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RESEARCH ARTICLE

Vitamin D Alleviates Obesity in Insulin Resistance Rat Model via Adipokine-Gut Microbiome Regulation

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Abstract

Introduction: It was established that vitamin D reduced insulin resistance in prediabetic rats in a model induced by a high-fat, high-glucose diet and low-dose streptozotocin (HFDS). However, it remained unclear whether vitamin D could assist with obesity and dysbiosis in the insulin-resistant rat model induced by HFDS. Materials and Methods: Twenty-four male Wistar rats in the study were randomly divided into two groups. Six rats were fed a standard diet, while 18 were fed an HFDS diet. Once obesity and insulin resistance were established, the 18 HFDS rats were randomly assigned to three treatment groups for 12 weeks: one group received no treatment, another received 100 IU/kg BW of vitamin D3, and the third received 1000 IU/kg BW of vitamin D3. Body weight and the Lee index were assessed before and after treatment. At the end of the experiment, microbiome profiles of colon segments and epididymal fat adipose tissues were analyzed. Results and Discussion: We found that in a rat model induced by HFDS, vitamin D3 supplementation at both doses reduced body weight, the Lee index, and adipose tissue size, and increased the adiponectin/leptin ratio. The richness and relative abundance of gut microbiome richness and composition alongside improvements in obesity profiles. The main species modulated by vitamin D3 supplementation; notably *Lactobacillus* spp. and *Allobaculum* increased, whereas *Blautia* spp. decreased, particularly at the dose of 1000 IU/kg BW. Conclusions: Vitamin D3 supplementation was associated with improvements in obesity-related parameters, accompanied by changes in the adiponectin/leptin ratio and shifts in gut microbiome diversity and species composition.

Keywords: Adipokines, *Allobaculum*, 25-hydroxy-vitamin D, *Lactobacillus*, Lee index

Introduction

Over the past 50 years, global obesity rates have significantly tripled, leading to a widespread and serious public health issue often referred to as an epidemic. This ongoing situation has developed from various factors, including genetic, metabolic, behavioral, and environmental influences. Obesity substantially contributes to the global burden of chronic diseases, resulting in serious consequences that impact

individuals across different age groups and socioeconomic statuses [1,2].

Obesity has been associated with a dietary pattern marked by a high intake of processed carbohydrates and saturated fats. The increased risk is attributed to chronic, low-grade inflammation induced by these nutrients [3]. In various well-established animal models, even a short period of a high-fat, high-sugar diet leads to obesity and insulin resistance [4-6]. While obesity contributes to insulin resistance, evidence suggests vitamin D deficiency may increase fat accumulation and worsen insulin resistance [7,8]. Epidemiological research indicates that the incidence of vitamin D deficiency in obese children is closely associated with adiposity [9,10]. In obesity, lower circulating 25-hydroxyvitamin D is often reported and may reflect reduced bioavailability due to sequestration in adipose tissue and volumetric dilution [11,12].

Furthermore, studies have indicated a probable three-way interaction between vitamin D, gut dysbiosis, and a higher risk of cardiovascular disease [13,14]. In addition, findings revealed that individuals with obesity and hypertension had altered gut microbiomes, potentially influenced partly by vitamin D insufficiency [14-16].

We previously established that vitamin D supplementation improved insulin resistance in prediabetic rats within a model induced by high-fat, high-glucose diets and low-dose streptozotocin [17]. However, it remained unclear whether vitamin D could mitigate issues with obesity and dysbiosis in this insulin-resistant rat model. Therefore, the present study aimed to investigate the effects of vitamin D3 supplementation on obesity in an insulin-resistant rat model, with a focus on the gut microbiome.

