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Characterization of Fecal Microbiomes of Osteoporotic Patients in Korea

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Abstract

An imbalanced gut microbiome has been linked to a higher risk of many bone-related diseases. The objective of this study was to discover biomarkers of osteoporosis (OP). So, we collected 76 stool samples (60 human controls and 16 OP patients), extracted DNA, and performed 16S ribosomal ribonucleic acid (rRNA) gene-based amplicon sequencing. Among the taxa with an average taxonomic composition greater than 1%, only the *Lachnospira* genus showed a significant difference between the two groups. The Linear Discriminant Effect Size analysis and qPCR experiments indicated the *Lachnospira* genus as a potential biomarker of OP. Moreover, a total of 11 metabolic pathways varied between the two groups. Our study concludes that the genus *Lachnospira* is potentially crucial for diagnosing and treating osteoporosis. The findings of this study might help researchers better understand OP from a microbiome perspective. This research might develop more effective diagnostic and treatment methods for OP in the future.

K e y w o r d s: osteoporosis, microbiomes, gut microbiota, fecal microbiomes, 16S rRNA gene-based metagenomics, *Lachnospira*

Introduction

Osteoporosis (OP), the most common bone-related disease, is characterized by a loss of bone mass, increased bone fragility, damage to bone tissue microstructure, and increased fracture risk. It affects men and women (1–8% and 9–38%, respectively) (Wade et al. 2014; Cannarella et al. 2019). Throughout life, human bone continues the remodeling process. One remodeling cycle consists of four stages (initiation, resorption, reversal, and formation) (Ding et al. 2020). When bone resorption outpaces bone formation, bone integrity is compromised, leading to OP (Tang 2020). The pathophysiology of OP is linked to heredity, hormonal levels, diet, lifestyle, and inflammatory factors (Peng et al. 2018; Zheng et al. 2019; Li et al. 2020; Tang 2020). OP is more common in women than in men. The primary cause of OP has been linked to estrogen deprivation after menopause (Manolagas 2010). The secondary cause of OP includes smoking, type 1 diabetes (T1D), parathyroid disorder, inflammatory bowel disease (IBD), arthritis, and glucocorticoid medication (Zaheer and LeBoff 2000). Many pharmacological, hormonal, antibody and inhibitor-based therapies are currently being practiced to cure OP. However, all available treatments are associated with severe side effects like gastrointestinal diseases, rhinitis, dermatological reactions, musculoskeletal pain, dizziness, nausea, headache,

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stroke, and hypercholesterolemia (Camacho et al. 2016; Tu et al. 2018). Thus, a new OP therapy with minimum or no adverse effects is urgently needed.

It has been estimated that there are 10 trillion bacteria in the human intestinal microbiota (Yan et al. 2016). Based on their involvement in human health, intestinal microorganisms are classified as beneficial, opportunistic, and commensal microbes. The beneficial bacteria (probiotics) confer health benefits to hosts (Guarner and Schaafsma 1998). Some commonly used probiotics are *Bifidobacteria*, *Lactobacillus reuter*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*-group, *Bacillus coagulans*, *Escherichia coli* strain Nissle 1917, *Enterococcus faecium*, and certain enterococci (Pandey et al. 2015). The opportunistic microbes utilize the opportunity of weakened defense mechanisms of the host to inflict damage. Some opportunistic bacteria are *Corynebacterium equi*, *Staphylococcus aureus*, *Mycoplasma pneumoniae*, and *Salmonella* spp. The commensal bacteria defend against foreign pathogens directly by competing for living space or nutrients by toxins (bacteriocins, acids, and phenols) (Guarner and Malagelada 2003; Wang et al. 2018). Moreover, some commensal bacteria act on the host immune system (Stecher and Hardt 2008), and several commensal bacteria reside inside the human gut, like *Bacteroides fragilis*, *Bacteroides uniformis*, and *Clostridium ramosum*.

Previously, multiple studies have demonstrated the link between gut microbiome compositions and bone metabolism. Also, bone-related mineral absorption is involved in OP under different physiological conditions (Scholz-Ahrens et al. 2007; Sjögren et al. 2012; Charles et al. 2015; Li et al. 2016; D'Amelio and Sassi 2018; Uchida et al. 2018; Tavakoli and Xiao 2019; Cheng et al. 2020). Xu et al. (2017) showed that intestinal microbiota composition and structure could be influenced by both host (genetic background and gender) and environmental factors (diet, lifestyle, hygiene, antibiotics, and probiotics). A new genome-wide associated study found that the order Clostridiales and family *Lachnospiraceae* are positively related to bone mass variation, implying a linkage between microbiota and bone formation (Ni et al. 2021).

Several recent studies have investigated the effects of microbiomes on primary or secondary OP between OP patients and healthy controls (HCs) (Wang et al. 2017; Das et al. 2019; Li et al. 2019; Wei et al. 2021b). However, these previous studies were limited to Chinese, Latin American, and European populations. Thus, this study aimed to investigate the bacterial community structure and diversity alterations of gut microbiota in OP patients among Korean people. Variations in the gut microbial composition of OP patients compared to HCs were obtained based on in-depth research of microbial components connected to OP. These findings were correlated with clinical parameters. We expect that our study could serve as a platform for future research into new microbe biomarkers and processes behind the impact of gut microbiota on OP.

Experiment

Materials and Methods

Sample collection, DNA extraction, amplification, and sequencing. The present study was performed from May 2020 to November 2021 in the Healthcare Center affiliated with the Probiotics Microbiome Convergence Center at Soonchunyyang University, Asan, South Korea. It was approved by the Institutional Review Board (IRB) (IRB No. 2019-10-017-005). Seventy-six (33–82 years) adults were enrolled in this study, including 60 human controls (HC) and 16 OP patients (Table SI). OP was diagnosed by bone density test using dual-energy X-ray absorptiometry (DEXA) based on the World Health Organization (WHO) recommendations (Kanis 2008). Participants of this study were informed about the sampling method and risks involved. All of them agreed to laboratory tests and gave written consent. The first fecal samples before breakfast (5–10 g) were collected by each participant individually at the recruitment site at RT and placed at −80°C immediately. Then, samples were transported to the laboratory with dry ice (temperature ~ −78°C) and kept at −80°C until further processing. All 76 samples were used for the 16S rRNA gene V4 region sequencing.

