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Improvement of Piperine on the Disturbed Intestinal Flora and Leaky Gut in Obese Mice

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Abstract: Disturbed intestinal flora and leaky gut play the key roles in the occurrence of obesity-induced diabetes. Piperine can reduce the metabolic inflammation in obese mice and delay the occurrence of diabetes, but the mechanism is not clear. We aim to explore the potential mechanism of piperine in terms of intestinal barrier integrity and flora composition. Here, the obese mice model was induced by feeding 60% high-fat diet, and the feces of each mice were collected for 16s analysis of intestinal flora after intragastric administration of piperine (15 mg/kg/d) for 8 weeks. We found that piperine treatment decreased the serum contents of LPS, TNF-α and FFA, inhibited the high expression of inflammation-related proteins (TLR4, IL-1 β and CD11C) and upregulated the permeability-related proteins (ZO-1 and occludin) of ileum. Piperine also adjusted the ratio of Firmicutes to Bacteroides, changed the diversity and community richness of intestinal flora and regulated the content of short-chain fatty acids. Taken together, piperine can inhibit the occurrence of intestinal leakage by improving the disturbance of intestinal flora and the intestinal barrier integrity in obese mice.

Keywords: Piperine, Intestinal Flora, Intestinal Barrier, Obesity, Diabetes

Introduction:

The global wave of obesity, physical inactivity and high-calorie diet has led to an unprecedented increase of diabetes. The 10th edition of the International Diabetes Federation (IDF) Diabetes Atlas showed that there were about 537 million people with diabetes in the world in 2021 (10.5% of the total population), and it was estimated to be 643 million by 2030. Diabetes is a complex disease with multiple factors, and its pathogenesis is complex, including intestinal flora disorder and intestinal leakage associated with high-calorie diet[1]. Intestinal flora is a general term for microorganisms parasitic in the intestines of humans or animals, which is composed of more than 3500 species of bacteria, of which more than 95% can be classified into 3 phyla, Firmicutes, Bacteroides, Actinobacteria. Among the human intestinal flora, Firmicutes and Bacteroides are absolutely dominant, which is composed of more than 200 genera, such as Lactobacillus, Mycoplasma, Bacillus, Clostridium and so on. Pseudomonas includes more than 20 species of bacteria[2, 3]. Intestinal pathogens and lipopolysaccharides (LPS) can pass through the intestinal epithelium through a variety of pathways, including activating the Toll-like receptor 4 (TLR4) and leading to M1-like polarization of macrophages in the lamina propria of the intestine [4, 5]. In addition, thinning of the intestinal mucosal layer and decrease of tight junctions (ZO-1 and occludin) can increase the intestinal epithelial permeability[6]. The "leaky gut" may further allow LPS and pathogenic organisms to transdermal transfer in the intestine[7, 8]. They can even transfer from submucosa to systemic circulation and to surrounding tissues, including adipose tissue and omentum [5]. It is suggested that maintaining the composition of normal intestinal flora and the integrity of intestinal barrier may delay the occurrence of obesity-related diabetes.

The close relationship between the disturbed intestinal flora and diabetes has been widely recognized and valued by medical researchers, but so far there are few studies based on natural drugs. Piperine is a natural plant alkaloid extracted from pepper (Piper nigrum Linn), a famous edible spice in the world[9]. Our previous studies found that piperine has a strong delaying effect on metabolic inflammation induced by obesity and the occurrence of diabetes[10]. In view of the important role of chronic low-grade inflammation caused by intestinal leakage in the pathological progression of obesity-induced diabetes, the purpose of this study is to explore whether piperine plays an anti-diabetic role by improving intestinal barrier integrity and intestinal flora composition.

