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Research Article
Lactobacillus reuteri can Improve BMD in Patients Having Bone Fragility

[1]Moetazza M. Alshafei, [1]Seham S. Kassem, [2]Azzat B. El-Khalek, [2]Mohamed T. Fouad, [3,6]Enas N. Danial, [4]Emtenan M. Hanafi, [5]Maha M. Saber and [5]Eitedal M. Daoud

[1]Department of Food Science and Nutrition, National Research Centre, Dokki, Giza, Egypt
[2]Department of Dairy Science, National Research Centre, Dokki, Giza, Egypt
[3]Department of Biochemistry, Faculty of Science Alfaisalia Campus, King Abdulaziz University, Saudi Arabia
[4]Departmentof Animal Reproduction and Artificial Insemination, National Research Centre, Dokki, Giza, Egypt
[5]Department of Complementary Medicine, National Research Centre, Dokki, Giza, Egypt
[6]Chemistry of Natural and Microbial Products Department, Division of Pharmaceutical Industries, National Research Centre, Cairo, Egypt

Abstract

Background: Osteopenia or osteoporosis translates as "porous bones" or when the holes between bone become bigger, making it fragile and liable to break easily. Practically, osteoporosis is translated to Bone Mineral Density (BMD). Aged women are in higher risk due to amenorrhea, higher calcium loss in urine, immobilization, low mineral supplementation and absorption. Some antinutritive factors such as phytate accumulates in the seeds (wheat, maize, rice, soya bean, etc) during the ripening period. The unique structure of phytic acid offers it the ability to strongly chelate with cations such as calcium, magnesium, zinc, copper, iron and potassium to form insoluble salts. It therefore adversely affects the absorption and digestion of these minerals and consequently affects bone formation in monogastric animals and human because they lack the intestinal digestive enzyme phytase. Foods supplementation of some enzyme such as phytase and acid phosphatase in diets results in increase in mineral absorption. Phytase and acid phosphatase are two enzymes produced by some *Lactobacillus* bacteria, *L. reuteri*. **Objective:** The current study was to investigate the valuable effect of dairy product (yoghurt) enriched with phytase producing bacteria in improving mineralization of bone in postmenopausal women. **Methodology:** Two enzymes (phytase and acid phosphatase) have been collected from *Lactobacillus reuteri*. Both enzymes activity were evaluated in stored yoghurt for 11 days to estimate the duration of enzyme activity. Twelve patients proved to have either osteopenia or osteoporosis were enrolled in the study. Patients accepted to eat our dairy product for 2 months, with diet rich in grains. They gave full data about their nutritional habits. They provided blood samples and carried out DEXA for estimation of BMD at the start and end of the study period. **Results:** The study showed that both enzymes, phytase and acid phosphatase were active for long time and wide range of acidity suggesting high activity in both stomach and intestine. The DEXA showed improved BMD in patients under investigation, elevation in serum mineral concentration and elevation of serum antioxidants markers also were demonstrated. **Conclusion:** The study concluded that consumption of our dairy product may be promising treatment for patients having bone fragility.

Key words: Osteoporosis, dairy product, *L. reuteri*, phytase, bone mineral density

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Corresponding Author: Emtenan M. Hanfi, Department of Animal Reproduction and Artificial Insemination, Veterinary Research Division, National Research Center, Dokki, Giza, Egypt

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Osteoporosis is a reduction in bone mass per unit volume, it is clinically known as reduction in Bone Mineral Density (BMD). Any degree of skeletal fragility sufficient to increase the risk of fracture is considered osteoporosis. After the forty, cortical bone is lost at a rate 0.3-0.5% year^{-1} with acceleration to 5-7% year^{-1} in postmenopausal women and the cumulative bone loss can be 40-50% in some women[1].

Osteoporosis foundation estimates that over 20 million postmenopausal white women have either osteoporosis or low bone density at the hip and the patients having hip fracture having mortality rate[2] 10-20% year^{-1}. Proper nutrition is associated with the prevention of osteoporosis.

