Title

Combination of α-glycosyl-isoquercitrin and soybean fiber promotes quercetin bioavailability, glucagon-like peptide-1 secretion, and improves glucose homeostasis in rats fed a high-fat and high-sucrose diet

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Abstract

This study examined the effects of a combination of soybean fiber and α-glycosyl-isoquercitrin (AGIQ) on improving quercetin bioavailability and glucose metabolism in rats fed an obesogenic diet. For 9 weeks, rats were individually fed a control diet, a high-fat high-sucrose (H) diet, H with soybean fiber (HS), or with AGIQ (HQ), or with both (HSQ). Quercetin derivatives in plasma, feces, urine, and cecal content were quantified by HPLC to assess the bioavailability of quercetin, and meal tolerance tests were performed to assess postprandial glycemia and glucagon-like peptide-1 (GLP-1) responses. HSQ group had higher plasma quercetin levels than HQ. The postprandial glycemia was attenuated in HSQ group when compared to H group. The basal plasma GLP-1 concentrations positively correlated with plasma quercetin derivatives concentrations. Hence, the combination of soybean fiber and AGIQ could be beneficial for reducing the risk of glucose intolerance, possibly involving enhanced quercetin bioavailability and GLP-1 secretion.

Keywords: Diabetes, Diet-induced obesity, Fermentable fiber, Glucose tolerance

Introduction

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a flavonoid belonging to the flavonol class and is widely distributed in various kinds of vegetables and fruits, mainly as in a glycosylated form.[1] Reportedly, quercetin possesses protective effects against numerous diseases such as; cancer in *in vitro* and animal studies;[2] cardiovascular diseases in animal and human studies;[3] obesity in *in vitro*, high-fat diet fed animal and obese human studies;[4,5] and diabetes in db/db mice and streptozotocin induced rat studies.[6] Numbers of *in vitro* and animal studies suggest the beneficial potential of quercetin, but studies demonstrating its efficacy in human subjects are limited.[5,7]

After ingestion, hydrolysis of glycoside into aglycone by the intestinal enzyme is thought to be an essential step for its absorption into intestinal epithelial cells. After absorbed, the conjugation for quercetin metabolites mainly occurred in the liver. However, intestinal absorbability of quercetin is known to be low.[2-7] Concomitantly, unabsorbed quercetin aglycone and some metabolites were transported to the large intestine, the main site of gut fermentation.[8] In here, the intestinal bacteria with various enzymatic activities can deconjugate (transform) quercetin metabolites and further degrade the quercetin aglycone, which results in loss of biological activities from the original compound.[9] An *in vitro* fermentation study revealed that quercetin can be degraded by the anaerobic pathogen. Along with this degradation, a reduction in antioxidant activities and increment in a degraded product of quercetin, 3,4-dihydroxyphenylacetic acid were demonstrated.[10] Thus, the degradation by large intestinal bacteria and poor intestinal absorption of quercetin limit quercetin's bioavailability, and health beneficial effects of this flavonol.

Recently, we have reported that soybean fiber is the best candidate, among other tested water-soluble dietary fibers such as pectin and guar gum, to improve quercetin

bioavailability in a long-term feeding study performed in rats.[11] This study has revealed that cecal fermentation promoted by feeding the dietary fiber is linked with the bioavailability of quercetin. Accordingly, it was expected that the increased quercetin bioavailability would prevent the impairment of glucose homeostasis induced by chronic excessive energy intake.

Currently, type 2 diabetes is turning into an epidemic worldwide.[12] This disease gradually develops owing to impaired glucose homeostasis, comprised of glucose intolerance and insulin resistance.[13] For the treatment of type 2 diabetes, incretin-based therapy has been developed and successfully used.[14] Glucagon-like peptide-1 (GLP-1) is an incretin hormone released by enteroendocrine L cells in response to luminal nutrients.[15] GLP-1 plays a crucial role in glucose homeostasis by enhancing insulin secretion in a glucose dependent manner and possibly promoting the beta-cell mass.[16] Furthermore, we have demonstrated the protective role of enhanced GLP-1 secretion during the development of glucose intolerance in diet-induced obese rats.[17] Recently, manipulating GLP-1 secretion by various strategies has been gaining momentum as a promising strategy for preventing glucose intolerance.[18,19] In our previous study using rats, the combination of a fructo-oligosaccharide and quercetin-3-O-β-d-glucoside (isoquercitrin) demonstrated an improvement in glucose tolerance and insulin sensitivity, with increased GLP-1 secretion and plasma quercetin concentration.[20]

Thus, in the present study, we examined whether the combined ingestion of α-glycosyl-isoquercitrin (AGIQ) and soybean fiber promotes quercetin bioavailability, affects GLP-1 secretion, and prevents glucose intolerance in rats continuously fed an obesogenic high-fat high-sucrose diet.

78

79 ## Materials and methods

80 ### Animals and diets

81 Thirty-five male Wistar-ST rats 5 weeks old, weighing about 130-150 g (purchased from
82 Japan SLC, Inc., Shizuoka, Japan) were housed in individual stainless-steel cages with wire mesh
83 bottoms. Rats were maintained in a temperature-controlled room (22 ± 2°C) with a 12 h light-dark
84 cycle (light period 08.00–20.00) and fed using a modified American Institute of Nutrition-93G
85 (AIN-93G) diet,[21] by changing the cellulose content from 5% to 7% (2% of cellulose substituted
86 with corn starch, Table 1). In the test diet, α-glycosyl isoquercitrin (AGIQ, kindly provided by
87 San-Ei Gen F.F.I., Inc., Osaka, Japan), mainly consisting of quercetin-3-O-β-d-glucoside (Q3G,
88 31.8%), mono (23.3%), and di (20.3%) D-glucose adducts with an α-1,4-linkage, was used as a
89 quercetin source. Soybean fiber (kindly provided by Fuji Oil Co., Ltd, Osaka, Japan), a low
90 viscosity highly fermentable fiber, was used as supplemental fiber. After one week of acclimation,
91 the rats were divided into five groups (n=7) based on body weight and basal plasma glucose
92 concentration. For 9 weeks, each group of rats was respectively fed the modified AIN-93G as a
93 control (C) diet, a high-fat high-sucrose (H) diet containing 30% fat and 40% sucrose,[17,22] a high-
94 fat high-sucrose diet with either soybean fiber (HS) or AGIQ (HQ), and high-fat high-sucrose with
95 both soybean fiber and AGIQ (HSQ), ad libitum. The supplementary doses of AGIQ and soybean
96 fiber were selected based on a previous study.[11] In addition, the usage of higher concentrations
97 (0.7%) of quercetin in both mice and human studies had been reported.[23,24] Body weights and food
98 intakes were measured every 1 or 2 days. Nine weeks after the start of test feeding, rats were fasted
99 overnight and euthanized by whole blood withdrawal. Furthermore, the visceral adipose tissue
100 (mesenteric, retroperitoneal, and epididymal adipose tissue) was carefully dissected and weighed.

The whole cecum was collected and stored at -80°C until measurement. The experiments were conducted according to the guidelines of the Hokkaido University for the care and use of laboratory animals, and the study was approved by the Hokkaido University Animal Committee (approval reference number: 19-0072).

Because we designed to elucidate the impact of soybean fiber on quercetin bioavailability and glycemic response in a diet-induced obesity rat model, together with the role of GLP-1 in insulin secretion is well established in normal conditions, the effect of soybean fiber and/or AGIQ was examined only in a high-fat high-sucrose diet (not in the control diet fed rats).

Cecal content treatment for pH and short-chain fatty acid measurement

The frozen cecum was separated into cecal tissue and contents. The contents were diluted with four times weight of deionized water, and the pH was measured using a pH meter (B-211 twin waterproof pH, Horiba, Ltd., Kyoto, Japan). The remaining diluted sample (700 μl) was mixed with 200 μL of the internal standard (25 mM crotonic acid in 50 mM sodium hydroxide) and centrifuged at 15600*g* for 10 min at 4°C. The supernatant (600 μL) was mixed with the same volume of chloroform and centrifuged again. The supernatant was filtrated through a 0.2 μm membrane, followed by short-chain fatty acid measurement using high-performance liquid chromatography (HPLC)[25,26] with two shim-pack SCR-102H (8 mm I.D.×300 mm L, Shimadzu Corp., Kyoto, Japan) and a conductivity detector CCD-6A (polarity: +, response: slow, temperature: 45°C; Shimadzu Corp., Kyoto, Japan).

