Probiotic treatment using a mix of three *Lactobacillus* strains for lumbar spine bone loss in postmenopausal women: a randomised, double-blind, placebo-controlled, multicentre trial

Per-Anders Jansson, Dan Curiac, Irini Lazou Ahrén, Fredrik Hansson, Titti Martinsson Niskanen, Klara Sjögren, Claes Ohlsson

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Wallenberg Laboratory, Department of Molecular and Clinical Medicine, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden (P-A Jansson MD); **CTC, Gothia Forum, Sahlgrenska University Hospital, Gothenburg, Sweden** (D Curiac MD); **Probi, Lund, Sweden** (I Lazou Ahrén PhD, T Martinsson Niskanen PhD); **CTC Clinical Trial Consultants, Uppsala, Sweden** (F Hansson PhD); **and Centre for Bone and Arthritis Research, Department of Internal Medicine and Clinical Nutrition, Institute of Medicine, Sahlgrenska Academy at University of Gothenburg, Gothenburg, Sweden** (K Sjögren PhD, C Ohlsson MD)

Correspondence to: Prof Claes Ohlsson, Centre for Bone and Arthritis Research, Sahlgrenska University Hospital, S-413 45 Gothenburg, Sweden **claes.ohlsson@medic.gu.se**

Summary

Background Postmenopausal bone loss in the spine is associated with an increased risk of vertebral fractures. Certain probiotic treatment protects rodents from ovariectomy-induced bone loss. The aim of the present study was to determine if treatment with a combination of three bacterial strains protects against the rapid spine bone loss occurring in healthy early postmenopausal women.

Methods This randomised, double-blind, placebo-controlled, multicentre trial was done at four study centres in Sweden. Early postmenopausal women were randomly assigned in a 1:1 ratio to receive probiotic treatment consisting of three *Lactobacillus* strains (*Lactobacillus paracasei* DSM 13434, *Lactobacillus plantarum* DSM 15312, and *Lactobacillus plantarum* DSM 15313; 1×10^{10} colony-forming units per capsule) or placebo once daily for 12 months. The primary outcome was the percentage change from baseline in lumbar spine bone mineral density (LS-BMD) at 12 months. The primary analysis was done in all participants with BMD measurements available both at baseline and at 12 months. Analyses of adverse events and safety included all participants who had taken at least one capsule of placebo or *Lactobacillus*. This trial is registered with ClinicalTrials.gov, NCT02722980, and is completed.

Findings Between April 18 and Nov 11, 2016, 249 participants were randomly assigned to receive probiotic product or placebo, and 234 (94%) completed the analyses required for the primary outcome. *Lactobacillus* treatment reduced the LS-BMD loss compared with placebo (mean difference 0·71%, 95% CI 0·06 to 1·35). The LS-BMD loss was significant in the placebo group (–0·72%, –1·22 to –0·22), whereas no bone loss was observed in the *Lactobacillus*-treated group (–0·01%, –0·50 to 0·48). The adverse events were similar between the two groups.

Interpretation Probiotic treatment using a mix of three *Lactobacillus* strains protects against lumbar spine bone loss in healthy postmenopausal women.

Funding Probi.

Introduction

Osteoporosis can lead to fragility fractures, which results in clinical burden and increased mortality.[1,2] Osteoporotic fractures are very common, affecting every second white woman older than age 50 years, the majority of whom are postmenopausal.[3] The rapid bone loss occurring during and early after menopause contributes to the lower bone mass and higher risk of fractures in women compared with men.[4,5] In particular, a substantial spine bone loss is observed during and early after menopause.[6] Estrogen deficiency reduces lumbar spine bone mineral density (LS-BMD) by enhancing osteoclastogenesis and increasing bone turnover, resulting in increased risk of vertebral fractures.[6]

The importance of the gut microbiota for both health and disease has been intensively studied. Experimental studies[7–11] show that manipulation of the composition of the gut microbiota might alter bone homeostasis in rodents. We hypothesised that treatment with probiotics might protect mice from ovariectomy-induced bone loss.[12] A selected mixture of three probiotic strains with anti-inflammatory properties (*Lactobacillus paracasei* DSM 13434, *Lactobacillus plantarum* DSM 15312, and *L plantarum* DSM 15313)[13] was given to ovariectomised mice, and we observed that this treatment protected the mice from ovariectomy-induced bone loss.[12] Several subsequent independent studies have confirmed that different probiotic treatments can protect rodents from ovariectomy-induced bone loss.[7,14,15]

A 2019 cross-sectional observational study[16] showed that *Lactobacillus* abundance was positively correlated with LS-BMD in a Chinese population, suggesting a possible link between *Lactobacillus* and bone mass not only in rodents, but also in humans. Three small single-centre trials[17–19] have evaluated the effect of different probiotic treatments on bone health in women. Two studies[17,19] indicated that probiotic treatment might affect serum bone-turnover markers, but none of these short-term

