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Novel role of hesperidin improve obesity in HFD mice by modulating the composition of the gut microbiota

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Abstract

Background: Hesperidin is a plant-derived dihydroflavone derivative with multiple pharmacological functions. Obesity is associated with low-grade chronic inflammation and intestinal dysbiosis. We examined the possibility that hesperidin may prevent diet-induced obesity by modulating the composition of the gut microbiota. High-fat diet (HFD)-fed mice were treated with hesperidin. Its effects on the gut microbiota were assessed by horizontal faecal microbiota transplantation (FMT) and 16S rRNA gene amplicon sequencing-based microbiota analysis. Results: Gut microbiota analysis revealed that hesperidin selectively promoted the growth of beneficial Lactobacillus salivarius and harmful Staphylococcus sciuri and Desulfovibrio C21_c20 and inhibited that of beneficial Bifidobacterium pseudolongum and Mucispirillumschaedleri and harmful Helicobacter ganmani and Helicobacter hepaticus . However, hesperidin reversed obesity and inflammation and improved gut integrity in HFD-fed mice. The anti-obesity effects and hesperidin-modulated Lactobacillus salivarius, Desulfovibrio C21_c20, Mucispirillumschaedleri and Helicobacter hepaticus were transmissible via horizontal faeces transfer from hesperidin-treated mice to HFD-fed mice. Conclusions: Hesperidin plays a dual role in both beneficial and harmful microbes. However, its overall effects reduce body weight and reverse HFD-related disorders in HFD-fed mice.

Background

Obesity is a disease condition considered to be associated with a high risk of numerous health problems. The increasing prevalence of obesity has become a major threat to public health, and addressing obesity is therefore a critical challenge in modern societies [1]. Obesity is characterized by fat mass accumulation, chronic subclinical inflammation and an imbalanced gut microbiota. Unfit life styles (especially the consumption of high-fat diets and inadequate exercise), neuronal and hormonal factors, and genetic and epigenetic mechanisms all contribute to obesity development [2]. The gut microbiota plays a role in obesity development [3].

A number of bioactive chemicals have been reported to alleviate disease symptoms by modulating the gut microbiota. Hesperidin is a flavanone glycoside (a subclass of flavonoids) that shows high levels in citrus fruits and quite a few vegetables [4]. Previous studies have shown that hesperidin exhibits various biological activities, including vitamin-like activity and antioxidant, anti-inflammatory, anticarcinogenic, anti-hyperglycaemic, anti-hypolipidaemic and antiallergic properties [4-6]. Although a large number of studies have been published describing the novel pharmacological activities, molecular targets and mechanisms of action of hesperidin, none have reported its effects on HFD-induced obesity or the gut microbiota.

The gut microbiota is a potential target through which hesperidin may intervene in HFD-induced obesity. In the present study, we examined whether hesperidin can decrease obesity in HFD-fed mice. Our results indicate that hesperidin reduces obesity and inflammation, improves gut integrity and modulated several gut microbiota species in HFD-fed mice. The anti-obesity effects and most of the hesperidin-modulated

gut microbiota species were transmissible through horizontal faecal transplantation. Our data demonstrated that hesperidin plays a role in reducing body weight and reversing HFD-related disorders in HFD-fed mice by enriching beneficial and inhibiting harmful microbes.

Results

Hesperidin prevents HFD-induced obesity in mice.

HFD feeding for 10 weeks led to significant increases in body weight, epididymal and visceral fat accumulation, plasma total cholesterol, triglycerides, high-density lipoprotein, and low-density lipoprotein and a slight increase in liver weight **(Figure 1A–1I)**. Hesperidin (2%) did not produce any significant effects in the normal diet-fed mice except for a decrease in plasma low-density lipoprotein **(Figure 1A–1I)**. Supplementation with hesperidin decreased weight gain, fat accumulation and plasma lipids in a dose-dependent manner in HFD-fed mice **(Figure 1A–1I)**. The effects of hesperidin on body weight and obesity parameters were not due to reduced food consumption or energy extraction according to our weekly feeding records. These results implied that hesperidin reduced weight gain, fat accumulation and plasma lipids in HFD-fed mice.

Hesperidin reduces inflammation in HFD-fed mice.

Studies have shown that obesity is characterized by low-grade inflammation with increases in pro-inflammatory cytokines including tumour necrosis factor-alpha (TNF-α), interleukin-1-beta (IL-1β), and interleukin-6 (IL-6) [7]. We measured the plasma levels of the TNF-α and IL-6 proteins and the colonic messenger RNA (mRNA) expression levels of these cytokines after 10 weeks of HFD feeding with or without hesperidin supplementation. IL-1β, TNF-α and IL-6 levels were higher in the plasma and colons of HFD-fed mice than in those of normal diet-fed mice **(Figure 2A–E)**. The expression level of these cytokines was reduced in a dose-dependent manner by hesperidin treatment **(Figure 2A–E)**. Inducible NO synthase (iNOS) is a key pro-inflammatory mediator. iNOS mRNA expression increased in the colons of HFD-fed mice compared to ND-fed mice but decreased following treatment with hesperidin **(Figure 2F)**. These results indicate that hesperidin reduced inflammation in HFD-fed mice.

Hesperidin maintains intestinal integrity in HFD mice.

Previous studies have shown that gut microbiota dysbiosis caused by an HFD increases gut permeability and subsequently results in the release of bacterial endotoxin into the circulation [8]. We examined the effects of hesperidin on gut integrity. Colon length, lipid binding protein (LBP) and intestinal fatty acid binding protein (iFABP) are key markers of intestinal integrity; muc2 is an important indicator of gut barrier function; and claudin 2, occludin and zonula occludens-1 (ZO-1) are three main tight junction components. HFD feeding reduced colon length and the expression of the tight junction components and increased plasma LBP and iFABP levels, and all of these effects were reversed by hesperidin supplementation **(Figure 3A-3G)**. These results suggested that hesperidin improved intestinal barrier integrity in HFD-fed mice.

Hesperidin reverses HFD-induced gut dysbiosis to some extent.

The gut microbiota of obese humans and HFD-fed mice is characterized by an increased Firmicutes-to-Bacteroidetes ratio, elevated levels of endotoxin-producing Proteobacteria, and reduced levels of immune-homeostatic *Akkermansia muciniphila* [9 10]. We examined the effects of hesperidin on the gut microbiota composition by performing a pyrosequencing-based analysis of bacterial 16S rRNA (V3–V4 region) in caecal faeces. A total of 36 211 258 effective reads were obtained from all faecal samples. Based on the 99% similarity level, the reads were clustered into 343273 OTUs. HFD feeding reduced the number of OTUs compared to that in ND-fed mice. Hesperidin reversed the HFD-induced OTU decrease in a dose-dependent manner **(Figure S1A)**. Microbiota richness and evenness were increased by hesperidin, as indicated by α-diversity analysis **(Figure S1B)**. Unifrac-based principal coordinates analysis (PCoA) showed a distinct clustering of the microbiota composition for each treatment group **(Figure S1C)**. Hesperidin also decreased the Firmicutes-to-Bacteroidetes ratio **(Figure S1D)**.

The OTUs could be annotated to 8 phyla, 13 classes, 15 orders, 22 families, 29 genera and 19 species **(Figure 4)**. We detected 8 species that were significantly different between ND-fed and HFD-fed mice. Four of these species decreased in HFD-fed mice, including *Lactobacillus salivarius* in the Firmicutes phylum, Bacilli class, Lactobacillales order, Lactobacillaceae family; *Staphylococcus sciuri* in the Firmicutes phylum, Bacilli class, Bacillales order, Staphylococcaceae family; *Desulfovibrio C21_c20*in the Proteobacteria phylum, Deltaproteobacteria class, Desulfovibrionales order, Desulfovibrionaceae family and *Akkermansiamuciniphila* in the Verrucomicrobia phylum, Verrucomicrobiae class, Verrucomicrobiales order, and Verrucomicrobiaceae family. The other four were increased in HFD-fed mice, which included *Helicobacter ganmani*and *Helicobacter hepaticus* in the Proteobacteria phylum, Epsilonproteobacteria class, Campylobacterales order, Helicobacteraceae family; *Bifidobacterium pseudolongum* in the Actinobacteria phylum, Actinobacteria class, Bifidobacteriales order, Bifidobacteriaceae familyand*Mucispirillumschaedleri* in the Deferribacteres phylum, Deferribacteres class, Deferribacterales order, and Deferribacteraceae family. A closer look at the microbial community revealed the specific influence of hesperidin from the phylum to species levels. *Lactobacillus salivarius*, *Staphylococcus sciuri* and *Desulfovibrio _C21_c20* were enriched in the hesperidin-supplemented HFD-fed mice **(Figure 4, Figure S2A-2C)**; *Helicobacter ganmani*, *Helicobacter hepaticus*, *Bifidobacterium pseudolongum* and*Mucispirillumschaedleri* were decreased in the hesperidin-supplemented HFD-fed mice **(Figure 4, Figure S2D-2G)**. *Akkermansiamuciniphila* was not altered by hesperidin **(Figure 4, Figure S2H**). These results implied that hesperidin modified the composition of the gut microbiota and partially reversed HFD-induced gut dysbiosis.

