Diversity analysis of gut microbiota in osteoporosis and osteopenia patients

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Several evidences suggest that bone health can be regulated by gut microbiome. However, no researches have been reported to explore the gut microbiota changes in osteoporosis patients. In this study, we performed 16S ribosomal RNA sequencing to analyze the intestinal microbial diversity in primary osteoporosis (OP) patients, osteopenia (ON) patients and normal controls (NC). We observed an inverse correlation between number of bacterial taxonomy and value of bone mineral density . The diversity estimators in OP and ON groups were increased compared with that in NC group, hierarchical clustering and principal coordinate analysis (PCoA) in beta diversity could discriminate the NC samples from OP and ON samples. Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria constituted the four dominant phyla in all samples. Proportion of Firmicutes was significantly higher and Bacteroidetes significantly lower in OP samples than that in NC samples ($p < 0.05$), Gemmatimonadetes and Chloroflexi were significantly different between OP and NC group as well as between ON and NC group ($p < 0.01$). A total of 21 genera with proportions above 1% were captured and Bacteroides accounted for the largest proportion in all samples. The Blautia, Parabacteroides and Ruminococcaceae genera differed significantly between the OP and NC groups ($p < 0.05$). Linear discriminant analysis (LDA) results showed 1 and 7 phylum communities were enriched in ON and OP group, respectively, 35, 5 and 2 genus communities were enriched in OP, ON and NC group, respectively. The results of this study indicate that gut microbiota may be a critical factor in osteoporosis development, and further help us understanding the interaction between gut microbiota and bone health.

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ABSTRACT

Several evidences suggest that bone health can be regulated by gut microbiome. However, no researches have been reported to explore the gut microbiota changes in osteoporosis patients. In this study, we performed 16S ribosomal RNA sequencing to analyze the intestinal microbial diversity in primary osteoporosis (OP) patients, osteopenia (ON) patients and normal controls (NC). We observed an inverse correlation between number of bacterial taxonomy and value of bone mineral density. The diversity estimators in OP and ON groups were increased compared with that in NC group, hierarchical clustering and principal coordinate analysis (PCoA) in beta diversity could discriminate the NC samples from OP and ON samples. Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria constituted the four dominant phyla in all samples. Proportion of Firmicutes was significantly higher and Bacteroidetes significantly lower in OP samples than that in NC samples ($p < 0.05$), Gemmatimonadetes and Chloroflexi were significantly different between OP and NC group as well as between ON and NC group ($p < 0.01$). A total of 21 genera with proportions above 1% were captured and Bacteroides accounted for the largest proportion in all samples. The Blautia, Parabacteroides and Ruminococcaceae genera differed significantly between the OP and NC groups ($p < 0.05$). Linear discriminant analysis (LDA) results showed 1 and 7 phylum communities were enriched in ON and OP group, respectively, 35, 5 and 2 genus communities were enriched in OP, ON and NC group, respectively. The results of this study indicate that gut microbiota may be a critical factor in osteoporosis development, and further help us understanding the interaction between gut microbiota and bone health.

Keywords: Osteoporosis, Gut microbiota, Diversity analysis, 16S ribosomal RNA, Bone mineral density

INTRODUCTION

Osteoporosis is a type of bone-thinning disorder, characterized by a reduction in bone mass, microarchitecture deterioration and an increased risk of fragility fracture. Osteoporosis

represents a serious health burden among the elderly. As the population grows and ages, the number of patients with osteoporosis is expected to increase. A decline in bone mineral density (BMD) is the primary cause of fragility fracture (Lu et al. 2016). As a metabolic procedure, bone homeostasis relies on a balance between bone formation (osteoblast-regulated) and bone resorption (osteoclast-regulated) (Chung et al. 2014; Harada & Rodan 2003). Hereditary characteristic and environmental factors can regulate the complex process of bone metabolism, contributing significantly to age-related bone loss (Pollitzer & Anderson 1989). The effective treatment and prevention of osteoporosis will require that we consider potential factors affecting bone metabolism.

Recently, the gut microbiota have attracted attention in connection with bone health. The gut microbiota establish a rich ecosystem in the human gastrointestinal tract. Populations of bacteria living in the gut have critical effects in the emergence of metabolic disorders including obesity, diabetes, and osteoporosis (Ejtahed et al. 2016). These bacterial populations regulate food intake, immune activation, lipid accumulation, short-chain fatty acid production and bone mass regulation. Therefore, factors affecting the gut microbiota may represent a novel approach for the diagnosis and treatment of metabolic disorders (Steves et al. 2016). Several reports have identified the gut microbiota as a regulator of bone mass (McCabe et al. 2015; Sjogren et al. 2012; Weaver 2015). Bacterial populations living in the gut act through effects on the immune system that affect osteoclastogenesis, intestinal calcium absorption and the release of neurotransmitters (e.g, serotonin). However, no previous study has performed a diversity analysis of the gut microbiota in osteoporosis patients. The results of such a study represent an important foundation for studying gut microbiota and bone metabolism.

Traditional methods for research on bacterial community inhabitants include isolation, cultivation, and optical microscopy. These approaches are insufficient to obtain relatively full-scale and accurate results about the structure and diversity of microbiota communities in specific samples, because the vast majority of bacteria in fecal samples are anaerobic and cannot be recovered in the laboratory (Perry et al. 2010). High-throughput sequencing has recently been used for bacterial diversity analysis (Li et al. 2016a; Li et al. 2016b). This approach overcomes the limitations of traditional technology and can effectively capture the genomic information of uncultured microorganisms, which may be pathogenic or important for biological processes.

