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Original Article

The gut microbiome is associated with bone turnover markers in postmenopausal women

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Abstract: Objective: The association of the gut microbiome with bone turnover markers (BTMs) in postmenopausal women is poorly understood. Methods: Fecal samples were collected from 97 Chinese postmenopausal women, and the serum CTX and P1NP were determined. Individuals with serum CTX lower or higher than the median value were divided into LCTX and P1NP groups; and individuals with serum P1NP lower or higher than the median value were grouped into LP1NP and HP1NP groups. Microbiota profiles were determined by high-throughput 16S rRNA gene sequencing. Results: In postmenopausal women, only Faecalibacterium showed significant alteration in the HCTX group compared with the LCTX group (P=0.004, q=0.143). Linear discriminant analysis effect size (LEfSe) analysis revealed that Clostridiaceae (P=0.015, LDA=2.89), Faecalibacterium (P=0.017, LDA=4.60), Prevotella (P=0.040, LDA=3.61) and Clostridium (P=0.007, LDA=2.79) were abundant in the LCTX group, and Facklamia (P=0.044, LDA=3.10) was enriched in the HCTX group. Peptostreptococcaceae (P=0.048, LDA=2.83) and the SMB53 (P=0.028, LDA=2.05) genus were enriched in the LPINP group, and Veillonellaceae (P=0.025, LDA=4.43) and the S24_7 (P=0.023, LDA=3.08) family were enriched in the HPINP group. Six taxa correlated with BTMs in all subjects, including Clostridium (Clostridiaceae) that was negatively correlated with serum CTX amounts significantly (r=-0.34, P<0.001). Conclusion: This study identified taxa-specific differences in the intestinal microflora associated with BTMs, notably CTX. These findings may help in uncovering the roles of gut microbiota on bone metabolism.

Keywords: Gut microbiota, postmenopausal women, 16S ribosomal RNA, bone turnover marker

Introduction

Postmenopausal osteoporosis (PMO), a systemic skeletal disease associated with estrogen deficiency affecting women's health, features a decreased bone mass, bone microstructure alteration, and elevated fracture risk. Hip fractures result in 17% mortality in the initial year and approximately 12%-20% in the subsequent two years [1, 2]. The main pathogenesis of PMO is the cessation of ovarian function, promoting bone resorption and minimal bone generation, causing rapid bone loss [3]. There are genetic factors in PMO development, but environmental factors also have pivotal functions in this process. Recently, the association of bone mass with gut microbiota (GM) has attracted extensive attention.

The intestinal microbiota comprises 10^{14} bacterial organisms that represent 5000 species and 5 million genes [4]. The composition of the GM may be influenced by many factors, including host genetics, geography, age, diet, use of certain medications, and host immune status, though it remains relatively stable in adulthood [5, 6]. Gender may be another factor affecting GM [7-9]. The GM is considered to regulate bone mass, mainly by the immune system, as well as through the endocrine system and calcium balance [10, 11]. Germ-free (GF) C57Bl6/J mice colonized with the GM show elevated bone amounts of pro-inflammatory cytokines and induced bone resorption [12]. Another animal study showed that reduced levels of sex steroids in GF mice failed to increase osteoclastogenic cytokine production and stimulate bone

resorption, indicating a central role for the GM in trabecular bone loss caused by low levels of sex steroids [13]. Therefore, it was speculated that a detrimental GM composition would result in enhanced osteoclastic activity, which leads to higher bone turnover and, subsequently, greater bone loss.

A considerable number of studies have assessed the association between GM and bone mass [14-17]. Moreover, recent efforts demonstrated a causal association of GM with bone mineral density [18]. However, few reports have described GM's effects on bone turnover markers (BTMs) in PMO patients, which offer a more dynamic, albeit imperfect, perspective of bone metabolism [19]. Therefore, the association of GM with BTMs should be further evaluated.

We hypothesized that the levels of BTMs may be modulated by the GM in postmenopausal women. Therefore, this work aimed to explore the GM composition and diversity in postmenopausal women by high-throughput sequencing and to assess the possible function of the GM in the regulation of bone metabolism. The findings provide novel insights into PMO pathogenesis.

Materials and methods

Subjects

The trial obtained approval from the Ethics Committee of Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine (approval No. 2020-29). Signed informed consent was obtained from each participant prior to enrollment.

Eligible participants were recruited at Osteoporosis Department and Health Examination Center, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China from Apr 2020 to Jun 2020. They were aged 50-69 years old and had natural menopause for at least one year. All participants had no smoking history. Volunteers filled out a questionnaire that included information on menstrual history, fracture history, personal history, family history, medical history, smoking and/or drinking statuses, and eating habits. None of them took antibiotic drugs in the 3 months preceding fecal specimen collection, or ingested prebiotics, probiotics, or yogurt during sample collection. The subjects had no history of gastrointestinal surgery and were administered no current medications or the same drugs for ≥3 months. We excluded women with conditions or medications potentially interfering with either bone metabolism or the GM population such as secondary osteoporosis, a comorbidity that may impact bone metabolism (e.g., chronic liver disease, kidney disease, diabetes, heart disease, thyroid or parathyroid dysfunction, rheumatoid arthritis, and malignancy), stress status (e.g., severe trauma and serious infections), urinary tract infection, chronic gastrointestinal diseases, acute condition with nausea, vomiting or diarrhea in the past month; new fractures in the past year; use of active vitamin D, anti-osteoporosis medicines (e.g., bisphosphonates, raloxifene, calcitonin, teriparatide, and denosumab) and glucocorticoids within 36 months before enrollment; use of hormone replacement therapy, Vitamin K antagonists (e.g., warfarin), heparin, thiazide diuretics, anticonvulsants, aromatase inhibitors, and drug or alcohol addiction in the past 12 months. Totally 101 postmenopausal women met these eligibility criteria. Because of failure to provide stool samples on time, 4 of them were excluded, and 97 individuals were finally assessed.

Bone density measurements

DXA (Lunar Prodigy, GE, USA) was carried out for detecting bone mineral density (BMD) at the lumbar spine (LS) and the left proximal femur including the femoral neck (FN) and total hip. All operations were carried out by the same technician on the same machine. Coefficient of variation (CV) values for repositioning were 1.5%, 1.7% and 1.2% for lumbar spine BMD, femoral neck and total hip, respectively.

Biochemical data

All participants were required to avoid intense exercise the day before sampling. Venous blood specimens were obtained from 7 Am to 9 Am following an overnight fast, at ambient for 30 min, and centrifuged (3000 g for 20 min at 4°C) to yield the serum. C-terminal cross-linking telopeptide of type I collagen (CTX) was assessed as a bone resorption biomarker while procollagen type 1 N-terminal propeptide (P1NP), osteocalcin (OC) and bone-specific alkaline phos-

phatase (BAP) were analyzed as bone formation biomarkers [20-22].

