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# Fecal microbiota transplantation from blueberry and blackberry anthocyanins-supplemented mice ameliorated metabolic syndrome by regulating gut microbiota in a high-fat diet model

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ABSTRACT

Blueberry anthocyanins (VA) and blackberry anthocyanins (RA) showed benefits on metabolic syndrome (MS) induced by high-fat diet (HFD) in mice. In this study, we investigated whether the therapeutic effects of VA and RA were achieved by the gut microbiota regulation and whether these effects could be replicated through fecal microbiota transplantation (FMT) using the HFD caused MS model in pseudo-germ-free mice. The results demonstrated that the beneficial effects of VA and RA on MS, including reducing body weight gain and fat accumulation, improving glucose and lipid metabolism, and mitigating intestinal barrier damage, were attributed to the gut microbiota and could be replicated by FMT. 16S rRNA sequencing analysis suggested that FMT from donor mice supplemented with VA and RA could regulate the gut microbiota composition. Particularly, FMT from RA supplemented mice displayed the potential to restore the diversity of gut microbiota and the ratio of Firmicutes to Bacteroidetes. Meanwhile, FMT from VA supplemented mice appeared to exert its effects by selectively influencing specific gut microbiota, such as the genus *Akkermansia.* Furthermore, our analysis identified 10 common differential amplicon sequence variants (ASVs) among groups compared to HFD-HFD group. Notably, ASV_36450 was negatively associated with metabolic parameters, suggesting that *Lactobacillus* might be the potential bacteria in regulating MS. Overall, our study demonstrated that FMT from VA and RA supplemented mice could ameliorate MS induced by HFD in mice through regulating specific gut microbiota.



## 1. Introduction

Metabolic syndrome (MS) is a disorder characterized by the simultaneous presence of several risk factors, including abdominal obesity, high blood pressure, high blood glucose and high triglycerides[1]. MS has been defined as a highly prevalent disease worldwide by various epidemic studies, which is highly related with obesity, type 2 diabetes and cardiovascular diseases[2-3]. The unhealthy western diet pattern, characterized by high calories, high fat, high sugar and low-fiber foods, has been proven to increase the risk of MS[4]. On the contrary, the healthy diet patterns rich in fruits, vegetables and high fiber have demonstrated preventive and therapeutic effects of MS[5]. With the continuous advancement of research, numerous interventions have been discovered to intervene in the progression of MS, such as medical treatment, lifestyle modification, diet or fecal

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microbiota transplantation (FMT)[6-8]. However, effective lifestyle changes require substantial resource investment, and medical interventions can be costly and may lead to adverse effects[9-10]. In comparison, FMT is an attractive approach because it offers significant therapeutic benefits with fewer side effects, personalization, and convenience[11-12].

Diet as a key approach to regulate or treat MS has been attracting increasing attentions in recent years. Among the various mechanisms involved, the modulation of the host's gut microbiota emerges as a vital factor in MS regulation and treatment. The gut microbiota, consisting of approximately 10–100 trillion microorganisms residing in the gastrointestinal tract, has been considered as a complex ecosystem[13]. The balance of gut microbiota is crucial for host health, as it plays key roles in digestion, energy expenditure, systemic immunity and so on[14-15]. Different dietary patterns can lead to different gut microbiota profiles and metabolic functions. For instance, evidence indicated that dietary patterns rich in fiber, and low in saturated fatty acids could lead to an increase in the proportion of Bacteroidetes and *Prevotella*, while reducing the level of Firmicutes in the gut[16]. Conversely, diets characterized by low fiber and high saturated fatty acids have been found to significantly increase the proportion of Firmicutes and Proteobacteria, while reducing the proportion of Bacteroidetes[17]. In addition, fruits rich in polyphenols, flavonoids and other nutrients, are also considered to be crucial for alleviating or treating MS[18]. In a recent study, Petersen et al.[19] explored the effects of strawberry consumption in diabetic mice, and found that supplementation with frozen strawberries could prevent diabetes in mice by increasing the abundance of *Bifidobacterium*. Another study revealed that gavaging obese mice induced by a high-fat, high-sucrose diet with cranberry extract could reduce weight gain and visceral obesity compared with the model group through regulating gut microbiota composition, especially by increasing the population of *Akkermansia* and *Oscillibacter*[20].

With the development of high-throughput technologies and ongoing research, our understanding of gut microbiota and its impact on host health has significantly deepened. FMT is a medical procedure that involves the transfer of gut microbiota from a healthy fecal sample (donor) into the intestines of another patient (recipient) to treat a range of conditions related to gut microbiota-associated diseases or imbalances[21]. The earliest application of FMT can be traced back to the 4$^{th}$ century when Chinese doctors used "yellow soup" to treat diarrhea[22]. In modern medical research, FMT has been successfully applied in the treatment of *Clostridium difficile* infection, which helps to restore the diversity of gut microbiota in patients and alleviate clinical symptoms[23]. In addition, FMT is also the subject of growing research into its use in the treatment of obesity and other metabolic diseases. Ridaura et al.[24] conducted a groundbreaking study in which they first transplanted human fecal samples into germ-free (GF) C57BL/6J mice. The results demonstrated that mice receiving fecal samples from the obese individual of twins significantly gained more body weight, confirming that the phenotype (weight gain) could be replicated through FMT. In animal studies, Wang et al.[25] found that FMT could lessen the rise in blood glucose, enhance glucose tolerance, and alleviate insulin resistance in diabetic mice induced by streptozotocin. In addition, dysbiosis can lead to an increased release of gram-negative bacteria and the production of large amounts of lipopolysaccharides, causing chronic inflammation in the body. Conversely, the transplantation of microbiota from healthy mice into diabetic mice can reduce the secretion of inflammatory factors[25-26]. In a recent study, epigallocatechin-3-gallate-FMT was proven to have the ability to improve inflammatory response and oxidative stress, thereby alleviating the colonic barrier damage[27].

However, conducting FMT in mice can be a complex process, and one of the considerations is the preparation of recipient mice. According to recent studies, recipient mice can be divided into specific pathogen-free (SPF) mice, GF mice and pseudo-germ-free (PGF) mice[28-29]. The PGF mouse model was created by treating mice with broad-spectrum antibiotics to eliminate a significant portion of the gut microbiota. Unlike SPF mice with a more natural microbial community and GF mice with a completely sterile status, the experimental results obtained using PGF mouse model were similar to those of GF mouse model but did not require the high cost of maintaining GF conditions[30-31]. Therefore, the use of PGF mice model has become a key approach in studying FMT.

In our previous study, we found that blueberry (*Vaccinium* ssp.) anthocyanins (VA) and blackberry (*Rubus* L.) anthocyanins (RA) could ameliorate body weight gain, impaired glycemic and lipid metabolism, and inflammatory status in C57BL/6J mice induced by high-fat diet (HFD)[32]. Additionally, they restored the imbalance in gut microbiota caused by the HFD, promoting microbial diversity and reducing the Firmicutes/Bacteroidetes (F/B) ratio in the mice. In the present study, to investigate whether the therapeutic effects of VA and RA on MS were mediated through the gut microbiota and look into whether these effects could be replicated through FMT, a HFD induced MS model and the PGF mice was utilized and FMT was performed.

## 2. Materials and methods

### 2.1 Donor mice

All animal experiments had been approved by the Committee for Animal Research of China Pharmaceutical University, and all procedures were conformed to the Guide for the Care and Use of Laboratory Animals. The donor mice were prepared according to our previous method[32]. Six-week-old male C57BL/6J mice were supplied by Huachuang Xinnuo Pharmaceutical Technology Co., Ltd. (Taizhou, China). Then the mice were kept with 4 mice per cage in standard laboratory conditions (12-h light/dark circle, 45%–75% relative humidity, (22 ± 2) ℃). After an adaptation period of 7 days, mice were randomly divided into 2 groups: chow diet (Chow) group ($n$ = 8) and HFD group ($n$ = 24). After 8 weeks, mice in HFD group were further divided into the following 3 subgroups: 1) HFD group (HFD + water); 2) HVA group (HFD + 100 mg/kg VA); 3) HRA group (HFD + 100 mg/kg RA). After the second round of grouping, the experiment continued for another 8 weeks. During this period, fresh fecal samples were collected daily from each mouse among groups for microbiota transplantation. The entire experimental process lasted for 16 weeks, during which all mice were given free access to food and water. All food was purchased from Junke Bioengineering Co., Ltd. (Nanjing, China).

### 2.2 Recipient mice and FMT

A total of 32 male C57BL/6J mice were purchased from the same company as the donor mice. The feeding conditions of recipient mice were kept the same as that of donor mice. After acclimation for

7 days, mice were randomly divided into 2 groups: 1) Chow-R group ($n$ = 8) fed with normal diet; 2) HFD-R group ($n$ = 24) fed with HFD. After 6 weeks, 4 antibiotics were added to the drinking water in the following proportions: 1 g/L ampicillin, 1 g/L neomycin sulfate, 1 g/L metronidazole, and 500 mg/L vancomycin. The antibiotics were refreshed every 2 days and administered for 2 weeks, then the water was substituted with distilled water[33-34]. Then all mice started receiving fecal transplantation at a dose of 200 μL bacterial suspension per mouse, the Chow-R group mice received fecal transplantation by oral gavage from donor Chow group mice and was named Chow-Chow group. The HFD-R mice were divided into 3 groups, each consisting of 8 mice, and received fecal transplantation by oral gavage from donor HFD group, HVA group, and HRA group mice, respectively, and were named HFD-HFD, HVA-HFD, and HRA-HFD groups, respectively. In this study, the FMT method was performed according to previous research with slight modifications[35]. In brief, freshly collected fecal samples were mixed with sterile phosphate-buffered saline (PBS) at a ratio of 100 mg feces per 1 mL PBS. The mixture was then vortexed for 2 min and centrifuged at 8 000 r/min for 5 min at 4 ℃. The supernatant was filtered through gauze to obtain the transplant material. The FMT process lasted for 8 weeks, and the entire experimental procedure is illustrated in Fig. 1A. During the period, food consumption and body weight were recorded weekly. After the experiments, samples were collected from each mouse, including varies various organs, adipose tissues and serum, for subsequent analysis. A portion of these samples was rapidly frozen in liquid nitrogen and kept at –80 ℃ before analysis, while the remaining samples were preserved in 4% formalin for histological analysis.