In this study, we performed 16S rRNA gene sequencing using the Illumina Miseq platform to explore the bacterial community structure and diversity of gut microbiota in patients with primary osteoporosis and primary osteopenia as well as in healthy control subjects.

METHODS

Subject recruitment

Participants in this study were recruited from Hong Hui Hospital, Xi'an Jiaotong University College of Medicine, Xi'an, China. We included only subjects who had undergone dual X-ray absorptiometry (DXA). We excluded all patients with any malignancy, chronic liver disease, heart disease, kidney disease, or diabetes. Finally, a total of 18 subjects including six with primary osteoporosis (OP), six with primary osteopenia (ON), and six normal controls (NC; as

determined by physical examination) were selected for further research (Table 1). The study was approved by Hong Hui Hospital, Xi'an Jiaotong University, Biomedical research ethics committee. Each participant provided his or her written informed consent.

Fecal sample collection and DNA extraction

None of the 18 participants ingested yogurt, prebiotics, or probiotics during the fecal collection period, nor had they used medication (e.g., antibiotics) within one month of sample collection. Fresh stool samples were collected in sterile boxes, then frozen and stored at -80℃ for further use. The microbial genome was extracted using QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sample DNA purity and concentration were tested using a Nanodrop 2000 Spectrophotometer.

16S rRNA PCR and Illumina sequencing

We amplified the bacterial 16S ribosomal RNA gene V3-V4 region using the TransGen AP221-02 Kit (TransGen, Beijing, China). The following PCR primers were used: 338F 5'-ACTCCTACGGGAGGCAGCAG-3' and 806R 5'-GGACTACHVGGGTWTCTAAT-3'. The reaction volume (20 μl) comprised 5 × FastPfu Buffer (4 μl), 2.5 mM dNTPs (2 μl), forward primer (0.8 μl), 5 μM reverse primer (0.8 μl), FastPfu Polymerase (0.4 μl), and template DNA (10 ng). Cycling proceeded as follows: 3 min at 95℃; 27× (30s at 95°C, 30s at 55 ℃, 45s at 72℃); 10 min at 72℃. After amplicons extraction, samples were purified and quantified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, CA, U.S.) and QuantiFluor™-ST (Promega, U.S.), respectively. Purified amplicons were pooled in equimolar proportions and paired-end sequenced (2 × 250) on the Illumina MiSeq platform.

16S rRNA gene sequencing analysis

We applied QIIME (version 1.17) software to analyze the raw fastq sequence data (Caporaso et al. 2010), UPARSE（version 7.1) software clustered operational taxonomic units (OTU) at a 97% similarity cutoff (Edgar 2013). Sequences were aligned to SILVA database(Quast et al. 2013). The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier (Wang et al. 2007). Alpha diversity at the OTU level (e.g., ace, chao, shannon and simpson index) were calculated. The significance of the estimators between groups was evaluated. Rarefaction curves were generated based on the four estimators. The unweighted_unifrac algorithm was applied for hierarchical clustering and principal co-ordinates analysis at the OTU level to analyze beta diversity. The collinearity diagram was illustrated to visualize the corresponding abundance relationship between samples and bacterial communities at the phylum and genus levels. The enriched and significant bacteria in each group were identified by linear discriminant analysis (LDA) combined with effect-size measurements (LEfSe), with $p < 0.05$. For the Kruskal-Wallis test, LDA values > 2 were considered significant (Szafranski et al. 2015).

120
121 **Statistical analysis**
122 Clinicopathological information, alpha estimators and relative bacterial abundance are expressed
123 as means ± standard deviations. Results analysis and figure generation were performed using
124 SPSS 21.0 and GraphPad Prism 5.0 software. Student's t-test and the Mann-Whitney U-test were
125 performed, with $p < 0.05$ indicating a significant difference between groups.
126
127 **RESULTS**
128 **Illumina sequencing data characteristics**
129 The clinicopathological information for each of the three groups included in the study is
130 presented in Table 1. There were no significant differences in terms of age or gender, while
131 BMD, T-score and Z-score differed significantly among groups. Illumina sequencing captured a
132 total of 694232 high-quality sequences, with an average of 38568.44 sequences/sample. Detailed
133 information on the sequence results obtained for each sample are presented in Table S1.
134
135 **Inverse correlation between number of bacterial taxonomy and value of BMD**
136 Based on the sequencing data, the gut microbiota of all samples were classified to 507 OTUs,
137 367 species, 235 genera, 99 families, 63 orders, 38 classes, 5 phyla, and 1 kingdom. The number
138 of bacterial taxonomies tended to increase at each level in accordance with the reduction in BMD,
139 as shown in Table 2 and Figure S1. Figure 1 presents a Venn diagram for the OP, ON and NC
140 groups (at the OTU level). There were 455, 378, and 282 OTUs present in the OP, ON, and NC
141 group, respectively. In addition, 208 OTUs (41%) were shared by all samples; 154 OTUs (30.4%)
142 were shared between the OP and ON groups. For the remaining components (28.6%), the OP
143 group (13.6%) accounted for nearly half of all OTUs.
144
145 **Diversity analysis of gut microbiota in osteoporosis and osteopenia patients**
146 To determine alpha diversity, we calculated the mean ace index, chao index, shannon index, and
147 simpson reciprocal index. This process allowed us to fully characterize the bacterial community
148 diversity in samples. Detailed information on the estimators in each sample is presented in Table
149 S2. The OTU-level rarefaction curves of diversity estimators reached plateau phase (Figure S2),
150 indicating that most bacterial species had been captured by sequencing in all samples. Higher
151 numbers of the estimators represent greater diversity, which suggests that alpha diversity index
152 was inversely correlated with BMD, although there were no significant differences between the
153 OP and ON groups, as shown in Figure 2.
154 With regard to beta diversity, unweighted UniFrac analysis indicated that hierarchical
155 clustering and principal co-ordinate analysis (PCoA) could discriminate the NC samples from
156 OP as well as ON samples. However, there was substantial overlap between the OP and ON
157 groups, and most ON samples were positioned in the middle of the OP and NC samples, as
158 Figure 3 illustrates. Results of the diversity analysis suggest that a study of the gut microbiota
159 may help researchers to understand osteoporosis and osteopenia disease, which result from
160 abnormal bone metabolism.