Materials and methods

Animal grouping and treatment

Male C57BL/6C mice (7-week age) were purchased from Vital River Laboratory Animal Technology. 60% high-fat diet (HFD) was purchased from Beijing Huafukang Biotechnology Co., Ltd. All animals were divided into three groups (n = 10) based on the body weight: normal group, HFD group and piperine group (15 mg/kg/d). All the mice were allowed free access to water, mice in the normal group were given basic feed, while mice in HFD and piperine groups were fed with 60% HFD. After one-week induction of 60% HFD, piperine were given by gavage once a day for 8 consecutive weeks. In the last 3 days, the fresh feces

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of mice in each group were collected and stored at -80℃ after quick freezing in liquid nitrogen for subsequent intestinal flora analysis and detection of short-chain fatty acids (SCFAs). At the end, blood was collected after anesthesia for serum index detection, ileal tissue were separated, and fixed with 4% paraformaldehyde for pathological analysis and target protein determination. All animal protocols were implemented in accordance with the guidelines for the care and use of laboratory animals prepared and approved by the animal protection committee of Qingdao University.

Hematoxylin and eosin (HE) staining

The ileal tissue of mice was fixed for one week and then were paraffin-embedded, sliced(5μm), dewaxed and stained with hematoxylin and eosin according to the standard protocol. Morphological changes of ileum in each group were observed under microscope.

Elisa detection

Serum LPS, TNF- α, FFA and zonulin were monitored by Elisa method (Mlbio, Shanghai, China). The operation is briefly described as follows. The serum, sample diluent and HRP-labeled reagent were added in turn in the sample holes and incubated at 37℃ for 60 minutes. After washing with detergent, chromogenic agent was added to develop color, and measure OD value after terminating the reaction. Concentrations of LPS, TNF- α, FFA and zonulin in each sample were calculated according to the standard curve.

Analysis of gut microbiota

Microbial community genomic DNA was extracted from fecal samples using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.). Next, the hypervariable region V3-V4 of the bacterial 16S rRNA gene was amplified with primer pairs 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') with an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq PE300 platform/NovaSeq PE250 platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered by fastp version 0.20.0 and merged by FLASH version 1.2.7. Operational taxonomic units (OUTs) with 97% similarity cut off was clustered using UPARSE version 7.1, and chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analysed with the RDP Classifier version 2.2 against the 16S rRNA database (eg. Silva v138) using confidence threshold of 0.7.

SCFAs detection

The analysis was performed using an Agilent 8890B gas chromatography coupled to an Agilent 5977B mass selective detector with an inert electron impact ionization (EI) source and ionization voltage was 70Ev (Agilent, USA). Analyte compounds were separated with a HP-FFAP (30 m×0.25mm×0.25 μm) capillary column, using 99.999% helium as a carrier gas at a constant flow rate (1 mL/ min). The GC column temperature was programmed to hold at 80 °C and rise to 120°C at a rate of 40°C per minute, then rise to 200°C at a rate of 10°C per minute, finally hold at temperature of 230°C for 6 min. The injection volume of samples was 1 μL and introduced in splitting mode (10:1) with the inlet temperature of 260°C. The ion sources temperature was 230°C and the quadrupole temperature was 150°C. Data acquisition was conducted on full scan mode with a range of m/z 30-300. Compounds were identified and quantified by software of Masshunter (v10.0.707.0, Agilent).

Western blot (WB) analysis

After ultrasonic fragmentation, the protein was extracted from the ileum of mice. The protein concentration was determined by BCA (NCM Biotech, Suzhou, China) kit and was adjusted to the appropriate concentration. According to the standard protocol, the tissue homogenate was subject to 10–12% SDS-PAGE electrophoresis, transferred to a PVDF membrane, which was then incubated with the primary antibody of IL-1β (1:1000; Abcam USA), CD11c and Toll like receptor-4 (TLR-4) (1:1000; Affinity, USA), ZO-1 and occludin (1:500; Affinity, USA) overnight at 4 ℃. Next, the membrane was washing with TBST and incubated with HRP-linked secondary antibody at 25 °C for 2 h. An ECL WB kit (CWBIO, Beijing, China) was used to detect the target proteins.

