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Gut Microbiota Composition and Fecal Short-Chain Fatty Acids in Osteopenia and Osteoporosis: A Pilot Cross-Sectional Study

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ABSTRACT

Background & Objective: Osteoporosis represents a metabolic bone disease distinguished by a decrease in bone density and deterioration of the skeletal framework. Some gut microbiota (GM) products lead to pH reduction, promote calcium absorption, and can play an important role in maintaining bone health. This pilot cross-sectional study explores the bacterial community composition in patients with osteoporosis and osteopenia compared to healthy controls.

Methods: Serum biochemical parameters, bone mineral density, fecal short-chain fatty acid composition, and gut bacterial patterns were measured based on 16S rRNA gene sequencing.

Results: Thirty-six subjects were involved (eight osteoporosis, eight osteopenia, and 20 healthy controls). *Firmicutes, Bacteroidetes, Proteobacteria*, and *Actinobacteria* constituted the four dominant phyla in all samples. Alpha diversity indices decreased from the control group to the patients but it was not statistically significant among the groups (p-value> 0.05). Intestinal enterotypes, *Bacteroides 2* were the highest in osteopenia patients while *Bacteroides 1* was higher in the healthy controls. The genus of *Faecalibacterium* had the highest relative abundance in the three groups. An exploratory difference in the relative abundance of *Butyricicoccus* was observed; however, adjusted p-values could not be independently verified.

Conclusion: This pilot study provides preliminary data on GM and SCFA profiles in relation to bone health. The observed findings are exploratory and hypothesis-generating and should be interpreted with caution given the small sample size and study limitations. Larger, adequately powered prospective studies are needed to validate these preliminary observations and to further investigate the relationship between gut microbiota, SCFAs, and bone health.

KEYWORDS Gut microbiota composition, Diversity analysis, Short-chain fatty acids (SCFAs), Osteoporosis and Osteopenia, Intestinal microbiome enterotypes

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INTRODUCTION

Osteoporosis-related fracture risk is substantial in the Western world: approximately 50 out of every 100 women and about 20 out of every 100 men are at risk (1). The incidence of osteoporosis in Iran is 22% for women and about 11% for men (2), and fractures in the elderly are connected to notable rates of mortality and morbidity. Twin and family studies have shown that there is a hereditary element as well in the decline of bone density related to age, although environmental factors appear to exert a comparatively more marked influence in this latter process (3, 4). The process of bone remodeling that results in the

deterioration of bone is characterized by an overall disparity between the creation of new bone tissue and the breakdown of existing bone material. Numerous factors have the potential to exacerbate this imbalance and hasten the loss of bone density. Among these factors, body mass, age, postmenopausal status, and genetic variables are particularly influential (5). The significance of the intestinal bacterial community (gut microbiota) in both wellness and illness has been extensively researched in recent times. GM is pivotal in maintaining homeostasis, modulating the immune system, and regulating metabolic and neurobehavioral pathways (6). Disruptions in this delicate ecosystem have been linked to a wide range of conditions, including obesity, diabetes, inflammatory bowel diseases, and mental health disorders such as depression and anxiety (7). Groundbreaking studies have highlighted the clinical importance of GM, particularly its role in obesity and microbial dysbiosis in type 2 diabetes. Furthermore, fecal transplantation research has demonstrated the causative role of GM in weight regulation, providing valuable insights into its therapeutic potential (8). This bacterial community is made up of a vast number of bacteria, estimated to be in the range of trillions, that collectively possess around 150 times as many genes as what is present in the human genome (9). Although functioning as an independent entity, it has undergone parallel development with the human genome and can be categorized as a multi-cellular organ that engages in communication and influences its host through various means. Additionally, it is subject to regulation by multiple factors such as dietary intake, administration of antibiotics, comorbidities, level of physical activity, genetics, delivery method, and environmental factors (10).

