



Research Article

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Changes in the gut microbiota of osteoporosis patients based on 16S rRNA gene sequencing: a systematic review and meta-analysis

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Abstract: Background: Osteoporosis (OP) has become a major public health issue, threatening the bone health of middle-aged and elderly people from all around the world. Changes in the gut microbiota (GM) are correlated with the maintenance of bone mass and bone quality. However, research results in this field remain highly controversial, and no systematic review or meta-analysis of the relationship between GM and OP has been conducted. This paper addresses this shortcoming, focusing on the difference in the GM abundance between OP patients and healthy controls based on previous 16S ribosomal RNA (rRNA) gene sequencing results, in order to provide new clinical reference information for future customized prevention and treatment options of OP. Methods: According to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA), we comprehensively searched the databases of PubMed, Web of Science, Embase, Cochrane Library, and China National Knowledge Infrastructure (CNKI). In addition, we applied the R programming language version 4.0.3 and Stata 15.1 software for data analysis. We also implemented the Newcastle-Ottawa Scale (NOS), funnel plot analysis, sensitivity analysis, Egger's test, and Begg's test to assess the risk of bias. Results: This research ultimately considered 12 studies, which included the fecal GM data of 2033 people (604 with OP and 1429 healthy controls). In the included research papers, it was observed that the relative abundance of *Lactobacillus* and *Ruminococcus* increased in the OP group, while the relative abundance for *Bacteroides* of Bacteroidetes increased (except for Ireland). Meanwhile, Firmicutes, *Blautia*, *Alistipes*, *Megamonas*, and *Anaerostipes* showed reduced relative abundance in Chinese studies. In the linear discriminant analysis Effect Size (LEfSe) analysis, certain bacteria showed statistically significant results consistently across different studies. Conclusions: This observational meta-analysis revealed that changes in the GM were correlated with OP, and variations in some advantageous GM might involve regional differences.

Key words: Osteoporosis; Microbiome; Intestinal; 16S ribosomal RNA (rRNA) sequencing

1 Introduction

As the global issue of population aging is becoming increasingly prominent, the comprehensive prevalence of osteoporosis (OP) has grown to 21.7%. In particular, its value has reached 24.3% among the Asian

population. As a result, OP can be considered a major public health crisis that is severely threatening the bone health of middle-aged and elderly people worldwide (Anonymous, 1993; Salari et al., 2021). Moreover, osteoporotic fractures cause patients to suffer from high treatment costs, severe lifelong disability, and a heightened risk of death. Therefore, the prevention and treatment of OP has become of particular concern and must be urgently addressed.

Tens of thousands of gut microbiota (GM) evolve along with their host organism, creating a mutually beneficial symbiotic relationship (Danne et al., 2017; Wei et al., 2019). GM comprise a complicated and large microbial metabolic system that participates

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in regulating the stability of the internal and external environment, and also has a key role in healthy human physiological processes and disease development (Juárez-Fernández et al., 2020; Shi et al., 2020). If the homeostasis between the human body and GM is damaged, it could lead to many types of human chronic diseases such as metabolic, mental, neurodegenerative, cardiovascular, and cancer diseases (Baker et al., 2011; Li et al., 2017; Hou et al., 2021). In recent years, researchers have established that changes in the GM are also related to the maintenance of bone mass and quality, while GM impact the dynamic balance between bone formation maintained by osteoblasts and bone resorption maintained by osteoclasts, which relies on the gut-bone axis (Liang et al., 2018). Sjögren et al. (2012) found that the bone mass of germ-free mice was abnormally high. When implanting GM into these mice, they were able to induce and activate cluster of differentiation-positive (CD⁺) T cells. GM triggered the excretion of several cytokines in the bone to promote osteoclast activation and initiate the bone resorption mechanism, and normalized the bone mass of ovariectomized (OVX) mice. Since 2017, five clinical randomized controlled trials have shown that replenishing probiotics can prevent and treat OP as well as improve bone quality in OP, and fecal microbiota transplantation (FMT) could become a new therapeutic standard for OP (Jafarnejad et al., 2017; Lambert et al., 2017; Nilsson et al., 2018; Takimoto et al., 2018; Jansson et al., 2019). However, as different populations have unique GM abundance characteristics, applying beneficial and customized GM-mixed preparations may be a safer and more effective choice (Tyagi et al., 2018). As a result, identifying advantageous bacterial strains or metabolites with positive bone effects, as well as constructing customized and precise GM therapies based on different OP, may provide new, safe, and effective OP treatment options.

