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Exercised gut microbiota improves vascular and metabolic abnormalities in sedentary diabetic mice through gut–vascular connection

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Abstract

Background: Exercise elicits cardiometabolic benefits, reducing the risks of cardiovascular diseases and type 2 diabetes. This study aimed to investigate the vascular and metabolic effects of gut microbiota from exercise-trained donors on sedentary mice with type 2 diabetes and the potential mechanism.

Methods: Leptin receptor-deficient diabetic (db/db) and nondiabetic (db/m^{+}) mice underwent running treadmill exercise for 8 weeks, during which fecal microbiota transplantation (FMT) was parallelly performed from exercise-trained to sedentary diabetic (db/db) mice. Endothelial function, glucose homeostasis, physical performance, and vascular signaling of recipient mice were assessed. Vascular and intestinal stresses, including inflammation, oxidative stress, and endoplasmic reticulum (ER) stress, were investigated. RNA sequencing analysis on mouse aortic and intestinal tissues was performed. Gut microbiota profiles of recipient mice were evaluated by metagenomic sequencing.

Results: Chronic exercise improved vascular and metabolic abnormalities in donor mice. Likewise, FMT from exercised donors retarded body weight gain and slightly improved grip strength and rotarod performance in recipient mice. Exercise-associated FMT enhanced endothelial function in different arteries, suppressed vascular and intestinal stresses, and improved glucose homeostasis in recipient mice, with noted microRNA−181b upregulation in aortas and intestines. Altered gut microbiota profiles and gut-derived factors (e.g., short-chain fatty acids and glucagon-like peptide-1) as well as improved intestinal integrity shall contribute to the cardiometabolic benefits, implying a gut–vascular connection.

Conclusion: This proof-of-concept study indicates that exercised microbiota confers cardiometabolic benefits on sedentary db/db mice, extending the beneficial mechanism of exercise through gut–vascular communication. The findings open up new therapeutic opportunities for cardiometabolic diseases and shed light on the development of exercise mimetics by targeting the gut microbiota.

Keywords: Diabetes mellitus; Endothelial dysfunction; Exercise; Fecal microbiota transplantation; microRNA

1. Introduction

Exercise training elicits systemic benefits on the body, reducing the risks of chronic diseases and premature mortality.[1] Diabetes mellitus is a significant risk factor of cardiovascular complications, highly associated with all-cause and cardiovascular mortality.[2] The effectiveness of exercise training has been implicated in the primary and secondary prevention of cardiovascular diseases and diabetes mellitus.[3,4] Regular exercise training alleviates a variety of cardiovascular and metabolic abnormalities, particularly insulin resistance, hyperlipidemia, chronic low-grade inflammation, and vascular dysfunction.[5] Nevertheless, the comprehensive mechanism of

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exercise contributing to cardiometabolic health is an expanding universe, requiring further extensive study.

Considered an "invisible endocrine organ", the gut microbiota is critical to host metabolism and homeostasis.[6] Perturbation of homeostatic gut microbiota composition, termed "dysbiosis", has been implicated in the pathologies of numerous diseases, including cardiovascular diseases and diabetes.[7] Dysbiosis alters the profile of gut-derived substances, significantly contributing to dysregulation of the immune system and chronic inflammation.[8] Meanwhile, exercise training potentially elicits positive health effects by altering gut microbiota composition and improving intestinal integrity.[9] In humans, exercise is believed to enrich the diversity of the gut microbiota, increasing the abundance of short-chain fatty acid (SCFA)-producing bacteria, in particular.[10] However, whether chronic exercise improves cardiometabolic health through targeting dysbiosis remains elusive, as do the underlying mechanisms.

Notably, due to the close proximity between intestine and blood circulation, the vasculature is often the first-line barrier vulnerable to dysbiosis.[8] Exercise training directly elicits beneficial effects on vasculature by increasing blood flow rate to generate higher shear stress on endothelial cells.[11] Exercise training improves endothelium-dependent vasodilation, which is potently protective against the development of cardiovascular diseases (e.g., atherosclerosis and cardiomyopathy) and diabetes.[12] Because of the tight gut–vascular connection, it is reasonable to postulate that exercise might indirectly alter vascular function through the gut microbiota. To study the indirect benefits of exercise training on vasculature, we therefore performed fecal microbiota transplantation (FMT) from exercised donor to sedentary mice to see whether the exercised gut microbiota alone is sufficient to exert vascular benefits. We anticipated an enriched microbial diversity and a partially similar microbial shift in sedentary recipient mice after exercise-associated FMT. Due to disability, injury, or illness, certain individuals cannot exercise regularly. Whether exercised microbiota potentially serves as a partial "exercise mimetic" remains doubtful.

Therefore, we performed the current study to (a) explore the cardiometabolic benefits of exercised gut microbiota, (b) extend the mechanism of exercise training, (c) uncover microbiota alterations and underlying mechanism that are potentially pivotal, and (d) elucidate the potential of exercised microbiota as a partial exercise mimetic. Our findings suggest the gut–vascular connection as a potential intervention target, opening up new therapeutic opportunities against cardiometabolic diseases.

2. Methods

All animal experiments were performed in compliance with the procedures and ethical polices established by the Animal Research Ethics Sub-Committee of City University of Hong Kong (approval No. AN-STA-00000132). All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals established by the National Institutes of Health and Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines.

Detailed methods are available in the online-only Supplementary Materials.

3. Results

3.1. FMT from exercised donors improves metabolic parameters and physical performance in sedentary diabetic mice

To study the effects of exercised microbiota on the vasculature and metabolism of sedentary mice, we performed FMT from different groups of donor mice ($n = 20$ per group): sedentary nondiabetic (Sed db/m$^+$), sedentary diabetic (Sed db/db), exercised nondiabetic (Ex db/m$^+$), and exercised diabetic (Ex db/db) to sedentary diabetic mice (All db/db), constituting 4 groups of recipient mice ($n = 20$ per group): Sed db/m$^+$-R, Ex db/m$^+$-R, Sed db/db-R, and Ex db/db-R (Fig. 1A). The db/db mice were chosen to understand the cardiometabolic benefits and therapeutic potential of exercised microbiota against diabetes mellitus. FMT from Sed db/db mice was performed as a control experiment. FMT from nondiabetic mice (Sed db/m$^+$ and Ex db/m$^+$) was also conducted to study whether exercised microbiota from lean exercised donors exert better cardiometabolic benefits in recipient db/db mice.

To verify the fitness of donor mice, we evaluated their metabolic parameters and physical performance. The 8-week exercise protocol lowered final body weights and retarded body weight gain in exercised groups compared to corresponding sedentary groups of donor mice (Sed db/m$^+$ *vs.* Ex db/m$^+$ and Sed db/db *vs.* Ex db/db; Supplementary Fig. 1A and 1B), although the amount of food intake in the Ex db/db group was slightly increased (Supplementary Fig. 1C). At Weeks 4 and 8 of the exercise protocol, glucose tolerance and insulin resistance were found improved in exercised donor mice, as shown by oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) (Supplementary Fig. 1D–1 G). Exercised donor mice demonstrated higher grip strength and better rotarod performance than their sedentary counterparts at Weeks 4 and 8 of the exercise protocol (Supplementary Fig. 1H and 1I). Before exercise protocol, the initial fasting glucose levels were comparable in all donor db/db mice (Supplementary Fig. 2). The 8-week exercise training improved lipid profiles in db/m$^+$ and db/db mice (Supplementary Fig. 3A–3D). The donor mice were certified to donate exercised microbiota.

During the 8-week FMT, db/db mice receiving FMT from Sed db/m$^+$, Ex db/m$^+$, and Ex db/db mice showed lower final body weights (lowest in Ex db/m$^+$-R) and retarded body weight gain compared to those transplanted from Sed db/db mice (Fig. 1B and 1C). The amount of food intake was slightly higher in the Sed db/db-R group (non-significant; Fig. 1D), partially accounting for body weight changes and hinting at altered metabolism upon FMT. Before FMT, the initial fasting glucose levels were comparable in all recipient db/db mice (Supplementary Fig. 2). Compared to the Sed db/db-R group, Sed db/m$^+$-R and Ex db/m$^+$-R groups started to show improved