Research in context

Evidence before this study

Probiotic treatments have the capacity to protect rodents from ovariectomy-induced bone loss, but less is known about the effect of probiotic treatments on bone health in humans. We searched PubMed for studies published before May 16, 2019, with the search criteria "probiotics" AND "bone mineral density" AND "clinical trial". The search found two studies indicating that probiotic treatment might affect serum bone-turnover markers, but none of these small short-term studies had bone mineral density as a predefined primary endpoint. However, in one small, singe-centre clinical trial treatment with a *Lactobacillus* strain was shown to increase bone mineral density in the distal tibia, but that study was not powered for the secondary endpoint of lumbar spine bone mineral density. Thus, although there is evidence of an effect of probiotic treatments on bone health in women, there is no previous large, randomised clinical study with a predefined primary endpoint to evaluate the effect of probiotic treatment on clinically relevant lumbar spine bone loss in early postmenopausal women.

Added value of this study

This randomised, double-blind, placebo-controlled, multicentre trial shows that probiotic supplementation with certain *Lactobacillus* strains naturally occurring in the human gut microbiota prevents lumbar spine bone loss in healthy early postmenopausal women. This is, to our knowledge, the first randomised clinical trial reporting the beneficial effect of probiotic bacteria on bone mineral density at the lumbar spine.

Implications of all the available evidence

Further long-term studies are warranted to explore the beneficial effects of probiotic treatment on lumbar spine bone loss in postmenopausal women. Probiotic treatment might be useful for the prevention of lumbar spine bone loss in postmenopausal women.

studies had bone mineral density (BMD) as a predefined primary endpoint. However, in the clinical trial by Nilsson and colleagues,[18] the predefined primary endpoint was relative change in volumetric BMD in the distal tibia at 12 months versus baseline. 90 older (age 75–80 years) Swedish women with osteopenia were randomly assigned a study product, and 34 participants in the *Lactobacillus reuteri*-treated group and 36 participants in the placebo-treated group completed the study with regards to this primary endpoint.[18] The probiotic treatment significantly reduced bone loss in the distal tibia, but the study was not powered for the secondary endpoint of LS-BMD.[18]

Thus, although there is evidence of an effect of probiotic treatments on bone health in women, no previous large, randomised clinical multicentre study exists with a predefined primary endpoint to evaluate the effect of probiotic treatment on clinically relevant lumbar spine bone loss in postmenopausal women. The aim of our study was to determine if treatment with a combination of three bacterial strains, with the same bacterial composition as that shown to protect mice from ovariectomy-induced bone loss,[12] protects against the spine bone loss occurring in healthy early postmenopausal women.

Methods

Study design

The ProBone study was a randomised, double-blind, placebo-controlled, multicentre trial to evaluate the efficacy on percentage change from baseline in LS-BMD of a probiotic treatment, consisting of three *Lactobacillus* strains, as compared with placebo in healthy early postmenopausal women. We screened participants at four study centres in Sweden (Gothenburg, Uppsala, Linköping, and Stockholm). The trial was approved by the ethical review board in Gothenburg. The protocol is available in the appendix.

Participants

Healthy women in the early post-menopausal phase (≥2 years and ≤12 years since the last menstruation and ≥1 year since the last intake of hormone replacement therapy) with a T score of more than –2·5 at the lumbar spine (L1–L4), as measured by dual-energy x-ray absorptiometry (DXA), and a body-mass index (BMI) of between 18 and 30 were eligible for participation. Women were recruited using advertisements in local newspapers. The criterion of at least 2 years after last menstruation was selected because we wanted to be sure that the participant had passed menopause, but because we hypothesised that the preventive effect would be largest early after menopause, we also added a limit of maximal 12 years after last menstruation. The criterion of more than 1 year from last intake of HRT was chosen to avoid possible confounding effects of exogenous sex hormones. The criterion of more than –2·5 in T score was chosen because we did not want to include patients with osteoporosis. The BMI criteria of not being underweight (BMI <18) or obese (BMI >30) were chosen to avoid the effect of abnormal weight on BMD. Detailed exclusion criteria and restrictions during study are provided in the appendix (p 3). Each participant provided written informed consent before inclusion.

Randomisation and masking

Participants were randomly assigned in a 1:1 ratio to receive the probiotic product or placebo. The randomisation into one of the two study groups was done in blocks of four using a computerised random number generator in Excel. An independent statistician not otherwise involved in the study generated the sequence. Medical personnel at the participating centres, not involved in the data analyses or the interpretation of the data enrolled the participants and assigned them to the trial groups.

