



Association between gut microbiota, bone metabolism, and fracture risk in postmenopausal Japanese women

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Abstract

Introduction We investigated the relationship between gut microbiota composition and osteoporosis/fracture risk in Japanese postmenopausal women using 16S rRNA gene sequencing, FRAX, bone mineral density, biochemical bone parameters, and a self-administered questionnaire. Variation in abundance of specific microbiota was found to be significantly associated with fracture risk and vitamin K levels.

Gut microbiota data with respect to bone metabolism and fracture risk is limited. Vitamin K is produced by certain intestinal bacteria and has been reported to play a role in maintaining bone quality.

Purpose We investigated relationships among gut microbiota composition, bone metabolism, and fracture risk in postmenopausal Japanese women.

Methods Bone mineral density (BMD) was evaluated in 38 postmenopausal women (mean age 62.9 years) using forearm dual-energy X-ray absorptiometry. We collected and analyzed serum bone turnover markers (vitamin K fraction and tartrate-resistant acid phosphatase 5b; TRACP-5b), gut microbiota profiling (16S rRNA gene sequencing), and self-administered questionnaire data, including fracture history and vitamin K intake. Vitamin K2, BMD, and TRACP-5b data were divided into high- and low-level groups using cutoff values of 0.06 ng/mL, 87.05%, and 420 mU/dL, respectively; the proportions of bacteria were analyzed. Fracture incidence and relative risk were investigated for each bacterium.

Results The genus *Bacteroides* was predominant in the high vitamin K2 group (29.73% vs 21.58%, $P = 0.022$). Fracture incidence was significantly higher in the low *Bacteroides* group, with a 5.6-times higher risk ratio of fracture history.

The family *Rikenellaceae* was more abundant in the low BMD group and more abundant in the high TRACP-5b group (2.15% vs 0.82%, $P = 0.004$; 2.38% vs 1.12%, $P = 0.013$, respectively).

Conclusion *Bacteroides* and *Rikenellaceae* may be involved in bone metabolism and fracture risk. Further investigations of the underlying microbiota-related pathways in bone metabolism may reveal treatment strategies, and facilitate the prevention of osteoporosis.

Keywords Bone metabolism · Fracture · Gut microbiota · Osteoporosis · Vitamin K

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Introduction

In recent years, the Japanese population has been rapidly aging with the elderly population aged 65 years and above accounting for more than 27.7% of the total population. Thus, the incidence of osteoporosis is steadily increasing. The prevalence of osteoporosis in Japanese patients older than 40 years was reported to be 3.4% in men and 19.2% in women, in addition to 12.4% and 26.5% in men and women with an osteoporotic lumbar vertebral L2–L4 region, respectively [1]. The estimated number of patients with osteoporosis nationwide has exceeded 13 million, particularly in women. In postmenopausal women, osteoporosis progresses due to

increased bone resorption due to estrogen deficiency and decreased bone density. Osteoporosis is widely considered a major risk factor for fractures. Osteoporosis-related fractures, such as fractures of the vertebral body, forearm, and proximal femur, are thus more likely to occur [2]. The Fracture Risk Assessment Tool (FRAX®) has been developed by the World Health Organization to assess the 10-year probability of major osteoporotic fracture in patients at risk of hip fractures and major osteoporotic fractures by evaluating bone mineral density (BMD) and other risk factors relating to BMD and lifestyle [3]. The Japanese practice guidelines on osteoporosis consider fracture risk $\geq 15\%$ as the criterion for initiating pharmaceutical treatment. Hip fracture increases mortality risk by 10 to 20% within 1 year after the fracture. Functional status of patients with hip fracture progressively worsens in 60% of these patients compared with functional status before the fracture. In addition, vertebral fractures contribute to increased risk of mortality even among those detected using radiography. Among the Japanese osteoporotic population, hip fracture occurs in 130,000 of these patients annually; of these, 20,000 patients die and 60,000 experience functional decline [4]; thus, prevention has become an important issue not only in terms of medical treatment but also for society at large.

The human microbiome consists of an estimated 100 trillion microbes, and the intestines are host to approximately 1000 different bacterial species [5, 6]. This intestinal bacterial population is collectively referred to as the gut microbiota.

The human gut microbiota comprises 4 major co-existing phyla, including Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. These phyla represent over 90% of the gut microbiota [7].

The gut microbiota maintains intestinal homeostasis via a complex mechanism. Even if this homeostasis is somewhat disturbed by various stimuli, such as stress, aging, or other external factors, it has a strong tendency to return to the original condition. Moreover, oral ingestion of live beneficial bacteria (probiotics) has the potential to alter the gut microbiota. However, disruption of intestinal homeostasis (referred to as microbial dysbiosis), due to either host genetic predisposition or excessive influence of external environmental factors, can lead to loss of innate intestinal tract defense against infection. This can ultimately result in gastrointestinal disorders, such as inflammatory bowel disease (IBD) [8] and colorectal cancer [9], metabolic diseases, such as obesity [10] and diabetes mellitus [11], and various other disorders, such as depression [12], Parkinson's disease [13], and allergies [14]. This association between microbial dysbiosis and the above-mentioned diseases has been widely reported.

