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RESEARCH PAPER

Retinol metabolism signaling participates in microbiota-regulated fat deposition in obese mice

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Abstract

Obesity is a global pandemic threatening public health, excess fat accumulation and overweight are its characteristics. In this study, the interplay between gut microbiota and retinol metabolism in modulating fat accumulation was verified. We observed gut microbiota depletion reduced the body weight and the ratios of white adipose tissues (WATs) to body weight in high-fat diet (HFD) fed-mice. The kyoto encyclopedia of genes and genomes (KEGG) analysis and protein-protein interaction (PPI) network of RNA-seq results indicated that retinol metabolism signaling may be involved in the microbiota-regulated fat deposition. Furthermore, activated retinol metabolism signaling by all-trans retinoic acid (atRA) supplementation reduced body weight and WAT accumulation in obese mice. 16S rRNA gene sequencing of the ileal microbiota suggested that atRA supplementation increased the microbial diversity and induced the growth of beneficial bacteria including *Parabacteroides, Bacteroides, Clostridium_XVIII, Bifidobacterium, Enterococcus, Bacillus, Leuconostoc*, and *Lactobacillus* in obese mice. Spearman correlation showed that the microbiota altered by atRA were associated with body and WAT weights. Together, this study reveals the interaction between the gut microbiota and retinol metabolism signaling in regulating adipose accumulation and obesity. It is expected of this finding to provide new insights to prevent and develop therapeutic measures of obesity-related metabolic syndrome.

Keywords: Gut microbiota; Obesity; All-trans retinoic acid (atRA); Retinol; Adipose tissue; Fat deposition.

1. Introduction

Obesity is a chronic disease that has been considered a pandemic due to its increasing prevalence and its association with increased risks of various chronic diseases, including type 2 diabetes, cardiovascular disease, neurodegenerative disease, and even cancer [1]. Obesity is caused by an imbalance between energy intake and expenditure that promotes excessive lipid accumulation. White adipose tissue (WAT) is the primary site for the storage of lipids within lipid droplets [2]. WAT is subdivided into two major depots, subcutaneous (SAT) and visceral (VAT), based on their location and function [3]. The expansion and dysfunction of WAT are hallmarks of obesity and contribute to the development of metabolic syndrome such as diabetes and atherosclerosis [4].

Recently, the gut microbiota has emerged as a target for therapeutic modulation of obesity [5]. Furthermore, accumulating evidence indicates that the gut microbiota is a vital endogenous factor in modulating the physiology of WAT [3]. The depletion of gut microbiota via different cocktails of antibiotics (Abx) decreased the body, subcutaneous white adipose tissue (sWAT), and epididymal white adipose tissue (eWAT) weights and alleviated metabolic disorders [6]. Furthermore, studies showed that gut microbiota is associated with the browning and inflammation of WAT, thus mediating high-fat diet (HFD)-induced metabolic syndromes in mice [7,8]. Thus, the gut microbiota is a potential target in the management of obesity-associated adipose tissue dysfunction.

All-trans retinoic acid (atRA) is the active metabolite of vitamin A (retinol). It has been reported that atRA supplementation reduced the body weight and inhibited adiposity in mice fed with chow diet [9,10]. Additionally, when administered to HFD-fed mice, atRA ameliorated obesity-related metabolic impairments, including hepatic steatosis, insulin resistance, and adipogenesis

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[11,12]. Moreover, the crosstalk between gut microbiota and retinol metabolism has been reported by previous studies [13,14]. However, the correlation between gut microbiota and atRA in obesity and whether gut microbiota mediates adiposity in response to atRA are not clear.

To further investigate the impact of the bidirectional modulation between atRA and gut microbiota on adipose tissue accumulation, the objective of this study was to investigate the influence of gut microbiota on WAT accumulation and retinol metabolism, as well as to characterize the impact of atRA supplementation on the gut microbiota and WAT accumulation. Compared to germ-free mice, pseudo-germ-free mice might be a more proper model to explore the impacts of gut microbiota on host metabolism, since pseudo-germ-free mice still retain some gut microbiota, which helps maintain immune system development and host metabolism due to these microbiota [15]. What is more, the pseudo-germ-free mouse model is a more cost-effective and accessible alternative than the germ-free (GF) mouse model [16]. In this study, we used a pseudo-germ-free mouse model to reveal the important role of gut microbiota in WAT accumulation. Additionally, we showed that the depletion of gut microbiota influences retinol metabolism. Furthermore, atRA supplementation was able to inhibit WAT accumulation and alter microbial composition. Our findings have important implications for understanding the bidirectional relationship between gut microbiota and retinol metabolism in obesity, expanding our knowledge about the functional attributes of gut microbiota in obesity. This study may hold promise for developing novel nutritional interventions for alleviating metabolic disorders.

2. Materials and methods

2.1. Animal treatments

The animal studies were complied with all relevant ethical regulations and approved by the Experimental Animal Welfare and Ethical Committee of the Institute of Animal Science, Chinese Academy of Agricultural Sciences (IAS2020-86). C57BL/6J mice (Specific pathogen-free grade, male, 3 weeks old) were purchased from the Peking University Health Science Center (Beijing, China) and allowed 1-week of acclimatization. Mice were maintained under a constant temperature of 22±2°C on a 12/12 h light/dark cycle. The chow diet (D12450B, 10% calories from fat, Beijing Keao Xieli Feed Co., Ltd., Beijing, China) or HFD (D12492, 60% calories from fat, Beijing Keao Xieli Feed Co., Ltd., Beijing, China) were used in all mice experiments.

In the pseudo-germ-free mice study, a total of 18 mice were randomly divided into three groups (n=6 per group) named Chow, HFD, and HFD-GM separately. The Chow group was fed with chow diet, whereas the HFD and HFD-GM groups were fed with HFD. For the generation of pseudo-germ-free mice, mice in the HFD-GM group were administered with an antibiotics cocktail (Abx: 1 g/L ampicillin, 1 g/L neomycin, 1 g/L metronidazole, and 0.5 g/L vancomycin hydrochloride) in drinking water during the whole experimental period. The mice in the Chow and HFD groups were supplemented with drinking water without antibiotics. Water bottles were changed twice weekly to contain fresh Abx. Body weight and dietary intake were monitored during the experiments. After 10 weeks, mice were fasted overnight and then killed by cervical dislocation. The WAT (iWAT: inguinal white adipose tissue; eWAT: epididymal white adipose tissue; pWAT: perirenal adipose tissue) weights were monitored and the liver tissues were quickly frozen in liquid nitrogen and then stored at -80°C for further analysis.

In the atRA supplementation study, a total of 64 mice were divided randomly into four groups (n=16/group) named Chow, Chow+atRA, HFD, and HFD+atRA. The mice were fed with either standard chow diet (D12450B, 10% calories from fat) or HFD (D12492, 60% calories from fat). Mice in the atRA treatment groups (Chow+atRA and HFD+atRA) received one daily i.g. of 10mg/kg atRA body weight for 3 weeks before they were sacrificed; other mice received an equal volume of vehicle (corn oil). The dose and treatment time of atRA used in this study was determined based on previous studies showing that 10 or 15 mg/kg atRA was able to reduce body weight in mice [17,18]. At the end of the 11th week, mice were fasted overnight and then sacrificed. The WAT weights were monitored and the ileal content samples were quickly frozen in liquid nitrogen and then stored at -80°C for further analysis.