### 2.3 Chemicals

Fasting blood glucose (FBG), total cholesterol (TC), triacylglycerol (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), alanine transaminase (ALT) and aspartate transaminase (AST) kits were purchased from Nanjing Jiancheng Biological Engineering Institute (Nanjing, China). Superoxide dismutase (SOD), malondialdehyde (MDA), and enzyme-linked immunosorbent assay (ELISA) kits of fasting insulin (FIN), tumor necrosis factor-alpha (TNF-α) and interleukin 1 beta (IL-1β) were purchased from Beyotime Biotechnology (Haimen, China).

### 2.4 Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT)

In the last week, all mice were fasted for 12 h before OGTT, then orally gavaged with 2 g/kg glucose solution based on the body weight. After that, blood samples were collected from inner orbit using capillary tubes at 5 different time points (0, 30, 60, 90, and 120 min) to assess the level of blood glucose. After 3 days, ITT was conducted, following a similar procedure to OGTT. After 12 h fasting, a dose of 0.5 U/kg of insulin was injected to each mouse based on their body weight. Then the blood samples were collected by using capillary tubes at the same time intervals as OGTT to assess the level of blood glucose. All blood glucose levels during OGTT and ITT were measured by commercial kits according to the manufacturers' instructions purchased from Nanjing Jiancheng Biological Engineering Institute (Nanjing, China). Finally, the area under the curve (AUC) of OGTT/ITT was calculated by multiplying each blood glucose level by its corresponding time.

### 2.5 Biochemical analysis

Serum levels of FBG, TC, TG, LDL-C, HDL-C, SOD and MDA were measured by commercial kits according to the manufacturers' instructions.

### 2.6 ELISA analysis

The levels of TNF-α, IL-1β and FIN were assessed by ELISA kits, and the index of homeostasis model assessment of insulin resistance (HOMA-IR) and the index of homeostasis model assessment of insulin sensitivity (HOMA-IS) were calculated according to the previous study[25].

### 2.7 Histological analysis

The freshly collected adipose tissue samples and small intestine were fixed in 4% paraformaldehyde for 24 h. After fixation, the samples were dehydrated, embedded, and sectioned into 5-μm thick sections, which were then subjected to hematoxylin and eosin (H&E) staining. Then to assess the quantity of goblet cells in the small intestine, alcian blue and periodic acid-schiff (AB-PAS) staining was performed on the small intestinal tissues, where goblet cells were stained blue, followed by cell counting.

### 2.8 Oil red O (ORO) staining

The frozen liver slicers were stained with ORO working solution. Subsequently, the sections were rinsed with 60% isopropanol and stained with H&E for nucleus visualization. After washing with PBS, the sections were placed under a microscope (Olympus, Tokyo, Japan) for visualization and capturing.

### 2.9 DNA extraction, 16S rRNA gene sequencing and microbiota analysis

Total microbial genomic DNA of each fecal sample were extracted using the OMEGA Soil DNA Kit (M5635-02) (Omega Bio-Tek, Norcross, GA, USA) based on the manufacturer's instructions. Then the DNA quantity and quality were checked by NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively. Then the paired-end 2 × 250 bp sequencing was performed at Suzhou PANOMIX Biomedical Tech Co., Ltd. (Suzhou, China) using the Illlumina TruSeq Nano DNA LT Library Prep.

Raw data was performed with QIIME2 2019.4 with slight modifications following the official tutorials. In brief, the raw sequence was filtered, denoised, merged, and chimeric sequences were removed using the DATA2 plugin, followed by annotation of each amplicon sequence variant (ASV). QIIME2 was used to evaluate $\alpha$- and $\beta$-diversity. Subsequently, the principal co-ordinates analysis (PCoA) results were visualized using the vegan and ggplot2 packages in R software (version 4.3.0) (https://www. r-project.org/). Additionally,

the metagenomeSeq package was used to filter differentially abundant ASVs, and then the Spearman correlation analysis was performed using the psych and ComplexHeatmap packages to assess the correlations between the top 10 phyla and genera with metabolic indicators, as well as the correlations between differentially abundant ASVs and metabolic indicators. The results were visualized accordingly. Finally, the correlation network and Sankey diagrams were constructed using the OmicStudio tools athttps://www.omicstudio.cn/tool.

### *2.10 Statistical analysis*

All experimental data were analyzed using Graphpad Prism 9.0 software (Graphpad SoftwareInc., CA, USA) and presented as the mean ± standard error of the mean (SEM). Statistical significances were analyzed using ANOVA with Dunnett's test for more than 2 group. In all data analysis, $P < 0.05$ was considered to be statistically significant.

## 3. Results

### *3.1 FMT from VA and RA supplemented mice prevented obesity induced by HFD*

It has been proven that transplanting fecal microbiota from obese individuals into GF-mice could transfer the host's obese phenotype[36]. Conversely, transplanting fecal microbiota from lean individuals into mice showed potential to improve obesity and metabolic functions[37-38]. To identify whether FMT from mice in HVA and HRA groups have beneficial effects on MS, we conducted a comprehensive analysis of recipient PGF mice. Our horizontal fecal transfer results indicated that FMT from donor Chow mice to Chow-R mice effectively maintained the mice in a lean state. In contrast, HVA-HFD and HRA-HFD groups showed a significantly improved in obesity symptoms induced by HFD and reduced weight gain compared to HFD-HFD group, without affecting food intake (Figs. 1B-E). However, there were significant differences observed in white fat weight and brown fat weight among Chow-Chow, HFD-HFD, HVA-HFD, and HRA-HFD groups (Fig. 1F). Similarly, FMT from HVA group and HRA group prevented obesity induced by HFD as supported by significant decrease in white fat weight and increase in brown fat weight. Interestingly, the white fat weight in HRA-HFD group was lower than that in HVA-HFD group (Figs. 1F and G). In addition, compared to FMT from HFD group, histological analysis of white fat in HVA-HFD and HRA-HFD groups showed a reduction in the expansion of adipocytes induced by HFD, decreased the proportion of large-sized cells, and increased the proportion of small-sized cells (Figs. 1H and I). These results suggested that FMT from donor mice supplemented with VA and RA significantly improved obesity symptoms induced by HFD.

### *3.2 FMT from VA and RA supplemented mice improved glycemic and lipid metabolism induced by HFD*

Disorders in glycemic and lipid metabolism, such as impaired glucose tolerance, insulin resistance, abnormal levels of TC and TG, were important contributors in MS. These metabolic disturbances contribute to the development of cardiovascular diseases and other complications associated with MS[39-40]. To ascertain whether FMT from HVA and HRA groups had favorable effects on glycemic and

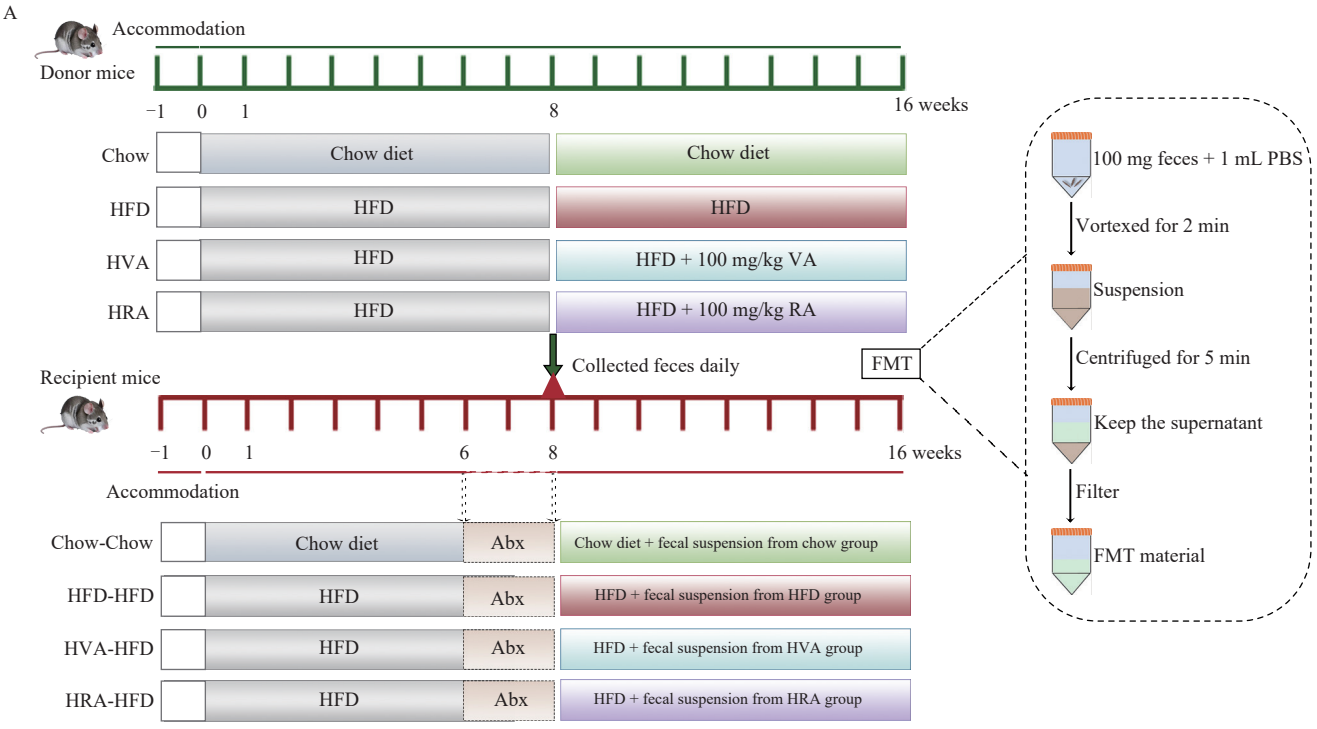


**Fig. 1** FMT from VA and RA supplemented mice prevented obesity induced by HFD. (A) Experimental design; (B) Body weight changes of 16 weeks; (C) Food intake; (D) Weight gain; (E) Morphological observations of body; (F) Organ weight; (G) Morphological observations of white fat; (H) H&E staining of white fat; (I) Adipocyte size distribution. Data are expressed as means ± SEM, $n = 8$. $^{\#}$Significant difference between Chow-Chow and HFD-HFD groups ($^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$); *Significant difference between HFD-HFD and HVA-HFD or HRA-HFD groups (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