However, the effect of gut microbiota alteration in patients with osteoporosis remains unknown. Wang et al. analyzed the diversity of gut microbiota in patients with primary osteoporosis, osteopenia, and in normal controls. They found that in patients with osteoporosis, the proportion of Firmicutes was

increased compared with that of the normal control group, and the proportion of Bacteroidetes was reported to be significantly decreased in patients with osteoporosis [15].

In terms of classification, intestinal bacteria are subdivided into class, order, family, and genus from phyla represented by Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria (Fig. 1).

Even in the same phylum, each group such as family or genus has various functions.

For example, the Firmicutes family *Ruminococcaceae* consists of some butyric acid-producing bacteria such as *Faecalibacterium* and *Butyrivibrio*. Butyrate-producing bacteria are promising probiotic candidates targeting microbiota modulation in gastrointestinal disorders such as IBD [16]. Similarly, *Ruminococcus gnavus*, a member of the *Ruminococcaceae* family, has been reported to be implicated in infection following artificial hip joint replacement [17], liver abscess due to *Ruminococcus gnavus*, and infective endocarditis. In addition, reports have shown that *Ruminococcus* is involved in the development of cerebral and myocardial infarction [18]. Thus, bacteria with different functions constitute the same population in terms of classification.

This study was conducted to clarify the effects of changes in the gut microbiota, especially at the family and genus levels, in patients with osteoporosis. A better understanding of the roles of gut microbiota may lead to the development of new therapies for osteoporosis. Thus, we aimed to determine whether particular gut microbes are associated with osteoporosis by investigating and analyzing gut microbiota and bone metabolism in postmenopausal Japanese women.

Methods

Subjects

In this study, data were collected from 38 postmenopausal women (mean age 62.9 (range, 50–82) years) who visited the Musashiurawa Orthopedic and Internal Medicine Clinic for outpatient treatment from January 2017 to April 2017. All subjects provided informed consent to participate, and sufficient ethical consideration was given to avoid personal identification of each participant's data. Those with diabetes, malignant tumors, fracture in the acute phase (within 2 months), and IBD, or those who had undergone dialysis or had taken medication for osteoporosis, as well as warfarin, steroids, and antibiotics within 3 months before sample collection, were excluded. This was because these conditions and drugs could possibly affect bone metabolism and gut microbiota.

BMD was measured on the proximal 1/3 of the forearm using dual-energy X-ray absorptiometry (DCS 600EXV; Hitachi Aloka Medical, Tokyo, Japan). Bone density was