Emerging evidence suggests that gut dysbiosis may contribute to the pathophysiology of osteoporosis through inflammatory processes. Dysbiosis can increase gut permeability, leading to systemic inflammation via the translocation of microbial endotoxins (e.g., lipopolysaccharides) into circulation (11, 12). This inflammatory state influences osteoclastogenesis, promoting bone resorption and reducing bone formation. The gut-bone axis represents a bidirectional relationship, where changes in gut microbiota (GM), mediated by factors such as diet, antibiotics, and host genetics, affect skeletal health through various mechanisms (13). These mechanisms include modulation of the immune system, endocrine signaling, and production of metabolites like short-chain fatty acids (SCFAs), which play a role in calcium absorption and bone mineralization (14). The GM through non-digestible oligosaccharides and dietary fiber fermentation produces SCFAs, which lead to pH reduction and enhance mineral solubility in the distal gastrointestinal tract. The low pH promotes calcium absorption and elevates the calcium content of the bone so it can play an important role in bone health maintenance (15). Additionally, Alterations in specific microbial taxa have been linked to low bone mineral density, highlighting the potential role of gut microbiota (GM) as a therapeutic target for osteoporosis, while calcium's role in bone health extends beyond absorption by participating in bone remodeling through the modulation of osteoblast and osteoclast activity. The elevation in calcium levels can directly impact bone mineral density and reduce the risk of conditions like osteoporosis, with the interplay between pH, calcium absorption, and bone health involving several other factors, such as vitamin D, parathyroid hormone, and osteoprotegerin (16).

The current study explores the bacterial community composition in patients with osteoporosis and osteopenia compared to healthy controls based on 16S rRNA sequencing. This pilot study may provide preliminary data on GM and SCFA profiles in relation to bone health and may contribute to future research investigating the relationship between the gut microbiota and skeletal health.

MATERIALS AND METHODS

Ethics, setting, and study design

The current study was performed according to the ethical rules of the Helsinki Declaration and the study protocol is approved by the ethical committee of the National Institute for Medical Research Development of Iran (IR.PII.REC.1395.52). Participants were recruited from Amir al-Momenin Hospital in Tehran, Iran, based on predefined inclusion and exclusion criteria. Each participant provided written informed consent. This study was a prespecified secondary analysis of stored samples collected under the original approved protocol, which investigated changes in the abundance of fecal Akkermansia muciniphila in patients with osteoporosis and osteopenia. (17). The samples were stored at -80°C and subsequently analyzed for microbiome composition using the next-generation sequencing method.

Study population

A total of 102 individuals were approached for participation in the study, of whom 82 met the inclusion criteria. Of these, 9 declined to participate and 73 provided consent, which provided consent participants include 51 patients and 22 healthy controls. Due to incomplete data or other confounding conditions, 35 patients and duo to lost to follow-up 2 healthy controls were excluded. The final samples include 20 healthy controls and 16 patients, which patient group was categorized as follows: eight with osteoporosis and eight with osteopenia. The details of the participant selection process are presented in Figure 1. We excluded patients taking antibiotics over the past three months, a BMI greater than 35 kg/m2 or less than 18.5 kg/m2, diabetes, chronic inflammatory bowel diseases, hypertension, autoimmune diseases, hyperlipidemia, and cancer, or specific physiological conditions like pregnancy. Participants with a BMI of less than 18.5 were excluded from the study to reduce confounding factors associated with underweight individuals and their distinct GM composition. Previous studies have demonstrated that low BMI is independently associated with altered gut microbial diversity, systemic inflammation, and a higher risk of osteoporosis, which could confound the interpretation of the relationship between GM and bone health in our study population (18). Cases were identified based on bone mineral density (BMD) measurements using dual-energy X-ray absorptiometry (DXA), with osteoporosis and osteopenia defined according to World Health Organization (WHO) criteria (19). Controls were individuals with normal BMD. No randomization was performed, as the study aimed to compare GM composition between these predefined groups. Cases (osteoporosis and osteopenia) were identified during routine clinical evaluations and referred for BMD measurements. Healthy controls were also recruited from individuals attending the hospital for general health check-ups, ensuring they met the inclusion criteria for normal BMD and the absence of other confounding conditions.

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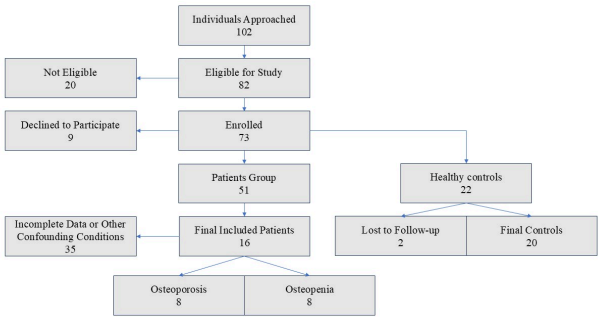


Fig 1. The flow chart shows the details of the participant selection process.