Significance analysis of gut bacterial community abundance in osteoporosis and osteopenia patients

At the phylum level illustrated in Figure 4, Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria constituted the four dominant phyla in all samples. The average ratios of Firmicutes/Bacteroidetes were 3.326, 1.755 and 1.290 in the OP, ON, and NC groups, respectively. Furthermore, we calculated the significance of the 10 most dominant phyla of microbial community structure among the OP, ON, and NC groups. Differences among the four dominant phyla (Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria) were not statistically significant for comparisons between the OP and ON group or the ON and NC group ($p > 0.05$). Proportion of Firmicutes was significantly higher and Bacteroidetes proportion was significantly lower in OP samples than that in the NC group ($p < 0.05$) (Figure 4B). As for other bacterial communities with small proportions, most were almost non-existent in the NC group but increased in the OP and ON groups. Gemmatimonadetes and Chloroflexi were significantly different between the OP and NC groups ($p < 0.01$) as well as between the ON and NC groups ($p < 0.01$).

At the genus level, a total of 21 genera with proportions above 1% were captured, as visualized in Figure 5. *Bacteroides* accounted for the largest proportion in all samples. In the NC group, 3 genera (*Bacteroides*, *Faecalibacterium* and *Prevotella*) contributed more than half of the bacterial community. In the ON and OP groups, 5 and 11 genera, respectively, accounted for 50% of the bacterial community. Differentiation analysis of the 21 genera is presented in Figure 5B. The *Blautia*, *Parabacteroides* and *Ruminococcaceae* genera differed significantly between the OP and NC groups. Figure S3 depicts the collinearity diagram for the bacterial community and samples from all three groups.

We further applied linear discriminant analysis (LDA) combined effect size measurements (LEfSe) to explore the significant changes and relative richness of the bacterial community in the OP, ON, and NC groups, at phylum and genus levels. The Kruskal-Wallis test was performed, with $p < 0.05$ and LDA value > 2 considered as significant. Figure 6 summarizes the enrichment and variations in bacterial community for all three groups. At the phylum level, 1 and 7 phylum communities were enriched in the ON and OP group, respectively, while no community in the NC group was enriched. At the genus level, 35, 5 and 2 genus communities were enriched in the OP, ON and NC groups, respectively. The significance and variance of bacterial communities, as determined by sequencing analysis, may help discriminate OP or ON patients from NC subjects.

DISCUSSION

The human microbiome, referred to as our second genome, can influence genetic diversity, immunity and metabolism (Grice & Segre 2012; Solt et al. 2011). All of the bacteria in specific samples can now be detected based on microbiota DNA. Research on the correlation between gut microbiota and bone metabolism has recently emerged. Our study is among the first surveys on the composition and differences in the gut microbiota of osteoporosis, osteopenia patients and healthy controls using metagenomic sequencing. The results indicate that bacterial component

structure and diversity are altered in osteoporosis and osteopenia patients as compared with normal controls, further suggesting that gut microbiota affect bone mass.

From the phylum to OTU levels, the complexity of bacterial community taxonomy was inversely correlated with BMD value. The number of groups at each taxonomic level was greatest in the OP group, followed by the ON group, and then by the NC group (Table 2, Figure S1). Microbiota diversity analysis is valuable for quantifying the bacterial component and relative richness of a specific community. Our investigation of alpha diversity revealed an elevation of diversity estimators in the OP and ON groups. Hierarchical clustering and PCoA analysis of beta diversity was able to discriminate the NC group from the OP & ON groups but could not distinguish the OP from the ON group (Figure 3). These results suggest that a rich diversity of gut microbiota may be related to bone mass reduction.

In comparison to the NC group, the proportion of Firmicutes phyla increased, and the proportion of Bacteroidetes decreased significantly ($p < 0.05$). Several communities present at low levels in the OP and ON groups were absent in the NC group (e.g., Gemmatimonadetes Chloroflexi and Synergistetes) (Figure 4). At the genus level, 21 genera with proportions over 1% were identified. *Bacteroides*, *Faecalibacterium* and *Prevotella* were the top 3 genera in the NC group, while Prevotella was not observed in the ON and was present at low levels in the OP group. The *Lachnoclostridium* and *Klebsiella* genera were more abundant in the OP and ON groups as compared to the NC group (Figure 5). We identified the enriched and significant community in each group (Figure 6). At the phylum level, 1 and 7 communities were enriched in the ON and OP groups, respectively. At the genus level, more communities were screened in the OP, ON and NC groups. We speculated that these communities may be considered as disease-specific biomarkers in OP and ON patients. According to recent reports, metabolomic studies in microbiota research have increased, which focusing on exploring novel biomarkers for disease(Castro-Nallar et al. 2015; Vernocchi et al. 2016).

