Plasma quercetin metabolites are affected by intestinal microbiota of human microbiota-associated mice fed with a quercetin-containing diet

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Original Article

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**Animals and diets.** Germ-free BALB/cA female mice were bred and maintained in the Laboratory of Veterinary Public Health, University of Tokyo. Before feeding the mice the experimental diets, animals were fed the AIN-93M pellet diet purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). At the start of the experiment, 18-weeks-old germ-free mice were randomly divided into four groups of six animals each. Fecal samples (HF1, HF2, HF3, HF4) were thawed and weighed into sterile glass homogenizers to prepare 100-fold dilutions with the anaerobic medium in an anaerobic chamber. The mice were orally inoculated with these dilutions into the stomach with a metal feeding needle in the flexible vinyl isolator. All mice were fed the AIN-93M pellet diet for three weeks, followed by a 0.05% quercetin-containing pellet diet for four weeks. The composition of the quercetin-containing diet was 14% casein, 0.18% L-cysteine, 38.8179% corn starch, 16.7% α-corn starch, 10% sucrose, 3% soy bean oil, 7.5% lard, 5% cellulose powder, 3.5% AIN-76 mineral mix, 1% AIN-93 vitamin mix, 0.25% choline bitartrate, 0.0021% tert-butylhydroquinone, and 0.05% quercetin. Both the AIN-93M and 0.05% quercetin-containing diets were sterilized by γ-irradiation at 50 kGy and the final body weight was measured. Three days before the end of the experimental diet-feeding, feces were collected individually from all mice and stored at −80°C until analysis of bile acids. After the experimental diet-feeding period, the mice were anesthetized and sacrificed by exsanguination and blood samples were taken from the heart and placed in heparinized tubes. The plasma was separated from whole blood by centrifugation and stored at −80°C until analysis of plasma quercetin metabolites and plasma lipids. The liver, visceral fat, and cecal contents were collected and weighed. Liver samples were stored at −80°C until liver lipids analysis. All procedures involving mice in this study were approved by the Animal Care Committee of the University of Tokyo, in accordance with guidelines from the “Guidelines for Animal Care and Experimentation” of the University of Tokyo.

**Measurement of plasma cholesterol, triglyceride, non-esterified fatty acid (NEFA).** The following tests were performed with kits purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Total plasma cholesterol concentrations were measured using a cholesterol E-test kit based on cholesterol oxidase. Plasma triglyceride concentrations were determined using a triglyceride E-test kit based on the glycerol-3-phosphate oxidase method. Plasma non-esterified fatty acid (NEFA) concentrations were measured using a NEFA C-test kit involving the acyl-coenzyme A (CoA) synthase, acyl-CoA oxidase, and FAD-dependent MS/MS parameters was performed by direct infusion of standards. Each analyte dissolved in MeOH (1–10 mg/L) was supplied at the rate of 10 μl/min with a syringe pump, mixed via a “mixing T” with the carrier solvent adjusted to 50% B, and introduced into the mass spectrometer at a flow rate of 0.2 ml/min. MS and MS/MS parameters (such as the selection of the most abundant MRM transitions, declustering potentials, collision energies, and cell exit potentials) were optimized for all analytes in the positive APIC mode.

**DNA extraction from cecal contents.** Procedures of DNA extraction from cecal contents were conducted according to Matsuki’s method. Cecal samples (20 mg) were washed twice by resuspending them in 0.2 ml of PBS and centrifuging each preparation at 14,000 × g to remove possible PCR inhibitors. Following the second centrifugation, the cecal pellets were resuspended in a solution consisting of 0.2 ml of PBS, 250 μl of extraction buffer (200 mM Tris-HCl, 80 mM EDTA, pH 9.0), and 50 μl of 10% sodium dodecyl sulfate. A total of 300 mg of glass beads (diameter 0.1 mm) and 500 μl of buffer-saturated phenol were added to the suspension, and the mixture was vortexed vigorously for 180 s using a MicroSmash (Tomy Seiko Co., Ltd, Tokyo, Japan) at 4,000 rpm. After centrifugation at 14,000 × g for 5 min, 400 μl of the supernatant was collected. Phenol–chloroform–isoamyl alcohol extraction was then performed, and 250 μl of the supernatant was subjected to isopropanol precipitation. Finally, the obtained DNA was dissolved in 1 ml of 10 mM Tris-EDTA buffer, pH 8.0. The DNA solution was adjusted to a final concentration of 10 μg/ml in the same buffer.
Real-time PCR. qPCR was carried out on a Real-Time QPCR System MX3000p (Agilent Technologies Ltd., Santa Clara, CA) to determine Bifidobacterium, Clostridium leptum subgroup, Clostridium cocoides group, Bacteroides fragilis group, Atopobium cluster, and Enterobacteriaceae cell counts by means of specific primers (Table 1). Next, 10 ng of DNA from cecal contents (1 μl) was added to 19 μl of the reaction mix [0.4 μl of each 10 μM primer, 8.2 μl of distilled water and 10 μl of 2× KAPA SYBER FAST qPCR Master Mix Universal (Kapa Biosystems Inc., Wilmington, MA)].