2.2. Hepatic metabolomic profiling analysis

A total of 50 mg liver tissues was weighted, and the metabolites were extracted using 400 μL methanol: water (4:1, v/v) solution with an internal standard (0.02 mg/mL L-2-chlorophenylalanin). The mixture was settled at -10°C and treated by High throughput tissue crusher Wonbio-96c (Shanghai wanbo biotechnology co., LTD) at 50 Hz for 6 min, then followed by ultrasound at 40 kHz for 30 min at 5°C. The samples were placed at -20°C for 30 min to precipitate proteins and then centrifuged at 13000 g at 4°C for 15 min. After that, the supernatants were transferred to sample vials for LC-MS/MS analysis. Chromatographic separation was performed using a Thermo UHPLC system equipped with an ACQUITY UPLC HSS T3 (100 mm×2.1 mm i.d., 1.8 μm; Waters, Milford, USA). The samples were analyzed using a Thermo UHPLC -Q Exactive HF-X Mass Spectrometer equipped with an electrospray ionization (ESI) source operating in either positive or negative ion mode. After UPLC-MS analyses, the raw data were imported into the Progenesis QI 2.3 (Nonlinear Dynamics, Waters, USA) for peak detection and alignment. The metabolites with variable importance in the projection (VIP) value >1 and P value $< .05$ were considered as different metabolites.

2.3. Hepatic transcriptomics analysis

The Total RNA of each liver tissue was extracted by using TRIzol® reagent (Invitrogen, USA). The purity, concentration, and quality of the total RNA samples were detected and only high-quality RNA was used for further analysis. Oligo (dT)-enriched mRNA was fragmented, and the cleaved RNA fragments were reverse transcribed to establish the final cDNA library using a SuperScript double-stranded cDNA synthesis kit (Invitrogen, USA) with random hexamer primers (Illumina, California, USA).

The synthesized cDNA was subjected end-repaired, adenylated, and ligated to the sequencing adaptors. The RNA-seq sequencing library was sequenced with an Illumina Hiseq xten/NovaSeq 6000 sequencer (Illumina, San Diego, CA, USA) at Majorbio BioPharm Technology Co. Ltd. (Shanghai, China). After quality control, the clean reads were mapped to the reference genome with orientation mode using TopHat (http://tophat.cbcb.umd.edu/, version 2.0.0) software. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: PRJNA1089170). The data were analyzed using the online platform of Majorbio Cloud Platform (www.majorbio.com).

2.4. Gut microbiota analysis

The Total genome DNA of each ileal content sample was extracted using an E.Z.N.A.® Stool DNA Kits (Omega Bio-Tek Inc., Norcross, GA, United States) according to the manufacturer's instructions. The DNA concentration and purity were monitored on 1% agarose gels. 16S rRNA gene V3-V4 region was amplified by PCR using the primer set 338F/806R

(338F, 5′-ACTCCTACGGGAGGCAGCAG-3′; 806R, 5′-GGACTACHVGGGTWTCTAAT-3′). Amplicons were detected using 2% agarose gel electrophoresis and purified using an AxyPrep DNA gel extraction kit (Axygen Bioscience, CA, USA). After purification, amplicons were sequenced by the Illumina MiSeq platform (Illumina, San Diego, CA, USA) at Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: PRJNA1087312). The sequences were analyzed and assigned to operational taxonomic units (OTUs; 97% identity). A-diversity was analyzed using QIIME (Version 1.7.0). β-diversity was estimated by using Principal Component Analysis (PCA).

2.5. Statistical analysis

All statistical analysis was performed with SPSS 21 software. Multigroup data were compared using one-way ANOVA with Duncan's posthoc test. Spearman correlation analysis was conducted using GraphPad Prism 10.0. Data are expressed as the mean±SEM. A *P*-value less than .05 was considered significant.

3. Results

3.1. Gut microbiota modulated the body weight and WAT accumulation in obese mice

To determine the effects of gut microbiota on adiposity, mice were subjected to HFD with or without gut microbiota depletion for 10 weeks (Fig. 1A). The results showed that compared with chow diet, HFD significantly increased body weight from the 3rd week ($P < .05$; Fig. 1B and C), which is consistent with the higher energy intake ($P < .05$; Fig. 1D). However, gut microbiota depletion caused significantly lower body weight in mice fed an HFD ($P < .05$; Fig. 1B and C). Meanwhile, the energy intake was significantly decreased in the 1st week, but increased in the 2nd and 3rd weeks by depleted gut microbiota in HFD-fed mice ($P < .05$; Fig. 1D).

We further examined whether the altered body weight was caused by different WAT accumulation. It has been reported that WAT can be further subdivided into SAT and VAT. SAT is located near the body surface, especially within the gluteal region, abdomen, and thighs [19]. In male mice, the fat depots around the testes (epididymal fat) and kidney (perirenal fat) are considered a model of VAT [2]. Thus, in this study, WAT mass was segmented into iWAT, eWAT, and pWAT and then were measured. As expected, HFD markedly elevated the indices (fat weight/body weight ratios) of iWAT, eWAT, and pWAT ($P < .05$; Fig. 1E and F). However, gut microbiota depletion markedly decreased the indices of iWAT, eWAT, and pWAT in HFD-fed mice ($P < .05$; Fig. 1E and F). These data indicate that the HFD-treated mice showed characteristics (overweight and WAT accumulation) of obesity, and gut microbiota depletion had a certain alleviation effect on obesity induced by HFD.

3.2. Gut microbiota altered hepatic metabolites related to lipid metabolism in obese mice

Since the liver is the main site for lipid metabolism, we next investigated whether gut microbiota depletion induced alterations in hepatic metabolism by performing hepatic metabolome. After peak alignment and removal of missing values, a total of 329 negative ions and 444 positive ions were identified in the liver. We then evaluated the differential metabolites between different comparison groups based on the criteria of *P*-value < .05 (Student's t-test) and VIP > 1 (OPLS-DA model). The preliminary