Materials and methods

Animal experiment

This study was conducted in accordance with the Animal Experimental Guidelines of the Animal Research Facility, IMERI, Faculty of Medicine, Universitas Indonesia. The Faculty of Medicine Ethics Committee approved the study at Universitas Indonesia, with no. KET-701/ UN2.F1/ETIK/PPM.00.02/2020. The research was conducted on 24 male Wistar rats, which were randomly assigned to two groups: A control group of 6 healthy rats fed a standard diet, and an experimental group of 18 rats exposed to a high fat (TestDiet 58V8), 20% glucose in drinking water and a low dose of streptozotocin (HFDS). In brief, the high-fat, high-glucose regimen was given for 3 weeks, followed by a single intraperitoneal injection of streptozotocin (30 mg/kg BW) to induce an insulin-resistant/prediabetic phenotype, as previously described [17]. As indicated in the previous study, the 18 rats were confirmed to be insulin-resistant, as described in Krisnamurti *et al.* [17]. Once insulin resistance was confirmed, 18 rats given HFDS were assigned randomly to three treatment groups for 12 weeks: A control group receiving no treatment, a group receiving vitamin D3 at a dose of 100 IU/kg body weight, and a group receiving vitamin D3 at a dose of 1,000 IU/kg body weight. Throughout the 12-week treatment period, the experimental rats were fed high-fat, high-glucose diets. Body weight and Lee index were measured at the beginning and end of the treatment period. After 12 weeks of treatment, colon segments were analyzed for their microbiome profiles. Adipose tissues from epididymal fat were collected and quantified for adipokine concentration and microscopically examined after staining with Hematoxylin-Eosin. Metabolic profiles, glucose tolerance test and serum 25-hydroxyvitamin-D3 of the rats before and after vitamin D3 supplementation were presented in the previous manuscript [17].

Leptin and adiponectin assays

For the analysis of leptin and adiponectin concentrations, epididymal adipose tissue was weighed, rinsed in ice-cold phosphate buffered saline, homogenized on ice in lysis buffer containing protease inhibitors. The homogenates were then centrifuged, and the supernatants were used for the analysis of leptin and adiponectin levels using enzyme-linked immunoassay (ELISA) kits according to the manufacturer's instructions. Rat adiponectin and leptin ELISA kits were sourced from MyBioSource (Rat Adiponectin, Cat No. MBS2708313; Leptin, Cat No. MBS761173).

Lee index calculation

The rats' body weight and nasoanal length were measured at the beginning and end of the treatment period. The Lee index was calculated by dividing the

cube root of body weight (in grams) by nasoanal length (in centimeters), then multiplying the result by 1,000.

Microbiota profile analysis from the rats' colon

DNA extraction was conducted on colon segments obtained from the intestines. Following the manufacturer's instructions, the ZymoBIOMICS DNA Miniprep kit was used to extract bacterial DNA. The collected DNA was then stored at –20 °C for microbiome analysis. DNA quantity was measured using the NanoDrop ND-1000 Spectrophotometer from NanoDrop Technologies and the Qubit 2.0 Fluorometer from Life Technologies.

Subsequently, polymerase chain reaction (PCR) amplification was performed on the V3/4 region of the 16S ribosomal RNA (rRNA) using primers specifically designed for this purpose. Afterward, a secondary PCR was conducted to incorporate the index barcode. Then, a quality control assessment of the PCR products was performed, followed by normalization of the library to 4 nM. According to the manufacturer's instructions, the library pooling and sequencing procedures were completed using MiSeq reagent kits (Illumina, San Diego, CA, USA).

Data analysis

The microbiome sequencing data were analyzed with MiSeq Reporter and the Greengenes database. Furthermore, the raw data obtained were subjected to bioinformatic analysis. Operational Taxonomic Units (OTUs) (97% similarity) were constructed using the USEARCH pipeline available at https://www.drive5.com/userach/. These OTUs were compared using the 16S sequence database obtained from the Ribosomal Database Project. After the experiment, an examination was conducted to assess alpha diversity, beta diversity, and OUT-level relative abundances in the samples. The outcomes were presented using the Statistical Analysis of Metagenomic Profiles (STAMP) software.

The differences between groups were analyzed using a one-way ANOVA followed by a post-hoc Tukey test. Differences were considered significant at $p < 0.05$. The graphs shown in the manuscript were created using GraphPad Prism 10.

Results and Discussion

Vitamin D normalized obesity in high-fat, high-glucose, and streptozotocin-induced rats

High-fat, high-glucose diets (HFDS) led to a significant increase in both body weight and Lee index ($p < 0.05$ compared to the normal group). The Lee index of all the rats in the untreated HFDS group was above the obesity cut-off point. We found that Vitamin D treatment at both dosages significantly reduced body weight and the Lee index after high-fat diet and streptozotocin administration ($p < 0.05$ compared to HFDS for 100 and 1,000 IU/kg BW) and was comparable to the healthy group (**Figure 1**).

