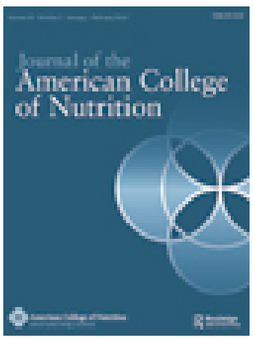

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Effects of a Multispecies Probiotic Supplement on Bone Health in Osteopenic Postmenopausal Women: A Randomized, Double-blind, Controlled Trial

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ABSTRACT

Objective: The development of alternative approaches to prevent and/or treat osteoporosis, as a chronic progressive bone disease, is being considered currently. Among dietary supplements, probiotics may have favorable effects on bone metabolism. Therefore, the aim of this study was to evaluate the effects of a multispecies probiotic supplementation on bone biomarkers and bone density in osteopenic postmenopausal women.

Methods: This randomized double-blind placebo-controlled clinical trial was performed on 50 patients with osteopenia aged 50–72 years. Participants were randomly assigned to take either a multispecies probiotic supplement (GeriLact; $n = 25$) or placebo ($n = 25$) for 6 months. GeriLact contains 7 probiotic bacteria species. Participants received 500 mg Ca plus 200 IU vitamin D daily. Bone mineral density (BMD) of lumbar spine and total hip and blood biomarkers including bone-specific alkaline phosphatase (BALP), osteocalcin (OC), collagen type 1 cross-linked C-telopeptide (CTX), deoxypyridinoline (DPD), parathyroid hormone (PTH), 25-OH vitamin D, and serum pro-inflammatory cytokines (tumor necrosis factor [TNF]-α and interleukin [IL]-1β) were assessed at baseline and at the end of the study.

Results: The multispecies probiotic significantly decreased BALP ($p = 0.03$) and CTX ($p = 0.04$) levels in comparison with the control group but had no effect on BMD of the spine and total hip. Moreover, there was a statistically significant decrease in serum PTH ($p = 0.01$) and TNF-α ($p = 0.02$) in the intervention group compared to the placebo group.

Conclusions: These results may suggest the favorable effects of the multispecies probiotic supplementation for 6 months on bone health in postmenopausal women due to slowing down the rate of bone turnover.

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Probiotic; postmenopausal women; osteopenia; osteoporosis; bone biomarkers; pro-inflammatory cytokines

Introduction

Osteoporosis, as a major public health problem in postmenopausal women, is a chronic progressive bone disease and a potentially debilitating condition characterized by decreased bone mass and quality resulting in fragility fractures [1]. Osteoporosis affects 75 million people in Europe, Japan, and the United States and is estimated to affect 200 million women worldwide [2]. Based on a recent meta-analysis on Iranian studies, estimated prevalence of osteoporosis is 3% among premenopausal women and 19% among postmenopausal women [3].

The most common cause of bone loss among postmenopausal women is hormonal deficiency, which occurs following an abrupt cessation of estrogen production, with a consequent drop in circulating levels of estrogen. This leads to an imbalance between bone formation and bone resorption [4].

There are currently several drug therapies that are commonly used to manage osteoporosis by either increasing bone formation or reducing bone resorption. Nonetheless, these anti-resorptive agents (such as raloxifene, calcitonin, and bisphosphonates) are known to have side effects (such as atypical femoral fractures, atrial fibrillation, esophageal cancer, and gastrointestinal issues [5]), poor compliance, and high costs; in addition, based on research, these drugs can prevent the incidence of fractures maximally by 50% [6,7]. Nevertheless, some patients prefer alternative and complementary therapies, including dietary supplements [8]. Studies have consistently shown that supplementation with calcium and vitamin D improved bone metabolism and health, but it seems that supplementation alone is not sufficient to fully prevent the menopausal bone loss [9]. Hence, the development of alternative approaches to prevent and/or treat osteoporosis is being considered.

Among dietary supplements, probiotics may have favorable effects on bone metabolism. The Food and Agriculture Organization/World Health Organization define probiotics as live

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microorganisms that, when administered in adequate amounts, confer a health benefit on the host [10]. Probiotics have several functional properties, including resistance to gastric acidity and bile salts, the ability of immune response modulation, adhesion to gut tissues, and production of antimicrobial agents. Probiotics modify physiological homeostasis of the intestinal flora, which has an important role in preventing diseases [11]. Several studies have investigated the changes of gut microbiota during aging, which may alter mineral absorption [12]. Moreover, it has been shown that levels of inflammatory mediators and cytokines, including interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α increase with aging even without the presence of acute infections or physiologic stress [13].

On the other hand, some studies have indicated that systemic inflammation or gut inflammation is associated with enhanced production of potent osteoclastogenic cytokines as the key contributors of bone loss [14,15]. Therefore, controlling the mediators of inflammation, especially in aging, may lead to balanced bone remodeling. Probiotics may improve absorption of nutrients and prevent or ameliorate systemic inflammation followed by gut inflammation, due to restoration of the composition of the gut microbiota [16].

To date, there is limited evidence regarding the association of bone metabolism with the state of the gastrointestinal microbiota that could be altered by probiotic bacteria. Britton et al. [17] demonstrated the protective effect of *Lactobacillus reuteri* on ovariectomized (ovx)-related bone loss. They showed an altered microbiota and prevention of ovx-induced trabecular bone loss [17]. A multistrain probiotic containing *Lactobacillus casei, Lactobacillus reuteri*, and *Lactobacillus gasseri* showed higher bone weight among the probiotic-fed group compared to the control group [18]. In another study, the effect of 2 strains probiotics on bone mass was examined in chickens over 6 weeks and an increased bone mass in the tibia was reported [19]. In several studies, the effect of gut microbiota modulation on bone loss in sex steroid–deficient female mice was investigated. It is mostly hypothesized that the probiotics attenuated the bone loss in ovx-induced mice [20–23].

To the best of our knowledge, there are no human data available to determine the effect of probiotics on bone health or metabolism. Hence, we carried out a 6-month experiment to evaluate their efficacy on modulation of bone metabolism in postmenopausal women with osteopenia who were not on drugs or any agents known to significantly influence bone metabolism.

