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# BMJ Open

## Probiotic supplements and bone health in postmenopausal women: a meta-analysis of randomized controlled trials

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4 1 **Probiotic supplements and bone health in postmenopausal**  
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6 2 **women: a meta-analysis of randomized controlled trials**  
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## 30 **Abstract**

31 **Objective:** Osteoporosis is a common disease in postmenopausal women. Several  
32 studies have analyzed the associations between dietary supplement of probiotics and  
33 bone health in postmenopausal women, but the results are still controversial. We  
34 conducted this meta-analysis to assess the effects of probiotics supplement on bone  
35 mineral density (BMD) and bone turnover markers for postmenopausal women.

36 **Design:** systematic review and meta-analysis.

37 **Methods:** We systematically searched PubMed, EMBASE and the Cochrane Library  
38 from their inception to May 2019 for randomized controlled trials (RCTs) assessing  
39 probiotic supplements and osteoporosis in postmenopausal women. Study-specific  
40 risk estimates were combined using fixed-effect or random-effect models.

41 **Results:** Four RCTs (n = 218) were included. Probiotic supplements were associated  
42 with a significantly higher BMD in both hips (SMD (standardized mean difference) =  
43 0.37, 95% CI: 0.12–0.62) and lumbar spine (SMD = 0.28, 95% CI: 0.03–0.53) than in  
44 the control ( $P = 0.004, 0.029$  respectively). Collagen type 1 cross-linked  
45 C-telopeptide (CTX) levels in the treatment groups were significantly lower than  
46 those of the placebo group (SMD = -0.34, 95% CI: -0.60– 0.09). In subgroup  
47 meta-analysis, levels of bone-specific alkaline phosphatase (BALP), osteoprotegerin  
48 (OPG), osteocalcin (OC) and tumor necrosis factor (TNF) did not differ between the  
49 probiotic and placebo groups.

50 **Conclusions:** Supplementation with probiotics can increase lumbar and hip BMD,  
51 and reduce bone resorption. Probiotics retard osteoporosis in postmenopausal women.

## 53 **Strengths and limitations of this study**

54 1. We conduct a systematic review and meta-analysis of high-quality randomized  
55 controlled trials. We find probiotics could retard osteoporosis in postmenopausal  
56 women.

57 2. To our knowledge, this is the first meta-analysis describing the evidence of the  
58 association of probiotic supplements and bone status in postmenopausal women.

59 3. There is little heterogeneity between included articles and fixed-effects model used

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4 60 to calculate the results.

5 61 4. Only four randomized controlled trials satisfied our inclusion criteria. The limited  
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7 62 number of reports prevented us from conducting subgroup analysis. Furthermore,  
8  
9 63 insufficient number of estimates inflate the impact of the results of a particular study.  
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11 64

12  
13 65 **Keywords:** probiotics supplement; bone mineral density; bone turnover markers;  
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15 66 postmenopausal; meta-analysis  
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## 90 Introduction

91 Osteoporosis is characterized by low bone mineral density (BMD) and deteriorated  
92 bone microstructure, leading to reduced bone strength and increased susceptibility to  
93 fractures <sup>1</sup>. Osteoporosis and fracture are particularly common in postmenopausal  
94 women, who experience a natural decline in endogenous estrogen, reducing BMD (on  
95 average 2%–5% BMD/y) <sup>2</sup> and leading to adverse effects on bone microarchitecture.

96 Currently, many medications are used in osteoporosis to decrease bone resorption  
97 or increase bone formation. Large randomized controlled trials (RCTs) showed that  
98 estrogen therapy was effective for the prevention and treatment of osteoporosis in  
99 postmenopausal women<sup>3-5</sup>. However, this remains controversial because of the  
100 increased risk of cancer, including endometrial, breast and ovarian cancer <sup>6</sup>.  
101 Nevertheless, other anti-resorptive agents are not widely used because of their  
102 side-effects, high prices and poor compliance on the part of patients; these include  
103 bisphosphonates, calcitonin and raloxifene. Therefore, complementary and dietary  
104 therapies are more acceptable to some patients. It was shown that calcium and vitamin  
105 D supplement effectively improved bone microarchitecture and health <sup>7</sup>; however,  
106 supplementation with calcium and vitamin alone is not sufficient to halt menopausal  
107 bone loss <sup>8</sup>.

108 Therefore, alternative ways to prevent and/or treat osteoporosis are sought.  
109 Probiotics are popular dietary therapies that have favorable effects on the skeletal  
110 system.<sup>9</sup> Probiotics are “live microorganisms that when administered in adequate  
111 amounts will confer a health benefit on the host” defined by the Food and Agricultural  
112 Organization/World Health Organization (FAO/WHO) <sup>10</sup>. They are affordable and  
113 have fewer side-effects.

114 To our knowledge, there has been no systematic review or meta-analysis of RCTs  
115 with probiotics in the treatment arms, analyzing the effect of probiotics in  
116 postmenopausal-related osteoporosis. Therefore, this systematic review and  
117 meta-analysis was performed to provide an overview of the effects of dietary  
118 probiotic supplements in postmenopausal related bone resorption in women and to  
119 inform researchers of new potential sources of bias to be addressed in future clinical

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4 120 trials.

5 121 **Methods and analysis**

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7 122 **Data sources and search strategies**

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9 123 A literature search of relevant studies was performed in PubMed, EMBASE and the  
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11 124 Cochrane Library. A comprehensive search strategy was developed. The key words  
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13 125 were as follows: ‘probiotics,’ ‘bone,’ ‘osteoporosis,’ ‘osteopenia,’ ‘bone mineral  
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15 126 density,’ ‘bone turnover,’ ‘menopause,’ ‘postmenopausal,’ and ‘post-menopause.’  
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17 127 References of retrieved articles were also scanned to identify and additional relevant  
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19 128 studies. Two independent reviewers (Jiawei Yu and Gaoyang Cao) conducted this  
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21 129 work. Discrepancies were resolved by consensus of the two reviewers. If required,  
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23 130 final disposition was determined by Ming Cai.

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25 131 **Inclusion and exclusion criteria**

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27 132 Inclusion criteria are as follows: (1) randomized controlled trials; (2) consideration  
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29 133 of dietary probiotic supplement as baseline exposure, and bone status (BMD and bone  
30  
31 134 turnover markers) as outcomes; (3) postmenopausal women administered probiotics  
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33 135 for more than 6 months; and (4) English language original articles indexed up to May  
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35 136 2020.

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37 137 Exclusion criteria are as follows: (1) absence of key data for meta-analysis; and (2)  
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39 138 low-quality articles according to Cochrane checklist.

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41 139 **Data extraction and quality assessment**

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43 140 The characteristics of the relevant articles were extracted and recorded  
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45 141 independently by two reviewers (Jiawei Yu and Gaoyang Cao) as follows: first  
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47 142 author’s name, year, area, age (mean or range), type of probiotic supplement, dose  
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49 143 design, course of treatment, number of cases, number of controls, and bone status (as  
50  
51 144 shown in Table 1). The Cochrane Collaboration’s tool <sup>11</sup> was used for assessing risk  
52  
53 145 of bias, and results were displayed as low risk, unclear risk or high risk of bias.

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55 146 **Statistical analysis**

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57 147 Meta-regression was conducted to verify whether different types of probiotic  
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59 148 supplement would introduce sources of heterogeneity. The mean relative change from  
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149 baseline to the end of course and standard deviation (SD) were used to express the



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4 150 effect of probiotic supplement on bone status in postmenopausal women. If the  
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6 151 original studies did not provide the mean relative change and standard deviation, we  
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8 152 converted the data using a common method<sup>12-13</sup>. The pooled effects of included  
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10 153 studies were expressed in terms of standardized mean difference (SMD) with 95%  
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12 154 confidence interval (CI). Q test and  $I^2$  index were used to evaluate heterogeneity  
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14 155 among the included results. If the Q test and  $I^2$  index did not show heterogeneity ( $P >$   
15  
16 156 0.05 and  $I^2 \leq 50\%$ ), a fixed-effects model was used; otherwise, a random-effects model  
17  
18 157 was used. Forest plots and funnel plots were produced and publication bias was tested  
19  
20 158 using Begg's test and the weighted Egger test<sup>14-15</sup>. Sensitivity analysis was conducted  
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22 159 to verify the impact of each individual study on the pooled results. All analysis was  
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24 160 performed using STATA 12.0 (StataCorp LP, College Station, TX, USA).

## 161 **Results**

### 162 **Search results and characteristics of identified studies**

163 A total of 524 articles were identified from the initial search in PubMed and  
164 EMBASE, 468 articles were removed because of no relevance to the topic. Then, 8  
165 articles were retained after reviewing the abstract according to the exclusion criteria.  
166 Finally, 4 randomized controlled trials<sup>16-19</sup> satisfied the inclusion criteria and entered  
167 this meta-analysis after full-text review. A detailed overview of the selection process  
168 is outlined in Figure 1.

169 A total of 218 postmenopausal women completed these trials. Among the four  
170 trials, half of the trials were conducted in Asia (one in Japan<sup>16</sup>, the other in Iran<sup>18</sup>),  
171 and the other two trials were in Europe (one in Sweden<sup>17</sup>, the other in Denmark<sup>19</sup>).  
172 All trials were randomized with the double-blinded method. Each trial identified the  
173 type of probiotic supplements used and described the dosage design. Three studies  
174 had treatment with probiotics only<sup>16-18</sup>, while another study included treatment with  
175 combined isoflavone and probiotics<sup>19</sup>. All studies provided BMD data from DXA  
176 scans at the lumbar spine and total hip. Collagen type 1 cross-linked C-telopeptide  
177 (CTX), bone-specific alkaline phosphatase (BALP), osteoprotegerin (OPG),  
178 osteocalcin (OC) and tumor necrosis factor (TNF) were used as bone turnover  
179 markers. Details of the characteristics are displayed in Table 1.

## 180 **Probiotics supplements and total hip BMD**

181 Overall, four estimates of the association between probiotics supplement and hip  
182 BMD were included in the meta-analysis. The results of meta-regression revealed that  
183 various types of probiotics were not a source of heterogeneity ( $P = 0.927$ ). Therefore,  
184 we brought the four estimates into the pooled analysis. We found that probiotic  
185 supplements gave higher hip BMD of the supplementary group than did the placebo  
186 group (SMD = 0.37, 95% CI: 0.12–0.62), with no heterogeneity ( $P = 0.404$ ;  $I^2 = 0.0$ )  
187 (Figure 2). The funnel plot is shown in Supplementary Figure 1a; it was symmetrical,  
188 excluding publication bias (Begg's test  $z_c = 1.02$ ,  $P = 0.308$ ; Egger's test  $t = -1.42$ ,  $P =$   
189  $0.291$ ). Sensitivity analyses indicated that the positive result was affected by the  
190 Lambert trial<sup>19</sup> (Supplementary Figure 2a).

## 191 **Probiotic supplements and lumbar spine BMD**

192 A total of four estimates were included in the meta-analysis. The results of  
193 meta-regression also showed no source of heterogeneity from various types of  
194 probiotics ( $P = 0.813$ ). Therefore, the four estimates were incorporated into the  
195 pooled analysis. Compared to the placebo group, the lumbar spine BMD level of the  
196 supplementary group was higher (SMD = 0.28, 95% CI: 0.03–0.53), with no  
197 heterogeneity ( $P = 0.661$ ;  $I^2 = 0.0$ ) (Figure 3). The funnel plot was symmetrical  
198 (Supplementary Figure 1b) and excluded publication bias (Begg's test  $z_c = 1.02$ ,  $P =$   
199  $0.308$ ; Egger's test  $t = -2.07$ ,  $P = 0.174$ ). Sensitivity analyses indicated that the  
200 positive result was affected by the Takimoto<sup>16</sup> and Lambert trials<sup>19</sup> (Supplementary  
201 Figure 2b).

## 202 **Probiotic supplements and bone turnover markers**

203 Four estimates of CTX, and two estimates of BALP, OPG, OC and TNF were  
204 incorporated into the pooled analysis. The results suggested that probiotic  
205 supplements help decrease body CTX level of the supplementary group when  
206 compared with the placebo group (SMD = -0.34, 95% CI: -0.60 – -0.09), with  
207 substantial heterogeneity. There was no evidence that probiotic supplements were  
208 associated with the levels of BALP, OPG, OC and TNF (Figure 4).

## 209 **Discussion**

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4 210 This meta-analysis provides evidence that dietary probiotics supplement can slow  
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6 211 bone resorption in postmenopausal women. Daily supplementation with probiotics for  
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8 212 24 weeks to 12 months significantly decreased levels of bone turnover marker CTX  
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10 213 (compared to placebo) in postmenopausal women. BMD loss at total hip and lumbar  
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12 214 spine was significantly lower in the treatment group.

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14 215 Bone loss occurs throughout life following maturation, and is accelerated following  
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16 216 menopause in women <sup>20</sup>. Postmenopausal women have an increased risk of fragility  
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18 217 fractures. Using a naturally-occurring bacterium to significantly reduce the annual  
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20 218 bone loss in this group of patients is a new concept that could lead to a paradigm shift  
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22 219 in osteoporosis prevention. Previous studies in rodents have demonstrated that  
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24 220 supplementation with specific bacterial strains decreased bone loss and improved  
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26 221 bone mineral density <sup>21-23</sup>. Kim et al. reported that the administration of *Lactobacillus*  
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28 222 *casei* 393 significantly increased BMD in ovariectomized rats <sup>24</sup>. For the first time, the  
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30 223 present meta-analysis systemically demonstrated that this probiotic also works in  
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32 224 humans.

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34 225 The vertebrae and metaphyses of long bones, rich in trabecular bone, have a higher  
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36 226 turnover rate than do cortical bones in the axis of long bones. Therefore, medications  
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38 227 and diseases affecting lumbar spine and hip are identified earlier than in other skeletal  
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40 228 segments <sup>25</sup>. The vertebrae and hips are easily accessible for measuring BMD.  
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42 229 Therefore, the lumbar spine and hip BMD were suitable primary outcome variables in  
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44 230 the present studies. McCabe et al. <sup>26</sup> showed that oral administration of *Lactobacillus*  
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46 231 probiotics identified a 45% increase in hip and vertebral trabecular bone volume  
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48 232 fraction in male mice. In another study, the administration of *Lactobacillus plantarum*  
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50 233 and *Lactobacillus paracasei* to ovariectomized mice showed increased trabecular  
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52 234 number compared to sham-ovariectomized control groups <sup>27</sup>. Our meta-analysis  
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54 235 showed, in the probiotics group, both total hip and lumbar vertebrae BMD were at a  
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56 236 significantly high levels than those of the control.

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58 237 Because BMD depends on the dynamic balance of bone formation and resorption,  
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60 238 bone turnover markers are also very important parameters analyzed in our  
239 meta-analysis. The measurement of CTX has been taken as a marker of bone

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4 240 resorption; it is produced by osteoclasts during bone resorption <sup>28</sup>. Therefore, the  
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6 241 increased levels of serum CTX indicated increased bone resorption. Subgroup  
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8 242 included 3 RCT studies, suggesting that ingestion of probiotic supplements  
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10 243 significantly reduced the bone resorption marker CTX. Another study from Japan <sup>16</sup>  
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12 244 showed that the probiotics group had significantly lower uNTx (urinary type I  
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14 245 collagen cross-linked N-telopeptide) levels than did the placebo group at 12 weeks of  
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16 246 treatment. uNTx is another fragment of type I collagen generated during resorption  
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18 247 detected in urine; therefore, this also suggested that probiotics inhibit bone resorption  
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20 248 by suppressing osteoclast activity. BALP is another well-known bone turnover  
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22 249 marker, an indicator of osteoblast proliferation that is thought to be a marker of bone  
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24 250 formation <sup>29</sup>. However, the present meta-analysis showed no significant changes in  
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26 251 BALP. Similarly, no differences were detected in levels of biochemical markers for  
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28 252 bone metabolic indices (OPG, OC).

29 253 Probiotics have many functional properties in humans. They function in the  
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31 254 gastrointestinal system by modifying the microbiota composition, intestinal barrier  
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33 255 function, and the immune system which feeds back systemic benefits to the host,  
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35 256 including bone health. Some can be used in intestinal infections and treatment of  
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37 257 diarrhea, because they not only tolerate low PH environment but also colonize the  
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39 258 human colon, adhering to the gastrointestinal tract, with antimicrobial effects <sup>30</sup>.  
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41 259 Moreover, probiotic function modifying physiological homeostasis of the intestinal  
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43 260 flora can also benefit bone metabolism <sup>31</sup>. Many studies have looked at changes of  
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45 261 gastrointestinal flora during aging, which may alter mineral absorption.  
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47 262 Gastrointestinal inflammation and systemic inflammation are closed related to  
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49 263 enhanced generation of potent osteoclastogenic cytokines as the main cause of bone  
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51 264 loss <sup>32-33</sup>. Probiotics can restore balance of the gut microbiota, preventing or  
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53 265 moderating gut and systemic inflammation and allowing absorption of nutrients,  
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55 266 especially in elderly people <sup>34</sup>. Probiotics may restore microbiota composition through  
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57 267 several mechanisms. They act in the gastrointestinal tract simply by proliferation, as  
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59 268 well as by ability inhibiting other flora. Furthermore, probiotics turn complex  
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269 carbohydrates to oligosaccharides <sup>35</sup>, which can be used by other bacteria, indirectly

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4 270 improving the balance of microflora. Furthermore, probiotics decrease the levels of  
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6 271 inflammatory mediators and cytokines in the gut and bone marrow <sup>36</sup>. These changes  
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8 272 give signals to bone cells, including osteoblasts, osteoclasts and stem cells,  
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10 273 significantly affecting bone homeostasis. Endocrine factors (such as serotonin and  
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12 274 incretins) secreted by intestine also remarkably affect bone cells <sup>37</sup>.