The beneficial effects of hesperidin are transferable by faecal transplantation.

It has been reported that diet-induced obesity and associated metabolic disorders may be caused by the gut microbiota [3]. The anti-obesogenic effects of some Chinese herbs, such as polysaccharides from *Ganoderma lucidum* and *Hirsutellasinensis*, are mediated by the gut microbiota [11 12]. We tested whether the beneficial effects of hesperidin may also be mediated by the gut microbiota. Faecal microbiota from

ND-fed mice treated with saline and hesperidin were transplanted into HFD-fed recipients. To further confirm that our method of FMT is effective, one additional control was conducted: faecal microbiota from HFD-fed mice treated with saline were transplanted into ND-fed recipients **(Figure 5A)**. FMT from HFD-fed mice increased obesity traits, inflammation and gut integrity in ND recipients, although the changes in most of the indicators were not significant **(Figure 5B-5G, Figure 6A-6F, Figure 7A-7G)**. In contrast, FMT from ND-fed groups reduced obesity traits, inflammation and gut integrity in HFD recipients compared with the controls **(Figure 5B-G, Figure 6A-F, Figure 7A-G)**. Furthermore, FMT from hesperidin-treated ND-fed groups had more significant effects on reducing obesity traits, inflammation and gut integrity in HFD recipients **(Figure 5B-G, Figure 6A-F, Figure 7A-G)**. These results proved that the gut microbiota mediates the beneficial effects of hesperidin.

FMT transmits specific intestinal microbial taxa.

To examine whether the beneficial effects of hesperidin result from the specific microbes that it regulates and whether the modified microbiota can be transmitted to recipients by FMT, we sequenced the gut microbiota after FMT. FMT from hesperidin-treated ND-fed groups increased the number of OTUs in HFD recipients **(Figure S2A)**. FMT increased the total species diversity, as indicated by the Chao1 value, but decreased the richness and evenness of the main microbiota, as shown by the Shannon and Simpson values **(Figure S2B)**. UniFrac-based principal coordinates analysis (PCoA) showed distinct clustering of the microbiota composition for each treatment group **(Figure S2C)**. FMT failed to reverse the HFD-induced increase in the Firmicutes-to-Bacteroidetes ratio **(Figure S2D)**. Among the seven microbiomes specifically regulated by hesperidin, *Lactobacillus salivarius, Staphylococcus sciuri, Desulfovibrio C21_c20, Mucispirillumschaedleri* and *Helicobacter hepaticus* were transmitted from donor to recipient mice **(Figure 8, Figure S4A, 4B, 4C, 4E, 4G)**, while *Helicobacter ganmani* and *Bifidobacterium pseudolongum*were not **(Figure 8, Figure S4D, 4F)**. *Akkermansiamuciniphila* also failed to be transmitted from ND-fed donors to HFD-fed recipient mice **(Figure 8, Figure S4H)**.

Discussion

Polyphenols have been reported to modulate metabolism and/or inflammation related to obesity [13]. As a bioactive chemical belonging to the polyphenols, hesperidin has been extensively studied for its effects on cancer and cardiovascular diseases but not obesity [4 5]. In vitro studies have indicated that citrus polyphenols, including hesperidin, cause a reduction in adipocyte differentiation, the lipid content in the cell and adipocyte apoptosis, revealing a positive role in the management of obesity [14]. The evidence from animal studies has not been entirely consistent, but most of these studies indicate a reduction in adipose tissue, increased gene expression resulting in the stimulation of β-oxidation, improvement of the lipid profile and glycaemia as well as an improved inflammatory status [15]. Our experiments on HFD-fed mice also indicated a reduction in adipose tissue and improvements of the lipid profile and inflammatory status. Moreover, hesperidin improved intestinal barrier function in HFD-fed mice. The role of hesperidin in decreasing intestinal inflammation and restoring intestinal barrier function has also been proven in

DSS-induced colitis mice [16]. However, solid clinical evidence is very limited. A systematic review and meta-analysis concluded that hesperidin might not affect the lipid profile and blood pressure based on 10 randomized controlled clinical trials [17]. Therefore, well-designed trials in humans are still needed to confirm the anti-obesity effects of hesperidin.

The trillions of microbes in the gut microbiota play important roles in ingesta digestion, immunity regulation and energy equilibrium. Innumerable studies have indicated that changes in the composition of the gut microbiota are related to the development of various diseases, including obesity [18]. Lower diversity and richness, an increased ratio of the major phyla Firmicutes/Bacteroidetes and changes in several bacterial species are common characteristics of both obese mice and human faecal samples [10]. Moreover, obese animals with gut dysbiosis exhibit impaired intestinal integrity [19].

The gut microbiota, which exhibits 10 times the number of human cells in an individual and 150 times the number of genes of the human genome, is considered a “hidden organ” [20]. New findings from this field and their importance for human health have opened up a new frontier for understanding the occurrence and development of various diseases as well as the mechanisms of drugs, traditional herb medicines, bioactive chemicals and functional foods [21 22]. In obesity, research has shown that the gut microbiota of obese humans and HFD-fed animals differ from those of lean and ND-fed animals, and studies on obese subjects or those undergoing weight loss have revealed numerous microbes from the phylum to species levels that are positively or negatively associated with obesity. However, there have been few consistent conclusions about the characteristics of the obese microbiota profile except for an increased Firmicutes-to-Bacteroidetes ratio and elevated relative abundance of *Akkermansiamuciniphila*. [23]

Different studies identified different obesity-related microbes, probably because of differences in the experimental organisms and background diets since the gut microbiota differs between species, and diet is the most important factor shaping it [24 25]. Second, not all microbes within a lower taxon, such as a genus or species, play the same role within a given higher taxon, such as a phylum. Different bacterial species present different characteristics, which may be related to beneficial or harmful traits. For example, Lactobacillus species such as *Lactobacillus plantarum* and *paracasei* have been associated with thinness, while species such as *Lactobacillus reuteri* have been associated with obesity.[26] These findings suggest that the specific physiological effects of microbes are dependent on the strain. Therefore, it may be inaccurate to conclude that traits are related to microbe taxa higher than the species or strain level. Third, as members of a complicated ecological system, one microbe may be regulated by another according to specific physiological conditions. For example, both *Bifidobacterium pseudolongum* and *Akkermansiamuciniphila* are beneficial microbes; however, rats fed with the *Bifidobacterium pseudolongum*strain Patronus exhibit a large increase in mucus thickness associated with a decrease in *Akkermansiamuciniphila,* which loses to bifidobacteria in the competition for the mucus niche [27]. Therefore, when a trait is reported to be related to a specific microbe, it is under specific physiological conditions with specific microbial community characteristics. Fourth, experimental procedures including

sampling, sequencing and bioinformatic analysis may also contribute to the inconsistency between reports.

In the present study, using 16S rRNA sequencing, we detected 8 species that were significantly different between ND-fed and HFD-fed mice. *Desulfovibrio C21-c20* has been reported to be positively related to cisplatin-induced mucositis in male Wistar rats [28] and negatively related to the antihyperlipidaemic effects of *Rhizomacoptidis* alkaloids in high-fat and high-cholesterol-induced hyperlipidaemic B6 mice [29]. *Staphylococcus sciuri* has been reported as a human opportunistic pathogen in nosocomial diseases and related infections [30]. *Helicobacter hepaticus* is a pathogen that can cause typhlitis, colitis, and hepatitis [31]. *Helicobacter ganmani* may also be a pathogen since *H. ganmani* infection is associated with a significant increase in the expression of the pro-inflammatory cytokine IL12/23p40 in IL10-deficient mice [31]. These four microbes are considered harmful microbes. *Lactobacillus salivarius* is a promising probiotic since it has been reported to possess the ability to enhance of the immune system and attentuate gut inflammation and to exert antimicrobial activity against pathogenic bacteria such as *Staphylococcus aureus* [32 33]*. Akkermansiamuciniphila* has been proven to presenta negative correlation with overweight, obesity, untreated type 2 diabetes mellitus and hypertension, and beneficial effects of this species on obesity have been reported in a clinical trial.[34 35] *Bifidobacterium pseudolongum*is a beneficial microbe that plays a role in protecting the gut barrier function; however, it competes for the mucus niche with *Akkermansiamuciniphila*. *Mucispirillum schaedleri* has been reported to confer protection against *Salmonella* colitis in mice by competing for anaerobic respiration substrates in the gut [36]. These four species are considered to be beneficial microbes.

