RESEARCH ARTICLE

Intermittent Parathyroid Hormone Alters Gut Microbiota in Ovariectomized Osteoporotic Rats

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Objective: To investigate the effect of intermittent parathyroid hormone (PTH) on gut microbiota (GM) in ovariectomized (OVX) osteoporotic rats.

Methods: Thirty female Sprague–Dawley rats were divided into three groups: sham-operation (SHAM) group, OVX group and PTH treatment group. After 3 months of treatment, the femurs, serum and feces were acquired for micro-CT, biochemical analysis and 16S rRNA sequencing, respectively. For 16S rRNA sequencing, after raw reads filtrated and chimera sequences removed, the clean reads were obtained. According to these clean reads, the operational taxonomic units (OTUs) were clustered. Venn diagram analysis was conducted to explore common and unique GM among the three groups. The α-diversity analysis including Shannon and Simpson indexes were used to evaluate the richness and diversity of the GM. The β-diversity analysis was performed to estimate the structure of GM. The metabolic function was predicted by Tax4Fun analysis.

Results: With micro-CT and biochemical analysis, significant improvements were found in the PTH group compared with the OVX group. In Venn diagram analysis, more unique OTUs were found in the SHAM and PTH groups than the OVX group. According to the rank abundance curve, the SHAM and PTH groups had similar richness and evenness, which were higher than the OVX group. Simpson and Shannon indexes were higher in the SHAM and PTH groups compared with the OVX group, indicating that the SHAM and PTH groups had higher microbiota complexity than the OVX group. In β-diversity analysis, apparent separation was found in the OVX group from the PTH and SHAM groups, which suggested that osteoporosis is the critical factor influencing the GM composition and PTH treatment and can restore the structure of GM. Compared with the OVX group, treatment with PTH increased the abundances of GM which were reported to increase bone mass, such as *Lactobacillus_reuteri*, *Muribaculaceae*, *Ruminococcaceae*, and *Clostridia*, and inhibited the relative abundance of *Rikenellaceae*, which was reported to be potentially related to osteoporosis. GM function analysis showed that PTH could promote butyrate synthesis. In Tax4Fun analysis, the function of butanoate metabolism is more vital in the PTH group than the OVX and SHAM groups, suggesting PTH treatment could regulate microbial metabolic function, including butanoate metabolism.

Conclusion: Intermittent PTH can interact with GM through increasing the abundance of probiotics and reducing the abundance of the pathogenic bacteria to enhance the bone mass.

Key words: Bone metabolism; Butyrate; Gut microbiota; Parathyroid hormone; Postmenopausal osteoporosis

Introduction

Osteoporosis, a metabolic bone disorder characterized by reduced bone mass and disruption of bone architecture, leads to significant morbidity and mortality for patients.[1] It affects more than 10 million people and accounts for over 2 million bone breaks in the U.S.[2] A large amount of human and financial resources need to be invested in osteoporosis associated treatment and nursing, bringing huge economic burden to patients and society. By 2025, it is estimated that fragile fractures and costs are increasing by 50% per year to more than 3 million and 25 billion, respectively.[3,4] Therefore, intervention to prevent osteoporosis is particularly critical.

Postmenopausal osteoporosis (PMOP) is a common type of osteoporosis. After menopause, the serum estradiol levels drop by 85% to 90%.[5] With the decrease of estrogen levels, bone remodeling rates increase by two to four times. Increased bone resorption can lead to bone remodeling imbalances, causing calcium from the bone to escape into extracellular fluid, resulting in bone loss.[5] The animal model is an effective measure to mimic osteoporosis and evaluate therapeutic efficacy, and the most commonly used model is ovariectomized (OVX) rat model. The US Food and Drug Administration (FDA) pointed out that the OVX rat model is a good model for studying postmenopausal osteoporosis.[6] The OVX model can simulate bone loss caused by estrogen deficiency and show clinical manifestations of postmenopausal osteoporosis.[7] In addition, currently commonly used therapeutic drugs for osteoporosis have been studied in ovariectomized rat models.[8–11]

Recent years, the "gut-bone axis" theory has been proposed, which has contributed to the exploration of bone metabolization.[12] The homeostasis of gut microbiota (GM) has been regarded as a key regulator of bone metabolic function.[13,14] The trillions of microbes in the gastrointestinal tract are called the second gene pool of the human body containing about 150-fold more genes than the human genome.[15] GM can regularize synthesis and decomposition of bone by influencing host immune status, synthesis of short-chain fatty acids, and endocrine milieu.[16–19] Jia et al. found that berberine can improve periodontal bone loss induced by estrogen-deficiency via modulating GM.[20] Xie et al. found the effect of neuropeptide Y1 receptor antagonist in GM and concluded it is a potential therapeutic strategy for osteoporosis.[21]

