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Research Article

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**Polydatin combined with hawthorn flavonoids alleviate high fat diet induced atherosclerosis
by remodeling the gut microbiota and glycolipid metabolism**

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ABSTRACT

Background: Atherosclerosis is a widely studied pathophysiological foundation of cardiovascular diseases. The gut microbiota and their metabolites are considered independent risk factors for atherosclerosis. Polydatin combined with hawthorn flavonoids (PH), as the extracts of *Polygonum cuspidatum* Sieb. et Zucc. (*P. cuspidatum*) and *Crataegus pinnatifida* Bunge (*C. pinnatifida*), have

shown excellent cardiovascular protective effects. However, the underlying mechanism requires further investigation. Our study aimed to explore the anti-atherosclerotic mechanism of PH through gut microbiota and their metabolites.

Methods: ApoE^{-/-} mice were fed either a normal-chow diet or a high-fat diet. The PH group received varied doses of polydatin and hawthorn flavonoids: a high dose (polydatin 200 mg/kg daily; hawthorn flavonoids 100 mg/kg daily), a medium dose (polydatin 100 mg/kg daily; hawthorn flavonoids 50 mg/kg daily), and a low dose (polydatin 50 mg/kg daily; hawthorn flavonoids 25 mg/kg daily). The control and model groups were administered distilled water (0.2 mL daily). The experiment lasted for 24 weeks.

Results: PH administration significantly reduced lipid and inflammatory cytokine levels, meanwhile, the atherosclerotic lesions in a high-fat diet-induced ApoE^{-/-} mice were significantly decreased. Additionally, PH also inhibited the enhancement of trimethylamine N-oxide (TMAO), trimethylamine (TMA) levels of HFD-induced ApoE^{-/-} mice by regulating the expression of hepatic flavin-containing enzyme monooxygenase 3 (FMO3). 16S rRNA sequencing results demonstrated that high-dose PH treatment reduced *Firmicutes/Bacteroidetes* ratio, increased the abundance of *Actinobacteriota*, *Atopobiaceae* and *Coriobacteriaceae*, and decreased the abundance of *Desulfobacterota*. *Norank_f_Muribaculaceae* was enriched in the medium-dose PH and simvastatin groups, and *Lactobacillus* was mainly increased in the simvastatin and the low-dose PH groups. According to the metagenetic results, functional annotations also suggested that the biological processes of each group mainly focused on metabolism-related processes. Specifically, PH may regulate the abundance of TMA-producing bacteria (*Coriobacteriaceae*, *Desulfovibrio*, *Muribaculum*, and *Clostridium*) and related enzymes in glycolipid metabolic pathways to exert an

important effect on the prevention of atherosclerosis.

Conclusions: Our results suggested that PH could regulate the glucolipid metabolism-related pathway, attenuate inflammatory cytokine levels, and reduce atherosclerotic plaques by remodeling gut microbiota.

Trial registration: Not applicable.

Keywords: atherosclerosis, gut microbiota, trimethylamine N-oxide, polydatin, hawthorn flavonoids

1. Introduction

Cardiovascular diseases (CVDs) are the leading cause of mortality worldwide (1). Atherosclerosis is the pathological basis of CVDs and is characterized by chronic inflammatory artery disease accompanied by disturbances in glucose and lipid metabolism (2). Inflammation spreads throughout the process of atherosclerosis, and in combination with lipid deposition, aggravates the progression of atherosclerotic plaques (3). Risk factors, including dyslipidemia and diabetes, promote atherosclerosis progression (4). There has been increasing evidence that gut microbiota can regulate gut microbial homeostasis in the human body (5). The gut microbiota is responsible for converting different complex substances into bioactive metabolites (6) and is considered an indicator of host pathology, including CVDs (7). Thus, on one hand, gut microbiota may indirectly affect atherosclerosis by increasing the risk of glucose and lipid metabolism disorders, such as obesity and diabetes (8); on the other hand, gut microbiota influences atherosclerosis by enhancing the direct risk factors of atherosclerotic lesions through its metabolites (9).

Trimethylamine-N-oxide (TMAO) is generated from dietary sources, which are widely present in high-protein diets and dairy products (10). Intriguingly, trimethylamine (TMA)-producing

bacteria, a type of specific gut bacteria (11), may be involved in the metabolism of these foods by producing TMA-lyse (mainly choline-TMA lyase [CutC/D]) (12) , and the oxidation of the hepatic flavin-containing enzyme monooxygenase 3 (FMO3) also participates in the microbial pathways involved in TMA/TMAO formation (13). Recent studies have reported that choline utilization (cut) gene clusters, such as *Lachnoclostridium*, *Desulfovibrio*, and *Clostridium*, promote the conversion of choline to TMA, which is associated with elevated TMAO levels (14). The gut microbial alterations by TMA-producing bacteria are often accompanied by elevated TMAO levels, plaque lipid deposition, high lipid levels, and damaged cholesterol transport (15). In addition, inflammatory cytokines have been highlighted as a central driving force that is triggered by TMAO (16) and aggravate genes related to gluconeogenesis and glucose transport (17), thus indicating the microbial mechanism of atherosclerosis (18). Collectively, the gut microbiota, acting as a virtual endocrine organ (19), accompanied by TMAO, contribute to the pathologic processes of atherosclerosis through various mechanisms, mainly via inflammation (20) and glycolipid metabolism disorders (21). These effects occur when there is an imbalance occurs in the host's gut microbiota.

It is widely known that statins may prevent the progression of atherosclerosis to a certain extent (22). However, myopathy, renal disease, rhabdomyolysis, and other muscle-related adverse diseases associated with statins should not be overlooked (23). In contrast, traditional Chinese medicine presents a good impetus and vast potential for future development (24). Modern pharmacological studies (25) and clinical trials (26) have confirmed that specific Chinese herbs or extracts have shown excellent potential therapeutic effects in CVDs owing to their prominent detoxifying and blood-activating effects (27). *Polygonum cuspidatum* Sieb. et Zucc. (*P. cuspidatum*) (28) and *Crataegus pinnatifida* Bunge (*C. pinnatifida*) as representative detoxifying and blood-activating

herbs (29) , are known for their efficacy in blood activation and resolving stasis, clearing heat, and detoxification (30). Extensive evidence has shown that polydatin (31) or hawthorn flavonoids (32) could exert cardioprotective effects and alleviate metabolic disorders, partially via the gut microbiota. Previously, our team combined hawthorn to promote blood circulation and resolving blood stasis with *P. cuspidatum* to clear heat and detoxify (33), applied it to the treatment of acute coronary syndrome, and achieved great clinical efficacy (34). In addition, our previous clinical trial also confirmed the remarkable anti-atherosclerotic efficacy of polydatin (the extract of *P. cuspidatum*) combined with hawthorn flavonoids (the extract of hawthorn) (35). However, the intimate associations and mechanisms were unknown. Therefore, we hypothesized that the anti-atherosclerotic mechanism of polydatin combined with hawthorn flavonoids (PH), compatibility of traditional Chinese medicines, is linked with the inhibition of TMAO (36), related pathways, and the regulation of gut microbiota disorders (37). Taking these factors into consideration, we evaluated the indicators of lipids, inflammation, and plaque area, provided an integrated metagenomic sequencing (38), and targeted metabolomics (39) analysis of the metabolite TMAO to further explore the specific therapeutic mechanism of PH.

2. Materials and methods

2.1. Drugs and diets

Polydatin (purity $\geq 98\%$, Xi'an Guanjie BioTech Co. Ltd., Xi'an, China) and hawthorn flavonoids (purity $\geq 90\%$, no. AKH12-2; Linyi Aikang Pharmaceutical Co., Ltd., Shandong, China) were mixed to prepare a solution. simvastatin was purchased from Hangzhou MSD Pharmaceutical Co. Ltd (Zhangzhou, China; U010049). The high-fat diet (HFD) containing 21% saturated fat, 0.15% cholesterol and 1% choline was purchased from Keao Xieli Feed Co., Ltd (SCXK [Beijing] 2019–

0003).

2.2. Animal experiments

Specific pathogen-free (SPF) male C57BL/6J mice (8-week-old, 19–21g) and male apolipoprotein E knockout (ApoE^{-/-}) mice of the same age and genetic background were obtained from Vital River Laboratory Animals (Beijing, China). The mice were bred in a SPF laboratory and had free access to food and water. After 13 weeks, aortic atherosclerotic plaque formation was confirmed by microscopic observation after dissection, indicating that the atherosclerotic model was successfully prepared. Subsequently, all mice were randomly assigned to six groups: (1) control: C57BL/6J mice received normal diet; (2) model: ApoE^{-/-} mice received HFD; (3) low dose of PH group (PHL): HFD supplemented with polydatin (50 mg/kg daily) and hawthorn flavonoids (25 mg/kg daily); (4) medium dose of PH group (PHM): HFD supplemented with polydatin (100 mg/kg daily) and hawthorn flavonoids (50 mg/kg daily); (5) high dose of PH group (PHH): HFD supplemented with polydatin (200 mg/kg daily) and hawthorn flavonoids (100 mg/kg daily); (6) simvastatin: HFD supplemented with simvastatin (5 mg/kg daily). The control and model groups received distilled water (0.2 mL daily). The experiment lasted for 24 weeks, and all mice were fasted overnight and euthanized using isoflurane inhalation at the end of the experiment. The experimental protocol was approved by Institutional Animal Care and Use Committee (IACUC), Guang'an men Hospital, China Academy of Chinese Medical Sciences.

2.3. Histology

The thorax of the exposed mice was dissected. Their hearts and aortas were lavaged with a slow injection of 10 mL normal saline, and 5 mL 4% paraformaldehyde was slowly injected for internal fixation. For enface staining of the plaque areas in the mice, the whole aorta was soaked

with Oil Red O (ORO) solution and photographed against a black background. The aortic sinus specimens were fixed with 4% paraformaldehyde, dehydrated in layers, and embedded in paraffin wax. Continuous sections (5 μ m) were taken from the aortic sinus to the aortic arch at intervals of 40 mm, and atherosclerotic aortic sinus lesions were evaluated using hematoxylin-eosin (H&E) staining. The remaining aortic sinus samples were buried at the optimal cutting temperature, and frozen sections were used for ORO staining to evaluate lipid deposition areas. Quantitative analysis of aortic plaques was performed using the ImageJ software.

2.4. Lipid measurements

The plasma concentrations of cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), very low-density lipoprotein cholesterol (VLDL-C), and high-density lipoprotein cholesterol (HDL-C) were measured using an automatic biochemical instrument (BackmanAU5821, USA).

2.5. AimPlex multiplex immunoassays of inflammatory cytokines

Serum levels of interleukin-1-beta (IL-1 β), interleukin-2 (IL-2), interleukin-6(IL-6), interleukin-17A (IL-17A), tumor necrosis factor alpha (TNF- α) and hs-CRP were measured using the Aimplex Mice Custom Premixed Analyte kit (Beijing Quantobio, China).