In our study, we found that beneficial *Lactobacillus salivarius* and *Akkermansiamuciniphila* and harmful *Staphylococcus sciuri* and *Desulfovibrio C21_c20* were decreased in HFD-fed mice, while beneficial *Bifidobacterium pseudolongum* and *Mucispirillumschaedleri* and harmful *Helicobacter ganmani* and *Helicobacter hepaticus* were increased in HFD-fed mice compared to ND-fed controls. Beneficial *Lactobacillus salivarius* and harmful *Staphylococcus sciuri* and *Desulfovibrio C21_c20* were enriched in the hesperidin-supplemented HFD-fed mice **(Figure 4, Figure S2A-2C)**, while beneficial *Bifidobacterium pseudolongum*and *Mucispirillumschaedleri* and harmful *Helicobacter ganmani* and *Helicobacter hepaticus* were decreased in the hesperidin-supplemented HFD-fed mice **(Figure 4, Figure S2D-2G)**. Beneficial *Akkermansiamuciniphila* was unchanged by hesperidin **(Figure 4, Figure S2H)**. These results implied that the HFD did not enrich all harmful microbes or inhibit all beneficial microbes and that hesperidin did not enrich all beneficial microbes or inhibit all harmful microbes. This dual role of hesperidin inboth beneficial and harmful microbes may explain the unstable and inconsistent anti-obesity results obtained in animal and clinical tests.

To further study the causal relationship between the gut microbiota and disease, FMT (faecalmicrobiota transplantation) is usually conducted. Human faecal microbiota transplants from obese twins to germ-free mice resulted in an increase in body fat compared to mice receiving FMT from lean twins, which proved that the gut microbiota could be the cause of obesity [37]. FMT is also one way to study the causal

relationship between the gut microbiota and the effects of drugs, traditional herbal medicines, bioactive chemicals and functional foods. The traditional Chinese medicine *Ganoderma lucidum mycelium* has been reported to reduce body weight, inflammation and insulin resistance in HFD-fed mice. Faecal microbiota transplants from mice treated with water extracts of *Ganoderma lucidum mycelium* to HFD-fed mice also transmitted the anti-obesity effects, which proved that the anti-obesity effects of *Ganoderma lucidum mycelium*are mediated by the gut microbiota [11]. Our research also indicated that FMT transmitted donor traits to the receptors, which provided additional evidence of the gut microbiota-mediated effects of bioactive chemicals and FMT as an effective therapy for diseases.

Interestingly, although FMT from healthy donors often brings about good results in recipients, comparison of the recipient's gut microbiota before and after FMT and the evaluation of its similarity to the donors' gut microbiota showed that the recipient's gut microbiota was obviously changed by FMT; however, the similarity of the recipient's gut microbiota after FMT to the donor's was not as high as expected, especially when the recipients were not germ-free subjects, such as humans or SPF animals [38]. This may be attributed to the colonization ability of the microbes; some may easily win out in competition with intrinsic microbes or colonize the receipts' gut, and some may exhibit difficulty in winning an ecological niche. The colonization ability of gut microbes has not been well studied and is worthy of additional investigation in the exploration of the mechanism of the beneficial effects of FMT therapy for various health problems.

In our study, close consideration of the specific microbes that are transmitted to recipients by FMT revealed that *Lactobacillus salivarius,Staphylococcus sciuri, Desulfovibrio C21_c20, Mucispirillumschaedleri* and *Helicobacter hepaticus* can be transmitted from donor to recipient mice **(Figure 8, Figure S4 A, B, C, E, G)**, while *Helicobacter ganmani, Bifidobacterium pseudolongum*and *Akkermansiamuciniphila* cannot **(Figure 8, Figure S4D, F, H)**. *Akkermansiamuciniphila* is known to colonize the mucosa layer, and *Bifidobacterium pseudolongum* shows strong adhesion to porcine colonic mucin [39]. It may be more difficult for these species to replace the original microbes that occupy the mucus niche.

Since there are thousands of species in the gut microecosystem, scientists believe there are key players among the gut microbiota and have been screening the driving species that contribute to the development of disease and the beneficial effects of intervention [40]. It is a common goal for scientists in this field to find the key players and genetic or environmental factors that regulate key microbes. For example, *Parabacteroides goldsteinii*was found to be enriched by *Hirsutella sinensis* mycelium, which produces anti-obesogenic and antidiabetic effects in obese mice. Thus, the oral treatment of obese mice with live *P. goldsteinii* bacteria produces anti-obesogenic and antidiabetic effects [12]. Therefore, *P. goldsteinii*isthe key microbe that contributes to the beneficial effects of *Hirsutellasinensis* mycelium. Furthermore, the impacts of microbes on the host rely mostly on their metabolites. *Lactobacillus, Bifidobacterium,* and *Faecalibacterium prausnitzii* are negatively correlated with cardiovascular disease and type 2 diabetes because they are SCFA-producing species [41 42]. When an *Enterobacter* strain

screened and isolated from a morbidly obese human was inoculated into germfree mice, it induced obesity and insulin resistance because it was an endotoxin-producing bacterium [43]. These insightful findings indicate the potential application of the gut microbiota and their metabolites as novel biomarkers for disease diagnosis and new probiotics for disease therapy. The species that were found to be significantly changed by HFD and hesperidin treatment in the screens performed in the present study, require further verification by live strain supplementation, especially for *Staphylococcus sciuri*, *Desulfovibrio C21_c20, Mucispirillumschaedleri, Helicobacter hepaticus* and *Helicobacter ganmani*, which have not been reported to be obesity related in the literature. Their metabolites and molecular mechanisms suggested to be related to obesity require further exploration.

Conclusions

In conclusion, our results indicated that hesperidin reduced obesity and inflammation, improved gut integrity and modified several gut microbiota species in HFD-fed mice. The anti-obesity effects and most of the hesperidin-modified gut microbiota species were transmissible through horizontal faecal transplantation. Our data demonstrated that hesperidin plays a role in the reduction of body weight and the reversal of HFD-related disorders in HFD-fed mice by enriching beneficial and inhibiting harmful microbes.

Materials And Methods

Murine.

Animal experiments were approved and performed in accordance with the guidelines of the Laboratory Animal Center of Guangzhou Medical University. Eight-week-old male C57BL/6 mice were purchased from Guangdong Medical Laboratory Animal Center (GDMLAC) and kept under controlled temperature and light conditions (25°C, 12 h light–dark cycle) with free access to food and water. The mice were randomly distributed into eight groups containing six animals each. The mice were housed in groups of three animals per cage and were fed either a normal diet (13.5% of energy from fat; D12450; GDMLAC, China) or a high-fat diet (40% of energy from fat; D12451; GDMLAC, China). The formula of the diet is shown in **Supplementary Table 1**. Each group of mice was fed a chow diet or HFD for 10 weeks with free access to either water or a saturated hesperidin (Aladdin, CAS#520-26-3) solution at 0.1 or 0.2% (w/v).

The mice were supplementedevery other day with sterile saline (vehicle)or hesperidin (100, 200 mg/kg BW) via intragastric gavage from the fifth week of feeding. Faecal microbiota transplantation (FMT) was initiated in the fifth week of feeding. At the tenth week, the animals were fasted for 12 h before being killed. Mice were deeply anaesthetized with a 1% pentobarbital sodium (50 mg/kg BW), and whole blood was withdrawn through the ventral aorta in tubes containing KEDTA anticoagulant. Visceral adipose tissues, epididymal white adipose tissue and the liver were removed and weighed. The colorectum was removed, and its length was measured. The faecal tissue in the caecum was squeezed out. All samples were immersed in liquid nitrogen and stored at −80°C for further analysis.

Faecal microbiota transplantation.

Stools from donor mice of each dietary group were collected under alaminar flow hood in sterile conditions, and 100 mg of the sample was suspended in 3 ml of sterile saline. The solution was vigorously mixed and centrifuged at 2000 g for 3 min. The deposit was resuspended in 3 ml of sterile saline and used as transplant material. Fresh transplant material was prepared on the same day as transplantation within 10 min before oral gavage (10 ml/kg BW) to prevent changes in the bacterial composition. Recipient mice were inoculated every other day with fresh transplant material via oral gavage for 6 weeks before being killed for subsequent analysis.

Measurement of plasma cytokines

Whole blood was withdrawn through the ventral aorta into tubes containing KEDTA anticoagulant. Blood was centrifuged at 500 g for 5 min, and the supernatants (plasma) were collected. Plasma interleukin (IL)-6, tumour necrosis factor-alpha (TNF-α), intestinal fatty acid binding protein (iFABP), and lipopolysaccharide-binding protein (LBP) were determined with commercial ELISA kits: mouse IL-6 high sensitivity ELISA kit (Cat# EK206HS-96, Multi Sciences, China), mouse TNF-α high sensitivity ELISA kit (Cat# EK282HS-96, Multi Sciences, China), mouse LBP ELISA kit (Cat# CSB-EL012775MO, CUSABIO biotech CO.,LTD,China), mouse iFABP ELISA kit (Cat# CSB-E08025m, CUSABIO biotech CO.,LTD,China), according to the manufacturer's instructions.

