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Hop-derived prenylflavonoid isoxanthohumol suppresses insulin resistance by changing the intestinal microbiota and suppressing chronic inflammation in high fat diet-fed mice

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Abstract. – OBJECTIVE: To assess whether the hop-derived polyphenol isoxanthohumol suppresses insulin resistance by changing the intestinal microbiota.

MATERIALS AND METHODS: Male C57BL/6J mice (7 weeks of age) were divided into five groups (n = 9-10): Normal Diet (ND), High Fat Diet (HFD), HFD + low dose isoxanthohumol (0.01%IX), HFD + medium dose isoxanthohumol (0.03% IX), and HFD + high dose isoxanthohumol (0.1% IX). Oral glucose tolerance tests (OGTTs) were performed at 4 and 8 weeks, and insulin tolerance tests (ITTs) were performed at 13 weeks. 16S rRNA gene sequencing analyses revealed the fecal microbiota profiles, and the relative abundance of *Akkermansia muciniphila* and *Clostridium* cluster XI was calculated by qRT-PCR. Plasma lipopolysaccharide (LPS) levels were measured by ELISA, and mRNA expression levels of tumor necrosis factor (TNF)-α, and interleukin (IL)-1β in epididymal adipose tissues were measured by qRT-PCR.

RESULTS: Isoxanthohumol showed antibacterial activity towards several bacterial species and mitigated impaired glucose tolerance and insulin resistance induced by the HFD in a dose-dependent manner, as shown by OGTTs and ITTs. The concentration of phylum *Verrucomicrobia* bacteria dramatically increased in the 0.1% IX group, the relative abundance of *A. muciniphila* increased, and that of Clostridium cluster XI decreased. Moreover, the intake of isoxanthohumol decreased the levels of plasma LPS and mRNA expression of TNF-α and IL-1β in epididymal adipose tissues.

CONCLUSIONS: We found that isoxanthohumol can suppress HFD-induced insulin resistance by changing the intestinal microbiota and reducing the expression of inflammation factors.

Key Words:
Isoxanthohumol, Anti-hyperglycemia effect, Insulin resistance, Intestinal microbiota, Inflammation.

Introduction

Diabetes mellitus is a major problem globally, and it is estimated that by 2030, diabetes will affect 522 million people worldwide[1]. Including patients with pre-diabetes, a substantial proportion of the world population is at risk. Diabetes is divided into type 1 and type 2. A characteristic of type 2 diabetes (T2D) is insulin resistance, in which the body responds poorly to insulin and glucose is poorly metabolized, resulting in chronic hyperglycemia. Impaired glucose tolerance (IGT) patients showing insulin resistance generally develop T2D within several years[2]. Insulin resistance typically leads to various diseases, such as hyperglycemia, obesity, hypertension, and hyperlipidemia[3-6]; thus, addressing insulin resistance before disease onset is very important for maintaining health. Insulin resistance is likely caused by lifestyle and can be prevented or mitigated by improving lifestyle habits, such as diet and exercise. In addition, we believe that the intake of functional food ingredients can help overcome insulin resistance.

Insulin resistance is mainly caused by chronic inflammation[7]. Chronic inflammation is triggered by inflammatory cytokines produced from adipocytes and hepatocytes in obese patients and from immune cells generated as a response to foreign materials. The relationship between chronic inflammation and the intestinal microbiota has recently been the focus of much researches. For example, high fat diet (HFD)-induced obesity causes insulin resistance[8] and is accompanied by changes in the gut microbiota, with *Bacteroides* predominating over *Firmicutes* through an as-yet poorly understood mechanism[9]. However, experiments with germ-free mice have shown

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that the presence of intestinal microbiota affects the onset of obesity[10] and the induction of insulin resistance[11]. Several reports show that glycometabolism is improved in HFD-fed mouse models following a reduction in the number of intestinal microbiota cells. Lipopolysaccharides (LPS) and branched-chain amino acids produced by intestinal microbiota cause insulin resistance, accompanied by changes in the intestinal microbiota[12]. In addition, short-chain fatty acids metabolized by intestinal microbiota are reported to suppress insulin resistance[13]. Furthermore, some studies[14,15] have shown that *Akkermansia muciniphila* suppresses insulin resistance and improves glucose metabolism both in *in vivo* experiments and in an exploratory human trial, clearly demonstrating that the gut microbiota is involved in the induction of insulin resistance. The intestinal microbiota is significantly influenced by food factors. Prebiotics, such as oligosaccharides, and polyphenols, such as resveratrol[16] and curcumin[17], alter the intestinal microbiota, reduce insulin resistance, and help prevent obesity. Various investigations into the mechanisms underlying these effects have yet to identify the predominant factor(s) (e.g., changing the intestinal microbiota, polyphenol metabolites generated by intestinal microbiota, other metabolites, or specific bacteria).

Hops (*Humulus lupulus*) are agricultural products used since antiquity as raw materials for brewing beer. Hops are used both for flavor and bitterness and also to control microbial growth in beer. Several polyphenols in hops are reported to have antibacterial activity[18,19]. Physiologically active ingredients in hops, such as α-acids, isohumulone, and xanthohumol, are reported to have anti-obesity, anti-hyperlipidemia, and anti-Alzheimer activities[20-22], and xanthohumol has antibacterial activity[23]. Isoxanthohumol is a component of hops and is derived from xanthohumol, but little is known regarding its physiological action compared to other components in hops. Isoxanthohumol is a major component in hops, and as it is more stable in beer than xanthohumol, we anticipated that isoxanthohumol has various physiological activities. If isoxanthohumol has antimicrobial activity, it may reduce the number of intestinal bacteria or the levels of metabolites that cause insulin resistance.