The underlying mechanisms of gut microbiota changes in osteoporosis and osteopenia patients remain to be explained. We hypothesize that the immune-inflammatory axis may act as the key bridge joining the gut microbiome to bone metabolism. Studies have shown that bone mass increased in germ-free (GF) mice compared with conventionally raised mice. The authors reported fewer osteoclasts, osteoclast precursor cells, CD4 (+) cells and inflammatory cytokines in the bone and bone marrow of GF mice. The authors also found that bone mass could be normalized after gut microbiota transplantation in GF mice. Moreover, certain pre- and probiotics have been shown to increase bone mass (Bindels et al. 2015; Maekawa & Hajishengallis 2014; Scholz-Ahrens et al. 2007). Research suggests that gut microbiota and specific probiotics may regulate IGF-1, TNF-α and IL-1β, resulting in changes in bone formation and growth (Ohlsson et al. 2014; Yan et al. 2016).

Notably, this study does have certain limitations. The sample size may not have been large enough. The average age in the experimental and control groups was 70 years. We therefore considered the relevant hormonal changes, with corresponding effects on bone metabolism, because postmenopausal women are at high risk for osteoporosis (Cappola & Shoback 2016). Researchers have reported that prebiotics improve calcium absorption, calcium accretion in bone

and BMD in adolescents as well as postmenopausal female subjects (Roberfroid et al. 2010). Thus, dietary intake (e.g., pre- or probiotics) may alter bone metabolism in both pre- and post-menopausal women.

In summary, we explored gut microbiota diversity in primary osteoporosis and osteopenia patients. To accurately identify osteoporosis-specific microbiota, additional studies with a larger sample size are required. The knowledge obtained would allow us to modify the gut microbiome and thus bone metabolism, with meaningful effects for human health.

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Figures and Tables

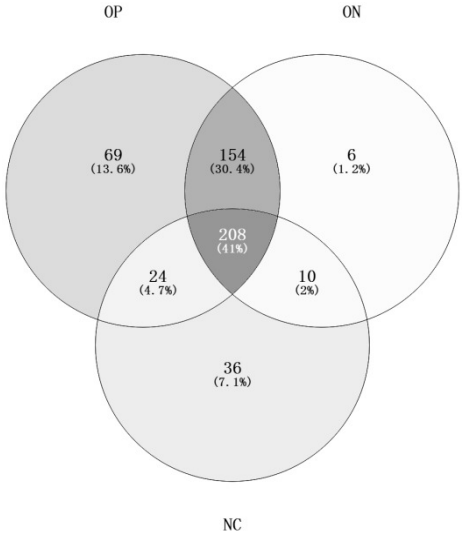

Figure 1. Venn diagram of OP, ON and NC groups at OTU level.

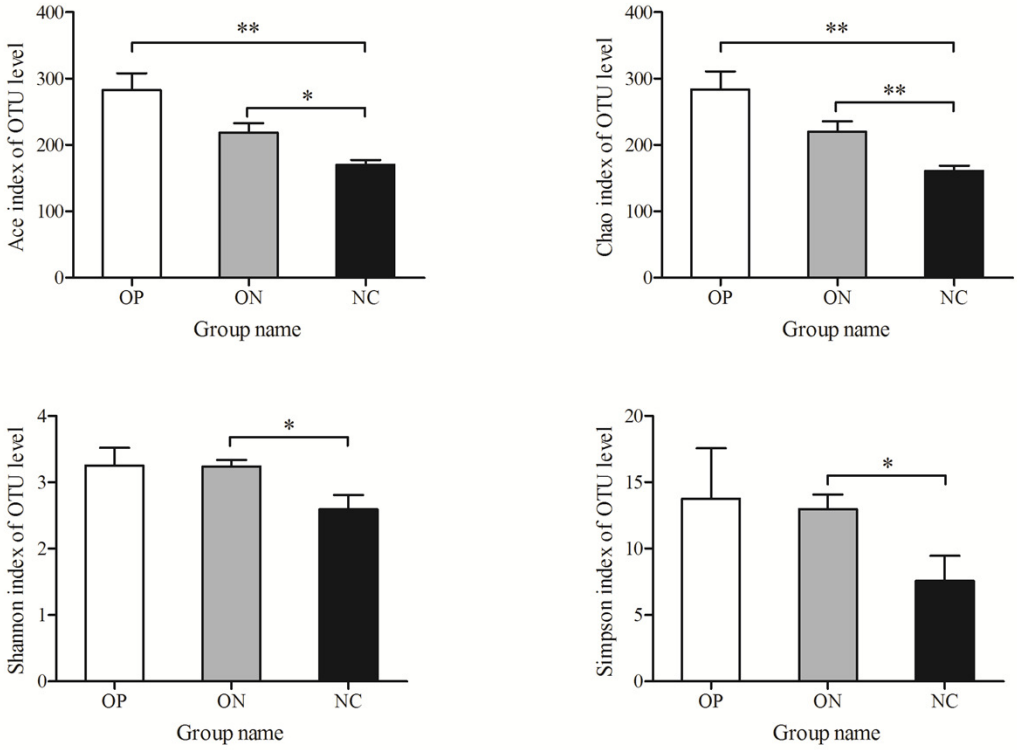

Figure 2. Significance of alpha diversity estimators between different groups. $0.01<p\leq0.05$, $^{**}0.001<p\leq0.01$.