Serum CTX, P1NP, OC, BAP, and 25OHD were assessed by electro-chemiluminescent immunoassay (ECLIA) on a Cobas e601 (Roche Diagnostics, Germany). Inter-assay and intra-assay CVs of serum CTX were 2.2% and 1.6%, respectively. The assay sensitivity was 0.07 ng/mL. Inter-assay and intra-assay CVs of serum P1NP were 2.6% and 1.7%, respectively. The assay sensitivity was 5 ng/mL. Inter-assay and intra-assay CVs of serum OC were 1.2% and 0.8%, respectively. The assay sensitivity was 0.50 ng/mL. Inter-assay and intra-assay CVs of serum BAP were 3.3% and 1.5%, respectively. The assay sensitivity was 0.1 μg/L. Inter-assay and intra-assay CVs of serum 25OHD were 3.5% and 2.7%, respectively. The assay sensitivity was 7.5 nmol/L. Serum calcium (Ca) was measured using the Azo arsenic III method on an AU5800 (Beckman Coulter). Inter-assay and intra-assay CVs of serum Ca were 1.67% and 0.95%, respectively. The assay sensitivity was 0.03 mmol/L. Serum phosphorus (P) was measured using the phospho-molybdate method on an AU5800 (Beckman Coulter). Inter-assay and intra-assay CVs of serum P were 3.33% and 0.96%, respectively. The assay sensitivity was 0.05 mmol/L.

Fecal specimen collection, DNA extraction, and PCR

Fresh fecal samples were collected in sterile fecal collection devices and kept at -80°C until the time of analysis. Microbial DNA extraction utilized OMEGA Soil DNA Kit (D5625-01; Omega BioTek, USA), as directed by the manufacturer, and DNA was kept at -20°C until use. DNA quality and amounts were evaluated on a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA) and agarose gel electrophoresis. PCR was performed on an ABI 2720 Thermal Cycler (Thermo Fisher Scientific). The V3-V4 region of bacterial 16S rRNA was amplified with the 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') as sense and antisense primers, respectively. The reaction volume (25 μL) comprised 5x reaction buffer (5 μL), 5x GC buffer (5 μL), 2.5 mM dNTPs (2 μL), 1 μL each primer (10 μM), DNA template (2 μL), ddH_2O (8.75 μL) and Q5 DNA Polymerase (0.25 μL). Cycling proceeded as follows: initial denaturation at 98°C for 2 min, denaturation at 98°C for 15 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, final extension at 72°C for 5 min, and 10°C hold (25-30 cycles). PCR amplicons were purified with Vazyme VAHTSTM DNA Clean Beads (Vazyme, China) and quantitated with Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, USA). Quantitated amplicons were combined in equal amounts and pair-end 2*300 bp sequencing was carried out on an Illumina NovaSeq platform with NovaSeq 6000 SP Reagent Kit at Shanghai Personal Biotechnology (China).

Sequencing analysis

Microbiome bioinformatics was performed with QIIME2 2019.4 based on publicly available tutorials (https://docs.qiime2.org/2019.4/tutorials/), with slight modifications. In brief, raw sequencing reads underwent demultiplexing and primer cutting with the demux and cutadapt plugins, respectively. Sequences underwent quality filtration, denoising, merging, and chimera removal with the DADA2 plugin. Non-singleton amplicon sequence variants (ASVs) were aligned using mafft (via q2-alignment) and utilized for phylogenetic tree building (via q2-phylogeny) with fasttree 2. Alpha-diversity parameters (Chao1 estimator, present species, Shannon and Simpson indexes, Faith's PD, Pielou's evenness, and Good's coverage) and beta-diversity parameters (Bray-Curtis dissimilarity) were evaluated with the diversity plugin. ASVs were assigned to taxa with the classify-sklearn naïve Bayes taxonomy classifier in the feature-classifier plugin based on the Greengenes database.

Bioinformatics analysis and statistical analysis

Sequences were examined with QIIME2 and R v3.2.0. ASV-level alpha-diversity indexes indicating within-sample richness were calculated using the AVS table in QIIME2 and visualized as box plots. ASV-level abundance curves were built for comparing ASV richness and evenness among specimens. Kruskal-Wallis Rank-Sum test and Dunn' test were used to verify the significance of differences in alpha diversity between groups. Beta-diversity was assessed for investigating microbial communities in terms of structure across specimens based on Bray-Curtis metrics. Visualization was carried out by principal coordinate analysis (PCoA) and

nonmetric multidimensional scaling (NMDS). PERMANOVA (Permutational multivariate analysis of variance) was utilized for assessing the significance of microbiome structure differences using QIIME2. Venn diagrams were built for visualizing common and unique ASVs in various groups with R's "VennDiagram". Taxa abundances at the ASV level were compared by MetagenomeSeq. The differences in the relative abundance of flora between groups were assessed by Wilcoxon rank-sum test and adjusted by False Discovery Rate (FDR), an approach to multiple comparisons correction. Linear discriminant analysis (LDA) effect size (LEfSe) was carried out for detecting taxa with differential abundance levels among groups. LEfSe combines a nonparametric Kruskal-Wallis test or pairwise Wilcoxon rank-sum test with LDA. The logarithmic LDA score >2 and P<0.05 were considered significant. Spearman correlation analysis was conducted for evaluating associations of different taxa with BTMs, BMD measurements, as well as clinical variables. Independent-samples t-test, the Mann-Whitney U test, and/or the Fisher's exact test were performed for comparing anthropometric and clinical variables between the groups. SPSS 21.0 was utilized for analysis, and two-sided P<0.05 indicated statistical significance.

Results

Patient features

Because CTX-I and PINP were recommended by the International Osteoporosis Foundation (IOF) as markers of bone resorption and bone formation, respectively, participants were grouped by the median of CTX or P1NP in this study [23]. Individuals with serum CTX lower and higher than the median were classified into the LCTX (n=48) and HCXT (n=49) groups, respectively. Similarly, participants with serum P1NP lower and higher than the median were classified into the LP1NP (n=48) and HP1NP (n=49) groups, respectively. The levels of BTMs, including CTX, P1NP, OC, and BAP, were significantly higher in the HCTX and HP1NP groups compared with the LCTX and LP1NP groups, respectively (P<0.001, **Table 1**). The fracture percentages were 16% (8/49), 4% (2/48), 14% (7/49) and 6% (3/48) in the HCTX, LCTX, HP1NP and LP1NP groups, respectively. There was no significant difference in fracture rate between the HCTX and LCTX groups, or between the HP1NP and LP1NP groups (P>0.05). HCTX and HP1NP individuals tended to have lower BMD and T-scores in the LS, FN, and total hip compared with the LCTX and LP1NP groups, but differences did not achieve significance (P>0.05). The remaining data, including age, BMI, years since menopause (YSM), and calcium, and vitamin D amounts, did not reach significance between the HCTX and LCTX groups, or between the HP1NP and LP1NP groups (P>0.05).