2.6. Immunological biomarkers analysis

Paraffin-embedded fixed aortic arch tissues were sliced into approximately 10 μ m sections. These sections were incubated with nuclear factor- κ B (NF- κ B) p65 Rabbit PolyClonal antibody (Proteintech; 10745-1-AP, 1:500); tumor necrosis factor alpha (TNF- α) antibody (Proteintech; 60291-1-Ig, 1:1000); and Anti-FMO3 Antibody (abcam; ab126711, 1:5000) for overnight at 4 $^{\circ}$ C. After being washed, slices were incubated with horseradish peroxidase (HRP)-conjugated anti-

rabbit secondary antibody (Proteintech; SA00001-2, 1:300) for 1 h at 37 °C. Hematoxylin was added to the slices for 30 s and the slices were counterstained after washing with PBS. The area of positive staining represented the levels of NF- κ B and TNF- α in the aortic arch tissue, which was calculated and evaluated using the ImageJ software.

2.7. Western blot

Protein concentrations in the aorta and liver tissues were quantified using a BCA kit (Thermo Scientific). Proteins were separated by 5% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore, Burlington, MA). The membrane was blocked with 5% skimmed milk, for 1 h, and then incubated with primary antibody overnight at 4°C: TNF- α , NF- κ B p65 and Anti-FMO3 antibody were same as the above (as shown in 2.6); β -actin (ABclonal, AC026, 1:1000). The blots were incubated with HRP-conjugated anti-rabbit IgG for 1 h. After washing, the membranes were analyzed using chemiluminescence detection kit.

2.8. Quantitative RT-PCR

Total RNA was extracted from liver tissues using TRIzol reagent. The cDNA was synthesized using SuperScript II (TaKaRa Bio). RT-PCR for mRNA amplification was performed using a SYBR Green Master Mix kit. Details of the real-time PCR conditions are shown in **Table S1**. The target mRNA level was normalized to the β -actin level. All primer sequences were designed using the PrimerBank software and are listed in **Table S2**.

2.9. TMA and TMAO detected by HPLC-MS/MS

Serum TMAO and TMA levels were measured using the Waters ACQUITY ultra-high-performance liquid chromatography system (Waters Corporation, Milford, MA, USA). Samples

were separated using a mobile phase gradient consisting of a mixture of 5 mM ammonium formate as solvent A and acetonitrile as solvent B. The analytes were eluted using the following gradient profile: 90% B, 10% A for 1 min; 3.5 min, 50% A, 50% B; and reconditioning at 90% B, 10% A for 8.6 min. The detailed contents of the mobile phase gradient and mass spectrometry conditions are shown in **Table S3** and **Table S4**. Data processing was completed using the TargetLynx software.

2.10. DNA extraction and 16S rRNA sequencing

Bacterial DNA was extracted from 0.5 g fecal samples utilizing the FastDNA® Spin Kit for SoilDNA (Qiagen, Limburg). DNA integrity was detected using 1% agarose gel electrophoresis, and the concentration and purity of DNA were detected using a NanoDrop 2000 microspectrophotometer (Thermo Fisher Scientific, USA). The forward primer 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used to amplify the V3-V4 variable region of the bacterial 16S rRNA gene. Amplification was performed according to the standard protocols. PCR products were detected by 2% agarose gel electrophoresis and purified using an AxyPrep DNA Gel Extraction Kit (Invitrogen, Carlsbad, CA, USA). A NEXTFLEX Rapid DNA-Seq Kit (Bioo Scientific, USA) was used to prepare the sequencing library. The amplified products were collected and sequenced on an Illumina MiSeq PE300/NovaSeq PE250 platform (Illumina, San Diego, USA).

2.11. Biochemical analyses

The quality control and splicing process of the raw sequencing sequences were based on the fastp software that was merged with FLASH. The UPARSE software was used to cluster operational taxonomic units (OTUs) with a 97% similarity cutoff. Each sequence was classified using the RDP classifier and compared to the Silva 16S rRNA database (V128) at a threshold of 70%. Alpha

diversity and beta diversity analysis was used to show the distribution and similarity using principal coordinate analysis (PCoA). The differences at different taxonomic levels were evaluated using linear discriminant analysis effect size (LEfSe). A correlation heatmap was utilized to visualize the relationships between the different species and other parameters.

2.12. Metagenome sequence and taxonomic annotation

Quality control and optimization were performed using software fastp, and filtered reads were assembled (contigs ≥ 300 bp). In addition, open reading frames (ORFs) prediction and translation of contigs were performed using MetaGene to obtain the corresponding amino acid sequences. CD-HIT was used to cluster the predicted genetic sequences, and the non-redundant gene set was constructed. Diamond was used to align the amino acid sequences with the GENES database using BLASTP (version 2.2.28) and annotate the kyoto encyclopedia of genes and genomes (KEGG) databases (version 94.2). The amino acid sequences were aligned to the carbohydrate-active enzymes (CAZymes) database using HMMER (version 3.1b2). The abundance and differences in the corresponding species, CAZy, and KEGG functional categories were estimated using normalized RPKM values.

2.13. Statistical analyses

Statistical analyses were performed using GraphPad Prism 9.0. All data were presented as the mean $\pm$ standard deviation (SD), and multiple comparisons were analyzed using ANOVA. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Polydatin and hawthorn flavonoids (PH) reduced body weight and lipid levels in HFD-fed

ApoE^{-/-} mice

(E) VLDL-C. (F) HDL-C. All data are shown as the mean $\pm$ SD (n=6). * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001, vs Model; # P <0.05, ## P <0.01, ### P <0.001, #### P <0.0001, vs. Control; ^a P <0.05 vs. simvastatin; ^b P <0.05 vs. PHL; ^c P <0.05 vs. PHH

3.2. PH attenuates HFD-induced atherosclerosis in HFD-fed ApoE^{-/-} mice

Atherosclerotic plaque size was measured to assess the pathological changes in atherosclerosis. The ratio of the plaque area to the entire aorta or aortic root in the model group increased after HFD feeding. The results of ORO staining revealed that the rising trend in atherosclerotic plaque area was inhibited, and even reduced in different doses of the PH or simvastatin groups as compared with the model group (**Fig. 2A, 2C**). The aorta inner wall was smooth without any evidence of obvious plaques attached in the control group, whereas lipid deposition was more prominent with an amount of foam cells within the plaque and aortic vessel stenosis in the model group (**Fig. 2A, 2C**). Specifically, quantitative analysis revealed that the proportion of aortic lesions to the total aortic vessel area in the PH group showed a descending trend compared to that of the model group (P <0.0001) (**Fig. 2B**). The therapeutic effects of PHM and PHH on atherosclerosis were significant. Consistently, the proportion of cross-sectional lesions of the aortic roots reduced in the PHM and PHH groups, compared to the model group (P <0.01) (**Fig. 2D**).

The results of H&E staining also suggested that the aortic root vessel wall intima was significantly thickened, with a large number of lipid deposits; inflammatory cell infiltration, smooth muscle cell proliferation, and foam cells appeared under the intima in the model group. The inner wall surface of the aortic root of mice was smooth, and the endothelial cells were neatly arranged, with no obvious atherosclerotic plaques was formed in the control group. However, the inner wall of the aortic root was thickened with lipid deposition in mice fed an HFD. In addition, soakage of

inflammatory cells, proliferation of smooth muscle cells and foam cells, and increased collagen content in the aortic roots contributed to plaque attachment within the aortic wall and a significantly narrowed the lumen of the vessel (**Fig. 2E**). The lesion area in the aortas in the PH and simvastatin groups mice was significantly reduced ($P<0.001$) (**Fig. 2F**). In summary, PHM, PHH, and simvastatin had exerted significant effects on atherosclerosis and delayed plaque deterioration.

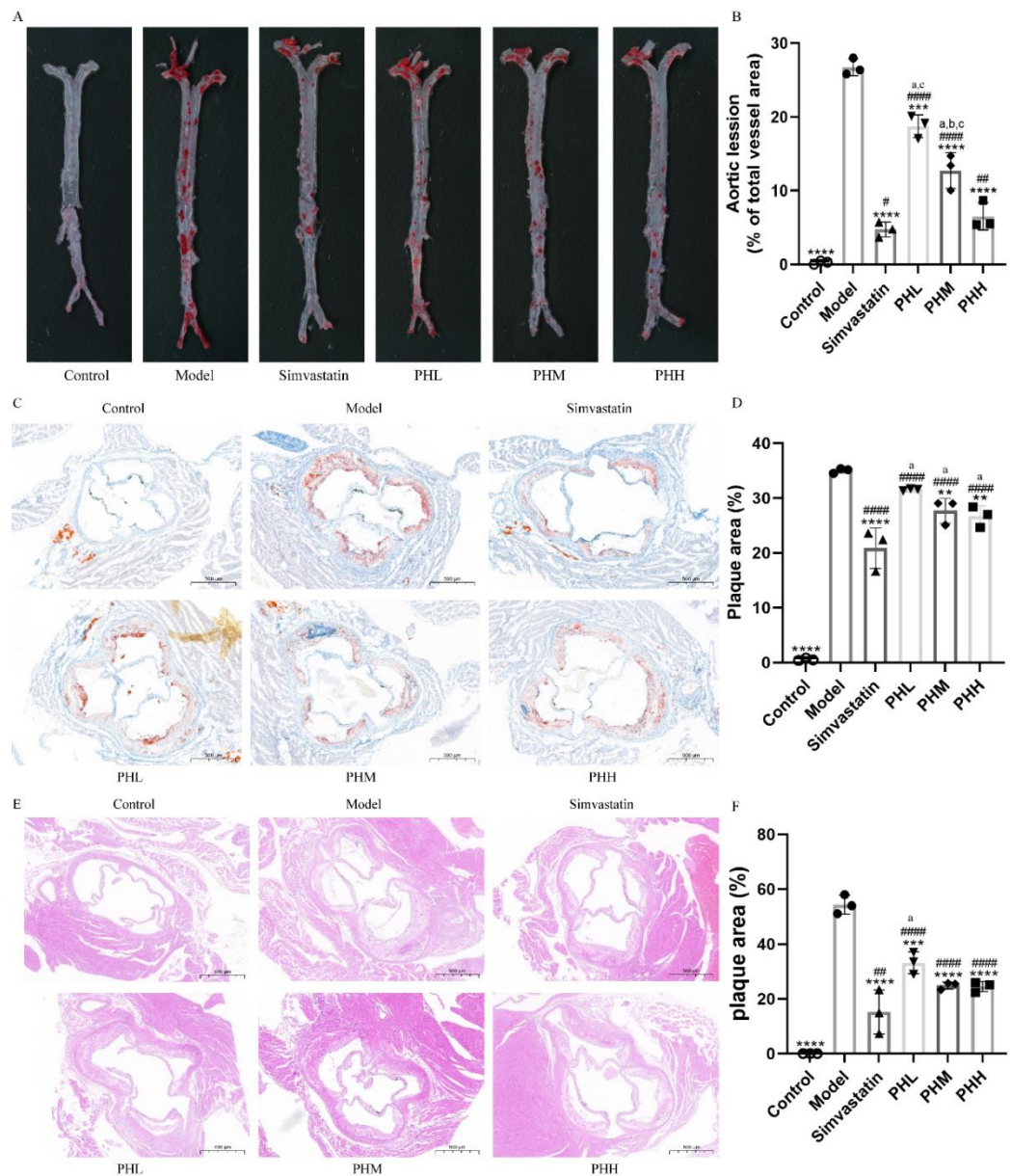


Fig. 2. PH attenuated atherosclerosis injury in HFD-fed ApoE^{-/-} mice. (A) and (B) Image of enface aortas stained with ORO, and quantitative analysis of the aortic lesion ratio. (C) and (D)

