A Novel Phytase Enzyme for Poultry Feed

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Abstract: During the past decade, the inclusion of microbial phytase in poultry diets has increased remarkably, mainly in response to heightened concerns over phosphorus (P) pollution of the environment. By releasing phytate-bound P and reduce P excretion. Based on limited studies, it appears that exogenous phytase hydrolyses less than 0.35 of dietary phytate in chicks at the ileal level. If so, there is considerable scope to enhance phytate degradation by the introduction of more effective phytate-degrading enzymes or enzyme combinations and facilitative nutritional and management strategies. This study aimed to investigate the effect of phytases derived from different sources [lactobacillus reuteri (L.reuteri) and Aspergillus niger (A.niger)] on feed utilization and the state of body mineralization and tracing and to what extent they protect the environment from phosphorus pollution. Forty cup chicks (1 day old) were purchased and divided into 4 groups (10 each). Control group fed on basal diet (G1), birds fed on Basal diet + bacterial phytase enzyme derived from L. reuteri 600 FTU/ Kg (G2), birds fed on basal Diet + bacterial suspension of l. reuteri (G3), birds fed on basal Diet+ commercial phytase derived from fungus(A.niger)(G4). Birds in G3 given bacterial suspension estimated to produce the same units of the enzyme just to compare between bacterial flora and enzyme supplementation. At the end of the experiment (after 6 weeks). Birds were slaughtered and the collected plasma was analyzed for the level of iron, phosphorus, zinc, total antioxidant, Malondialdehyde, Gamma glutamyltransferase, creatinine, bilirubin(total and direct). The study revealed better effect of bacterial enzyme and bacterial emulsion than fungus derived enzyme expressed in better feed efficiency ratio organ functions, oxidative status and state of mineralization, if compared to G1 and G4. Also the rate of mineral liberation from phytin-P was higher that means less phosphorus pollution to the environment.

Key words: Poultry Industry · Phytase · Lactobacillus reuteri · Mineralization · Meat Production

INTRODUCTION

Phytate is a topic of great interest in human nutrition, medical science, food and feed technology [1]. This has been fortuitous for poultry researchers, as this interest has generated a wealth of relevant information. In particular, the negative influence of phytate on the availability of Ca and trace minerals, particularly zinc, in human food stuffs has been extensively investigated. However, the presence of phytate in human diets is also claimed to have potential benefits, including anti-carcinogenic properties, as reviewed by Harland and Morris [2]. Animal nutritionists have long regarded phytate as both indigestible and an anti-nutritional factor for non-ruminant animals [3]. Because poultry possess insufficient inherent phytase activity, phytate-P is only partially available and this availability is also variable. Phytate is a polyanionic molecule with the potential to chelate positively charged nutrients, which is almost certainly fundamental to the anti-nutritive properties of phytate. These anti-nutritive properties require further investigation, but phytate probably compromises the utilisation of protein/amino acids, energy, calcium and trace minerals. Phytase, which occurs widely throughout
nature, is the requisite enzyme to degrade and release inorganic P [4]. If the practical acceptance of microbial phytase in poultry diets continues, it is likely that phytase feed enzymes will re-define nutrient requirements for sustainable poultry production in the future. Nevertheless, three decades elapsed before an Aspergillus niger-derived phytase feed enzyme, with the capacity to liberate phytate-bound P and reduce P excretion, was commercially introduced in 1991 [5].

Two big goal for this study, first is to find new microbial phytase compete the currently used in poultry feed. Second is to clarify to what extent the phytaseenzyme may encourage feed utilization, keep the environment from phosphorus contamination using enzymes of different microbial sources.

**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, 5g skimmed milk and 1 g 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 [6].

**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 [7, 8]. 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.

**Determination of Phytate in Feed and Ilealdigesta:** complete diets, ilealdigesta have been collected to estimate the phytin-phosphorus and phytate levels [9]. 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% NHSCN(aq) and 53.5 cm of distilled water were mixed together and titrated against standard FeCl(aq) solution containing 0.00195 g Fe/cm³ until a brownish yellow colour persisted for five minutes.

**Preparation of Feed:** The formulated feed were analyzed for minerals, carbohydrate, protein, fat, ash (Table 1). 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%, cooking oil 4%) [10].