In this study, we first determined the antibacterial activity of isoxanthohumol. Next, we studied its anti-insulin resistance activity in HFD-fed mice using an oral glucose tolerance test (OGTT) and an insulin tolerance test (ITT). We investigated the underlying mechanism of action by measuring the plasma concentration of LPS, which causes inflammation, and the mRNA expression levels of the inflammation markers tumor necrosis factor (TNF)-α and interleukin (IL)-1β. Given the involvement of intestinal microbiota, we also measured the relative abundance of *Clostridium* cluster XI as a representative of changes in the phylum *Firmicutes*, and of *A. muciniphila*, an intestinal microorganism that likely improves insulin resistance.

Materials and Methods

Preparation of Isoxanthohumol

Isoxanthohumol for the antibacterial activity test was purified from commercial hop extract purchased from Asama Chemical Co., Ltd. (Tokyo, Japan) using normal and reversed phase column chromatography. The purity was confirmed by UV absorbance at 280 nm using high performance liquid chromatography. The purified product with an isoxanthohumol peak area of over 95% of the overall absorbance was used for the antibacterial activity test. We used Isoxanthoflav (Hopsteiner, Mainburg, Germany) containing 95% isoxanthohumol for the animal tests.

Antibacterial Activity Test

The experiment was carried out on five strains: *Alicyclobacillus acidoterrestris* (ATCC49025, American Type Culture Collection, ATCC, Manassas, VA, USA), *Bacillus cereus* (IFO13494), *Clostridium perfringens* (JCM129, JCM, Ibaraki, Japan), *Clostridium difficile* (ATCC9689, ATCC), and *Staphylococcus aureus* subsp. *Aureus* (NBRC12732, NBRC, Chiba, Japan). We determined the minimum inhibitory concentration (MIC) for these strains by dissolving isoxanthohumol in ethanol at 3.13, 6.25, 12.5, 25, 50, and 100 mg/mL. The agar medium and incubation temperature and time for each strain are shown in Table I. The MIC endpoint was indicated as the lowest concentration at which no bacterial growth was observed.

Animal Experiments

Male C57BL/6J mice (7 weeks of age) were purchased from Japan SLC, Inc. (Shizuoka, Japan). Throughout the experiment, the mice had free access to food and water and were housed at 25 ± 1°C and 60 ± 5% humidity under a 12-h light-dark cycle. The mice were fed a normal diet (ND) (D12450JN, Research Diets, New Brunswick, NJ, USA) for 1 week, and then, were ran-

domly divided into seven groups (n=9-10/group) based on body weight and 4-h fasting blood glucose. The groups were: ND Group, mice given ND; HFD Group, 60% kcal high fat diet (HFD; D12492NM, Research Diets); 0.01% IX Group, HFD containing 0.01% Isoxanthoflav; 0.03% IX Group, HFD containing 0.03% Isoxanthoflav; and 0.1% IX Group, HFD containing 0.1% Isoxanthoflav. Isoxanthoflav contains 95% isoxanthohumol. The experimental protocol used in this model study is summarized in Figure 1. Mice were allowed free access to food and water for 14 weeks. OGTTs were performed at 4 and 8 weeks, and ITTs were performed at 13 weeks. Feces were collected on the same days as the OGTT. After 14 weeks, all mice were euthanized with isoflurane and the epididymal adipose tissue, perirenal adipose tissue, and liver were collected and weighed. Each sample was placed separately into a 2.0 mL tube, freeze-dried, and stored at -80°C until analysis. Body weights were measured twice a week throughout the 14 weeks of the experiment using an electronic scale. The experiments were conducted in 2018. All protocols for the animal procedures were approved by the Ethics Committee for Animal Experiments in accordance with the Internal Regulations on Animal Experiments at Suntory and are based on the Law for the Humane Treatment and Management of Animals (Law No. 105, 1 October 1973, as amended on 2 June 2017).

OGTTs and ITTs

OGTTs were carried out in mice after 6 h of fasting. Blood glucose levels were determined using a Glutest Neo Sensor (Sanwa Kagaku Kenkyusho, Aichi, Japan) for blood samples taken from the tail vein 0, 15, 30, 60, 90, and 120 min following oral injection of glucose (1 g/kg body weight). The plasma insulin levels in OGTT samples were measured using an Ultra-Sensitive Mouse Insulin ELISA kit (Morinaga Institute of Biological Science Inc., Kanagawa, Japan). ITTs were performed after 4 h of fasting. After an intraperitoneal (i.p.) injection of insulin (0.5 IU/kg body weight), blood glucose levels were measured at 0, 30, 60, 90, and 120 min. The area under the curve (AUC) for both blood glucose and insulin was calculated using the trapezoidal rule. Homeostatic model assessment (HOMA-IR) was calculated according the formula: HOMA-IR = 6-h fasting blood glucose concentration (mg/dL) × 6-h fasting insulin concentration (μU/mL)/405.

Analysis of Gut Microbiota by 16S rRNA Sequencing

We used MiSeq-based high throughput 16S rRNA gene sequencing (Illumina, San Diego, CA, USA) to analyze the composition of the microbiome in feces. Genomic DNA was extracted using a NucleoSpin Microbial DNA kit (Macherey-Nagel, Bethlehem, PA, USA). We generated the sequencing library by amplifying the V3-V4 region of 16S rRNA with specific primers, purified the PCR products using AMPureXP (Beckman Coulter, Brea, CA, USA), and performed phylogenetic analyses of the microbiomes using the MiSeq system.