Sample treatment for quercetin measurement

123 At week 2, 4, 6, and 8 of the feeding periods, urine and feces were collected for 2 and 3
124 days, respectively. On the day after feces collection, tail vein blood samples were collected into a
125 tube containing heparin (final concentration of 50 IU/mL blood; Ajinomoto Company, Inc., Tokyo,
126 Japan) and aprotinin (final concentration of 500 kallikrein inhibitor units (kIU)/ml blood; Wako
127 Pure Chemical Industries, Ltd., Osaka, Japan). The collected blood samples were centrifuged at
128 2300*g* for 10 min at 4°C to separate the plasma before storage at -80°C. During urine collection,
129 bacterial degradation of quercetin metabolites was prevented by adding 0.05% sodium azide
130 (Sigma Aldrich, Saint Louis) into a collection tray. After two days, the collected urine was filled
131 up to 100 mL, and 15 mL of diluted urine samples were stored at -40°C. Fecal samples were
132 collected by placing a steel sieve mesh under the rat cage for three days, followed by storage at -
133 80°C. After the urine and feces were collected, the rats were rested for one day before overnight
134 fasting, and the meal tolerance test (MTT) or plasma collection was performed on the next day as
135 mentioned above.

136 On extraction, plasma and urine samples (100 μL) were acidified with 10 μL of 0.58 mol/L
137 acetic acid. In both samples, quercetin and its metabolites have been detected in substantial
138 amounts as conjugate metabolites.[27] To obtain quercetin and methylquercetin aglycone derivatives,
139 7.5 μL of β-glucuronidase and sulfatase (from *Helix pomatia* extract; Sigma Aldrich, Saint Louis),
140 and 7.5 μL of sulfatase (from *Aerobacter aerogenes*; Sigma Aldrich, Saint Louis), were added and
141 incubated at 37°C for 1 h. The reaction mixture was mixed with 100 μL of the internal standard
142 (naringenin 10 μM at final), heated at 100°C for 1 min, and centrifuged at 9300*g* for 5 min at 4°C.
143 The supernatant was collected and the precipitate was twice re-extracted with methanol to recover
144 aglycone of quercetin derivatives in the precipitant. The collected supernatant was loaded into a
145 C18 cartridge (Oasis HLB; Waters Co. Ltd., Massachusetts) and eluted with 1 mL methanol.

For feces, frozen samples were dried and milled into a powder. The feces powder (0.1 g) was mixed with 1 mL of 80% methanol and 100 μL of internal standard (naringenin 50 μM at final), then homogenized using an ultrasonic homogenizer (VP-050, ULTRAS homogenizer; Taitec Co. Ltd., Nagoya, Japan) with 30% power for 20 s. The homogenized sample was incubated at 60°C for 1 h and centrifuged at 2300*g* for 10 min at 4°C. This extraction procedure was repeated twice. In the case of cecal contents, one gram of the content was suspended in 5 mL of deionized water. The suspended sample (100 μL) was mixed with 100 μL of internal standard (naringenin 40 μM at final) and heated at 100°C for 1 min. The supernatant was collected by centrifugation at 9300*g* for 5 min at 4°C. This extraction procedure was repeated two times by adding methanol instead of the internal standard as in the previous step. The accumulated supernatant, feces, or cecal content, was loaded into a C18 cartridge similar to plasma and urine samples. As fecal and cecal contents usually appear in the aglycone form, these samples were extracted without deconjugation enzyme treatment. All eluted samples were dried by evaporation and then reconstituted with 50% methanol on the measurement day.

Quercetin measurement using HPLC

The sum of quercetin aglycone and methylquercetin aglycone was considered as the total quercetin derivative, which was quantified using HPLC[28] (Thermo Scientific Dionex UltiMate™ 3000 HPLC system; Thermo Fisher Scientific Inc., Massachusetts). The analyte was separated using a ZORBAX RRHD Eclipse Plus Phenyl-Hexyl C18 (2.1×100 mm, 1.8 μm; Agilent Technologies Japan, Ltd., Tokyo, Japan) column with setting temperature at 50°C. The mobile phases were methanol with formic acid (99.99:0.01 as solvent A) and water with formic acid (99.99:0.01 as solvent B). For measurement, the linear gradient started with solvent A and B at a

ratio of 70:30, steadily change to solvent B 100% in 5 min (0-5 min), isocratically hold for 1 min (5-6 min), then decreased back to 70:30 within 1 min (6-7 min), followed by re-equilibration for 1 min (7-8 min). Naringenin and quercetin (quercetin aglycone and methylquercetin aglycone) were detected at 280 nm and 370 nm, respectively. The concentrations of quercetin derivatives were calculated from the peak area based on the calibration curve of standard compounds.

Meal tolerance test

MTT was performed instead of an oral glucose tolerance test because nutrients from a mixed meal can provide relevant postprandial responses regarding glycemia, GLP-1, and insulin secretions.[17,29,30]

During the feeding period, MTT was performed after overnight fasting (16 h) every 4 weeks (at week 4 and 8). Here, tubes containing anti-coagulant, as described in plasma collection for quercetin measurement, were used. Blood samples from the tail vein of rats were collected before (0 min) and after (15, 30, 60, 90, and 120 min) oral gavage administration of the liquid meal (15 kcal/ 10 ml per kg body weight; Ensure H, Abbott, Japan). The collected blood samples were centrifuged, then the plasma was separated and stored at -80°C. The collected plasma was used for glucose, insulin, and total GLP-1 measurements using the Glucose CII Test Kit (Wako Pure Chemical Industries, Osaka, Japan), Insulin ELISA (U-E type; Shibayagi Company Limited, Gunma, Japan), and Total GLP-1 ELISA Kit (Wako Pure Chemical Industries, Osaka, Japan), respectively. All analytical procedures, using the respective test kits, were performed in accordance with the instructions provided with the assay kit.

GLP-1 measurement in cecal tissue

GLP-1 concentrations in cecal tissue were measured as described before.[30] Briefly, A segment of cecal tissue was homogenized in a mixture of ethanol:water:12 M HCl (74:25:1, 5 mL/g tissue), and extracted at 4°C for 24 hr. The homogenized sample was centrifuged at 2000*g* for 20 min at 4°C. Then, the supernatants were collected and diluted (500-fold) with saline to measure total GLP-1 concentrations by ELISA.

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Before analyses with statistical test, normal distribution of the variables and there was homogeneity of variances were checked. Differences among treatment groups were analyzed with the Tukey–Kramer's test or Student's t-test (described in the legend of each figure) after one-way ANOVA. A *P* value of less than 0.05 was considered significant. Pearson's correlation coefficient (r) and the significance of correlations were analyzed between various parameters. The area under the curve (AUC) for postprandial glucose, insulin, and GLP-1 was calculated using the trapezoidal rule. Additionally, the AUC of HOMA-IR[29,31] was calculated to compute the degree of insulin resistance resulting from the feeding diet as follows:

HOMA-IR AUC = (AUC postprandial glucose (mg/dL×2hr) × AUC postprandial insulin (μU/mL×2hr))/2430

where 1 mg insulin = 26 IU.

All statistical analyses were performed using the JMP Pro version 13.0 software (SAS Institute, Inc., North Carolina).

Results

Body and tissue weights, energy intake, and pH of cecal content

Although no difference was observed in the energy intake and the final body weight among groups (Fig. 1a, b), the highest visceral fat pad weight was observed in the H group (Fig. 1c). Rats fed with a soybean fiber supplemented high-fat high-sucrose diet demonstrated a markedly decreased accumulation of visceral fat when compared with H group ($P < 0.05$; Tukey–Kramer's test).

Soybean fiber-fed groups (HS and HSQ) had higher cecal tissue and cecal content weights, as well as reduced pH of the cecal content (Fig. 1d-f), when compared to groups without soybean fiber (C, H, and HQ). These results suggested that cecal fermentation was promoted by soybean fiber supplementation.

Short-chain fatty acids (SCFAs) in cecal content

The amount of SCFAs (Fig. 2) represents the degree of cecal fermentation and metabolic activity of the intestinal microbiota.[32] A diet with soybean fiber increased the amount of both acetic and propionic acids, with the highest amounts of acetic acid (53.4 ± 2.3 μmol/total cecum) and propionic acid (16.6 ± 2.6 μmol/total cecum) observed in the HSQ group. Correspondingly, an extensive elevation of total SCFAs was also observed in both soybean fiber fed groups (HS and HSQ).

Total quercetin derivatives in plasma, urine, feces, and cecal content

Quercetin and its derivatives were not detected in C, H, and HS groups (data not shown). The total quercetin derivatives represent the sum of quercetin aglycone and methylquercetin aglycone (isorhamnetin and tamarixetin) derivatives. In rats fed the diet containing AGIQ, almost

all the quercetin derivatives detected in the plasma were methylated form. From week 2 to 8 of the feeding period, the HSQ group showed higher total quercetin derivative levels than the HQ group. (Fig. 3a).