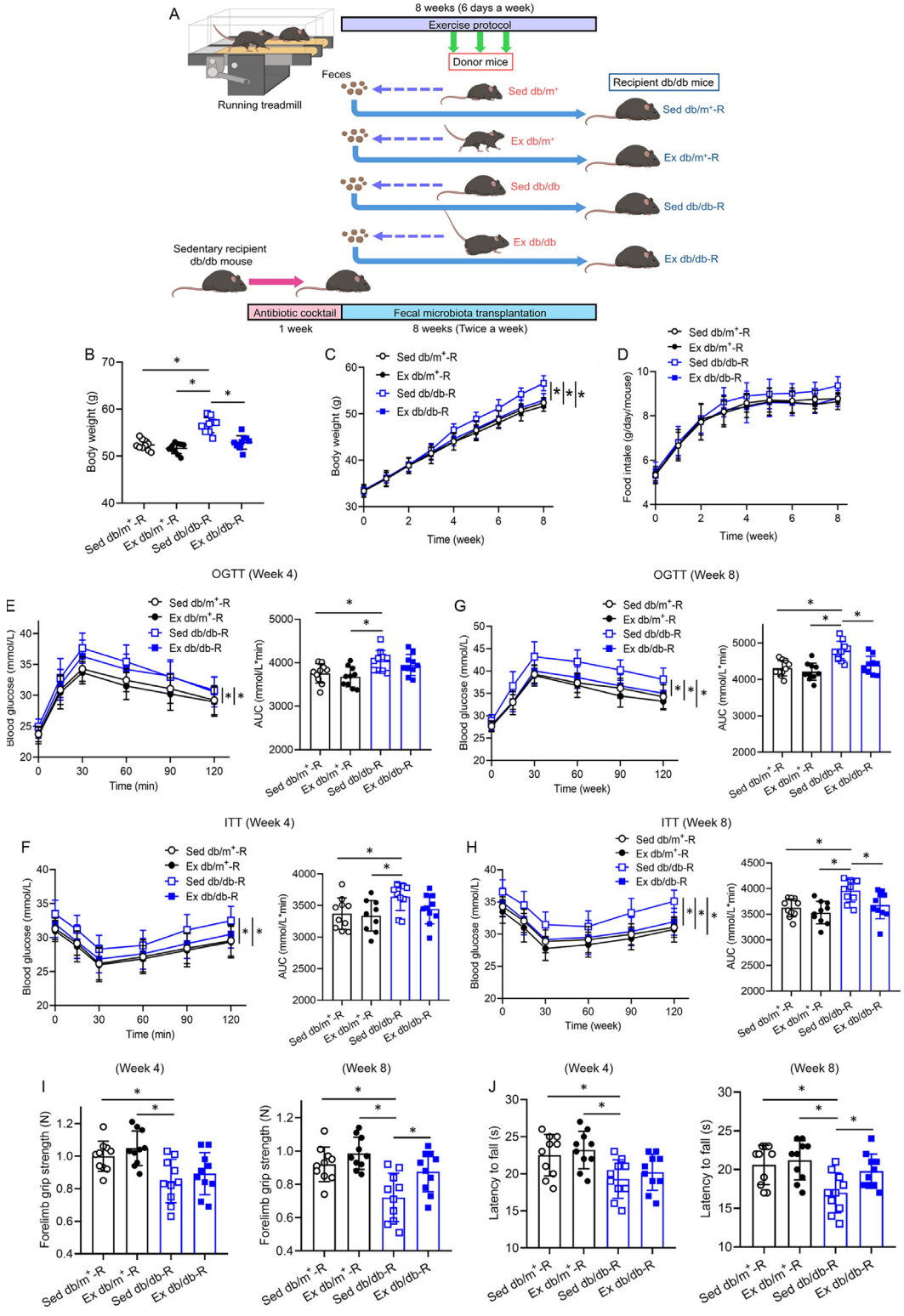

Fig. 1. Effects of exercised microbiota on body parameters and physical performance of sedentary diabetic mice. (A) Schematic overview on FMT protocol from different donor mice to sedentary diabetic mice. The donor mice include sedentary nondiabetic (Sed db/m$^+$), exercised nondiabetic (Ex db/m$^+$), sedentary diabetic (Sed db/db), and exercised diabetic (Ex db/db). (B) Body weights of different groups of recipient mice, including Sed db/m$^+$-R, Ex db/m$^+$-R, Sed db/db-R, and Ex db/db-R, after the 8-week FMT protocol. (C) Body weight changes of recipient mice during the 8-week FMT. Statistical significance was determined by AUC analysis. (D) Changes in food intake amount of recipient mice during the 8-week FMT. Statistical significance was evaluated by AUC analysis. OGTT and corresponding AUC analysis on recipient mice at (E) Week 4 and (G) Week 8 of FMT. ITT and corresponding AUC analysis on recipient mice at (F) Week 4 and (H) Week 8 of FMT. (I) Forelimb grip strength performance of recipient mice at Week 4 and Week 8 of FMT. (J) Rotarod performance of recipient mice at Week 4 and Week 8 of FMT. $n = 10$ per group. Data are mean ± SD. * $p < 0.05$ (Brown-Forsythe and Welch analysis of variance, and Dunnett T3 test). AUC = area under curve; FMT = fecal microbiota transplantation; ITT = insulin tolerance test; OGTT = oral glucose tolerance test.

glucose homeostasis at the 4th week of FMT (Fig. 1E and 1F), whereas the Ex db/db-R group showed improved glucose homeostasis at the 8th week of FMT (Fig. 1G and 1H). A similar improvement trend was observed in grip strength and rotarod performance of recipient mice (Fig. 1I and 1J). The 8-week FMT slightly lowered total triglyceride (TG) and low-density lipoprotein (LDL) levels while raising high-density lipoprotein (HDL) levels in the Ex db/m$^+$-R group (Supplementary Fig. 3E−3H). The findings implied that microbiota from a lean exercised donor might be of higher efficacy in improving metabolism and physical performance.

3.2. Exercised microbiota improves endothelial function in different arteries of recipient mice

Since diabetes-associated hyperglycemia and dyslipidemia are key contributors to endothelial dysfunction,[13] we studied whether exercised microbiota beneficially counteracts diabetic endothelial dysfunction in different arteries by wire myography. Regular exercise ameliorates endothelial dysfunction.[14] We confirmed that 8 week of exercise improved acetylcholine (ACh)-induced endothelium-dependent relaxations (EDRs) in aortas, mesenteric arteries, and renal arteries of donor mice (Supplementary Fig. 4), further confirming their fitness to donate microbiota. To different extents, FMT from Sed db/m$^+$, Ex db/m$^+$, and Ex db/db mice improved ACh-induced EDRs in aortas, mesenteric arteries, and renal arteries of recipient mice (Fig. 2A, 2C, and 2E). FMT did not alter sodium nitroprusside (SNP)-induced endothelium-independent relaxations in aortas and renal arteries from different mouse groups (Fig. 2B and 2F), indicating that improved vascular function was mainly due to endothelium-derived nitric oxide (NO). Besides, relaxations in mesenteric arteries depend on both endothelial nitric oxide synthase (eNOS)-derived NO and endothelium-derived hyperpolarizing factor (EDHF). Mesenteric arteries were incubated with N^G-nitro-L-arginine methyl ester (L-NAME) to eliminate eNOS-derived NO production to study the role of EDHF in relaxations.[15] EDHF-dependent relaxations were similar in mesenteric arteries among mouse groups after L-NAME incubation (Fig. 2D), indicating that increased NO bioavailability mainly contributed to enhanced mesenteric vasorelaxation.

Notably, the Ex db/m$^+$-R group demonstrated the best relaxation in aortas, mesenteric arteries, and renal arteries (Fig. 2A, 2C, and 2E). The differential efficacy on vasorelaxation enhancement could be reflected by the vascular NO production. Nitrite assay revealed levels of NO production in aortas of recipient mice in the following order: Ex db/m$^+$-R > Sed db/m$^+$-R > Ex db/db-R > Sed db/db-R (Fig. 2H). Since exercise can activate adenosine monophosphate-activated protein kinase (AMPK)/eNOS signaling to boost vascular NO production by promoting phosphorylation of AMPK (at threonine 172) and eNOS (at serine 1177),[16] we wondered whether exercise-associated FMT causes vascular AMPK/eNOS activation. Upregulated phosphorylated adenosine monophosphate-activated protein kinase at threonine 172 (p-AMPK^{Thr172}) and phosphorylated endothelial nitric oxide synthase at serine 1177 (p-eNOS^{S1177}) were observed in aortas of Ex db/m$^+$-R, Sed db/m$^+$-R, and Ex db/db-R groups as compared to the Sed db/db-R group (Fig. 2G).

Uncoupling protein 2 (UCP2) from mitochondria, which is upregulated by AMPK activation,[17] plays antioxidant and anti-inflammatory roles in counteracting endothelial dysfunction and atherosclerosis.[18] Importantly, aortic UCP2 levels were found to be upregulated in Ex db/m$^+$-R, Sed db/m$^+$-R, and Ex db/db-R groups (Fig. 2G), hinting at an activated AMPK/UCP2 axis upon exercise-associated FMT. Exercise is associated with ATP depletion and ADP release, as reflected by an increased ADP/ATP ratio in different tissues.[19] Notably, increased ADP/ATP ratios were observed in mouse aortas upon exercise-associated FMT (Fig. 2I), which is consistent with our previous finding that UCP2 upregulation and AMPK activation in aortas are correlated with an elevated ADP/ATP ratio.[18] This finding implied a similar energy stress between exercise training and exercise-associated FMT. Endothelial dysfunction promotes blood pressure elevation.[20] Consistent with the wire myography results, both chronic exercise and exercise-associated FMT lowered mean arterial pressures in db/db mice at Week 8 (Supplementary Fig. 5).