Data Analysis

All data were analyzed by using Graphpad prism 8.0 and were expressed as means ± SD. In the analysis of intestinal flora data, t-test was used for the comparison between groups. The differences of other data between groups were compared by One-way ANOVA. $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$ were considered to be statistically significant.

Results

Piperine improves the overall state of HFD-induced obese mice.

In the experiment, we found that HFD mice were in poor condition after feeding 60% HFD for 4 weeks, accompanied by marked abdominal obesity, sparse back hair and decreased motolity and response. However, the mice in the piperine group were always in good condition, the coat color was bright and

smooth, and there was no death during intragastric administration for 8 weeks.

Piperine reduces metabolic inflammation in obese mice.

Results as shown in figure 2, the contents of LPS and TNF- α in the HFD group were significantly higher than those in the normal group ($P < 0.001$). After 8 weeks of administration of piperine, the contents of LPS and TNF- α in serum were decreased significantly. At the same time, we detected that serum FFA had the same trend. Compared with the normal group, the content of FFA in the HFD group increased markedly, and it decreased significantly after piperine treatment ($P < 0.01$). Combined with the above results, piperine can reduce the levels of inflammatory factors LPS and TNF-α and enhance the degradation of FFA, and finally reduce the degree of inflammation in obese mice.

Piperine reduces intestinal inflammatory infiltration in obese mice.

The results of WB showed that piperine could improve the intestinal inflammation of obese mice. As shown in figure 3, compared with the normal group, the expression of intestinal inflammatory factors CD11c, IL-1 β and TLR-4 in the HFD group were significantly up-regulated ($P < 0.01$). It reflected the increase of intestinal inflammatory mediators, the activation of inflammatory pathway and the accumulation of M1-like macrophages in the intestine. Compared with the HFD group, the levels of CD11c and IL-1 β protein in the intestine were markedly decreased after treatment with piperine, and the activation of TLR4 inflammatory pathway was inhibited. Taken together, intragastic administration of piperine can markedly inhibit the inflammatory infiltration in the intestines of obese mice induced by 60% HFD.

Piperine protects the integrity of intestinal barrier in obese mice.

According to the WB results in figure 4, the expression of tight junction proteins ZO-1 and occludin in ileum of HFD group was significantly lower than that of normal group, reflecting the damage of intestinal structural integrity in obese mice. After treatment with piperine, the expression of ZO-1 and occludin in the ileum was significantly higher than that in the HFD group, which was consistent with the results of serum level of zonulin (figure 4B), indicating that piperine plays a positive role in maintaining the integrity of intestinal structure.

And the ileum HE staining (figure 4C) showed the changes of intestinal structural integrity. In the normal group, the ileal structure was intact and the intestinal villi were arranged neatly. While, the ileal structure was incomplete in the HFD group, the villi were irregular, the cells were broken and the local epithelium fell off. In the piperine group, the intestinal structure was relatively intact and the intestinal villi were neatly arranged and maintained a relatively stable structure, which reflected that piperine had a protective effect on the intestinal epithelium of HFD-induced obese mice.

Effects of piperine on the diversity of intestinal flora in obese mice

Venn diagram of OTU distribution Venn diagram is used to statistically analyze the number of common and unique OTUs in multiple groups, which intuitively shows the independence and overlap of OTUs in each group. The distribution of OTUs of 11 samples in the three groups is shown in figure 5A. There are common 250 OTUs distributed in the three groups of samples, that is, the three groups of samples have a high degree of similarity. In addition, there were 110 unique OTUs only in the normal group, 22 unique OTUs were distributed only in the HFD group, and 11 specific OTUs were present in the piperine group. It suggested the differences in species distribution among the three groups.

As shown in figure 5B and C, there were significant differences in the ACE index among the three groups, indicating that the species diversity of the piperine group was different from the normal group and the HFD group. It was also found that there were significant differences in Shannon index ($P<0.05$) among the three groups, indicating that the community richness of the piperine group was changed compared with the HFD group or the normal group.