Image of aortic roots sections stained with ORO, and quantitative analysis of the aortic roots cross-sectional lesions ratio. **(E) and (F)** Image and quantitative analysis of H&E staining, illustrating the proportion of the cross-sectional lesions in the aortic roots. All data are shown as the mean $\pm$ SD (n=3). ** P <0.01, *** P <0.001, **** P <0.0001, vs Model; # P <0.05, ## P <0.01, #### P <0.0001, vs. Control; ^a P <0.05 vs. Simvastatin; ^b P <0.05 vs. PHL; ^c P <0.05 vs. PHH

3.3. PH improved inflammatory cytokine levels in HFD-fed ApoE^{-/-} mice

Besides lipid abnormalities, the progression of atherosclerosis is also influenced by systemic inflammation to a certain extent. Given that inflammatory cytokines have been recognized as an index of atherosclerosis in epidemiological studies, immunohistochemical staining of aortic root plaques was conducted. We observed that the expression of TNF- α and NF- κ B was more prominent in mice fed an HFD than in mice fed a chow diet. Nevertheless, dietary supplementation with PH and simvastatin diminished the intensity of the staining, implying that a strong antagonistic action was exerted against the inflammatory reaction during the process of atherosclerotic plaque formation (**Fig. 3A, 3C**). Quantitative analysis was utilized to assess the specific efficacy of PH and simvastatin. As shown in **Fig. 3B** and **3D**, the ratio of TNF- α and NF- κ B positive area to the aortic sinus plaques in PH and simvastatin groups were dramatically decreased (P <0.0001), especially obvious in PHH group. Thus, the therapeutic effect of PHH in down-regulating TNF- α and improving inflammation factor levels is superior to that of simvastatin (**Fig. 3B**). Consistently, we observed that the down-regulation of NF- κ B protein in aortic plaque in PHM, PHH, and simvastatin group was more significant than that in model group (P <0.0001) (**Fig. 3E**), with the lowest protein expression in the PHH group (**Fig. 3F, 3G**).

Furthermore, we measured the levels of other important inflammatory mediators to confirm

the effects of PH on atherosclerosis. The results were consistent with the previously reported changes, demonstrating that the serum levels of hs-CRP, TNF- α , IL-1 β , IL-2, IL-6, and IL-17A in model group mice dramatically increased in mice fed an HFD (**Fig. 3H-3M**). After PH and simvastatin administration, a dose-dependent decrease in the inflammatory cytokine levels was observed, with the lowest level in PHH group ($P<0.05$) (**Fig. 3M**). This suggested that inflammatory cytokines, represented by TNF- α and NF- κ B, are closely related to the progression of atherosclerotic plaques. PH reduces inflammatory factor levels, thereby improving atherosclerotic plaque formation, with the most pronounced effects found in the PHH group.

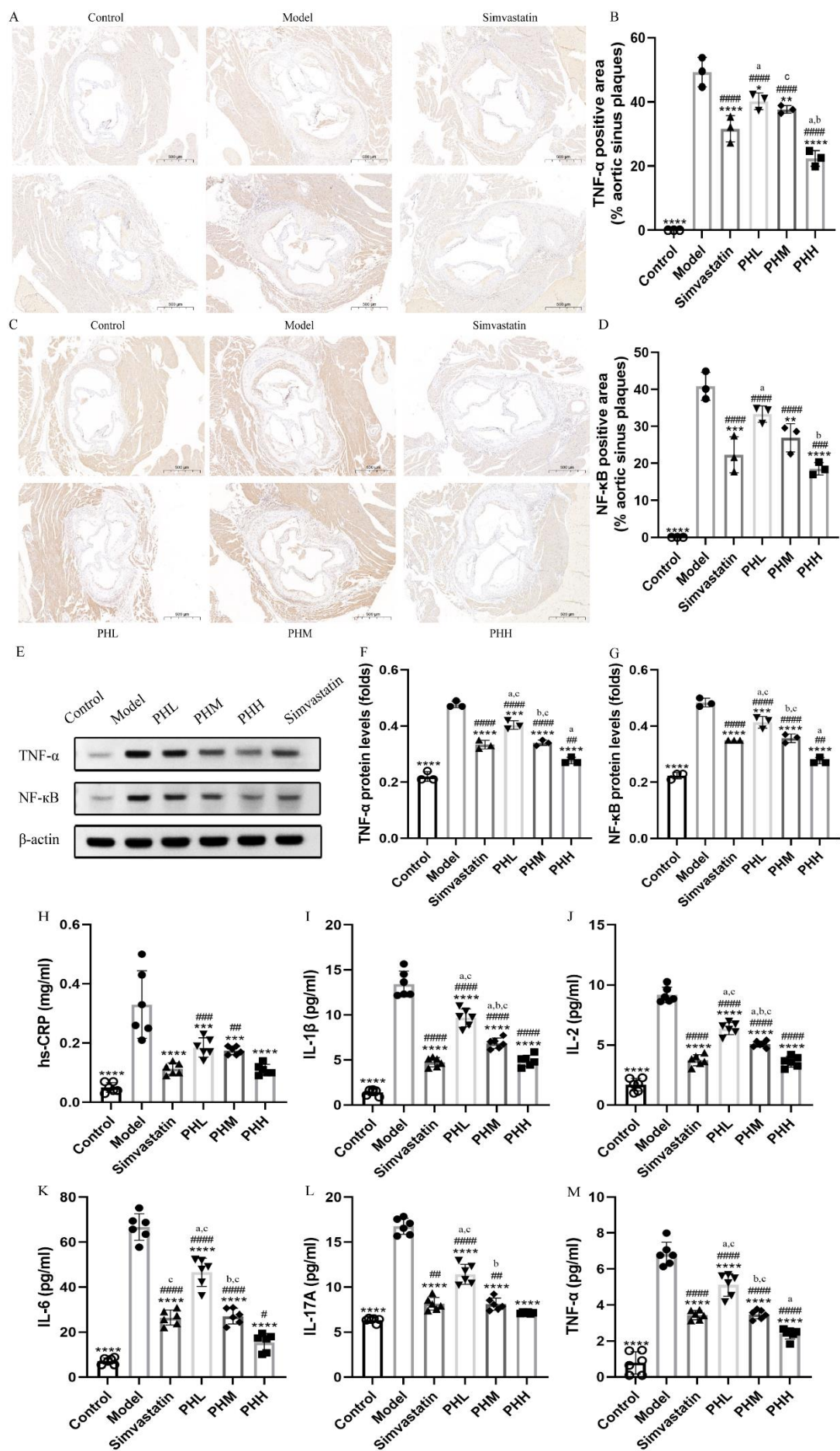


Fig. 3. PH inhibited the activation of inflammatory cytokine in HFD-fed ApoE^{-/-} mice. (A) and (B) Image and quantitative analysis of immunohistochemical staining of the aortic sinus and the proportion of the TNF- α positive area to the aortic sinus plaques (n=3). (C) and (D) Image and quantitative analysis of immunohistochemical staining of the aortic sinus and the proportion of the NF- κ B positive area to the aortic sinus plaques (n=3). (E) Protein expression of TNF- α and NF- κ B. (F) and (G) Quantitative analysis of TNF- α and NF- κ B protein levels in the aortic sinus plaques (n=3). (H-M) Plasma hs-CRP, IL-1 β , IL-2, IL-6, IL-17A, and TNF- α levels (n=6). * P <0.05, ** P <0.01, * P <0.001, **** P <0.0001, vs Model; # P <0.05, ## P <0.01, ### P <0.001, #### P <0.0001, vs. Control; ^a P <0.05 vs. Simvastatin; ^b P <0.05 vs. PHL; ^c P <0.05 vs. PHH**

3.4. PH inhibited the enhancement of TMA and TMAO level in HFD-fed ApoE^{-/-} mice

To investigate the underlying mechanism of PH on atherosclerosis, atherosclerotic markers TMAO, and the TMAO metabolic pathway-related proteins were assessed. We observed that the HFD containing 1% choline induced an elevation of TMA and TMAO levels in the model group mice. Similar to the findings of previous studies (40), choline accelerated atherosclerotic progression in the present study. We observed the attenuation of TMA and TMAO in the PHH group, compared to the model group (P <0.001). Notably, the overexpression of TMA and TMA levels was reversed by PH supplementation at high doses, indicating that the therapeutic effect of PHH was more prominent than that of the others (**Fig. 4A,4B**).

Hepatic FMO3 is a crucial enzyme involved in the conversion of TMA to TMAO. Thus, we focused on assessing the dynamic changes in FMO3 expression and activity. In line with previous findings, the results demonstrated that compared to the control group, the protein and mRNA levels of FMO3 in mice were up-regulated in the model group (P <0.01). PH downregulated the protein

and mRNA expression of FMO3, which was more evident in terms of protein expression of FMO3. Similarly, a high dose of PH exhibited an unexpected effect on TMA/TMAO metabolism (Fig. 4C,4D,4E). In conclusion, we speculated that activation of the TMA/FMO3/TMAO pathway is an important incentive for atherosclerosis, and PH inhibits TMAO generation through the involvement of hepatic FMO3.