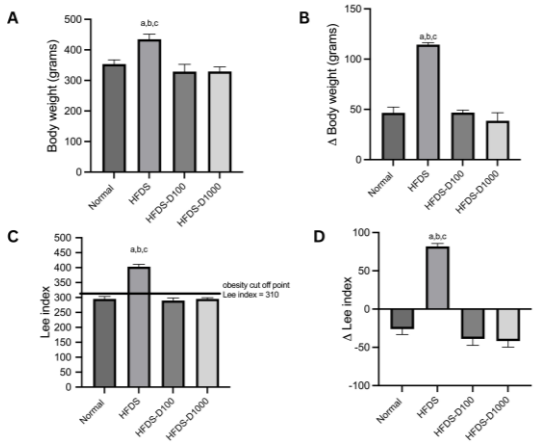

Figure 1 (A) Body weight after 12 weeks of treatment; (B) difference in body weight after and before treatment; (C) Lee index after 12 weeks of treatment; (D) difference in Lee index after and before treatment. HFDS: High-fat, high-glucose, and streptozotocin-induced rats; HFDS-D100: HFDS-induced rats + vitamin D 100 IU/kg BW; HFDS-D1000: HFDS-induced rats + vitamin D 1,000 IU/kg BW. a: $P < 0.05$ vs normal group; b: $P < 0.05$ vs. HFDS-D100; c: $P < 0.05$ vs. HFDS-D1000.

Our results confirmed previous research that high-fat-induced obesity affects vitamin D metabolism, leading to epigenetic modifications in liver vitamin D-metabolizing enzymes and resulting in vitamin D insufficiency. Vitamin D is fat-soluble and is typically absorbed with dietary fat through passive diffusion in the jejunum and ileum. Dietary vitamin D is packaged into chylomicrons with cholesterol, triglycerides, and other lipids within the intestinal wall, and this process depends on microsomal triglyceride transfer protein (MTP). Consequently, dietary vitamin D absorption in the small intestine may be influenced by fat consumption [18-20]. Therefore, supplemental vitamin D3 may be appropriate for obese individuals with excess abdominal fat on a high-fat diet to reduce obesity [19].

Vitamin D increases the adiponectin/leptin ratio by reducing leptin concentrations in the adipocytes

A high-fat, high-glucose diet markedly reduced adiponectin and the adiponectin/leptin ratio ($p < 0.05$). Supplementation with vitamin D at both doses reduced leptin concentration, thereby improving the adiponectin/leptin ratio. However, the reduction did not achieve the expected normal value (**Figure 2**).

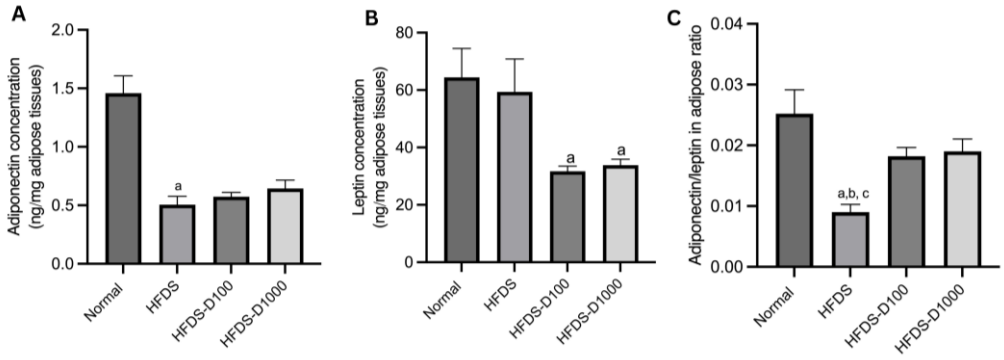

Figure 2 (A) adiponectin concentration; (B) leptin concentration; (C) adiponectin/leptin ratio, quantified 12 weeks after treatment. HFDS: High-fat, high-glucose, and streptozotocin-induced rats; HFDS-D100: HFDS-induced rats + vitamin D 100 IU/kg BW; HFDS-D1000: HFDS-induced rats + vitamin D 1,000 IU/kg BW. a: $P < 0.05$ vs. normal group; b: $P < 0.05$ vs. HFDS-D100; c: $P < 0.05$ vs. HFDS-D1000 HFDS: High-fat, high-glucose, and streptozotocin-induced rats; HFDS-D100: HFDS-induced rats.