Sequence classification analysis of intestinal flora at the phylum level

As shown in figure 6A, the abundance of three species of bacteria at the phylum level had certain differences in the intestinal flora of mice in each group. Compared with the normal group, the abundance of Firmicutes in the intestinal tract in the HFD group was significantly increased, while the abundance of Verrucomicrobia and Bacteroidetes was markedly decreased. Compared with the HFD group, the abundance of Verrucomicrobia and Bacteroidetes in the piperine group was obviously increased, but there was still a certain gap compared with the normal group. The results suggest that piperine can reduce the abundance of Firmicutes and increase the abundance of Verrucomicrobia and Bacteroidetes in the gut of obese mice. At the same time, we calculated the ratio of Firmicutes to Bacteroidetes in the intestinal flora of mice in each group(figure 6B), showing that the ratio of Firmicutes/Bacteroidetes in the HFD group was significantly higher than that in the normal group ($P<0.001$); and the ratio of

Firmicutes/Bacteroidetes in the piperine group decreased markedly ($P<0.05$).

Classification of intestinal flora at the class and genus level

The top four relative abundances of species at the class level are Clostridia, Bacterodia, Bacilli and Deltaproteobacteria (figure 7A). The total relative abundances of the three in the normal group, HFD group and piperine group are 92.1%, 95% and 94.2%, respectively. Compared with the normal group, Bacteroidia and Deltaproteobacteria in the HFD group decreased by 75.9% and 7.12%, respectively, while Clostridia and Bacteroidia increased by 158% and 235%, respectively; after 8 weeks of piperine intervention, compared with the HFD group, Bacilli and Deltaproteobacteria decreased by 64.42% and 50.31% in piperine group, and Bacterodia and Clostridia increased by 65.51% and 24.56%, respectively (figure 7B). The top four relative abundances of species at the order level are Clostridiales, Bacteroidales, Lactobacillales and Desulfovibrionales (figure 7C). Compared with the normal group, the Bacteroidales and Desulfovibrionales in the HFD group decreased by 75.95% and 7.84%, respectively, while the Clostridiales and Lactobacillales increased by 158.4% and 233.7%, respectively. After 8 weeks of piperine intervention, compared with the HFD group, Lactobacillales and Desulfovibrionales in the piperine group were decreased by 64.6% and 51.06%, respectively, while Clostridiales and Bacteroidales were increased by 24.78% and 65.5%, respectively (figure 7D). The results showed that the contents of Bacteroidetes, Bacteroidia, Bacteroidales and other species decreased in high-fat diet-induced obese mice, while the contents of Bacteroidetes, Bacterodia, Bacteroidales and other species increased after 8 weeks of piperine intervention, which changed the composition of intestinal flora. Helps alleviate the effects of high-fat diet-induced obesity.

Piperine increase the content of SCFA in intestinal feces of obese mice.

As shown in figure 8, compared with the normal group, the contents of acetic acid, propionic acid, butyric acid, isobutyric acid, isovaleric acid and valeric acid in the feces of the HDF group decreased by 59.56%, 84.31%, 92.70%, 31.80%, 60.79%, 73.08%, respectively, and the total amount decreased by 70.07%. After piperine treatment, the contents of acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid and isovaleric acid in the Piperine group increased by 16.56%, 106.40%, 107.22%, 6.89%, 66.16%, 102.54% compared with those of the HDF group, and the total amount increased by 36.97%. Therefore, long-term intervention with piperine can increase the contents of SCFA in the feces of HFD- induced obese mice.

Discussion

Obesity, especially abdominal obesity, is a common cause of diabetes. High calorie intake can lead to obvious intestinal inflammatory stress in obese patients, including the over-expression of inflammatory factors such as IL-1 β and LPS[11], suggesting the damage of intestinal barrier. The intestinal pathogenic bacteria and inflammatory mediators are easy to leak through the impaired intestinal wall into extra-intestinal tissues, induce low-degree metabolic inflammation, aggravate the systemic insulin resistance and oral glucose intolerance, then increase islet workload and eventually lead to the occurrence of diabetes[12]. In this study, it was found that long-term intervention with piperine could significantly reduce the levels of serum level of Gram-negative bacteria metabolite LPS, TNF- α and FFA, suggesting that metabolic inflammation was obviously alleviated in obese mice. Subsequently, the WB results showed that piperine decreased the over-expression of inflammation-related proteins such as IL-1β, TLR4 and CD11c, and up-regulated the tight junction proteins including ZO-1 and occludin in the ileum of obese mice, and the intestinal mucosal barrier was repaired to some extent.