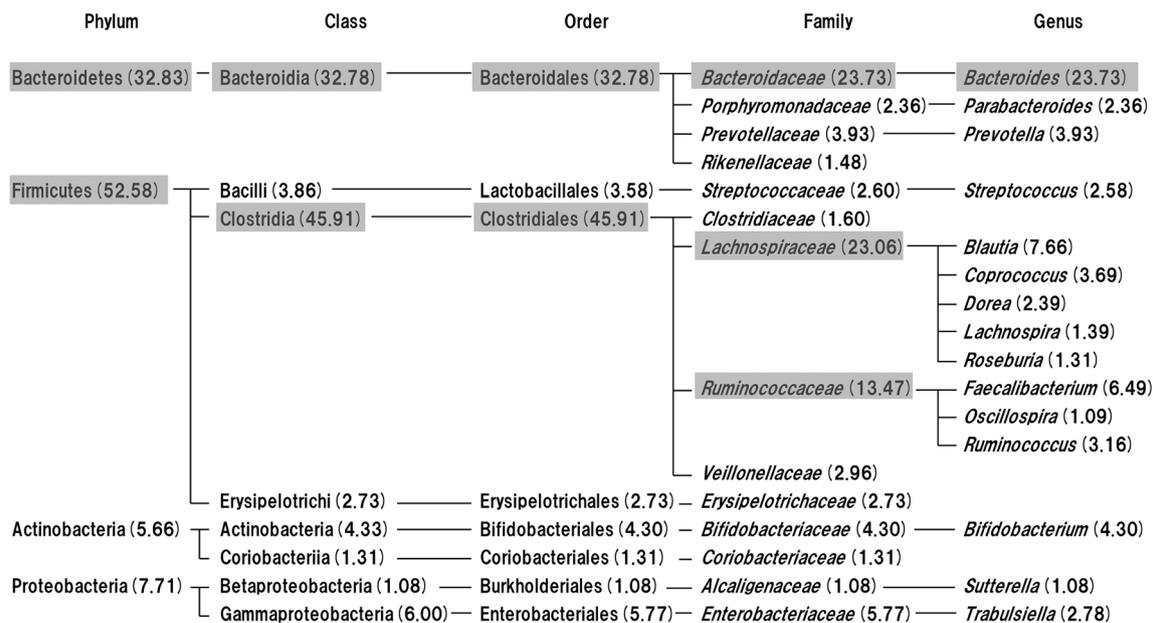


Fig. 1 Bacterial species targeted for statistical analysis. List of bacteria with their taxonomical classification (phylum-class-order-family-genus) with a mean value of > 1% ($n = 38$). Numbers are presented as averages

(%). Bacterial species highlighted in gray indicate bacterial species with mean value of $\geq 10\%$

determined based on the Young Adult Mean (YAM: 100% of mean value bone density at age 20–44 years) value.

Biochemical parameters

Due to the setup of the clinic, it was difficult to unify the timing of blood sampling, and so we opted to use tartrate-resistant acid phosphatase 5b (TRACP-5b) as the bone resorption marker, because it has less diurnal variation than the conventional carboxy-terminal collagen crosslinks [19]. Also, we used undercarboxylated osteocalcin (ucOC) as a measure of vitamin K deficiency; serum levels of vitamins K1, K2, 1α , 25-(OH) $_2$ vitamin D, alkaline phosphatase (ALP), calcium (Ca), and phosphorous (P) were measured as biochemical indices of bone metabolism.

Questionnaire survey

Menopausal age, fracture history, and FRAX score were recorded. To clarify the relationships with bacterial composition, we conducted a randomized questionnaire survey on exercise frequency, defecation frequency, frequency of alcohol intake, and dietary intake of grains, vegetables, yogurt, lactic acid bacteria beverages, and a fermented soybean product (natto), a rich source of vitamin K2 that is common in Japan. No dietary restrictions or lifestyle control measures were applied to the subjects during the study. Two groups were defined: a high intake group and a low intake group. Each group was surveyed about the number of meals per week (0, 1–3, 4–6, daily) and the frequency of natto intake.

Regarding fracture history, high-energy injuries such as those resulting from traffic accidents and falls from a height were excluded. Relationships between the questionnaire information and gut microbiota were analyzed statistically (Table 1).

Analysis of the gut microbiota: fecal sampling, DNA extraction, and sequencing

Fecal samples were collected using a brush-type collection kit containing guanidine thiocyanate solution (Feces Collection kit®; Techno Suruga Laboratory, Shizuoka, Japan) and stored at 4 °C until analysis. DNA was extracted from fecal samples using an automated DNA extraction machine (GENE PREP STAR PI-480; Kurabo Industries Ltd., Osaka, Japan) according to the manufacturer's instructions. The 16S ribosomal RNA (rRNA) regions (V1–V2) were amplified using a forward primer (16S_27Fmod: TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG AGR GTT TGA TYM TGG CTC AG) and reverse primer (16S_338R: GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GTG CTG CCT CCC GTA GGA GT) with KAPA HiFi HotStart ReadyMix PCR kit (Roche, Basel, Switzerland). To sequence 16S amplicons using the Illumina MiSeq platform (Illumina, San Diego, CA), dual index adapters were attached using the Nextera XT Index kit (Illumina). Each library was diluted to 5 ng/ μ L, and equal volume aliquots were mixed to generate a library pool of 4 nM each. The DNA concentration of the mixed libraries was quantified using qPCR with the KAPA SYBR FAST qPCR Master mix (KK4601, KAPA Biosystems, Wilmington, MA) using primer 1 (AAT GAT