3.3. Exercised microbiota alleviates vascular stresses in recipient mice

Diabetes-associated hyperglycemia induces vascular reactive oxygen species (ROS) accumulation to cause endothelial dysfunction,[21] and excess ROS triggers NO quenching to lower NO bioavailability.[22] We therefore wondered whether exercised microbiota confers the ROS-lowering benefit of exercise on vasculature. Lucigenin-enhanced chemiluminescence assay showed that chronic exercise (Ex db/m$^+$ and Ex db/db groups) and FMT from exercised donors (Ex db/m$^+$-R and Ex db/db-R groups) suppressed oxidative stress in aortas (Fig. 3A and Supplementary Fig. 6A), consistent with the FMT-upregulated UCP2 in aortas (Fig. 2G). In addition, aortic nicotinamide adenine dinucleotide phosphate oxidase (NOX) activity was reduced upon chronic exercise and exercise-associated FMT, albeit to different extents (Fig. 3B and Supplementary Fig. 6B), implying a NOX-dependent ROS-lowering effect of exercise and exercised microbiota. Our previous study has shown that exercise can upregulate miR-181b, an AMPK-downstream microRNA (miRNA) that elicits ROS-lowering and anti-inflammatory effects on the vasculature of diabetic mice.[11] We therefore postulated that exercised microbiota might confer an miR-181b-elevating effect of exercise to partially contribute to the ROS-lowering effect. Notably, aortic miR-181b levels were found to be upregulated upon exercise training and FMT from exercised donors (Fig. 3C and Supplementary Fig. 6C). RNA sequencing analysis of aortas from Sed db/db-R and Ex db/db-R mice, along with corresponding Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, revealed enriched pathways of upregulated and downregulated genes upon exercise-associated FMT, which are crucial to the

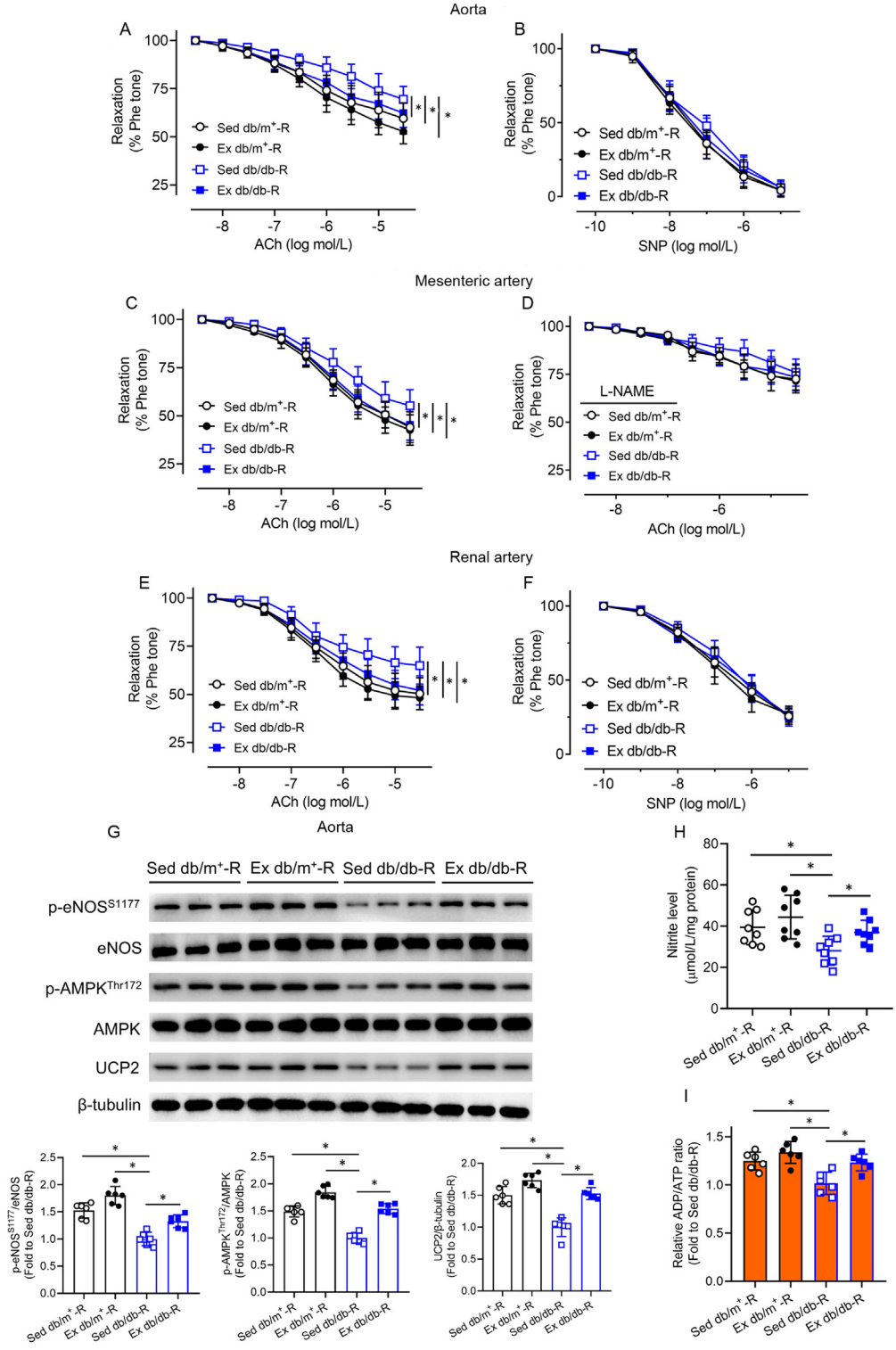

Fig. 2. Effects of exercised microbiota on vascular function of recipient mice. Wire myography on (A) ACh-induced EDRs and (B) SNP-induced vasorelaxation of aortas from different groups of recipient mice. Wire myography on ACh-induced EDRs of mesenteric arteries from recipient mice (C) in the absence and (D) in the presence of L-NAME pre-treatment. Wire myography on (E) ACh-induced EDRs and (F) SNP-induced vasorelaxation of renal arteries from recipient mice. $n = 8$ per group. (G) Representative Western blots and corresponding quantification on expression of vascular function-related proteins. $n = 6$ per group. (H) Nitrite levels in aortas of recipient mice. $n = 8$ per group. (I) Relative ADP/ATP ratios in aortas of recipient mice. $n = 6$ per group. Data are mean ± SD. * $p < 0.05$ (Brown-Forsythe and Welch analysis of variance, and Dunnett T3 test). Ach = acetylcholine; ADP/ATP = adenosine diphosphate/ adenosine triphosphate; AMPK = adenosine monophosphate-activated protein kinase; β-tubulin = beta-tubulin; EDR = endothelium-dependent relaxation; eNOS = endothelial nitric oxide synthase; L-NAME = NG-nitro-L-arginine methyl ester; p-AMPK^{Thr172} = phosphorylated adenosine monophosphate-activated protein kinase at threonine 172; p-eNOS^{S1177} = phosphorylated endothelial nitric oxide synthase at serine 1177; SNP = sodium nitroprusside; UCP2 = uncoupling protein 2.

