Acupuncture and Herbal Medicine

Formononetin mitigates atherosclerosis by reducing trimethylamine N-oxide production through modulation of gut microbiota in LDLR$^{-/-}$ mice

Guobin Zheng[1], Wenwen Zhang[2], Tao Jiang[3], Yuanli Chen[4], Yanfei Cheng[3], Jia Shi[3], Jie Ji[3], Linna Zhao[3], Zhenyu Zhao[1,*], Shangjing Liu[3,*], Chuanrui Ma[3,*]

[1]NHC Key Lab of Hormones and Development and Tianjin Key Lab of Metabolic Diseases, Tianjin Medical University Chu Hsien-I Memorial Hospital & Institute of Endocrinology, Tianjin, China; [2]Tianjin Institute of Gynecology Obstetrics, Tianjin Central Hospital of Gynecology Obstetrics, Tianjin, China; [3]First Teaching Hospital of Tianjin University of Traditional Chinese Medicine, National Clinical Research Center for Chinese Medicine, Tianjin, China; [4]Key Laboratory of Metabolism and Regulation for Major Diseases of Anhui Higher Education Institutes, Hefei University of Technology, Hefei, China

Abstract

Objective: Atherosclerosis is the primary cause of cardiovascular disease. Trimethylamine N-oxide (TMAO) is a gut microbiota-derived metabolite that contributes to atherogenesis. Formononetin is an isoflavonoid compound that exhibits potential anti-atherogenic properties, but there are no reports of its role in mitigating atherosclerosis by modulating gut microbiota to reduce TMAO production. In this study, formononetin demonstrated anti-atherosclerotic effects by modulating the gut microbiota to reduce TMAO production.

Methods: Eight-week-old male low-density lipoprotein receptor (LDLR)$^{-/-}$ mice were fed on a high-fat diet to induce atherosclerosis. Oil Red O staining was used to assess whether formononetin attenuated atherosclerosis. Fecal transplantation was used to determine whether the anti-atherosclerotic effect of formononetin was mediated by modulation of the gut microbiota. Metagenomic sequencing of the mouse gut microbiota and enzyme-linked immunosorbent assay were used to evaluate whether formononetin reduced TMAO production by modulating gut microbiota.

Results: Formononetin significantly reduced the plaque area in the entire aorta and aortic root and increased plaque stability. Fecal transplantation confirmed that the anti-atherosclerotic effect of formononetin was associated with modulation of the gut microbiota. Metagenomic sequencing of the mouse gut microbiota showed that formononetin reduced the relative abundance of Firmicutes, increased the relative abundance of bifidobacteria, and reduced serum TMAO levels. Moreover, formononetin reduced the expression of the *cutC* gene in the mouse gut microbiota.

Conclusions: Formononetin reduced TMAO production by modulating the gut microbiota, thus contributing to its anti-atherosclerotic effects. Modulation of the gut microbiota to reduce TMAO production may represent one of the mechanisms by which formononetin mitigates atherosclerosis.

Keywords: Atherosclerosis, Formononetin, Gut microbiota, Trimethylamine N-oxide, lipoprotein receptor (LDLR)$^{-/-}$ mice

Graphical abstract: https://links.lww.com/AHM/A211

Introduction

Cardiovascular disease has become the leading cause of death worldwide and is associated with high morbidity[1–2]. Atherosclerosis is the pathological basis of cardiovascular disease, characterized by chronic progression and refractory features[3–4]. Multiple risk factors contribute to atherosclerosis, such as inflammation, hyperlipidemia, and hyperglycemia[2]. The gut microbiota plays an important role in the development of atherosclerosis[5]. Some metabolites derived from the gut microbiota, such as short-chain fatty acids, significantly lower serum cholesterol[6] and

Guobin Zheng, Wenwen Zhang, and Tao Jiang contributed equally to this article.

**Corresponding author. Chuanrui Ma, E-mail: chuanruima2013@mail.nankai.edu.cn; Shangjing Liu, 18309149530@163.com; Zhenyu Zhao, E-mail: zhaozhenyu0858@163.com.*

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blood glucose[7], and downregulate inflammation[8]. Conversely, other metabolites, such as trimethylamine (TMA) N-oxide (TMAO), increase the risk of cardiovascular disease[9]. Thus, the gut microbiota has become an important target for the development of anti-atherosclerotic drugs[10].

The gut microbiota is a collective term for microorganisms that live in the host's intestinal tract, and their number far exceeds that of host cells. The microbial community, consisting of more than 1,000 species, is mutually beneficial and symbiotic with the host. Although there are individual differences in gut microbiota composition, the core populations remain in dynamic balance and help maintain intestinal homeostasis. The gut microbiota aids in food digestion; synthesizes essential vitamins; regulates the immune system; eliminates pathogens, toxins, and carcinogens; and preserves intestinal function. Disruption of this balance can trigger various diseases[11–12].

TMA is a microbiota-derived metabolite. Diets rich in eggs, meat, and fish products contain large amounts of choline, which is converted to TMA by the gut microbiota. TMA enters the liver *via* the portal circulation, where it is oxidized to TMAO by flavin monooxygenases (FMOs). One study showed that serum TMAO concentration was positively correlated with hypertension, hyperglycemia, and related conditions[13]. Clinical studies have also shown that elevated circulating TMAO concentrations are associated with significantly increased risks of cardiovascular disease[14–15], kidney disease[16], type 2 diabetes[17], and cancer[18]. Reducing TMAO production can ameliorate atherosclerosis[1], and TMAO has been identified as a novel and independent risk factor for this condition. TMAO is a metabolite of TMA, which is primarily derived from the degradation of dietary choline by the gut microbiota[19]. Therefore, modulation of the gut microbiota to reduce TMAO production has emerged as a promising strategy for anti-atherosclerotic therapy.