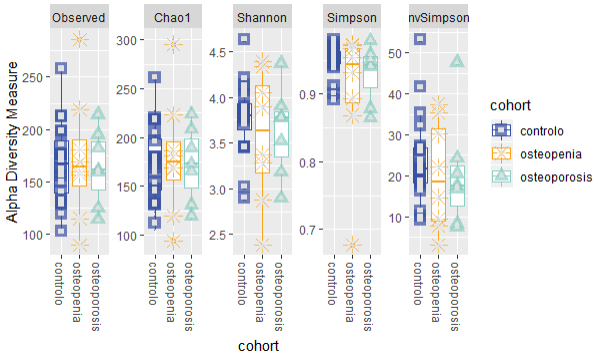


Fig 2. Comparison of alpha-diversity indices in subjects with osteoporosis, osteopenia, and healthy controls.

Blood samples and serum biomarkers

Venous blood samples (10 mL) were collected from each participant following an overnight fast of at least 8 hours. Blood was drawn into EDTA-coated and serum separator tubes and immediately stored on ice. Samples were centrifuged at 3,000×g for 10 minutes at 4°C to separate plasma or serum. The following parameters were analyzed: fasting blood sugar (FBS), triglyceride, cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), and vitamin D_3 using standardized and validated laboratory methods. All assays were performed at the clinical laboratory of Amir al-Momenin Hospital, adhering to strict quality control procedures.

Bone mineral density

Dual-energy X-ray absorptiometry (DXA) was utilized to measure BMD at the total hip and lumbar vertebrae. All measurements were performed using the Hologic Discovery QDR system (Hologic Inc., Bedford, MA, USA), a widely recognized and validated device for bone density assessments in clinical and research settings. Osteoporosis was defined as a BMD T-score lower than −2.5, and osteopenia was defined as a BMD T-score between −1 and −2.5, in accordance with the World Health Organization (WHO) criteria. The coefficient of variation (CV) for BMD measurements in our laboratory was 1.2% for the lumbar spine and 1.5% for the femoral neck, as determined through routine quality assurance procedures, including repeated scans of a phantom and healthy volunteers. These values reflect the precision and reliability of the DXA scanner used in this study (20, 21).

Fecal sample collection and DNA extraction

Fresh fecal specimens were collected using sterile containers from all participants and were transported to the laboratory in ice packs. The microbial genome was extracted using the Power Microbiome RNA Isolation Kit (MOBIO Laboratories Inc., Germany) according to the manufacturer's instructions following repeated bead-beating and heating to increase DNA yield. The analysis of DNA extraction quality and concentration was performed using agarose gel electrophoresis and NanoDrop ND-8000 from Thermo Scientific, based in the United States.

Gut Microbiota Profiling

515F/806R primer set designed to specifically target the V4 hypervariable region of 16S rRNA genes was used for amplification and it has covered gut bacterial diversity. 16S rRNA sequencing was performed utilizing the Illumina MiSeq platform at the Nucleomics core, KU Leuven, Belgium. Raw reads were subjected to quality filtering using LotuS and DADA2 pipelines and taxonomy assignment was performed with the RDP classifier v 2.12. After the exclusion of reads categorized as Chloroplast, mitochondria, and unclassified bacteria, the dataset was standardized to 10,000 reads for each sample. Measures of alpha diversity were computed utilizing the *vegan* and *phyloseq* R packages. The alpha-diversity measures microorganism diversity within one sample. Here, two alpha-diversity indices were applied at the operational taxonomic unit (OTU) level: observed Richness (number of OTUs) and Simpson diversity index (includes also relative abundance). Beta-diversity (distance between samples) was measured using the Bray–Curtis dissimilarity index. All further assessments were performed at the genus level. Enterotyping was performed in R using the DMM approach. With the aim of improving precision, enterotyping was done on a merged genus-abundance matrix that incorporated study and disease cohort samples, along with an extra set of 1,106 samples sourced from the FGGP.