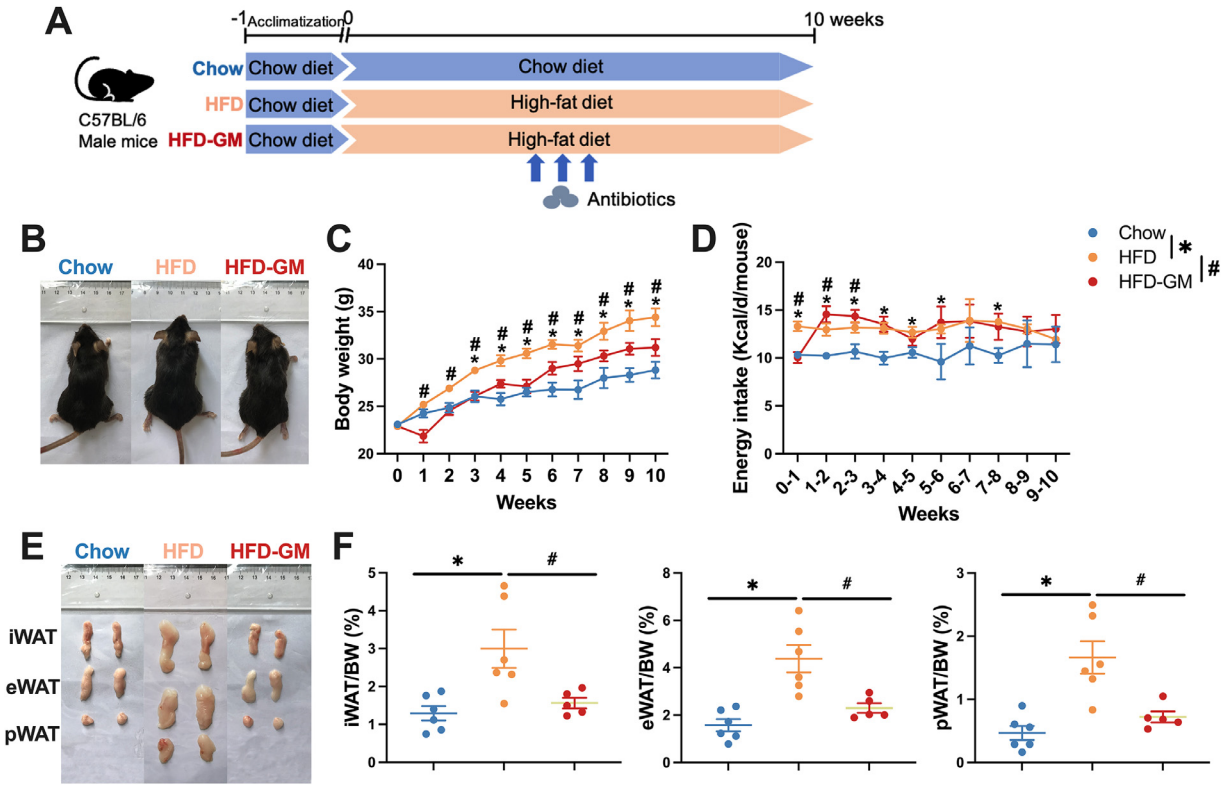

Fig. 1. Body weight and WAT accumulation were reduced by gut microbiota depletion in HFD-fed mice. (A) Experimental scheme; (B) Representative mice in the Chow, HFD, and HFD-GM groups; (C) Body weight; (D) Energy intake; (E) Representative pictures for WAT in mice from the Chow, HFD, and HFD-GM groups; (F) The ratios of iWAT, eWAT, and pWAT to body weight. Data were expressed as the mean±SEM. $^{*}P < .05$: Chow vs. HFD; $^{\#}P < .05$: HFD-GM vs. HFD.

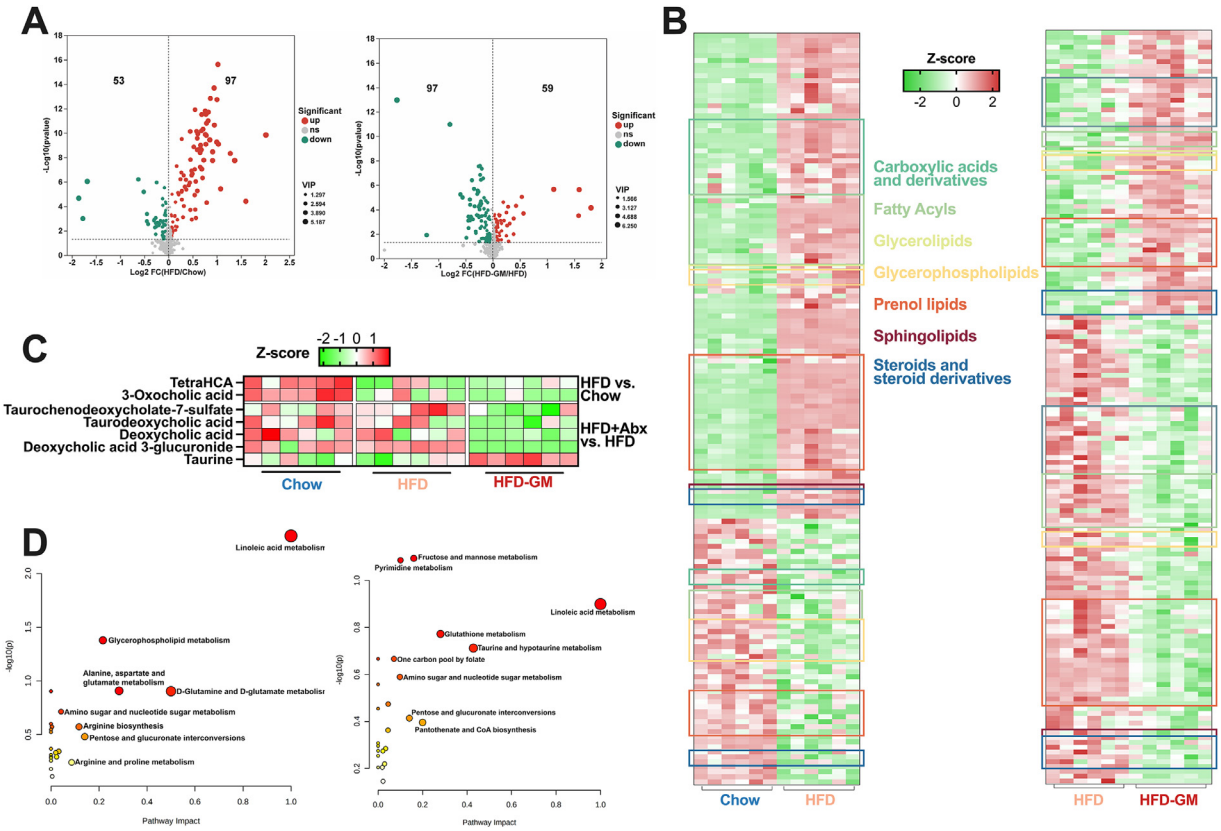

Fig. 2. Hepatic metabolites related to lipid metabolism were altered by gut microbiota depletion in HFD-fed mice. (A) Volcano plot of differential metabolites between mice fed with chow diet and HFD (left) and differential metabolites between mice with and without gut microbiota depletion when fed with HFD (right); (B) Heatmap of the differential metabolites between mice fed with chow diet and HFD (left) and the differential metabolites between mice with and without gut microbiota when fed with HFD (right); (C) Heatmap of differential metabolites related to bile acid metabolism between the Chow, HFD, and HFD-GM groups; (D) MetaboAnalyst-computed pathway analysis of the differential metabolites between mice fed with chow diet and HFD (left) and the differential metabolites between mice with and without gut microbiota depletion when fed with HFD (right).

