half, from 70% to 35%, and also reduced the activity against methylquercetin by

a similar degree, from 40% in the control group to 20% in the FOS and DFA groups. These findings suggest that the enzyme or bacterium involved in the breakdown of quercetin or methylquercetin is the same but that methylquercetin is more resistant to degradation by this enzyme. This may be one reason for the fact that the major portion of quercetin derivatives present in the feces or in the cecal contents was in the methylated form. During the metabolism of quercetin, others have reported that aglycone is cleaved into phloroglucinol and 3,4-dihydroxyphenylacetic acid by intestinal bacteria. *Clostridium orbisciandens* (*17*) and *Eubacterium ramlus* have both been identified as the bacteria involved in aglycone metabolism in human feces (*28*). *Bifidobacterium* spp. and *Lactobacillus* spp. are known to be the major contributors to

9468 *J. Agric. Food Chem.,* Vol. 57, No. 20, 2009

Matsukawa et al.

FOS fermentation in the large intestine (*27*), and *Ruminococcus productus* has been identified as a DFA-assimilating bacteria (*29*). These intestinal bacteria or their fermentation products may commonly suppress proliferation of the quercetin degrading bacteria described above.

In order that the restored quercetin derivatives in the cecum contribute to the increase of the bioavailability of Q3G, the cecum must have a considerably high absorptive activity for quercetin derivatives. We examined the absorptive rates for quercetin metabolites, mainly in the form of unconjugated methylquercetin, in the cecal lumen compared with those in the small intestine. After an instillation of the quercetin metabolites into the cecal segment of anesthetized rats, the increase in the concentration of quercetin derivatives in the cecal vein was much higher than that in the mesenteric vein of the ileum. Absorptive speed per mucosal surface area was 70% greater in the cecum than in the ileum. These findings clearly show that the cecum has a high absorptive activity for the unconjugated form of quercetin derivatives that is comparable to that of the small intestine.

In conclusion, FOS and DFAIII equally promote the bioavailability of a quercetin glycoside, Q3G, in rats. Suppression of the degradation of quercetin aglycone by cecal bacteria contributes to the promotive effect in association with the high absorptive capacity of the cecum.

ABBREVIATIONS USED

Q3G, quercetin-3-*O*-β-D-glucoside; FOS, fructooligosaccharides; DFAIII, di-D-fructose anhydride III.

Supporting Information Available: Individual organic acid pools in the cecal contents of rats fed the test diet for 2 weeks. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Sakakibara, H.; Honda, Y.; Nakagawa, S.; Ashida, H.; Kanazawa, K. Simultaneous determination of all polyphenols in vegetables, fruits, and teas. *J. Agric. Food Chem.* **2003**, *51*, 571–581.

(2) Manach, C.; Scalbert, A.; Morand, C.; Remesy, C.; Jimenez, L. Polyphenols: food sources and bioavailability. *Am. J. Clin. Nutr.* **2004**, *79*, 727–747.

(3) Bischoff, S. C. Quercetin: potentials in the prevention and therapy of disease. *Curr. Opin. Clin. Nutr. Metab. Care* **2008**, *11*, 733–740.

(4) Hertog, M. G.; Kromhout, D.; Aravanis, C.; Blackburn, H.; Buzina, R.; Fidanza, F.; Giampaoli, S.; Jansen, A.; Menotti, A.; Nedeljkovic, S.; et al. Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. *Arch. Intern. Med.* **1995**, *155*, 381–386.

(5) Chun, O. K.; Chung, S. J.; Claycombe, K. J.; Song, W. O. Serum C-reactive protein concentrations are inversely associated with dietary flavonoid intake in U.S. adults. *J. Nutr.* **2008**, *138*, 753–760.

(6) Morand, C.; Manach, C.; Crespy, V.; Remesy, C. Quercetin 3-O-beta-glucoside is better absorbed than other quercetin forms and is not present in rat plasma. *Free Radical Res.* **2000**, *33*, 667–676.

(7) Hollman, P. C.; Bijsman, M. N.; van Gameren, Y.; Cnossen, E. P.; de Vries, J. H.; Katan, M. B. The sugar moiety is a major determinant of the absorption of dietary flavonoid glycosides in man. *Free Radical Res.* **1999**, *31*, 569–573.

(8) Hollman, P. C.; van Trijp, J. M.; Buysman, M. N.; van der Gaag, M. S.; Mengelers, M. J.; de Vries, J. H.; Katan, M. B. Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man. *FEBS Lett.* **1997**, *418*, 152–156.

(9) Day, A. J.; Gee, J. M.; DuPont, M. S.; Johnson, I. T.; Williamson, G. Absorption of quercetin-3-glucoside and quercetin-4′-glucoside in the rat small intestine: the role of lactase phlorizin hydrolase and the sodium-dependent glucose transporter. *Biochem. Pharmacol.* **2003**, *65*, 1199–1206.

(10) Sesink, A. L.; Arts, I. C.; Faassen-Peters, M.; Hollman, P. C. Intestinal uptake of quercetin-3-glucoside in rats involves hydrolysis by lactase phlorizin hydrolase. *J. Nutr.* **2003**, *133*, 773–776.