Materials and methods

Participants

This randomized, double-blind, placebo-controlled study was performed in Shahrekord, Iran, from May 2016 to October 2016. The protocol was developed according to the Consolidated Standards of Reporting Trials (CONSORT) declaration [24]. According to the sample size formula suggested by Vanlint and Ried, we considered a type I error of 5% ($\alpha = 0.05$) and a type II error of 20% ($\beta = 0.2$; power = 80%) [25]. Based on the collagen type 1 cross-linked C-telopeptide (CTX) level as a key variable, we reached a sample size of 20 patients in each group. A total of 142 postmenopausal women were assessed for eligibility. The diagnosis of osteopenia was made based upon World Health Organization criteria [26] (T-score between −1.0 and −2.5). Of the volunteers, 50 women aged 50–72 years with mild bone loss were recruited in this study.

Women with osteoporosis (bone mineral density [BMD] T-score at any site below 2.5 standard deviations of the mean) were excluded from the study. Furthermore, subjects treated with calcitonin, bisphosphonates, raloxifene, and/or anabolic agents such as growth hormone, parathyroid hormone, and steroids within 3 months before the start of the study were excluded. Any other medications or supplements that could influence bone mass were also not permitted.

In addition, participants with chronic diseases of the bone, renal disease, liver disease, cardiovascular disease, diabetes mellitus, gastrointestinal disease, respiratory disease, smokers, and subjects with body mass index (BMI) > 40 were excluded. Participants were randomly assigned to receive either Gerilact capsules (n = 25) or placebo capsules (n = 25) for 6 months. Both probiotic and placebo capsules were made by Zist Takhmir Co, Tehran, Iran.

The study protocol was approved by the Tehran University of Medical Sciences Ethical Committee (ID: IR.TUMS.REC.1394.785) and registered on www.irct.ir as IRCT2015092024103N1. Written informed consent was obtained from all participants before the intervention. Complete medical and nutritional histories were obtained from the subjects before initiating the treatments. All clinical investigations were conducted according to the Declaration of Helsinki and Good Clinical Practice guidelines.

Study design

Randomization was performed using a computerized random number generator, which generated the random sequence, based on simple randomization. All subjects were identified by a trial identification number and assigned a treatment code. The researchers and participants were blinded to the treatment code until the end of the study, when data were collected and evaluated. The groups were decoded after completion of the densitometry, biochemical, and data analyses. Nevertheless, all investigators, including the physician, staff who collected anthropometric data, laboratory technician, and statistician, were blind to the treatment. The probiotic and placebo groups were given either a multispecies probiotic supplement (Gerilact capsule) or placebo capsule. The capsules were stored at 2 to 8°C prior to distribution. The patients were instructed to keep the capsules refrigerated. They were asked to take one capsule a day before any meal for a period of 6 months. Moreover, both groups were provided with 500 mg Ca plus 200 IU vitamin D daily. They were asked not to consume any other probiotic-containing products such as probiotic-rich milk or yogurt, supplements containing vitamin D, or calcium during the study and were asked not to alter their usual diets or routine physical activity.

Multispecies probiotic capsules and placebo capsules were provided for subjects every month. Compliance with consumption of capsules was monitored every 2 weeks through phone

interviews. The participants were asked to return the unused capsules as part of monitoring compliance.

Anthropometric data

The participants completed a 3-day dietary recall (2 weekdays and one weekend day) by interview monthly throughout the study period. Nutrient analysis was performed by a trained dietitian using Nutritionist IV software (First Databank, San Bruno, CA) modified for Iranian foods. Similarly, physical activity levels were assessed at baseline and at 3 and 6 months and were estimated through daily physical activity questionnaires validated by Kelishady et al. [27]. Physical activity levels were calculated as metabolic equivalents/day.

Anthropometric measurements were also assessed at baseline and after 6 months of intervention. Body weight was measured wearing light clothes without shoes using digital scales with 100-g precision. Height was measured using a stadiometer with 0.5-cm precision in a normal standing position without shoes (Seca, Hamburg, Germany). BMI was calculated as weight in kilograms divided by height in meters squared.

Bone mineral density measurements

BMD of the L1–L4 spine and total hip was measured by dual-energy X-ray absorptiometry (Hologic QDR4500, Hologic Inc., Waltham, MA). Before measurement, the equipment was calibrated according to the manufacturer's manual. Measurements were assessed at the beginning and at the end of the study.

Laboratory analysis

Fasting blood samples (10 mL) were taken at baseline and the end of the trial early in the morning after an overnight fast. Serum was