DNA extraction. Using the QIAamp DNA fast Stool Mini Kit (Qiagen, Germany), microbial DNA was extracted from 180–220 mg fecal samples following the manufacturer's protocol. The DNA concentration was measured with a Qubit-4 fluorometer (Thermo Fisher Scientific, UK). The quality of DNA was checked by 0.8% agarose gel electrophoresis. All DNA samples were stored at −20°C until further use.

PCR amplification of the 16S rRNA gene. The 16S bacterial rRNA (V4 hypervariable region) was amplified using Illumina 16S amplicon primer set (5 μM each) (Forward primer: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG-3', Reverse primer: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGG-GTATCTAATCC-3') with 10 ng of template DNA and KAPA HiFi HotStart ReadyMix (Kapa Biosystems, USA) following the previously described protocol by our team (Kim et al. 2021). Briefly, the PCR was performed on a Veriti 96-well Thermal cycler (Applied Biosystems, Thermo Fisher, USA) with all 76 samples, including negative control (no template DNA) and positive control (5 ng of mouse stool DNA). Amplification conditions for all

samples were: initial denaturation at 95°C for 3 min, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a final extension step at 72°C for 5 min. PCR products were purified using AMPure beads (Beckman Coulter, UK) following the manufacturer's protocol. Indexed PCR was performed using Nextera XT DNA Library Prep Kit (Illumina, USA) according to the recommended protocol, followed by PCR clean-up using AMPure beads. Each sample was diluted to 1 nM final concentration, and samples were pooled together.

The 16S rRNA gene-based sequencing and data analysis. Pooled library (50 pMol) was used for sequencing with 30% PhiX spiking on an iSeqTM100 platform (Illumina, USA). Data were analyzed following the procedures described previously by our team (Kim et al. 2021; ul-Haq et al. 2022). Briefly, we analyzed data using the EzBioCloud server (http://www.ezbiocloud.net). Trimmomatic (version. 0.32) was used for quality checking and filtering of low-quality reads (< Q25). Primer trimming was done with Myers and Miller's alignment algorithm (Myers and Miller 1988). Samples without 16S rRNA encoding were identified using HMMER software and nhmmer (package ver. 3.2.1) (Wheeler and Eddy 2013). The unique reads and redundant reads were clustered using the derep_full length command in VSEARCH (Rognes et al. 2016). We employed EzBioCloud's 16S rRNA database (Yoon et al. 2017) for taxonomic assignment with VSEARCH (Myers and Miller 1988; Rognes et al. 2016). Chimeric reads were filtered using UCHIME (Edgar et al. 2011). To identify sequences at the low taxonomic level, the cluster_fast command (Rognes et al. 2016) was used to create operational taxonomic units (OTUs). Single-read OTUs were removed from further analysis. Sequences were deposited in Sequence Read Archive (SRA) (BioProject ID: PRJNA795857, accessible at https://www.ncbi.nlm.nih.gov/bioproject/795857).

Quantitative PCR. qPCR was used for comparative quantification of *Lachnospira* using BioRad CFX Connect Real-Time-System thermocycler equipment (BioRad, USA) and iQ SYBR® Green Supermix (BioRad, USA) with *Lachnospira*-specific primers (Forward primer 5'-CCTGACTAAGAAGCTCCGGC-3'; Reverse primer: 5'-CAAAAGCAGTTCCGGGGTTG-3') according to Liu et al. (2022). A total of 32 samples (16 OP patients and 16 HCs) with positive control (mouse stool DNA) and negative control (no template DNA) were used for this experiment. Triplicate qPCR was performed using 10 ng of genomic DNA from each sample, 10 µl of SYBR Mixture, 1 µl forward primer (1 µM), and 1 µl reverse primer (1 µM) for each PCR reaction. PCR conditions were as follows: pre-denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, and annealing/extension at 56°C for 30 seconds. Quantitation cycle (Cq) values from HC and OP patient groups were compared using GraphPad Prism software (ver. 8.0.1, USA).

Statistical analysis. Alpha diversities of the samples were calculated for samples based on Chao1 (Chao 1987), ACE (Chao and Lee 1992), Shannon/Simpson (Magurran 2013), Jackknife (Burnham and Overton 1979), NPShannon (Chao and Shen 2003), and Phylogenetic diversity (Faith 1992). On the other hand, beta diversity distances were analyzed based on Generalized UniFrac (Chen et al. 2012), Fast UniFrac (Hamady et al. 2010), Jenson-Shannon (Lin 1991), and Bray-Curtis (Beals 1984). Permutational multivariate analysis of variance (PERMANOVA) was used to determine the beta set significance between OP and HC. The taxonomic biomarkers were found using statistical comparison algorithms of LEfSe (Linear discriminant analysis Effect Size) (Segata et al. 2011) and Kruskal-Wallis *H* tests (Kruskal and Wallis 1952). The Student's *t*-test was performed to evaluate the statistical significance of comparing Cq (quantification cycle) values of OP patients and HCs. Functional profiles were predicted based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Ye and Doak 2009) using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) (Douglas et al. 2020). The differences between the groups were assessed using STAMP (statistical analysis of taxonomic and functional profiles) software and Welch's *t*-test. The $p < 0.05$ was taken as statistically significant for all the analyses.

Results

Characteristics of the study population. Table SI shows a demographic data comparison between the two groups. The number of participants was 60 in the HC group and 16 in the OP group. The mean ages of the HC and OP groups were 59.1 ± 9.8 years and 66.3 ± 8.9 years, respectively. Baseline characteristics, such as age, gender, and body mass index (BMI), showed no statistically significant differences between the two groups. Mean T-scores of HCs and OP patients were – 0.75 ± 1.1 and – 2.85 ± 0.3, respectively. Lifestyle factors such as smoking and drinking did not significantly differ between OP and HC groups. Hypertension, diabetes mellitus, and blood chemistry measurements (glucose, triglyceride, protein, albumin, and blood urea nitrogen (BUN)) were not significantly different between the two groups, suggesting that these parameters did not seem to have a significant relationship with OP.