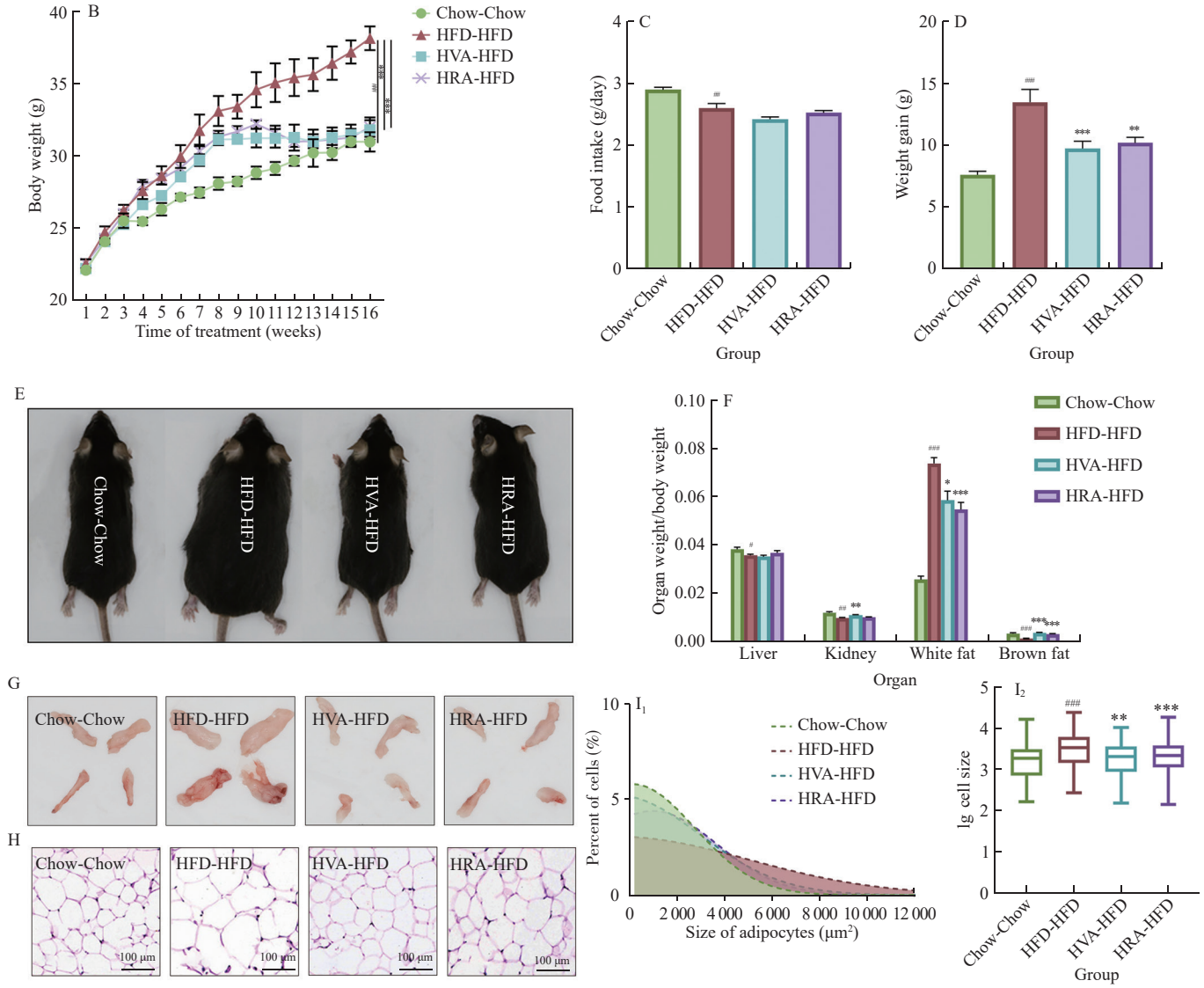


**Fig. 1** (*Continued*)

lipid metabolism, we further measured the related parameters. As shown in Fig. 2, HFD elevated the levels of FBG, FIN and HOMA-IR and decreased the level of HOMA-IS compared with Chow-Chow group. In contrast, FMT from HRA group could reverse the levels of all 4 parameters. Similarly, FMT from HVA group was able to reverse the levels of FBG, HOMA-IR and HOMA-IS, but had no significant effects on FIN (Figs. 2A-D). Furthermore, HFD could induce the impaired glucose tolerance and insulin resistance. However, mice in HVA-HFD and HRA-HFD groups showed lower AUC of OGTT and ITT compared to those in HFD-HFD group (Figs. 2E and F).

In terms of lipid metabolism, the levels of TC, TG and LDL-C in HFD-HFD group were significantly higher compared to those in Chow-Chow group. However, the level of HDL-C showed a totally different trend. FMT both from HVA and HRA groups resulted in decreased levels of TC and TG. Additionally, HVA-HFD group had no significant effects on LDL-C and HDL-C, whereas HRA-HFD group enhanced the level of HDL-C (Figs. 2G-J). In addition, the morphological observations of liver showed hepatomegaly and yellowing in HFD-HFD group, while the livers in HVA-HFD and HRA-HFD groups exhibited morphological characteristics similar to those in Chow-Chow group (Fig. 2K). This finding was further supported by ORO staining of liver samples, which demonstrated a reduction in the proportion of lipid droplets after FMT that both from HVA and HRA groups, similar to those observed in Chow-Chow group, in comparison to those in HFD-HFD group (Fig. 2L). Furthermore, the serum levels of ALT and AST were significantly increased in HFD-HFD group. However, FMT from HVA group could significantly reduce the elevated levels of ALT and AST induced by the HFD (Figs. 2M and N). Meanwhile, the level of AST was significantly decreased in HRA-HFD group, but the effect on ALT level was not significant (Figs. 2M and N). Results above suggested that FMT from donor mice supplemented with VA and RA had beneficial effects on disorders in glycemic metabolism, lipid metabolism and liver function induced by HFD. Moreover, the effects of FMT from HRA group were superior to those from HVA group.

### *3.3 FMT from VA and RA supplemented mice improved oxidative stress, inflammation and intestinal barrier damage induced by HFD*

Inflammation and oxidative stress exhibit a synergistic effect, as they can mutually enhance each other, influencing glycemic and lipid metabolism, thereby promoting the formation and development of metabolic disorders[41]. Additionally, oxidative stress and the release of pro-inflammatory cytokines can lead to barrier dysfunction[42]. Our findings revealed that in HFD-HFD group, both the levels of MDA and pro-inflammatory cytokines such as TNF-α and IL-1β were increased, whereas the SOD level was reduced. However, these trends were totally reversed in HRA-HFD group, while, HVA-HFD group did not show significant difference in levels of SOD, MDA and IL-1β (Figs. 3A-D). Furthermore, the H&E staining of intestines showed inflammatory cell infiltration and intestinal villi damage in HFD-HFD group, and FMT from HVA and HRA groups improved the symptoms induced by HFD. The ratio of villus height to crypt depth (V/C) was reduced by HFD, causing the loss of villus, whereas FMT from HVA and HRA groups reversed the downregulation trend with HRA-HFD group having a better effect. The AB-PAS staining showed distinct variations of goblet cells in the intestines. Specifically, HFD-HFD group showed a reduced number of goblet cells, while in contrast, HVA-HFD and HRA-HFD groups displayed a remarkable increase in the abundance of goblet cells (Figs. 3G and H). These results indicated that FMT from donor mice supplemented with VA and RA could potentially ameliorate oxidative stress, inflammation and intestinal barrier impairment induced by HFD. Furthermore, in these aspects, FMT from HRA group demonstrated superior effectiveness, consistent with our previous results[32].