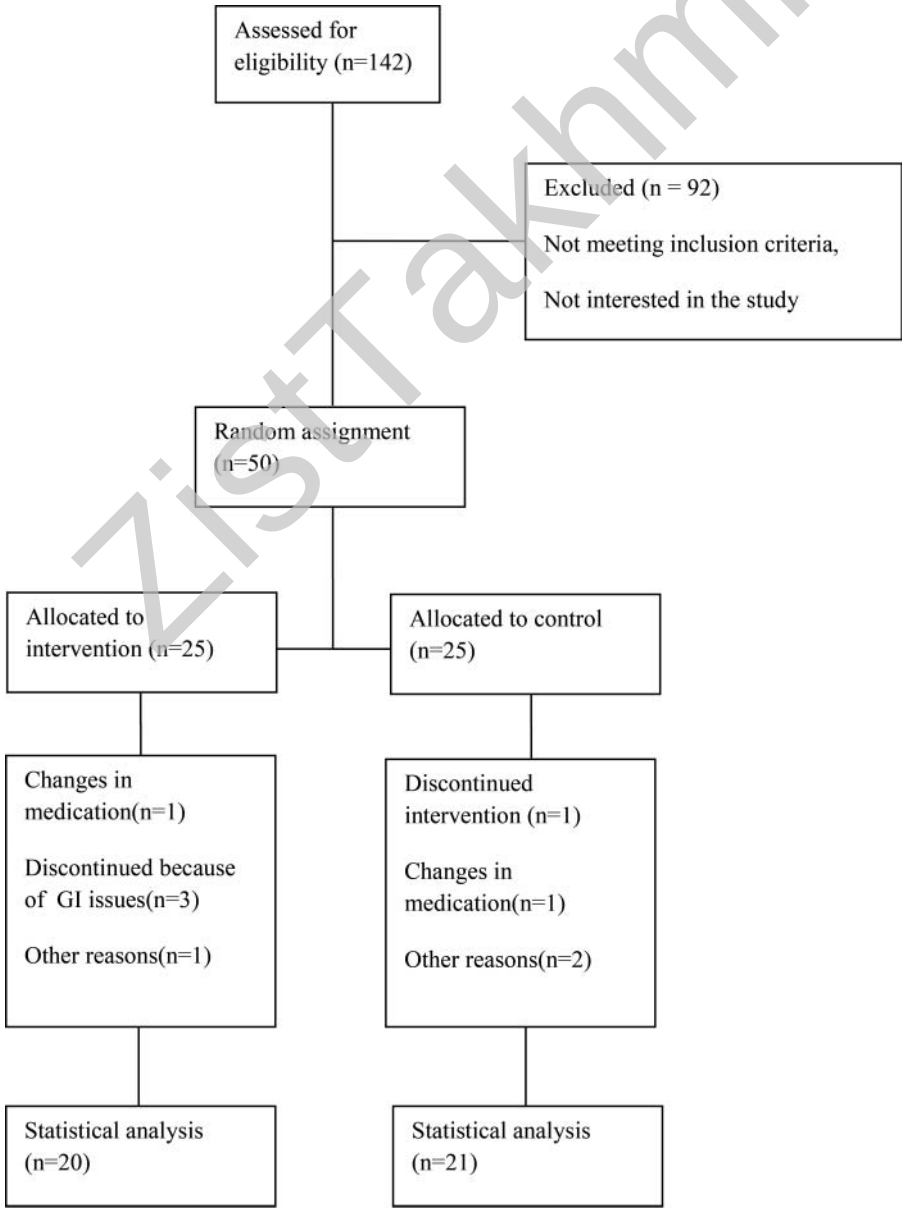

Figure 1. Flowchart of the study design and subject participation.

Table 1. Baseline characteristics of the study participants.[a]

Variables	Probiotic group ($n = 20$)	Control group ($n = 21$)	p Value
Age (years)	58.85 ± 0.68	57.29 ± 0.72	0.125
Weight (kg)	64.25 ± 1.12	62.53 ± 0.91	0.239
Height (cm)	160.85 ± 1.29	162.14 ± 1.19	0.464
BMI (kg/m^2)	24.86 ± 0.41	23.82 ± 0.38	0.070
Time since menopause ± years	7.35 ± 0.73	6.38 ± 0.67	0.332
T-score (L1–L4)	−1.41 ± 0.05	−1.45 ± 0.05	0.535

BMI = body mass index.
[a]Data are means ± SEM obtained from an independent t test.

immediately separated by centrifugation at 3500 rpm for 15 minutes and stored at −80°C until the assay. Twenty-four-hour urine specimens were collected, excluding the first void, at baseline and 6 months and were stored at −80°C for later analysis.

Bone and pro-inflammatory biomarkers were assessed at baseline and 6 months using commercially available enzyme-linked immunosorbent assay (ELISA) kits. Total serum levels of bone-specific alkaline phosphatase (BALP), osteocalcin (OC), CTX, and vitamin D were measured with ELISA kits from IDS (Boldon, UK). Intra-assay (within-run) and interassay (between-run) coefficients of variation (CVs) were <4.5% and <6.4% for BALP, 1.8% and 4% for OC, 2.2% and 7.7% for CTX, and 5.9% and 6.6% for vitamin D. Serum total receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegrin (OPG) levels were measured with an ELISA kit from BIOMEDICA (Vienna, Austria; intra-assay CV 7.5% and interassay CV 4% for RANKL; intra-assay CV 7.5% and interassay CV 7% for OPG). Serum TNF-α and IL-1β levels were measured using ELISA kits from Bender MedSystems GmbH (Vienna, Austria). Intra-assay and interassay coefficients of variation were 8.5% and 9.8% for TNF-α and 5.1% and 8.6% for IL-1β. Serum parathyroid hormone (PTH) level was measured using commercial ELISA kits from Diametra (Segrate, Italy). The overall intra-assay and interassay CVs were calculated to be 5.1% and 8.6%, respectively. Urinary deoxypyridinoline (DPD) level was measured using ELISA kits from Crystal Day Biotech (Shanghai, China; interassay CV 12%, intra-assay CV 10%). Serum calcium, phosphorus, magnesium, albumin, creatinine, alkaline phosphatase, and urinary amounts of calcium, phosphorus, magnesium, and creatinine were analyzed using Pars Azmoon kits (Pars Azmoon Co., Tehran, Iran) at baseline and 6 months.

Characteristics of supplements

Multispecies probiotic capsules (GeriLact) contained 7 bacteria species (Lactobacillus casei 1.3 × 10^{10} colony-forming units [CFU], Bifidobacterium longum 5 × 10^{10} CFU, Lactobacillus acidophilus 1.5 × 10^{10} CFU, Lactobacillus rhamnosus 3.5 × 10^9 CFU, Lactobacillus bulgaricus 2.5 × 10^8 CFU, Bifidobacterium breve 1 × 10^{10} CFU, and Streptococcus thermophilus 1.5 × 10^8 CFU per 500 mg). Placebo capsules were produced in a same size, shape, odor, color, and packing as the probiotic capsules and contained 500 mg of corn starch. The multispecies probiotic and placebo capsules are manufactured by ZistTakhmir Co., Tehran, Iran.

