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Original Article

Gut microbiota can affect bone quality by regulating serum estrogen levels

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Abstract: Germ-free (GF) animals and animal models of the antibiotic disruption of gut microbiota are widely used to explore studies of gut microbiota-host interactions. The role of gut microbiota in bone growth and development has been well explained in studies on GF mice, indicating that changes in the gut microbiota may affect normal bone developmental processes. The mechanisms, however, are yet unclear. This study aims to clarify the effect of antibiotic treatment disrupting the gut microbiota on bone development in mice and investigate the possible causes of this effect. Our results show that long-term antibiotic feeding significantly alters gut microbiota composition in mice, reduces the bone mineral density of the spinal region, and leads to changes in trabecular microstructure. Interestingly, we found a significant decrease in the serum estrogen levels in mice treated with antibiotics, suggesting that gut microbiota may affect bone quality by regulating serum estrogen levels. These results may help understand how gut ecological dysregulation affects sex hormones and provide a new conception for the clinical treatments of osteoporosis.

Keywords: Antibiotic, intestinal microbiota, bone, osteoporosis, estrogen

Introduction

Osteoporosis is a common metabolic disease of the skeletal system characterized by decreased bone mass and changes in bone microstructure [1]. The current anti-osteoporosis treatment consists mainly of calcium, estrogen, or bisphosphonates, but the therapeutic effect of these drugs is unsatisfactory, and there are safety and tolerance problems in long-term treatment [2, 3].

For the past few years, the gut microbiota (GM) has been a vital regulator for host physiology, including bone health. The GM is defined as "all microorganisms that colonize our intestine, including bacteria, viruses, fungi, and protozoa" [4, 5]. GM is acquired from the mother during birth, then established in the first few years of life, and composition remains relatively stable [5, 6]. However, many factors (i.e., diet, age, travel, and medications) can shift or change the gut microbiota composition. Previous studies have revealed that GM can be thought of as a multicellular organ that interacts with and influences the host in multiple ways, including gut physiology, metabolic and immune-system functions, inflammatory processes, etc. [7-10]. Alterations in the composition of the GM have been associated with bone remodeling, for example, GF mice have lower levels of osteoclastogenesis and higher bone mass [11], and male mice with intestinal infections caused by pathogenic bacteria have increased bone loss [12]. Further, probiotic treatment reduces bone loss in ovariectomized female mice [13, 14], and type 1 diabetic male mice [15]. These studies suggest that GM dysbiosis can cause bone loss, and probiotic treatment can prevent bone loss due to some factors.

Estrogen is an essential regulator of bone mass, and postmenopausal osteoporosis is the most common type of osteoporosis, caused mainly by the cessation of ovarian function, in which decreased estrogen levels lead to the stimulation of bone resorption and bone formation. Bone resorption, however, is stimulated to

a much greater degree than bone formation, which leads to rapid bone loss [16]. It is known that bone loss in mice due to ovariectomy is associated with dysbiosis of the GM and that probiotic treatment can alleviate this bone loss [13, 17]. A recent study has shown that estrogen deficiency does not cause bone loss in GF mice but can lead to significant bone loss in GF mice with recolonized gut microbiota, which implies the regulation of bone mass by estrogen is dependent on GM [18]. Therefore, we hypothesized that bone loss due to gut microbiota dysbiosis is correlates with estrogen levels and may cause osteoporosis in mice by decreasing serum estrogen levels.

The purpose of this study is to explore whether bone loss caused by dysbiosis of the gut microbiota is correlated with estrogen levels. Although previous studies have used germ-free mice as a standard tool to demonstrate the effects that gut microbiota has on bone structure and density, defects in the immune system development in germ-free mice are an unavoidable problem [11]. To have a better characterization the effects of gut microbiota on bone density, some laboratories have used antibiotics to disrupt the GM [19-22]. For this reason, we use an antibiotic-treated animal model in this study. Our results demonstrate that long-term antibiotic treatment leads to significant changes in the gut microbiota composition with bone loss. Furthermore, we present evidence that serum estrogen levels decrease following gut microbiota dysbiosis, which suggests a strong correlation between gut microbiota, estrogen, and osteoporosis.

Materials and methods

Animals and study design

Four week-old Kunming mice (n=36) were obtained from Southwest Medical University and randomized into two groups, the treated group (n=18) and the control group (n=18). Mice were maintained in a special pathogen-free environment and fed with sterilized food and autoclaved water, ad libitum. In the treatment group, starting from 4 weeks of age, broad-spectrum antibiotics (0.5 g/L neomycin, 1.0 g/L ampicillin) were added to the drinking water [20, 22]. The bioavailability of ampicillin and neomycin is very low, limiting their effect outside the intestine [23]. The drinking water, with or without antibiotics, was renewed every two days. After a treatment of 12 weeks (mice age: 16 weeks), the animals reached skeletal maturity [23], mice were euthanized by cervical dislocation, and femurs were collected immediately after euthanasia. Blood and fecal samples were collected one hour before euthanasia. All animal experiments were approved by the Southwest Medical University Ethics Committee (SWMU-20220045).

Bone mineral density (BMD) measurement

BMD of the lumbar spine (L3-L4) in mice was measured using a dual-energy X-ray (GE Healthcare, Piscataway, NJ, USA) with a scanning speed of 1 mm/s and a resolution of 0.5×0.5 mm. before measurement, the instrument was calibrated using a companion model. The mean BMD of L3 and L4 was considered to be the BMD of the lumbar spine.