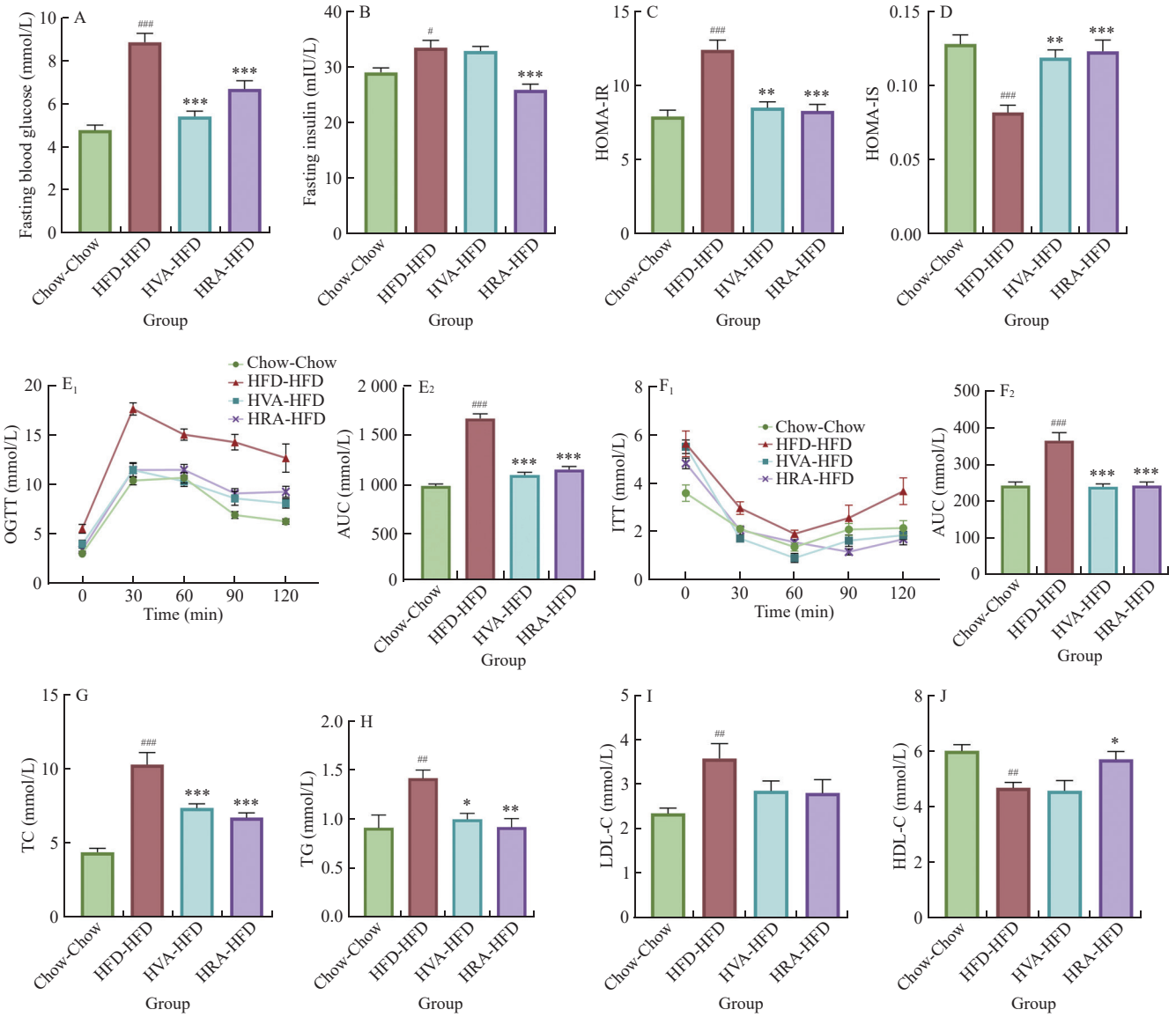


**Fig. 2** FMT from VA and RA supplemented mice improved glycemic and lipid metabolism induced by HFD. (A) FBG; (B) FIN; (C) HOMA-IR index; (D) HOMA-IS index; ($E_1$) OGTT and ($E_2$) AUC; ($F_1$) ITT and ($F_2$) AUC; (G) TC; (H) TG; (I) LDL-C; (J) HDL-C; (K) Morphological observations of liver; (L) Liver lipid content assessed using ORO staining; (M) ALT; (N) AST. Scale bar, 100 μm; Data are expressed as means ± SEM, $n = 8$. $^{\#}$ Significant difference between Chow-Chow and HFD-HFD groups ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$); * Significant difference between HFD-HFD and HVA-HFD or HRA-HFD groups (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

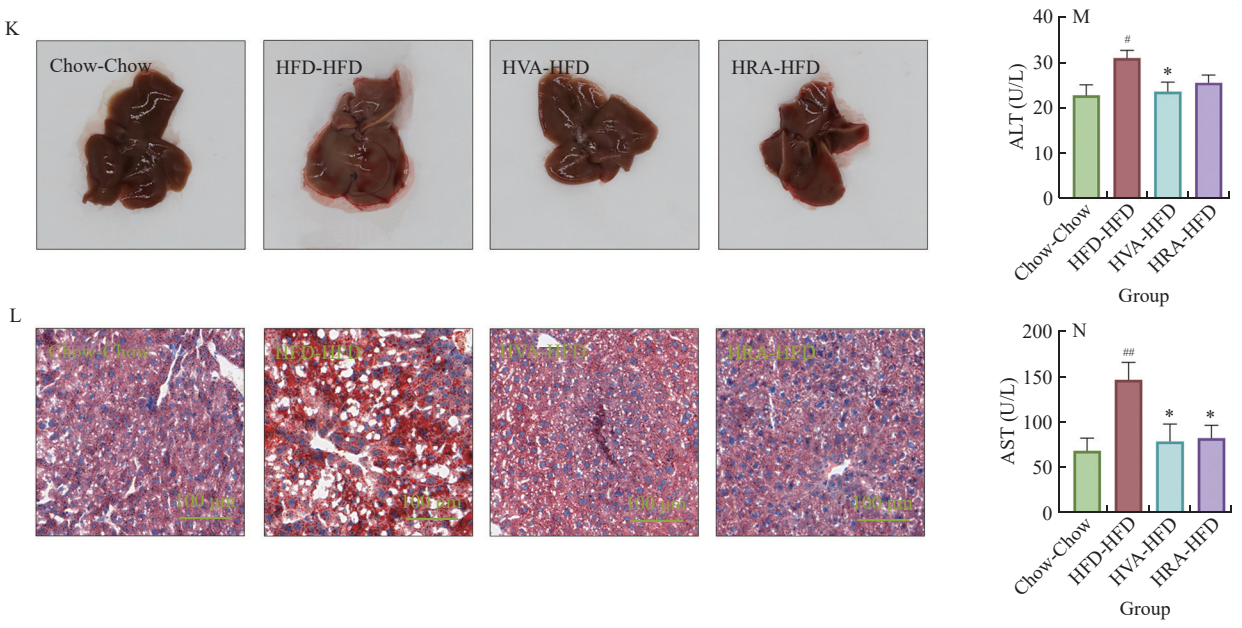


**Fig. 2** (*Continued*)

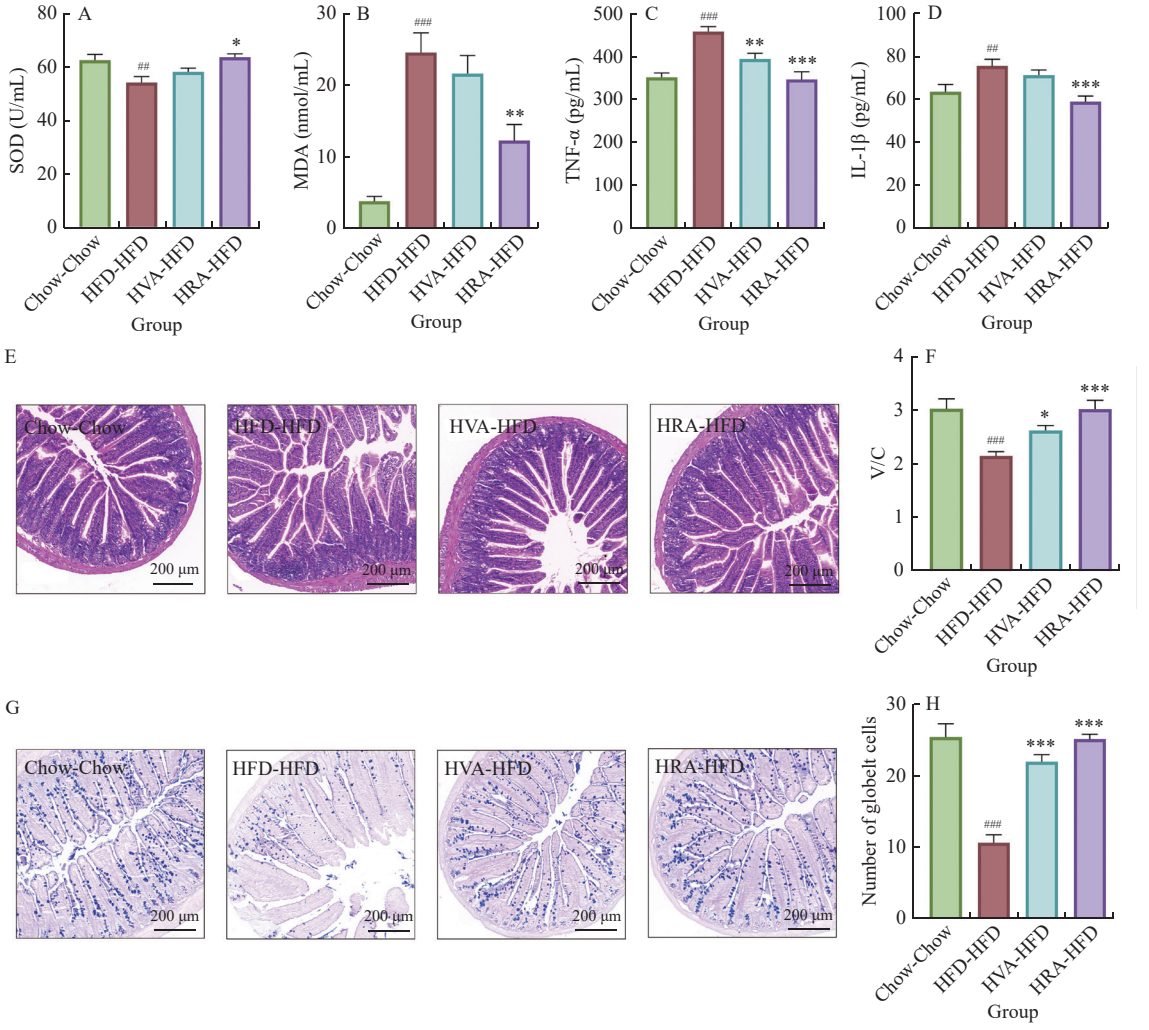


**Fig. 3** FMT from VA and RA supplemented mice improved oxidative stress, inflammation and intestinal barrier damage induced by HFD. Serum levels of (A) SOD; (B) MDA; (C) TNF-α; (D) IL-1β; (E) H&E staining of intestine samples; (F) V/C; (G) AB-PAS staining of intestine samples; (H) Number of goblet cells, 3 samples per group and 3 fields per sample were measured. Scale bar, 200 μm; Data are expressed as means ± SEM, $n = 8$. #Significant difference between Chow-Chow and HFD-HFD groups ($^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$); *Significant difference between HFD-HFD and HVA-HFD or HRA-HFD groups (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