Short-Chain Fatty Acids measurement

SCFAs including acetate, propionate, and butyrate were quantified using gas chromatography. A stool specimen weighing 100 milligrams was dissolved in a single milliliter of a sodium chloride solution with a concentration of 36%. 2-ethylbutyric acid as an internal standard (Merck, München, Germany) was added and the samples were homogenized using glass beads. After the addition of 150 µL of H2SO4 96%, the extraction of SCFAs was conducted using 3 mL of ether. Subsequently, the collected ether layer underwent the drying process with Na2SO4. The ensuing supernatant was subjected to analysis through gas chromatography utilizing flame ionization detection (Agilent, Santa Clara, California, USA). The gas chromatography system was equipped with a DB FFAP analytical column (30m x 0.53 mm ID, 1.0 µm; Agilent). Helium GC grade was employed as the carrier gas, maintaining a constant flow rate of 4.2 ml/min. Initially, the oven temperature was maintained at 100 °C for a duration of 3 min. It was then gradually increased at a rate of 4 °C/min, reaching 140 °C where it remained isothermal for 5 min. Subsequently, the temperature was further increased at a rate of 40 °C/min until it reached 235 °C, where it remained isothermal for 15 min. The quantification of SCFAs was achieved by

Table 1. Characteristics of the study participants (Results are presented as mean ± SD).

	Osteoporosis (N=8)	Osteopenia (N=8)	Healthy control (N=20)	p-value
Age	61.71 ± 13.62	45 ±15.52	45.05 ± 7.46	0.02
BMI	24.63 ± 3.95	25.27 ± 3.20	27.41 ± 4.16	0.35
T-score	-3.02 ± 0.66	-1.62 ± 0.24	-0.07 ± 0.70	<0.01
Z-score	-2.38 ± 0.94	-1.39 ± 0.23	0.04 ± 0.70	<0.01

N: Number, BMI: Body mass index, Note: p-value < 0.05 was considered statistically significant.

implementing appropriate calibration curves derived from the utilization of internal standard measures.

Statistical analysis

Statistical analyses were conducted utilizing R version 3.5.0 and SPSS software. Continuous variables are presented as mean ± standard deviation (SD), while categorical variables are expressed as frequency and percentage. The normality of the distribution of variables was assessed through the Kolmogorov-Smirnov test. The chi-square test was employed to ascertain associations between categorical variables. A comparison of quantitative variables among three groups was performed using the Kruskal-Wallis test. Differences in alpha diversity indices and relative abundance of taxa among groups were evaluated using the Wilcoxon rank-sum test, Kruskal Wallis test, and DESeq test. The Benjamini-Hochberg false discovery rate adjustment was used for multiple testing corrections.

RESULTS

Demographic characteristics

In the current study, a total of 36 samples including 20 samples of healthy individuals, eight samples of osteopenia, and eight samples of osteoporosis were examined. All participants were selected from Tehran Iran, where the climate is generally temperate. The socioeconomic status of targeted participants was mostly moderate and their education was undergraduate level or below. The mean age of osteoporosis, osteopenia individuals, and healthy controls was significantly different (osteoporosis: 61.71 ± 13.62 vs osteopenia: 45 ± 15.52 vs healthy controls: 45.05 ± 7.46; p-Value= 0.02). The baseline BMI in the patient group was similar to the control group (osteoporosis: 24.63 ± 3.95 vs osteopenia: 25.27 ± 3.20 vs healthy controls: 27.41 ± 4.16; p-Value= 0.35). Two osteopenia and three healthy controls were male (13.88% of all participants). There were significant differences in terms of T-score (osteoporosis: -3.021 ± 0.66 vs osteopenia: -1.62 ± 0.24 vs healthy controls: -0.072 ± 0.70; p-Value< 0.01) and Z-score among groups (osteoporosis: -2.38 ± 0.94 vs osteopenia: -1.39 ± 0.23 vs healthy controls: 0.049 ± 0.70; p-Value< 0.01). Patient characteristics and clinical data of each group are presented in Table 1. Blood sample analysis of subjects with osteoporosis, osteopenia, and healthy individuals was shown in Table 2. The serum biochemical parameters of the healthy subjects did not show any significant differences from the disease state (p-Value> 0.05).