The 16S ribosomal RNA (rRNA) genes are present in the genome of all bacteria. Consequently, 16S rRNA gene sequencing has become widely applied in the research on microbial phylogeny and accurate species classifications. In addition, it has significant value when explaining the relationship between GM and disease. Several clinical studies based on populations have used GM 16S rRNA gene sequencing to analyze the abundance of GM in patients (Rettedal et al., 2021; Wang ZX et al., 2021; Xu et al., 2021). These

results verified that changes occurred in the relative abundance of several species of microorganisms, which may be related to GM impacting the bone metabolism. However, these 16S rRNA gene sequencing results were dispersed and inconsistent, lacked systematic reviews and summaries, and were unable to provide new reference values for current and future large-scale clinical trials. Prompted by this deficiency, in this paper, we conducted a systematic review and meta-analysis of the differences in GM abundance between OP and healthy control (HC) from previous 16S rRNA gene sequencing results, to more effectively explain the role of GM in OP, and to provide new clinical reference information for customized OP prevention and treatment.

2 Results

2.1 Search flow and overview of studies

A total of 103 items of data were selected through database retrieval. Among them, 35 repetitive studies were excluded, 29 studies with inconsistent data were excluded, and the remaining 39 articles were evaluated. After carefully reading the titles and abstracts, 26 articles were excluded because they did not meet the inclusion criteria. Two articles were excluded as they did not provide relevant data on the relative abundance of GM, including one with a sample size fewer than ten patients. Two articles were excluded for not providing sufficient data on analyses of alpha diversity. One study on *Bacteroides* was excluded as it had high heterogeneity. One article was included by the combination of manual retrieval and literature tracking. After reading the full texts carefully and comparing the selection criteria, 12 studies were finally included (Fig. 1) (Das et al., 2019; Li C et al., 2019; Li LS, 2019; He et al., 2020; Palacios-González et al., 2020; Xu et al., 2020; Ling et al., 2021; Lyu et al., 2021a, 2021b; Rettedal et al., 2021; Wang ZX et al., 2021; Wei et al., 2021). The basic data and sequencing methods of the population are shown in Table S1. The meta-analysis included studies covering a period from 2019 to 2021, including 604 OP patients and 1429 HC. Among them, five studies included all female participants (He et al., 2020; Palacios-González et al., 2020; Lyu et al., 2021b; Rettedal et al., 2021; Wang ZX et al., 2021), while all participants in another