Microbiota characteristics. A total of 1,419,302 high-quality reads were generated among the 76 fecal samples, with 4,279 median values per sample. Compared to the HC group, OP patients had no difference

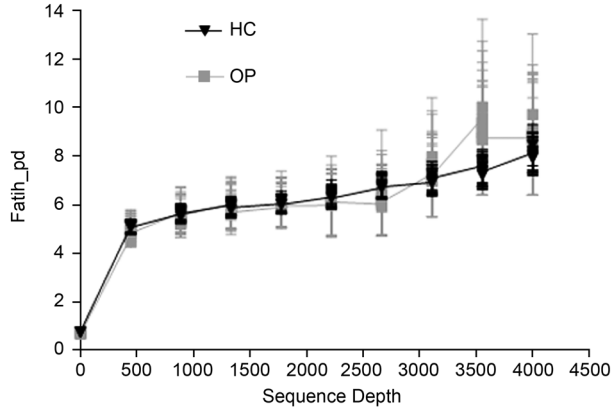

Fig. 1. Rarefaction curve for sequence depth. The Faith phylogenetic diversity (Faith_pd) rarefaction curve shows that there is no difference in species abundance and diversity between the healthy control (HC) group and the osteoporotic (OP) patient group.

in species richness and diversity, as shown in Faith phylogenetic diversity (Faith_pd) rarefraction curve (Fig. 1). Along with that, average taxonomic compositions in feces of osteoporotic patients and HCs are presented in Table SII and Fig. 2. Our data showed that average taxonomic compositions of OP patients and HC group were not significantly different at the phylum or class level. However, at the phylum level, Firmicutes showed the highest average percentage in both OP patients and HCs (HC = 50%, OP = 47%), followed by Bacteroidetes (HC = 35, OP = 39%), Proteobacteria (HC = 7.7%, OP = 8.4%), and Actinobacteria (HC = 6.2%, OP = 5.5%). Similarly, at the class level, Clostridia had the highest percentage in both OP patients and HCs (HC = 45%, OP = 44%), followed by Bacteroidia (HC = 35.6%, OP = 39%), Gam-

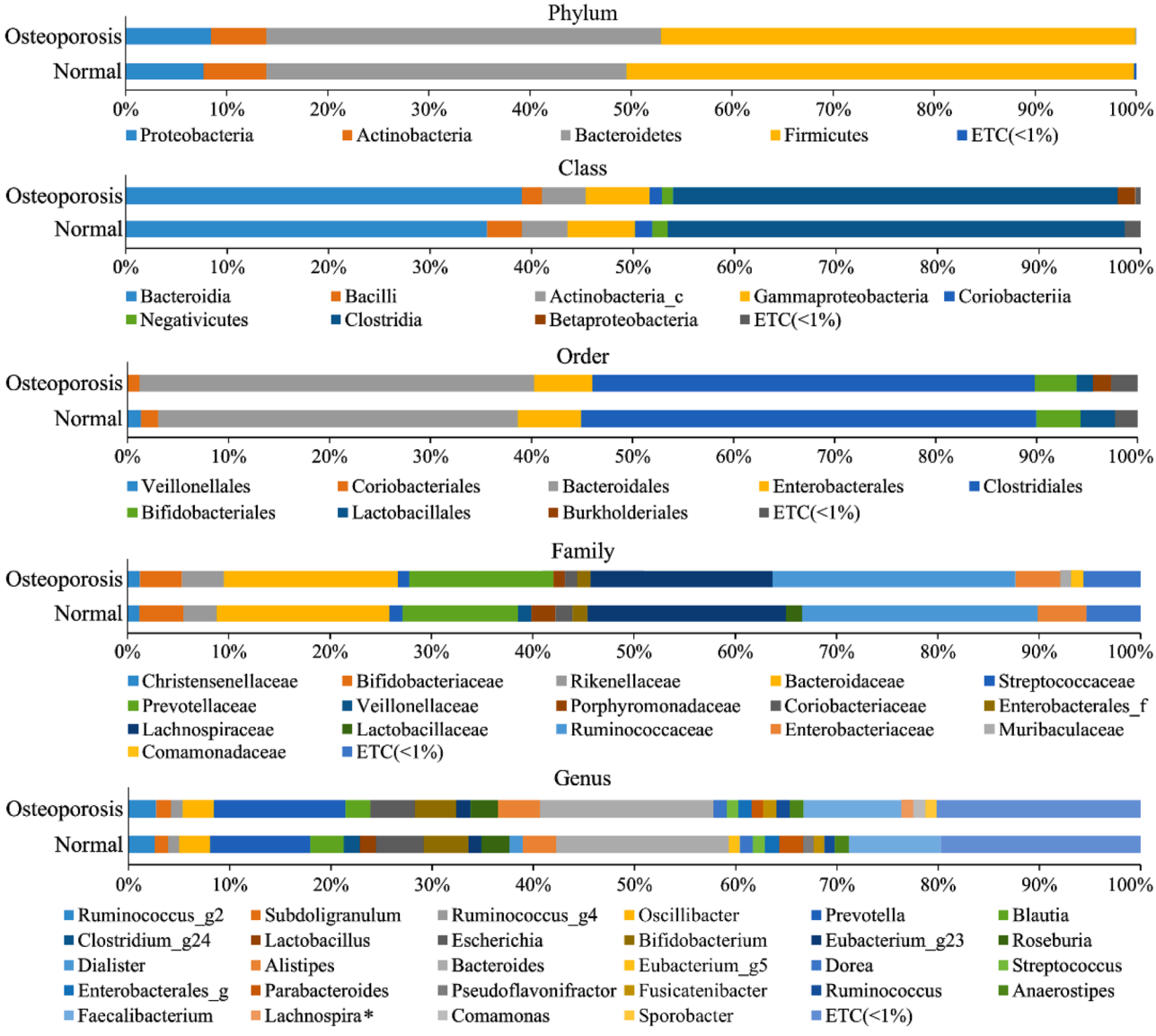

Fig. 2. Average taxonomic compositions of healthy controls (normal) and osteoporosis patient groups. The normal group and osteoporosis (OP) patients were further classified at the phylum,-class, order, family, and genus levels. Those with relative abundances less than 1% were expressed as ETC. Only the *Lachnospira* genus showed a significant difference between the two groups among taxa of all ranks. Statistical significance between groups was analyzed using the Wilcoxon rank-sum test. *$p < 0.05$.

