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Moderate-Intensity Exercise Improves Endothelial Function by Altering Gut Microbiome Composition in Rats Fed a High-Fat Diet

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Background: Obesity changes gut microbial ecology and is related to endothelial dysfunction. Although the correlation between gut microbial ecology and endothelial dysfunction has been studied in obese persons, the underlying mechanisms by which exercise enhances endothelial function in this group remain unclear. This study investigated whether exercise improves endothelial function and alters gut microbiome composition in rats fed a high-fat diet (HFD).

Methods: Obesity was induced by an HFD for 11 weeks. Whole-body composition and endothelium-dependent relaxation of mesenteric arteries were measured. Blood biochemical tests were performed, and gut microbiomes were characterized by 16S rRNA gene sequencing on an Illumina HiSeq platform.

Results: Exercise training for 8 weeks improved body composition in HFD-fed rats. Furthermore, compared with the untrained/HFD group, aerobic exercise significantly increased acetylcholine-induced, endothelium-dependent relaxation in mesenteric arteries ($P < 0.05$) and circulating vascular endothelial growth factor levels ($P < 0.01$) and decreased circulating C-reactive protein levels ($P < 0.05$). In addition, exercise and HFD resulted in alterations in the composition of the gut microbiome; exercise reduced the relative abundance of Clostridiales and *Romboutsia*. Moreover, 12 species of bacteria, including *Romboutsia*, were significantly associated with parameters of endothelial function in the overall sample.

Conclusions: These results suggest that aerobic exercise enhances endothelial function in HFD-fed rats by altering the composition of the gut microbiota. These findings provide new insights on the application of physical exercise for improving endothelial function in obese persons.

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Key words: exercise, obesity, gut microbiota, *Romboutsia*, endothelial function

Introduction

Obesity is a major public health concern and has attracted global attention because of its impact on human health. Obesity can cause cardiovascular disorders such as dyslipidemia, hypertension, and atherosclerosis. Endothelial dysfunction is the starting point of many cardiovascular disorders and a key link in the development of cardiovascular risk factors associated with obesity and metabolic disorders[1]. Hemodynamic changes associated with increased systemic inflammation, oxidative stress, and weight gain may directly cause vascular endothelial injury and dysfunction, a cascade that may represent the pathogenesis of atherosclerosis in obese individuals[2]. Data from many epidemiological studies indicate that exercise has systemic effects and many benefits, such as reducing chronic disease risk factors[3]. However, the underlying mechanism by which exercise enhances endothelial function in obese individuals remains unclear.

Increasing evidence indicates that obesity is closely related to the host's gut microbiota[4], which is essential in

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the pathogenesis of obesity and diabetes and affects key pathways such as energy homeostasis and inflammation[5]. Zhong and colleagues found reduced alpha diversity in the gut of an obese population and altered ecology of the gut microbiota[6]. A high-fat diet (HFD) altered the composition of the gut microbiota by reducing the abundance of beneficial bacteria in the intestines and increasing factors that trigger inflammation[7]. This, in turn, also negatively affected gut microbiota diversity and reduced production of short-chain fatty acids[8]. Gut microbiota implanted from high-fat-induced or *ob/ob* mice produced more body fat components in germ-free mice than in conventional or wild-type mice[9]. Nirmalkar et al. reported a correlation between intestinal flora and markers of endothelial dysfunction in Mexican children and adolescents with obesity[10]. A clinical study of 617 middle-aged women revealed that vascular function is closely related to intestinal flora; in addition, 7 operational taxonomic units (OTUs) were associated with arterial stiffness[11]. These studies indicate that the gut microbiota is correlated with obesity status and endothelial function in obese persons.

Previous studies have described the positive effects of exercise on gut microbiota[12,13]. Exercise increases alpha and beta diversity in the gut microbiota[14–20]. It also modulates gut microbiota profiles and increases levels of butyrate-producing bacteria and fecal butyrate, which are independent of diet, in murine species and humans[12]. However, the effect of exercise on gut microbiota, including prevention of HFD-induced vascular endothelial dysfunction, is unclear. Furthermore, no study has identified the specific taxa involved in the development of endothelial dysfunction. Therefore, this study investigated the associations of gut microbiota, aerobic exercise, and endothelial function. We hypothesized that aerobic exercise would alter the composition of intestinal microbes and improve vascular endothelial function in rats with HFD-induced obesity and that improved vascular endothelial function may be related to exercise-induced changes in the gut microbiota.

Materials and Methods

Animals and Exercise Training Protocol

Four-week-old male Sprague-Dawley rats (weight range: 109.3-144.1 g) were obtained from the Guangdong Medical Laboratory Animal Center (SCXK, 2013-0002) and randomly assigned to a standard diet (SD, n = 16) or HFD (n = 40) for 11 weeks. Rats were fed in an SPF environment with four rats per cage. The formulas of the SD and HFD (Product No. D12492) are shown in **Supplementary Table 1** (https://doi.org/10.1272/jnms.JNMS.2022_89-307). Food and water were given *ad libitum*, and the animals were maintained in a facility with a 12-h light-dark cycle, humidity of 60%, and temperature of 23 ± 1°C. After HFD feeding for 11 weeks, the animals were randomly divided into an exercise-trained group (HF trained, n = 8) or untrained group (HF control, n = 10). These animals continued to be fed on HFD. Rats in the SD group were also randomly divided into an exercise-trained group (SD trained, n = 8) and untrained group (SD control, n = 8) and continued on an SD. Both the HF trained group and SD trained group were subjected to a moderate-intensity exercise training protocol (20 m/min without inclination, 60 min/day, 6 days/week for 8 weeks) on a motor-driven treadmill (Model LE8710TRS, PanLab, Harvard Apparatus, Holliston, MA, USA)[21]. Change in average 24-h energy intake and body weight during the exercise intervention are shown in **Supplementary Figure 1, 2**, respectively.