Diversity analysis of the gut microbiota

Illumina sequencing detected a total of 5,791,689 high-quality reads in the 97 fecal specimens (averaging 59,708 reads/sample). The fecal bacteria belonged to 203 species, 235 genera, 121 families, 77 orders, 52 classes and 21 phyla (Supplementary Table 1). There were 50410, 48954, 50204, and 49241 ASVs in the HCTX, LCTX, HP1NP and LP1NP group, respectively. Only 9695 ASVs (10.81%) were shared between the HCTX and LCTX groups, while 9776 ASVs (10.90%) were shared between the HP1NP and LP1NP groups (**Figure 1**).

Alpha-diversity indexes were assessed to reflect bacterial diversity in specimens; the higher the value, the greater the diversity. Alpha-diversity indexes (Chao1, Simpson, Shannon, Pielou's evenness, observed species, Faith's PD, and Good's coverage) were similar in the HCTX and LCTX groups, and in the HPINP and LPINP groups (**Figure 2**). With regard to beta-diversity, neither the CTX group nor the P1NP group could be distinguished by PCoA, NMDS using Bray-Curtis distance (Supplementary Figure 1) or PERMANOVA test (P=0.234, q=0.234 and P=0.127, q=0.127 for the CTX and P1NP groups, respectively). The ASV level rarefaction curves of diversity indexes all plateaued, suggesting that the majority of bacterial organisms were included.

Taxonomic composition of gut bacterial communities

Enterobacteria mainly consisted of Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria, which constituted the four major phyla in various specimens (Supplementary Figure 2). Firmicutes accounted for the largest proportion, averaging 54% among groups, fol-

Table 1. Patient features

Variable	HCTX (n=49)	LCTX (n=48)	statistical values between CTX groups	*P* value for CTX groups	HP1NP (n=49)	LP1NP (n=48)	statistical values between P1NP groups	*P* value for P1NP groups
Age (years)	60.5±5.1	61.8±4.0	*t*=1.418	*P*=0.160	61.1±5.2	61.2±4.0	*t*=0.091	*P*=0.928
BMI	22.56±3.38	23.80±2.76	*t*=1.976	*P*=0.051	23.11±3.20	23.24±3.10	*t*=0.192	*P*=0.849
YSM	9.8±6.1	11.1±5.0	*t*=1.154	*P*=0.251	10.1±6.2	10.8±4.8	*t*=0.568	*P*=0.572
Fracture, n (%)	8 (16)	2 (4)	χ^2=2.674	*P*=0.102	7 (14)	3 (6)	χ^2=0.936	*P*=0.333
LS BMD (g/cm^2)	0.935±0.159	0.992±0.187	*t*=1.629	*P*=0.107	0.948±0.156	0.979±0.191	*t*=0.856	*P*=0.394
LS T-score	-1.5±1.3	-1.0±1.6	*t*=1.778	*P*=0.079	-1.3±1.3	-1.1±1.6	*t*=0.790	*P*=0.431
FN BMD (g/cm^2)	0.766±0.101	0.774±0.106	*t*=0.382	*P*=0.703	0.760±0.917	0.780±0.114	*t*=0.929	*P*=0.355
FN T-score	-1.4±0.9	-1.3±0.9	*t*=0.405	*P*=0.687	-1.4±0.8	-1.3±1.0	*t*=0.921	*P*=0.359
Total hip BMD (g/cm^2)	0.811±0.110	0.844±0.133	*t*=1.272	*P*=0.206	0.811±0.111	0.845±0.132	*t*=1.384	*P*=0.170
FN T-Total hip	-1.2±0.8	-1.0±1.0	*t*=1.131	*P*=0.261	-1.2±0.8	-1.0±1.0	*t*=1.241	*P*=0.218
βCTX (pg/mL)	731.43±132.66	415.88±81.79	*t*=-14.067	*P*<0.0005	681.04±185.03	467.32±132.30	*t*=-6.532	*P*<0.0005
P1NP (ng/mL)	75.62±21.58	51.38±14.03	*t*=-6.534	*P*<0.0005	79.81±18.08	47.10±9.46	*t*=-11.131	*P*<0.000
OC (ng/mL)	24.04±5.35	16.85±3.91	*t*=-7.549	*P*<0.0005	24.07±16.82	5.36±3.82	*t*=-7.652	*P*<0.0005
BAP (μg/L)	16.29±4.64	12.56±2.85	*t*=-4.712	*P*<0.0005	15.98±4.42	12.88±3.50	*t*=-3.782	*P*<0.0005
Ca (mmol/L)	2.39±0.07	2.37±0.08	*t*=-1.216	*P*=0.227	2.38±0.08	2.38±0.07	*t*=0.276	*P*=0.783
25OHD (nmol/L)	53.21±20.44	56.27±18.22	*t*=0.779	*P*=0.438	54.35±21.27	55.11±17.34	*t*=0.193	*P*=0.847

Note: Independent-samples t-test, the Mann-Whitney U test, and/or Fisher's exact test were performed for comparisons. HCTX: women with serum βCTX higher than the median; LCTX: women with serum βCTX lower than the median; HP1NP: women with serum P1NP higher than the median; LP1NP: women with serum P1NP lower than the median; SD: standard deviation; BMI: body mass index; YSM: years since menopause; LS: lumbar spine 1-4; BMD: bone mineral density; FN: femoral neck; βCTX: type I collagen crosslinked beta C-telopeptide; P1NP: type I procollagen-N-propeptide; OC: osteocalcin; BAP: bone-specific alkaline phosphatase; Ca: calcium; 25OHD: total vitamin D.

