

Article

Exploring the Underlying Mechanisms of Aerobic Exercise—Improving Cardiovascular Function by Integrating Microbiome, Metabolome, and Proteome Analysis in a High-Fat Diet-Induced Obesity Rat Model

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Abstract

Objective: This study aimed to investigate the potential mechanisms by which moderate-intensity aerobic exercise improves cardiovascular dysfunction in high-fat diet-induced obese rats through integrated multi-omics analysis. **Methods:** Animals were assigned to three groups: normal diet, HFD, and HFD with exercise. Cardiovascular function was assessed by echocardiography and vascular tension measurement. Gut microbiota, serum metabolites, and protein expression were analyzed using 16S rRNA sequencing, untargeted metabolomics, and proteomics, respectively. Integrated multi-omics analysis was performed using Mantel tests and mediation effect analysis. **Results:** Eight weeks of aerobic exercise significantly improved cardiovascular function in obese rats, including enhanced acetylcholine-induced vasodilation and increased left ventricular ejection fraction. Furthermore, exercise also reshaped the gut microbiota composition, notably altering the relative abundances of *Lactobacillus* and *Ruminiclostridium_9*. Metabolomics revealed that exercise shifted the metabolic phenotype from high-fat diet-induced basal metabolic disorder toward beneficial pathways, including fatty acid biosynthesis and ubiquinone biosynthesis. Proteomics identified key differentially expressed proteins such as APOE, FN1, and Lap3. Integrated multi-omics analysis for the first time revealed a core regulatory axis: exercise may influence Lap3 expression, modulate the abundance of *Lactobacillus*, and thereby systematically regulate the level of palmitoyl lysophosphatidylcholine, ultimately improving cardiovascular function. **Conclusions:** Aerobic exercise counteracts HFD-induced cardiovascular dysfunction through systemic remodeling of the gut microbiota–host metabolism–protein network. The discovery of the Lap3–*Lactobacillus*–palmitoyl lysophosphatidylcholine axis provides new molecular insights into the exercise-mediated protective mechanisms of the gut–cardiovascular system axis.

Keywords: aerobic training; gut microbiota; metabolomics; proteomics; cardiometabolic health; rat model



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1. Introduction

Cardiovascular diseases (CVDs) represent a major public health issue worldwide, accounting for substantial morbidity and mortality. Amidst the myriad of risk factors, obesity, usually exacerbated by sedentary lifestyles and high-fat diets (HFD), has emerged as a substantial contributor to the escalating burden of CVDs [1]. The intricate relationship between obesity and cardiovascular health has prompted a growing interest in interventions capable of mitigating these risks.

As obesity rates continue to rise globally, exploring effective strategies to alleviate the cardiovascular burden associated with obesity becomes imperative. Among the interventions under scrutiny, aerobic exercise has gained prominence for its potential to counteract the deleterious effects of HFD on cardiovascular health. A robust body of evidence supports the notion that exercise serves as a potent intervention for ameliorating CVDs, showcasing its multifaceted impact on core physiological processes [2]. These encompass the modulation of gut microbiota, alterations in serum metabolites, and the regulation of key target proteins [3–5], collectively contributing to a holistic strategy for cardiovascular health.

Recent years have positioned the gut microbiota as a central mediator between lifestyle interventions and host metabolic health. Exercise enriches short-chain fatty acid (SCFA)-producing bacteria such as *Lactobacillus* and *Blautia*, reinforcing intestinal barrier integrity and attenuating systemic inflammation [6–8]. Concurrently, the serum metabolome serves as a functional readout of host–microbiota crosstalk; lipid species like lysophosphatidylcholines and branched-chain amino acids are robustly linked to obesity-related cardiovascular risk [9]. Furthermore, proteins are the direct executors of biological functions; exercise-induced proteomic signatures have been shown to encompass pathways involved in immune regulation and metabolism. Large-scale population-based studies have identified multiple circulating proteins associated with physical activity, including lipoproteins (LPL, HDL-related proteins), inflammatory mediators (IL6, GDF15), and adipokines (leptin), with these proteomic alterations linked to reduced risk of cardiometabolic diseases [10]. Despite these advances, most studies have examined each molecular layer in isolation, precluding a systems-level understanding of how exercise coordinates multi-dimensional biological networks. Integrated multi-omics analysis—combining microbiome, metabolome, and proteome data—offers a powerful strategy for decoding such complex regulatory circuits. Therefore, the present study employed 16S rRNA sequencing, untargeted metabolomics, and label-free proteomics to comprehensively elucidate the ‘gut–metabolite–protein’ axis through which aerobic exercise improves obesity-related cardiovascular dysfunction.

This study seeks to extend our understanding of exercise’s impact on cardiovascular health by unraveling the complex interactions within the gut microbiota, metabolic pathways, and protein regulation. By focusing on these interconnected elements, we aim to provide valuable insights into the mediating effects of aerobic exercise on cardiovascular dysfunction induced by HFD. Through this research, we aspire to contribute not only to the scientific understanding of exercise’s role but also to the development of targeted interventions that can alleviate the cardiovascular burden associated with obesity and HFD.

2. Materials and Methods

2.1. Experimental Animals

Male Sprague–Dawley rats were obtained from VitonLihua Ltd. (Beijing, China). All experimental protocols received approval from the Ethics Review Committee of Guangzhou Sport University and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Animals were maintained in the Animal Facility of the Scientific Research Center at Guangzhou Sport University under controlled conditions (12 h light/dark cycle, 22 ± 2 °C, $50\% \pm 5\%$ humidity) and with free access to chow and

water. Following a 1-week acclimatization period, rats were randomly assigned to three groups: normal diet sedentary (NC), high-fat diet sedentary (HC), and high-fat diet with exercise (HE). To establish a high-fat diet-induced obesity rat model, 6-week-old male SD rats were fed a high-fat diet (20.2% protein, 45.4% fat and 34.5% carbohydrate) for 20 weeks. Rats fed a standard maintenance diet (20.6% protein, 12% fat and 67.4% carbohydrate) served as the control group. Body weight and length were recorded weekly to compute body mass index (BMI) and Lee's index. BMI was calculated according to the formula: $BMI = \text{weight (kg)} / \text{height (m}^2\text{)}$. Lee's index was calculated according to the formula: $\text{Lee's index} = \text{body weight (g)}^{1/3} \times 1000 / \text{body length (cm)}$. After confirming successful obesity model construction (high-fat diet-fed rats weighing more than 10% of the body weight of standard diet-fed rats and with Lee's index higher than 1.5%), the rats were subjected to an 8-week exercise training using a motor-driven treadmill.

2.2. Aerobic Exercise Training

Rats were subjected to motor-driven treadmill training as previously described [11]. The training period lasted for 8 weeks, including one week of adaptive training. Treadmill conditions were as follows: warming up at 5 m/min for 5 min; running at 5–15 m/min for 50 min to achieve the target intensity in the first week; increasing the speed to 15 m/min for the next 7 weeks; and relaxing at 8 m/min for 5 min. Training was conducted 5 times a week.

2.3. Echocardiography

Cardiac function was assessed by echocardiography using a VINNO6VET system (Visual Sonic, Bothell, WA, USA) under 2% isoflurane anesthesia. Vascular wall thickness, vascular diameter, left ventricular posterior wall (LVPW), LV fractional shortening (FS), LV ejection fraction (EF), maximum flow velocity, and stroke volume were calculated [12]. All values were averaged from five consecutive cardiac cycles.

2.4. In Vitro Arterial Tension Measurement

Mesenteric artery function was evaluated via vascular tension measurements following established methods [13]. Arterial segments were dissected in ice-cold Krebs buffer (composition in mmol/L: NaCl 118, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄·7H₂O 1.2, NaHCO₃ 25.2, glucose 11.1; pH 7.4) continuously aerated with 95% O₂/5% CO₂. Vessels were cut into 2 mm rings and mounted in a myograph chamber (DMT 620M, Danish Myo Technology, Aarhus, Denmark) for isometric tension recording using LabChart 8.0 software. After pre-contraction with phenylephrine (1 µmol/L), cumulative concentrations of acetylcholine (ACh) were applied to generate concentration-relaxation curves.

2.5. Cecal Sample Collection and 16S rRNA Sequencing

To investigate the effect of aerobic exercise on the gut microbial community structure in HFD-induced obese rats, cecal contents were collected and subjected to 16S rRNA gene sequencing. Cecal contents were collected and immediately frozen in liquid nitrogen. The methods of total bacterial DNA extraction and sequencing of the V3–V4 region of the 16S rRNA genes, as well as the construction of the sequencing library, were illustrated in previous publications [14,15]. The 16S rRNA gene amplicon was sequenced on the Illumina MiSeq platform based on the manufacturer's instructions. The resulting data were concatenated and filtered using Trimmomatic (version 0.35) to obtain a set of high-quality sequences for downstream analysis [16]. The qualified reads were clustered into operational taxonomic units (OTUs) with USEARCH (version 10.0) based on a 97% sequence similarity cut-off value [17]. Next, alpha diversities including the Chao, ACE, Shannon, and Simpson were performed using R software v4.2.3 (vegan package), while beta diversity

based on Bray–Curtis dissimilarity was assessed using QIIME v1.9.1. Rarefaction curves were generated with USEARCH. Furthermore, Linear discriminant analysis Effect Size (LEfSe) was applied to identify differentially abundant taxa [18]. Finally, species taxonomy was acquired by annotating feature sequences with a naive Bayes classifier based on the reference database provided by Silva (<http://www.arb-silva.de>).

