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Lactobacillus reuteri reduces bone loss in older women with low bone mineral density – a randomized, placebo-controlled, double-blind, clinical trial

Prevention of bone loss with Lactobacillus reuteri

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Abstract

The importance of the gut microbiome for bone metabolism in mice has recently been demonstrated, but no studies are available in humans. *Lactobacillus reuteri* ATCCPTA 6475 (*L. reuteri* 6475) has been reported to increase bone mineral density (BMD) in mice but its effect on the human skeleton is unknown. The objective of this trial was to investigate if *L. reuteri* 6475 affects bone loss in older women with low BMD. In this double-blind, placebo-controlled study, women from the population who were 75 to 80 years old and had low BMD were randomized to orally receive 10^{10} colony-forming units of *L. reuteri* 6475 daily or placebo. The predefined primary end-point was relative change after 12 months in tibia total volumetric BMD (vBMD).

Ninety women were included and 70 completed the study. *L. reuteri* 6475 reduced loss of total vBMD compared to placebo both in the intention-to-treat (ITT) analysis (-0.83% [95% confidence interval [CI], -1.47 to -0.19%] vs. -1.85% [95% CI, -2.64 to -1.07%]; mean difference 1.02% [95% CI, 0.02 to 2.03]) and per protocol analysis (-0.93% [95% CI, -1.45 to -0.40] vs. -1.86% [95% CI, -2.35 to -1.36]; mean difference 0.93% [95% CI, 0.21 to 1.65]. In general, similar but smaller effects were observed in the secondary bone variable outcomes, but these differences did not reach statistical significance in the ITT population. Adverse events did not differ between groups. In conclusion, supplementation with *L. reuteri* 6475 should be further explored as a novel approach to prevent age-associated bone loss and osteoporosis.

Introduction

Osteoporosis is a disease characterized by loss of bone density and deteriorated bone microstructure, resulting in reduced bone strength and increased risk of fracture.[1, 2] Osteoporotic fractures are usually caused by low energy trauma and are very common, affecting every second woman and every fourth man after 50 years of age.[3] In 2005, there were over 2 million fractures, costing almost USD 17 billion, in the United States alone.[4] Pharmacological treatment that effectively reduces the risk of fracture[5] is available but treatment rates and adherence to medication in patients with high fracture risk remain low, possibly due to fear of rare side effects.[6]

The human gastrointestinal tract is the host for several trillions of microbial cells, suggested to have many important physiological functions in the host, including regulation of the immune system, intestinal endocrine signaling, protection against pathogen overgrowth, biosynthesis of vitamins and serving as a source of energy biogenesis.[7, 8] Dysbiosis of the gut microbiota has been implicated in diseases such as obesity, diabetes mellitus and autoimmune diseases.[8] It has become increasingly clear that that the gut microbiome also modulates bone homeostasis in mice, potentially by regulating the immune system and osteoclast formation,[9, 10] suggesting that modulating the microbiome and/or inflammatory tone may provide means to modulate bone mass and prevent osteoporosis.

Lactobacillus reuteri ATCC PTA 6475 (*L. reuteri* 6475) is a microbe indigenous to the human gastro-intestinal tract[11] and has potential host benefits including anti-inflammatory properties.[12, 13] Inflammation leads to accelerated bone loss due to stimulation of osteoclastogenesis and increased bone resorption.[14] *L. reuteri* 6475 supplementation was recently reported to prevent ovariectomy-induced bone loss in mice[15] and increases trabecular bone density in female mice

with active inflammation.[16] The effect of probiotic supplementation on the human skeleton is unknown.

In this randomized, double-blind, placebo-controlled trial, it was investigated if daily supplementation with *L. reuteri* 6475 could reduce bone loss in older women with low BMD.

Methods

Study design

The study "Effects of *Lactobacillus reuteri* on Bone in Older Women" (ELBOW) was a double-blind, randomized, placebo-controlled, single center trial, performed at the Geriatric Medicine Unit at Sahlgrenska University Hospital in Mölndal, Sweden. The study was approved by the Regional Ethics Committee in Gothenburg, D-nr 075-15 (February 26th, 2015) and addendum T229-16 (March 10th, 2016). The study was registered at Clinicaltrials.gov (number NCT02422082) prior to study start.

