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 derivatives with multiple pharmacological function. 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 rDNA-based microbiota analysis.

Results: Gut microbiota analysis revealed that hesperidin selectively promoted the growth of beneficial *Lactobacillus salivarius* and harmful *Staphylococcus sciuri, Desulfovibrio C21_c20* and inhibiting beneficial *Bifidobacterium pseudolongum, Mucispirillum schaedleri* and harmful *Helicobacter ganmani*, *Helicobacter hepaticus*.

in HFD-fed mice. However, hesperidin reverses obesity, inflammation and improves gut integrity in HFD-fed mice. The anti-obesity effects and hesperidin-modulated *Lactobacillus salivarius, Desulfovibrio C21_c20, Mucispirillum schaedleri* and *Helicobacter hepaticus* were transmissible via horizontal faces transfer from hesperidin-treated mice to HFD-fed mice.

Conclusions: Hesperidin has a role to reduce body weight and reverse HFD-related disorders in HFD-fed mice by enriching some

beneficial and inhibiting harmful microbes.

Key words: gut microbiota; hesperidin; obesity; faecal microbiota transplantation

Background

Obesity is considered to be a disease condition associated with high risk of numerous health problems. The increasing prevalence of obesity has becoming a major threat to public health, which makes administration of obesity a main challenge for modern societies.[1] Obesity is characterized by fat mass accumulation, chronic subclinical inflammation and imbalanced gut microbiota. Unfit life style especially high fat diet and inadequate exercises, neuronal and hormonal factors, genetic and epigenetic mechanisms all contribute to obesity development.[2] Gut microbiota plays roles in obesity development.[3]

A number of bioactive chemicals have been reported to alleviate disease symptoms by modulating gut microbiota. Hesperidin is a flavanone glycoside (a subclass of flavonoids) that is rich in citrus fruits and quite a few vegetables.[4] Previous studies have shown that hesperidin has various biological activities including vitamin-like activity, antioxidant, anti-inflammatory, anticarcinogenic, anti-hyperglycemic, anti-hypolipidemic and antiallergic properties.[4-6]

Although a large number of studies have been published describing its new pharmacological activities, molecular targets and mechanisms of action. None reported its' effects on HFD induced obesity and gut microbiota.

In brief, gut microbiota is a potential target for hesperidin to intervene HFD resulted obesity. In the present study, we examined whether hesperidin can decrease obesity in HFD-fed mice. Our results indicate that hesperidin reduced obesity, inflammation, improved gut integrity and modified a few gut microbiota species in HFD-fed mice. The anti-obese effects and most hesperidin modified gut microbiota species were transmissible through horizontal fecal transplantation. Our data demonstrate that hesperidin has a role to reduce body weight and reverse HFD-related disorders in HFD-fed mice by enriching some 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, triglyceride, high-density lipoprotein, low-density lipoprotein and slight increases in liver weight **(Figure 1A–1I)**. 2% hesperidin did not produce any significant effects in normal diet mice except for decreasing 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 reduced inflammation in HFD-fed mice.

Studies have shown that obese was characterized by low grade inflammation with higher pro-inflammatory cytokines including tumor necrosis factor-alpha (TNF-α), interleukin-1-beta (IL-1β), interleukin-6 (IL-6).[7] We measured plasma levels of TNF-α and IL-6 proteins and 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 plasma and colons of HFD-fed mice compared with normal diet feeding mice **(Figure 2A–2E)**. while the expression level of these cytokines was reduced in a dose-dependent manner by hesperidin treatment **(Figure 2A–2E)**. Inducible NO-synthase (iNOS) is a key pro-inflammatory mediator. iNOS mRNA expression increased in 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 HFD increased gut permeability and subsequently resulted in releasing of bacterial endotoxin into the circulation.[8] We examined 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 claudin2, occluding and zonula occludens-1 (ZO-1) are three main tight junction components. HFD feeding reduced colon length, expression of the tight junction components,

increased plasma LBP and iFABP, while all 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 part of HFD-induced gut dysbiosis.

The gut microbiota of obese humans and HFD-fed mice is characterized by an increased Firmicutes-to-Bacteroidetes ratio, elevated endotoxin producing Proteobacteria, reduced immuno-homeostatic *Akkermansia muciniphila*.[9 10] We examined the effects of hesperidin on gut microbiota composition by performing a pyrosequencing-based analysis of bacterial 16S rRNA (V3–V4 region) in caecal feces. A total of 36211258 effective reads were obtained from all fecal samples. Based on 99% similarity level, the reads were clustered into 343273 OTUs. HFD reduced OTUs compared to ND-fed mice. Hesperidin reversed HFD-induced OTU decreasing 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 microbiota composition for each treatment group **(Figure S1C)**. Hesperidin also decreased Firmicutes-to-Bacteroidetes ratio **(Figure S1D)**.