Gut Microbiota Abundance Analysis by 16S rDNA PCR

Quantitative Real Time-PCR (qRT-PCR) reactions were carried out with 2.5 μL of each above DNA sample, 200 nM primers, and 12 μL of KOD SYBR qPCR MIX and 50×ROX reference dye (Toyobo, Osaka, Japan) in a final volume of 25 μL using an Applied Biosystems QuantStudio 3 (Ther-

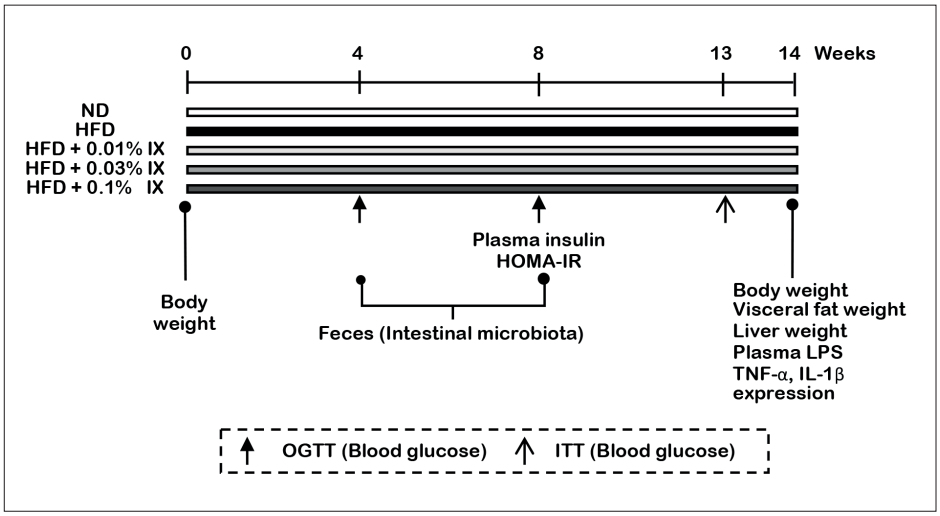

Figure 1. Experimental protocol for the HFD-induced insulin resistance model.

mo Fisher Scientific, Waltham, MA, USA). Relative abundance is shown as fold change. The following qRT-PCR primers were constructed: *Akkermansia muciniphila* Fw: 5' CAGCACGTGAAGGTGGGGAC 3', *Akkermansia muciniphila* Rv: 5' CCTTGCGGTTGGCTTCAGAT 3', *Clostridium* cluster XI Fw: 5' ACGCTACTTGAGGAGGA 3', *Clostridium* cluster XI Rv: 5' GAGCCGTAGCCTTTCACT 3'.

RNA Extraction and QPCR for Analysis of MRNA Expression

Epididymal adipose tissues were collected and subjected to qRT-PCR. Total RNA was isolated from liver tissues using QIAzol reagent and an RNeasy kit (QIAGEN, Hilden, Germany). A High-capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to synthesize cDNA. qRT-PCR was performed using TaqMan Fast Universal PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and specific primers for the TNF-α and IL-1β genes. The gene expression level was correlated with that of the 18S rRNA genes.

Measurement of LPS

Plasma LPS levels were analyzed using a Mouse LPS enzyme-linked immunosorbent assay (ELISA) kit (Cusabio, Wuhan, China).

Statistical Analysis

All data are presented as means ± SE. We used SPSS statistics (IBM, Chicago, IL, USA) for statistical analysis, and Dunnett's test was used to compare more than two groups. Student's *t*-test was used to compare two independent groups, such as ND and HFD. The difference was considered to be statistically significant at $p < 0.05$.

Results

Antibacterial Effect of Isoxanthohumol

We confirmed the antibacterial effect of isoxanthohumol against five bacterial strains: *Alicyclobacillus acidoterrestris*, *Bacillus cereus*, *Clostridium perfringens*, *Clostridium difficile,* and *Staphylococcus aureus* subsp. *Aureus* using the agar diffusion method. The MIC value of isoxanthohumol against *A. acidoterrestris* was 25 mg/mL and was 50 mg/mL against the other bacterial strains (Table I).

Effect of Intake of Isoxanthohumol for 14 Weeks on Body and Organ Weights in a HFD-Fed Mouse Model

Data for the body and organ weights are shown in Table II. The body, epididymal adipose tissue, and perirenal adipose tissue weights increased significantly in HFD-fed mice. The body weight gain induced by the HFD was significantly suppressed in the 0.1% IX-fed group. The increase in perirenal adipose tissue and liver weights was slightly but not statistically significantly decreased by the intake of isoxanthohumol.

Effect of Isoxanthohumol on Insulin Resistance in a HFD-Fed Mouse Model

We confirmed the anti-hyperglycemia effect of isoxanthohumol on HFD-fed mice by measuring the blood glucose levels after OGTTs (Figure 2A-2D). After 4 and 8 weeks of HFD intake, the blood glucose levels at each time point and the glucose AUC in each OGTT were significantly increased. Isoxanthohumol improved glucose tolerance in a dose-dependent manner, and the intake of 0.1% IX in particular significantly suppressed the increase in blood glucose level at each time point and in each

Table I. Antibacterial experimental conditions and the MIC of isoxanthohumol.

Microorganism	Medium	Temperature (°C)	Incubation time
Alicyclobacillus acidoterrestris (ATCC49025)	YSG agar	50 ± 1	5 days
Bacillus cereus (IFO13494)	Mueller-Hinton agar	37 ± 1	18-20 h
Clostridium perfringens (JCM129)	GAM agar	35 ± 1	24 h
Clostridium difficile (ATCC9689)	Taurocholic acid–containing BHI agar	37 ± 1	18-20 h
Staphylococcus aureus subsp. Aureus (NBRC12732)	Mueller-Hinton agar	37 ± 1	18-20 h

Antibacterial experimental conditions are shown for each strain, including agar medium, incubation temperature, and time. The isoxanthohumol concentrations were 3.13, 6.25, 12.5, 25, 50, and 100 μg/mL. The MIC endpoint was determined as the lowest concentration at which no bacterial growth was observed.