Statistical analyses

Statistical analysis of data was performed using SPSS for Microsoft Windows (Ver 19.0; SPSS Inc., Chicago, IL). According to the Shapiro-Wilk test, all quantitative parameters had normal distributions. For descriptive statistics, the mean and standard error of the variables were calculated. To compare the 2 groups for baseline measures, the independent sample t test was used. Comparisons between the baseline and final results were performed using paired sample t tests to identify within-group differences. Changes from baseline of variables including biochemical and bone parameters were analyzed by analysis of covariance using treatment group as the main factor and baseline values as covariates. Because dietary and anthropometric parameters were measured 7 times and 3 times during the study period, respectively, analysis of variance for repeated measurements was used to compare data among these time intervals. A type I error (α) of 0.05 was considered to be statistically significant.

Results

Baseline characteristics, dietary intake, and physical activity status

Forty-one of the 50 women (20 on probiotics and 21 on placebo) completed the study. The attrition rate for the intervention and control groups was 18%. Common reasons for not finishing the study included alteration in medications; claims of medical and health-related issues, especially gastrointestinal issues; and personal reasons (Figure 1). All participants who completed the study were compliant with their treatments.

Baseline characteristic data for subjects who completed the study are presented in Table 1. Baseline characteristics were not significantly different for subjects who completed the study. Age, years since menopause, body weight, height, and BMI were similar at baseline between the treatment groups.

Level of physical activity was assessed at baseline and at 3 and 6 months. There were no significant differences in physical activity levels throughout the study (Table 2). Analysis of the 3-day food recalls indicated that the participants' food intakes,

Table 2. Physical activity patterns assessed at baseline, 3 months, and 6 months[a].

	Probiotic group ($n = 20$)			Control group ($n = 21$)			
Variables	Baseline	3 Months	6 Months	Baseline	3 Months	6 Months	p Value
PA (MET.h/d)	35.94 ± 0.35	36.09 ± 0.41	35.93 ± 0.37	34.93 ± 0.49	35.05 ± 0.46	35.10 ± 0.37	NS

PA = physical activity, MET = metabolic equivalent.
[a]Data are means (SEM). There were no statistical significant differences observed between the baseline values of the intervention and control groups or between the baseline and corresponding values at 3 and 6 months based on analysis of variance for repeated measurements.

Table 3. Daily nutrient intake calculated from 3-day food recall at baseline and 1, 2, 3, 4, 5, and 6 months.[a]

	Probiotic group ($n = 20$)							Control group ($n = 21$)							
Variables	Baseline	1 Month	2 Months	3 Months	4 Months	5 Months	6 Months	Baseline	1 Month	2 Months	3 Months	4 Months	5 Months	6 Months	*p* Value
Macronutrients															
Energy (kcal)	1818 ± 56	1842 ± 54	1814 ± 47	1813 ± 42	1807 ± 37	1806 ± 40	1802 ± 28	1750 ± 47	1814 ± 28	1966 ± 52	1788 ± 46	1819 ± 51	1772 ± 29	1835 ± 38	NS
Protein (g)	70.1 ± 4.3	69.3 ± 4	65.6 ± 4.3	66.3 ± 4.5	63.6 ± 2.8	64.4 ± 3.5	68.2 ± 4	65 ± 3.6	63.2 ± 3.4	66.8 ± 4	66.4 ± 3.4	63.2 ± 3.7	64.3 ± 3.8	67.5 ± 3.9	NS
Carbohydrate (g)	218 ± 13	214 ± 10	219 ± 9	225 ± 12	227 ± 7	224 ± 12	215 ± 10	211 ± 11	223 ± 12	217 ± 14	203 ± 10	203 ± 11	218 ± 10	222 ± 9	NS
Total fat (g)	76.9 ± 4.2	72.2 ± 5.4	69.2 ± 5.1	70.6 ± 3.3	75.1 ± 4.6	66.2 ± 3.9	62.9 ± 4.2	72.4 ± 4.5	65.2 ± 3.5	66.1 ± 4.2	70.8 ± 3.8	72 ± 4.1	64.5 ± 5.1	65.7 ± 4.4	NS
Dietary fiber (g)	15.5 ± 1.4	15.7 ± 1.1	14.6 ± 1.2	15.1 ± 1.5	14.2 ± 1.2	13.3 ± 1.3	13.7 ± 1	13 ± 1	14.6 ± 1.4	14.5 ± 1.4	13.8 ± 1.1	12.6 ± 0.9	13.4 ± 0.9	13.6 ± 0.7	NS
Total cholesterol (mg)	210.1 ± 16.1	193.7 ± 15.3	222.8 ± 16.1	195.6 ± 16.8	211.8 ± 18.3	216 ± 16.6	213.2 ± 11.9	204 ± 14.7	209.6 ± 14.8	199.7 ± 14.1	199.9 ± 14.1	189.2 ± 12.9	192.8 ± 13.9	190 ± 12.7	NS
SFAs (g)	19.6 ± 1.7	21.3 ± 1.7	22.1 ± 2	17.9 ± 1.5	18.6 ± 1.6	22 ± 1.9	19.7 ± 1.1	20.1 ± 1.7	19.1 ± 1.5	22.1 ± 1.3	18.2 ± 1.6	20.6 ± 1.2	16.1 ± 1.2	17.8 ± 1	NS
PUFAs (g)	9.7 ± 0.7	8.6 ± 0.8	8.9 ± 1.1	6.9 ± 0.6	7.4 ± 0.8	10.2 ± 1.2	9.1 ± 1.3	8.8 ± 0.7	8.1 ± 0.9	7.8 ± 0.6	7.2 ± 0.9	10.1 ± 0.7	10.5 ± 0.8	8.7 ± 0.6	NS
MUFAs (g)	17.5 ± 2.1	15.1 ± 1.5	14.8 ± 1.9	16.2 ± 1.5	19.4 ± 2.2	14.1 ± 3.4	15.2 ± 3.1	18.4 ± 0.9	18.1 ± 2.1	16.1 ± 2.4	16.7 ± 2.2	17.6 ± 2.7	18.1 ± 2.9	15.2 ± 1.7	NS
Vitamins															NS
K (μg)	166.2 ± 32.9	183.3 ± 30.2	166.2 ± 34.2	130.1 ± 24.5	121.2 ± 15.7	161.2 ± 31.2	155 ± 10.3	120.6 ± 17.1	173.3 ± 25.7	187.9 ± 29.4	139.5 ± 15.4	131.5 ± 21.3	116.6 ± 20.5	144.6 ± 13.5	NS
D (μg)	2.6 ± 0.8	2.9 ± 0.9	3.2 ± 0.9	3 ± 0.8	2 ± 0.5	1.5 ± 0.4	1.3 ± 0.3	2.4 ± 0.7	2.3 ± 0.6	2.5 ± 0.7	1.9 ± 0.7	1.7 ± 0.6	1.4 ± 0.3	1.6 ± 0.3	NS
E (mg)	8.4 ± 2.2	7.6 ± 2.2	6.8 ± 1.6	6.6 ± 1.5	5.5 ± 1.6	7.4 ± 1.9	8.8 ± 0.9	6.3 ± 2.1	8 ± 2.1	7.6 ± 2.1	5.9 ± 1	5.2 ± 1.5	5.6 ± 1.5	6.9 ± 0.7	NS
C (mg)	71 ± 12	86 ± 13	112 ± 17	73 ± 11	68 ± 12	75 ± 9	107 ± 13	94 ± 16	103 ± 13	90 ± 10	73 ± 12	75 ± 9	89 ± 13	72 ± 7	NS
Minerals															NS
Ca (mg)	618.2 ± 38	740 ± 51.8	721.2 ± 73.6	629.3 ± 38.4	652.2 ± 37	706.5 ± 43.7	642.3 ± 34.2	655.4 ± 62.8	664.4 ± 50.8	730.1 ± 52.4	600.3 ± 32.2	618.8 ± 53.1	625.8 ± 53	598 ± 29	NS
Mg (mg)	264.6 ± 17.4	286.3 ± 17.2	262.6 ± 15.9	252.2 ± 16.1	262.5 ± 15.2	242.6 ± 16.5	233.4 ± 12.5	232.8 ± 13.4	254.2 ± 15.3	275.6 ± 20.6	249.4 ± 13.7	235.3 ± 12.5	250.9 ± 12.9	261.9 ± 11.9	NS
P (mg)	950 ± 40	1001 ± 54	981 ± 58	933 ± 42	968 ± 32	938 ± 50	823 ± 36	915 ± 54	943 ± 43	993 ± 54	917 ± 40	876 ± 50	938 ± 49	901 ± 46	NS
Fe (mg)	14.1 ± 1.1	13.7 ± 0.9	12.6 ± 0.8	13.4 ± 1	12.7 ± 0.9	13.3 ± 1	15.1 ± 0.8	12.1 ± 0.8	13.7 ± 0.8	14.3 ± 1.1	13.2 ± 0.9	12.5 ± 0.8	13.8 ± 1	14.8 ± 1	NS
Zinc (mg)	8.4 ± 0.5	9 ± 0.6	8.4 ± 0.6	7.8 ± 0.5	7.9 ± 0.4	7.9 ± 0.5	7.7 ± 0.3	8 ± 0.5	7.9 ± 0.4	8.7 ± 0.5	8 ± 0.5	7.8 ± 0.5	7.7 ± 0.5	7.7 ± 0.3	NS