Measurement of plasma lipids

Whole blood was withdrawn through the ventral aorta into tubes containing KEDTA anticoagulant. The blood was centrifuged at 500 g for 5 min, and the supernatants (plasma) were collected. Total cholesterol (Tcho), triglyceride (Trig), low-density lipoprotein (LDL)and high-density lipoprotein (HDL) levels were determined with commercial ELISA kits: Cholesterol Gen 2 (Cat# 05168538190, Roche Diagnostics,USA), Triglycerides (Cat# 05171407190, Roche Diagnostics, USA), HDL-Cholesterol plus 3rd generation (Cat# 05168805190, Roche Diagnostics USA), and LDL-Cholesterol Gen 3 (Cat# 07005768 190, Roche Diagnostics, USA), according to the manufacturer's instructions.

Caecal microbiota analysis

Caecal microbiota DNA was extracted using a Stool DNAKit (Guangzhou IGE Biotechnology, China) and subjected to the amplification of the V3-V4 regions of 16S rRNA for gene amplicon sequencing. The caecal microbiota composition was assessed via Illumina 2500 sequencing of the 16S rRNA gene amplicon and QIIME-based microbiota analysis. High-quality reads for bioinformatics analysis were selected, and all of the effective reads from all samples were clustered into OTUs based on 99% sequence similarity according to Qiime Uclust. OTUs were annotated with RDP Classifier (Version 2.2) with a confidence cutoff of 0.8 according to the GreenGene database. Then, the composition and relative abundance information of each sample at different classification levels was statistically summarized.

Quantitative real-time reverse-transcription qRT-PCR.

Total RNA was isolated using a UNIQ-10 Column TRIzolTotal RNA Isolation Kit (Sangon Biotech, China). Equal amounts of total RNA were used to synthesize cDNA with the PrimeScript^{TM} RT reagent kit with gDNA Eraser (Cat# RR047A, TAKARA, Japan).qRT–PCR was performed in triplicate using TB Green^{TM} premix Ex Taq^{TM} II (Cat# RR820A, TAKARA, Japan), 96-well plates and a7500 Real-Time PCR System (Applied Biosystems). Each well was loaded with a total volume of 20 μl containing 2 μl of cDNA, 2μl of the target primers, 6 μl of water and 10 μl of TAKARATB green premix. Hot-start PCR was performed for 40 cycles, with each cycle consisting of denaturation for 15s at 95°C, annealing for 30 s at 60°C and elongation for 10s at 72°C. Applied Biosystems software (Life Technologies) was used for data analysis. Relative quantification was performed using the $2^{-\Delta\Delta c(t)}$ method. Expression was normalized against the housekeeping gene β-actin. The mean expression levels of ND-fedmice were set as 100%. The primers used are shown in Supplementary **Table 2**.

Statistical analysis

Statistical analyses of the data were performed using GraphPad Prism Version 7.00. Unless otherwise indicated, comparisons of two groups in which both groups passed the Shapiro-Wilke normality test were compared with the two-tailed Student's t-test. Those in which one or both groups did not pass the Shapiro Wilke normality test were compared with the nonparametric Mann–Whitney U-test.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

Please contact author for data requests.

Competing interests

The authors declare that they have no competing interests.

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Contributors Ting Liu and Wei-qi Song conceived the project, contributed to the experimental design, performed experiments, interpreted the results, and prepared the figures; Ting Liu wrote the manuscript; Chao Lei, Chen Li, Rong Fang, Hui Chen and Qinghong Huang performed experiments; Zhihua Liu, Yanlei Ma and Ning Sun conceived and supervised the project and interpreted the results; Xue Liang, Huihui Ti and Xiao-mei Li revised the manuscript; all authors discussed the results and approved the manuscript.

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Figures

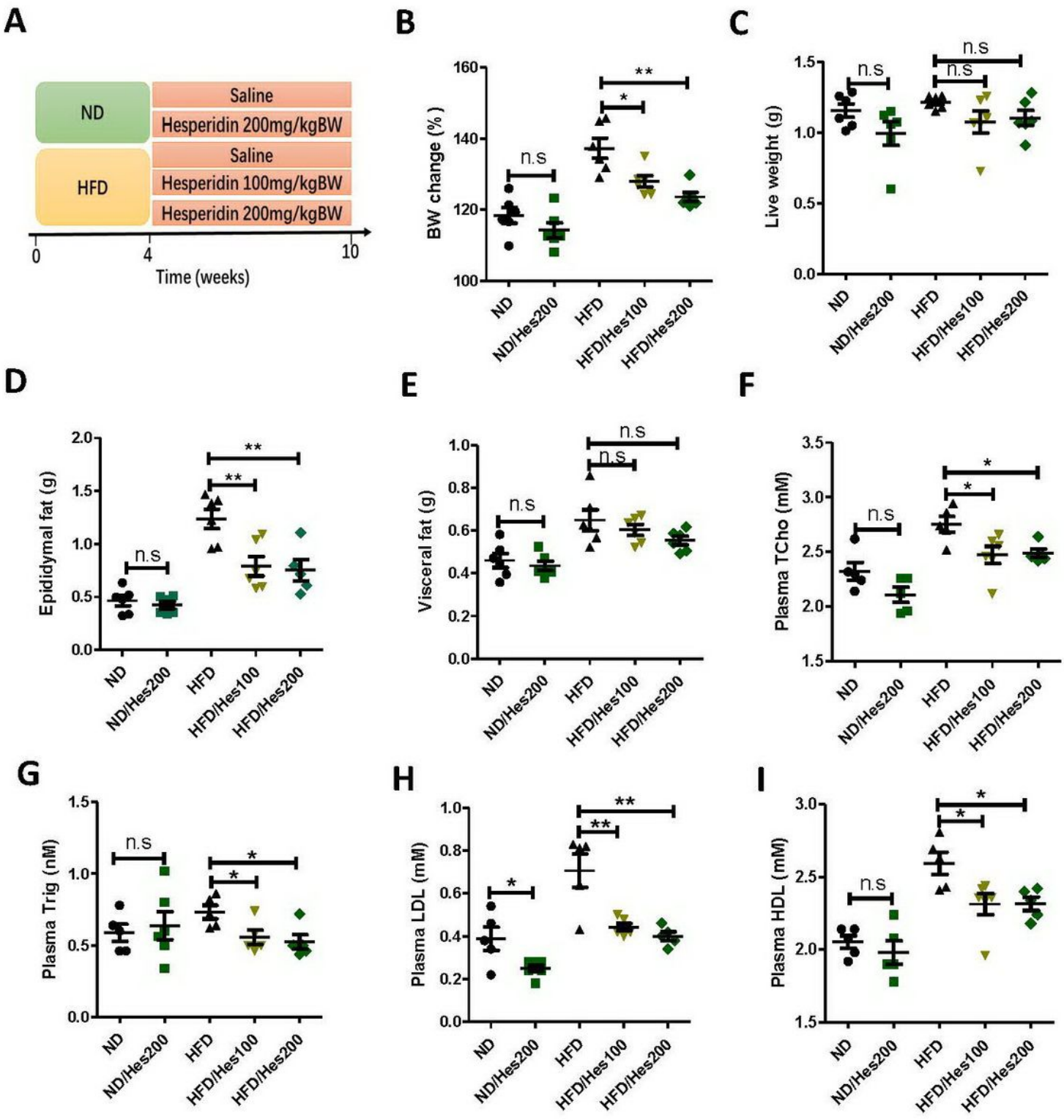

Figure 1

Hesperidin reduced body weight, fat accumulation and plasma lipids in HFD-fed mice. (A) ND- and HFD-fed mice were treated every other day with 10ul/g BW of either saline or hesperidin at 1 or 2% (w/v) by intragastric gavage for 6 weeks (n=6/5 for each group). (B) Body weight gain (C) Liver weight (D) Epididymal fat (E) visceral fat (F) Plasma total cholesterol (G) Plasma triglyceride (H) Plasma low-density

lipoprotein (l) Plasma high-density lipoprotein. Data are expressed as mean ± SEM. All differences were analysed using unpaired two-tailed student's t-test(*P<0.05, **P<0.01) .