All animals (n = 34) were euthanized at the end of the study with a solution of 3% sodium pentobarbitone administered intraperitoneally. Body composition was measured, and blood, second-order mesenteric arteries, and cecal samples were collected and stored for later analysis.

Body Composition Measurement

All rats were immobilized in a prone position and then scanned by micro-computed tomography (Model LCT-200, Hitachi, Aloka, Japan). All scans continued for 20 to 25 min, and the data were processed with ultra-high-resolution analysis software. Body composition—including body weight, fat mass, fat-free mass, fat-free mass percentage, and fat mass percentage—was assessed.

Isometric Tension Measurement

Vessel tension measurements were performed as previously described[22,23]. In brief, the second-order mesenteric arteries were dissected in a Petri dish filled with ice-cold Krebs solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 25.2 mM $NaHCO_3$, and 11.1 mM glucose; pH 7.4) that was continuously bubbled with 95% oxygen (O_2) and 5% carbon dioxide (CO_2) mixed gas. Vessel segments were cut into pieces approximately 2 mm in length and mounted in a DMT myograph chamber (Model 620M, Danish Myo-Technology, Aarhus, Denmark) containing Krebs solution (pH 7.4) saturated with 95% O_2 plus 5% CO_2 at 37°C. The tensile vascular ring produced an initial passive tension of 3 mN, and changes in vascular tone were recorded

with a Powerlab system. After a 30-min equilibration, the contractile function of the vessel was tested by replacing the Krebs solution with 60 mM K^+ solution (prepared by substituting NaCl with equimolar KCl). After washout three times and a 20-min equilibration period, the vessels were pre-contracted with 1 μM phenylephrine. When the contraction response reached a plateau, a gradient of acetylcholine (ACh) was cumulatively added to induce relaxation of the rings, to obtain a concentration-response curve for ACh-mediated relaxation.

Biochemical Testing

For serum preparation, blood collected from the abdominal aorta was placed in tubes without anticoagulants. We measured levels of inflammatory factor indicators, including tumor necrosis factor-α (TNF-α) and C-reactive protein (CRP), as well as vascular endothelial function biomarkers, namely vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS), with enzyme-linked immunosorbent assay kits (Cusabio, Biotech. Co., Ltd., Wuhan, China) used in accordance with the manufacturer's instructions. The optical density of each indicator was read by a microplate reader (EPOCH2, BioTek, Winooski, VT, USA).

Cecal Sample Collection and 16S rRNA Sequencing

Cecal samples were collected and snap frozen in liquid nitrogen. DNA was extracted from approximately 100 mg of cecal sample with a QIAamp DNA stool Mini Kit. DNA concentration was determined using an ND-1000 NanoDrop and Qubit (Thermo Scientific, Waltham, MA, USA). Biological data, such as the abundance and diversity of intestinal microbes, were analyzed for each group by 16S rRNA sequencing based on the Illumina HiSeq platform[24]. Details of the analysis are described elsewhere[25–28]. Briefly, the raw reading was processed with Quantitative Insights Into Microbial Ecology (QIIME) software to create an OTU table. Uclust's open reference selection strategy was used to cluster sequences into a single OTU with a default similarity level of 97%, followed by chimeric detection utilizing the UCHIME method. The Ribosomal Database Project Classifier was used to align with the Greengenes Database, using a single representative sequence from each OTU cluster. Alpha diversity was assessed by using QIIME to calculate five indices, i.e., the observed species (Sobs), Chao, Ace, Shannon, and Simpson indices. Metastat analysis, which was used to identify the difference, was evaluated by false discovery rate. Linear discriminant analysis (LDA) was used to estimate the effect size of the differentially rich genera. The threshold logarithmic LDA score for the distinguishing feature was set to 2.5.

Co-Occurrence Analysis

Co-occurrence analysis was conducted by using *out_table.biom* files in the CoNet (Co-occurrence Network Inference) plugin tool[29]. The resulting co-occurrence networks were generated and analyzed with Cytoscape (v3.6.1) software[10].

Statistical Analysis

The effective concentration that induced 50% of the maximum effect (EC_{50}) was calculated from each concentration-relaxation curve by using the following logistic, curve-fitting equation: $E = MA^p/(A^p + K^p)$, where M is the maximal relaxation, E is the response, K is the EC_{50} concentration, A is the concentration, and p is the slope parameter[30]. Analyses were conducted using SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Differences between experimental groups were assessed using two-way ANOVA (diet × exercise), and *in vitro* assays were analyzed using one-way ANOVA. Pearson's correlation analysis was performed to measure correlations between two variables, and correlations were screened for $R > 0.8$ and deemed significant at $P < 0.05$. All data are expressed as mean ± standard deviation. Differences with a *P*-value of <0.05 were considered to be statistically significant. To avoid false positives, the false discovery rate (FDR) (*q*-value) was estimated with the Benjamini-Hochberg method.

Ethics approval

All animal experiments were performed in accordance with the guidelines established by the US National Institutes of Health (NIH Publication No. 8523) and received approval from the Animal Experimentation Ethics Committee of Guangzhou Sport University (NO. 2020DWLL-17).