Recent study concluded that dairy dietary pattern (consisting of high intake of milk, dairy products and green tea) decreased the risk for osteoporosis while the traditional meals were associated with greater risk for osteoporosis in postmenopausal women[3].

Plant-based food products, despite of being important source of carbohydrates, protein, dietary fiber, vitamins and minerals, they have antinutrients[4]. Among all the antinutritional components, phytic acid is of prime concern for human nutrition and health management. It has the ability to strongly chelate cations such as calcium, magnesium, zinc, copper, iron and potassium, which are essential for bone formation and rigidity to form insoluble salts. It therefore adversely affects the bioavailability of these minerals by monogastric animals and human being[5] because they lack the intestinal digestive enzyme phytase and acid phosphatase[6]. *Lactobacillus reuteri* is frequently found in fermented and probiotic food[7]. Consumption of 10^9-10^{10} living *L. reuteri* cells per day is considered safe and well tolerated, even in immune deficient individuals[8]. The *L. reuteri* has been added as adjuvant to the starter culture of dairy products and was able to survive in cheese and yoghurt and produce reuter in (antimicrobial compound) which improves the digestion and absorption of nutrients[9]. Also, the bacteria showed high production of phytase and acid phosphatase which have been reported to improve mineral bioavailability necessary for bone formation[10].

The current study was to investigate the effect of *L. reuteri* (probiotic bacterial) suspended in yoghurt as dairy product contains species *Streptococcus thermophilus* and *Lactobacillus bulgaricus* for treatment of postmenopausal women having osteoporosis or oseopenia.

MATERIALS AND METHODS

Preparation of the dairy product: Fresh raw milk, obtained from Faculty of Agriculture, Cairo University, Egypt was used in manufacturing of yoghurt and fortified with 2% skim milk powder. Milk is heated at 90°C for 10 min then cooled to 42°C. The samples were inoculated with pure lyophilized yoghurt culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp., *Bulgaricus* (at rates of 2%) and *Lactobacillus reuteri* (obtained from Chr. Hansen Lab., Copenhagen, Denmark) was added at rates of 2% and dispensed into plastic cups 100 mL. Batches were incubated at 42°C for 3-4 h to complete solidation. Then they were immediately transferred to a cold store (4±1°C)[11].

Measurements of the pH value: Both initial and final pH of the fermentation broth and the buffer pH were measured using a radiometer pH meter (Copenhagen).

Enzyme assay: Two enzymes (phytase and acid phosphatase) have been collected from *Lactobacillus reuteri*. The enzymes activity was evaluated in the stored yoghurt for eleven days to estimate the duration of the optimal enzyme activity in our product. The enzymes were produced in modified tomato skimmed milk broth where 200 mL of tomato juice, 5 g skimmed milk and 1 g of sodium phytate were mixed in one litter of distilled water and the pH was adjusted to 6.5. The inoculated flasks were incubated in 37°C for 72 h under anaerobic condition[12].

The phytase enzyme assay was done by preparation of mixture of 0.9 mL of acetate buffer (0.2 M, pH 5.5), containing 1 mm sodium phytate and 0.1 mL of the enzyme solution and incubation of the mixture for 30 min at 37°C. The reaction was stopped by addition of 1 mL of 10% trichloroacetic acid. Phytase activity was determined by measuring the amount of liberated inorganic phosphate[13]. This carried out by addition of 1.5 mL of colour reagent (consists of 1:4 v/v of 2.75% ferrous sulphate: 2.5% ammonium molibdate dissolved in 5.5% sulphuric acid). One unit of the phytase activity was expressed as the amount of enzyme required to liberate 1 μmol of phosphate min^{-1} from sodium phytate. The enzyme activity and its stability were estimated every day for consecutive eleven days of dairy product storage.