Table 1 Relationship between questionnaire factors and gut microbiota

	<i>n</i>	<i>Bacteroides</i>	<i>Rikenellaceae</i>	<i>Lachnospiraceae</i>	<i>Blautia</i>	<i>Oscillospira</i>	<i>Ruminococcus</i>	<i>Veillonellaceae</i>	<i>Erysipelotrichaceae</i>	<i>Bifidobacterium</i>	<i>Sutterella</i>	
Alcohol (over 350-ml beer)	More than twice/month	16	17.75	1.55	25.51	7.77	1.03	3.06	3.22	4.03	4.96	1.31
	Less than twice/month	22	28.07	1.43	25.73	7.59	1.14	3.23	2.77	3.48	3.82	0.92
Exercise (more than 30 min)	<i>P</i> value		0.0054**	0.383	0.657	0.976	0.976	0.965	0.767	0.515	0.214	0.406
	More than once/week	17	24.41	0.95	29.76	10.40	1.01	3.17	2.94	2.79	1.79	0.81
Stool frequency	Less than once/day	21	23.17	1.91	22.30	5.44	1.16	3.15	2.97	4.46	6.33	1.30
	<i>P</i> value		0.965	0.150	0.0019**	0.0015**	0.780	0.953	0.714	0.067	0.0006***	0.190
Rice, bread, noodles, mochi	More than once/day	25	22.15	1.42	26.19	8.50	0.76	2.79	3.36	3.69	4.31	1.19
	Less than once/day	13	26.75	1.60	24.58	6.05	1.73	3.86	2.19	3.75	4.27	0.88
Unpurified cereals (unpolished rice, millet rice, bran)	<i>P</i> value		0.236	0.538	0.451	0.181	0.006**	0.317	0.317	0.770	0.559	0.331
	More than twice/day	23	26.22	1.71	25.87	7.56	0.91	3.10	1.90	3.59	4.03	1.14
Green and yellow vegetables	Less than once/week	15	19.90	1.13	25.28	7.82	1.38	3.24	4.59	3.90	4.71	0.99
	<i>P</i> value		0.038*	0.420	0.731	0.881	0.347	0.881	0.021*	0.823	0.858	0.799
Yogurt, lactic acid bacteria beverage	More than once/2-days	18	23.89	1.37	26.92	8.16	1.56	3.83	2.20	3.88	3.37	0.85
	Less than once/2-days	18	24.89	1.46	24.55	7.35	0.68	2.63	4.03	3.19	4.90	1.08
Unpurified cereals (unpolished rice, millet rice, bran)	<i>P</i> value		0.825	0.949	0.411	0.812	0.012*	0.342	0.133	0.728	0.071	0.427
	More than once/2-days	21	25.66	1.41	24.88	7.25	0.94	2.92	2.28	3.75	3.73	0.97
Yogurt, lactic acid bacteria beverage	Less than once/2-days	17	21.33	1.57	26.57	8.18	1.28	3.44	3.80	3.66	5.01	1.22
	<i>P</i> value		0.419	0.769	0.607	0.628	0.872	0.780	0.110	0.918	0.355	0.353
Unpurified cereals (unpolished rice, millet rice, bran)	More than once/2-days	26	23.99	1.64	25.69	7.64	1.09	3.02	2.78	3.89	4.30	1.02
	Less than once/2-days	12	23.16	1.13	25.52	7.72	1.10	3.45	3.34	3.32	4.30	1.22

Table 1 (continued)

	<i>n</i>	<i>Bacteroides</i>	<i>Rikenellaceae</i>	<i>Lachnospiraceae</i>	<i>Blautia</i>	<i>Oscillospira</i>	<i>Ruminococcus</i>	<i>Veillonellaceae</i>	<i>Erysipelotrichaceae</i>	<i>Bifidobacterium</i>	<i>Sutterella</i>
Less than once/2-days											
<i>P</i> value	0.753	0.717	0.753	0.802	0.362	0.672	0.722	0.615	0.925	0.571	
More than once/2-days	11	16.97	26.41	7.04	1.05	4.05	2.88	3.62	3.14	0.50	
Less than once/2-days											
<i>P</i> value	0.0079**	0.923	0.664	0.782	0.898	0.500	0.949	0.809	0.509	0.090	
	27	26.48	25.32	7.92	1.11	2.79	2.99	3.75	4.77	1.32	

**P* < 0.05

***P* < 0.01

****P* < 0.001

ACG GCG ACC ACC) and primer 2 (CAA GCA GAA GAC GGC ATA CGA). Library preparation was performed according to the 16S library preparation Illumina protocol (Illumina). Libraries were sequenced using the MiSeq Reagent Kit v2 (500 Cycles) for 250-bp paired-ends (Fig. 2).

Analysis of gut microbiota: taxonomy assignment based on the 16S rRNA gene sequence

The paired-end reads of partial 16S rRNA gene sequences were clustered by 97% nucleotide identity, and then assigned taxonomic information using the Greengenes database (v13.8) (Second Genome, South San Francisco, CA) [20] through the Quantitative Insights into Microbial Ecology (QIIME) pipeline (v1.8.0) (Knight Lab, University of Colorado, Boulder, CO) [21]. The steps for data processing and assignment based on the QIIME pipeline were as follows: (i) joining paired-end reads, (ii) quality filtering with an accuracy of Q30 (> 99.9%) and a read length of > 300 bp, (iii) random extraction of 10,000 reads per sample for subsequent analysis, (iv) clustering of operational taxonomic units (OTUs) with 97% identity by UCLUST (v1.2.22q) [22], and (v) assignment of taxonomic information to each OTU using RDP classifier [23] with the full-length 16S gene Greengenes data (v13.8) to determine the identity and composition of the bacterial genera. The analysis of the gut microbiota was contracted to Cykinso Inc., Tokyo, Japan.