Results

Changes in Body Composition with Exercise Intervention

Changes in body weight and body composition have been previously described[21]. The results of micro-computed tomography (**Supplementary Table 2**) indicate that body weight, fat mass, and fat mass percentage were significantly higher in the SD control group than in the HF control group (all $P < 0.01$), while fat-free mass percentage was significantly lower in the HF control group ($P < 0.01$). After 8 weeks of aerobic exercise, body weight and fat-free mass were lower in the SD trained group than in the SD control group ($P < 0.05$). Moreover, fat mass ($P < 0.05$), body weight, fat mass percentage, and

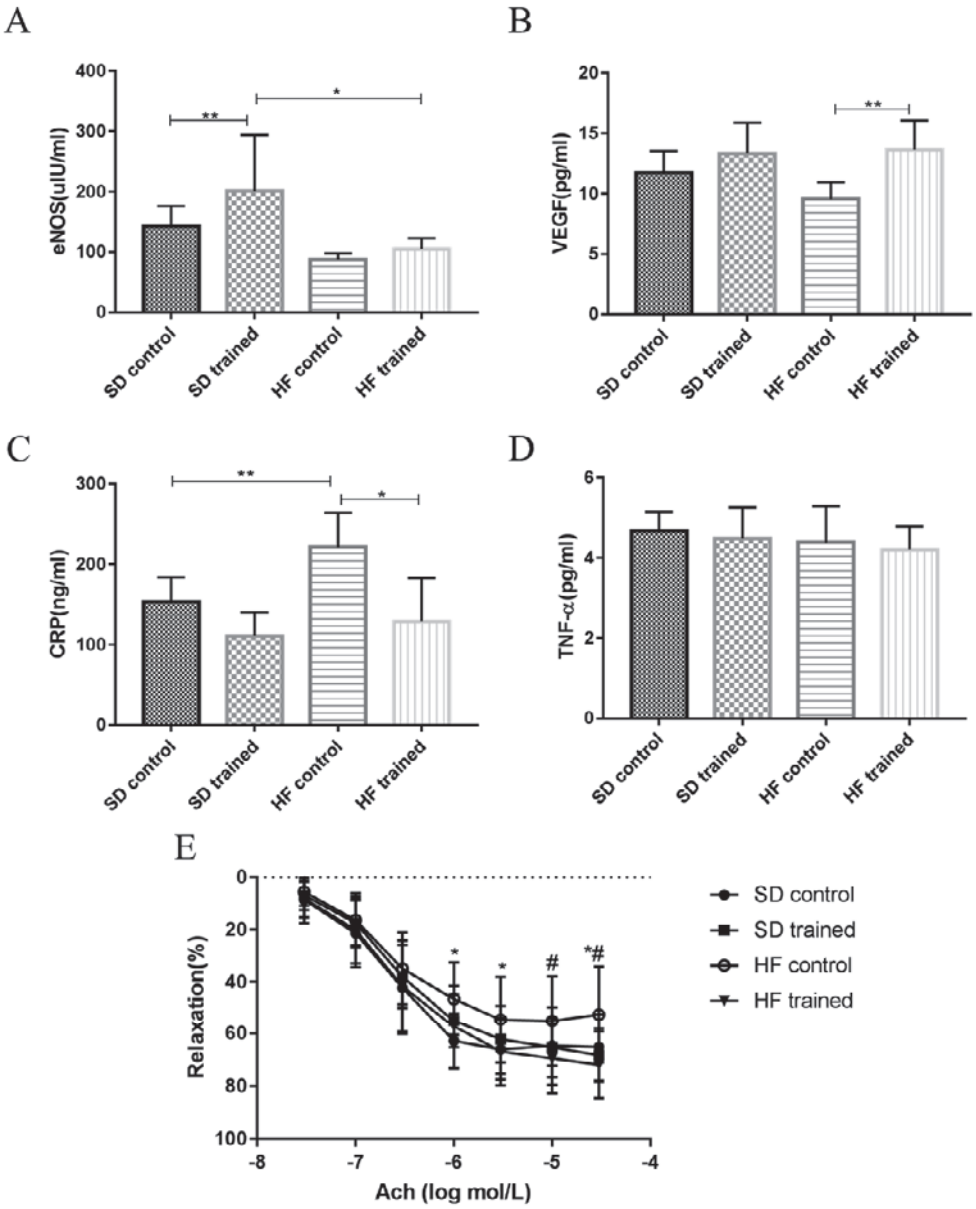

Fig. 1 Effects of the exercise intervention on circulating endothelial function (A and B) and inflammatory (C and D) markers, and acetylcholine (Ach)-induced mesenteric arterial vasodilation (E) in rats fed a high-fat diet.
*P<0.05, **P<0.01 compared with SD control group; #P<0.05 compared with HF trained group. SD control, n = 8; SD trained, n = 8; HF control, n = 8; HF trained, n = 6.

fat-free mass (P < 0.01) were significantly lower in the HF trained group than in the HF control group. In contrast, the HF trained group had a higher fat-free mass percentage than the HF control group (P < 0.01; **Supplementary Table 2**). These results suggest that the 8-week exercise intervention improved body composition in HFD-fed rats.