Table II. Effect of isoxanthohumol on the final body, epididymal adipose tissue, perirenal fat, and liver weights of HFD-fed mice.

	ND (n = 9)	HFD (n = 10)	HFD +0.01%IX (n = 10)	HFD +0.03%IX (n = 10)	HFD +0.1%IX (n = 10)
Starting body weight	21.7 ± 0.3	21.7 ± 0.3	21.7 ± 0.3	21.6 ± 0.2	21.6 ± 0.2
Final body weight	31.7 ± 0.7	44.0 ± 1.6^{++}	40.5 ± 1.2	41.8 ± 1.4	38.3 ± 1.7*
Epididymal adipose tissue	0.89 ± 0.08	2.66 ± 0.17^{++}	2.66 ± 0.12	2.66 ± 0.18	2.20 ± 0.27
Perirenal adipose tissue	0.25 ± 0.04	1.03 ± 0.07^{++}	0.88 ± 0.05	0.87 ± 0.05	0.75 ± 0.09
Liver	1.20 ± 0.02	1.41 ± 0.12	1.25 ± 0.06	1.28 ± 0.05	1.20 ± 0.05

Mice were fed ND, HFD or HFD + 0.01-0.1%IX for 14 weeks. The final body and organ weights were measured as described in the Materials and Methods. Data are presented as means ± S.E. (g) of 9-10 mice. +; significant difference between the two groups; the ND group and HFD group were compared by Student's t-test (++; $p<0.01$). *; significant difference between the HFD and 0.1% IX groups by Dunnett's test (*; $p<0.05$).

OGTT (4 and 8 weeks). After 4 weeks' ingestion of isoxanthohumol, the blood glucose levels of the 0.01% IX and 0.03% IX groups at 15 min in the OGTT were significantly lower than those in the HFD group; at 8 weeks, the blood glucose levels in the 0.01% IX and 0.03% IX groups at 120 min in the OGTT were lower than those in the HFD group. At 8 weeks, we also measured plasma insulin levels at the same time as blood glucose during the OGTT and calculated the insulin AUC. The insulin AUC of the HFD group dramatically increased compared with the ND group, and that of the 0.1% IX group was significantly lower than that of the HFD group (Figure 2E). In addition, the HOMA-IR at 8 weeks was calculated using the blood glucose and plasma insulin levels after 4 h of fasting. The intake of 0.1% IX significantly suppressed the increase in HOMA-IR induced by the HFD (Figure 2F). We confirmed the effect of isoxanthohumol on insulin resistance induced by HFD by measuring the blood glucose levels after i.p. administration of insulin at 13 weeks (Figure 3). The decrease in blood glucose level after insulin administration was significantly suppressed in HFD-fed mice compared to ND-fed mice. Isoxanthohumol decreased the blood glucose levels after insulin administration in a dose-dependent manner, and the intake of 0.1% IX, in particular, significantly decreased the relative blood glucose level at each time point, indicating that the intake of isoxanthohumol helps mitigate insulin resistance induced by a HFD.

Plasma LPS and Fat Gene Expression Related to Inflammation

Since chronic inflammation is a major factor in insulin resistance, we measured three inflammatory factors: LPS in plasma, and the mRNA expression levels of TNF-α and IL-1β in epididymal adipose tissue. Isoxanthohumol decreased plasma LPS in HFD-fed mice in a dose-dependent manner, and the intake of 0.1% IX, in particular, significantly suppressed the increase in plasma LPS (Figure 4A). Feeding a HFD to mice significantly enhanced the mRNA expression levels of TNF-α and IL-1β (Figures 4B and 4C), whereas the intake of isoxanthohumol suppressed the expression of these genes in a dose-dependent manner, similar to the decrease in plasma LPS levels.

Analysis of the Intestinal Microbiota in Feces

We evaluated the effect of isoxanthohumol on the microbiome composition by MiSeq-based high throughput 16S rRNA gene sequencing of fecal samples from mice after 4 and 8 weeks of HFD intake. The relative levels of phylum *Verrucomicrobia* dramatically increased in the 0.1% IX group compared to the HFD group, whereas the levels of phylum *Firmicutes* decreased (Figure 5A). The intake of 0.03% IX slightly increased the ratio of phylum *Verrucomicrobia*. *Akkermansia muciniphila* is a member of the *Verrucomicrobia* known to have an anti-obesity effect[14]. We therefore examined whether isoxanthohumol changes the abundance of *A. muciniphila* by determining its relative abundance by qRT-PCR analysis using species-specific primers. The results showed that 0.1% IX significantly increased both the abundance ratio of phylum *Verrucomicrobia* and the relative abundance of *A. muciniphila* (Figure 5B). The antibacterial effect of isoxanthohumol in the intestine was confirmed by measuring the relative abundance of *Clostridium* cluster XI, a member of the phylum *Firmicutes*. As expected, isoxanthohumol dose-dependently suppressed the increase in *Clostridium* cluster XI induced by the HFD (Figure 5C).