The amplification program for the Atopobium cluster consisted of one cycle of 95°C for 3 min and then 40 cycles of 95°C for 3 s, 62°C for 60 s. The cycling conditions for the Bacteroides fragilis group involved one cycle of 95°C for 3 min and then 40 cycles of 95°C for 3 s and 58°C for 60 s. The amplification program for Bifidobacterium consisted of one cycle of 95°C for 3 min and then 40 cycles of 95°C for 3 s and 58°C for 60 s. The amplification program for Clostridium leptum subgroup consisted of one cycle of 95°C for 3 min and then 40 cycles of 95°C for 3 s, 60°C for 60 s. The amplification program for the Clostridium cocoides group consisted of one cycle of 95°C for 3 min and then 40 cycles of 95°C for 3 s and 60°C for 60 s. The thermal cycling conditions for Enterobacteriaceae included one cycle of 95°C for 3 min and then 40 cycles of 95°C for 5 s, 66°C for 20 s, and 72°C for 40 s. To check the specificity of PCR, a melting curve analysis was conducted after the amplification. The melting curves were obtained by heating at temperatures from 55 to 95°C with continuous fluorescence monitoring. DNA samples extracted from Clostridium clindamycin JCM1291T, Bacteroides thetaiotaomicron Toky-10, Bifidobacterium longum subsp. longum JCM1217T, Eggerthella lenta JCM9979T, Escherichia coli JCM 20335, and Faecalibacterium prausnitzii JCM 31915 served as real-time PCR standard for the group-specific g-Coc, g-Bfra, g-Bifido, c-Atypo, Eco, sg-Clept primers, respectively. A standard curve was generated with the RT-qPCR data and the corresponding standard cell count. For enumeration of standard bacteria, we used the Baestain DAPI solution (Dojindo Laboratories., Kumamoto, Japan) based on 4,6-diamidino-2-phenylindole (DAPI) staining according to the manufacturer instructions and then trapped between a glass slide and a square coverslip. The cells were imaged with a fluorescence microscope (BZ-8000; KEYENCE CORPORATION, Osaka, Japan). Images were then produced by using the image analysis software ImageJ 1.52a (National Institute of Health, Bethesda, Maryland), and the total number of cells detected in each field was calculated.

Statistics. Data are expressed as mean ± SE. All data were analyzed using Sigma Plot 11 (Systat Software, Inc., San Jose, CA) by the one-way analysis of variance. In the multiple comparison, the Tukey test was used. Statistical significance was assumed at a p value <0.05. For correlation analysis, we used the Spearman Rank Order Correlation. We performed principal component analysis of the data using the online website of Gunma University (http://aokii2.si.gunma-u.ac.jp/BlackBox/BlackBox.html) for statistical analysis.

Results

No significant differences were observed among four groups in final body weight [g; HF1 (27.4 ± 1.5), HF2 (28.8 ± 0.9), HF3 (26.7 ± 0.5), HF4 (29 ± 1.2)], liver weight [g; HF1 (1.34 ± 0.08), HF2 (1.35 ± 0.04), HF3 (1.33 ± 0.06), HF4 (1.31 ± 0.06)], visceral fat [g; HF1 (1.50 ± 0.36), HF2 (2.00 ± 0.19), HF3 (1.32 ± 0.09), HF4 (1.90 ± 0.31)], food consumption [g; HF1 (3.6 ± 0.06), HF2 (3.6 ± 0.04), HF3 (3.7 ± 0.02), HF4 (3.6 ± 0.20)]. Cecal contents (g) were HF1 (0.16 ± 0.01), HF2 (0.19 ± 0.02), HF3 (0.28 ± 0.01), HF4 (0.19 ± 0.01). The cecal contents of HF3 group was significantly greater than the other three groups (p<0.05).

