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ORIGINAL ARTICLE

Gut microbiota composition and bone mineral loss—epidemiologic evidence from individuals in Wuhan, China

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Abstract

Summary We explored the association between gut microbiota composition and bone mineral loss in Chinese elderly people by high-throughput 16S ribosomal RNA (rRNA) gene sequencing. Compared with controls, a smaller number of operational taxonomic units (OTUs), several taxa with altered abundance, and specific functional pathways were found in individuals with low-bone mineral density (BMD).

Introduction Gut microbiota plays important roles in human health and associates with a number of diseases. However, few studies explored its association with bone mineral loss in human.

Methods We collected 102 fecal samples from each eligible individual belonging to low-BMD and control groups for high-throughput 16S rRNA gene sequencing.

Results The low-BMD individuals had a smaller number of OTUs and bacterial taxa at each level. At the phylum level, Bacteroidetes were more abundant in the low-BMD group; Firmicutes were enriched in the control group; Firmicutes and Actinobacteria positively correlated and Bacteroidetes negatively correlated with the BMD and T-score in all subjects. At the family level, the abundance of Lachnospiraceae in low-BMD individuals reduced and positively correlated with BMD and T-score; meanwhile, BMD increased with increasing Bifidobacteriaceae. At the genus level, low-BMD individuals had decreased proportions of *Roseburia* compared with control ones ($P < 0.05$). *Roseburia*, *Bifidobacterium*, and *Lactobacillus* positively correlated with BMD and T-score. Furthermore, BMD increased with rising abundance of *Bifidobacterium*. Functional prediction revealed that 93 metabolic pathways significantly differed between the two groups (FDR-corrected $P < 0.05$). Most pathways, especially pathways related to LPS biosynthesis, were more abundant in low-BMD individuals than in control ones.

Conclusions Several taxa with altered abundance and specific functional pathways were discovered in low-BMD individuals. Our findings provide novel epidemiologic evidence to elucidate the underlying microbiota-relevant mechanism in bone mineral loss and osteoporosis.

Keywords Bone mineral loss · Gut microbiota · Osteoporosis · 16S Ribosomal RNA · Sequencing

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Introduction

Low bone mineral density (BMD) is the main character of osteoporosis (OP), which results in increased bone fragility and even fractures [1]. OP is responsible for the increased mortality and reduced life quality among elderly [2]. In the USA, OP is prevalent in 26% of women aged ≥ 65 years and over 50% of those aged ≥ 85 years [2]. In China, the prevalence of OP rises dramatically due to population aging [3]. The quantity and quality of bone mass depend on the balance between bone formation (regulated by osteoblasts) and bone resorption (regulated by osteoclasts) [4, 5]. Bone mass decreases when bone resorption overrides bone formation [4, 5]. Bone metabolism is influenced by modifiable factors (e.g., smoking, lack of physical activity, and low calcium intake) [6, 7] and hereditary characteristics (e.g., genes in the WNT signaling pathway) [8, 9].

The human gut is populated by trillions of microorganisms, known as the gut microbiota [10]. The gut microbiota influences the host in diverse ways [10, 11]. The gut microbiome is highly heterogeneous, consisting of 10^{14} bacteria coded by 5 million genes [12, 13]. The human gut microbiota comprises over 1000 distinct microbial species, many of which are not well characterized yet. About two-thirds of microbial species constituting the gut microbiota is unique to each individual [14]. The gut microbiota contributes to many human chronic diseases, such as obesity, metabolic disease, malnutrition, neurological disorders, cardiovascular disease, and cancer [15]. Alterations in the microbiome are also associated with the maintenance of bone mass and bone quality [15]. An animal study suggested that manipulation of the microbiome or its metabolites may afford opportunities to optimize bone growth and health [16]. Another study also observed that the gut microbiota can regulate bone mass in mice by altering immune status in the bone and affecting osteoclast-mediated bone resorption [10]. Three main areas in which the microbiota is being investigated for its impact on the bone are nutrient acquisition (calcium and phosphate), immune regulation, and direct effects through the production of small molecules such as serotonin or estrogen-like molecules [17]. To date, epidemiologic evidence for the role of gut microbiota in bone mineral loss remains inadequate. Only one study used 16S ribosomal RNA (rRNA) gene sequencing to investigate the link between gut microbiome and OP in human beings [18]. However, the report is clearly limited by a small sample size ($n = 6$ per group). Hence, the associations of gut microbiota with low BMD in human are worthy of further investigation.

To this end, fecal sample of 102 aged participants eligible for strict inclusion criteria was collected for bacterial diversity analysis using 16S rRNA gene high-throughput sequencing. The bacterial community composition and diversity were compared between the control and low-BMD groups.