SFA = saturated fatty acid, PUFA = polyunsaturated fatty acid, MUFA = monounsaturated fatty acid.

[a]Data are presented as mean ± SEM. There were no statistically significant differences observed between the baseline values of the 2 treatment groups or between the baseline values and corresponding values at 1, 2, 3, 4, 5, and 6 months based on analysis of variance for repeated measurements.

Table 4. Characteristics of patients who received probiotic or placebo, at baseline and after 6 months.[a]

Variables	Probiotic group ($n = 20$)		Control group ($n = 21$)		p Value*
	Baseline	6 Months	Baseline	6 Months	
Spinal BMD (g/cm^2)	0.919 ± 0.08	0.920 ± 0.07	0.912 ± 0.06	0.914 ± 0.07	0.892
Total hip BMD (g/cm^2)	0.837 ± 0.07	0.822 ± 0.08	0.808 ± 0.1	0.792 ± 0.08	0.725
BALP (U/L)	19.65 ± 1.66	16.53 ± 0.90†	17.81 ± 1.35	18.63 ± 1.29	0.033
CTX (ng/ml)	0.41 ± 0.02	0.35 ± 0.02	0.45 ± 0.02	0.42 ± 0.02	0.046
RANKL (pg/ml)	5.2 ± 0.45	4.07 ± 0.50	3.98 ± 0.43	4.59 ± 0.61	0.068
OPG (pg/ml)	103 ± 1.9	100.4 ± 2	98.6 ± 1.3	101.6 ± 2	0.734
RANKL/OPG ratio	0.044 ± 0.005	0.053 ± 0.007	0.041 ± 0.005	0.045 ± 0.006	0.455
DPD (nmol/L)	7.9 ± 0.93	8.55 ± 1.08	8.86 ± 0.72	9.49 ± 0.9	0.722
OC (ng/ml)	18.06 ± 0.8	18.9 ± 0.73	19.47 ± 0.8	17.85 ± 1	0.470
TNF-α (pg/ml)	4.24 ± 0.5	3.73 ± 0.43	3.83 ± 0.47	4.32 ± 0.5	0.041
IL-1 (pg/ml)	2.17 ± 0.28	2.29 ± 0.34	1.79 ± 0.27	2.36 ± 0.23	0.975
Vitamin D (ng/ml)	29.53 ± 1.76	28.20 ± 0.97	31.74 ± 1.40	29.08 ± 1	0.720
PTH (pg/ml)	31.92 ± 1.39	29.05 ± 1.53†	30.65 ± 1.44	32.81 ± 1.72	0.012
Serum calcium (mg/dl)	9.40 ± 0.18	10.15 ± 0.30	9.86 ± 0.21	9.34 ± 0.25	0.067
24-Hour urinary calcium (mg/d)	169.6 ± 15	184.1 ± 15.5	174.4 ± 17.8	178.2 ± 18.1	0.250
Serum phosphorus (mg/dl)	3.61 ± 0.18	3.9 ± 0.14	3.77 ± 0.14	3.72 ± 0.14	0.344
24-Hour urinary phosphorus (mg/d)	562.7 ± 37	512.5 ± 25	642.4 ± 37.8	590.7 ± 31.6	0.222
Serum magnesium (mg/dl)	1.96 ± 0.09	1.81 ± 0.1	1.75 ± 0.09	1.72 ± 0.08	0.864
24-Hour urinary magnesium (mg/d)	88.8 ± 8	100.2 ± 6.4	94.8 ± 6	97 ± 5.5	0.658
Serum creatinine (mg/dl)	0.89 ± 0.03	0.83 ± 0.04	0.82 ± 0.05	0.85 ± 0.04	0.307
24-Hour urinary creatinine (mg/d)	1040 ± 61	1016 ± 55	1162 ± 52	1137 ± 33	0.269
ALP (U/L)	189.3 ± 8.2	196.7 ± 7.5	184.9 ± 8.9	192.4 ± 8.5	0.861
Albumin (mg/dl)	4.22 ± 0.06	4.32 ± 0.06	4.27 ± 0.06	4.19 ± 0.07	0.144