2.6. Metagenomic Pathway Prediction by PICRUSt2

PICRUSt2 was utilized to predict the metagenomic functional compositions [19]. Pathways that were different in abundance between the HC vs. NC and HE vs. NC groups were obtained using Student's *t*-test, and the Storey FDR was used to correct for multiple tests. STAMP software (v2.1.3) was utilized for statistical analyses and visualization of the identified pathways.

2.7. Non-Targeted Serum Metabolomics Analysis

To profile the systemic metabolic changes associated with exercise intervention, serum samples were analyzed using untargeted UPLC-MS/MS. Blood was collected into tubes devoid of anticoagulant for serum isolation. Following centrifugation ($1500 \times g$, 15 min, 22 °C), serum supernatants were stored at -80 °C until analysis. The metabolite profiles of the liver were detected by a UPLC-MS/MS system (Thermo Fisher Scientific, Waltham, MA, USA) via Novogene (Novogene, Beijing, China). Sample preparation for metabolomic analysis has been described previously [20]. The chromatographic and mass spectrometry settings established in the work were based on the device parameters of Yu et al. [21]. Subsequently, the raw data were preprocessed for further multivariate analysis. Detailed procedures for data processing and analysis included de-noising based on the relative standard deviation (RSD), quality evaluation, missing value imputation, normalization, transformation, and scaling. Processed data (peak identifiers, sample names, normalized peak areas) were imported into SIMCA 16.0.2 (Sartorius Stedim Data Analytics AB, Umea, Västerbotten, Sweden) for multivariate analysis. Metabolites with variable importance in projection (VIP) >1 and raw $p < 0.05$ were deemed significantly different between groups. For visualization, z-score-normalized intensities of differential metabolites were displayed as heatmaps generated with the R package pheatmap (version 4.4.0). Metabolite identification was achieved by matching fragment spectra against HMDB, Metlin, Lipid Maps, and in-house databases (PPM < 20). The Small Molecule Pathway Database (SMPDB) (<https://smpdb.ca>) was used to identify and interpret the change patterns of metabolites in important biological pathways for the different metabolites identified.

2.8. Sample Preparation for Label-Free Proteomics and Analysis

To identify exercise-responsive proteins and their associated biological functions, serum proteomic profiling was performed using label-free quantification. Serum proteins were extracted using RIPA lysis buffer (Thermo Scientific, Waltham, MA, USA) and quantified via a BCA assay. Aliquots containing 250 µg protein were processed using a modified filter-aided sample preparation method [22]. Samples were reduced with dithiothreitol, denatured with 10 mol/L urea and alkylated with iodoacetamide, and then centrifuged through a high molecular weight cut-off centrifugal filter (Millipore Sigma, Burlington, MA, USA, 10 k MWCO). Next, the digested peptides were collected after overnight digestion with sequencing-grade trypsin (Promega, Madison, WI, USA) at 37 °C and cleaned with a C18 ProtoTM 300 Å ultramicrocentrifugal column. Digested peptide samples (60 µg) were labeled with a tandem mass tag (TMT) 10plex TM isomeric labeling reagent group (Thermo Fisher Scientific). Finally, the Oasis HLB extraction column (Waters Corporation, Milford, MA, USA) was used for concentration and desalination, and the digested peptides were extracted [23]. The peptides were analyzed using LC-MS/MS on Q ExactiveTM HF-X

(Thermo Fisher Scientific) coupled to an EASY-nLC 1000 UPLC system (Thermo Fisher Scientific). Raw data were processed with Proteome Discoverer v2.2.0.388.

Proteomic data were analyzed using R software. First, the raw data were transformed by taking logarithmic base 2 followed by quantile normalization. Next, differential proteins ($p < 0.05$) were further filtered by fold-change (FC) criteria ($-0.6 < \log_2\text{FC} < 0.6$), yielding significant results for the differential proteins. Moreover, the search tool for recurring instances of neighboring genes (STRING) (<https://cn.string-db.org/>) was used to analyze protein–protein interactions. Finally, functional enrichment analysis was performed with Gene Ontology (GO) terms (<http://geneontology.org/>). Statistical significance was set at $p < 0.05$.

2.9. Integrative Analysis of Multi-Omic Data

To systematically elucidate the crosstalk among gut microbiota, serum metabolites, and host proteins, integrative correlation analysis, Mantel testing, and mediation analysis were performed. Correlations between the differentially expressed metabolites and differentially expressed proteins, and between the DEMs and differential microbiota were analyzed using the Pearson statistical method; the correlation coefficient R and p value were calculated. Correlations between differentially expressed metabolites and differential microbiota were visualized using the pheatmap package. Additionally, we used Cytoscape (version 3.9.1) [24] to visualize the correlation relationships between the DEMs and differential microbiota. Finally, to investigate the causal role of gut bacteria in contributing to the key proteins through key metabolites, a mediation analysis was conducted using the “mediate” function from the R package “mediation”.

2.10. Statistical Analysis

The present study adhered to rigorous statistical methods to ensure the validity and reliability of the results. All continuous parameters were first examined for normality using the Shapiro–Wilk test. Specifically, data were presented as mean $\pm$ standard deviation. Baseline differences between the two groups were analyzed using a two-tailed independent t -test, while a paired t -test was used to compare the means within each group. Pearson correlation analysis was conducted to evaluate the linear relationship among differentially expressed proteins, metabolites and genera among the groups [25]. To further explore the relationship between differential proteins, differential metabolites, and differential bacterial genera, the Mantel test was used to evaluate the correlation between them [26]. Furthermore, a mediation analysis was conducted to examine whether the effect of Palmitoyl lysophosphatidylcholine on Lap3 was mediated by Lactobacillus, with the significance of the mediation effect tested using the bootstrap method [27]. The false discovery rate (FDR) was calculated for multiple testing; an FDR-corrected p -value of less than 0.05 was considered statistically significant. All statistical analyses and visualizations were computed using R software (version 4.4.0).

3. Results

3.1. Exercise Ameliorated Obesity-Induced Cardiovascular Dysfunction

During the 20-week dietary intervention, both HC and HE rats exhibited rapid weight gain and maintained significantly higher body weights than the NC rats. From Week 20 to Week 28, rats in the HE group were subjected to the exercise training while continuously being fed the high-fat diet. The body weight of the HE group tended to stabilize, while that of the HC group continued to increase (Figure 1A). To minimize the confounding effects of individual size variation, Lee’s index and BMI were calculated at Weeks 20 and 28 (Figure 1B,C). At Week 20, both the HC and HE groups displayed elevated Lee’s index and

BMI relative to the NC controls. However, at Week 28, the Lee's index and BMI of the HE group were significantly lower than those of the HC group. These results indicated that the animal model of obesity was successfully established, and exercise training significantly reduced the body weight and BMI of obese rats.