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14 275 Anti-inflammatory effects are among the underlying mechanisms by which  
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16 276 probiotics benefit bone metabolism. There is evidence that arginine deiminase,  
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18 277 produced by the probiotic *Lactobacillus brevis* CD2, has an anti-inflammatory effect  
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20 278 <sup>38</sup>. Supplementation of probiotics may reduce expression of pro-inflammatory and  
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22 279 osteolytic cytokines, including TNF- $\alpha$ . These cytokines alter anti-osteoclastogenic  
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24 280 cytokine expression, leading to enhanced osteoclast formation and inhibited osteoblast  
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26 281 activity <sup>39</sup>. Some studies found that probiotic supplementation reduces TNF $\alpha$ , IL-17,  
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28 282 and RANKL expression levels in ovariectomized mice <sup>40</sup>. These changes give signals  
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30 283 to bone cells, such as osteoblasts, osteoclasts and stem cells, which significantly affect  
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32 284 bone homeostasis. In this meta-analysis, TNF- $\alpha$  was reported by two RCTs. One  
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34 285 reported <sup>17</sup> that serum levels of TNF- $\alpha$  were significantly lower in the probiotic-treated  
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36 286 group; however, another study <sup>18</sup> showed there was no differences between probiotic  
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38 287 and control groups. More clinical trials are needed in the future to elucidate the  
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40 288 relationship between administration of probiotics and anti-inflammatory effects.

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42 289 Our study has some limitations. First, only four randomized controlled trials  
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44 290 satisfied our inclusion criteria. The limited number of reports focusing on the  
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46 291 association between probiotic supplement and BMD and bone turnover markers  
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48 292 prevented us from conducting subgroup analysis and drawing conclusive summaries.  
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50 293 Furthermore, insufficient number of estimates inflate the impact of the results of a  
51  
52 294 particular study. Second, although, meta-regression was used to determine that  
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54 295 various types of probiotic supplement did not have an impact on the pooled results,  
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56 296 dosage design and course of treatment could also introduce bias. Third, in Lambert's  
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58 297 study <sup>19</sup>, probiotics plus soflavones were used as a treatment regimen, rather than  
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60 298 probiotics alone. This may cause some bias; however, we did not want to ignore this  
299 valuable study. Third, the units describing BMD change were inconsistent among the

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4 300 four reports. Nilsson's study <sup>17</sup> applied T score to describe BMD change, while other  
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6 301 three studies used g/cm<sup>2</sup> instead. We could only calculate SMD rather than weighted  
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8 302 mean difference (WMD). Thus, our results of meta-analysis should be interpreted  
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10 303 with caution.

11 304 Our research also has some strengths. First, to our knowledge, this is the first  
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13 305 meta-analysis describing the evidence of the association of probiotic supplements and  
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15 306 bone status in postmenopausal women. Second, there is little heterogeneity between  
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17 307 included articles and fixed-effects model used to calculate the results. Third, all  
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19 308 included randomized controlled trials were of high quality for analysis.

### 21 309 **Conclusion**

22  
23 310 Our systematic review and meta-analysis showed that probiotic supplementations in  
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25 311 postmenopausal women were associated with preserving BMD and attenuating bone  
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27 312 resorption. Appropriate supplement of probiotic could be recommended to improve  
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29 313 bone status in postmenopausal women.

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43  
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45  
46 322 analysis

### 48 323 **Contributors**

49  
50 324 M Cai and J Yu conceived and designed the meta analysis; J Wu, G Cao, S Yuan and  
51  
52 325 Cong Luo searched the literature; J Yu, G Cao, and S Yuan analysed the data; X Cai  
53  
54 326 contributed analysis tools; J Yu and G Cao wrote the paper; X Cai and M Cai revised  
55  
56 327 the manuscript.

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59  
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330 **Competing interests**

331 The authors declare no conflicts of interest. This study does not contain human  
332 participants or animals.

333 **Patient consent for publication**

334 Not required

335 **Provenance and peer review**

336 Not commissioned; externally peer reviewed

337 **Data availability statement**

338 All data relevant to the study are included in the article or uploaded as supplementary  
339 information. No additional data available.

340 **Patient and public involvement**

341 No patient involved

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Table 1. Characteristics of included randomized controlled trials in the meta-analysis

| Study      | Year | Area    | Age (year)         | Blinding     | Type of probiotic supplement                  | Number of T | Number of P | Course of treatment (months) | dose design                      | Minerals intake                                  | BMD                 | BTM                             |
|------------|------|---------|--------------------|--------------|---|-------------|-------------|------------------------------|----------------------------------|--|---------------------|---------------------------------|
| TAKIMOTO   | 2018 | Japan   | T: 57.5<br>P: 57.8 | double-blind | bacillus subtilis C-3102                      | 31          | 30          | 6                            | 3.4×10 <sup>9</sup> CFU /d       | Estimated by BDHQ                                | hip<br>lumbar spine | CTX                             |
| Nilsson    | 2018 | Sweden  | T: 76.4<br>P: 76.3 | double-blind | lactobacillus reuteri 6475                    | 32          | 36          | 12                           | 5x10 <sup>9</sup> CFU twice/d    | Estimated by astandardize d questionnaire        | hip<br>lumbar spine | CTX<br>BALP<br>TNF              |
| Jafarnejad | 2017 | Iran    | T: 58.9<br>P: 57.3 | double-blind | seven probiotic bacteria species <sup>#</sup> | 20          | 21          | 6                            | one Gerilact capsule /d          | 500 mg Ca plus 200 IU vitamin D daily            | hip<br>lumbar spine | CTX<br>BALP<br>OPG<br>OC<br>TNF |
| Lambert    | 2017 | Denmark | T: 60.8<br>P: 62.9 | double-blind | lactic acid bacteria and soflavones           | 38          | 40          | 12                           | 60mg isoflavone and probiotics/d | 1200 mg Ca, 550 mg Mg, and 25mg calcitriol daily | hip<br>lumbar spine | CTX<br>OPG<br>OC                |

BDHQ: a brief-type self-administered diet history questionnaire; BMD: bone mineral density; BTM: bone turnover marker; CFU: colony-forming unit; CTX: collagen type 1 cross-linked C-telopeptide; BALP: bone-specific alkaline phosphatase; OPG: osteoprotegerin; OC: osteocalcin; P: placebo group; T: treat group; TNF: tumor necrosis factor; RCE: red clover extract which is rich in isoflavone aglycones and probiotics; # Lactobacillus casei 1.3 x 10<sup>10</sup> colony-forming units[CFU], Bifidobacterium longum 5 x 10<sup>10</sup> CFU, Lactobacillus acidophilus 1.5 x 10<sup>10</sup> CFU, Lactobacillus rhamnosus 3.5 x 10<sup>9</sup> CFU, Lactobacillus bulgaricus 2.5 x 10<sup>8</sup>

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5 CFU, Bifidobacterium breve  $1 \times 10^{10}$  CFU, and Streptococcus thermophilus  $1.5 \times 10^8$  CFU per 500 mg.  
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3 Figure 1. Flow diagram of the studies search process

4 Figure 2. Forest plots of meta-analysis on probiotics supplements and total hip BMD

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6 Figure 3. Forest plots of meta-analysis on probiotics supplements and lumbar spine BMD

7 Figure 4. Forest plots of meta-analysis on probiotics supplements and bone turnover markers

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10 Supplementary Figure 1. Funnel plots of meta-analysis on probiotics supplements and BMD: A.  
11 total hip BMD; B. lumbar spine BMD.

12 Supplementary Figure 2. Sensitivity analyses of meta-analysis on probiotics supplements and  
13 BMD: A. total hip BMD; B. lumbar spine BMD.  
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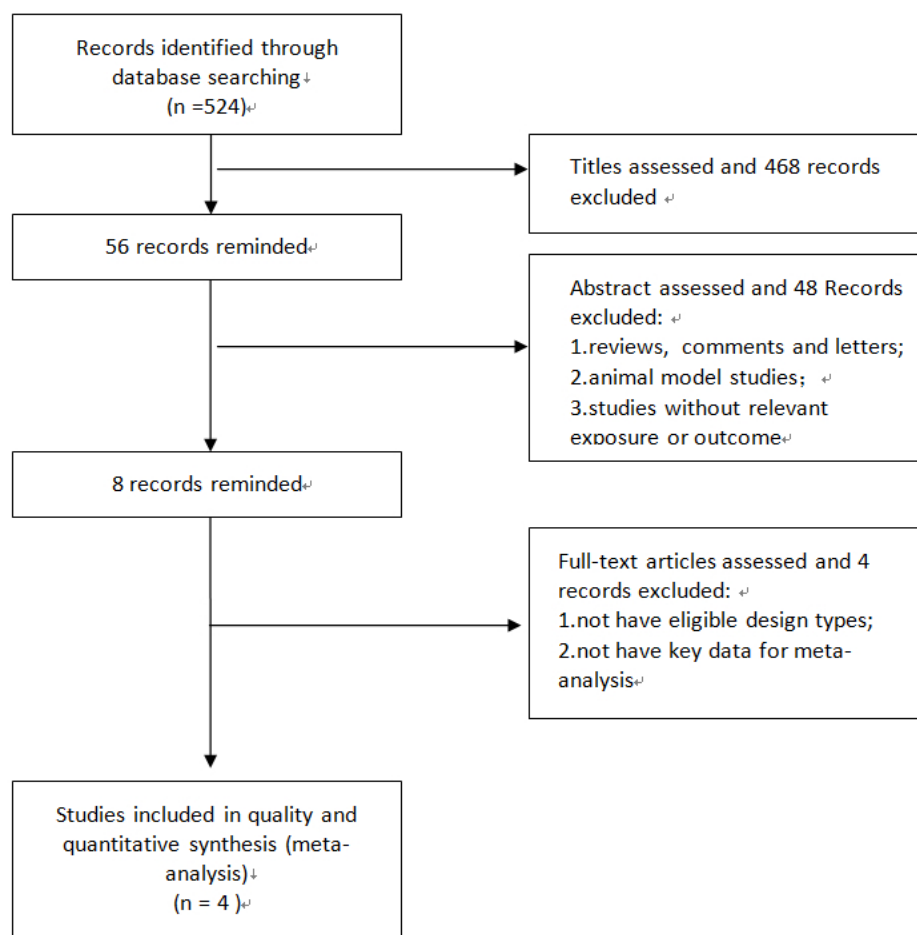


Figure 1. Flow diagram of the studies search process

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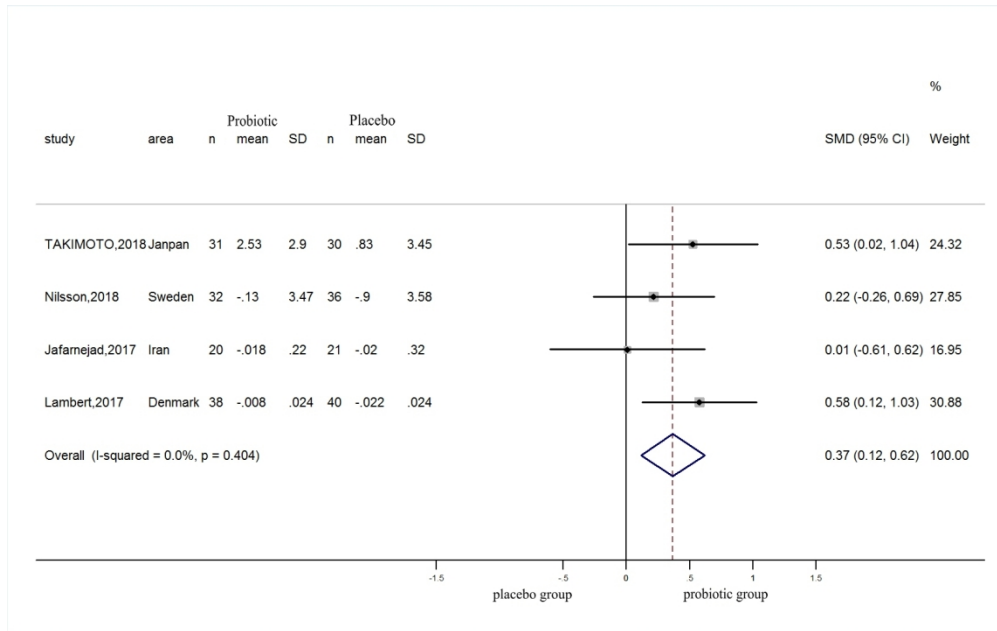


Figure 2. Forest plots of meta-analysis on probiotics supplements and total hip BMD

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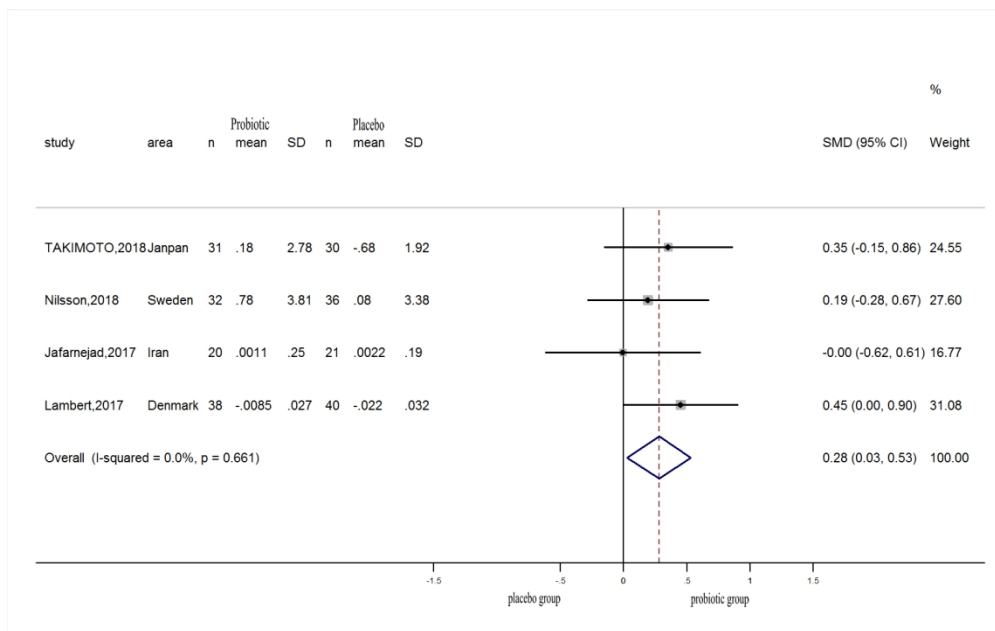


Figure 3. Forest plots of meta-analysis on probiotics supplements and lumbar spine BMD



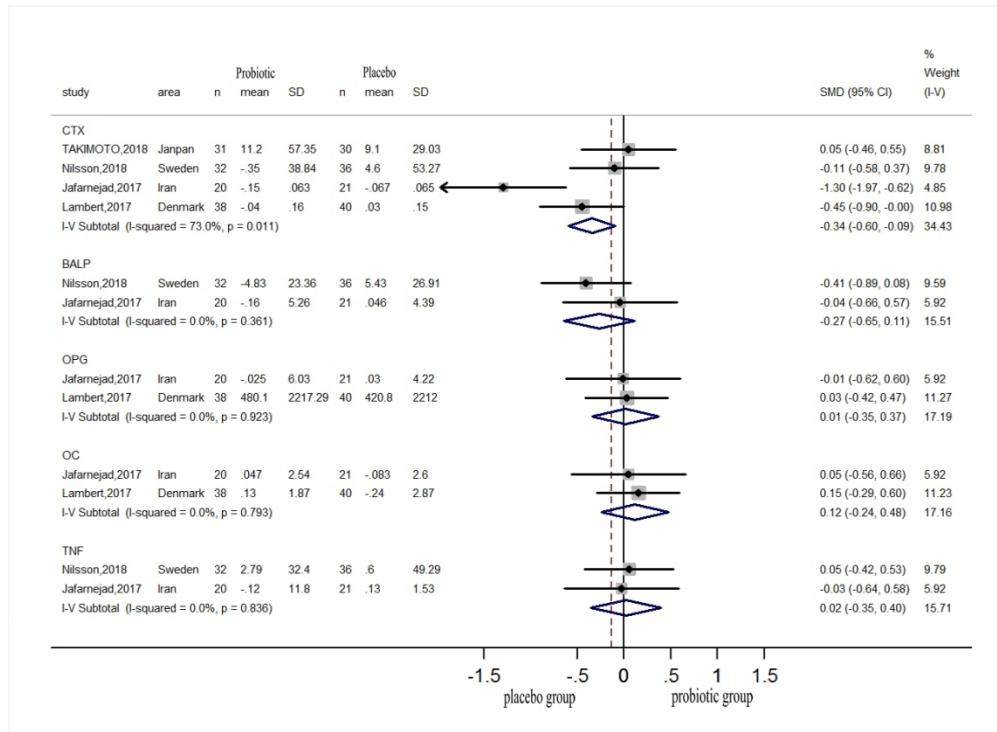
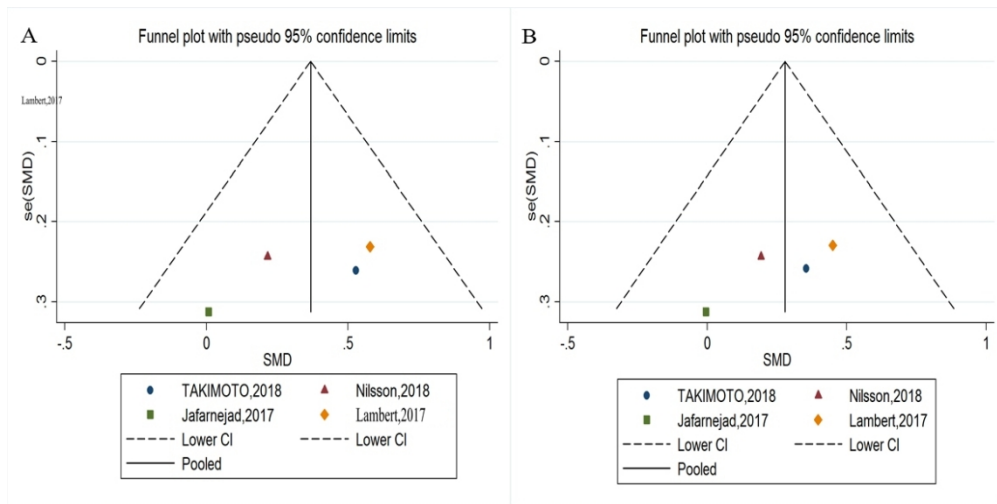
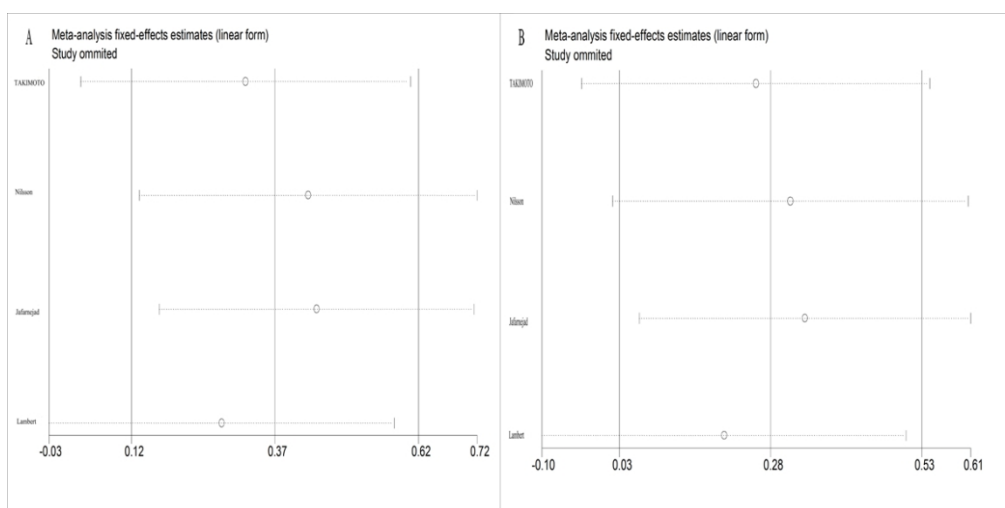