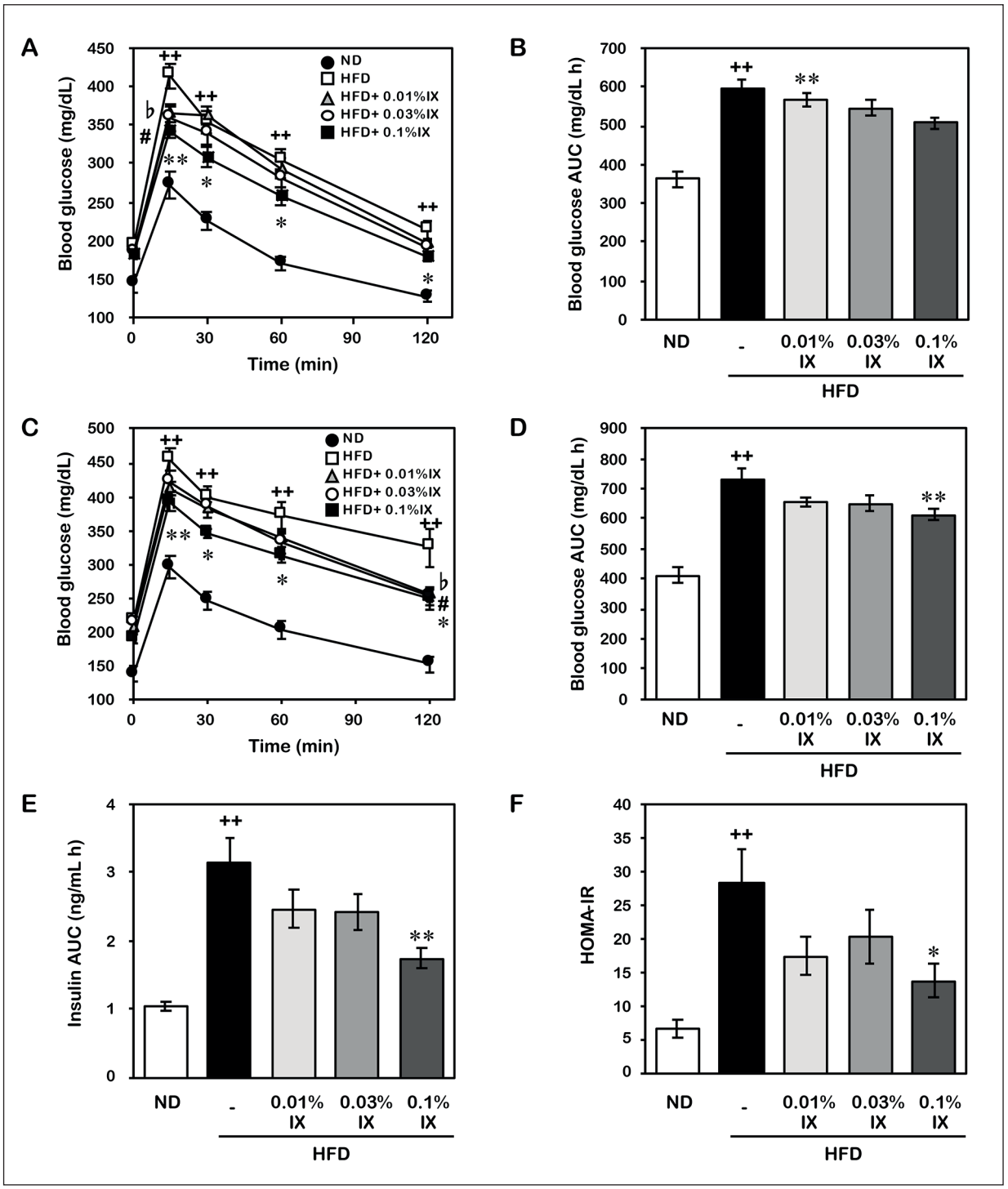

Figure 2. Suppressive effect of isoxanthohumol on HFD-induced glucose intolerance. OGTTs were performed after 4 and 8 weeks of HFD intake, and the blood glucose and plasma insulin levels were measured. **A**, Changes in blood glucose levels in the OGTT at 4 weeks. **B**, AUC of blood glucose at 4 weeks. **C**, Changes in blood glucose levels in the OGTT at 8 weeks. **D**, AUC of blood glucose at 8 weeks. **E**, AUC of plasma insulin in the OGTT at 8 weeks. **F**, HOMA-IR at 8 weeks. HOMA-IR was calculated using the 6-h fasting blood glucose level and 6-h fasting plasma insulin level. Data are presented as means ± S.E. of 10 mice. +; significant difference between the ND group and HFD group by Student's t-test (++; $p<0.01$). ♭, #, *; significant difference between the HFD and 0.01%IX, 0.03%IX and 0.1%IX groups, respectively, by Dunnett's test (one symbol; $p<0.05$, two symbols; $p<0.01$).

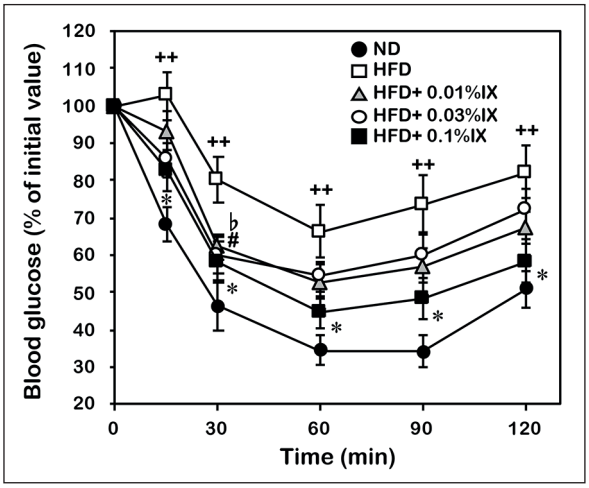

Figure 3. Suppressive effect of isoxanthohumol on HFD-induced insulin resistance. ITTs were performed 13 weeks after the initiation of HFD intake, and the relative blood glucose levels were measured. Data are presented as means ± S.E. of 10 mice. +; significant difference between the ND group and HFD group by Student's t-test (++;$p<0.01$). ♭, #, *; significant difference between the HFD and 0.01%IX, 0.03%IX and 0.1%IX groups, respectively, by Dunnett's test (one symbol; $p<0.05$).

