Gut Microbial Community and Fecal Metabolomic Signatures in Different Types of Osteoporosis Animal Models

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Abstract

Background

The gut microbiota (GM) constitutes a critical factor in the maintenance of physiological homeostasis. Numerous studies have empirically demonstrated that the GM is closely associated with the onset and progression of osteoporosis (OP). Nevertheless, the characteristics of the GM and its metabolites related to different forms of OP are poorly understood. In the present study, we examined the changes in the GM and its metabolites associated with various types of OP as well as the correlations among them.

Methods

We simultaneously established rat postmenopausal, disuse-induced, and glucocorticoid-induced OP models. We used micro-CT and histological analyses to observe bone microstructure, three-point bending tests to measure bone strength, and enzyme-linked immunosorbent assay (ELISA) to evaluate the biochemical markers of bone turnover in the three rat OP models and the control. We applied 16s rDNA to analyze GM abundance and employed untargeted metabolomics to identify fecal metabolites in all four treatment groups. We implemented multi-omics methods to explore the relationships among OP, the GM, and its metabolites.

Results

The 16S rDNA sequencing revealed that both the abundance and alterations of the GM significantly differed among the OP groups. In the postmenopausal OP model, the bacterial genera g__Bacteroidetes_unclassified, g__Firmicutes_unclassified, and g__Eggerthella had changed. In the disuse-induced and glucocorticoid-induced OP models, g__Akkermansia and g__Rothia changed, respectively. Untargeted metabolomics disclosed that the GM-derived metabolites significantly differed among the OP types. However, a Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis showed that it was mainly metabolites implicated in lipid and amino acid metabolism that were altered in all cases. An association analysis indicated that the histidine metabolism intermediate 4-(β-acetylaminoethyl)imidazole was common to all OP forms and was strongly correlated with all bone metabolism-related bacterial genera. Hence, 4-(β-acetylaminoethyl)imidazole might play a vital role in OP onset and progression.

Conclusions

The present work revealed the alterations in the GM and its metabolites that are associated with OP. It also disclosed the changes in the GM that are characteristic of each type of OP. Future research should
endeavor to determine the causal and regulatory effects of the GM and the metabolites typical of each form of OP.

**Introduction**

Osteoporosis (OP) is characterized by low bone mineral density, bone architecture deterioration, and increased risk of fracture, and has become a major global health problem[1]. Approximately 200 million people worldwide suffer from OP and nine million OP-related fractures occur annually[2]. Bone fracture is the main complication of OP and is associated with increased morbidity and mortality[3]. The incidence of OP is expected to continue to rise. The disease will diminish the quality of life of the aging global population and impose a huge socioeconomic burden on society at large[4, 5]. OP is classified as primary or secondary[6]. Postmenopausal, disuse, and glucocorticoid-induced OP are the major forms of the disorder in humans. Of these, postmenopausal OP is primary while the other two are secondary[7]. At present, the treatment of osteoporosis is still based on drug therapy[8]. Long-term anti-osteoporotic drug administration may cause mandibular osteonecrosis and atypical femoral fracture[9, 10]. Therefore, the development of novel therapeutic approaches against bone loss is a priority.

The gut microbiota (GM) comprises the commensal microorganisms that inhabit the human intestines and function as a secondary gene pool[11, 12]. The GM helps regulate various physiological functions and is associated with various diseases of muscle and bone metabolism[12–16]. Dynamic GM homeostasis is vital to health. When the GM is altered and this balance is perturbed, the host may develop certain pathological conditions. The GM may strongly influence bone metabolism, and GM modulation could reverse bone loss. Hence, the GM is a potential target of OP treatment[17, 18]. The GM strongly affects metabolism and the immune system in humans and animals[19]. The correlation between the GM and the immune system is crucial as the latter helps regulate bone density[20]. GM dysbiosis is closely associated with an increased risk of bone loss[17]. Hence, it is necessary to explore the relationship between bone health and the GM, study the role of the latter in osteoporosis, and apply it in the clinical treatment of this disorder[21].