Micro-computed tomography (μCT) bone imaging

Six mice randomly selected from each group were used for a micro-CT assay. The femurs were collected on the day of harvesting, fixed in 10% formalin for 24 h, and the bone was transferred to a 70% ethanol solution and scanned using the Siemens Inveon Micro-PET/CT system, with a voxel resolution of 20 μm. Each run included skeletons from mice under experimental conditions and a calibration model to standardize grayscale values and maintain consistency between the analyses. Separation of bone from bone marrow was made with a fixation threshold (980). Bone mineral density (BMD), trabecular bone thickness (Tb.Th), trabecular bone number (Tb.N), and spacing (Tb.Sp) were assessed in the secondary trabecular regions at 2-4 mm away from the growth plate, and hand-drawn contours were used to separate the metaphyseal region of interest from the trabecular compartment. The data were then analyzed and recorded using Inveon Research Workplace Software (Siemens, USA). Surface images such as femoral trabeculae were taken from an area of the femur and 1.0 mm in length and 1.0 mm in diameter were analyzed. All bone analyses were performed without regard to experimental conditions.

Microbial community analysis

Fresh fecal samples (>0.1 g per animal) were collected in sterile tubes from 12 mice (n=6 per

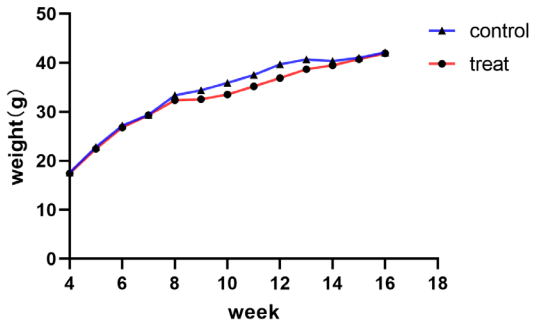

Figure 1. Bodyweight gain of mice in two groups. (Data are presented as mean ± SD).

group) and immediately stored at -80°C. The absolute quantification of 16S rRNA gene sequencing was conducted by TinyGene Bio Inc. (Shanghai, China). The samples were sent for 16S rRNA [25] sequencing using MiSeq technology. Briefly, the DNA was extracted, and 16S 'V4-V5' region was amplified using the specific primer (515F 5'-GTG CCA GCM GCCGCG GTAA-3'; 926R 5'-CCG TCA ATT CMT TTG AGT TT-3'). The amplified products were purified, quantified to establish a 16S rRNA library, and sequenced (Illumina). Quality trimming of sequences using Trimmomatic and mothur was done to identify and remove chimeric sequences. Sequences were classified using USEARCH software, and those classified as Eukarya, Archaea, chloroplast, mitochondria, or unknown were removed. The sequence data were then filtered to remove any sequence appearing only once in the data set. The clean tags processed above were subjected to OTU clustering, and the sequences were clustered into operable classification units (OTUs) with 97% similarity using USEARCH.

Serum estrogen

Twelve mice (n=6 per group) were randomly selected for an analysis of their serum estrogen levels. Whole blood was collected by cardiac puncture at the time of euthanasia. After complete coagulation, the tube was centrifuged (2500 rpm, 15 min), and the serum layer was collected and kept at -20°C. For estrogen assays, the samples were thawed, maintained at room temperature for 0.5 h, and assayed with mouse 17α-E2 ELISA kits (Qiao-Du Biotech, Shanghai, China) following the manufacturer's instructions.

Bone histology

Six mice randomly selected from each group were used for the histomorphometric assay. The distal parts of the collected femurs were stripped of soft tissues and fixed in 10% neutral formalin for 4 h. Subsequently, they were rinsed repeatedly with physiological saline and immersed in an ethylenediaminetetraacetic acid solution (10%) for one month (sample volume:solution volume >20:1), with the solution changed once every 3 days. The decalcified samples were embedded in paraffin, cut into slices (thickness: 10 μm; 1 slice/sample), and stained with hematoxylin-eosin (H&E). The stained sections were studied with a light microscope and analyzed for histomorphometric indices (Image-Pro Plus 6.0, Media Cybernetics, Bethesda, MD, USA), including the percentage of bone trabecula (%.Tb.Ar), trabecular bone area (Tb.Ar), average bone trabecular spacing (Tb.Sp), and average bone trabecular width (Tb.Wi) [33].

Statistical analysis

Statistical analysis was performed by using Graph Pad Prism. Measurement data conforming to the normal distribution were compared using t-test between two groups. For measurement data that did not meet normal distribution, the Wilcoxon test was performed for the comparison between the two groups. A difference of $P<0.05$ was considered statistically significant.

Results

Effect of antibiotic treatment on the growth of mice

The difference in mean body weight between both groups of mice before and after antibiotic treatment was not statistically significant. Notably, there was a decreasing trend in bodyweight growth rate in the control mice after three weeks (7 weeks of age) of antibiotic treatment and a statistically significant difference after six weeks (10 weeks of age); but this difference disappeared rapidly after two weeks (12 weeks of age). These data suggest that antibiotic treatment delays weight gain in mice (**Figure 1**).

Effect of antibiotic treatment on the gut microbiota of mice

To investigate the effect of antibiotic treatment on the gut microbiota, the composition of the mice's gut microbiota was analyzed by 16S RNA sequencing of fecal samples at multiple taxonomic levels from phylum to order (**Figures 2**-**4**). The results showed that the composition of the gut microbiota changed profoundly at several taxonomic levels after 12 weeks of antibiotic treatment. At the phylum level, the relative abundance of *Tenericutes* phylum significantly decreased after antibiotic treatment; at the class level, the relative abundance of *Clostridia* significantly increased, while *Bacilli* significantly decreased; at the order level, the relative abundance of *Clostridiales* significantly increased, while the relative abundance of *Lactobacillales* significantly decreased (**Figure 2A**). Non-parametric test was used to explore the populations that differed the most between the two groups. The results showed that at the phylum level, the greatest differences were between *Verrucomicrobia* and *Cyanobacteria*; at the class level, between *Erysipelotrichia* and *Betaproteobacteria*; and at the order level, between *Erysipelotrichales* and *Burkholderiales* (**Figure 2B**).