Traditional Chinese medicine has been shown to exert anti-atherosclerotic effects[20]. Formononetin is an isoflavonoid extracted from plants, such as *Dalbergia ecastophyllum*. The chemical structure of formononetin is 7-hydroxy-4'-methoxyisoflavone, which is structurally similar to that of estrogen[21]. Formononetin possesses antioxidant, anti-inflammatory, and anticancer properties[22–24]. Formononetin can attenuate the development of atherosclerosis by regulating the interplay between KLF4 and SRA[25]. Another study reported that formononetin can reshape the gut microbiota, prevent the progression of obesity, and improve host metabolism[26]. However, it has not yet been established whether formononetin exerts anti-atherosclerotic effects by modulating the gut microbiota to reduce TMAO production. In our study, fecal transplantation (FT) confirmed that the anti-atherosclerotic effect of formononetin was associated with modulation of the gut microbiota. Metagenomic sequencing of the mouse gut microbiota showed that formononetin increased the relative abundance of bifidobacteria, reduced serum TMAO levels, and thereby ameliorated atherosclerosis in low-density lipoprotein receptor (LDLR)$^{-/-}$ mice.

Materials and methods

Reagents

Mouse anti-α-smooth muscle actin (α-SMA; CAT# 14395-1-AP) polyclonal antibody was purchased from Proteintech Group, Inc (Wuhan, China). Oil Red O (CAT# 1320-06-5) was purchased from Sigma-Aldrich (Shanghai, China). Hematoxylin (CAT# G1005-1), Differentiation Solution (CAT# G1039, 500 mL), and alcoholic eosin (CAT# G1005-2) were purchased from Wuhan Service Bio Technology Co., Ltd (Wuhan, China). Sirius Red staining solution (CAT# G1472) was purchased from Solarbio Science & Technology Co., Ltd (Beijing, China). RAW264.7 complete medium (CAT# CM1002L) was purchased from Cellcook Biotech Co., Ltd (Guangzhou, China). Formononetin (CAT# S2299, purity: 99.97%) was purchased from Selleck Chem (Shanghai, China). A TMAO enzyme-linked immunosorbent assay (ELISA) kit (CAT# ELK0789) was purchased from ELK Biotechnology Co., Ltd (Wuhan, China). A stool DNA extraction kit (CAT# 18820ES50) and RNA extraction kit (CAT# 19221ES50) were purchased from Yeasen Biotechnology (Shanghai, China) Co., Ltd. The reverse transcription reagent (CAT# R233-01) was purchased from Vazyme Biotech Co., Ltd (Nanjing, China), and the Ultra SYBR Mixture (Low ROX) (CAT# CW2601H) was purchased from Jiangsu Cowin Biotech Co., Ltd (Jiangsu, China).

Animal studies

All animal care and experimental protocols for *in vivo* studies conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH; NIH Publication No. 85-23, revised 1996) and were approved by the Chu Hsien-I Memorial Hospital of Tianjin Medical University (DXBYY-IACUC-2023014). Eight-week-old male LDLR$^{-/-}$ mice were purchased from Changzhou Kavens Laboratory Animal Co., Ltd (Jiangsu, China) and maintained at the Animal Center of Chu Hsien-I Memorial Hospital of Tianjin Medical University. Mice were allowed free access to food and water under alternating light and dark cycles of 12 hours. After 1 week of acclimatization, all mice were randomly divided into three groups: Ctrl group, low-dose formononetin treatment (FNT-L group), and high-dose formononetin treatment (FNT-H group). Atherosclerosis was induced by feeding a high-fat diet (HFD; MD12015HL, Medicience

Table 1

The nutritional and ingredient composition of the diet

Composition	Ratios (%)
Casein	19.50
Corn starch	15.00
Sucrose	33.95
Cellulose	5.00
Anhydrous milk fat	21.00
Cholesterol	0.15
Multivitamin and multimineral	5.40
Total	100