Short-chain fatty acid analysis

The mean concentration of total SCFA levels was higher among osteopenia followed by osteoporosis and the healthy control groups (osteopenia: 136.30 ± 41.85 vs osteoporosis: 120.35 ± 45.26 vs healthy controls: 115.23 ±36.31; p-Value > 0.05). Also, the osteopenia group showed the highest level of acetate, propionate, and, butyrate concentration. The healthy control group had the lowest level of acetate concentration, while the lowest levels of propionate and butyrate were observed among the osteoporosis group (p-Value > 0.05). The amounts of short-chain fatty acids in each group are shown in Table 3.

Diversity analysis of gut microbiota

To determine alpha diversity, we calculated the Observed, Chao, Shannon, Simpson, and Inv Simpson reciprocal indices. This process allowed us to fully characterize the bacterial community diversity in all samples which are shown in Figure 2. Although alpha diversity indices decreased from the control group to patients, it was not statistically significant among groups (p-Value > 0.05).

Gut microbiota enterotypes

Intestinal enterotypes, Bacteroides 2 was the highest in osteopenia patients while its proportion was low in the control group. Bacteroides 1 was higher in the healthy controls in comparison to the patients. The differences in intestinal enterotypes were observed between the studied groups which are shown in Figure 3. Although, these differences were not

Table 2. Serum biochemical parameters of the study participants (Results are presented as mean ± SD).

	Osteoporosis (N=8)	Osteopenia (N=8)	Healthy control (N=20)	p-value
FBS (mg/dL)	84.00 ± 9.28	80.20 ± 9	86.5± 23.60	0.64
TG (mg/dL)	102 ± 31.70	89.10 ± 33.70	110 ± 41.60	0.46
Chol (mg/dL)	180 ± 29.60	196 ± 15.40	172 ± 38.30	0.21
HDL (mg/dL)	65.50 ± 39.0	50.50 ± 7	47.09 ± 8.86	0.19
LDL (mg/dL)	115 ± 22.70	125 ± 15.70	93.04 ± 36	0.21
VLDL (mg/dL)	22.62 ± 9.10	19 ± 8	22.90 ± 90.90	0.58
Vitamin D3 (ng/mL)	45.91 ± 21.5	25.90 ± 11.40	31.01 ± 14.50	0.06

N: Number, FBS: fasting blood sugar, TG: Triglyceride, Chol: Cholesterol, HDL: High-density lipoprotein, LDL: Low-density lipoprotein, VLDL: Very low-density lipoprotein, Note: p-value < 0.05 was considered statistically significant

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Table 3. Short-chain fatty acids concentration in the stool samples of the study participants (Results are presented as mean ± SD).

	Osteoporosis (N=8)	Osteopenia (N=8)	Healthy control (N=20)	p-value
Acetate	83.41± 28.44	88.17 ± 22.36	76.27 ± 21.49	0.51
Propionate	18.91 ± 10.36	23.74 ± 16.15	19.28 ± 8.68	0.87
Butyrate	18.03 ± 9.14	24.38 ± 8.85	19.67 ±8.88	0.44
Total SCFAs	120.35 ± 45.26	136.30 ± 41.85	115.23 ±36.31	0.71

N: Number, SCFA: Short chain fatty acids, Note: p-value < 0.05 was considered statistically significant.

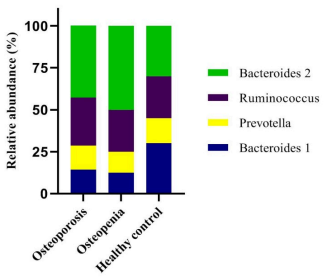


Fig 3. Proportion of intestinal enterotypes in subjects with osteoporosis, osteopenia, and healthy controls.