The gut microbial community and the fecal metabolomic signatures related to postmenopausal, disuse, and glucocorticoid-induced osteoporosis remain unknown. Animal models are currently being established and implemented to explore gut-bone interaction as the gut metagenome has been characterized[22]. In the present study, we applied various techniques to construct animal models of ovariectomized (OVX), disuse-induced (DIO), and glucocorticoid-induced (GIO) osteoporosis. We then performed 16S rDNA gene sequencing and untargeted liquid chromatography-mass spectrometry (LC-MS)-based metabolomics on feces to explore the GM and the modifications to metabolites in various osteoporosis models. Understanding the GM and its metabolites characteristic of each type of osteoporosis could facilitate the development and administration of novel therapeutic approaches against this condition.

**Materials and Methods**
Animals

Female Sprague-Dawley (SD) rats aged 12 wks were obtained from the Ying Ze District Campus Animal Testing Center of Shanxi Medical University, Shanxi, China. They were housed under specific-pathogen-free (SPF) conditions and a 12 h light/12 h dark cycle, and had *ad libitum* access to sterile food and autoclaved water. They were subjected to 1 wk of adaptive feeding and randomly divided into four groups of six rats per group. The treatments included (1) bilateral ovariectomy-induced postmenopausal osteoporosis (OVX) [23], (2) right leg sciatic neurotomy-induced disuse osteoporosis (DIO) [24], (3) glucocorticoid-induced osteoporosis established by 1 mg kg\(^{-1}\) intramuscular dexamethasone saline injection every other day (GIO) [25], and (4) an untreated control (CON). After 10 wks, all rats were euthanized and samples were collected from them.

Micro-CT

Micro-CT (vivaCT80; SCANCO Medical AG, Wangen-Brüttisellen, Switzerland) was used to scan the distal femurs and compare the trabecular bones among the models. The parameters evaluated were bone mineral density (BMD), bone volume per tissue volume (BV/TV), trabecular spacing (Tb.Sp), trabecular number (Tb.N), trabecular thickness (Tb.Th), and the structure model index (SMI).

Histological Analysis

Femoral samples were excised, fixed in 4% (v/v) paraformaldehyde (PFA), decalcified in 20% (w/v) ethylenediaminetetraacetic acid (EDTA), and cut into 5-mm sagittal sections that were stained with hematoxylin and eosin (H&E) and examined under a light microscope.

Mechanical tests

A three-point bending test (ElectroForce 3200 Series; TA Instruments, New Castle, DE, USA) was performed to measure mechanical stress on the femurs. The metrics evaluated included maximum displacement, fracture load, peak load, and stiffness.

ELISA

Blood was drawn from the abdominal aorta and centrifuged at 3,000 rpm for 15 min to obtain the serum. ELISA kits (Lunchang Shuo Biotechnology, Xiamen, China) were used to measure the serum N-terminal propeptide of type I procollagen (PINP) and C-terminal telopeptide of type I collagen (CTX-I) levels.

Fecal sampling

After 10 wks of animal maintenance, sufficient fecal samples were collected from the rats and subjected to microbial and metabolic analyses. All fecal samples were placed in sterile centrifuge tubes, immediately frozen in liquid nitrogen, and stored at -80°C until sequencing.

16S rDNA sequencing and microbial community analysis
The 16S rDNA sequencing was conducted at Lc-Bio Technologies Co. Ltd., Hangzhou, Zhejiang, China. The cetyltrimethylammonium ammonium bromide (CTBA) method was used to extract total DNA from all samples. Polymerase chain reaction (PCR) amplification of the V3–V4 region of the 16S rRNA gene was performed using the 341F (5′-CTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTNNGYCC-3′) primers. The PCR was performed as follows: initial denaturation at 98 °C for 30 s, 32 denaturation cycles at 98 °C for 10 s, annealing at 54 °C for 30 s, extension at 72 °C for 45 s, and final extension at 72 °C for 10 min. The PCR product size was confirmed by 2% agarose gel electrophoresis. The PCR products were purified with AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA) and quantified by Qubit (Invitrogen, Carlsbad, CA, USA). The libraries were sequenced on the NovaSeq 6000 platform (Illumina, San Diego, CA, USA) by Lc-Bio Technologies Co. Ltd. according to the manufacturer's recommendations. Paired-end reads were assigned to the samples based on their unique barcodes, truncated by cutting off the barcode and primer sequences, and merged with FLASH v. 1.2.8 (http://ccb.jhu.edu/software/FLASH/). The raw reads were quality filtered with fqtrim v. 0.94 (http://ccb.jhu.edu/software/fqtrim/) and high-quality clean tags were generated. The clean reads were then analyzed with DADA2 in QIIME2 v. 2019.7 (https://qiime2.org/), and amplicon sequence variant (ASV) feature tables and sequences were obtained. BLAST (Basic Local Alignment Search Tool; https://blast.ncbi.nlm.nih.gov/) was used to align the sequences. Those that were characteristic of each representative sequence were annotated against the NT-16s database. All graphs were plotted using the package in R v. 3.5.2 (R Core Team, Vienna, Austria).