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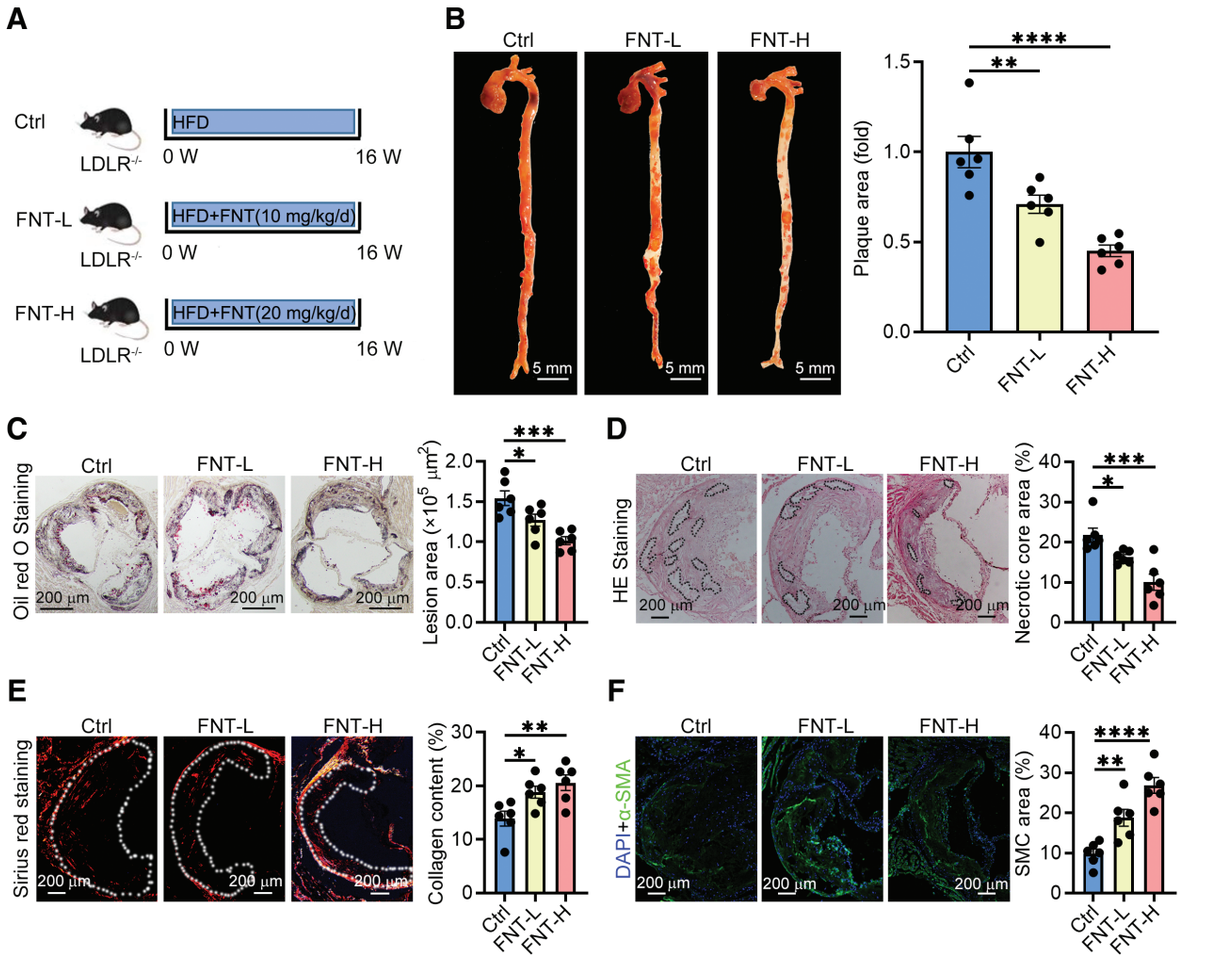


Figure 1. Formononetin ameliorated atherosclerosis in LDLR$^{-/-}$ mice and increased plaque stability. (A) LDLR$^{-/-}$ mice were randomized into three groups and the experimental protocol is shown (n = 6). (B and C) Lesion in whole aorta and aortic root cross-sections were assessed by Oil Red O staining and analyzed quantitatively (n = 6). (D) Necrotic core size was determined by HE staining, and the positive area was analyzed quantitatively (n = 6). (E) Collagen content in plaques was assessed by Sirius Red staining, and the collagen area as a proportion of plaque area was calculated (n = 6). (F) Expression of α-SMA in plaques was detected by immunofluorescence staining (n = 6). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared with the Ctrl group. All data are presented as mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. α-SMA: α-Smooth muscle actin; ANOVA: Analysis of variance; FNT-H: High-dose formononetin treatment; FNT-L: Low-dose formononetin treatment; HE: Hematoxylin & eosin; LDLR: Low-density lipoprotein receptor; SEM: Standard error of the mean; SMC: Smooth muscle cell.

Ltd, Yangzhou, Jiangsu, China), the nutritional and ingredient composition of which is shown in Table 1. The Ctrl group served as the model control, whereas the FNT-L and FNT-H groups received formononetin by gavage at the doses of 10 and 20 mg/kg·d respectively. All mice were fed and treated for 16 weeks (Figure 1A).

Cell culture

Mouse peritoneal macrophages were extracted and seeded in 24-well plates. Each well received 500 μL of RAW264.7 complete medium containing 1% penicillin/streptomycin. After 24 hours, adherent cells were stained with Oil Red O staining.

Oil Red O staining

An Oil Red O stock solution was prepared by dissolving 0.5 g Oil Red O powder in 100 mL of isopropanol. The stock solution was then diluted with purified water at a ratio of 3:2 to obtain a working solution. Whole aortas and frozen sections of the aortic root were stained with the working solution for 1 hour. Adherent cells were washed three times with phosphate-buffered saline (PBS), immersed in the working solution for 1 hour, and finally sealed with glycerol gelatin. The whole aorta was observed using a stereomicroscope (OFormononetin C, SZ680), and frozen sections and cells were imaged using a light microscope (Olympus BX43). The lesion area of the whole aorta and aortic root and number of foam cells were calculated using ImageJ software.

Hematoxylin & eosin staining

Frozen sections of the aortic root were immersed in purified water for 10 minutes before staining, then immersed in hematoxylin for 5 minutes, and gently rinsed with running water. Subsequently, the sections were immersed in eosin for 3 minutes, rinsed again with running water, dehydrated, cleared, sealed, and imaged using a light

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Table 2

The sequences of primers for qPCR analysis

Gene	Forward	Reverse
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
FMO3	ACTGGTGGTACACAAGGCAG	ATGGTCCCATCCTCAAACACA
cutC	CAGATCGACGACTTCGGC	GTGTTGATCCAGGTCCAGTT
16S rRNA	GTGTGGGAGAGGTAGGTGGA	TGCGTTAGCTGCATTACTGG

qPCR: Quantitative polymerase chain reaction.

microscope (Olympus BX43). Plaque stability was assessed by calculating the necrotic core area.

Immunofluorescence staining

Frozen sections of the aortic root were immersed in purified water for 10 minutes before staining. Tissues were permeabilized with an immunofluorescence permeabilization solution for 5 minutes and blocked with 2% bovine serum albumin for 1 hour. The primary antibody was applied to fully cover the tissue, and sections were placed in a humidified chamber and stored at 4°C overnight. The next day, sections were washed three times with PBS for 10 minutes each, incubated with the secondary antibody at 37°C for 1 hour, washed three additional times with PBS, and sealed with a coverslip in the dark. Images were captured using an orthogonal fluorescence microscope (Leica, Wetzlar, Germany, DM6000B).