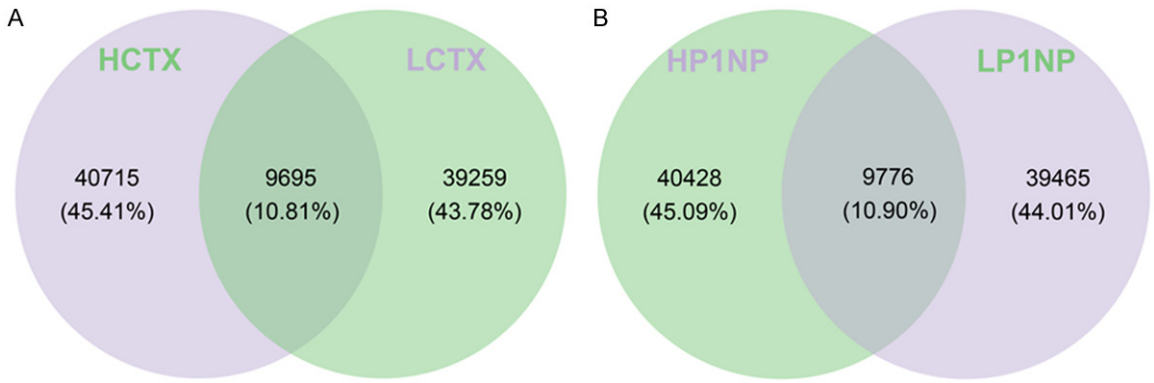

Figure 1. Venn diagrams at the ASVs level. A: Venn diagram of the HCTX and LCTX groups; B: Venn diagram of the HPINP and LPINP groups. HCTX: women with serum βCTX higher than the median; LCTX: women with serum βCTX lower than the median; HP1NP: women with serum P1NP higher than the median; LP1NP: women with serum P1NP lower than the median; ASVs: amplicon sequence variants.

lowed by Bacteroidetes, Proteobacteria and Actinobacteria, accounting for 32%, 10% and 3%, respectively. Other phyla accounted for only less than 1%. The four major phyla had similar abundance levels in the HCTX and LCTX groups, and in the HPINP and LPINP groups (P>0.05, q>0.25; **Figure 3A**, **3B**).

Concerning genera, Bacteroides was the most abundant, with a mean relative abundance of 24% in all samples, followed by Faecalibacterium, Roseburia, Prevotella and Shigella, which accounted for 14%, 6%, 5% and 5%, respectively (Supplementary Figure 2). Differentiation analysis showed that Faecalibacterium had a significant difference between the HCTX and LCTX groups (P=0.004, q=0.143; **Figure 3C**, **3D**). Differences among the top 20 genera showed no statistical significance between the HPINP and LPINP groups (P>0.05).

We further performed LEfSe analysis to detect differentially abundant taxa across groups at different levels (**Figure 4**). The results revealed that Clostridiaceae (P=0.015, LDA=2.89), and Faecalibacterium (P=0.017, LDA=4.60), Prevotella (P=0.040, LDA=3.61) and Clostridium (P=0.007, LDA=2.79) were enriched in the LCTX group, and Facklamia (P=0.044, LDA=3.10) was enriched in the HCTX group. Peptostreptococcaceae (P=0.048, LDA=2.83) and SMB53 (P=0.028, LDA=2.05) were enriched in the LPINP group, and Veillonellaceae (P=0.025, LDA=4.43) and S24_7 (P=0.023, LDA=3.08) were enriched in the HPINP group.

Associations of gut microbiome abundance with BTMs

Spearman correlation analysis was performed for evaluating the associations of the gut bacteria with clinical parameters and BTMs. The results showed that Clostridium (Clostridiaceae) and (Clostridium) were negatively correlated with serum CTX levels significantly (r=-0.34, P<0.001 and r=-0.21, P=0.040, respectively; **Figure 5**). Clostridium (Ruminococcaceae) was positively correlated with serum CTX amounts (r=0.20, P=0.049). Bacteroides and (Clostridium) were negatively correlated with serum PINP levels (r=-0.23, P=0.024 and r=-0.22, P=0.033, respectively), while Chryseobacterium and Dehalobacterium had positive correlations with PINP levels (r=0.23, P=0.023 and r=0.25, P=0.014, respectively).

Discussion

This study investigated the intestinal microbiota in 50- to 69-year-old postmenopausal women with respect to BTMs. High-throughput sequencing was carried out for analyzing gut microbiome composition and diversity, and various intestinal microbial genera were associated with the levels of BTMs in this well-characterized cohort.

Despite the long clinical success of DXA, it is limited in assessing fracture risk [24, 25]. BTMs are useful in assessing the bone turnover rate, which provides an improved understanding of