Materials and methods

Study subjects

In general, healthy adults aged ≥ 60 years were recruited at Wuhan Union Hospital and two communities in Wuhan City from 2016 to 2017. BMD values at skeleton sites of the lumbar spine (LS), femoral neck (FN) and total hip were obtained using dual-energy X-ray absorptiometry (DXA) (Lunar Prodigy, GE, USA) assessment. All participants were measured on the same DXA. According to the reference [19], the T-score is the BMD difference expressed by the standard deviation of a reference BMD distribution from 20 to 40-year-old individuals of the same sex, and the Z-score is the BMD difference expressed by the standard deviation of the BMD distribution from age- and sex-matched controls. Participants with BMDs higher than the median of that at the skeleton site of FN were classified into the control group ($n = 51$); whereas participants with BMDs lower than the median of that at the skeleton site of FN were classified into the low-BMD group ($n = 51$). Demographic information (e.g., sex and age), lifestyle factors (e.g., cigarette smoking, alcohol drinking, and physical activity), prevalent diseases (e.g., diabetes, renal, gastrointestinal and thyroid disease, fracture, and diseases of the uterus and/or ovary), and medication history (e.g., use of antibiotics and hormone) were collected with a self-made questionnaire. Cigarette smoking was defined as the use of tobacco products for more than 6 months continuously or cumulatively in a lifetime [20]. Weight and height were measured in light indoor clothes without shoes by an ultrasonic height and weight measuring instrument (HW–900Y). Weight was recorded in kilograms to one decimal place and height was recorded in centimeters to one decimal place. Body mass index (BMI) was calculated by dividing weight in kilograms by height in meters squared. We adopted the following exclusion criteria: (1) use of antibiotics or hormone within 6 months before fecal sample collections; (2) previous medical treatment of hysterectomy and/or ovariectomy; (3) disease history of hyperthyroidism or hypothyroidism; (4) prevalent diseases of diabetes and gastrointestinal diseases. Finally, 102 participants (42 men and 60 women) were included in our analysis (Suppl. Fig. 1).

This study was approved by the Ethics Committee of Tongji Medical College of Huazhong University of Science and Technology. All participants presented written informed consent prior to enrollment.

Sample collection, DNA extraction, and PCR

Fecal samples were collected in sterile plastic cups, frozen, and then transported within 4 h to the laboratory, where they were stored at − 80 °C until further processing. Fecal microbial DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). PCR amplification was carried out in an ABI 2720 Thermal Cycler (Thermo Fisher Scientific, USA). We used

the bacterial genomic DNA as the template for amplification of the V3–V4 hypervariable region of the 16S rRNA gene in three replicate reactions with the primer pair F (Illumina adapter sequence 1 + CCTACGGGNGGCWGCAG) and R (Illumina adapter sequence 2 + GACTACHVGGGTATCTAATCC). The replicate PCR products were pooled and purified with Agencourt AMPure XP magnetic beads (Beckman Coulter, USA). The TopTaq DNA Polymerase kit (Transgen, China) was used. The purity and concentration of sample DNA were tested using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA).

Microbiota sequencing and sequence quality control

PCR products were sequenced using the Illumina Miseq platform with the 2 × 250 bp paired-end method after the library was quantified, mixed, and quality checked [21]. The raw reads were filtered by several steps to remove low-quality reads. Firstly, TrimGalore was used to filter raw reads at Q20 and adapter sequence and reads with length < 100 bp were removed. Secondly, pairs of reads from the original DNA fragments were merged using FLASH2 [22] and low-quality sequences were further removed after merging. Third, Mothur was used to find and remove primers in the sequences, as well as to remove sequences including N–base/homopolymer > 6 bp. Finally, reads with an error rate of > 2 and reads with length < 100 bp were removed using usearch to obtain clean reads for further analyses. Operational taxonomic units (OTUs) were assigned by clustering the sequences with a threshold of 97% pairwise identity and chimeras were removed using UPARSE. OTUs were taxonomically assigned at a confidence threshold of 80% based on the Ribosomal Database Project database by Mothur [21]. All samples were sequenced at once and at the same laboratory. Bioinformatics analysis was performed by Genasky Biotechnology Inc. (Shanghai, China).

Analysis of microbial community

Alpha- and beta-diversity estimates, which respectively indicate within-sample richness and between-sample dissimilarity, were computed using the R software. PERMANOVA test was performed using the adonis2 function in vegan in *R* with 9999 permutations. Principal component analysis (PCA) was used to depict the beta-diversity at an OTU level. Linear discriminant effect size (LEfSe) analysis was applied to identify microorganism features distinguishing fecal microbiota specifically for biomarker discovery. LEfSe combines Kruskal–Wallis test or pairwise Wilcoxon rank-sum test with linear discriminant analysis (LDA). LEfSe analysis was performed given two indispensable situations: threshold on the logarithmic LDA score for discriminative features equaling to 2.0 and an alpha significance level of 0.05. Two independent-sample *t* test and Mann–Whitney *U* test were performed to detect the significant differences of abundances among taxa. Spearman correlation analysis was conducted to evaluate the correlations between microbial abundance and DXA-derived BMD measurements. Considering the collinear problem, we used the ridge regression analysis—a penalized regression approach that offers good performance in multivariate prediction problems [23, 24]—to explore the association between gut microbiota relative abundance and bone mass measurements (i.e., BMD, T-score, and Z-score) while adjusting for covariates such as age, sex, cigarette smoking, and BMI.

Function profile analysis

We used Phylogenetic Investigation of Communities by Reconstruction of Unobserved States to estimate metagenome composition and Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs to predict functions. The 16S rRNA amplicon data were referred to for existent gene predications, abundance calculations, metabolic pathway assignments (using KEGG), and comparison of differences between two groups. Reaching significant difference of KEGG ortholog abundances between groups was defined as a FDR-corrected *P* value of < 0.05.

Statistical analyses

Two independent-sample *t* test was used to compare the differences of variables conforming to normal distribution, which were expressed as means ± standard deviation. Mann–Whitney *U* test was used to compare the differences of variables not conforming to normal distribution between groups, which were expressed as median ± interquartile range. The relative abundances in each sample were calculated on the basis of the sum of 16S rRNA sequences. The R software and SPSS version 21.0 were used for statistical analyses. A two-sided $P < 0.05$ was considered to achieve statistical significance.

