Polygonum tinctorium leaf extract ameliorates high-fat diet-induced intestinal epithelial damage in mice

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Abstract. Dietary fat strongly influences the intestinal mucosal barrier, which protects against invading pathogenic bacteria. A high-fat diet (HFD) compromises the integrity of epithelial tight junctions (TJs) and reduces mucin production, leading to intestinal barrier disruption and metabolic endotoxemia. It has been shown that the active constituents of indigo plants can protect against intestinal inflammation; however, their protective role in HFD-induced intestinal epithelial damage remains unknown. The present study aimed to investigate the effects of *Polygonum tinctorium* leaf extract (indigo Ex) on HFD-induced intestinal damage in mice. Male C57BL6/J mice were fed a HFD and injected intraperitoneally with either indigo Ex or phosphate-buffered saline (PBS) for 4 weeks. The expression levels of TJ proteins, zonula occludens-1 and Claudin-1, were analyzed by immunofluorescence staining and western blotting. The colon mRNA expression levels of tumor necrosis factor-α, interleukin (IL)-12p40, IL-10 and IL-22 were measured by reverse transcription-quantitative PCR. The results revealed that indigo Ex administration attenuated the HFD-induced shortening of the colon. Colon crypt length was shown to be significantly greater in the indigo Ex-treated group mice compared with that in the PBS-treated group mice. Moreover, indigo Ex administration increased the number of goblet cells, and ameliorated the redistribution of TJ proteins. Notably, indigo Ex significantly increased the colon mRNA expression levels of IL-10. Indigo Ex displayed little effect on the gut microbial composition of HFD-fed mice. Taken together, these results suggested that indigo Ex may protect against HFD-induced epithelial damage. The leaves of indigo plants contain promising natural therapeutic compounds that could be used to treat obesity-associated intestinal damage and metabolic inflammation.

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Introduction

The intestinal mucosal barrier comprises epithelial cells, mucus, immune cells in the lamina propria, and commensal microorganisms. Epithelial cells are tightly bound together via intercellular junctional structures such as tight junctions (TJ). TJ are composed of transmembrane proteins including claudins and cytosolic scaffolding proteins including zonula occludens-1 (ZO-1). Claudin-1, a member of the claudin protein family, is widely expressed in the intestinal epithelia and regulates the paracellular permeability (1,2). ZO-1 interacts with claudins and cytoskeletal actin and plays an important role in TJ assembly (3). Additionally, mucus that covers the epithelia contains several antibacterial peptides and functions as a physical barrier. The commensal microbiota and host immune cells interact with each other, which is essential for protection against invading pathogenic bacteria and toxins (4). Importantly, the function of the intestinal barrier is strongly influenced by dietary contents (5). High-fat diet (HFD) increases the intestinal permeability by inducing the expression of barrier-disrupting cytokines and reducing mucus secretion (6). Moreover, growing evidence suggests that HFD is associated with abnormal intestinal flora and gut dysbiosis (5,7). A high-fat and high-sugar diet alters the gut microbial structure in only 3.5 days (8). The host mucosal barrier disruption occurs even more rapidly. The epithelial expression of ZO-1 has been reported to decrease after 48 h of HFD consumption (9). Moreover, the number of intestinal immune cells in mice has been observed to have significantly decreased after 24 h of HFD consumption (10). These findings

suggest that HFD-induced intestinal damage occurs simultaneously with or prior to the development of obesity-associated metabolic disorders. An impaired intestinal barrier facilitates the invasion of enteric microbe-derived antigens into blood vessels, and metabolic endotoxemia modulates systemic inflammation (11). Therefore, proper functioning of the gut barrier is critical to prevent the progression of metabolic inflammation.

The leaves of indigo plants, such as *Polygonum tinctorium* contain a wide variety of bioactive molecules. Tryptanthrin and kaempferol, isolated from *P. tinctorium*, possess antibacterial properties (12). Indirubin and its analogues exert anti-tumor effects by inhibiting cyclin-dependent kinases (13). It was recently reported that *P. tinctorium* leaves exerted anti-inflammatory effects by modulating the interleukin (IL)-10-related pathway in a mouse model of chemically induced colitis (14). However, the effectiveness of indigo leaf extract in HFD-induced intestinal damage has not yet been fully elucidated. Therefore, this study aimed to investigate the effects of the indigo leaf extract on intestinal damage in HFD-fed mice.

Materials and methods

Reagents. Rabbit polyclonal anti-ZO-1 (#21773-1-AP) and anti-Claudin-1 (#28674-1-AP) antibodies were purchased from Proteintech. The rabbit polyclonal anti-β-actin antibody (#4967) was purchased from Cell Signaling Technology. Alexa Fluor 488-conjugated anti-rabbit IgG antibody (#A11008) and ProLong Gold antifade regent with 4',6-diamidino-2-phenylindole (DAPI) were purchased from Thermo Fisher Scientific. Alcian Blue (AB) solution (pH2.5) and BCA protein assay kit were purchased from FUJIFILM Wako Pure Chemical Co. Periodic acid-Schiff (PAS) stain kit and NucleoSpin RNA kit were obtained from Muto Pure Chemicals Co. and Macherey-Nagel GmbH and Co. KG, respectively. Moloney murine leukemia virus (M-MLV) reverse transcriptase and oligo $(dT)_{18}$ primers were purchased from Invitrogen; Thermo Fisher Scientific. Thunderbird™ Next SYBR® qPCR mix was purchased from Toyobo (Osaka, Japan). Polyvinylidene fluoride (PVDF) membranes and Luminata Crescendo Western HRP substrate were purchased from Merck Millipore. Enzyme-linked immunosorbent assay (ELISA) kit for IL-10 was obtained from LSBio.

Preparation of indigo Ex. For the purpose of this study, *P. tinctorium* was cultivated without pesticides in Aomori. A voucher specimen was deposited in the herbarium of the Medical Herbal Garden of the Tohoku Medical and Pharmaceutical University. The indigo Ex was prepared as previously described (15). Briefly, the air-dried leaves were powdered and extracted with *d*-limonene for 48 h. After filtration, a pale-yellow extract was obtained. In this study, we used the original indigo Ex AOMORI-BLUE (Japanese Patent no. 6389492). This extract is rich in the principal ingredient, tryptanthrin (15). The stock solution, diluted 10,000-fold with water, was provided by the Aomoriai-Sangyo Co. This solution was diluted 50-fold with phosphate-buffered saline (PBS) and filter sterilized through a 0.33 μm membrane.