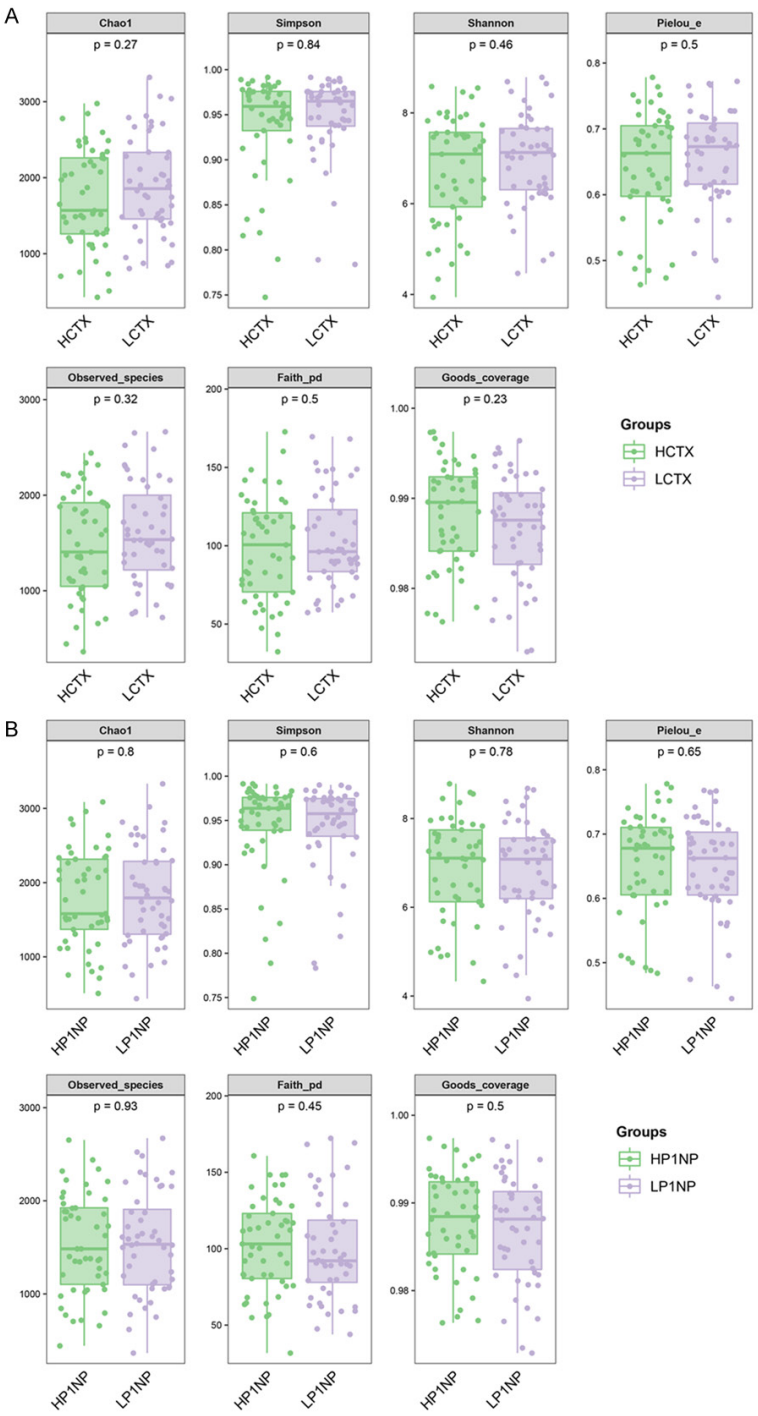

Figure 2. Alpha diversity indices in various groups. A: Comparison of alpha diversity between the HCTX and LCTX groups; B: Comparison of alpha diversity between the HPINP and LPINP groups. Various panels are alpha diversity indexes (top gray areas in panels). Abscissa, group names; ordinate, value of the alpha diversity index. *P*-value based on the Kruskal-Wallis test. HCTX: women with serum βCTX higher than the median; LCTX: women with serum βCTX lower than the median; HP1NP: women with serum P1NP higher than the median; LP1NP: women with serum P1NP lower than the median.

the pathogenesis and enables the early assessment of osteoporosis. We found that the levels of all BTMs, including CTX, PINP, OC, and BAP, were markedly increased in groups with elevated bone turnover (HCTX and HPINP groups) compared with low-bone turnover groups (LCTX and LPINP groups), while BMI, YSM, calcium and vitamin D amounts were similar among groups. Increased concentrations of BTMs in postmenopausal women are associated with rapid bone loss [26]. Moreover, evidence suggests that BTMs can predict the risk of vertebral and hip fractures [27-29]. This study observed a similar fracture rate among groups, and BMD values were only slightly lower in the HCTX and HPINP groups compared with the LCTX and LPINP groups, respectively, and the differences did not achieve significance. On the one hand, this may be due to the fact that all participants were relatively young and healthy postmenopausal women. Even though the bone turnover is increased in this period, bone loss was not yet obvious because BTMs have serological fluctuations prior to changes in BMD. On the other hand, the inverse relationship between BTMs and BMD is not strong enough to be a diagnostic marker [30].

The microbiota and BMD have been shown to be associated in older adults and PMO individuals [14-17]. Here, we demonstrated different taxonomic compositions of the gut bacterial community between the high-turnover and low-turnover groups, which confirms our hypothesis that bone turnover is influenced by the intestinal microbiome. We observed that Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria constituted the major phyla in healthy post-

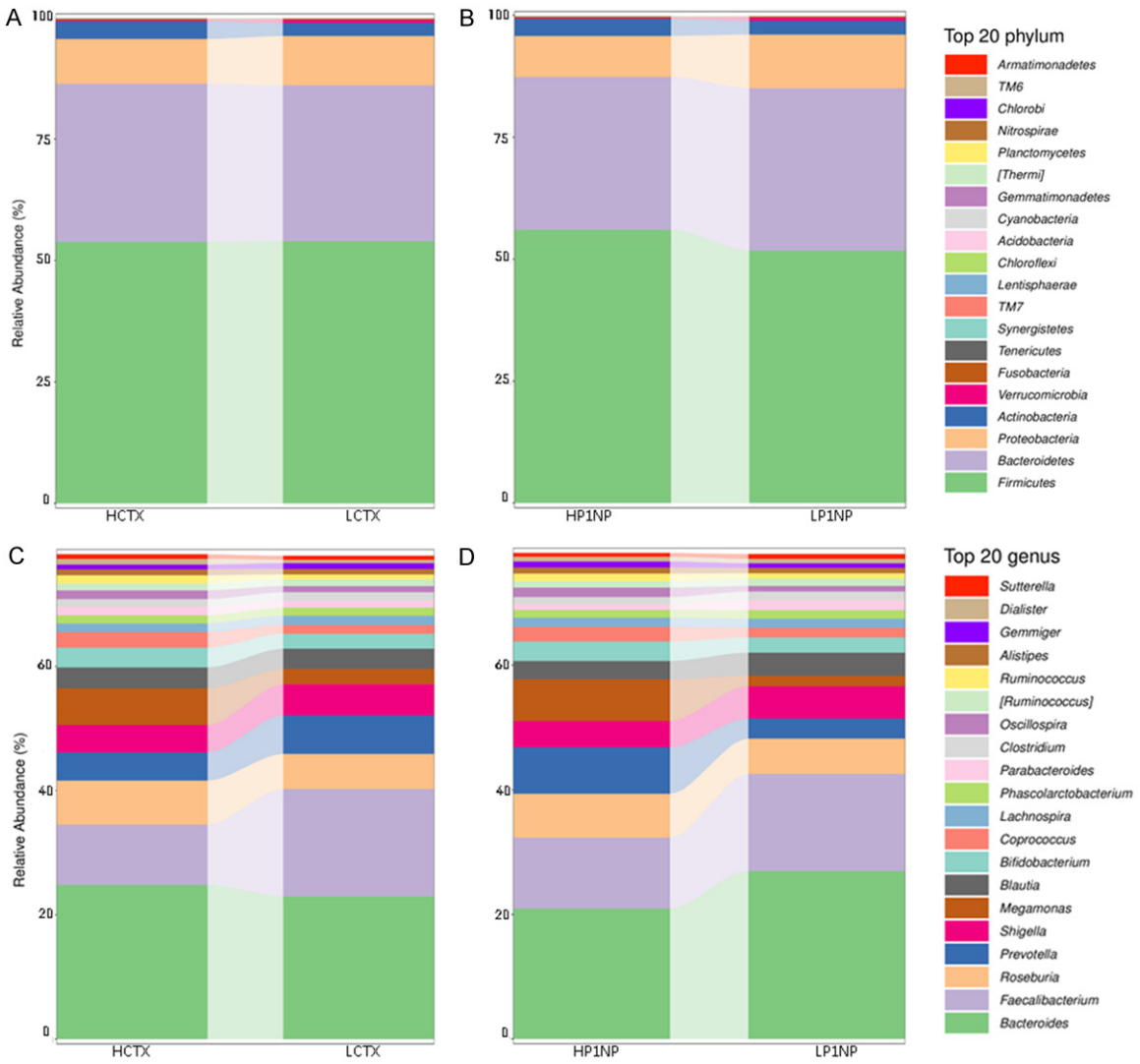

Figure 3. Bacterial community abundance levels. A and B: Abundance of bacterial communities (bar plots) at the phylum level. C and D: Abundance of bacterial communities (bar plots) at the genus level. HCTX: women with serum βCTX higher than the median; LCTX: women with serum βCTX lower than the median; HP1NP: women with serum P1NP higher than the median; LP1NP: women with serum P1NP lower than the median.

menopausal women, which are relatively consistent in most normal GM profiles though their contents in each individual's gut vary [31, 32]. Interestingly, the genus Faecalibacterium differences were found in both differentiation analysis and LefSe analysis, suggesting that Faecalibacterium may be a marker between CTX groups (bone resorption). Recently, a study reported Faecalibacterium as a member of the core microbiota in healthy Chinese individuals, with reduced abundance in frailer females [33, 34].