In 16SrRNA high-throughput sequencing, piperine adjusted the composition of intestinal microorganisms in HFD-induced obese mice. It not only makes the intestinal flora diversity and community richness of obese mice close to the normal mice, but also makes the ratio of thick-walled bacteria to Bacteroides significantly lower than that of HFD group. The ratio of high thick-wall bacteria to Bacteroides is usually related as a biological marker of obesity[13], and the decrease of the ratio of the two bacteria affects the content of SCFAs, a metabolite in the intestinal tract of obese mice. SCFAs is the glycolysis product of incompletely digested carbohydrates by anaerobes in the intestinal tract, which regulates the absorption of various nutrients and the production of hormones in the intestinal tract, maintains the acidic environment in the intestinal tract, is conducive to the growth of beneficial bacteria, and contributes to the regulation of intestinal flora homeostasis and energy balance.

Piperine is the main pharmacological component of edible spice pepper. It has definite effects on weight loss and regulation of glucose and lipid metabolism in many obese mice models. Our previous studies found that piperine could inhibit M1-like polarization of macrophages in visceral adipose tissue of obese mice to a certain extent, significantly reduce metabolic inflammation, and delay the process of obesity-induced diabetes[14]. The reason is that the occurrence of metabolic inflammation is closely related to the disturbance of intestinal flora and intestinal leakage in the state of obesity. Based on the

results of this experiment, the regulation of piperine on intestinal flora and the protective effect of intestinal barrier may be potential mechanism of piperine in anti-obesity-related diabetes.

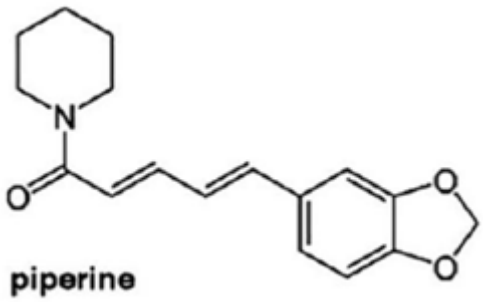

Figure1. Molecular structure of piperine

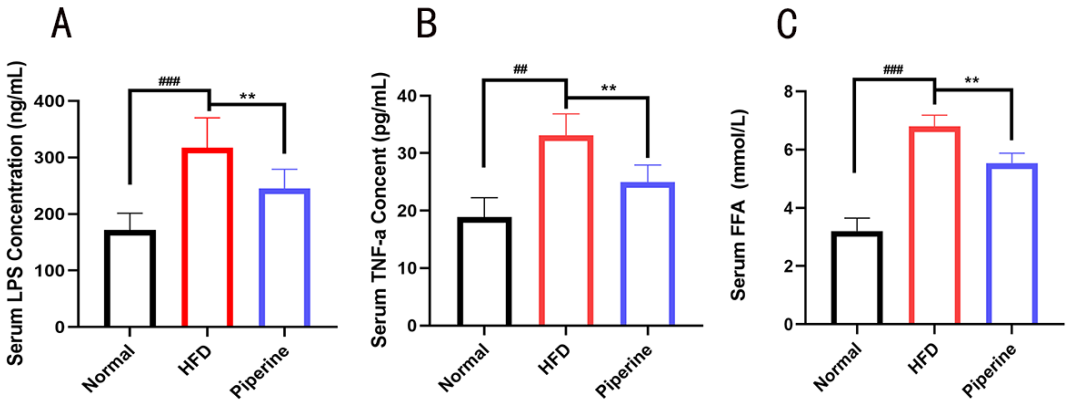

Figure 2. Piperine reduces the serum levels of inflammation related factors. A： serum LPS level； B： serum TNF-α level; C, serum FFA level. n=6-8. (## P<0.01, ### P<0.001 v.s. Normal group； **P<0.01 v.s. HFD group).