Rats fed with HSQ demonstrated a higher urinal quercetin level from week 4 to 8 (Fig. 3b) than HQ group. Regarding the fecal sample, higher quercetin levels were observed in HQ group from week 2 to 4, than in HSQ group. The decreased tendency in fecal quercetin derivatives of HQ group was observed in a later period, while HSQ group demonstrated a reciprocal trend with a five-times greater level than HQ group at week 8 of the feeding period. (Fig. 3c).

The quercetin content of cecum in HSQ fed rats was greater (Fig. 4) than that in HQ group at the end of the experimental period. In HQ fed rats, quercetin aglycone levels (0.34 ± 0.18 μmol/cecum) were considerably lower than those observed in HSQ fed rats (6.5 ± 0.75 μmol/cecum).

Degradation of quercetin and methylquercetin derivatives

The effect of test diets on quercetin degradation was calculated as described in Table 2.[33] To determine the precise aglycone levels ingested, we averaged the diet intake data from the day before urine and feces collection until the day after plasma collection (complete week period) for the calculation. In addition, the ingested aglycone weight is calculated by subtracting glycoside weight from ingested quercetin glycoside weight. The sum of excreted aglycone (quercetin aglycone + methylquercetin aglycone of urine and feces) can be considered as quercetin that escaped from degradation by intestinal bacteria.[34]

In the present study, HQ group had a higher ingested aglycone weight than HSQ group. At week 2, HQ group showed a lower percentage of degraded aglycone, but it gradually rose from

week 4 to 8. Markedly, two-way repeated measures ANOVA for degraded aglycone revealed a significant interaction between the duration of feeding period and the diet ($P = 0.0034$).

In contrast, we observed fluctuations in the degraded percentage in HSQ group (80-86%). In addition, the HSQ group demonstrated significantly lower percentage of degraded aglycone compared to HQ group at week 8 of feeding period. These results indicate that soybean fiber supplementation in long-term feeding could potentially suppress quercetin degradation induced by intestinal bacteria.

Glycemic, insulin, and GLP-1 responses under MTT

Because statistically significant differences were not detectable in time course changes of glucose, insulin, and GLP-1 (Fig. 5a, 5c, 5e and 6a, 6c, 6e), results are explained based on AUC values (Fig. 5b, 5d, 5f, 5g and 6b, 6d, 6f, 6g). At week 4 of the feeding period, the postprandial glycemic response in the H group was significantly higher than that observed in the C group, while the HSQ group showed a markedly lower glycemic response than the H group (Fig. 5b). No significant differences were seen in insulin responses between groups (Fig. 5d). The highest levels of basal and postprandial GLP-1 (Fig. 5e, f) were seen in the HSQ group among experimental groups.

Regarding the MTT at week 8, HSQ group showed a reduction in the postprandial glycemic responses when compared to H group (Fig. 6b). A significant elevation was observed in the AUC of postprandial insulin in the H group than the C group (Fig. 6d). Among all treatments, the basal GLP-1 concentration in the HSQ group was the greatest (16.07 ± 1.00 pM), while that in the H group was the lowest (11.24 ± 0.58 pM) (Fig. 6e). The HSQ group also maintained substantially higher postprandial GLP-1 AUC (Fig. 6f). Rats fed the H diet demonstrated significantly higher

284 HOMA-IR AUC when compared with C group, indicating the development of insulin resistance
285 after continuous feeding with a high-fat high-sucrose diet. Markedly, the HSQ group had a
286 HOMA-IR AUC value almost similar to that observed for the C group (Fig. 6g).
287
288 **Cecal tissue GLP-1 content**
289 No significant differences were observed in intestinal GLP-1 concentrations (Fig. 7a).
290 Total amount of GLP-1 in the cecal tissue was highest in HSQ group than HQ and other groups,
291 while HS group had almost same level as H group (Fig. 7b).
292
293 **Correlation between concentrations of basal GLP-1 and total quercetin derivatives in plasma**
294 **or cecal tissue GLP-1 content**
295 Correlations between plasma quercetin concentrations and various parameters, including
296 basal and postprandial glucose/insulin/GLP-1, were analysed. Basal GLP-1 concentrations (Fig. 8
297 a and b) were positively and significantly associated with total plasma quercetin derivatives at
298 week 4 ($r = 0.60$, $P = 0.0289$) and 8 ($r = 0.77$, $P = 0.0081$).
299 Correlation analysis were performed between basal plasma GLP-1 at week 8 and tissue
300 GLP-1 content (total amount in the cecum), separatory in Q-fed groups (HQ and HSQ), and non-
301 Q-fed groups (H and HS). A significant and positive correlation was observed for data from Q-fed
302 groups, but not for data from non-Q-fed groups (Fig. 8c, d).
303
304 **Discussion**
305 Previously, we have shown a substantial elevation in the total quercetin derivatives
306 detected in the plasma of rats fed a diet supplemented with a combination of soybean fiber and

AGIQ.[11] Thus, we examined here whether the combined supplementation of soybean fiber and AGIQ promotes quercetin bioavailability in rats fed an obesogenic high-fat high-sucrose diet and whether this supplementation exerts a preventive effect against glucose intolerance. At the end of the feeding test, the combination of soybean fiber and AGIQ in a high-fat high-sucrose (HSQ) diet gave the highest concentration of total quercetin derivatives in plasma and promoted cecal fermentation. The combined supplementation of soybean fiber and AGIQ also attenuated the glucose intolerance induced by the continuous feeding of the high-fat high-sucrose diet. Furthermore, plasma quercetin concentrations were positively associated with plasma GLP-1 concentrations. These results indicated that the co-ingestion of soybean fiber promotes quercetin bioavailability, following by enhanced GLP-1 secretion even under an obesogenic dietary status to prevent the development of glucose intolerance.

Although apparent effects of experimental diets on body weight were not observed in the present study, visceral fat accumulation caused by high-fat high-sucrose diet has been mitigated by soybean fiber or AGIQ or both components. Consistent with these findings, the downregulation of leptin and TNF-alpha genes in mice fed a high-fat diet containing soybean fiber, resulting in reduced epididymal adipose tissue weight.[35] Another study has demonstrated that quercetin improves energy expenditure and reduces fat accumulation in mice fed a high-fat diet by promoting white adipocyte browning.[36]

Elevated cecal fermentation from soybean fiber ingestion was confirmed by an increase in SCFAs and a decrease in pH.[37,38] As previously reported, we proposed that quercetin bioavailability can be promoted by soybean fiber supplementation, which in turn enhances cecal fermentation in normal rats.[11] This modification in the large intestine can reduce the degradation of luminal quercetin, increased the available quercetin which can be absorbed in the large intestine.

In the present study, total quercetin derivatives in fecal and cecal samples were higher in the HSQ group (Fig. 3 and 4), which correlated with the percentage of quercetin degradation that was lower than the HQ group in the later experimental period (Table 2). These results revealed that the proposed mechanism by which soybean fiber enhances quercetin bioavailability is maintained even under an obesogenic dietary condition. Notably, the proposed mechanism is not related to the absorption rate, but possibly depends on the absorptive capacity with the expansion of the cecal tissues and the available amount of quercetin and its derivatives in the intestine.

Previous studies demonstrated that a diet containing a high percentage of lard oil (17%) increased quercetin bioavailability,[39] and that quercetin can be transported into lymph circulation along with fat.[40,41] One possible mechanism underlying these findings was the increased formation of chylomicrons, that incorporates quercetin together with fat in the diet.[42] In the present study, quercetin degradation gradually increased over time in HQ group, which may be linked with increased quercetin degrading bacteria such as *Bacteroides fragilis*.[43] In lard fed mice, increases in *Bacteroides* and *Bilophila* bacteria have been reported.[44] Enhancement of quercetin bioavailability by soybean fiber could relate to the modification of intestinal microflora. Promotive effect of dietary fiber on the growth of intestinal bacteria such as *Bifidobacteria* and *Lactobacilli* has been shown.[45] This, in turn, controls or reduces the growth of harmful bacteria such as *Clostridium perfringens* and *Escherichia coli* that are known as quercetin degrading bacteria.[43]

At the beginning of the experimental period (week 2), the higher percentage of aglycon degradation in HSQ group than in HQ group seems inconsistent with the proposed mechanism for increasing plasma quercetin (quercetin bioavailability). However, amounts of degraded aglycon are very similar in these groups, indicating that both of groups had almost equivalent “degradation capacity”. Therefore, in this case, higher percentage of degradation in HSQ than HQ group is

353 owing to lower intake of aglycon in HSQ than in HQ group. After 4 weeks, amounts of degraded
354 aglycon gradually increased in HQ group while decreased in HSQ group accompanied with
355 significant differences between two groups ($P < 0.05$ at week 4, $P < 0.01$ at week 6 and 8), and
356 resulted in a significant interaction between the duration and the diet ($P = 0.0034$ by two-way
357 repeated measures ANOVA). The former is likely chronic effect of high fat diet, and the latter is
358 chronic effect of soybean fiber supplementation. Thus, the supplementation of soybean fiber is an
359 alternatively effective approach to enhance quercetin bioavailability even under a chronic feeding
360 of high-fat diet.