### 3.4 FMT from VA and RA supplemented mice improved gut microbiota composition

A growing body of research has demonstrated that the mechanism of disease treatment through FMT involves modulating the gut microbiota[43]. In order to explore whether FMT from HVA and HRA groups have the potential to regulate gut microbiota, we conducted 16S rRNA sequencing to analyze the gut microbiota composition in different groups. As illustrated in Fig. 4, 4 different indexes (Chao1, Shannon, Simpson and Observed_species) were used to show $\alpha$-diversity among 4 groups, assessing the richness and evenness. As expected, HFD significantly decreased the levels of all 4 indexes compared to Chow-Chow group, while FMT from HRA group could significantly alleviate the decline induced by HFD. However, the 4 indexes

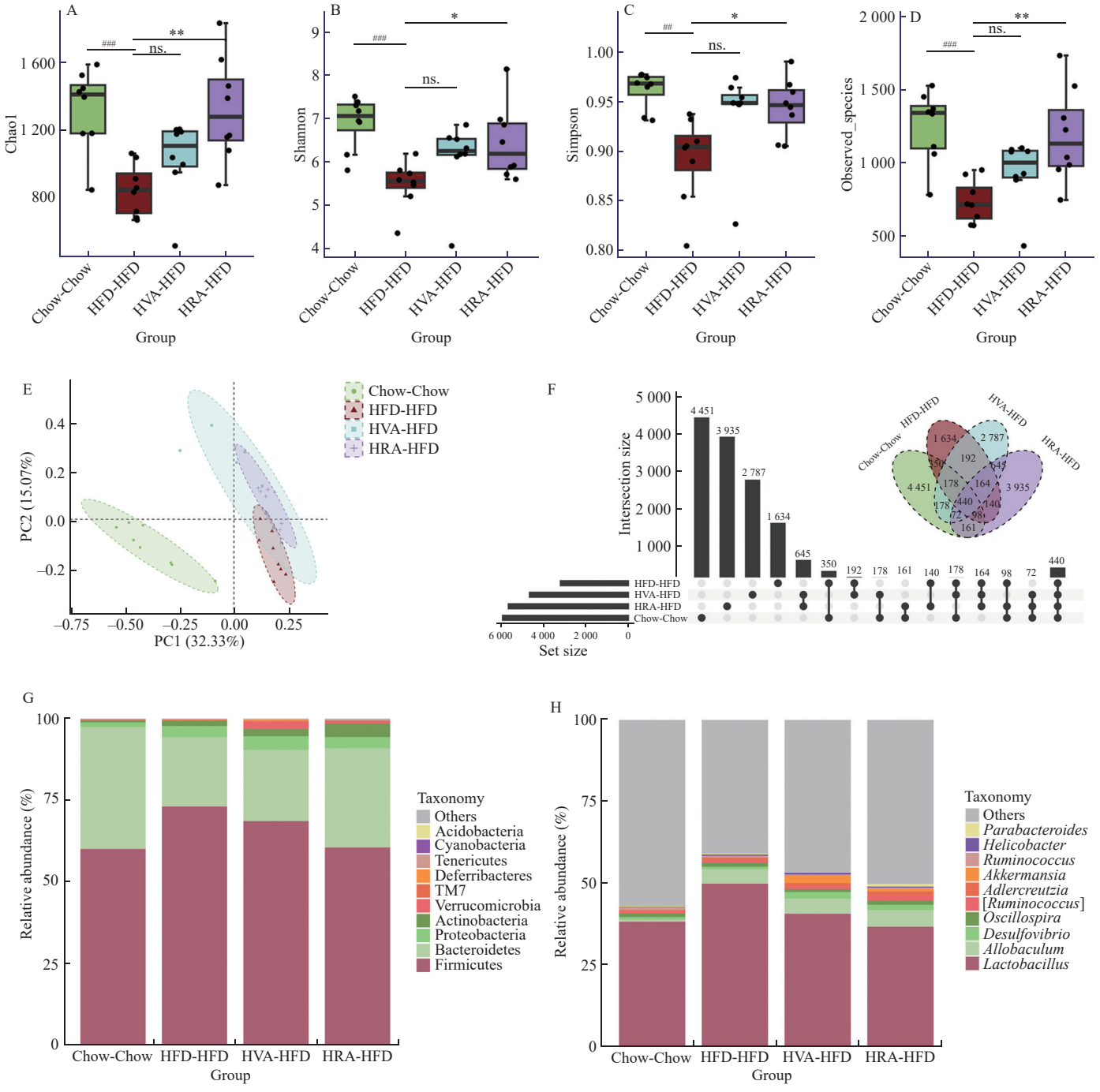


**Fig. 4** FMT from VA and RA supplemented mice improved gut microbiota composition. $\alpha$-Diversity indicated by (A) Chao1 index; (B) Shannon index; (C) Simpson index; (D) Observed_species; (E) PCoA based on bray_curtis distance metric; (F) Venn diagram; (G) Gut microbial composition at phylum level; (H) Gut microbial composition at genus level; (I) The relative abundance of Firmicutes; (J) The relative abundance of Bacteroidetes; (K) Ratio of F/B; (L) Abundance of *Akkermansia*; (M) Taxonomic diagrams obtained from LefSe analysis; (N) Histogram of LDA effect values for biomarker species. Data are expressed as means ± SEM, $n = 8$. $^{\#}$Significant difference between Chow-Chow and HFD-HFD groups ($^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$); *Significant difference between HFD-HFD and HVA-HFD or HRA-HFD groups (*$P < 0.05$; **$P < 0.01$); ns. means not significant.

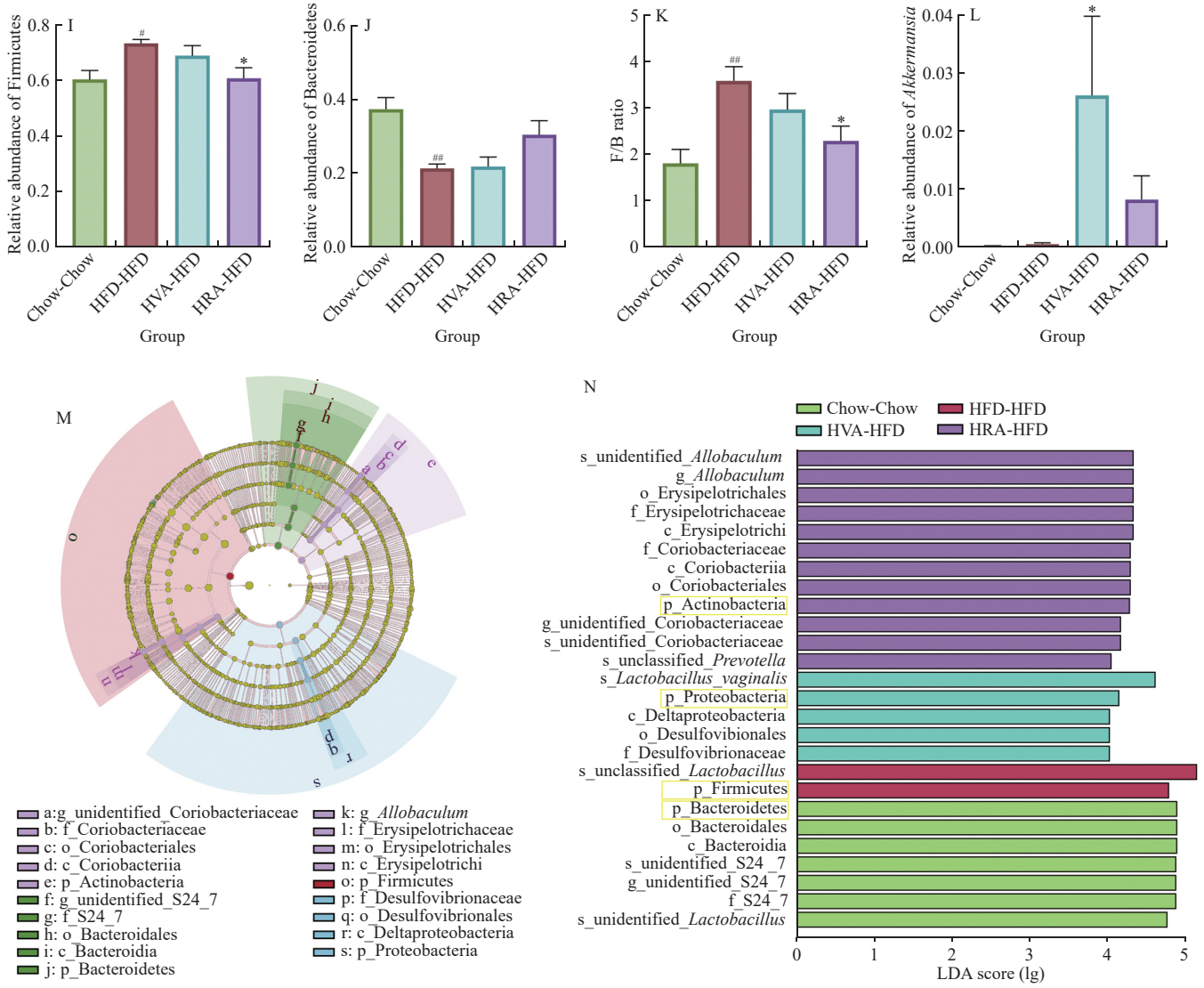


**Fig. 4** (*Continued*)

in HVA-HFD group showed no significant alleviating effects compared to HFD-HFD group (Figs. 4A-D). The PCoA analysis revealed the *β*-diversity of microbial communities, indicating the diversity differences among different groups. There was clear separation between Chow-Chow group and HFD-HFD group, indicating the microbial communities were changed by HFD. However, HVA-HFD group and HRA-HFD group also showed distinct clusters in comparison with HFD-HFD group. Furthermore, the combined effect of the first 2 principal components (PCs) explained 47.7% of the overall variation, with PC1 contributing 15.07% and PC2 contributing 32.33% of the variation (Fig. 4E).