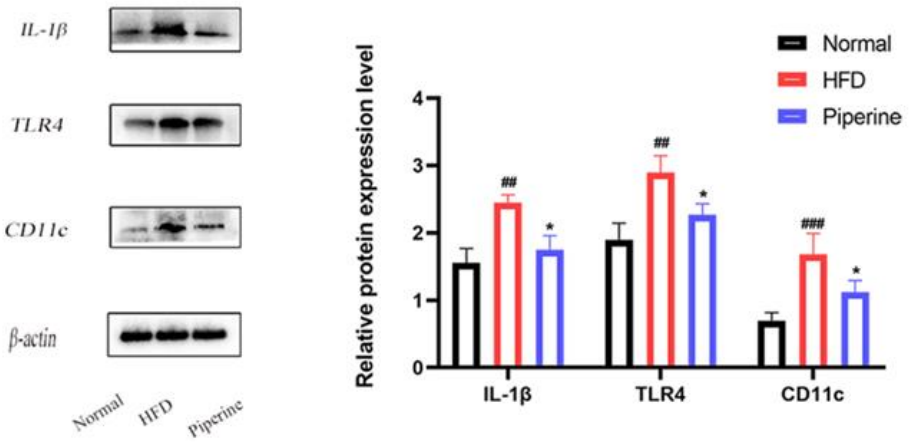

Figure 3. Piperine inhibits the over-expression of inflammation-related target proteins of ileum in obese mice. n=3-4, ## P<0.01, ### P<0.001v.s. Normal group； *P<0.05 v.s. HFD group.

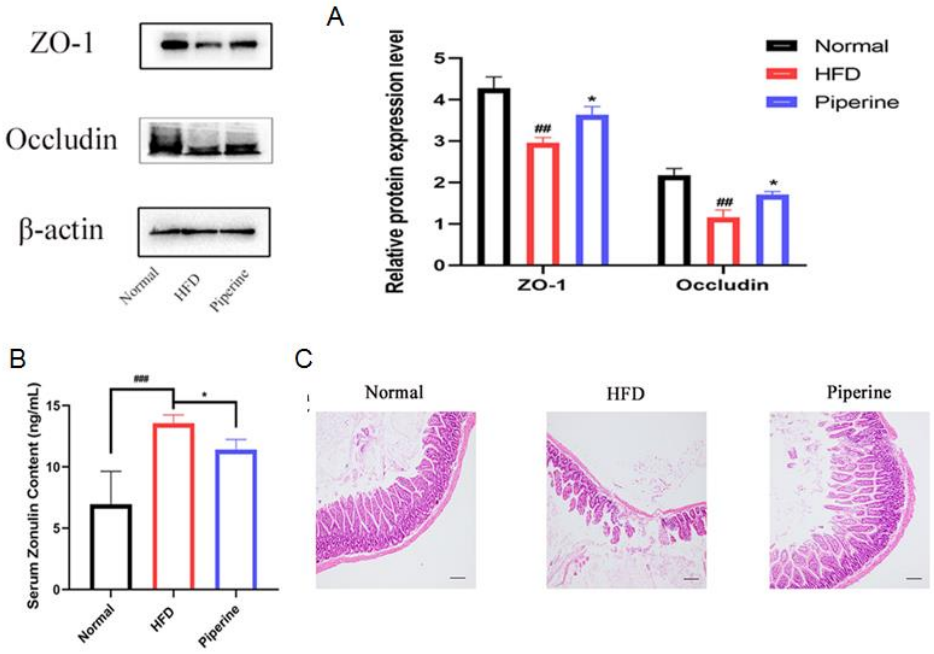

Figure 4. Piperine alleviates the damage of intestinal barrier in obese mice. A, expression and analysis of target proteins in ileum; B, serum zonulin level; C, morphological characteristics of ileum. n=3-4, ## P<0.01, ###P<0.001v.s. Normal group； *P<0.05 vs. HFD group.