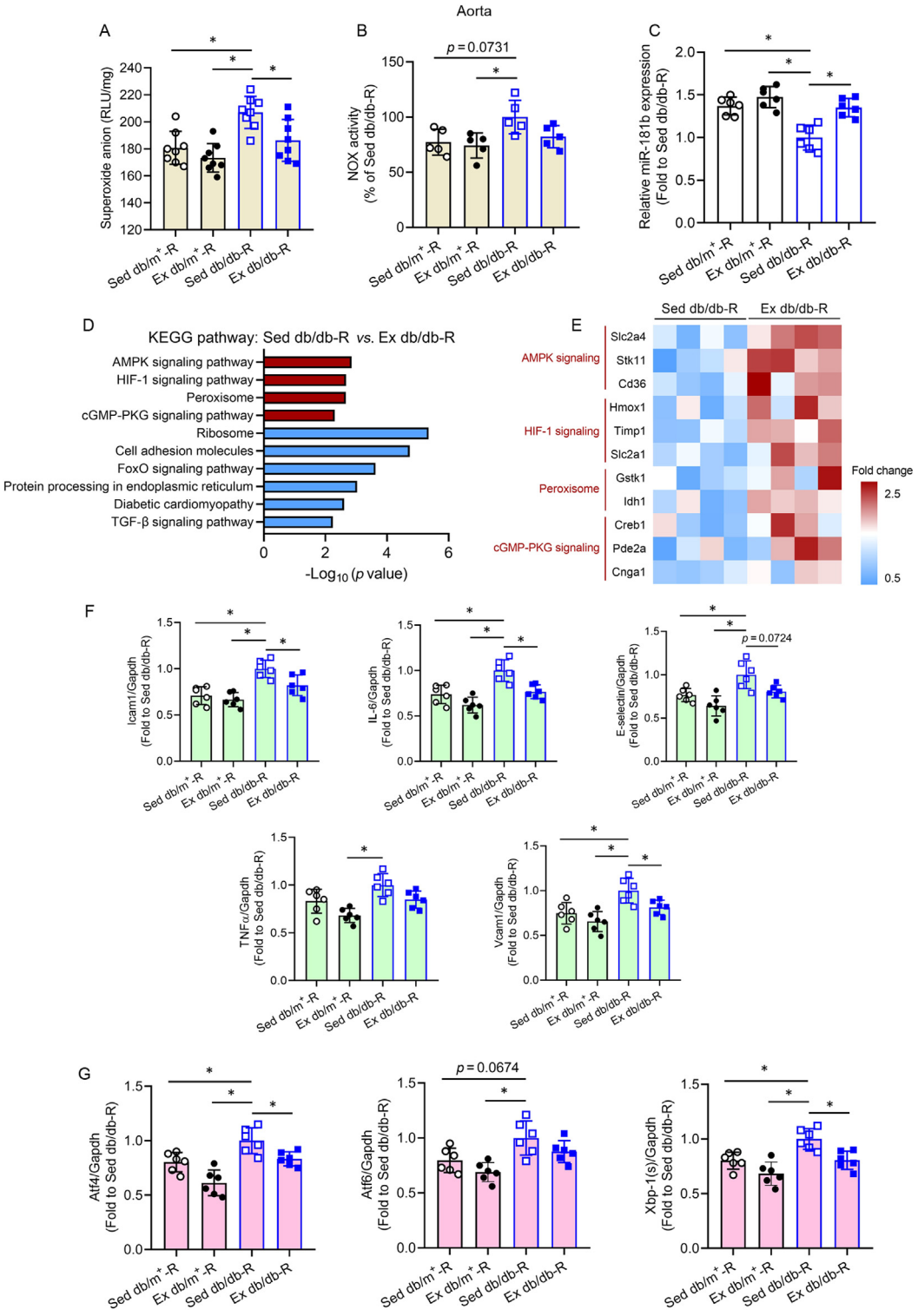

Fig. 3. Effects of exercised microbiota on vascular stresses of receipt mice. (A) Lucigenin-enhanced chemiluminescence on ROS levels in aortas of recipient mice. $n = 8$ per group. (B) NOX activity assay in aortas of recipient mice. $n = 5$ per group. (C) RT-PCT on relative miR-181b expression in aortas of recipient mice. $n = 6$ per group. (D) Enriched KEGG pathways for upregulated and downregulated genes in aortas from Sed db/db-R and Ex db/db-R groups. (E) Heatmap showing differentially expressed genes related to vascular function regulation between Sed db/db-R and Ex db/db-R groups. $n = 4$ per group. RT-PCT on mRNA levels of (F) pro-inflammatory genes and (G) ER stress markers in aortas of recipient mice. $n = 6$ per group. Data are mean ± SD. * $p < 0.05$ (Brown-Forsythe and Welch analysis of variance, and Dunnett T3 test). AMPK = adenosine monophosphate-activated protein kinase; Atf4 = activating transcription factor 4; Atf6 = activating transcription factor 6; cGMP-PKG = cyclic guanosine monophosphate-dependent protein kinase; ER = endoplasmic reticulum; E-selectin = endothelial selectin; FoxO = forkhead box O; HIF-1 = hypoxia-inducible factor 1; Icam1 = intercellular adhesion molecule 1; IL-6 = interleukin-6; KEGG = Kyoto Encyclopedia of Genes and Genomes; miR-181b = microRNA-181b; NOX = nicotinamide adenine dinucleotide phosphate oxidase; RLU = relative light unit; ROS = reactive oxygen species; RT-PCR = reverse transcription polymerase chain reaction; TGF-β = transforming growth factor-beta; TNFα = tumor necrosis factor-alpha; Vcam1 = vascular cell adhesion molecule 1; Xbp-1(s) = X-box Binding Protein 1 (spliced form).

regulation of vascular function (e.g., AMPK signaling pathway), antioxidant activity (e.g., hypoxia-inducible factor 1 (HIF-1) signaling pathway), vascular inflammation (e.g., forkhead box O (FoxO) and transforming growth factor-beta (TGF-beta) signaling pathways), and endoplasmic reticulum (ER) stress (e.g., protein processing in the endoplasmic reticulum) (Fig. 3D and 3E), consistent with the improved EDRs and diminished oxidative stress in the vasculature of recipient mice.

Reciprocal causalities exist among oxidative stress, inflammation, and ER stress during the progression of endothelial dysfunction,[23] and exercise was previously shown to attenuate ER stress and oxidative stress in aortas of db/db mice.[24] We therefore measured the expression of inflammation and ER stress markers in aortas of recipient mice. Compared to the Sed db/db-R group, the Sed db/m$^+$-R, Ex db/m$^+$-R, and Ex db/db-R groups showed downregulated expression of inflammation and ER stress markers to different extents; the Ex db/m$^+$-R group demonstrated the lowest expression of these stress markers (Fig. 3F and 3G). Additionally, exercised microbiota insignificantly upregulated the expression of Foxp3, the regulatory T cell (Treg) marker, while downregulating the expression of retinoid-related orphan receptor γ (RORγ), the T helper (Th)17 marker in aortas of recipient mice (Supplementary Fig. 7), where Treg/T17 balance in the vasculature critically influences endothelial function, blood pressure, and vascular inflammation.[25] These results suggest that exercised microbiota might exert cardioprotective effects by altering T cell infiltration to the vasculature, potentially implying a gut-immune-vascular axis. Exercised microbiota might also alter certain circulating factors in recipient mice to counteract these vascular stresses during endothelial dysfunction.

3.4. FMT from exercised donors causes microbial shifts in recipient mice

The analysis of microbial diversity and community structure across different FMT groups in sedentary diabetic mice revealed significant differences when compared to the control group (Sed db/db-R) (Fig. 4). All Sed db/m$^+$-R, Ex db/m$^+$-R, and Ex db/db-R groups exhibited significantly higher Chao diversity indices, indicating increased species richness compared to the Sed db/db-R group (Fig. 4A). The non-metric multidimensional scaling (NMDS) analysis further highlighted distinct clustering patterns, with the Sed db/db-R group showing a unique microbial community structure that was markedly different from the other FMT groups (Fig. 4B). This suggests that the microbial composition in sedentary diabetic mice can be modulated by FMT from lean and exercised donors, potentially altering the gut microbiota toward a more diverse and stable state.

A comprehensive analysis of gut microbiota profiles across different experimental groups, using the Sed db/db-R group as a control, revealed significant changes in abundance at both phylum and genus levels (Fig. 4C−4G). In the Ex db/db-R group, the mean abundance of *Bacteroidetes* increased by approximately 12%, while that of Firmicutes decreased by about 10%. The abundance of *Deferribacteres* was significantly lower than that of the Sed db/db-R group (Fig. 4D). Additionally, 38 genera exhibited significant shifts, with many remaining unclassified. Among the identified genera, 11 specific taxa exhibited notable shifts: *Acutalibacter, Afipia, Alistipes, Anaerotruncus, Bacteroidales_unclassified, Duncaniella, Faecalicatena, Limosilactobacillus, Mucispirillum, Rhizobiales_unclassified,* and *Roseburia*. Specifically, *Bacteroidales_unclassified, Duncaniella, Limosilactobacillus,* and *Roseburia* were significantly enriched, while the others, including *Acutalibacter*, *Anaerotruncus*, *Mucispirillum*, and *Faecalicatena,* were decreased (Fig. 4E).

In the Ex db/m$^+$-R group, at the phylum level, only *Deferribacteres* showed a significant decrease in abundance, while that of *Firmicutes* increased slightly (Fig. 4D). Additionally, there were 12 classified taxa: *Afipia, Anaerotruncus, Candidatus Arthromitus, Clostridiaceae_unclassified, Desulfovibrionaceae_unclassified, Emergencia, Faecalicatena, Lachnospiraceae_unclassified, Mucispirillum, Rhizobiales_unclassified, Rikenellaceae_unclassified,* and *Roseburia*. Among these, *Lachnospiraceae_unclassified, Clostridiaceae_unclassified,* and *Roseburia* were increased, while others, including *Rikenellaceae_unclassified, Candidatus Arthromitus, Anaerotruncus, Mucispirillum,* and *Faecalicatena,* were decreased (Fig. 4F).

In the Sed db/m$^+$-R group, the abundance of *Tenericutes* was significantly decreased when compared to the Sed db/db-R group (Fig. 4D). At the genus level, 46 genera exhibited significant changes, with 15 specific genera identified: *Acetatifactor, Afipia, Candidatus Arthromitus, Clostridia_unclassified, Clostridiaceae_unclassified, Desulfovibrionaceae_unclassified, Dorea, Duncaniella, Eggerthellaceae_unclassified, Faecalicatena, Lachnospiraceae_unclassified, Limosilactobacillus, Prevotella, Rhizobiales_unclassified,* and *Roseburia*. Among these, *Lachnospiraceae_unclassified, Duncaniella, Limosilactobacillus, Prevotella*, *Clostridiaceae_unclassified, Roseburia, Desulfovibrionaceae_unclassified, Acetatifactor, Dorea*, and *Clostridia_unclassified* were enriched, while others, including *Candidatus Arthromitus* and *Faecalicatena,* were decreased (Fig. 4G).