LEfSe analysis showed that Clostridium was enriched in the LCTX group. Furthermore, Clostridium was negatively correlated with CTX. While some Clostridium spp. cause severe human and animal infections, others present in the intestinal microbiome mostly promote health and well being [35]. The potential mechanisms of Clostridium's effects on bone turnover may involve the immune system, endocrine system, and products of bacterial metabolism. The immune-skeletal axis is critical for maintaining skeletal integrity through balancing bone resorption and bone formation [36]. Clostridium promotes T-regulator cell accumulation and differentiation, which play important roles in maintaining bone homeostasis [37]. Clostridium may also be involved in the metab-

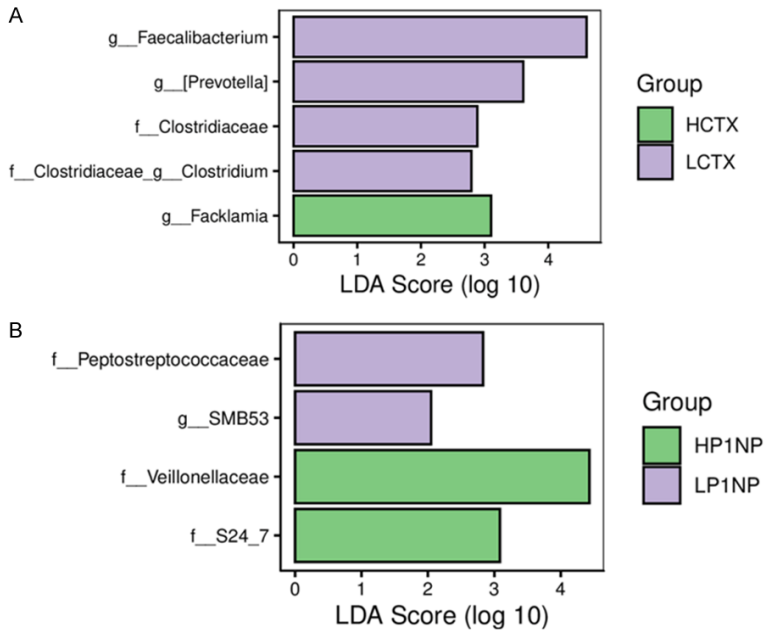

Figure 4. LEfSe analysis to detect differentially abundant taxa across groups at different levels (taxa with P<0.05 and LDA >2 are shown). Ordinate, taxa with significant differences among groups; abscissa, logarithm score of LDA analysis for each taxon. A: Differentially taxa between the HCTX and LCTX groups; B: Differentially taxa between the HPINP and LPINP groups. LDA: linear discriminant analysis; LefSe: LDA Effect Size; f: family; g: genus.

olism of non-ovarian estrogens. It has been reported that non-ovarian systemic estrogens have tight and significant associations with fecal Clostridia taxa in Firmicutes both in men and postmenopausal women [8]. Therefore, Clostridium may inhibit osteoclasts by affecting the production of non-ovarian estrogens. In addition, Clostridium may inhibit bone resorption by producing short-chain fatty acids (SCFAs). Indeed, Clostridium spp. are able to synthesize SCFAs such as propionate and butyrate [38-40]. Recent evidence suggests that the protective effects of propionate and butyrate on bone mass involve reduced osteoclast differentiation *in vitro* and *in vivo* [41].

We did not find marked differences in overall diversity among postmenopausal women, between the high-turnover and low-turnover groups. The correlation between gut microbiota diversity and BMD remains debatable, with some reports detecting no marked diversity difference between low BMD and normal BMD individuals [15, 16]. Others demonstrated varying bacterial diversities among the osteoporosis, osteopenia and control groups [14, 17]. This inconsistency may be due to the differences in study populations and sample sizes. Microbiota diversity is considered a valuable indicator of health [16]. It was previously reported that overall microbial diversity is not significantly altered in elderly individuals, and microbiome profiles in the healthy elderly and young individuals of the same population show negligible differences [33]. This work enrolled healthy elderly postmenopausal women, and the subjects were comparable because of strict inclusion criteria, which may be the main reason why there was no significant difference in diversity between the high- and low-bone turnover groups.

No taxa showed significantly different abundance levels between the HP1NP and LP1NP groups in this work. Moreover, the biomarkers iden-tified by LEfSe analysis were not consistent with the results of correlation analysis. On one hand, the gut microbiota may mainly affect the process of bone resorption. It was reported that SCFAs only inhibited bone resorption while bone formation was not affected [41]. On the other hand, bone resorption is the major bone turnover status in estrogen deficiency. Generally, all BTMs are elevated after menopause but the increasing extent of resorption markers is higher than that of formation markers. Gossiel and collaborators reported elevated CTX and PINP amounts in postmenopausal women, by 80% and 33% within 10 years postmenopause, respectively [42]. Hence, CTX represents a more sensitive index of bone turnover in the study of microflora.

This study had strict inclusion and exclusion criteria in order to exclude conditions that may affect intestinal microflora and bone turnover as much as possible. Only one study touched on the association of GM with BTMs in postmenopausal women [17]. Nevertheless, the study mainly focused on the GM's association with bone mass by grouping based on bone mineral density and did not exclude diseases