361 Significant differences in energy consumption were not detected by Tukey–Kramer's test
362 (Fig. 1a), but HSQ group had approximately 10% lower energy intake than HQ group, which
363 resulted in significant differences in aglycon ingestion between HQ and HSQ group, when
364 calculated by Student's t-test (Table 2). Such small difference in energy intake might also
365 contribute improved glucose tolerance in HSQ group compared to HQ group. Lack of comparison
366 under iso-energetic conditions such as pair-fed design may be one of limitations of the present
367 study. However, it is valuable finding that the quercetin bioavailability is higher in HSQ than HQ
368 group, even though HSQ had slightly lower quercetin intake than HQ group.

369 The long-term feeding of HSQ diet can increase GLP-1 secretion, especially at the basal
370 level, but the lower concentration of glucose in plasma may not be sufficient to activate the
371 function of GLP-1 to enhance insulin secretion in a glucose dependent manner. According to this
372 assumption, the combination of AGIQ and soybean fiber also improved glucose tolerance and
373 insulin sensitivity in rats fed a high-fat high-sucrose diet (Fig. 5 and 6). Relatively, but
374 insignificantly, lower energy consumption and visceral fat accumulation cannot be used for
375 explanation because both HS and HSQ groups have shown similar results for these parameters

(Fig. 1). Soybean fiber supplementation has been shown to mitigate plasma glucose and triglyceride levels without body weight changes in both male random-bred albino control and streptozotocin-induced diabetic rats.[46] Reportedly, quercetin acts on 5' AMP-activated protein kinase (AMPK) stimulation and glucose transporter type 4 (GLUT4) translocation, promoting glucose uptake in skeletal muscle and liver.[47] These data partly explain the preventive effects of combined soybean fiber and AGIQ against metabolic impairments induced by excessive energy intake. Possibly, an extension on the feeding period may show clearer effects, therefore, a longer chronic feeding of this combination diet is needed in the future.

Throughout the experiment, we found a significant correlation between basal GLP-1 levels and total quercetin derivatives in plasma (Fig. 8). The basal GLP-1 level was possibly promoted by increased methylquercetin metabolites because a high concentration of these metabolites was detected in the plasma, cecum, urine, and feces of AGIQ fed rats (Fig. 3 and 4). It is well known that methylquercetin metabolites are resistant to degradation by intestinal bacteria,[33,48] and long-term quercetin consumption can extend the methylation of quercetin.[49] The stimulatory effect of AGIQ on GLP-1 secretion has been demonstrated following an acute infusion into the rat small intestine, as well as following acute exposure in a murine GLP-1-producing cell line (GLUTag cells).[50] Luminal amount (Fig. 4) and concentrations (0.62 ± 0.17 mM in HQ, 5.57 ± 0.67 mM in HSQ) of quercetin in the cecum were much higher in HSQ group than HQ group, but significant correlations between plasma GLP-1 concentrations were not observed ($r = 0.36$, $P = 0.2080$ for amount of luminal quercetin; $r = 0.37$, $P = 0.1869$ for concentration of luminal quercetin). We did not measure luminal quercetin in the small intestine, but luminal quercetin in the ileum might be involved in the increment of basal GLP-1 secretion. Another possible explanation is the involvement of increased mass of the cecal tissue for the increment of GLP-1 secretion. There

399 were no differences in tissue GLP-1 concentrations among treatments (Fig. 7a), however, the
400 amount of GLP-1 in the whole cecum was higher than in HSQ group than in HQ group (Fig. 7b).
401 This suggests that the number of GLP-1 producing cells was simultaneously increased together
402 with the increased mass of cecal tissue (Fig. 1d). The correlation analysis (Fig.8c, d) revealed the
403 significant and positive correlation between plasma GLP-1 concentrations and total amount of
404 cecal tissue GLP-1 ($r = 0.72$, $P = 0.0037$) in data from Q-fed groups (HQ vs HSQ), but not in the
405 data from non-Q-fed groups (H vs HQ). Therefore, not only tissue expansion by soybean fiber but
406 the presence of quercetin (in the intestinal lumen and/or plasma) could be responsible for
407 increasing basal GLP-1 secretion. Further studies are needed to clarify the molecular mechanisms
408 by which quercetin and its metabolites promote GLP-1 secretion.

409 In this work, the supplementation with soybean fiber promoted quercetin bioavailability in
410 rats fed a high-fat high-sucrose diet by enhancing cecal fermentation. The combination of soybean
411 fiber and AGIQ prevented the high-fat high-sucrose diet induced glucose intolerance,
412 accompanied by an increase in plasma GLP-1 levels. Furthermore, plasma GLP-1 levels positively
413 correlated with plasma quercetin concentrations. These findings reveal effects of soybean fiber
414 and quercetin (AGIQ) on reducing the risk of glucose intolerance and subsequent diabetes.

415

416 **Abbreviations**

417	AGIQ	α-glycosyl-isoquercitrin
418	C	Control
419	GLP-1	Glucagon-like peptide-1
420	H	High-fat high-sucrose
421	HS	High-fat high-sucrose + Soybean fiber

422	HSQ	High-fat high-sucrose + Soybean fiber + α-glycosyl-isoquercitrin
423	HQ	High-fat high-sucrose + α-glycosyl-isoquercitrin
424	MTT	Meal tolerance test
425	SCFA(s)	Short-chain fatty acid(s)

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566

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570

571 Author Contributions

572 A. T., T. H. and H. H. designed the research; A. T. conducted the research and analysed the data;
573 T. H. provided essential reagents; A. T. and T. H. wrote the paper; T. H. had primary responsibility
574 for the final content. All authors read and approved the final manuscript.

575

576 Conflicts of interest

577 The authors declare that they have no conflicts of interest.

Figure captions

Figure 1. a) energy intake, b) final body weight, c) visceral fat pad weight, d) cecal tissue weight, e) cecal content weight, and f) pH of cecal content after feeding test diets for 9 weeks. Values represent means with standard errors indicated by vertical bars (n = 7). Mean values not sharing a common alphabetical letter differ significantly ($P < 0.05$, Tukey–Kramer's test). N.S. indicates the absence of a significant difference among treatments.

Figure 2. Amount of individual/total SCFA(s) of cecal content after feeding test diets for 9 weeks. Values represent means with standard errors indicated by vertical bars (n = 7). Mean values not sharing a common alphabetical letter differ significantly ($P < 0.05$, Tukey–Kramer's test).

Figure 3. Concentrations or amounts of total quercetin derivatives (quercetin aglycone and methylquercetin aglycone) in the a) plasma, b) urine, and c) feces of rats fed with AGIQ-containing diets (HQ, and HSQ) after 2,4, 6, and 8 weeks. Values represent means with standard errors indicated by vertical bars (n = 7). Asterisk indicate a significant difference between mean values of HQ vs. HSQ (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.005$, Student's t test).

Figure 4. Concentration of total quercetin derivatives (quercetin aglycone and methylquercetin aglycone) in the cecal content of rats fed AGIQ-containing diets (HQ, and HSQ) after 9 weeks. Values represent means with standard errors indicated by vertical bars (n = 7). Asterisk indicate a significant difference between mean values of HQ vs. HSQ (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.005$, Student's t test).

Figure 5. Postprandial glucose, insulin, glucagon-like peptide-1 level, and homeostatic model assessment of insulin resistance during the meal tolerance test after 4 weeks of feeding the test diet. Values represent means with standard errors indicated by vertical bars (n = 7). Mean values not sharing a common alphabetical letter differ significantly ($P < 0.05$, Tukey–Kramer's test) among all treatments (panels b, d, f, and g). N.S. indicates the absence of a significant difference among treatments.

Figure 6. Postprandial glucose, insulin, glucagon-like peptide-1 level, and homeostatic model assessment of insulin resistance during the meal tolerance test after 8 weeks of feeding the test diet. Values represent means with standard errors indicated by vertical bars (n = 7). Mean values not sharing a common alphabetical letter differ significantly ($P < 0.05$, Tukey–Kramer's test) among all treatments (panels b, d, f, and g). N.S. indicates the absence of a significant difference among treatments.