The concentrations of triglyceride, total cholesterol, and NEFA, and amounts of liver lipids, liver cholesterol, and liver triglyceride were shown in Table 2. Plasma total cholesterol levels were significantly lower in HF3 group (133.3 ± 6.4) than in the HF2 group (163.8 ± 6.1). There were no significant differences in plasma triglyceride and NEFA levels between the four groups. There were also no significant differences in liver lipid, liver cholesterol and liver triglyceride levels between the four groups.

Fecal bile acid concentrations are shown in Fig. 1. There was significant difference in the fecal bile acid concentration between four groups. Average fecal bile acid concentration (nmol/g feces) was greatest in HF4 group amongst the four groups. Fecal bile acid concentration was significantly greater in HF4 group (5,124.7 ± 887.4) than in the HF2 group (1,616.9 ± 253.9).

There were significant differences in the quercetin metabolites between four groups (Fig. 2). Plasma quercetin concentration (μmol/L) was significantly greater in the HF3 group (3.25 ± 0.37) than in the HF1 group (2.24 ± 0.16). Isohamnetin (3'-methyl-quercetin) is one of quercetin metabolites found in the liver and plasma isorhamnetin concentration was significantly greater in the HF3 (3.44 ± 0.26) than in HF4 group (2.6 ± 0.13). Plasma quercetin plus isorhamnetin concentration was significantly greater in the HF3 (6.69 ± 0.63) than in the HF1 group (4.98 ± 0.25).

The cell number of cecal Atopobium cluster, Bacteroides fragilis group, Bifidobacterium, Clostridium cocoides group Clostridium leptum subgroup, and Enterobacteriaceae per μg of DNA from cecal contents are shown in Fig. 3. The intestinal microbiota was different among the 4 groups. Cecal Atopobium cluster counts were significantly greater in the HF3 group than in the other three groups. Cecal Bacteroides fragilis group counts

Table 1. 16S rRNA gene-targeted group-specific primers

| Target organism            | Primers         | Sequences (5’ to 3’)            | Size (bp) | References |
|---------------------------|-----------------|--------------------------------|-----------|------------|
| Atopobium cluster         | c-Atypo-F       | GGTTGAGAGACGCGACC              | 190       | (21)       |
|                           | c-Atypo-R       | CGGRCCTCTCTCCTGAGG             |           |            |
| Bacteroides fragilis group| g-Bfra-F        | ATAGCCCTTCGAAAGRAAGAT          | 495       | (21)       |
|                           | g-Bfra-R        | CCAATACAACTGCAATTTTA           |           |            |
| Bifidobacterium           | g-Bifid-F       | CTTCGGAAAGCGGTTG              | 549–563   | (22)       |
|                           | g-Bifid-R       | GGTCTTCCCGATCATCTACA           |           |            |
| Clostridium leptum subgroup| sg-Clept-F     | GCACAGGACGTTGAG               | 239       | (21)       |
|                           | sg-Clept-R3     | CTTCTTCCGGTTTTTGCA            |           |            |
| Clostridium cocoides group| g-Ccoc-F        | AATAGCGTACCTGACATTAA          | 438–441   | (22)       |
|                           | g-Ccoc-R        | CCTCTTACCTCTTGGCGA            |           |            |
| Enterobacteriaceae        | Eco1457F        | CATTGACGGTACCGACGAAGAAGC       | 195       | (23)       |
|                           | Eco1652R        | CTCTACGAGACTCCTAAGCTGC        |           |            |
Table 2. The concentrations of plasma cholesterol, triglyceride, NEFA, amounts of liver lipids, liver cholesterol, and liver triglyceride

|                        | HF1 group | HF2 group | HF3 group | HF4 group |
|------------------------|-----------|-----------|-----------|-----------|
| Plasma cholesterol (mg/dl) | 152.0 ± 4.8<sup>a,b</sup> | 163.8 ± 6.1<sup>*</sup> | 133.3 ± 5.4<sup>b</sup> | 143.1 ± 7.7<sup>a,b</sup> |
| Plasma triglyceride (mg/dl) | 170.8 ± 10.0 | 176.1 ± 10.7 | 170.8 ± 4.3 | 212.2 ± 51.9 |
| Plasma NEFA (mEq/L)    | 2.5 ± 0.1  | 2.2 ± 0.06 | 2.5 ± 0.1  | 2.7 ± 0.4  |
| Liver lipids (g)       | 0.11 ± 0.01| 0.11 ± 0.01| 0.10 ± 0.01| 0.12 ± 0.01|
| Liver triglyceride (mg) | 53.8 ± 7.9 | 62.7 ± 0.8  | 51.2 ± 7.2 | 66.3 ± 10.0 |
| Liver cholesterol (mg)  | 7.0 ± 2.9  | 6.1 ± 1.0  | 5.6 ± 0.9  | 6.0 ± 0.5  |