From the results, the average of the 38 postmenopausal women was calculated at the phylum, class, order, family, and genus levels. Due to the exploratory nature of this study, only bacteria with an average proportion of 1% or more were subjected to statistical analysis, and bacteria with an average proportion of less than 1% were excluded (Fig. 1).

Grouping by measurements of clinical parameters

We divided age, menopausal age, BMD, fracture history, FRAX score, ucOC, TRACP-5b, vitamin K1, and vitamin K2 levels, into two groups as follows.

Age and BMD were divided based on the average values. This is because it was difficult to divide BMD based on the standard value for osteoporosis; only a few patients met the diagnostic criteria for osteoporosis after excluding those treated for osteoporosis during data collection. For menopausal age, the average value [24] for Japanese women (50.2 years) was used as the cutoff. Fracture history data was divided into groups with or without fracture history. For ucOC and TRACP-5b, the cutoffs of normal values in Japan were used. For vitamin K2, we used the lower limit of 0.06 ng/mL because the majority exceeded this value. In Japan, the recommended cutoff value of FRAX score is 15%, but this study had only 3 out of 38 patients, and so it was difficult to divide them into 2 groups. The cutoff value was then taken as the average

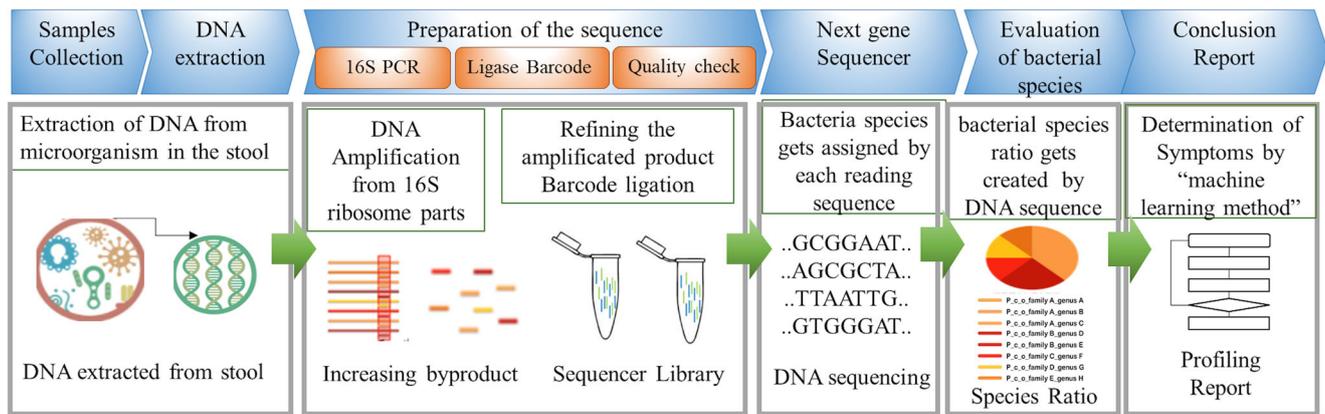


Fig. 2 Overview of fecal sample genetic profiling using the 16S rRNA gene to obtain a representation of gut microbiota composition

value. For natto, one intake was calculated as 40 g (vitamin K intake: 375.6 μg [25]), and the average intake of the subjects, 150 μg , was used as the cutoff value. From the results of the questionnaire, those who ate 40 g or more of natto 4 to 6 times a week were classified as the group with high frequency of natto intake, and those who ate natto 1 to 3 times a week were classified as the low frequency of natto intake group.

Statistical analysis

Comparisons were made to assess for significant differences in gut bacteria between groups. Statistical analysis was carried out using the Mann-Whitney *U* test and $P < 0.05$ was considered significant because more than half of the gut microbiota did not show a normal distribution. Fracture risk for each genus was determined from the two groups of gut microbiota and the fracture history, and odds ratios and relative risks were calculated. No correction for multiple testing was performed due to the exploratory nature of our study.

Results

The mean BMD was $87.05\% \pm 11.78\%$ for the YAM value and that for menopausal age was 51.79 ± 4.65 years. Bone metabolism markers (ucOC, TRACP-5b) and all assessed bone nutrients (vitamins D, K1, K2, Ca, and P) were within normal levels except for active vitamin D, which was slightly lower at 12.59 ± 4.59 pg/mL. FRAX score was $8.37\% \pm 3.95\%$ (Table 2). Among the 11 patients with a fracture history, 2 patients had fragility fracture (Fig. 3a). The questionnaire data showed that 17 of the 38 patients exercised at least once a week, 16 consumed alcohol more frequently, and 11 of them ate natto frequently (Table 1).