screening of the altered metabolites is visualized in the form of a volcano plot (Fig. 2A). We identified 150 changed metabolites in obese mice compared with chow diet-fed mice, including 97 up-regulated metabolites and 53 down-regulated metabolites ($P < .05$; Fig. 2A and B). These differential metabolites mainly belonged to carboxylic acids and derivates, fatty acyls, glycerophospholipids, prenol lipids, steroids and steroids derivates. Meanwhile, gut microbiota depletion up-regulated 59 and down-regulated 97 metabolites in obese mice ($P < .05$; Fig. 2A and B). These differential metabolites mainly belonged to carboxylic acids and derivates, fatty acyls, glycerophospholipids, prenol lipids, steroids and steroids derivates, which might suggest that gut microbiota depletion influenced hepatic lipid metabolism in obese mice. Additionally, gut microbiota depletion significantly altered the levels of bile acids, including Taurochenodeoxycholate-7-sulfate, Taurodeoxycholic acid (TDCA), Deoxycholic acid (DCA), and Deoxycholic acid 3-glucuronide in obese mice ($P < .05$; Fig. 2C).

To understand the functional characteristics and classification of these changed metabolites, we conducted pathway enrichment analysis based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The results showed that HFD treatment mainly changed the metabolites involved in linoleic acid and amino acid metabolism (Fig. 2D). Gut microbiota depletion mainly affected the metabolites related to linoleic acid metabolism, fructose and mannose metabolism, and taurine and hypotaurine metabolism (Fig. 2D).

Collectively, these findings reveal the role of gut microbiota in modulating lipid metabolism, further supporting the contribution of gut microbiota to altered fat deposition.

3.3. Gut microbiota altered the expression of genes involved in retinol and lipid metabolism in obese mice

To discern possible process and cell signaling pathways involved in microbiota depletion-altered lipid metabolism, we investigated the alterations of hepatic gene expression profile by performing RNA-seq analysis. We conducted functionally organized networks using Cytoscape based on the differentially expressed genes (DEGs, fold change >1.5, *P*-adjust value < .05) between HFD and HFD-GM groups (Fig. 3A). Notably, gut microbiota depletion altered the genes mainly involved in the retinol metabolism, lipid metabolism, and amino acid metabolism (Fig. 3A). Additionally, Cyp2b10 and Cyp3a11 have been reported to be involved in bile acid synthesis [20]. In this study, gut microbiota depletion significantly decreased the expression of *Cyp2b10* and *Cyp3a11* (Fig. 3B), which is consistent with the altered bile acid profile. Interestingly, the KEGG enrichment analysis also showed gut microbiota depletion-induced alterations in genes involved in retinol metabolism and primary bile acid biosynthesis in obese mice (Fig. 3C). These data led us to investigate the role of retinol metabolism signaling in lipid metabolism.

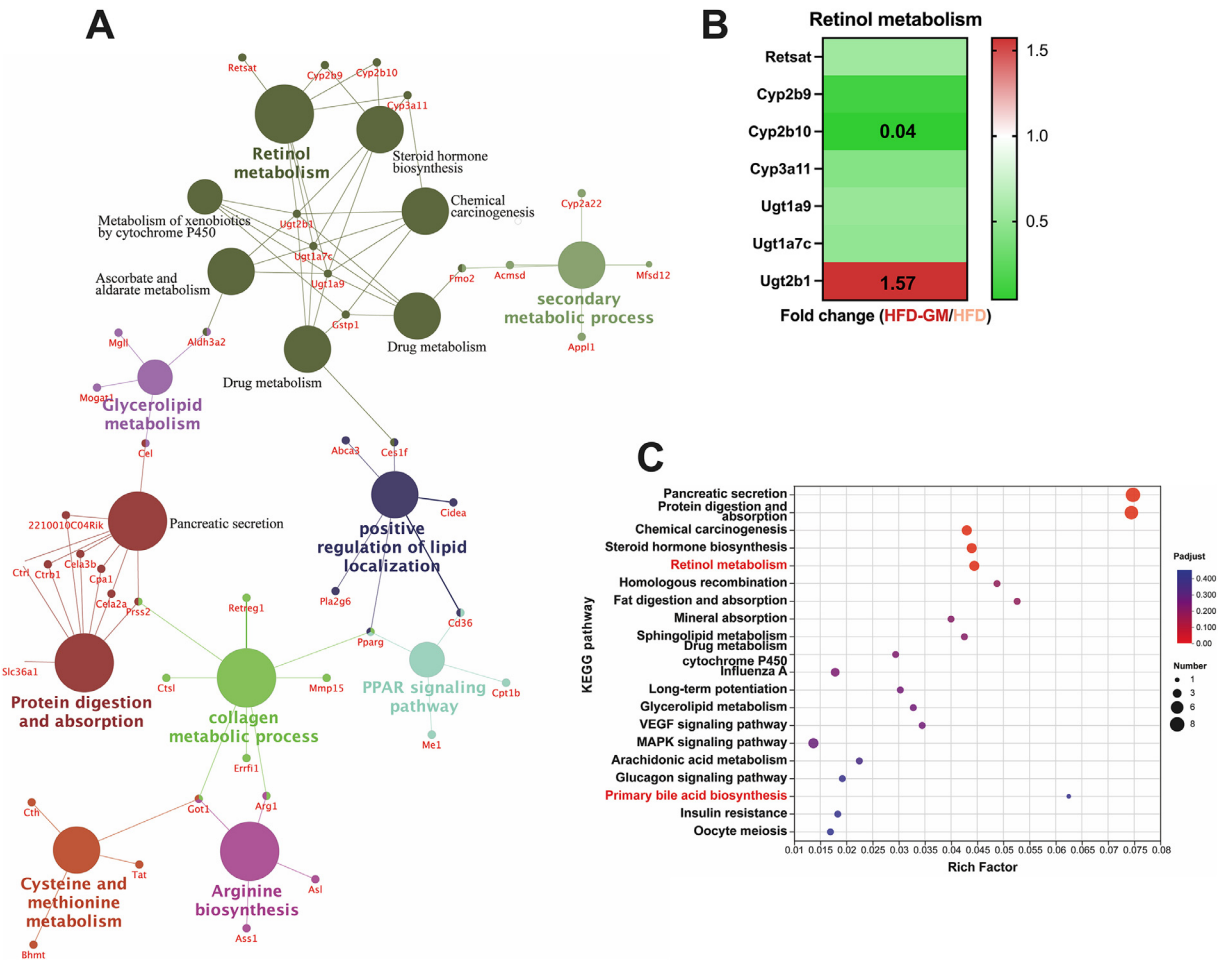

Fig. 3. Gut microbiota depletion altered the expression of genes involved in retinol metabolism. (A) Functionally organized networks generated by Cytoscape based on the differential genes between HFD and HFD-GM groups; (B) Differential genes related to retinol and bile acid metabolism between mice with and without gut microbiota depletion when fed with HFD; (C) Go analysis of differential genes that were altered by gut microbiota depletion in HFD-fed mice; (D) KEGG analysis of differential genes that were altered by gut microbiota depletion in HFD-fed mice.