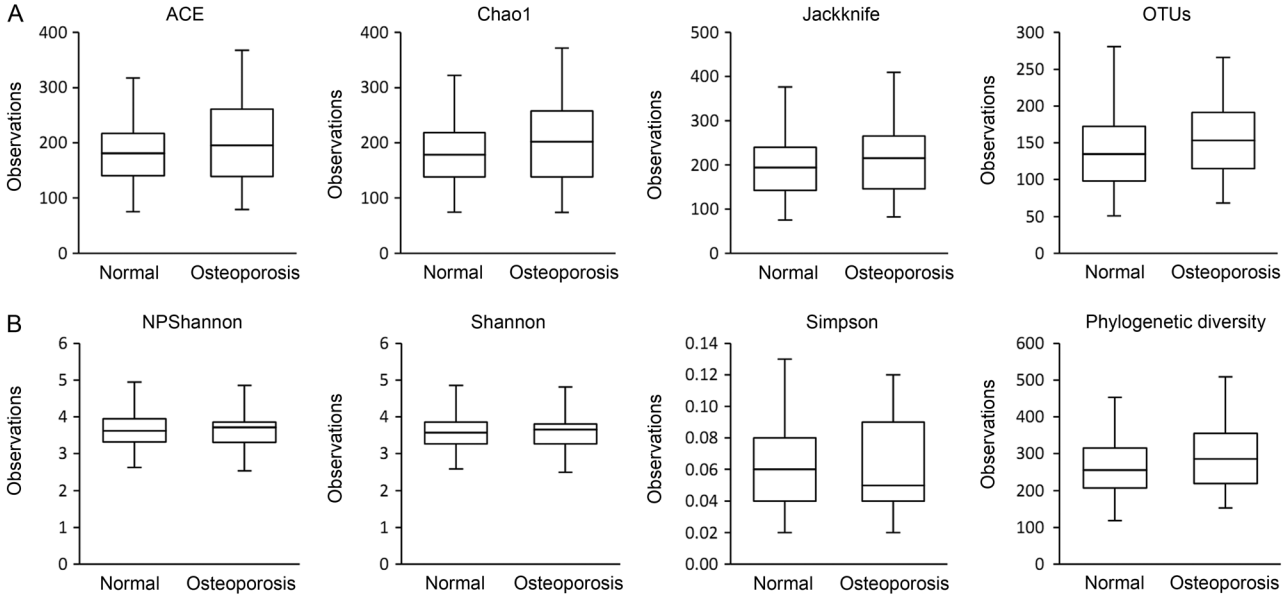

Fig. 3. Alpha diversity indices for stool samples of healthy controls (normal) and osteoporosis (OP) patients. A) Species richness was analyzed with Ace, Chao1, Jackknife, OTUs, and B) Species diversity was analyzed with NPShannon, Shannon, Simpson, and Phylogenetic diversity. The horizontal thick black band represents the median value, and boxplot margins indicate the first and third quartiles. There was no significant difference between the two groups in any analysis results.

maproteobacteria (HC = 6.6%, OP = 6.3%), Actinobacteria (Class) (HC = 4.5%, OP = 4.3%), and Bacilli (HC = 3.4%, OP = 2%). At the order level, Clostridiales (HC = 45%, OP = 43.8%), Bacteroidales (HC = 35.6%, OP = 39%), Enterobacterales (HC = 6.3%, OP = 5.8%), and Bifidobacterailes (HC = 4.4%, OP = 4.1%) were abundant in HC and OP groups. Furthermore, at the family level, *Ruminococcaceae* (HC = 23.3%, OP = 24%) had the highest percentage in abundance, followed by *Lachnospiraceae* (HC = 19.6%, OP = 17.9%), *Bacteroidaceae* (HC = 17.1%, OP = 17.2%), and *Prevotellaceae* (HC = 11.4%, OP = 14.2%). Different genera also showed varied abundance in the two groups of subjects. The five most popular genera in both study groups were *Bacteroides* (HC = 17.05%, OP = 17.7%), *Prevotella* (HC = 9.91%, OP = 13.07%), *Faecalibacterium* (HC = 9.09%, OP = 9.72%), *Escherichia* (HC = 4.75%, OP = 4.41%), and *Bifidobacterium* (HC = 4.38%, OP = 4.12%). The rest of the genera were in lower abundance (Table SI). Our data showed that the genus *Lachnosipra* had a significantly higher abundance in OP patients than in HCs according to ranks of all taxa.

Alpha diversity analysis. To determine the alpha diversity index for HC and OP patients' stool samples, we performed multiple statistical analyses (Fig. 3). The species richness was analyzed with Ace, Chao1, Jackknife, and OTUs (Fig. 3A). The species diversity was analyzed with NPShannon, Shannon, Simpson, and Phylogenetic diversity (Fig. 3B). We found that differences between the HC and OP groups were not statistically significant in any analysis. Hence, our data indicated that both groups do not differ in species load.

Variations of microbiota in OP Patients and HCs. Our beta set-significance analysis by Jensen-Shannon, Bray-Curtis, Generalized UniFrac, and UniFrac revealed no differences between OP patients and HC at the genera level (Table I). Changes in microbiota between HC and OP patients were also investigated using principal coordinate analysis (PCoA) (Fig. 4). PCoA plots were based on Jensen-Shannon divergence (Fig. 4A), Bray-Curtis (Fig. 4B), generalized UniFrac (Fig. 4C), and uniFrac (Fig. 4D) in two dimensions. Furthermore, OP patients and HCs were categorized individually according to cluster analysis based on the unweighted pair group method with arithmetic means (UPGMA) hierarchical clustering analysis (Fig. 5), including analysis by Jensen-Shannon (Fig. 5A), Bray-Curtis (Fig. 5B), generalized UniFrac (Fig. 5C), and UniFrac (Fig. 5D). UPGMA analysis resulted in no characteristic distinction between OP patients and HCs.

Taxonomic biomarker discovery. The results of Kruskal-Wallis *H* tests and LEfSe analysis showed that

Table I
Results of beta set-significance analysis.