BMD = bone mineral density, BALP = bone-specific alkaline phosphatase, CTX = collagen type 1 cross-linked C-telopeptide, RANKL = receptor activator of nuclear factor kappa-B ligand, OPG = osteoprotegerin, DPD = deoxypyridinoline, OC = osteocalcin, TNF-α = tumor necrosis factor-alpha, IL-1 = interleukin-1, PTH = parathyroid hormone, ALP = alkaline phosphatase.

[a]All continuous variables are expressed as means ± SEM.

†Significant difference within group throughout the study ($p < 0.05$, obtained from a paired t test).

*p Values obtained from analysis of covariance, which shows the changes from baseline in comparison with placebo. No significant differences were observed between the experimental and placebo groups at baseline (obtained from an independent t test).

including macronutrients and micronutrients, were not significantly different between the 2 treatment groups at baseline and throughout the study period (Table 3).

Effect of probiotic supplementation on BMD

Mean BMD measures are shown in Table 4. No significant effect was found as a result of probiotic supplementation with regard to L1–L4 vertebrae and total hip BMD measures (Table 4, Figure 2).

Effect of probiotic supplementation on serum biomarkers of inflammation and bone metabolism

Mean serum concentrations of biomarkers of bone metabolism, BALP, CTX, and OC, and mean concentration of urinary DPD were assessed and are presented in Table 4. The intervention group showed a significant reduction in BALP compared to the placebo group ($p = 0.033$; Table 4). Serum CTX level, as a marker of bone turnover, was significantly lower in the intervention group compared to the placebo group at the end of the study ($p = 0.046$; Table 4).

Mean serum levels of OC and mean concentration of urinary DPD were not significantly different at the end of the study (Table 4).

There were no significant differences in mean serum levels of RANKL, OPG, RANKL/OPG ratio, 25-OH vitamin D, alkaline phosphatase, albumin, magnesium, and phosphorous between intervention and placebo groups after the 6-month period (Table 4). Similarly, no significant changes were observed for urinary calcium, phosphorus, and magnesium and both serum and urinary creatinine (Table 4). Moreover, there was a nonsignificant but marginal effect of probiotic supplementation on increasing serum calcium ($p = 0.067$). There was a statistically significant decrease in PTH in the probiotic group compared to the placebo group ($p = 0.012$). Serum TNF-α, as a major pro-inflammatory cytokine, decreased significantly in the probiotic group compared to the placebo group ($p = 0.027$). However, there was no significant difference in serum IL-1β between probiotic and placebo groups (Table 4).

Discussion

This study revealed that consumption of the multispecies probiotic supplement for 6 months among postmenopausal women with osteopenia had significant effects on serum BALP, CTX, PTH, and TNF-α; however, we failed to find any significant effect on bone density compared to the control group.

Previous studies conducted in hormone-deficient and ovariectomized rats have demonstrated that different strains of probiotics, including *Lactobacillus* and *Bifidobacterium*, can prevent and even restore bone loss due to hormone deficiency [22,23,28–30]. As will be described below, the protective effects of probiotics have been demonstrated mostly in animal studies.

In a study by Chiang and Pan, the administration of *Lactobacillus plantarum* (NTU 102) and *Lactobacillus paracasei* (NTU 101) fermented milk to ovx mice resulted in higher trabecular number (Tb.N) compared to ovx and sham-ovariectomized control groups [31]. In another study, oral administration of *Lactobacillus* probiotics showed a 45% increase in femoral and vertebral trabecular bone volume fraction in male mice [32]. In parallel with previous studies, Kim