Figure 7. a) concentrations and b) amounts of GLP-1 contents in cecal tissue of rats after feeding test diets for 9 weeks. Values represent means with standard errors indicated by vertical bars (n = 7). Mean values not sharing a common alphabetical letter differ significantly ($P < 0.05$, Tukey–Kramer's test). N.S. indicates the absence of a significant difference among treatments.

Figure 8. Correlation between basal GLP-1 concentrations and total quercetin derivative concentrations in the plasma at a) week 4 and b) week 8 in rats fed AGIQ-containing diets (HQ and HSQ). Correlation between basal GLP-1 at week 8 and amount of GLP-1contents in cecal

tissue of rats fed c) non AGIQ-containing diets (H and HQ) and d) AGIQ-containing diets (HQ and HSQ).

Table 1 Composition of test diets

Ingredients (g/kg)	Normal diet	High-fat high-sucrose diets			
	C	H	HS	HQ	HSQ
Corn starch [a]	377.5	-	-	-	-
Casein [b]	200	200	200	200	200
Dextrinized corn starch [c]	132	-	-	-	-
Soybean oil	70	70	70	70	70
Sucrose	100	379.5	379.5	372.5	372.5
Lard oil	-	230	230	230	230
Cellulose [d]	70	70	20	70	20
Mineral (AIN-93G-MX) [e]	35	35	35	35	35
Vitamin (AIN-93-VX) [e]	10	10	10	10	10
L-Cystine	3	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5	2.5
Tertiary butylhydroquinone	0.014	0.014	0.014	0.014	0.014
AGIQ (Q) [f]	-	-	-	7	7
Soybean fiber (S) [g]	-	-	50	-	50
Total	1000	1000	1000	1000	1000
Energy per gram diet (kcal/g)	4.04	5.19	5.19	5.16	5.16

AIN, American Institute of Nutrition; C, control diet; H, high-fat high-sucrose diet; HS, high-fat high-sucrose diet + soybean fiber; HSQ, high-fat high-sucrose diet + soybean fiber + α-glycosyl-isoquercitrin; HQ, high-fat high-sucrose diet + α-glycosyl-isoquercitrin.

[a] Amylalpha (Chuo Foods Co., Ltd., Aichi, Japan)

[b] Acid casein (Fonterra, Ltd., Tokyo, Japan)

[c] TK-16 (kindly supplied by Matsutani Chemical Industry Co., Ltd., Hyogo, Japan)

[d] Avicel PH102 (Asahi Kasei Chemicals Corporation, Tokyo, Japan)

[e] Mineral and vitamin mixtures were prepared according to the AIN-93G formulation.

[f] α-glycosyl isoquercitrin (AGIQ; Q, kindly supplied by San-Ei Gen F.F.I., Inc., Osaka, Japan)

[g] Soybean fiber (kindly supplied by Fuji Oil Co., Ltd., Osaka, Japan)

Table 2 The amount of quercetin aglycone degraded per day, and the rate (%) of excreted aglycone derivatives in rats fed with AGIQ-containing diets (HQ, and HSQ) after 2, 4, 6, and 8 weeks.

Group		Ingested aglycone (μmol/day)	Excreted aglycone [a] (μmol/day)	Degraded aglycone [b] (μmol/day)	Excreted aglycone [c] (%)	Degraded aglycone [d] (%)
Week 2	HQ	188.51± 5.33	41.64± 2.20	146.87± 5.59	22.18± 1.29	77.82± 1.29
	HSQ	167.33± 6.91 **	23.84± 1.69 ***	143.50± 6.17	14.24± 0.82 ***	85.76± 0.82 ***
Week 4	HQ	181.38± 5.36	32.61± 1.98	148.77± 5.33	18.04± 1.12	81.96± 1.12
	HSQ	167.20± 6.86 *	31.23± 1.55	135.98± 6.29 *	18.76± 0.91	81.24± 0.91
Week 6	HQ	177.82± 4.84	21.68± 2.35	156.14± 5.51	12.25± 1.37	87.75± 1.37
	HSQ	165.49± 6.31 *	26.09± 1.67	139.39± 6.50 **	15.91± 1.10 *	84.09± 1.10 *
Week 8	HQ	186.22± 3.50	17.40± 0.70	168.82± 3.44	9.36± 0.38	90.64± 0.38
	HSQ	164.83± 6.39 *	29.83± 2.95 **	135.00± 5.29 ***	18.04± 1.48 ***	81.96± 1.48 ***

Values represent mean ± SEM (n = 7). Asterisk indicate a significant difference between mean values of HQ vs. HSQ (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.005$, Student's t test).

[a] Excreted aglycone (μmol/day) = total aglycone derivatives in urine + total aglycone derivatives in feces

[b] Degraded aglycone (μmol/day) = Ingested aglycone - Excreted aglycone

[c] Excreted aglycone (%) = (Excreted aglycone / Ingested aglycone) × 100

[d] Degraded aglycone (%) = [100 - (Excreted aglycone / Ingested aglycone)] × 100

Figure 1

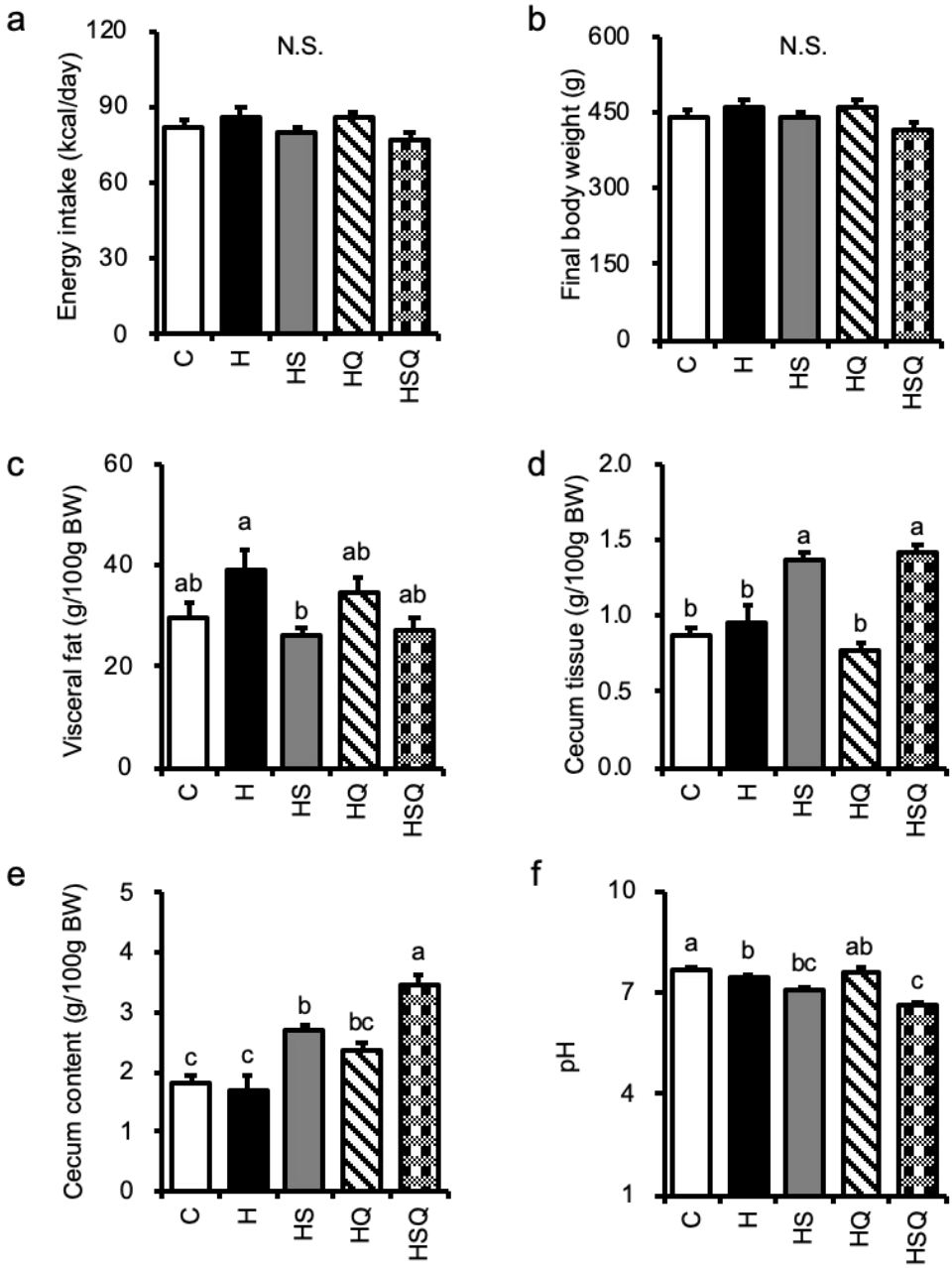

a
Energy intake (kcal/day)
120
90
60
30
0
N.S.
C
H
HS
HQ
HSQ
b
Final body weight (g)
600
450
300
150
0
N.S.
c
Visceral fat (g/100g BW)
60
40
20
0
ab
a
b
ab
ab
d
Cecum tissue (g/100g BW)
2.0
1.5
1.0
0.5
0.0
b
b
a
b
a
e
Cecum content (g/100g BW)
5
4
3
2
1
0
c
c
b
bc
a
f
pH
10
7
4
1
a
b
bc
ab
c