Venn diagrams showed the unique ASVs in each group and common ASVs among groups. In total, 440 ASVs were determined as common ASVs among 4 groups, and the total ASVs in each group of Chow-Chow, HFD-HFD, HVA-HFD, HRA-HFD were 5 928, 3 196, 4 656 and 5 655, respectively. Moreover, the unique ASVs in each group Chow-Chow, HFD-HFD, HVA-HFD, HRA-HFD were 4 451, 3 935, 2 787 and 1 634, respectively (Fig. 4F). Then we further explored the relative microbial abundance of each group at both phylum and genus levels (Figs. 4G and H). The top 10 phyla were presented in Fig. 4G, with Firmicutes, Bacteroidetes and Proteobacteria being the 3 most prominent ones. In addition, Firmicutes was the most abundant phylum among all groups, which in HFD-HFD group accounted for 73.13% and was higher than the percentage observed in Chow-Chow, HVA-HFD and HRA-HFD groups. Moreover, FMT from HRA group significantly reduced the proportion of Firmicutes in mice (Fig. 4I). The relative abundance of Bacteroidetes was the second dominant phylum, the proportion in each group of Chow-Chow, HFD-HFD, HVA-HFD, HRA-HFD were 37.25%, 21.21%, 21.72% and 30.32%, respectively. HFD could significantly decrease the relative abundance of Bacteroidetes compared to chow diet and increase the ratio of F/B (Fig. 4J and K). However, FMT from HRA group could reverse the change of F/B induced by HFD. Interestingly, the relative abundance of Firmicutes, Bacteroidetes and the ratio of F/B in HVA-HFD group showed no significant difference compared to HFD-HFD group. At genus level, *Lactobacillus*, *Allobaculum* and *Desulfovibrio* were the top 3 dominant genus. Notably, *Lactobacillus* was the first dominant genus, counting for 38.30%, 49.92%, 40.69%, and 36.75% in the Chow-Chow,

HFD-HFD, HVA-HFD, and HRA-HFD groups, respectively (Fig. 4H). Interestingly, FMT from HVA group significantly increased the abundance of *Akkermansia* (Fig. 4L). These results suggested that FMT from HRA group exhibited a better performance on gut microbiota diversity caused by HFD. In contrast, FMT from HVA group primarily influenced the gut microbiota by enriching the specific taxa, such as the genus of *Akkermansia*, rather than by restoring the structure to Chow-Chow group.

To identify the specific biomarkers in different groups, the linear discriminant analysis effect size (LEfSe) analysis was used (LDA score > 4). As shown in Figs. 4M and N, in Chow-Chow group, 7 taxa were enriched, including the phylum of Bacteroidetes. Consistent with expectations, the phylum of Firmicutes could be taken as a biomarker in HFD-HFD group. Notably, FMT from HVA group and HRA group significantly and specifically enriched the phylum of Proteobacteria and Actinobacteria, respectively. In conclusion, the LEfSe analysis indicated the specific alterations in the gut microbiota induced by FMT from HVA group and HRA group.

### *3.5 Correlation between metabolic parameters and dominant bacterial phyla and genus*

To elucidate the correlation between the top 10 bacterial phyla and genus and metabolic parameters, Spearman's correlation analysis was utilized. As shown in Fig. 5, at the phylum level, Bacteroidetes was negatively correlated with IL-1β, FIN, TG, LDL-C, ALT, MDA, TC, white fat weight, FBG, OGTT_AUC, TNF-α, AST, ITT_AUC and HOMA-IR, and positively with brown fat weight, HOMA-IS, HDL-C and SOD. As expected, Firmicutes showed totally different trends with Bacteroidetes. In addition, the correlation between Cyanobacteria or Acidobacteria and parameters exhibited similar trends with those observed in Bacteroidetes and parameters, while the correlation between TM7 and parameters showed similar trends with those observed in Firmicutes. Furthermore, Actinobacteria, Deferribacteres and Proteobacteria all had a significantly positive correlation with white fat weight and TG. At the genus level, *Lactobacillus* showed a positive correlation with nearly all parameters, except for brown fat weight, HOMA-IS, HDL-C, and SOD. Furthermore, *Allobaculum* exhibited a significantly positive correlation with TC and white fat weight. Additionally, *Adlercreutzia* demonstrated a significant positive correlation with TC, white fat weight and OGTT_AUC, while being negatively associated with liver weight. These results showed that the dominant phyla and genus regulated by FMT from HVA group and HRA group were related to metabolic parameters. This correlation implied that changes in gut microbiota composition resulting from FMT from donor mice supplemented with VA and RA strongly correlated with metabolic parameters, indicating that the alterations of the specific bacterial taxa might have potential effects on MS.

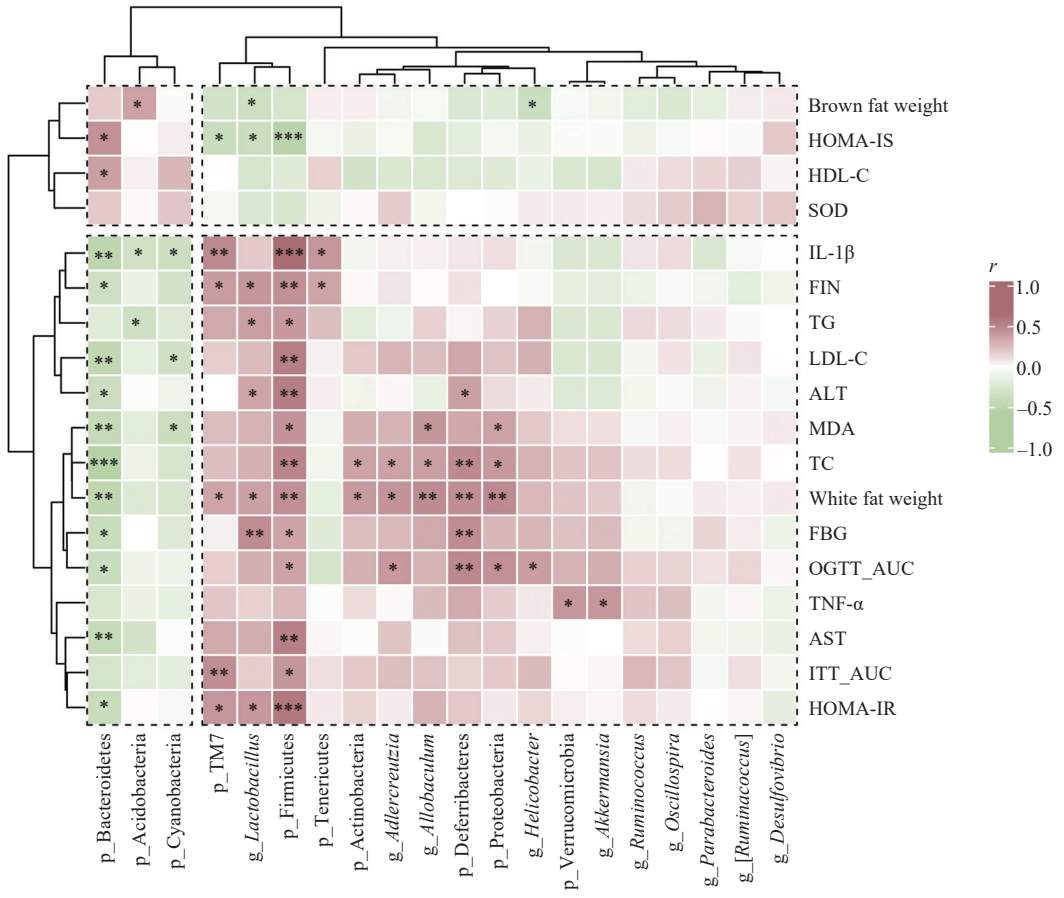


**Fig. 5** Correlation heatmap of metabolic parameters and dominant bacterial phyla and genus. The correlation coefficients (*r*) are displayed in different colors on the right side of the legend, with red indicating positive correlation and green indicating negative correlation. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 indicate significant correlation.