FT experiment

Eight-week-old male LDLR$^{-/-}$ mice were randomly divided into two groups and gavaged with a mixture of vancomycin (50 mg/kg), neomycin (100 mg/kg), and metronidazole (100 mg/kg) twice daily for 2 weeks to deplete the endogenous gut microbiota of the mice. Fresh feces were collected from mice in the Ctrl and FNT-H groups, homogenized, and gavaged into antibiotic-treated mice for 12 weeks. All mice were fed an HFD.

Metagenomic sequencing of mouse gut microbiota

After 16 weeks of treatment, fresh feces were collected from mice in the Ctrl, FNT-L, and FNT-H groups, immediately frozen in liquid nitrogen, and transferred on dry ice to Shenzhen Wekemo Information Technology Co. (Shenzhen, China) for metagenomic sequencing and data analysis.

The sequencing procedure, provided by Shenzhen Wekemo Information Technology Co., was as follows: DNA was extracted from mouse fecal samples using the cetyltrimethylammonium bromide method, DNA integrity, potential contamination, and concentration were assessed using an Agilent 5400. A sequencing library was generated using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA; Catalog#: E7370L) according to the manufacturer's recommendations, and index codes were added to each sample. Genomic DNA was fragmented to an average size of 350 bp by sonication. The fragments were end-polished, A-tailed, and ligated with a full-length adapter for Illumina sequencing, followed by polymerase chain reaction (PCR) amplification. PCR products were purified using the AMPure XP system (Beverly, MA, USA). Library quality was assessed with the Agilent 5400 system (Agilent, Santa Clara, CA, USA) and quantified by quantitative polymerase chain reaction (qPCR, 1.5 nM). Qualified libraries were pooled and sequenced on Illumina platforms using the PE150 strategy, according to the effective library concentration and data requirements.

Enzyme-linked immunosorbent assay

TMAO levels in mouse serum were determined using an ELISA kit according to the manufacturer's instructions.

Quantitative polymerase chain reaction

Fresh feces were collected and frozen in liquid nitrogen. Approximately 200 mg of feces was used to extract DNA from the gut microbiota with a stool DNA extraction kit. RNA was extracted from the mouse liver using an RNA extraction kit and reverse-transcribed into cDNA. The mRNA expression levels of *cutC* and FMO3 were determined by qPCR. Primer sequences are shown in Table 2.

Statistical analysis

Data were analyzed with GraphPad Prism (version 9.0.0) for Windows. Continuous variables with normal distributions are presented as mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was performed, followed by the Tukey *post hoc* test. An unpaired Student *t* test was used for comparison between two groups. Statistical significance was set at $P < 0.05$.

Results

Formononetin reduced atherosclerotic lesion area and increased plaque stability

Plaque formation is the primary feature of atherosclerosis[27]. Oil Red O staining of the whole aorta and aortic root sections showed that the plaque area was significantly reduced by formononetin treatment (Figure 1B and C). The most common cause of arterial thrombosis is plaque rupture, and high plaque vulnerability is associated with cardiovascular events[28]. Hematoxylin & eosin (HE) staining of aortic root sections showed that formononetin significantly reduced the necrotic

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core area and increased the thickness of the fibrous cap (Figure 1D). In plaques, smooth muscle cells secrete collagen to cover the lipid core and increase stability. Sirius Red staining and immunofluorescence staining of α-SMA showed that collagen content and smooth muscle cell numbers within plaques were significantly increased by formononetin treatment (Figure 1E and F). These findings indicate that formononetin suppresses atherogenesis and promotes the plaque stability.

Formononetin attenuated atherosclerosis by modulating the gut microbiota

The oral bioavailability of formononetin is low[29–30]. Therefore, we focused on whether its anti-atherosclerotic effect was mediated by modulation of the gut microbiota. To test this, we performed an FT experiment (Figure 2A). The results showed that the aortic plaque area was significantly reduced in the HFD + FNT-FT group compared with that in the HFD-FT group (Figure 2B and C). ELISA results demonstrated that serum TMAO levels in the HFD + FNT-FT group were significantly lower than those in the HFD-FT group (Figure 2D). Foam cell formation, a hallmark of early atherosclerosis[31], was assessed by Oil Red O staining of mouse peritoneal macrophages. The number of foam cells was significantly reduced in the HFD + FNT-FT group compared with that in the HFD-FT group (Figure 2E). Collectively, the FT experiment confirmed that formononetin attenuates atherosclerosis by modulating the gut microbiota.

Formononetin modulated gut microbiota to reduce TMAO production

Metagenomic sequencing was used to evaluate the effect of formononetin on gut microbiota. The results showed that the number of species with linear discriminant analysis (LDA) >2 in the relative abundance of phyla was seven in the Ctrl group, two in the FNT-L group, and four in the FNT-H group. The Ctrl group contained Firmicutes, Nitrospirae, Mucoromycota, Proteobacteria, Basidiomycota, Fungi, and Ascomycota, with Firmicutes showing the highest relative abundance (LDA >5). The FNT-L group contained Peploviricota and Candidatus_Melainabacteria, whereas the FNT-H group contained Actinobacteria, Bacteroidetes, Phixoviricota, and Sangervirae (Figure 3A). The gut microbiota that mainly increase the TMA synthesis are *Escherichia* (Proteobacteria), *Clostridium* (Firmicutes), and *Bacteroides* (Bacteroidetes), whereas Bifidobacterium (Actinobacteria) decreases the TMA synthesis[32]. The stacked histogram of the relative abundance of Firmicutes showed that Firmicutes were most abundant in the Ctrl group. After formononetin treatment, the relative abundance of Firmicutes was reduced, whereas the relative abundance of Actinobacteria increased (Figure 3B and E). Analysis at the genus level revealed that the relative abundance of *Bifidobacterium* increased significantly after formononetin treatment (Figure 3C, D, and F). Subsequently, we measured TMAO content in mouse serum and found that serum TMAO levels were significantly reduced by formononetin treatment (Figure 3G). Bifidobacterium reduces serum TMAO[33–34]. Our results verified that formononetin lowers serum TMAO, which is associated with an increased relative abundance of Bifidobacterium.