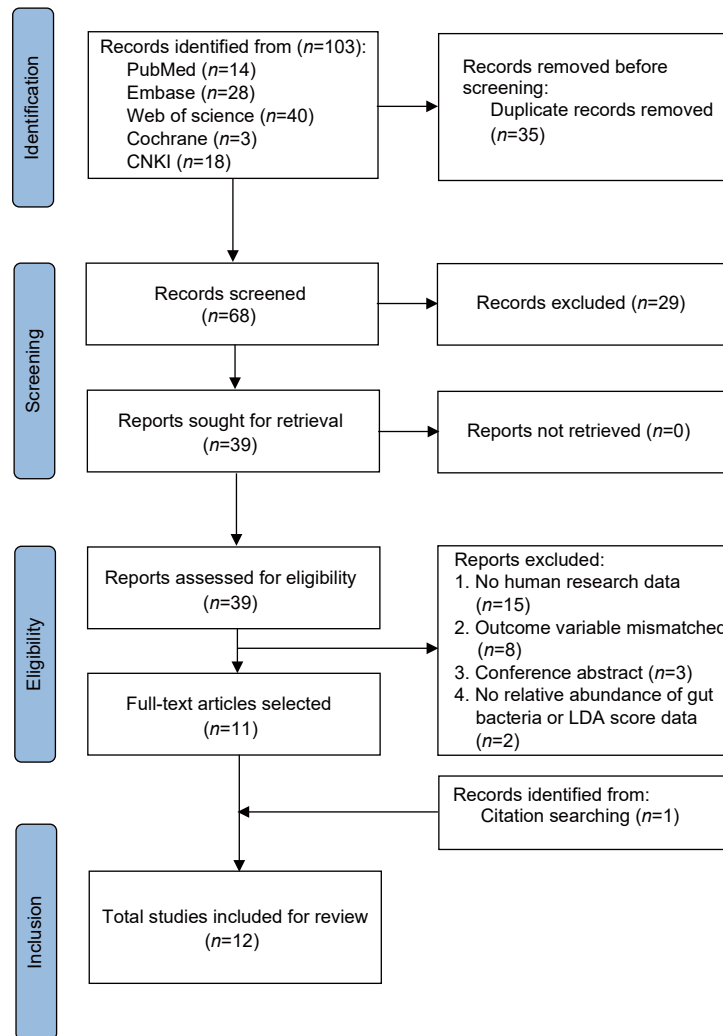


Fig. 1 Search and selection procedures of the literature for the systematic review, described in detail by Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flowchart. CNKI: China National Knowledge Infrastructure; LDA: linear discriminant analysis.

were exclusively male (Lyu et al., 2021a). The total proportion of female participants was 67.8%, except that Li et al. (2019) did not adjust for gender-related confounding factors. The results of other included studies showed significant differences in the intestinal flora among different groups after adjusting for sex-related confounding factors. As for population areas, one study was conducted in Ireland (Das et al., 2019), one in Mexico (Palacios-González et al., 2020), one in New Zealand (Rettedal et al., 2021), and nine in China (Li C et al., 2019; Li LS, 2019; He et al., 2020; Xu et al., 2020; Ling et al., 2021; Lyu et al., 2021a, 2021b; Wang ZX et al., 2021; Wei et al., 2021). All studies measured bone mineral density (BMD) using dual-energy X-ray (DXA), and all of them used

sequencing schemes, including 16S rRNA sequencing, to evaluate intestinal flora samples. One study utilized 16S rRNA to measure the V3–V5 regions (Das et al., 2019), two studies measured the V4 region (Li, 2019; Palacios-González et al., 2020), and seven studies measured the V3 and V4 regions (Li et al., 2019; He et al., 2020; Xu et al., 2020; Ling et al., 2021; Lyu et al., 2021a, 2021b; Wei et al., 2021). One study did not specify the sequencing region (Rettedal et al., 2021). Two studies defined patients as people with low BMD (Li et al., 2019; Palacios-González et al., 2020). Nine studies reported data on the relative abundance of microflora (Das et al., 2019; Li, 2019; He et al., 2020; Palacios-González et al., 2020; Xu et al., 2020; Lyu et al., 2021a, 2021b; Wang ZX et al., 2021;

Wei et al., 2021), and ten studies reported data on the linear discriminant analysis (LDA) scores (Li C et al., 2019; Li LS, 2019; He et al., 2020; Palacios-González et al., 2020; Xu et al., 2020; Ling et al., 2021; Lyu et al., 2021a, 2021b; Rettedal et al., 2021; Wang ZX et al., 2021). Seven studies reported the alpha diversity data (Das et al., 2019; Li, 2019; Palacios-González et al., 2020; Xu et al., 2020; Ling et al., 2021; Rettedal et al., 2021; Wei et al., 2021). Two studies reported the specific value of Firmicutes and Bacteroidetes (F/B) (Li et al., 2019; Palacios-González et al., 2020). Three studies used Spearman's correlation analysis to evaluate the correlation between GM at the phylum and genus levels and the BMD measurements (Li et al., 2019; Xu et al., 2020; Wei et al., 2021). The average overall quality score of these studies was eight stars (Table S2).