### 3.6 Discovery and identification of key ASVs and correlation with metabolic parameters

It is known that FMT from different donor individuals leads to distinct gut microbiota in recipient mice, resulting in divergent phenotypes that are associated with the donor individual's phenotypes[44]. In order to further investigate how FMT from HVA group and HRA group contributes to the amelioration of MS through specific gut microbiota, we employed metagenomeSeq to identify the shared significant differential ASVs present in the 4 groups (Fig. S1A). As shown in Fig. 6A, when comparing Chow-Chow, HVA-HFD, and HRA-HFD groups with HFD-HFD group, a total of 10 differentially abundant ASVs were identified. These ASVs are: ASV_36450, ASV_22293, ASV_36525, ASV_9642, ASV_19857, ASV_44647, ASV_14884, ASV_39801, ASV_12446, and ASV_34982. These ASVs belonged to phyla Firmicutes and Bacteroidetes, as well as genes *Lactobacillus*, *Odoribacter*, and *Turicibacter*. Among these 10 ASVs, the abundance trends in Chow-Chow, HVA-HFD, and HRA-HFD groups were consistent. Except for ASV_36450 from the genus *Lactobacillus* within the Firmicutes phylum, which was more abundant. The remaining ASVs exhibited lower abundance, including ASV_44674 and ASV_14884 from the phylum Bacteroidetes. To further identify the potential roles of these ASVs to MS, the Spearman correlation analysis was conducted. As seen in Fig. 6B, ASV_36450 was distinctly clustered separately, displaying a trend entirely opposite to the other 9 ASVs. Specifically, it was negatively correlated with TC, FBG, OGTT_AUC, TNF-α, TG, ITT_AUC, IL-1β, white fat weight, HOMA-IR, ALT, FIN, MDA, LDL-C and AST, while positively correlated with brown fat weight, HOMA-IS, HDL-C, and SOD. The correlation network also demonstrated consistent findings. Among them, ASV_44647, ASV_14884, ASV_34982, and ASV_39801 exhibited notably stronger correlations with these indicators, suggesting that these

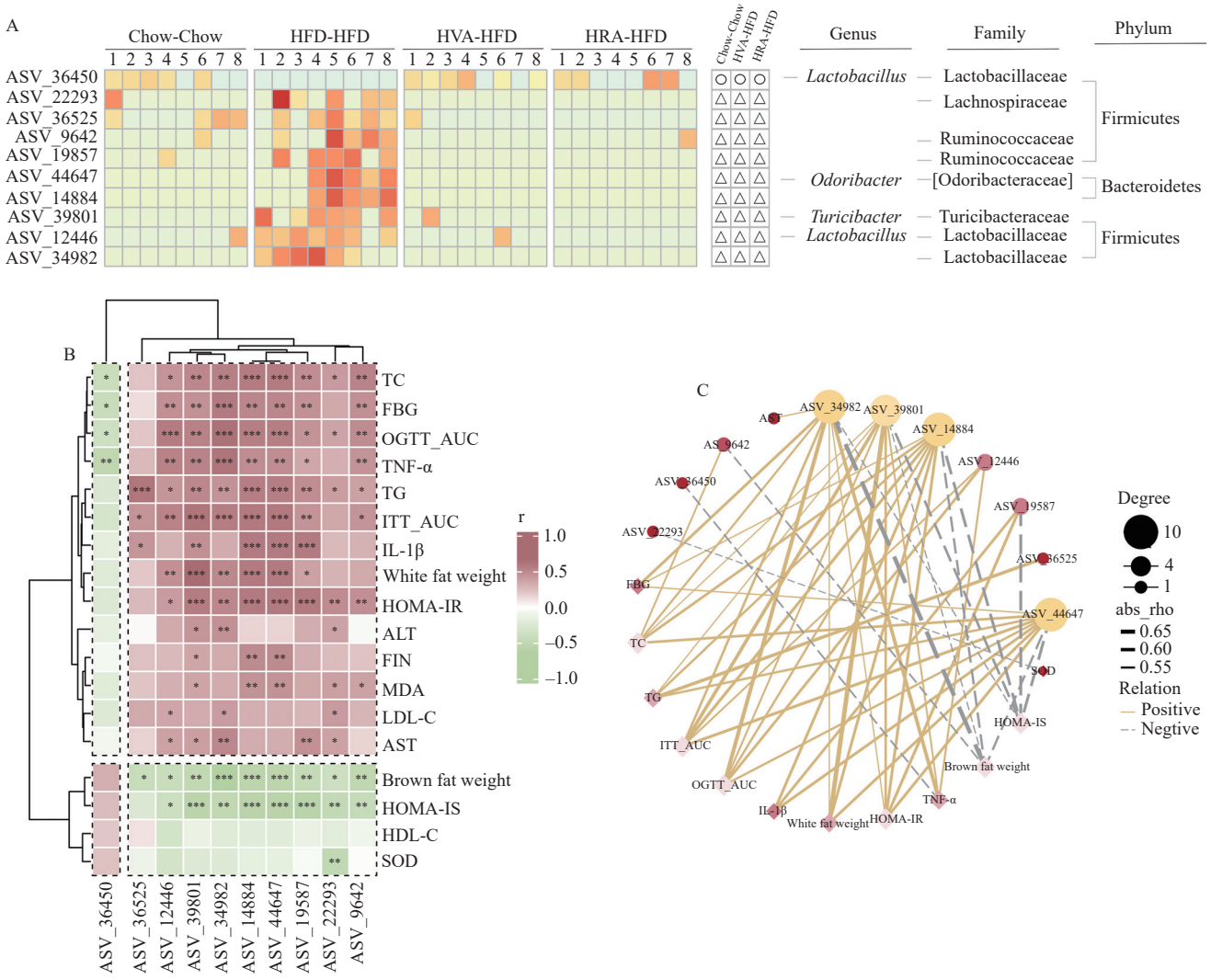


**Fig. 6** Discovery and identification of key ASVs and correlation with metabolic parameters. (A) Heatmap of 10 common ASVs among groups altered by HFD. The color of the spots in the left panel represents the relative abundance of the ASVs in each group. In the middle, circles represent more abundant ASVs in Chow-Chow, HVA-HFD and HRA-HFD groups compared with HFD-HFD; triangles represent less abundant ASVs in Chow-Chow, HVA-HFD and HRA-HFD groups compared with HFD-HFD; The phylum, family, and genus names of the ASVs are shown on the right panel. (B) Correlation heatmap between the 10 ASVs and metabolic parameters. The correlation coefficients ($r$) are displayed in different colors on the right side of the legend, with red indicating positive correlation and green indicating negative correlation. *$P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ indicate significant correlation. (C) Correlation network of the 10 ASVs and metabolic parameters. Nodes with higher degree indicate stronger connections; Yellow solid lines represent positive correlations, gray dashed lines represent negative correlations, and the thickness of the lines indicates the strength of the correlation.

specific ASVs might play a more prominent role in influencing the observed relationships with these indicators (Fig. 6C). These findings indicated that FMT from different donors significantly influenced the recipient's gut microbiota composition, subsequently impacting the relevant parameters of metabolic syndrome. These differential ASVs may serve as potential microbial biomarkers or targets, holding promise for playing a significant role in the prevention and treatment of MS.

In addition, in order to identify the distinct bacterial taxa that played a role in HVA-HFD and HRA-HFD groups, we first identified the differential ASVs in Chow-Chow, HVA-HFD, and HRA-HFD groups compared to the HFD-HFD group. Subsequently, we compared the distinct differential ASVs in HVA-HFD and HRA-HFD groups with Chow-Chow group, yielding unique sets of differential ASVs for HVA-HFD and HRA-HFD groups. These specific ASVs might contribute to the different effects on MS compared to Chow-Chow group. Finally, we employed principal component analysis to determine the top 20 contributing differential ASVs within HVA-HFD and HRA-HFD groups, followed by visual representation using Sankey diagrams. As depicted in Figs. S1A-C, there were 54 differential ASVs exclusively observed in HVA-HFD group, with the top 20 ASVs encompassing phyla Bacteroidetes, Firmicutes, Proteobacteria, and Verrucomicrobia. These ASVs belonged to families Clostridiaceae, Enterobacteriaceae, Lactobacillaceae, S24-7, and Verrucomicrobioceae. Notably, the S24-7 family exhibited the highest representation. In HRA-HFD group, a total of 193 unique differentially abundant ASVs were identified, with the top 20 ASVs involving phyla Bacteroidetes and Firmicutes. These ASVs were associated with families Lactobacillaceae and S24-7, with S24-7 still being the most prominently represented. These findings indicated differences in gut microbiota composition between HVA-HFD and HRA-HFD groups compared to Chow-Chow group. This might explain the varying effects of FMT from VA and RA supplemented mice on the outcomes of MS.

## 4. Discussion

The prevalence of MS has already jeopardized the health of approximately one billion people worldwide and led to many other complications, such as diabetes, cardiovascular diseases and obesity[45-46]. Therefore, searching for methods to improve or treat MS has become an urgent task. FMT, an innovative approach for disease treatment, has shown significant efficacy in addressing metabolic disorders. The underlying principle of using FMT to treat diseases is to restore the imbalanced gut microbiota[47]. For instance, FMT from healthy Chinese Kazak ethnic group could downregulate the levels of FBG, TC, TG and LDL-C. This was achieved by decreasing the abundance of *Desulfovibrio* and *Clostridium coccoides*, ultimately contributing to the improvement of diabetes in *db*/*db* mice[48]. In addition, fecal transplantation from mice supplemented with ginger could significantly prevent the increase of body weight and fat accumulation caused by HFD, while improving glucose tolerance as well. Furthermore, FMT significantly elevated the abundance of the genus *Allobaculum* and *Bifidobacterium*[49]. Zhang et al.[50] demonstrated that through FMT the anti-obesity effect of phlorizin was achieved by restoring gut microbiota and intestinal barrier integrity. In summary, FMT has been an effective therapy for treating diseases by regulating gut microbiota beyond *C. difficile* infection[51].