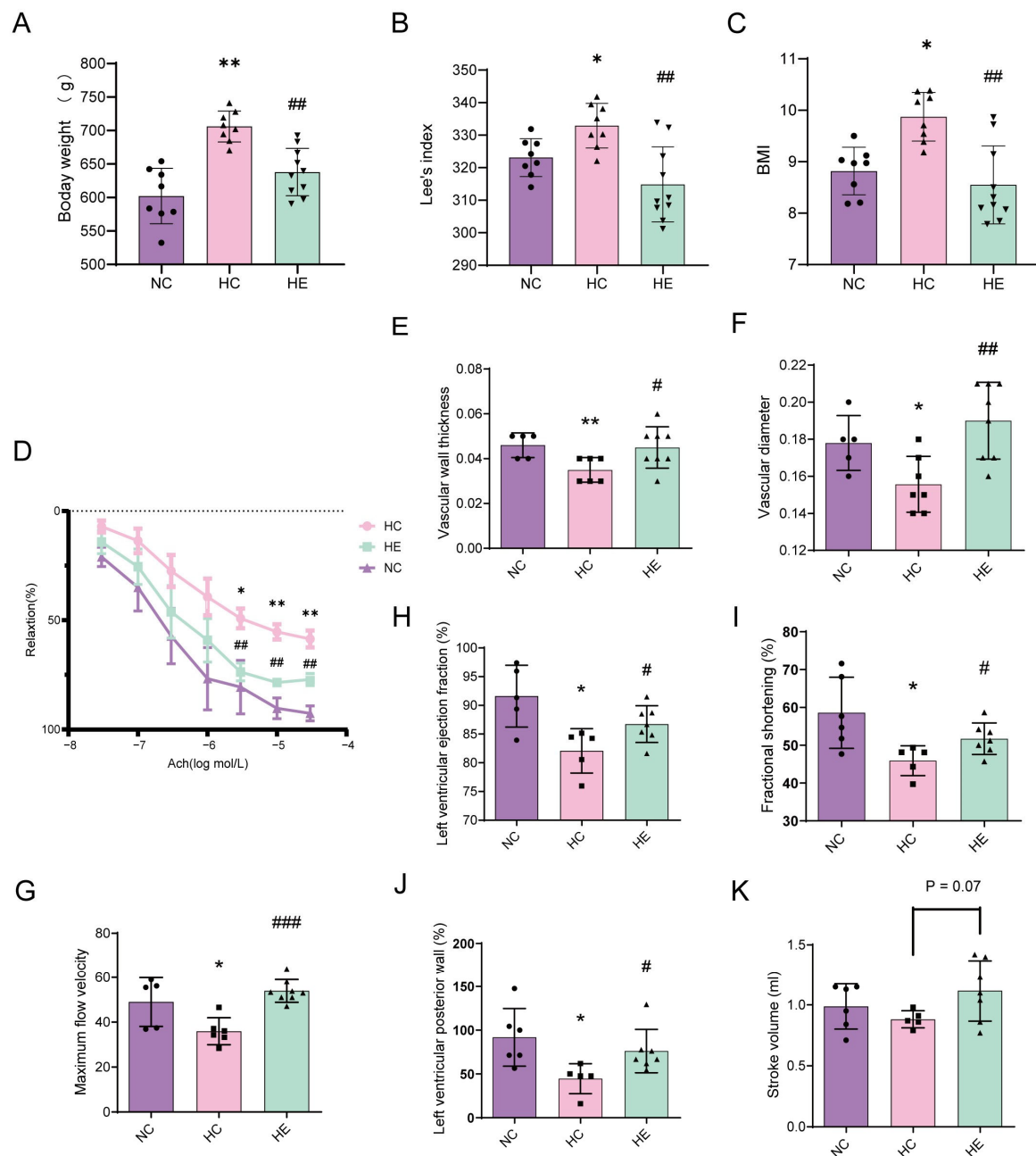


Figure 1. Exercise restored obesity-induced cardiovascular dysfunction. (A–C) Box line chart showing body weight (A), Lee's index (B) and BMI (C) of the NC (n = 8), NE (n = 10), HC (n = 8), and HE (n = 10) rats. (D) Line chart showing mesenteric artery vasodilatory capacity after Ach stimulation in the indicated groups. (E–G) Maximum flow velocity (G), vascular wall thickness (E) and vascular diameter (F) in the indicated groups. (H,I) The content of cardiac function, left ventricular ejection fraction (LVEF) (H), fractional shortening (FS) (I), left ventricular posterior wall (LVPW) (J), and stroke volume (SV) (K) in the indicated groups. * $p < 0.05$, ** $p < 0.01$, #: compare to HC, # $p < 0.05$ ## $p < 0.01$, ### $p < 0.01$: same group before and after comparison. All data are shown as the mean $\pm$ SD. NC, normal control; HC, high-fat diet control; HE, high-fat diet exercise.

Assessment of vascular function revealed that ACh-induced vasorelaxation was markedly impaired in the HC group compared to the NC controls; this impairment was significantly ameliorated in the HE group (Figure 1D). Consistently, the EC₅₀ for ACh was higher in the HC rats than in the NC rats, and this increase was blunted by exercise training (Figure 1D). Furthermore, compared to rats in the NC group, rats in the HC group showed a significant decrease in vascular wall thickness, vascular diameter, and maximum flow velocity, and these reductions were significantly reversed in the HE group. These results indicate that long-term aerobic exercise protected against vascular dysfunction, especially endothelial dysfunction, in rats with obesity (Figure 1E–G).

In addition, the left ventricular ejection fraction (LVEF), fractional shortening (FS), and left ventricular posterior wall (LVPW) were significantly lower in the HC rats than in the NC controls; however, these reductions were significantly reversed in the HE group. Meanwhile, compared to the HC group, stroke volume (SV) in the HE group also increased after exercise training ($p = 0.07$). These results suggest that long-term exercise training can restore obesity-induced cardiac insufficiency in rats (Figure 1H–K).

3.2. Exercise Restored the Gut Microbiota Imbalance Caused by HFD

The rarefaction curves (Figure S1A) of all samples supported the adequacy of the sequencing depth. The OTUs flower Venn diagram showed 214 OTUs commonly shared by the NC, HC, and HE groups (Figure 2A). We first analyzed the differential changes in gut microbiota among the three groups using α diversity. Although no significant differences were observed in α -diversity values among the groups (Figure 2B), we noted a gradually increasing trend in species richness across the three groups, demonstrating that HFD can alter the composition of the rat gut microbiota. Next, we performed Principal Coordinate Analysis (PCoA) on the three sample groups (Figure 2C) and simultaneously assessed differences among them using Adonis analysis. Results indicated significant alterations in bacterial community structure among the NC, HC, and HE groups ($p < 0.001$). As shown in Figure 2F, we found that *g__Lactobacillus* had the highest LDA score in the NC group, *g__Ruminiclostridium_9* had the highest score in the HC group, and *g__Blautia* had the highest score in the HE group. Additionally, we conducted an in-depth analysis of the relative abundance of changes in the microbial phylum and genera across groups. The Firmicutes were the most dominant phylum, with a relative abundance of 68.5% in the NC group, 73.3% in the HC group, and 75.5% in the HE group (Figure 2E, Table S1). The second most dominant phylum was Bacteroidetes (NC: 23.9%, HC: 15.6%, HE: 15.4%). Interestingly, we observed a significant decrease in probiotics (such as *g__Lactobacillus*) in the HC group, demonstrating that HFD markedly disrupts the gut microbiota in rats (Figure 2F and Table S2). Through LEfSe analysis (Figure 2D), we identified three sets of potential gut microbiota biomarkers: the relative abundance of *g__Lactobacillus* was significantly elevated in the NC group ($p < 0.01$), *g__Ruminiclostridium_9* showed significantly increased relative abundance in the HC group ($p < 0.01$), and the relative abundance of *g__Blautia* was significantly increased in both the HC and HE groups ($p < 0.05$) (Figure 2G). Finally, the functional information of the differential gut microbiota in each group was predicted based on PICRUSt2, which illustrated that compared to the NC group, the HC and HE groups were significantly enriched in the bile acid metabolic pathway to varying degrees (Supplementary Figure S1B,C). Based on the above results, we found that HFD significantly disrupted the gut microbiota of rats, leading to a decrease in the abundance of probiotic bacteria such as *g__Lactobacillus* and an increase in the abundance of pathogenic bacteria like *g__Ruminiclostridium_9*. Additionally, we identified the beneficial bacterium *g__Blautia* as a significantly differential genus in the HE group, suggesting its potential as an obesity biomarker for cardiovascular and metabolic disease risk [28].