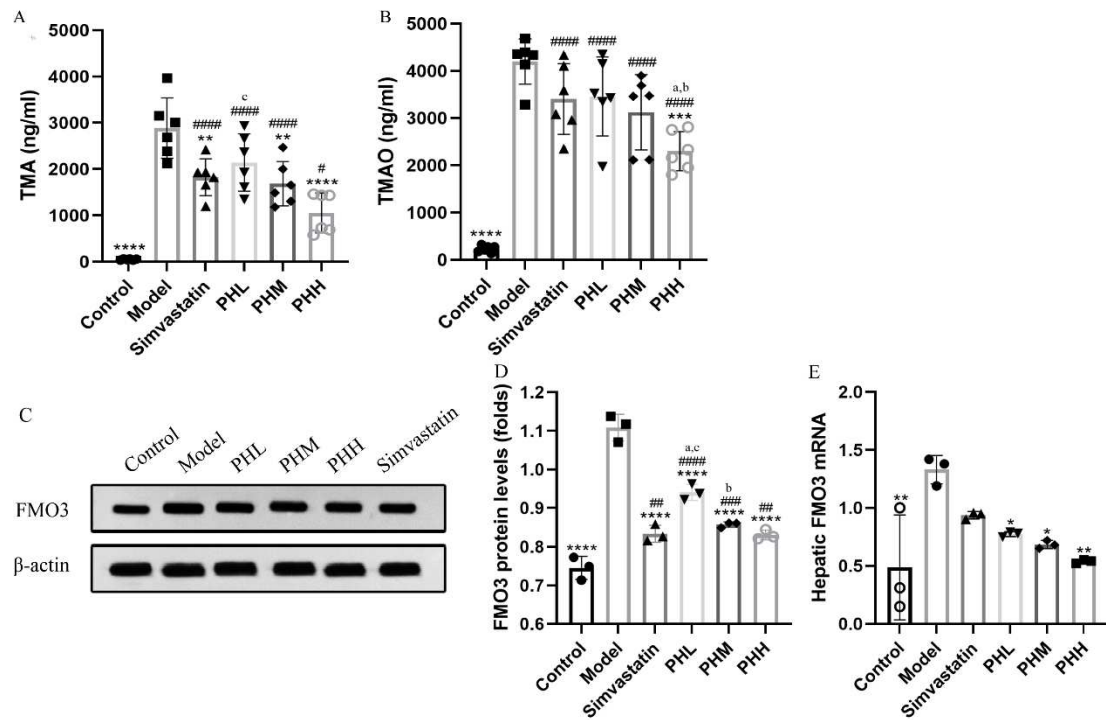


Fig. 4. PH regulated TMA/TMAO metabolism involving hepatic FMO3 in HFD-fed ApoE^{-/-} mice. (A) and (B) TMA and TMAO levels (n = 6). (C) and (D) Western blot detection and quantitative analysis of FMO3 protein expression (n = 3). (E) mRNA levels of FMO3 quantified by qPCR (n = 3). All data are shown as the mean ± SD. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001, vs. Model; #*P*<0.05, ##*P*<0.01, ###*P*<0.001, ####*P*<0.0001, vs. Control; ^a*P*<0.05 vs. Simvastatin; ^b*P*<0.05 vs. PHL; ^c*P*<0.05 vs. PHH

3.5. The effect of PH on microbial diversity and distribution during the process of atherosclerosis

To explore the microbial mechanism, 16S rRNA gene sequencing with a 97% similarity

criterion for operational taxa was acquired from 36 samples. After sequence quality control and
 splicing, 3375 operational taxonomic units (OTUs) were identified. The rank-abundance curve
 declined smoothly and eventually flattened (**Fig. 5A**). The taxonomy cumulative and rarefaction
 curve revealed that the sequences per sample were sufficient and the sequencing depth was qualified
 for analysis later (**Fig. 5B, 5C**). Alpha (α) diversity analysis revealed that microbial diversity and
 abundance were reduced after an HFD intervention (**Fig. S1 and S2, Table S5**); however, this trend
 was reversed or attenuated after PH administration (**Fig. 5D, 5E, 5F**). Similar alterations were seen
 in the number of OTUs in all groups (**Fig. 5G**). The gut microbiota characteristics were explored
 and analyzed at different levels, and 11 phyla, 14 classes, 32 orders, 50 families, 116 genera, and
 201 species were identified. Moreover, the detail of distribution of different species were shown in
Fig. S3, S4 and S5. At the phylum level, the proportion of *Desulfobacterota* markedly increased in
 the model group, while decreased in the PHM, PHH and simvastatin groups (**Fig. 5H**). At a more
 specific level, the abundance of *norank_f_Muribaculaceae*, *Lactobacillus* and
Coriobacteriaceae_UCG-002 in the model group was lower than that in the simvastatin and PH
 groups (**Fig. 5I, 5J**). Consistently, the *Firmicutes/Bacteroidetes* (F/B) ratio dramatically increased
 in mice fed an HFD, suggesting a gut dysbiosis (**Fig. 5K, 5L, 5M**).

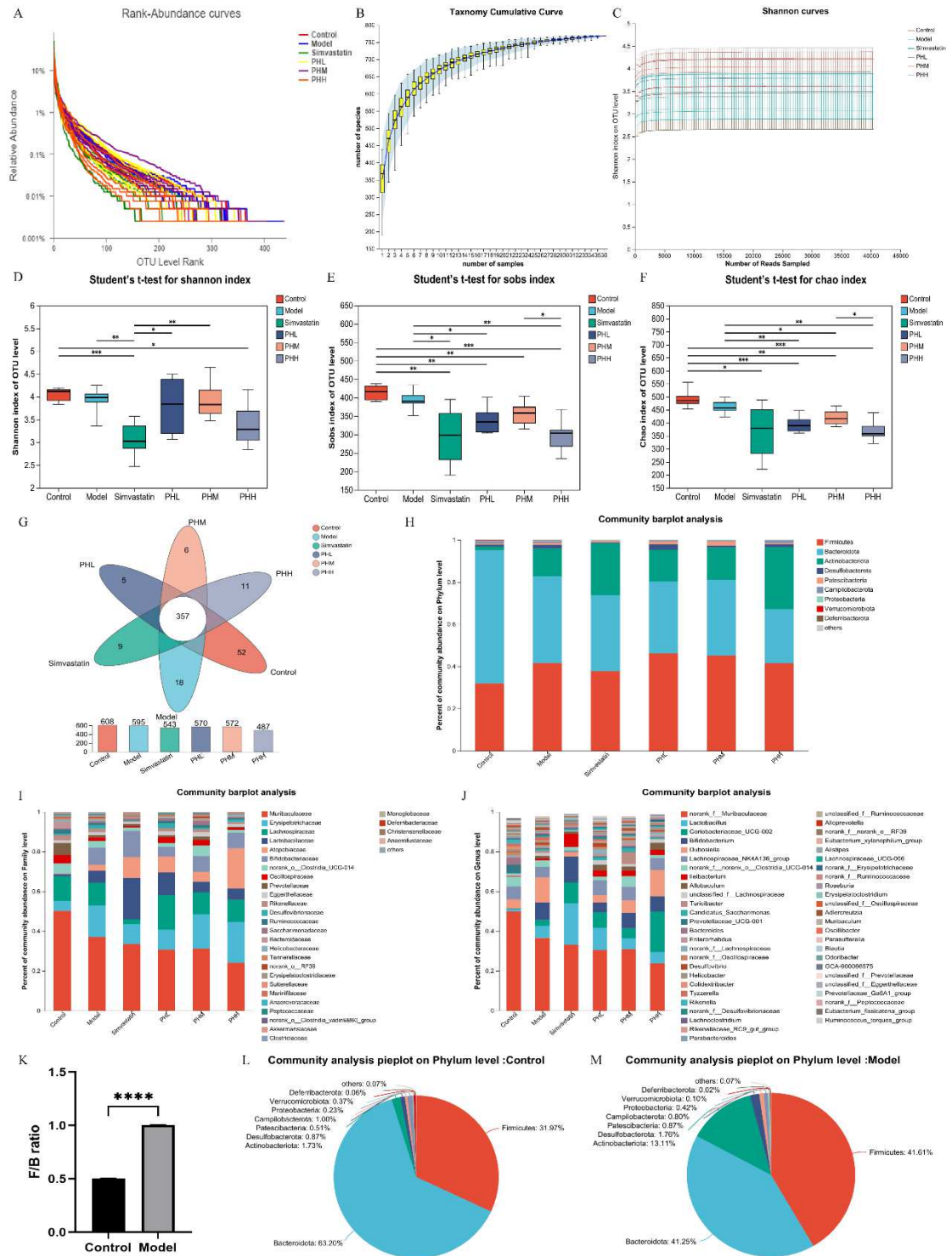


Fig. 5. (A) Rank abundance curves; **(B)** Species accumulation curves; **(C)** Shannon rarefaction curve; **(D), (E) and (F)** Alpha diversity was analyzed by the Shannon, Sobs, Chao index; **(G)** Venn diagrams of OTUs; **(H), (I) and (J)** Taxonomic composition/microbial compositions at the phylum, family, genus level; **(K)** Quantification analyzes of the of F/B ratio in control and HDF groups (n=6);

(L) and (M) Pie chart of the predominant bacteria. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$,
**** $P < 0.0001$

3.6. PH protected *ApoE*^{-/-} mice from HFD-induced atherosclerosis via remodeling microbiota

Diversity analysis was performed to identify the alterations of gut microbial structure. The PCoA plot showed a remarkable separation among the four groups (control, model, PHH, and simvastatin) along the PC2 axis, which explained 13.96% of the variation in the microbial structure (Fig. 6A). The segregated microbial structure among the three groups (control, PHH, and simvastatin) was consistently visualized using non-metric multidimensional scaling (NMDS) analysis (Fig. 6B). The microbiota composition was clustered by partitioning around the medoids, and three enterotypes were identified. We identified that the proportion of *Lactobacillus* increased with the administration in PH or simvastatin (Fig. 6C). Notably, the enterotypes in the PHH and simvastatin groups were mainly clustered into *g_Lactobacillus* and *g_norank_f_Muribaculaceae_1* group, indicating that PHH and simvastatin reversed the HFD-induced enterotype switch (Fig. S6). Presumably, PHH and simvastatin protected the atherosclerotic mice against microbes. Accumulating evidence indicates that the HFD-induced decrease in gut microbial diversity can be restored to some extent by PH treatment. *Bacteroidetes* and *Bacteroides* were enriched in the control group, whereas *Alistipes*, *f_Clostridiaceae*, and *f_Defluviitaleaceae* were the predominant genera in the model group. PH administration significantly improved the proportion of *p_Actinobacteria*, *f_Atopobiaceae*, and *g_Coriobacteriaceae_UCG-002*, whereas it decreased the proportions of *f_Clostridiaceae* and *f_Defluviitaleaceae* in the model group mice (Fig. 6D). Finally, the multigroup difference analysis suggested that HFD increased the abundance of *Antinobacteriota*, *Desulfobacterota*, *Coriobacteriaceae_UCG-002*, and *Atopobiaceae* in the model group and reduced

the abundance of *Bacteroidetes*, *Bacteroides*, and *norank_f_Mribaculaceae*, whereas PH treatment reversed these changes (Fig. 6E-L).