Untargeted metabolomics data analysis

Fifty milligrams of each frozen sample was set aside, transferred to a 1.5-mL Eppendorf (EP) tube (Eppendorf GmbH, Hamburg, Germany), and thawed on ice. The metabolites were extracted with 50% (v/v) methanol buffer-acetonitrile and centrifuged at 4,000 × g for 20 min. The supernatants were stored at -80 °C until they were subjected to liquid chromatography-mass spectrometry (LC-MS). The LC-MS was performed in a Thermo Scientific UltiMate 3000 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a high-resolution tandem Q-Exactive MS (Thermo Fisher Scientific) operated in positive and negative ion modes. An online Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome.jp/kegg/) annotated the metabolites by matching their exact molecular mass data, names, and formulae with those in the database. A principal component analysis (PCA) was performed to detect outliers in the preprocessed dataset. Differential metabolites had variable influence of projection (VIP) > 1, P < 0.05, and ratio ≥ 2 or ≤ ½, and a KEGG enrichment analysis was performed on them. Correlations between significant differential genera and metabolites were analyzed by Spearman's rank correlation test.

Statistical Analysis

All data were presented as means ± standard deviation (SD). Differences between group pairs were analyzed by Student's t-test. Multiple group comparisons were performed by one-way analysis of variance (ANOVA) with the Bonferroni correction. Wilcoxon's rank-sum and Kruskal-Wallis tests were applied to identify differences in the bacterial taxa between group pairs and multiple groups, respectively.
Student's $t$-test and fold change (FC) analysis were used to identify differences in metabolites between groups.

**Results**

Alterations in the femoral bone microarchitecture of various rat induced osteoporosis models

We performed micro-CT analyses on different rat induced osteoporosis models. The 3D micro-CT images revealed obvious changes in the distal femoral metaphysis bone microstructure in the OVX, DIO, and GIO groups compared with the CON group (Fig. 1A). The micro-CT disclosed that the femoral BMD, BV/TV, Tb.N, and Tb.Th values were lower for the OVX, DIO, and GIO groups than the CON group (Fig. 1B–1F). In contrast, the Tb.Sp and SMI values were higher for the OVX, DIO, and GIO groups than the CON group (Fig. 1D and 1G).

Changes in the bone histomorphology, mechanical properties, and serum bone turnover markers of various rat induced osteoporosis models

Hematoxylin-eosin (H&E) staining, a three-point bending test, and an enzyme-linked immunosorbent assay (ELISA) were used to examine and compare the bone histomorphology, mechanical properties, and serum bone turnover indices among the rat induced OP models. The H&E staining showed that compared with the CON group, the numbers of bone trabeculae were significantly reduced and the bone trabeculae were rod-shaped rather than plate-like in the OVX, DIO, and GIO groups. These findings were consistent with the SMI values determined by micro-CT. Moreover, the bone marrow adipocyte counts were higher in the OVX, DIO, and GIO groups than in the CON group (Fig. 2A). The bone resorption and formation markers include CTX-I and PINP, respectively. The ELISA showed that the serum PINP was relatively lower in the DIO and GIO models and higher in the OVX model because high bone turnover is associated with postmenopausal osteoporosis. The serum CTX-I levels were higher in the OVX, DIO, and GIO groups than in the CON group (Fig. 2B and 2C). The three-point bending test disclosed that the fracture load, peak load, and stiffness were significantly lower in the OVX, DIO, and GIO groups than in the CON group. However, there were no significant differences among treatments in terms of the maximum displacement (Fig. 2D–2G).