Adipokines are considered the link between obesity and insulin resistance. They play active roles in energy balance as well as physiological functions such as immunity and inflammation. Adipokines include leptin, adiponectin, resistin and many others. Adiponectin is an insulin-sensitizing and anti-inflammatory adipokine, while leptin predominantly regulates satiety and energy equilibrium. In obesity, hyperleptinemia and leptin resistance frequently associated with pro-inflammatory signaling [21]. Thus, the adiponectin/leptin ratio has been suggested as an estimate of dysfunctional adipose tissue and cardiometabolic risk [22].

To regulate inflammation and insulin resistance, adipocytes release a range of pro-inflammatory and anti-inflammatory adipokines. The levels of pro-inflammatory adipokines are elevated in obese individuals and animal models, contributing to insulin resistance [23-25]. Vitamin D has been reported to inhibit inflammatory pathways and adipokine synthesis in human adipocytes through the VDR. Increasing vitamin D levels may help alleviate obesity-related metabolic issues by reducing adipose tissue inflammation [26,27].

Vitamin D restores the size of adipocytes in obese rats

Adipocytes in the obese groups were significantly larger than in the normal group. Obesity Vitamin D at 1,000 IU/kg BW resulted in the smallest adipocyte size compared to the healthy group or the treatment with Vitamin D at 100 IU/kg BW (**Figure 3**).

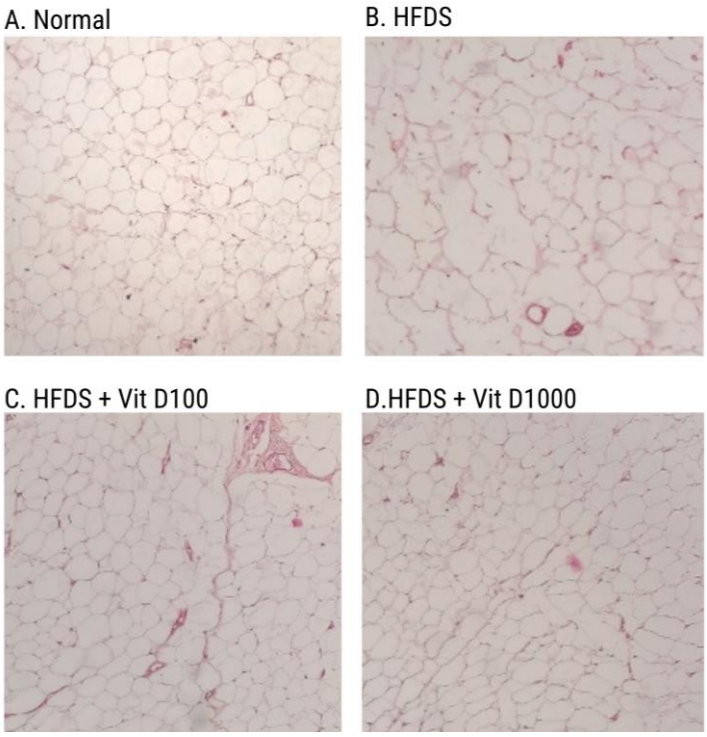

Figure 3 Histology of adipocytes taken from rats' epididymis. Magnification at 10×. HFDS: High-fat, high-glucose, and streptozotocin-induced rats; HFDS-D100: HFDS-induced rats + vitamin D 100 IU/kg BW; HFDS-D1000: HFDS-induced rats + vitamin D 1,000 IU/kg BW.

High-fat diets increase adipocyte size and number, which can contribute to adipose tissue dysfunction, limiting adipose tissue development, altering brown/beige ratios, and promoting irregular lipid deposits and insulin resistance [28]. In obesity, low circulating 25-hydroxyvitamin D has been associated with adipose tissue dysfunction, potentially reducing bioavailability due to the sequestration in adipose tissue [11]. Additionally, research in obese patients showed that impaired adipose tissue function was associated with low levels of vitamin D [29].

In our study, vitamin D3 supplementation was associated with reduced size of adipocytes, lower body Lee index, and a higher adiponectin/leptin ratio, leading to reduced body weight. Mechanistic links to systemic metabolic outcomes can be explained by the involvement of vitamin D in regulating energy metabolism in adipose tissue by influencing fatty acid oxidation, insulin resistance, and adipokine synthesis. Inflammation in adipose tissue can profoundly influence the metabolic problems commonly linked to obesity, while vitamin D may regulate the inflammatory response of immune cells and adipocytes in adipose tissue [30]. However, the links to the metabolic outcomes should be interpreted cautiously, in the absence of markers of adipocyte inflammatory remodeling, such as macrophage infiltration. Infiltration of adipose macrophages has been associated with insulin resistance and vascular endothelial dysfunction in obesity [31].