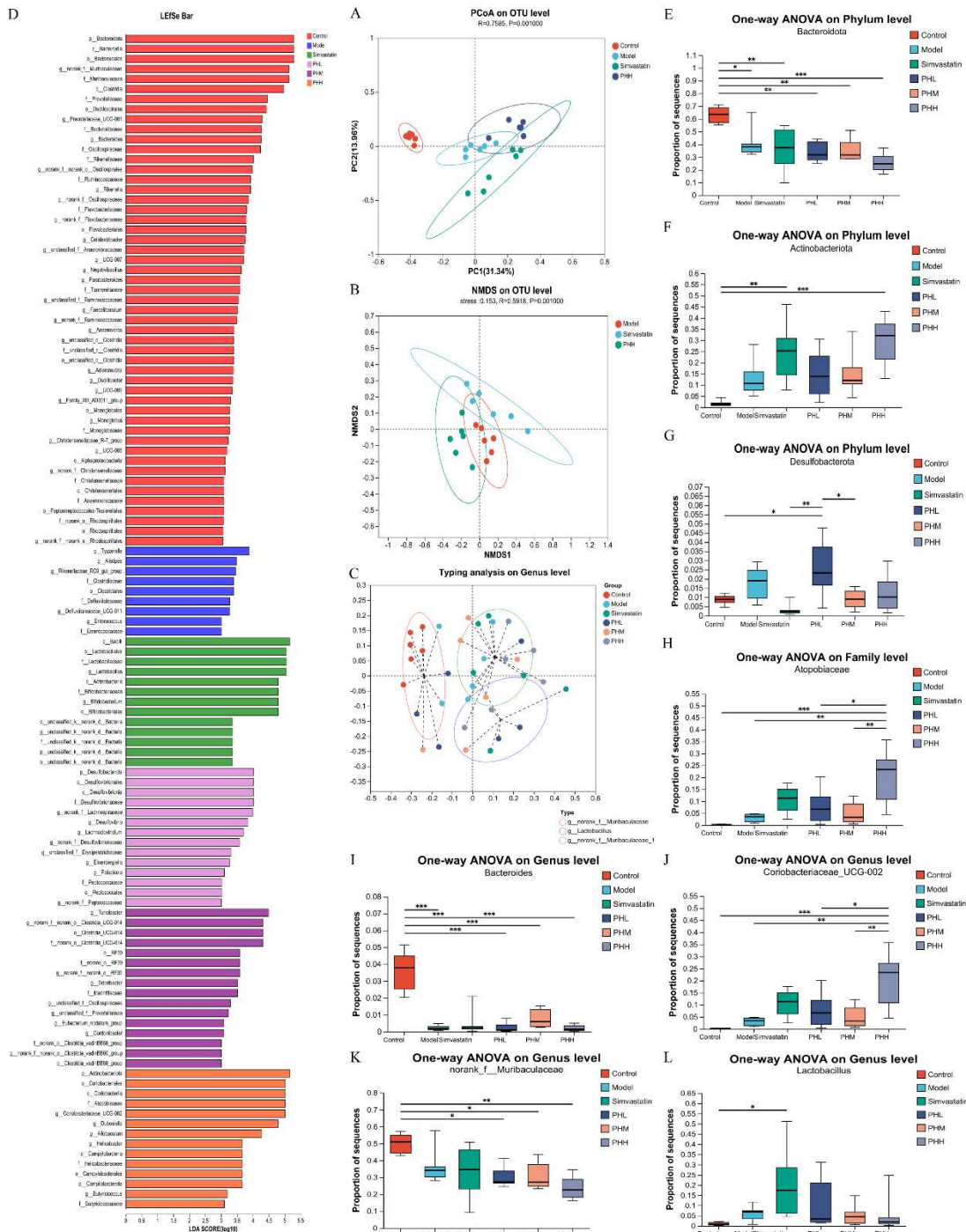


Fig. 6. (A) PCoA plot; (B) NMDS analysis; (C) Effects of PH or simvastatin on enterotype of mice; (D) LEfSe analysis; (E)-(L) Multigroup difference analysis. n = 6. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

382 3.7. *Uncovering the anti-atherosclerotic mechanism of PH: targeting the functional capacity of gut*
383 *microbiota*

384 We further conducted the metagenomic sequencing analysis to investigate the core functional
385 capacity of different genes. Based on the LEfSe analysis, we observed that 37 differential KEGG
386 pathways were identified in PHL and PHH groups, and ribosome, protein export, and RNA
387 polymerase pathways were enriched in the model group. Most of the 37 differentially expressed
388 functional pathways were highly enriched in the carbohydrate metabolism and membrane transport-
389 related pathways. Among these, ABC transporters, carbon metabolism, glycerolipid metabolism,
390 and glucagon signaling pathways were identified in the PHL group, and the biosynthesis of amino
391 acids, starch, and sucrose metabolism were increased in the PHH group (**Fig. 7A**). Additionally,
392 fatty acid metabolism, fatty acid and lipopolysaccharide biosynthesis were enriched in the control
393 group, whereas the enrichment was reduced in the PHH group. A similar trend was observed in the
394 multi-group comparison analysis (**Fig. 7D-K**). Based on these results, we investigated the
395 enrichment of CAZymes by LEfSe analysis. At the class level, glycosyl transferases (GTs) and
396 carbohydrate esterases (CEs) were highly enriched in the PHL and PHH groups, whereas glycoside
397 hydrolases (GHs) were mainly present in the simvastatin group (**Fig. 7B**). The HFD-induced
398 abundance of CAZymes was restored by PH administration, at the family level. Several CAZymes,
399 including GT2_Glyco_tranf_2_3 and CE1, were enriched in the PHH group (**Fig. 7C**).

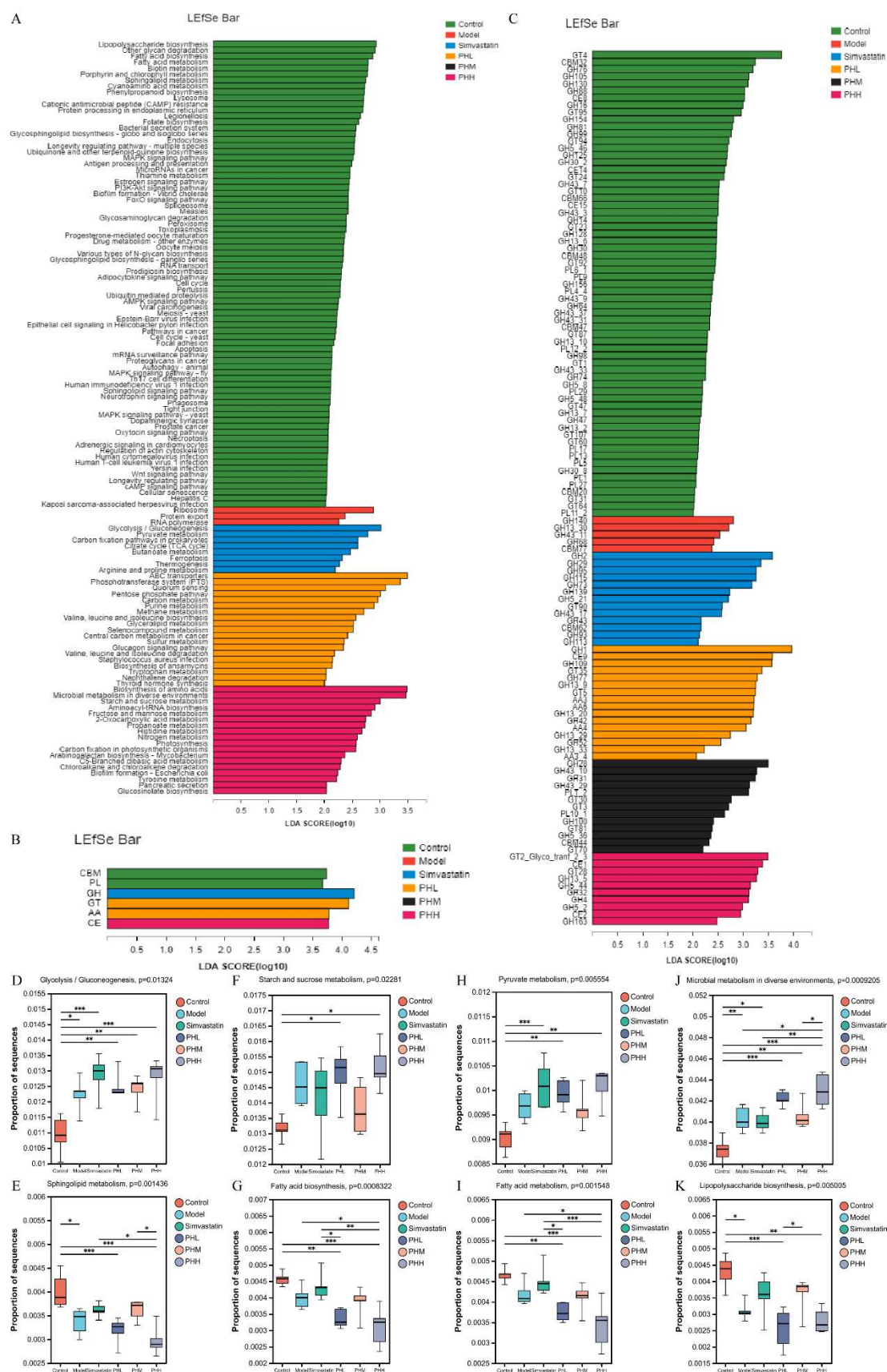


Fig. 7. (A) LefSe analysis of KEGG pathways (LDA>2.5); **(B)** LefSe analysis of CAZymes at the

class level (LDA>3); **(C)** LEfSe analysis of the differential enrichment of CAZymes at the family level (LDA>3); **(D)-(K)** Multigroup comparison analysis of KEGG pathways; * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

3.8. Association analysis among atherosclerotic parameters, microbiota species, and microbial functions

We constructed a heatmap to visualize the associations between the gut microbiota and biochemical parameters. *Desulfobacterota* demonstrated a significant positive correlation with lipid levels (TG, TC, and LDL-C) and inflammatory cytokines (hs-CRP, IL-2, IL-1 β , and IL-17A). Additionally, *Firmicutes* displayed a similar trend with TG, LDL-C, IL-2, and IL-1 β (**Fig. 8A**). Notably, *Firmicutes*, *Lachnospirillum* and *Turicibacter* demonstrated significant positive correlations with TMA, TMAO, lipid, and inflammatory cytokine levels. *Desulfovibrio*, *Muribaculum*, and *Allobaculum* also correlated with lipid levels and inflammatory cytokines. *Bacteroides*, *Prevotellaceae_Ga6A1_group*, and *Eubacterium_fissicatena_group* negatively correlated with TMA, TMAO, lipid levels, and inflammatory cytokines (**Fig. 8B**), thereby indicating that these microbial biomarkers may be involved in the development of atherosclerosis.