We first constructed a rarefaction curve to evaluate our sequencing depth, and the end of the curve flattens out, as shown in **Figure 3A**, which indicates that our sequencing depth is reasonable. The overall abundance of the gut microbiota was then assessed by rank-abundance plots, and no significant difference was found between the two groups (**Figure 3B**). Venn diagrams were used to analyze the unique and common operative taxonomic units (OTUs) in the two groups. We observed 56 and 64 unique OTUs in the treatment and control groups, respectively, while 244 OTUs were shared by both groups (**Figure 3C**). We then assessed alpha diversity with the ACE estimator, Chao estimator, and Shannon index and found a decreasing trend in the antibiotic-treated group, indicating less alpha diversity in the gut microbiota after antibiotic treatment (**Figure 3D**, **3E**). In addition, the increase in the Simpson's index indicated less alpha diversity in the antibiotic-treated group (**Figure 3D**, **3E**). To determine beta diversity after antibiotic treatment, ecological distances were visualized using an OTU-based variability matrix heat map and Principal Coordinate Analysis (PCoA). We found that samples from the same group were well-clustered, while samples from different groups were well-separated (**Figure 4A**, **4B**). Correspondingly, UniFrac trees clustered and differentiated equally well (**Figure 4C**). In conclusion, the above data suggest that antibiotic treatment significantly alters the gut microbiota composition and reduces its complexity.

Effect of antibiotic treatment on bone quality in mice

To investigate the effect of antibiotic treatment on bone quality in mice, we assessed the bone mineral density (BMD) of the spine by using dual-energy X-rays and the morphology and micro parameters of the femur using H&E staining and Micro-CT scanning.

Dual-energy radiography showed that the greyness of the spine in the antibiotic-treated mice (**Figure 5A**) was significantly lower than that in the control group (**Figure 5B**). Correspondingly, the results of quantitative analysis of the L3-L4 showed a 24% decrease in BMD in the treated group compared to the control group (**Figure 5C**).

Staining of the distal femoral epiphysis was used to evaluate its histomorphology; representative photomicrographs are shown in **Figure 6A**. Compared with the control group, the number of trabeculae in the cancellous bone was lower in the treatment group, and the cavity area in the medullary cavity was larger. The trabeculae were small, sparse, and disconnected. In addition, resorption pits produced by osteoclast activity were observed on the tiny trabeculae in the treated group. Quantitative analysis showed a significant decrease in Tb.Ar, %Tb.Ar, and Tb.Wi and a significant increase in Tb.Sp in the treatment group compared with the control group (**Figure 6B-E**).

A micro-CT scan of the femur showed a significantly decreased amount of bone mass in trabeculae of the treated group (**Figure 7A**), and analysis of trabecular parameters showed significant decrease in BV/TV, Tb.Th, Tb.N in the treated group, whereas Tb.Sp (**Figure 7B-E**) significantly increased compared with the control group. These data suggest that antibiotic treatment causes bone loss and alters bone trabecular microarchitecture in mice.