See Online for appendix

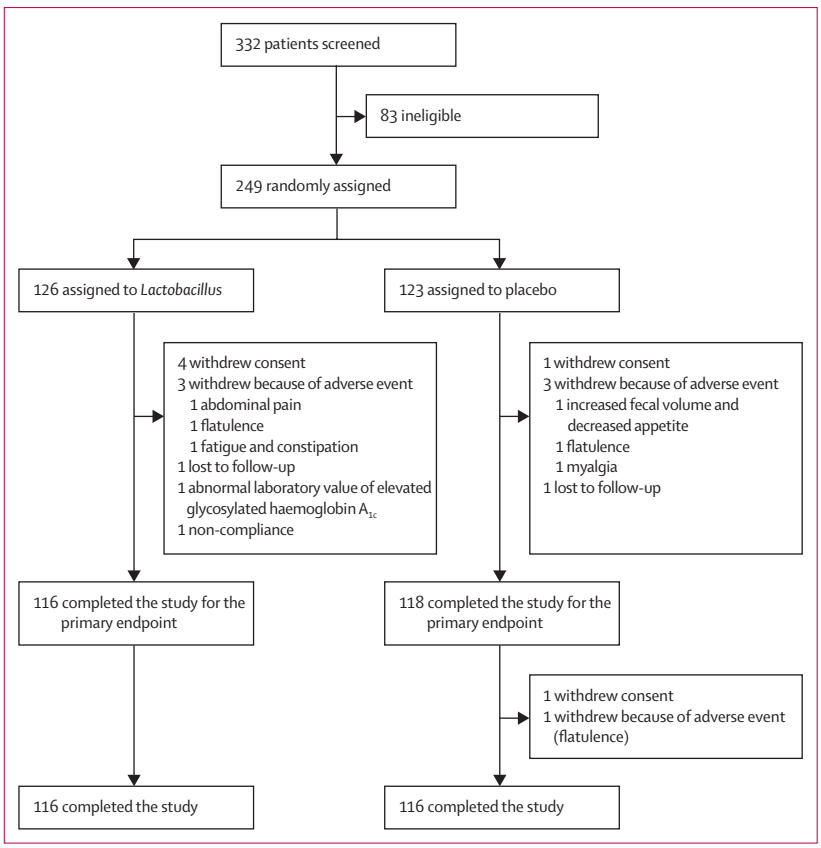

Figure 1: **Trial profile**

The study product was prepared by personnel at Probi, not otherwise involved in the study. The product was labelled according to the generated numbers in the randomisation list that was stored in a sealed envelope by the randomiser. The same people prepared the corresponding intervention code envelopes that were sealed and safely stored by the local primary investigators at each recruiting site. All study products were identical, both in terms of package and with regards to the capsules. Both the probiotic-containing capsules and those with placebo had the same appearance, taste, and texture. Only the randomisation number on the packages differed. The recruitment of study participants was done by medical personnel at the four recruiting centres (the same personnel that met the participants at the predefined study visits). Each participant was allocated the next available study product and randomisation number at the site. None of the personnel who met the study participants throughout the clinical trial were involved in data management or statistical analysis. Masking to group assignment was maintained throughout the study both for the participants and the personnel involved in the recruitment, study visits, or data management. Following the database lock, the assignment was revealed to the statistician responsible for the analysis of data.

Procedures

During a screening visit, signed informed consent forms for participation in the study were collected and the participants were checked for compliance with the eligibility criteria (excluding the DXA criteria), based on a medical history questionnaire. Eligible participants were scheduled for a DXA scan, and if eligibility for the study was confirmed, they were scheduled for their first visit (baseline visit; randomisation) within 2 weeks. Additional visits were done at months 1, 3, 6, and 12 after the baseline visit, and the study participants were contacted by phone 2 months and 9 months after the baseline visit to confirm that they were taking the study product as planned and to collect information on any adverse events.

The active investigational product consisted of a combination of the three probiotic bacterial strains *L paracasei* 8700:2 (DSM 13434), *L plantarum* Heal 9 (DSM 15312), and *L plantarum* Heal 19 (DSM 15313). The investigational product was supplied in capsules containing a powder with freeze-dried bacteria and maize starch used as filler. Each bacterial strain was equally represented in the total bacterial dose of 1×10^{10} colony-forming unit (CFU) per capsule. The equal representation of the three strains and the total dose of 1×10^{10} CFU per capsule were selected because we have previously observed that the combination of these three *Lactobacillus* strains protected mice from ovariectomy-induced bone loss.[12] Several previous randomised controlled studies evaluating the efficacy and safety of *Lactobacillus* treatment on a variety of health outcomes have used doses in the range of 1×10^{8} to 1×10^{10} CFU per day.[17,18,20–23] Well-designed clinical dose-response studies of the efficacy of different *Lactobacillus* doses on health outcomes are few, but, in general, daily doses up to 1×10^{10} CFU are well tolerated. We selected a high dose (ie, 1×10^{10} CFU per capsule per day), of *Lactobacillus* because we hypothesised that this dose would be likely to exert an effect on LS-BMD without having any major adverse effects. The participants were instructed to consume one capsule daily for the total length of the study (12 months). Compliance to intake of the investigational product was evaluated by counting the number of unused capsules returned.

BMD was measured at the lumbar spine and proximal femur (total hip, trochanter, and femoral neck) by means of DXA (Lunar iDXA and Lunar Prodigy Advance, GE Healthcare, USA) at baseline and 12 months. Bioclinica was responsible for the calibration of the scanning equipment used at the different sites (standardisation with the same phantom), the quality control, and central reading of DXA imaging (Bioclinica, London, UK). Analyses of serum and urine markers are described in the appendix (pp 3–4). Adverse events reported by the participants, observed or elicited based on non-leading questions by the investigator or medical personnel were collected from the time of signing the informed consent until completion of the study.