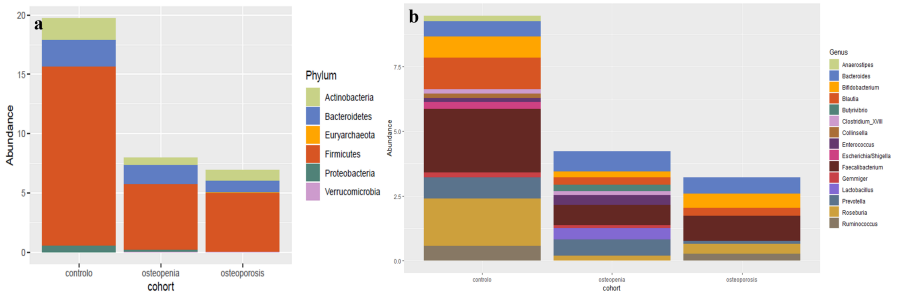


Fig 4. Mean relative abundance of, phyla (a) and genera (b) in subjects with osteoporosis, osteopenia, and healthy controls.

statistically significant between the studied groups (p-Value> 0.05).

On Spearman's rank analysis across intestinal enterotypes with stool SCFAs concentration, a negative correlation was found between B*acteroides1, Ruminococcus,* and *Prevotella* with all three SCFAs concentrations (r= -0.50 for acetate, propionate, and butyrate vs. *Bacteroides1* and *Prevotella*, and r= -0.86 for acetate, propionate, and butyrate vs. *Ruminococcus*) while a positive correlation was found between *Bacteroides 2* and all SCFAs proportion (r= 0.50 for acetate, propionate, and butyrate). The correlations between intestinal enterotypes and stool SCFAs concentration were not statistically significant (*p*-Value> 0.05). Because none of the correlations reached statistical significance (all *p*-Value> 0.05), they should not be interpreted as evidence of a significant association between intestinal enterotypes and fecal SCFA concentrations.

Gut bacterial composition

At the phylum level illustrated in Figure 4-a, Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria constituted the four dominant phyla in all samples. The relative abundance of different genera is presented in Figure 4-b. The genus of Faecalibacteriumhad the highest relative abundance in three groups. Only the abundance of Butyricicoccus was significantly different in healthy control compared to osteopenia (p-Value: 0.02) and osteoporosis (p-Value: 0.03) groups. The supplementary file presents the details of analysis across the groups.

DISCUSSION

Evidence shows that GM regulates bone homeostasis and affects bone formation and resorption (22). We characterized GM patterns in osteoporosis, osteopenia, and healthy controls and found significant differences in one genus between these three groups. A relationship between decreased taxonomic diversity and disease state has been found in many diseases such as inflammatory bowel disease and non-alcoholic fatty liver disease (23, 24). The GM diversity and composition also have a significant association with bone mass (25). Our results show that the alpha diversity decreases in osteoporosis and osteopenia compared to controls. Comparable to our observation, Jihan Wang *et al.* has been documented that alpha diversity was inversely correlated with BMD (26). In a cohort study conducted by Mrinmoy Das *et al.*, they showed a significant correlation between GM composition and bone density reduction (27). Because information regarding multiple-testing correction and adjusted p-values was unavailable for independent verification, this finding should be considered exploratory and interpreted with caution.

In the present pilot study, we characterized GM patterns in participants with osteoporosis, osteopenia, and healthy controls and observed a difference in the relative abundance of one genus (Butyricicoccus) among the study groups. However, because details regarding multiple-testing correction were unavailable, this finding should be considered exploratory. In addition, alpha diversity tended to decrease in the osteoporosis and osteopenia groups compared with healthy controls. Similar associations between gut microbiota diversity and bone mineral density have been reported in previous studies (25–27), although our findings require confirmation in larger, adequately powered cohorts.

SCFAs, produced by GM during dietary fiber fermentation, influence bone remodeling by promoting osteogenesis and reducing inflammation. Specifically, acetate and propionate upregulate osteogenic markers such as alkaline phosphatase (ALP), while butyrate suppresses osteoclast activity, enhances bone regeneration, and balances Treg/Th17 cell ratios. Reduced levels of SCFAs, often associated with gut dysbiosis, are linked to chronic inflammation and the activation of inflammatory pathways like NF-κB, contributing to bone loss in conditions such as osteoporosis and osteopenia (28). In our study, we observed a higher SCFA levels in individuals with osteopenia but not osteoporosis. This is the outcome from our study, and we acknowledge that the underlying reasons remain unclear. We

suggest that further research is needed to explore and clarify the mechanisms behind this observation.