2.2 Alpha diversity and Firmicutes/Bacteroidetes (F/B) ratio

Alpha diversity can reflect the richness and diversity of microbial communities in samples. We analyzed the alpha diversity indices (Chao and Shannon) between OP and HC, and found that the two indices did not significantly differ between the Western and the Chinese populations, within the Chinese population, or within the Western population (Fig. 2). The contents of F/B play a dominant role in intestinal microbial composition, and the F/B ratio was associated with the dietary intake, energy metabolism, and GM imbalance (Rinaldi et al., 2019; Zhao et al., 2019). Of the 12 included studies, two studies reported the F/B ratios, and our analysis showed no significant difference in F/B ratios between OP and HC (Fig. 3).

2.3 Bacterial phyla

As shown in Table 1, the relative abundance of Firmicutes in the OP group was decreased in five groups (Li et al., 2019; Xu et al., 2020; Lyu et al., 2021a, 2021b; Wei et al., 2021). It should be noted that in the Irish and Mexico results, the relative abundance of Firmicutes increased in the OP group. Meanwhile, the relative abundance of Bacteroidetes increased in the OP group in China and Mexico (Li, 2019; Palacios-González et al., 2020; Xu et al., 2020; Lyu et al., 2021a, 2021b; Wei et al., 2021). We found that the relative abundance of Bacteroidetes decreased only in the OP group in Ireland, but not in China or Mexico.

2.4 Bacterial genera

A total of eight studies reported the relative abundance data at the genus level, as shown in Table 2 (Das et al., 2019; Li, 2019; He et al., 2020; Xu et al., 2020; Lyu et al., 2021a, 2021b; Wang ZX et al., 2021; Wei et al., 2021). *Blautia* were decreased in five Chinese OP groups (Li, 2019; He et al., 2020; Xu et al., 2020; Lyu et al., 2021b; Wei et al., 2021); research by He et al. (2020) also indicated that *Blautia* was directly correlated with lumbar BMD in postmenopausal osteopenia patients, and was negatively correlated with metabolite L-lysine. The relative abundance of *Alis-tipes* was decreased in four Chinese OP groups (Li, 2019; He et al., 2020; Wang ZX et al., 2021; Wei et al., 2021), and increased in an Irish OP group (Das et al., 2019). The relative abundance of *Megamonas* was decreased in two OP groups in China (Li, 2019; Xu et al., 2020). The relative abundance of *Anaerostipes* was increased in one Irish OP group (Das et al., 2019) and decreased in two Chinese OP groups (Li, 2019; Wei et al., 2021). The relative abundance of *Lactobacillus* and *Ruminococcus* was increased in the OP groups (Das et al., 2019; Li, 2019; Lyu et al., 2021a, 2021b; Wei et al., 2021). Li (2019) found that *Lactobacillus* was positively correlated with BMD, while *Bacteroides* was negatively correlated with BMD (Fig. S1). The relative abundance of *Bacteroides* was increased in seven OP groups (Li et al., 2019; He et al., 2020; Xu et al., 2020; Lyu et al., 2021b; Rettedal et al., 2021; Wang ZX et al., 2021; Wei et al., 2021), and decreased in an Irish OP group (Das et al., 2019).

2.5 Controversial results

For certain bacteria, contradictory results were found in studies from the same or different countries; however, these bacteria were still associated with bone health (Table 3). For example, Xu et al. (2020) indicated that the OP changes were related to *Faecalibacterium* and found a negative correlation between *Bifidobacterium* and BMD, while Li et al. (2019) found a positive correlation between *Bifidobacterium* and *Roseburia* and BMD (Fig. S1). A Chinese study by Wang ZX et al. (2021) revealed that *Prevotella* was less abundant in postmenopausal women with OP.