Figure 4. Forest plots of meta-analysis on probiotics supplements and bone turnover markers

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# PRISMA 2009 Checklist

| Section/topic                      | #  | Checklist item  | Reported on page #  |
|------------------------------------|----|---|---|
| <b>TITLE</b>                       |    |   |   |
| Title                              | 1  | Identify the report as a systematic review, meta-analysis, or both.   | 1   |
| <b>ABSTRACT</b>                    |    |   |   |
| Structured summary                 | 2  | Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number. | 2   |
| <b>INTRODUCTION</b>                |    |   |   |
| Rationale                          | 3  | Describe the rationale for the review in the context of what is already known.  | 3-4   |
| Objectives                         | 4  | Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).  | 3-4   |
| <b>METHODS</b>                     |    |   |   |
| Protocol and registration          | 5  | Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.   | <a href="http://www.crd.york.ac.uk/PROSPERO/">http://www.crd.york.ac.uk/PROSPERO/</a> |
| Eligibility criteria               | 6  | Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.  | 4   |
| Information sources                | 7  | Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.  | 4   |
| Search                             | 8  | Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.   | 4   |
| Study selection                    | 9  | State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).   | 4   |
| Data collection process            | 10 | Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.  | 4   |
| Data items                         | 11 | List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.   | 4   |
| Risk of bias in individual studies | 12 | Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.  | 4   |
| Summary measures                   | 13 | State the principal summary measures (e.g., risk ratio, difference in means).   | 4-5   |



# PRISMA 2009 Checklist

|                      |    |   |     |
|----------------------|----|---|-----|
| Synthesis of results | 14 | Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., $I^2$ ) for each meta-analysis. | 4-5 |
|----------------------|----|---|-----|

Page 1 of 2

| Section/topic                 | #  | Checklist item   | Reported on page # |
|-------------------------------|----|--|--------------------|
| Risk of bias across studies   | 15 | Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).   | 4-5                |
| Additional analyses           | 16 | Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.   | 4-5                |
| <b>RESULTS</b>                |    |  |                    |
| Study selection               | 17 | Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.  | 5                  |
| Study characteristics         | 18 | For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.   | 5                  |
| Risk of bias within studies   | 19 | Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).  | 5-6                |
| Results of individual studies | 20 | For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot. | 5-6                |
| Synthesis of results          | 21 | Present results of each meta-analysis done, including confidence intervals and measures of consistency.  | 5-6                |
| Risk of bias across studies   | 22 | Present results of any assessment of risk of bias across studies (see Item 15).  | 5-6                |
| Additional analysis           | 23 | Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).  | 5-6                |
| <b>DISCUSSION</b>             |    |  |                    |
| Summary of evidence           | 24 | Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).                     | 6-10               |
| Limitations                   | 25 | Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).  | 6-10               |
| Conclusions                   | 26 | Provide a general interpretation of the results in the context of other evidence, and implications for future research.  | 6-10               |
| <b>FUNDING</b>                |    |  |                    |
| Funding                       | 27 | Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.   | n/a                |



# PRISMA 2009 Checklist

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Page 2 of 2

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# BMJ Open

## Probiotic supplements and bone health in postmenopausal women: a meta-analysis of randomized controlled trials

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| <b>Primary Subject Heading</b>: | Evidence based practice   |
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1 **Probiotic supplements and bone health in postmenopausal**  
2 **women: a meta-analysis of randomized controlled trials**

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13 # The first two authors contributed equally to the work.

## 30 **Abstract**

31 **Objective:** Osteoporosis is a common disease in postmenopausal women. Several  
32 studies have analyzed the associations between dietary supplementation with  
33 probiotics and bone health in postmenopausal women, but the results are still  
34 controversial. We conducted this meta-analysis to assess the effects of probiotics  
35 supplement on bone mineral density (BMD) and bone turnover markers for  
36 postmenopausal women.

37 **Design:** systematic review and meta-analysis.

38 **Methods:** We systematically searched PubMed, EMBASE, and the Cochrane Library  
39 from their inception to November 2020 for randomized controlled trials (RCTs)  
40 assessing probiotic supplements and osteoporosis in postmenopausal women.  
41 Study-specific risk estimates were combined using random-effect models.

42 **Results:** Five RCTs (n = 497) were included. Probiotic supplements were associated  
43 with a significantly higher BMD in the lumbar spine (standardized mean difference,  
44 SMD = 0.27, 95% CI: 0.09–0.44) than in control. There was no difference between  
45 probiotic supplements and BMD in hips (SMD = 0.22, 95% CI: -0.07 – 0.52).  
46 Collagen type 1 cross-linked C-telopeptide (CTX) levels in the treatment groups were  
47 significantly lower than those of the placebo group (SMD = -0.34, 95% CI: -0.60 –  
48 -0.09). In subgroup meta-analysis, levels of bone-specific alkaline phosphatase  
49 (BALP), osteoprotegerin (OPG), osteocalcin (OC), and tumor necrosis factor (TNF)  
50 did not differ between the probiotic and placebo groups.

51 **Conclusions:** Supplementation with probiotics increases lumbar BMD and reduces  
52 bone resorption. More randomized controlled trials are recommended to validate these  
53 results.

## 52 **Strengths and limitations of this study**

54 This is the first meta-analysis on the effectiveness of probiotic supplements on bone  
55 status in postmenopausal women.  
56

57 We included only high-quality randomized controlled trials to improve the level of  
58 evidence.  
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4 60 These results provide new insights into the association between probiotic supplements  
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6 61 lumbar spine bone mineral density

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8 62 The limited number of reports prevented us from conducting subgroup analysis and  
9  
10 63 made it difficult to draw firm conclusions.

11 64

12  
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14  
15 66 **Keywords:** probiotics supplement; bone mineral density; bone turnover markers;  
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17 67 postmenopausal; meta-analysis

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## 90 Introduction

91 Osteoporosis is characterized by low bone mineral density (BMD) and deteriorated  
92 bone microstructure, leading to reduced bone strength and increased susceptibility to  
93 fractures<sup>1</sup>. Osteoporosis and fracture occur commonly in postmenopausal women,  
94 who experience a natural decline in endogenous estrogen, reducing BMD (on average  
95 2%–5% BMD/y)<sup>2</sup> and adverse effects on bone microarchitecture.

96 Currently, many medications are used in osteoporosis to decrease bone resorption  
97 or increase bone formation. Large randomized controlled trials (RCTs) showed that  
98 estrogen therapy (such as red clover isoflavone supplementation) was effective for  
99 preventing and treating osteoporosis in postmenopausal women<sup>3-5</sup>. However, this  
100 remains controversial because of the increased risk of cancer, including endometrial,  
101 breast, and ovarian cancer<sup>6</sup>. Nevertheless, other anti-resorptive agents are not widely  
102 used because of their side-effects, high prices, and poor compliance on the part of  
103 patients; these include bisphosphonates, calcitonin, and raloxifene. Therefore,  
104 complementary and dietary therapies are more acceptable to some patients. Also,  
105 natural treatments are increasingly requested by patients.<sup>7</sup> It was shown that calcium  
106 and vitamin D supplements effectively improved bone microarchitecture and health<sup>8</sup>;  
107 however, supplementation with calcium and vitamin alone is not sufficient to halt  
108 menopausal bone loss<sup>9</sup>.

109 Therefore, alternative ways to prevent and treat osteoporosis are sought. Probiotics  
110 are popular dietary therapies that have favorable effects on the skeletal system.<sup>10</sup>  
111 Probiotics are "live microorganisms that when administered in adequate amounts will  
112 confer a health benefit on the host" defined by the Food and Agricultural  
113 Organization/World Health Organization (FAO/WHO)<sup>11</sup>, such as bacillus subtilis,  
114 lactobacillus, and other mixed strains. They are affordable and have fewer  
115 side-effects.

116 To our knowledge, there has been no systematic review or meta-analysis of RCTs  
117 with probiotics in the treatment arms, analyzing the effect of probiotics in  
118 postmenopausal-related osteoporosis. Therefore, this systematic review and  
119 meta-analysis were performed to provide an overview of the effects of dietary

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4 120 probiotic supplements in postmenopausal related bone resorption in women and to  
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6 121 inform researchers of new potential sources of bias to be addressed in future clinical  
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8 122 trials.

## 9 123 **Methods and analysis**

### 10 124 **Data sources and search strategies**

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13 125 A literature search of relevant studies was performed in PubMed, EMBASE, and  
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15 126 the Cochrane Library. A comprehensive search strategy was developed. The protocol  
16  
17 127 was drafted according to the PRISMA statement<sup>12</sup>. The keywords were as follows:  
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19 128 'probiotics', 'probiotic supplement', 'bone,' 'osteoporosis', 'osteopenia', 'bone mineral  
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21 129 density', 'bone turnover', and 'postmenopausal' (search queries available in  
22  
23 130 Supplementary Table 1). References of retrieved articles were also scanned to identify  
24  
25 131 any additional relevant studies. Two independent reviewers (Jiawei Yu and Gaoyang  
26  
27 132 Cao) conducted this work. Discrepancies were resolved by consensus of the two  
28  
29 133 reviewers. If required, the final disposition was determined by Ming Cai.

### 30 134 **Inclusion and exclusion criteria**

31  
32  
33 135 Inclusion criteria are as follows: (1) randomized controlled trials and prospective  
34  
35 136 cohort studies; (2) consideration of postmenopausal women as patients, consideration  
36  
37 137 of probiotic supplement as interventions, consideration of placebo as a comparison,  
38  
39 138 and consideration of the change of BMD and bone turnover markers (BTM) as  
40  
41 139 outcomes; (3) BMD was measured by dual-energy X-ray absorptiometry (DXA) and  
42  
43 140 BTM was measured using blood tests at baseline, and the end of trial; (4)  
44  
45 141 administered probiotics for more than 6 months; and (5) English language original  
46  
47 142 articles indexed up to November 2020.

48  
49 143 Exclusion criteria are as follows: (1) absence of critical data for meta-analysis; and  
50  
51 144 (2) low-quality articles according to Cochrane checklist.

### 52 145 **Data extraction and quality assessment**

53  
54 146 The characteristics of the relevant articles were extracted and recorded  
55  
56 147 independently by two reviewers (Jiawei Yu and Gaoyang Cao) as follows: first  
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58 148 author's name, year, area, age (mean or range), type of probiotic supplement, dose  
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60 149 design, course of treatment, number of cases, number of controls, and bone status (as

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4 150 shown in Table 1). The Cochrane Collaboration's tool <sup>13</sup> was used for assessing the  
5  
6 151 risk of bias. Six domain-based evaluations (selection bias, performance bias, detection  
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8 152 bias, attrition bias, reporting bias, and other bias) were used in the tool to assess the  
9  
10 153 possible bias of randomized controlled trials. The results were displayed as low risk,  
11  
12 154 unclear risk, or high risk of bias (available in Supplementary Table 2).

### 13 155 **Statistical analysis**

14  
15 156 The mean relative change from baseline to the end of the course and standard  
16  
17 157 deviation (SD) were used to express the effect of the probiotic supplement on bone  
18  
19 158 status in postmenopausal women. If the original studies did not provide the mean  
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21 159 relative change and standard deviation, we converted the data using a common  
22  
23 160 method <sup>14-15</sup>. The pooled effects of included studies were expressed in terms of  
24  
25 161 standardized mean difference (SMD) with 95% confidence interval (CI). Q test and  $I^2$   
26  
27 162 index were used to evaluate heterogeneity among the included results.  
28  
29 163 Meta-regression was conducted to determine whether different types of probiotic  
30  
31 164 supplements would introduce sources of heterogeneity. Random-effects model and  
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33 165 subgroup analysis were used in the face of heterogeneity. Forest plots and funnel plots  
34  
35 166 were produced, and publication bias was tested using Begg's test and the weighted  
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37 167 Egger test <sup>16-17</sup>. Sensitivity analysis was conducted to verify the impact of each study  
38  
39 168 on the pooled results. In the sensitivity analyses, each study was omitted to recalculate  
40  
41 169 the pooled estimates. All analysis was performed using STATA 12.0 (StataCorp LP,  
42  
43 170 College Station, TX, USA).

### 44 171 **Patient and public involvement**

45  
46 172 Patient and public involvement is not applicable for this meta-analysis.

### 47 173 **Results**

#### 48 174 **Search results and characteristics of identified studies**

49  
50 175 A total of 604 articles were identified from the initial searches of PubMed and  
51  
52 176 EMBASE, and 547 articles were removed because of absence of relevance. Nine  
53  
54 177 articles were retained after reviewing the abstract according to the exclusion criteria.  
55  
56 178 Finally, five randomized controlled trials<sup>18-22</sup> satisfied the inclusion criteria and  
57  
58 179 entered this meta-analysis after full-text review. A detailed overview of the selection  
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180 process is outlined in Figure 1.

181 A total of 497 postmenopausal women completed these trials. Among the five  
182 trials, two were conducted in Asia (one in Japan<sup>18</sup>, the other in Iran<sup>20</sup>), and the other  
183 three were in Europe (two in Sweden<sup>19 22</sup>, the last one in Denmark<sup>21</sup>). All trials were  
184 randomized using the double-blinded method. Each trial identified the type of  
185 probiotic supplements used and described the dosage design. Three studies considered  
186 treatment with probiotics only<sup>18-20</sup>, while the other two studies included treatment  
187 with combined isoflavone and probiotics<sup>21 22</sup>. All studies provided BMD data from  
188 DXA scans at the lumbar spine and total hip. Collagen type 1 cross-linked  
189 C-telopeptide (CTX), bone-specific alkaline phosphatase (BALP), osteoprotegerin  
190 (OPG), osteocalcin (OC), and tumor necrosis factor (TNF) were used as bone  
191 turnover markers. Details of the characteristics are displayed in Table 1 and  
192 Supplementary Table 3.

### 193 **Probiotic supplements and lumbar spine BMD**

194 A total of five estimates were included in the meta-analysis. The meta-regression  
195 results also showed no source of heterogeneity from various types of probiotics ( $P =$   
196  $0.987$ ). Therefore, the five estimates were incorporated into the pooled analysis.  
197 Compared to the placebo group, the lumbar spine BMD level of the supplementary  
198 group was higher (SMD = 0.27, 95% CI: 0.09 – 0.44), with no heterogeneity ( $P =$   
199  $0.805$ ;  $I^2 = 0.0$ ) (Figure 2). The funnel plot was symmetrical (Supplementary Figure 1)  
200 and excluded publication bias (Begg's test  $z_c = 0.73$ ,  $P = 0.462$ ; Egger's test  $t = -0.22$ ,  
201  $P = 0.843$ ). Sensitivity analyses indicated that the positive result was robust.  
202 (Supplementary Figure 2).

### 203 **Probiotics supplements and total hip BMD**

204 Overall, five estimates of the association between probiotics supplement and hip  
205 BMD were included in the meta-analysis. The meta-regression results revealed that  
206 various types of probiotics were not a source of heterogeneity ( $P = 0.237$ ). Therefore,  
207 we brought the five estimates into the pooled analysis. There was no difference  
208 between probiotic supplements and BMD in hips (SMD = 0.22, 95% CI: -0.07 –  
209 0.52), with no heterogeneity ( $P = 0.055$ ;  $I^2 = 56.8$ ) (Figure 3). The funnel plot is

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4 210 shown in Supplementary Figure 3; it was symmetrical, excluding publication bias  
5  
6 211 (Begg's test  $z_c = -0.24$ ,  $P = 1.00$ ; Egger's test  $t = 1.59$ ,  $P = 0.209$ ). Sensitivity analyses  
7  
8 212 indicated that the positive result was affected by the Jansson trial (Supplementary  
9  
10 213 Figure 4).

#### 214 **Probiotic supplements and bone turnover markers**

215 Four estimates of CTX and two estimates of BALP, OPG, OC, and TNF were  
216 incorporated into the pooled analysis. The results suggested that probiotic  
217 supplements help decrease the supplementary group's body CTX level compared with  
218 the placebo group (SMD = -0.34, 95% CI: -0.60 – -0.09) with substantial  
219 heterogeneity. There was no evidence that probiotic supplements were associated with  
220 BALP, OPG, OC, and TNF (Figure 4).

#### 221 **Discussion**

##### 222 **Main findings**

223 This meta-analysis provides evidence that dietary probiotics supplement can slow  
224 bone resorption in postmenopausal women. Daily supplementation with probiotics for  
225 24 weeks to 12 months significantly decreased bone turnover marker CTX (compared  
226 to placebo) in postmenopausal women. BMD loss at the lumbar spine was  
227 significantly lower in the treatment group.