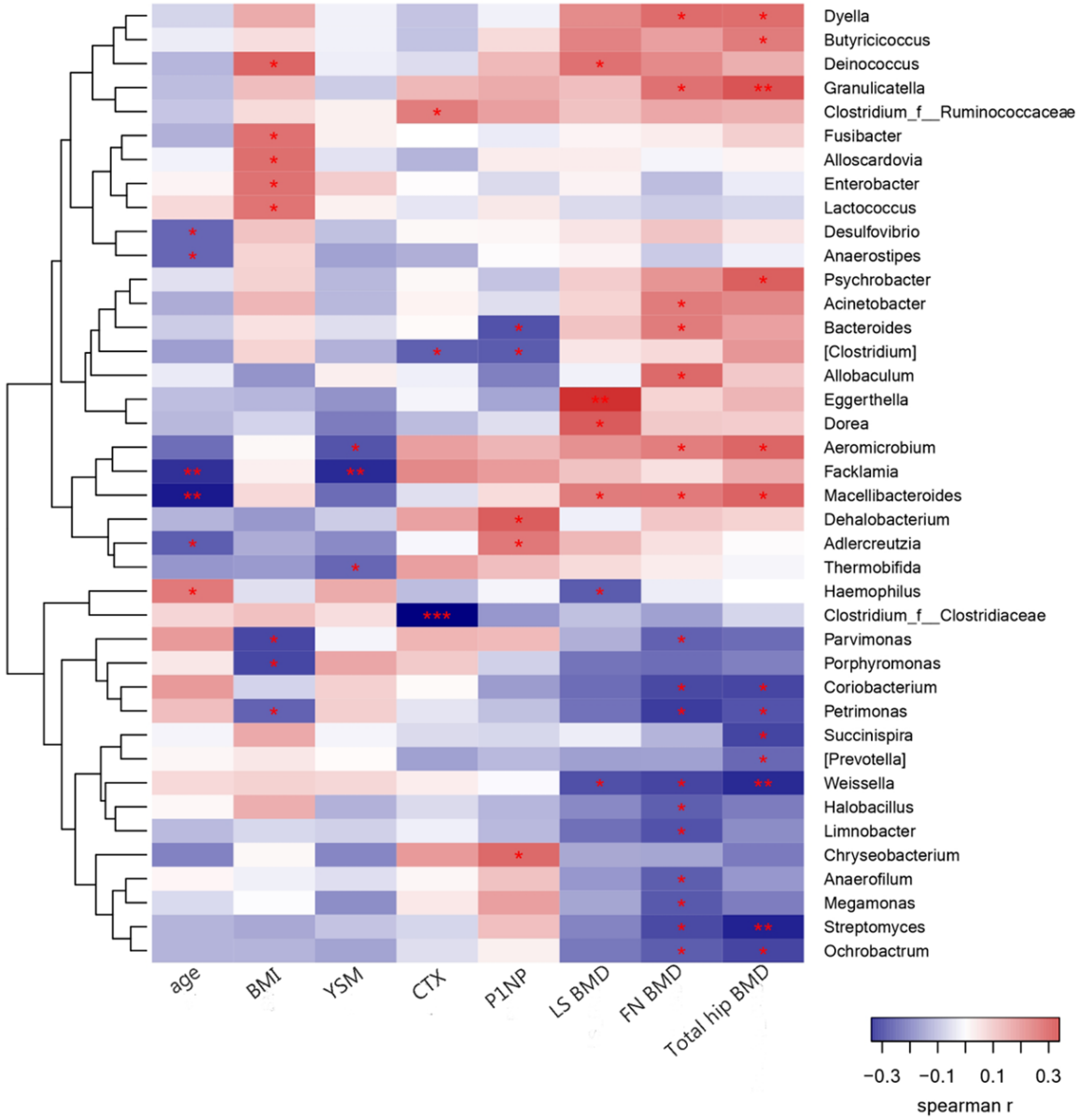

Figure 5. Associations of gut bacteria with clinical indexes and BTMs. Red: positive correlation; blue: negative correlation. The deeper the color, the closer the correlation. The Spearman correlation coefficient achieved significance at *P<0.05, **P<0.01 or ***P<0.001. BMI: body mass index; YSM: years since menopause; LS: lumbar spine 1-4; BMD: bone mineral density; FN: femoral neck; CTX: type I collagen crosslinked beta C-telopeptide; P1NP: type I procollagen-N-propeptide.

such as diabetes, which would affect BTMs. More recently, a small sample study investigated the relationship between gut microbiota composition and bone metabolism in women aged 50 to 82 [43]. Due to the age range, the subjects of this study included not only PMO but also patients with senile osteoporosis, which often occurs after 70 years old with a low-turnover level. Furthermore, age is another important factor that influences the composition of intestinal flora [5].

However, the current study had certain limitations. First, it had a cross-sectional design, and causality could not be inferred from microbial community changes to bone turnover. Another potential issue is that dietary factors could affect the results under certain circumstances, though individuals were recruited from the same region and required to take a normal diet to minimize this effect. Furthermore, the current study was based on a relatively small sample in a single region. Therefore, prospective,

randomized controlled trials in larger populations are warranted to further validate the current findings.

In conclusion, this study identified taxa-specific intestinal microflora differences associated with BTMs, especially CTX. Further studies are required to explore the mechanisms of these specific genera in bone metabolism.

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Disclosure of conflict of interest

None.