Relative differences in the gut microbiota among rat induced osteoporosis models

A Venn diagram visualized the common and unique amplicon sequence variants (ASVs) and the changes in the GM among the various treatment groups. The CON, OVX, DIO, and GIO groups had 2,449, 2,689, 2,683, and 1,176 unique ASVs while all four groups shared 553 ASVs (Fig. 3A). We calculated the ASV abundances and plotted and compared the rarefaction curves for different samples to display bacterial species diversity. Flat rarefaction curves indicate reasonable sequencing data. An $\alpha$-diversity analysis revealed that the rarefaction curves for the Shannon, Chao1, Goods_coverage, observed_species, and Simpson indices were all smooth (Figs. 3B and S1A–S1D). Beta-diversity analyses disclose and compare species diversity among various environmental communities. A principal coordinate analysis (PCoA) is a
type of β-diversity analysis. Here, a PCoA was performed to demonstrate the differences among the four treatment groups in terms of their gut microbiota. The PCoA clustered samples with highly similar microbial community structures. The PCoA of the 2D and 3D models indicated that each treatment group was induced by different factors distributed across various regions. Though the samples from each OP group were not identical, they were nonetheless localized to approximately the same area (Fig. 3C and 3D). We then analyzed the GM community structure. We obtained the phylum-, class-, order-, family-, genus-, and species-level abundances and displayed them in the form of stacked bar charts and heat maps. The distributions of the various bacterial taxa of the OVX, DIO, and GIO groups differed from those of the CON group (Figs. 4A–4F and S2A–S2F). We also investigated the significant bacterial phylum- to species-level differences among the OVX, DIO, GIO, and CON groups (Figs. 4G–4L and S2G–S2O). At the phylum level, p__Candidatus_Saccharibacteria, p__Firmicutes, and p__Tenericutes were more abundant while p__Deferrribacteres, p__Candidatus_Melainabacteria, and p__Bacteroidetes were less abundant in the OVX group compared with the CON group. Relative changes in p__Firmicutes and p__Bacteroidetes abundance were characteristic of the OVX group (Table S1). However, p__Candidatus_Saccharibacteria and p__Tenericutes were more abundant while p__Candidatus_Melainabacteria, p__Verrucomicrobia, and p__Deferrribacteres were less abundant in the DIO group compared with the CON group. Relative change in p__Verrucomicrobia abundance was characteristic of the DIO group (Table S1). Moreover, p__Tenericutes and p__Candidatus_Saccharibacteria were more abundant while p__Candidatus_Melainabacteria and p__Deferrribacteres were less abundant in the GIO group compared with the CON group (Table S1). There were 36, 34, and 36 differential bacterial genera in the OVX, DIO, and GIO groups compared to the CON group (Table S2). The taxonomic cladogram generated by linear discriminant analysis (LDA) effect size (LEfSe) visualized the relative differences among groups in terms of bacterial species abundance (Figure S3A–S3F).

Differentially abundant metabolites among the rat induced osteoporosis models

A principal component analysis (PCA) is a commonly used type of multivariate analysis that identifies potential metabolomic markers within a large amount of data. Each point on a PCA graph represents a sample, and the similarity among samples decreases with increasing distance between points on the plot. Point clustering and separation indicate that the observed variables have high and low degrees of similarity, respectively. For the OVX, DIO, and GIO groups, the metabolites in positive and negative ion modes were in two distinct regions compared to the CON group. Thus, the metabolites were significantly altered in the OVX, DIO, and GIO groups relative to the CON group (Fig. 5A and 5B). The heat maps and volcano maps generated the same results (Fig. 5C–5H). In positive ion mode, and compared to the CON group, there were (a) 4,942 differential metabolites of which 2,755 were upregulated and 2,178 were downregulated in the OVX group; (b) 4,579 differential metabolites of which 2,620 were upregulated and 1,959 were downregulated in the DIO group; and (c) 5,041 differential metabolites of which 2,857 were upregulated and 2,178 were downregulated in the GIO group. In negative ion mode, and compared to the CON group, there were (a) 2,190 differential metabolites of which 1,134 were upregulated and 1,056 were downregulated in the OVX group; (b) 2,110 differential metabolites of which 1,111 were upregulated and 999 were downregulated in the DIO group; and (c) 2,398 differential metabolites of which 1,278 were
upregulated and 1,120 were downregulated in the GIO group. A KEGG enrichment analysis was then performed on the differential metabolites and the top ten metabolic pathways and metabolites in the various rat induced OP models (Fig. 5I–5K; Table S3–S8). Both lipid and amino acid metabolism may play important roles in osteoporosis progression.