Results

Characteristics of participants involved in this study

The low-BMD individuals had lower BMD, T-scores, and Z-scores in LS, FN, and total hip than the control group (all $P < 0.05$) (Table 1). The low-BMD group had higher proportions of females and smoking individuals than the control group (all $P < 0.05$). Differences in other variables, such as age, BMI, alcohol drinking, and the presence of common chronic diseases (e.g., fracture, renal disease, and osteoarthritis), did not achieve significance between the two groups.

Table 1 Characteristics of included subjects in the study

Variables	Low-BMD group ($n = 51$)	Control group ($n = 51$)	P value
Female, n (%)*	41 (80.4)	19 (37.3)	*< 0.001*
Age (years; means ± SD)#	65.5 ± 6.0	66.6 ± 6.0	0.362
BMI (means ± SD)#	23.9 ± 3.0	24.6 ± 3.0	0.248
Smoking, n (%)*	27 (52.9)	17 (33.3)	*0.046*
Drinking, n (%)*	26 (51.0)	17 (33.3)	0.071
Renal Disease, n (%)*	7 (14.0)	7 (14.0)	0.968
Fracture, n (%)*	16 (31.4)	9 (17.6)	0.107
Osteoarthritis Disease, n (%)*	13 (26.0)	9 (17.6)	0.309
LS Z-score#	− 0.4 ± 1.0	0.7 ± 1.7	*< 0.001*
LS T-score#	− 2.1 ± 1.1	− 0.4 ± 1.7	*< 0.001*
LS BMD (g/cm^2)#	0.9 ± 0.1	1.2 ± 0.2	*< 0.001*
FN Z-score#	− 0.6 ± 0.6	0.5 ± 0.8	*< 0.001*
FN T-score#	− 2.2 ± 0.8	− 0.7 ± 0.9	*< 0.001*
FN BMD (g/cm^2)#	0.7 ± 0.06	0.9 ± 0.09	*< 0.001*
Total hip Z-score#	− 0.6 ± 0.7	0.6 ± 0.8	*< 0.001*
Total hip T-score#	− 1.9 ± 0.7	− 0.5 ± 0.8	*< 0.001*
Total hip BMD (g/cm^2)#	0.8 ± 0.09	1.0 ± 0.1	*< 0.001*

Z-score, T-score, and BMD were measured using dual X-ray absorptiometry method

LS lumbar spine 1–4, *FN* femoral neck, *BMD* bone mineral density, *BMI* body mass index, *SD* standard deviation

*Chi-square test

Two independent-sample t test

Difference achieved significance of $P < 0.05$

Comparisons of gut microbial diversity between two groups

A total of 17,881,129 high-quality sequences were detected among the 102 fecal samples from the participants with an average of 175,305 sequences per sample. The distribution of clean sequences clustered at length 422 bp. The fecal bacteria were composed of 247 species, 182 genera, 75 families, 37 orders, 24 classes, and 15 phyla in total. Compared with controls, the low-BMD group had smaller number of bacterial taxa at each level (species, genus, family, order, class, and phylum) (Table 2). A total of 969 OTUs were quantified in this study. The Venn diagram showed that the number of OTUs was smaller in the low-BMD group (783) than in the control group (872) (Fig. 1). The gut microbiota shared 70.8% of OTUs in common across the two groups, 10.0% of OTUs were only quantified in the low-BMD individuals, and 19.2% of OTUs were only quantified in the controls. The difference of alpha-diversity indexes (ACE, Chao1, Shannon, and Simpson) did not achieve significance.

The two groups were not completely distinguished by PCA (Suppl. Fig. 2) and neither did the PERMANOVA test ($R^2 = 0.013$, $P = 0.173$). LEfSe analysis revealed that Bacteroidetes (phylum), Bacteroidaceae (family), and *Bacteroides* (genus) were enriched in the low-BMD individuals and Firmicutes (phylum), Lachnospiraceae (family), and *Roseburia* (genus) were enriched in the controls (Fig. 2). The rarefaction curves reached a plateau phase, suggesting that most bacterial species of the microbiota were captured by our sequencing (Suppl. Fig. 3).

Taxonomic composition of gut microbial community abundance

The gut microbiota was dominated mainly by Bacteroidetes, Firmicutes, Proteobacteria, Fusobacteria, and Actinobacteria

Table 2 Bacterial compositions in each group at different levels

	Phylum	Class	Order	Family	Genus	Species	OTU
Low BMD group	12	20	34	70	165	211	783
Control group	15	24	36	72	174	228	872
Total	15	24	37	75	182	247	969

Values in the "total" row indicate a summarized number of phylum, class, order, family, genus, species, and OTU, respectively, across all fecal samples in this study