**Experimental Design:** Forty cup chicks (1 day old) were purchased and divided into 4 groups (10 each) Control group fed on basal diet (G1), birds fed on Basal diet + phytase Enzyme derived from lactobacillus reuteri (G2), birds fed on basal Diet + bacterial suspension of lactobacillus reuteri (G3) birds fed on basal Diet+ commercial phytase derived from A. niger funugs (G4). Chicks were weighed before the start of the experiment and twice weekly. All chicks had free access to feed and water. The daily consumed feed were calculated and the feed efficiency ratio were estimated (Body weight gain/consumed feed). Enzymes was given to G2 and G4 and justified according to feed intake to be 600 FTU/ Kg.

| Table 1: Analysis of the nutritional components (%)of poultry feed |
|---------------------------------|------|------|------|------|------|------|------|------|
| 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 | 26.51 |
| DM= Dry matter,NEF= Nan estrified fatty acids, EE= Ether extract, CP= crude protein, OM= organic matter. |
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 bacterial flora and enzyme supplementation. At the end of the experiment (after 6 weeks) birds were slaughtered and the collected blood samples were centrifuged and the harvested plasma was analyzed for the levels of iron, phosphorus, zinc, total antioxidant, malondialdehyde, gama glutamyltransferase, creatinin and bilirubin(total and direct). Due to PM finding of dilated and engorged gall bladder and yellowish discoloration of plasma suggesting cholecystitis, sample of liver was kept in formalin and sent for histopathological investigation and the intestinal contents were sent for screening the presence of aflatoxin to exclude the onset of fungal toxemia [11].

RESULTS

Screening of twenty two lactobacillus Species for phytase production revealed that lactobacillus reuteri was the highest producing species (200 U/ml) so that it was selected to be compared to A. niger, used for production of commercial phytase, when given to the birds. The analysis of feed ingredients showed positive correlation between phytate(mg/g dry weight) and phytin phosphorus concentration(mg/100g dry weight). Also Soya bean and concentrate showed higher phytate content more than maize (Fig 1). Feeding chicks on diets supplemented with phytase enzyme revealed that the group of birds supplemented with lactobacillus bacterial suspension (G3) showed the highest amount of enzyme activity expressed as liberation of the bound phosphorus leaving the least amount of phytin-phosphorus in bird’s ingesta followed by those given the commercial phytase(G4) if compared to levels of phytin-phosphorus in the birds feed (Fig 2). These results confirm that using phytase enzymes in poultry feed protects the environment from phosphorus pollution. Estimation of the serum level of minerals showed elevated zinc, iron and phosphorus levels(Table 2). The most efficient treatment was in G3 followed by G4 and G2 as compared to G1.

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<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
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<tr>
<td>Total bilirubin(mg/dl)</td>
<td>0.94±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.92±0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.93±0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.27±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Direct bilirubin(mg/dl)</td>
<td>0.79±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.77±0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.84±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.00±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>GGT (µ/L)</td>
<td>110.96±8.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37.24±4.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>52.63±5.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>57.07±5.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Creatinine(mg/dl)</td>
<td>1.82±0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.89±0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.02±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.98±0.08&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Total antioxidant(µµ/L)</td>
<td>0.65±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.71±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.57±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.68±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>MDA (nmol/ml)</td>
<td>6.64±0.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.64±0.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.10±0.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.19±0.53&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Fe (ug/dl)</td>
<td>63.64±3.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>70.30±3.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>102.3±6.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>88.76±4.59&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Pi (mg/dl)</td>
<td>17.32±1.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.51±0.70&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.77±0.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.9±0.42&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Zn (µg/dl)</td>
<td>46.07±4.47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.34±2.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41.18±6.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>87.84±6.5&lt;sup&gt;a&lt;/sup&gt;</td>
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Different superscripts within the same row means significant difference p=0.05.
Fig. 3: Feed efficiency ratio (body weight gain/Feed intake) throughout 6 weeks

Fig. 4: Disorganization of hepatic plates and portal infiltration with mononuclear inflammatory cells (H and E; X 400).

Fig. 5: Hyperplasia and vacuolization of epithelial lining bile duct (H and E; X 400).

Monitoring the rate of growth and feed intake of the growing chicks and calculation of feed efficiency ratio (FER), results revealed that the highest FER was in the group of birds fed on the *lactobacillusreuteri* (G3) while those given phytase enzymes either derived from *lactobacillus* (G2) or *A. Niger* (G4) showed little bit higher FER if compared to control group (G1) (Fig. 3). Unfortunately towards the end of the experiment [sixth week] the group of chicks fed on commercial phytase (G4) showed greenish diarrhea with decline in FER and when slaughtered showed yellowish serum discoloration and engorged gall bladder that may suggest the presence of gallstone or cholelithiasis.

By analysis of liver function, results revealed markedly higher level of bilirubin and MDA in this group (Table 2). Examination of visceral content for the presence of afla toxins showed negative results that negates the presence of fungal toxicity. Also the histopathological examination of liver showed disorganization of hepatic plates and portal infiltration with mononuclear inflammatory cells (Fig. 4) and hyperplasia and vacuolization of epithelial lining bile duct associated with fine fibroblasts proliferation (Fig. 5).