All study participants were instructed by a physician to refrain from using other products, functional food, or

dietary supplements containing added probiotic bacteria. The list of products not to be used also included various types of fermented vegetables. Participants received, as an example, a list with products to be avoided but were also instructed to carefully check the ingredients of the products they were consuming paying special attention and avoiding products with added bacterial cultures. They were asked not to consume more than five cups of coffee per day or corresponding amount of other caffeine-containing products. In addition, they were instructed not to use calcium or vitamin D supplements.

Outcomes

The primary endpoint was the percent change from baseline in BMD at the lumbar spine at 12 months in the *Lactobacillus*-treated group compared with the placebo-treated group. Because we hypothesised that the possible effect of the treatment would be most pronounced early after menopause, a subgroup analysis was predefined, including participants having below the median time since menopause onset at baseline (<6 years after menopause).

The secondary endpoints were the percent change from baseline in BMD at the total hip and femoral neck at 12 months; serum concentrations of procollagen type I N-terminal propeptide and osteocalcin (markers of bone formation); serum concentrations of β-isomer of the C-terminal telopeptide of type I collagen (β-CTX; marker of bone resorption); and urine concentrations of bone resorption N-terminal telopeptide/creatinine (NTx/Cr; marker of bone resorption) at months 1, 3, 6, and 12 in the *Lactobacillus* group compared with the placebo group. The key exploratory endpoints included the percent change from baseline in BMD at the hip trochanter at 12 months, in concentrations of high sensitivity C-reactive protein (hsCRP) at months 1, 3, 6, and 12 and in concentrations of TNF-α at months 1, 3, and 12 in the *Lactobacillus* group compared with the placebo group. Safety was assessed based on physical examination and analysis of blood samples at each study visit. It was also based on registered adverse events that were reported by the subjects or identified by the medical personnel during the scheduled study visits and telephone contacts. Adverse events were collected from start until end of study.

Statistical analysis

When doing the power calculations before the study start, an SD of 5% for change in LS-BMD was anticipated, resulting in an 80% power to detect a statistically significant ($p<0·05$) difference of 1·98% between the probiotic group and placebo group if a sample size of 100 participants per group was used. Allowing for a withdrawal rate of 20% for the primary outcome, we decided to aim to randomly assign 250 participants to receive either active product or placebo at a ratio of 1:1.

When approximately 50 participants had completed the study, a masked interim analysis was done with the aim to confirm the validity of the calculated sample size. This was done by verifying that the SD, with regards to the primary endpoint, did not exceed the one used in the primary sample size calculation. The interim analysis was done according to a prespecified statistical analysis plan and confirmed that the SD of the primary endpoint was not larger than the value used for the power analyses. A statistical analysis plan was developed before unmasking. The difference between the treatment groups for BMD efficacy variables was tested by analysis of covariance (ANCOVA) with relative change from baseline to 12 months as dependent variable; treatment group as fixed effect; and site, baseline age, and baseline time from menopause as covariates. From these models, least square means (LSM) with 95% CI are presented. The p values given for the within-group comparison (12 months

	Lactobacillus (N=126)	Placebo (N=123)
Age (years)	59·1 (3·8)	58·1 (4·3)
Height (cm)	167 (5)	168 (6)
Weight (kg)	67·4 (8·2)	67·3 (9·0)
Body-mass index (kg/m²)	24·2 (2·7)	23·9 (2·6)
Time in the study (days)	343 (336–351)	342 (336–347)
Number of doses	338 (329–348)	336 (327–347)
Race		
White	125 (99%)	121 (98%)
Hispanic or Latino	1 (1%)	1 (1%)
Asian	0 (0%)	1 (1%)
Bone mineral density (T score)		
Lumbar spine	−0·63 (1·13)	−0·64 (1·21)
Total hip	−0·72 (0·76)	−0·60 (0·89)
Hip trochanter	−0·95 (0·78)	−0·86 (0·90)
Hip femoral neck	−1·13 (0·68)	−1·06 (0·77)
Osteopenia	54 (43%)	54 (44%)
Time since menopause (years)	6·55 (2·70)	6·50 (2·77)
Serum bone markers*		
PINP (μg/L)	68·3 (53·3–81·8)	68·1 (50·4–84·4)
Osteocalcin (μg/L)	24·9 (19·9–31·7)	24·8 (18·7–31·8)
β-CTX (ng/L)	520 (390–720)	490 (370–710)
Urine bone marker†		
NTx/Cr (nM/μM)	61·0 (51·9–82·0)	63·1 (51·1–78·6)
Inflammatory markers†		
hsCRP (mg/L)	0·66 (0·44–1·60)	0·71 (0·42–1·52)
TNF-α (pg/mL)	1·18 (0·93–1·47)	1·19 (0·88–1·43)
Glucose metabolism		
Glycosylated haemoglobin A_{1c}‡ (mmol/mol)	35·1 (3·4)	34·6 (3·1)

Data are mean (SD), median (IQR), or n (%). PINP=procollagen type I N-terminal propeptide. β-CTX=β-isomer of the C-terminal telopeptide of type I collagen. NTx/Cr=N-terminal telopeptide/creatinine. hsCRP=high sensitivity C-reactive protein. TNF-α=tumor necrosis factor-α. *n=115 in the *Lactobacillus* group, and n=118 in the placebo group. †n=117 in the *Lactobacillus* group, and n=118 in the placebo group. ‡Normal range 31–46 mmol/mol.