BMD bone mineral density, *OTU* operational taxonomic unit

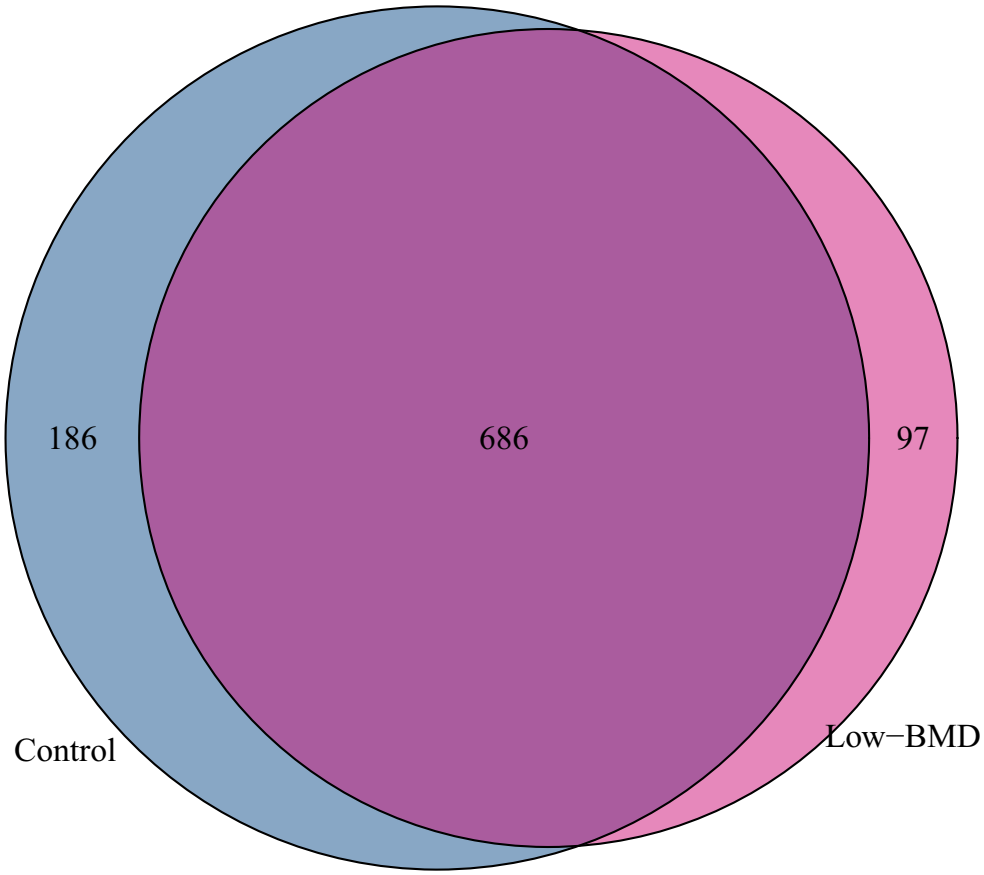


Fig. 1 Comparison of gut microbiota diversity between the two groups by Venn diagram at the operational taxonomic units (OTUs) level. Low–BMD, low-bone mineral density (BMD) group; Control, control group

at the phylum level in all samples. The proportion of Bacteroidetes was significantly higher and the Firmicutes-to-Bacteroidetes ratio was lower in the low-BMD group than in the control group (all $P < 0.05$) (Table 3). At the family level, the low-BMD individuals had a higher level of Bacteroidaceae and a lower level of Lachnospiraceae than the controls (all $P < 0.05$). At the genus level, *Bacteroides* accounted for the largest proportion in all samples. The proportion of *Bacteroides* was higher and the proportion of *Roseburia* was lower in the low-BMD group than in the control group (all $P < 0.05$).

Correlations between gut microbiota abundance and BMD measurements

At the phylum level, Firmicutes and Actinobacteria positively correlated to the BMD and T-score (all $P < 0.05$), and Bacteroidetes negatively correlated to the BMD in total hip, FN, LS, and T-score in LS (all $P < 0.05$) (Table 4). At the family level, Lachnospiraceae, Lactobacillaceae, and Bifidobacteriaceae positively correlated with the BMD and T-score, and the Bacteroidaceae negatively correlated to the BMD in FN (all $P < 0.05$). At the genus level, *Roseburia*, *Bifidobacterium*, *Anaerobacterium*, and *Lactobacillus* positively correlated with the BMD and T-score and *Bacteroides, Anaerostipes,* and *Clostridium_XlVb* negatively correlated with the BMD and T-score (all $P < 0.05$).

Associations between gut microbiota relative abundance and BMD measurements

Ridge regression analysis showed that the BMD in FN increased with rising Actinobacteria abundance after adjusting for age, sex, cigarette smoking, and BMI ($P < 0.05$) (Table 5). At the family level, the BMD in LS and FN increased in response to the increase in Bifidobacteriaceae community abundance (all $P < 0.05$). At the genus level, the T-score and Z-score in total hip and Z-score in FN decreased with increasing *Clostridium_XlVb* abundance after adjusting for confounders (all $P < 0.05$). The BMD in FN increased with increasing *Bifidobacterium* abundance after adjusting for age, sex, cigarette smoking, and BMI ($P < 0.05$).