Participants

Study subjects were recruited from women who participated in a larger population-based study (Sahlgrenska University hospital Prospective Evaluation of the Risk of Bone fractures, SUPERB).[17] Inclusion criteria were willingness to participate, availability throughout the study period, and a T-score ≤-1 standard deviation (SD) for bone mineral density (BMD) at the spine, hip or femoral neck measured by dual energy x-ray absorptiometry (DXA). Exclusion criteria were: osteoporosis defined as T-score <-2.5 at the spine or the total hip; untreated hyperthyroidism; rheumatoid arthritis; a diagnosis during the last year of chronic obstructive pulmonary disease, inflammatory bowel disease, celiac disease, or diabetes; malignancy diagnosed during the last 5 years; or medication with oral

glucocorticoids, antiresorptive therapy, teriparatide during the last 3 years, or antibiotics during the last 2 months. All study subjects gave their written informed consent prior to inclusion.

Randomisation and masking

The sponsor (BioGaia AB) provided 120 sequentially numbered containers, based on a randomization of 120 study subjects into 20 blocks (6 in each block) and was generated by using the Web site Randomization.com ⟨http://www.randomization.com⟩. Study subjects were consecutively assigned to study numbers. The study product and placebo were given in identical stick-packs and was provided by research nurses to study subjects, both without knowledge of supplement type (double-blinded). Blinding was maintained for all until study end, completion of the statistical analysis plan and database lock.

Procedures

The study product was freeze-dried *L. reuteri* 6475 (BioGaia AB, Stockholm, Sweden) in doses of $5x10^9$ colony-forming units (CFU) mixed with maltodextrin powder, filled in stick packs, and was taken twice daily, yielding a total daily dose of $1x10^{10}$ CFU/day. The placebo product consisted of maltodextrin powder. Study participants were instructed to ingest the powder after it was mixed with a cold and non-alcoholic containing food or drink.

A standardized questionnaire was used to collect information about smoking habits, calcium supplementation, alcohol intake, medical history, medications and physical activity.[17] Volumetric BMD (vBMD) and bone microstructure were investigated, at baseline and after 12 months, in the distal tibia with high-resolution peripheral quantitative computed tomography (HR-pQCT; XtremeCT, Scanco medical AG, Brüttisellen, Switzerland) with a protocol described earlier.[18] After processing

the images, as previously described[19], the following variables were obtained: total volumetric BMD (vBMD, mg/cm^3), cortical thickness (Ct.Th, mm), cortical volumetric BMD (vBMD, mg/cm^3), trabecular bone volume fraction (%). The coefficients of variation (CVs) at our unit, assessed in 30 women aged 75-80 years, were 0.2% (total vBMD), 0.5% (Ct.Th), 0.3% (cortical vBMD), and 0.5% (trabecular bone volume fraction). Areal BMD (aBMD, g/cm^2) was measured at the total hip, femoral neck and lumbar spine (L1-L4), and body composition of the total body, using a Hologic Discovery A DXA (Hologic, Waltham, MA, USA). The CVs for aBMD measurements at our unit for women aged 75 to 80 years were 0.8% for total hip, 1.3% for femoral neck and 0.7% for lumbar spine.

Serum samples were immediately frozen after collection. Analysis of HbA1c was conducted on HPLC (ion exchange liquid chromatography) with the Mono-S 5/50 GL Tricorn column (GE Healthcare). The coefficient of variation for reproducibility was 1.17% and the correlation coefficient for linearity with IFCC reference was 0.9992. All other serum analyses were performed in duplicate using immunoassays by TECO medical AG (Sissach, Switzerland): N-telopeptide of type I collagen (NTx; Osteomark®; Alere Scarborough, Inc., Scarborough, ME, USA; intra- and inter-assay variability 4.6 and 6.9%, respectively), bone-specific alkaline phosphatase (BAP; Microvue™; Quidel Corporation, Athens, OH, USA; intra- and inter-assay variability 5.0 and 5.9%, respectively), high sensitivity c-reactive protein (hsCRP; BioMerica, Inc., Irvine, CA, USA; intra- and inter-assay variability 4.4 and 3.3%, respectively), and tumor necrosis factor-α (TNF-α; Quantikine®; R&D Systems, Inc., Minneapolis, MN, USA; intra- and inter-assay variability 2.0 and 6.7%, respectively).