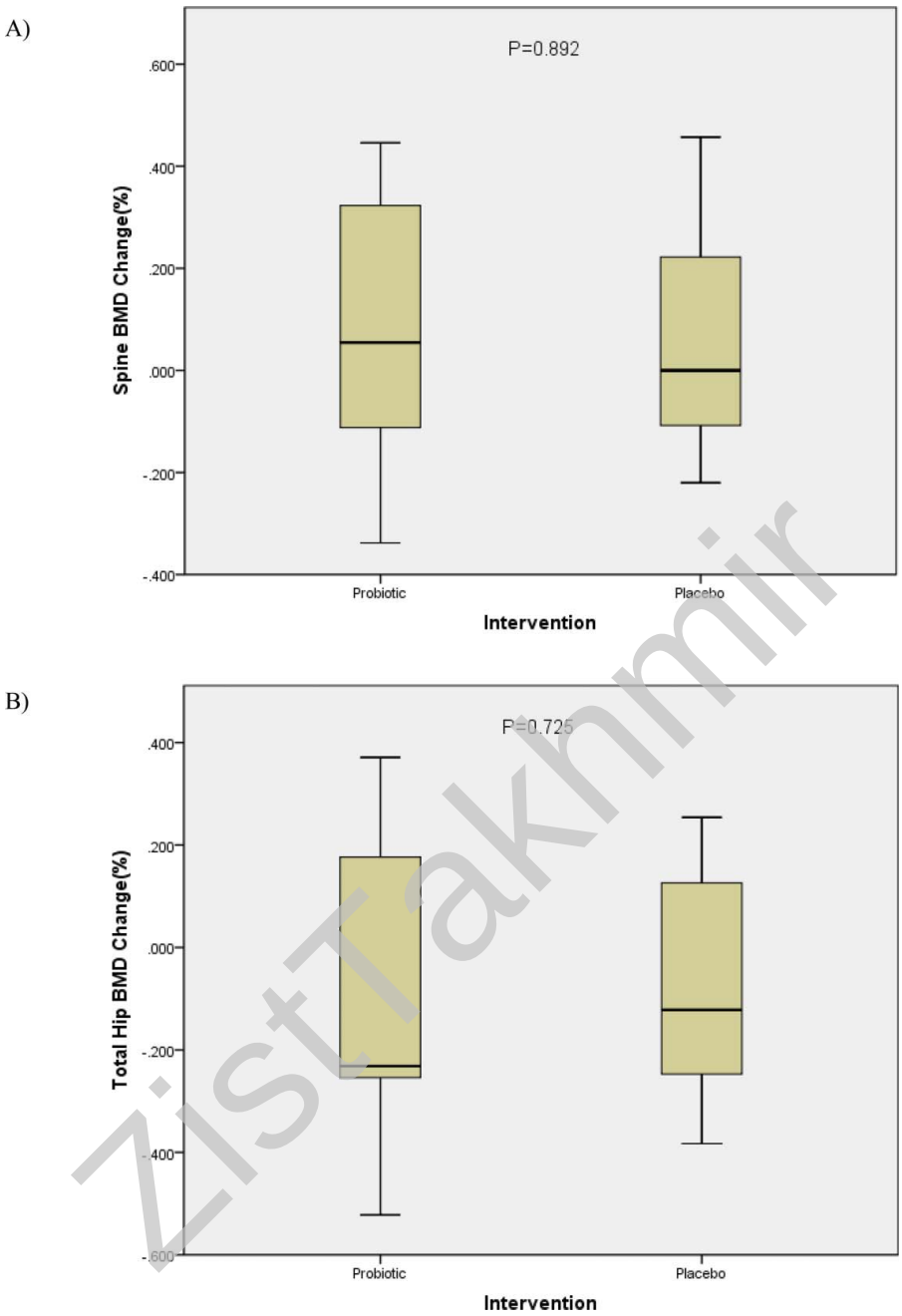

Figure 2. Bone mineral density changes (%) from baseline in the (A) spine and (B) total hip after 6 months. Values are means, with standard deviations represented by vertical bars.

et al. reported a significantly increased level of BMD in ovx rats by administration of *Lactobacillus casei* 393 from fermented milk [28]. However, in our study, the results did not show any significant changes in BMD and bone mineral count (BMC). Therefore, it is suggested that the BMD is not influenced by administration of the probiotic supplementation. However, the findings of bone biomarkers showed favorable effects of probiotics on bone protection.

The results of bone biomarkers, especially CTX and BALP, have shown that administration of a multispecies probiotic supplement may influence the activity of both osteoblasts and osteoclasts by modifying bone formation as well as bone resorption.

BALP as an indicator for osteoblast proliferation and activity or for increased bone turnover is generally increased during growth period and reduced after a delayed bone formation or a slowed-down bone turnover [30]. In previous studies, BALP was considered to be a marker of bone formation; however, more recent studies have suggested that this should be noted as a marker of bone turnover rather than bone formation [33–36]. Scholz-Ahrens et al. reported that BALP was decreased after symbiotic administration [37]. Another study showed that intervention with *Lactobacillus reuteri* decreased BALP and resulted in diminished bone turnover [17], which is in agreement with the present study. This is compatible with the study of Adolphi et al., which showed a reduction in bone turnover

after consumption of fermented milk supplemented with calcium [38].

The measurement of CTX and OC has been considered as a marker of bone resorption and bone formation, respectively. Serum CTX is produced by osteoclasts during the bone resorption process, whereas serum OC is secreted only by osteoblast and osteocyte cells during bone formation [39]. Thus, the increase in the bone resorption process is accompanied by increasing osteoclast activity and increased levels of serum CTX, whereas an increase in the bone formation process is accompanied by an increase in osteoblast activity and serum levels of OC [40]. Therefore, the activity of osteoclast and osteoblast cells can be evaluated by measuring both OC and CTX in serum [29]. In our study, serum levels of CTX were decreased by administration of probiotics but not in the placebo group, suggesting that bone mass is protected from enhanced bone resorption. The result is in agreement with the study by Foureaux et al., which showed that the use of *Bifidobacterium subtilis* (CH201) decreased levels of CTX in both unstressed and stressed animals [41]. In parallel with the study, Parvaneh et al. reported that *Bifidobacterium longum* supplementation decreased the ratio of osteoclast/osteoblast and serum levels of CTX [29].

In the present study, serum levels of OC and urinary DPD, as a bone resorption marker, did not change during the 6-month intervention period. This agrees with the conclusions reached in Ohlsson et al., which showed that bone formation, as indicated by serum osteocalcin, was not significantly affected by probiotic treatment during 6 weeks of intervention [42].

Based on the mentioned findings, although administrating probiotics did not appear to affect bone formation, it resulted in diminished bone turnover and thus diminished bone resorption, as observed after intervention with the multispecies probiotic. Therefore, our overall findings suggest that probiotics improve bone mass by slowing down the rate of bone turnover.

Parathyroid hormone is the most important regulator of calcium concentration in the blood. A high PTH concentration increases calcium release from bone and also bone resorption. It has been shown that oral calcium supplementation led to a decrease in PTH concentrations, which resulted in decreased bone resorption [43,44]. One of the potential effects of various strains of probiotics on bone possibly occurs via producing several short-chain fatty acids, which decrease PTH followed by an increase in mineral absorption by solubilization [45,46].