Functionally predicted metabolic pathways underlying the observed associations

A total of 93 KEGG metabolic pathways were identified to show significantly different microbiota abundances

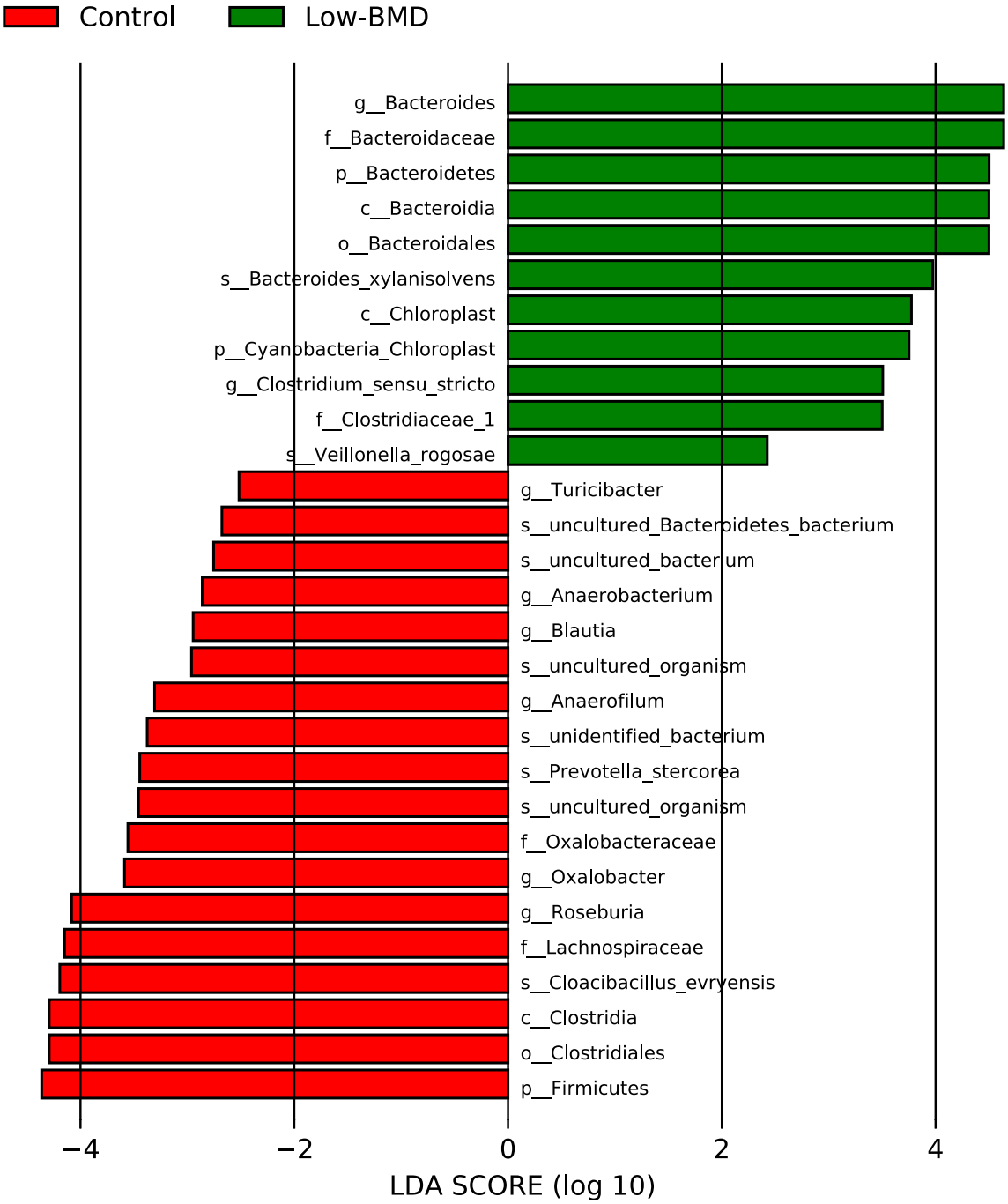


Fig. 2 LEfSe indicating differences in the bacterial taxa at different levels (p, phylum; c, class; o, order; f, family; g, genus; s, species), only the taxes having a $P < 0.05$, LDA value > 2 are shown in the figure. Low–BMD, low-bone mineral density (BMD) group; Control, control group

between the two groups (all corrected $P < 0.05$) (Fig. 3). About 72.0% of those pathways, particularly those relevant to LPS biosynthesis and glycan degradation, had more abundance in low-BMD group than in the control group. Left 26 pathways, including those related to saccharide metabolism and biosynthesis, lipid metabolism, transporters, signal transduction, and secretion system, had more abundance in the control group than in the low-BMD group.

Discussion

In this study, we used high-throughput sequencing to analyze the composition and diversity of the gut microbiota and compared them between low-BMD individuals and controls. The low-BMD group had fewer OTUs and bacterial taxa at each level compared with the control group. At the phylum level, Bacteroidetes were enriched in the low-BMD individuals and Firmicutes were enriched in the control group. Firmicutes and

Table 3 Comparison of gut microbiota compositions (abundance of sequences) between two groups

Taxonomic level	Low-BMD group	Control group	*P* value
Phylum			
Firmicutes*	11,200 ± 6709	13,514 ± 6160	0.073
Bacteroidetes*	27,599 ± 7227	24,409 ± 8013	*0.037*
F/B[#]	0.340 ± 0.443	0.454 ± 0.467	*0.013*
Family			
Lachnospiraceae[#]	3310 ± 3385	4693 ± 3906	*0.010*
Bacteroidaceae*	21,635 ± 8252	17,203 ± 8299	*0.008*
Genus			
Roseburia[#]	387 ± 1061	1130 ± 1805	*0.005*
Bacteroides[*]	21,635 ± 8252	17,203 ± 8299	*0.008*

F/B the ratio of Firmicutes-to-Bacteroidetes, *BMD* bone mineral density

*Data followed a normal distribution and were expressed in mean ± standard deviation and the difference tested by two independent-sample *t* test;