Outcomes

Primary outcome was relative change after 12 months in tibia total vBMD and secondary outcomes were relative changes after 12 months in: aBMD measured at the hip and spine; trabecular bone volume fraction; cortical vBMD; cortical thickness; serum markers for bone turnover (N-terminal telopeptide and bone-specific alkaline phosphatase); serum markers for inflammation (C-reactive

protein and tumor necrosis factor alpha); serum HbA1c; and body composition (total fat and lean mass). Study subjects visited the clinic every third month in order to collect more study product and for assessment of adherence and adverse events.

Statistical analyses

A statistical analysis plan was developed before unblinding. Missing data were handled by multiple imputation methodology with 50 study samples including patient characteristics, medical history and prior medications that were associated with at least $r \geq 0.40$ to any of the outcome variables or absolute values of those at 12 months or with missingness of any of the outcome variables at ≤ 0.10 significance level. Baseline measurements for the outcome variables were also included in the imputation regression model, but no follow-up data. Hence, the following variables were used for imputation: age, height, secondary osteoporosis, fracture after age of 50 and all baseline variables for the studied efficacy outcomes. A few variables, for example diabetes, inflammatory bowel disease, menopause before 45 years, that lead to model convergence problems, were excluded. The imputations were performed separately per treatment group to avoid cross-contamination of imputation models and separately for each outcome using PROC MI procedure in SAS Software with fully conditional specification with regression method. The results were pooled using PROC MIANALYZE procedure. A seed was decided ahead of performed analyses.

The difference between the treatment groups with respect to efficacy variables were tested by Analysis of Covariance (ANCOVA) with relative change from baseline to 12 months as dependent variable, treatment group as fixed effect and baseline value as covariate. From this model Least Square Means (LSM) with 95% Confidence Intervals (CI) with p-values were

presented. The p-value for the relative change from baseline within the two groups were obtained from the same model and normality checks were performed. For tests between the two treatment groups, Fisher's Exact test was used for dichotomous variables, two-sample t-test or Mann-Whitney U-test for normally and not normally distributed continuous variables, respectively.

The primary and all secondary variables were analyzed both for the intention-to-treat (ITT; all randomized subjects) and per protocol (PP) population (those who completed the study and had not used medication in violation of the exclusion criteria). Sensitivity analysis of the primary variable was complete case analysis, adjusted for baseline value and any significant confounders on ITT population. Anticipating a difference of 1.1% in tibia total vBMD change over 12 months between the placebo and treatment group, a standard deviation of 1.5%, an alpha of 5%, and a statistical power of 80%, the number of study subjects needed per group was 30. All tests were two-tailed and conducted at 0.05 significance level. All analyses were performed using SAS Software version 9.4 (SAS Institute Inc., Cary, NC, USA).

Role of the funding source

The funders had no role in design and conduct of the study; collection, management, analysis, and interpretation of the data; or in preparation, review, or approval of the manuscript. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

On the basis of BMD criteria, a total of 329 out of 969 screened women were eligible for the study (Fig. 1). Of the 253 women who could be reached, 157 declined participation and 6 met exclusion criteria, resulting in 90 women enrolled in the study. Study subjects were included between May 8th 2015 and May 26th 2016 and completed their 12-month visit between May 9th 2016 and May 24th 2017.

The ITT analysis included all study subjects (n=90) who were randomized to treatment with *L. reuteri* 6475 or placebo. The study groups were well balanced with regard to baseline characteristics (Table 1). All study subjects were Caucasian. A total of 70 (76%) patients completed the study, of whom 34 had been randomized to *L. reuteri* 6475 and 36 to placebo. Two study subjects (both randomized to *L. reuteri* 6475) who had completed the study were excluded from the PP population before unblinding due to glucocorticoid treatment during the study. No study subjects began treatment with osteoporosis medication during the study. Also in the per protocol population, the study groups were balanced at baseline (Supplementary appendix, Table S1).

The mean relative change in the primary outcome, tibia total vBMD, after study completion was -0.83% (95% confidence interval [CI], -1.47 to -0.19%) in subjects randomized to *L. reuteri* 6475 and -1.85% (95% CI, -2.64 to -1.07%) in those randomized to placebo in the ITT population. The mean difference between the groups was 1.02% (95% CI, 0.02 to 2.03; adjusted p-value=0.047; Fig. 2A). Similar results were seen in the PP population with a mean change in tibia total vBMD of -0.93% (95% CI, -1.45 to -0.40) in the *L. reuteri* 6475 group and -1.86% (95% CI, -2.35 to -1.36) in the placebo group (adjusted p-value 0.013; Fig. 2B).