Parathyroid hormone (PTH) is a key endocrine regulator of extracellular calcium and phosphate levels.[22] When continuous administration of PTH (cPTH), it stimulates bone resorption greater and promotes bone formation lesser, leading to net loss of bone.[23] In contrast, when injected once daily, intermittent PTH (iPTH) 1–34 can increase bone formation and enhance bone mass, and is an FDA-approved pattern for osteoporosis.[24,25] It could reduce the risk of fracture through promoting bone formation.[26–28] In addition, for PMOP patients with Kümmell's disease, PTH could prevent progressive vertebral collapse.[29] Recently, Li et al. found that PTH could increase bone mass in conventionally housed mice. However, a similar effect was not found in germ-free mice. Then, they made the conclusion that the butyrate is essential for PTH to induce bone anabolism.[30]

Although the pivotal role of GM in iPTH-associated bone formation was explored, the effect of iPTH on GM composition in osteoporosis has not been studied. In this study, we hypothesize that intermittent treatment with PTH could enhance the bone mass in OVX rats through regulating GM. Therefore, the aim of the present study was to investigate whether iPTH can influence GM in OVX rats and explore a new perspective for PTH mechanism in osteoporosis.

Methods

Animals

The current study obtained permission by the Animal Care and Use Committee of Tianjin Medical University General Hospital (IRB-2021-DWFL-329). Thirty six-month-old female Sprague–Dawley rats (n = 30) were housed in an animal lab with a temperature of 20–24 °C, 50% relative humidity, 12 h' light cycle, and food and water were available *ad libitum*. After 1 week of adaptive feeding, the rats were randomly divided into three groups (n = 10 for each group): (i) the rats received sham-operation (SHAM group); (ii) the rats received OVX surgery (OVX group); and (iii) the rats received OVX surgery and treated by PTH (subcutaneously, 30 μg/kg/day) (PTH group). Twelve weeks following SHAM or OVX surgery, placebo or PTH was administered to rats once daily for 3 months. Then, the femurs were acquired for micro-CT scanning to evaluate bone quality and the feces were collected for 16S rRNA sequencing.

Micro-CT Analysis

The femur specimens were vertically fixed in the sample fixator along the long axis to analyze the micro-CT imaging. Three-dimensional CT scans of rat femur specimens were reconstructed under the same conditions using Skyscan-1174 micro-CT (Bruker, Kartuizersweg, Belgium). The following data were analyzed: bone mineral density (BMD, g/cm^3), trabecular number (Tb.N; mm^{-1}), trabecular thickness (Tb.Th; mm), trabecular separation (Tb.Sp; mm), percentage of bone volume fraction (BV/TV; %) and structural model index (SMI).

Biochemical Analysis

Serum levels of BTMs (bone turnover markers) were measured by ELISA kits (AMEKO, Shanghai, China), including bone alkaline phosphatase (BALP), osteocalcin (OC) and tartrate-resistant acid phosphatase 5b (TRACP-5b). In addition, butyric acid in the feces was also determined by a high-performance liquid chromatography (HPLC) system (model 1260; Agilent).

Extraction of Bacterial DNA

CTAB/SDS method was used to extract total genome DNA from the samples. DNA concentration and purity were

detected on 1% agarose gels. According to the concentration, DNA was diluted to 1 ng/μL by sterile water.

16S rRNA High-throughput Sequencing

Sequencing library was constructed through Ion Plus Fragment Library Kit 48 rxns (Thermo Scientific, Waltham, MA, USA) and the quality was evaluated by Qubit 2.0

Fluorometer (Thermo Scientific). Then, Ion S5TM XL platform was used for library sequencing.