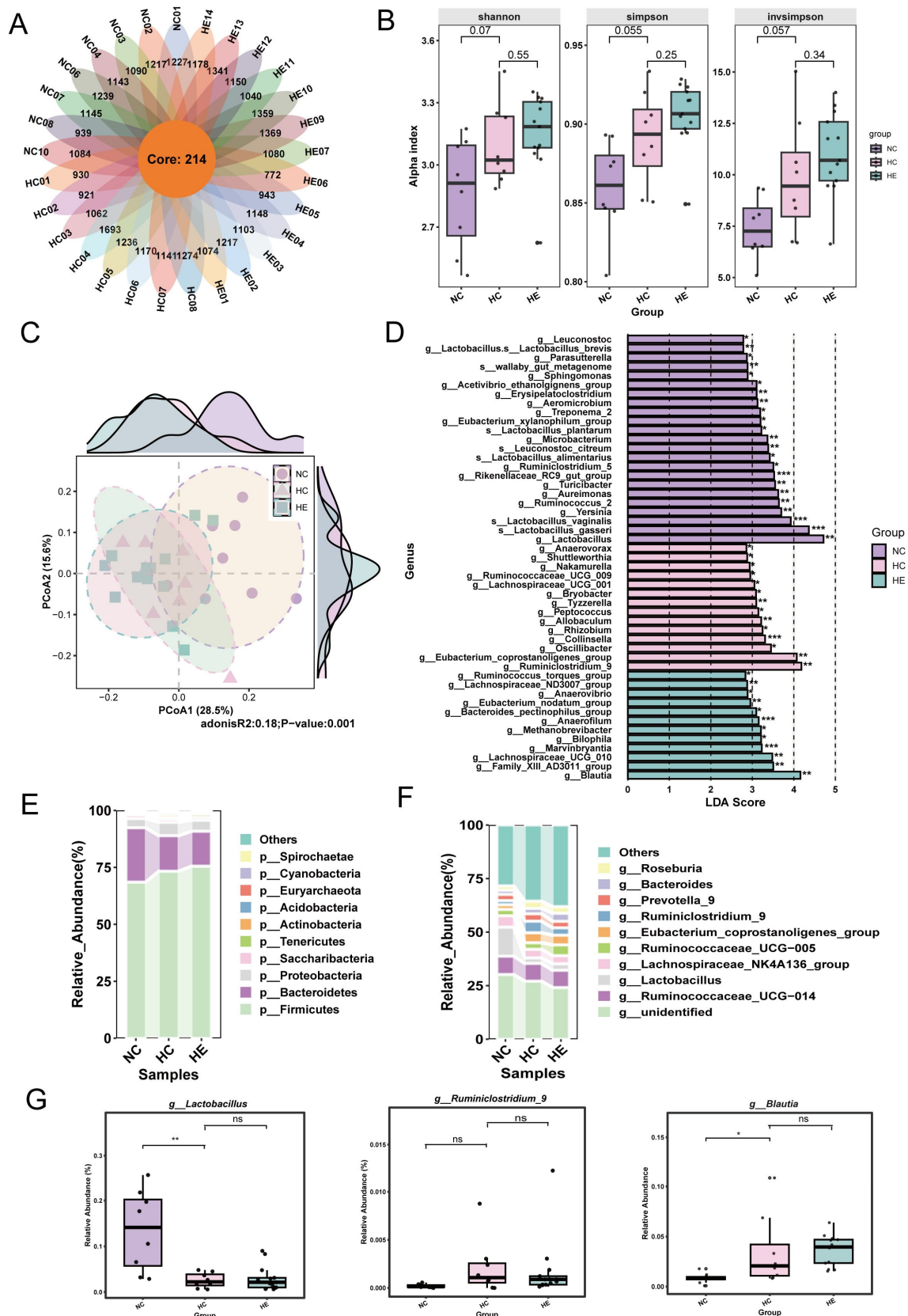


Figure 2. Microbial analysis using rat colonic flora. (A) Flower Venn diagram showing the distribution of operational taxonomic units (OTUs). The numbers in the center represent the OTUs common to all samples (i.e., Core OTUs), and the numbers on the petals represent the total OTUs of each sample. (B) Alpha diversity was analyzed using the observed Shannon, Simpson, and Invsimpson indices. (C) Principal Coordinates Analysis (PCoA) based on β -diversity distances. Each point represents one

sample (NC: circles; HC: triangles; HE: squares), and colored ellipses indicate 95% confidence intervals for each group. Density plots along the top and right margins illustrate the distribution of samples along each coordinate axis. (D) Linear discriminant analysis Effect Size (LEfSe) analysis of gut microbiota differences among groups at the genus level. The horizontal axis represents the LDA score, indicating the effect size and contribution of each genus to group discrimination. The vertical axis lists the genera with significant differences. Different colors correspond to different groups (NC, HC, and HE). Asterisks indicate statistical significance levels (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (E) Relative abundance of gut microbiota at the phylum level among different groups. (F) Relative abundance of gut microbiota at the genus level among different groups. (G) The relative abundance of *g__Lactobacillus*, *g__Ruminiclostridium_9*, and *g__Anaerofilum* among different groups. LDA, Linear discriminant analysis. NC, normal control; HC, high-fat diet control; HE, high-fat diet exercise. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3. Analyses of Serum Metabolites

Volcano plots illustrate the distribution of differentially expressed metabolites (DEMs) based on p -values and fold changes (Figure 3A–C). Among them, a total of 49, 83, and 6 different expressed metabolites (DEMs) were identified between the HC and NC groups, the HE and NC groups, and the HE and HC groups, respectively (Tables S3–S5). Subsequently, we conducted metabolism correlation analysis to evaluate changes in metabolites in different groups. Hierarchical clustering of the top 50 DEMs clearly separated the three groups (Supplementary Figure S2A,B). Notably, we found 3 DEMs that were associated with the NC, HC, and HE groups and 5 unique DEMs in the HC group vs. the NC groups, 1 unique DEM in the HE group vs. the HC group, and 39 DEMs in the HE group vs. the NC group (Figure 3D). Next, the heatmaps show the expression of differential metabolites among the three groups (Figure 3E). Finally, our enrichment analysis indicated that pathways mainly enriched in the NC group vs. the HC group were methylhistidine metabolism, vitamin B6 metabolism, phospholipid biosynthesis, beta-alanine metabolism, and histidine metabolism, and pathways enriched in the HC group vs. the HE group included fatty acid biosynthesis, thiamine metabolism, ubiquinone and other terpenoid-quinone biosynthesis, caffeine metabolism, and phenylalanine metabolism (Figure 3F,G).

3.4. Identification of Different Expressed Proteins (DEPs)

We identified DEPs in the HC group vs. the NC group, the HE group vs. the HC group, and the HE group vs. the NC group with a $|\log_2(\text{fold change})| \geq 0.6$ and a $p\text{-value} < 0.05$. Significantly up-regulated and down-regulated DEPs in the indicated groups were labeled in Figure 4A–C and Tables S6–S8. Among them, there are 35 DEPs in the HC group vs. the NC group, 28 DEPs in the HE group vs. the HC group, and 18 DEPs in the HE group vs. the NC group (Figure 4D). Subsequently, we showed the expression of three groups of DEPs in the form of heatmaps (Figure 4E). Interestingly, compared to HC group, 11 proteins were significantly down-regulated while 7 proteins were significantly up-regulated after exercise. Furthermore, the GO enrichment analysis of DEPs in the HC group vs. the NC group revealed several enriched pathways, including peptide cross-linking, immune system process, positive regulation of B cell activation, immunoglobulin complex, cornified envelope, immunoglobulin receptor binding, and antigen binding (Figure 4F). DEPs in the HE group vs. the NC group mainly enriched in the retinol metabolic process, low-density lipoprotein particle remodeling, extracellular space, heparin binding, receptor binding, and peptide hormone binding (Figure 4G). To fully understand the functional enrichment of DEPs in the HE group vs. the HC group, we selected the TOP 7 GO enrichment pathway to be shown in Figure 4H, which included the enrichment of innate immune response, retinol metabolism, complement activation, extracellular space, calcium binding and calcium ion binding.

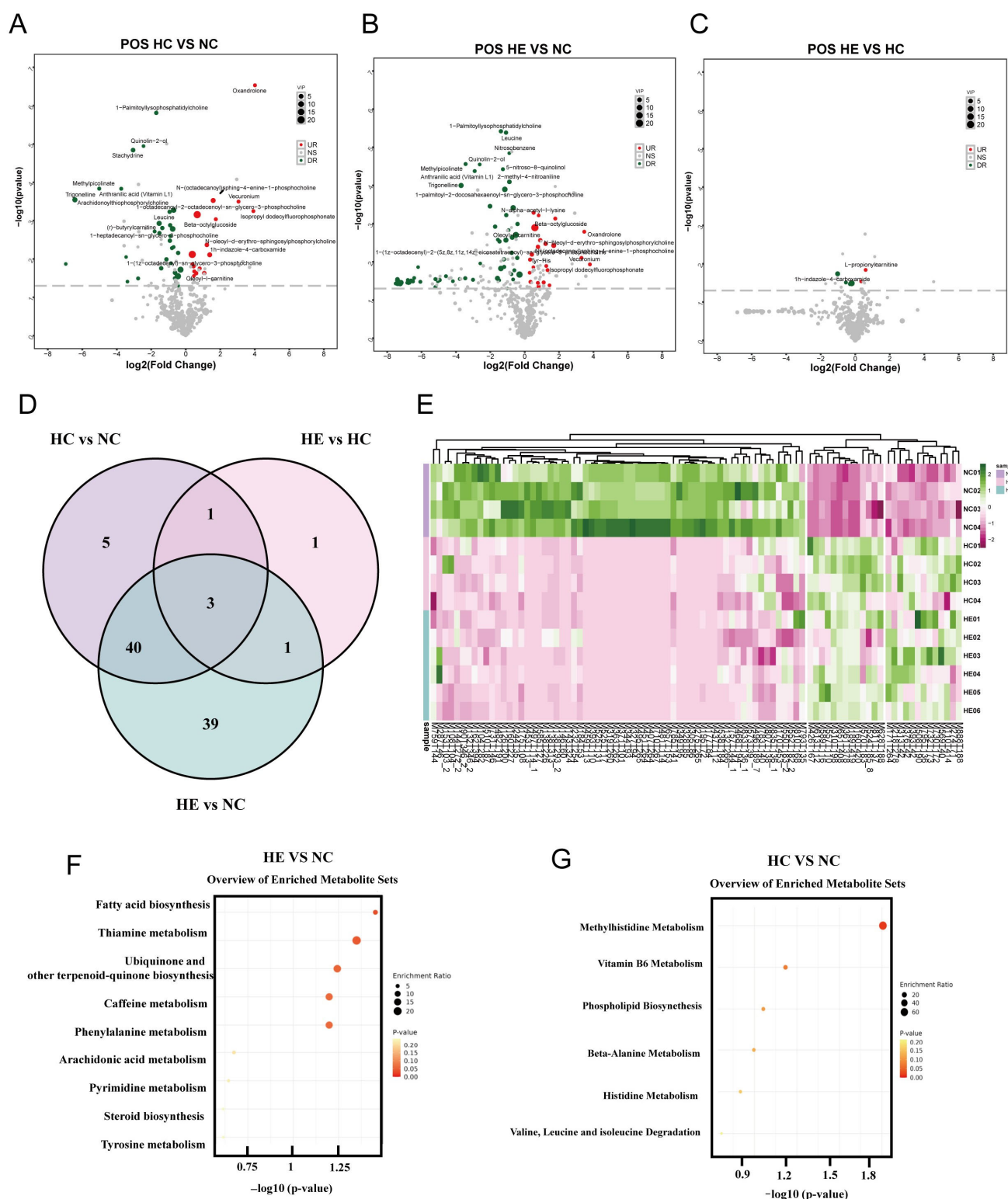


Figure 3. The serum metabolites of rats were determined by the metabolomics analysis method. (A–C) Volcano plots of DEMs between HC and NC, NC and HE, and HC and HE groups. (D) Comparative analysis of DEMs among the three groups. (E) Heatmap plot shows that 64 metabolites were significantly higher in abundance in the NC group and 26 metabolites were significantly higher in abundance in the HC and HE groups. (F) SMPDB analysis indicated the top 25 enriched metabolite sets between the NC and HC groups. (G) SMPDB analysis indicated the top 25 enriched metabolite sets between the HC and HE groups. NC, normal control; HC, high diet control; HE, high diet exercise; DEMs, different expressed metabolites.

