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Dietary Role of Phytase to Improve Minerals Bioavailability for Bone Conformation

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Abstract: 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 affect bone farmation in mongastric animals because they lack the intestinal digestive enzyme phytase. It also negatively impacts protein and lipid utilisation. It is of major concern for individuals who depend mainly on plant derivative foods. Supplementation of phytase in diets results in increase in mineral absorption. This study aims to throw light on the possible use of kind of lactobacillus species, *Lactobacillus reutrei,* to get rid of phytate bonds entrapping minerals and traces in cereals and make it available for our bodies and bone conformation. The Experiment was designed on Forty cup chicks of both sexes (1 day old) were divided into 4 groups (10 each) Control group fed on basal diet (G1), birds fed on Basal diet beside phytase Enzyme derived from *lactobacillus reuteri* (G2), birds fed on basal Diet beside bacterial suspension of *lactobacillus reuteri* (G3) birds fed on basal Diet beside commercial phytase derived from *A.niger* funugs (G4). All chicks had free access to feed and water. Enzymes were given to G2 and G4 and justified according to feed intake to be 600 FTU/ Kg. At the end of the experiment (after 6 weeks) Birds were slaughtered, classified to males (M) and females(F). The collected blood samples were centrifuged and the harvested plasma was analyzed for the levels of Calcium, phosphorus, Iron, zinc. Transverse section of the bone of femur of both males and females were taken from the different groups, dried and sent for evaluation of porosity using Automated Helium Pycnometer. The same bone samples were fired and ground to be analyzed for the minerals Ca, pi, Zn and Fe concentration using atomic absorption. Results revealed that the serum showed elevated level of calcium, phosphorus, iron and zinc in groups of birds supplemented with phytase enzyme specially G3 if compared to those not supplied by the enzyme. This reflected on bone where the same groups showed less bone porosity and higher level of mineralization. The study concluded that use of phytase enzymes or the producing bacteria in monogastric animal may improve the bioavailability of minerals and controls bone porosity.

Key words: Osteoporosis · Bone Density · Minerals · Maize · Phytase Enzyme

INTRODUCTION

Osteoporosis literally translates as “porous bones” or when the holes between bone become bigger, making it fragile and liable to break easily. Osteoporosis is a progressive systematic skeletal disease characterized by low bone mass and micro-architectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture. Osteoporosis is a silent disease until it is completed by fractures that can occur following minimal trauma [1].

Fractures are claimed to affect 50% of women and 30% of men aged over 50 years [2]. Bone is made up of bone tissue, bone marrow, periosteum, endosteum, nerves and blood vessels. Bone tissue contains an extracellular matrix with an organic and inorganic matrix.

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The organic matrix ensures flexibility and elasticity while, bone minerals provide strength and mechanical rigidity to the bone [3, 4]. The inorganic matrix contains hydroxyapatite crystals and small amounts of carbonate, citrate, Mg, Na, K, Cl and F. Hydroxyapatite crystals are formed by osteoblasts, which combine collagen with calcium (Ca) and phosphorus (P) to form these crystals. The formation of hydroxyapatite crystals is necessary for overall bone size and bone width [3]. so, an inadequate supply of calcium over the lifetime is thought to play a significant role in contributing to the development of osteoporosis [5, 6]. An adequate amount of dietary protein is essential to maintain production of hormones and growth factors that modulate bone synthesis [7]. Low serum phosphate will limit bone formation and mineralization [8] where it limit osteoblast function and enhance osteoclastic bone resorption [9]. At any age, the ratio of phosphorus to calcium is probably more important than the intake of phosphorus alone [7, 8] where the high phosphorus and low calcium intake may lead to secondary hyperparathyroidism and bone loss.

Plant-based food products are the main staple food for human beings in many parts of the world. They constitute an important source of carbohydrates, protein, dietary fibre, vitamins and non nutrients [10]. Among all the antinutritional components, phytic acid is of prime concern for human nutrition and health management. The unique structure of phytic acid offers it the ability to strongly chelate 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 by animals [11]. Phytate accumulates in the seeds (wheat, maize, rice, soya bean, etc) during the ripening period and is the main storage form of both phosphate and inositol in plant seeds and grains [12]. Phosphorus, in this form, is not utilized by human beings and monogastric animals like dogs and pigs or birds because they lack the intestinal digestive enzyme phytase [13]. Besides, phytate has also been reported to form complexes with proteins at both low and high pH values. These complex formations alter the protein structure, which may result in decreased protein solubility, enzymatic activity and proteolysis (digestibility). Phytase is an acid phosphohydrolase, which catalyzes the hydrolysis of phosphate from phytic acid to inorganic phosphate and myo-inositol phosphate derivatives. The big goal of this study is to investigate the possible role of different sources of exogenous dietary phytase (fungal and bacterial) to improve the bioavailability of minerals and traces necessary for bone formation and control the incidence of osteoporosis taking poultry as a model for monogastric animals.