Data Analysis

The V4 region was amplified through custom barcoded primers. Raw reads were filtrated through Cutadapt software, and then the reads were compared with the Silva database
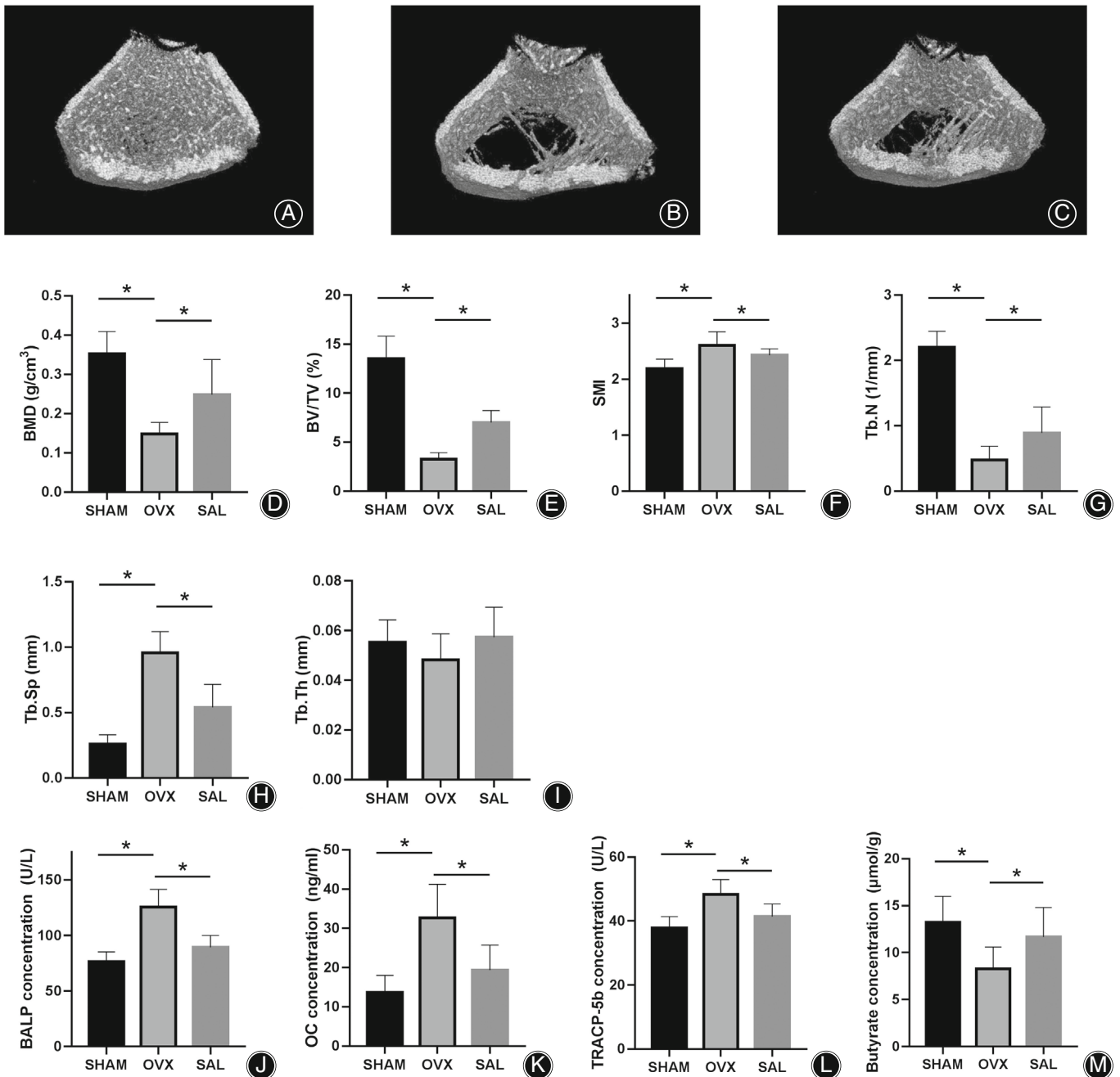

Fig. 1 Intermittent PTH improved bone mass, bone turnover markers and butyrate in OVX rats. (A–C) Representative micro-CT images of femurs; (D–I) Comparisons of the BMD, BV/TV, SMI, Tb.N, Tb.Sp, and Tb.Th among the three groups; (J-L) Comparisons of the serum BALP, OC AND TRACP-5b among the three groups; (M) Comparisons of the concentration of butyrate in feces among the three groups. *P < 0.05

for removing the chimera sequences. Finally, the clean reads were obtained. Clean reads were spliced into tags and the tags were clustered into operational taxonomic units (OTUs) with at least 97% similarity through Uparse software. The α-diversity analysis including Shannon and Simpson indexes were conducted to evaluate the richness and diversity of GM. The β-diversity analysis was performed through principal coordinate analysis (PCoA) plots by QIIME software. Furthermore, the metabolic function was predicted by Tax4Fun analysis through Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Statistical Analysis

The data are presented as the means ± standard deviation (SD) and analyzed by SPSS 25.0 (IBM, Armonk, NY, USA). One-way analysis of variance (ANOVA) was conducted to compare the difference among the three groups. Data visualization was performed through R software (Version 3.5.0, R Foundation for Statistical Computing, Vienna, Austria). A P-value less than 0.05 was considered significant.

Results

iPTH Attenuates Bone Loss in OVX Rats

The femurs of rats were analyzed through micro-CT, and representative images were shown in Fig 1A–C. As expected, the trabecular bone was thinner in OVX group, and the OVX group exhibited significant reductions of BMD, BV/TV, Tb.N, and increases of Tb.Sp and SMI. These data were all significantly improved in PTH group except for Tb.Th (Fig 1D–I).