Acid phosphatase assay was done using 2 mM of P-nitrophenylphosphate as substrate. Acid phosphatase activity was estimated using the release of P-nitrophenol as colour density measured at 400 nm[14].

Patients: Thirty patients visited complementary medicine clinic Center of excellence in National Research Center, Egypt between 2015 and 2016. They were seeking advice for their back pain and arthritis. Medical history, 24 h dietary recall "to calculate their dairy and calcium intake" and complete clinical examination was done after their consent. All patients were sent for radiology center to estimate Bone Mineral Density (BMD) by dual energy x-ray absorptiometry scan (DEXA) and provided blood samples for analysis for some parameters (level of serum minerals and oxidative status using commercial Biodiagnostic kits). They have done Dexa for three parts (the vertebral spine, right and left femur) which is the most predictive of fracture risk[15]. Before and after treatment. Every patient is considered as control to himself before treatment. Twenty postmenopausal women proved to have osteopenia (T-score from -1 to -2.5) or osteoporosis (score less than -2.5) were enrolled in the study. They received our dairy product (yoghurt enriched with *L. reuteri*) but only 12 patients completed the treatment course for 2 months and provided complete data while the other 8 patients did not complete the treatment course and their data were excluded. The remaining 10 patients were either with normal DEXA (7 patients) and considered as control group or excluded (3 patients) due to health problem. All volunteers were 52±8 years old and 80±14 kg b.wt. All of them have sedentary life and with minimal activity. Our patients showed T score of spine from 1-3.5 below normal. They were kept on normal balanced diet (bread, rice, meat and vegetables) beside the enriched yoghurt with 10^9 CFU mL^{-1} *L. reuteri*[10].

RESULTS

Data presented in Fig. 1 showed that the optimal activity of both enzymes was at the pH 6.5 (the fresh yogurt pH), that means the enzymes have the ability to elicit activity in duodenum (pH 6.5-7.5). Phytase enzyme showed decrease in activity with elevated acidity while acidphospatase enzymeshowed more stability. This is a notable characteristic for application as animal feed additive.

Analysis of patient's dietary recall revealed that all patients did not consume the recommended amount of calcium intake where they consume in average 2±0.8 servings of dairy product per day which contain about 504±208 mg calcium, vitamin D 186.4±77 IU and that is considered much less than recommended daily allowance in such age (Ca: 1200 mg day^{-1}, vitamin D: 600 IU day^{-1})[15,16]. Treated patients showed improved BMD (Fig. 2) especially in those having osteopenia more than patients having osteoporosis. This coincide the results of Geer and Krebs[17] T-scores after treatment were significantly elevated in spine, right femur and left femur ($p<0.01$). Also, there were significant elevation in serum calcium, phosphorus ($p<0.01$) and serum zinc levels ($p<0.001$) if compared to normal healthy participants. Also, values of magnesium elevated towards normal (Table 1).

Estimation of oxidative status of patients (Table 1) showed that the level of lipid peroxide is significantly ($p<0.01$) lowered and the level of total antioxidant markedly elevated at the end of the treatment.

Table 1: Level of serum minerals and oxidative markers in patients having low BMD before and after treatment

	Treatments		
Serum parameters	Before	After	Normal healthy
Calcium (mg dL^{-1})	9.17±0.17	12.61±0.16	12.66±0.20
Phosphorus (mg dL^{-1})	6.69±0.10	8.48±0.09	7.67±0.08
Zinc (µmol L^{-1})	72.71±3.58	115.63±3.56	141.00±3.50
Magnesium (mg dL^{-1})	1.55±0.07	1.88±0.043	2.10±0.05
Malondialdhyde (µmol mL^{-1})	19.36±0.46	11.07±0.26	10.60±0.30
Total antioxidant (µmol L^{-1})	1.5±0.02	2.22±0.032	2.10±0.03