Table 1: **Participants' baseline characteristics**

	Within-group comparison (month 12 *vs* baseline)		Difference between groups
	Lactobacillus (n=116)	Placebo (n=118)	
Primary outcome			
LS-BMD	−0·01% (−0·50 to 0·48)	−0·72% (−1·22 to −0·22)*	0·71% (0·06 to 1·35)†
Secondary or explorative outcomes			
Total hip BMD	−1·18% (−1·54 to −0·82)*	−1·00% (−1·37 to −0·63)*	−0·18% (−0·65 to 0·29)
Trochanter BMD	−1·29% (−1·94 to −0·64)‡	−1·27% (−1·92 to −0·61)*	−0·02% (−0·87 to 0·82)
Femoral neck BMD	−1·39% (−1·84 to −0·95)*	−0·74% (−1·20 to −0·29)*	−0·65% (−1·23 to −0·07)†

Data are least square mean (95% CI). The primary outcome was the difference between groups for relative change after 12 months in lumbar spine bone mineral density (LS-BMD). Adjustments for site, baseline age, and baseline years after menopause were done using analysis of covariance (ANCOVA) for comparisons between groups. BMD=bone mineral density. *p<0·001 for within-group comparison using Wilcoxon signed rank-sum test. †p<0·05 for between-group comparison using ANCOVA. ‡p<0·01 for within-group comparison using Wilcoxon signed rank-sum test.

Table 2: **Analyses of the relative change in the primary and secondary BMD outcomes**

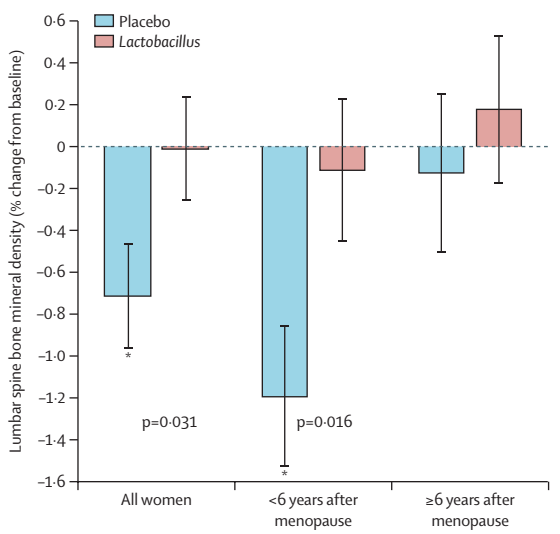

Figure 2: **Relative change in lumbar spine BMD at 12 months versus baseline**
Results are presented as least square means with standard errors for all women with BMD measurements available both at baseline and at 12 months (all women) and for a predefined subanalysis of participants with less than or at least 6 years after menopause. The between group p values given within the figure (*Lactobacillus vs* placebo) were calculated using ANCOVA. *p<0·001 for within-group comparison (month 12 *vs* baseline) using Wilcoxon signed-rank sum test.

vs baseline) of BMD parameters were calculated using Wilcoxon signed-rank sum test. The BMD statistical analyses were done for all randomly assigned participants with BMD measurements available both at baseline and at 12 months (234 participants, 116 *Lactobacillus* treated, and 118 placebo treated). As the percent change of hsCRP, TNF-α, and bone-turnover markers were not normally distributed, Wilcoxon rank-sum test was used to assess the treatment difference with regards to the percentage change from baseline for these markers. Handling of missing data are described in the appendix (p 4).

For comparisons of adverse events between the treatment groups, Fisher's exact test was used. Analyses of adverse events and safety included all participants who were randomly assigned and had taken at least one capsule of placebo or *Lactobacillus*.

The study was registered at ClinicalTrials.gov (NCT02722980) before the start of the study.

Role of the funding source

Representatives of the funding source Probi (ILA and TMN) were involved in the design of the study, contributed to the writing of the Article, and the final decision to submit for publication.