Formononetin regulated the gene cutC in mouse gut microbiota

Analysis of altered genes in the FNT-H and Ctrl groups showed that a total of 171,927 genes were detected by metagenomic sequencing, of which 59,749 were upregulated and 44,773 were downregulated (Figure 4A). Gene enrichment analysis indicated that the nitrogen compound metabolic process was the most affected pathway. Because the chemical structure of choline is 2-hydroxy-N,N,N-trimethylethanaminium, this pathway may be related to choline metabolism (Figure 4B). Further analysis of the top 20 genes with the largest changes showed five regulated genes and 15 downregulated genes. Among these 20, only four genes were identified in the database_UniRef90_K7VRF0, UniRef90_P35881, UniRef90_K7VWI5, and UniRef90_H2A9K5_all annotated as transposases. The remaining 16 genes lacked corresponding database information. Transposases are key enzymes that catalyze the migration of transposons within or between genomes. Their core function is to mediate the excision of transposons from their original location (cut-and-paste mechanism) or the insertion of new copies after replication (copy-and-paste mechanism), thereby potentially interfering with gene synthesis[35–36]. A literature review did not reveal any reports linking transposases directly to TMA production; however, they may influence the genomic stability of TMA synthesis-related genes and thereby indirectly affect TMA synthesis. We further analyzed the sequencing data by fold change and filtering and found that formononetin significantly reduced the expression of *cutC* gene (ranked 4301 among all differential genes, UniRef90_Q9CE53), which encodes choline TMA-lyase, an enzyme that converts choline to TMA[37]. To more intuitively demonstrate the effect of formononetin on *cutC*, we created a volcano plot containing *cutC,* the 20 most differentially expressed genes and the 20 least differentially expressed genes (Figure 4C). In addition, qPCR validation showed that *cutC* expression was significantly downregulated by formononetin treatment (Figure 4D). Because FMO3 is a key enzyme in the conversion of TMA to TMAO[38], we examined FMO3 expression in mouse livers and found that neither the FNT-L nor FNT-H group affected FMO3 expression (Figure 4E). These results suggest that formononetin reduces TMA production, and consequently TMAO production, by downregulating *cutC* in the gut microbiota.

Discussion

Atherosclerosis is a major cause of cardiovascular disease and is associated with most deaths worldwide[4]. Inflammation and disorders of lipid metabolism are the main features of atherosclerosis, and traditional therapies have focused on cholesterol-lowering and anti-inflammation. TMAO, a product of dietary choline metabolism, is

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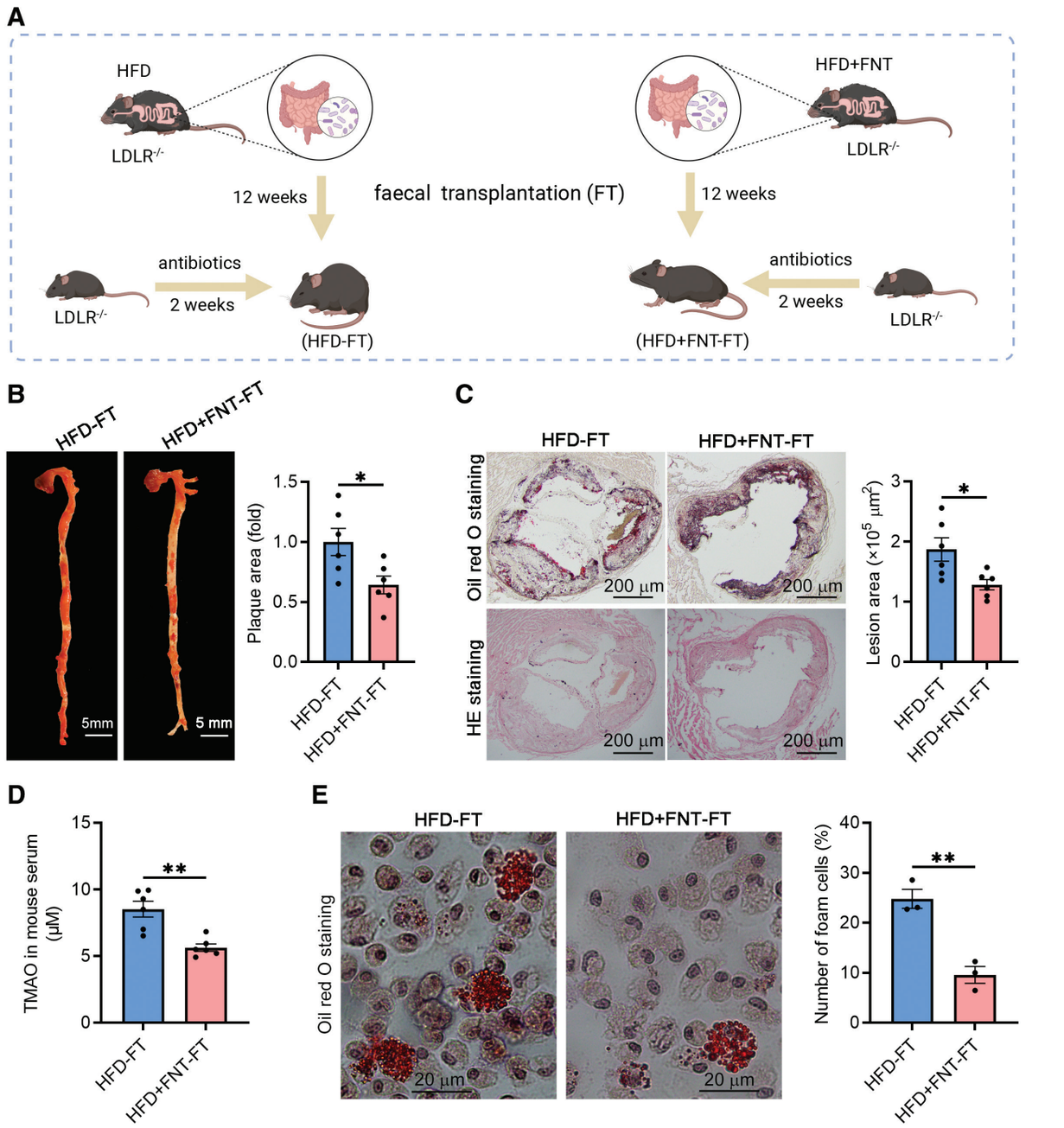