**DISCUSSION**

The original phytase feed enzymes were produced mainly from fungi. But recent developments in the production and/or expression of enzymes in other forms of microorganisms, such as bacteria and yeast, have resulted in new exogenous phytases. There is suggestive evidence that bacterial phytase may be more efficacious in breeding of broilers where bacterial phytase derived from *E. coli* liberated more P in broilers than two recombinant fungal phytases [13]. The bacterial phytase was more resistant to pepsin activity than fungal phytases [14]. Also, when Palacios *et al.* [15] investigated that the enzyme production by some bacterial isolates from different parts of the gastrointestinal tract of chickens, they found that the species *Bifidobacteriumdentium*, *Lactobacillus reuteri* (L-M15) and *Lactobacillus salivarius* (L-ID15) had the highest phytase production and phytate degrading activity when used as starter in whole wheat breadmaking process. The present study declared that *lactobacillusreuteri*, out of twenty two species, produced the highest level of phytase enzyme and proved to be more efficacious than commercial enzyme derived from the fungus to liberate the phosphorus from its bound form expressed as lower level of phytin-p in the ileac contents as compared to its level in the feed as shown in figure (2).

The study revealed better activity in G2 and G3 than G4 Expressed as higher FER of chicks especially towards the end of the experiment. This may be explained in the light of the better activity of bacterial suspension or derived enzyme than fungus derived phytase under the GIT condition where the ideal enzyme would have good thermo stability during feed processing, high activity under wide ranges of gut pH, resistance to proteolysis and good stability under ambient temperatures [12].
Previous studies showed that phytase activity of lactic acid bacteria was optimally active at pH 4.0 and 45 °C. The enzyme was thermo-stable after exposure to 70 °C for 30 min [16]. Also L. reuteri produced a novel broad-spectrum antibiotic substance via the organism’s fermentation of glycerol, called reuterin [17]. This antimicrobial effect act as growth promoter for the growing birds in G3 [Fig. 3].

Among all the antinutritional components, phytic acid is of prime concern for human and all monogastric animals’ nutrition and health management. The chemical description for phytic acid is myoinositol (1,2,3,4,5,6) hexakisphosphoric acid. 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 as it acts as strong negatively charged ion. It therefore adversely affects the absorption and digestion of these minerals by animals [18].

It has been suggested that negatively charged phytate interacts with basic amino acids (lysine, histidine, arginine) to form binary protein-phytate complexes when gut pH is less than the isoelectric point of proteins whereby phytate depresses the digestibility of dietary amino acids [17]. Phytase enzyme was reported to improve protein and amino acid utilization through breakdown of phytin-protein complexes [19]. The other possible mode of action is that phytate may induce increases in endogenous amino acid flows [20]. Both mechanisms would depress apparent ileal digestibility of amino acids in poultry diets, which should be countered, at least in part, by phytase supplementation.

At present, the mechanisms underlying the protein-associated responses to added phytase remain largely speculative. However, growth performance responses to phytase supplementation showed that the addition of phytase to P inadequate diets entirely or almost entirely based on plant protein sources has been shown to enhance growth performance and feed efficiency of broilers [21, 22]. The current study confirm these results where FER of chicks supplemented with phytase showed better results than control group.

Analysis of organ function and estimation of oxidative status and the mineral level in blood plasma of chicks fed on bacterial phytase and bacterial emulsion showed better values if compared to control group and those given commercial enzyme derived from the fungus, the thing recommends the use of lactobacillus as a source of phytase enzyme in poultry feed.

The capacity of phytase to increase total P digestibility in broilers has frequently been demonstrated. For example, Ravindran et al. [23] found that phytase increased ileal phosphorus digestibility by 14.7% and may correct Ca:P imbalances [24]. Concerning the surprising finding of distended gall bladder and elevated plasma bilirubin and the hyperplastic epithelial lining of bile ducts in all birds of G4 (Fig 4) suggesting the presence of cholelithiasis. Considering the negative results of examination of the visceral content for the presence of aflatoxin that negates the incidence of fungal toxicity, we cannot give brief explanation for this finding without further studies. However, phytase has been demonstrated to be effective against four types of renal stones [25], namely calcium oxalate that is characterised by induction of subepithelial calcifications [26]. The mechanism is that phytate can interfere with formation of calculi (crystals) of calcium oxalate and phosphate [27]. Further studies should focus on what is the case with adding phytase enzyme to the feed and if the fungus derived enzyme differ than bacteria derived enzyme in this respect.

CONCLUSION

It is concluded that Lactobacillus reuteri bacterial emulsion would be better source for phytase than A. niger in poultry feed. And we do not recommend to use the fungus derived phytase for long period in such dose (600 FTU/kg).

REFERENCES


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