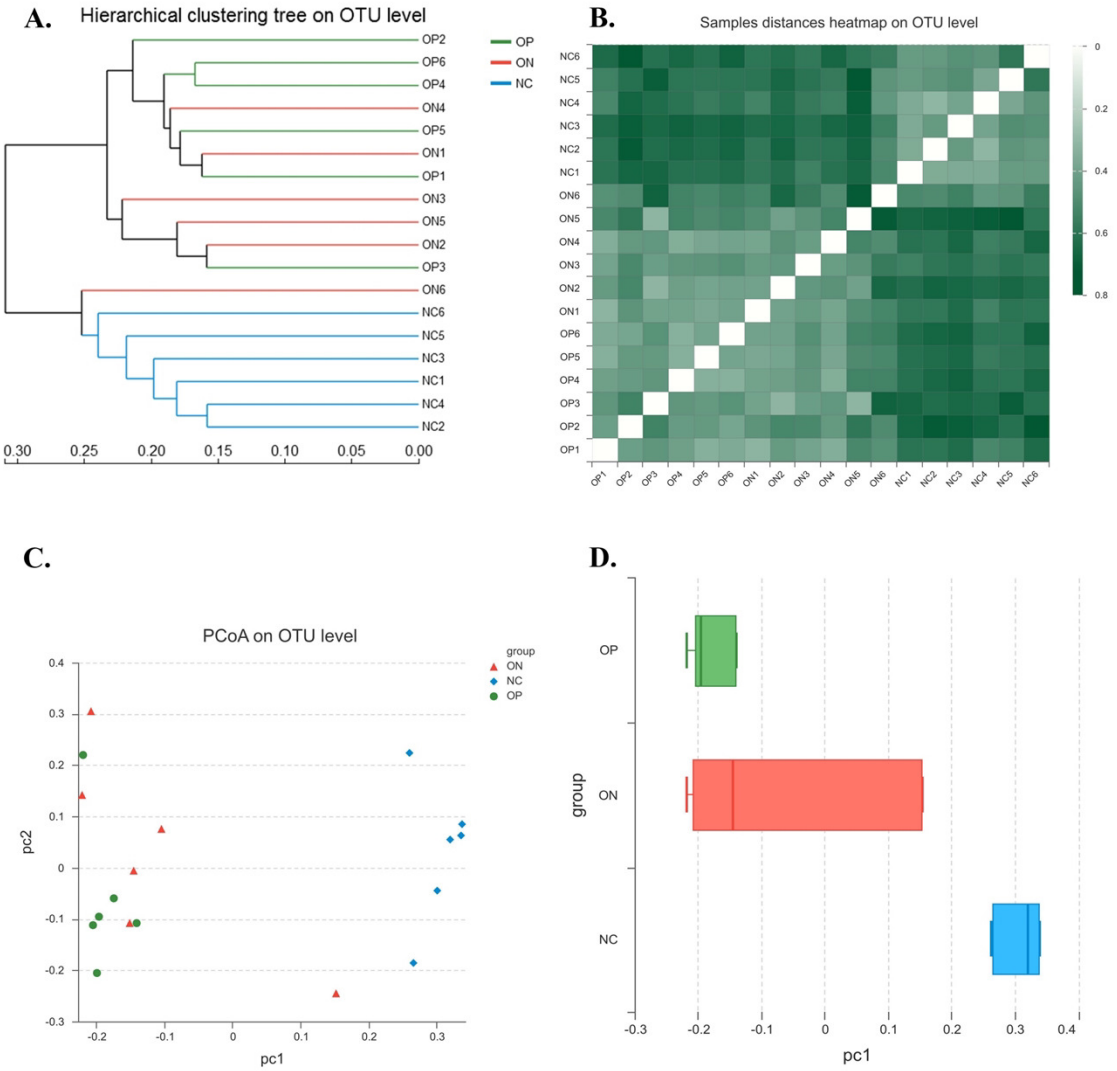

Figure 3. Beta diversity analysis of OP, ON and NC group at OTU level. (*A*) the hierarchical clustering tree. (*B*) Sample distance heatmap based on color similarity coefficient. (*C*) Principal co-ordinate analysis (PCoA) scatter plot. (*D*) PCoA box plot.