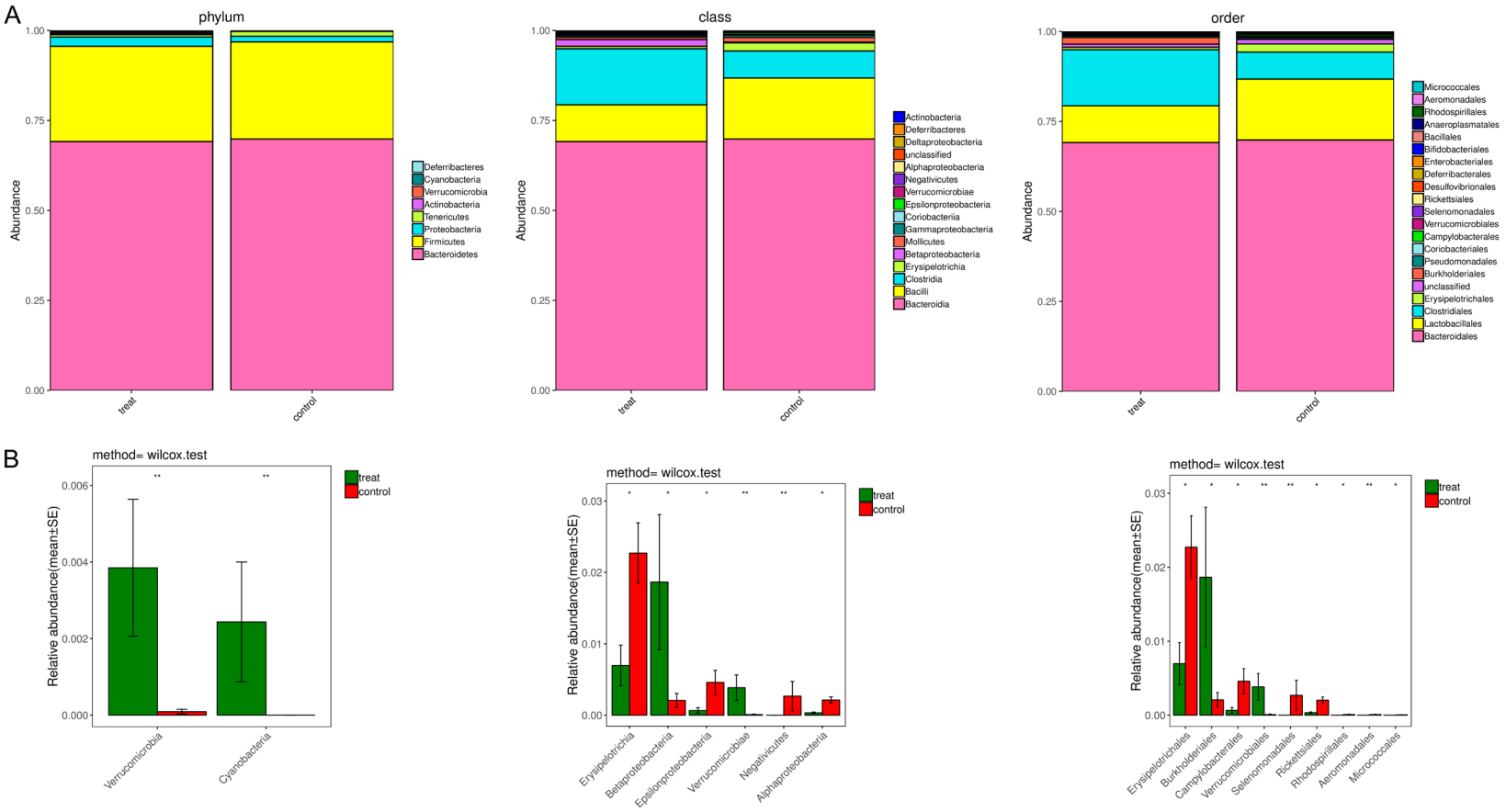

Figure 2. Effect of 12 weeks antibiotics treatment on the composition of gut microbiota in mice (n=6 per group). A. The abundance of fecal bacterial communities at the phylum, class, and order levels; B. Wilcoxon test for significance of differences in OTUs between the two groups at the phylum, class, and order levels. *P<0.05, **P<0.01. Data are presented as mean ± SD.

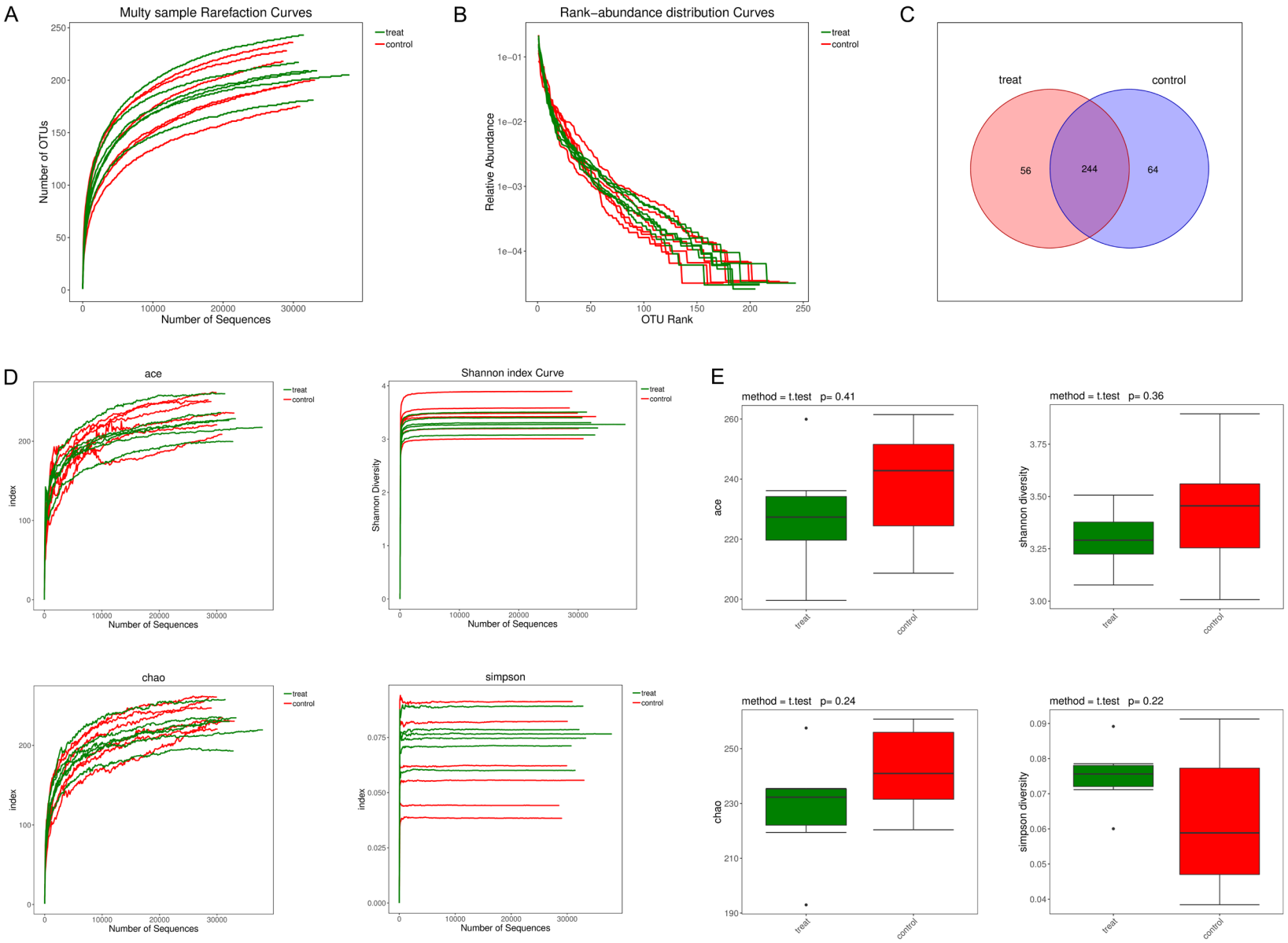

Figure 3. Alterations in the gut microbiota of mice (n=6 per group). A. Multy sample Rarefaction Curves; B. Rank-abundance diagram; C. Venn diagram of OTU with 97% similarity level; D. Curves of the ACE estimator, Chao estimator, Shannon index, and Simpson index; E. Analysis of variability between the two groups on the ACE estimator, Chao estimator, Shannon index, and Simpson index. Values were calculated using one-way ANOVA. Data are presented as mean ± SD.