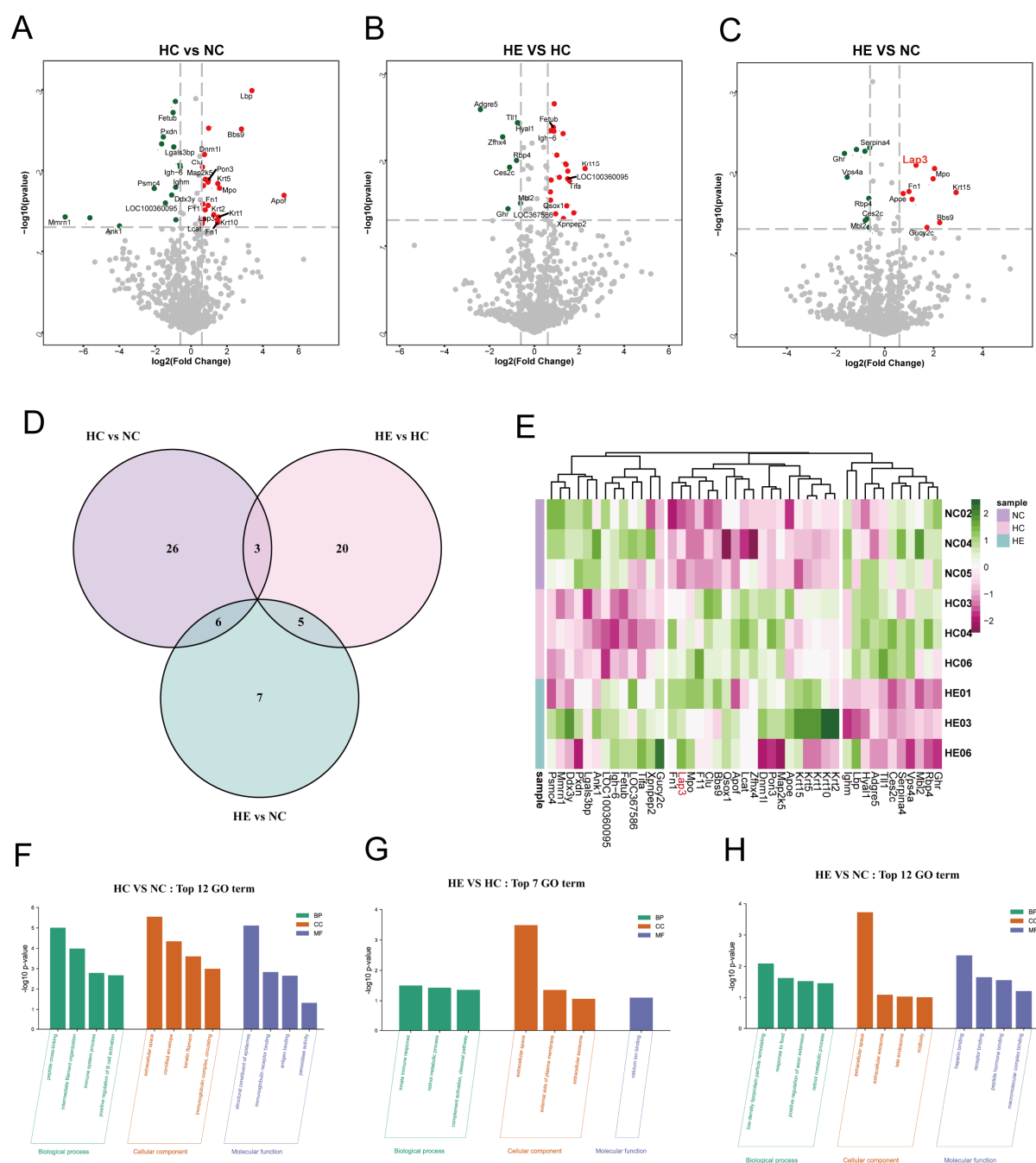


Figure 4. Exercise changed the overall protein composition. (A–C) Volcano plots of DEPs between the HC and NC, HE and NC, and HE and HC groups. (D) Comparative analysis of DEPs among the three groups. (E) Comparison of the HE and HC groups; the heatmap plot shows that 7 proteins were significantly higher in abundance in the HE group, while 11 proteins were significantly higher in abundance in the HC group. (F) PPI network was used to show the interaction of DEPs. (G) The top 12 significantly enriched GO annotations between HC and NC rats. (H) The top 12 significantly enriched GO annotations between HE and NC rats. DEPs, Different Expressed Proteins; NC, normal control; HC, high-fat diet control; HE, high-fat diet exercise; PPI, protein–protein interaction; GO, Gene Ontology.

3.5. Integrated DEMs and Differential Gut Microbiota

As shown in Figure 5A, a total of 47 metabolites were correlated with 22 genera in the HC group vs. the NC group. Interestingly, 35 out of 47 metabolites were negatively correlated with genera. However, 45 out of 47 metabolites were positively correlated with genera (Table S9). Notably, we observed that *g__Bacteroides* was remarkably negatively

correlated with 1-Palmitoyl-lysophosphatidylcholine (M538T184) and *g__Lactobacillus* was remarkably negatively correlated with Quinolin-2-ol (M146T60). Meanwhile, correlations between the genera and differential metabolites in the HE group vs. the NC group are shown in Figure 5B. In total, 40 out of 82 metabolites were negatively correlated with genera, and 74 out of 82 metabolites were positively correlated with genera (Table S10). Interestingly, we observed that *g__Yersinia* was remarkably positively correlated with Ectoine (M143T324), 2-piperidinecarboxylic acid, and Acetamide (M497T114_1). Oleoyl-l-carnitine (M426T167) was remarkably negatively correlated with *g__Aureimonas*. Furthermore, consistent with previous results, only 6 metabolites were correlated with 6 genera in the HE group vs. the HC group (Figure 5C, Table S11). We observed that *g__Peptoclostridium*, *g__Marvinbryantia*, and *g__Blautia* were correlated with Oxandrolone (M613T112). In addition, an unidentified genus was significantly associated with N-alpha-acetyl-l-lysine (M171T264), L-propionylcarnitine (M218T278), 1h-indazole-4-carboxamide (M570T183), and 2-oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine (M783T140).

3.6. Integrated DEPs and Differential Metabolites

We conducted a Pearson correlation analysis using network diagrams of expression correlations between DEPs and differential metabolites (Supplementary Figure S3A–C). In total, we identified 41 significant correlations between DEPs and differential metabolites in the HC group vs. the NC group (Figure 6A). Among them, LBP, Igh-6, Lap3, and Fetub had cross-sectional relationships with 1h-indazole-4-carboxamide (M570T183), Leucine (M132T266), Quinolin-2-ol (M146T60), 2-epahpa (M628T188), and Stachydrine (M144T272). In the HE group vs. the NC group (Figure 6B), the DEPs, such as Mpo, Serpina4, Mbl2, and Gucy2c, had cross relationships with the differential metabolites, such as Leucine, Quinolin-2-ol, N-Docosanoyl-4-sphingenyl-1-O-phosphorylcholine (M810T168), N-(octadecanoyl)-sphing-4-enine-1-phosphocholine (M732T173), N-stearoylsphinganine (M569T40), and N-oleoyl-d-erythro-sphingosylphosphorylcholine (M730T172). In addition, in the HE group vs. the HC group (Figure 6C), we observed that there were close relationships between DEPs such as Mbl2, LOC367586, Ghr, Osox1, Tifa, Fetub, Tll1, Rbp4, Igh-6, Kr15, LOC100360095, Ifhx4, Ces2c, Hyal1, and Adgre5 and metabolites such as 1h-indazole-4-carboxamide (M570T183), Oxandrolone (M613T112), L-propionylcarnitine (M218T278), 2-oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine (M783T140), 1-palmitoyl-sn-glycero-3-phosphocholine (M496T189_7), and N-alpha-acetyl-l-lysine (M171T264).