Biochemical Analysis

For biochemical analysis, higher BALP, OC and TRACP-5b were found in OVX group compared with SHAM group. After PTH treatment, significant improvement of BALP, OC and TRACP-5b were detected (Fig 1J–L). For butyrate in feces, lower concentration of butyrate was found in OVX group compared with SHAM group. However, higher concentration of butyric acid was determined in PTH group (Fig. 1M).
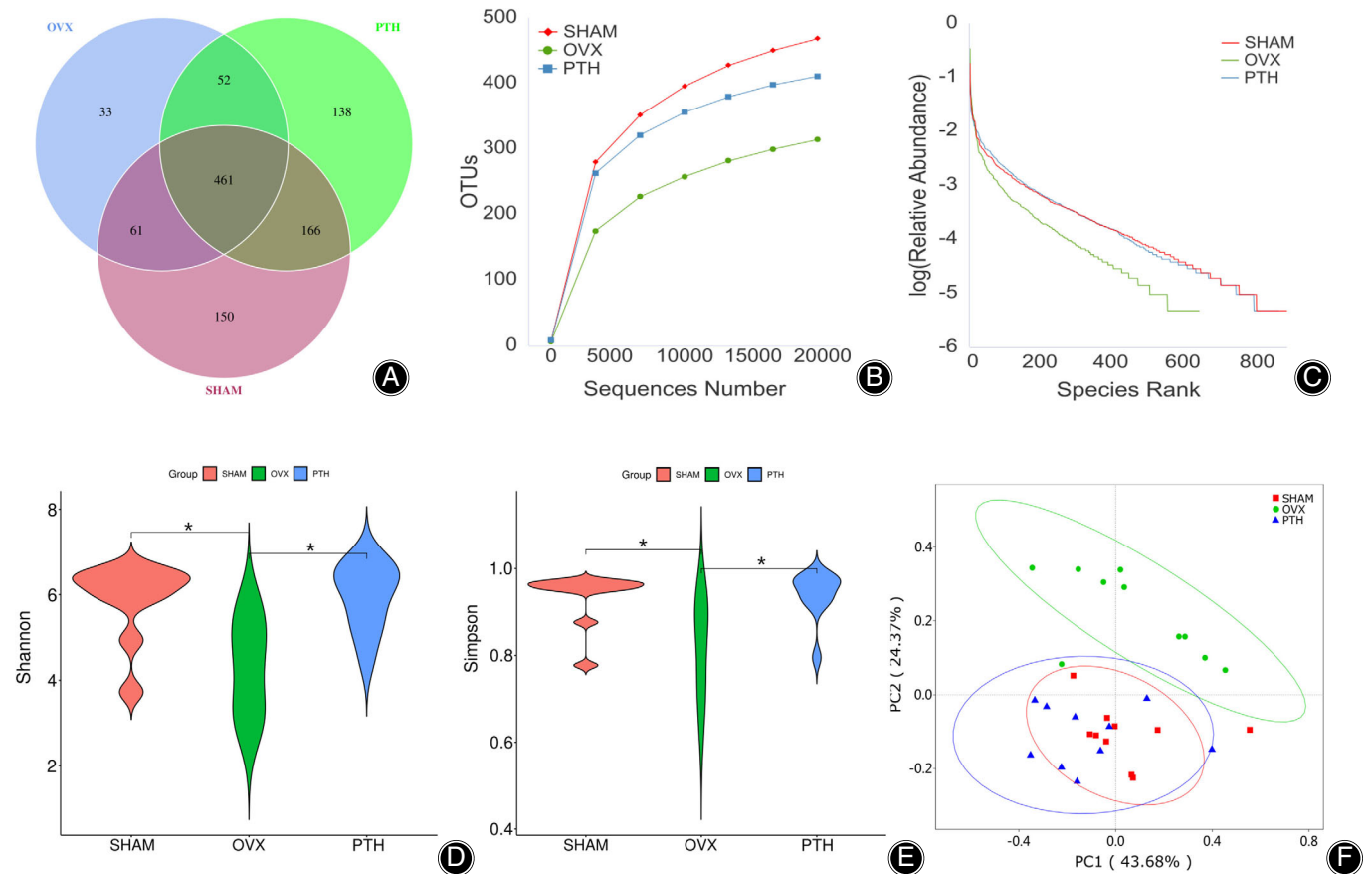

Fig. 2 Composition of gut microbiota. (A) Venn diagram; (B) Rarefaction of samples; (C) Rank abundance curves; (D) α-complexity: Shannon index; (E) α-complexity: Simpson index; (F) β-complexity: principal coordinate analysis (PCoA) based on an weighted UniFrac between the groups. * $P < 0.05$

Analyses of Gut Microbiota

Overview of the Data

A total of 1,519,391,302 good reads were obtained from 30 samples. In Venn diagram analysis, 463 common OTUs were found among the three groups; the PTH group shared 48 OTUs with the OVX group; the PTH group shared 166 OTUs with the SHAM group; the OVX group shared 54 OTUs with the SHAM group. In addition, more unique OTUs were found in the PTH and SHAM groups than the OVX group (134 *vs* 148 *vs* 38) (Fig. 2A). The rarefaction curves of samples tend to be flat, indicating the samples for the study were adequate (Fig. 2B). The rank abundance curve can directly describe the species richness and evenness of microbial communities of the different groups. According to the rank abundance curve, the PTH and SHAM groups had similar species richness and evenness, which was higher than the OVX group (Fig. 2C).

Alpha-diversity Analysis

In α-diversity analysis, Simpson and Shannon indexes were higher in the SHAM and PTH groups compared with the OVX group, and no significant differences were found between the SHAM and PTH groups (Figure 2D,E). These results indicated that the SHAM and PTH groups have higher microbiota complexity than the OVX group.