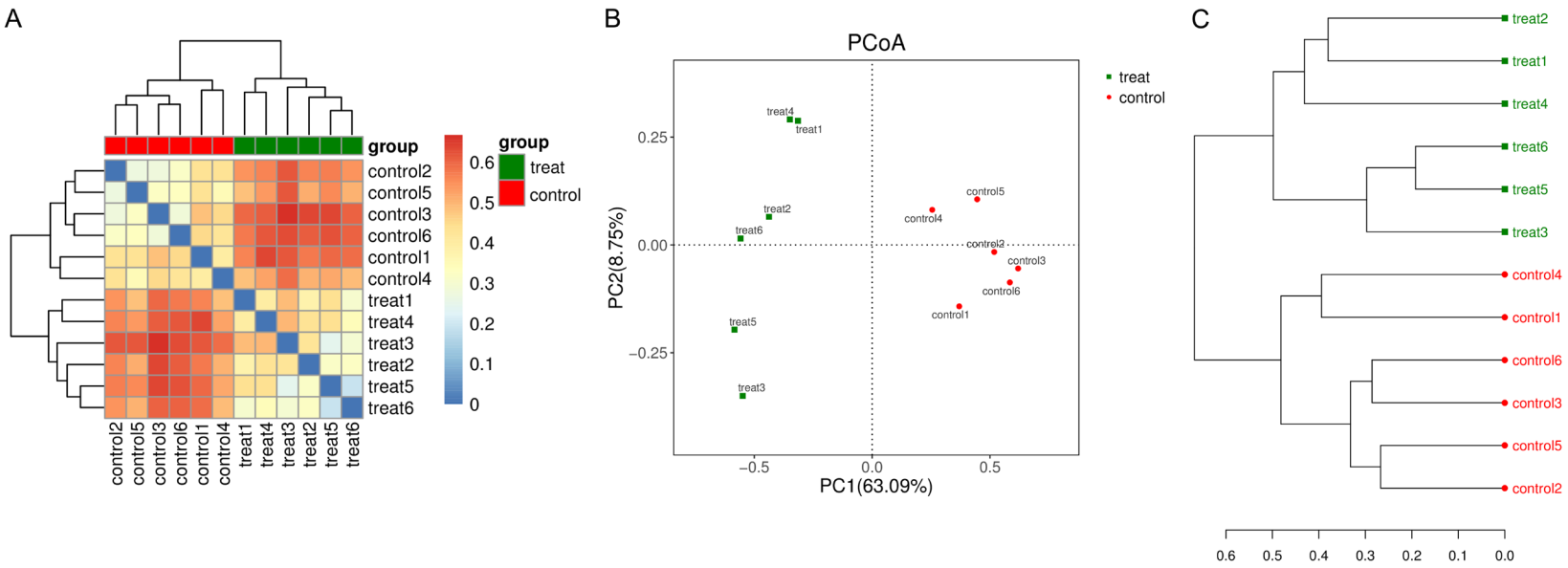

Figure 4. Beta diversity assessment of samples (n=6 per group). A. Matrix Heat Map; B. PCoA analysis; C. UniFrac trees. Data are presented as mean ± SD.

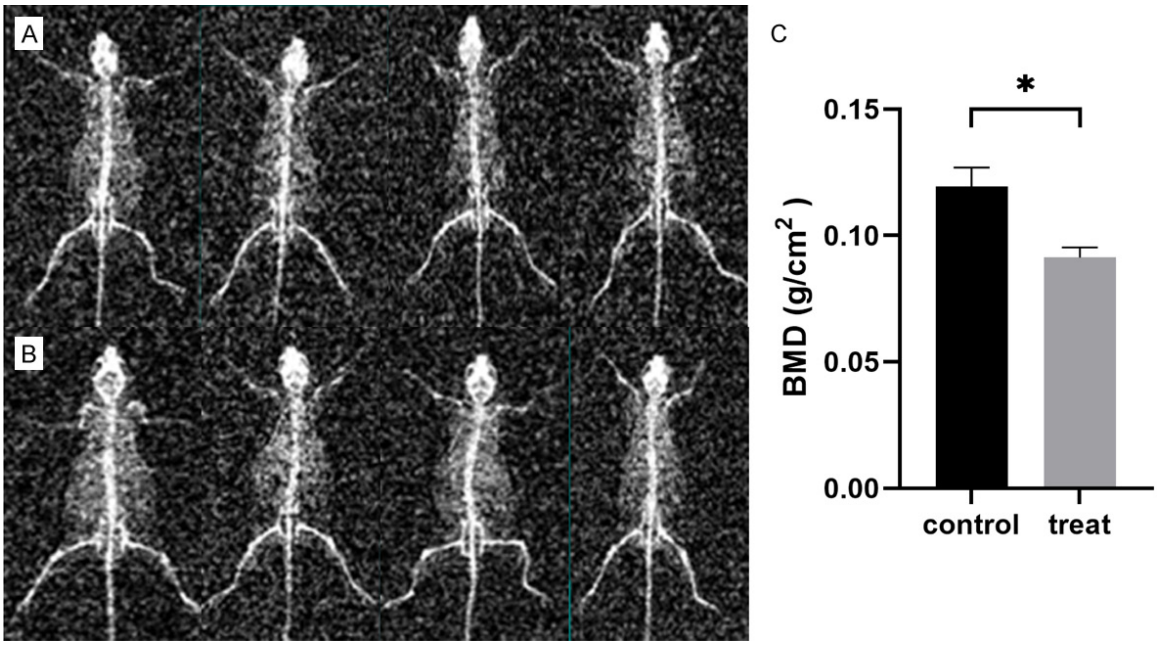

Figure 5. Dual Energy X-Ray images (n=18 per group). Of (A) Treated group and (B) Control group; (C) Comparison of bone mineral density between two groups (p<0.0001). Data points are presented as mean ± SD and t-test was used for comparison between groups. *P<0.05.