Correlation analyses of differential genus-level gut microbiota abundance and fecal metabolomes associated with lipid and amino acid metabolism

To investigate the microbiota-metabolite interactions associated with each type of osteoporosis, we evaluated the correlations among differential bacterial genera and the top ten fecal metabolites related to lipid and amino acid metabolism according to the KEGG enrichment analysis. We plotted a correlation heat map (Fig. 6A–6C; (|r| > 0.6, P < 0.05). Compared with the CON group, the differential bacterial genera included (a) g__Bacteroidetes_unclassified, g__Ruminococcaceae_unclassified, g__Parabacteroides, g__Firmicutes_unclassified, and g__Eggerthella in the OVX group, (b) g__Proteus, g__Akkermansia, g__Ruminococcaceae_unclassified, g__Roseburia, and g__Prevotella in the DIO group, and (c) g__Rothia, g__Roseburia, g__Proteus, and g__Prevotella in the GIO group. The foregoing taxa were closely related to bone metabolism. Changes in g__Bacteroidetes_unclassified, g__Firmicutes_unclassified, and g__Eggerthella were exclusive to the OVX group, a change in g__Akkermansia was specific to the DIO group, and a change in g__Rothia was unique to the GIO group. We then constructed a correlation network to disclose the major interactions among the differential bacterial genera associated with bone metabolism and the differential metabolites related to lipid and amino acid metabolism (Fig. 6D–6F).

The correlation heat map and network map revealed that the differential metabolite 4-β-acetylaminoethyl)imidazole was negatively correlated with g__Bacteroidetes_unclassified (r = -0.783; P = 0.004), g__Akkermansia (r = -0.832; P = 0.001), and g__Rothia (r = -0.636; P = 0.030) but positively correlated with g__Firmicutes_unclassified (r = 0.727; P = 0.01) and g__Eggerthella (r = 0.748; P = 0.007). Hence, 4-(β-acetylaminoethyl)imidazole may be a principal metabolite associated with osteoporosis.