Significant difference between alphabets with different superscript (<sup>p</sup> < 0.05).
were significantly greater in the HF3 group than in the HF1 and HF2 groups. Cecal Bifidobacterium counts were significantly greater in the HF3 group than in the other three groups. Cecal Clostridium leptum subgroup counts were significantly lower in the HF3 group than in the other three groups. Cecal Clostridium coccoides group counts were significantly greater in the HF1 group than in the HF2 and HF3 groups. Cecal Enterobacteriaceae counts were significantly greater in the HF3 group than in the other three groups.

Positive correlation \( (r = 0.618, p = 0.007) \) was observed between the Log_{10} Enterobacteriaceae count and the plasma quercetin metabolites (quercetin and isorhamnetin) (circle, HF1 group; triangle, HF2 group; square, HF3 group; rhombus, HF4 group).

Positive correlation \( (r = 0.606, p = 0.008) \) was observed between the Log_{10} Enterobacteriaceae count and the plasma quercetin metabolites. Positive correlation \( (r = 0.606, p = 0.008) \) was observed between the ratio of Log_{10} Clostridium coccoides group count and the plasma quercetin metabolites. Positive correlation \( (r = 0.606, p = 0.008) \) was observed between the ratio of Log_{10} Clostridium leptum subgroup count/Log_{10} Enterobacteriaceae count and the plasma quercetin metabolites. Negative correlation \( (r = -0.642, p = 0.005) \) was observed between the ratio of Log_{10} Clostridium leptum subgroup count/Log_{10} Enterobacteriaceae count and the plasma quercetin metabolites (Fig. 6).

Principal component analysis was performed by using the data of cecal number of bacteria, plasma total cholesterol, triglyceride, NEFA, liver lipids, liver cholesterol, liver triglyceride, fecal bile acids, plasma quercetin, plasma isorhamnetin, and plasma quercetin plus isorhamnetin. Contribution rate was 20.65 for principal component 1, 19.61 for principal component 2, 19.61 for principal component 3, and 12.28 for principal component 4. Distribution on the third principal component (y axis) and the second principal component (x axis) of the principal component score of HF1 group, HF2 group, HF3 group and HF4 group are clearly divided into four groups on the correlation diagram (Fig. 7).

Discussion

In our experiment, although the quercetin content of the experimental diet was the same for all groups, plasma quercetin metabolites in HF3 was significantly more than in HF1 group.
significant negative correlation with amounts of visceral fat ($r = -0.544$) (Table 3) although plasma isorhamnetin or quercetin concentration did not (Table 3). It has been reported that accumulation of visceral fat correlates with the metabolic syndrome occurrence.\(^{(27)}\) The present study suggests that relative concentration of isorhamnetin against quercetin might relate to the occurrence of metabolic syndrome. Isorhamnetin is suggested to be a quercetin metabolite formed by a liver enzyme.\(^{(16)}\) Quercetin circulating in plasma would be metabolized to a greater extent to isorhamnetin in the liver when quercetin circulates longer in blood. So, higher ratio of plasma isorhamnetin/quercetin might mean longer blood circulation time. On the other hand, HMA mice with a high ratio of plasma isorhamnetin/quercetin might have higher quercetin methylation enzyme activity in liver than those with a low ratio.

NAFLD is considered as a hepatic complication of metabolic syndrome.\(^{(28)}\) Higher ratio of plasma isorhamnetin/quercetin might contribute to the suppression of the development of NAFLD. It has been reported that quercetin alleviates metabolic syndrome.\(^{(15)}\) Our results suggest that the ratio of plasma isorhamnetin/quercetin, but not merely the concentration of quercetin or isorhamnetin in plasma, is more relevant to anti-metabolic syndrome effects of dietary quercetin. Further studies on this mechanism would be necessary in future.

Fecal bile acid concentrations were significantly greater in the HF4 group than those in the HF2 groups. Bile acids are reabsorbed from the ileum as conjugates.\(^{(29)}\) Some kinds of intestinal bacteria deconjugate the bile acid conjugate and affect the resorption of bile acid.\(^{(29,31)}\) Thus, the composition of intestinal microbiota may affect fecal excretion of bile acid.