3.7. Multi-Omics Integration Analysis Revealed the Crosstalk Among Differential Gut Microbiota, Serum Metabolites, and Proteins

We further utilized the Mantel test to explore the potential correlations among DEPs, DEMs, and differential genera. We first correlated distance-corrected dissimilarities of metabolites and genera with those of proteins. As shown in Figure 7A, Fn1, Mpo, and Lap3 were the strongest correlates of these metabolites and genera. A previous study had shown that cholesterol-induced upregulation of Lap3 inhibited autophagy in the pathogenesis of NAFLD [29]. Subsequently, the hub metabolites were identified according to Lap3 and genera (Figure 7B). We found that Palmitoyl-lysophosphatidylcholine and Leucine had significant correlations with Lap3 and genera. Palmitoyl-lysophosphatidylcholine has been widely reported as a novel lipid biomarker for cardiovascular dysfunction [30], while leucine is known to influence glucose tolerance and lipid metabolism [31]. We next used Lap3 and Palmitoyl-lysophosphatidylcholine to further reveal the key microbiota and surprisingly found that *g__Lactobacillus* had a potentially important link with both Lap3 and Palmitoyl-lysophosphatidylcholine (Figure 7C). In addition, a negative association between Lap3 and Palmitoyl-lysophosphatidylcholine was revealed ($R = -0.77$, $p = 0.015$) (Figure 7D). Particularly, there was also a negative correlation between Lap3 and

g__Lactobacillus ($R = -0.83$, $p = 0.0054$) (Figure 7E). In addition, the positive correlation between *g__Lactobacillus* and Palmitoyl-lysophosphatidylcholine was observed ($R = 0.95$, $p = 7 \times 10^{-5}$) (Figure 7F). Our mediation analysis showed that Lap3 causally contributed to Palmitoyl-lysophosphatidylcholine accumulation through promoting *g__Lactobacillus* ($P_{\text{mediation}} = 0.04$, 71%) (Figure 7G). In summary, these results suggest that exercise may improve obesity-induced cardiovascular dysfunction through regulating the crosstalk among Lap3, Palmitoyl-lysophosphatidylcholine, and *g__Lactobacillus*.

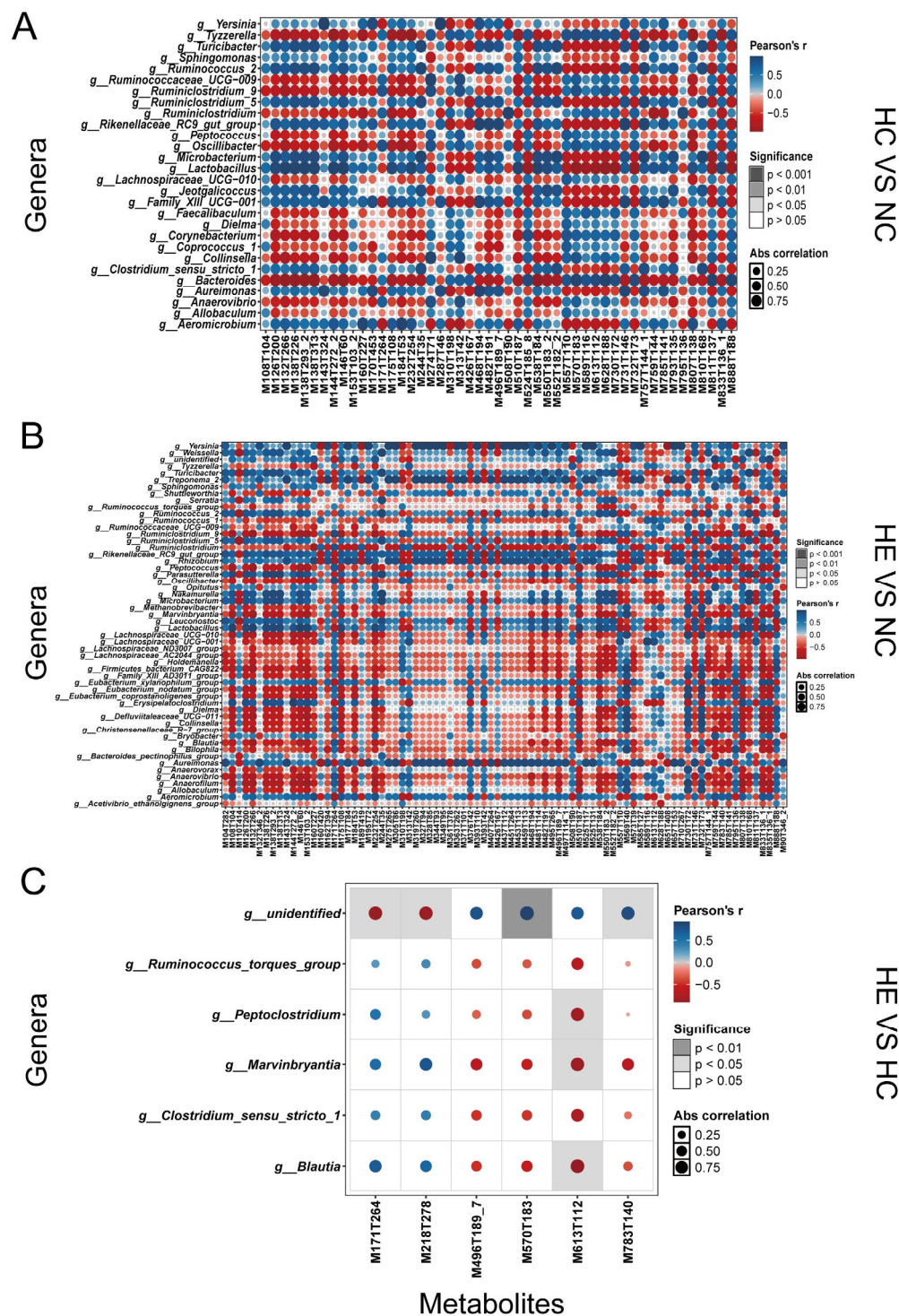


Figure 5. The correlation of metabolites with the gut microbiota. (A–C) Pearson correlations between the metabolites of interest and the bacteriophage: NC and HC groups (A), NC and HE groups (B),

HC and HE groups (C); correlations are highlighted in blue (positive) and red (negative). Horizontal coordinates are metabolites; vertical coordinates are microbiota. NC, normal control; HC, high-fat diet control; HE, high-fat diet exercise.