Importantly, certain genera displayed consistent trends across multiple comparisons. Specifically, *Roseburia* was consistently enriched while *Faecalicatena* were decreased in the Ex db/db-R, Ex db/m$^+$-R, and Sed db/m$^+$-R groups. *Lachnospiraceae_unclassified* was consistently increased in both the Sed db/m$^+$-R and Ex db/m$^+$-R groups (both lean donors), while *Mucispirillum* was consistently decreased in both the Ex db/db-R and Ex db/m$^+$-R groups (both exercised donors) (Fig. 4E−4G). These findings underscore the distinct microbial shifts induced by FMT of different donor profiles, illustrating both the diversity of responses in sedentary diabetic mice and the stable alteration of specific microbial taxa across different FMT groups. The enriched diversity of gut microbiota and increased abundance of SCFA-producing genera, including *Roseburia*[10] and *Lachnospiraceae,*[26] suggests a partially similar microbial shift as that induced by exercise training in recipient mice.

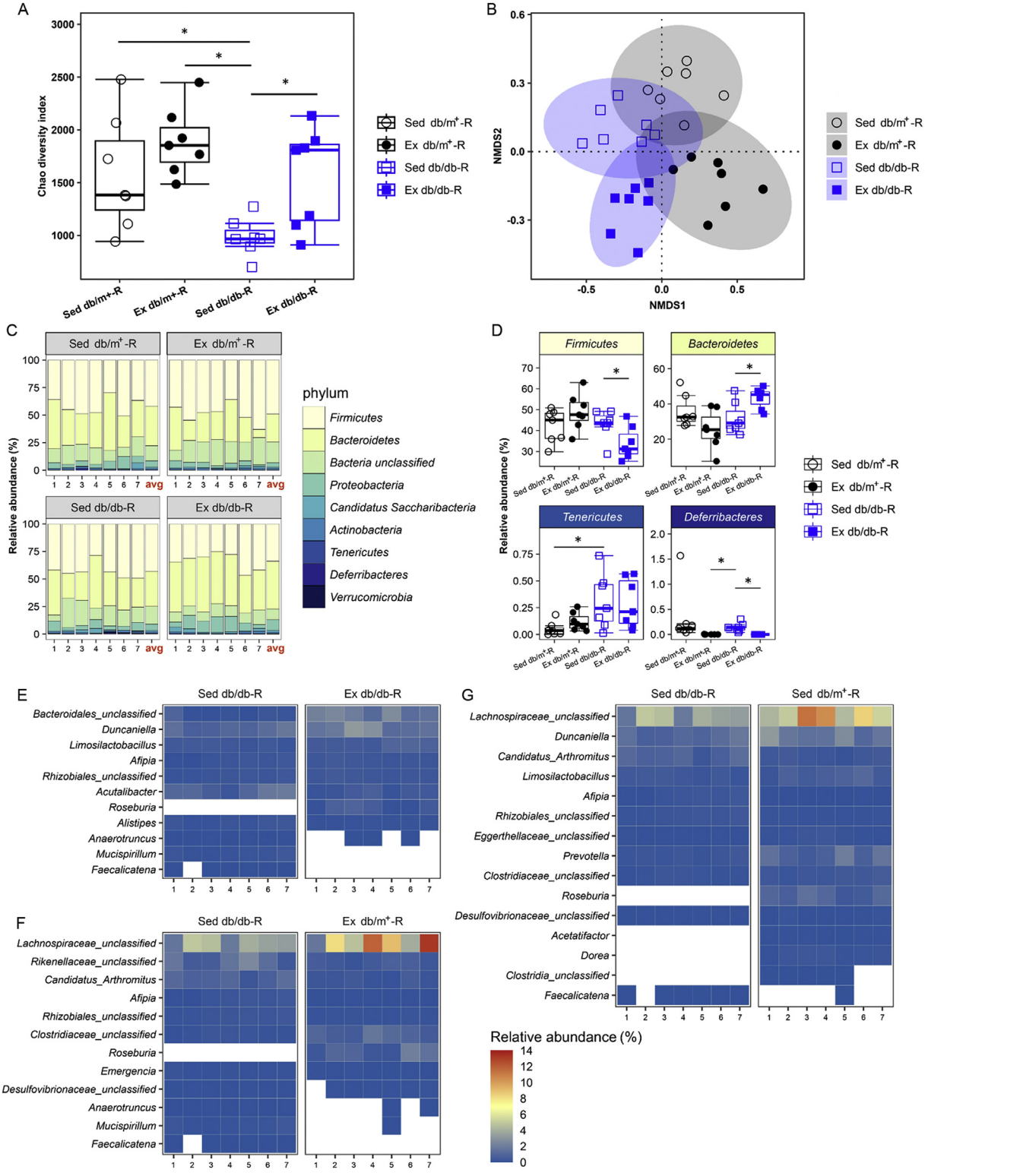

Fig. 4. Effects of exercise-associated FMT on gut microbial diversity and community structure. (A) Chao diversity index of microbial diversity in recipient sedentary diabetic mice (Sed db/db) receiving FMT from different donor mice, resulting in Sed db/m$^+$-R, Ex db/m$^+$-R, Sed db/db-R, and Ex db/db-R groups. Data are mean ± SD. * $p < 0.05$ (Brown-Forsythe and Welch analysis of variance, and Dunnett T3 test). (B) NMDS plot showing the microbial community structure across the 4 groups. Shading: 5% confidence intervals for each group. (C) Relative abundance plots at phylum levels for each group sample and the mean value for each group. The phyla represented include: *Firmicutes, Bacteroidetes, Proteobacteria, Candidatus Saccharibacteria, Actinobacteria, Tenericutes, Deferribacteres,* and *Verrucomicrobia*. (D) Box plots illustrating the significant differences at phylum levels among mouse groups. * $p < 0.05$ (Brown-Forsythe and Welch analysis of variance, and Dunnett T3 test). (E−G) Heatmaps highlighting the relative abundance of significantly different bacterial genera in different groups of recipient mice. $n = 7$ per group. FMT = fecal microbiota transplantation; NMDS = non-metric multidimensional scaling.

3.5. Exercised microbiota mitigates intestinal stresses in recipient mice

Since the intestine of the digestive system is one of the primary organs that harbors the exercised microbiota from exercised donors, we postulated that the exercised microbiota would elicit beneficial effects on the intestines of the recipient mice before bringing benefits to the vasculature through gut−vascular communication. We suspected that exercised microbiota could reduce intestinal stresses, like inflammation, oxidative stress, and ER stress. RNA sequencing analysis on intestines from the Sed db/db-R and Ex db/db-R groups, along with corresponding KEGG pathway enrichment analysis, revealed enriched pathways of upregulated and downregulated genes, which are important to the regulation of nutrient metabolism (e.g., fatty acid biosynthesis, and fat digestion and absorption), inflammation (e.g., cytokine−cytokine receptor interaction and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway), and ER stress (e.g., protein processing in the endoplasmic reticulum) (Fig. 5A and 5B).

We further found that chronic exercise (Ex db/m$^+$ and Ex db/db groups) and FMT from exercised donors (Ex db/m$^+$-R and Ex db/db-R groups) suppressed expression of inflammation markers in mouse intestines, although to different extents (Fig. 5C and Supplementary Fig. 8A). Notably, chronic exercise and exercise-associated FMT (Ex db/m$^+$-R and Ex db/db-R groups) downregulated expression of ER stress markers in mouse intestines, though to different extents (Fig. 5D and Supplementary Fig. 8B). In contrast, chronic exercise did not reduce intestinal oxidative stress or NOX activity in donor mice, although lower intestinal ROS levels were observed in db/m$^+$ mice compared to db/db mice (Supplementary Fig. 8C and 8D). Likewise, recipient mice undergoing FMT from db/m$^+$ mice showed lower intestinal oxidative stress and NOX activity than those transplanted from db/db mice (Fig. 5E and 5F), suggesting that exercised microbiota did not exert an ROS-lowering effect on the intestine. Similar downregulation trends of inflammation and ER stress markers were observed in the intestines of donor and recipient mice, where an ER stress-inflammation loop often aggravates intestinal pathogenesis.[27] These findings imply differential regulation mechanisms underlying oxidative stress, ER stress, and inflammation between the vasculature and intestine.

One of the most highly expressed members of the miR-181 family in human and mouse intestines is miR-181b, which is protective against intestinal inflammation.[28] We therefore measured the miR-181b expression in intestines of donor and recipient mice. Importantly, chronic exercise, and exercise-associated FMT elevated intestinal miR-181b levels to different extents (Fig. 5G and Supplementary Fig. 8E), consistent with the downregulation trend observed in the expression of inflammation markers (Fig. 5C and Supplementary Fig. 8A). These findings indicate that exercised microbiota might elicit an anti-inflammatory effect on the intestine partially through miR-181b regulation.