Changes in Vascular Endothelial Function and Inflammatory Markers after the Exercise Intervention

To investigate the effects of the exercise intervention on vascular endothelial function, vascular endothelial function biomarkers and endothelium-dependent relaxation were measured and calculated. As shown in **Figure 1**, circulating eNOS levels were significantly higher in the SD trained group than in the SD control (P < 0.01) and the HF trained groups (P < 0.05) (**Fig. 1A**). In addition, circulating VEGF levels were significantly higher in the HF trained group than in the HF control group (P < 0.01; **Fig. 1B**). Analysis of inflammatory factor indices indicated that CRP levels were significantly higher in the HF control group than in the SD control group (P < 0.01), an effect that was abolished by the exercise intervention

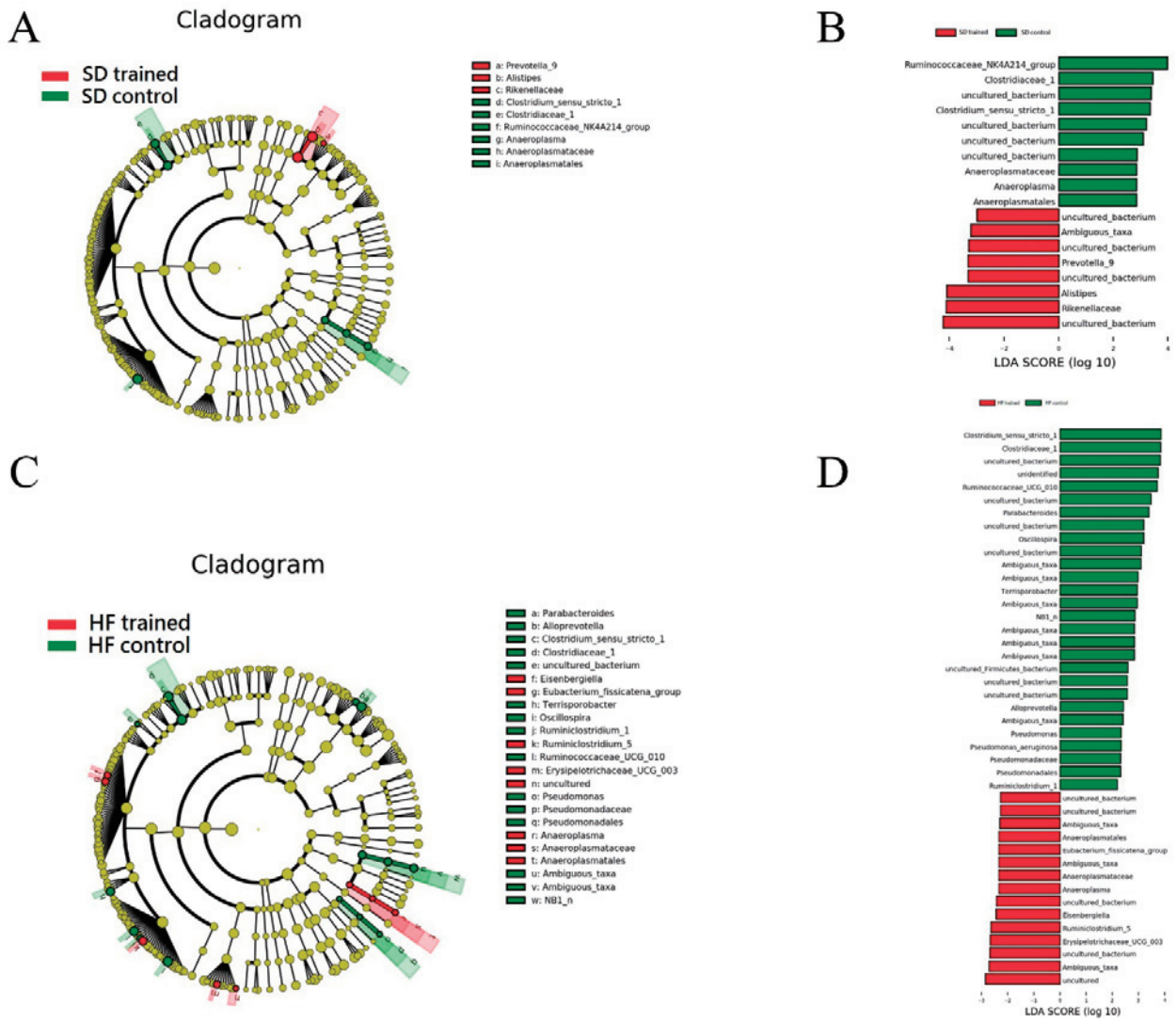

Fig. 2 Cladogram and differential features were selected according to LDA effect size between the exercise-trained and control rats in the standard diet (A, B) and high-fat diet (C, D) groups.

(**Fig. 1C**). No significant change was detected in circulating TNF-α levels in any group (**Fig. 1D**). Furthermore, Ach resulted in a dose-dependent relaxation of rat mesenteric arteries. **Figure 1E** shows that Ach-induced relaxation was greater in the HF control group than in the SD control group, and EC_{50} exhibited a significant shift to the right (−4.64 ± 0.38 *vs.* −5.04 ± 0.32 log M; $P < 0.01$), indicating that endothelium-dependent relaxation was diminished in obese rats. However, this attenuation was completely reversed by the exercise intervention (−4.93 ± 0.23 *vs.* −4.64 ± 0.38 log M, $P < 0.05$).

Changes in Microbiome Composition with the Exercise Intervention

OTU (97% similarity) levels were sequenced and analyzed. **Supplementary Figure 3** shows the resulting species accumulation curve. As the sample size gradually increased, there was no significant increase in microbial species, indicating that the sample size ($n = 29$) was sufficient to capture species diversity.

Alpha diversity was used to assess abundance of intestinal flora with the Wilcoxon rank-sum test. No significant differences were observed among groups (**Supplementary Table 3**). Beta diversity composition, which was analyzed with the Bray-Curtis and weighted UniFrac methods, exhibited differences in community composition in the SD control group and HF control ($P < 0.05$; **Supplementary Figure 4C**).