[#] Data did not follow a normal distribution and were expressed in median ± interquartile range and the difference tested by Mann–Whitney U test

Difference achieved significance of $P < 0.05$

Only phyla, family, and genera with relative abundance greater than 0.1% were included in this analysis

Actinobacteria positively correlated and Bacteroidetes negatively correlated with the BMD and T-score in all individuals. At the family level, the proportion of Lachnospiraceae was low in the low-BMD group and positively correlated with BMD and T-score. At the genus level, the low-BMD group had lower proportion of *Roseburia* and higher proportion of *Bacteroides* compared with the control group. *Roseburia*, *Bifidobacterium*, and *Lactobacillus* positively correlated with BMD and T-score. Moreover, BMD increased with increasing *Bifidobacterium*. KEGG metabolic pathways related to LPS biosynthesis showed more abundance in the low-BMD individuals than in the controls.

To date, only one similar study of fecal microbiota and osteoporosis on human beings was found [18]. The authors reported an inverse correlation between the number of bacterial taxa and BMD, which contradicted with our findings. The inconsistency may be due to several factors such as sample size, sex ratio of participants, number of sequence reads, and uneven coverage of microbes by different PCR primers. The sample size, sex ratio of participants (female to male), and number of sequence reads were 102 (vs. 18), 60:42 (vs. 5:1), and 17,881,129 (vs. 694,232) in our study (vs. that of Wang et al.) [18]. The PCR primers also differed in the two studies.

The underlying mechanisms for gut microbiota variations associated with BMD declines remain unclear. Bacteroidetes were enriched in the low-BMD individuals and Firmicutes were enriched in the controls. Lachnospiraceae reduced in the low-BMD group and positively correlated with the BMD

Table 4 Spearman correlation analyses of gut microbiota abundance and BMD measurements at phylum, family, and genus levels

Taxonomic level	LS BMD	LS T-score	LS Z-score	FN BMD	FN T-score	FN Z-score	Hip BMD	Hip T-score	Hip Z-score
p_Bacteroidetes	*− 0.260***	*− 0.234**	− 0.154	*− 0.229**	− 0.172	− 0.132	*− 0.235**	− 0.189	− 0.118
p_Firmicutes	*0.260***	*0.239**	0.163	*0.248**	*0.208**	0.141	*0.285***	*0.238**	0.149
p_Actinobacteria	0.153	0.132	0.069	0.176	*0.204**	0.073	0.192	0.161	0.090
f_Bacteroidaceae	− 0.151	− 0.130	− 0.058	*− 0.220**	− 0.166	− 0.126	− 0.157	− 0.090	− 0.051
f_Lachnospiraceae	*0.279***	*0.272***	0.185	*0.256***	*0.217**	0.143	*0.326***	*0.300***	*0.197**
f_Lactobacillaceae	0.220	*0.205**	0.113	*0.225**	*0.221**	0.101	*0.201**	0.152	0.076
f_Peptostreptococcaceae	0.160	0.164	0.097	*0.214**	0.137	0.084	*0.224**	*0.198**	0.116
f_Bifidobacteriaceae	*0.204**	0.189	0.137	0.156	*0.203**	0.097	*0.214**	*0.199**	0.142
g_Bacteroides	− 0.151	− 0.130	− 0.058	*− 0.220**	− 0.166	− 0.126	− 0.157	− 0.090	− 0.051
g_Roseburia	*0.233**	*0.200**	0.072	*0.328***	*0.237**	0.188	*0.345***	*0.288***	*0.199**
g_Lactobacillus	*0.220**	*0.205**	0.113	*0.225**	*0.221**	0.101	*0.201**	0.152	0.076
g_Gemmiger	0.191	0.172	0.089	0.156	0.168	0.087	*0.208**	0.189	0.133
g_Bifidobacterium	*0.201**	0.186	0.134	0.155	*0.201**	0.096	*0.212**	*0.197**	0.140
g_Romboutsia	0.172	0.171	0.089	*0.227**	0.169	0.121	*0.223**	*0.202**	0.114
g_Anaerostipes	*− 0.218**	− 0.179	− 0.064	− 0.167	− 0.088	− 0.067	− 0.158	− 0.077	− 0.043
g_Clostridium_XlVb	− 0.103	− 0.134	− 0.187	− 0.099	− 0.184	− 0.092	− 0.158	*− 0.217**	*− 0.212**
g_Anaerobacterium	0.187	0.174	0.120	*0.208**	*0.204**	0.143	*0.234**	*0.197**	0.189
g_Sporobacter	0.161	0.144	0.078	*0.197**	0.142	0.103	*0.220**	0.175	0.133

The correlation coefficient achieved a significance of $*P < 0.05$ or $**P < 0.01$. *LS*, lumbar spine 1–4; *FN*, femoral neck; *BMD*, bone mineral density

p, phylum; f, family; g, genus

Only phyla, family, and genera with relative abundance greater than 0.1% were included in this analysis

Difference achieved significance of $P < 0.05$

and T-score. At the genus level, the low-BMD individuals had lower proportions of *Roseburia* (from Lachnospiraceae) than controls, and *Roseburia* positively correlated with BMD and T-score. Lachnospiraceae, emerging from the Firmicutes phylum, are important producers of short-chain fatty acids (SCFAs) such as acetate, propionate, and butyrate. SCFAs are major end products of bacterial metabolism in the human large intestine and play important roles in the maturation of host immune system, including protection from infection, induction of peripheral regulatory T cells, energy homeostasis, and modulation of metabolic rate [25–29]. Any decrements in key SFCA producers such as the Firmicutes phylum can reduce SCFA production [30]. SCFAs mediate microbiota-induced changes in IGF-1 levels and contribute to the effects of colonization on bone turnover [16]. Several animal studies also evidenced that the probiotics containing species from the Firmicutes phylum Lactobacillaceae family promote bone maintenance in case of sex-steroid deficiency [31–33].