In the ITT population, there were no significant differences in the secondary outcomes, including aBMD, trabecular bone volume fraction or bone microarchitectural indices, bone biomarkers, body composition, inflammatory markers, or HbA1c between the *L. reuteri* 6475 group and the placebo group (Table 2). In the PP population, there was a significantly lower reduction of trabecular bone volume fraction in the *L. reuteri* 6475 group compared to placebo (-0.49% [95% CI, -0.97 to -0.01%] vs. -1.29 [95% CI, -1.74 to -0.83], respectively, with a mean difference of 0.80% (95% CI, 0.13 to 1.46; adjusted p-value 0.02; Supplementary appendix, Table S2). In the other secondary outcomes, there were no differences between the groups in the PP population.

During the study period, 80% of the subjects randomized to *L. reuteri* 6475 and 87% of those randomized to placebo reported any adverse event (Table 3, Table S4). Gastrointestinal symptoms were most common, reported by 47 % in the *L. reuteri* 6475 group and 51% in the placebo group. Change in bowel habit and flatulence were the most frequent events in both groups. Adverse events considered to be related to the treatment were similar between the groups (40% in *L. reuteri* 6475 and 44% in placebo). The main reason for discontinuation was adverse events (91 % in the *L. reuteri* 6475 group and 78% in the placebo group, p=0.57). Five serious adverse events occurred during the study: one patient in each study group was diagnosed with a breast tumor; two patients in the placebo group suffered from cerebral infarction; and a transient cerebral ischemic attack occurred in a patient in the *L. reuteri* 6475 group.

As sensitivity analysis, primary and secondary outcomes were also compared between the *L. reuteri* 6475 group and the placebo group for all subjects who completed the study. In the analysis of complete cases, a significantly reduced loss of total vBMD and trabecular bone volume fraction was observed in those treated with *L. reuteri* 6475 compared to those treated with placebo (Supplementary appendix, Table S3).

Discussion

In this randomized, placebo-controlled, double-blind, clinical trial, supplementation with *L. reuteri* 6475 for 12 months resulted in reduced bone loss in older women with low bone density. The reduction in total vBMD in the distal tibia was nearly half as large in women taking $1x10^{10}$ CFU/day of *L. reuteri* 6475 than in women taking placebo. The two groups were well balanced regarding all anthropometric and life-style factors and over all, the pattern of adverse events was similar.

After attainment of peak bone mass, bone loss occurs throughout life and is accelerated following menopause in women.[20] Older women are at a very high risk for fragility fractures,[3] and to substantially reduce the yearly bone loss in this group of patients using a naturally occurring bacteria is a new concept which could lead to a paradigm shift in the prevention of osteoporosis. Previous studies in rodents have suggested that treatment with specific bacterial strains can improve bone density[15, 16, 21] but the present study demonstrates for the first time that this may also be the case in humans.

Trabecular bone, present in the vertebras and metaphyses of the long bones, has a much higher turnover than cortical bone, present primarily in the shafts of the long bones. As a result, diseases and medication affecting the skeleton are identified earlier at skeletal sites rich in trabecular bone.[22] The distal tibia is rich in trabecular bone and is easily accessible for measurement with HRpQCT, a technique with the ability to capture even small alterations in trabecular and cortical bone traits, which both contribute to the total vBMD. Based upon this knowledge, a rapid response to any treatment would most likely be detected as a change in HRpQCT-derived tibia total vBMD, which was therefore chosen as the primary outcome variable in the present trial. Confirming this hypothesis, *L. reuteri* 6475 supplementation reduced loss of total vBMD in all analyses and of

trabecular bone volume fraction in the per protocol and sensitivity analyses. Given the relatively smaller group to group differences in most of the other secondary outcome variables, such as change in spine and hip BMD, in combination with the greater method-dependent coefficients of variations (0.7-1.3% for areal BMD measurements), the statistical power to detect significant treatment dependent differences for these outcomes was insufficient. With effects of treatment of the observed magnitude, over 140 women would be needed in each arm to detect statistically significant effects on total hip BMD.