The metabolites originating from the microorganisms in the gastrointestinal tract may exert positive or negative effects on the endocrine cells within the gastrointestinal tract, the permeability of the gastrointestinal tract, the immune system, and the enteric nervous system (29). The endocrine cells, primarily known as enteroendocrine cells (EECs), play a crucial role in sensing microbial metabolites and regulating various physiological functions. Key types of EECs include L cells, which secrete glucagon-like peptide-1 (GLP-1) and peptide YY (PYY); K cells, which produce gastric inhibitory peptide (GIP); G cells that release gastrin; D cells that secrete somatostatin; and enterochromaffin cells that produce serotonin (30). These cells are integral to maintaining metabolic homeostasis, and alterations in their function due to microbial metabolites can have significant implications for metabolic diseases (31). For instance, studies indicate that SCFAs derived from microbial fermentation stimulate EECs to secrete hormones like GLP-1 and PYY, which are essential for regulating glucose metabolism and appetite (32).

In the current study, the frequency of *Bacteroides* enterotype in patients and *Prevotella* enterotype in the control group was higher, as expected serum cholesterol and LDL levels were higher in the patients than control group. The association between enterotypes and lower LDL levels was shown in individuals with *Prevotella* entropy than in other enterotypes (33). The Enterotypes were first described by Arumugam *et al.* in 2011, and contain three groups: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2), and *Ruminococcus* (enterotype 3), they are directly related to diet (34). *Bacteroides* are divided into *Bacteroides 1* and *Bacteroides 2*, associated with various diseases (35). Our data shows the frequency of *Bacteroides 2* was higher in the osteoporosis and osteopenia group accompanied by SCFA concentrations, the opposite result was observed in the *Bacteroides 1*. Studies show that the *Bacteroides* enterotype is associated with the Western diet and the *Prevotella* enterotype is associated with a simple carbohydrate-rich diet (36). Numerous studies have shown that the ratio of *Prevotella* and *Bacteroides* is altered in disease conditions compared to healthy individuals. Finally, the effect of diet on human intestinal enterotypes may be an important environmental factor in the pathogenesis of various diseases that are observed in industrialized countries (37). Thus, enterotypes can become a biomarker as an intestinal factor, although further studies and interventions are needed to evaluate their utility (38).

Analysis of SCFAs in patients and healthy groups revealed an increase in patients with osteoporosis and osteopenia, however, these increases were not significant compared to the control group. Recent *in vivo* studies have shown that SCFAs modulate bone metabolism and ultimately decrease bone resorption. This protective effect of SCFAs on bone health is associated with osteoclast inhibition, not bone formation (39). However, it should be noted that in animal studies all conditions are under control and it cannot be generalized to clinical studies, moreover, our study was the first study in Iran that determined the fecal concentration of SCFAs in osteoporosis patients and healthy controls. It seems that the balanced levels of these compounds in the intestine have beneficial effects and excessive levels may impair the metabolism of osteoclasts. To our knowledge, data regarding GM composition and fecal SCFA profiles in individuals with osteoporosis and osteopenia from the Iranian population remain limited. Therefore, this pilot study provides preliminary data that may contribute to the growing body of evidence in this field.

Two general mechanisms have been reported for the GM and bone health association: Immuno-regulatory effect and osteoclast/osteoblast bone remodeling. The reports suggested that resident gut microbes have an immunomodulatory effect and consequently cause bone health through up-regulating toll-like receptor-2 signaling in the liver known as Gut-Liver-Bone Axis (40). Studies showed that the administration of probiotics (*Lactobacillus plantarum*) or prebiotics like inulin could improve bone health via insulin-like growth factor 1 induction (41). The probiotic treatment also showed decreased TNF-α and interleukin-1β production and inhibited osteoclastogenesis in the mice model (42). Given the importance of using probiotics in the prevention of osteoporosis and considering the microbiota as an important factor contributing to several disease mechanisms, the microbiome diversity and microbiome-derived metabolite analysis is valuable as fundamental research to determine the bacterial pattern and relative frequency in a particular community. It will be supportive for future studies in the probiotic or post-biotic design for preventing or treating osteoporosis.