Beta-diversity Analysis

In β-diversity analysis, apparent separation was found in the OVX group from the PTH and SHAM groups (Fig. 2F),
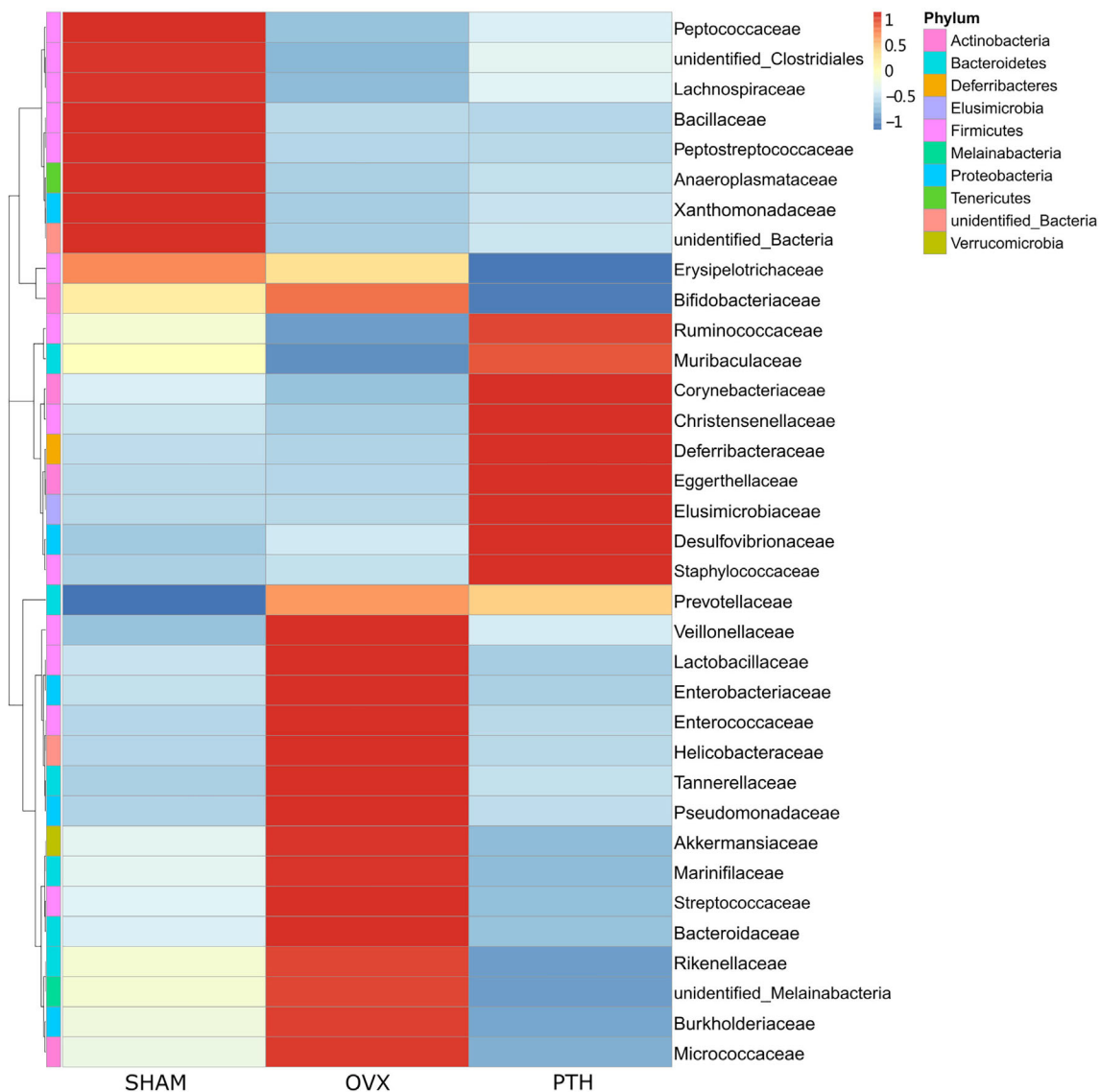

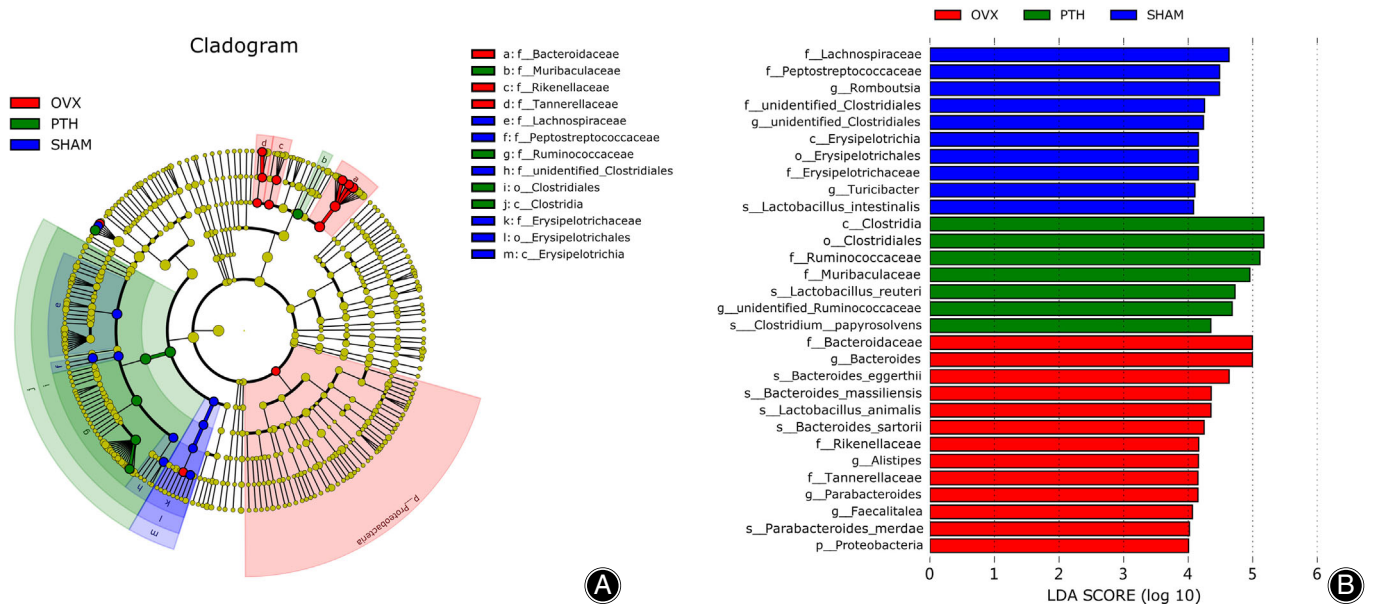**Fig. 3** The heatmap of microbiota based on family level