In our previous study, we established the MS model using HFD and investigated the potential benefits of VA and RA interventions. Our findings demonstrated that supplementation with VA and RA could ameliorate the metabolic disorders[32]. However, it remains unknown that whether the anti-MS effects attributed to VA and RA were indeed mediated through regulations of gut microbiota and whether the effects could be replicated by FMT. Herein, we utilized the PGF mice to evaluate the potential effects of FMT from donor mice in Chow, HFD, HVA and HRA groups. The results indicated that HFD similarly induced MS, characterized by significant weight gain and fat accumulation[52]. Compared to FMT from HFD donor mice, FMT from HVA and HRA groups significantly ameliorated HFD-induced weight gain, increase in white fat weight, and fat accumulation, bringing these parameters closer to the levels observed in Chow-Chow group. More importantly, these effects were not due to the reduction in food intake, indicating that the anti-obesity effects of FMT from VA and RA supplemented mice were not attributed to the decreased food intake.

Furthermore, HFD also induced abnormalities in glycemic and lipid metabolism, thereby causing cardiovascular diseases and diabetes[53]. However, FMT from both HVA and HRA groups could remarkably decrease the levels of FBG, and improve glucose tolerance and insulin resistance, showing potential protection in glucose metabolism. Moreover, elevated levels of LDL-C are considered a major risk factor for cardiovascular diseases[54]. However, it is noteworthy that in this study, we observed that FMT from HVA and HRA groups did not significantly impact LDL-C levels, despite significant improvements in other metabolic parameters such as TC, TG, and HDL-C. In addition, FMT from HVA and HRA groups ameliorated HFD-induced liver damage and hepatic lipid accumulation, reducing serum levels of ALT and AST, demonstrating potential hepatoprotective effects. Moreover, current research has demonstrated that HFD can induce oxidative stress, elevate levels of MDA, and reduce levels of SOD, which is consistent with our study findings[55]. Oxidative stress can trigger an inflammatory response, inducing the release of pro-inflammatory cytokines such as TNF-α and IL-1β, which in turn facilitates oxidative stress, ultimately resulting in the onset of chronic inflammation within the body[56]. Such an inflammatory state may result in an elevation of intestinal mucosal permeability, consequently causing impairment of barrier function[57]. In our study, FMT from HRA group demonstrated a significant improvement in inflammation and oxidative stress induced by HFD, as evidenced by the increase in SOD levels and the reduction in MDA, TNF-α, and IL-1β levels. These effects were consistent with the results of FMT from *Rosa roxburghii* Tratt fruit supplemented mice, as reported previously[35]. Meanwhile, FMT from HVA group exhibited a notable regulatory effect only on TNF-α. Furthermore, in comparison to HFD-HFD group. The ratio of V/C significantly increased in HVA-HFD and HRA-HFD groups, the higher V/C ratio indicates a healthier state of intestine. Simultaneously, the quantity of goblet cells, which secrete mucus to safeguard the intestinal mucosa, was notably reduced in HFD-HFD group[58]. However, following interventions with FMT from HVA and HRA groups, the quantity of these goblet cells exhibited a significant increase. Taken together, the results indicated that VA and RA could exert their anti-MS effects through FMT. Particularly, FMT from HRA group demonstrated superior effectiveness than FMT from HVA group.

Subsequently, we conducted a further detailed analysis of the microbial composition in each group of Chow-Chow, HFD-HFD, HVA-HFD and HRA-HFD. In our previous study, both VA and RA were found to significantly restore the reduced gut microbiota diversity induced by HFD, which was consistent with the results by Zeng et al.[52]. In this study, compared to FMT from HFD group, FMT from HRA group was found to significantly restore the decreased diversity of gut microbiota, as indicated by increased Chao1, Shannon, Simpson indices, and observed_species. Additionally, FMT from HRA group resulted in a significant decrease in the abundance of Firmicutes and a reduction in the F/B ratio, and these results were consistent with those of donor mice. However, unexpectedly, despite the improvement in these indicators with FMT from HVA, none of them reached a significant level. This might explain why FMT from HVA was less effective in improving metabolic parameters compared to FMT from HRA, as it failed to restore gut microbiota diversity to the levels observed in Chow-Chow group. This further confirmed that VA and RA exerted their metabolic protective effects through regulating gut microbiota. Therefore, the improvement in MS of FMT from HVA might not necessarily be achieved by restoring gut microbiota diversity. Instead, it might be achieved through the modulation of specific microbial populations, such as an increase in the proportion of *Akkermansia*. In our previous study, the gut microbiota analysis in donor mice revealed a significant increase in the abundance of *Akkermansia* in HVA group compared to HFD group, which was not observed in HRA group[32]. The increased abundance of *Akkermansia* was believed to be beneficial for mitigating obesity to increase the number of goblet cells and enhance mucin secretion[59-60]. Furthermore, at the phylum level, Firmicutes dominates in abundance, followed by Bacteroidetes. At the genus level, *Lactobacillus* is the most abundant, followed by *Allobaculum*[61]. While *Lactobacillus* is considered a beneficial bacterium because of its role in health-promoting effects, such as enhancing intestinal barrier function and inhibiting the growth of some pathogenic bacteria[62]. However, the role of *Lactobacillus* has not been fully elucidated, as research has found associations between it and obesity[63].

Furthermore, the LefSe results (LDA > 4) showed that the phylum of Firmicutes was specifically enriched in HFD-HFD group, and the phylum of Bacteroidetes was specific in Chow-Chow group, which is consistent with our previous research and some other studies[64-65]. Our data also revealed that the phylum of Actinobacteria and the genus of *Allobaculum* were specifically enriched in HRA-HFD group. Actinobacteria and *Allobaculum* have been demonstrated to possess anti-inflammatory effects, which are associated with the production of short-chain fatty acids (SCFA)[66]. Unexpectedly, Proteobacteria was enriched in HVA-HFD group. A study has previously demonstrated that Proteobacteria was more abundant in diabetes patients than in healthy individuals and are associated with the release of endotoxin, which promots inflammatory processes in the body[67]. However, Proteobacteria, as one of the major phyla constituting the gut microbiota, has not been fully defined in terms of its role. Subsequently, the Spearman correlation indicated the negative correlation between Bacteroidetes, Acidobacteria, Cyanobacteria, and parameters related to obesity or inflammation, as well as the positive correlation between Firmicutes, Proteobacteria and indicators associated with obesity or inflammation, which had been confirmed previously[68]. These results suggested that the specific bacterial taxa could contribute to the improvements of MS.

Additionally, our research revealed that the abundance of ASV_36450 decreased, which belonged to the genus *Lactobacillus* within the Lactobacillaceae family of the Firmicutes phylum, in HFD-HFD group. Conversely, its abundance significantly increased in Chow-Chow, HVA-HFD, and HRA-HFD groups. *Lactobacillus* is known to induce the production of SCFA which are important for maintaining the integrity and functionality of the gut barrier[69]. Furthermore, the consistent trends of the remaining 9 ASVs suggested a similar potential impact of FMT from both HVA and HRA groups on the gut microbiota. Specifically, this implied that FMT from HVA and HRA groups exerted the effects on MS through the modulation of gut microbiota. The findings were further substantiated by Spearman correlation analysis and correlation network, which provided additional support for the involvement of the 10 ASVs in regulating MS. Moreover, ASV_36450 exhibited a negative correlation with obesity or inflammatory-related indices, indicating the potential significance in metabolic disorders. However, further experiments are warranted to validate these findings. Moreover, the Sankey diagrams illustrated the differential ASVs compared to HFD-HFD group, displaying the distinct top 20 differential ASVs in both HVA-HFD and HRA-HFD groups when compared to Chow-Chow group. These ASVs might contribute to the observed different improvements in MS through FMT from both HVA and HRA groups, with FMT from HRA demonstrating superior effectiveness. Among the 20 ASVs in HVA-HFD group, the Proteobacteria phylum is noteworthy. The presence of Proteobacteria has previously been linked to conditions such as endotoxemia and chronic inflammation[70]. However, it is worth noting that the S24-7 family within the Bacteroidetes phylum, shared by both groups, was the most significantly enriched. S24-7 is a representative of the Bacteroidetes phylum and plays a role in carbohydrate metabolism. Interestingly, its abundance tends to be lower in individuals with obesity and diabetes[71-72]. Therefore, the S24-7 family might play a crucial role in improving metabolic disorders. However, the exact mechanisms underlying its role require further in-depth investigations through both *in vivo* and *in vitro* studies for confirmation.

## 5. Conclusion

In summary, by establishing a MS model through HFD and then conducting FMT from donor mice intervened with VA and RA, aiming to investigate whether the therapeutic effects of VA and RA on MS were mediated through gut microbiota and whether these effects could be replicated through FMT. The results revealed that compared to FMT from HFD group, FMT from both HVA and HRA groups could significantly improve MS induced by HFD. The 16S rRNA analysis demonstrated that FMT from both HVA and HRA groups improved the gut microbiota composition, with FMT from HRA group showing better enhancement in microbial diversity, whereas FMT from the HVA group exerted its beneficial effects on MS by increasing the abundance of *Akkermansia*. The Spearman analysis further confirmed the correlation between specific microbial taxa and MS. Specifically, Bacteroidetes, Acidobacteria, and Cyanobacteria exhibited a negative correlation with MS, while Firmicutes showed a positive correlation with MS. Additionally, the key differential ASVs were identified by using metagenomeSeq, among which ASV_36450 exhibited a consistent trend of increased abundance in Chow-Chow, HVA-HFD, and HRA-HFD groups compared to HFD-HFD group, indicating a potential role of the *Lactobacillu*s genus in treating MS.

This was further confirmed by Spearman correlation analysis, where ASV_36450 was significantly negatively correlated with obesity and inflammation-related parameters. Furthermore, the family of S24-7 might also be a key microbial through which VA and RA exerted their anti-MS effects. Despite these meaningful findings, further research is necessary to comprehensively understand the mechanisms of action of these microbial communities and their exact relationship with MS.

## Conflict of interest

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

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## Data availability

The data presented in the study are submitted in the NCBI Sequence Read Archive (SRA) repository, accession number is PRJNA1032709.

## Appendix A. Supplementary information

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2024.9250238.

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