The OTUs can be annotated to 8 phylums, 13 classes, 15 orders, 22 families, 29 genuses and 19 species **(Figure 4)**. We detected 8 species that were significantly different between ND-fed and HFD-fed mice. Four of them 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 Proteobacteria phylum, Deltaproteobacteria class, Desulfovibrionales order,

Desulfovibrionaceae family and *Akkermansia muciniphila* in Verrucomicrobia phylum, Verrucomicrobiae class, Verrucomicrobiales order, Verrucomicrobiaceae family were decreased in HFD-fed mice. The other four including *Helicobacter ganmani* and *Helicobacter hepaticus* in the Proteobacteria phylum, Epsilonproteobacteria class, Campylobacterales order, Helicobacteraceae family; *Bifidobacterium pseudolongum* in Actinobacteria phylum, Actinobacteria class, Bifidobacteriales order, Bifidobacteriaceae family and *Mucispirillum schaedleri* in Deferribacteres phylum, Deferribacteres class, Deferribacterales order, Deferribacteraceae family were increased in HFD-fed mice. A closer look at the microbial community revealed 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 *Mucispirillum schaedleri* were decreased in the hesperidin supplemented HFD-fed mice (Figure 4, **Figure S2D-2G**). While *Akkermansia muciniphila* failed to be changed by hesperidin **(Figure 4, Figure S2H)**. Those results implied that hesperidin modified composition of the gut microbiota and reverses part of HFD-induced gut dysbiosis.

The beneficial effects of hesperidin were transferable by fecal transplantation.

It was reported that diet-induced obesity and associated metabolic disorders may resulted from gut microbiota.[3] Anti-obesogenic effects of Chinese herbs such as polysaccharides from *Ganoderma lucidum* and *Hirsutella sinensis* were mediated by the gut microbiota.[11 12] We tested whether the beneficial effects of hesperidin may also be mediated by the gut microbiota. Fecal microbiota from ND-fed mice

treated with saline, hesperidin was transplanted into HFD-fed recipients. To further confirm that our method of FMT works, one more control was conducted: fecal 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, though most of the indicators were not significant **(Figure 5B-5G, Figure 6A-6F, Figure 7A-7G)**. On the contrary, FMT from ND-fed groups reduced obesity traits, inflammation and gut integrity in HFD recipients compared with the controls **(Figure 5B-5G, figure 6A-6F, figure 7A-7G)**. Furthermore, FMT from hesperidin treated ND-fed groups had more significant effects in reducing obesity traits, inflammation and gut integrity in HFD recipients **(Figure 5B-5G, Figure 6A-6F, Figure 7A-7G)**. These results proved that the gut microbiota mediates the beneficial effects of hesperidin.

FMT transmitted specific intestinal microbial taxa.

To check whether the beneficial effects of hesperidin are resulted from the specific microbe it regulated 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 OTUs in HFD recipients **(Figure S2A)**. FMT increased total species diversity as indicated by Chao1 value, but decreased richness and evenness of the main microbiota as shown by Shannon and Simpson value **(Figure S2B)**. UniFrac-based principal coordinates analysis (PCoA) showed a distinct clustering of microbiota composition for each treatment group **(Figure S2C)**. FMT failed to reverse HFD induced increasing of Firmicutes-to-Bacteroidetes ratio **(Figure S2D)**. Among the seven microbiome that specifically regulated by hesperidin, *Lactobacillus salivarius, Staphylococcus sciuri, Desulfovibrio C21_c20, Mucispirillum*

schaedleri and *Helicobacter hepaticus* can be transmitted from donor to recipient mice **(Figure 8, Figure S4A, 4B, 4C, 4E, 4G)** while *Helicobacter ganmani* and *Bifidobacterium pseudolongum* can not **(Figure 8, Figure S4D, 4F)**. *Akkermansia muciniphila* was also failed to be transmitted from ND-fed donor to HFD-fed recipient mice **(Figure 8, Figure S4H)**.