3.6. Exercised microbiota suppresses endotoxemia and systemic inflammation in recipient mice

Diabetes is associated with chronic systemic inflammation and elevated circulating levels of pro-inflammatory cytokines,[29] which aggravate vascular inflammation. We therefore measured circulating levels of inflammatory cytokines in diabetic recipient mice by Luminex multiplex immunoassay. Compared to the Sed db/db-R group, the Sed db/m$^+$-R, Ex db/m$^+$-R, and Ex db/db-R groups demonstrated lower levels of multiple inflammatory cytokines to different extents (Fig. 6A and Supplementary Fig. 9), hinting at the inhibitory effect of exercised microbiota on systemic inflammation in diabetic hosts.

Since diabetes is often associated with endotoxemia, translocation of endotoxin (bacterial lipopolysaccharide (LPS)) into host circulation, and impaired intestinal barrier integrity,[30] we wondered whether exercised microbiota could reduce endotoxemia and enhance intestinal integrity. We observed that both chronic exercise and FMT from exercised donors (Ex db/m$^+$-R and Ex db/db-R groups) significantly reduced the fecal and serum levels of endotoxin as well as the serum level of LBP (Fig. 6B−6D and Supplementary Fig. 10A−10C), another marker of bacterial endotoxemia, indicating suppressed endotoxemia. We evaluated intestinal permeability by measuring circulating levels of intestinal fatty acid-binding protein (I-FABP), the surrogate biomarker of elevated intestinal permeability.[31] Lower serum I-FABP levels suggest improved intestinal integrity upon chronic exercise and exercise-associated FMT (Fig. 6E and Supplementary Fig. 10D).

Recognized by toll-like receptor 4 (TLR4) on the endothelial cell surface, bacterial LPS promotes endothelial dysfunction and vascular inflammation, and modulates endothelial oxidative stress through the TLR4/NOX pathway.[32] In particular, NOX1 and NOX4, 2 NOX isoforms, are downstream of TLR4 activation in endothelial cells.[32,33] Consistent with reduced endotoxemia, chronic exercise and exercise-associated FMT reduced aortic expression of *Tlr4*, *Nox1*, and *Nox4* to varying degrees (Supplementary Fig. 11). Hence, exercise and exercised microbiota are likely to lower vascular ROS production by inhibiting NOX expression and activity (Fig. 3B and Supplementary Fig. 11). Consistent with the results on circulating I-FABP levels, exercise-associated FMT enhanced the expression of tight junction proteins, including claudin-1 and occludin,[34] in the intestines of recipient mice (Supplementary Fig. 12), indicating improved intestinal integrity. Taken together, enhanced intestinal integrity limits the entry of harmful factors, including bacterial LPS, into host circulation to alleviate endothelial dysfunction.

3.7. Exercised gut microbiota alter gut-derived and blood-borne factors in recipient mice

Since microbial alterations often induce shifts in pools of metabolites, peptides, and cytokines in host circulation to affect host vasculature,[8] we hypothesized that exercised gut microbiota might boost certain gut-derived and blood-borne factors beneficial to host vasculature and metabolism. As a

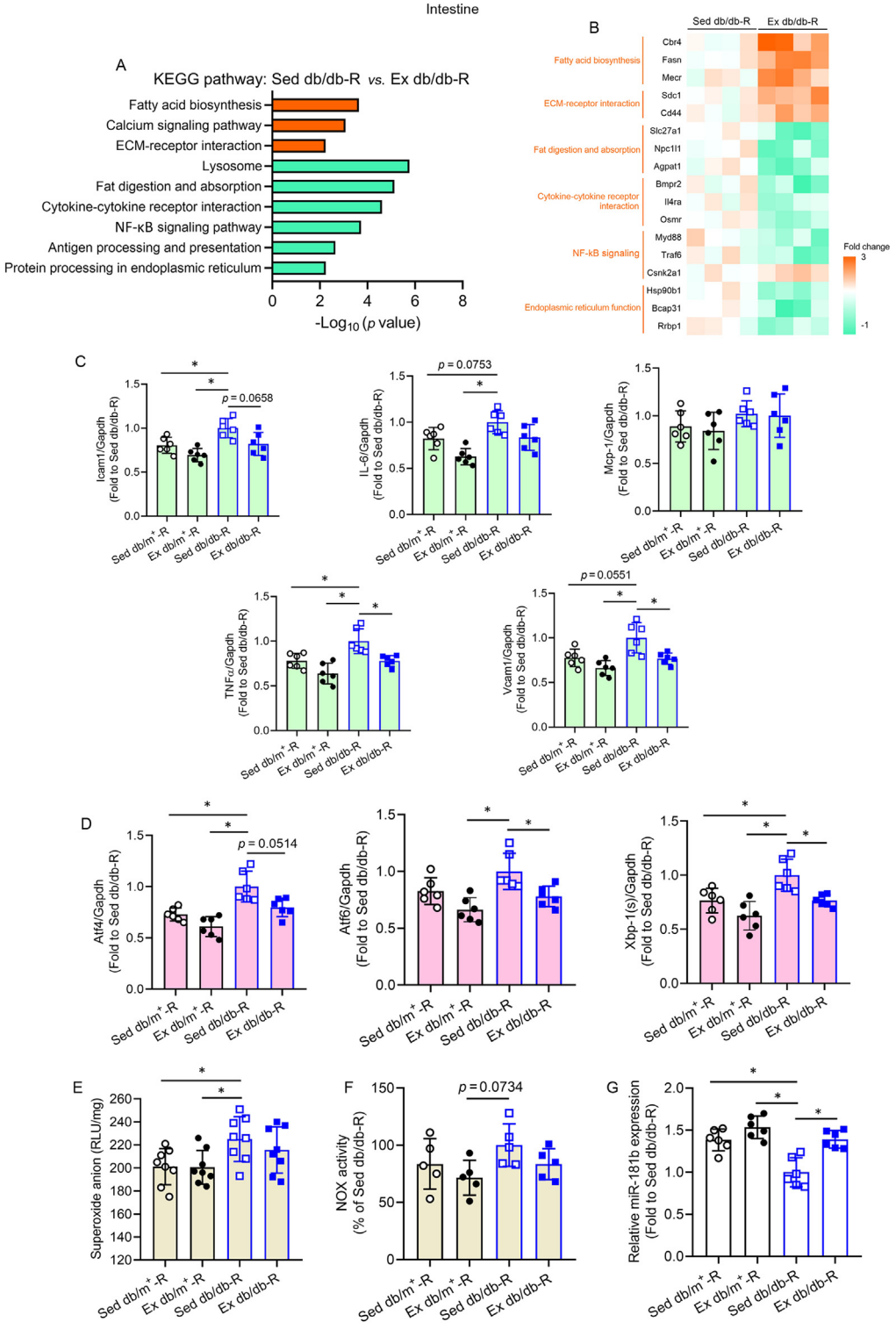

Fig. 5. Effects of exercised microbiota on intestinal stresses of receipt mice. (A) Enriched KEGG pathways for upregulated and downregulated genes in intestines from Sed db/db-R and Ex db/db-R groups. (B) Heatmap showing differentially expressed genes related to the regulation of intestinal metabolism, inflammation, and ER stress between Sed db/db-R and Ex db/db-R groups. $n = 4$ per group. RT-PCT on mRNA levels of (C) pro-inflammatory genes and (D) ER stress markers in intestines of recipient mice. $n = 6$ per group. (E) Lucigenin-enhanced chemiluminescence on ROS levels in intestines of recipient mice. $n = 8$ per group. (F) NOX activity assay in intestines of recipient mice. $n = 5$ per group. (G) RT-PCT on relative miR-181b expression in intestines of recipient mice. $n = 6$ per group. Data are mean ± SD. * $p < 0.05$ (Brown-Forsythe and Welch analysis of variance, and Dunnett T3 test). Atf4 = activating transcription factor 4; Atf6 = activating transcription factor 6; ECM = extracellular matrix; ER = endoplasmic reticulum; E-selectin = endothelial selectin; Icam1 = intercellular adhesion molecule 1; IL-6 = interleukin-6; KEGG = Kyoto Encyclopedia of Genes and Genomes; miRNA-181b = microRNA-181b; NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells; NOX = nicotinamide adenine dinucleotide phosphate oxidase; RLU = relative light unit; TNFα = tumor necrosis factor-alpha; Vcam1 = vascular cell adhesion molecule 1; Xbp-1(s) = X-box Binding Protein 1 (spliced form).