Animal model. Forty-five male C57BL6/J mice (6-7 weeks old) were purchased from CLEA Japan and maintained on a 12-12 h light/dark cycle at 22˚C and 50% relative humidity in a specific pathogen-free environment. After a week of acclimation, the mice were divided into three groups (n=15 per group). One group was fed a normal chow (NC) diet and injected intraperitoneally with 10 mg/kg of PBS daily (NC/PBS group). The others were fed an HFD (High Fat Diet 32: 56.7% of calories from fat, CLEA Japan) and injected intraperitoneally with either PBS (HFD/PBS group) or indigo Ex (HFD/indigo Ex group). Body weights were measured weekly, and after 4 weeks, the mice were euthanized by cervical dislocation and the tissue samples were collected. Blood samples obtained by cardiac puncture were incubated for 30 min at room temperature. The sera were collected by centrifugation at 3,000 rpm for 10 min at 4˚C, and the serum levels of total cholesterol (T. Chol), triglycerides (TG), and glucose were analyzed using a biochemical analyzer (SPOTCHEM EZ SP-4430; Arkray). All animal experiments were conducted in accordance with the Guidelines for Animal Experimentation of the Hirosaki University.

Histological analysis. The colon, epididymal fat, and liver tissues collected from the mice were fixed in 10% formalin and embedded in paraffin. Sections (3-5 μm thick) were cut and conventional hematoxylin and eosin (H&E) staining was performed for morphological evaluation. Goblet cells were histologically assessed by AB/PAS staining using a standard protocol.

Immunofluorescence (IF) staining. IF staining was performed to examine the epithelial expression of TJ-associated proteins ZO-1 and Claudin-1. Tissue sections were immersed in 10 mM citrate buffer (pH 6.0) and heat-induced antigen retrieval was performed using an autoclave. Blocking of the endogenous peroxidase of the tissue sections with 3% hydrogen peroxide was followed by that with 5% normal goat serum for 1 h at room temperature. Thereafter, sections were incubated with the anti-ZO-1 (1:250) or anti-Claudin-1 (1:250) antibody overnight at 4˚C. After washing with PBS, the tissue sections were incubated with Alexa Fluor 488-conjugated anti-rabbit IgG antibody (1:250) for 1 h at room temperature. The slides were mounted with ProLong Gold antifade reagent with DAPI, and visualized using a confocal laser scanning microscope (C1si; Nikon).

Western blotting. Murine colon tissues were lysed with RIPA buffer containing 0.2% proteinase inhibitor cocktail, and the lysates were centrifuged at 12,000 rpm for 10 min at 4˚C. The supernatants were collected, and equal amounts of the protein (15 mg) were loaded onto a 10-20% sodium dodecyl sulfate (SDS) polyacrylamide gel for electrophoresis. The separated proteins were transferred onto a PVDF membrane. After blocking with Tris-buffered saline with Tween 20 (TBS-T, pH 7.4) containing 5% nonfat dry milk, the membranes were incubated overnight at 4˚C with a primary antibody against ZO-1 (1:1,000), Claudin-1 (1:1,000), or β-actin (1:2,000). Membranes were then incubated with an HRP-conjugated secondary antibody for 1 h at room temperature. Immunodetection was performed using Luminata Crescendo substrate. The density

of each band was measured using the ImageJ software (16). The levels of ZO-1 and Claudin-1 proteins were normalized to β-actin.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the colon tissues using the NucleoSpin RNA kit according to the manufacturer's instructions. Single-stranded complementary DNA (cDNA) was synthesized using oligo $(dT)_{18}$ primers and M-MLV reverse transcriptase. RT-qPCR was performed using a Bio-Rad CFX real-time PCR thermocycler and Thunderbird™ Next SYBR® qPCR mix. The results were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels. Relative gene expression was calculated using the delta-delta CT method. All assays were performed in triplicates. The primer sequences used were as follows: MUC2-F: 5'-CCATTGAGTTTGGGA ACATGC-3', R: 5'-TTCGGCTCGGTGTTCAGAG-3', spliced X-box binding protein 1 (sXBP-1)-F: 5'-GAGTCCGCAGCA GGTGC-3', R: 5'-CAAAAGGATATCAGACTCAGAATC TGAA-3', ZO-1-F: 5'-AGGACACCAAAGCATGTGAG-3', R: 5'-GGCATTCCTGCTGGTTACA-3', Claudin-1-F: 5'-CTG GAAGATGATGAGGTGCAGAAGA-3', R: 5'-CCACTAATG TCGCCAGACCTGAA-3', tumor necrosis factor (TNF)-α-F: 5'-GATCTCAAAGACAACCAACATGTG-3', R: 5'-CTCCAG CTGGAAGACTCCTCCCAG-3', IL-12p40-F: 5'-GGAAGC ACGGCAGCAGAATA-3', R: 5'-AACTTGAGGGAGAAGTAG GAATGG-3', IL-10-F: 5'-TGGCCCAGAAATCAAGGAGC-3', R: 5'-CAGCAGACTCAATACACACT-3', IL-22-F: 5'-GTCAAC CGCACCTTTATGCT-3', R: 5'-CATGTAGGGCTGGAACCT GT-3', GAPDH-F: 5'-TGAAGGTCGGTGTGAACGGATTTG G-3', R: 5'-ACGACATACTCAGCACCAGCATCAC-3'.

ELISA. Total protein was extracted from the colon tissues using RIPA buffer containing 0.2% proteinase inhibitors cocktail as described above, and the concentration was determined using a BCA protein assay kit. Colon IL-10 levels were measured using an ELISA kit, according to the manufacturer's instructions.

Gut microbiota analysis. Gut microbiota analyses was performed at the Bioengineering Lab. Co., Ltd. (Kanagawa, Japan). Fecal samples were collected from mice and stored at -20˚C. Total DNA was extracted using the MPure-12 system and MPure bacterial DNA extraction kit (MP Bio Japan K.K.). The V3-V4 regions of the 16S ribosomal RNA (rRNA) genes were amplified using the primers 341F (5'-ACACTCTTTCCC TACACGACGCTCTTCCGATCT-NNNNN-CCTACGGGN GGCWGCAG-3') and 805R (5'-GTGACTGGAGTTCAG ACGTGTGCTCTTCCGATCT-NNNNNGACTACHVGG GTATCTAATCC-3'). Amplicons were sequenced using the 2x300-bp paired-end method on a MiSeq system (Illumina). The microbiome was analyzed using QIIME2 (ver. 2022.2).