Figure 2. Formononetin ameliorated atherosclerosis by modulating gut microbiota. (A) LDLR$^{-/-}$ mice were randomly divided into two groups for fecal transplantation experiments (n = 6). (B and C) Lesions in whole aortas and aortic root cross-sections were assessed by Oil Red O staining and analyzed quantitatively (n = 6). (D) Serum TMAO levels were determined by ELISA (n = 6). (E) Peritoneal macrophages were stained with Oil Red O to quantify foam cells (n = 3). *P < 0.05, **P < 0.01, compared with the HFD-FT group. All data are presented as mean ± SEM. Data were analyzed by an unpaired Student t test. ELISA: Enzyme-linked immunosorbent assay; FNT: Formononetin; HFD-FT: High-fat diet-fecal transplantation; LDLR: Low-density lipoprotein receptor; SEM: Standard error of the mean; TMAO: Trimethylamine N-oxide.

inextricably linked to gut microbiota. Over the past few years, TMAO has been identified as a novel and independent risk factor for atherosclerosis[39]. TMAO is derived from the oxidation of TMA by FMOs in the liver, whereas TMA is mainly produced by gut microbiota that metabolize dietary choline. Therefore, regulating gut microbiota can reduce TMA production and ultimately decrease TMAO synthesis. Accordingly, the gut microbiota has become a promising therapeutic target for atherosclerosis[40]. Formononetin is an isoflavone derived from plants, and our research confirmed that it attenuates atherosclerosis and increases plaque stability in LDLR$^{-/-}$ mice. However, whether formononetin ameliorates atherosclerosis, specifically modulation of the gut microbiota to reduce TMAO production, requires further investigation.

The gut microbiota comprises a large number of microorganisms present in the intestinal tract of the host that rely on the host's intestinal tract to live and assist the host in performing various physiological and

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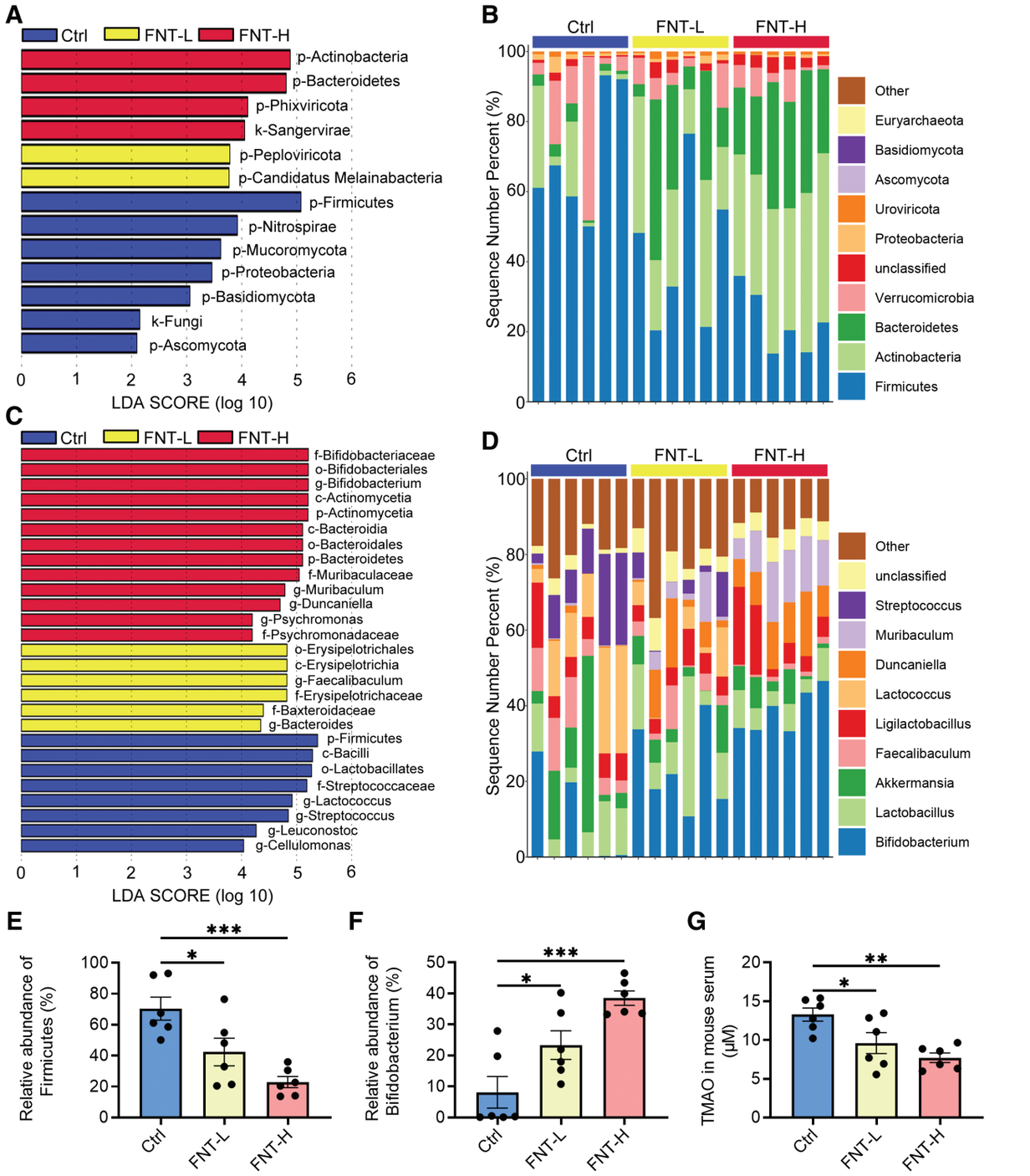