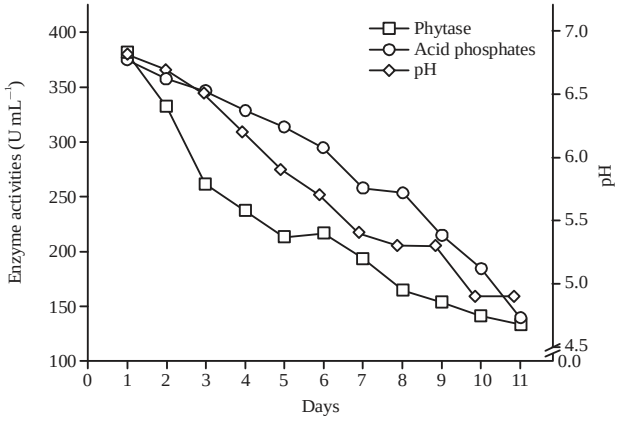
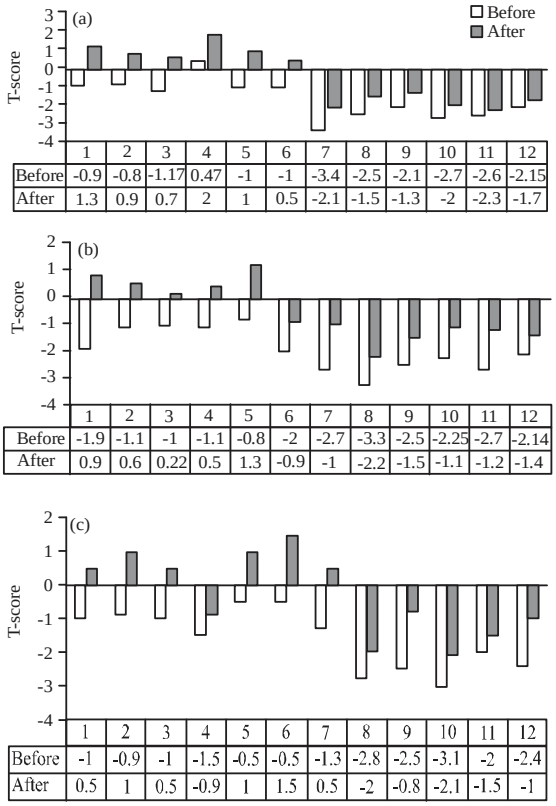


Fig. 1: Enzyems activity under different pH values



	1	2	3	4	5	6	7	8	9	10	11	12
Before	-0.9	-0.8	-1.17	0.47	-1	-1	-3.4	-2.5	-2.1	-2.7	-2.6	-2.15
After	1.3	0.9	0.7	2	1	0.5	-2.1	-1.5	-1.3	-2	-2.3	-1.7

	1	2	3	4	5	6	7	8	9	10	11	12
Before	-1.9	-1.1	-1	-1.1	-0.8	-2	-2.7	-3.3	-2.5	-2.25	-2.7	-2.14
After	0.9	0.6	0.22	0.5	1.3	-0.9	-1	-2.2	-1.5	-1.1	-1.2	-1.4

	1	2	3	4	5	6	7	8	9	10	11	12
Before	-1	-0.9	-1	-1.5	-0.5	-0.5	-1.3	-2.8	-2.5	-3.1	-2	-2.4
After	0.5	1	0.5	-0.9	1	1.5	0.5	-2	-0.8	-2.1	-1.5	-1

Fig. 2(a-c): BMD of patients before and after treatment, (a) T-score of right femur, (b) T-score of spine and (c) T-score of left femur

The improved mineral density in patients towards normal control levels suggesting that our dairy product can be used to alleviate the mineral scarcity and elevate its absorption or bioavailability that elevate the BMD in postmenopausal women having bone fragility.