Figure 2

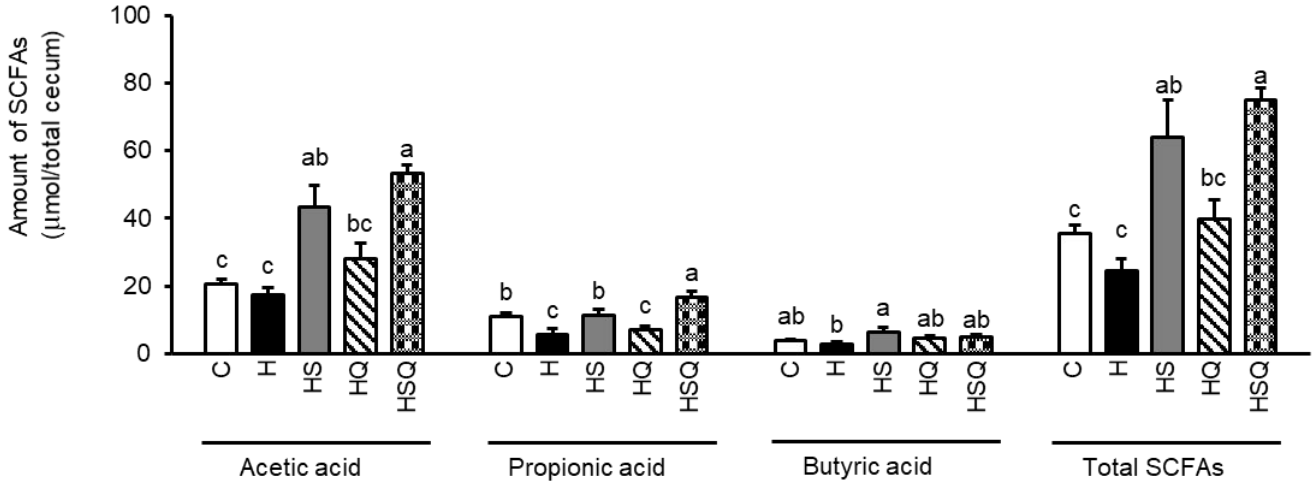

Amount of SCFAs (μmol/total cecum)
100
80
60
40
20
0
c
c
ab
bc
a
b
c
b
c
a
ab
b
a
ab
ab
c
c
ab
bc
a
C
H
HS
HQ
HSQ
Acetic acid
Propionic acid
Butyric acid
Total SCFAs

Figure 3

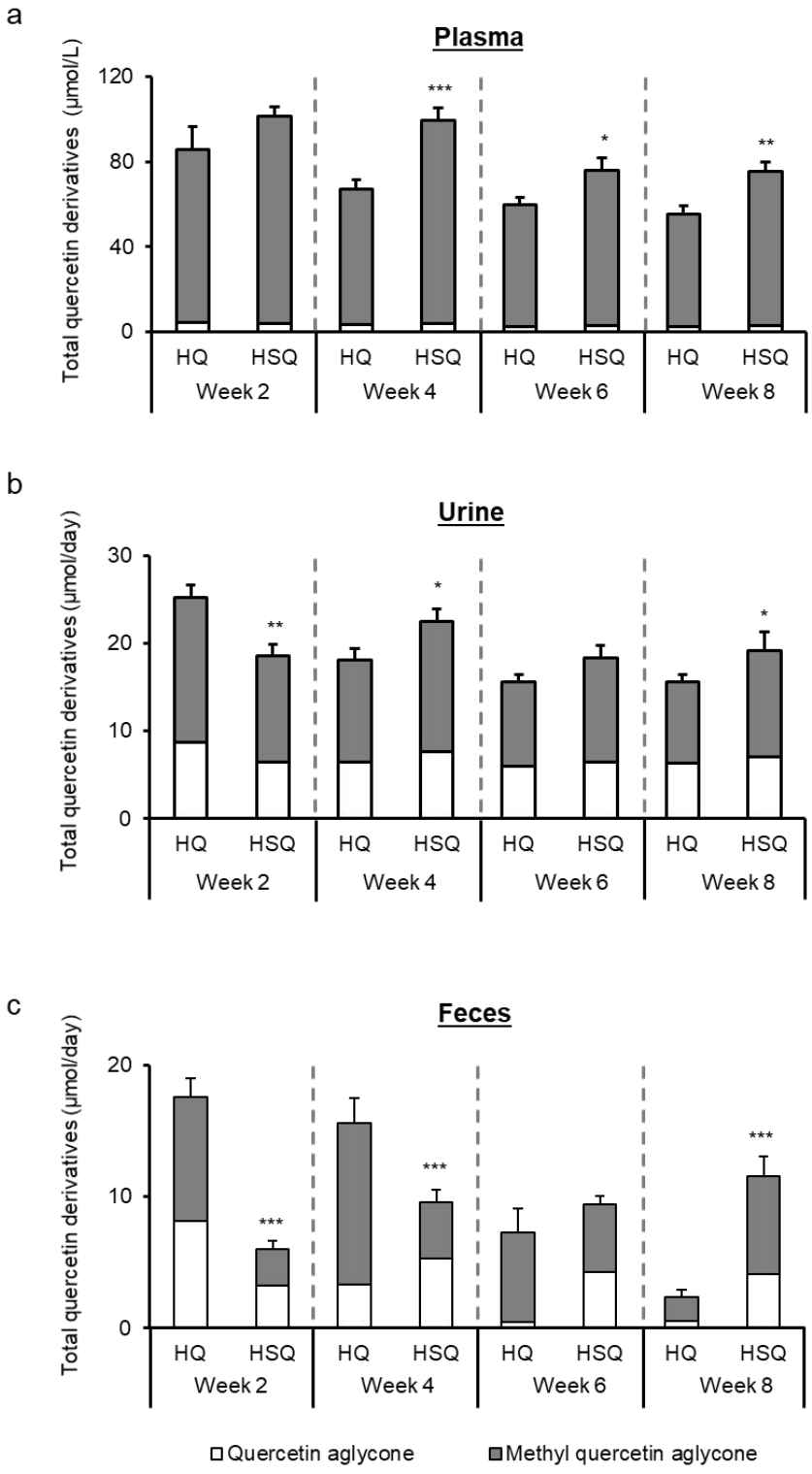

a
Plasma
Total quercetin derivatives (µmol/L)
120
80
40
0
***
*
**
HQ HSQ HQ HSQ HQ HSQ HQ HSQ
Week 2 Week 4 Week 6 Week 8
b
Urine
Total quercetin derivatives (µmol/day)
30
20
10
0
**
*
*
HQ HSQ HQ HSQ HQ HSQ HQ HSQ
Week 2 Week 4 Week 6 Week 8
c
Feces
Total quercetin derivatives (µmol/day)
20
10
0
***
***
***
HQ HSQ HQ HSQ HQ HSQ HQ HSQ
Week 2 Week 4 Week 6 Week 8
Quercetin aglycone
Methyl quercetin aglycone