3.4. atRA supplementation attenuated the adiposity in obese mice

Next, we questioned whether gut microbiota exerts its effects on fat deposition by altering retinol metabolism signaling. It has been reported that atRA is a bioactive metabolite of retinol that regulates numerous physiological processes [21,22]. In this study, mice were supplemented with atRA to clarify the role of retinol metabolism signaling in lipid metabolism (Fig. 4A). Compared with the chow diet, HFD induced increased body weight from the 1st week ($P < .05$; Fig. 4B and C). Meanwhile, atRA treatment for 2 weeks significantly reduced the body weight in mice fed with chow diet ($P < .05$; Fig. 4B and C). 1-week atRA supplementation significantly reduced the body weight in HFD-fed mice ($P < .05$; Fig. 4B and C). The energy intake of mice was also measured after atRA treatment. As shown in Fig. 4D, mice in the HFD group had significantly higher energy intake than those in the Chow group ($P < .05$; Fig. 4D). However, there was no significant difference in energy consumption between the HFD-fed mice with and without atRA supplementation, suggesting that the effect of atRA on body weight was independent of the change in energy intake ($P < .05$; Fig. 4D).

To clarify whether the reduced body weight induced by atRA treatment was due to the inhibited accumulation of adipose tissue, we next measured the WAT weight. The results showed that the ratios of the mass of iWAT, eWAT, and pWAT to the body weight were significantly higher in HFD-fed mice compared to those in mice fed with chow diet ($P < .05$; Fig. 4E and F). However, atRA supplementation markedly lowered the indices of iWAT, eWAT, and pWAT in obese mice, but not those in the mice fed with chow diet ($P < .05$; Fig. 4E and F).

These data suggest that atRA can alleviate obesity, further revealing that altered retinol metabolism signaling may be partially responsible for the reduced WAT accumulation in obese mice after gut microbiota depletion.

3.5. atRA altered the gut microbiota in obese mice

To investigate the association between the atRA and gut microbiota, we analyzed the microbiota with 16S rRNA gene sequencing in ileal content from the mice. Based on quality control, in total, clean tags were clustered into OTU based on 97% identity the sobs, Shannon, Ace, and Chao1 indices were used to calculate α-diversity. As shown in Fig. 5A., compared with the chow diet, HFD had no significant effects on the sobs, Shannon, Ace, and Chao1 indices ($P > .05$; Fig. 5A). atRA supplementation significantly increased the Shannon index in mice fed with chow diet ($P < .05$; Fig. 5A). Meanwhile atRA supplementation significantly increased the Sobs and Shannon indices in obese mice ($P < .05$; Fig. 5A). Fur-

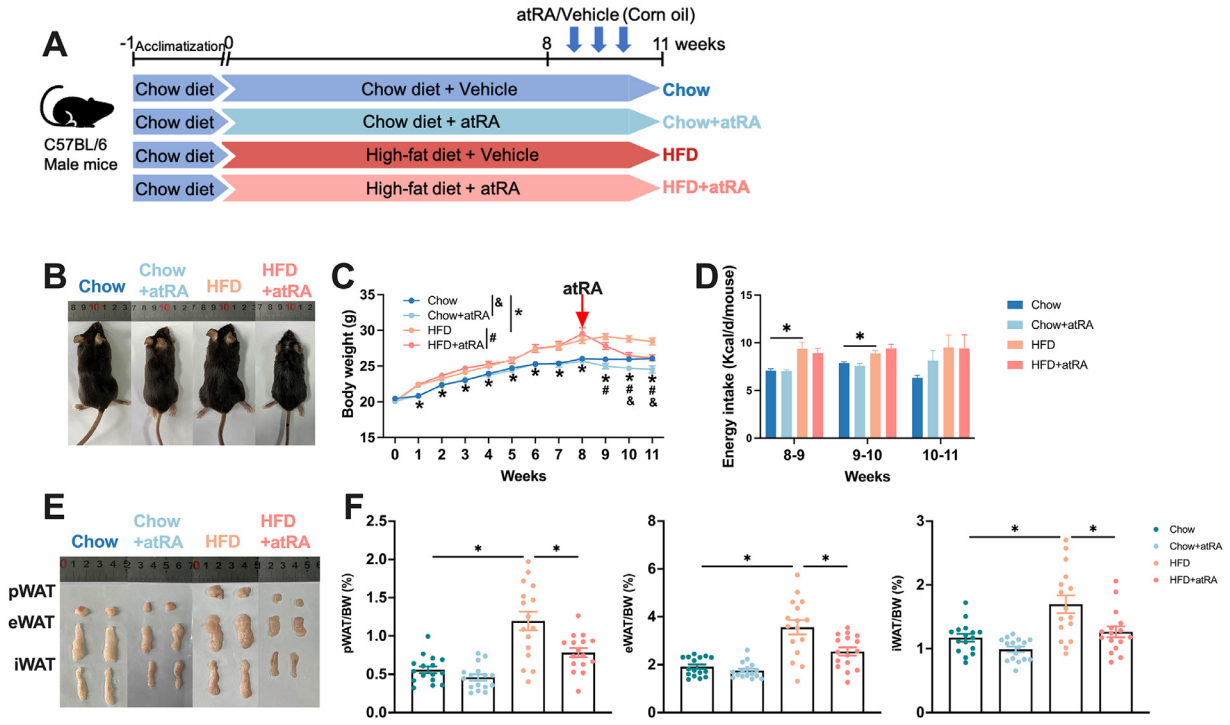

Fig. 4. atRA supplementation reduced the body weight and WAT accumulation in HFD-fed mice. (A) Experimental scheme; (B) Representative mice in the Chow, Chow+atRA, HFD, and HFD+atRA groups; (C) Body weight; (D) Energy intake; (E) Representative pictures for WAT in mice from the Chow, Chow+atRA, HFD, and HFD+atRA groups; (F) The ratios of pWAT to body weight, eWAT, and iWAT;. Data were expressed as the mean±SEM. $^{\&}P < .05$: Chow+atRA vs. Chow; $^{*}P < .05$: HFD vs. Chow; $^{\#}P < .05$: HFD+atRA vs. HFD.