Results

332 women were screened, and 249 postmenopausal women who fulfilled the inclusion criteria and agreed to participate in the study were randomly assigned to either placebo (n=123) or *Lactobacillus* treatment (n=126; figure 1). Study participants were included between April 18, and Nov 11, 2016, and completed their 12-month visit between March 30, and Oct 19, 2017. 118 (96%) of the 123 participants who were assigned to receive placebo and 116 (92%) of the 126 participants who received *Lactobacillus* completed the two DXA analyses (baseline and month 12) required for the primary outcome (change in LS-BMD). Two additional participants in the placebo group discontinued the study after they had completed the second DXA analysis, but before the final visit including the biochemical serum and urine analyses at 12 months (figure 1). The study groups were well balanced with regards to baseline characteristics (table 1; appendix p 8–9). The mean lumbar spine T score at baseline was –0·64 in the placebo group and –0·63 in the *Lactobacillus*-treated group (table 1). Neither the number of days in the study nor the number of doses consumed differed significantly between the two treatment groups (table 1).

The predefined primary analysis was done for all randomly assigned participants who completed the two DXA analyses (baseline and month 12) required for the primary outcome change in LS-BMD (n=234). LS-BMD (relative change after 12 months) was significantly reduced in the placebo group (–0·72%, 95% CI –1·22 to –0·22%), while no change was observed in the *Lactobacillus*-treated group (–0·01%, –0·50 to 0·48; table 2; figure 2). *Lactobacillus* treatment reduced the LS-BMD loss compared with placebo (p=0·031; mean difference 0·71%, 95 CI 0·06 to 1·35; table 2; figure 2). *Lactobacillus* treatment also reduced the LS-BMD loss compared with placebo when the absolute change was evaluated using ANCOVA adjusted for site, baseline age, baseline BMD and baseline years after menopause (mean difference 7·44 mg/cm², 95% CI 0·38 to 14·50). Furthermore, a secondary intention-to-treat analysis of all participants (n=249) who were randomly assigned, which included the 15 participants who did not have a second LS-BMD measurement available, was done using imputed values for missing LS-BMD data at month 12 revealing similar results for the between-group difference (p=0·033; 0·65%, 0·05 to 1·25).

Because we hypothesised that the possible protective effect of the *Lactobacillus* treatment would be most pronounced early after menopause, a predefined subgroup analysis of participants with below the median time since menopause at baseline (<6 years) was done (figure 2; table 3). The protective effect of *Lactobacillus* treatment was significant for participants below (mean difference 1·08%, 95% CI 0·20 to 1·96; table 3), but not above (0·31%, –0·62 to 1·23), the median time since menopause (figure 2). However, a formal test of interaction did not reveal a statistically significant different treatment effect on the relative change in LS-BMD between these two groups (p=0·76 for interaction term).

The secondary BMD endpoints at the hip were either not affected (total hip BMD and trochanter BMD) or reduced (femoral neck BMD) by *Lactobacillus* treatment compared with placebo (table 2). For the within-group comparison of month 12 versus baseline, a significant bone loss in total hip, trochanter, and femoral neck BMD was observed for both the placebo-treated and the *Lactobacillus*-treated group (table 2). No significant differences between the groups were observed with respect to the secondary outcome markers of bone formation (serum osteocalcin and serum P1NP; appendix p 5), or bone resorption (serum CTX and urine NTX/Cr; appendix p 5), or in the prespecified exploratory outcome inflammatory markers (hsCRP and TNF-α; appendix pp 6–7).

The proportions of participants reporting adverse events and serious adverse events were similar in both groups (table 4). During the study period, 83% of the participants randomly assigned to *Lactobacillus* and 80% of those randomly assigned to placebo reported any adverse events (table 4). The number of adverse events considered to be related to the treatment were similar between the groups (24% in *Lactobacillus* and 26% in placebo). No clinical vertebral fractures were reported in any of the treatment groups.

	Within-group comparison (month 12 *vs* baseline)		Difference between groups
	Lactobacillus (n=45)	Placebo (n=47)	
LS-BMD	–0·11% (–0·78 to 0·55)	–1·20% (–1·86 to –0·53)*	1·08% (0·20 to 1·96)†
Total hip BMD	–1·01% (–1·65 to –0·37)‡	–1·16% (–1·80 to –0·53)*	0·15% (–0·69 to 0·99)
Trochanter BMD	–1·13% (–2·27 to 0·20)	–1·54% (–2·68 to –0·39)§	0·41% (–1·10 to 1·92)
Femoral neck BMD	–1·34% (–2·09 to –0·58)*	–0·88% (–1·64 to –0·13)‡	–0·46% (–1·45 to 0·54)

Data are least square mean (95% CI). Adjustments for site and baseline age were done using analysis of covariance (ANCOVA) for comparisons between groups. LS-BMD=lumbar spine bone mineral density. BMD=bone mineral density. *p<0·001 for within-group comparison using Wilcoxon signed-rank sum test. †p<0·05 for between-group comparison using ANCOVA. ‡p<0·01 for within-group comparison using Wilcoxon signed-rank sum test. §p<0·05 for within-group comparison using Wilcoxon signed-rank sum test.