Composition of gut microbial community

The mean value for gut microbiota composition at the phylum level was $52.58\% \pm 9.87\%$ for Firmicutes, $32.83\% \pm 11.64\%$ for Bacteroidetes, $7.71\% \pm 9.16\%$ for Proteobacteria, and $5.66\% \pm 4.51\%$ for Actinobacteria (Fig. 3b).

Age

The patients were divided into groups aged ≥ 62.9 and < 62.9 years, ($n = 20$ and $n = 18$, respectively). The older group had significantly lower proportions of *Lachnospiraceae* and *Blautia* ($P = 0.047$, 0.002 , respectively) (Table 3).

Menopausal age

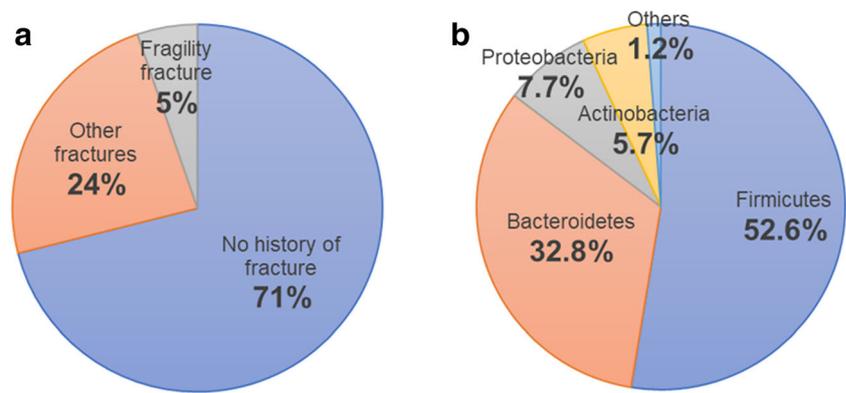
Age at menopause for Japanese women is 50.2 years, and so patients were divided into a high menopausal age ($n = 22$) and a low menopausal age group ($n = 16$). *Oscillospira* and *Ruminococcus* were significantly more abundant in the high

Table 2 Characteristics of the participants

Average value	
Age	62.87 \pm 6.22
Menopausal age	51.79 \pm 4.65
BMD (YAM: %)	87.05 \pm 11.78
ucOC (ng/ml)	4.38 \pm 2.16
TRACP (mU/dL)	371.45 \pm 126.77
Vitamin D (pg/mL)	12.59 \pm 4.59
Vitamin K1 (ng/mL)	1.08 \pm 0.75
VitaminK2 (ng/mL)	0.08 \pm 0.08
FRAX (%)	8.38 \pm 3.94

BMD, bone mineral density; ucOC, uncarboxylated osteocalcin; TRACP-5b, tartrate-resistant acid phosphatase 5b; ALP, alkaline phosphatase; FRAX, Fracture Risk Assessment

Fig. 3 (a) Fracture history of the 38 patients in this study. **b** Mean values of gut microbiota composition at the phylum level



menopausal age group ($P=0.018$, 0.014 , respectively). In contrast, *Erysipelotrichaceae* and *Bifidobacterium* were significantly abundant in the low menopausal age group ($P=0.011$, 0.014 , respectively) (Table 3).

BMD

BMD was compared between the group with higher than the average BMD (high BMD group) and the group with lower than the average BMD (low BMD group; $n=19$ each). *Rikenellaceae* was significantly abundant in the low BMD group ($P=0.004$) (Table 3).

Fracture history

Fracture history was observed in 11 of the 38 patients; 2 of these had fragility fractures. *Lachnospiraceae* was less abundant in the group with fracture history ($P=0.02$). Fracture history was not significantly affected by age (Table 3).

ucOC

The normal value for ucOC was set at <4.5 ng/mL, and thus ≥ 4.5 ng/mL was designated the high ucOC group and <4.5 ng/mL was the low ucOC group ($n=17$ and $n=11$, respectively). *Sutterella* was predominant in the high ucOC group ($P=0.044$) (Table 3).

TRACP-5b

Regarding TRACP-5b (normal value <420 mU/dL), patients were divided into a high TRACP-5b group and low TRACP-5b group with a cutoff value of 420 mU/dL ($n=11$ and $n=27$, respectively). *Rikenellaceae* and *Bifidobacterium* were predominant in the high TRACP-5b group ($P=0.013$, 0.016 , respectively) (Table 3).

Vitamin K2

The lower limit of blood vitamin K2 measurement was 0.06 ng/mL, and so this was used as the cutoff value based on which patients were divided into high vitamin K2 groups ($n=10$) and low vitamin K2 groups ($n=28$). *Bacteroides* and *Sutterella* were predominant in the high vitamin K2 group ($P=0.022$, 0.026 , respectively) (Table 3). In addition, there was no correlation between vitamin K2 and natto intake.