Statistical analyses. Data obtained are presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) with post-hoc Tukey test was performed to analyze the statistical significance among the three groups, and an unpaired two-tailed t-test was performed to compare the differences in gut microbiota between the HFD/PBC and HFD/indigo Ex groups. Statistical significance was set at $P<0.05$.

Results

Indigo Ex has no effect on HFD-induced obesity. First, we investigated the effect of indigo Ex on diet-induced obesity. The results showed that the body weight of the HFD-fed mice was significantly higher than that of the NC-fed mice at 4 weeks. There was no significant difference in the body weights of the HFD/PBS and HFD/indigo Ex groups (Fig. 1A). Daily food intake in the HFD/indigo Ex group was similar to that in the HFD/PBS group (Fig. S1A). Epididymal fat weight was higher in the HFD/PBC group than in the NC/PBC group, while indigo Ex had little effect on this increase (Fig. S1B). Histological results confirmed that HFD induced adipocyte hypertrophy, with indigo Ex having little effect on them (Fig. S1C). Four weeks of HFD feeding did not lead to apparent steatosis of the liver, and indigo Ex had no effect on the liver (Fig. S1D). There were no differences in the serum levels of T. Chol, TG, and glucose between the HFD/PBS and HFD/indigo Ex groups (Fig. S1E).

Indigo Ex ameliorates HFD-induced intestinal epithelial damage. Our results showed that indigo Ex ameliorated the HFD-induced shortening of the colon in mice (Fig. 1B and C). Moreover, the colon crypt length in the HFD/indigo Ex group mice was significantly greater than that in the HFD/PBS group mice (Fig. 1D and E). There was no difference in the number of crypts between any of the three groups, and we could not detect mucosal destruction or increased infiltration of immune cells (Fig. 1D). To further evaluate the protective effect of indigo Ex against HFD-induced intestinal damage, we performed AB/PAS staining to assess the number of goblet cells. Goblet cells play an essential role in mucus production, and it has been reported that HFD causes mucin depletion at a relatively early stage (6,17). Consistently, we observed that the number of goblet cells was lower in the HFD/PBS group than in the NC/PBS group, and that indigo Ex administration prevented this decrease in HFD-fed mice (Fig. 1F). Because MUC2 is the major component of mucin (18,19), we performed RT-qPCR to analyze the expression of MUC2 mRNA in the colon tissue of mice from different groups. The results showed that there was no significant difference in MUC2 mRNA levels between the HFD/PBS and HFD/indigo Ex groups (Fig. 2A). Dietary fat-induced endoplasmic reticulum (ER) stress is associated with impaired goblet cell function and mucin depletion (17,20). Therefore, we evaluated the mRNA levels of sXBP-1, an unfolded protein response (UPR) signaling molecule. Results showed that HFD and indigo Ex had no effect on sXBP-1 mRNA expression (Fig. 2B).

As indigo Ex had a protective effect on goblet cells, we speculated that the extract may improve HFD-induced redistribution of TJ-associated proteins. We performed IF staining to assess the expression of TJ-associated proteins, ZO-1 and Claudin-1. The results showed that the expression of these proteins in the mucosal surface layer was markedly decreased in the HFD/PBS group compared to that in the NC/PBS group, and indigo Ex administration restored the expression of these proteins (Fig. 2C and D). The results of western blotting showed that the expression level of ZO-1 protein in the HFD/indigo Ex group mice was significantly increased compared to that in the HFD/PBS group mice, whereas there