Pair-wise	Species	Genus
Jensen-Shannon	N.S. ($p = 0.725$)	N.S. ($p = 0.796$)
Bray-Curtis	N.S. ($p = 0.463$)	N.S. ($p = 0.173$)
Generalized UniFrac	N.S. ($p = 0.616$)	N.S. ($p = 0.631$)
UniFrac	N.S. ($p = 0.757$)	N.S. ($p = 0.732$)

Permutational multivariate analysis of variance (PERMANOVA) was used to determine the beta set significance between osteoporosis (OP) and the normal group (HC).

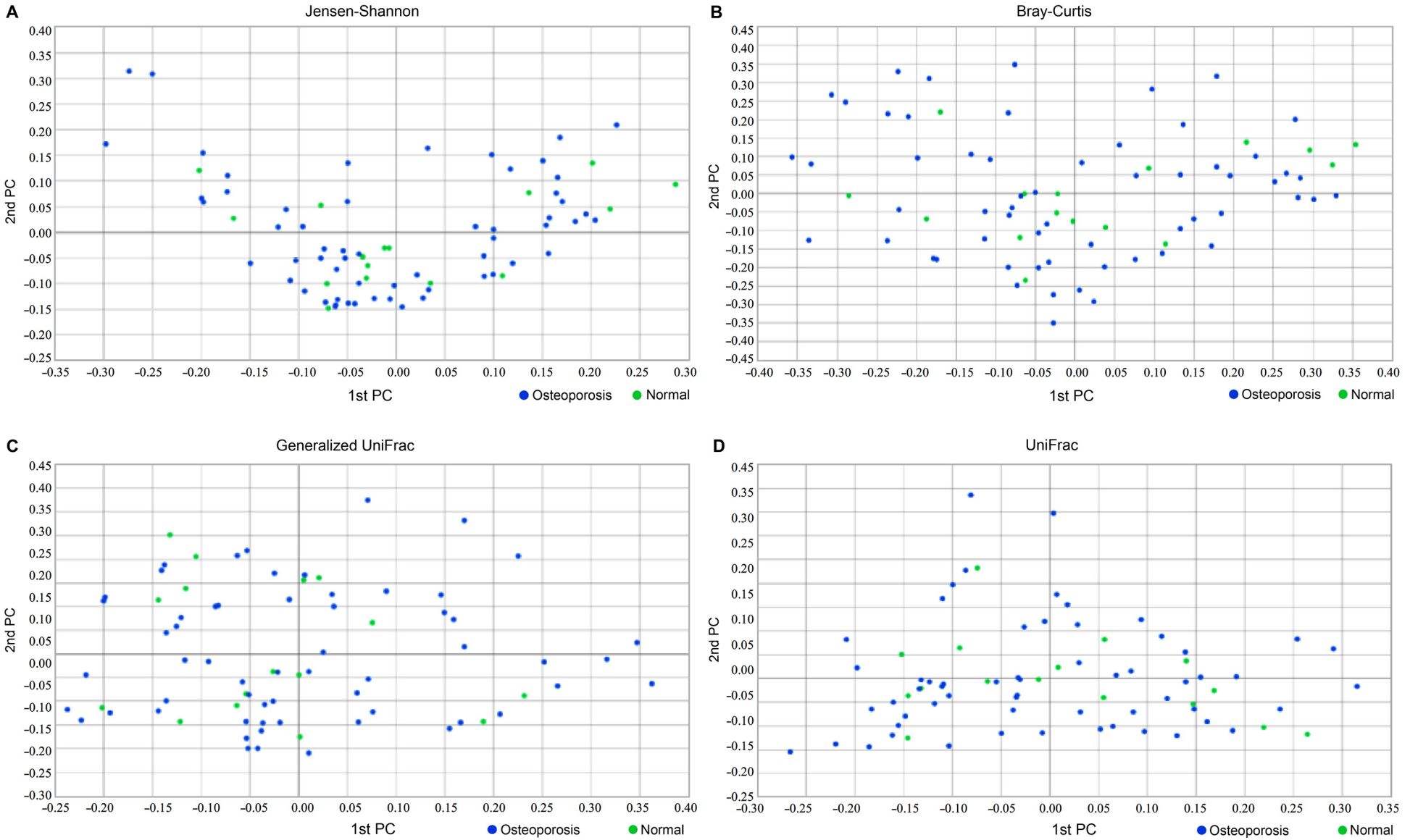

Fig. 4. Principal coordinate analysis (PCoA) of bacterial communities. Clustering using the Unweighted Pair Group Method with Arithmetic mean (UPGMA). Healthy controls (normal) and osteoporosis (OP) patients were analyzed by A) Jensen-Shannon, B) Bray-Curtis, C) Generalized UniFrac, and D) UniFrac.