Figure 4

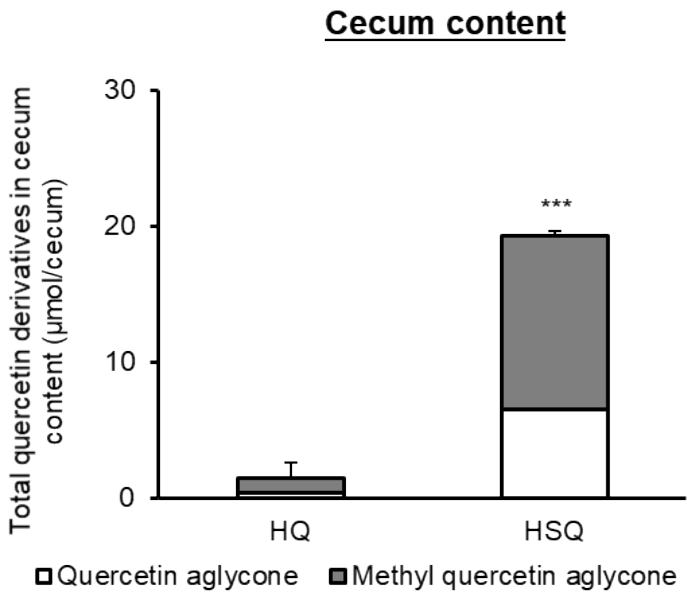

Cecum content
Total quercetin derivatives in cecum content (µmol/cecum)
30
20
10
0
***
HQ
HSQ
Quercetin aglycone
Methyl quercetin aglycone

Figure 5

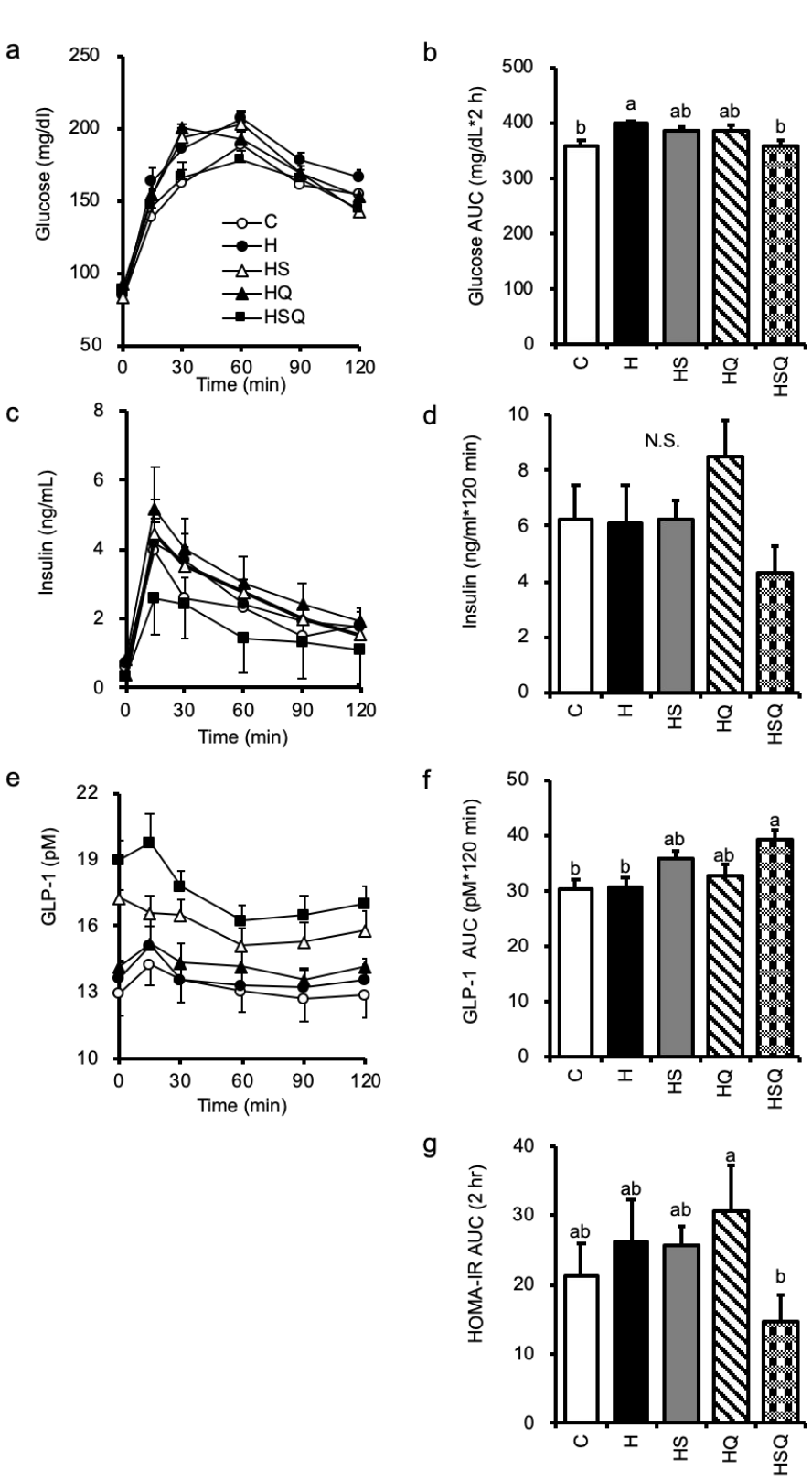

Week 4
a
Glucose (mg/dl)
Time (min)
C
H
HS
HQ
HSQ
b
Glucose AUC (mg/dL*2 h)
c
Insulin (ng/mL)
Time (min)
d
Insulin (ng/ml*120 min)
N.S.
e
GLP-1 (pM)
Time (min)
f
GLP-1 AUC (pM*120 min)
g
HOMA-IR AUC (2 hr)

Figure 6

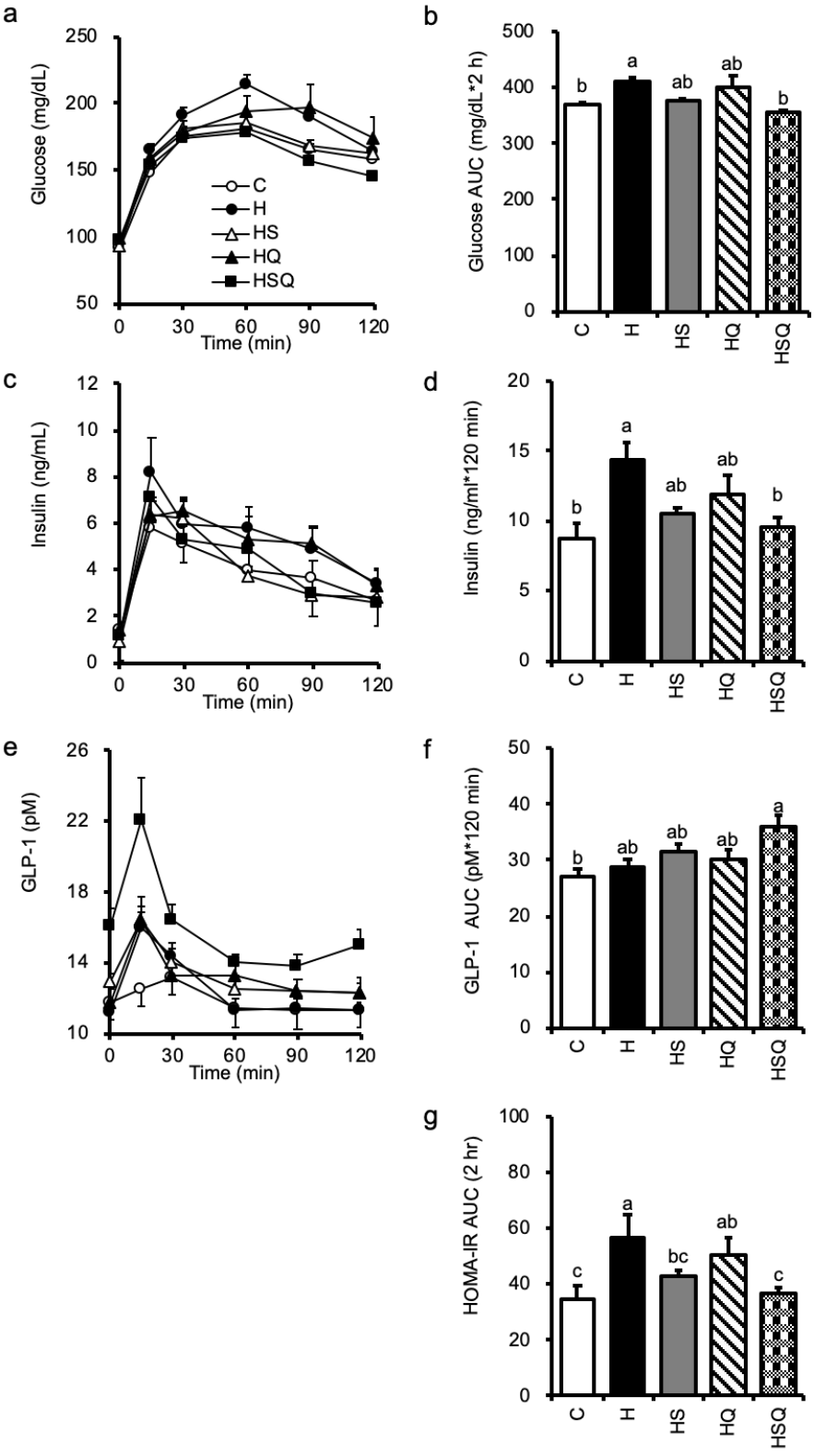

Week 8
a
Glucose (mg/dL)
Time (min)
C
H
HS
HQ
HSQ
b
Glucose AUC (mg/dL*2 h)
c
Insulin (ng/mL)
d
Insulin (ng/ml*120 min)
e
GLP-1 (pM)
f
GLP-1 AUC (pM*120 min)
g
HOMA-IR AUC (2 hr)