FRAX

The cutoff value for FRAX scores is 8.37%; thus, patients were divided into a high and a low FRAX score groups ($n=9$ and $n=29$, respectively). *Veillonellaceae* was predominant in the low FRAX score group ($P=0.044$) (Table 3).

There was no significant difference between groups in age except for FRAX score. The high FRAX score group was predominantly older, because the FRAX tool also includes age as an item.

No significant difference was found in vitamin D, vitamin K1, ALP, Ca, and P.

Fracture risk

The incidence of fracture in the high fracture risk and low fracture risk groups was compared among the above bacteria. In the genus *Bacteroides*, the low-fracture risk group had a fracture incidence of 52.9%, while the high-fracture risk group had a fracture incidence of 9.5% (odds ratio 10.7, 95% confidence interval: 1.88, 60.9, $P=0.0049$, relative risk 5.57. But the other bacteria showed no significant difference. *Lachnospiraceae* tended to show a lower risk of fracture, similar to the FRAX results above, and *Rikenellaceae* and *Erysipelotrichaceae* tended to show a slightly higher risk of fracture (Fig. 4).

Table 3 (continued)

(ng/mL)	0.849	0.528	0.692	0.286	0.895	0.860	0.044*
TRACP	5.88	0.87	3.81	2.58	3.65	5.98	1.73
	8.39	1.19	2.89	3.30	3.73	3.61	0.82
(mU/dL)	0.209	0.573	0.215	0.373	0.664	0.016*	0.103
Vitamin K2	7.07	1.10	3.18	3.48	4.69	5.63	1.88
	7.88	1.09	3.15	2.82	3.36	3.82	0.80
(ng/mL)	0.921	0.740	1.000	0.816	0.336	0.312	0.026*
FRAX	5.97	1.02	3.20	2.12	3.71	2.99	1.05
	9.04	1.15	3.12	3.64	3.71	5.36	1.11
(%)	0.081	0.419	0.547	0.044*	0.587	0.277	0.606

* $P < 0.05$ *** $P < 0.005$ BMD, bone mineral density; μcOC , uncarboxylated osteocalcin; TRACP-5b, tartrate-resistant acid phosphatase 5b; ALP, alkaline phosphatase; FRAX, Fracture Risk Assessment

Discussion

In this study, the results of the statistical analysis suggest that gut bacteria affect bone mineral density and risk of fracture. *Bacteroides* showed a significant difference in vitamin K2 levels and fracture risk, and *Rikenellaceae* showed a significant difference in BMD and TRACP-5b levels. In addition, we would like to consider *Lachnospiraceae*, which is of importance in terms of both age and fracture history.

Bacteroides

Bacteroides synthesize vitamin K. Essentially, vitamin K is a group of several structurally similar, fat-soluble compounds required by humans for complete synthesis of certain proteins including phylloquinone (vitamin K1) and menaquinone (MKn; vitamin K2). Vitamin K is a cofactor in the production of blood coagulation factors in the liver, osteocalcin in bone, and matrix Gla protein in cartilage and the vascular wall [26]. Furthermore, certain proteins in bone are vitamin K-dependent, such as osteocalcin and matrix Gla protein, and thus vitamin K also plays an important role in regulating bone matrix quality [27].

Menaquinone-4 (MK-4, menatetrenone), a vitamin K2 homolog, is used in the treatment of osteoporosis in Japan. It is known to have well-recognized preventive effects in terms of bone resorption and fracture. In addition, the incidence of femoral, cervical, and vertebral compression fracture is higher among elderly women with low blood vitamin K concentrations and in those with high serum ucOC levels [28].

MKn contains a large amount of MK-7 found in Japanese natto, the well-known fermented soybean product containing *Bacillus subtilis*. MK-7 is also found in other fermented foods. MKn is also produced by the gut microbiota, while MK-10 and MK-11 are synthesized by *Bacteroides* [29].

In our study, *Bacteroides* was significantly more abundant in the high-level vitamin K2 group than in the low-level vitamin K2 group ($P = 0.022$) (Table 3), but there was no significant difference in terms of natto intake between these two groups. Therefore, we surmised that dietary intake did not affect vitamin K2 level and that vitamin K2-producing *Bacteroides* exerted some influence.

Given that there was no significant difference in BMD, we postulated that *Bacteroides* does not affect bone strength. In addition, *Bacteroides* is believed to have some effect on bone quality and is associated with fracture unrelated to osteoporosis, rather than fragility fracture.