DISCUSSION

Bone mass density is affected when imbalance in bone remodeling takes places[14]. Nutritional factors, is one of several factors (gender, race, genetics, calcium intake, exercise, oxidative status and overall health) that may affect the peak bone mass and ultimately osteoporosis[18]. People consuming cereals (wheat, rice, barely) in great amount showed greater liability to lower BMD[19] because cereals beside having the principal food constituents have also a significant amount of anti-nutritional factor (phytic acid)[20] which chelates and compromise the minerals (Ca, P, Mg and Zn) required for bone formation[21] and protein bioavailability[22]. Phytase and acid phosphatase which occur widely throughout nature are the requisite enzymes to degrade and release these nutrients to increase its bioavailability[23].

The present dairy product (Yoghurt) contained culture of *Streptococcus thermophilus*, *Lactobacillus bulgaricus* and *Lactobacillus reuteri*. They act as probiotic bacteria and the latest had high phytase and acid phosphatase production ability.

Data presented in Fig. 1 showed that the enzymes have broad pH stability from 6.8-4.8. The enzymes activity decreased gradually by time where the pH value decrease by storage. In this respect, Gargova and Sariyska[24] found that the optimal pH value for phytase and acid phosphatase obtained from *Aspergillus niger* was 6.5 and with increased acidity, the enzymes stability or activity decreased. Also the enzyme derived from *L. reuteri* proved higher activity than that was derived from *Aspergillus niger* reflected as higher serum and bone mineralization and less bone porosity in experimental chicks[25].

Results of serum analysis (Table 1) revealed marked elevation in serum calcium, phosphorus and zinc levels that was reflected as improved BMD (Fig. 2) in treated patients.

Previous studies showed that probiotic bacteria have positive effect on bone through several mechanisms such as, production of short chain fatty acids which decrease parathyroid hormone (PTH) followed by an increase in mineral absorption[26], production of bioactive peptides or enzymes which may support release of minerals from insoluble ion and thus enhance mineral absorption[27] and increase bone density[25]. Another possible mechanism of these peptides is through preventing the formation of angiotensin II from angiotensin I[28] which stimulate bone resorption[29] and may act as a vasoconstrictor in bone vasculature[30]. Also probiotic bacteria syntheses vitamins necessary for calcium metabolism and bone formation[31-33]. The casein-o-phosphopeptides (CPP) produced during digestion of milk in the intestine, increase the bioavailability of calcium and controlled osteoporosis in ovariectomized rats[34,35].

Menopause is associated with an unequivocal and sustained rise in calcium excretion. The increase in calcium intake is required to meet this obligatory loss[3]. Results showed significant elevation in serum calcium after treatment accompanied by elevated bone density. This may be due to elevated calcium bioavailability or absorption due to phytase and acid phosphatase production[36].

Zinc is an essential trace element for bone formation where it is a component of 200 enzymes necessary for normal mineralization and collagen synthesis in bone. It has a stimulatory effect on osteoblastic bone formation and an inhibitory effect on osteoclastic bone resorption[36,37]. The decline in the estradiol levels after menopause might upregulate the production of several cytokines and activation of osteoclast and cause bone resorption[38,39].

Previous studies recorded that zinc supplementation to the ovariectomized animals elevated estrogen levels suggesting the role of zinc in the secretion of estrogen from glands other than ovary like suprarenal glands and adipose tissues[40]. Zinc directly improves the antioxidant defense system of bone and indirectly decreases oxidative stress by recovering estrogen levels[41].

Oxidative stress stimulates osteoclast differentiation[42] *Lactobacillus reuteri* reduces proinflammatory cytokine levels systemically, which leads to increased bone volume fraction, similarly, it reduces the expression of proinflammatory cytokine in Jejunum and ileum and reduces intestinal inflammation and increases calcium uptake and bone mass density[43].

CONCLUSION

The study concluded that microbiota present in dairy product and phytase producing *Lactobacillus* has possible curative effect on bone disorders. Further study are needed to throw light on the mechanism of bacteria on bone health, larger scale of patients is needed.

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