228 Bone loss occurs throughout life following maturation and is accelerated following  
229 menopause in women<sup>23</sup>. Postmenopausal women have an increased risk of fragility  
230 fractures. Using a naturally-occurring bacterium to significantly reduce the annual  
231 bone loss in this group of patients is a new concept that could lead to a paradigm shift  
232 in osteoporosis prevention. Previous studies in animals demonstrated that  
233 supplementation with specific bacterial strains increases bone density and protect  
234 against osteoporosis<sup>24-26</sup>. Kim et al. reported that the administration of *Lactobacillus*  
235 *casei* 393 significantly increased BMD in ovariectomized rats<sup>27</sup>. For the first time, the  
236 present meta-analysis systemically demonstrated that this probiotic also works in  
237 humans.

238 The lumbar spine and hip are the most suitable organs to assess bone metabolism.  
239 The vertebrae and metaphyses of long bones, rich in trabecular bone, have a higher



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4 240 turnover rate than cortical bones in the axis of long bones. Therefore, medications and  
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6 241 diseases affecting the lumbar spine and hip are identified earlier than in other skeletal  
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8 242 segments<sup>28</sup>. The vertebrae and hips are easily accessible for measuring BMD.  
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10 243 Therefore, the lumbar spine and hip BMD were suitable primary outcome variables in  
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12 244 the present studies. McCabe et al<sup>29</sup> showed that oral administration of *Lactobacillus*  
13  
14 245 probiotics identified a 45% increase in hip and vertebral trabecular bone volume  
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16 246 fraction in male mice. In another study, the administration of *Lactobacillus plantarum*  
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18 247 and *Lactobacillus paracasei* to ovariectomized mice showed increased trabecular  
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20 248 number compared to sham-ovariectomized control groups<sup>30</sup>. Our meta-analysis  
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22 249 showed, in the probiotics group, both total hip and lumbar vertebrae BMD were at  
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24 250 significantly higher levels than those of the control.

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26 251 CTX and BALP were chosen as critical bone turnover markers. Because BMD  
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28 252 depends on the dynamic balance of bone formation and resorption, bone turnover  
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30 253 markers are also important parameters analyzed in our meta-analysis. The  
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32 254 measurement of CTX has been taken as a marker of bone resorption; osteoclasts  
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34 255 produce it during bone resorption<sup>31</sup>. Therefore, the increased levels of serum CTX  
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36 256 indicated increased bone resorption. Subgroup included 3 RCT studies, suggesting  
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38 257 that probiotic supplements' ingestion significantly reduced the bone resorption marker  
39  
40 258 CTX. Another study from Japan<sup>18</sup> showed that the probiotics group had significantly  
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42 259 lower uNTx (urinary type I collagen cross-linked N-telopeptide) levels than the  
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44 260 placebo group at 12 weeks of treatment. uNTx is another fragment of type I collagen  
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46 261 generated during resorption detected in urine; therefore, this also suggested that  
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48 262 probiotics inhibit bone resorption by suppressing osteoclast activity. BALP is another  
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50 263 well-known bone turnover marker, an indicator of osteoblast proliferation that is  
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52 264 thought to be a bone formation marker<sup>32</sup>. However, the present meta-analysis showed  
53  
54 265 no significant changes in BALP. Similarly, no differences were detected in levels of  
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56 266 biochemical markers for bone metabolic indices (OPG, OC).

### 267 **The mechanism of action**

57  
58 268 The mechanisms of action of probiotics are as follows. Probiotics have many  
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60 269 functional properties in humans. They function in the gastrointestinal system by

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4 270 modifying the microbiota composition, intestinal barrier function, and the immune  
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6 271 system, which feeds back systemic benefits to the host, including bone health.  
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8 272 Moreover, probiotic function modifying physiological homeostasis of the intestinal  
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10 273 flora can also benefit bone metabolism<sup>33</sup>. Gastrointestinal inflammation and systemic  
11  
12 274 inflammation are close to enhanced generation of potent osteoclastogenic cytokines as  
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14 275 the leading cause of bone loss<sup>34-35</sup>. Probiotics can restore the balance of the gut  
15  
16 276 microbiota, preventing or moderating gut and systemic inflammation and allowing  
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18 277 absorption of nutrients, especially in older adults<sup>36</sup>.

19 278 Furthermore, probiotics decrease levels of inflammatory mediators and cytokines  
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21 279 in the gut and bone marrow<sup>37</sup>. These changes give bone cell signals, including  
22  
23 280 osteoblasts, osteoclasts, and stem cells, significantly affecting bone homeostasis.  
24  
25 281 Endocrine factors (such as serotonin and incretins) secreted by the intestine also  
26  
27 282 remarkably affect bone cells<sup>38</sup>. Anti-inflammatory effects are among the underlying  
28  
29 283 mechanisms by which probiotics benefit bone metabolism. There is evidence that  
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31 284 arginine deiminase, produced by the probiotic *Lactobacillus brevis* CD2, has an  
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33 285 anti-inflammatory effect<sup>39</sup>. Supplementation of probiotics may reduce the expression  
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35 286 of pro-inflammatory and osteolytic cytokines, including TNF- $\alpha$ . These cytokines alter  
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37 287 anti-osteoclastogenic cytokine expression, leading to enhanced osteoclast formation  
38  
39 288 and inhibited osteoblast activity<sup>40</sup>. Some studies found that probiotic supplementation  
40  
41 289 reduces TNF $\alpha$ , IL-17, and RANKL expression levels in ovariectomized mice<sup>41</sup>.  
42  
43 290 These changes give bone cell signals, such as osteoblasts, osteoclasts, and stem cells,  
44  
45 291 significantly affecting bone homeostasis. More clinical trials are needed in the future  
46  
47 292 to elucidate the relationship between the administration of probiotics and  
48  
49 293 anti-inflammatory effects.

#### 50 294 **Limitations and Strengths**

51  
52 295 Our study has some limitations. First, only five randomized controlled trials with  
53  
54 296 specific population groups satisfied our inclusion criteria. The limited number of  
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56 297 reports and specific population groups focusing on the association between the  
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58 298 probiotic supplement and BMD and bone turnover markers prevented us from  
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60 299 conducting subgroup analysis and drawing conclusive summaries. Furthermore, the

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4 300 insufficient number of estimates inflates the impact of the results of a particular study.  
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6 301 Second, although meta-regression was used to determine that various types of  
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8 302 probiotic supplements did not impact the pooled results, dosage design and course of  
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10 303 treatment could also introduce bias. Third, in Lambert's study <sup>21</sup>, probiotics plus  
11  
12 304 isoflavones were used as a treatment regimen, rather than probiotics alone. This may  
13  
14 305 cause some bias; however, we did not want to ignore this valuable study. Third, the  
15  
16 306 units describing BMD change were inconsistent among the five reports. Nilsson's  
17  
18 307 study <sup>19</sup> and Jansson's study <sup>22</sup> applied T score to describe BMD change, while the  
19  
20 308 other three studies used g/cm<sup>2</sup> instead. We could only calculate SMD rather than the  
21  
22 309 weighted mean difference (WMD). Fifth, unfortunately, we did not find a relevant  
23  
24 310 prospective cohort for this meta-analysis. Thus, our results of a meta-analysis should  
25  
26 311 be interpreted with caution.

27 312 Our research also has some strengths. First, to our knowledge, this is the first  
28  
29 313 meta-analysis describing the evidence of the association of probiotic supplements and  
30  
31 314 bone status in postmenopausal women. Second, there is little heterogeneity between  
32  
33 315 the included articles and the fixed-effects model used to calculate the results. Third,  
34  
35 316 all included randomized controlled trials were of high quality.

#### 36 317 Implications and future research

37  
38 318 This systematic review and meta-analysis are useful for multidisciplinary clinicians  
39  
40 319 to evaluate their practices and make a proper clinical decision. The beneficial effects  
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42 320 of probiotic supplements may infect probiotic indication in postmenopausal women  
43  
44 321 with osteoporosis. More RCT studies from different regions are needed to validate our  
45  
46 322 argument and help answer research questions about probiotic supplements, dose, and  
47  
48 323 the optimal duration.

#### 49 324 **Conclusion**

50  
51 325 Our systematic review and meta-analysis showed that probiotic supplementations in  
52  
53 326 postmenopausal women were associated with preserving **lumbar spine** BMD and  
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55 327 attenuating bone resorption. An appropriate supplement of probiotics could be  
56  
57 328 recommended to improve bone status in postmenopausal women.  
58  
59 329

330

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333 the analysis.

**334 Contributors**

335 MC and JY(Jiawei Yu) conceived and designed the meta-analysis; GC, SY and CL  
336 searched the literature; JY(Jiawei Yu), GC, and SY analyzed the data; JY(Jiawei Yu)  
337 contributed analysis tools; JY(Jiawei Yu) and GC wrote the paper; JY(Jiafeng Yu)  
338 and MC revised the manuscript.

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**341 Competing interests**

342 The authors declare no conflicts of interest. This study does not contain human  
343 participants or animals.

**344 Patient consent for publication**

345 Not required

**346 Provenance and peer review**

347 Not commissioned; externally peer-reviewed

**348 Data availability statement**

349 All data relevant to the study are included in the article or uploaded as supplementary  
350 information. No additional data are available.

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For peer review only



Table 1. Characteristics of included randomized controlled trials in the meta-analysis

| Study      | Year | Area    | Age                | Blinding     | Type of probiotic supplement        | Number of Treatment | Number of Placebo | Course of treatment | BMD                 | BTM                             |
|------------|------|---------|--------------------|--------------|-------------------------------------|---------------------|-------------------|---------------------|---------------------|---------------------------------|
| Jansson    | 2019 | Sweden  | T: 59.1<br>P: 58.1 | double blind | three Lactobacillus strains*        | 126                 | 123               | 12 months           | lumbar spine<br>hip | N/A                             |
| Takimoto   | 2018 | Japan   | T: 57.5<br>P: 57.8 | double blind | bacillus subtilis C-3102            | 31                  | 30                | 6 months            | lumbar spine<br>hip | CTX                             |
| Nilsson    | 2018 | Sweden  | T: 76.4<br>P: 76.3 | double blind | lactobacillus reuteri 6475          | 32                  | 36                | 12 months           | lumbar spine<br>hip | CTX<br>BALP<br>TNF              |
| Jafarnejad | 2017 | Iran    | T: 58.9<br>P: 57.3 | double blind | seven probiotic bacteria species#   | 20                  | 21                | 6 months            | lumbar spine<br>hip | CTX<br>BALP<br>OPG<br>OC<br>TNF |
| Lambert    | 2017 | Denmark | T: 60.8<br>P: 62.9 | double blind | lactic acid bacteria and soflavones | 38                  | 40                | 12 months           | lumbar spine<br>hip | CTX<br>OPG<br>OC                |

BMD: bone mineral density; BTM: bone turnover marker; CTX: collagen type 1 cross-linked C-telopeptide; BALP: bone-specific alkaline phosphatase; OPG: osteoprotegerin; OC: osteocalcin; TNF: tumor necrosis factor; N/A: not available; \* Lactobacillus paracasei DSM 13434, Lactobacillus plantarum DSM 15312, and Lactobacillus plantarum DSM 15313; # Lactobacillus casei, Bifidobacterium longum, Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacillus bulgaricus, Bifidobacterium breve, and Streptococcus thermophilus.

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4 Figure 1. Flow diagram of the studies search process

5 Figure 2. Forest plots of meta-analysis on probiotics supplements and lumbar spine BMD

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7 Figure 3. Forest plots of meta-analysis on probiotics supplements and hip BMD

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9 Figure 4. Forest plots of meta-analysis on probiotics supplements and bone turnover markers

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13 Supplementary Figure 1. Funnel plots of meta-analysis on probiotics supplements and lumbar  
14 spine BMD

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17 Supplementary Figure 2. Sensitivity analyses of meta-analysis on probiotics supplements and  
18 lumbar spine BMD

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20 Supplementary Figure 3. Funnel plots of meta-analysis on probiotics supplements and hip  
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23 Supplementary Figure 4. Sensitivity analyses of meta-analysis on probiotics supplements and  
24 hip BMD

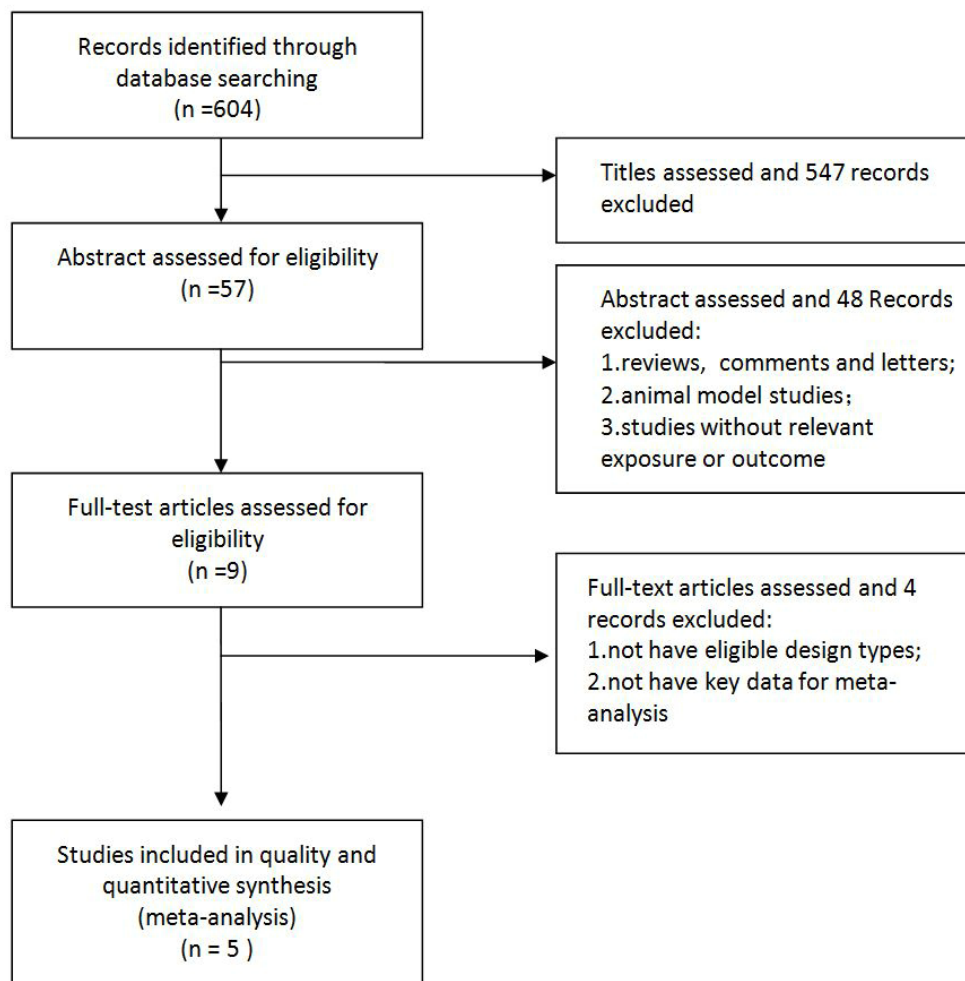


Figure 1. Flow diagram of the studies search process

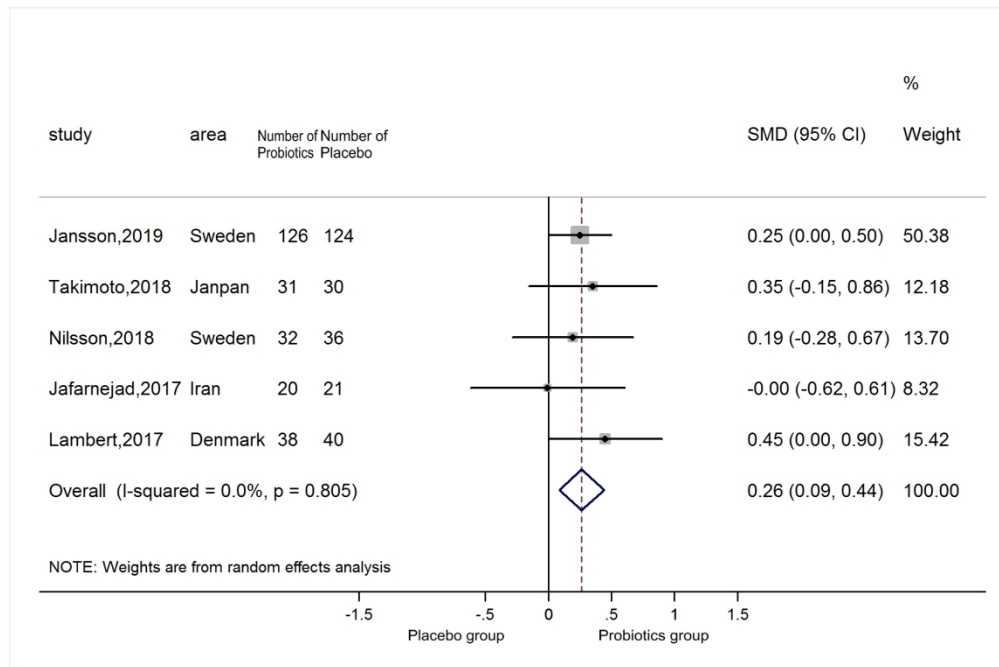


Figure 2. Forest plots of meta-analysis on probiotics supplements and lumbar spine BMD