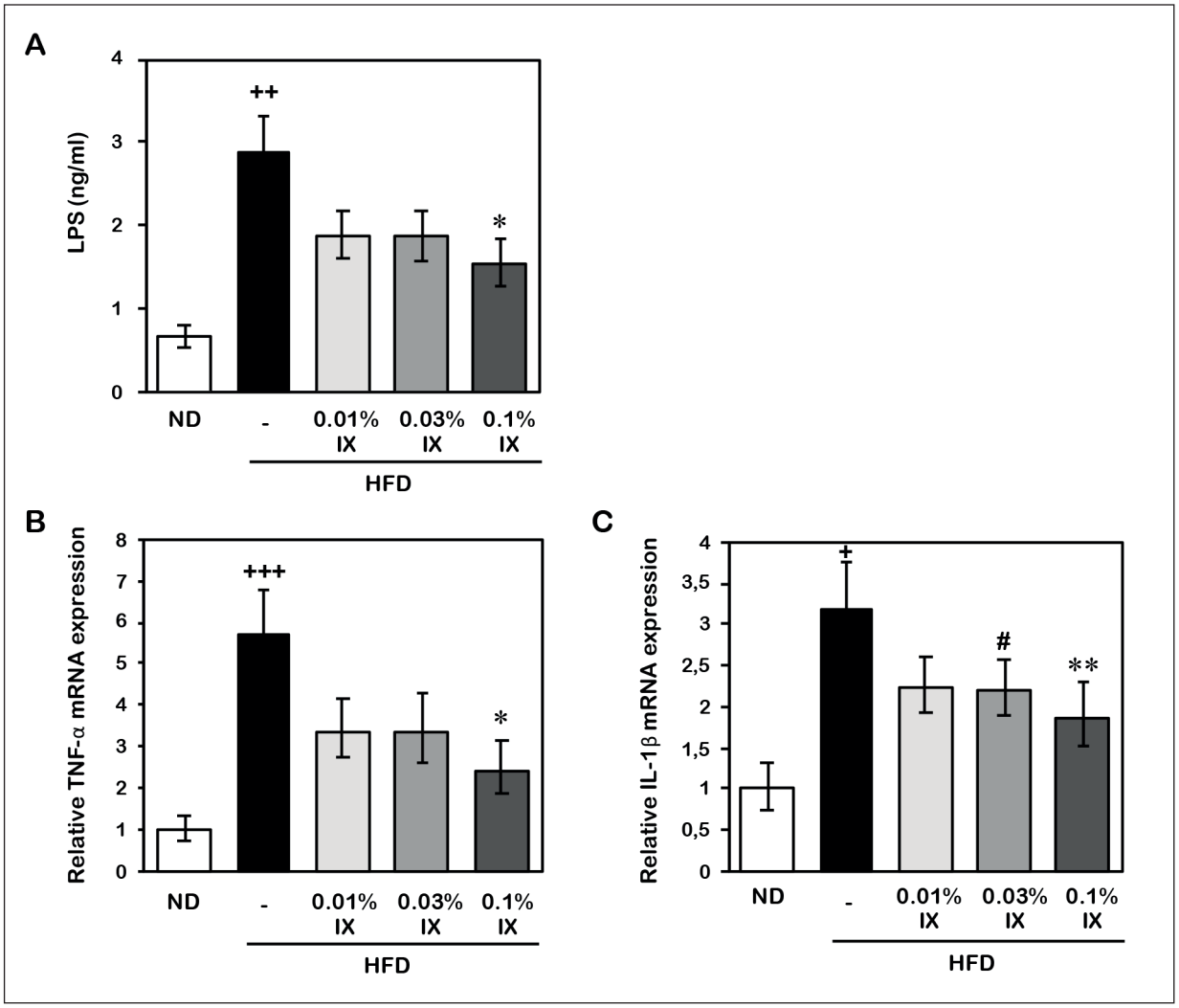

Figure 4. Anti-inflammatory effect of isoxanthohumol. Plasma and epididymal adipose tissue were collected at 14 weeks after initiation of HFD intake. **A**, Plasma LPS and relative mRNA expression of (**B**) TNF-α and (**C**) IL-1β in epididymal adipose tissue were measured. Data are presented as means ± S.E. of 9-10 mice. +; significant difference between the ND group and HFD group by Student's t-test (+; $p<0.05$, ++; $p<0.01$). #, *; significant difference between the HFD and 0.03%IX and 0.1%IX groups, respectively, by Dunnett's test (one symbol; $p < 0.05$).