Figure 3. Formononetin modulated gut microbiota to inhibit TMAO production. (A) LEfSe analysis of the relative abundance of phyla in mouse gut microbiota ($n = 6$). (B) Stacked histogram of the relative abundance of phyla in mouse gut microbiota ($n = 6$). (C) LEfSe analysis of the relative abundance of genera in mouse gut microbiota ($n = 6$). (D) Stacked histogram of the relative abundance of genera in mouse gut microbiota ($n = 6$). (E) Relative abundance of Firmicutes at the phylum level ($n = 6$). (F) Relative abundance of Bifidobacterium at the genus level ($n = 6$). (G) Serum TMAO content was determined using an ELISA kit ($n = 6$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared with the Ctrl group. All data are presented as mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. ANOVA: Analysis of variance; ELISA: Enzyme-linked immunosorbent assay; FNT-H: High-dose formononetin treatment; FNT-L: Low-dose formononetin treatment; LDA: Linear discriminant analysis; LEfSe: Linear discriminant analysis Effect Size; SEM: Standard error of the mean; TMAO: Trimethylamine N-oxide.

biochemical functions simultaneously. The rich intestinal environment of the host provides the gut microbiota with a habitat[41], which form mutually beneficial symbiotic relationships with the host, plays an extremely important role in maintaining the host's normal physiological functions. The number of intestinal microorganisms far exceeds the number of host cells, and they possess metabolic functions that the host does not; therefore, the gut microbiota and host constitute a superorganism[42]. Under normal conditions, the gut microbiota

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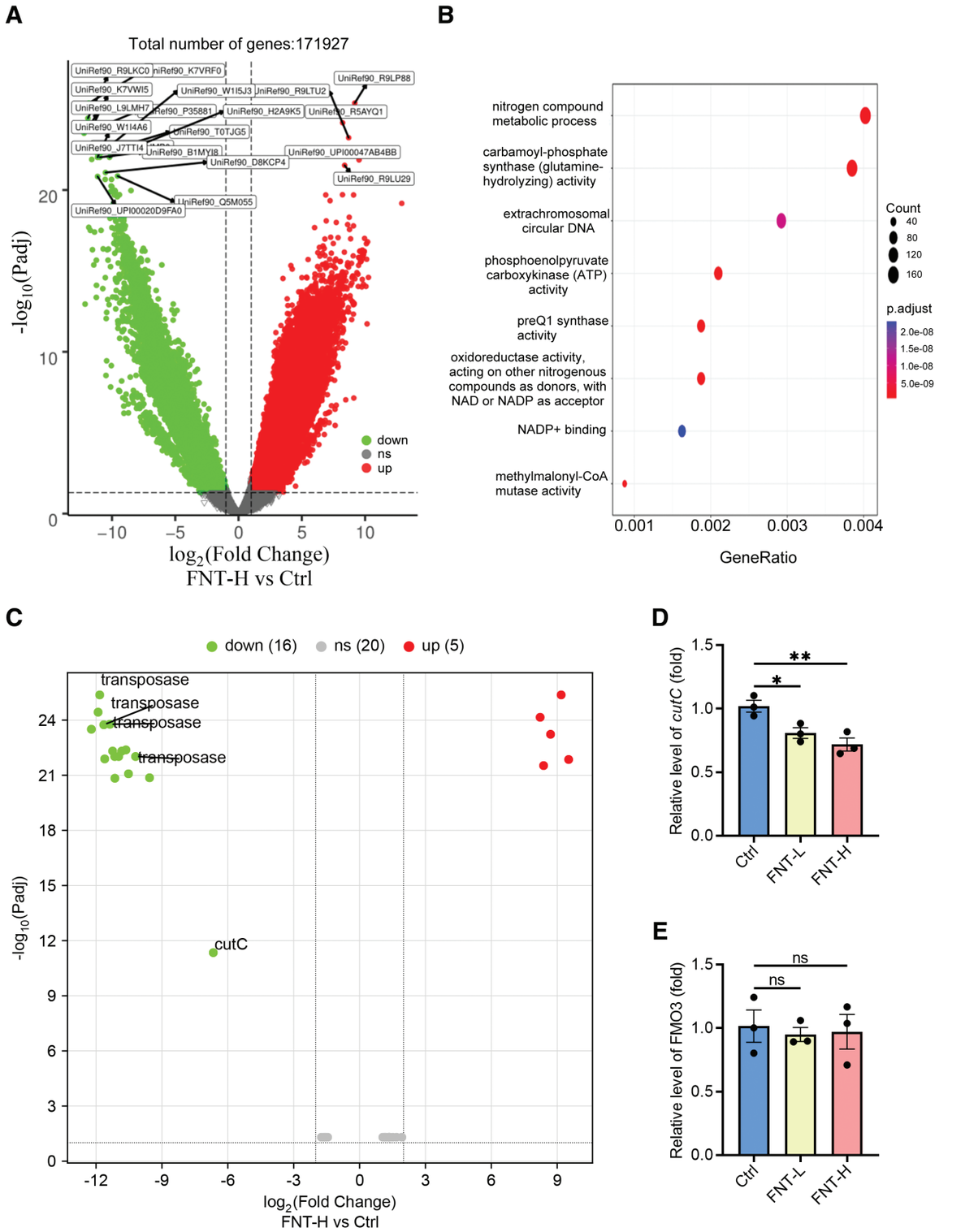