The inflammation system is critical to bone loss. Inflammation activation within the bone is a key driver of disruption to normal bone remodeling and initiation of bone loss [34–36]. In vitro studies showed that high osteoporosis risk corresponded to increasing levels of tumor necrosis factor (TNF) [36]. Resolution to this inflammatory cytokine (possibly with anti-inflammatory agents) is beneficial to bone health maintenance, consequently reducing osteoporosis risk [37, 38]. *Lactobacillus reuteri* can suppress basal TNF-α mRNA levels in the ileum and jejunum of male mice and increase trabecular bone parameters (e.g., BMD, trabecular number, bone volume fraction, and trabecular thickness) in the distal femur metaphyseal region and in the lumbar vertebrae [36]. Our findings of positive correlations of *Lactobacillus* with BMD and T-score are in accordance with those observations.

Bifidobacterium were associated with BMD and T-score positively in our study. Bifidobacterium is reportedly involved in intestinal microbial homeostasis, gut barrier, and lipopolysaccharide (LPS) reduction [39]. The enrichment of the modules for LPS biosynthesis and export in patients suggests that the gut microbiota plays a potential role in causing low-grade inflammation [40, 41]. Function predictions in our study observed that the abundance of LPS biosynthesis and LPS biosynthesis proteins significantly increased in the low-BMD group compared with

Fig. 3 Predicted functional differences between two groups. *P* value was Benjamini–Hochberg false discovery rate (FDR) corrected. Low, low-bone mineral density (BMD) group; Control, control group. Mean proportions are shown in stacks for the low-BMD group (orange) and the control group (blue). Difference in mean proportions = mean proportion in the control group minus mean proportion in low-BMD group. Pathways which were more abundant in low-BMD group were on the negative side (orange circle with 95% confidence interval) and those which were more abundant in control group were on the positive side (blue circle)

Table 5 Ridge regression analysis of the effects of gut microbiota relative abundance on BMD measurements

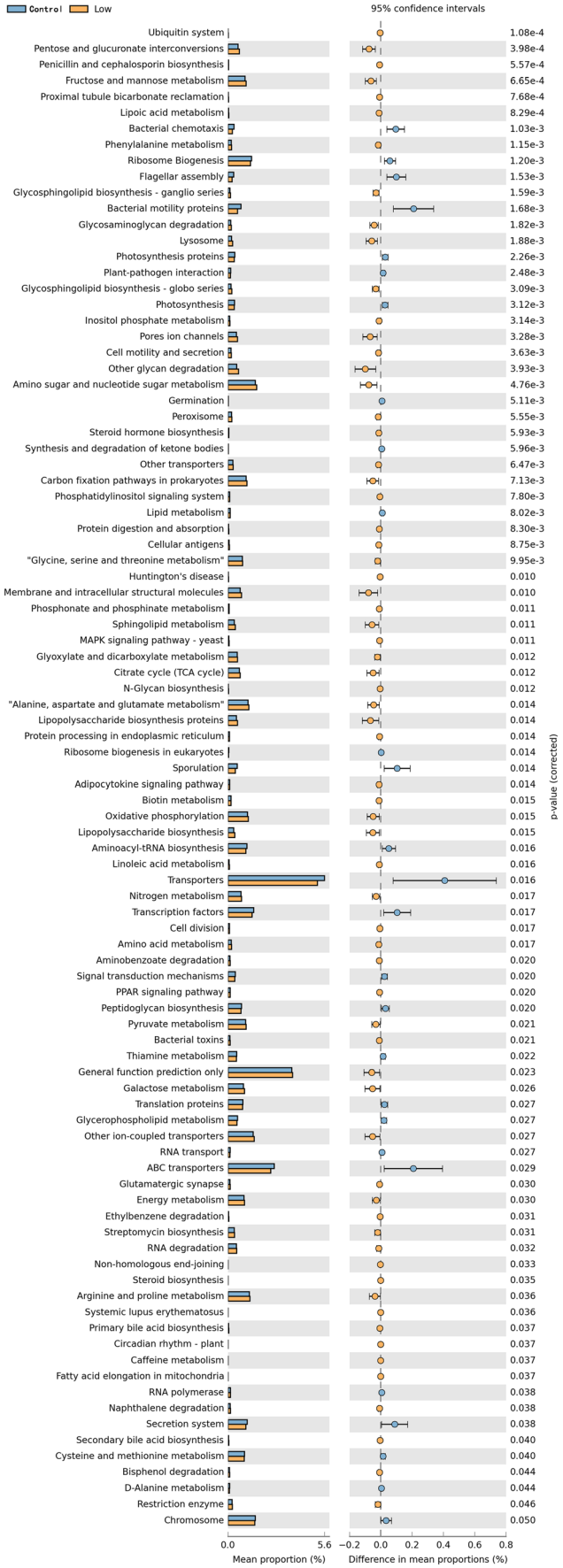
Taxonomic level	LS BMD	LS T-score	LS Z-score	FN BMD	FN T-score	FN Z-score	Hip BMD	Hip T-score	Hip Z-score
Phylum									
Bacteroidetes	−0.013	−0.011	0.013	−0.012	0.031	−0.001	−0.007	0.063	0.203
Firmicutes	0.024	0.023	0.014	0.050	0.075	0.022	0.060	0.104	0.195
Actinobacteria	0.128	0.123	0.146	*0.156**	0.140	0.148	0.062	0.070	0.092
Family									
Bacteroidaceae	−0.033	−0.025	−0.032	−0.095	−0.098	−0.083	−0.016	−0.002	−0.003
Lachnospiraceae	0.063	0.091	0.067	0.064	0.101	0.027	0.140	0.158	0.135
Lactobacillaceae	0.018	0.004	−0.002	0.035	0.009	0.023	−0.007	−0.004	−0.038
Bifidobacteriaceae	*0.134**	0.145	0.148	*0.142**	0.134	0.142	0.107	0.091	0.124
Genus									
Bacteroides	−0.054	−0.055	−0.086	−0.080	−0.055	−0.099	0.009	0.027	0.012
Roseburia	−0.034	−0.033	−0.072	0.114	0.067	0.093	0.106	0.119	0.094
Bifidobacterium	0.107	0.100	0.125	*0.114**	0.111	0.099	0.084	0.071	0.079
Romboutsia	−0.043	−0.043	−0.039	−0.014	−0.013	0.007	−0.004	−0.001	0.022
Anaerostipes	−0.054	−0.015	−0.046	0.002	0.035	−0.001	0.020	0.067	0.004
Clostridium_XlVb	−0.080	−0.102	−0.155	−0.116	−0.201	*−0.215**	−0.165	*−0.209**	*−0.244**
Anaerobacterium	0.026	0.019	0.004	0.095	0.074	0.082	0.108	0.110	0.110
Sporobacter	0.059	0.068	0.045	0.030	0.017	−0.005	0.080	0.089	0.055