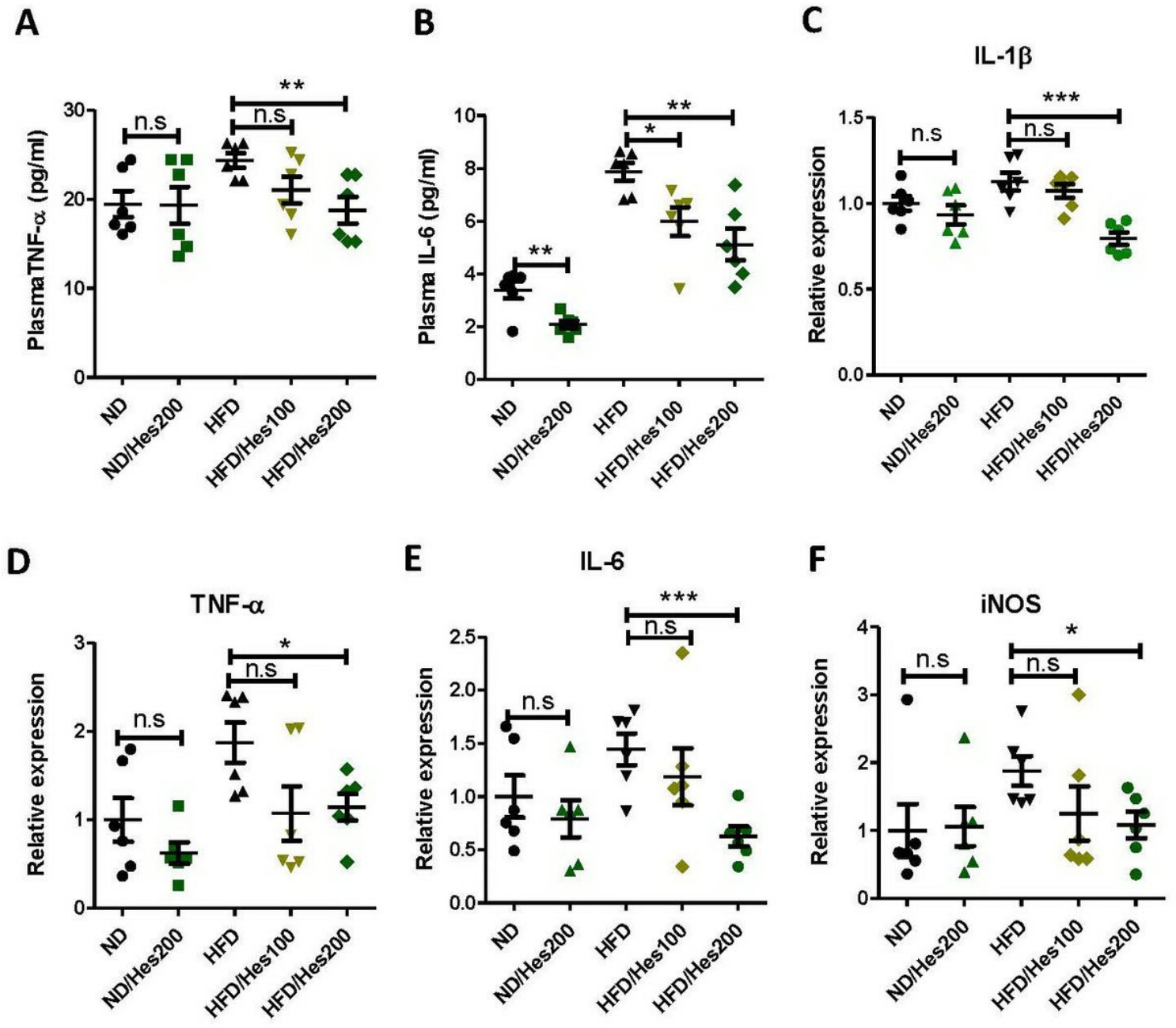

Figure 2

Hesperidin reduced system and colon pro-inflammatory cytokines in HFD-fed mice. ND- and HFD-fed mice were treated every other day with 10ul/g BW of either saline or hesperidin at 1 or 2% (w/v) by intragastric gavage for 6 weeks (n=6 for each group). (A) Levels of TNF-α in plasma. (B) Levels of IL-6 in plasma. (C) Relative mRNA expression levels of IL-1β in colon. (D) Relative mRNA expression levels of TNF-α in colon. (E)) Relative mRNA expression levels of IL-6 in colon. (F) Relative mRNA expression levels of iNOS in colon. Data are expressed as mean ± SEM. All differences were analyzed using unpaired two-tailed student's t-test(*P<0.05, **P<0.01, ***P<0.001).

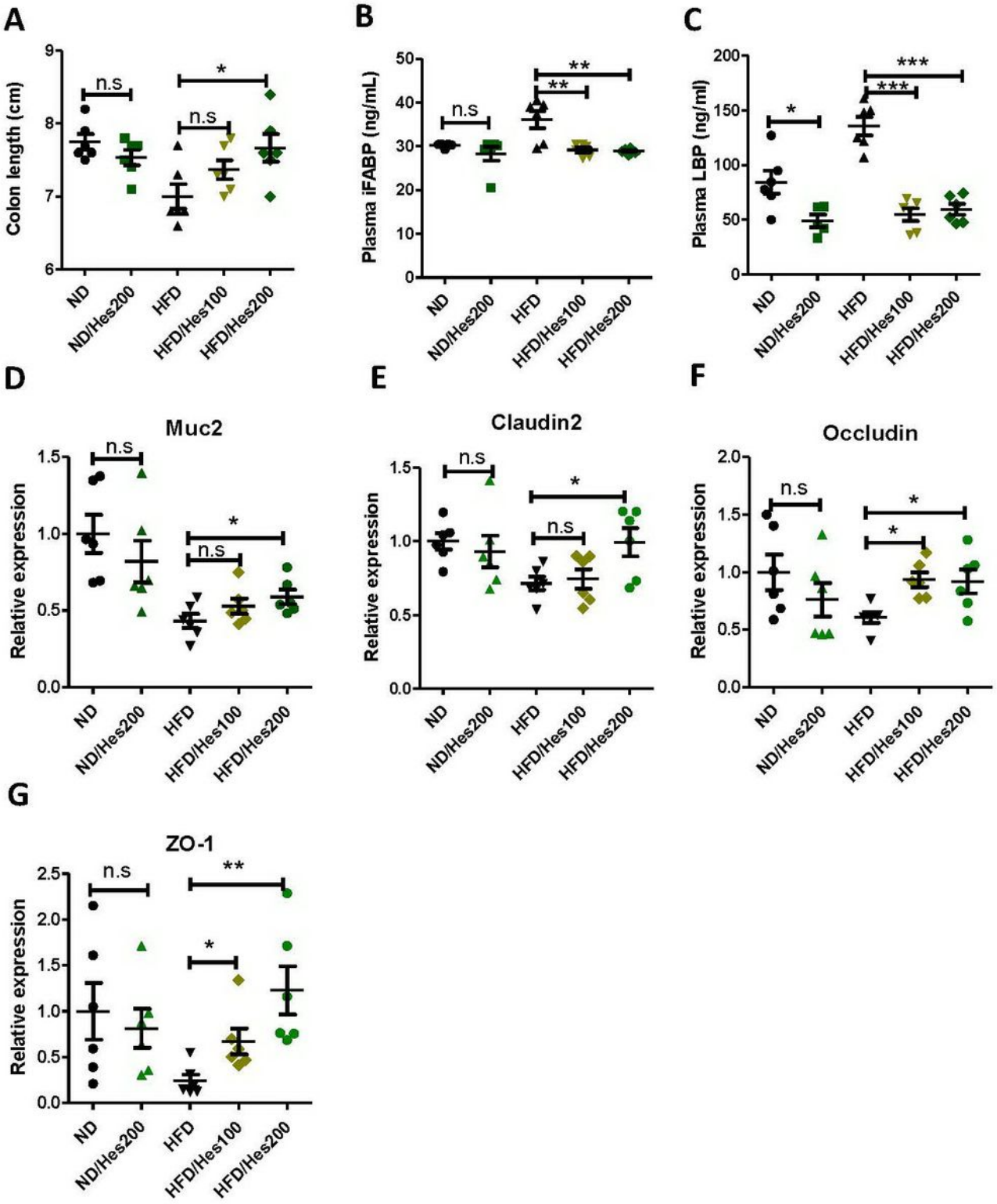

Figure 3

Hesperidin reduced Intestinal barrier permeability in HFD-fed mice. ND- and HFD-fed mice were treated every other day with 10ul/g BW of either saline or hesperidin at 1 or 2% (w/v) by intragastric gavage for 6 weeks (n=6 for each group). (A) Colon length. (B) Levels of Lipid binding protein(LBP) in plasma. (C) Levels of intestinal fatty acid binding protein (iFABP) in plasma. (D) Relative mRNA expression levels of Muc2 in colon. (E) Relative mRNA expression levels of Claudin2 in colon. (F) Relative mRNA expression

levels of Occludin in colon. (G) Relative mRNA expression levels of ZO-1 in colon. Data are expressed as mean ± SEM. All differences were analyzed using unpaired two-tailed student's t-test(*P<0.05, **P<0.01, ***P<0.001).