Figure 4. Genetic changes identified by metagenomic sequencing of the mouse gut microbiota. (A) Volcano plot of differentially expressed genes identified by metagenomic sequencing. (B) Gene enrichment analysis of differentially expressed genes. (C) Volcano plot highlighting *cutC*, the 20 most upregulated genes, and the 20 most downregulated genes. (D) Expression level of *cutC* in mouse gut microbiota determined by qPCR ($n = 3$). (E) Expression level of FMO3 in mouse liver determined by qPCR ($n = 3$). *$P < 0.05$, **$P < 0.01$, compared with the Ctrl group. All data are presented as mean ± SEM. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test. ANOVA: Analysis of variance; FMO: Flavin monooxygenase; qPCR: Quantitative polymerase chain reaction; SEM: Standard error of the mean.

maintains homeostasis. A chronic HFD reduces the overall gut microbiota, alters the abundance of bacterial species, and increases intestinal permeability[43–44], which disrupts gut microbiota homeostasis, leading to altered intestinal metabolites and chronic inflammation, and ultimately causing various diseases[45].

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We performed an FT experiment to confirm that formononetin attenuates atherosclerosis by modulating the gut microbiota. TMA is the precursor for the synthesis of TMAO and is mainly derived from the breakdown of choline in food by gut microbiota choline-TMA-lyase, in which Firmicutes play an important role[46–48]. We performed metagenomic sequencing of the mouse gut microbiota, and the results showed that the relative abundance of Firmicutes was reduced, whereas the relative abundance of Actinobacteria was increased following treatment with formononetin. Further analysis of the relative abundance at the genus level revealed that Bifidobacterium significantly increased after treatment with formononetin. Bifidobacterium is a probiotic bacterium[49], and it not only lowers cholesterol[50], but also reduces serum TMAO[34]. Our results confirmed that serum TMAO levels were significantly decreased in mice treated with formononetin. *CutC* is a choline TMA-lyase that converts choline to TMA. Further analysis of altered genes in the FNT-H and Ctrl groups showed that formononetin significantly reduced the expression of the *cutC* gene. In addition, TMA is oxidized to TMAO in the liver by FMOs. We examined the gene expression of FMO3 in mouse livers and found that neither the FNT-L nor the FNT-H group affected FMO3 expression. It must be acknowledged that our experiment has certain limitations. There may be differences between the relative abundances of gut microbiota species in humans and those in mice. Additionally, the experiment did not include a validation study involving the repopulation of the model group mice with the differential microbiota.[51]

Conclusion

In conclusion, our study revealed that formononetin reduced TMAO production by increasing the relative abundance of Bifidobacterium and reducing expression of cutC in the mouse gut microbiota, there by attenuating atherosclerosis. Undeniably, formononetin also has anti-inflammatory effects. Inflammation initiates atherosclerosis and persists throughout its progression, and it persists its progression. Therefore, reducing TMAO production is not the sole mechanism by which formononetin alleviates atherosclerosis; it may exert multiple effects to achieve anti-atherosclerotic activity.

Conflict of interest statement

The authors declare no conflict of interest.

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Author contributions

Guobin Zheng participated in the performance of the research, writing of the paper, and data analysis. Wenwen Zhang participated in the performance of the research and data analysis. Tao Jiang and Linna Zhao participated in data analysis. Yuanli Chen contributed new reagents or analytic tools. Yanfei Cheng and Jia Shi participated in the performance of the research. Jie Ji contributed new reagents or analytic tools. Zhenyu Zhao and Shangjing Liu participated in the writing of the paper and contributed new reagents or analytic tools. Chuanrui Ma participated in research design and writing of the paper and contributed new reagents or analytic tools.

Ethical approval of studies and informed consent

All animal care and experimental protocols for *in vivo* studies conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH; NIH Publication No. 85-23, revised 1996) and were approved by the Chu Hsien-I Memorial Hospital of Tianjin Medical University (DXBYY-IACUC-2023014).

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Data availability

Raw data of metagenomics sequencing has been uploaded to the National Library of Medicine (submission: SUB14819930), website: https://submit.ncbi.nlm.nih.gov/subs/sra/. The data that support the findings of this study are available from the corresponding author, Chuanrui Ma, upon reasonable request.

Declaration of generative AI in scientific writing

The conception and writing of this article were completed by all authors. Artificial intelligence (www.deepl.com) was used to polish the sentence coherence and revise punctuation. All references in this section were manually searched and inserted. After using the above tools/services, the authors have reviewed and revised the content as needed and take full responsibility for the content of this publication.

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