In vitro studies indicate that *L. reuteri* 6475 has the ability to interfere with TNF-α-mediated propagation of inflammatory responses in human macrophages.[12, 23] However, *L. reuteri* 6475 treatment did not significantly affect the levels of inflammation markers (TNF-α and hsCRP) in this trial. Other strains of *L. reuteri* have also been reported to reduce blood glucose levels in diabetic mice,[24] and prevent obesity in a mouse model of the metabolic syndrome.[25] Many studies have pointed to an association between gut microbiota and systemic metabolism,[26] but in the present study, treatment with *L. reuteri* 6475 had no significant effect on HbA1c or body composition. As no differences were detected in the biochemical markers for inflammation, bone metabolism (NTX and BALP) or metabolic indices, the present trial does not provide any explanation regarding the mechanism of the reduced bone loss observed with *L. reuteri* 6475 treatment.

The strengths of the present study include the randomized and double-blind study design with a pre-specified analysis plan and the fact that the study population was well characterized with regard to life-style factors and consisted of older women who are at the highest risk for fragility fractures. The limitations include the relatively small sample size, and that the key secondary outcome variables were not significantly altered by the *L. reuteri* 6475 supplementation in the primary analyses of the ITT cohort, and that the analyses did not reveal any potential mechanisms for the observed reduced

bone density loss with *L. reuteri* 6475 treatment. The non-significantly higher proportion of prevalent fractures in the placebo group at baseline, could indicate poorer bone health and perhaps greater bone loss in this group which could have affected the trial results. Also, it cannot be ruled out that the proportion of e.g. vitamin D deficiency was higher in the placebo arm than in the control arm, which could have resulted in augmented bone loss in the placebo arm. However, arguing against this hypothesis is the fact that there were no baseline differences in bone turnover markers, which would likely be affected by vitamin D deficiency. The trial may still serve as a proof of concept and elicit further studies in the field. Treatment with bisphosphonates, the first-line osteoporosis therapy, results in much larger effects on bone density[27, 28] than what was observed with *L. reuteri* 6475. However, prolonged bisphosphonate treatment more than 3-5 years is not recommended for patients with low to moderate fracture risk, limiting long-term use of these drugs in many patients.[29]

In conclusion, daily supplementation with *L. reuteri* 6475 for 12 months reduced loss of tibia total vBMD in older women with low BMD. The underlying mechanism for this remains to be elucidated, and further studies are needed to evaluate the clinical usefulness of *L. reuteri* 6475 supplementation. Probiotic supplementation could be a new and promising concept for prevention of bone loss and osteoporosis.

Contributors

Anna G. Nilsson, Daniel Sundh and Mattias Lorentzon had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. The first draft of the manuscript was written by Anna G. Nilsson and Mattias Lorentzon. All authors contributed to the

design of the study and have critically revised the manuscript for important intellectual content and gave final approval of the submitted manuscript.

Other contributors

Aldina Pivodic, MSc and Anders Pehrson (Statistiska Konsultgruppen, www.stat-grp.se, Stigbergsliden 5, Gothenburg, Sweden) have performed and contributed with all statistical analyses specified in the statistical analysis plan, in exchange for financial compensation.

Declaration of interests

The authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Dr Nilsson has received lecture fees from Shire and Pfizer. Dr. Sundh states he has no conflicts of interest. Professor Bäckhed is co-founder and shareholder of Metabogen AB, a company partly owned by BioGaiaAB. Professor Lorentzon has received lecture fees from Amgen, Lilly, Meda, Renapharma, UCB Pharma, and consulting fees from Amgen, Radius Health, UCB Pharma, Renapharma and Consilient Health.

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Figure legends

Figure 1. Patient flow through phases of a randomized trial of *L. reuteri 6475* vs. placebo for the prevention of bone loss in older women. Screening, randomization and number of women in the intention-to-treat (ITT) and the per-protocol (PP) population. *L. reuteri 6475* denotes *Lactobacillus reuteri* 6475.

Figure 2. Effect of Lactobacillus reuteri 6475 on total vBMD in older women. Relative change from baseline in tibia total volumetric bone mineral density (vBMD) after 12 months of treatment with *Lactobacillus reuteri* 6475 (*L. reuteri* 6475) or placebo in the intention-to-treat (A) and the per-protocol (B) population of older women with low bone density. Differences between the groups are given as mean (95% confidence interval).