Discussion

Polyphenols have been reported to modulate the metabolism and/or inflammation related to obesity.[13] As a bioactive chemical belong to polyphenols, hesperidin was extensively studied for its effects on cancer and cardiovascular diseases but not obesity.[4 5] In vitro studies indicated that citrus polyphenols including hesperidin caused a reduction in adipocyte differentiation, lipid content in the cell and adipocyte apoptosis, which showed positive role in the management of obesity.[14] Animal evidence were not entirely consistent, but most of them indicated a reduction in adipose tissue, increased genes expression resulted in stimulation to β-oxidation, improved lipid profile and glycemia as well as improved inflammatory status.[15] Our experiments on HFD-fed mice also indicated a reduction in adipose

tissue, improved lipid profile and inflammatory status. Moreover, hesperidin improved intestinal barrier function in HFD-fed mice. Role of hesperidin on decreasing intestinal inflammation and restoring intestinal barrier function was also proved in DSS-induced colitis mice.[16] However, solid clinical evidences were very limited. A systematic review and meta-analysis concluded that hesperidin might not affect lipid profile and blood pressure based on 10 randomized controlled clinical trials.[17] Therefore, well-designed trials on human are still needed to confirm anti-obesity effects of hesperidin.

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

Gut microbiota that has 10 times the number of human cells and 150 times number of genes of the human genome was considered a “hidden organ”.[20] New findings from this field and their importance for human health has providing a new frontier to understand occurrence and development of various diseases, as well as mechanism of drugs, traditional herb medicines, bioactive chemicals and functional foods.[21] [22] For obesity, research found that the gut microbiota of obese humans and HFD-fed animals were different from lean and ND-fed animals, and different studies on obesity or weight losing subjects found quite a few microbes from phylum to species level that were positively or negatively associated with

obesity, however, there are not many consistent conclusions for an obese microbiota profile except for an increased Firmicutes-to-Bacteroidetes ratio, elevated abundance of *Akkermansia muciniphila*.[23]

Different studies found different obesity related microbes probably because of different experimental objects and background diet since gut microbiota was different between species and diet was the most important factor shaping it.[24] [25] Second, not all microbes that in a lower taxon like genera and species play the same role within a given higher taxon like phylum. Different bacterial species present different characteristics, which may be related to beneficial or harmful traits. For example, Lactobacillus such as *Lactobacillus plantarum* and *paracasei* have been associated with thinness, while species such as *Lactobacillus reuteri* that have been associated with obesity.[26] This suggests that the physiological effects of microbes are detailly dependent on the strain. Therefore, it may be inaccurate to conclude traits related microbe that at higher than species or strains level. Third, as members in a complicated ecological system, one microbe may be regulated by another according to specific physiology conditions, for example, both *Bifidobacterium pseudolongum* and *Akkermansia muciniphila* were beneficial microbes, however, rats fed with *Bifidobacterium pseudolongum* strain Patronus led to a large increase of mucus thickness associated with a decrease of *Akkermansia muciniphila* which was lost to bifidobacteria in the competition for the mucus niche.[27] Therefore, when state a traits related microbe, it is under the specific physiology conditions with a specific microbial community characteristic. Forth, experiment procedures including sampling, sequencing and bioinformatic analysis may also contribute to the inconsistency among 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* was reported to be positively related to cisplatin-induced mucositis of Male Wistar rats[28] and negatively related to antihyperlipidemic effects of *Rhizoma Coptidis* alkaloids in high-fat and high-cholesterol induced hyperlipidemic 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 was associated with a significant increase in the expression of the proinflammatory cytokine IL12/23p40 in IL10-deficient mice.[31] Those four microbes were considered to be harmful microbes. *Lactobacillus salivarius* is a promising probiotic since it has been reported to possess certain abilities such as enhancement of the immune system, attenuation of gut inflammation and to have antimicrobial activity against pathogenic bacteria *like Staphylococcus aureus.*[32] [33] *Akkermansia muciniphila* has been proved to have a negative correlation to overweight, obesity, untreated type 2 diabetes mellitus or hypertension studies and its beneficial effects on obesity have been reported by a clinical trial.[34] [35] *Bifidobacterium pseudolongum* is a beneficial microbe that has a role to protect gut barrier, however it competes mucus nich with *Akkermansia muciniphila. Mucispirillum schaedleri* was reported to confers protection against Salmonella colitis in mice by competing for anaerobic respiration substrates in the gut.[36] Those four were considered to be beneficial microbes.