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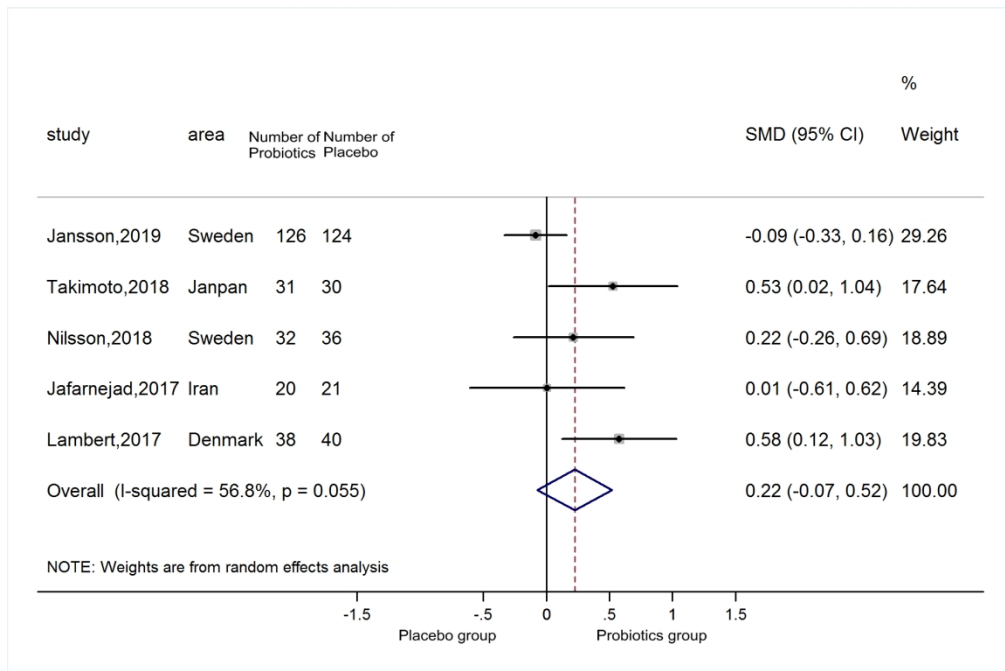
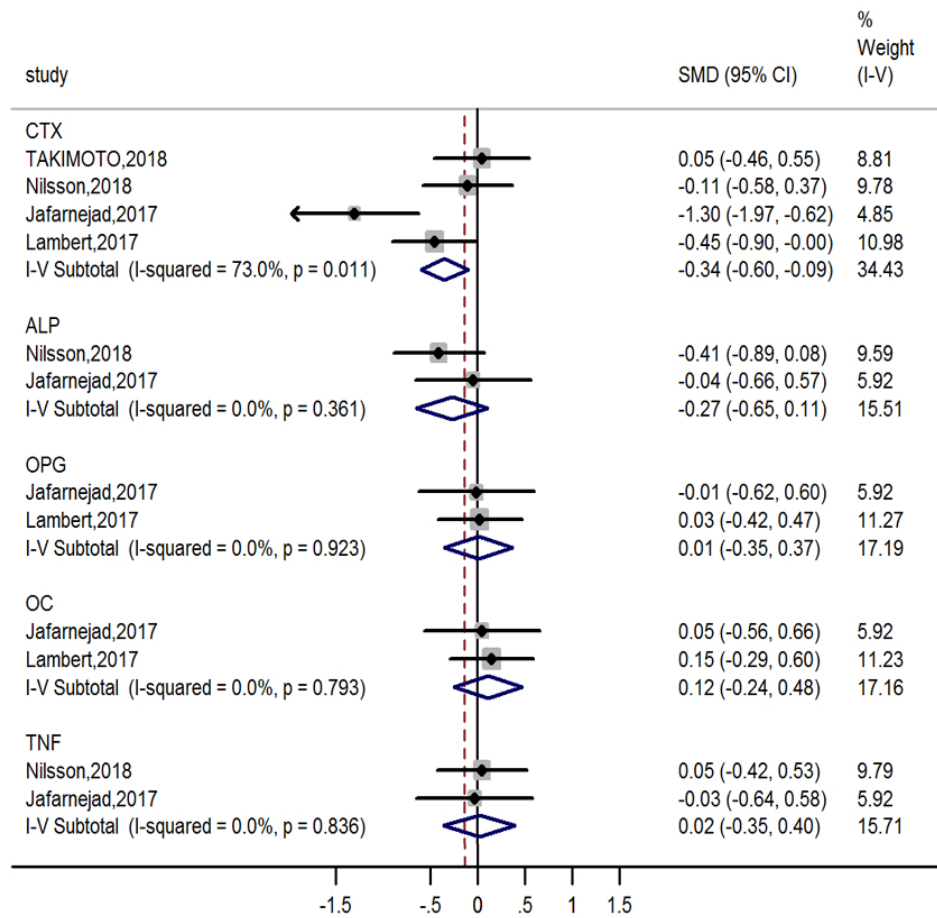


Figure 3. Forest plots of meta-analysis on probiotics supplements and hip BMD



37 Figure 4. Forest plots of meta-analysis on probiotics supplements and bone turnover markers  
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Supplementary Table 1 Search strategy of Medline

| #   | Searches             |
|-----|----------------------|
| 1   | Probiotics           |
| 2   | Probiotic supplement |
| 3   | Bone                 |
| 4   | Osteoporosis         |
| 5.  | Osteopenia           |
| 6.  | Bone mineral density |
| 7.  | Bone turnover        |
| 8.  | Postmenopausal       |
| 9.  | 1 and 3 and 8        |
| 10. | 1 and 4 and 8        |
| 11. | 1 and 5 and 8        |
| 12. | 1 and 6 and 8        |
| 13. | 1 and 7 and 8        |
| 14. | 2 and 3 and 8        |
| 15. | 2 and 4 and 8        |
| 16. | 2 and 5 and 8        |
| 17. | 2 and 6 and 8        |
| 18. | 2 and 7 and 8        |

The same strategy for other databases

Supplementary Table 2. Assessment of risk bias of the studies included in the meta-analysis

| Study      | Selection bias             | Selection bias         | Performance bias                       | Detection bias                 | Attrition bias          | Reporting bias      | Overall |
|------------|----------------------------|------------------------|--|--------------------------------|-------------------------|---------------------|---------|
|            | Random sequence generation | Allocation concealment | Blinding of participants and personnel | Blinding of outcome assessment | Incomplete outcome data | Selective reporting |         |
| Jansson    | Low                        | Low                    | Low                                    | Low                            | Low                     | Low                 | Low     |
| Takimoto   | Low                        | Low                    | Low                                    | Low                            | Low                     | Low                 | Low     |
| Nilsson    | Low                        | Unclear                | Low                                    | Low                            | Low                     | Low                 | Low     |
| Jafarnejad | Low                        | Low                    | Low                                    | Low                            | Low                     | Low                 | Low     |
| Lambert    | Low                        | Unclear                | Low                                    | Low                            | Low                     | Low                 | Low     |

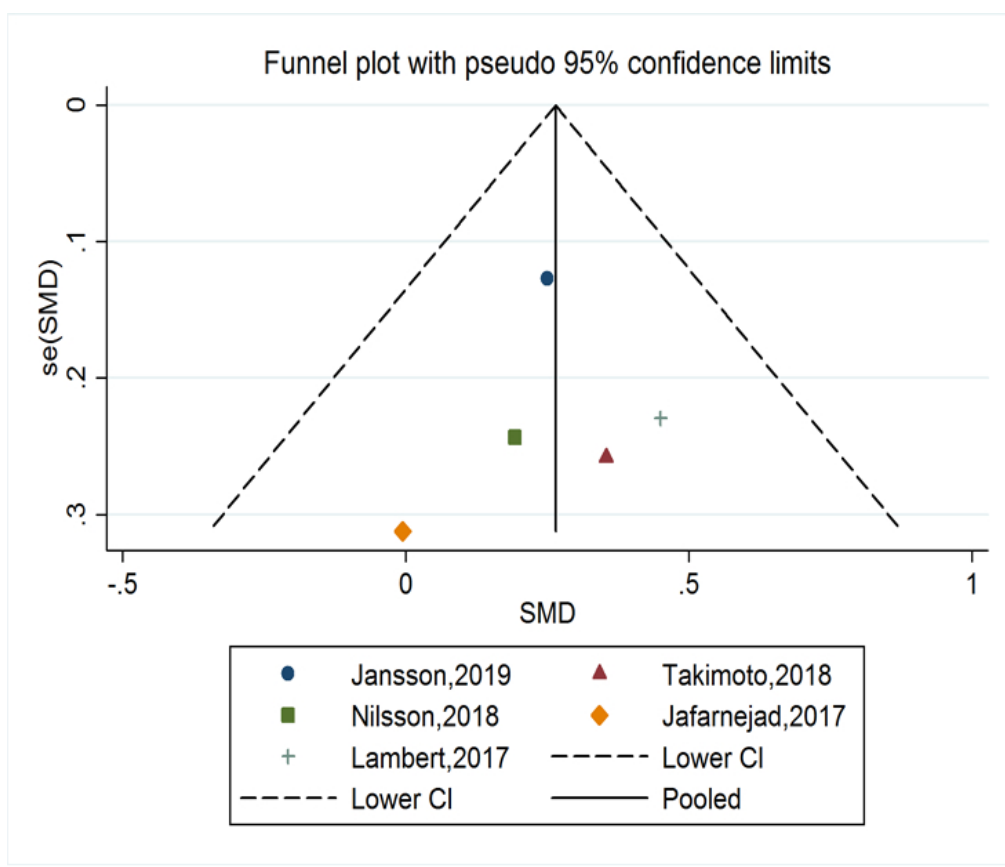


Supplementary Table 3. Other characteristics of included randomized controlled trials in the meta-analysis

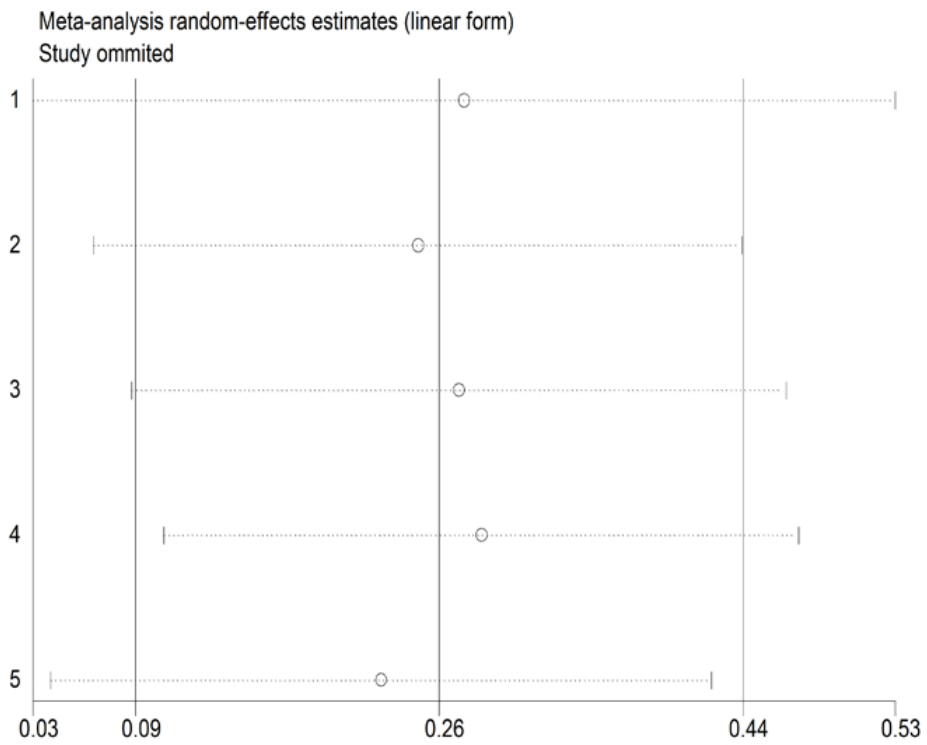
| Study      | Year | Area    | dose design                      | Minerals intake                                  | Measurement of outcome |
|------------|------|---------|----------------------------------|--|------------------------|
| Jansson    | 2019 | Sweden  | 1 x10 <sup>10</sup> CFU/d        | N/A  | After 12 months        |
| Takimoto   | 2018 | Japan   | 3.4x10 <sup>9</sup> CFU /d       | BDHQ   | After 6 months         |
| Nilsson    | 2018 | Sweden  | 5x10 <sup>9</sup> CFU twice/d    | A standardized questionnaire                     | After 12 months        |
| Jafarnejad | 2017 | Iran    | one Gerilact capsule /d*         | 500 mg Ca plus 200 IU vitamin D daily            | After 6 months         |
| Lambert    | 2017 | Denmark | 60mg isoflavone and probiotics/d | 1200 mg Ca, 550 mg Mg, and 25mg calcitriol daily | After 12 months        |

BDHQ: a brief-type self-administered diet history questionnaire; CFU: colony-forming unit; \*Lactobacillus casei 1.3 x 10<sup>10</sup> CFU, Bifidobacterium longum 5 x 10<sup>10</sup> CFU, Lactobacillus acidophilus 1.5 x 10<sup>10</sup> CFU, Lactobacillus rhamnosus 3.5 x 10<sup>9</sup> CFU, Lactobacillus bulgaricus 2.5 x 10<sup>8</sup> CFU, Bifidobacterium breve 1 x 10<sup>10</sup> CFU, and Streptococcus thermophilus 1.5 x 10<sup>8</sup> CFU per 500 mg.

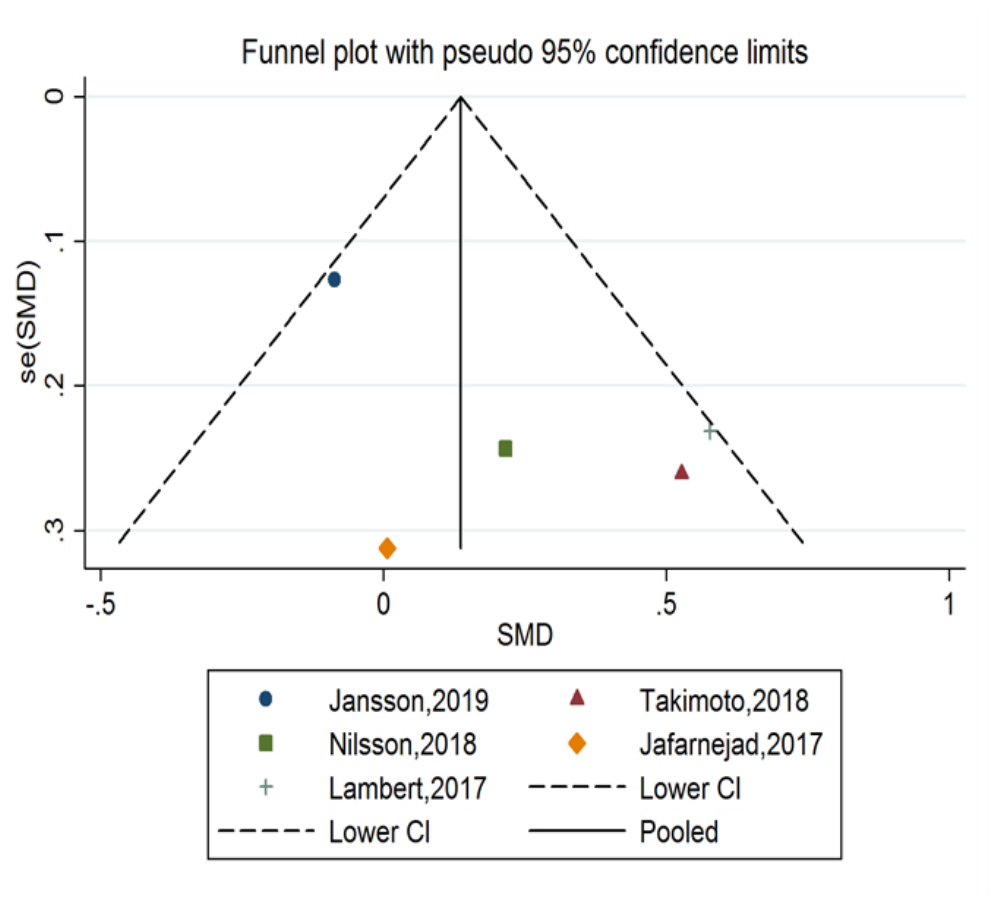
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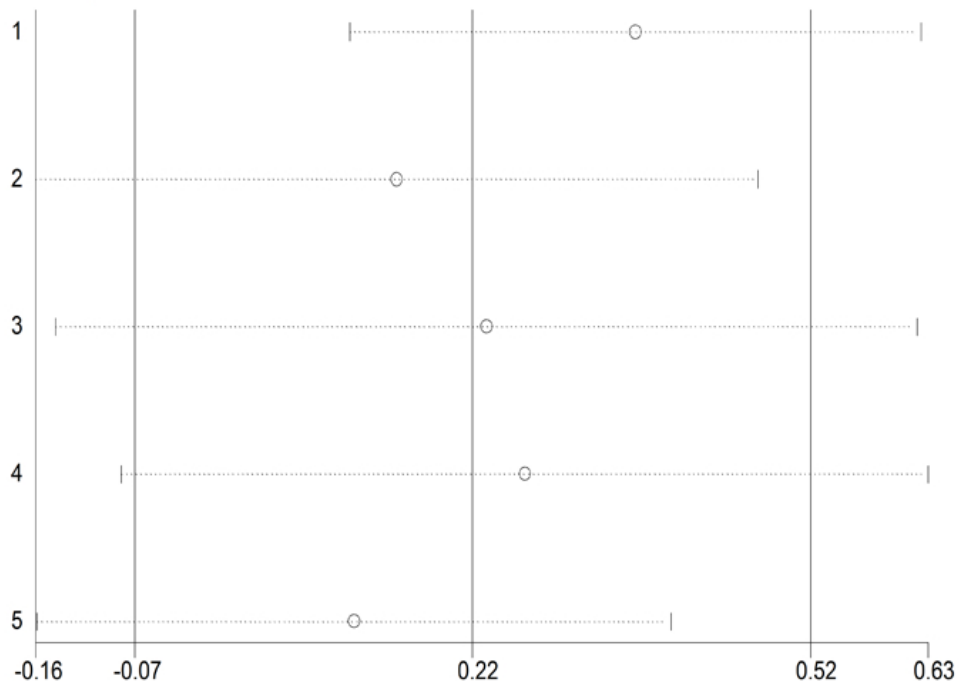


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Meta-analysis random-effects estimates (linear form)