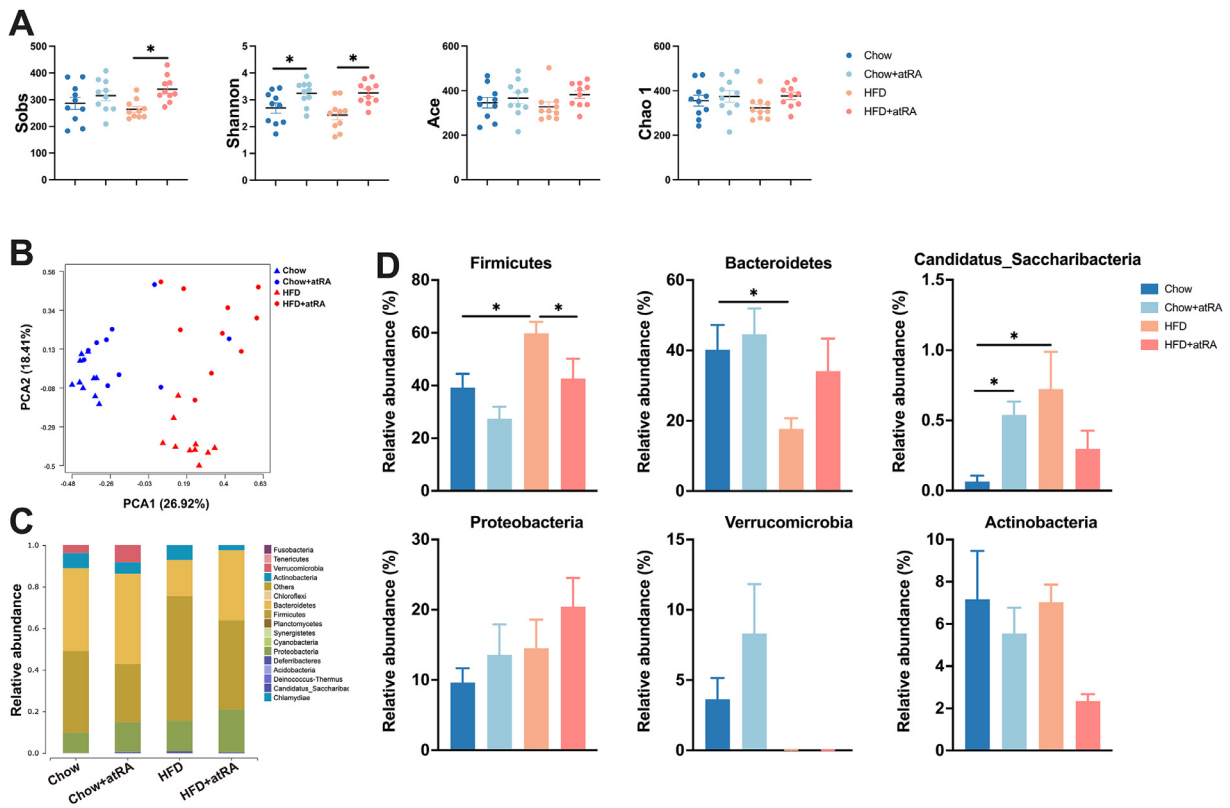

Fig. 5. atRA supplementation increased the α-diversity and changed the composition of ileal microbiota in HFD-fed mice. (A) α-diversity; (B) β-diversity with PCA and NMDS score plots for discriminating the ileal micorbiota from Chow, Chow+atRA, HFD, and HFD+atRA groups; (C and D) Bacterial taxonomic profiling at the phylum level of ileal microbiota from different groups. Data were expressed as the mean±SEM. $^{*}P < .05$.

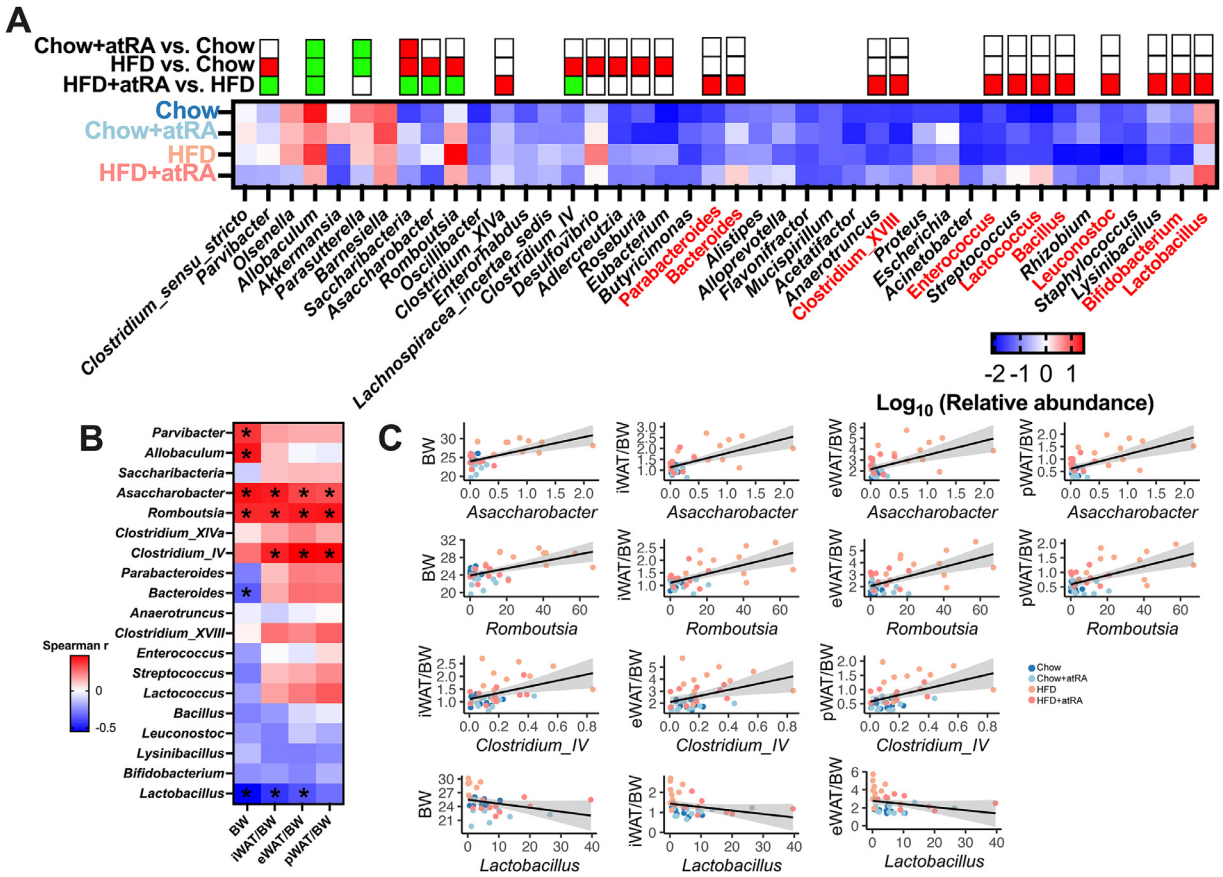

Fig. 6. atRA supplementation changed the microbial genus asoicated with body weight and the rations of WAT to body weight in obese mice. (A) Bacterial taxonomic profiling at the genus level of ileal microbiota from different groups; (B) Heatmap of Spearman correlation between the gut microbial population and body weight and the indices of WAT; (C) Scatter plot with a linear regression showing the association between gut microbiota and the body weight and indices of WAT. Data were expressed as the mean±SEM. $^{*}P < .05$ were significantly negative (green) or positive (red) Spearman correlation.

thermore, β-diversity was calculated to generate PCA at the OTU level. The results showed that the clustering of the microbial community in Chow, Chow+atRA, HFD, and HFD+atRA groups were clearly separated (Fig. 5B).