Table 1 Baseline characteristics for the intention-to-treat population[a]

Characteristic	***L. reuteri* 6475** **(N = 45)**	**Placebo** **(N = 45)**
Age — yr	76.4±1.0	76.3±1.1
Height — cm	162.8±4.8	164.5±5.4
Weight — kg	67.6±8.6	68.6±10.0
Body mass index — kg/m^2[b]	25.5±3.5	25.3±3.3
Run-in period — days[c]	55.0 (33.5 to 74.5)	57.0 (40.5 to 71.5)
Follow-up time — days[d]	367 (361 to 375)	366 (362 to 380)
Physical health — PSC[e]	48.6±10.1	49.6±10.0
Mental health — MSC[e]	55.1±7.7	54.8±8.3
Physical activity — PASE[f]	103 (78.6 to 138)	111 (78.6 to 146)
Current smoking — no. (%)	2 (4.4)	1 (2.2)
Parental hip fracture — no. (%)	8 (18)	10 (22)
Previous glucocorticoids > 3 months — no. (%)[g]	1 (2.2)	0 (0.0)
High alcohol intake — no. (%)[h]	0 (0.0)	0 (0.0)
Previous fracture — no. (%)[i]	12 (27)	20 (44)

Diseases associated with secondary osteoporosis — no. (%)[j]	11 (24)	10 (22)
FRAX score[k]	20.2±6.7	22.0±9.4
Bone mineral density — T score		
Lumbar spine	-0.90±0.95	-1.00±0.95
Total hip	-1.14±0.65	-1.24±0.53
Femoral neck	-1.68±0.59	-1.69±0.62
HR-pQCT-derived bone variables		
Total volumetric bone mineral density — mg/cm^3	233±39.9	227±43.6
Trabecular bone volume fraction — %	12.4±2.1	12.5±2.4
Cortical volumetric bone mineral density — mg/cm^3	759±66.4	733±70.6
Cortical thickness — mm	0.78±0.2	0.72±0.3
Serum markers		
N-terminal telopeptide — nM	14.2±3.8	15.6±7.3
Bone-specific alkaline phosphatase — U/L	17.0±3.8	18.9±7.6
C-reactive protein — mg/L	1.65 (0.87 to 2.98)	1.51 (0.80 to 3.82)
Tumor necrosis factor alpha — pg/mL	1.34±0.4	1.38±0.4
HbA1c — mmol/mol	37.9±6.2	37.0±3.8

Body composition — kg		
Total fat mass	25.3±5.7	26.2±6.6
Total lean mass	42.7±4.0	42.7±4.9

[a] Mean±SD. For categorical variables numbers and percentages are presented. Non-normally distributed variables are presented as median with interquartile range. For comparison between groups, Fisher´s Exact test was used for dichotomous variables and t-test or Mann-Whitney U-test were used as appropriate for continuous variables. There were no significant differences in any of the baseline characteristics between the trial groups consisting of all subjects randomized. HR-pQCT=high-resolution peripheral quantitative computed tomography, *L. reuteri* 6475=*Lactobacillus reuteri* 6475.

[b] The body-mass index is the weight in kilograms divided by the squared height in meters.

[c] Run in period was the number of days between bone measurements and first dose of treatment.

[d] Follow-up time is presented as median and interquartile range. Data on follow-up time is presented for complete cases (n=34 in the treatment group and n=36 in the placebo group).

[e] Mental and physical composite score derived from SF-12 (version 1.0).

[f] The physical activity score was estimated using the PASE (physical activity in the elderly) questionnaire. Data on PASE-score was missing for one participant in the *L. reuteri* 6475 group.

[g] Previous glucocorticoid treatment was defined as oral glucocorticoids in a daily dose equivalent to 5 mg prednisolone for more than 3 months.

[h] A high alcohol intake was defined as drinking more than 3 daily standard drinks (330 ml beer, 90 ml wine, 4 cl spirits) per day over a period of seven days.

[i] Previous fracture after the age of 50.

[j] Diseases associated with secondary osteoporosis included any of the following: diabetes, chronic liver disease, untreated hyperthyroidism, menopause before age 45 years of age, malnutrition, malabsorption, chronic obstructive pulmonary disease and irritable bowel disease.

[k] The fracture risk assessment tool FRAX score, was calculated with femoral neck bone mineral density and presented as percentage for the 10-year probability of a major osteoporotic fracture. Data for all FRAX variables and prior use of bone affecting medications is presented in Table S5 and Table S6, respectively.