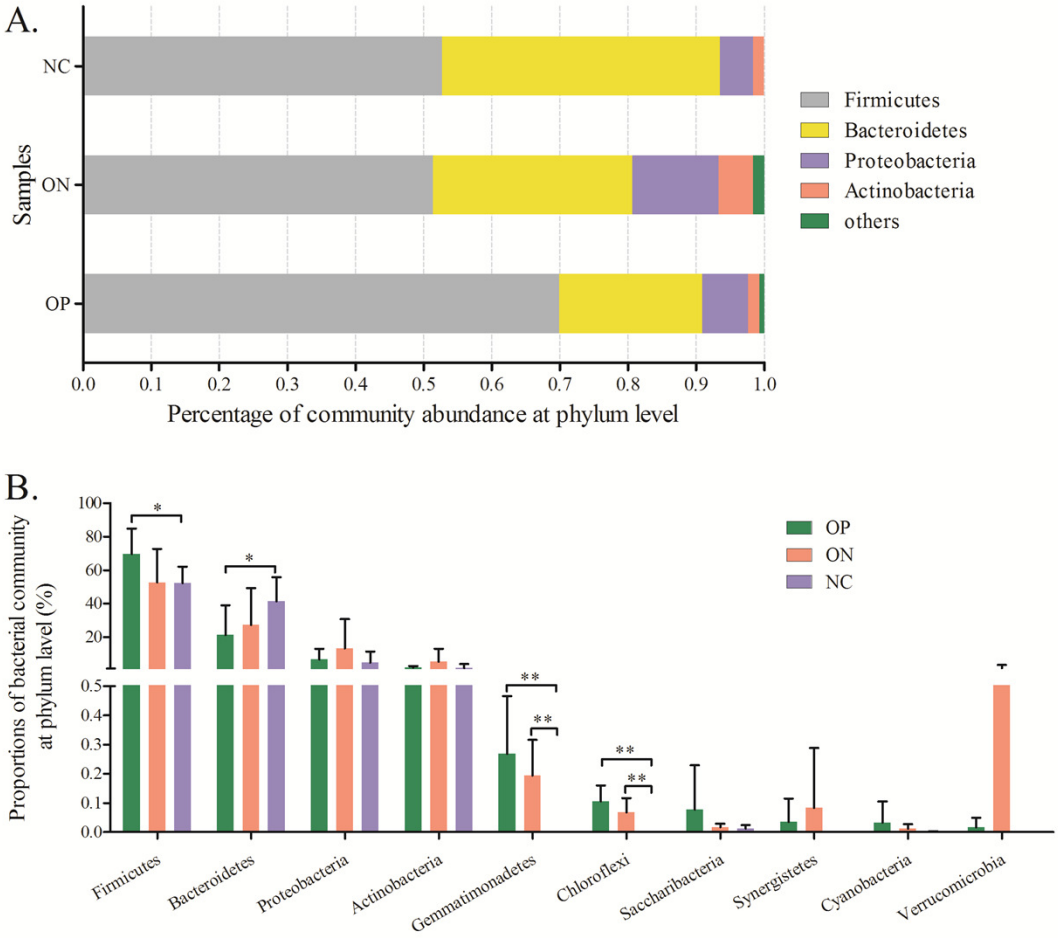

Figure 4. Bacterial community abundance at phylum level of each group. (*A*) Bacterial community abundance barplot at phylum level. (*B*) Significance of the top 10 bacterial community abundance at phylum level. *0.01<p≤0.05, **0.001<p≤0.01.

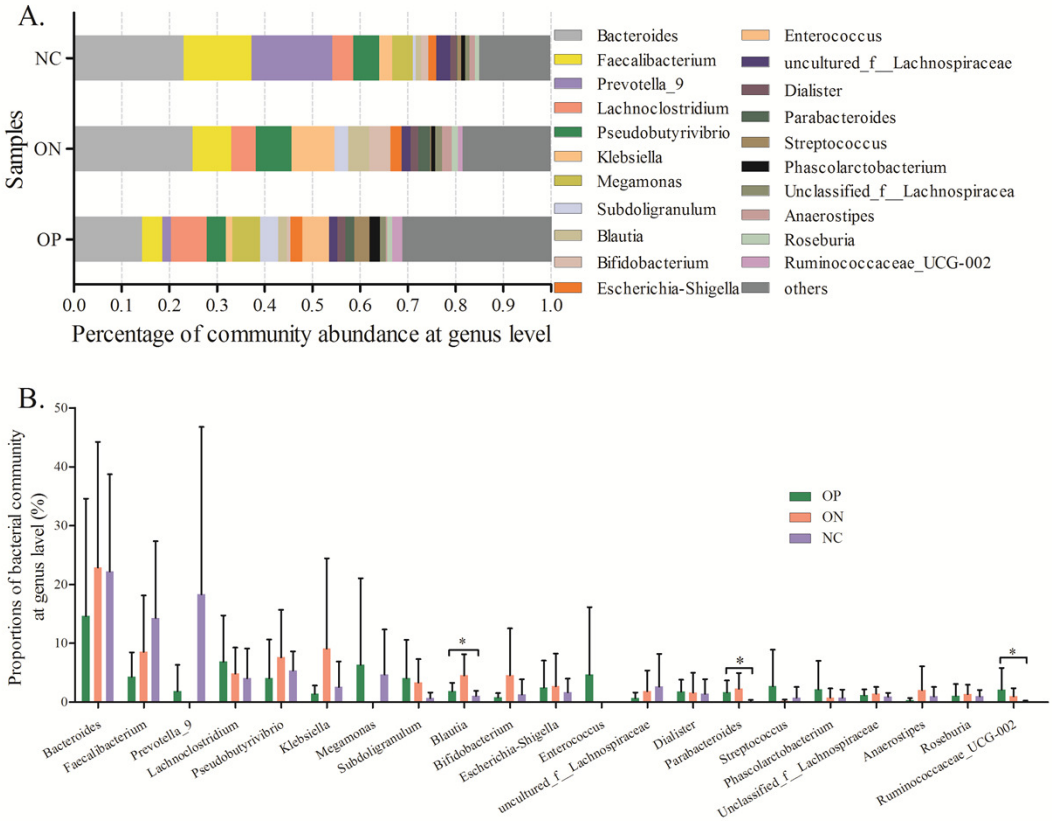

Figure 5. Bacterial community abundance at genus level of each group. (*A*) Bacterial community abundance barplot at genus level. (*B*) Significance of the 10 bacterial community abundance at genus level. *$0.01<p\leq0.05$.