In our study, we found beneficial *Lactobacillus salivarius*, *Akkermansia muciniphila* and harmful *Staphylococcus sciuri*, *Desulfovibrio C21_c20* were decreased in HFD-fed mice, while

beneficial *Bifidobacterium pseudolongum*, *Mucispirillum schaedleri* and harmful *Helicobacter ganmani*, *Helicobacter hepaticus* were increased in HFD-fed mice compared to ND-fed controls. Beneficial *Lactobacillus salivarius* and harmful *Staphylococcus sciuri, Desulfovibrio C21_c20* were enriched in the Hesperidin supplemented HFD-fed mice, while beneficial *Bifidobacterium pseudolongum, Mucispirillum schaedleri* and harmful *Helicobacter ganmani*, *Helicobacter hepaticus* were decreased in the hesperidin supplemented HFD-fed mice. Benificial *Akkermansia muciniphila* failed to be changed by hesperidin. Those results implied that HFD did not enrich all harmful or inhibit all beneficial microbes and hesperidin did not enrich all the beneficial or inhibit all harmful microbes. This double role of hesperidin on both beneficial and harmful microbes may explain the unstable and inconsistent anti-obesity results in the animal and clinical tests.

To further study causal relationship between gut microbiota and disease, FMT (fecal microbiota transplantation) was usually conducted. Human fecal microbiota transplants from obese twins to germ-free mice resulted in the increase of body fat, compared to those mice receiving FMT from lean twins, which proved that gut microbiota could be the cause of obesity.[37] FMT is also the way to study causal relationship between gut microbiota and effects of drugs, traditional herb medicines, bioactive chemicals and functional foods. A traditional Chinese medicine *Ganoderma lucidum mycelium* was reported to reduce body weight, inflammation and insulin resistance in HFD-fed mice. Fecal microbiota transplants from mice treated with water extracts of *Ganoderma lucidum mycelium* to HFD-fed mice transmitted the anti-obesity effects coincidentally, which proved that anti-obesity effects of Ganoderma lucidum mycelium was mediated by gut microbiota.[11] Our research

also indicated that FMT transmitted donors' traits to the receptors, which provided one more evidence for gut microbiota mediated effects of bioactive chemicals and FMT as an effective therapy for diseases.

Interestingly, although FMT from healthy donors often bring about a good result to receipts, when comparing receipts gut microbiota before and after FMT and check the similarity to the donors' gut microbiota, it found that the receipts gut microbiota was obviously changed by FMT, however, the similarity of the receipts' gut microbiota after FMT to the donor's was not as high as expected, especially when the receipts were not germ-free subjects like human or SPF animals.[38] This may attribute to colonization ability of the microbes, some may easy to win out in the competition with intrinsical microbes or set down in the receipts' gut, some may not easy to win an ecological niche. The colonization ability of gut microbes has not been well studied and worth more research when exploring the mechanism of benefit effects of FMT therapy for virous health problems.

In our study, taking a close look to the specific microbes that be transmitted to recipients by FMT, *Lactobacillus salivarius, Staphylococcus sciuri, Desulfovibrio C21_c20, Mucispirillum schaedleri* and *Helicobacter hepaticus* can be transmitted from donor to recipient mice while *Helicobacter ganmani, Bifidobacterium pseudolongum* and *Akkermansia muciniphila* can not. *Akkermansia muciniphila* was known to set down in the mucosa layer, *Bifidobacterium pseudolongum* also shown high adhesion to porcine colonic mucin.[39] It may be harder for them to replace the original microbes that occupied the mucus niche.

Since there are thousands of species in the gut microecosystem, scientists believe there are key players among the gut microbiota

and they have been screening the driving species that contribute to the development of disease and beneficial effects of intervention.[40] It's a common goal for scientists in this field to find the key players and genetic or environment factors that regulate the key microbes. For example, *Parabacteroides goldsteinii* was found to be enriched by *Hirsutella sinensis* mycelium that produce anti-obesogenic and antidiabetic effects in obese mice. Oral treatment of obese mice with live *P. goldsteinii* bacteria thus produce anti-obesogenic and antidiabetic effects.[12] So *P. goldsteinii* was the key microbe that contribute to beneficial effects of *Hirsutella sinensis* mycelium. Further on, impacts of the microbes on the host rely on mostly their metabolites. *Lactobacillus, Bifidobacterium, Faecalibacterium prausnitzii* are negatively correlated with cardiovascular disease and type 2 diabetes because they are SCFA-producing species.[41 42] When the strain *Enterobacter* was screened and isolated from a morbidly obese human and inoculated to germfree mice, it induced obesity and insulin resistance because it's an endotoxin-producing bacterium.[43] Those delighting findings provide potential application of gut microbiota and their metabolites as novel biomarkers for disease diagnosis and new probiotics for disease therapy. The species that were screened to be significantly changed by HFD and hesperidin in the present study, especially *Staphylococcus sciuri*, *Desulfovibrio C21_c20, Mucispirillum schaedleri, Helicobacter hepaticus* and *Helicobacter ganmani* that have not been reported to be obese related in previous literatures need further verification by live strain supplementation. Their metabolites and molecular mechanisms hinted for obese need further exploration.