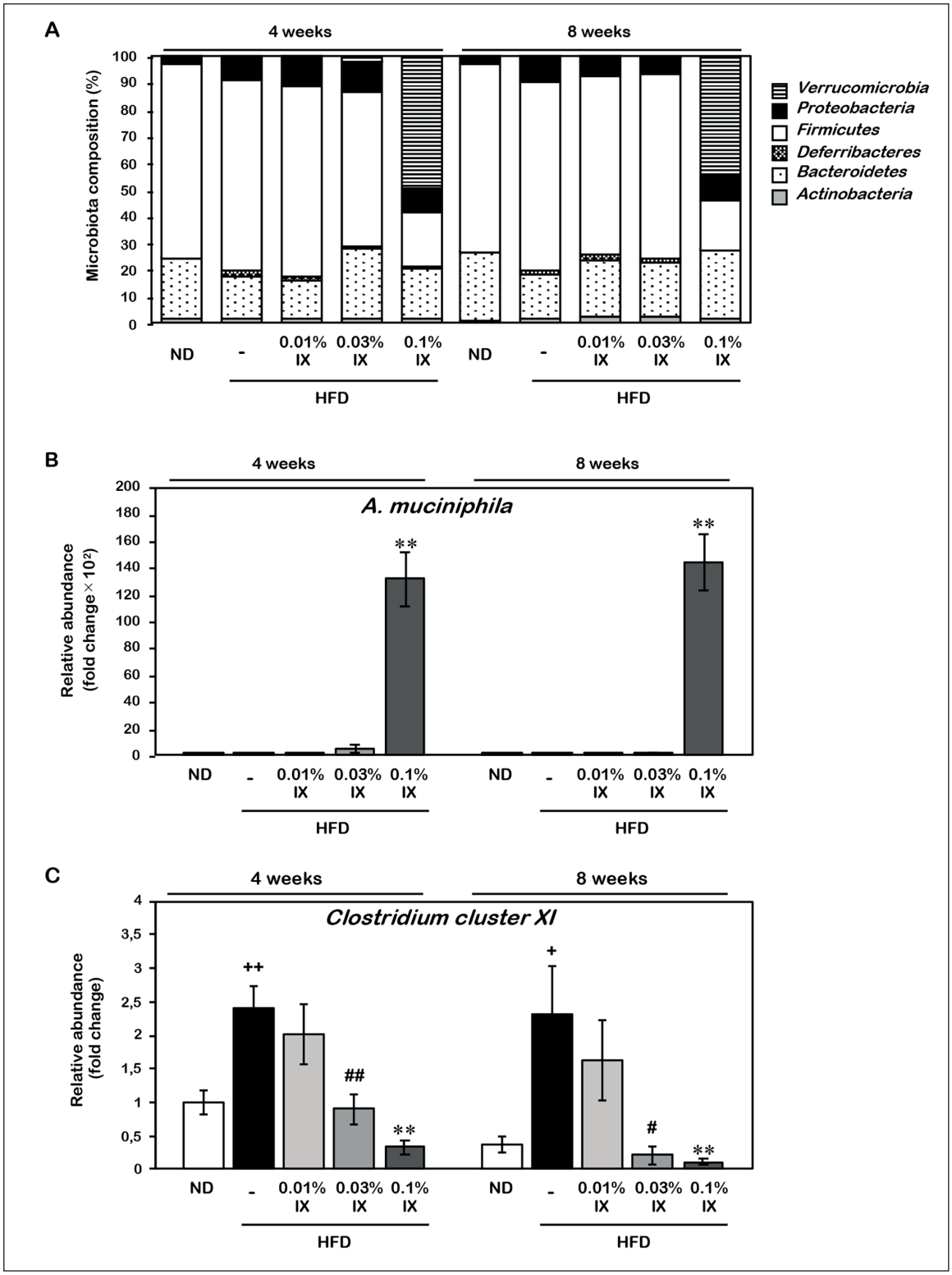

Figure 5. Effect of isoxanthohumol on the intestinal microbiota of HFD-fed mice. Feces were collected at 4 and 8 weeks after initiation of HFD intake. **A**, The microbial composition of the feces at the phylum level was measured by 16S rRNA sequencing. Data are presented as means of 10 mice. **B**, Relative abundance of A. muciniphila and (**C**) Clostridium cluster XI were determined by qPCR analysis using species-specific primers. Data are presented as means ± S.E. of 10 mice. +; significant difference between the ND group and HFD group by Student's t-test (+; $p<0.05$, ++; $p<0.01$). #, *; significant difference between the HFD and 0.03%IX and 0.1% IX groups, respectively, by Dunnett's test (one symbol; $p<0.05$, two symbols; $p<0.01$).

Discussion

T2D is associated with many metabolic disorders[24-26]. The International Diabetes Federation[27] reported 425 million diabetes patients in 2017 and estimated that globally there will be about 629 million diabetics by 2045. Whiting et al[1] estimated that 522 million people worldwide will develop diabetes by 2030. The onset of diabetes is particularly related to poor dietary and exercise habits, which cause insulin resistance and reduced glucose tolerance[28]. IGT characterized by insulin resistance develops into T2D within a few years[2] and hence, mitigating insulin resistance is important for maintaining health. To this end, control of the intestinal microbiota is attracting worldwide attention. In the present study, we found that isoxanthohumol suppressed insulin resistance in HFD-fed mice (Figures 2 and 3), suppressed inflammation, (Figure 4), and caused changes in the intestinal microbiota, in particular increasing *A. muciniphila* and decreasing *Clostridium* cluster XI levels (Figure 5).

Hops have been used in beer as an antibacterial material since antiquity, whereas to date, the antibacterial activity of isoxanthohumol is unknown. We found that isoxanthohumol showed antibacterial activity against several species, such as *A. acidoterrestris*, *B. cereus*, *C. perfringens*, *C. difficile*, and *S. aureus* (Table I). Several antibiotics have been reported[29] to mitigate insulin resistance and improve glucose metabolism. We thus hypothesized that insulin resistance can be improved by continual intake of isoxanthohumol and indeed found that isoxanthohumol intake suppressed HFD-induced depression of glucose metabolism (Figure 2A-2D) and mitigated insulin resistance (Figure 2F and Figure 3). These effects are likely due to both an anti-obesity effect (Table II) and to changes in the composition of the intestinal microbiota.