Associations between atherosclerotic variables, the KEGG functional pathways, and gut microbiota were analyzed. At Level 3, ABC transporters demonstrated positive correlations with lipid levels (TG, TC, LDL-C, and VLDL-C) and inflammatory cytokines (IL-2, IL-6, IL-17A, IL-1 β , hs-CRP, and TNF- β). In particular, LDL-C demonstrated a positive correlation with the metabolic pathways, including carbon, starch, and sucrose metabolism (**Fig. 8D**). At level 2, ko00520, ko00030, and ko00550 demonstrated similar associations with the lipid and inflammatory cytokine levels (**Fig. 8E**). At module level, M00001 (Glycolysis), M00016 (Lysine biosynthesis),

424 and M00051 (De novo pyrimidine biosynthesis) demonstrated positive correlations with lipid and
425 IL-1 β levels (**Fig. 8F**). *Uncultured_bacterium_g_Turicibacter*, and
426 *uncultured_bacterium_g_Desulfovibrio* exhibited positive correlations with TMAO, lipids, and
427 inflammatory cytokine levels, whereas *Bacteroides_acidifaciens* exhibited negative correlations
428 with TMAO, lipid, and inflammatory cytokine levels (**Fig. 8C**). In addition, we observed that
429 metabolic pathways, including starch and sucrose metabolism, and ABC transporters, including
430 M00001, M00051, and M00003, exhibited a positive correlation with
431 *s_Desulfovibrio_fairfieldensis* and *s_Allobaculum_stercoricanis*, but a negatively correlation with
432 with *Bacteroides* spp. and *s_Lactobacillus_murinus* (**Fig. 8G, 8H**).



Fig. 8. (A), (B) and (C) Correlation heatmaps of gut microbiota and atherosclerotic parameters at the phylum, genus, and species levels. **(D), (E) and (F)** Correlation between atherosclerotic parameters and KEGG functional pathways at level 3, level 2, and modules; **(G) and (H)** Correlation between different species and KEGG functional pathways at level 3 and modules. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.9. PH regulated the glycolipid metabolic pathways via TMA-producing bacteria related to atherosclerosis

Considering the pathways involved in the choline metabolism, TMAO, and associated gut microbiota, TMA-producing bacteria were selected for gene set construction. The gene set included CutC-related bacteria, which may have contributed to the therapeutic effects of PH. Critical KEGG pathways among different groups were identified using linear discriminant analysis. Glycolysis/gluconeogenesis, fluid shear stress, atherosclerosis, drug metabolism, and the HIF-1 signaling pathway were highly enriched in the PHH group (**Fig. 9F, Fig. S7**). Further multigroup comparison analysis revealed that the abundance of glycolysis/gluconeogenesis and HIF-1 signaling pathway markedly increased after PHH treatment (**Fig. 9B-E**). Thus, an analysis of intergroup differences in metabolic pathways was conducted based on these findings. The abundance of 6-phospho-beta-glucosidase [EC:3.2.1.86], which is involved in the glycolysis/gluconeogenesis metabolic pathway. The abundance of phosphoglycerate mutase [EC:5.4.2.11] and fructose-bisphosphate aldolase [EC:4.1.2.13] were increased in the model group. In addition, other related enzymes, such as 6-phosphofructokinase [EC:2.7.1.90] and fructose-bisphosphatase [EC:3.1.3.11], may also exerted an important effect (**Fig. 9G**). We hypothesized that phosphoglycerate mutase and fructose-bisphosphate aldolase were the main contributors of glycolysis/gluconeogenesis pathway.

Moreover, the gene ontology (GO) functional annotation results suggested that the biological processes (BP) of each group were mainly enriched in metabolic and biosynthetic processes (**Fig. 9A**).

Correlation analyses between metabolic pathways and TMA/TMAO revealed that ko00240, ABC transporters, and auxiliary activities positively correlated with TMA, suggesting that TMA-producing bacteria were mainly involved in the progression of atherosclerosis via metabolic pathways and related enzymes (**Fig. 9H, 9I, 9J**). Moreover, we identified the top 20 species that contributed to the differences in pathways at level 3. Metabolic functions are involved in different pathways, including glycolysis/gluconeogenesis, microbial metabolism, biosynthesis of secondary metabolites, and carbon metabolism. *Desulfovibrio*, *Prevotella*, *Muribaculum*, *Clostridium*, *unclassified_f_Lachnospiraceae*, *unclassified_o_Bacteroidales*, *unclassified_f_Muribaculaceae*, and *unclassified_f_Coriobacteriaceae* were contributed to these metabolic pathways (**Fig. 9K, 9L**).

Finally, we further investigated the association between the TMA-producing bacteria and TMA/TMAO levels. Interestingly, *Lachnospiraceae_bacterium*, *Muribaculaceae_bacterium_Isolate-104_(HZI)*, *Clostridiales_bacterium_bacterium_0.1xD8-71*, *Helicobacter_ganmani*, *Desulfovibrio_sp.*, *Clostridium_sp._CAG:417*, and *Clostridium_sp._CAG:451* exhibited positive correlations with TMA and TMAO. However, *Coriobacteriaceae_bacterium*, *Ileibacterium_valens*, *Dubosiella_newyorkensis*, *Olsenella_sp._KGMB02461*, *unclassified_g_Parabacteroides*, and *Lachnoclostridium_sp._An181* expression exhibited negative correlations with TMA and TMAO (**Fig. 10M, 10N**). Specifically, the abundance of *s_Clostridium_sp._CAG:557*, *s_Clostridium_cuniculi*, and *s_Clostridium_disporicum* increased in the model group, while the HFD-induced increase in the

478 abundance of *Clostridium* spp. was partially restored by PH administration. In addition, similar
479 changes were observed in certain gut microbial species, such as *s_Lachnoclostridium_sp._An118*,
480 *s_Muribaculaceae_bacterium_Isolate-104_(HZI)*, and *s_unclassified_g_Desulfobibrio* (**Fig. 10.**
481 **A-L**). We speculated that CutC-related bacteria, such as *Lachnospiraceae*, *Muribaculaceae*,
482 *Clostridium*, and *Desulfovibrio* prompted the progression of atherosclerosis via glycolipid
483 metabolic pathways.

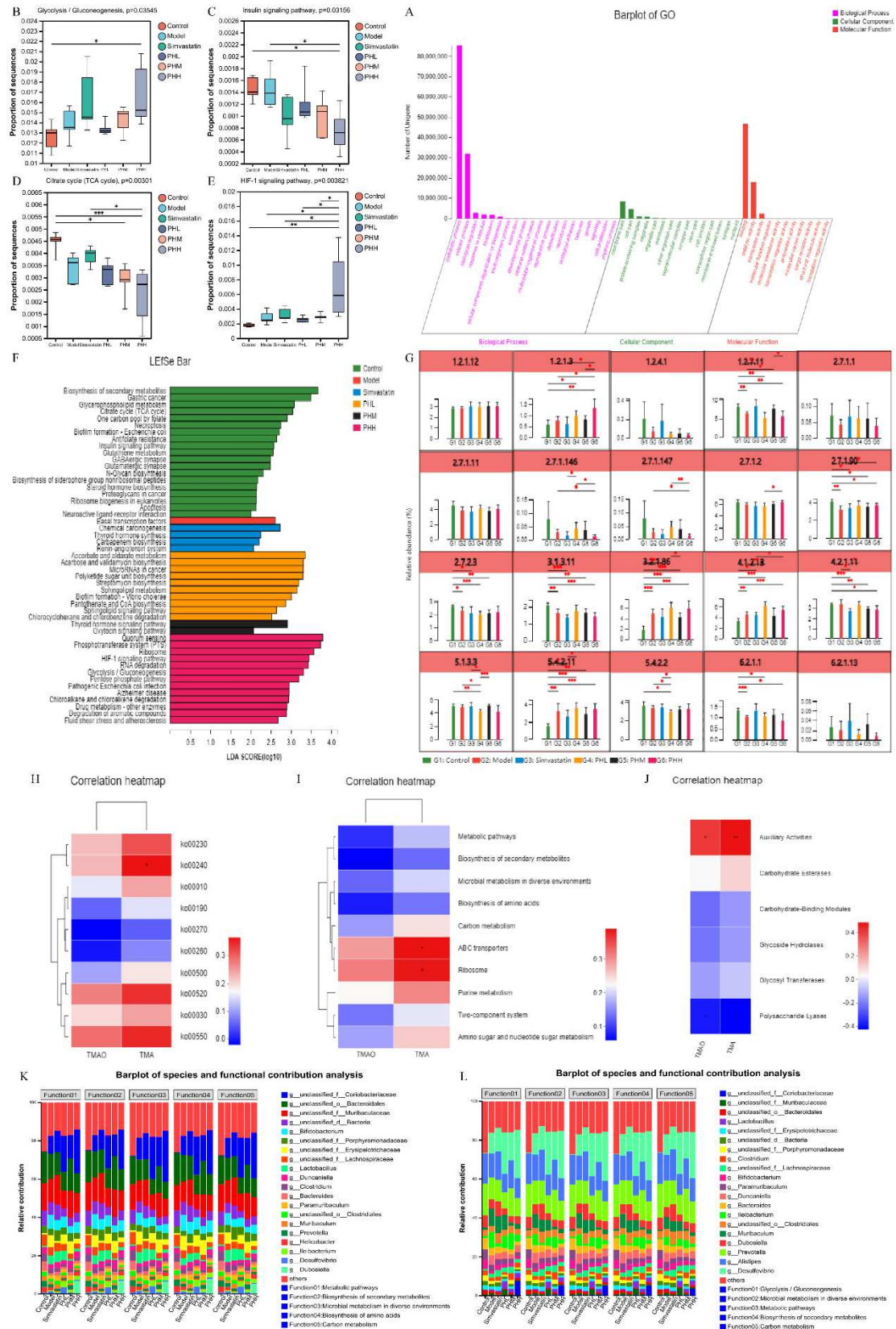


Fig. 9. (A) GO function annotation analysis; (B), (C), (D) and (E) Multigroup variance analysis of KEGG pathways based on TMA-producing bacteria gene set; (F) Linear discriminant analysis of

487 TMA-producing bacteria gene set; **(G)** Analysis of intergroup difference in metabolic pathways;
488 **(H), (I) and (J)** Spearman's correlation analysis between metabolic pathways and TMA/TMAO;
489 **(K) and (L)** Analysis of species function contribution. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