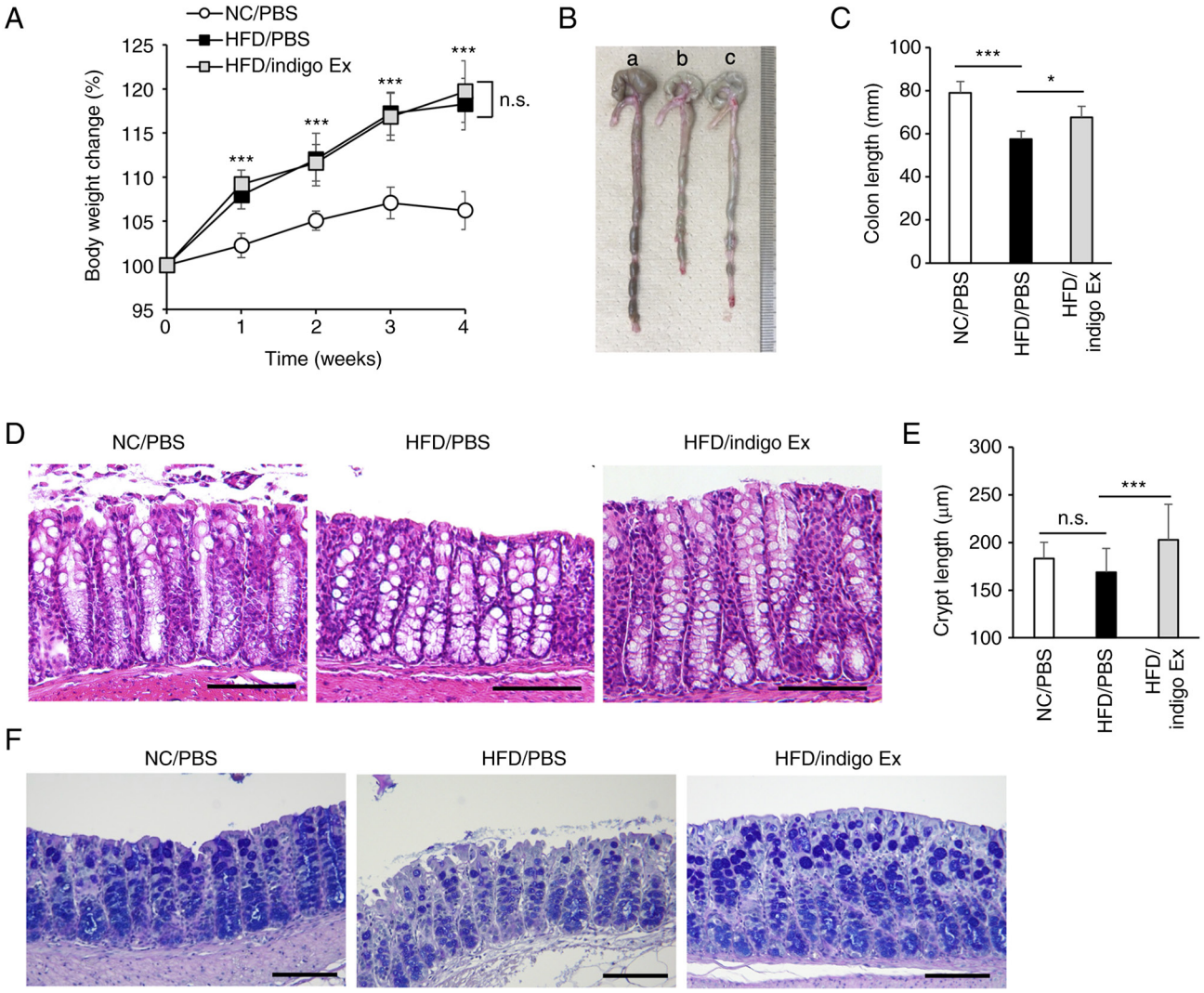

Figure 1. Effects of *P. tinctorium* leaf extract (indigo Ex) on HFD-induced intestinal damage in mice. The mice were divided into three groups. The NC/PBS group was fed an NC diet and intraperitoneally injected with PBS. The HFD/PBS and HFD/indigo Ex groups were fed with a HFD and injected with either PBS or indigo Ex, respectively. (A) Percentage change in the body weight after 4 weeks. Data are presented as mean ± SD, n=8-9 in each group. ***P<0.001 vs. NC/PBS; n.s., not statistically significant. (B) Representative images of the colons from mice in the NC/PBS (a), HFD/PBS (b), and HFD/indigo Ex (c) groups. (C) Bar graph depicting the colon length. Data are presented as mean ± SD, n=8-9 in each group. ***P<0.001, *P<0.05. (D) H&E staining of the colon sections. Original magnification: 200x. Scale bar, 100 μm. (E) Bar graph showing the crypt length. Results are presented as mean ± SD, n=8-9 in each group. ***P<0.001; n.s., not statistically significant. (F) AB/PAS staining was performed to assess the number of goblet cells. Scale bar, 100 mm. NC, normal chow; HFD, high-fat diet; PBS, phosphate-buffered saline; H&E, hematoxylin and eosin; AB/PAS, Alcian Blue/Periodic acid Schiff; ns, not significant.

was no difference in the expression level of Claudin-1 between HFD/PBS group and HFD/indigo Ex group (Fig. 2E-G). The expression of ZO-1 and Claudin-1 mRNA was not affected by the indigo Ex (Fig. 3A and B).

Next, we examined the mRNA levels of TNF-α, IL-12p40, IL-10, and IL-22. The results showed no significant difference in TNF-α and IL-12p40 levels between the NC/PBS, HFD/PBS, and HFD/indigo Ex groups (Fig. 3C and D). Interestingly, indigo Ex significantly increased the mRNA levels of IL-10 but not IL-22 (Fig. 3E and F). We performed ELISA for IL-10 using colon tissue homogenates, and the results indicated that the IL-10 levels were significantly higher in the HFD/indigo Ex group than in the NC/PBS group (Fig. 3G).

Indigo Ex does not alter the gut microbial composition in HFD-fed mice. We performed 16S rRNA gene sequencing to clarify whether the effects of the indigo Ex were accompanied by changes in the gut microbial structure. As shown in Fig. 4A, there were no significant differences in the microbial composition between the HFD/PBS and HFD/indigo Ex groups at the phylum level. Similarly, there was no significant difference in the *Firmicutes*/*Bacteroides* ratio between the two groups (Fig. 4B). At the genus level, indigo Ex had no effect on the relative abundance of *Allobaculum*, *Akkermansia*, *Bacteroides*, and *Ruminococcus* (Fig. 4C).

Discussion

The molecular mechanisms underlying HFD-induced impairment of the intestinal mucosal barrier are complex and not fully understood. Recent studies have demonstrated that long-term HFD consumption negatively affects TJ integrity and enhances the intestinal permeability (21,22). Moreover, increased expression of proinflammatory cytokines and intestinal inflammation