We next investigated the specific changes in gut microbial composition at different levels. At the phylum level, atRA supplementation significantly increased the relative abundance of Candidatus_Saccharibacteria in mice fed with chow diet ($P < .05$; Fig. 5C and D). Compared with those in the Chow group, the relative abundances of Firmicutes and Candidatus_Saccharibacteria were significantly higher, but the relative abundance of Bacteroidetes was significantly lower in the HFD group ($P < .05$; Fig. 5C and D). However, atRA supplementation significantly reduced the relative abundance of Firmicutes in obese mice ($P < .05$; Fig. 5C and D). At the genus level, atRA supplementation significantly increased the relative abundance of *Saccharibacteria*, but significantly decreased the relative abundances of *Allobaculum* and *Parasutterella* in mice fed with chow diet ($P < .05$; Fig. 6A). Compared with the chow diet, HFD significantly increased the relative abundances of *Parvibacter, Saccharibacteria, Asaccharobacter, Romboutsia, Clostridium_IV*, which were restored by atRA treatment ($P < .05$; Fig. 6A). Additionally, compared with those in mice fed with chow diet, the relative abundances of *Desulfovibrio, Adlercreutzia, Roseburia*, and *Eubacterium* were significantly higher, whereas the relative abundances of *Allobaculum* and *Parasutterella* were significantly lower in obese mice ($P < .05$; Fig. 6A). Meanwhile, atRA supplementation significantly increased the relative abundances of *Clostridium_XlVa, Parabacteroides, Bacteroides, Anaerotruncus, Clostridium_XVIII, Enterococcus, Streptococcus, Lactococcus, Bacillus, Leuconostoc, Lysinibacillus, Bifidobacterium*, and *Lactobacillus*, but decreased the relative abundance of *Allobaculum* in obese mice ($P < .05$; Fig. 6A).

To further explore the association between the atRA-altered gut microbiota and WAT accumulation, we detected the correlation between some key genus and body weight and the indices of WAT. The results showed that there were significantly positive correlations between the abundances of *Parvibacter, Allobaculum, Asaccharobacter*, and *Romboutsia* and body weight ($P < .05$; Fig. 6B and C). The abundances of *Asaccharobacter, Romboutsia*, and *Clostridium_IV* were positively correlated with body weight and the indices of WAT ($P < .05$; Fig. 6B and C). However, there were significantly negative correlations between the abundances of *Bacteroides* and *Lactobacillus* and body weight ($P < .05$; Fig. 6B and C). Additionally, the abundance of *Lactobacillus* was negatively correlated with the indices of iWAT and eWAT ($P < .05$; Fig. 6B and C).

Collectively, these data suggest that atRA can alter the gut microbial composition, which might be associated with reduced body weight and inhibited WAT accumulation.

4. Discussion

Obesity is accompanied by the accumulation of WAT. WAT not only displays energy-storing function but also regulates numerous physiological processes that maintain energy balance [23]. It has been reported that the dysregulated accumulation of WAT

contributes to the occurrence and development of numerous metabolic syndromes [24,25]. In this study, we observed gut microbiota depletion decreased the indices of WAT in obese mice, suggesting that gut microbiota mediates WAT accumulation. Hepatic metabolomics and transcriptomics analyses confirmed the role of gut microbiota in modulating lipid metabolism. Interestingly, KEGG analysis and PPI network of RNA-seq results indicated that altered retinol metabolism may be a mechanism by which gut microbiota-mediated fat deposition. Furthermore, atRA (an active metabolite of retinol) supplementation suppressed the WAT accumulation, as well as altered microbial composition in obese mice. Overall, this study demonstrates the bidirectional modulation of retinol metabolism and gut microbiota and this intricate relationship might mediate the WAT accumulation in obese mice.

In this study, we isolated three primary WATs, including iWAT, eWAT, and pWAT to evaluate the respective adipose tissue alterations. We found that gut microbiota depletion reduced the body weight and the indices of WAT in obese mice. Similarly, a previous study showed that Abx-depleted gut microbiota reduced the indices of subcutaneous adipose tissue (sWAT) and eWAT in HFD-fed mice [6]. These data suggest that gut microbiota depletion can inhibit the accumulation of WAT, leading to reduced body weight.

The liver acts as the main organ for lipid metabolism. We also detected the metabolome of the liver to investigate the effects of gut microbiota on hepatic metabolism. In this study, gut microbiota depletion mainly altered the metabolites belonged to carboxylic acids and derivates, fatty acyls, glycerophospholipids, prenol lipids, steroids and steroids derivates, suggesting that gut microbiota depletion influences lipid metabolism. Meanwhile, the KEGG enrichment analysis revealed that the metabolic pathways that differed significantly between Chow and HFD groups were linoleic acid, glycerophospholipid, and amino acid (e.g. alanine, aspartate, glutamate, and arginine) metabolism, which was consistent with a previous study [26]. Additionally, gut microbiota depletion mainly altered the metabolites involved in linoleic acid and taurine metabolism. Notably, our results showed that gut microbiota depletion reduced the concentration of TDCA and DCA in the liver of HFD-fed mice. It has been reported that the primary bile acid, cholic acid (CA), is synthesized and then conjugated with taurine to transform into taurocholic acid (TCA) in the liver of mice [27]. TCA will be transported into the gut, where it can be metabolized by gut microbiota and transformed into CA, DCA, and TDCA, subsequently [27]. The majority of bile acid metabolites derived from gut microbiota will then be re-absorbed by the liver. Thus, we suspected that the decreased TDCA and DCA might because the depletion of bacterial bile acid metabolism. Meanwhile, the increased taurine in the liver indicates reduced bile acid conjugation, which might be a result of the reduced microbial bacterial deconjugation in the intestine of mice with gut microbiota depletion. Consistently, a previous study using GF mice showed that the absence of gut microbiota enhanced hepatic taurine levels [28]. Furthermore, it has been reported that bile acids play a critical role in regulating lipid metabolism [29]. Collectively, these data further confirm the contribution of gut microbiota in modulating lipid metabolism and fat deposition.

Additionally, hepatic RNA-seq analysis showed that gut microbiota depletion significantly altered the genes related to retinol metabolism, including *Retsat, Cyp2b9, Cyp2b10*, *Cyp3a11, Ugt1a9, Ugt1a7c*, and *Ugt2b1*. RetSat, expressed in metabolically active organs (i.e., liver and WAT), is responsible for catalyzing the saturation of retinol [30]. A previous study showed that *Cyp2b* and *Cyp3a* were involved in atRA metabolism [31]. Additionally, *Ugt1a7* and *Ugt1a9* were shown to be involved in the 13-cis retinoic acid metabolism [32]. Moreover, it has been postulated that gut microbiota plays multiple roles in regulating retinol metabolism. For example, some bacteria strains can encode the proteins involved in retinol and retinoic acid metabolism [33]. In addition, gut microbiota influences the metabolism of carotenoids and retinoid absorption and metabolism via producing secondary bile acids [13,34]. Thus, it is reasonable to speculate that the depletion of gut microbiota was able to alter the retinol metabolism in this study. Furthermore, previous studies have revealed the association between retinol metabolism and obesity, although the relationship is still unclear [35]. Collectively, these data indicate that altered retinol metabolism may be proposed as a mechanism by which gut microbiota mediates lipid metabolism.