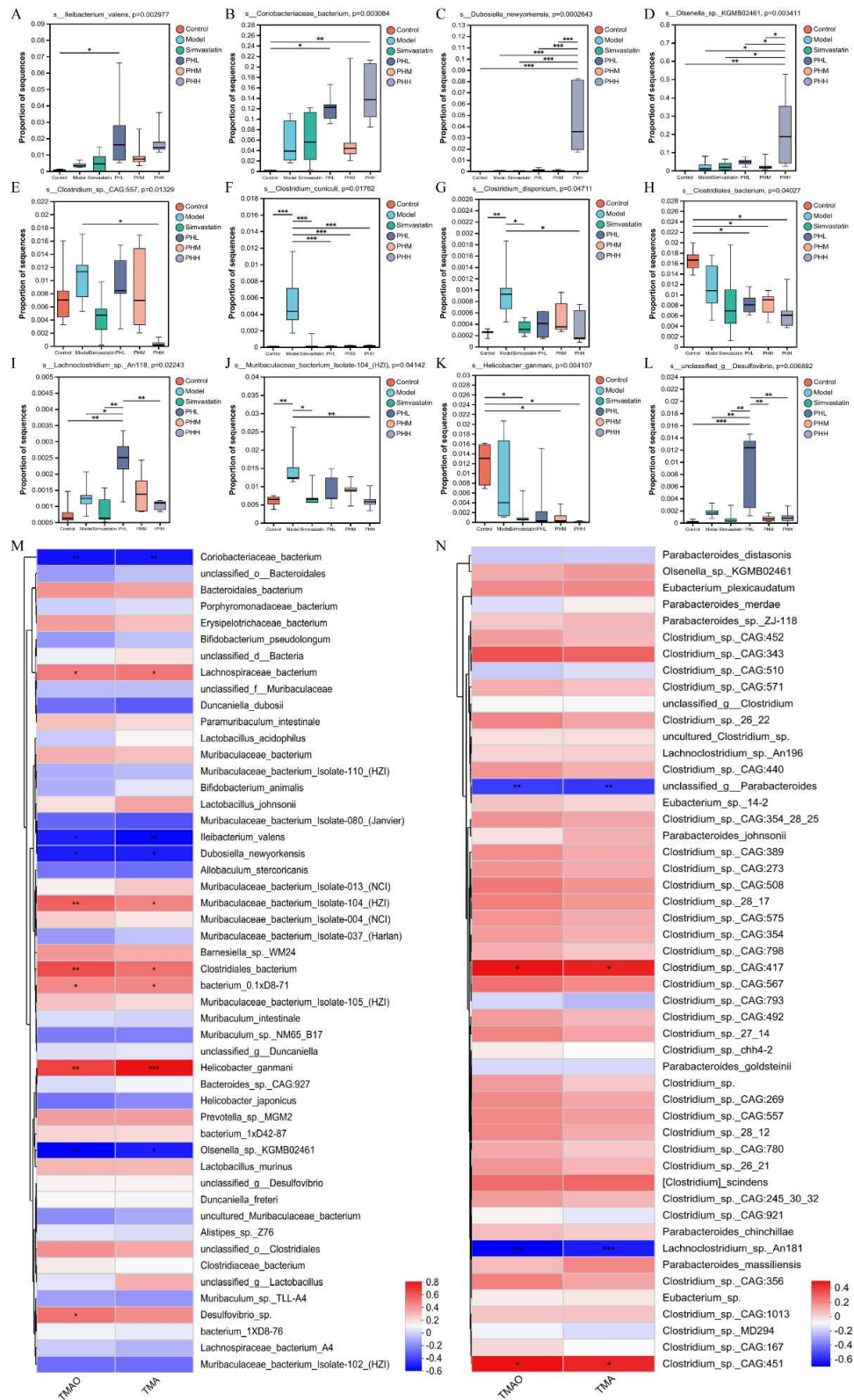


Fig. 10. (A)-(L) Multigroup variance analysis of TMA-producing bacteria gene set; **(M)** and **(N)**

Spearman's correlation analysis between TMA-producing bacteria and TMA/TMAO. * $P<0.05$,

** $P<0.01$, *** $P<0.001$.

4. Discussion

In this study, we uncovered the anti-atherosclerotic mechanism of PH through gut microbiota and metabolites. Our results suggested that PH could regulate the glucolipid metabolism-related pathway, attenuate inflammatory cytokine levels, and reduce atherosclerotic plaques by remodeling gut microbiota. Thus, 16S rRNA sequencing and metagenomics were utilized to identify the microbial markers. We observed that beneficial bacteria were markedly enriched, and certain TMA-producing bacteria were reduced. Disorders of intestinal homeostasis were induced by HFD feeding and were reversed by PH treatment by modulating the abundance of the specific bacterial species and level of gut microbiota-derived metabolites.

Polydatin, a monocrystalline polyphenol, also known as resveratrol glycoside (the chemical structure was shown in **Fig. S8**), is a stilbene compound isolated from *P. cuspidatum* that appears as a white needle-like crystalline powder with a molecular formula $C_{20}H_{22}O_8$ and molecular weight of 390.384 (41). Trans-polydatin and trans-resveratrol are primary derivatives of polydatin (42). Modern pharmacological studies have shown that polydatin is extensively used for the treatment of atherosclerosis and systemic metabolic disorders owing to its medicinal properties, including anti-inflammatory (43), antioxidant (44), and lipid metabolism modulation (45). Similarly, hawthorn is an excellent plant resource for both medicine and food (46). To date, more than 70 flavonoids and their glycosides have been isolated from hawthorn, which are mainly found in its leaves (47). According to the different aglycones, these flavonoids can be divided into luteolin, kaempferol, quercetin, hyperoside and vitexin, to name a few (48). Since the 1950s, flavonoids present in hawthorn discovered to have pharmacological effects on the cardiovascular system (49). Currently, hawthorn flavonoids and polydatin are officially included in the Chinese Pharmacopoeia (50).

hawthorn flavonoids are the main chemical components of these plants (the chemical structure is shown in **Fig. S9**) and are extracted from its leaves. They have additional medicinal properties, such as hypotensive, anti-inflammatory, hypocholesterolemic, and antihyperglycemic properties (51). Furthermore, evidence have shown that hawthorn flavonoids alleviate metabolic hyperlipidemia by altering bacterial and gut-derived metabolites related to cholesterol homeostasis (32). hawthorn flavonoids (the extract of hawthorn), as a pharmaceutical intervention, have been applied for our previous clinical trial (35) and research (52). In particular, our clinical trial confirmed that polydatin combined with hawthorn flavonoids can exert anti-atherosclerotic effect on patients with carotid atherosclerosis by promoting plaque stability and decreasing the inflammatory factors levels (35), which suggested that the combination of polydatin and hawthorn flavonoids could exhibit an amazing anti-atherosclerotic effect.

An HFD intensifies excess cholesterol accumulation, thereby leading to disorders of glycolipid metabolism (53). Our previous research confirmed that the mice in the model group may have experienced a decrease in the serum TC, TG, and LDL-C levels; however, hawthorn flavonoids and simvastatin reversed these changes and increased HDL-C levels (52). Furthermore, polydatin moderates the lipid metabolism via inhibiting the formation of peritoneal macrophage-derived foam cells in ApoE^{-/-} mice (54). It is reasonable to speculate that polydatin combined with hawthorn flavonoids may have excellent lipid-regulating effects. Our data revealed that PH could reverse dyslipidemia by reducing lipid levels (TC, TG, LDL-C, and VLDL-C) and body weight and increasing HDL-C levels.

The systemic inflammatory state appears to be not only an indicator of the progression of atherosclerosis, but also involved in the entire process of atherosclerosis (55). NF-κB, as a classical

inflammatory signaling pathway, regulates the expression of IL-6 and TNF- α (56). IL-6, IL-17, and TNF- α are proinflammatory and proatherogenic cytokines (57). Considering these inflammatory cytokines in the modulation of atherosclerotic plaque formation, immunohistochemical staining and the quantitative analyzes of protein expression in the aortic plaque were used to identify the inflammation pathological degree. As anticipated, the elevation of TNF- α and NF- κ B expression clearly promoted the progression of atherosclerosis, while PH reduced inflammatory cytokines levels, thereby improving atherosclerotic plaques. IL-6 plays a potential role in acute coronary syndrome and other complications related to atherosclerotic thrombosis (58). Although hs-CRP and LDL cholesterol levels are important biomarkers for predicting residual cardiovascular risk, several studies have revealed that the inhibition of the IL-6 signaling system and IL-1 β are key targets for improving atherosclerosis in an inflammatory cascade from CRP to IL-6 and IL-1 β (59). Additionally, the IL-17 receptor A, a differential regulator of atherosclerosis, may influence the atherosclerotic plaque formation (60).

Polyphenols are abundant in polydatin and hawthorn flavonoids and exert potential anti-inflammatory effects by attenuating HFD-induced inflammatory reactions (61). Our previous results demonstrated that polydatin could reduce TNF- α and IL-1 β levels in the peritoneal macrophages of ApoE^{-/-} mice (54), and hawthorn flavonoids could inhibit the elevation of hs-CRP and IL-1 β (52). Additionally, our clinical trial data confirmed that 15 g of *P. cuspidatum* combined with 10 g of hawthorn granule formula exerted cardioprotective effects by reducing hs-CRP, TNF- α , and IL-6 levels. We also observed that the plaque score decreased, and the level of plaque stability was strongly enhanced in patients with carotid atherosclerosis (34). Furthermore, our previous research revealed that hawthorn flavonoids (52) and polydatin (62) could individually improve

atherosclerotic plaques in ApoE^{-/-} mice. Consistent with these findings, we observed that PH could reduce serum inflammatory factors (IL-1 β , IL-2, IL-6, IL-17A, hs-CRP and TNF- α) levels and improve atherosclerotic plaques. The current study provided further evidence that the combination of polydatin and hawthorn flavonoids could enhance the improvement of atherosclerotic plaques by significantly reducing the levels of lipid and inflammatory factors.

Previous studies have revealed that the F/B ratio is an indicator of obesity-induced gut microbiota dysbiosis and a therapeutic target for metabolic disorders (63). *Firmicutes* induce lipopolysaccharide and deoxycholic acid production, leading to the development of hepatic steatosis (64), whereas supplementation of *Bacteroidetes* is beneficial to protect against obesity through the reduction of branched-chain amino acid levels in brown fat (65). Our study also confirmed that the abundance of *Firmicutes* increased in mice fed an HFD, whereas PH treatment exerted an anti-atherosclerotic effect by reducing the F/B ratio. Metabolic disorders in the intestinal microenvironment of the host can lead to reduction of microbial diversity, and certain bacterial species are closely associated with the levels of TC and TG (66). *Desulfovibrio*, a gram-negative bacterium, is involved in lipid metabolism and inflammation by producing lipopolysaccharide to trigger the release of IL-1 β , IL-6, and TNF- β and alter intestinal permeability and microbial composition (67). Previous research revealed that HFD-induced gut microbial alterations in atherosclerotic mice are mainly reflected in the enrichment of *Desulfovibrio* (68). In our study, the alpha diversity analysis revealed that *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Desulfobacterota* accounted for the majority of species in our study, and the microbial diversity and abundance were reduced after the HFD intervention. In particular, we observed that the proportion of *Desulfobacterota* was increased in the model group, however, it was decreased in the PHM, PHH

and simvastatin groups.

Muribaculaceae (S24-7) can inhibit the inflammation (69), gut microbial dysbiosis, and intestinal dysfunction by degrading mucin (70). Dingxin Recipe IV, a Chinese medicinal formula, alleviates atherosclerosis by moderating lipid metabolism; this effect is possibly associated with the elevation of *Muribaculaceae* (71). Simvastatin attenuates HFD-induced hyperlipidemia by increasing the abundance of *Lactobacillus* and reducing the level of TG and TC (72). Most studies have confirmed that *Lactobacillus* spp. can moderate lipid metabolism (73) and reduce cholesterol availability (74). Our findings revealed that *norank_f_Muribaculaceae* was enriched in the PHM and simvastatin groups. Additionally, *Lactobacillus* was mainly increased in the simvastatin and PHL groups. Furthermore, PHH and simvastatin administration also switched the enterotype to *g_Lactobacillus* and *g_norank_f_Muribaculaceae_1* to optimize the gut microbiota composition. *Atopobiaceae* bacterial strain P1 has been isolated from mouse feces (75). *In vivo* studies have revealed that the inhibition of C-C chemokine motif ligands can reduce TMAO levels and the abundance of *Muribaculaceae*, increase the abundance of *Atopobiaceae* to improve glucose and lipid metabolism disorders (76). *Coriobacteriaceae_UCG-002* belongs to the *Coriobacteriaceae* family, which accelerates cholesterol absorption and exhibits a positive correlation with the levels of TG (77). However, a recent report had suggested that *Coriobacteriaceae_UCG-002* can improve obesity (78). The β -diversity analysis in our study revealed that PHH and simvastatin improved the abundance of *Actinobacteria*, *Atopobiaceae* and *Coriobacteriaceae_UCG-002*.