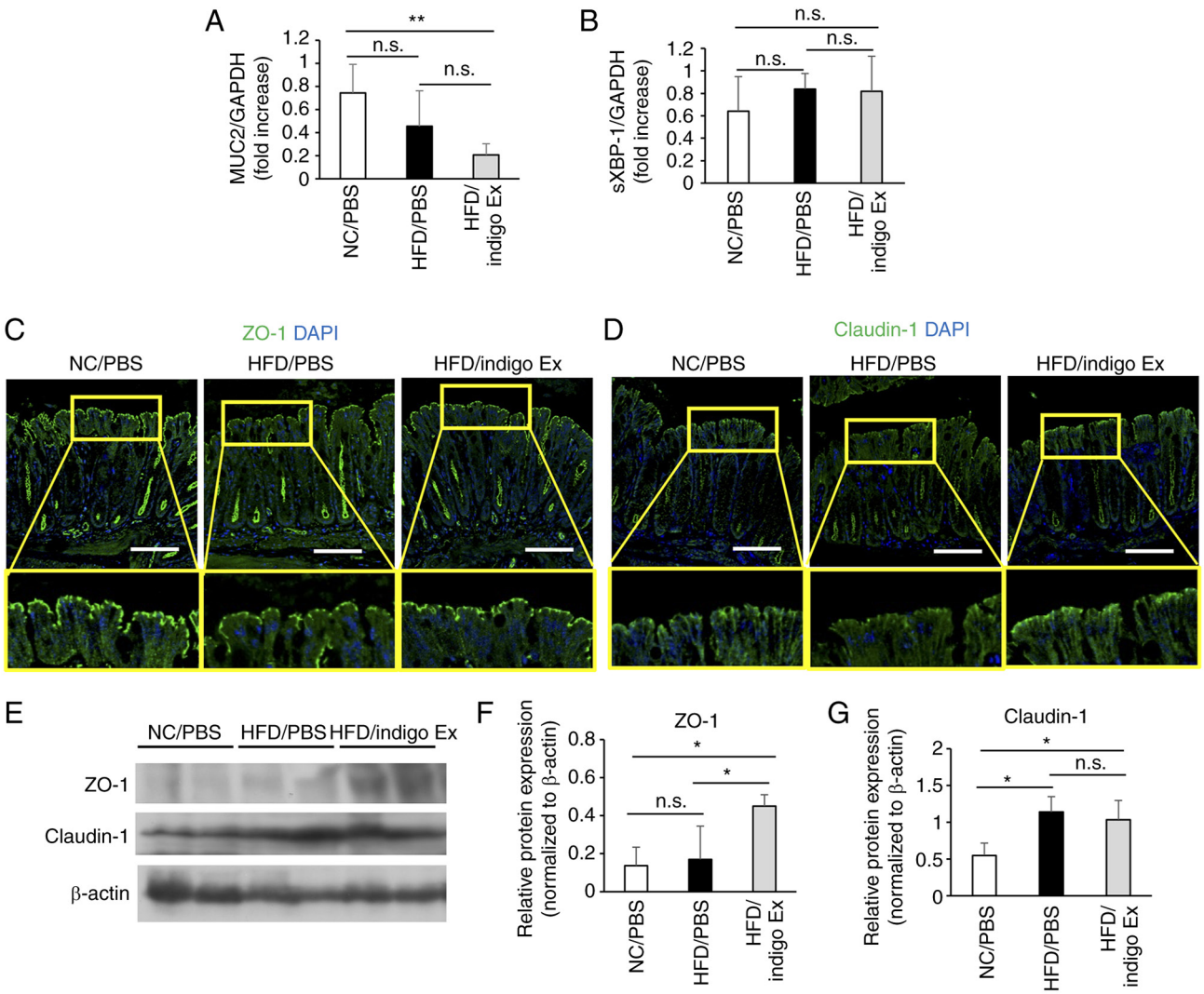

Figure 2. The mRNA levels in the colon of mice from NC/PBS, HFD/PBS, and HFD/indigo Ex groups. Total RNA was extracted from the colon tissue and cDNA was synthesized. Diagrammatic representations of RT-qPCR performed to analyze the mRNA levels of (A) MUC2 and (B) sXBP-1. Data are presented as mean ± SD, n=3-5 in each group. **P<0.01; n.s., not statistically significant. (C and D) Diagrammatic representation of results obtained for immunofluorescence staining performed to analyze the expression of (C) ZO-1 and (D) Claudin-1 in the colon mucosa. Both proteins were expressed in the luminal surface of the epithelial cells (green). Cell nuclei were stained with DAPI (blue). Representative images for 4-5 mice in each group. Scale bar, 100 μm. (E) Colon tissue homogenates were prepared, and the expressions of ZO-1, Claudin-1, and β-actin were analyzed by western blotting. (F and G) The optical density of each band was measured using the ImageJ software. Results are presented as mean ± SD, n=4 in each group. *P<0.05; n.s., not statistically significant. NC, normal chow; HFD, high-fat diet; PBS, phosphate-buffered saline; RT-qPCR, reverse transcription-quantitative PCR; MUC2, mucin-2; sXBP-1, spliced X-box binding protein-1; ZO-1, zonula occludens-1; DAPI, 4',6-diamidino-2-phenylindole.

reduce the expression of TJ-associated proteins (6,23). A previous study showed that saturated fatty acids induced ER stress in epithelial cells (24). Elevated ER stress in goblet cells reduces mucin secretion, leading to intestinal barrier disruption (17). Furthermore, alterations in the structure of the gut microbiota affect the function of both epithelial and immune cells. In this study, we demonstrated that indigo Ex ameliorates the decrease in the number of goblet cells in the colon of HFD-fed mice. Additionally, indigo Ex restored the expression of ZO-1 and Claudin-1 proteins, whereas that of mRNA was not affected, suggesting that the extract modulated the expression of these proteins in a post-transcriptional manner. These results indicated that indigo Ex could protect against HFD-induced intestinal epithelial damage. It has been reported that the TJ-associated proteins are regulated post-translationally by phosphorylation, palmitoylation, and glycosylation in response to various stimuli (25,26). We speculated that indigo Ex might affect the post-translational modulation of TJ proteins. Alternatively, the indigo Ex might indirectly attenuate the epithelial cell damage by increasing mucus production. The results of IF staining showed that the epithelial expression of ZO-1 and Claudin-1 was markedly decreased in the HFD-fed mice, while the protein level of Claudin-1 in colon tissue homogenates was increased, as shown by western blotting. We speculate that this discrepancy may be ascribed to the results obtained using whole tissue homogenates, while failing to reflect the changes in the expression pattern of the TJ proteins in the mucosal surface layer. Histologically, our study neither detected mucosal destruction nor immune cell infiltration; moreover, the mRNA levels of the proinflammatory cytokines were not increased in the colons of HFD-fed mice. In addition, the level of sXBP-1 mRNA in HFD-fed mice was similar to that in the NC-fed mice. According to