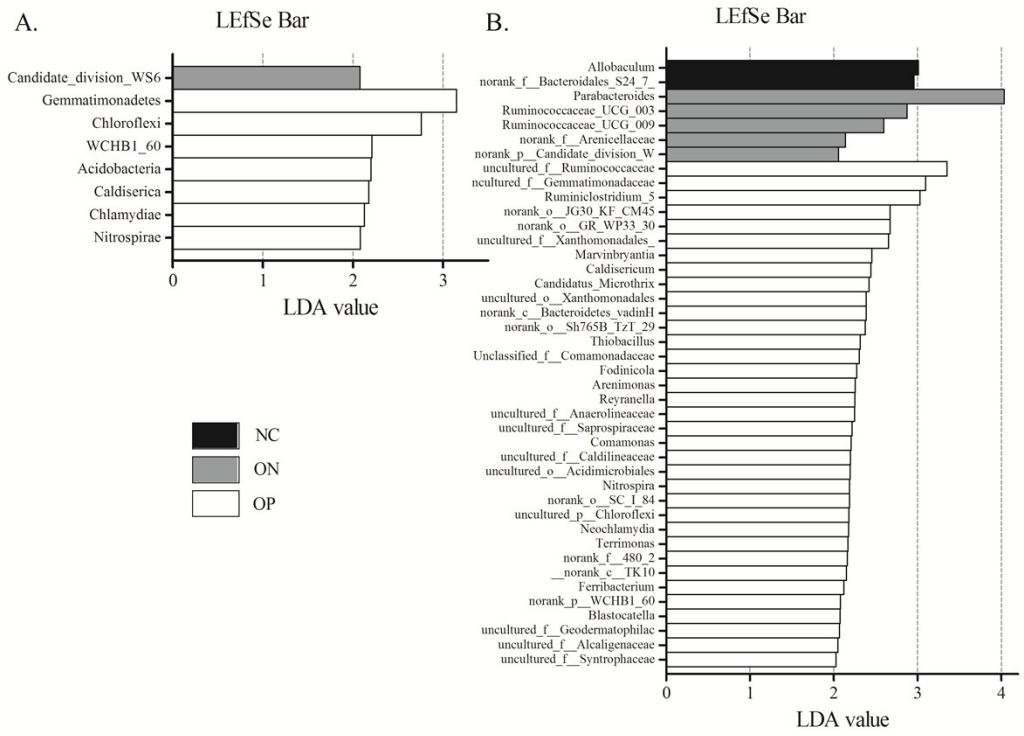

Figure 6. LEfSe at the phylum and genus level of each group. (*A*) LEfSe bar at phylum level. (*B*) LEfSe bar at genus level. $P < 0.05$, LDA value > 2.

355 **Table 1.** Clinicopathological information of the study participants.

Group	Case	Gender	Age	BMD L_{1-4} (g/cm^2)	Z-score L_{1-4}	T-score L_{1-4}
Normal control (NC)	6	Female: 5 Male: 1	64.80±5.93	0.81±0.08	0.12±0.45	-0.42±0.26
Osteopenia (ON)	6	Female: 5 Male: 1	67.17±8.30	0.75±0.04*	-0.22±0.50	-2.15±0.34**
Osteoporosis (OP)	6	Female: 5 Male: 1	70.00±7.77	0.61±0.06$^{**\#\#}$	-1.18±0.73$^{**\#}$	-3.57±0.46$^{**\#\#}$

356 Compares with NC group: $^{*}P < 0.05$, $^{**}P < 0.01$. Compares with ON group: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

358 **Table 2.** Bacterial taxonomy in each group at different levels.

	Kingdom	Phylum	Class	Order	Family	Genus	Species	OTU
NC	1	8	14	20	41	134	218	282
ON	1	21	33	56	88	195	296	378
OP	1	23	35	58	92	219	335	455
Total	1	25	38	63	99	235	367	507

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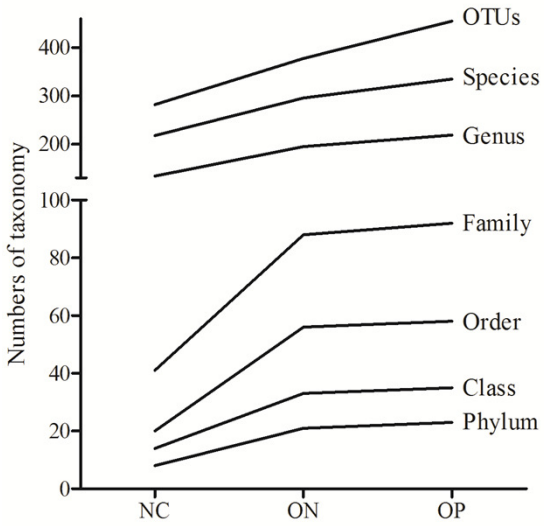
361 **Supplemental Figures and Tables**



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363 **Figure S1** Numbers of species in each group at different taxonomy levels.