Through principal component analysis, we found that all groups were distributed in independent regions by using the correlation diagram with the second and third principal component. It is inferred that each of these four groups have different characteristics and no intersection of regions. Principal component 3 is defined as a plasma quercetin metabolite lowering factor, and principal component 2 is defined as a microbiota related factor. The HF1 and HF4 groups have similarity in the plot distribution in the correlation diagram with the third principal component. In fact, these two groups share similar tendency in plasma quercetin and isorhamnetin level.

There were significant differences in the quercetin metabolites between four groups. Just as there are individual differences in intestinal microbiota, there might be individual differences in quercetin metabolism. As the result of principal component analysis, we suppose that different type of microbiota differently affects the plasma quercetin metabolites. Positive correlation was observed between the Log$_{10}$ Enterobacteriaceae count and the plasma quercetin metabolites (quercetin and isorhamnetin). It has been reported that Enterobacteriaceae were positively correlated with quercetin concentration in the human fecal incubation solution with quercetin.\(^{(26)}\) Positive correlation was observed between the ratio of Log$_{10}$ Enterobacteriaceae count/Log$_{10}$ Clostridium cocoides group count and the plasma quercetin metabolites. On the other hand, negative correlation was observed between the ratio of Log$_{10}$ Clostridium leptum subgroup count/Log$_{10}$ Enterobacteriaceae count and the plasma quercetin metabolites. It has been reported that the fate of quercetin in the lower gut depends on the composition of microbiota that metabolize this compound.\(^{(26)}\) The composition of the microbiota may be related to

![Fig. 7. Distribution on the third principal component (y axis) and the second principal component (x axis) of the principal component score using the correlation diagram. All four groups are clearly divided into four groups. (circle, HF1 group; triangle, HF2 group; square, HF3 group; rhombus, HF4 group).](image)

### Table 3. Correlation between plasma quercetin, isorhamnetin, the ratio of plasma isorhamnetin/quercetin with visceral fat

| Correlation with Visceral fat | Quercetin vs Visceral fat | Isorhamnetin vs Visceral fat | Isorhamnetin/Quercetin vs Visceral fat |
|-----------------------------|--------------------------|-----------------------------|--------------------------------------|
| Correlation coefficient     | 0.147                    | -0.108                      | -0.544*                              |
| $p$ value                   | 0.529                    | 0.644                       | 0.013                                |

*Significantly different ($p<0.05$).
the bioavailability of quercetin metabolites. Intestinal microbiota affects the quercetin degradation.\(^{(11-13)}\) Quercetin undergoes glucuronidation in the intestinal cells\(^{(36)}\) and direct biliary excretion of enterically or extrahepatically derived flavonoid glucuronides has been reported.\(^{(35)}\) Intestinal microbiota has glucuronidase activity and deconjugation of quercetin-glucuronide occurs by these bacterial glucuronidases.\(^{(34)}\) Thus, intestinal bacteria would affect the quercetin absorption in the lower gut.

Quercetin ameliorates high fat diet-induced NAFLD in male Sprague-Dawley rats.\(^{(35)}\) Protective effects of quercetin on high-fat diet-induced NAFLD in mice are reported.\(^{(36)}\) On the other hand, development of NAFLD is affected by the intestinal microbiota.\(^{(35)}\) Modulation of intestinal microbiota leads to a new therapeutic approach for NAFLD.\(^{(36)}\) In our experiment, plasma quercetin concentration in HF3 group was the highest in all groups. Average visceral fat weight was the lowest in HF3 group among all groups. The intestinal microbiota was significantly different among the 4 groups. The intestinal microbiota of HF3 group may be the most effective in preventing NAFLD. Prebiotic dietary fiber helps improve constipation and gut dysbiosis symptoms and behavioral irritability in children with autism spectrum disorder.\(^{(32)}\) Quercetin supposed to have a prebiotic-like effect.\(^{(30)}\) The present study has indicated that intestinal microbiota of human microbiota-associated mice inoculated with fecal suspensions from different donors react to dietary quercetin in different ways. Thus, it is speculated that intestinal microbiota strongly affects the bioavailability of quercetin. Intestinal microbiota that improves the bioavailability of quercetin may be effective in preventing NAFLD.

**Author Contributions**

MT and KH designed research. KH, MT, HN, SH, TS conducted research and analyzed data. MT wrote paper. All authors read and approved the final manuscript.

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**Conflict of Interest**

No potential conflicts of interest were disclosed.

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