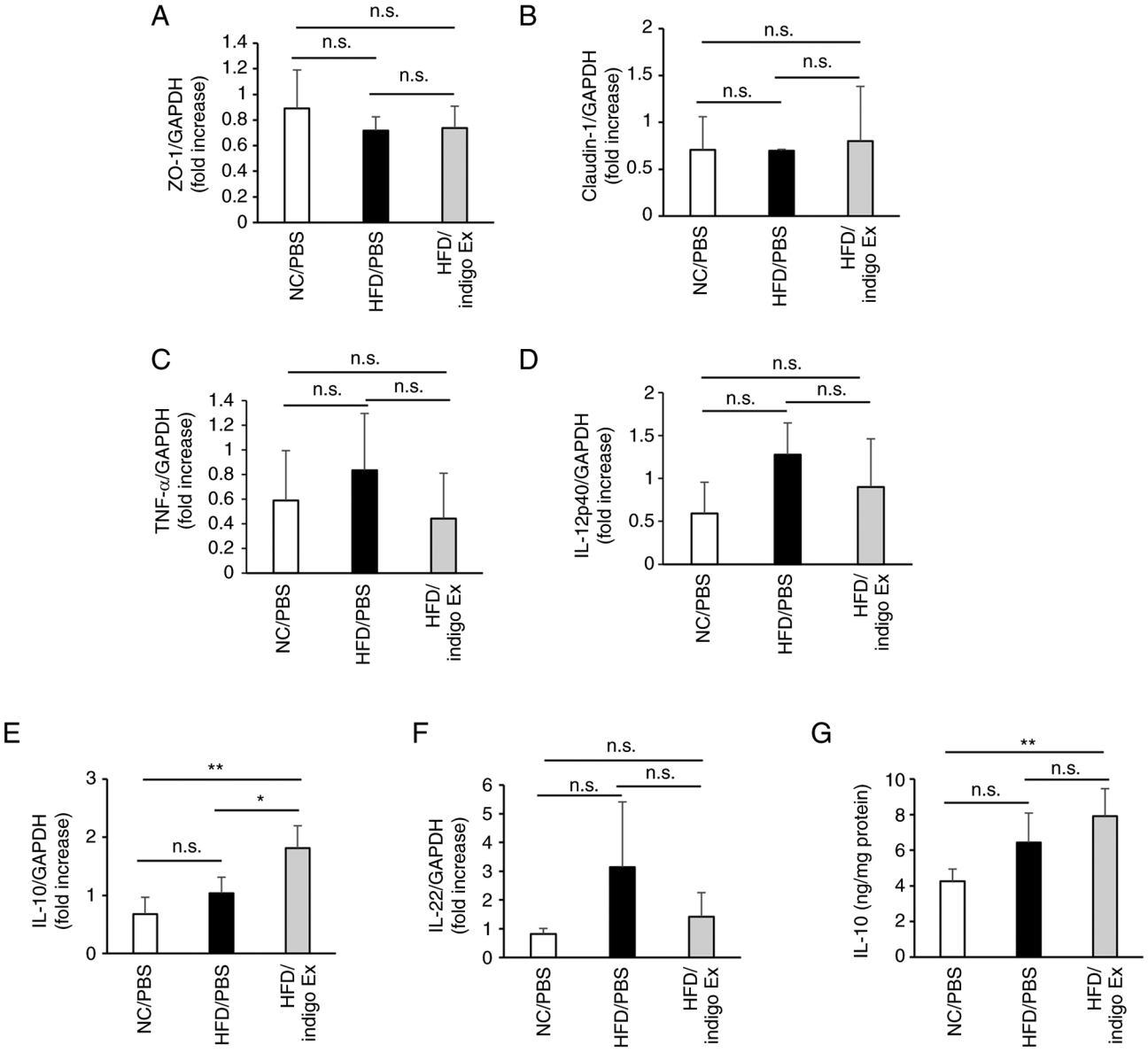

Figure 3. Diagrammatic representations of RT-qPCR performed to analyze the mRNA levels of (A) ZO-1, (B) Claudin-1, (C) TNF-α, (D) IL-12p40, (E) IL-10 and (F) IL-22. Data are presented as mean ± SD, n=3-5 in eac h group. **P<0.01, *P<0.05; n.s., not statistically significant. (G) The protein level of IL-10 in murine colon tissues was determined by ELISA. The results are presented as mean ± SD, n=5 in each group. **P<0.01; n.s., not statistically significant. NC, normal chow; HFD, high-fat diet; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; ZO-1, zonula occludens-1; TNF, tumor necrosis factor; IL, interleukin.

previous reports, ER stress occurs at least 11 weeks after HFD consumption (17,20). Our results indicated that indigo Ex exerts a protective effect independently of intestinal inflammation or ER stress. Further studies are however required to determine the precise mechanisms through which indigo Ex exerts a protective effect on epithelial cells.

Indigo Ex increased the mRNA levels of IL-10, but not IL-22. Using chemical compounds, indigo is known to act as an aryl hydrocarbon receptor (AhR) agonist and induce IL-22 expression, leading to the improvement of intestinal barrier function under HFD-fed conditions (27). Our results showed that indigo Ex did not induce IL-22 mRNA expression, suggesting that the protective effects of indigo Ex were not mediated by IL-22. Some beneficial effects of indigo leaves may be unrelated to AhR signaling activation. Previously, we reported that *P. tinctorium* leaves improved murine colitis independently of AhR (14). 3,5,4'-trihydroxy-6,7-methylenedioxyflavone-*O*-glycosides and their aglycones, which are present in *P. tinctorium*, have been reported to suppress experimental colitis by inducing IL-10 (28). IL-10 plays a key role in decreasing epithelial stress and enhancing TJ integrity (23,29). Our results revealed that the colon IL-10 level was enhanced in the HFD/indigo Ex group mice, while there was no significant difference between HFD/PBS and HFD/indigo Ex groups. We speculate that the induction of IL-10 is modestly associated with the effects of indigo Ex in HFD-fed mice. In the present study, we were unable to identify the cellular source of IL-10 and the extract components responsible for the IL-10 increase. These important issues need to be addressed in future research.

A growing body of evidence suggests that commensal microbes influence the epithelial expression of TJ-associated