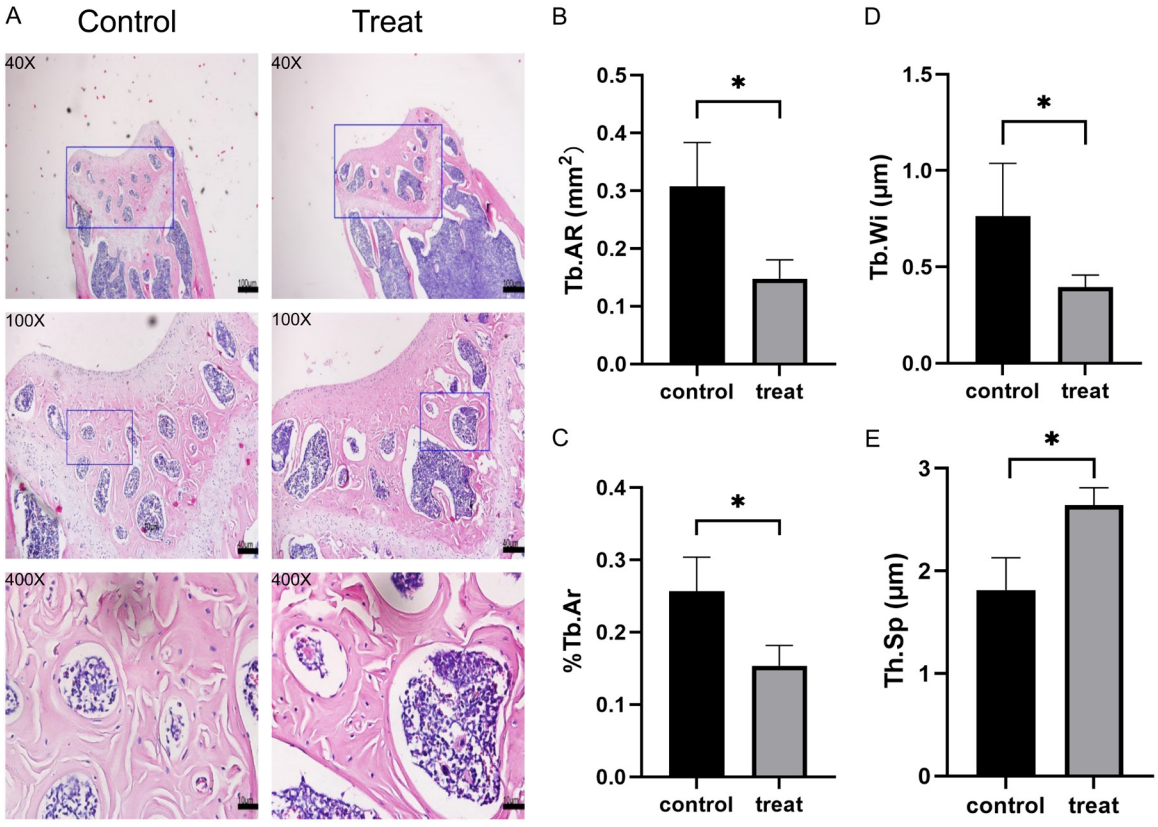

Figure 6. Histomorphological analysis on femur (n=6 per group). A. Representative photomicrographs of H&E staining of the femur; B. Trabeculae area (Tb.Ar), (P<0.0001); C. Percentage of trabecular area (%Tb.Ar), (P<0.0001); D. Trabeculae width (Tb.Wi), (P=0.0025); E. Trabeculae separation (Tb.Sp), (P<0.0001); Data points are presented as mean ± SD and t-test was used for comparison between groups. *P<0.05.