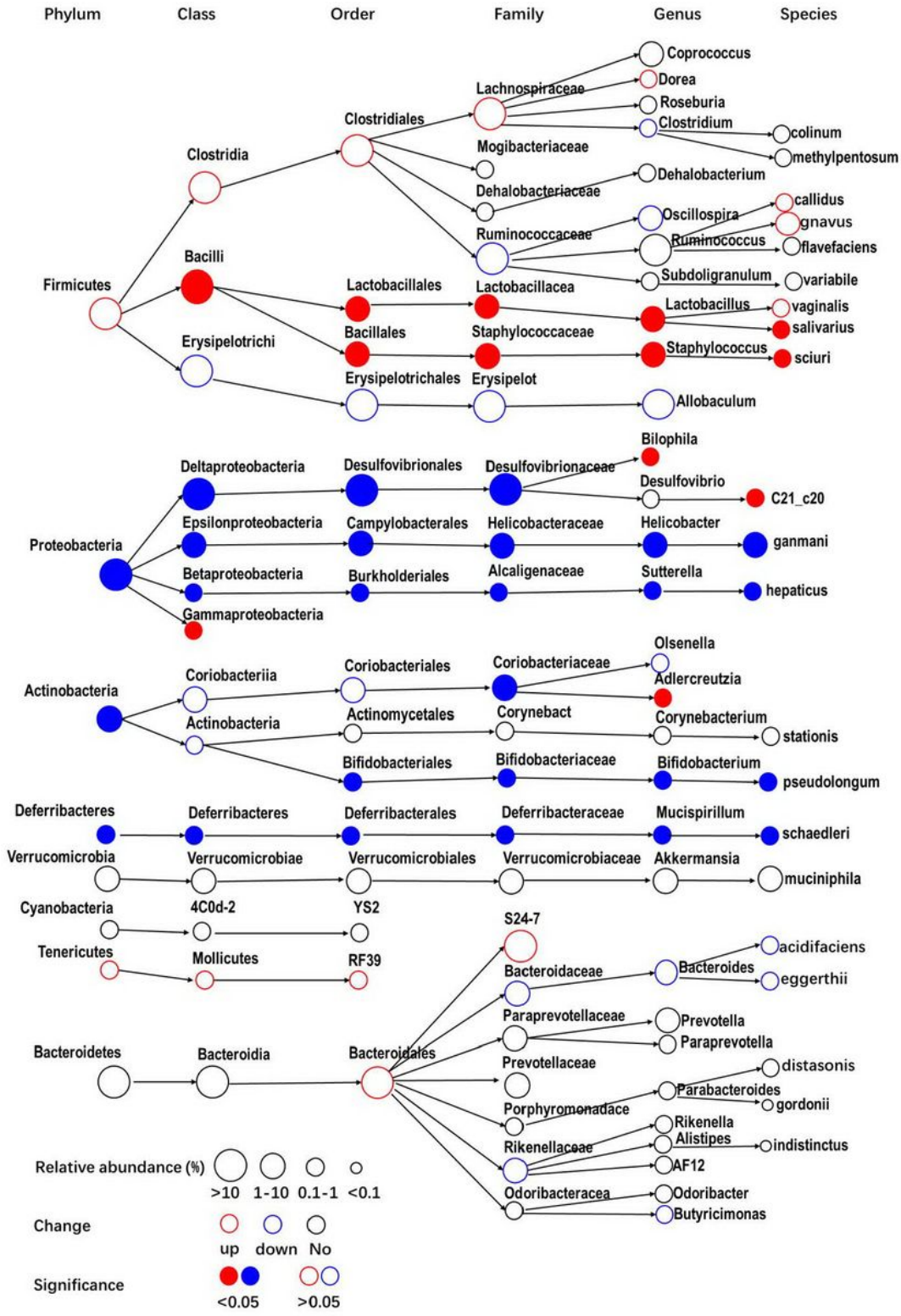

Figure 4

Hesperidin changes relative abundance of specific intestinal microbial taxa. Phylogenetic tree created manually showing specific changes in intestinal microbial community at different taxonomic levels

caused by hesperidin supplementation to HFD mice. Nodes represent taxa, and the size of each node represents its relative abundance. The color red indicates an increase, blue represents a decrease and black means no change of relative abundance in HFD-Hes200 compared with HFD mice. The full color of the nodes indicates the statistically significant difference and the hollow nodes indicate the statistically non-significant difference by unpaired two-tailed student's t-test. See also additional Fig. S2.

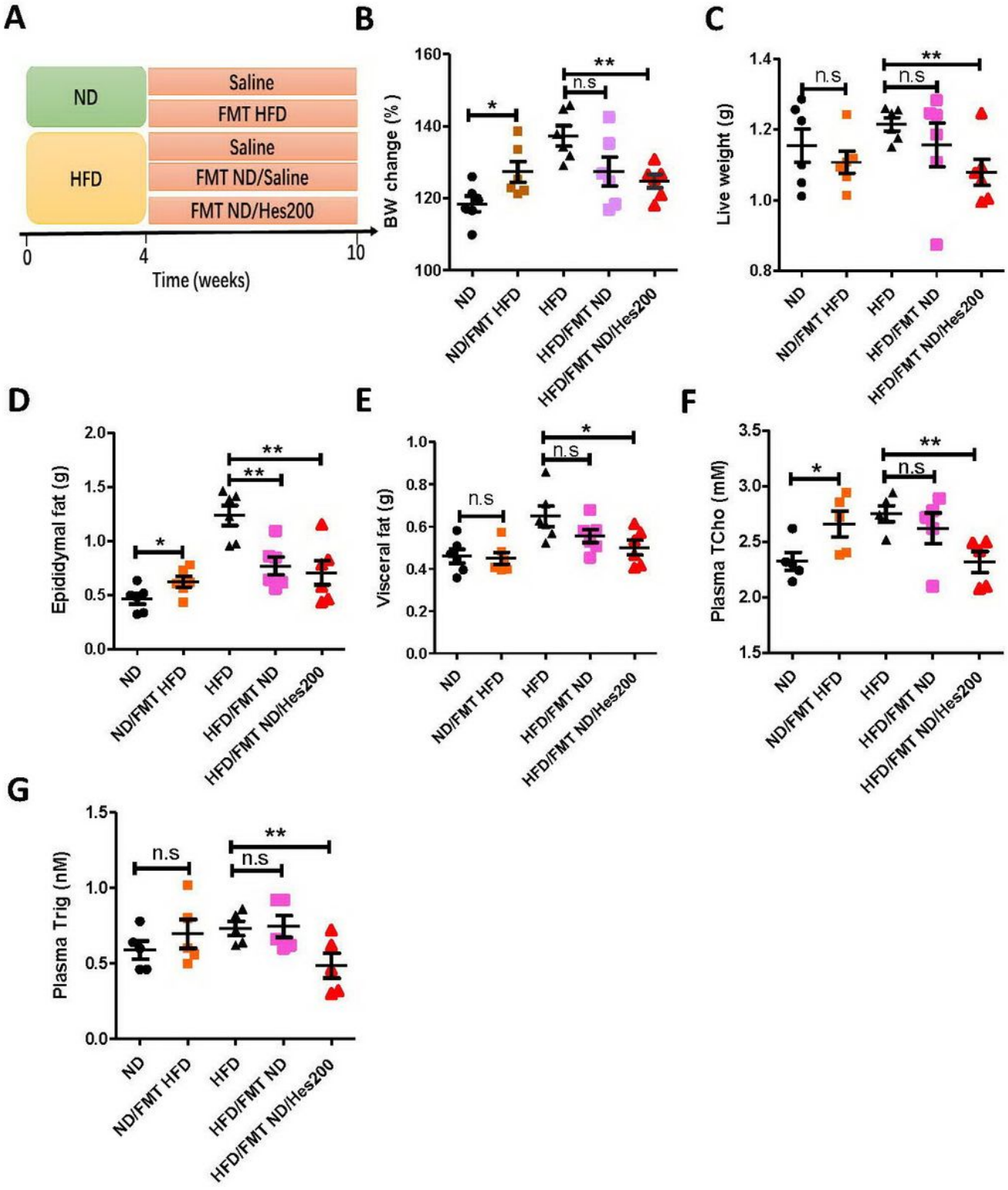

Figure 5

Body weight, fat accumulation and plasma lipid were reversed by fecal transplantation from hesperidin-treated mice to HFD-fed mice. (A) ND- and HFD-fed mice were treated every other day with 10ul/g BW of either saline or faecal bacteria from donor mice at 2% (w/v) by intragastric gavage for 6 weeks (n=6 for each group).(B) Body weight gain (C) Liver weight (D) Epididymal fat (E) visceral fat (F) Plasma total cholesterol (G) Plasma triglyceride. Data are expressed as mean ± SEM. All differences were analysed using unpaired two-tailed student's t-test(*P<0.05, **P<0.01) .