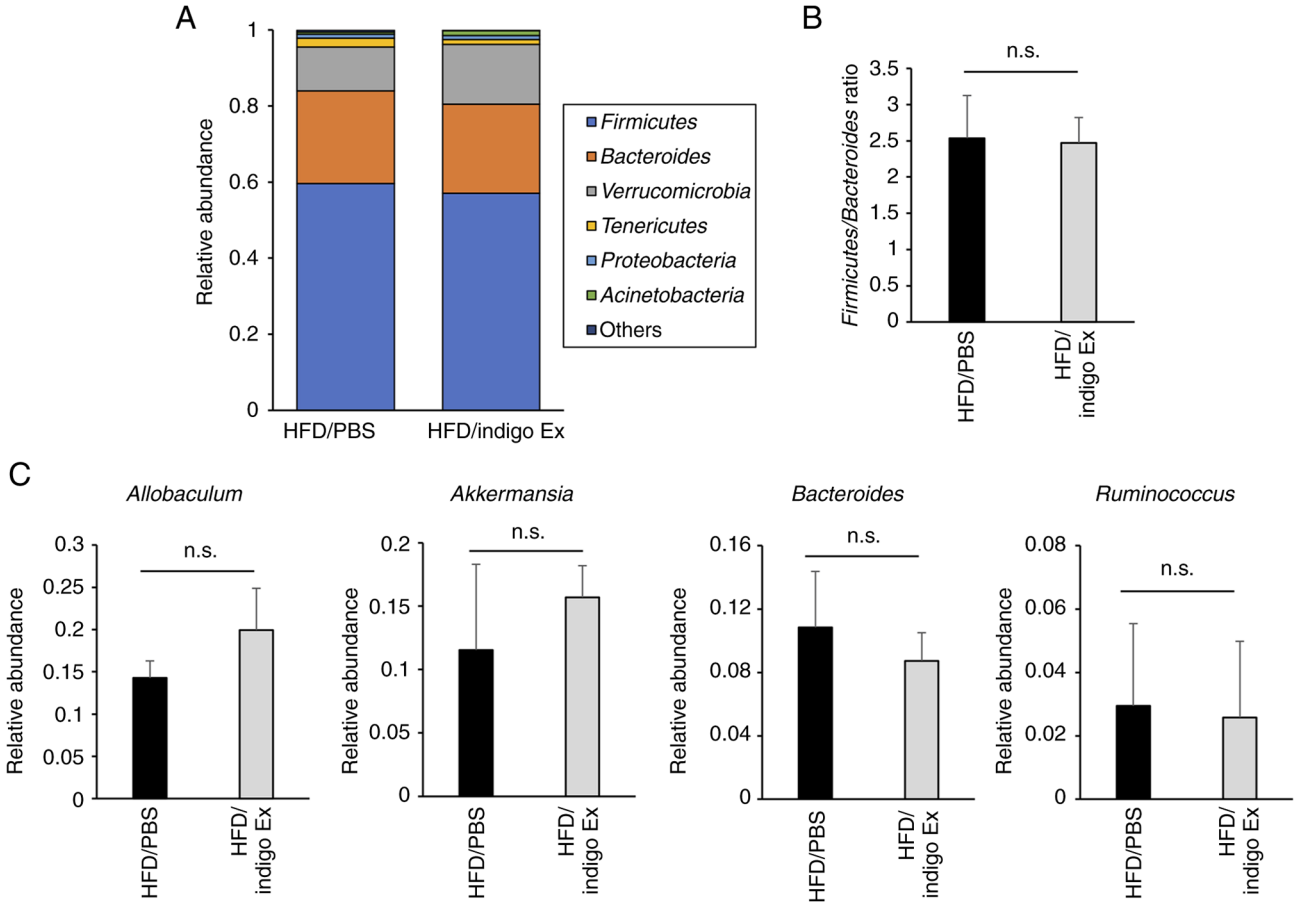

Figure 4. Results for 16S rRNA gene sequencing performed on fecal samples collected from mice of HFD/PBS and HFD/indigo Ex groups are presented as mean ± SD, n=4 in each group. (A) Relative abundance of microbiota at the phylum level. (B) Firmicutes/Bacteroides ratio. (C) Relative abundance of *Allobaculum*, *Akkermansia*, *Bacteroides* and *Ruminococcus*. HFD, high-fat diet; PBS, phosphate-buffered saline; n.s. not significant.

proteins. Dietary fat-induced alterations in the gut microbiota structure lead to epithelial cell dysfunction. Specifically, HFD decreases the abundance of barrier-promoting microbes, such as *Lactobacillus* spp., *Bifidobacterium* spp., and *Akkermansia muciniphila* (6). In addition, it is well-known that HFD leads to an increase in the *Firmicutes*/*Bacteroidetes* ratio (30,31). Our findings demonstrated that indigo Ex had little effect on the gut microbial composition at 4 weeks after HFD consumption, suggesting that the protective effects of indigo Ex are not mediated by alterations in the gut microbiota structure.

Moreover, we could not confirm the prophylactic effects of indigo Ex on obesity itself. Presumably, the 4-week treatment period was too short to evaluate the effects of indigo Ex on obesity-associated metabolic disorders, and long-term treatment may be required. Nevertheless, indigo Ex may prevent the exacerbation of metabolic inflammation, along with exerting favorable effects on the metabolic profile.

In conclusion, *P. tinctorium* leaf extract increased the number of goblet cells and ameliorated the redistribution of TJ proteins in the colon mucosa of HFD-fed mice, thereby preventing the HFD-induced intestinal damage. *P. tinctorium* leaf extract may contain natural compounds for treating obesity-associated intestinal damage, and could be developed as a potential therapeutic agent for intestinal barrier dysfunction and metabolic inflammation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The metagenome data are available in the DDBJ Sequenced Read Archive using the accession numbers DRR433110-DRR433117 (https://ddbj.nig.ac.jp/resource/bioproject/PRJDB15098).