Study omitted





# PRISMA 2009 Checklist

| Section/topic                      | #  | Checklist item  | Reported on page #  |
|------------------------------------|----|---|---|
| <b>TITLE</b>                       |    |   |   |
| Title                              | 1  | Identify the report as a systematic review, meta-analysis, or both.   | 1   |
| <b>ABSTRACT</b>                    |    |   |   |
| Structured summary                 | 2  | Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number. | 2   |
| <b>INTRODUCTION</b>                |    |   |   |
| Rationale                          | 3  | Describe the rationale for the review in the context of what is already known.  | 3-4   |
| Objectives                         | 4  | Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).  | 3-4   |
| <b>METHODS</b>                     |    |   |   |
| Protocol and registration          | 5  | Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.   | <a href="http://www.crd.york.ac.uk/PROSPERO/">http://www.crd.york.ac.uk/PROSPERO/</a> |
| Eligibility criteria               | 6  | Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.  | 4   |
| Information sources                | 7  | Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.  | 4   |
| Search                             | 8  | Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.   | 4   |
| Study selection                    | 9  | State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).   | 4   |
| Data collection process            | 10 | Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.  | 4   |
| Data items                         | 11 | List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.   | 4   |
| Risk of bias in individual studies | 12 | Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.  | 4   |
| Summary measures                   | 13 | State the principal summary measures (e.g., risk ratio, difference in means).   | 4-5   |



# PRISMA 2009 Checklist

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| Synthesis of results | 14 | Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., $I^2$ ) for each meta-analysis. | 4-5 |
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Page 1 of 2

| Section/topic                 | #  | Checklist item   | Reported on page # |
|-------------------------------|----|--|--------------------|
| Risk of bias across studies   | 15 | Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).   | 4-5                |
| Additional analyses           | 16 | Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.   | 4-5                |
| <b>RESULTS</b>                |    |  |                    |
| Study selection               | 17 | Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.  | 5                  |
| Study characteristics         | 18 | For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.   | 5                  |
| Risk of bias within studies   | 19 | Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).  | 5-6                |
| Results of individual studies | 20 | For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot. | 5-6                |
| Synthesis of results          | 21 | Present results of each meta-analysis done, including confidence intervals and measures of consistency.  | 5-6                |
| Risk of bias across studies   | 22 | Present results of any assessment of risk of bias across studies (see Item 15).  | 5-6                |
| Additional analysis           | 23 | Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).  | 5-6                |
| <b>DISCUSSION</b>             |    |  |                    |
| Summary of evidence           | 24 | Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).                     | 6-10               |
| Limitations                   | 25 | Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).  | 6-10               |
| Conclusions                   | 26 | Provide a general interpretation of the results in the context of other evidence, and implications for future research.  | 6-10               |
| <b>FUNDING</b>                |    |  |                    |
| Funding                       | 27 | Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.   | n/a                |



# PRISMA 2009 Checklist

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For peer review only



# BMJ Open

## Probiotic supplements and bone health in postmenopausal women: a meta-analysis of randomized controlled trials

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| Secondary Subject Heading:      | Nutrition and metabolism  |
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4 1 **Probiotic supplements and bone health in postmenopausal**  
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6 2 **women: a meta-analysis of randomized controlled trials**  
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9 3 Jiawei Yu<sup>1#</sup>, Gaoyang Cao<sup>2#</sup>, Shuohui Yuan<sup>1</sup>, Cong Luo<sup>1</sup>, Jiafeng Yu<sup>1</sup>, Ming Cai<sup>1\*</sup>  
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## 30 **Abstract**

31 **Objective:** Osteoporosis is a common disease in postmenopausal women. Several  
32 studies have analyzed the associations between dietary supplementation with  
33 probiotics and bone health in postmenopausal women, but the results are still  
34 controversial. We conducted this meta-analysis to assess the effects of probiotics  
35 supplement on bone mineral density (BMD) and bone turnover markers for  
36 postmenopausal women.

37 **Design:** systematic review and meta-analysis.

38 **Methods:** We systematically searched PubMed, EMBASE, and the Cochrane Library  
39 from their inception to November 2020 for randomized controlled trials (RCTs)  
40 assessing probiotic supplements and osteoporosis in postmenopausal women.  
41 Study-specific risk estimates were combined using random-effect models.

42 **Results:** Five RCTs (n = 497) were included. Probiotic supplements were associated  
43 with a significantly higher BMD in the lumbar spine (standardized mean difference,  
44 SMD = 0.27, 95% CI: 0.09–0.44) than in control. There was no difference between  
45 probiotic supplements and BMD in hips (SMD = 0.22, 95% CI: -0.07 – 0.52).  
46 Collagen type 1 cross-linked C-telopeptide (CTX) levels in the treatment groups were  
47 significantly lower than those of the placebo group (SMD = -0.34, 95% CI: -0.60 –  
48 -0.09). In subgroup meta-analysis, levels of bone-specific alkaline phosphatase  
49 (BALP), osteoprotegerin (OPG), osteocalcin (OC), and tumor necrosis factor (TNF)  
50 did not differ between the probiotic and placebo groups.

51 **Conclusions:** We conclude cautiously that supplementation with probiotics could  
52 increase lumbar BMD. More randomized controlled trials are recommended to  
53 validate or update these results.

## 52 **Strengths and limitations of this study**

54 This is the first meta-analysis on the effectiveness of probiotic supplements on bone  
55 status in postmenopausal women.

56 We included only high-quality randomized controlled trials to improve the level of  
57 evidence.  
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4 60 The limited number of reports prevented us from conducting subgroup analysis and  
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6 61 made it difficult to draw firm conclusions.  
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11 64 **Keywords:** probiotics supplement; bone mineral density; bone turnover markers;  
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13 65 postmenopausal; meta-analysis  
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## 90 Introduction

91 Osteoporosis is characterized by low bone mineral density (BMD) and deteriorated  
92 bone microstructure, leading to reduced bone strength and increased susceptibility to  
93 fractures<sup>1</sup>. Osteoporosis and fracture occur commonly in postmenopausal women,  
94 who experience a natural decline in endogenous estrogen, reducing BMD (on average  
95 2%–5% BMD/y)<sup>2</sup> and adverse effects on bone microarchitecture.

96 Currently, many medications are used in osteoporosis to decrease bone resorption  
97 or increase bone formation. Large randomized controlled trials (RCTs) showed that  
98 estrogen therapy (such as red clover isoflavone supplementation) was effective for  
99 preventing and treating osteoporosis in postmenopausal women<sup>3-5</sup>. However, this  
100 remains controversial because of the increased risk of cancer, including endometrial,  
101 breast, and ovarian cancer<sup>6</sup>. Nevertheless, other anti-resorptive agents are not widely  
102 used because of their side-effects, high prices, and poor compliance on the part of  
103 patients; these include bisphosphonates, calcitonin, and raloxifene. Therefore,  
104 complementary and dietary therapies are more acceptable to some patients. Also,  
105 natural treatments are increasingly requested by patients.<sup>7</sup> It was shown that calcium  
106 and vitamin D supplements effectively improved bone microarchitecture and health<sup>8</sup>;  
107 however, supplementation with calcium and vitamin alone is not sufficient to halt  
108 menopausal bone loss<sup>9</sup>.

109 Therefore, alternative ways to prevent and treat osteoporosis are sought. Probiotics  
110 are popular dietary therapies that have favorable effects on the skeletal system.<sup>10</sup>  
111 Probiotics are "live microorganisms that when administered in adequate amounts will  
112 confer a health benefit on the host" defined by the Food and Agricultural  
113 Organization/World Health Organization (FAO/WHO)<sup>11</sup>, such as bacillus subtilis,  
114 lactobacillus, and other mixed strains. They are affordable and have fewer  
115 side-effects.

116 To our knowledge, there has been no systematic review or meta-analysis of RCTs  
117 with probiotics in the treatment arms, analyzing the effect of probiotics in  
118 postmenopausal-related osteoporosis. Therefore, this systematic review and  
119 meta-analysis were performed to provide an overview of the effects of dietary

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4 120 probiotic supplements in postmenopausal related bone resorption in women and to  
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6 121 inform researchers of new potential sources of bias to be addressed in future clinical  
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8 122 trials.

## 9 123 **Methods and analysis**

### 10 124 **Data sources and search strategies**

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13 125 A literature search of relevant studies was performed in PubMed, EMBASE, and  
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15 126 the Cochrane Library. A comprehensive search strategy was developed. The protocol  
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17 127 was drafted according to the PRISMA statement<sup>12</sup>. The keywords were as follows:  
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19 128 'probiotics', 'probiotic supplement', 'bone,' 'osteoporosis', 'osteopenia', 'bone mineral  
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21 129 density', 'bone turnover', and 'postmenopausal' (search queries available in  
22  
23 130 Supplementary Table 1). References of retrieved articles were also scanned to identify  
24  
25 131 any additional relevant studies. Two independent reviewers (Jiawei Yu and Gaoyang  
26  
27 132 Cao) conducted this work. Discrepancies were resolved by consensus of the two  
28  
29 133 reviewers. If required, the final disposition was determined by Ming Cai.

### 30 134 **Inclusion and exclusion criteria**

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33 135 Inclusion criteria are as follows: (1) randomized controlled trials and prospective  
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35 136 cohort studies; (2) consideration of postmenopausal women as patients, consideration  
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37 137 of probiotic supplement as interventions, consideration of placebo as a comparison,  
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39 138 and consideration of the change of BMD and bone turnover markers (BTM) as  
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41 139 outcomes; (3) BMD was measured by dual-energy X-ray absorptiometry (DXA) and  
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43 140 BTM was measured using blood tests at baseline, and the end of trial; (4)  
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45 141 administered probiotics for more than 6 months; and (5) English language original  
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47 142 articles indexed up to November 2020.

48  
49 143 Exclusion criteria are as follows: (1) absence of critical data for meta-analysis; and  
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51 144 (2) low-quality articles according to Cochrane checklist.

### 52 145 **Data extraction and quality assessment**

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54 146 The characteristics of the relevant articles were extracted and recorded  
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56 147 independently by two reviewers (Jiawei Yu and Gaoyang Cao) as follows: first  
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58 148 author's name, year, area, age (mean or range), type of probiotic supplement, dose  
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60 149 design, course of treatment, number of cases, number of controls, and bone status (as

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4 150 shown in Table 1). The Cochrane Collaboration's tool <sup>13</sup> was used for assessing the  
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6 151 risk of bias. Six domain-based evaluations (selection bias, performance bias, detection  
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8 152 bias, attrition bias, reporting bias, and other bias) were used in the tool to assess the  
9  
10 153 possible bias of randomized controlled trials. The results were displayed as low risk,  
11  
12 154 unclear risk, or high risk of bias.

### 13 155 **Statistical analysis**

156 The mean relative change from baseline to the end of the course and standard  
157 deviation (SD) were used to express the effect of the probiotic supplement on bone  
158 status in postmenopausal women. If the original studies did not provide the mean  
159 relative change and standard deviation, we converted the data using a common  
160 method <sup>14-15</sup>. The pooled effects of included studies were expressed in terms of  
161 standardized mean difference (SMD) with 95% confidence interval (CI). Q test and  $I^2$   
162 index were used to evaluate heterogeneity among the included results.  
163 Meta-regression was conducted to determine whether different types of probiotic  
164 supplements would introduce sources of heterogeneity. Random-effects model and  
165 subgroup analysis were used in the face of heterogeneity. Forest plots and funnel plots  
166 were produced, and publication bias was tested using Begg's test and the weighted  
167 Egger test <sup>16-17</sup>. Sensitivity analysis was conducted to verify the impact of each study  
168 on the pooled results. In the sensitivity analyses, each study was omitted to recalculate  
169 the pooled estimates. All analysis was performed using STATA 12.0 (StataCorp LP,  
170 College Station, TX, USA).

### 171 **Patient and public involvement**

172 Patient and public involvement is not applicable for this meta-analysis.

### 173 **Results**

#### 174 **Search results and characteristics of identified studies**

175 A total of 604 articles were identified from the initial searches of PubMed and  
176 EMBASE, and 547 articles were removed because of absence of relevance. Nine  
177 articles were retained after reviewing the abstract according to the exclusion criteria.  
178 Finally, five randomized controlled trials<sup>18-22</sup> satisfied the inclusion criteria and  
179 entered this meta-analysis after full-text review. All the five RCTs had low risk of



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4 180 bias (available in Supplementary Table 2).

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6 181 A detailed overview of the selection process is outlined in Figure 1.

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8 182 A total of 497 postmenopausal women completed these trials. Among the five  
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10 183 trials, two were conducted in Asia (one in Japan<sup>18</sup>, the other in Iran<sup>20</sup>), and the other  
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12 184 three were in Europe (two in Sweden<sup>19 22</sup>, the last one in Denmark<sup>21</sup>). All trials were  
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14 185 randomized using the double-blinded method. Each trial identified the type of  
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16 186 probiotic supplements used and described the dosage design. Three studies considered  
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18 187 treatment with probiotics only<sup>18-20</sup>, while the other two studies included treatment  
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20 188 with combined isoflavone and probiotics<sup>21 22</sup>. All studies provided BMD data from  
21  
22 189 DXA scans at the lumbar spine and total hip. Collagen type 1 cross-linked  
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24 190 C-telopeptide (CTX), bone-specific alkaline phosphatase (BALP), osteoprotegerin  
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26 191 (OPG), osteocalcin (OC), and tumor necrosis factor (TNF) were used as bone  
27  
28 192 turnover markers. Details of the characteristics are displayed in Table 1 and  
29  
30 193 Supplementary Table 3.

#### 31 194 **Probiotic supplements and lumbar spine BMD**

32  
33 195 A total of five estimates were included in the meta-analysis. The meta-regression  
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35 196 results also showed no source of heterogeneity from various types of probiotics ( $P =$   
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37 197  $0.987$ ). Therefore, the five estimates were incorporated into the pooled analysis.  
38  
39 198 Compared to the placebo group, the lumbar spine BMD level of the supplementary  
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41 199 group was higher (SMD = 0.26, 95% CI: 0.09 – 0.44), with no heterogeneity ( $P =$   
42  
43 200  $0.805$ ;  $I^2 = 0.0$ ) (Figure 2). The funnel plot was symmetrical (Supplementary Figure 1)  
44  
45 201 and excluded publication bias (Begg's test  $z_c = 0.73$ ,  $P = 0.462$ ; Egger's test  $t = -0.22$ ,  
46  
47 202  $P = 0.843$ ). Sensitivity analyses indicated that the positive result was robust.  
48  
49 203 (Supplementary Figure 2).

#### 50 204 **Probiotics supplements and total hip BMD**

51  
52 205 Overall, five estimates of the association between probiotics supplement and hip  
53  
54 206 BMD were included in the meta-analysis. The meta-regression results revealed that  
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56 207 various types of probiotics were not a source of heterogeneity ( $P = 0.237$ ). Therefore,  
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58 208 we brought the five estimates into the pooled analysis. There was no difference  
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60 209 between probiotic supplements and BMD in hips (SMD = 0.22, 95% CI: -0.07 –

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4 210 0.52), with no heterogeneity ( $P = 0.055$ ;  $I^2 = 56.8$ ) (Figure 3). The funnel plot is  
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6 211 shown in Supplementary Figure 3; it was symmetrical, excluding publication bias  
7  
8 212 (Begg's test  $z_c = -0.24$ ,  $P = 1.00$ ; Egger's test  $t = 1.59$ ,  $P = 0.209$ ). Sensitivity analyses  
9  
10 213 indicated that the positive result was affected by the Jansson trial (Supplementary  
11  
12 214 Figure 4).

### 215 **Probiotic supplements and bone turnover markers**

15 216 Four estimates of CTX and two estimates of BALP, OPG, OC, and TNF were  
16  
17 217 incorporated into the pooled analysis. The results suggested that probiotic  
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19 218 supplements help decrease the supplementary group's body CTX level compared with  
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21 219 the placebo group (SMD = -0.34, 95% CI: -0.60 – -0.09) with substantial  
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23 220 heterogeneity. There was no evidence that probiotic supplements were associated with  
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25 221 BALP, OPG, OC, and TNF (Figure 4).

### 222 **Discussion**

#### 223 **Main findings**

30  
31 224 This meta-analysis included five randomized controlled trials with low risk of bias  
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33 225 and 497 postmenopausal women. The results provides evidence that dietary probiotics  
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35 226 supplement can slow bone resorption in postmenopausal women. Daily  
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37 227 supplementation with probiotics for 24 weeks to 12 months significantly decreased  
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39 228 bone turnover marker CTX (compared to placebo) in postmenopausal women. BMD  
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41 229 loss at the lumbar spine was significantly lower in the treatment group.

42  
43 230 Bone loss occurs throughout life following maturation and is accelerated following  
44  
45 231 menopause in women<sup>23</sup>. Postmenopausal women have an increased risk of fragility  
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47 232 fractures. Using a naturally-occurring bacterium to significantly reduce the annual  
48  
49 233 bone loss in this group of patients is a new concept that could lead to a paradigm shift  
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51 234 in osteoporosis prevention. Previous studies in animals demonstrated that  
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53 235 supplementation with specific bacterial strains increases bone density and protect  
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55 236 against osteoporosis<sup>24-26</sup>. Kim et al. reported that the administration of *Lactobacillus*  
56  
57 237 *casei* 393 significantly increased BMD in ovariectomized rats<sup>27</sup>. For the first time, the  
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59 238 present meta-analysis systemically demonstrated that this probiotic also works in  
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239 humans.

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4 240 The lumbar spine and hip are the most suitable organs to assess bone metabolism.  
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6 241 The vertebrae and metaphyses of long bones, rich in trabecular bone, have a higher  
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8 242 turnover rate than cortical bones in the axis of long bones. Therefore, medications and  
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10 243 diseases affecting the lumbar spine and hip are identified earlier than in other skeletal  
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12 244 segments <sup>28</sup>. The vertebrae and hips are easily accessible for measuring BMD.  
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14 245 Therefore, the lumbar spine and hip BMD were suitable primary outcome variables in  
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16 246 the present studies. McCabe et al <sup>29</sup> showed that oral administration of *Lactobacillus*  
17  
18 247 probiotics identified a 45% increase in hip and vertebral trabecular bone volume  
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20 248 fraction in male mice. In another study, the administration of *Lactobacillus plantarum*  
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22 249 and *Lactobacillus paracasei* to ovariectomized mice showed increased trabecular  
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24 250 number compared to sham-ovariectomized control groups <sup>30</sup>. Our meta-analysis  
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26 251 showed, in the probiotics group, both total hip and lumbar vertebrae BMD were at  
27  
28 252 significantly higher levels than those of the control.