MATERIALS AND METHODS

Preparation of Phytase Enzymes

Microorganism: Twenty two lactobacillus spp. were tested for their ability to produce phytase (data is not shown). The screening was carried out by inoculated lactobacillus spp in fresh MRS medium supplemented with 0.1% sodium phytate. Inoculated flasks were incubated in 37°C for 72h under anaerobic condition. Lactobacillus species were sub-cultured in MRS broth and preserved in glycerol solution (20%) at working cell bank at -80 °C for further use. This was an important step to ensure that the starter culture of each experiment of the same generation number. Among the tested lactobacillus Spp. *lactobacillus reuteri* showed the highest production of phytase and was selected for further investigation. 0.6 ml of bacterial suspension was having million CFU/ ml that was presumably producing 600 FTU

Phytase Production: Production of phytase derived from *lactobacillus reuteri* was carried out in modified tomato skimmed milk broth consisted of 200 ml of tomato juice, 5gm skimmed milk and 1 gm of sodium phytate in one litter of distilled water the pH was adjusted to 6.5. The inoculated flasks were incubated in 37°C for 72h under anaerobic condition. The enzyme was given to birds at 600FTU/Kg the same dose used in poultry farms using commercial phytase which has been derived from *A.niger* [14].

Phytase Assay: Phytase activity was determined by measuring the amount of liberated inorganic phosphate. The reaction mixture consisted of 0.9 ml of acetate buffer (0.2 M, pH 5.5) containing 1 mM phytate and 0.1 ml of the enzyme solution. After incubation for 30 min at 37°C, the reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid. The aliquot was subsequently analyzed for inorganic phosphate as described earlier [15]. 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 1µmol of phosphate per min from sodium phytate.

Table 1: concentration of phytin phosphorus and phytate in feed ingredient

	Phytin-phosphorus (mg/100g DW)	Phytate (mg/100g DW)
Maize	19.04	67.59
Soya bean	11.30	40.13
concentrates	173.74	616.77
Maize stuff	16.66	59.14

Table 2: Analysis of the nutrients' level (%) of poultry feed [17]

	Moisture	DM	OM	CP	CF	EE	NEF	Ash
Maize	8.58	91.42	83.79	6.63	4.10	5.42	67.64	16.21
Soya bean	8.44	91.56	94.95	43.10	11.79	3.66	36.40	5.05
Concentrate	8.99	91.01	73.49	40.34	5.63	13.30	14.22	26.51

DM= Dry matter, NEF = Nitrogen free extract, EE= Ether extract, CF = crude fiber, CP= crude protein, OM= organic matter.

Table 3: Analysis of Minerals in poultry feed

	Phosphorus (mg/g)	Calcium (mg/g)	Iron (ppm)	Zinc (ppm)
maize seeds	43.4	34	850	37.4
Maize stuff	6.2	2.48	54.1	21.5

Determination of Phytate in Feed and Ileal Digesta: Complete diets and ileal digesta have been collected to estimate the phytin-phosphorus and phytate levels [16]. Powdered sample (4 g) were soaked in 100 cm of 2% HCl (v/v) for 3 h and filtered. To 25 cm of the filtrate in a conical flask, 5 cm of 0.3% NH_4SCN(aq) and 53.5 cm of distilled water were mixed together and titrated against standard $FeCl_3$(aq) solution containing 0.00195 g Fe/cm^3 until a brownish yellow colour persisted for five minutes. Blank was treated in a similar manner. Phytin Phosphorus (1 cm Fe = 1.19 mg Phytin-Phosphorus) was determined and the phytate content calculated by multiplying the value of Phytin-Phosphorus by 3.55

Preparation of Feed: The formulated feeds were analyzed for minerals, carbohydrate, protein, fat and ash (Table 1) according to AOA [17]. The formulated feed were justified to have 21% protein for the growing chicks (Maize 55%, Soya bean 33%, concentrates 5%, vitamin mix 0.3%, sodium chloride0.3%, Calcium phosphate 2.5% and cooking oil 4%) [18].