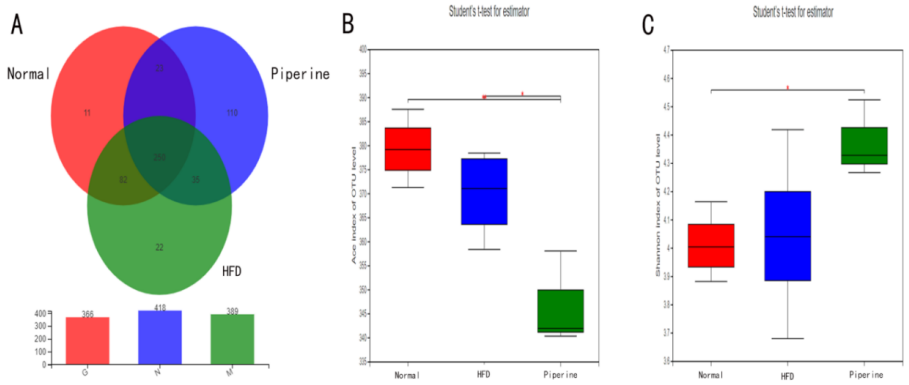

Figure 5 Piperine improves the disturbance of gut flora in HFD mice. A, Venn diagram of gut microbiota composition. B-C, Analysis of Ace and Shannon index of intestinal flora composition. Data are expressed as means ± SD. *P <0.05 vs. Normal group (Figure 5B-C).

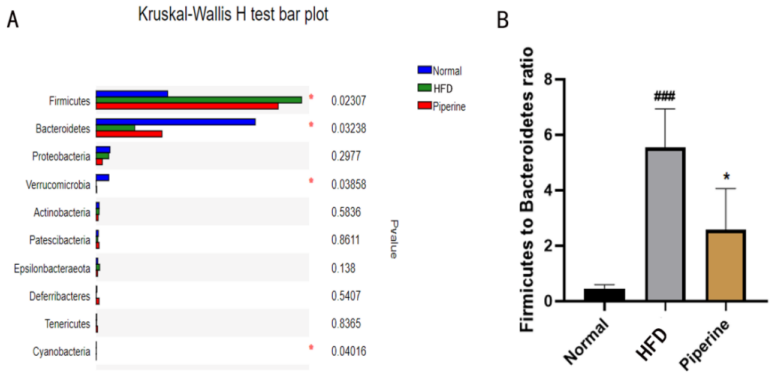

Figure 6 Relative abundance in phylum level (%) and the ratio of Firmicutes/Bacteroidetes in three groups. *P<0.05 vs HFD group (Figure 6A); ###P<0.001 vs Normal group (Figure 6B).