Isoxanthohumol only showed an anti-obesity effect in the 0.01% IX group; no effect was observed at lower doses. However, 0.03% IX and 0.01% IX significantly lowered blood glucose levels at 120 min in the OGTT at 8 weeks (Figure 2C) and the blood glucose level at 30 min in the ITT at 13 weeks (Figure 3). An important characteristic of insulin resistance is that the blood glucose level after 120 min in the OGTT is difficult to decrease. It is likely that the inhibitory effect of isoxanthohumol towards insulin resistance and its suppression of growth in inflammatory markers are somewhat related. The expression level of TNF-α at 14 weeks in the 0.01% IX and 0.03% IX groups tended to decrease, although not significantly ($p = 0.097$ and $p = 0.086$, respectively) (Figure 4B), whereas the expression level of IL-1β was essentially unchanged (0.01% IX, $p = 0.065$) (Figure 4C). Inflammatory cytokines are expressed as immune responses to an immune stimulant, such as LPS. The continual intake of isoxanthohumol decreased LPS plasma levels (Figure 4C) and may be associated with a decreased expression of inflammatory cytokines. Events that can be predicted from decreased expression of inflammatory factors, such as TNF-α and IL-1β in adipose tissue include increased adiponectin and decreased leptin levels in adipocytes. It has been shown[30-32] that increased adiponectin and decreased leptin levels suppress the inflammatory response. Therefore, it is also expected that one of the mechanisms of suppression of insulin resistance by isoxanthohumol involves suppression of the inflammatory response *via* increased adiponectin and decreased leptin levels. Our data suggested the involvement of a series of mechanisms, such as changes in the intestinal microbiota (Figure 5), suppression of inflammatory responses (Figure 4A-4C), and suppression of insulin resistance (Figure 2F, Figure 3). However, the detailed mechanism by which isoxanthohumol suppresses insulin resistance could be revealed by determining the involvement of other biomolecules, such as adipocyte-producing hormones.

The continual ingestion of isoxanthohumol significantly changed the intestinal microbiota profile (Figure 5A). The anti-inflammation effect of isoxanthohumol is thus likely related to this change in the intestinal microbiota through one of two mechanisms. One possible mechanism involves an increase in the phylum *Verrucomicrobia* (Figure 5A). *Akkermansia muciniphila* is a member of the *Verrucomicrobia*, and isoxanthohumol increased both the ratio of phylum *Verrucomicrobia* compared to five other phyla and the relative abundance of *A. muciniphila* (Figure 5B). Both live and dead *A. muciniphila* are reported to suppress inflammation and improve glycometabolism in *in vivo* experiments[33] and in humans[15]. The active components are believed to be membrane proteins[33] or extracellular vesicles[34]. These previous reports support this proposed mechanism: that isoxanthohumol mitigates insulin resistance due to an increase in *A. muciniphila*. However, in the 0.01% IX and 0.03% IX groups, the degree of insulin resistance mitigation and the decreases in plasma LPS and inflammatory cytokines did not parallel the increase in *A. muciniphila*, suggesting that a second mechanism may be involved, namely, a reduction in the number of harmful bacteria. This latter mechanism is

supported by scholars who show that antibiotics suppress chronic inflammation and increase glucose metabolism in HFD-fed mice[35]. We assessed the relative abundance of *Clostridium* cluster XI as a representative harmful bacterial family and confirmed that isoxanthohumol decreased the population of *Clostridium* cluster XI in a dose-dependent manner (Figure 5C). Since isoxanthohumol shows antibacterial activity, it likely shows antibacterial activity in the intestine. However, we cannot explain why isoxanthohumol increased the levels of *A. muciniphila*, but perhaps this population increased advantageously because of the decreased levels of other bacterial species caused by isoxanthohumol. The degree of *Clostridium* cluster XI reduction was isoxanthohumol dose dependent and paralleled the mitigation of insulin resistance, although it remains to be clarified whether this was due to the decrease in *Clostridium* cluster XI, the decrease in harmful bacteria in general, or to the reduction of metabolites that cause inflammation.

In this study, we revealed that isoxanthohumol improves insulin resistance by changing the intestinal microbiota. It is detected[36,37] that the intestinal structure and intestinal microbiota of mice and humans differ. In addition, daily dietary and lifestyle habits differ markedly between individuals. Therefore, the effect of isoxanthohumol in humans might not necessarily be observed, as well as in mice. However, isoxanthohumol could suppress insulin resistance in humans if it reduces plasma LPS, as LPS reportedly causes low-grade inflammation and induces insulin resistance in humans[38]. Similarly, insulin resistance could be suppressed if isoxanthohumol increases *A. muciniphila* in humans, as a recent clinical study showed that intake of *A. muciniphila* improves insulin resistance in humans[15]. We would like to examine the changes in the intestinal environment and insulin resistance-improving effects of long-term isoxanthohumol intake in humans. For that, it will be necessary to confirm the safety of isoxanthohumol for clinical studies.

Conclusions

In this study, we revealed that isoxanthohumol suppressed insulin resistance in HFD-fed mice. Continual intake of isoxanthohumol reduced in a dose-dependent manner the concentration of LPS in plasma that causes inflammation and reduced the mRNA expression levels of TNF-α and IL-1β, two inflammation markers closely related to insulin resistance. Isoxanthohumol exhibited an antibacterial effect, changed the component ratios of the intestinal microbiota, and changed the relative abundance of *Clostridium* cluster XI and *A. muciniphila*. These findings suggest that isoxanthohumol mitigates insulin resistance by reducing plasma LPS levels and suppressing inflammation by changing the intestinal microbiota through its antibacterial action.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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