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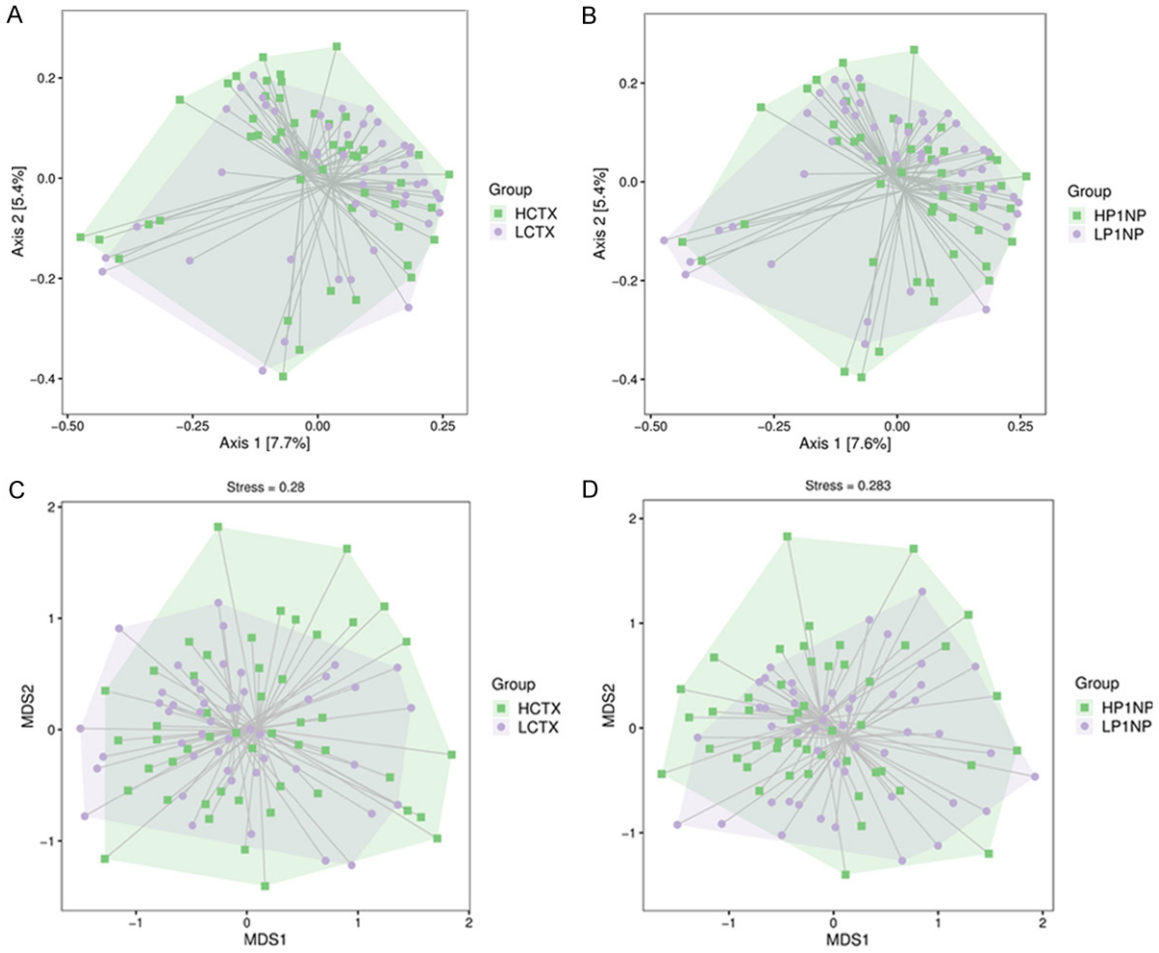
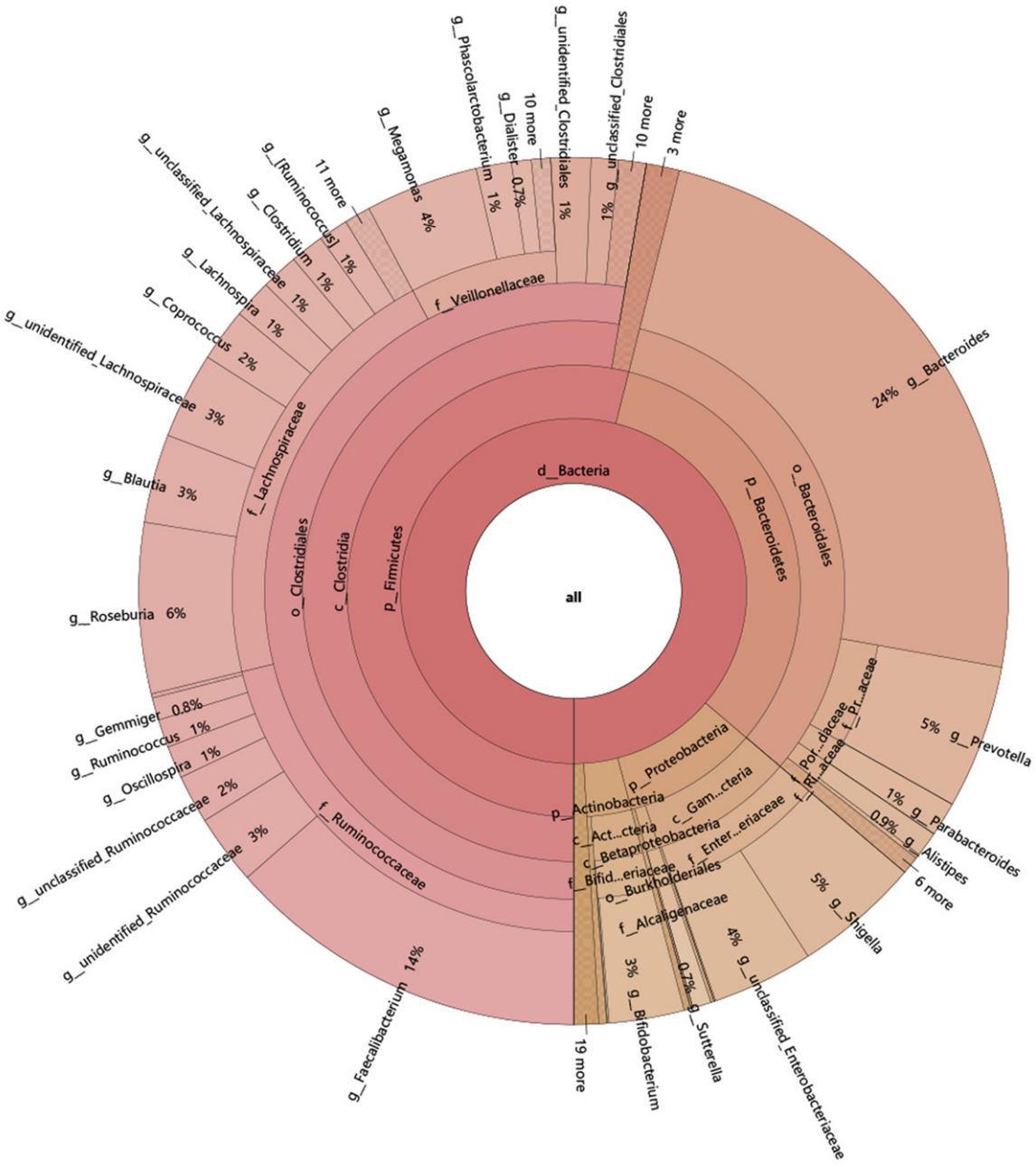
Supplementary Table 1. Bacterial taxa in various groups at distinct levels

	Phylum	Class	Order	Family	Genus	Species
HCTX	17	38	58	101	197	177
LCTX	19	43	63	104	179	158
HPINP	19	40	61	99	178	168
LPINP	18	41	60	105	195	165
Total	21	52	77	121	235	203

Note: Within the "Total" row, values indicate the numbers at different levels across all stool samples. HCTX: women with serum βCTX higher than the median; LCTX: women with serum βCTX lower than the median; HP1NP: women with serum P1NP higher than the median; LP1NP: women with serum P1NP lower than the median.



Supplementary Figure 1. Beta-diversity in the HCTX, LCTX, HPINP, and LPINP groups at the ASV level. A and B: PCoA's scatter plot; C and D: NMDS' scatter plot. PcoA: principal coordinate analysis; NMDS: nonmetric multidimensional scaling.



Supplementary Figure 2. Taxonomy composition diagram based on Krona. The circle represents the five taxonomic levels (phylum, class, order, family and genus) from inside to outside. The size of the fan reflects the relative abundance of the taxon. d: domain; p: phylum; c: class; o: order; f: family; g: genus.