Experimental Design: Forty cup chicks of both sexes (1 day old) were purchased and divided into 4 groups (10 each) Control group fed on basal diet (G1), birds fed on Basal diet beside phytase Enzyme derived from *lactobacillus reuteri* (G2), birds fed on basal Diet beside bacterial suspension of *lactobacillus reuteri* (G3) birds fed on basal Diet beside commercial phytase derived from *A.niger* funugs (G4). All chicks had free access to feed and water. Enzymes were given to G2 and G4 and justified according to feed intake to be 600 FTU/ Kg. it was prepared and added in small amount of drinking water in the early morning to be sure of complete consumption. Birds in G3 given bacterial suspension estimated to produce the same units of the enzyme, just to compare between supplementing bacterial flora and enzyme supplementation. At the end of the experiment (after 6 weeks) Birds were slaughtered, classified to males (M) and females (F). The collected blood samples were centrifuged and the harvested plasma was analyzed for the levels of calcium, phosphorus, iron and zinc. Transverse section of the bone of femur of both males and females were taken from the different groups, dried and sent for evaluation of porosity according to Nabawy [19]. The bulk and skeleton volume (v_b and v_{sk}) of the studied samples were measured using Automated Helium Pycnometer (Ultra Pyc 1200e by Quantachrome) at 19 psi and the ambient temperature. The bulk volume (v_b) was measured after covering and isolating the bone sample in a Para-film to avoid invading the pores by helium; where the skeleton volume was measured for the bare samples without coverage to enable injection of the helium into the pore of bone samples. Helium has the ability to invade the nano pore size, down to 10 ηm. Porosity '⌀' of the dry core-shaped samples was then calculated precisely by substituting for both the bulk and skeleton volume 'v_b, v_{sk}' in the following equation:-

$$\phi = 100 \times \frac{(v_b - v_{sk})}{v_b}$$

The same bone samples were fired and ground to be analyzed for the minerals Ca, pi, Zn and Fe using atomic absorption.

RESULTS

Estimation of phytin phosphorus in birds ingesta (fig 1) showed that feeding chicks on diets supplemented with phytase enzyme specially those given lactobacillus bacterial suspension (G3) showed high enzyme activity expressed as liberation of the bound phosphorus leaving the least amount of phytin-phosphorus in bird's ingesta if compared to levels of phytin-phosphorus in control group (G1).

Estimation of the serum level of minerals showed elevated calcium, phosphorus, iron and zinc levels (Table 4) in groups of birds supplemented with phytase enzyme specially G3 if compared to those not supplied by the enzyme.

Screening bone porosity (Fig 2) in groups of birds supplemented with phytase showed better condition specially those in G3. These results were confirmed by the higher level of bone mineralization in the same groups (Table 5).

DISCUSSION

Osteoporosis is a gradual loss of the number of cross bridges in the organic bone matrix, combined with a reduction in bone mineral density and a loss of bone mass. The diet is estimated to have contributed to as much as 30- 50% to peak bone mass and is significantly influenced by the dietary intake of calcium, phosphate and vitamin [20]. It has been widely reported that the presence of phytic acid may compromise mineral bioavailability from infant foods [21, 22]. Phytic acid (myoinositol hexa-phosphoric acid, IP6) is the major phosphorus storage compound of most seeds and cereal grains. It has a strong affinity to chelate multivalent metal ions, especially iron, zinc and calcium. The chelates thus formed, give rise to insoluble salts of the above minerals with poor absorption characteristics and hence low bioavailability [21]. Low absorption of these nutrients from cereals is considered to be a factor in the aetiology of mineral deficiencies in

Table 4: The mineral level (means ± SE) in serum of chicks supplemented with maize based diet and phytase enzyme

Group	Phosphorus (mg/dl)	Calcium (mg/dl)	Iron (ug/dl)	Zn (ug/dl)
G1F	17.32 ±1.01 ^{a}	8.16±0.20^{a}	63.64±3.9^{a}	46.07±4.47^{a}
G2F	18.51 ± 0.70 ^{ab}	8.48±0.19^{ab}	70.30 ±3.6^{a}	32.34±2.8^{a}
G3F	19.77 ± 0.39 ^{b}	8.82± 0.08^{b}	102.3 ± 6.3^{b}	41.18±6.02^{a}
G 4F	18.9 ± 0.42^{ab}	8.11± 0.17^{a}	88.76±4.59^{b}	87.84±6.50^{b}
G1M	18.0 ± 0.18^{a}	8.50± 0.13^{a}	74.00 ±1.50^{a}	27.07±0.58^{a}
G2M	18.8±0.39^{ab}	8.67±0.17^{a}	107.00±6.9^{b}	28.41±0.59^{a}
G3M	19.83±0.69^{b}	8.70±0.12^{a}	128.4±7.60^{b}	62.13±1.26^{b}
G4M	19.27±0.19^{ab}	9.30±0.59^{a}	119.2±6.70^{b}	90.60±2.15^{c}