Retinol is the bioactive form of vitamin A [34]. It has been reported that after absorption, vitamin A (retinol) can be transported to the liver and other organs with chylomicrons as retinyl ester. Retinol can be further converted into retinoic acid, a ligand for nuclear retinoic acid receptors that regulate numerous physiological processes, including growth, development, and metabolism [35]. Moreover, it has been reported that the adipose tissue is an important primary site for the storage of retinoids and retinoic acid. Furthermore, a previous study using genetically modified mice showed that deficient production of atRA promoted adiposity in mice fed with chow diet [36]. Similarly, an *in vitro* study using human adipose-derived stem cells showed that atRA treatment mediated the adiposity via regulating the apoptosis and differentiation processes [37]. Consistently, our data showed that atRA supplementation reduced body weight and suppressed the accumulation of WAT in obese mice. Taken together, our data suggest that the altered retinol metabolism signaling might be a cause for the inhibited WAT accumulation in mice with gut microbiota depletion.

Previous research reported that vitamin A has the ability to regulate gut microbiota [38], this led us to further test if atRA can modify gut microbiota population. In the present study, atRA supplementation increased the α-diversity of ileal microbiota in mice fed with chow or HFD, suggesting atRA could enhance the species diversity of gut microbiota. At the phylum level, obese mice exhibited a higher relative abundance of Firmicutes and a lower abundance of Bacteroidetes, which is consistent with previous studies [39,40]. However, atRA supplementation decreased the relative abundance of Firmicutes in obese mice. It has been reported that compared with Bacteroidetes, Firmicutes is more effective in extracting energy from diet [41], suggesting that atRA might decrease energy extraction via altering the gut microbiota. Additionally, HFD significantly increased the relative abundance of Candidatus_Saccharibacteria, which was slightly restored by atRA treatment. It has been reported that Candidatus_Saccharibacteria is positively associated with obesity markers, indicating that atRA had beneficial effects on the metabolism in mice fed with HFD [42,43]. Meanwhile, the mice in the HFD and HFD+atRA groups have almost no Verrucomicrobia. Consistently, previous studies showed that HFD reduced the relative abundance of Verrucomicrobia in mice [44,45]. Moreover, it has been reported that there was a negative association between the relative abundance of Verrucomicrobia and obesity status in children [46]. More specifically, in mice fed with chow diet, atRA supplementation significantly increased the relative abundance of *Saccharibacteria,* which has been reported to be associated with the production of retinal from β-carotene [47]. HFD increased the abundance of genus that were shown to be positively correlated with body and WAT weights, such as *Parvibacter* and *Romboutsia* [48,49], which were restored by atRA supplementation. In line with a previous study [50], HFD increased the abundance of *Clostridium_IV*. However, atRA supplementation reduced the abundance of *Clostridium_IV* in obese mice. Moreover, in this study, the correlation analysis also showed that

the abundance of *Romboutsia* and *Clostridium_IV* were positively correlated with the body weight and the indices of WAT. In addition, in obese mice, atRA supplementation increased the abundance of *Parabacteroides, Bacteroides, Clostridium_XVIII, Bifidobacterium, Enterococcus, Bacillus, Leuconostoc, Lactobacillus*, and *Lactococcus* which could improve lipid metabolism and obesity-related metabolic dysfunction [51–60]. Moreover, the correlation analysis showed that the *Bacteroides* and *Lactobacillus* were negatively correlated with the body weight and indices of WAT. It has been reported that *Allobaculum* was increased in the mice with HFD-induced obesity [61]. Our data showed that, in obese mice, atRA supplementation decreased the abundance of *Allobaculum*, which was positively associated with body weight. Collectively, our study demonstrates that atRA treatment increases the abundance of gut microbiota that have beneficial impacts on lipid metabolism, which might, at least partly, be the reason for inhibited WAT accumulation in HFD-fed mice.

In conclusion, our study explored the bidirectional relationship between gut microbiota and retinol metabolism in modulating fat deposition. Our data showed that gut microbiota depletion inhibited the WAT accumulation and altered the retinol metabolism in obese mice, suggesting the potential role of retinol metabolism signaling in gut microbiota-mediated lipid metabolism. Furthermore, supplementation with atRA, an active metabolite of retinol, resulted in suppressed WAT accumulation and promoted the growth of microbiota that contribute to improved lipid metabolism in obese mice. Further work is needed to thoroughly understand the specific role of gut microbiota in retinol metabolism and the exact impact of retinol metabolism on adiposity. Overall, our study identifies retinol metabolism as a mechanism linking gut microbiota and adiposity, providing potential opportunities to lessen the prevalence and consequences of obesity-related metabolic syndrome.

We acknowledged some limitations in our study. First, the sample size in the pseudo-germ-free mice study is indeed small. Initially, we conducted a 5-week experiment with 10 mice per group in the pseudo-germ-free mice study. We found that gut microbiota depletion for 5 weeks significantly reduced body weight and inhibited WAT accumulation [39]. To further validate our results and explore the long-term effects of gut microbiota depletion, we extended the experiment to 10 weeks with six mice per group. The results of the 10-week study, which are consistent with our previous findings, were presented in the current manuscript. Second, additional analysis such as serum biochemical parameters and histological analysis of the adipose tissue, could provide a more comprehensive understanding of the role of gut microbiota and microbiota-mediated retinol metabolism in lipid accumulation. Third, since body weight was measured on a weekly basis, we cannot pinpoint the exact day on which these changes occurred. The data in this study thus reflect the cumulative effect of 1 week of treatment, showing that atRA can significantly influence body weight over this short period. In our future studies, the body weight at shorter intervals, such as daily, should be measured to better capture the dynamics of body weight changes in response to atRA treatment. Final, the interplay between gut microbiota and retinol metabolic signals in regulating fat accumulation needs further investigation. In our future study, the role of the gut microbiota in modulating retinol metabolism could be further confirmed by comparing the differences in retinol metabolism between GF and conventionally raised mice; conducting fecal microbiota transplantation from conventionally raised mice with or without Abx treatment into GF mice. Additionally, to elucidate the specific role of retinol metabolic signals in modulating lipid accumulation, we should generate adipose- or hepatocyte -specific *retinoic acid receptor* (*Rar)* knockout mouse models and figure out whether the deficient retinol metabolic signals could influence lipid accumulation.

Declaration of competing interests

The authors declare that there is no conflicts of interest.

CRediT authorship contribution statement

Hui Han: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Shunfen Zhang:** Investigation, Formal analysis, Conceptualization. **Mengyu Wang:** Investigation, Formal analysis. **Bao Yi:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition. **Yong Zhao:** Investigation, Conceptualization. **Martine Schroyen:** Writing – review & editing, Conceptualization. **Hongfu Zhang:** Writing – review & editing, Supervision, Funding acquisition.

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