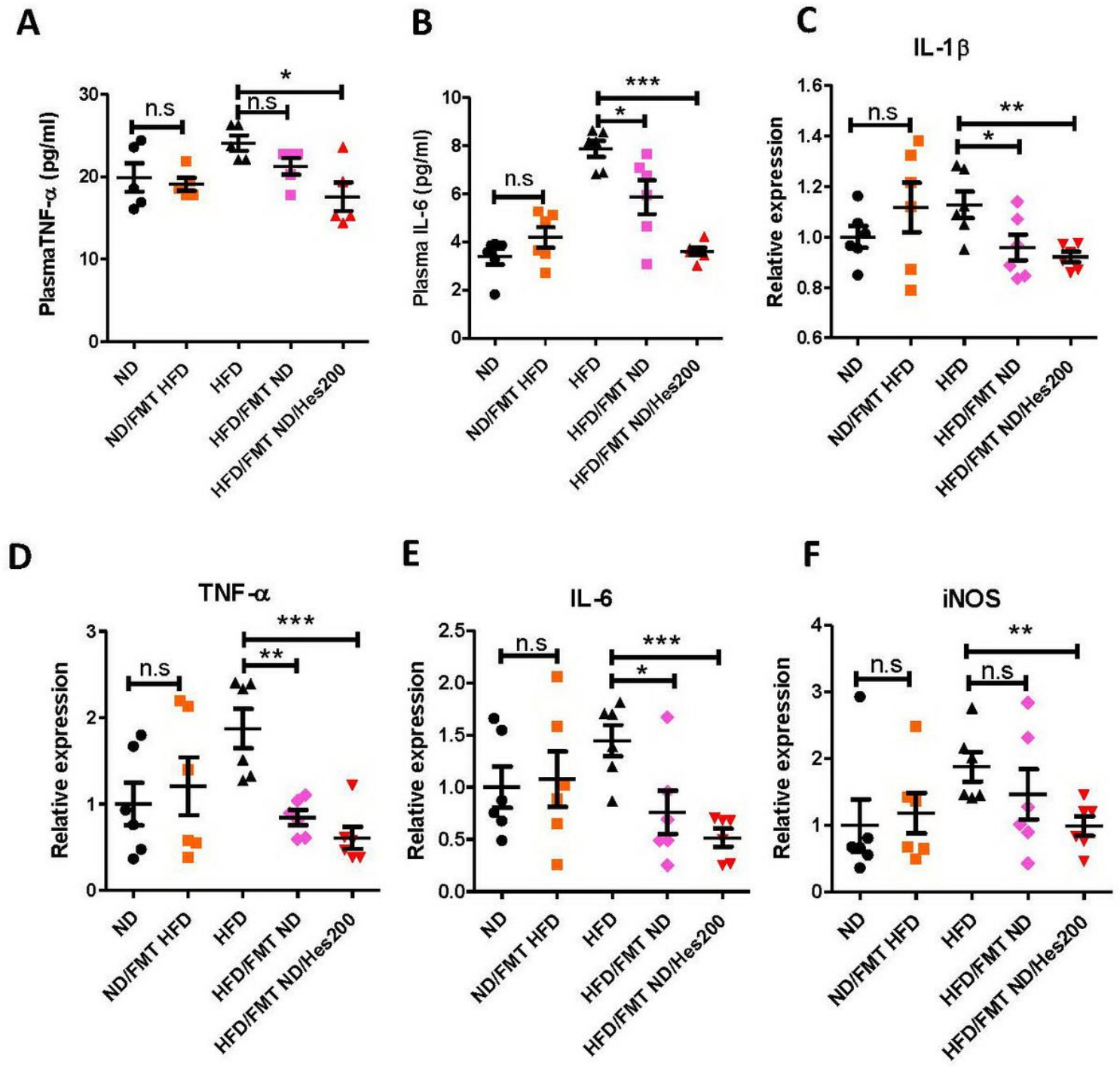

Figure 6

System and colon pro-inflammatory cytokines were reduced by fecal transplantation from hesperidin-treated mice to HFD-fed mice. ND- and HFD-fed mice were treated every other day with 10ul/g BW of either saline or faecal bacteria from donor mice at 2% (w/v) by intragastric gavage for 6 weeks (n=6 for each group). (A) Levels of TNF-α in plasma. (B) Levels of IL-6 in plasma. (C) Relative mRNA expression levels of IL-1β in colon. (D) Relative mRNA expression levels of TNF-α in colon. (E)) Relative mRNA expression levels of IL-6 in colon. (F) Relative mRNA expression levels of iNOS in colon. Data are expressed as mean ± SEM. All differences were analyzed using unpaired two-tailed student's t-test(*P<0.05, **P<0.01, ***P<0.001).

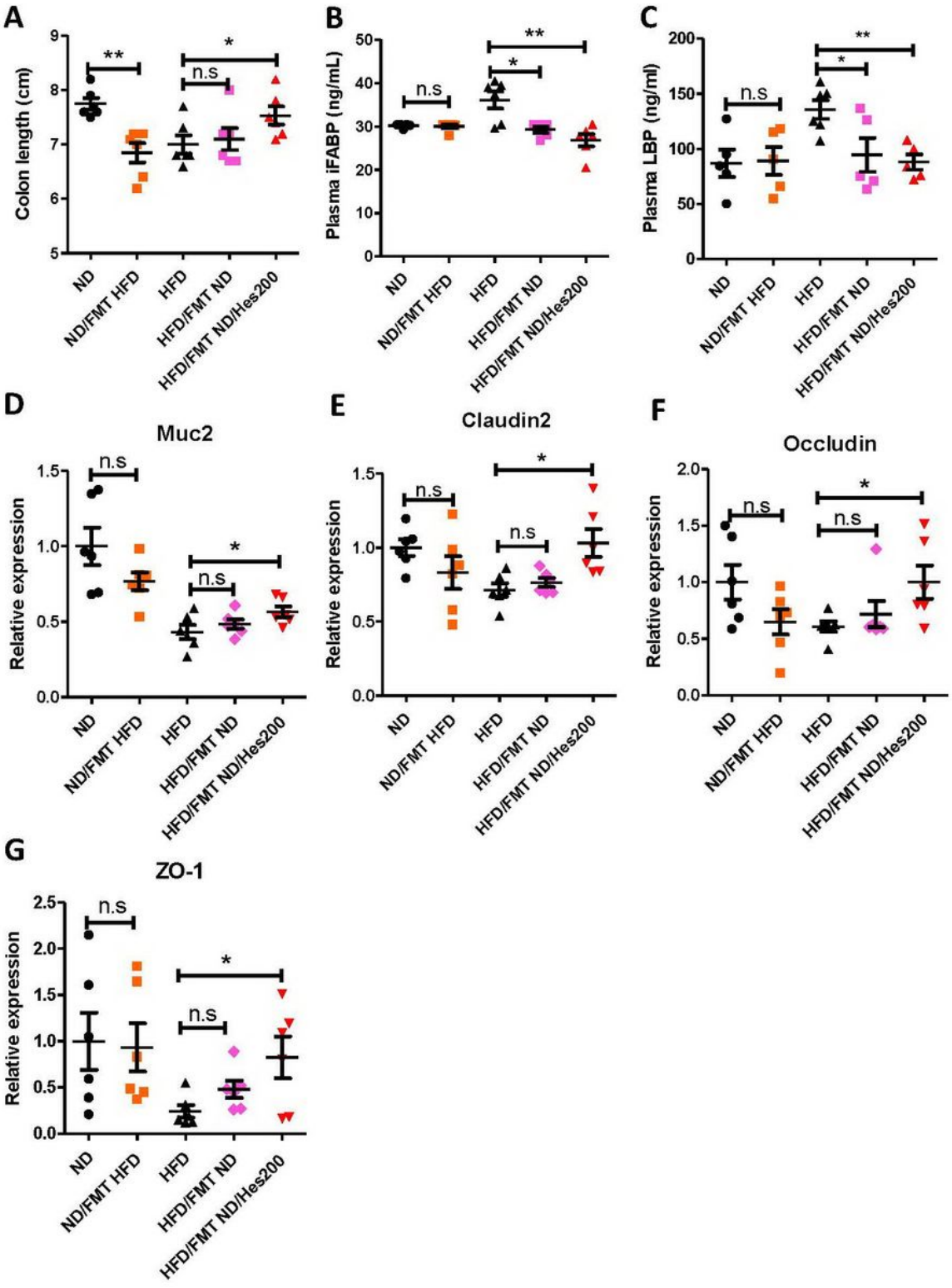

Figure 7

Intestinal barrier permeability were recovered by fecal transplantation from hesperidin-treated mice to HFD-fed mice. ND- and HFD-fed mice were treated every other day with 10ul/g BW of either saline or faecal bacteria from donor mice at 2% (w/v) by intragastric gavage for 6 weeks (n=6 for each group). (A) Colon length. (B) Levels of Lipid binding protein(LBP) in plasma. (C) Levels of intestinal fatty acid binding protein (iFABP) in plasma. (D) Relative mRNA expression levels of Muc2 in colon. (E) Relative

mRNA expression levels of Claudin2 in colon. (F) Relative mRNA expression levels of Occludin in colon. (G) Relative mRNA expression levels of ZO-1 in colon. Data are expressed as mean ± SEM. All differences were analyzed using unpaired two-tailed student's t-test(n.s. not significant, *P<0.05, **P<0.01).