2.6 Linear discriminant analysis (LDA)

LDA Effect Size (LEfSe) analysis can be used to compare the statistical differences between the

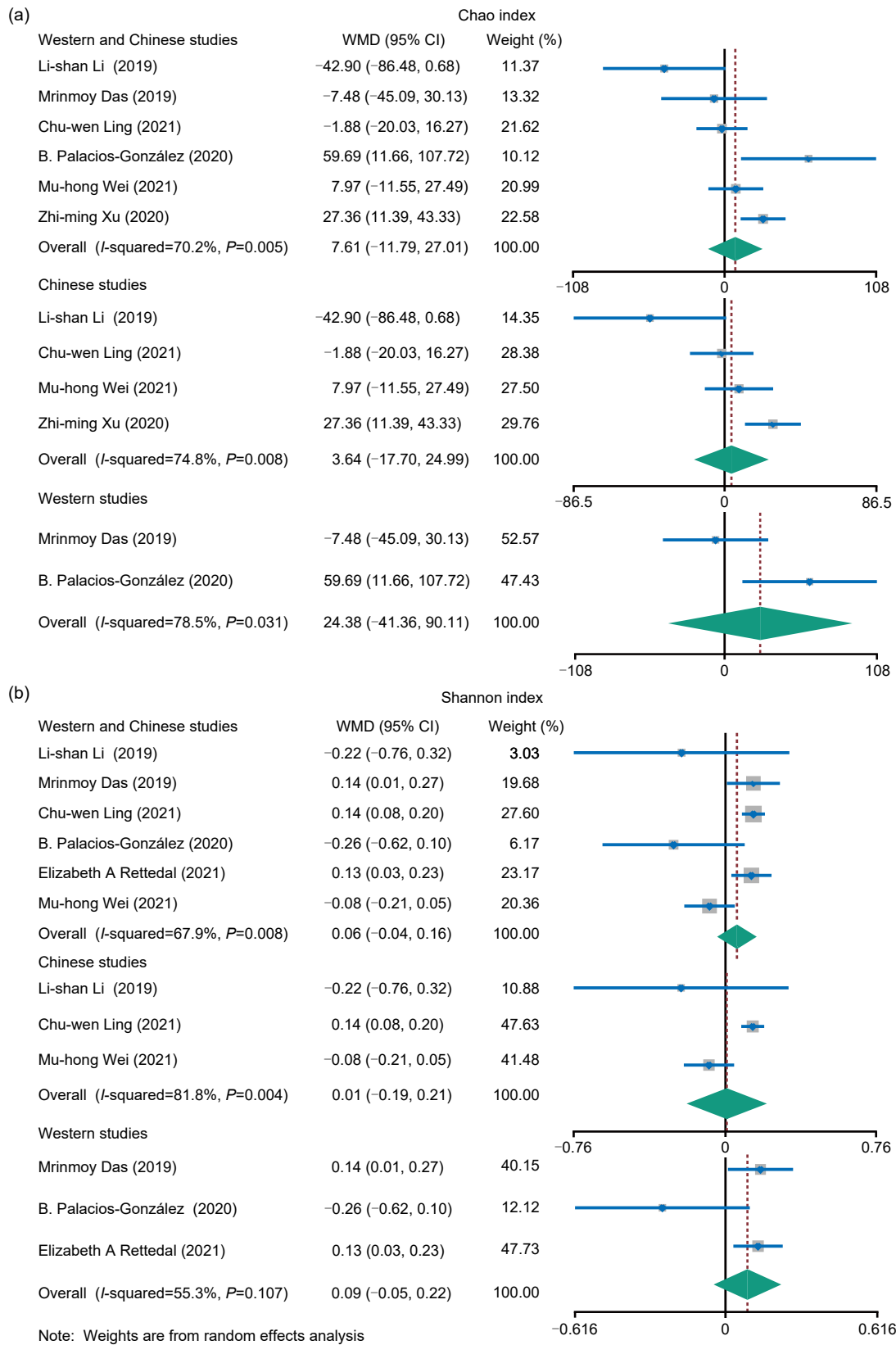


Fig. 2 Forest map of alpha diversity differences by Chao index (a) and Shannon index (b). WMD: weight mean difference; CI: confidence interval.