Examination of LDA effect size revealed that the relative abundances of *Prevotella-9*, Rikenellaceae, and *Alistipes* were greater in the SD trained group than in the SD control group. In contrast, the relative abundances of *Ruminococcaceae_NK4A214_group*, Clostridiaceae_1, *Clostridium_sensu_stricto_1*, Anaeroplasmataceae, *Anaeroplasma*, and Anaeroplasmatales were higher in the SD control

Table 1 Relative abundance of gut microbiota at the family level (Metastat analysis). To avoid false positives, the false discovery rate (FDR) was calculated using the Benjamini–Hochberg method

Family	SD control	HF control	*P*-value	FDR
Up-regulated				
Bacteroidaceae	8.409	22.266	0.002	0.052
Thermoanaerobacteraceae	0.007	0.067	0.001	0.031
Staphylococcaceae	0.005	0.025	0.03	0.192
Down-regulated				
Ruminococcaceae	18.202	12.515	0.04	0.213
Lactobacillaceae	6.156	0.299	0.001	0.031
Clostridiales_vadinBB60_group	0.840	0.073	0.03	0.192
Coriobacteriaceae	0.437	0.060	0.01	0.138
Anaeroplasmataceae	0.149	0.0005	0.03	0.192
Bifidobacteriaceae	0.065	0.005	0.01	0.122
	SD control	SD trained	*P*-value	FDR
Up-regulated				
Bacteroidaceae	8.409	13.746	0.02	0.429
Staphylococcaceae	0.005	0.031	0.03	0.429
Down-regulated				
Clostridiaceae_1	0.898	0.223	0.03	0.429
Anaeroplasmataceae	0.149	0.014	0.04	0.503
Bifidobacteriaceae	0.065	0.001	0.009	0.429
	HF control	HF trained	*P*-value	FDR
Up-regulated				
Micrococcaceae	0.005	0.012	0.02	0.384
Down-regulated				
Peptostreptococcaceae	2.349	1.161	0.02	0.370
Clostridiaceae_1	1.339	0.080	0.008	0.227

group than in the SD trained group (**Fig. 2A and B**). *Erysipelotrichaceae_UCG_003*, *Ruminiclostridium_5*, *Eisenbergiella*, and *Anaeroplasma* were significantly more abundant in the HF trained group than in the HF control group (**Fig. 2C and D**). In addition, the abundance percentages of Staphylococcaceae, Bacteroidaceae, and Thermoanaerobacteraceae, and of the bacterial genera *Lachnospira* and *Prevotellaceae_NK3B31_group*, were significantly higher in the HF control group than in the SD control group (**Supplementary Fig. 5A and B**).

Metastat analysis of differences in microbial community abundance percentages at the family (**Table 1**) and genus (**Table 2**) levels revealed that at the family level (**Table 1**), values for the abundance of Bacteroidaceae, Thermoanaerobacteraceae, and Staphylococcaceae ($P < 0.01$, $P < 0.01$, and $P < 0.05$, respectively) were significantly higher, and that values for the abundance of Ruminococcaceae ($P < 0.05$), Lactobacillaceae ($P < 0.01$), Clostridiales_vadinBB60_group ($P < 0.05$), Coriobacteriaceae ($P < 0.05$), Anaeroplasmataceae ($P < 0.05$) and Bifidobacteriaceae ($P < 0.05$) were significantly lower, in the HF control group than in the SD control group. However, exercise treatment significantly increased the relative abundance of Bacteroidaceae and Staphylococcaceae (both $P < 0.05$), as compared with the SD control group, and decreased the relative abundance of Clostridiaceae_1, Anaeroplasmataceae, and Bifidobacteriaceae ($P < 0.05$, $P < 0.05$ and $P < 0.01$, respectively). Moreover, the abundance of Micrococcaceae ($P < 0.05$) was significantly higher, and the abundance of Peptostreptococcaceae and Clostridiaceae_1 ($P < 0.05$ and $P < 0.01$, respectively) was significantly lower, in the HF trained group than in the HF control group.

At the genus level (**Table 2**), values for the abundance of *Bacteroides* ($P < 0.01$), *Desulfovibrio* ($P < 0.05$), *Prevotellaceae_NK3B31_group* ($P < 0.01$), *Romboutsia* ($P < 0.05$), *Ruminococcus_torques_group* ($P < 0.05$), *Lachnospira* ($P < 0.05$), *Anaerofilum* ($P < 0.05$), *Ruminiclostridium_1* ($P < 0.05$), and *Staphylococcus* ($P < 0.05$) were significantly higher, and values for the abundance of *Lactobacillus* ($P < 0.01$), *Ruminococcaceae_UCG-014* ($P < 0.05$), *Ruminococcaceae_NK4A 214-group* ($P < 0.01$), *Turicibacter* ($P < 0.05$), *Ruminococ-*

Table 2 Relative abundance of gut microbiota at the genus level (Metastat analysis). To avoid false positives, the false discovery rate (FDR) was calculated using the Benjamini–Hochberg method