Discussion

The gut microbiota (GM) plays vital roles in maintaining human health[21]. Disorders of the GM may cause various chronic conditions including obesity, metabolic dysfunction, neuropathies, malnutrition, cancers, and cardiovascular diseases (CVD)[26]. Evidence from clinical and animal studies indicates that changes in the composition of the GM and its metabolites are closely associated with osteoporosis (OP) [23, 27–37]. Based on the interaction between the GM and OP, it was proposed that the former is a potential therapeutic target of the latter[38]. However, there are several different types of OP, and the composition of the GM and the metabolites characteristic of each form of osteoporosis are unknown. In the present study, we constructed three animal models to simulate postmenopausal, disuse-induced, and glucocorticoid-induced osteoporosis. We integrated 16S rDNA sequencing and untargeted metabolomics to explore the GM composition and metabolites characteristic of each type of OP.
Bacterial high-throughput sequencing based on 16S rDNA is used to study the microbial community composition, diversity, abundance, and structure in an environmental sample. It also analyzes the relationship between microorganisms and the environment or host in which they reside. Traditional microbial research relies on laboratory culture. However, not all environmental or symbiotic microorganisms can be propagated or studied in this way. In contrast, 16S amplicon and other high-throughput sequencing can investigate these bacteria and their interactions with their hosts or ambient environment. Our 16S rDNA sequencing and β-diversity analyses revealed that the GM differed among the three types of rat induced OP models. Our subsequent species and significant difference analyses confirmed that the observed changes in the GM composition were unique to each type of osteoporosis. There were five genera related to bone metabolism between the OVX and CON groups, and the changes in g__Bacteroidetes_unclassified, g__Firmicutes_unclassified, and g__Eggerthella were unique to each of the induced osteoporosis models. Firmicutes and Bacteroidetes are the major phyla in the GM and comprise 80% of the total microbiome[39]. The animal OVX model was characterized by relatively higher Firmicutes and lower Bacteroidetes abundance than other OP models[35, 36]. These findings were consistent with the changes detected in the present study. The foregoing alterations may serve as biomarkers of postmenopausal osteoporosis[36]. *Eggerthella* is a component of normal human microflora. Imbalances in its abundance are related to various diseases[40]. *Eggerthella* abundance is comparatively higher in patients with osteoporosis[41]. Members of Family Eggerthellaceae played vital roles in a mouse postmenopausal osteoporosis model[35]. *Eggerthella* can activate Th17 lymphocytes in the gut[42]. These cells are the main effectors of OP pathogenesis. They secrete interleukin (IL)-17 which, in turn, induces the NF-κB ligand/receptor activator of NF-κB/osteoprotegerin (RANKL/RANK/OPG) system and, by extension, promotes osteoclastogenesis and bone resorption[43, 44]. Therefore, *Eggerthella* may play an important role in postmenopausal osteoporosis. There were five genera related to bone metabolism between the DIO and CON groups, and the observed changes in g__Akkermansia abundance were unique to each induced osteoporosis model. In the gut microbiota, *Akkermansia* (Phylum Verrucomicrobia) influences the onset and progression of several diseases[45] and maintains intestinal barrier homeostasis[46]. The loss of *Akkermansia* impairs intestinal integrity, increases intestinal leakage, and, by extension, promotes metabolic endotoxemia, inflammation, and insulin resistance[47]. *Akkermansia* abundance was reduced in patients with osteoporosis and osteopenia[48, 49]. Here, *Akkermansia* abundance was comparatively lower in the DIO model. *Akkermansia* is osteoprotective, is positively correlated with bone mass, and could serve as a probiotic for osteoporosis prevention or treatment[50]. Our findings suggest that a decrease in the abundance of *Akkermansia* might play a key role in the progression of disuse osteoporosis. There were four genera related to bone metabolism between the GIO and CON groups, and the changes in g__Rothia abundance were unique to each induced osteoporosis model. Abnormal *Rothia* abundance may be associated with various infectious and alcoholic liver diseases[51, 52]. *Rothia* abundance is positively correlated with an anti-osteoporosis effect. It transforms xylose, galactose, raffinose, and glucose into the short-chain fatty acid (SCFA) butyric acid[53]. In general, SCFAs participate in bone metabolism regulation. Butyric acid inhibits and promotes osteoclast and osteoblast differentiation, respectively, from bone marrow mesenchymal stem cells (BMSCs)[54, 55]. Here, we observed a relative decrease in *Rothia* abundance in the GIO group.
Liquid chromatography-mass spectrometry (LC-MS)-based metabolomics is used to identify and quantify the small molecules produced by normal microbial metabolism and elucidate their functions[56]. The GM secretes metabolites that link it to the skeletal system and regulate distant organs[57]. In the present study, we used untargeted LC-MS metabolomics to detect the fecal metabolites in the various rat induced osteoporosis models. We discovered that among the top ten metabolic pathways and metabolites, lipid and amino acid metabolism play important roles in OP progression. Osteoporosis and osteopenia often co-exist with disorders of lipid metabolism[58]. When adipocyte numbers and volumes increase in the bone marrow, alterations to the microenvironment within the bone marrow cavity may perturb lipid metabolism there, inhibit BMSC osteoblastogenesis, promote BMSC osteoclastogenesis, and eventually lead to osteoporosis[59]. Hence, the improvement of lipid metabolism promotes osteoblastogenesis while inhibiting osteoclastogenesis[60]. Here, the lipid metabolism disorders associated with the different induced OP models involved mainly the biosynthesis of primary and secondary bile acids and steroid hormones. Bile acids have been associated with OP[33, 35]. There might be a correlation between osteoporosis and the circulating amino acids that play important roles in bone metabolism[61]. Abnormal amino acid metabolism may promote the occurrence and development of OP, and patients with OP often exhibit it[62]. The GM may regulate OP-related amino acid metabolism and could, therefore, serve as a target for OP intervention[63]. Here, the various induced OP models were characterized by aberrant histidine, tryptophan, arginine, and proline metabolism. Intestinal bacteria decarboxylate the basic amino acid histidine to histamine which plays important roles in immunoregulation[64]. Histamine activates histamine H1 type receptor (H1R) which suppresses osteoblastogenesis and mineralization[65]. Histidine metabolism is at least partially implicated in bone formation[61]. In the present study, all three induced OP models presented abnormal histidine metabolism. Correlation heat maps and networks among the differential bacterial genera and metabolites in the induced OP models showed that the histidine metabolism intermediate 4-\((\beta\text{-acetylaminoethyl})\)imidazole was closely associated with the bacterial genera related to bone metabolism. Thus, the bone loss induced by the various types of OP might be connected to an increase in 4-(\(\beta\text{-acetylaminoethyl})\)imidazole.