Conclusions

In conclusion, our results indicate that hesperidin reduced obesity, inflammation, improved gut integrity and modified a few gut microbiota species in HFD-fed mice **(Figure 9)**. The anti-obese effects and most hesperidin modified gut microbiota species were transmissible through horizontal fecal transplantation. Our data demonstrate that hesperidin has a role to reduce body weight and reverse HFD-related disorders in HFD-fed mice by enriching some beneficial and inhibiting harmful microbes.

Materials and methods

Murine.

Animal experiments were approved and performed in accordance with the guidelines of Laboratory animal center of Guangzhou Medical University. Eight-week-old male mice of the C57BL/6 were purchased from Guangdong medical laboratory animal center (GDMLAC) and kept under controlled temperature and light conditions (25□, 12h light–dark cycle), with free access to food and water. Mice were randomly distributed into eight groups containing six animals each. Mice were housed in groups of three animals per cage, and were fed with 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 was shown in **Supplementary Table 1**. Each group of mice was fed for 10 weeks with chow diet or HFD, with free access to either water or saturated hesperidin (Aladdin, CAS#520-26-3) solution at 0.1 or 0.2% (w/v). Mice were supplemented every other day with sterile saline (vehicle), hesperidin (100, 200 mg/kg BW) by intragastric gavage from the fifth week of feeding. Fecal microbiota transplantation (FMT) was started from the fifth week of feeding. At the tenth week, animals were fasted for 12 hours before killing. Mice were deeply anaesthetized with 1% pentobarbital sodium (50 mg/kg BW) and whole blood was withdrawn through ventral aorta in tubes containing anticoagulant KEDTA. Visceral adipose tissues, epididymal white adipose tissues and the liver were removed and weighed. Colorectum were removed and its length was measured. Fecal in cecum was squeezed out. All samples were immersed in liquid nitrogen and stored at −80°C for

further analysis.

Fecal microbiota transplantation.

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

Measurement of plasma cytokines

Whole blood was withdrawn through ventral aorta in tubes containing anticoagulant KEDTA. Blood were centrifuged at 500 g for 5 min and supernatants (plasma) were collected. Plasma interleukin (IL)-6, tumour necrosis factor-alpha (TNF-α), intestinal fatty acid binding protein (iFABP), lipopolysaccharide-binding protein (LBP) were determined by commercial ELISA kits: mouse IL-6 high sensitivity ELISA kit (Cat# EK206HS-96, Multi Scences, China), mouse TNF-α high sensitivity ELISA kit (Cat# EK282HS-96, Multi Scences, 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 ventral aorta in tubes containing anticoagulant KEDTA. Blood were centrifuged at 500 g for 5 min and supernatants (plasma) were collected. Total cholesterol (Tcho), triglyceride (Trig), low-density lipoprotein (LDL)

high-density lipoprotein (HDL) were determined by 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), 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 DNA Kit (Guangzhou IGE biotechnology, China) and applied to amplification of V3-V4 regions of 16S rDNA. Caecal microbiota composition was assessed using Illumina2500 sequencing of 16S rDNA 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 through RDP Classifier (Version 2.2), confidence cutoff 0.8 according to the GreenGene database, then composition and abundance information of each sample at different classification levels were statistically summarized.

Quantitative real-time reverse-transcription qRT-PCR.

Total RNA was isolated using UNIQ-10 column trizol total RNA isolation kit (Sangon Biotech, China). Equal amounts of total RNA were used to synthesize cDNA with the PrimeScript™ RT reagent kit with gDNA Eraser (Cat# RR047A, TAKARA, Japan). qRT–PCR was performed in triplicate using TB Green™ premix Ex Taq™ (Cat# RR820A, TAKARA, Japan), 96-well plates and the 7500 Real-Time PCR System (Applied Biosystems). The Applied Biosysterm software (life technologies) was used for data analysis. Relative quantification was done using the $2^{-\Delta\Delta c(t)}$ method. Expression was normalized

against the housekeeping gene β-actin. Mean expression levels of ND-fed mice were set as 100%. The primers used are shown in **Supplementary Table 2**.

Statistical analysis

Statistical analyses of data were performed using GraphPad Prism Version 7.00. Unless otherwise indicated, comparisons of two groups in which both groups passed a Shapiro-Wilke normality test were compared by two-tailed Student t-test. Those in which one or both groups did not pass a Shapiro Wilke normality test were compared by a 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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Authors' contributions

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

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Figure legends

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 (I) 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.