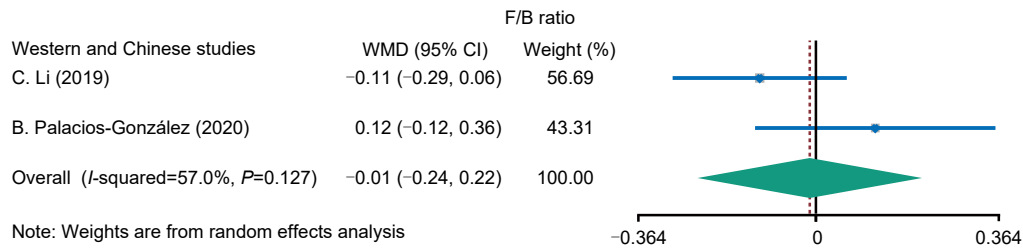


Fig. 3 Forest plot of Firmicutes/Bacteroidetes (F/B) ratio. WMD: weight mean difference; CI: confidence interval.

Table 1 Changes at the taxonomic level in phyla associated with intestinal flora

Phylum	Higher in osteoporosis		Higher in healthy control	
	Reference	Ratio (%) [#]	Reference	Ratio (%) [#]
Firmicutes	Das et al., 2019	79.13	Wei et al., 2021	54.51
	Palacios-González et al., 2020 ^{&}	38.01	Xu et al., 2020	84.14
			Lyu et al., 2021b	75.70
			Li, 2019	61.90
			Lyu et al., 2021a	67.86
Bacteroidetes	Li, 2019	29.57	Das et al., 2019	15.32
	Wei et al., 2021	38.93		
	Xu et al., 2020 [*]	12.03		
	Palacios-González et al., 2020 ^{&}	52.79		
	Lyu et al., 2021b	12.81		
	Lyu et al., 2021a	10.36		

[#] Approximation proportion of relative abundance histogram. [&] Patients characterized by low bone mineral density (BMD). ^{*} The difference was statistically significant.

OP and HC groups, to find the microflora with significant differences between groups. From the 12 studies included, ten reported the LDA scores of the OP and HC groups (Li C et al., 2019; Li LS, 2019; He et al., 2020; Palacios-González et al., 2020; Xu et al., 2020; Ling et al., 2021; Lyu et al., 2021a, 2021b; Rettedal et al., 2021; Wang ZX et al., 2021). Here, we describe the strains that have been reported more than twice. In the OP group, *g_Bacteroides*, *f_Bacteroidaceae*, *o_Bacteroidales*, *c_Bacteroidia*, *p_Bacteroidetes*, *o_Lactobacillales*, and *c_Bacilli* were significantly different microflora, whereas in the HC group, *f_Lachnospiraceae*, *g_Turicibacter*, and *g_Collinsella* were the significantly different microflora. Contradictory results were reported for the remaining strains (Fig. 4). Among them, *g_Lachnospira* was considered as significantly different microflora in low-BMD postmenopausal women in Mexico and significantly different microflora in the HC group in China (He et al., 2020; Palacios-González et al., 2020; Wang ZX et al., 2021). *f_Peptostreptococcaceae* was considered as significantly different microflora in the

OP group in China and significantly different microflora in the HC group in New Zealand (Lyu et al., 2021b; Rettedal et al., 2021).

2.7 Publication bias

We assessed the risk of bias for each of the included articles, and Begg’s and Egger’s regression tests indicated that there was no significant bias in the results of these meta-analyses (Fig. S2).

3 Discussion

Changes in the GM have an important impact on the physiological processes and disease prognosis of many organisms, and these changes differ between developed and developing countries (Vangay et al., 2018). As a result, assessing the correlation between GM and OP has a definite reference value for discovering the potential targets of GM regulating OP. Currently, research on the relationship between GM and bone health has become extremely popular. Based on

Table 2 Differences in the relative abundance of gut bacteria at the genus level between osteoporosis patients and healthy control