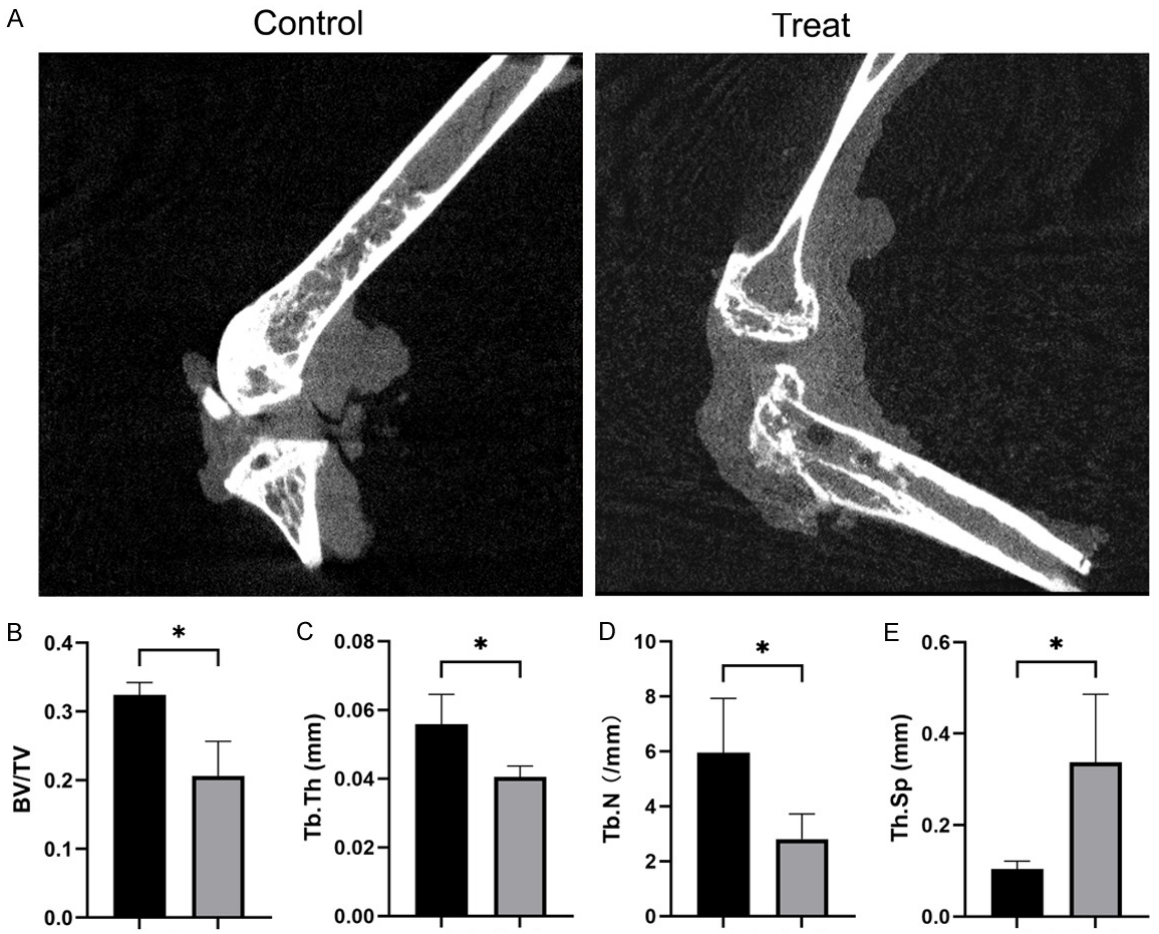

Figure 7. Micro-CT analysis on the trabecular architecture of distal femur metaphysis. A. Representative images of Two-dimensional images; B. Bone volume over total volume (BV/TV), (P=0.0128); C. Trabecular thickness (Tb.Th), (P=0.0156); D. Trabecular number (Tb.N), (P=0.0347); E. Trabeculae separation (Tb.Sp), (P=0.0466); Data points are presented as mean ± SD and t-test was used for comparison between groups. *P<0.05.

Effect of antibiotic treatment on serum estrogens in mice

To investigate whether antibiotic-induced bone loss is estrogen-related, we measured serum estrogen levels in mice with an ELISA test. The results showed that serum estrogen levels in the treated mice decreased by 29% compared with the control group (**Figure 8A**). Notably, BMD of mice in the treated group showed the same trend as the serum estrogen level, and we performed a correlation analysis between them, which showed a significant positive correlation between BMD and estrogen level (**Figure 8B**). These data suggest that antibiotic treatment can cause bone loss in mice by downregulating serum estrogen levels.

Discussion

We report that antibiotic treatment for 12 weeks leads to changes in the composition of gut microbiota and results in bone loss and decreased serum estrogen levels. Implying that estrogen may play an important role in bone loss due to gut microbiota dysbiosis.

It is well known that antibiotics are widely used to treat and prevent infections with bacteria. Some studies suggest that antibiotics can deplete the commensal bacteria in hosts as well, resulting in bacteria disorders and long-term changes and lasting negative effects on the host [24-26]. This provides an effective means of understanding the impact in microbial dysbiosis on bone health [19-21]. However, gut microbiota composition is in a dynamic state and is highly resistant to external influences. It has been shown that although 1-2 weeks of antibiotic treatment can affect the composition of gut microbiota, such changes tend to return rapidly to baseline levels after the treatment is discontinued [27]. Our study

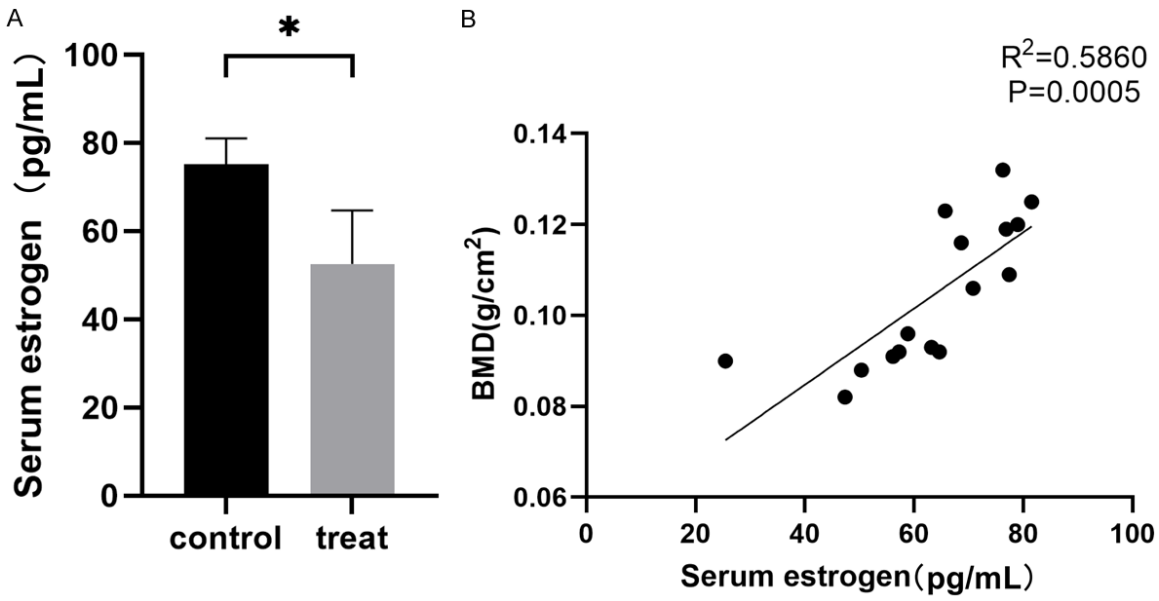

Figure 8. T-test of serum estrogen levels between groups and pearson correlation analysis with bone mineral density. Data points are presented as mean ± SD. *P<0.05.

used a 12-week treatment with neomycin and ampicillin to disrupt the gut microbiota. Long-term use of antibiotics can lead to prolonged and sustained disruption of the gut microbiota [28].

Neomycin and ampicillin are poorly absorbed in the intestine of rodents and can be effective in limiting extra-intestinal effects [23, 29] and, when used together, are effective in killing broad-spectrum bacteria and depleting the gut microbiota, establishing a new intestinal environment [30]. Although long-term treatment with antibiotics is rarely used in humans during the whole period of growth and development, the human gut microbiota does undergo less dramatic changes over longer periods due to diet or metabolic status [27].