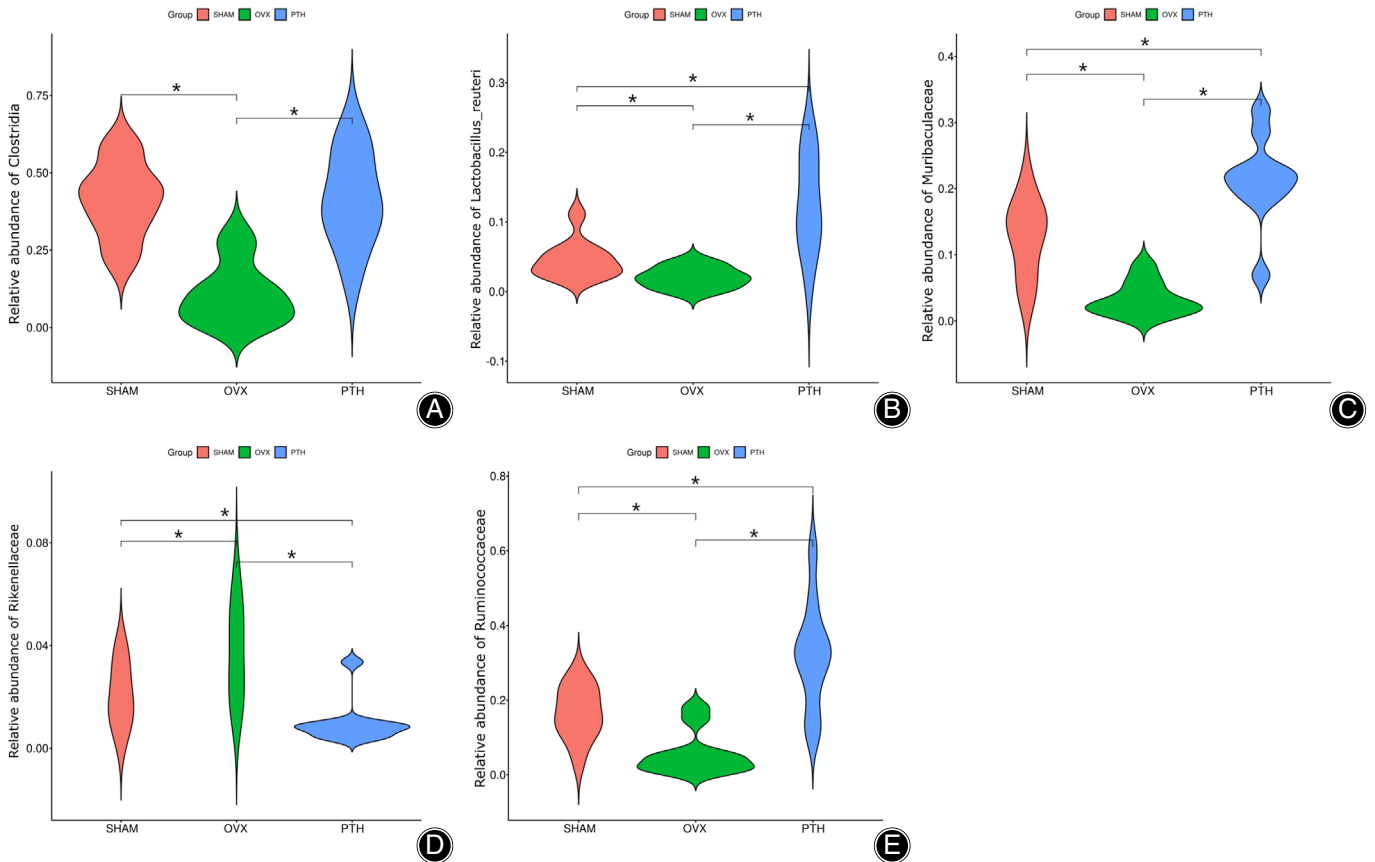**Fig. 4** Key bacterial alterations among the three groups. (A) Cladogram analysis; (B) LDA scores

Fig. 5 (A) Key microbiota among the three groups. (A-E) Abundance of *Clostridia, Lactobacillus_reuteri, Muribaculaceae, Rikenellaceae* and *Ruminococcaceae.* * *P* < 0.05

which suggested that osteoporosis is the critical factor influencing the GM composition and PTH treatment can restore the structure of GM.