Vitamin D increases the diversity of the gut microbiome

HFDS has decreased the variety of the gut microbiome, evidenced by reduced richness and Chao1 indexes alongside an increase in the Shannon index. Vitamin D therapy at 100 IU/kg BW and 1,000 IU/kg BW increased sample richness and diversity, as demonstrated by improvements across all three alpha-diversity indicators (**Figure 4**).

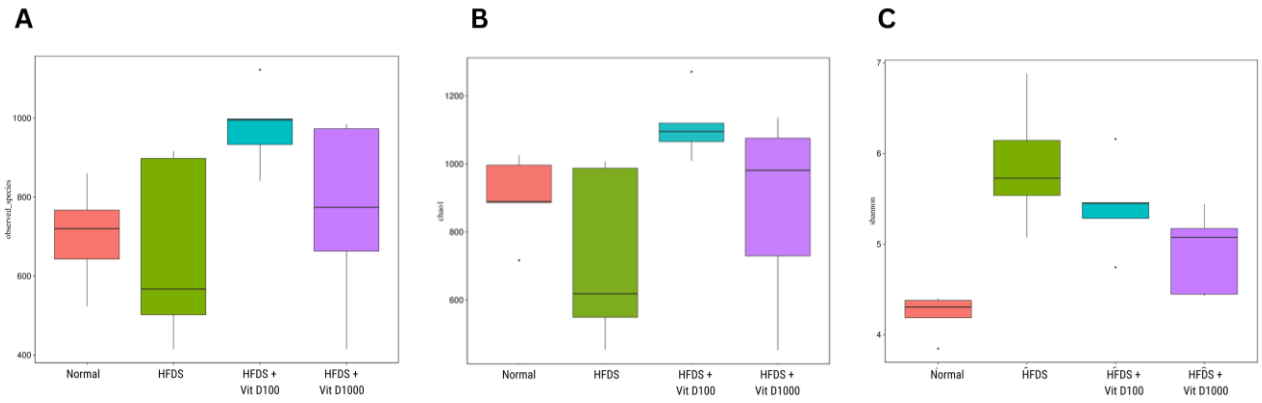

Figure 4 Alpha diversity of gut microbiomes as shown by (A) richness, (B) Chao1 index, and (C) Shannon index. HFDS: High-fat, high-glucose, and streptozotocin-induced rats; HFDS-D100: HFDS-induced rats + vitamin D 100 IU/kg BW; HFDS-D1000: HFDS-induced rats + vitamin D 1,000 IU/kg BW.

Beta diversity is a method used to analyze variations in the composition of microbial populations. These variations were evaluated using coordinate analysis (PcoA) with unweighted UniFrac and cluster analysis following principal component analysis (PCA). As shown in **Figure 5**, PCoA and PCA analyses indicate that administering vitamin D alongside a high-glucose, high-fat diet altered bacterial communities between treatment groups.