Genus	SD control	HF control	*P*-value	FDR
Up-regulated				
Bacteroides	9.438	25.224	0.001	0.054
Desulfovibrio	4.147	7.574	0.03	0.199
Prevotellaceae_NK3B31_group	1.324	5.097	0.008	0.122
Romboutsia	0.401	2.423	0.03	0.184
Ruminococcus_torques_group	0.104	2.139	0.03	0.185
Lachnospira	0.045	0.491	0.02	0.165
Anaerofilum	0.008	0.049	0.03	0.184
Ruminiclostridium_1	0.007	0.027	0.01	0.157
Staphylococcus	0.003	0.018	0.02	0.169
Down-regulated				
Lactobacillus	6.851	0.336	0.0008	0.037
Ruminococcaceae_UCG-014	3.335	1.172	0.01	0.157
Ruminococcaceae_NK4A214_group	3.104	0.655	0.001	0.056
Turicibacter	1.125	0.366	0.04	0.206
Ruminococcaceae_UCG-005	0.844	0.233	0.01	0.157
Marvinbryantia	0.695	0.091	0.02	0.185
Ruminococcus_2	0.298	0.004	0.01	0.157
Anaeroplasma	0.168	0.0005	0.03	0.184
Lachnospiraceae_UCG-006	0.104	0.005	0.02	0.181
Bifidobacterium	0.073	0.006	0.01	0.148
Butyricicoccus	0.047	0.002	0.03	0.185
Lachnospiraceae_FCS020_group	0.018	0.009	0.04	0.206
Ruminococcaceae_UCG-002	0.018	0.005	0.01	0.157
	SD control	SD trained	*P*-value	FDR
Up-regulated				
Bacteroides	9.438	15.295	0.03	0.764
Prevotella_9	0.001	0.004	0.01	0.527
Hydrogenoanaerobacterium	0.0009	0.009	0.009	0.363
Down-regulated				
Ruminococcaceae_NK4A214_group	3.104	0.805	0.004	0.363
Clostridium_sensu_stricto_1	0.776	0.248	0.008	0.363
Anaeroplasma	0.168	0.016	0.04	0.783
Bifidobacterium	0.073	0.001	0.008	0.363
	HF control	HF trained	*P*-value	FDR
Up-regulated				
Ruminiclostridium_5	0.045	0.137	0.04	0.634
Eisenbergiella	0.002	0.018	0.04	0.634
Down-regulated				
Romboutsia	2.423	1.245	0.04	0.634
Clostridium_sensu_stricto_1	1.574	0.086	0.007	0.393
Parabacteroides	1.354	0.796	0.01	0.620
Ruminiclostridium_1	0.027	0.003	0.006	0.393

caceae_UCG-005 ($P < 0.05$), *Marvinbryantia* ($P < 0.05$), *Ruminococcus_2* ($P < 0.05$), *Anaeroplasma* ($P < 0.05$), *Lachnospiraceae_UCG-006* ($P < 0.05$), *Bifidobacterium* ($P < 0.05$), *Butyricicoccus* ($P < 0.05$), *Lachnospiraceae_FCS020_group* ($P < 0.05$), and *Ruminococcaceae_UCG-002* ($P < 0.05$) were significantly lower, in the HF control group than in the SD control group. Compared with values for the SD control group, values for the abundance of *Bacteroides* ($P < 0.05$), *Prevotella_9* ($P < 0.05$), and *Hydrogenoanaerobacterium* ($P < 0.01$) were significantly higher, and values for the abundance of *Ruminococcaceae_NK4A214_group* ($P < 0.01$), *Clostridium_sensu_stricto_1* ($P < 0.01$), *Anaeroplasma* ($P <$

Table 3 Correlations of endothelial function indicators and inflammatory markers with relative abundance of bacterial taxa in the overall sample.

Parameter	Microbiota	*P*-value	*r*
EC_{50}	*Eubacterium_coprostanoligenes_group*	<0.001	0.781
EC_{50}	*Oscillibacter*	0.027	0.433
EC_{50}	*Romboutsia*	0.014	0.478
VEGF	*Anaerostipes*	0.048	0.399
VEGF	*Coprococcus_2*	0.004	0.559
VEGF	*Prevotellaceae_NK3B31_group*	0.026	–0.445
VEGF	*Romboutsia*	0.022	–0.457
eNOS	*Alistipes*	0.002	0.594
eNOS	*Escherichia-Shigella*	0.011	0.511
eNOS	*Morganella*	0.003	0.575
eNOS	*Proteus*	0.007	0.534
eNOS	*Ruminococcus_2*	0.040	0.422
CRP	*Escherichia-Shigella*	0.006	0.548
CRP	*Intestinimonas*	0.031	–0.440
CRP	*Lachnospiraceae_UCG-010*	0.026	0.454
CRP	*Morganella*	0.0008	0.636
TNF-α	*Alloprevotella*	0.039	0.415
TNF-α	*Lachnospiraceae_UCG-001*	0.025	0.446
TNF-α	*Papillibacter*	0.011	0.497
TNF-α	*Ruminiclostridium_6*	0.025	0.445

0.05), and *Bifidobacterium* ($P < 0.01$) were significantly lower, after the exercise intervention. Furthermore, compared with values for the HF control group, values for the abundance of *Ruminiclostridium_5* and *Eisenbergiella* (both $P < 0.05$) were significantly higher, and values for the abundance of *Romboutsia* ($P < 0.05$), *Clostridium_sensu_stricto_1* ($P < 0.01$), *Parabacteroides* ($P < 0.05$), and *Ruminiclostridium_1* ($P < 0.01$) were significantly lower, after the exercise intervention.