LEfSe Analysis

The microbiota heatmap of family was shown in Fig. 3. As shown in the taxonomic cladograms, the LEfSe analysis demonstrated the modulatory effects of PTH on different taxonomic levels of the GM in the three groups (Fig. 4A). Cladogram analysis showed *Bacteroidaceae, Rikenellaceae* and *Tannerellaceae* in OVX group, *Muribaculaceae, Ruminococcaceae,* u*nidentified_Clostridiales, Clostridiales* and *Clostridia* in PTH group, *Lachnospiraceae, unidentified_Clostridiales, Erysipelotrichaceae, Erysipelotrichales* and *Erysipelotrichia* in the SHAM group were the main cause for the difference in β-complexity.

The LEfSe method was used to explore the differences of GM abundance among the three groups (Fig. 4B). Using the linear discriminant analysis score, additional meaningful GM were explored, including *Parabacteroides_merdae, Faecalitalea, Parabacteroides, Tannerellaceae, Alistipes, Rikenellaceae, Bacteroides_sartorii, Bacteroides_massiliensis, Lactobacillus_animalis, Bacteroides_eggerthii, Bacteroides* and *Bacteroidaceae* in the OVX group, *Clostridium__papyrosolvens, unidentified_Ruminococcaceae, Lactobacillus_reuteri, Muribaculaceae, Ruminococcaceae, Clostridiales, Clostridia* in the PTH group, and *Turicibacter, Erysipelotrichaceae, Erysipelotrichales, Erysipelotrichia, Lactobacillus_intestinalis, unidentified_Clostridiales, unidentified_Clostridiales, Romboutsia, Peptostreptococcaceae* and *Lachnospiraceae* in the SHAM group. With regard to key bacteria, *Lactobacillus_reuteri, Muribaculaceae, Ruminococcaceae,* and *Clostridia* were more prevalent in the

PTH group, and *Rikenellaceae* was more prevalent in the OVX group (Fig. 5).

Tax4Fun Analysis

GM affects the host through their biological functions, which were encoded by the gene of GM. Therefore, the Tax4Fun was used to predict and calculate the functional pathways of GM functions. In the Tax4Fun analysis, similar predicted functions were found between SHAM-OVX analysis and PTH-OVX analysis (Fig. 6).

Discussion

In this study, significantly improved data in micro-CT and bone turnover markers were found for OVX rats received PTH treatment, indicating intermittent injection of PTH could promote bone formation. Concomitant with the improved indexes of osteoporosis, we observed that iPTH could improve the GM diversity in OVX rats. In addition, the key bacteria that may affect bone mass were found, including *Clostridia, Lactobacillus_reuteri, Muribaculaceae, Rikenellaceae* and *Ruminococcaceae.*

PTH and GM

Probiotics could prevent bone loss, diabetes and obesity.[31,32] Carbohydrate fermentation can generate the short-chain fatty acids (SCFAs) by GM, including acetate, propionate, butyrate, etc. Among these SCFAs, butyrate is the most deeply studied, which has been shown to inhibit pathogen growth, have anti-inflammatory properties, reinforce the colonic defense barrier, and improve minerals.[33]

The primary effect of intermittent application of PTH is promotion of bone formation. Tyagi *et al.* found that *Lactobacillus rhamnosus* (LGG) can increase bone mass in mice
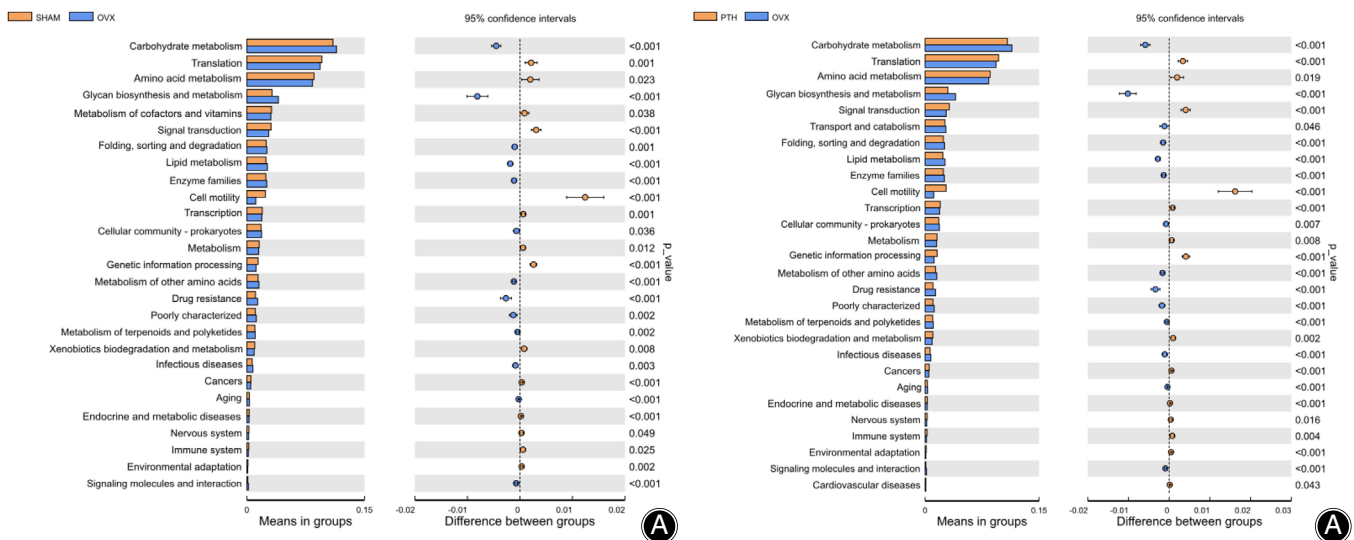