Table 3: **Predefined subanalyses of the relative change in BMD for early postmenopausal women, less than 6 years after menopause**

Discussion

No previous adequately powered study has evaluated the possible bone protective effect of probiotic treatment on the clinically relevant bone site LS-BMD in postmenopausal women. In this randomised, double-blind, placebo-controlled, multicentre trial, probiotic treatment using a mix of three *Lactobacillus* strains protected against lumbar spine bone loss in healthy, early postmenopausal women.

The menopausal and early postmenopausal lumbar spine bone loss is substantial in women, and by using a prevention therapy with bacteria naturally occurring in the human gut microbiota we observed a close to complete protection against lumbar spine bone loss in healthy postmenopausal women. The bone-protective effect of the probiotic treatment in the present study is in agreement with several previous experimental studies showing that different probiotic treatments protect rodents from ovariectomy-induced bone loss.[7,12,14,15] Rodent mechanistic studies suggest that the bone protective effect of probiotics might involve reduced gut permeability, increased levels of short-chain fatty acids, reduced inflammation in the gut, and reduced levels of proinflammatory cytokines in bone, and thereby reduced osteoclastic bone resorption.[8,9,24–26]

Effective osteoporosis treatments to reduce fracture risk exist, but, most likely because of fear of side-effects, the treatment rates and adherence to medication are low, and bisphosphonate treatment is not recommended as a preventive therapy to women with normal bone mass.[18,27] This underscores the need for the development of safe and inexpensive interventions.[26] Although the treatment was well tolerated, the effect size observed for the probiotic treatment on LS-BMD in the present 12-month study was of a minor magnitude compared with the effects of the first-line osteoporosis treatment bisphosphonates.[18,28] Thus, the short-term regimen with the *Lactobacillus* strains used in the present study cannot replace bisphosphonates for the treatment of women with established osteoporosis. However, in the present study, probiotic treatment was given as a prevention therapy to healthy, postmenopausal women, whereas the effects of bisphosphonates have generally been evaluated in postmenopausal women with osteoporosis or osteopenia.[29–31] Further long-term studies should be done to evaluate if the bone-protective effect becomes more pronounced with prolonged treatment with the *Lactobacillus* strains used in the present study. Future studies will inform us about the possible clinical usefulness of this treatment when given as a long-term prevention therapy to postmenopausal women not yet suffering from osteoporosis. Most of the fractures in absolute number occur in women who have not yet developed osteoporosis (with a T score of more than –2·5).[32]

In the study by Nilsson and colleagues,[18] bone loss in the distal tibia measured using high-resolution peripheral quantitative CT was shown to be significantly reduced by probiotic treatment. The distal tibia, similarly to lumbar spine, is a bone site with a relatively high trabecular bone content; the positive findings for the primary outcomes in the present study (lumbar spine bone loss) and in the

	Lactobacillus (N=126)	Placebo* (N=123)
Participants with any adverse event	104 (83%)	99 (80%)
Any adverse events†		
Infections and infestations	85 (67%)	71 (58%)
Nasopharyngitis	45 (36%)	44 (36%)
Gastroenteritis	10 (8%)	5 (4%)
Influenza	8 (6%)	5 (4%)
Urinary tract infection	2 (2%)	7 (6%)
Pyrexia	5 (4%)	3 (2%)
Tooth infection	4 (3%)	2 (2%)
Upper respiratory infection	4 (3%)	2 (2%)
Bronchitis	4 (3%)	1 (1%)
Borrelia infection	3 (2%)	2 (2%)
Musculoskeletal and connective tissue disorders	27 (21%)	32 (26%)
Arthralgia	11 (9%)	8 (7%)
Back pain	7 (6%)	9 (7%)
Ligament injury or musculoskeletal pain	5 (4%)	9 (7%)
Osteoporosis	2 (2%)	3 (2%)
Pain in extremity	2 (2%)	3 (2%)
Fracture in foot or wrist	0 (0%)	3 (2%)
Gastrointestinal disorder	35 (28%)	29 (24%)
Flatulence	8 (6%)	15 (12%)
Diarrhoea	10 (8%)	4 (3%)
Nausea	5 (4%)	2 (2%)
Vomiting	4 (3%)	0 (0%)
Abdominal pain	1 (1%)	4 (3%)
Constipation	1 (1%)	3 (2%)
Dyspepsia	3 (2%)	0 (0%)
Toothache	3 (2%)	1 (1%)
Nervous system disorders	13 (10%)	17 (14%)
Headache	13 (10%)	17 (14%)
Respiratory, thoracic and mediastinal disorders	5 (4%)	9 (7%)
Cough	3 (2%)	4 (3%)
Oropharyngeal pain	2 (2%)	5 (4%)
Injury, poisoning and procedure complications	3 (2%)	2 (2%)
Accident	3 (2%)	2 (2%)
Adverse events leading to discontinuation of trial agent‡		
Gastrointestinal disorder	3 (2%)	4 (3%)
Abdominal pain	1 (1%)	0 (0%)
Flatulence	1 (1%)	2 (2%)
Constipation	1 (1%)	0 (0%)
Decreased appetite	0 (0%)	1 (1%)
Faecal volume increased	0 (0%)	1 (1%)
Other	1 (1%)	1 (1%)
Myalgia	0 (0%)	1 (1%)
Fatigue	1 (1%)	0 (0%)
Participants with any treatment-related adverse event	30 (24%)	32 (26%)
Number of treatment-related adverse events	48	51
Any serious adverse event		
Pneumonia	1 (1%)	0 (0%)
Fracture	0 (0%)	1 (1%)
Any treatment-related serious adverse event	0 (0%)	0 (0%)