. Different superscript means significant difference $p \leq 0.05$

Table 5: mineral concentration in femur of male and female chicks supplemented with maize based diet and phytase of different sources

Group (F= female, M= male)	Phosphorus (mg/g)	Calcium (mg/g)	Iron (ppm)	Zinc (ppm)
G1F	83.9	32.35	589	700
G2F	126.5	49.6	623	827.1
G3F	210.1	69.01	892.8	827.1
G4F	78.08	40.0	607.8	650.4
G1M	14.7	82.7	716	240
G2M	36.3	103.8	346.6	223.8
G3M	38.3	94.5	295.25	183.5
G4M	17.0	150.6	639.1	459.1

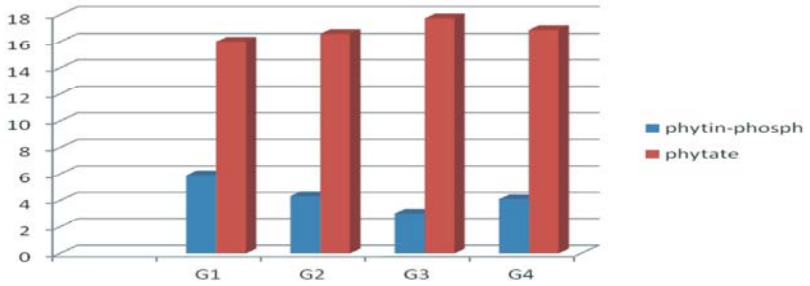

Fig. 1: Estimation of phytate and phytin-phosphorus in birds secretion

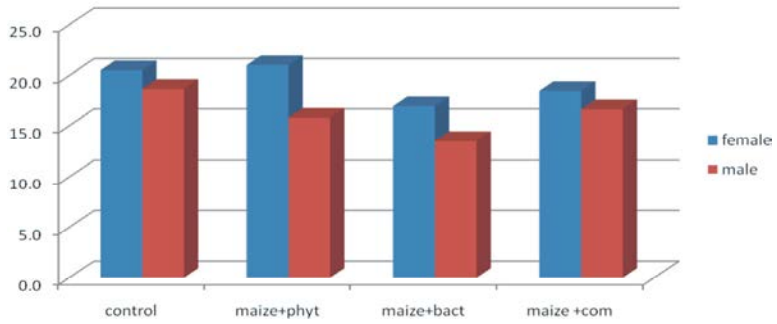

Fig. 2: Bone porosity of chicks fed on Maize based diet and phytase.

infants. Phytase, which occurs widely throughout nature, is the requisite enzyme to degrade and release inorganic P [23].

The practical acceptance of microbial phytase in poultry diets changed the nutritional requirement for mass poultry production. *Aspergillus niger*-derived phytase feed enzyme, with the capacity to liberate phytate-bound P, was commercially introduced in 1991 [24]. The bacterial phytase was more resistant to pepsin activity than fungal phytase [25]. *Lactobacillus reuteri* (L-M15) had the highest phytase production and phytate degrading activity when used as starter in whole wheat bread making process [26]. The present study declared that *lactobacillus reuteri,* produced higher level of phytase enzyme when given as bacterial emulsion and its phytase product proved to be more efficacious than that derived from *Aspergillus niger*. This high enzyme activity expressed as liberation of the bound phosphorus leaving the least amount of phytin-phosphorus in bird's ingesta in G3 if compared to levels of phytin-phosphorus in control group (G1). Estimation of minerals level in both serum and bone (table 4 and 5)showed elevated calcium, phosphorus, iron and zinc levels (Table 4) in groups of birds supplemented with phytase enzyme specially G3 if compared to those not supplied by the enzyme. The enzyme activity showed the least bone porosity in groups of birds supplemented with bacterial suspension (Fig.2). Use of phytase enzyme increased the bioavailability of iron which means protection from Iron deficiency complications such as anemia, significant decreases in psychomotor and mental development and reduced immune status [21]. The use of phytase with diets rich in cereals will also increase the absorption of calcium, affecting the attainment of peak bone mass, which may be important in reducing the risk of fractures and osteoporosis in later life [27]. In the same time the use of enzyme will increase bioavailability of Zinc which is necessary for bone formation and stimulation of growth hormone [28]. The study concluded that use of bacterial or fungal phytase in cereal rich diets improves mineral bioavailability and consequently the state of bone mineralization and controls bone fragility or osteoporosis. Also bacteria derived enzyme proved more efficient activity than fungal derived phytase.

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