Associations of Gut Microbiota Composition with Vascular Endothelial Function and Inflammatory Markers

Correlation analysis of gut microbiota composition and vascular endothelial function indicated that values for *Oscillibacter* and *Eubacterium_coprostanoligenes_group* abundance were positively correlated with EC_{50} ($P < 0.05$; **Table 3**). Furthermore, *Romboutsia* abundance was positively correlated with EC_{50} ($P < 0.05$) but negatively correlated with VEGF ($P < 0.05$). Values for *Anaerostipes* and *Coprococcus_2* were significantly positively correlated with VEGF ($P < 0.05$ and $P < 0.01$, respectively), whereas the abundance of *Prevotellaceae_NK3B31_group* was negatively correlated with VEGF ($P < 0.05$). Values for *Escherichia-Shigella* and *Morganella* were positively correlated with both eNOS and CRP. In addition, values for *Alistipes* ($P < 0.01$), *Proteus* ($P < 0.01$), and *Ruminococcus_2* ($P < 0.05$) were positively correlated with eNOS. There was also a significant positive correlation between the abundance of *Lachnospiraceae_UCG-010* and CRP ($P < 0.05$) and a significant negative correlation between the abundance of *Intestinimonas* and CRP ($P < 0.05$). Moreover, values for the abundance of *Alloprevotella*, *Lachnospiraceae_UCG-001*, *Papillibacter*, and *Ruminiclostridium_6* were positively correlated with TNF-α (all $P < 0.05$). In sum, these results suggest associations of gut microbiota with inflammatory markers and endothelial function.

Interactions between Gut Microbiota and Vascular Endothelial Function

Co-occurrence analysis of interactions between vascular endothelial function and gut microbiota showed that 12 species of bacteria significantly interacted with endothelial function parameters (EC_{50}, VEGF, eNOS) in the overall sample (**Fig. 3A**). Further analyses of the HF control group and HF trained group showed a positive association between the relative abundance of *Romboutsia* and EC_{50} ($P < 0.05$, $r = 0.7$; **Fig. 3B**).

Discussion

We found that moderate-intensity aerobic exercise increased vascular endothelial function, which was associated with altered gut microbiota composition in rats with HFD-induced obesity. Twelve species of bacteria, includ-

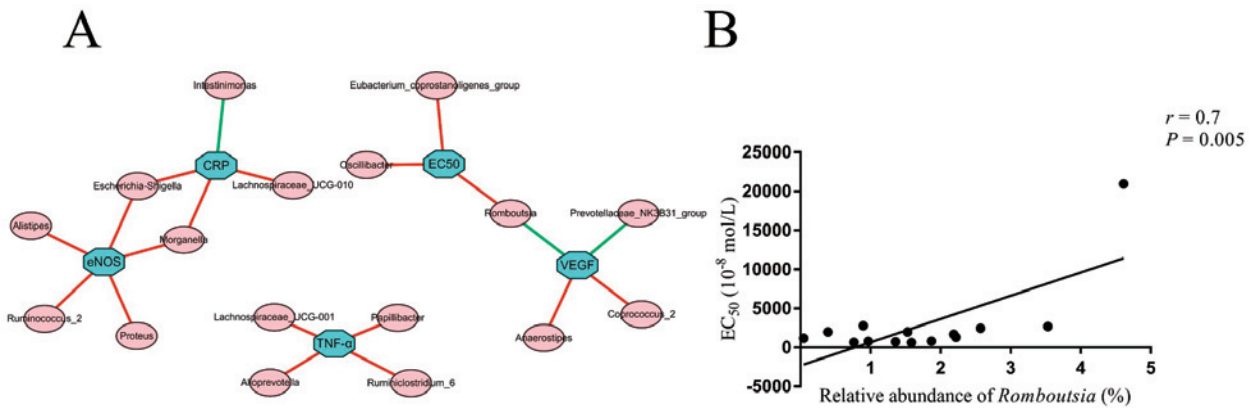

Fig. 3 (A) Analysis of significant co-occurrence between vascular endothelial function or inflammatory markers and gut microbiota in the overall sample. Selected interactions between vascular endothelial function and bacterial communities are illustrated (green lines, negative; red lines, positive). (B) Correlation of EC_{50} with relative abundance of *Romboutsia* in the HFD group. Values are mean ± SD. $r = 0.7$, $P = 0.005$.

ing *Romboutsia*, were significantly associated with variables related to endothelial function (EC_{50}, VEGF, eNOS) in the overall sample.

Regular exercise is an effective strategy for managing body weight and vascular endothelial function. Here, 8 weeks of aerobic exercise effectively enhanced body composition in HFD-fed rats. Moreover, EC_{50} was lower in the HF trained group than in the HF control group, indicating an improvement in endothelial function after the exercise intervention. Obesity increases oxidative stress and reduces NO bioavailability. eNOS and iNOS are the main effectors of endothelial cells and are essential for NO production, and iNOS expression is strong correlated with angiogenesis. We previously reported that exercise improved vascular endothelial function by decreasing oxidative stress, increasing expressions of eNOS and VEGF, and ameliorating inflammatory injury in obese animals and humans[31–33]. In agreement with these studies, our results indicate that exercise increased circulating levels of eNOS and VEGF and reduced CRP concentration in the SD control and HF control groups, respectively, whereas no change in TNF-α level was observed in any group. Obesity likely increases TNF-α; however, this increase may not be detectable in the circulation but only locally, in arteries. It would have been interesting if TNF-α expression had been measured in arteries.

Intestinal flora status was modified by the diet and exercise intervention. Alpha diversity was somewhat diminished in the HF control group, but the difference was not significant. This difference might have been due to the period assigned for HFD feeding. Moreover, microbiota composition can be influenced by host genetics. Because of the high homology of genes, it might be difficult to identify significant differences in alpha diversity between the HF control and SD control. Similar results have been reported previously[34]. Beta diversity of gut flora was decreased in HFD-fed rats. Furthermore, moderate-intensity exercise did not increase alpha or beta diversity in gut microbiota in the present SD trained and HF trained rats. A previous study reported that low-to-moderate exercise did not modulate the gut microbiota in mice fed a HFD[35]. Interestingly, moderate-intensity treadmill exercise had a minimal impact on change in an obesogenic diet-related gut microbiome[36]. However, another study found that high-intensity exercise training improved metabolic capacity and diversity of distal gut microbiota in mice with diet-induced obesity[19]. Nevertheless, intense exercise increases gastrointestinal epithelial wall permeability and decreases gut mucus thickness, potentially enabling pathogens to enter the bloodstream. This, in turn, may contribute to increased inflammation levels[37]. Our analysis suggests that exercise intensity is important in modifying the diversity of intestinal microbiota in diet-induced obesity.