Genus	Higher in osteoporosis		Higher in healthy control	
	Reference	Ratio (%) [#]	Reference	Ratio (%) [#]
<i>Blautia</i>			Wei et al., 2021	7.63
			Lyu et al., 2021b	9.36
			Li, 2019	14.46
			Xu et al., 2020*	4.21
			He et al., 2020**	
<i>Alistipes</i>	Das et al., 2019	1.23	Wang ZX et al., 2021	2.71
			Wei et al., 2021	2.05
			He et al., 2020	0.95
			Li, 2019	1.65
<i>Megamonas</i>			Xu et al., 2020	5.14
<i>Anaerostipes</i>	Das et al., 2019	2.21	Li, 2019	2.69
			Wei et al., 2021	1.63
<i>Lactobacillus</i>	Das et al., 2019	8.60		
	Li, 2019	1.65		
	Lyu et al., 2021a	8.75		
	Lyu et al., 2021b	5.94		
<i>Ruminococcus</i>	Wei et al., 2021	2.86		
	Das et al., 2019	3.68		
<i>Bacteroides</i>	He et al., 2020*	46.74	Das et al., 2019	16.42
	Wei et al., 2021	29.63		
	Xu et al., 2020	6.36		
	Lyu et al., 2021b	7.76		
	Wang ZX et al., 2021	48.12		
	Li et al., 2019 [*]			
	Rettedal et al., 2021			

[#] Approximation proportion of relative abundance histogram. * The difference was statistically significant. ** The difference was significant in postmenopausal osteopenia. ^{*} Patients characterized by low bone mineral density (BMD).

Table 3 Contradictory results of differences in the relative abundance of gut bacteria at the genus level between osteoporosis patients and healthy control

Genus	Higher in osteoporosis		Higher in healthy control	
	Reference	Ratio (%) [#]	Reference	Ratio (%) [#]
<i>Bifidobacterium</i>	Lyu et al., 2021b	27.40	Xu et al., 2020	4.66
	Wei et al., 2021	3.41	Li, 2019	4.96
	Lyu et al., 2021a	13.51		
	Das et al., 2019	2.45		
<i>Faecalibacterium</i>	Lyu et al., 2021b	16.90	Li, 2019	4.55
	Xu et al., 2020*	32.71	Lyu et al., 2021a	19.29
			He et al., 2020	1.87
			Wei et al., 2021	6.81
			Das et al., 2019	19.12
			Wang ZX et al., 2021	3.14
<i>Roseburia</i>	Wei et al., 2021	1.50	He et al., 2020	1.08
	Wang ZX et al., 2021	1.92	Das et al., 2019	12.75
<i>Prevotella</i>	Das et al., 2019	4.41	Wang ZX et al., 2021	26.81
	Wei et al., 2021	3.41		

[#] Approximation proportion of relative abundance histogram. * The difference was statistically significant.

osteoclast activity, changed the expression of IL-1 β , TNF- α , and other osteoclastic cytokines, and reduced bone loss in OVX mice.

We found that different studies had contradictory results for *Bifidobacterium*, *Faecalibacterium*, and *Roseburia*. *Bifidobacterium* belongs to Actinobacteria, which can mediate lipopolysaccharide (LPS)-induced inflammatory responses (Khokhlova et al., 2012). One study found that, when a low concentration of LPS induces bone mesenchymal stem cells (BMSCs), they could differentiate into osteoblasts, but a high concentration of LPS would reduce their differentiation ability (Ding, 2017). As a result, further research is needed to confirm how *Bifidobacterium* impacts bone quality. *Faecalibacterium* and *Roseburia* can generate butyrate bacteria (Crespo-Piazuelo et al., 2021). Butyrate can upregulate the expression of Wnt10b and enhance the osteogenic differentiation ability of BMSCs.

Ethnic or regional differences may be important factors affecting the composition and structure of GM. In this meta-analysis, the relative abundance of Firmicutes, Bacteroidetes, *Alistipes*, *Anaerostipes*, and *Bacteroides* in the gut of Irish OP patients showed inconsistent alterations compared with other regions. In the future, more population studies and meta-analyses are needed to explain this regional difference. Although the overall quality of the included original studies was good, we should not overlook the important fact that drugs can also influence GM composition and structure. It is well known that depression is one of the high-risk factors of OP. Wan et al. (2022) found that the new antidepressant drug *R*-ketamine can alleviate bone loss in OVX mice through the anti-inflammatory effect of GM, which reveals the potential therapeutic targets of OP based on the pathogenesis of GM. Thus, individualized treatment for specific patients can be achieved, which is expected to be beneficial for the treatment of OP. In addition, it should be noted that, although we were unable to incorporate GM metabolites into this systematic review and meta-analysis, clinical studies exploring the relationship between specific gut microbial-derived metabolites and OP may constitute valuable future research directions.