Fig. 6 The Tax4Fun analysis based on the KEGG database to predict microbial metabolic function and analyze the functional differences. (A) The differences between SHAM group and OVX group; (B) The differences between PTH group and OVX group

through enhancing the concentration of butyrate.[19] Butyrate could increase the number of Treg cells, which up-regulate the expression of Wnt10b, which eventually stimulates bone formation.[19] Li *et al.* found that PTH could increase bone mass in conventionally housed mice. However, similar effect was not found in germ-free mice.[25] They concluded that butyrate is necessary for PTH to induce bone anabolism. These findings suggest an essential role for the butyrate-producing bacteria in the efficacies of the PTH.

In the study of Yu *et al.*, continuous PTH could induce bone loss *via* microbial-dependent expansion of intestinal TNF⁺ T cells and Th17 cells.[34] However, the effect of iPTH in GM still need to be studied. We hypothesized iPTH can enhance the trabecular bone by regulating the GM. Then, we conducted this study. In the study, iPTH could restore the β-diversity consistent with the SHAM group and increase the abundance of butyrate-producing bacteria. Hence, PTH plays a role through modulating and optimizing the GM to enhance the abundance of butyrate-producing GM. We think that maybe GM is a helper for PTH to enhance the trabecular bone mass.

Key Bacteria

Clostridia

GM is crucial for the development of immune system.[35] It has been reported that *Clostridia* could relieve the inflammatory response and produce SCFA such as butyrate. As shown in the study by Kim *et al.*, *Clostridia* can restore the resistance of colonization to bacterial pathogens, diminish intestinal inflammation and protect neonatal mice from mortality induced by pathogen challenge.[36] In another study, increased proportion of SCFA producing *Clostridia* due to the supplementation of LGG could enhance the trabecular bone.[25] In our study, rich *clostridia* was found in the PTH group.

Lactobacillus_reuteri

The supplementation of *Lactobacillus reuteri* increased BMD in mice. In a randomized controlled trial study, the authors found additional supplementation of probiotics can significantly reduce bone loss in older women.[37] Similarly, from the perspective of Britton *et al.*, administration of *Lactobacillus reuteri* could protect OVX mice from bone loss. In their study, the bone resorption markers and osteoclastogenesis were significantly decreased following *Lactobacillus reuteri* treatment.[38]

Muribaculaceae

The *Muribaculaceae*, family S24-7 (phylum *Bacteroidetes*), was renamed by Ilias *et al.*[39] However, the prevalence of

Muribaculaceae might be under reported, because the Ribosomal Database Project[40] does not enroll the family.[39] Here, we used LEfSe analysis to assess the species with significant differences after PTH therapy and found *Muriebaculaceae* can significantly promote butyrate production.[41]

Rikenellaceae

Rikenellaceae has rarely been reported, but it is abundant in many diabetic patients.[42] *Rikenellaceae* may have a negative effect on BMD. Ozaki *et al.* found *Rikenellaceae* was richer in groups with lower BMD.[43] Similarly, in our study, consistent with the study of Ozaki *et al.*, *Rikenellaceae* was richer in the OVX group, and less in the PTH group.

Ruminococcaceae

Compared with the OVX group, family *Ruminococcaceae* were markedly enriched in PTH group. The *Ruminococcaceae* are butyrate-producing bacteria, which had a negative correlation with intestinal permeability. *Ruminococcaceae* is regarded as having a positive effect on gut barrier function.[44] In addition, it is highlighted as adjuvants to immune checkpoint inhibitors.[45]

Limitations

Although these results are promising, this study has some limitations. First, the effect on GM of PTH for SHAM rats was not studied. Second, sample size in each group was not large enough, which may induce potential bias. In addition, the PTH associated GM composition change was found in rats, however, this effect in human should be verified. Hence, further studies should be performed to explore the effect of PTH on GM.

Conclusions

Intermittent PTH improved the bone mass in OVX rats, which might be involved in the regulation of the GM composition through increasing the abundance of probiotics and reducing the abundance of the pathogenic bacteria. However, further studies are required to establish to what extent the anti-osteoporotic effects of intermittent PTH are dependent on the GM regulation.

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