LIMITATIONS

This study has several limitations that should be acknowledged. First, our study is a pilot study with low statistical power and small sample size. The study population was not matched in terms of sex, age, and BMI. One important limitation of this study is the significant age difference observed among the study groups. Participants in the osteoporosis group were older than those in the osteopenia and healthy control groups. Since age is independently associated with both bone mineral density and gut microbiota composition, residual confounding cannot be excluded. Furthermore, the relatively small sample size limited the feasibility and statistical reliability of multivariable adjusted analyses. Therefore, the observed associations should be interpreted cautiously and require confirmation in larger studies with appropriate adjustment for age, sex, BMI, and other potential confounders. Cohort and case-control studies are needed to support and optimize the data found in this study and determine the appropriate candidate for probiotics or prebiotics to prevent or treatment of osteoporosis and osteopenia. Second, we did not collect detailed data on participants' dietary data and calcium intake or their use of antiosteoporotic medications, both of which could influence bone health and GM composition. An additional limitation of this study is the incomplete availability of the original microbiome sequencing records. Raw sequencing metadata, sequencing quality-control information (including extraction and PCR controls), read-depth information before and after filtering, exact rarefaction parameters, and details regarding the original differential-abundance analysis, including adjusted p-values after multiple-testing correction, were not available for independent verification. These limitations should be addressed in future research to strengthen the validity and applicability of the findings.

CONCLUSION

This pilot cross-sectional study provides preliminary data on GM composition and SCFA profiles in individuals with osteoporosis, osteopenia, and healthy controls in Tehran, Iran. *Firmicutes, Bacteroidetes, Proteobacteria,* and *Actinobacteria* were the predominant bacterial phyla across all groups, and *Faecalibacterium* was the most abundant genus. An observed difference in the relative abundance of *Butyricicoccus* was identified between healthy controls and participants with osteopenia or osteoporosis. The mean concentration of total SCFAs level was higher among osteopenia. Also, the osteopenia group showed the highest level of acetate, propionate, and, butyrate concentration. Alpha diversity indices decreased from controls to patients. Bacteroides 2 enterotype was highest in osteopenia while Bacteroides 1 enterotype was higher in controls. These differences were not statistically significant and should therefore be interpreted with caution.

Given the pilot design, the small sample size, the age imbalance among study groups, and the absence of adjustment for important potential confounding factors, these findings are hypothesis-generating rather than confirmatory. Larger, adequately powered prospective studies with comprehensive clinical characterization and well-documented microbiome analytical pipelines are needed to validate these preliminary observations and to further investigate the relationship between GM, SCFAs, and bone health.

SUPPLEMENTARY INFORMATION

Supplementary File 1.pdf The results of the analysis in the control group *vs* osteoporosis and osteopenia groups.

ABBREVIATIONS

BMD: Bone mineral density
GM: Gut microbiota
HDL: High-density lipoprotein
LDL: Low-density lipoprotein
OTU: Operational taxonomic unit
SCFA: Short-chain fatty acids
TNF-α: Tumor necrosis factor-alpha
VLDL: Very low-density lipoprotein

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Conflict of interest

All authors have no conflicts of interest.

Data Availability

The data that support the findings of this study are not publicly available due to privacy reasons but are available from the corresponding authors (S.D.S., S.K.A.R., and M.S.) upon reasonable request.

Ethics Approval

The study protocol was approved by the ethical committee of the National Institute for Medical Research Development of Iran (ID: IR.PII.REC.1395.52). A written informed consent was obtained from all patients. Consent for publication was not applicable because no identifiable individual data are presented. Clinical trial number: Not applicable.

Authors' Contributions

S.K.A.R., S.S., and M.S. performed the experiments, sampling, and DNA extraction wrote the paper, prepared figures and tables; H.E., A.M., and M.S. 16S rRNA sequencing, SCFAs chromatography, data analysis and reviewed and edited drafts of the paper; A.T., S.S.S. and M.R. serum preparation and biochemical test and wrote original drafts of the paper; S.K., D.D., A.F., and M.Z. conceived and designed the experiments, reviewed drafts of the paper; S.D.S., S.K.A.R., and M.S. supervised the research, reviewed drafts of the paper. M.R., M.S., and A. M. are equally co-first authors.

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Declaration of AI Use

The authors declare that no generative AI or AI-assisted technologies were used in the writing process of this manuscript

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