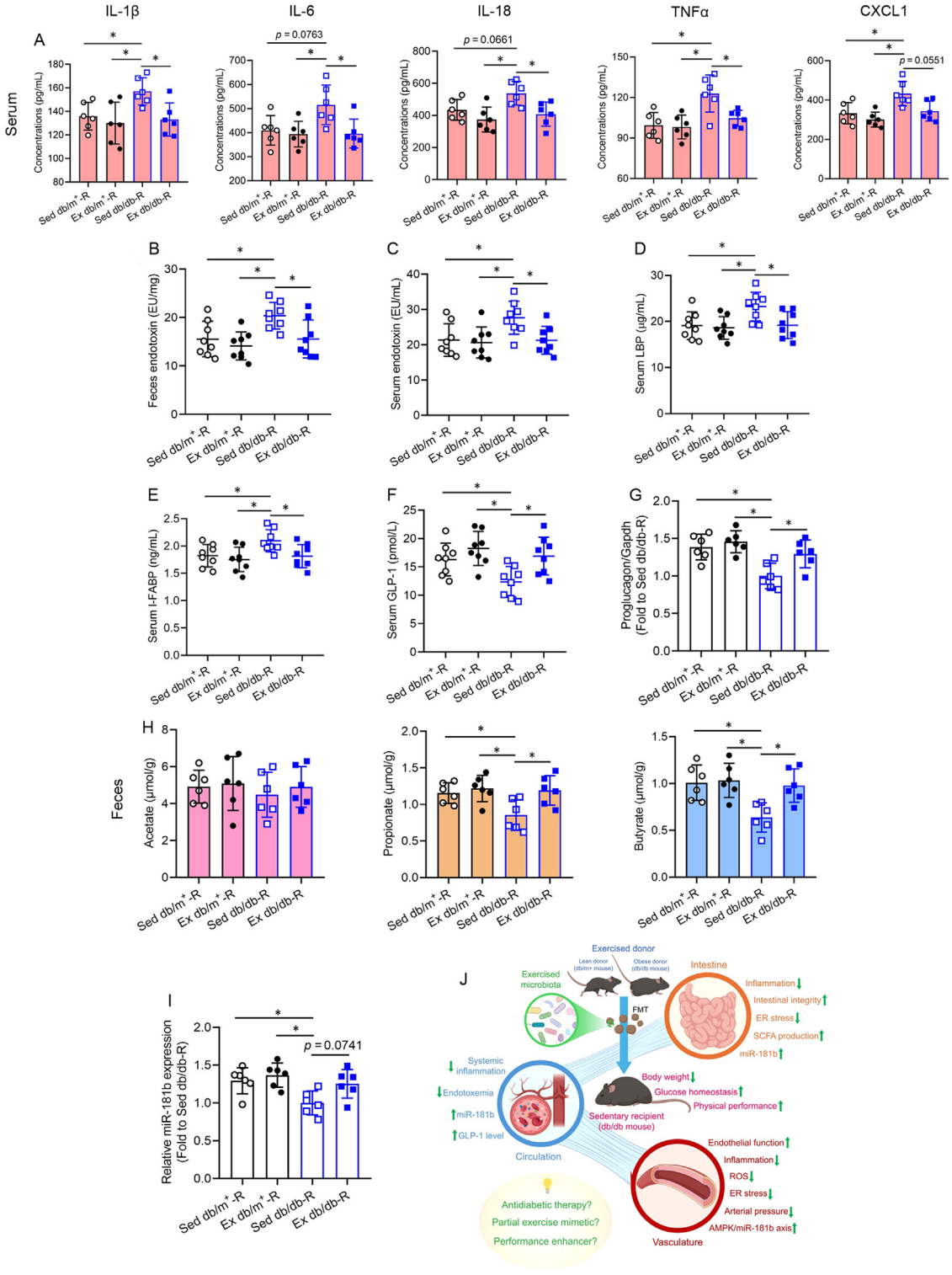

Fig. 6. Effects of exercised microbiota on gut-derived and blood-borne factors of receipt mice. (A) Luminex multiplex immunoassay showing the levels of inflammatory cytokines and chemokines in sera of recipient mice. $n = 6$ per group. Endotoxin levels in (B) feces and (C) sera of recipient mice. ELISA on (D) LBP, (E) I-FABP, and (F) GLP-1 levels in sera of recipient mice. $n = 8$ per group. (G) Proglucagon mRNA level in intestines of recipient mice. (H) Fecal concentrations of SCFAs—namely acetate, propionate, and butyrate—of recipient mice. (I) RT-PCT on relative miR-181b expression in sera of recipient mice. $n = 6$ per group. (J) Schematic diagram highlighting the potential benefits, mechanism, and insights of exercised microbiota on diabetic mice through the gut-vascular connection. Data are mean ± SD. * $p < 0.05$ (Brown-Forsythe and Welch analysis of variance, and Dunnett T3 test). AMPK = adenosine monophosphate-activated protein kinase; CXCL1 = C-X-C motif chemokine ligand 1; ELISA = enzyme-linked immunosorbent assay; ER = endoplasmic reticulum; EU = endotoxin unit; GLP-1 = glucagon-like peptide-1; I-FABP = intestinal fatty-acid binding protein; IL = interleukin; LBP = LPS-binding protein; miR-181b = microRNA-181b; ROS = reactive oxygen species; SCFA = short-chain fatty acid; TNFα = tumor necrosis factor-alpha.

gut-derived peptide, glucagon-like peptide-1 (GLP-1) is critical to glucose homeostasis and confers cardiovascular protection by improving endothelial function and lowering blood pressure.[35] Previous studies revealed that exercise (acute, moderate, and high-intensity) can raise GLP-1 levels in lean and obese individuals.[36] In addition to chronic exercise in donor mice, FMT from exercised donors (Ex db/m$^+$-R and Ex db/db-R groups) significantly increased circulating GLP-1 levels in diabetic mice (Fig. 6F and Supplementary Fig. 13A). The upregulated expression of intestinal proglucagon, the GLP-1 precursor, upon exercise and exercise-associated FMT in donor and recipient mice correlated to increased circulating GLP-1 levels (Fig. 6G and Supplementary Fig. 13B).

Gut-derived SCFAs stimulate intestinal GLP-1 secretion and are rapidly absorbed into the host's bloodstream to modulate host cardiometabolic homeostasis.[37] Diabetes downregulates intestinal SCFA production, while exercise upregulates it.[38] Chronic exercise consistently increased fecal and serum levels of SCFAs (namely acetate, propionate, and butyrate) in donor mice (Supplementary Fig. 13C and 13D). However, FMT from exercised donors only significantly upregulated fecal and serum levels of propionate and butyrate in recipient mice (Fig. 6H and Supplementary Fig. 14); note that these 2 SCFAs are found to be protective against endothelial dysfunction.[39] The increased SCFA production was consistent with the increased abundance of SCFA-producing bacterial genera (i.e., *Roseburia* and *Lachnospiraceae*) (Fig. 4E−4G) and enriched fatty acid biosynthesis pathway in the intestine, as shown by RNA sequencing analysis (Fig. 5A).

In vitro SCFA treatment at 5−10 mmol/L was previously shown to increase the expression of G protein-coupled receptors (GRR) GPR41 and GPR43 in rat endothelial cells.[39] However, our *in vivo* data suggests that chronic exercise slightly increased aortic levels of *Gpr41* and *Gpr43*, while only the Ex db/m$^+$-R group of the FMT experiment slightly increased aortic *Gpr41* levels ($p = 0.0875$; Supplementary Fig. 15). These results indicate that exercise-induced increases in SCFA levels might not be sufficient to significantly upregulate aortic *Gpr41* and *Gpr43*. Butyrate was shown to increase aortic miR-181b expression to improve endothelial function by upregulating peroxisome proliferator-activated receptor δ (PPARδ).[40] Notably, both chronic exercise and exercise-associated FMT increased aortic *Pparδ* expression (Supplementary Fig. 16), consistent with the higher circulating butyrate levels (Supplementary Figs. 13D and 14).

In addition to vascular and intestinal miR-181b levels, we also measured the levels of circulating miR-181b, which is a potential indicator of cardiometabolic health. Chronic exercise increased circulating miR-181b levels in donor mice (Supplementary Fig. 17), while only the Ex db/m$^+$-R and Sed db/m$^+$-R groups showed significant upregulation in circulating miR-181b levels (Fig. 6I), implying that FMT from lean donors might have greater cardiometabolic benefit.

4. Discussion

In the current study, we provided evidence that transplantation of exercised microbiota to sedentary diabetic mice induced metabolic and vascular improvements through the gut–vascular connection (Fig. 6J). Exercised microbiota confers certain cardiometabolic benefits of exercise. Exercise-associated FMT improved glucose homeostasis, physical performance, and endothelial function in sedentary diabetic mice. Furthermore, exercised microbiota mitigated vascular and intestinal stresses and induced microbial shifts in diabetic mice. Additionally, exercised microbiota enhanced intestinal integrity, reducing the entry of harmful factors, like endotoxin, to host circulation as well as systemic inflammation in diabetic mice. Exercised microbiota also enriches beneficial factors like GLP-1, SCFAs, and miR-181b in hosts to bring about cardiometabolic benefits. Notably, exercised microbiota from lean donors elucidated higher therapeutic efficacy on cardiometabolic abnormalities during diabetes.