Table 2 Main analysis of the relative change in the primary efficacy variable and secondary outcomes[a]

	***L. reuteri* 6475** **(N = 45)**	**Placebo** **(N = 45)**	**Difference between groups**
Primary outcome			
Total volumetric bone mineral density — (%)	-0.83 (-1.47 to -0.19)	-1.85 (-2.64 to -1.07)	1.02 (0.02 to 2.03)
Secondary outcomes			
Bone mineral density			
Lumbar spine — (%)	0.78 (-0.54 to 2.10)	0.08 (-1.02 to 1.19)	0.69 (-1.05 to 2.43)
Total hip — (%)	-0.13 (-1.33 to 1.07)	-0.90 (-2.07 to 0.27)	0.77 (-0.91 to 2.46)
Bone geometry and structure			
Trabecular bone volume fraction — (%)	-0.43 (-1.24 to 0.37)	-1.31 (-2.00 to -0.63)	0.88 (-0.17 to 1.93)
Cortical volumetric BMD — (%)	-0.67 (-1.37 to 0.03)	-1.35 (-2.15 to -0.54)	0.67 (-0.41 to 1.76)
Cortical thickness — (%)	-2.40 (-4.26 to -0.54)	-3.97 (-6.14 to -1.80)	1.57 (-1.28 to 4.42)
Serum markers			

N-terminal telopeptide — (%)	-0.35 (-13.8 to 13.1)	4.60 (-12.8 to 22.0)	-4.95 (-27.1 to 17.2)
Bone-specific alkaline phosphatase — (%)	-4.83 (-12.9 to 3.28)	5.43 (-3.38 to 14.2)	-10.3 (-21.9 to 1.40)
C-reactive protein — (%)	25.1 (-47.1 to 97.3)	18.2 (-87.4 to 124)	6.89 (-121 to 135)
Tumor necrosis factor alpha — (%)	2.79 (-8.44 to 14.0)	0.60 (-15.5 to 16.7)	2.19 (-17.2 to 21.6)
HbA1c — (%)	0.70 (-1.45 to 2.86)	1.53 (-0.30 to 3.36)	-0.83 (-3.63 to 1.98)
Body composition			
Total fat mass — (%)	-3.54 (-7.11 to 0.02)	-4.10 (-8.00 to -0.20)	0.56 (-4.67 to 5.79)
Total lean mass — (%)	3.22 (1.66 to 4.78)	2.78 (1.31 to 4.25)	0.44 (-1.69 to 2.57)

[a] The primary outcome was the relative change after 12 months in total volumetric bone mineral density measured at the tibia. This analysis was conducted in the intention-to-treat population, which consisted of all individuals who underwent randomization. Results presented as adjusted means with corresponding 95% confidence interval. Adjustments for variable baseline values were done using analysis of covariance (ANCOVA) after multiple imputation. *L. reuteri* 6475=*Lactobacillus reuteri* 6475.

Table 3 Adverse and serious adverse events

	***L. reuteri* 6475 (N = 45)**	**Placebo (N = 45)**
Any adverse event — no. (%)[a]	36 (80)	39 (87)
Gastrointestinal disorders	21 (47)	23 (51)
Change in bowel habit — no. (%)	9 (20)	8 (18)
Flatulence and related conditions — no. (%)	5 (11)	5 (11)
Nausea and vomiting — no. (%)	3 (6.7)	1 (2.2)
Other and unspecified abdominal pain — no. (%)	1 (2.2)	3 (6.7)
Other faecal abnormalities — no. (%)	4 (8.9)	1 (2.2)
Other specified symptoms and signs involving the digestive system and abdomen — no. (%)	2 (4.4)	1 (2.2)
Musculoskeletal and connective tissue disorders — no. (%)	5 (11)	8 (18)
Primary hip arthroplasty — no. (%)	0 (0.0)	3 (6.7)
Nervous system disorder — no. (%)	5 (11)	5 (11)
Dizziness and giddiness — no. (%)	1 (2.2)	2 (4.4)
Respiratory, thoracic and mediastinal disorders — no. (%)	10 (22)	6 (13)
Acute upper respiratory infection unspecified — no. (%)	3 (6.7)	4 (8.9)