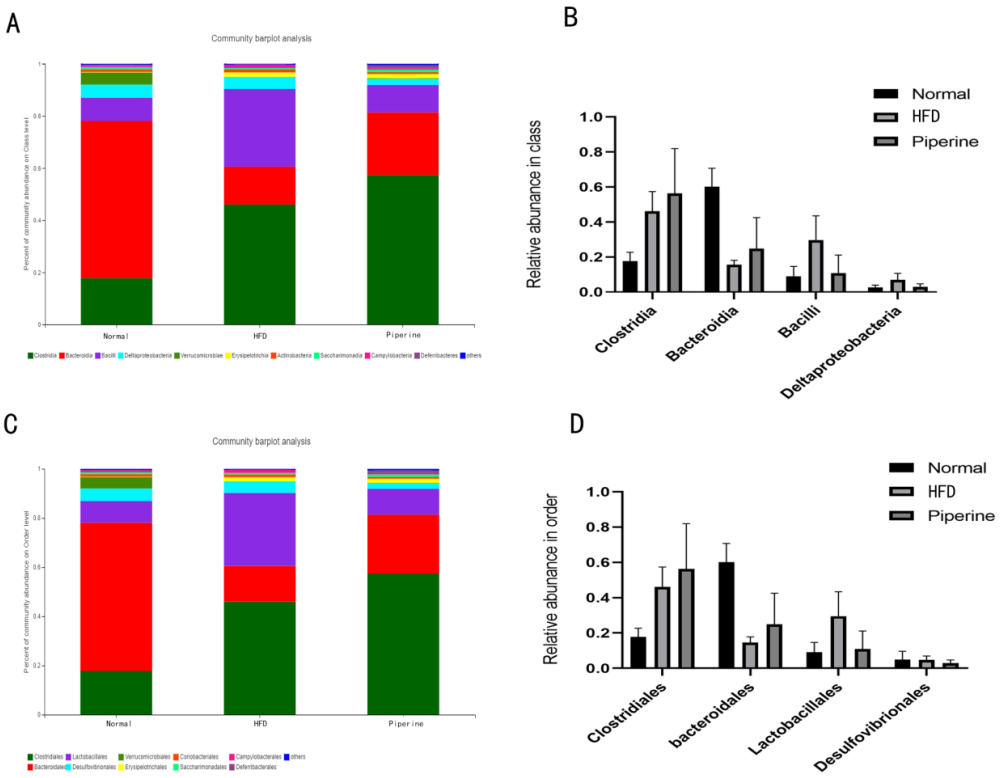

Figure7. Piperine alters HFD mice flora composition (class and genus levels). A, The composition of bacteria at the class level of each group; B, The proportion of the main bacterial species at the class level in each group; C, The composition of the flora at the genus level in each group; D, The proportion of main bacterial species in each group at the genus level.

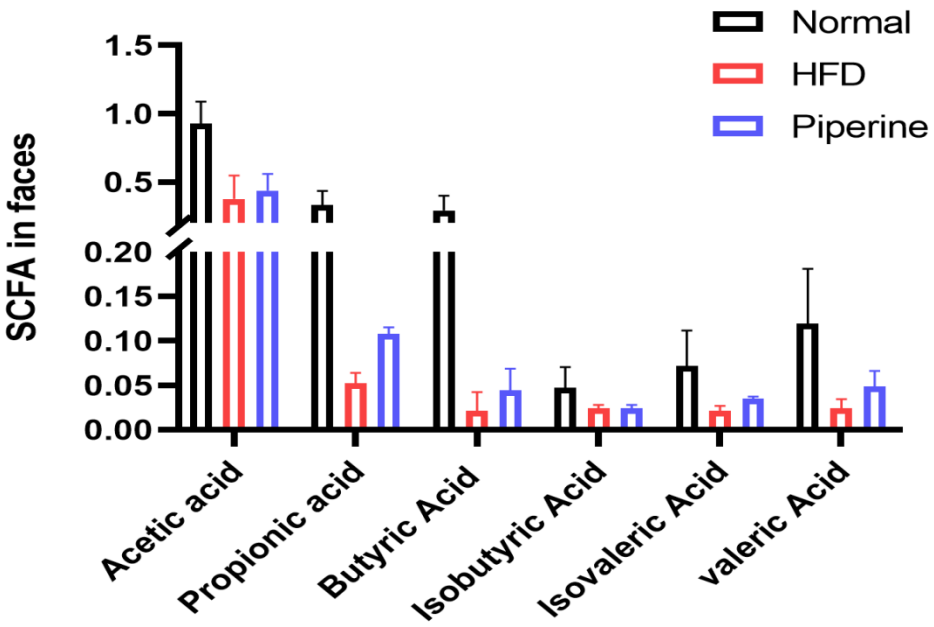

Figure 8 Effect of piperine intervention on SCFAs of feces in obese mice.

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Disclosure

The authors have declared that no conflict of interest exists. All the co-authors read and approved the final manuscript.

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