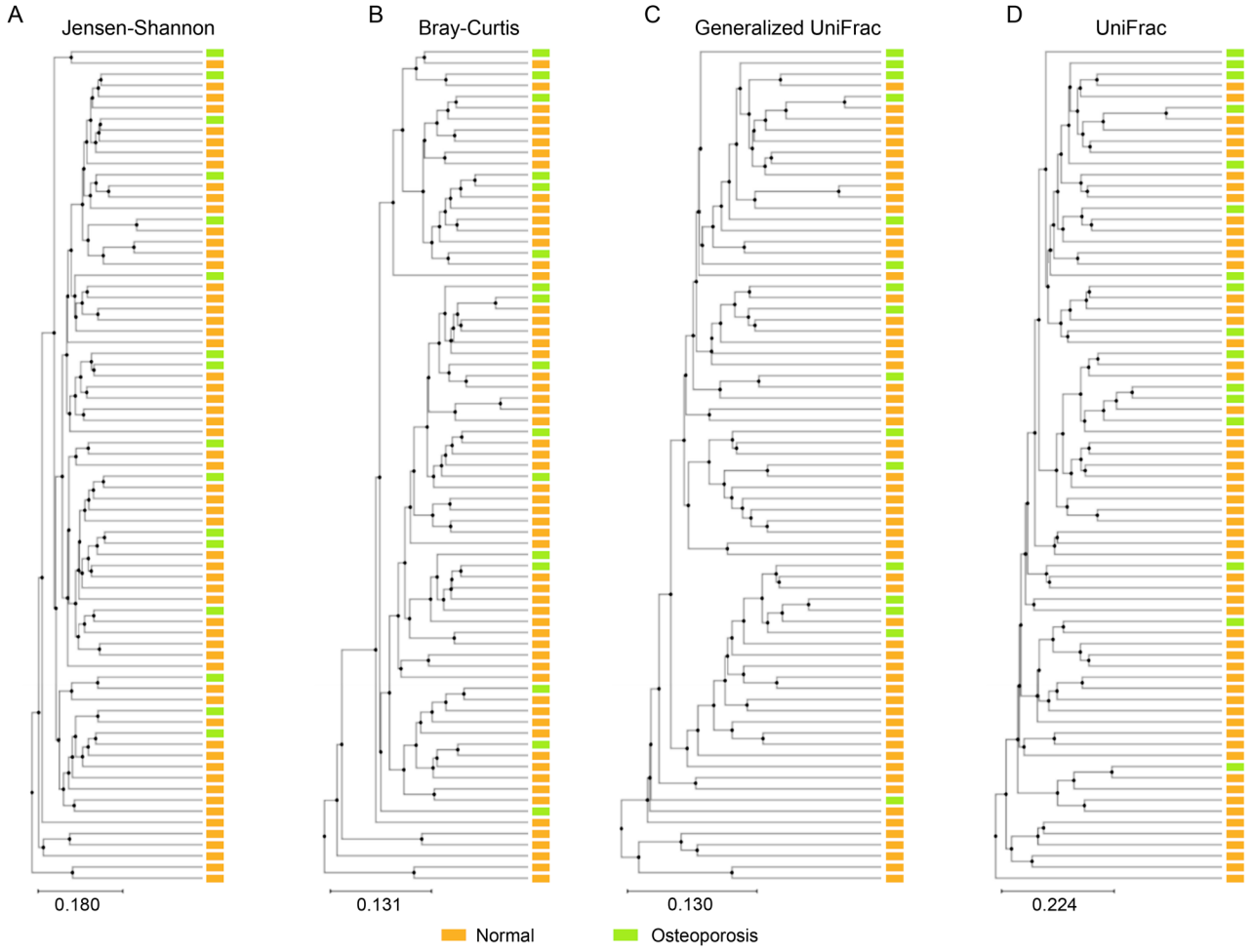

Fig. 5. Clustering using the Unweighted Pair Group Method with Arithmetic mean (UPGMA). Healthy controls (normal) and osteoporosis (OP) patients were analyzed by A) Jensen-Shannon, B) Bray-Curtis, C) Generalized UniFrac, and D) UniFrac.

one order, two families, and six genera were significantly different between the two groups (Fig. 6). The taxonomic groups with $p < 0.05$ and linear discriminant analysis (LDA) effect size > 2 are presented here. Distributions of order Micrococcales (HC = 0.02%, OP = 0.1%), family *Micrococcaceae* (HC = 0.02%, OP = 0.07%), family *Bacillaceae* (HC = 0.02%, OP = 0.06%), genus *Lachnospira* (HC = 0.74%, OP = 1.13%), genus *Solibacillus* (HC = 0.00%, OP = 0.20%), genus *PAC000195_g* (HC = 0.19%, OP = 0.30%), genus *PAC000741_g* (HC = 0.01%, OP = 0.06%), genus *PAC001435_g* (HC = 0.01%, OP = 0.04%), and genus *PAC001231_g* (HC = 0.02%, OP = 0.02%) were increased in OP patients compared to HCs. Our data showed that the *Lachnospira* and *Solibacillus* genera had LDA effect sizes exceeding three (3.26565 and 3.037, respectively). Among them, the *Lachnospira* genus had the highest LDA effect size. It was the only one that showed a significant change among taxa of all ranks.

To find out the relative abundance of *Lachnospira* in OP patients and HCs, we performed a percentage taxonomical abundance test and real-time PCR analysis (Fig. 7). After analyzing the relative taxonomic

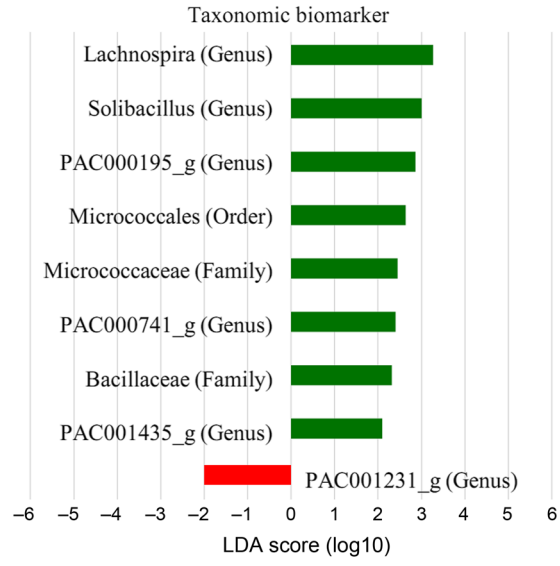

Fig. 6. Distinct taxa identified in healthy controls (normal) and osteoporosis (OP) patients using LEfSe (Linear discriminant analysis Effect Size) analysis. Taxonomic variations with linear discriminant analysis (LDA) scores greater than 2 and significance at $\alpha < 0.05$ as determined by the Kruskal-Wallis test are presented here. The raw data of the above analysis results are presented in Table SIII.

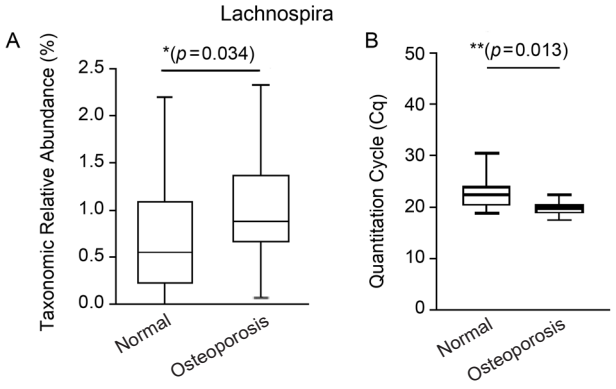

Fig. 7. The taxonomic abundance of the *Lachnospira* genus. Among taxa of all ranks, only the *Lachnospira* genus showed a significant difference in abundance between the two groups.