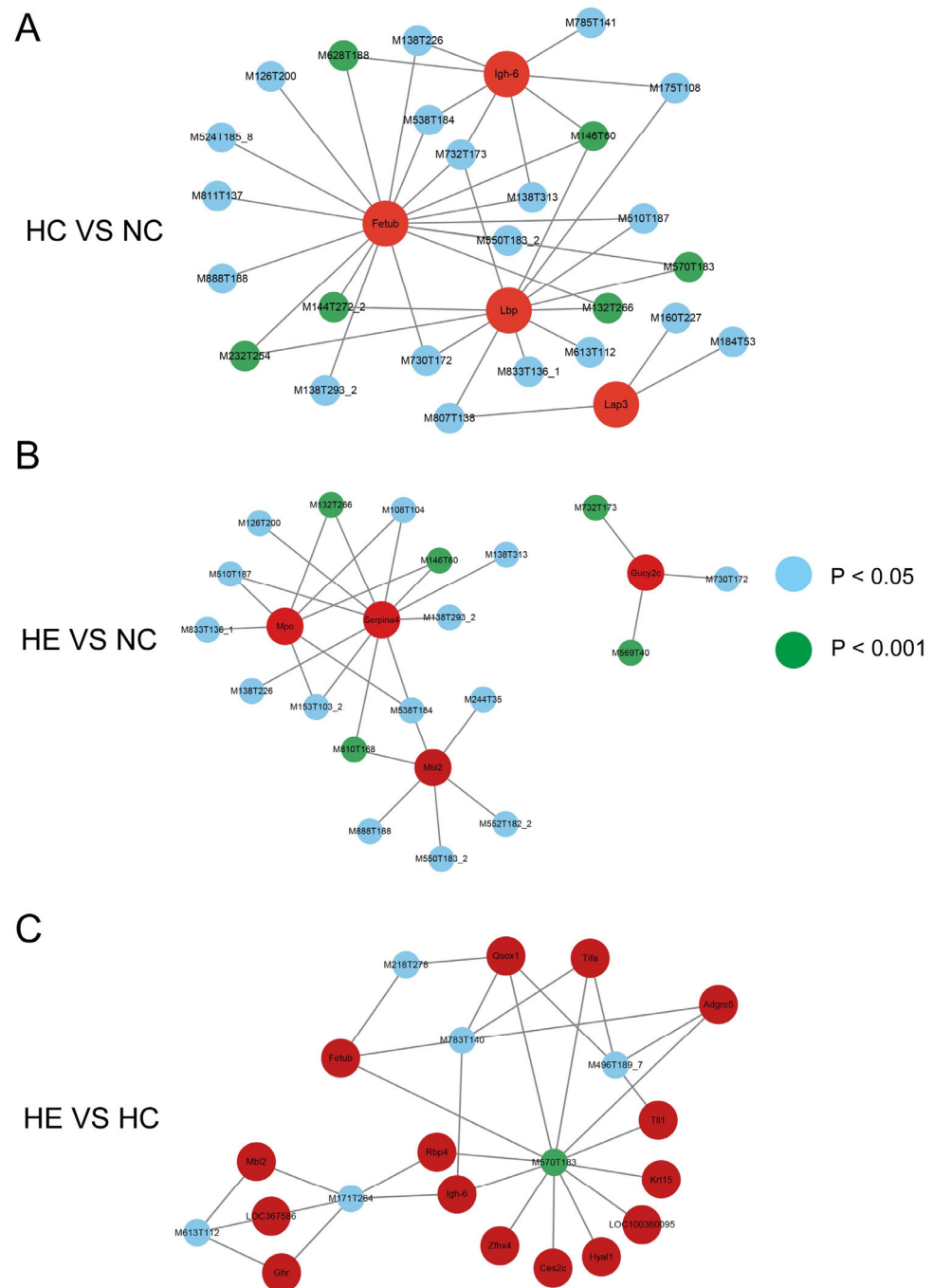


Figure 6. The differential metabolites and genus were integrated by a network diagram. (A) Network diagram of expression correlations between differential proteins and differential metabolites in the NC and HC groups. (B) Network diagram of expression correlation between differential proteins and differential metabolites in the NC and HE groups. (C) Network diagram of expression correlation between differential proteins and differential metabolites in the HC and HE groups. The figure shows network plots of correlations between proteins and metabolite response intensity data calculated based on Pearson correlation analysis, with pairs of relationships selected for $p < 0.05$ and $p < 0.001$. Red indicates proteins, and blue and green represent metabolites with $p < 0.05$ and $p < 0.001$, respectively. NC, normal control; HC, high-fat diet control; HE, high-fat diet exercise.

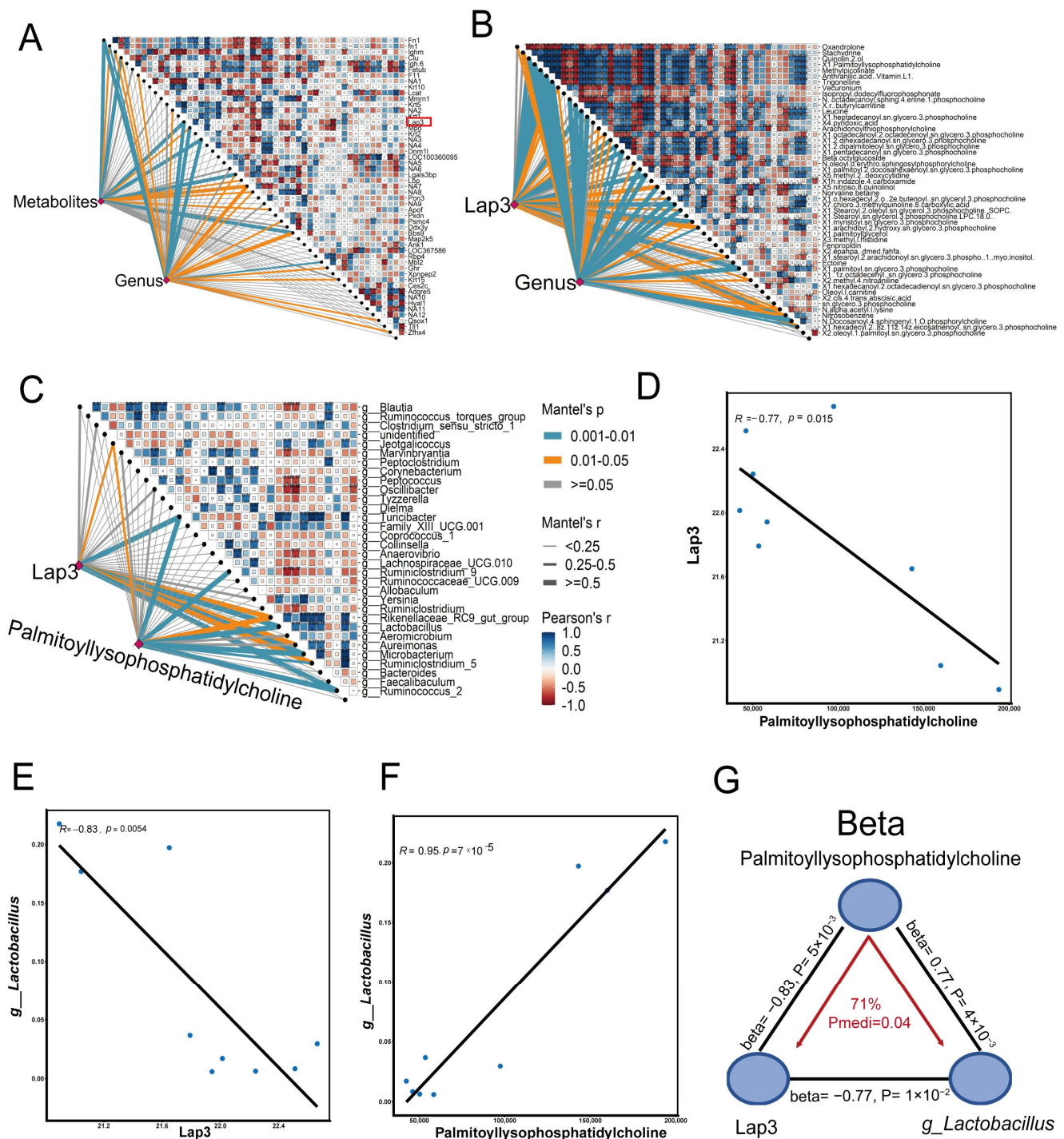


Figure 7. Differences between NC, HC, and HE groups in the crosstalk between differential colon genera, various serum metabolites, and key proteins. (A–C) Pairwise comparisons of proteins (A), metabolites (B) and microbes (C) are shown, with a color gradient denoting Pearson's correlation coefficient. Metabolites and genera (A); Lap3 and genera (B); and Lap3 and Palmitoyllysophosphatidylcholine (C) were related to each pairwise comparison of components by partial (geographic distance–corrected) Mantel tests. Edge width corresponds to Mantel's r statistic for the corresponding distance correlations, and edge color denotes the statistical significance based on permutations. (D–F) The scatter plot shows the Pearson's test correlation between Lap3, Palmitoyllysophosphatidylcholine and *g_Lactobacillus*. (G) Lap3 causally contributed to *g_Lactobacillus* enrichment by inhibiting Palmitoyl-lysophosphatidylcholine accumulation (Pmediation = 0.04, 71%). NC, normal control; HC, high-fat diet control; HE, high-fat diet exercise; Lap3, leucine aminopeptidase 3.

4. Discussion

This study employed an integrated multi-omics analysis in high-fat diet (HFD)-induced obese rats to elucidate the potential mechanisms by which moderate-intensity aerobic exercise improves cardiovascular dysfunction. Our findings demonstrated that exercise not only directly enhanced vascular endothelial function and cardiac pumping capacity—evidenced by improved acetylcholine-induced vasodilation, left ventricular ejection fraction (LVEF), and fractional shortening (FS)—but also systematically remodeled the gut microbiota structure, regulated the serum metabolome (e.g., palmitoyl-lysophosphatidylcholine and leucine), and modulated the expression of key proteins such as Lap3 and APOE. These alterations, through complex microbiota–metabolite–protein interactions, collectively constitute the cardiovascular-protective effects of exercise. The integrated multi-omics approach provides a comprehensive, multi-tiered perspective on how exercise improves cardiovascular health, highlighting its innovative value in deciphering complex physiological mechanisms.

Extensive evidence establishes exercise as an effective intervention for enhancing cardiovascular function [32,33]. Obesity, frequently accompanied by cardiovascular and metabolic risks, accelerates atherosclerosis and hypertension through mechanisms including visceral fat accumulation-induced dyslipidemia and increased vascular resistance. Consistent with this, our study confirmed that 8 weeks of moderate-intensity aerobic exercise significantly reduced body weight, body mass index (BMI), and Lee's index in HFD-fed rats, while improving endothelial function and cardiac parameters (e.g., LVEF and FS). These benefits align with the known capacity of exercise to restore nitric oxide (NO) balance and attenuate pathological vasoconstriction [34], supporting the established role of regular aerobic activity in counteracting obesity-related adverse effects and improving a spectrum of cardiovascular metrics [35].