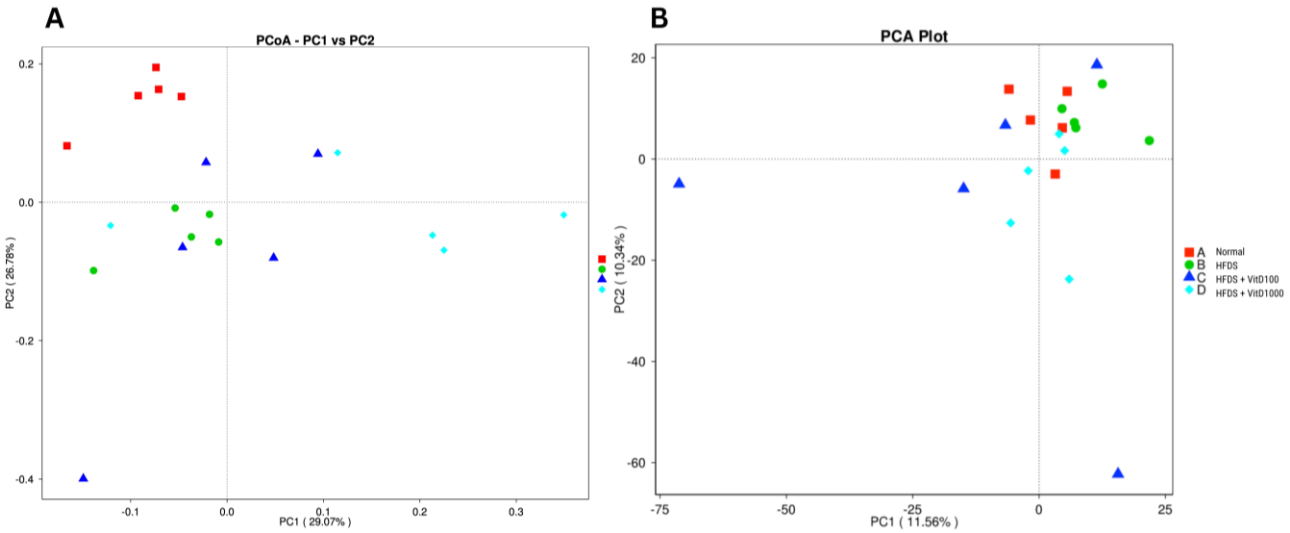

Figure 5 Beta diversity of gut microbiomes as shown by (A) Weighted Unifrac PCoA; (B) Weighted Unifrac PCA. Red points: Normal; green points (HFDS): High-fat, high-glucose, and streptozotocin-induced rats; blue points (HFDS-D100): HFDS-induced rats + vitamin D 100 IU/kg BW; light blue points (HFDS-D1000): HFDS-induced rats + vitamin D 1,000 IU/kg BW.

The microbiota in the human digestive system is essential for the body and comprises trillions of microorganisms. It is abundant in the gut and features thousands of distinct species. Changes in the composition and diversity of the microbiota have been associated with several disorders, including obesity and diabetes [32].

Alpha diversity reflects a sample's richness and diversity, as shown by the richness, Chao1, and Shannon indices. In contrast, beta diversity is a method used to assess variation in microbial population composition

[33]. In this study, high-fat, high-glucose delivery reduced the richness and Chao1 indexes compared to the control groups. In contrast, both levels of vitamin D treatment may enhance the microbial richness index. These findings support a prior study by Chen *et al*. [34] that found a decrease in the richness index in the prediabetic rat group [34]. Additionally, Ibrahim *et al*. [35] found a reduction in alpha diversity, as indicated by both the richness and Chao1 indices, in diabetic and obese rats compared to the control group [35].

Studies have shown that obesity-related gut microbiomes exhibit reduced diversity and altered species- or genus-level composition [36,37] Chen *et al*. [34] demonstrated that changes in alpha and beta diversity in diabetic rats induced by a high-fat diet may affect gut microbiota composition and the early stages of diabetes development [34] In the present study, the impact of high-fat, high-glucose administration also alters beta diversity, resulting in variations in the bacterial communities observed through PCoA analysis. These findings support recent work by Shan *et al*. [38] which identified that feeding prediabetic rats a high-fat diet led to distinct cluster regions in the Weighted UniFrac analysis [38].

Vitamin D modified the composition of the microbiome at the phylum and genus levels

A high-fat, high-glucose diet altered the intestinal microbiota composition at phylum and genus levels, particularly reducing *Blautia*, *Ruminococcus*, *Fusicatenibacter*, and *Allobaculum*. Compared to obese rats, vitamin D administration increased *Lactobacillus*, *Romboutsia*, and *Duboisiella* while decreasing *Fusicatenibacter*, UCG-005, and *Ruminococcus* (**Figure 6**).