A) Among 16S gene-based metagenomics analysis results, the relative taxonomic abundance of the *Lachnospira* genus was analyzed, and the Wilcoxon rank-sum test was used for statistical significance, B) this result was verified by real-time PCR. Unpaired Student's *t*-test was applied for statistical significance. The quantification cycle (Cq) value of the osteoporosis (OP) group was lower than that of the normal (HC) group, confirming that the osteoporosis (OP) group contained more *Lachnospira* than the normal group (HC). * $p < 0.05$; ** $p < 0.01$

abundance of the *Lachnospira* genus based on 16S rRNA amplicon sequencing results, it was found that OP patients were significantly rich in *Lachnospira* ($p = 0.034$) (Fig. 7A). Furthermore, qPCR results confirmed the higher abundance of the genus *Lachnospira* in OP patients than in HCs (Fig. 7B). So, these data indicate that the genus *Lachnospira* can be a candidate for taxonomic biomarker discovery of OP.

Functional pathway prediction. To investigate the possible functions of gut microbiota found in this investigation, PICRUST was used to identify KEGG functional pathways. Eleven KEGG pathways were projected to change between the osteoporosis and control groups, as illustrated in Fig. 8. HC had functionally ten improved pathways related to peptidoglycan maturation, purine metabolism, geranyl diphosphate biosynthesis, mevalonate pathway, PCO (photorespiratory carbon oxidation) cycle, glycerol degradation pathway, nicotinate pathway, L-valine degradation, creatinine degradation, and biphenyl degradation when compared OP. In contrast, the OP group had elevated pyrimidine biosynthesis than HCs ($p < 0.05$).

Discussion

The gut microbiota has been identified as a critical factor in several bone-related diseases like gout (Guo et al. 2016; Chu et al. 2021; Lin et al. 2021) and osteoporosis (Wang et al. 2017; Xu et al. 2017; Palacios-González et al. 2020; Rettedal et al. 2021; Wei et al. 2021a). Changes in gut microbiota have been linked to bone homeostasis and bone tissue quality (Sjögren et al. 2012; D'Amelio and Sassi 2018; Cheng et al. 2020; Ni et al. 2021). However, the precise link between gut microbiome composition and osteoporosis is unknown. In this work, the 16S rRNA gene sequencing method was employed to characterize gut microbiota compositions of OP and HC in the Korean population.

Representative indices for microbial richness were studied to explore the relationships between microbial compositions and OP risk in South Korean people. Our

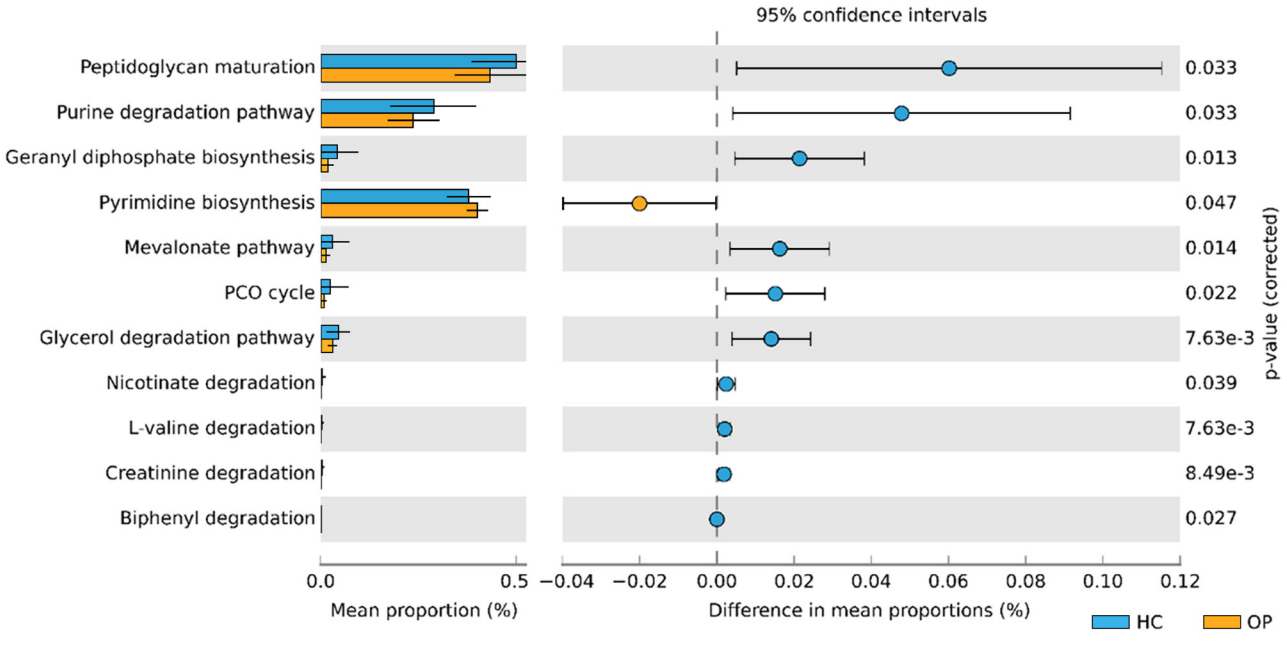

Fig. 8. Functional differences between OP and HC groups. A total of 11 metabolic pathways varied between the two groups. Tests were conducted at Kyoto Encyclopedia of Genes and Genomes (KEGG) using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUST) and MetaCyc webserver. PCO, photorespiratory carbon oxidation.

data showed no differences in the average taxonomic composition of OP and HC groups at higher taxa (phylum, class, order, and family) levels. However, only the *Lachnospira* genus was significantly higher among taxa of all ranks in the OP group. Several studies have investigated the relationship between gut microbiota based on taxa and OP proportionate abundances, yielding inconclusive results. Previous studies (Xu et al. 2020; Wei et al. 2021b) have reported increased phylum Bacteroidetes and genera *Bacteroides* in OP patients, while others (Wang et al. 2017) have shown a reduced population of phylum Bacteroidetes in OP patients. However, our data showed a slightly increased population of phylum Bacteroidetes in OP patients, although the increase was not statistically significant (HC = 35.63% vs. OP = 39.04%). Many Gram-negative bacteria of phylum Bacteroidetes have lipopolysaccharide (LPS) in their outer membrane (Eckburg et al. 2005). LPS-induced inflammation is reported to promote osteoclast and bone destruction (Abu-Amer et al. 1997; Zou and Bar-Shavit 2002). One cohort research has found that the relative abundance of the *Lachnospira* genus is increased in those with low bone marrow density (low-BMD) (Palacios-González et al. 2020), consistent with our findings.