Currently, the exact relationship between GM and OP is complicated and remains unclear. The numerous changes in GM abundance may be unable to directly explain the impact of GM on bone health. However, we

look forward to the application of this meta-analysis to provide reference information for future research designs. Applying GM as a target to improve bone health involves an enormous amount of potential, and research focusing on specific advantageous strains may be a direction worth exploring for improved diagnostic and treatment options for OP.

4 Limitations

In this work, we relied on established inclusion standards to comprehensively search for all of the studies that matched our requirements, and strictly implemented a systematic review according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guide (Moher et al., 2009). However, some limitations of this study could not be avoided. First, most of the included studies had a high level of heterogeneity. Different from the meta-analysis of randomized controlled trials, heterogeneity is a widely known and unavoidable problem of observational studies (Stroup et al., 2000). The included studies had a considerably large clinical and methodological heterogeneity, while specific situations among different individuals also exhibited relatively large differences. For instance, differences between factors such as the participants' sample size, ethnicity, place of residence, season, diet, medications, age and gender, physical exercise, and stress tend to impact the composition and structure of their GM (Gupta et al., 2017). The researchers' DNA extraction methods used to target the 16S rRNA gene area for sequencing, the sequencing platform, and sequencing depth were all reasons that could cause inconsistent results (Duvallat, 2018). We could not guarantee that the meta-analysis results display all of the strains correlated with OP. Furthermore, we were only able to identify the correlation between changes in GM and OP. However, neither does this definitively indicate that the two are causally related, nor can it conclusively verify that a particular advantageous bacterium can protect bones. At the same time, changes in relative abundance cannot always accurately react variations in absolute abundance (Smets et al., 2016). Furthermore, although 16S rRNA analysis is a powerful technique, it cannot provide reliable in-depth identification (Ravi et al., 2018).

5 Conclusions

Our meta-analysis results indicated no significant difference in alpha diversity (Chao and Shannon) indices or the F/B ratio between OP and HC. In the included research papers, it was observed that, compared with the HC group, the relative abundance of *Lactobacillus* and *Ruminococcus* increased, while the relative abundance for *Bacteroides* of Bacteroidetes increased (except for Ireland) in the OP group. Meanwhile, Firmicutes, *Blautia*, *Alistipes*, *Megamonas*, and *Anaerostipes* had reduced relative abundance in the included Chinese studies. In the LEfSe analysis, certain bacteria showed statistically significant results consistently across different studies. In addition, there may have been regional differences in the changes in some intestinal flora. The pathogenicity of GM is still controversial, while more basic and clinical research is needed to explain the role of microbes in bone microbiology, to help explore new strategies for using GM to prevent and treat OP.

Materials and methods

Detailed methods are provided in the electronic supplementary materials of this paper.

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Author contributions

Conceptualization: Gaofeng ZENG and Shaohui ZONG; Methodology: Jieqiong HUANG; Formal analysis: Rui HUANG and Huihua LI; Resources: Yeping SU; Writing – original draft preparation: Rui HUANG and Pan LIU; Writing – review and editing: Rui HUANG, Ruixin MA, and Quan ZHOU; Visualization: Rui HUANG and Yiguang BAI; Project administration: Rui PAN; Funding acquisition: Gaofeng ZENG and Shaohui ZONG. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Rui HUANG, Pan LIU, Yiguang BAI, Jieqiong HUANG, Rui PAN, Huihua LI, Yeping SU, Quan ZHOU, Ruixin MA, Shaohui ZONG, and Gaofeng ZENG declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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Supplementary information

Tables S1 and S2; Figs. S1 and S2; Materials and methods