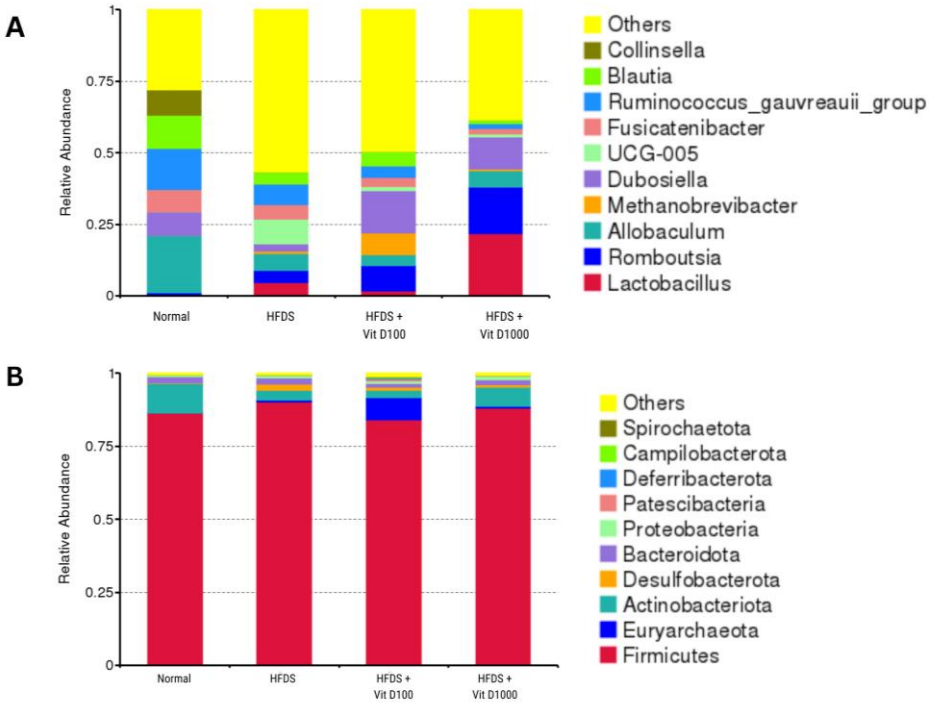

Figure 6 Proportion of gut microbiome in (A) phylum level; (B) genus level. HFDS: High-fat, high-glucose, and streptozotocin-induced rats; HFDS-D100: HFDS-induced rats + vitamin D 100 IU/kg BW; HFDS-D1000: HFDS-induced rats + vitamin D 1,000 IU/kg BW.

Compared with the healthy control group, HFDS treatment reduced the abundance of the *Actinobacteria* phylum. Previous studies have shown a decline in this phylum in people with obesity and type 2 diabetes. The *Actinobacteria* phylum has been linked to a lower incidence of obesity and diabetes [37,39]. *Actinobacteria* are gram-positive bacteria that produce inhibitor enzymes, such as amylase inhibitors,

associated with the etiology of obesity in diabetes [37,40]. A Study by Mandal demonstrated that administering 1,000 IU of vitamin D may boost the *Actinobacteria* phylum [40].

In the present study, vitamin D supplementation at 1,000 IU/kg BW was associated with higher *Lactobacillus* abundance compared with the HFDS groups. Prior studies aligned have reported associations between vitamin D status/vitamin receptor signaling and *Lactobacillus* abundance, suggesting that vitamin D-related immune pathways may influence this genus. Vitamin D supplementation may lower TLR2 and TLR4 expression, promoting *Lactobacillus*, enhancing glycemic response, strengthening the integrity of the intestinal barrier, and limiting bacterial translocation to the systemic circulation. This translocation could increase pro-inflammatory cytokines that contribute to the development of obesity and diabetes [41].

Our results indicate that *Allobaculum* abundance was lower in the obese rats than in healthy controls, while vitamin D supplementation resulted in minimal change. However, the response of *Allobaculum* to metabolic disturbances and vitamin D supplementation appears to be highly model-dependent [35,42]. A study by Q Ma *et al*. [42] showed that the quantity of *Allobaculum* in diabetic rat models was lower than predicted [42]. This varies from another research by Ibrahim *et al.* [35] which found that diabetic rats show a rise in *Allobaculum*, whereas obese rats do not [35]. A recent study by Lee *et al*. [43] indicates that vitamin D administration may enhance the number of *Allobaculum* in cirrhotic rats [43]. These discrepancies suggest that *Allobaculum* may not serve as a universal biomarker of metabolic improvement but rather reflects context-specific host-microbiome interactions**,** influenced by disease state, dietary composition, and inflammatory milieu [35,38]. *Allobaculum* is a beneficial bacterium found in the intestines of mice that may help reduce weight gain and produce short-chain fatty acids with anti-inflammatory properties by stimulating the release of butyrate and mucus in the gastrointestinal system [35].

Our study further identified a reduction in *Blautia* in obese rats compared to healthy rats. Treating obese rats with vitamin D3 at 1,000 IU/kg BW reduces the number of *Blautia*. Our findings are consistent with previous research that observed a reduction in *Blautia* in individuals with high vitamin D levels [44].

Blautia are gram-positive bacteria belonging to the phylum *Firmicutes*. In humans, these bacteria are predominant in hyperglycemia and play an essential role in glucose metabolism by converting acetate, lactate, hydrogen, ethanol, and succinate in the gut [45]. Obesity reduces *Blautia* and elevates pro-inflammatory cytokines associated with TLR2 and TLR4 [46].