FMT from different donors were shown to elicit beneficial effects on host metabolism and vasculature to different extents in the following order: Ex db/m$^+$-*R* > Sed db/m$^+$-*R* > Ex db/db-R. FMT from sedentary lean donors (Sed db/m$^+$ mice) could already mitigate cardiometabolic abnormalities in diabetic mice, consistent with previous reports that lean-to-obese FMT improved insulin sensitivity in human recipients with metabolic syndrome.[41] The slightly higher amount of food intake in exercised db/db mice might arise from compensatory eating after exercise.[42] Notably, exercise-associated FMT did not increase food intake amount in recipient mice, implying that exercised microbiota theoretically does not cause compensatory eating since recipient mice did not actually perform exercise.

Exercise can increase blood flow, which exerts shear stress on vascular endothelial cells to improve vascular function through activation of the AMPK/miR-181b axis.[11] Laminar flow can upregulate aortic UCP2 level,[18] and chronic exercise was previously shown to activate the aortic AMPK/UCP2 axis in old rats.[43] However, we showed that exercise-associated FMT, even without increasing blood flow in recipient mice, could still enhance vascular function in different arteries, lower arterial blood pressure, and suppress vascular stresses. Exercise-associated FMT was also shown to activate the AMPK/miR-181b and AMPK/UCP2 axes in vasculature. These findings implied that exercised microbiota might enhance endothelial function through an indirect and blood flow-independent mechanism, like altering gut-derived and blood-borne factors where these factors are potentially responsible for activating the signaling axes. The decreased levels of endotoxin, glucose, and pro-inflammatory cytokines, and increased levels of GLP-1 and miR-181b in circulation upon exercise-associated FMT might account for the activated AMPK/miR-181b and AMPK/UCP2 signaling and the improved vascular function. Future extensive study is needed to identify more exercised microbiota-related critical factors and immunomodulatory mechanisms contributing to cardiometabolic health.

Although chronic exercise and exercise-associated FMT suppressed ROS level in vasculature, they did not significantly suppress nor promote oxidative stress in intestines. Intensity and duration of exercise correspond to differential ROS-modulating capacities. Acute exercise induces oxidative stress in the

intestine to cause intestinal damage, while chronic regular exercise is associated with antioxidant defense adaptations for redox balance to maintain intestinal integrity,[44] which does not conflict with our findings that chronic exercise and exercise-associated FMT improved intestinal integrity in diabetic mice. Oxidative stress has been implicated in diabetic intestines.[45] We cannot exclude the possibility that the 8-week exercise protocol and FMT were not sufficient to reduce intestinal ROS in diabetic mice to nondiabetic levels. Conversely, intestinal ER stress and inflammation were significantly suppressed upon chronic exercise and exercise-associated FMT, hinting at differential modulation mechanisms among oxidative stress, ER stress, and inflammation between vasculature and intestine.

Previously, miR-181b was revealed to protect against intestinal inflammation upon epithelial injury.[28] We firstly provided clues that chronic exercise and exercised microbiota could upregulate intestinal miR-181b, though the detailed mechanism remains elusive. As a mechanosensitive miRNA, whether intestinal miR-181b can be upregulated by shear stress exerted by feces during exercise-induced intestinal motility remains unclear. Besides, it is reasonable to postulate that certain exercised microbiota-derived factors might upregulate miR-181b in a shear stress-independent manner. Clinically, downregulated miR-181b levels were noted in intestines of patients with inflammatory bowel disease (IBD).[28] The suppressed intestinal inflammation might also account for miR-181b upregulation.

Diabetes is associated with dysbiosis and reduced intestinal integrity.[30] Our results showed that exercise-associated FMT enriched microbial diversity and increased abundance of SCFA-producing bacterial genera, namely *Roseburia* and *Lachnospiraceae*, which is consistent with trends observed in previous human[10] and mouse studies[46,47] on microbial shifts after exercise training. However, depending on the host species, genetic and disease backgrounds, and treatment conditions (e.g., distinct exercise training modalities), the enriched microbial genera and species post-exercise can be largely distinct. Despite different subsets of SCFA-producing bacteria being enriched, butyrate-producing *Roseburia* has been found consistently enriched in this study and in previous human[48] and mouse studies,[46,47] suggesting a potentially critical role of *Roseburia* in mediating exercise-induced effects. Future extensive efforts are warranted to integrate exercise-induced microbial alterations under different exercise training modalities in human and animal models.

We showed that both chronic exercise and exercised microbiota reduced intestinal permeability and retarded endotoxemia to counteract systemic inflammation and diabetic vascular dysfunction. The suppressed intestinal inflammation due to exercise-induced microbial alteration might partially contribute to improved intestinal integrity.[49] Reduced endotoxemia potentially attenuates endothelial dysfunction and vascular inflammation through the TLR4/NOX axis. Exercise can enrich SCFAs in diabetic mice.[38] However, our results showed that transplantation of exercised microbiota only significantly upregulated propionate and butyrate levels, but not acetate levels, indicating that exercise-associated FMT could not fully mimic the benefits of exercise. We cannot rule out the possibility that prolonged FMT would further promote SCFA enrichment.

Propionate itself can enhance endothelial function by activating GPR41[50] and by stimulating intestinal GLP-1 secretion.[51] Butyrate can upregulate aortic miR-181b expression to improve endothelial function through PPARδ.[40] The present results imply a PPARδ/miR-181b axis in aortas induced by circulating butyrate. PPARδ is known to attenuate ER stress in the vasculature.[24] The higher *Pparδ* level might partially account for the ER stress-lowering effects in aortas after exercise and FMT. The increased butyrate level might contribute to improved intestinal integrity since butyrate was shown to upregulate the tight junction protein, claudin-1.[34] Whether butyrate can increase the intestinal miR-181b level by a similar mechanism remains unclear. The upregulated circulating miR-181b might represent another indicator of cardiometabolic health, but the source of increased circulating miR-181b, whether it originates from the vasculature or intestine after exercise and FMT, requires further exploration.

Notably, this study has limitations. Only male mice were used for this study, so the influence of gender on exercise-associated microbial alterations was not addressed. The enduring effect of exercised microbiota after halting the FMT protocol was not studied. In addition, this study did not evaluate the effect of exercise-associated FMT on bile acid metabolism. Future studies on the effects of exercised microbiota on recipient mice of different genetic and disease backgrounds (e.g., nondiabetic db/m^{+} mice) and treatment conditions (e.g., different exercise training modalities) shall further extend the beneficial mechanism of exercise. The duration and frequency of FMT protocol were chosen based on our previous study.[52] We cannot exclude the possibility that altering this FMT protocol, like lengthening the duration or increasing the frequency, might improve or worsen the beneficial effect of exercise-associated FMT.

Overall, our results suggested that exercised microbiota may be a partial exercise mimetic to promote host cardiometabolic health, and it is particularly suitable for disabled or diseased individuals who cannot regularly perform exercise. Future studies shall be conducted to uncover more benefits of exercised microbiota on other organ systems and diseases. For instance, whether exercised microbiota activates the gut-brain axis and alleviates IBD requires future investigation. Future efforts are needed to optimize the therapeutic efficacy of exercised microbiota. Differential cardiometabolic effects were observed upon FMT from different donors in this study, hinting that rigorous selection of potential donors might further boost the therapeutic efficacy of exercised microbiota. Whether exercised microbiota brings about similar cardiometabolic benefits in sedentary humans, and whether transplantation of exercised microbiota to regularly exercised humans could further enhance exercise performance are questions that require future study. Despite the proof-of-concept nature of this study, there is still a long way to go before we move exercised microbiota from bench to bedside.

5. Conclusion

The current study demonstrates that gut microbiota from exercise-trained donors can improve vascular and metabolic abnormalities in sedentary diabetic mice through the gut−vascular connection, thereby extending the mechanism of exercise training and suggesting exercised microbiota as a partial exercise mimetic. Exercised microbiota attenuates damage and stresses in intestinal tissues and alter gut-derived and blood-borne factors to exert vascular and metabolic benefits. Our findings open up new therapeutic opportunities against cardiometabolic diseases by targeting the gut−vascular connection.

Authors' contributions

CKC designed the study, carried out animal and molecular experiments, performed the statistical analysis, acquired funding, and drafted the manuscript; LY performed metagenomic and statistical analysis and drafted the manuscript; YW and YLW carried out animal experiments; YX provided resources to the study; SHSW and SC provided critical comments and revised the manuscript; YH supervised the study, acquired funding, participated in coordination, and revised the manuscript. All authors have read and approved the final version of the manuscript, and agree with the order of presentation of the authors.

Competing interests

The authors declare that they have no competing interests.

Data statement

The data that support the findings of the study are available from the corresponding author upon reasonable request.

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Supplementary materials

Supplementary materials associated with this article can be found in the online version at doi:10.1016/j.jshs.2025.101026.

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