The Firmicutes/Bacteroidetes ratio has been used as an indicator of obesity, as evidence indicates that obese individuals have a lower abundance of Bacteroidetes, a higher proportion of Firmicutes, and a higher Firmicutes/Bacteroidetes ratio than non-obese persons[10,38]. The present HF control group exhibited greater abundance of members of Bacteroidetes phyla, and lesser abundance of members of Firmicutes phyla, as compared with the SD control group (**Supplementary Fig. 6**). Our present results thus seem to contradict those of previous studies. However, a recent study indicated that Firmicutes/Bacteroidetes ratio may not correlate with a spe-

cific health status and might not be a hallmark of obesity[39]. Thus, rather than comparing phyla, studies should investigate specific species.

We analyzed the relative abundance of gut flora at the family level and genus level and found that exercise and diet altered the composition of intestinal microbiota. Abundances of the families Bacteroidaceae, Staphylococcaceae, and Thermoanaerobacteraceae and genera *Lachnospira* and *Prevotellaceae_NK3B31_group* were significantly higher in obese rats than in control rats. In contrast, abundances of Anaeroplasmataceae, Bifidobacteriaceae, Clostridiales_vadinBB60_group, Coriobacteriaceae, Lactobacillaceae, and Ruminococcaceae bacteria, at the family level, and *Butyricicoccus* and *Lactobacillus* bacteria, at the genus level, were lower in mice fed an HFD. The effect of an HFD on the abundance of Ruminococcaceae is consistent with the findings of a previous study[7].

Exercise did not affect alpha or beta diversity. We speculate that the effect on the microbiome is greater for a dietary intervention than for an exercise intervention. A recent study reported that 8 weeks of exercise training markedly improved insulin sensitivity and body composition in obese humans; however, this was not accompanied by improvements in gut microbiota alpha diversity or change in beta diversity; change in microbiome composition was limited[40]. A systematic review found that exercise is associated with alterations in gut microbial composition, increased abundance of butyrate-producing bacteria, and higher fecal butyrate concentrations, regardless of diet, in rodents and humans[12]. Previous studies reported that the relative abundance of the butyrate-producing bacteria Clostridiales was significantly higher in humans and animals with obesity and diabetes[10,41–45]. Similarly, the present study found that the abundance of *Ruminiclostridium_1* was higher in HFD-fed rats than in control rats. Moreover, exercise training significantly reduced the abundance of *Ruminiclostridium_1*, as well as Clostridiaceae_1 and *Clostridium_sensu_stricto_1*, in the SD control group and HF control group. These results suggest that Clostridiales play a major role in regulating obesity and diabetes by generating gut metabolites and that exercise effectively modulates these bacteria, which have been associated with vascular dysfunction and obesity and diabetes-related metabolic disorders, hence reducing the risk of these conditions.

Researchers recently reported a strong correlation between the gut microbiome and cardiovascular risk factors such as obesity, type 2 diabetes, and hypertension,[5,46,47]. Ruminococcaceae, *Lachnospira*, and *Prevotella* were associated with cardiac function[48]. Here, we found that values for the abundance of five OTUs in Ruminococcaceae were lower in obese rats. Moreover, *Ruminococcus_2* was positively correlated with eNOS and negatively correlated with body weight, fat-free mass, and fat mass percentage. In contrast, abundance of the *Ruminococcus_gnavus_group* was positively correlated with body weight, fat-free mass, and fat mass percentage. In addition, abundance of the *Prevotellaceae_NK3B31_group* was negatively correlated with VEGF, and abundance of Lachnospiraceae was positively correlated with CRP and TNF-α. These results indicate that Ruminococcaceae, *Prevotella*, and *Lachnospira* are important gut flora in regulating body composition and cardiovascular function in HFD-fed rats.

Previous studies reported a relationship between *Romboutsia* and obesity. The abundance of the genus *Romboutsia* was significantly higher in an obese group and positively correlated with lipid metabolism indicators[49–52]. The present results indicate that the exercise intervention markedly changed the abundance of intestinal bacterial flora. We observed that the abundance of *Romboutsia* was significantly lower in obese rats after the exercise intervention. Importantly, *Romboutsia* was positively correlated with EC_{50} and negatively correlated with VEGF. A study using L-NAME to induce hypertension and a disordered eNOS-nitric oxide pathway in treated rats found that the relative abundance of *Romboutsia* significantly increased in L-NAME-treated rats[53]. Another study, of the intestinal flora of healthy amateur half-marathon runners, showed that long-distance endurance running significantly reduced the abundance of *Romboutsia*[54]. These findings indicate that *Romboutsia* is a potential biomarker that links exercise with vascular endothelial function. We plan to examine the interaction between *Romboutsia* and endothelial function in a future study.

In summary, we observed that exercise-induced changes in intestinal flora composition were correlated with vascular endothelial function in HFD-fed rats. A program of physical exercise might thus be effective in improving gut flora and vascular endothelial function in obese persons.

Data availability: The raw data are available in the Supplemental File. Sequence data are available at the Sequence Read Archive (SRA): PRJNA715059.

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