(11) Wolffram, S.; Block, M.; Ader, P. Quercetin-3-glucoside is transported by the glucose carrier SGLT1 across the brush border membrane of rat small intestine. *J. Nutr.* **2002**, *132*, 630–635.

(12) Kottra, G.; Daniel, H. Flavonoid glycosides are not transported by the human Na+/glucose transporter when expressed in Xenopus laevis oocytes, but effectively inhibit electrogenic glucose uptake. *J. Pharmacol. Exp. Ther.* **2007**, *322*, 829–835.

(13) Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Remesy, C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **2005**, *81*, 230S–242S.

(14) Wiczkowski, W.; Romaszko, J.; Bucinski, A.; Szawara-Nowak, D.; Honke, J.; Zielinski, H.; Piskula, M. K. Quercetin from shallots (*Allium cepa* L. var. *aggregatum*) is more bioavailable than its glucosides. *J. Nutr.* **2008**, *138*, 885–888.

(15) Manach, C.; Morand, C.; Demigne, C.; Texier, O.; Regerat, F.; Remesy, C. Bioavailability of rutin and quercetin in rats. *FEBS Lett.* **1997**, *409*, 12–16.

(16) Keppler, K.; Hein, E. M.; Humpf, H. U. Metabolism of quercetin and rutin by the pig caecal microflora prepared by freeze-preservation. *Mol. Nutr. Food Res.* **2006**, *50*, 686–695.

(17) Schoefer, L.; Mohan, R.; Schwiertz, A.; Braune, A.; Blaut, M. Anaerobic degradation of flavonoids by *Clostridium orbiscindens*. *Appl. Environ. Microbiol.* **2003**, *69*, 5849–5854.

(18) Sakai, K.; Aramaki, K.; Takasaki, M.; Inaba, H.; Tokunaga, T.; Ohta, A. Effect of dietary short-chain fructooligosaccharides on the cecal microflora in gastrectomized rats. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 264–269.

(19) Uehara, M.; Ohta, A.; Sakai, K.; Suzuki, K.; Watanabe, S.; Adlercreutz, H. Dietary fructooligosaccharides modify intestinal bioavailability of a single dose of genistein and daidzein and affect their urinary excretion and kinetics in blood of rats. *J. Nutr.* **2001**, *131*, 787–795.

(20) Matsukawa, N.; Matsumoto, M.; Chiji, H.; Hara, H. Oligosaccharide promotes bioavailability of a water-soluble flavonoid glycoside, alpha G-rutin, in rats. *J. Agric. Food Chem.* **2009**, *57*, 1498–1505.

(21) Suzuki, T.; Hara, H.; Kasai, T.; Tomita, F. Effects of difructose anhydride III on calcium absorption in small and large intestines of rats. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 837–841.

(22) Tamura, A.; Mita, Y.; Shigematsu, N.; Hara, H.; Nishimura, N. Different effects of difructose anhydride III and inulin-type fructans on caecal microbiota in rats. *Arch. Anim. Nutr.* **2006**, *60*, 358–364.

(23) Hoshi, S.; Sakata, T.; Mikuni, K.; Hashimoto, H.; Kimura, S. Galactosylsucrose and xylosylfructoside alter digestive tract size and concentrations of cecal organic acids in rats fed diets containing cholesterol and cholic acid. *J. Nutr.* **1994**, *124*, 52–60.

(24) Braune, A.; Gutschow, M.; Engst, W.; Blaut, M. Degradation of quercetin and luteolin by *Eubacterium ramulus*. *Appl. Environ. Microbiol.* **2001**, *67*, 5558–5567.

(25) Mullen, W.; Rouanet, J. M.; Auger, C.; Teissedre, P. L.; Caldwell, S. T.; Hartley, R. C.; Lean, M. E.; Edwards, C. A.; Crozier, A. Bioavailability of [2-¹⁴C]quercetin-4′-glucoside in rats. *J. Agric. Food Chem.* **2008**, *56*, 12127–12137.

(26) Schneider, H.; Schwiertz, A.; Collins, M. D.; Blaut, M. Anaerobic transformation of quercetin-3-glucoside by bacteria from the human intestinal tract. *Arch. Microbiol.* **1999**, *171*, 81–91.

(27) Steer, T. E.; Johnson, I. T.; Gee, J. M.; Gibson, G. R. Metabolism of the soybean isoflavone glycoside genistin in vitro by human gut bacteria and the effect of prebiotics. *Br. J. Nutr.* **2003**, *90*, 635–642.

(28) Schneider, H.; Simmering, R.; Hartmann, L.; Pforte, H.; Blaut, M. Degradation of quercetin-3-glucoside in gnotobiotic rats associated with human intestinal bacteria. *J. Appl. Microbiol.* **2000**, *89*, 1027–1037.

(29) Minamida, K.; Ohashi, M.; Hara, H.; Asano, K.; Tomita, F. Effects of ingestion of difructose anhydride III (DFA III) and the DFA III-assimilating bacterium *Ruminococcus productus* on rat intestine. *Biosci. Biotechnol. Biochem.* **2006**, *70*, 332–339.