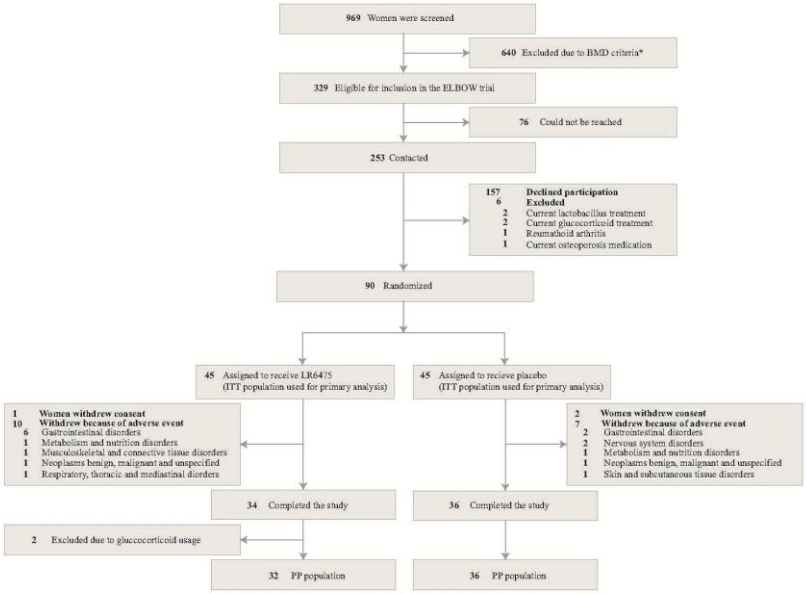
Skin and subcutaneous tissue disorders — no. (%)	2 (4.4)	7 (16)
Pruritus unspecified — no. (%)	1 (2.2)	3 (6.7)
Any fractures — no. (%)	1 (2.2)	2 (4.4)
Adverse events leading to discontinuation of trial agent — no. (%)[a]	10 (22)	7 (16)
Gastrointestinal disorders	6 (13)	2 (4.4)
Change in bowel habit	2 (4.4)	0 (0.0)
Nausea and vomiting	1 (2.2)	0 (0.0)
Other and unspecified abdominal pain	0 (0.0)	2 (4.4)
Other faecal abnormalities	1 (2.2)	0 (0.0)
Other specified symptoms and signs	2 (4.4)	0 (0.0)
Any treatment-related adverse event — no. (%)	18 (40)	20 (44)
Any serious adverse event — no. (%)	2 (4.4)	3 (6.7)
Neoplasms benign, malignant and unspecified (incl cysts and polyps) — no. (%)	1 (2.2)	1 (2.2)
Malignant neoplasm, breast unspecified — no (%)	1 (2.2)	1 (2.2)
Nervous system disorder	1 (2.2)	2 (4.4)
Cerebral infarction unspecified — no (%)	0 (0.0)	2 (4.4)
Transient cerebral ischaemic attack unspecified — no (%)	1 (2.2)	0 (0.0)

Any treatment-related serious adverse event — no. (%)	0 (0.0)	0 (0.0)
Death — no. (%)	0 (0.0)	0 (0.0)

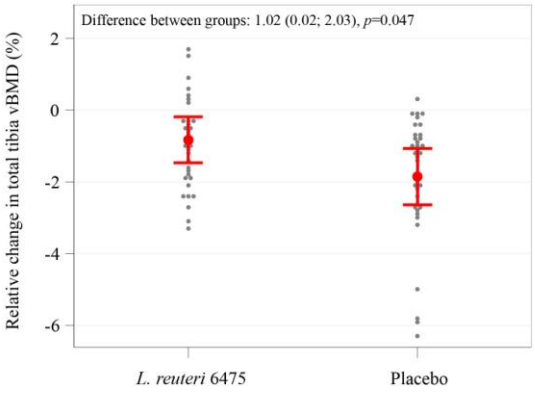
Results are presented for the safety population as number of events together with percentage. For comparison between groups, Fisher´s Exact test was used for dichotomous variables. No adverse or serious adverse events were significantly different between the two treatment groups. *L. reuteri* 6475=*Lactobacillus reuteri* 6475.

A more comprehensive presentation of adverse events is presented in Table S4.

[a] Adverse events are only reported for main categories occurring in at least 10% of the participants of a study group.



A) Intention-to-treat population



B) Per-protocol population