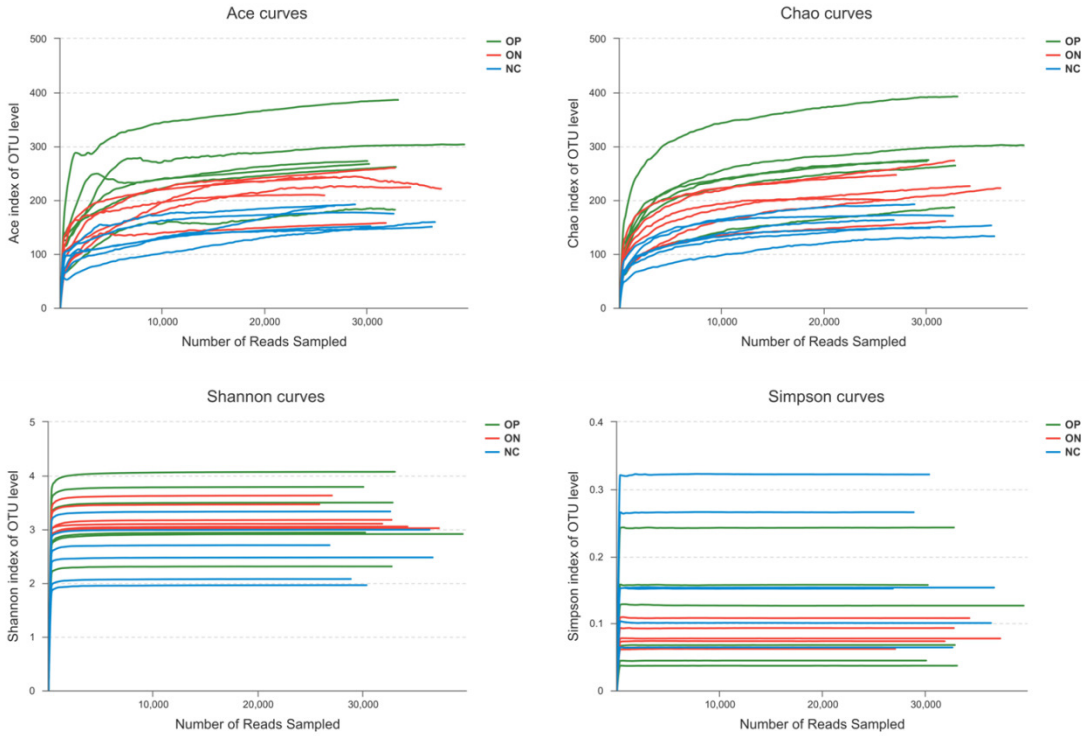

Figure S2 Rarefaction curves of diversity estimators at OTU level.

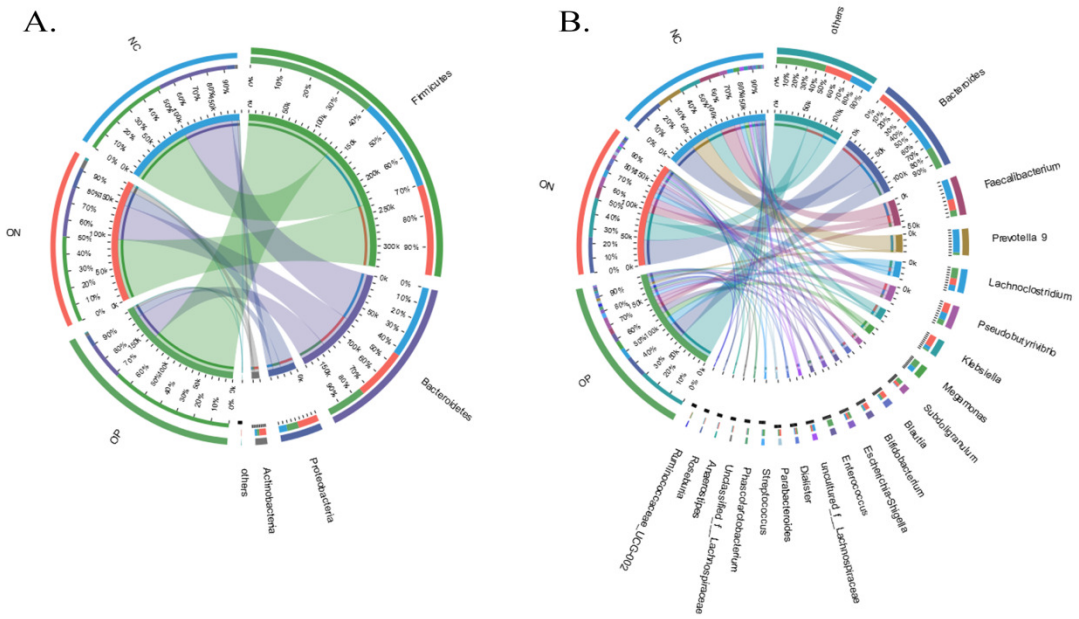

Figure S3 Collinearity diagram of samples and bacterial community. (*A*) Collinearity diagram at phylum level. (*B*) Collinearity diagram at genus level.

373 **Table S1** Illumina sequence information of each sample.

Sample\Info	Seq_num	Base_num	Mean_length	Min_length	Max_length
NC1	40711	17890180	439.4434	270	452
NC2	31129	13501857	433.7389	400	451
NC3	43906	19087002	434.7 242	338	452
NC4	34696	15257974	439.7618	420	452
NC5	31910	14136797	443.0209	411	453
NC6	42075	18179118	432.0646	338	464
ON1	30238	12996413	429.804	381	465
ON2	44314	19508304	440.2289	358	452
ON3	42600	18385245	431.5785	346	455
ON4	37892	16720433	441.2655	368	473
ON5	36209	15707157	433.7915	403	452
ON6	34623	15092595	435.9124	203	453
OP1	43489	18762550	431.4321	358	486
OP2	40925	17746681	433.6391	360	462
OP3	37909	16673545	439.8308	384	492
OP4	38323	16979717	443.0686	382	453
OP5	38564	16574675	429.7966	366	452
OP6	44719	19722882	441.0403	327	454
Total	694232	3.03E+08	7854.142	6413	8273

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377 **Table S2** Estimators of alpha diversity of each sample.

Sample\ Estimators	ace	chao	shannon	1/simpson
NC1	177.536	173	3.334414	15.57438
NC2	187.7208	162.0769	2.702705	6.57527
NC3	152.2616	158	2.994291	9.938678
NC4	151.9588	147.3529	1.961493	3.106246
NC5	191.4899	191.3333	2.078802	3.767656
NC6	160.0238	135.3333	2.478391	6.523072
ON1	205.3185	196.6071	3.458275	15.48419
ON2	227.361	228.4737	3.021578	12.8974
ON3	218.5306	220.0588	3.03805	9.204628
ON4	258.2515	268.05	3.177343	10.74483
ON5	157.6121	161.0769	3.105577	13.60082
ON6	243.1752	247.625	3.623966	16.05652
OP1	260.3745	265	3.492852	14.71432
OP2	390.3002	397.1818	4.074678	27.08046
OP3	204.614	192.4615	2.311026	4.118803
OP4	266.0439	271.2917	2.937315	6.344775
OP5	271.3796	274.4545	3.785153	22.34237
OP6	304.2477	303.0278	2.914457	7.90164

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