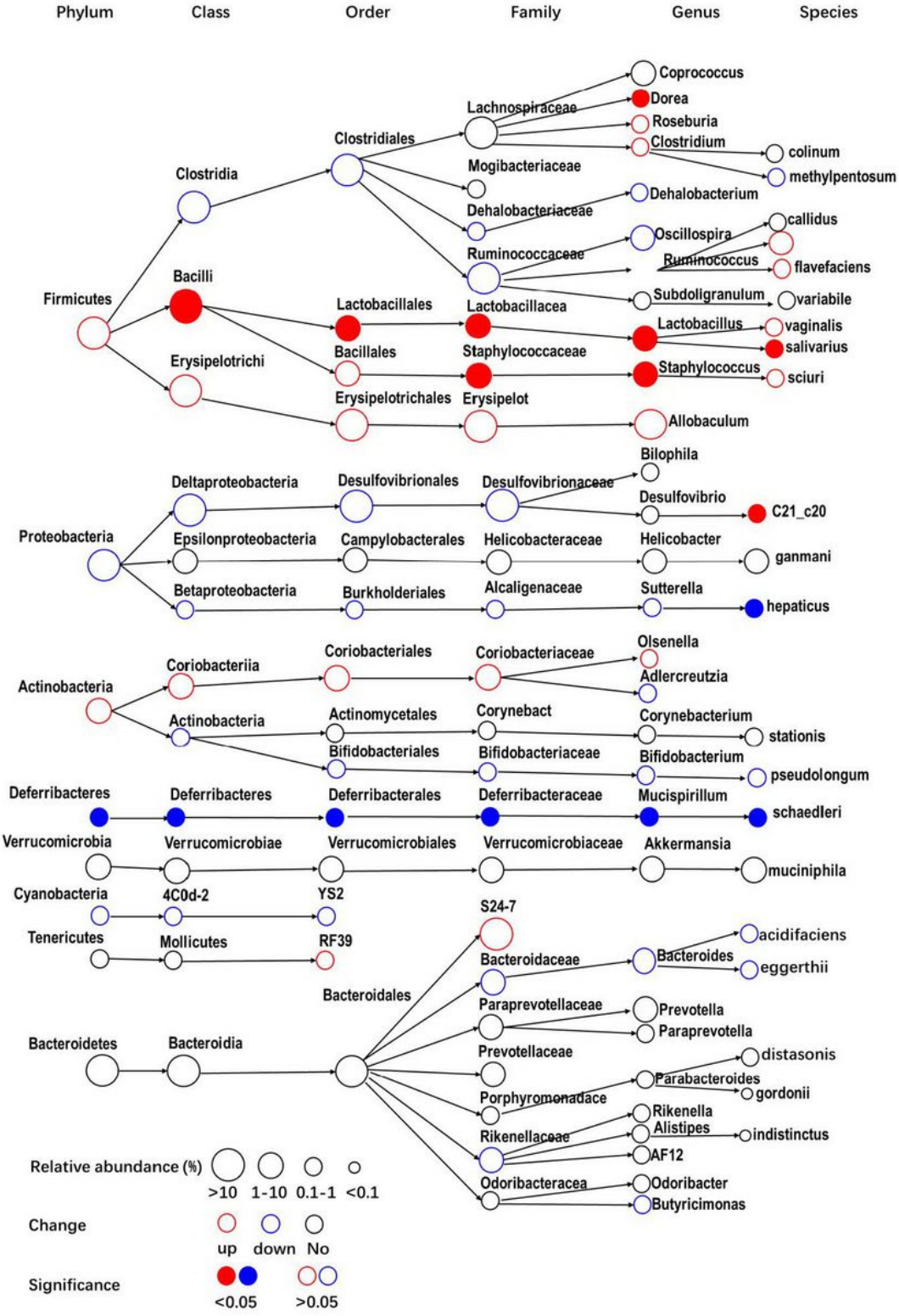

Figure 8

FMT changes relative abundance of specific intestinal microbial taxa. Phylogenetic tree created manually showing specific changes in intestinal microbial community at different taxonomic levels caused by FMT

from ND hes200 to HFD mice. Nodes represent taxa, and the size of each node represents its relative abundance. The color red indicates an increase, blue represents a decrease and black means no change of relative abundance in HFD-Hes200 compared with HFD mice. The full color of the nodes indicates the statistically significant difference and the hollow nodes indicates the statistically non-significant difference by unpaired two-tailed student's t-test. See also additional Fig. S4.

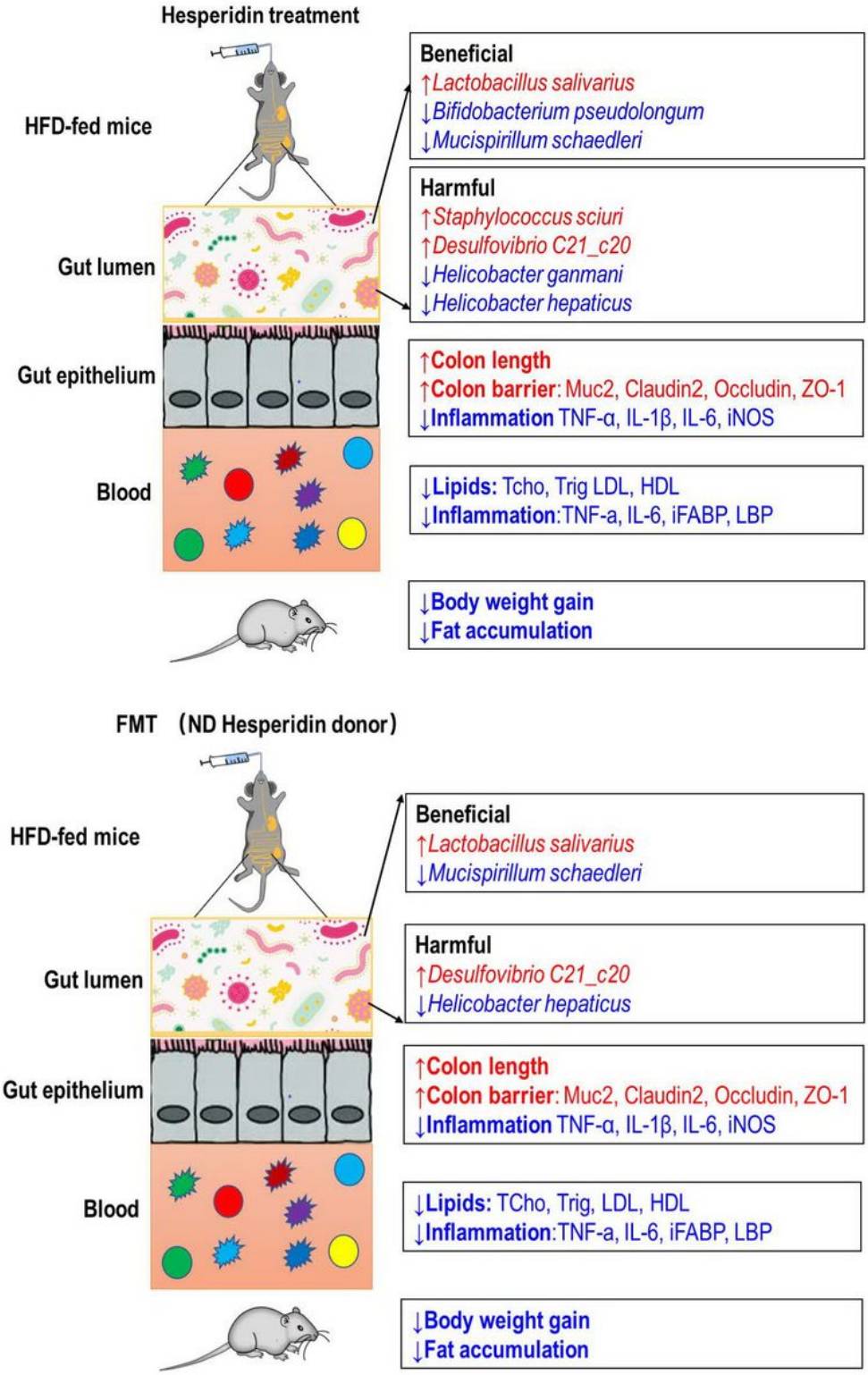

Figure 9

Proposed model for the anti-obesogenic effects of hesperidin and FMT in high-fat diet (HFD)-fed mice. Treatment with hesperidin produced dural changes on gut microbiota of HFD-fed mice, including enriching beneficial Lactobacillus salivarius and harmful Staphylococcus sciuri, Desulfovibrio C21_c20 and decreasing beneficial Bifidobacterium pseudolongum, Mucispirillum schaedleri and harmful Helicobacter ganmani, Helicobacter hepaticus. Horizontal faces transfer from hesperidin-treated mice to HFD-fed mice transfered hesperidin-modulated Lactobacillus salivarius, Desulfovibrio C21_c20, Mucispirillum schaedleri and Helicobacter. Both hesperidin treatment and FMT improved colon integrity and reducing inflammation, blood lipids, body weight gain and fat accumulation. IL, interleukin; TNF-α, tumour necrosis factor-alpha; ZO-1, zonula occludens-1; Tcho, Total cholesterol ; Trig, triglyceride; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

Supplementary Files

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