The ridge regression model was adjusted for age, sex, cigarette smoking, and body mass index. Difference achieved significance of *$P < 0.05$

LS lumbar spine 1–4, *FN* femoral neck, *BMD* bone mineral density

Only phyla, family, and genera with relative abundance greater than 0.1% were included in this analysis

Difference achieved significance of $P < 0.05$



the control group. Thus, we infer that the overproduction of LPS by the gut microbiota contributes to bone mineral loss possibly via inflammation-relevant pathways.

Evidence from animal studies remained inconsistent among microbiome and bone health associations. Sjogren et al. [10] found that the presence of microbiota reduces trabecular and cortical bone mass in conventionally raised mice probably due to the elevated bone resorption as a result of increment in osteoclast. However, Schwarzer et al. [42] found that conventionally raised neonatal mice have higher cortical bone mass and longer femur length compared with GF mice, although the BMD difference is not significant. In the present study, the low-BMD individuals had fewer OTUs and bacterial taxa at each level than the control group, which was in accordance with the study of Schwarzer et al.

Our study is characterized by several strengths. First, the sample size of our study is larger than that in the previous study [18]. Second, the gut microbiota composition is dynamic, complex, and influenced by non-modifiable factors, such as age and geographical location, and modifiable factors, such as diet, illness, and medication [43]. We have excluded subjects with exposures possibly influencing the gut microbiota composition, such as use of antibiotics or hormone within 6 months before fecal sample collection, medical history of hysterectomy and/or ovariectomy, and the presence of diseases, such as hyperthyroidism, hypothyroidism, diabetes, and gastrointestinal diseases. In addition, the risk factors for bone loss including smoking, lack of physical activity, and low calcium [6, 7]. For postmenopausal women, decreased estrogen level is an important risk factor for bone loss; for older men, age, vitamin D deficiency, hypogonadism, cigarette smoking, alcohol abuse, etc. are the main causes of low BMD [44]. We performed ridge regression to analyze the association between the gut microbiota abundance and BMD measurements controlling for covariates, such as age, sex, cigarette smoking, and BMI. Third, we conducted function predictions to evaluate the different roles of microbiomes in the two groups.

The main limitation of our study lies in the cross-sectional design, limiting a causality inference from the microbiome alterations to bone mineral loss. Furthermore, the participants were divided into two groups according to the BMD at FN. Considering this problem, we grouped the participants according to the BMD at the skeletal site of LS and conducted the same analyses, which yielded similar results. In addition, we recruited the participants in the same region, where individuals possibly have similar dietary habits, and the participants were requested to take a normal diet, as usual, avoiding diet changes and high-fat diet within a week before fecal sample collection. However, we failed to conduct a detailed energy intake investigation to the participants in this study. Potential dietary differences may still affect the results to some extent. Finally, our findings need validations in a larger population in other regions.

Conclusion

The composition and abundance of gut microbiota differ at the phylum, family, and genus levels between low-BMD individuals and controls. Several taxa associate with the BMD, T-score, and Z-score at skeleton sites of LS, FN, and total hip leading to different metabolic pathways with particular functions between the two groups. Our findings provide epidemiologic evidence to elucidate the underlying microbiota-relevant mechanism in bone mineral loss and OP pathologies and to develop microbiota modification therapy for the treatment and early prevention of such bone lesion.

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Compliance with ethical standards

Conflicts of interest None.

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