Furthermore, harmful bacteria such as *Alistipes*, *f_Clostridiaceae*, and *f_Defluviitaleaceae* were the predominant genera in the model group. *Alistipes* have been recognized as an inflammatory indicator in most studies. The increase in the HFD-induced abundance of *Alistipes* was associated

with TC and LDL-C levels, whereas red yeast rice treatment ameliorated atherosclerosis by decreasing their relative abundance (79). The class *Clostridia*, which includes *f_Clostridiaceae* and *f_Defluviitaleaceae*, has often been reported to prompt the progression of colitis in mice (80), which is consistent with our observations. In our study, *Desulfobacterota*, *Firmicutes*, *Desulfovibrio*, and *Muribaculum* have strong correlations with lipid and inflammatory cytokine levels.

Furthermore, the metagenomic analysis mainly focused on KEGG and CAZy functions to annotate the differences in the gut microbial function. The KEGG functional analysis revealed increased enrichment of glycerolipid metabolism and glucagon signaling pathways in the PHL group; additionally, biosynthesis of amino acids, starch, and sucrose metabolism demonstrated increased enrichment in the PHH group. Accordingly, ko00520, ko00030, and ko00550 were positively associated with lipid and inflammatory cytokine levels at Level 2 while *s_Desulfovibrio_fairfieldensis*, *s_Allobaculum_stercoricanis* exhibited positive correlation with the metabolic pathways and starch and sucrose metabolism. A decrease in *Desulfovibrio* may contribute to the improvement of glycolipid metabolism (81) and inflammation (82). Thus, the microbial therapeutic effects of PH administration may be attributed to glycerolipid metabolism. CAZymes are indicators of microbial functional diversity (83). Different types of gut microbiota possess variable CAZyme content and carbohydrate degrading capacity (84). Moreover, HFD generates energy through microbial enzymes that improve carbohydrate metabolism (85). GHs and PLs participate in glycan degradation and provide additional or alternative energy sources for the gut microbiota (83). According to the CAZy functional enrichment analysis, GTs and CEs were highly enriched in the PHL and PHH groups. Specifically, GT2_Glyco_tranf_2_3 and CE1 were enriched in the PHH group. Furthermore, *Bacteroides* spp. and *s_Muribaculaceae_bacterium* exhibited

positive correlation with GHs and GTs.

Accumulating evidences reveal that gut microbiota and the metabolite TMAO may mechanistically participated in the pathogenesis of atherosclerosis (86), although the specific mechanism has not yet been fully elucidated. The overactivation of FMO3 in the microbial TMAO pathway promotes FoxO1 levels in a PERK-dependent manner (87), followed by an increase in TMAO levels, glucose intolerance (88) and insulin resistance (89). Consistent with previous reports, our results confirmed that an HFD containing 1% choline increased TMA and TMAO levels in the model group mice (90). Interestingly, we observed that PH moderated the TMA/FMO3/TMAO pathway by downregulating the protein and mRNA expression of FMO3, which is consistent with previous findings. Nevertheless, a recent study reported that TMAO levels appear to have indirect associations with the expression of FMO3 (91). Considering these conflicting reports, these results require further investigation.

Accumulating evidence has documented that (92), cholesterol homeostasis serves as a crucial mediator of metabolic disorders. TMAO promotes the transformation of macrophages into foam cells via cholesterol accumulation involved in the scavenger receptors SR-AI and CD36 (93). Consistent with this mechanism, our correlation analysis revealed a positive correlation between TMA and ko00240, ABC transporters, and auxiliary activities Expression of hepatic Abcg5/g8 expression have been suggested to increase TMAO levels (94). In addition, our data suggested that the abundance of *Firmicutes*, *Turicibacter*, and *Lachnospirillum* might participate in the process of accelerating the TMAO levels. Consistently, *Turicibacter* and *Lachnospirillum* have been associated with butyric acid, which may promote imbalanced lipid metabolism and elevation of TMAO levels (95). At the species level, we observed positive correlations of

uncultured_bacterium_g_Desulfovibrio; *s_Desulfovibrio_fairfieldensis*; metabolic pathways, including starch and sucrose metabolism; ABC transporters, including M00001, M00051, and M00003, with *s_Allobaculum_stercoricanis*, but a negative correlation with beneficial bacteria, including *Bacteroides* spp., *s_Lactobacillus_murinus* and *s_Muribaculaceae_bacterium*. In line with previous findings, the observed alterations in the abundance of *Alloprevotella*, and *norank_f_Desulfovibrionaceae* could have contributed to an immunoprotective effect on ABC transporters, carbohydrate digestion and absorption, and glycerophospholipid metabolism pathways (96).

Since TMA-producing bacteria are the main contributors to the production of TMAO and atherosclerosis, they were identified for gene set construction to further investigate the relationship between TMAO and atherosclerosis. Glycolysis/gluconeogenesis, drug metabolism, and HIF-1 signaling pathway were highly enriched in the PHH group, suggesting that a high dose of PH could change the abundance of TMA-producing bacteria and regulate the above functional pathways to attenuate the pathological process of atherosclerosis. Unlike previous studies, we initially confirmed the importance of glycolysis/gluconeogenesis in glycolipid metabolic pathways. In addition, we observed that phosphoglycerate mutase [EC:5.4.2.11] and fructose-bisphosphate aldolase [EC:4.1.2.13] were increased in mice fed an HFD, whereas PH administration could reverse these changes. Consistent with previous research, aloe polysaccharides may produce health benefits by upregulating the gene expression of fructose-bisphosphate aldolase (97). The cell-free extract of *Clostridium sporogenes* exhibited a high fructose-bisphosphate aldolase activity (98). GO functional annotation also suggested that the BP of each group were mainly focused on metabolism-related processes. According to the results of the species contribution analysis, *Clostridium* was

among the top five contributors to metabolic pathways, and *Desulfovibrio*, *Prevotella*, *Muribaculum*, *unclassified_f_Lachnospiraceae*, *unclassified_o_Bacteroidales* were other important species involved in metabolic processes.

The genera *Clostridium* (*Clostridiaceae*) and *Collinsella* (*Coriobacteriaceae*) possess TMA lyase activity and influence the level of TMAO (99). *Clostridium*, *Desulfovibrio*, and *Collinsella* participate widely in the synthesis of TMA, which is generated from choline in dairy diets (100). A high-sugar/fat diet-induced imbalance in cholesterol metabolism and inflammation appears to correlate with the enrichment of *Coriobacteriaceae* (101). In addition, *Lachnospiraceae_bacterium*, *Muribaculaceae_bacterium_Isolate-104_(HZI)*, *Clostridiales_bacterium*, *Desulfovibrio_sp.*, *Clostridium_sp._CAG:417*, and *Clostridium_sp._CAG:451* exhibited positive correlations with TMA and TMAO levels. The abundance of *s_Clostridium_sp._CAG:557*, *s_Clostridium_cuniculi*, and *s_Clostridium_disporicum* increased in the model group, and the PH administration partially restored the HFD-induced abundance of these *Clostridium_spp*. To summarize, PH has the potential to regulate the abundance of TMA-producing bacteria (*Coriobacteriaceae*, *Desulfovibrio*, *Muribaculum*, and *Clostridium*), moderate the TMA-FMO3-TMAO pathway, and influence the related enzymes in glycolipid metabolic pathways, thereby exerting an important effect on the expression of TMAO, lipids, and inflammation, ultimately alleviating the progression of atherosclerosis.

Conclusion

In conclusion, our results suggested that PH administration could reduce inflammatory factors and lipid levels, thereby attenuating the development of atherosclerotic plaques in HFD-induced mice. In addition, PH exerts its beneficial effects by remodeling the gut microbiota and regulating

its derived metabolite, TMAO. In particular, PH-induced changes in TMA-producing bacteria and enzymes involved in the glycolipid metabolic pathways have important effects on atherosclerosis therapy. Nevertheless, the underlying mechanisms and new targets of gut microbial biomarkers require further investigation.

Abbreviations

PH, polydatin and hawthorn leaf flavonoids; *P. cuspidatum*, *Polygonum cuspidatum* Sieb. et Zucc; *C. pinnatifida*, *Crataegus pinnatifida* Bunge; CVDs, Cardiovascular diseases; TMAO, Trimethylamine N-oxide; TMA, trimethylamine; FMO3, flavin-containing enzyme monooxygenase 3; HFD, high-fat diet; H&E, hematoxylin-eosin; TC, cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; VLDL-C, very low density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; NF- κ B, nuclear factor- κ B; TNF- α , tumor necrosis factor alpha; IL-1 β , interleukin-1-beta; IL-2, interleukin-2; IL-6, interleukin-6; IL-17A, interleukin-17; OTUs, operational taxonomic units; ORFs, open reading frames; PCoA, principle coordinate analysis; NMDS, non-metric multidimensional scaling; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size; CAZymes, carbohydrate-active enzymes; GTs, glycosyl transferases; CEs, carbohydrate esterases; GHs, glycoside hydrolases; BP, biological processes.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contributions

M.W., D.L., L.L. designed the study. Dan Li reviewed the manuscript. M.W. and L.L. provided the experimental platform. D.L., Y. L., S.Y. C.Y., X.Z., X.J., J.L., and X.W. performed animal experiments. D.L. analyzed data and aided in the design of the illustrations. S.Y. helped revise the manuscript. All authors approved the manuscript.

Acknowledgments

Not applicable.

Appendix A. Supporting information

Supplementary material associated with this article: Table S1. Realtime PCR reaction condition; Table S2. Primer sequences; Table S3. Mobile phase gradient condition; Table S4. Mass spectrum condition; Table S5. Alpha diversity index comparison among groups; Figure S1. The distribution of coverage index and abundance index Sobs, Ace and chao; Figure S2. The distribution of diversity

index Shannon and Simpson; Figure S3. Venn analysis pie plot at phylum level; Figure S4. Venn analysis pie plot at family level; Figure S5. Venn analysis pie plot at genus level; Figure S6. PCoA and barplot of typing analysis; Figure S7. Glycolysis/Gluconeogenesis pathway; Figure S8. Chemical structure of polydatin; Figure S9. Chemical structure of the predominant hawthorn flavonoids.

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