Previous studies have demonstrated that treatment with antibiotics leads to changes in the composition of the gut microbiota [19-22]. The results of our study similarly demonstrate this. We have applied a long-term antibiotic treatment to mice and have used a 16s RNA analysis to examine the composition of the gut microbiota of mice. Our results showed that the composition of the gut microbiota of treated groups was significantly altered at the phylum level, and the relative abundance of *Lactobacillale*s was significantly lower in the treated group than in the control group at the level of order and class, while the relative abundance of *Clostridia* was significantly higher. Previous studies have shown that antibiotic treatment can cause a decrease in the abundance of the *Bacteroidete*s phylum, which is the major phylum in healthy mice and humans, and a decrease in *Bacteroidete*s abundance is related to many diseases, including IBD and type 2 diabetes [31, 32]. In our study, however, the change in the abundance of *Bacteroidete*s was not significant, while the abundance of *Lactobacillu*s was significantly altered; further, antibiotic treatment significantly lowered the abundance of *Lactobacillu*s. Many studies have demonstrated the benefit of different Lactobacillus treatments for bone density [13, 33, 34]. Therefore, we speculate that the decrease in the abundance of *Lactobacillu*s may be one of the reasons for the bone loss due to antibiotic treatment. However, we did not observe significant changes in the abundance of *Bacteroidete*s in our study, and we believe that this may be related to the type of antibiotic, the dose, the duration of treatment and the mice's strain, sex, and age.

It is now well known that dysbiosis of the gut microbiota can lead to bone loss, our study shows the same result. Our data show that antibiotic treatment significantly affects both bone mineral density (BMD) and trabecular parameters. BMD, BV/TV, Tb.Th, and Tb.N significantly

decreased, while Tb.Sp significantly increased. The histomorphological results were similarly to the micro-CT results, showing a decrease in Tb.Ar, %Tb.Ar and Tb.Wi, and an increase in Tb.Sp. Antibiotics have been used in different animal models to investigate its effects on bone, but we observe that different models have led to different conclusions. For example, Zhao et al. demonstrated female mice (C57BL/6J) showed a significant increase in BMD after 3 weeks of antibiotic treatment, but no effect after 7 weeks [19]. Another study showed that penicillin at low doses increased bone mineral density in female mice. Interestingly, male mice receiving the same treatment showed decreased bone mineral density [35]. In conclusion, these studies suggest that the administration of antibiotics affects bone mineral density, but the effect depends on the duration of antibiotic treatment and the age, sex, and strain of the mice.

How does dysbiosis caused by antibiotic treatments favor bone loss? There is no conclusive evidence. Previous studies have suggested that this phenomenon is related to intestinal permeability, inflammatory factors, and the immune system [22, 36]. However, in the present study, we surprisingly found that serum estrogen levels in mice showed a significant decrease after antibiotic treatment. Estrogen is known to play an essential role in the regulation of bone metabolism, and previous studies have found that bone loss due to estrogen deprivation requires the involvement of the gut microbiota and that probiotic treatment can alleviate this bone loss [18]. Another study thoroughly described changes in the gut microbiota in ovariectomized mice. It showed that the gut microbiota composition was significantly altered in the ovariectomized rats and that osteoporosis due to estrogen deprivation was associated with dysbiosis of the gut microbiota [37]. These studies suggest a strong correlation between gut microbiota and estrogen, and our findings suggest that estrogen may play an essential role in bone loss due to gut microbiota dysbiosis. However, it is unclear how intestinal microbiota dysbiosis leads to decreased serum estrogen levels. We speculate that this may be related to microorganism-associated molecular patterns (MAMP) and the role of the gut microbiota in the transformation of host metabolites. MAMP is a conserved component of microbial cells (e.g., peptidoglycan and lipopolysaccharide (LPS)). These microbial molecules can affect host bone metabolic processes by influencing the development of the immune system or activating systemic inflammation and cytokine production [38]. Studies on GF mice have shown that defective immune system development is the leading cause of their increased bone mass [11], and recently it has been shown that the defective immune system development in GF mice is caused by a lack of MAMPs [39]. In addition, inflammatory factors are one of the important regulatory mechanisms of bone metabolism, and it has been shown that LPS can contribute to the development of osteoporosis in rats by promoting the expression of inflammatory factors [40]. The presence, composition, and immunomodulatory activity of MAMPs are related to the species of bacteria, so alterations in GM composition may affect the bone metabolic activity of the host by affecting the levels of specific MPMAs. The role of the gut microbiota in the conversion of host metabolites should not be neglected. Estrogens are excreted into the gut after inactivation, and GM can reactivate estrogens with certain hydrolytic enzymes (e.g., β-glucuronidase) to continue their circulation and activity [41]. However, antibiotic treatment leads to dysbiosis of the gut microbiota, in which case the loss of enzymes may impair the recovery of this estrogen while increasing its excretion, leading to a decrease in serum estrogen and bone loss. Nevertheless, other factors related to the gut microbiota cannot be ignored, such as intestinal permeability, immune system, etc. [11, 18]. Therefore, the cause of the decrease in serum estrogen levels due to gut microbiota dysbiosis needs further investigation.

This study has several limitations. First, only one concentration of antibiotic treatment has been studied, which does not adequately determine the relationship between antibiotic treatment and bone loss/estrogen levels. Second, our results contradict previous studies. The effect of 12 weeks of antibiotic treatment on gut microbiota composition needs further investigation. Further, we did not measure the water consumption during the treatment period but only visually observed its approximate value to monitor the compliance of the treated group with the antibiotic treatment. Therefore, we suggest cautiously that gut microbiota can affect bone quality by regulating serum estrogen levels.

Conclusion

This study showed that chronic administration of antibiotics in mice results in gut microbiota disorder, reduced serum estrogen levels, and increased bone loss. These changes suggest that gut microbiota may affect bone quality by regulating serum estrogen levels.

Disclosure of conflict of interest

None.

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