In the present study, despite the nonstatistically significant effect of probiotic supplementation on serum calcium, we found a reduction in PTH. However, serum calcium concentration increased marginally, followed by a decrease in PTH and, as a consequence, reduced bone resorption in subjects. The results are in line with a study by Narva et al., which showed that fermentation with *Lactobacillus helveticus* bacteria had a positive effect on calcium metabolism by suppressing serum PTH and increasing serum calcium concentrations acutely compared to a normal fermented milk product [44].

Another potential mechanism of action of probiotics on bone health is modification of the RANKL/RANK/OPG pathway, which modulates osteoclastogenesis. RANKL is a key molecule in osteoclast activation, and OPG is a decoy receptor for RANKL. Therefore, the relative proportion of RANKL and OPG regulates the activity of bone resorption–mediated osteoclastogenesis [47]. Binding of RANKL on its receptor receptor activator of nuclear factor-κB (RANK), which is present on the surface of osteoclasts, led to enhanced osteoclastogenesis [48]. In the present study, serum levels of RANKL, OPG, and the ratio of RANKL/OPG were not significantly different from baseline, though a marginally significant decrease in the amount of serum RANKL was observed.

In an earlier animal study, the use of probiotics induced an increase in the expression of OPG but did not affect the expression of RANKL in rats with ligature-induced periodontitis [41]. Another study by Ohlsson et al. reported a decreased in the RANKL/OPG ratio by probiotic treatment, which was mainly caused by increased OPG expression [42]. In a recent study, it was shown that *Lactobacillus reuteri* modulated expression of RANKL and OPG and led to a significant reduction in the RANKL/OPG ratio, shifting the balance toward an anti-osteoclastogenic environment [49]. It is suggested that probiotics have an impact on bone turnover by downregulating the expression levels of RANKL and OPG. Such a reduction in the RANKL/OPG ratio was not demonstrated in the present study. This may be in part due to measurement of serum levels of both RANKL and OPG in the present study, whereas the above-mentioned studies mostly measured gene expression of RANKL and OPG in bone. Unfortunately, due to the specific limitation of available ELISA kits, interpreting serum levels is difficult [50–52]. Moreover, there are conflicting results among studies because of the complexity of the relationship between bone metabolism and RANKL/OPG ratio [53]. A previous study found that unlike serum levels of RANKL, gene expression of RANKL in bone correlated with the measurements of BMD [54]; hence, mRNA levels of RANKL and OPG may better manifest their activity in bone. Unfortunately, because of the invasive nature of measuring bone mRNA, we chose a noninvasive measurement of serum levels in the present study.

One of the underlying mechanisms conferring beneficial effects is the anti-inflammatory effect of probiotics [55]. It is suggested that the anti-inflammatory effects exerted by various probiotic strains are mediated via the induction of Treg cells [56]. There is an evidence that indicates that the anti-inflammatory effect of probiotics is likely mediated through arginine deiminase, produced by the bacteria [57]. Probiotic administration may reduce the expression of several pro-inflammatory and osteolytic cytokines (TNF-α and IL-1β). The cytokines alter expression of the OPG as an anti-osteoclastogenic cytokine, which is responsible for the altered RANKL/OPG ratio. This leads to enhanced activity and formation of osteoclasts and suppressed bone formation by osteoblasts, which results in bone loss [58–60]. The mentioned mechanism clearly signifies the effects of probiotic treatment on alteration of the status of immune cells, which results in attenuated bone resorption. In our study, serum levels of TNF-α as a pro-inflammatory mediator were significantly decreased in the probiotic-treated group. This agrees with the conclusions reached in McCabe et al. [32], which showed an anti-TNF-α activity of *Lactobacillus reuteri* ATCC PTA 6475 that could help suppress TNF-mediated bone resorption in mice. They concluded that *Lactobacillus reuteri* treatment was indeed able to improve bone health in healthy

male mice [32]. In another study, oral administration of *Saccharomyces cerevisiae* resulted in enhanced repair processes, including decreased pro-inflammatory cytokines TNF-α and IL-1β, and increased anti-inflammatory cytokine IL-10 [61], which is consistent with the findings of the present study. Wang et al. [62] indicated that probiotic supplementation in patients on peritoneal dialysis decreased levels of serum TNF-α, IL-5, and IL-6 after 6 months of treatment, which is in line with the present study. In a study by Britton et al. [17], suppression of the percentage of CD4+ T cells in bone marrow has been shown in a *Lactobacillus reuteri* treatment group. They concluded that gut microbiota have immune-modulatory properties and affect OCL-mediated bone resorption [17]. However, because probiotic strains can differ significantly in genotype and phenotype, they may show different metabolic functions, particularly with regard to immune function [63]. Therefore, overall findings of the present study are based on the administration of the 7 specific strains in the multispecies supplement.

Several limitations must be considered in the interpretation of our findings. First, the small sample size precluded small differences from reaching a level of statistical significance. Further trials with a large sample size would be needed to confirm our findings. In addition, due to budget limitations, the study period was 6 months. We only measured the levels of bone biomarkers and pro-inflammatory factors at 2 time points during the study (baseline and 6 months). Three-month interval assessment values for each serum biomarker would have provided a more accurate approach to assess the changes in indicators overtime. Moreover, as mentioned previously, expression levels of pro-inflammatory markers provide a more precise assessment of changes. Therefore, bone biopsy is a preferred method for sampling. Future research should be designed to measure bone levels of these biomarkers as opposed to serum levels alone.

In conclusion, multispecies probiotic supplementation among postmenopausal osteopenic women showed a possible role of OC, BALP, PTH, and TNF-α in suppressing bone resorption and bone turnover. The changes in bone density and other serum indicators did not reach a significant level. Further long-term investigations with different strains of probiotics are needed to dissect the mechanisms and effects of probiotics on bone resorption in humans.

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