29 253 CTX and BALP were chosen as critical bone turnover markers. Because BMD  
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31 254 depends on the dynamic balance of bone formation and resorption, bone turnover  
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33 255 markers are also important parameters analyzed in our meta-analysis. The  
34  
35 256 measurement of CTX has been taken as a marker of bone resorption; osteoclasts  
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37 257 produce it during bone resorption <sup>31</sup>. Therefore, the increased levels of serum CTX  
38  
39 258 indicated increased bone resorption. Subgroup included 3 RCT studies, suggesting  
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41 259 that probiotic supplements' ingestion significantly reduced the bone resorption marker  
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43 260 CTX. Another study from Japan <sup>18</sup> showed that the probiotics group had significantly  
44  
45 261 lower uNTx (urinary type I collagen cross-linked N-telopeptide) levels than the  
46  
47 262 placebo group at 12 weeks of treatment. uNTx is another fragment of type I collagen  
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49 263 generated during resorption detected in urine; therefore, this also suggested that  
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51 264 probiotics inhibit bone resorption by suppressing osteoclast activity. BALP is another  
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53 265 well-known bone turnover marker, an indicator of osteoblast proliferation that is  
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55 266 thought to be a bone formation marker <sup>32</sup>. However, the present meta-analysis showed  
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57 267 no significant changes in BALP. Similarly, no differences were detected in levels of  
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59 268 biochemical markers for bone metabolic indices (OPG, OC).

60 269 **The mechanism of action**

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4 270 The mechanisms of action of probiotics are as follows. Probiotics have many  
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6 271 functional properties in humans. They function in the gastrointestinal system by  
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8 272 modifying the microbiota composition, intestinal barrier function, and the immune  
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10 273 system, which feeds back systemic benefits to the host, including bone health.  
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12 274 Moreover, probiotic function modifying physiological homeostasis of the intestinal  
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14 275 flora can also benefit bone metabolism<sup>33</sup>. Gastrointestinal inflammation and systemic  
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16 276 inflammation are close to enhanced generation of potent osteoclastogenic cytokines as  
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18 277 the leading cause of bone loss<sup>34-35</sup>. Probiotics can restore the balance of the gut  
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20 278 microbiota, preventing or moderating gut and systemic inflammation and allowing  
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22 279 absorption of nutrients, especially in older adults<sup>36</sup>.

23  
24 280 Furthermore, probiotics decrease levels of inflammatory mediators and cytokines  
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26 281 in the gut and bone marrow<sup>37</sup>. These changes give bone cell signals, including  
27  
28 282 osteoblasts, osteoclasts, and stem cells, significantly affecting bone homeostasis.  
29  
30 283 Endocrine factors (such as serotonin and incretins) secreted by the intestine also  
31  
32 284 remarkably affect bone cells<sup>38</sup>. Anti-inflammatory effects are among the underlying  
33  
34 285 mechanisms by which probiotics benefit bone metabolism. There is evidence that  
35  
36 286 arginine deiminase, produced by the probiotic *Lactobacillus brevis* CD2, has an  
37  
38 287 anti-inflammatory effect<sup>39</sup>. Supplementation of probiotics may reduce the expression  
39  
40 288 of pro-inflammatory and osteolytic cytokines, including TNF- $\alpha$ . These cytokines alter  
41  
42 289 anti-osteoclastogenic cytokine expression, leading to enhanced osteoclast formation  
43  
44 290 and inhibited osteoblast activity<sup>40</sup>. Some studies found that probiotic supplementation  
45  
46 291 reduces TNF $\alpha$ , IL-17, and RANKL expression levels in ovariectomized mice<sup>41</sup>.  
47  
48 292 These changes give bone cell signals, such as osteoblasts, osteoclasts, and stem cells,  
49  
50 293 significantly affecting bone homeostasis. More clinical trials are needed in the future  
51  
52 294 to elucidate the relationship between the administration of probiotics and  
53  
54 295 anti-inflammatory effects.

#### 54 296 **Limitations and Strengths**

56 297 Our study has some limitations. First, only five randomized controlled trials with  
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58 298 specific population groups satisfied our inclusion criteria. The limited number of  
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60 299 reports and specific population groups focusing on the association between the

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4 300 probiotic supplement and BMD and bone turnover markers prevented us from  
5  
6 301 conducting subgroup analysis and drawing conclusive summaries. Furthermore, the  
7  
8 302 insufficient number of estimates inflates the impact of the results of a particular study  
9  
10 303 and the conclusions may change on the publication of future studies. Second, although  
11  
12 304 meta-regression was used to determine that various types of probiotic supplements did  
13  
14 305 not impact the pooled results, dosage design and course of treatment could also  
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16 306 introduce bias. Third, in Lambert's study <sup>21</sup>, probiotics plus isoflavones were used as a  
17  
18 307 treatment regimen, rather than probiotics alone. This may cause some bias; however,  
19  
20 308 we did not want to ignore this valuable study. Third, the units describing BMD  
21  
22 309 change were inconsistent among the five reports. Nilsson's study <sup>19</sup> and Jansson's  
23  
24 310 study <sup>22</sup> applied T score to describe BMD change, while the other three studies used  
25  
26 311 g/cm<sup>2</sup> instead. We could only calculate SMD rather than the weighted mean  
27  
28 312 difference (WMD). Fifth, unfortunately, we did not find a relevant prospective cohort  
29  
30 313 for this meta-analysis. Thus, our results of a meta-analysis should be interpreted with  
31  
32 314 caution.

33  
34 315 Our research also has some strengths. First, to our knowledge, this is the first  
35  
36 316 meta-analysis describing the evidence of the association of probiotic supplements and  
37  
38 317 bone status in postmenopausal women. Second, there is little heterogeneity between  
39  
40 318 the included articles and the fixed-effects model used to calculate the results. Third,  
41  
42 319 all included randomized controlled trials were of high quality.

#### 43 320 Implications and future research

44  
45 321 This systematic review and meta-analysis are useful for multidisciplinary clinicians  
46  
47 322 to evaluate their practices and make a proper clinical decision. The beneficial effects  
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49 323 of probiotic supplements may infect probiotic indication in postmenopausal women  
50  
51 324 with osteoporosis. More RCT studies from different regions are needed to validate our  
52  
53 325 argument and help answer research questions about probiotic supplements, dose, and  
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55 326 the optimal duration.

#### 56 327 **Conclusion**

57  
58 328 Our systematic review and meta-analysis showed that probiotic supplementations in  
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60 329 postmenopausal women were associated with preserving lumbar spine BMD. The

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4 330 results should be interpreted with caution and more high quality RCTs are needed to  
5 331 validate or update these results. An appropriate supplement of probiotics could be  
6 332 recommended to improve bone status in postmenopausal women.  
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11 334

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21 339

### 22 340 **Contributors**

23 341 MC and JY(Jiawei Yu) conceived and designed the meta-analysis; GC, SY and CL  
24 342 searched the literature; JY(Jiawei Yu), GC, and SY analyzed the data; JY(Jiawei Yu)  
25 343 contributed analysis tools; JY(Jiawei Yu) and GC wrote the paper; JY(Jiafeng Yu)  
26 344 and MC revised the manuscript.  
27  
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35

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37 348 The authors declare no conflicts of interest. This study does not contain human  
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40

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42 351 Not required  
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### 45 352 **Provenance and peer review**

46 353 Not commissioned; externally peer-reviewed  
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### 49 354 **Data availability statement**

50 355 All data relevant to the study are included in the article or uploaded as supplementary  
51 356 information. No additional data are available.  
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Table 1. Characteristics of included randomized controlled trials in the meta-analysis

| Study      | Year | Area    | Age                | Blinding     | Type of probiotic supplement        | Number of Treatment | Number of Placebo | Course of treatment | BMD                 | BTM                             |
|------------|------|---------|--------------------|--------------|-------------------------------------|---------------------|-------------------|---------------------|---------------------|---------------------------------|
| Jansson    | 2019 | Sweden  | T: 59.1<br>P: 58.1 | double blind | three Lactobacillus strains*        | 126                 | 123               | 12 months           | lumbar spine<br>hip | N/A                             |
| Takimoto   | 2018 | Japan   | T: 57.5<br>P: 57.8 | double blind | bacillus subtilis C-3102            | 31                  | 30                | 6 months            | lumbar spine<br>hip | CTX                             |
| Nilsson    | 2018 | Sweden  | T: 76.4<br>P: 76.3 | double blind | lactobacillus reuteri 6475          | 32                  | 36                | 12 months           | lumbar spine<br>hip | CTX<br>BALP<br>TNF              |
| Jafarnejad | 2017 | Iran    | T: 58.9<br>P: 57.3 | double blind | seven probiotic bacteria species#   | 20                  | 21                | 6 months            | lumbar spine<br>hip | CTX<br>BALP<br>OPG<br>OC<br>TNF |
| Lambert    | 2017 | Denmark | T: 60.8<br>P: 62.9 | double blind | lactic acid bacteria and soflavones | 38                  | 40                | 12 months           | lumbar spine<br>hip | CTX<br>OPG<br>OC                |

BMD: bone mineral density; BTM: bone turnover marker; CTX: collagen type 1 cross-linked C-telopeptide; BALP: bone-specific alkaline phosphatase; OPG: osteoprotegerin; OC: osteocalcin; TNF: tumor necrosis factor; N/A: not available; \* Lactobacillus paracasei DSM 13434, Lactobacillus plantarum DSM 15312, and Lactobacillus plantarum DSM 15313; # Lactobacillus casei, Bifidobacterium longum, Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacillus bulgaricus, Bifidobacterium breve, and Streptococcus thermophilus.

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4 Figure 1. Flow diagram of the studies search process

5 Figure 2. Forest plots of meta-analysis on probiotics supplements and lumbar spine BMD

6  
7 Figure 3. Forest plots of meta-analysis on probiotics supplements and hip BMD

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9 Figure 4. Forest plots of meta-analysis on probiotics supplements and bone turnover markers

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13 Supplementary Figure 1. Funnel plots of meta-analysis on probiotics supplements and lumbar  
14 spine BMD

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17 Supplementary Figure 2. Sensitivity analyses of meta-analysis on probiotics supplements and  
18 lumbar spine BMD

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20 Supplementary Figure 3. Funnel plots of meta-analysis on probiotics supplements and hip  
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23 Supplementary Figure 4. Sensitivity analyses of meta-analysis on probiotics supplements and  
24 hip BMD

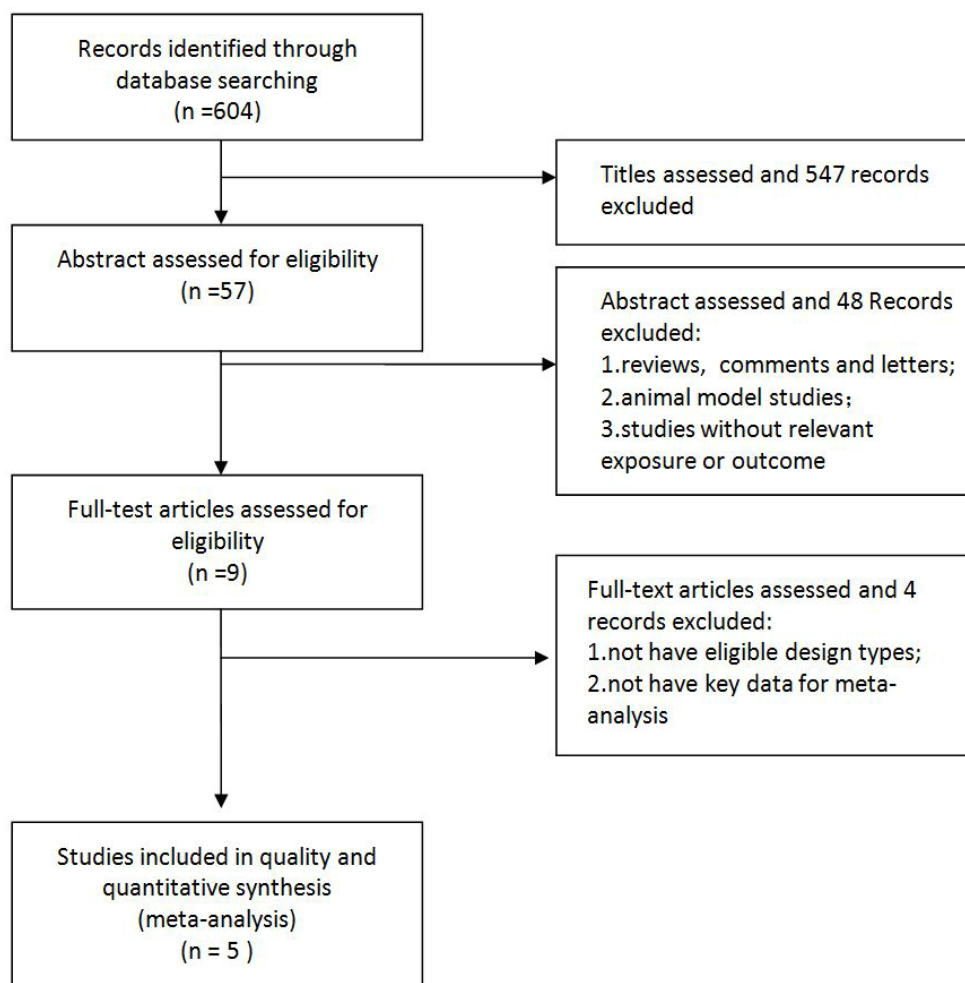


Figure 1. Flow diagram of the studies search process

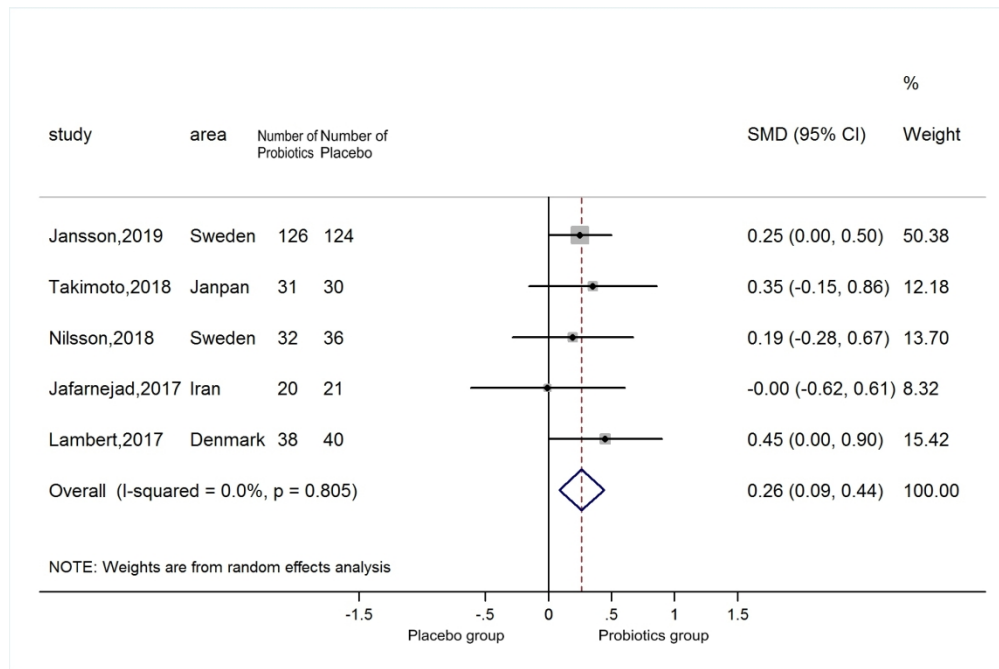


Figure 2. Forest plots of meta-analysis on probiotics supplements and lumbar spine BMD

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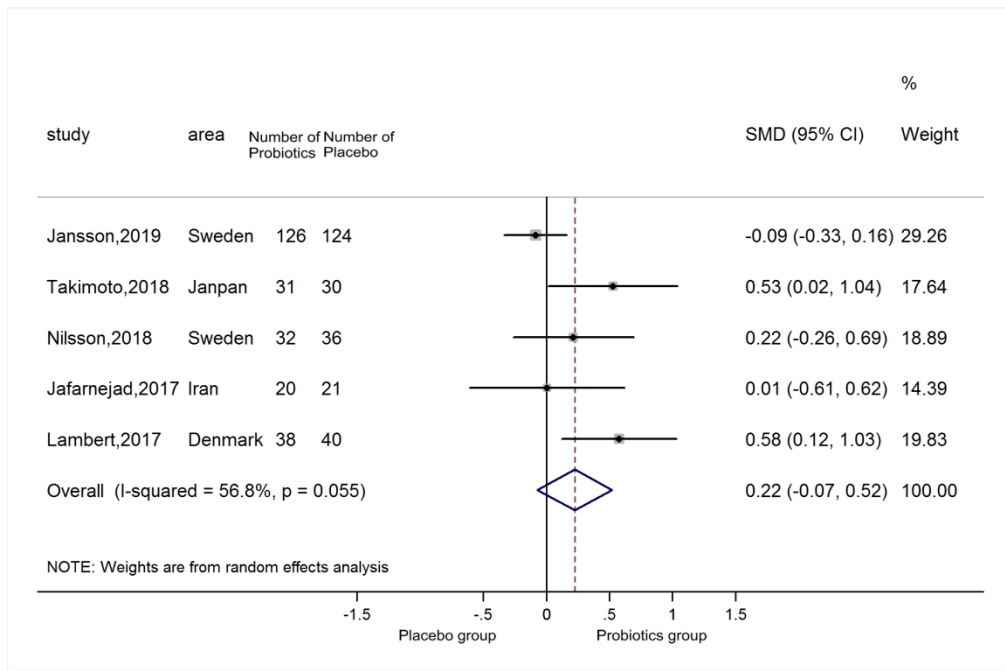