Our data showed no significant differences in species richness or diversity between OP and HC groups. This data is consistent with earlier research showing no change in Simpson or Shannon diversity based on the same 16S rRNA sequencing to identify community variations between OP patients and HCs (Das et al. 2019; Xu et al. 2020). One research has found variations in alpha diversity between HCs and OP patients (Wang et al. 2017). However, they studied only six subjects in each group. Thus, their conclusions should be cautiously considered (Wang et al. 2017). So, HC and OP groups can probably not be differentiated based on alpha and beta diversity analysis. Using the LEfse analysis, we identified some taxonomic differences between OP patients and HCs at order, family, and genus levels. After removing possible confounders, our data showed increased abundances for order Micrococcales, families *Micrococcaceae* and *Bacillaceae*, and genera *Lachnospira*, *Solibacillus*, *PAC000195_g*, *PAC000741_g*, and *PAC001435_g* might be linked to an increased risk of OP.

The *Lachnospira* genus is a prominent member of the *Lachnospiraceae* family. *Lachnospira* bacteria are anaerobic, fermentative, and chemoorganotrophic like other family members. Some species of *Lachnospira* have significant hydrolyzing enzymatic activities (Vacca et al. 2020). Furthermore, based on diet intake data and gastrointestinal OTUs, *Lachnospira* was favorably linked to vegetables, fiber consumption, and potassium intake. In contrast, it showed a negative relationship between an omnivorous diet and cholesterol (Di Iorio et al. 2019; De Angelis et al. 2020; Vacca et al. 2020). Naderpoor et al. (2019), in their clinical trials studies, have shown that the vitamin D dose group has a higher population of *Lachnospira* than the control groups. Whisner et al. (2018) have found that the *Lachnospiraceae* family and *Lachnospira* genus are important taxa in college students reporting moderate-to-vigorous physical activity. On the contrary, *Lachnospira* spp. is significantly more abundant in female subjects with obesity and obesity plus metabolic syndrome than in male subjects (Chávez-Carbajal et al. 2019). Based on previous studies, the exact role of the *Lachnospira* genus in different study groups remains unclear. However, our findings revealed that the population of genus *Lachnospira* increased significantly in OP patients. The role of other distinctively prevalent taxa (order Micrococcales, families *Micrococcaceae* and *Bacillaceae*, and genera *Solibacillus*, *PAC000195_g*, *PAC000741_g*, and *PAC001435_g*) in LEfse analysis were not linked to osteoporosis before.

The functional prediction data indicated that several KEGG pathways might play a role in osteoporosis pathogenesis. In our data, the peptidoglycan maturation showed the highest effect size of the other pathways. Many studies have established that peptidoglycan enhances osteoclastogenesis and bone resorption and synergizes osteoclast differentiation with LPS (Kishimoto et al. 2012; Kwon et al. 2021; Ozaki et al. 2021). Some studies indicate that peptidoglycan helps in the upregulation of bone density, facilitating osteoblast differentiation, and diminishing osteoclastogenesis by reducing the RANKL (receptor activator of NF-kB ligand)/OPG (osteoprotegerin) ratio (Sato et al. 2012; Ishida et al. 2015; Chaves de Souza et al. 2016). Our data shows the increased purine degradation pathway in the HC group, but purine metabolism is usually coupled with gout disease. However, some studies indicate that purines (ATP) regulate bone and cartilage metabolism as ATP increases intracellular Ca^{2+} (Yu and Ferrier 1993; 1994; Hoebertz et al. 2003) to facilitate the formation of osteoclast. Geranyl diphosphate and farnesyl pyrophosphate are necessary for protein prenylation and are produced by the mevalonate system. The increased protein prenylation promotes bone resorption rather than creation (Choi et al. 2010; Agabiti et al. 2017; Hasan et al. 2018). Our prediction showed the increased geranyl diphosphate and mevalonate system in HC, which contradicts previous studies for unknown reasons. Valine is a critical metabolic regulator of hematopoietic stem cell (HSCs) or bone marrow cell maintenance (Wilkinson et al. 2018), and Nakauchi (2017) demonstrated that dietary valine restriction emptied the mouse bone marrow niche within two weeks. A study by Huh et al. (2015) showed that creatinine is independently associated with low bone mineral density, affirming our prediction. We could not find the reasons for the elevation of other metabolisms (pyrimidine biosynthesis, PCO cycle,

glycerol degradation pathway, nicotine degradation, and biphenyl degradation) and their role in bone health.

We attempted to develop a flawless study. However, some limitations remained. Firstly, the sample size of OP patients was not large enough. Specially, we obtained only 16 OP patients and 60 HCs. Secondly, OP is more common in postmenopausal females than in males. It is the primary cause of OP (Manolagas 2010). However, we did not analyze the differences between OP males and OP females separately in the present study due to fewer OP patient samples. Furthermore, all participants were from Bucheon city and nearby areas. Because these patients came from a confined area, geographical and climatic parameter variations were minimal. Thus, our findings require confirmations from other locations. Moreover, this study did not perform metabolomics assays to determine the organic compounds involved in the metabolism. Finally, the 16S rRNA sequencing study showed insufficient depth for species identification. The weaknesses above must be addressed further by a future whole-genome sequencing (WGS) study. In addition, some studies indicate the relation between the oral microbiome and osteoporosis (Contaldo et al. 2020; 2021). So, a future study may correlate oral dysbiosis, gut dysbiosis, and osteoporosis. Despite these limitations, our findings provide essential information for the gut microbiota of Korean OP patients. They will have clinical significance for clinicians. However, these findings can be coupled with more precise and accurate techniques like whole genome sequencing and animal model studies.

Conclusions

Our data shows that a 16S rRNA amplicon sequencing study based on stool samples of OP patients can be used as a new diagnostic parameter for OP. Furthermore, OP patients and HC groups showed differences at the genera level, with OP patients showing a higher population of *Lachnospira*. Thus, *Lachnospira* might play an essential role in OP.

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Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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