In our study, the fracture risk was 5.6 times higher in the low-level *Bacteroides* group (Fig. 4b), suggesting a role for *Bacteroides* in bone metabolism and fracture risk. It has also been reported that intake of meals that contain resistant starch, high levels of soluble fiber, and red meat can increase

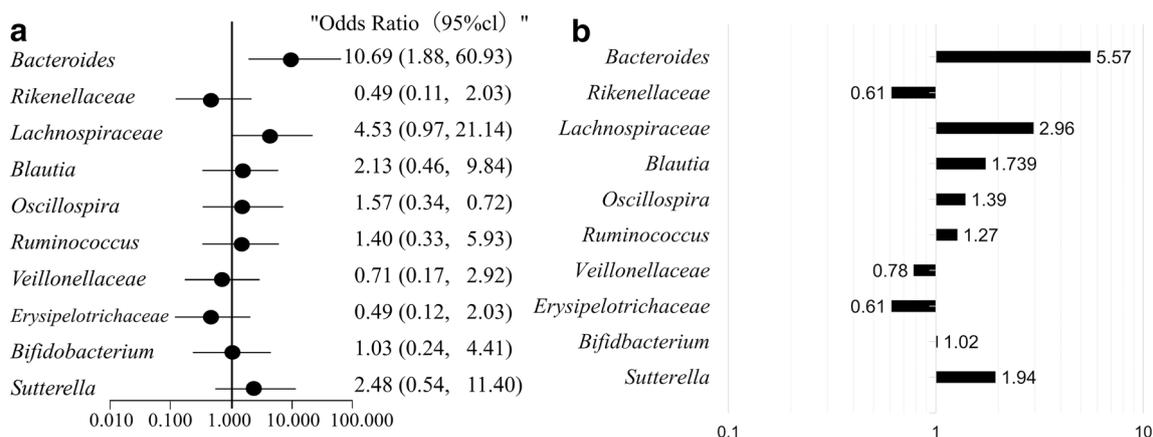


Fig. 4 Comparison of fracture incidence between the high- and low-isolation rate groups of gut microbiota. **a** Odds ratio **(b)** Relative risk: indicating fracture risk when these bacteria isolation rates are low. The higher the number [[isolation rate?]], the higher the fracture risk in the low-level group

the abundance of *Bacteroides* [7]. Therefore, ingestion of the above diets may reduce fracture risk.

Rikenellaceae

The functions of *Rikenellaceae* are not yet well understood. However, this family has been reported to be abundant in many diabetic patients [30]. Also, PD-1^{-/-} mice, which are less likely to develop ulcerative colitis, showed changes in the composition of the intestinal microbiota and significantly reduced proportions of *Rikenellaceae* [31]. In our study, *Rikenellaceae* was more abundant in the low BMD group and in the high TRACP-5b group, suggesting that this family may have a negative effect on bone resorption and bone density. Furthermore, mice fed a high-fat diet were reported to show increased proportion of *Rikenellaceae* [32], so refraining from a high-fat diet may have a positive effect on bone strength even though this is an animal study.

Lachnospiraceae

Lachnospiraceae is among the most abundant bacteria [33] in the human gut. *Lachnospiraceae* breaks down complex polysaccharides into short-chain fatty acids. The abundance of these bacteria boosts host immunity and is a strong indicator of gut health. They are considered to be among the useful bacteria, because they are decreased in the intestine of the elderly [34] and in individuals with various diseases, such as diabetes mellitus [35], liver cirrhosis [36], and colon cancer [37].

It has also been reported that *Lachnospiraceae* was less abundant in individuals with low BMD and was positively correlated with BMD and T-score [38]. In this study, there was no significant difference in BMD, but the fracture incidence was significantly higher while the relative risk was three times higher, in the low *Lachnospiraceae* group than in the high *Lachnospiraceae* group (Fig. 4b). This suggested that

Lachnospiraceae may act as useful bacteria for predicting fracture risk.

Conclusion

Our data suggested that the abundance of *Bacteroides* and *Lachnospiraceae* may play a positive role in bone metabolism and fracture risk. Moreover, *Rikenellaceae* may have a negative effect on bone metabolism and fracture risk.

Attention to new treatments targeting particular species in gut microbiota is warranted for osteoporosis and fracture prevention. Further research should focus on analyzing specific species and their potential roles in increasing bone strength or preventing fractures.

Limitations

This study has some limitations that must be acknowledged. First, the study was conducted at a single-center in Japan, and so the subjects were Japanese nationals only. Thus, our findings may not be generalizable to other nationalities. Second, no dietary restrictions or lifestyle control measures were applied, which might have introduced some level of bias and affected our results. Third, the sample size was small, and so our study may not have sufficient statistical power for a definite conclusion. Fourth, the microbial taxa data in this study had a high variance, and thus our methods for statistical analysis may not have been ideal. Therefore, further multi-center studies with a larger sample size, and taking into account these and other limitations are warranted.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

Ethical approval The study was performed in accordance with ethical standards as laid down in the World Medical Association's Declaration of Helsinki 1964 and its later amendments.

Informed consent Each participant gave written informed consent prior to inclusion in this study.

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