Figure 3. Forest plots of meta-analysis on probiotics supplements and hip BMD

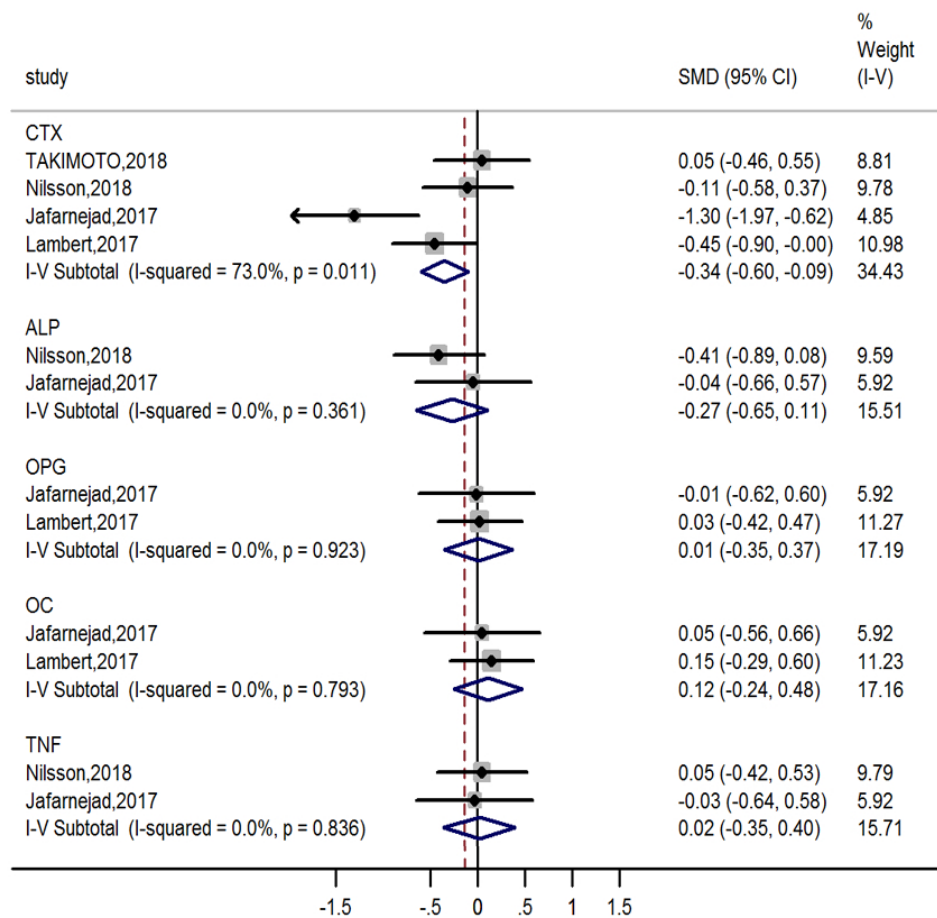


Figure 4. Forest plots of meta-analysis on probiotics supplements and bone turnover markers



Supplementary Table 1 Search strategy of Medline

| #   | Searches             |
|-----|----------------------|
| 1   | Probiotics           |
| 2   | Probiotic supplement |
| 3   | Bone                 |
| 4   | Osteoporosis         |
| 5.  | Osteopenia           |
| 6.  | Bone mineral density |
| 7.  | Bone turnover        |
| 8.  | Postmenopausal       |
| 9.  | 1 and 3 and 8        |
| 10. | 1 and 4 and 8        |
| 11. | 1 and 5 and 8        |
| 12. | 1 and 6 and 8        |
| 13. | 1 and 7 and 8        |
| 14. | 2 and 3 and 8        |
| 15. | 2 and 4 and 8        |
| 16. | 2 and 5 and 8        |
| 17. | 2 and 6 and 8        |
| 18. | 2 and 7 and 8        |

The same strategy for other databases

Supplementary Table 2. Assessment of risk bias of the studies included in the meta-analysis

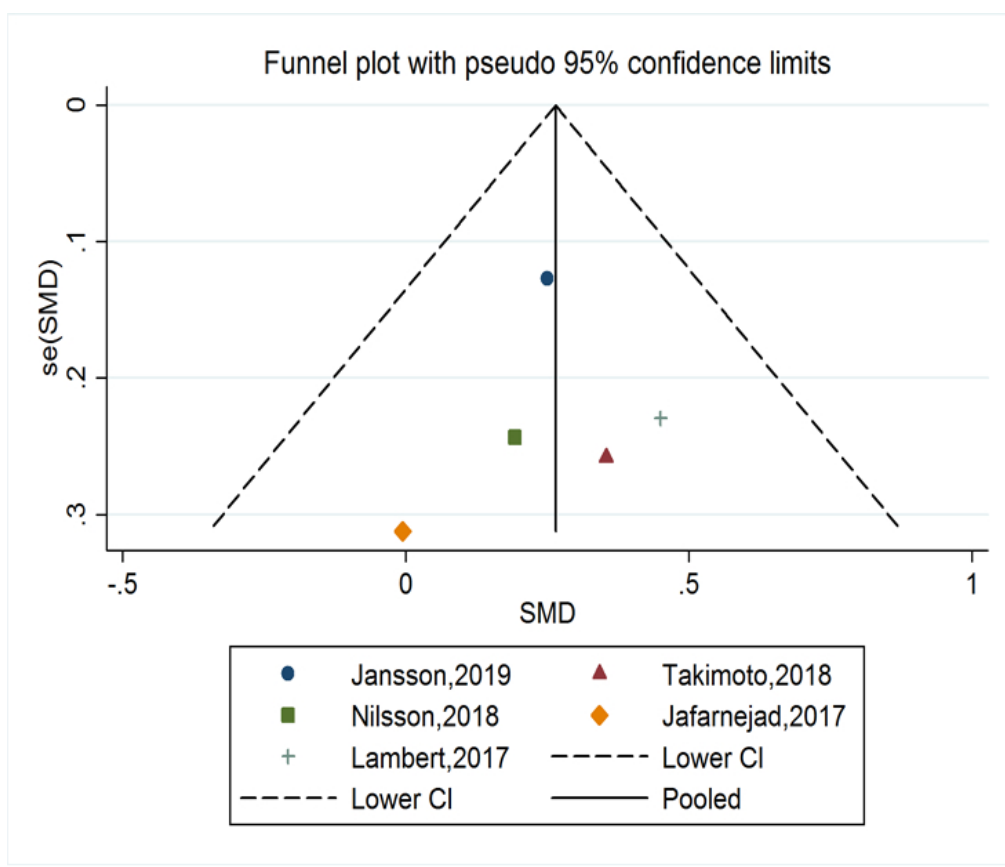
| Study      | Selection bias             | Selection bias         | Performance bias                       | Detection bias                 | Attrition bias          | Reporting bias      | Overall |
|------------|----------------------------|------------------------|--|--------------------------------|-------------------------|---------------------|---------|
|            | Random sequence generation | Allocation concealment | Blinding of participants and personnel | Blinding of outcome assessment | Incomplete outcome data | Selective reporting |         |
| Jansson    | Low                        | Low                    | Low                                    | Low                            | Low                     | Low                 | Low     |
| Takimoto   | Low                        | Low                    | Low                                    | Low                            | Low                     | Low                 | Low     |
| Nilsson    | Low                        | Unclear                | Low                                    | Low                            | Low                     | Low                 | Low     |
| Jafarnejad | Low                        | Low                    | Low                                    | Low                            | Low                     | Low                 | Low     |
| Lambert    | Low                        | Unclear                | Low                                    | Low                            | Low                     | Low                 | Low     |

Supplementary Table 3. Other characteristics of included randomized controlled trials in the meta-analysis

| Study      | Year | Area    | dose design                      | Minerals intake                                  | Measurement of outcome |
|------------|------|---------|----------------------------------|--|------------------------|
| Jansson    | 2019 | Sweden  | 1 x10 <sup>10</sup> CFU/d        | N/A  | After 12 months        |
| Takimoto   | 2018 | Japan   | 3.4x10 <sup>9</sup> CFU /d       | BDHQ   | After 6 months         |
| Nilsson    | 2018 | Sweden  | 5x10 <sup>9</sup> CFU twice/d    | A standardized questionnaire                     | After 12 months        |
| Jafarnejad | 2017 | Iran    | one Gerilact capsule /d*         | 500 mg Ca plus 200 IU vitamin D daily            | After 6 months         |
| Lambert    | 2017 | Denmark | 60mg isoflavone and probiotics/d | 1200 mg Ca, 550 mg Mg, and 25mg calcitriol daily | After 12 months        |

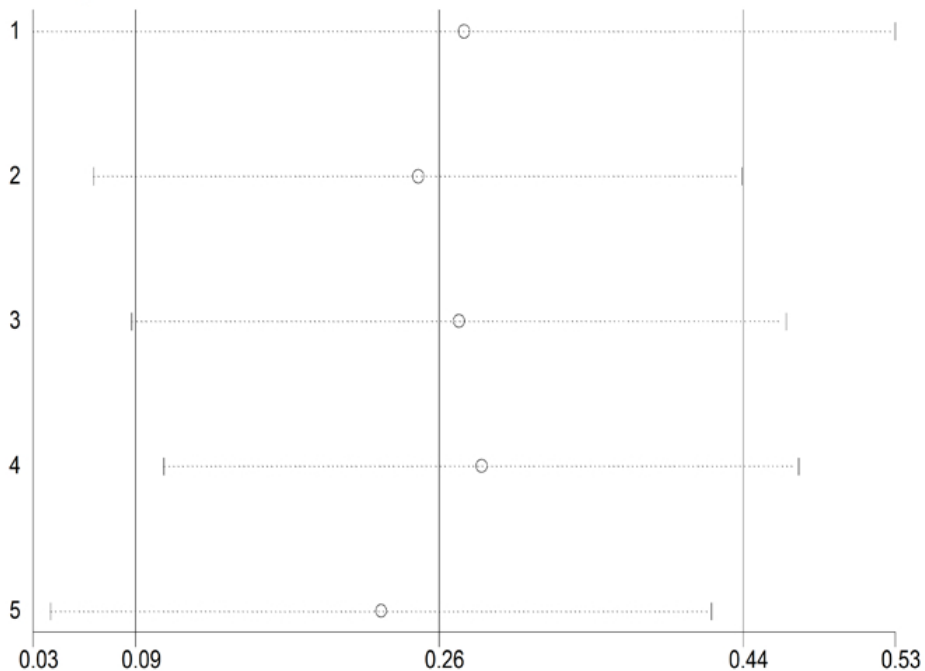
BDHQ: a brief-type self-administered diet history questionnaire; CFU: colony-forming unit; \*Lactobacillus casei 1.3 x 10<sup>10</sup> CFU, Bifidobacterium longum 5 x 10<sup>10</sup> CFU, Lactobacillus acidophilus 1.5 x 10<sup>10</sup> CFU, Lactobacillus rhamnosus 3.5 x 10<sup>9</sup> CFU, Lactobacillus bulgaricus 2.5 x 10<sup>8</sup> CFU, Bifidobacterium breve 1 x 10<sup>10</sup> CFU, and Streptococcus thermophilus 1.5 x 10<sup>8</sup> CFU per 500 mg.

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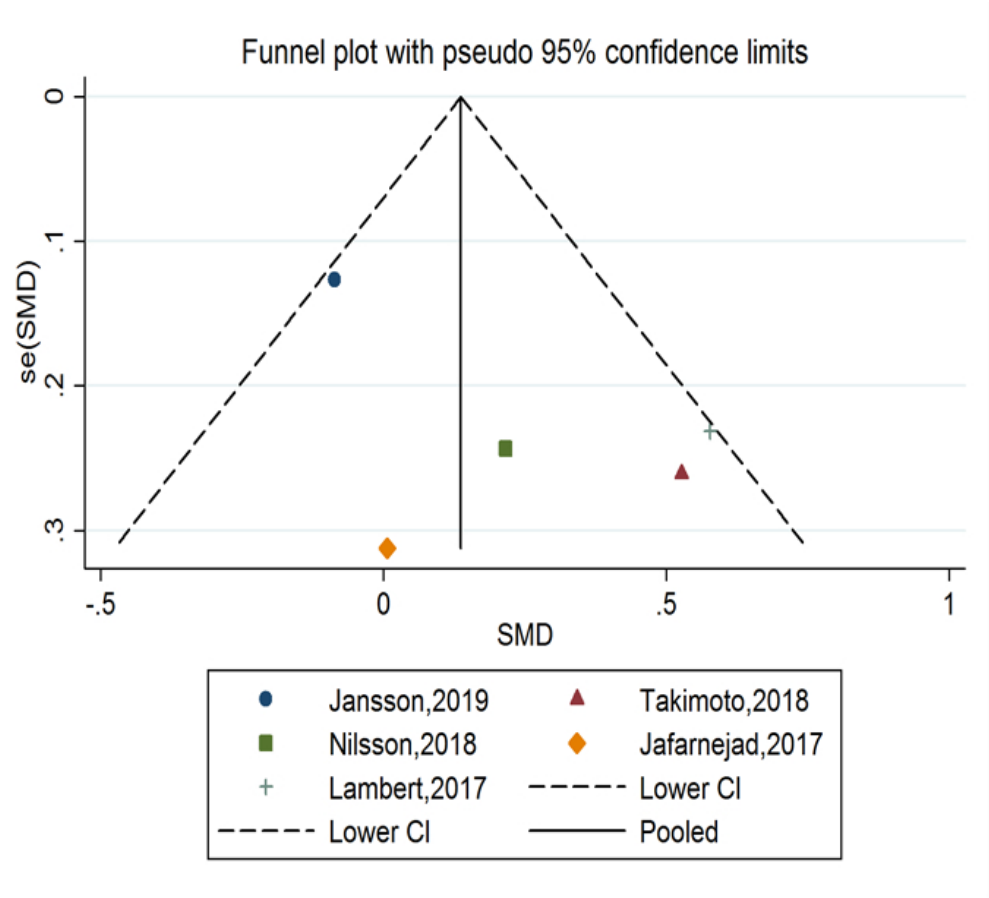


Meta-analysis random-effects estimates (linear form)

Study ommited

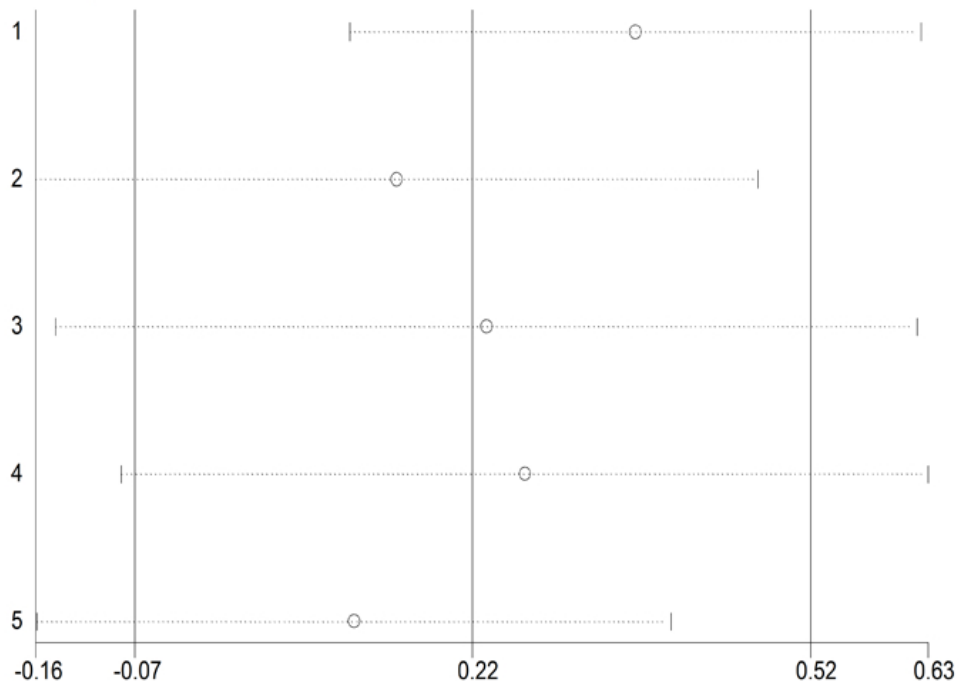


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Meta-analysis random-effects estimates (linear form)

Study ommited





# PRISMA 2009 Checklist

| Section/topic                      | #  | Checklist item  | Reported on page # |
|------------------------------------|----|---|--------------------|
| <b>TITLE</b>                       |    |   |                    |
| Title                              | 1  | Identify the report as a systematic review, meta-analysis, or both.   | 1                  |
| <b>ABSTRACT</b>                    |    |   |                    |
| Structured summary                 | 2  | Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number. | 2                  |
| <b>INTRODUCTION</b>                |    |   |                    |
| Rationale                          | 3  | Describe the rationale for the review in the context of what is already known.  | 4-5                |
| Objectives                         | 4  | Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).  | 4-5                |
| <b>METHODS</b>                     |    |   |                    |
| Protocol and registration          | 5  | Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.   | N/A                |
| Eligibility criteria               | 6  | Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.  | 5                  |
| Information sources                | 7  | Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.  | 5                  |
| Search                             | 8  | Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.   | 5                  |
| Study selection                    | 9  | State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).   | 5                  |
| Data collection process            | 10 | Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.  | 5-6                |
| Data items                         | 11 | List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.   | 5-6                |
| Risk of bias in individual studies | 12 | Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.  | 6                  |
| Summary measures                   | 13 | State the principal summary measures (e.g., risk ratio, difference in means).   | 6                  |
| Synthesis of results               | 14 | Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I <sup>2</sup> ) for each meta-analysis.  | 6                  |





# PRISMA 2009 Checklist

Page 1 of 2

| Section/topic                 | #  | Checklist item   | Reported on page # |
|-------------------------------|----|--|--------------------|
| Risk of bias across studies   | 15 | Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).   | 6                  |
| Additional analyses           | 16 | Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.   | 6                  |
| <b>RESULTS</b>                |    |  |                    |
| Study selection               | 17 | Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.  | 6-7                |
| Study characteristics         | 18 | For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.   | 6-7                |
| Risk of bias within studies   | 19 | Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).  | 7                  |
| Results of individual studies | 20 | For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot. | 7-8                |
| Synthesis of results          | 21 | Present results of each meta-analysis done, including confidence intervals and measures of consistency.  | 7-8                |
| Risk of bias across studies   | 22 | Present results of any assessment of risk of bias across studies (see Item 15).  | 7-8                |
| Additional analysis           | 23 | Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).  | 7-8                |
| <b>DISCUSSION</b>             |    |  |                    |
| Summary of evidence           | 24 | Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).                     | 8-11               |
| Limitations                   | 25 | Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).  | 8-11               |
| Conclusions                   | 26 | Provide a general interpretation of the results in the context of other evidence, and implications for future research.  | 8-11               |
| <b>FUNDING</b>                |    |  |                    |
| Funding                       | 27 | Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.   | n/a                |

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097

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