The present study had several limitations. Firstly, the sample size was relatively small. Hence, the results of this work provide only a few references to explore and compare intestinal microecology in different forms of OP. For this reason, future investigations must validate the findings of the work herein by using larger sample sizes. Moreover, future research should endeavor to elucidate the causal and regulatory relationships among the GM, its metabolites, and the various types of OP.

**Conclusion**

The present work empirically demonstrated that each type of OP is closely, characteristically, and uniquely related to the GM and its metabolites. To the best of our knowledge, the present study is the first to characterize the GM and the changes in their metabolites associated with different rat induced OP models. The results of this investigation may provide novel insights into the effects of the GM on the onset and progression of OP. Future research should aim to validate the findings made herein and
determine how they may be applied toward safe and efficacious clinical therapies against different forms of OP.

**Declarations**

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**Authors' contributions**

ZL, ZT, and YF contributed to the design of the experiment. KZ and RZ maintained the animals. XQ and LY performed the experiments. XL, ZW, and YP collected the fecal samples. XW, LL, CX and PL provided technical guidance. XQ drafted the manuscript. ZL, YF, and ZT revised the manuscript. All authors contributed to the manuscript and approved its final version.

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**Availability of data and materials**

The original data used in this study are publicly available at https://www.ncbi.nlm.nih.gov/sra/PRJNA973838.

**Declarations**

**Ethics approval and consent to participate**

We confirm that all animal experiments were performed in strict accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Experimental Animals and were approved by the Ethical Committee of Experimental Animal Care of Shanxi Medical University (No. 2021014).

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that the study was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.
References


Figures
Figure 1

(A) Representative 3D micro-CT reconstructions of femurs from each treatment group. (B–G) Trabecular bone at distal femoral metaphysis after 10 wks. Parameters included BMD, BV/TV, Tb.Sp, Tb.N, Tb.Th, and SMI. Data are means ± standard error of the mean (SEM). n = 6, *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001; ns, no significance.
Figure 2

(A) Trabecular bone at distal femoral metaphysis observed by H&E staining. (B–C) Serum levels of bone turnover biomarkers including PINP and CTX-I. (D–G) Comparison of three-point bending test parameters including maximum displacement, fracture load, peak load, and stiffness. Data are means ± SEM. n = 6, *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001; ns, no significance.
Figure 3

(A) Venn diagram showing numbers of amplicon sequence variants (ASVs) per group. (B) Rarefaction curves of Shannon index α-diversity analysis. (C) 2D model of gut microbiota PCoA. CON: orange; OVX: purple; DIO: light green; GIO: dark green. (D) 3D model of gut microbiota PCoA. CON: red; OVX: green; DIO: blue; GIO: yellow; n = 6.
Figure 4

Figure 5

Figure 6

(A–C) Correlation heat map between differential bacterial genera and fecal metabolites associated with lipid and amino acid metabolism in top ten KEGG enrichment analysis; |r| > 0.6; P < 0.05. (D–F) Correlation network map of differential bone metabolism-related bacterial genera and fecal metabolites associated with lipid and amino acid metabolism in top ten KEGG enrichment analysis; |r| > 0.6; P < 0.05; n = 6.

Supplementary Files

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