Although several studies have reported that *Blautia* spp. are associated with beneficial metabolic outcomes, including lower body weight, reduced fat accumulation, and improved lipid profiles, recent evidence indicates that *Blautia*'s metabolic role is highly context-dependent and influenced by host metabolic status, dietary composition, and glycemic status [46,47]. In metabolically impaired conditions such as obesity and insulin resistance, altered *Blautia* abundance has been associated with intestinal inflammation and dysregulated glucose metabolism [47,48]. Therefore, the reduction in *Blautia* observed following vitamin D3 supplementation at 1,000 IU/kg BW in the present study may reflect normalization of gut microbial composition rather than a detrimental effect. This microbial shift was accompanied by reduced body weight, smaller adipocyte size, and an improved adiponectin/leptin ratio, supporting a beneficial metabolic impact of vitamin D-mediated microbiome modulation [45].

In our study, vitamin D supplementation at both doses lowered the quantity of *Ruminococcus*. This is consistent with human research, showing that vitamin D administration reduces the amount of *Ruminococcus* [44]. Another study found that increased *Ruminococcus* in obese rats was associated with propionate metabolism and the development [47]. Furthermore, the level of UCG-005 in the vitamin D groups was lower than in the obese group. The results contrasted with prior research, which found a reduction in UCG-005 in diabetic patients. UCG-005 is claimed to be a beneficial gut bacterium that inhibits the development of diabetes and increases the synthesis of short-chain fatty acids with anti-inflammatory properties [48].

Although improvements in obesity parameters, adipokine profiles, and gut microbiome composition were observed concurrently following vitamin D supplementation, the present study does not allow causal

inference regarding the directionality or mechanistic hierarchy of these associations. The observed microbiome shifts may represent downstream effects, parallel adaptations, or bidirectional interactions with host metabolic changes rather than primary drivers of obesity improvement [33,38].

Our study has several limitations. Microbiome analysis was restricted to colon samples and did not encompass the entire gastrointestinal tract, which may not fully capture overall gut microbiome variability. In addition, the experimental model lacked heterogeneity in sex and age, potentially underestimating the biological complexity of human physiological systems. Sex differences are well-described in metabolic regulation and gut microbiome composition [49], therefore, extrapolation from male-only data should be cautious and future work should include both sexes.

In addition, the comprehensive metabolic panels (fasting glucose, insulin, HOMA IR, lipids) as well as serum 25-hydroxyvitamin D3 concentrations, which has been presented in our previous results, were not correlated with the present results, thus limiting direct inference about systemic vitamin D and metabolic outcomes with adipokine/microbiome findings.

Furthermore, the cross-sectional, endpoint-based design precludes the establishment of causal relationships between vitamin D supplementation, adipokine modulation, gut microbiome alterations, and obesity-related outcomes, as the observed associations may reflect parallel or downstream effects rather than direct mechanistic links. Methodologically, although OTU-based clustering was employed in the present study, ASV-based pipelines such as DADA2 or Deblur offer higher taxonomic resolution and improved reproducibility. Both OTU-based and ASV-based pipelines are considered appropriate depending on study objectives and data characteristics. In the present study, the OTU-based approach was suitable for genus-level ecological analyses, and given the modest sample size, higher sequence resolution may increase sparsity without substantially altering biological interpretation [50,51]. Future studies incorporating longitudinal designs, larger and more diverse cohorts, whole-gastrointestinal-tract sampling, and mechanistic approaches such as microbiota transplantation or targeted microbial manipulation are warranted to delineate causal pathways and to better define microbial signatures relevant to vitamin D supplementation and human obesity.

Conclusions

Vitamin D3 supplementation was associated with improvements in obesity-related parameters, accompanied by changes in the adiponectin/leptin ratio and shifts in gut microbiome diversity and species composition. These findings support a potential interaction between vitamin D status, host metabolic signaling, and the gut microbiome, which warrants further mechanistic investigation. Another study is needed to comprehensively explore the interactions between host and gut microbial ecosystems and potential comorbidities in insulin-resistant conditions. Lastly, validation of these findings in obese individuals experiencing insulin resistance is a must before formal implementation in humans.

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Declaration of generative AI in scientific writing

No content in the present manuscript was created using generative AI. We used Grammarly to improve the readability and language of a manuscript. All authors remain fully responsible for the authenticity of the content.

CRediT author statement

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