Figure 7

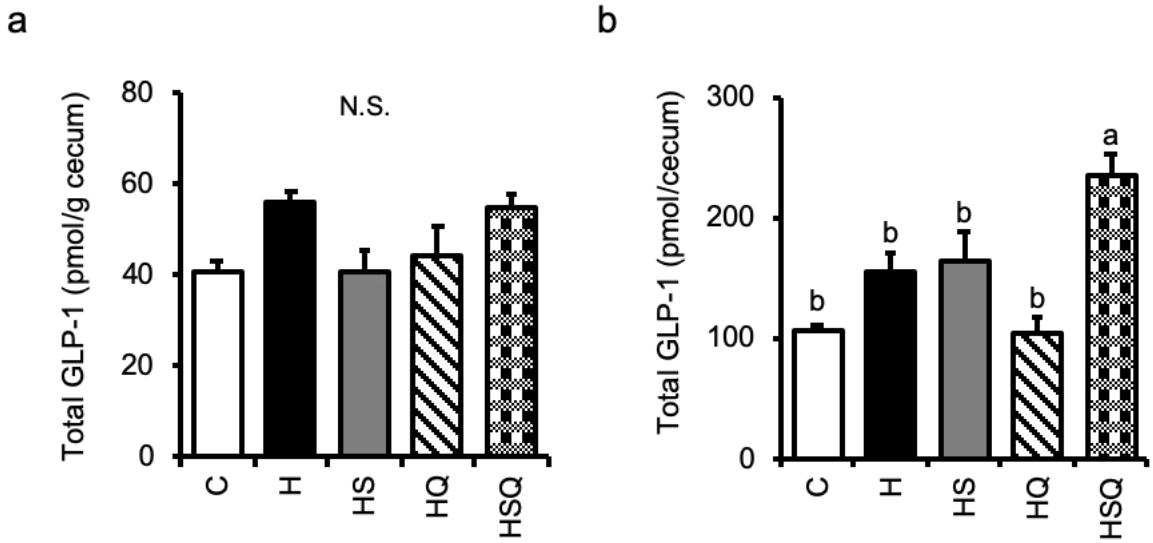
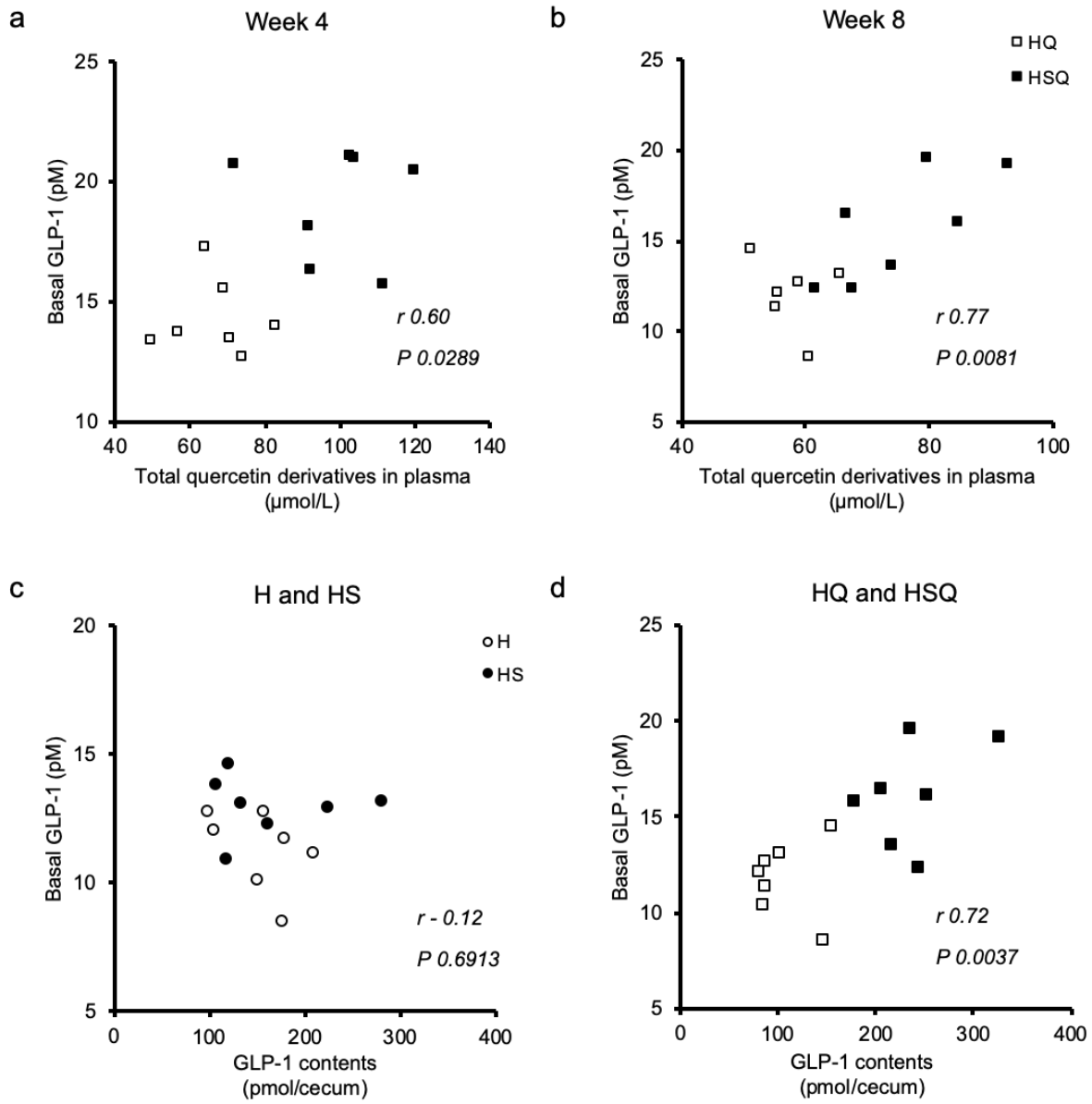
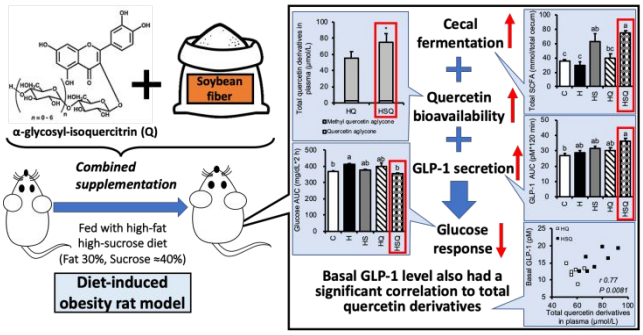

a
Total GLP-1 (pmol/g cecum)
80
60
40
20
0
N.S.
C
H
HS
HQ
HSQ
b
Total GLP-1 (pmol/cecum)
300
200
100
0
b
b
b
a
C
H
HS
HQ
HSQ

Figure 8


a
Week 4
Basal GLP-1 (pM)
Total quercetin derivatives in plasma (µmol/L)
r 0.60
P 0.0289
b
Week 8
HQ
HSQ
Basal GLP-1 (pM)
Total quercetin derivatives in plasma (µmol/L)
r 0.77
P 0.0081
c
H and HS
H
HS
Basal GLP-1 (pM)
GLP-1 contents (pmol/cecum)
r - 0.12
P 0.6913
d
HQ and HSQ
Basal GLP-1 (pM)
GLP-1 contents (pmol/cecum)
r 0.72
P 0.0037


Soybean fiber
α-glycosyl-isoquercitrin (Q)
Combined supplementation
Fed with high-fat high-sucrose diet (Fat 30%, Sucrose ≈40%)
Diet-induced obesity rat model
Cecal fermentation
Quercetin bioavailability
GLP-1 secretion
Glucose response
Basal GLP-1 level also had a significant correlation to total quercetin derivatives