The intricate relationship between obesity and cardiovascular disease (CVD) is well-documented, driving considerable research into exercise's potential to mitigate these conditions [36]. Our results further show that exercise remodeled the disrupted gut microecology: the HFD-induced decrease in beneficial bacteria (e.g., *Lactobacillus*) and increase in conditionally pathogenic bacteria (e.g., *Ruminiclostridium_9*) were partially reversed after exercise, consistent with previous reports [37,38]. Probiotics like *Lactobacillus* produce short-chain fatty acids (SCFAs) through fermentation, which exert anti-inflammatory and immunomodulatory effects, influence obesity-related pathways, and directly or indirectly impact CVD. Conversely, *Ruminiclostridium_9* is an inflammatory biomarker; its elevated abundance may compromise intestinal barrier function, increase gut permeability, and thereby promote endotoxin translocation and chronic inflammation [39,40]—a key driver of both obesity and CVD. Exercise likely helps maintain intestinal integrity by suppressing its overgrowth. Functional prediction of differential microbiota across groups indicated significant enrichment in bile acid metabolic pathways in both the HC and HE groups. This may stem from gut microbial dysbiosis, where reduced diversity diminishes SCFA synthesis and its consequent health benefits. Studies report an expanded bile acid pool and increased total circulating bile acid concentration in obese individuals [41]. Bile acids, primary products of cholesterol metabolism, play crucial roles in lipid homeostasis and regulate other gut microbiota-derived metabolites (e.g., SCFAs and TMAO), some of which modulate immunity and atherosclerosis. The bile acid pathway is a key component of the gut-liver axis, whose balance is vital for systemic health; its disruption is closely linked to various hepatic, intestinal, and metabolic disorders, including obesity, type 2 diabetes, and metabolic syndrome [42]. This finding underscores the systemic impact of exercise on metabolic pathways and offers a valuable direction for future research.

Multi-omics analysis further revealed exercise's influence on metabolic and protein networks. HFD and exercise affected metabolites through distinct pathways. In our study, HFD likely disrupted fundamental energy metabolism processes, with significant enrichment in pathways related to methylhistidine, vitamin B6, and phospholipid biosynthesis. Altered methylhistidine metabolism may reflect abnormal skeletal muscle protein function [43], while dysregulated vitamin B6 metabolism is closely associated with oxidative stress and inflammation—core drivers of atherosclerosis [44,45]. In contrast, the metabolic profile of the exercise group shifted fundamentally, showing enrichment in pathways such as fatty acid biosynthesis, thiamine metabolism, and ubiquinone biosynthesis. Fatty acids serve as fuel for energy supply; the onset of exercise markedly increases the uptake and oxidation of long-chain fatty acids in skeletal muscle, and the energy demands of aerobic activity can upregulate fatty acid synthesis to meet this demand [46]. Thiamine is essential for myocardial energy metabolism and has shown potential to improve LVEF in heart failure patients [47]—a finding paralleled by the significantly increased ejection fraction in the HE group (Figure 1). Moreover, exercise stimulates metabolism dependent on thiamine, riboflavin, and vitamin B6, potentially increasing the requirement for these vitamins in athletes and active individuals [48]. Ubiquinone deficiency is linked to various diseases, including heart failure, and its supplementation may improve myocardial mitochondrial function and reduce oxidative damage [49–51]. The enrichment of these pathways likely represents classic adaptive responses to exercise, supporting the concept that exercise counteracts HFD-induced cardiovascular injury by optimizing energy metabolism, enhancing mitochondrial antioxidant capacity, and regulating the metabolism of vasoactive substances.

At the protein level, key differentially expressed proteins—including MBL2, APOE, FN1, CLU, and Lap3—were implicated in pathways governing immune-inflammatory regulation, apolipoprotein synthesis, anti-inflammatory actions, and the promotion of vascular endothelial cell synthesis. APOE is a central regulator of lipid transport and metabolism, playing a core role in atherogenesis [52]. FN1 is an important modulator of tissue remodeling, a process implicated in atherosclerosis; higher plasma FN1 levels are associated with a reduced risk of coronary artery disease [53]. CLU, considered cardioprotective against damage from myocardial infarction or myocarditis, has a complex role in CVD [54]. MBL is a key component of the innate immune system and may be involved in the pathogenesis of atherosclerosis [55,56]. Collectively, these proteins and pathways suggest that exercise improves cardiovascular function by modulating systemic immune responses and maintaining lipid metabolic homeostasis.

Using Mantel tests and mediation effect analysis to integrate gut microbiome, serum metabolome, and proteomics data, we identified for the first time a regulatory axis linking a protein, gut microbiota, and a metabolite: Lap3–*Lactobacillus*–palmitoyllysophosphatidylcholine. Mediation analysis indicated that the physiological environment shaped by exercise allows changes in Lap3 expression to modulate the abundance of intestinal *Lactobacillus*, thereby influencing the levels of this lipid metabolite (mediation p -value = 0.04, explanatory proportion: 71%). This highlights the utility of exercise in regulating specific interactions and systematically reveals the multi-dimensional remodeling mechanisms through which aerobic exercise alleviates HFD-induced cardiovascular dysfunction. Previous research on Lap3 has focused on its role in cancer or in the pathogenesis of non-alcoholic fatty liver disease (NAFLD), where cholesterol-induced Lap3 upregulation inhibits autophagy, and its expression is significantly higher in severe NAFLD but decreases post-treatment [29,57,58]. Our study proposes that Lap3 may act as a factor conveying exercise-induced metabolic signals to the gut. Furthermore, while the cardiovascular benefits of *Lactobacillus* are often attributed to local effects like improving gut barrier function

and producing SCFAs, our findings connect its abundance to a specific exercise-mediated proteomic change [6]. Finally, palmitoyl-lysophosphatidylcholine is widely reported as a novel lipid biomarker for cardiovascular dysfunction. This axis provides new molecular evidence for elucidating the “gut-heart axis” mechanism by which aerobic exercise improves HFD-induced cardiovascular dysfunction.

The benefits of aerobic exercise extend beyond the regulation of single targets to encompass the remodeling of entire biomolecular interaction networks. We have partially elucidated how exercise contributes to cardiovascular protection by restoring gut microbiota composition, enhancing beneficial metabolites, and regulating immune-related proteins. However, several limitations of this study should be acknowledged. First, the experimental design did not include a normal diet with exercise group (NE). While the comparison between HE and HC groups effectively addresses our primary research question—whether exercise can ameliorate pre-existing HFD-induced cardiovascular dysfunction—the inclusion of an NE group would have allowed for a more complete assessment of exercise effects under physiological versus pathological conditions. This experimental paradigm has been widely adopted in recent studies investigating exercise-mediated cardioprotection in diet-induced obesity models [59]. Future studies incorporating an NE group are warranted to dissect the differential mechanisms of exercise in healthy and diseased states and should employ tools such as Lap3 conditional knockout animal models combined with fecal microbiota transplantation to directly verify the necessity of each component within this axis. By integrating diverse data from genomics, proteomics, and metabolomics, researchers can achieve a more holistic understanding of the interactions between different biological systems and the relationships among key molecules.

Beyond the mechanistic insights provided by this study, our findings hold potential implications for human health and clinical practice. The identification of the Lap3–Lactobacillus–palmitoyl-lysophosphatidylcholine axis as a key regulatory pathway suggests that this triad could serve as a biomarker panel for monitoring exercise efficacy in obesity-related cardiovascular dysfunction. Furthermore, the observed enrichment of beneficial bacteria such as *Blautia* and the restoration of metabolic pathways (e.g., fatty acid biosynthesis, ubiquinone metabolism) following exercise align with known cardioprotective mechanisms in humans [33,35]. These results support the notion that moderate-intensity aerobic exercise, which is accessible and low-cost, can exert systemic benefits through modulation of the gut–heart axis. From a public health perspective, our findings reinforce the importance of incorporating regular physical activity into lifestyle interventions for obesity management and cardiovascular disease prevention. Future translational studies should explore whether similar multi-omic signatures are detectable in human cohorts undergoing exercise interventions, and whether targeted modulation of the identified axis (e.g., through probiotics or dietary interventions) could enhance exercise-induced cardiovascular benefits.

In conclusion, our study illuminates the promising role of aerobic exercise in mitigating obesity-induced cardiovascular dysfunction by modulating gut microbiota, serum metabolites, and key proteins, highlighting its potential to influence systemic metabolism and immune responses. This understanding opens avenues for targeted interventions and emphasizes the importance of incorporating exercise into holistic approaches for preventing and managing CVDs.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/nu18050746/s1>, Figure S1: Sequencing quality and functional enrichment pathways of differential microbiota in NC, HC, and HE groups; Figure S2: Correlation assessment of differential metabolites between NC, HC, and HE groups; Figure S3: Correlation assessment between differential proteins and differential metabolites in the NC group, HC group, and

HE group; Table S1: Top 10 phylum relative abundance; Table S2: Top 10 genus relative abundance; Table S3: Information and classification of DEMs between HC and NC groups; Table S4: Information and classification of DEMs between HE and NC groups; Table S5: Information and classification of DEMs between HE and HC groups; Table S6: Information and function of DEGs between HE and HC groups; Table S7: Information and function of DEGs between HE and NC groups; Table S8: Information and function of DEGs between HE and HC groups; Table S9: The relationships between significant differential genus and differential metabolites between HC and NC groups; Table S10: The relationships between significant differential genus and differential metabolites between HE and NC groups; Table S11: The relationships between significant differential genus and differential metabolites between HE and HC groups.

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