Authors' contributions

SK, HS, HK, KM and KSa conceived and designed the study. SK and YH performed the experiments and wrote the original manuscript. JD, YT, KSe, TM, HH, SF and TI performed the data analyses and revised the manuscript. HS and HK confirm the authenticity of the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This study design was approved by the Animal Research Committee of Hirosaki University (approval no. M20016).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Garcia-Hernandez V, Quiros M and Nusrat A: Intestinal epithelial claudins: Expression and regulation in homeostasis and inflammation. Ann NY Acad Sci 1397: 66-79, 2017.
2. Chekakkot C, Ghim J and Ryu SH: Mechanisms regulating intestinal barrier integrity and its pathological implications. Exp Mol Med 50: 1-9, 2018.
3. Suzuki T: Regulation of intestinal epithelial permeability by tight junctions. Cell Mol Life Sci 70: 631-659, 2013.
4. Massier L, Blüher M, Kovacs P and Chakaroun RM: Impaired intestinal barrier and tissue bacteria: Pathomechanisms for metabolic diseases. Front Endocrinol (Lausanne) 12: 616506, 2021.
5. Forgie AJ, Fouhse JM and Willing BP: Diet-microbe-host interactions that affect gut mucosal integrity and infection resistance. Front Immunol 10: 1802, 2019.
6. Rohr MW, Narasimhulu CA, Rudeski-Rohr TA and Parthasarathy S: Negative effects of a high-fat diet on intestinal permeability: A review. Adv Nutr 11: 77-91, 2020.
7. Martinez KB, Leone V and Chang EB: Western diets, gut dysbiosis, and metabolic diseases: Are they linked? Gut Microbes 8: 130-142, 2017.
8. Carmody RN, Gerber GK, Luevano JM Jr, Gatti DM, Somes L, Svenson KL and Turnbaugh PJ: Diet dominates host genotype in shaping the murine gut microbiota. Cell Host Microbe 17: 72-84, 2015.
9. Mouries J, Brescia P, Silvestri A, Spadoni I, Sorribas M, Wiest R, Mileti E, Galbiati M, Invernizzi P, Adorini L, *et al*: Microbiota-driven gut vascular barrier disruption is a prerequisite for non-alcoholic steatohepatitis development. J Hepatol 71: 1216-1228, 2019.
10. Tanaka S, Nemoto Y, Takei Y, Morikawa R, Oshima S, Nagaishi T, Okamoto R, Tsuchiya K, Nakamura T, Stutte S and Watanabe M: High-fat diet-derived free fatty acids impair the intestinal immune system and increase sensitivity to intestinal epithelial damage. Biochem Biophys Res Commun 522: 971-977, 2020.
11. Cani PD, Bibiloni R, Knauf C, Waget A, Neurinck AM, Delzenne NM and Burcelin R: Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes 57: 1470-1480, 2008.
12. Kataoka M, Hirata K, Kunikata T, Ushio S, Iwaki K, Ohashi K, Ikeda M and Kurimoto M: Antibacterial action of tryptanthrin and kaempferol, isolated from the indigo plant (*Polygonum tinctorium* Lour.), against Helicobacter pylori-infected Mongolian gerbils. J Gastroenterol 36: 5-9, 2001.
13. Hoessel R, Leclerc S, Endicott JA, Nobel ME, Lawrie A, Tunnah P, Leost M, Damiens E, Marie D, Marko D, *et al*: Indirubin, the active constituent of a Chinese antileukaemia medicine, inhibits cyclin-dependent kinases. Nat Cell Biol 1: 60-67, 1999.
14. Asari T, Kikuchi H, Kawaguchi S, Sakuraba H, Yoshida S, Akemoto Y, Maeda T, Shinji O, Murai Y, Higuchi N, *et al*: *Polygonum tinctorium* leaves suppress sodium dextran sulfate-induced colitis through interleukin-10-related pathway. Biochem Biophys Rep 30: 101272, 2020.
15. Hagiyama M, Takeuchi F, Sugano A, Yoneshige A, Inoue T, Wada A, Kajiyama H, Takaoka Y, Sasaki K and Ito A: Indigo plant leaf extract inhibits the binding of SARS-CoV-2 spike protein to angiotensin-converting enzyme 2. Exp Ther Med 23: 274, 2022.
16. Schneider CA, Rasband WS and Eliceiri KW: NIH image to ImageJ: 25 Years of image analysis. Nat Methods 9: 671-675, 2012.
17. Gulhane M, Murray L, Lourie R, Tong H, Sheng YH, Wang R, Kang A, Schreiber V, Wong KY, Magor G, *et al*: High fat diets induce colonic epithelial cell stress and inflammation that is reversed by IL-22. Sci Rep 6: 28990, 2016.
18. Heazlewood CK, Cook MC, Eri R, Price GR, Tauro SB, Taupin D, Thornton DJ, Png CW, Crockford TL, Cornall RJ, *et al*: Aberrant mucin assembly in mice causes endoplasmic reticulum stress and spontaneous inflammation resembling ulcerative colitis. PLoS Med 5: e54, 2008.
19. Birchenough GM, Johansson ME, Gustafsson JK, Bergström JH and Hannson GC: New developments in goblet cell mucus secretion and function. Mucosal Immunol 8: 712-719, 2015.
20. Dai YJ, Liu WB, Abasubong KP, Zhang DD, Li XF, Xiao K, Wang X and Jiang GZ: The mechanism of lipopolysaccharide escaping the intestinal barrier in Megalobrama amblycephala fed a high-fat diet. Front sNutr 9: 853409, 2022.
21. Ghezzal S, Postal BG, Quevrain E, Brot L, Seksik P, Leturque A, Thenet S and Carrière V: Palmitic acid damages gut epithelium integrity and initiates inflammatory cytokine production. Biochim Biophys Acta Mol Cell Biol Lipids 1865: 158530, 2020.
22. Nascimento JC, Matheus VA, Oliveira RB, Tada SFS and Collares-Buzato CB: High-fat diet induces disruption of the tight junction-mediated paracellular barrier in the proximal small intestine before the onset of type 2 diabetes and endotoxemia. Dig Did Sci 66: 3359-3374, 2021.
23. Capaldo CT and Nusrat A: Cytokine regulation of tight junctions. Biochim Biophys Acta 1788: 864-871, 2009.
24. Escoula Q, Bellenger S, Narce M and Bellenger J: Docosahexaenoic and eicosapentaenoic acids prevent altered-Muc2 secretion induced by palmitic acid by alleviating endoplasmic reticulum stress in LS174T goblet cells. Nutrients 11: 2179, 2019.
25. Reiche J and Huber O: Post-translational modifications of tight junction transmembrane proteins and their direct effect on barrier function. Biochim Biophys Acta Biomembr 1862: 183330, 2020.
26. Butt AM, Khan IB, Hussain M, Idress M, Lu J and Tong Y: Role of post translational modifications and novel crosstalk between phosphorylation and O-beta-GlcNAc modifications in human claudin-1, -3 and -4. Mol Biol Rep 39: 1359-1369, 2012.
27. Lin YH, Luck H, Khan S, Schneeberger PHH, Tsai S, Clemente-Casares X, Lei H, Leu YL, Chan YT, Chen HY, *et al*: Aryl hydrocarbon receptor agonist indigo protects against obesity-related insulin resistance through modulation of intestinal and metabolic tissue immunity. Int J Obes (Lond) 43: 2407-2421, 2019.
28. Kimura H, Tokuyama-Nakai S, Hirabayashi Y, Ishihara T, Jisaka M and Yokota K: Anti-inflammatory and bioavailability studies on dietary 3,5,4'-trihydroxy-6,7-methylenedioxyflavone-O-glycosides and their aglycone from indigo leaves in a murine model of inflammatory bowel disease. J Pharm Biomed Anal 193: 113716, 2021.
29. Hasnain SZ, Tauro S, Das I, Tong H, Chen AC, Jeffery PL, McDonald V, Florin TH and McGuckin MA: IL-10 promotes production of intestinal mucus by suppressing protein misfolding and endoplasmic reticulum stress in goblet cells. Gastroenterol 144: 357-368.e9, 2013.
30. Hildebrandt MA, Hoffman C, Sherrill-Mix SA, Keilbaugh SA, Hamady M, Chen YY, Knight R, Ahima RS, Bushman F and Wu GD: High-fat diet determines the composition of the murine gut microbiome independently of obesity. Gastroenterol 137: 1716-1724.e1-e2, 2009.
31. Murphy EA, Velazquez KT and Herbert KM: Influence of high-fat diet on gut microbiota: A driving force for chronic disease risk. Curr Opin Clin Nutr Metab Care 18: 515-520, 2015.