(Table 4 continues in next column)

(Continued from previous column)

Data are n or n (%). *Non-significant *Lactobacillus* versus placebo. †Any adverse events are only reported if occurring in at least three participants in at least one of the treatment groups. For comparisons of adverse events between the treatment groups Fisher's exact test was used. ‡Three participants in the *Lactobacillus* group and four participants in the placebo group discontinued the trial agent.

Table 4: **Self-reported adverse and serious adverse events in study participants**

study by Nilsson and colleagues[18] (distal tibia bone loss) suggest that these probiotic treatments primarily protect trabecular bone in humans.[18]

In addition, we evaluated the effects on multiple secondary and exploratory endpoints in the present study. The secondary BMD endpoints at the hip were either not affected (total hip BMD and trochanter BMD) or reduced (femoral neck BMD) by probiotic treatment compared with placebo. In contrast to the lumbar spine region, the femoral neck region has a relatively high content of cortical bone and a rather low content of trabecular bone. There are several factors and mechanisms, including WNT16, sFRP4, and Wnt10b, that regulate the trabecular and cortical bone compartments differentially.[33–36] Therefore, one might speculate that the *Lactobacillus* strains used here target a mechanism with differential effect on trabecular and cortical bone, resulting in specific protection against spinal bone loss. However, further long-term studies are warranted to explore the apparent bone-site specific effects of probiotic treatment in postmenopausal women.

The global market for probiotics is predicted to expand from US$37 billion in 2015 to US$64 billion by 2023.[23] Hence, there is a need for high-quality, sufficiently powered, randomised, controlled trials that evaluate clinically useful and validated outcomes such as LS-BMD in relevant patient populations to provide guidance to consumers and clinicians. The present study is one of few long-term randomised trials to show a significant effect of probiotic treatment on a clinically relevant predefined primary outcome, especially in the bone health area. There are now two independent randomised clinical trials that show significant effects of two different *Lactobacillus*-based probiotic treatments on predefined bone-related primary outcomes; total volumetric BMD in distal tibia in the recent single-centre study by Nilsson and colleagues[18] and the clinically relevant bone site LS-BMD in the present multicentre study, suggesting that effects of probiotic

treatment on bone health in humans should be further explored in sufficiently powered randomised clinical trials.

The strengths of the present study include the relatively large sample size and the randomised, multicentre, double-blind design with a prespecified analysis plan, and a clinically relevant primary outcome. The secondary and exploratory analyses of bone-turnover markers and inflammatory markers did not reveal any underlying mechanism for the observed effect on LS-BMD. Circulating bone markers, reflecting overall bone turnover, were not significantly altered and this might be a result of the *Lactobacillus* treatment protecting against bone loss in the lumbar spine, but not in the hip. Recent studies have shown that short-chain fatty acids, which are generated by fermentation of complex carbohydrates by the gut microbiota, are important regulators of both bone formation and resorption.[26] It is a limitation of the present study that we did not explore whether short-chain fatty acids are involved in the observed effects of *Lactobacillus* treatment on LS-BMD. Furthermore, it might be regarded as a limitation of the present study that we only evaluated the effect of one dose of the *Lactobacillus* combination because it is possible that a higher dose might have resulted in a larger effect on the primary outcome.

In conclusion, probiotic treatment using a mix of three *Lactobacillus* strains naturally occurring in the human gut microbiota protects against lumbar spine bone loss in healthy postmenopausal women. Further long-term studies are warranted to explore the apparent bone site-specific effects of probiotic treatment in postmenopausal women.

Contributors

The study was designed by PAJ, ILA, TMN, and CO. FH did the analysis according to a prespecified statistical analysis plan. PAJ, DC, ILA, TMN, and CO take primary responsibility for the data and for the fidelity of the study to the protocol. PAJ and CO wrote the first draft of the Article. All authors contributed to subsequent drafts of the Article and made the decision to submit for publication.

Declaration of interests

ILA and TMN are employed by Probi. CO, KS, and ILA are listed as coinventors on a patent issued (WO 2014/163568) regarding the effects of probiotics in osteoporosis treatment. CO is listed as an inventor on a pending patent application regarding probiotic compositions and uses thereof. KS reports grants for probiotic-related research (mouse studies) from Probi, outside the submitted work.

Data sharing

Study protocols and the statistical analysis plan will be available with publication. Because of Swedish privacy laws, individual participant data of this clinical trial cannot be made publicly available, because it contains information that could compromise the privacy of the research participants. We did not state in the approved ethical application that individual participant data will be made publicly available.

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