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Effect of phytase supplementation on antibody titters against infectious bursal disease and Newcastle disease and lymphoid organs weight in broilers

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Abstract
A study investigated the impact of phytase enzyme supplementation on antibody titters against Infectious bursal disease (IBD) and Newcastle disease (ND), along with lymphoid organ weight in 120 Cobb broilers over 42 days of age. The experiment included a control group (T_1) with 0.45% available phosphorus and three low-phosphorus diets with added phytase: T_2 with 0.35% available phosphorus and 0.01% Phytase 5,000 FTU, T_3 with 0.30% available phosphorus and 0.02% Phytase 5,000 FTU and T_4 with 0.25% available phosphorus and 0.03% Phytase 5,000 FTU. Results showed no significant differences (p>0.05) in antibody titters against IBD and ND, as well as lymphoid organ weight among the groups on the 42nd day. Therefore, phytase enzyme supplementation in low-phosphorus diets did not affect the immune response against IBD and ND, nor did it alter immune organ weights in the broilers.

Keywords: Broilers, phytase, infectious bursal disease, Newcastle disease, lymphoid organs

1. Introduction
Feed costs constitute over 70% of the total production expenses in the poultry industry, posing a significant financial challenge, particularly in developing countries (Sharifi *et al*., 2012) [15]. The economic strain from these costs has a direct impact on the sustainability of poultry farming. Developing a balanced and cost-effective poultry feed involves careful selection of ingredients to ensure the inclusion of essential nutrients in the right proportions.

Plant-based ingredients, such as cereals, grain legumes, and oilseed meals, are the primary sources for poultry feed (Cowieson, 2006) [7]. Grains generally make up 50-70% of poultry diets, with seeds being the key source of plant-based nutrients. Corn and soybean meal are crucial to broiler diets, providing the necessary energy and protein that form the cornerstone of poultry nutrition (Butani and Parnerker, 2015) [6].

Phytate, the principal source of phosphorus in poultry feed, accounts for about 61–70% of the phosphorus content in feed ingredients (Aureli *et al*., 2011) [4]. Poultry, being monogastric animals, lack the endogenous enzyme phytase, which is required to effectively utilize phytate phosphorus, thereby necessitating supplementation with inorganic phosphates to meet their phosphorus requirements (Yu *et al*., 2004) [17].

Phytic acid is characterized by its stability and high phosphate content, maintaining a substantial negative charge over a wide pH range. This property affects the bioavailability of important mineral ions like Zn^{2+}, Fe^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, and Cu^{2+}. Phytate can also form complexes with proteins under varying pH conditions, potentially leading to alterations in protein structure. Such alterations can reduce protein solubility, enzymatic activity, and proteolytic digestibility (Kumar *et al*., 2010) [12].

To counter these challenges, microbial phytase is added to broiler diets. This enzyme breaks down phytate, releasing bound phosphorus, amino acids, and starch from plant-based sources. The release of these nutrients increases their bioavailability for broilers, improving the overall nutritional quality of the feed and enhancing the absorption of various cations (Mukhtar, 2013) [14]. Ultimately, the inclusion of microbial phytase not only improves feed efficiency but also reduces the need for inorganic phosphorus supplementation, contributing to a more sustainable and cost-effective approach to poultry production.

The antinutritional impact of phytic acid can lead to deficiency-related diseases, potentially compromising the quality of poultry meat.

Ghahri *et al*. (2012) [8] evaluated the impact of phytase on the immune system, blood mineral levels, enzyme activity and overall performance of broilers. They came to the conclusion that adding phytase to the feed improved (p<0.01) antibody production against the Newcastle disease virus in broiler chickens between the ages of 14 and 42 days. These findings showed phytase had a beneficial effect on the chicken immune system's reaction to vaccination. Ghosh *et al*. (2016) [9] investigated how broiler chicken immunity was affected by manganese supplementation with or without phytase. Showed that adding 75 mg of manganese per kg of diet without phytase or 50 mg of manganese per kg of diet with phytase could raise the antibody titters against the Newcastle disease virus. Attia *et al*. (2020) [3] investigated the impact of varying multienzyme levels including phytase on the immune response, blood haematology and biochemistry, antioxidant status and organ histology of broiler chicks fed standard and low-density diets. Their findings indicated a significant difference in the absolute and relative weight of the spleen when compared to the control group. There was no difference in the weights of the bursa Fabricius and the thymus.

2. Materials and Methods

A research project was designed with one-hundred and twenty-day-old Cobb broiler chicks to study approaches for improving feed quality and utilizing the bird's genetic potential to reduce phosphorus excretion. Cobb broilers were chosen for their feed efficiency and genetic traits that may lead to decreased phosphorus output. After initial weighing, the chicks were divided into four experimental groups through random allocation. Each group was further subdivided into three replicates, with ten chicks in each replicate. The birds were reared in a deep litter system from hatch to six weeks old. During this time, they had unrestricted access to feed and water, and were managed according to standard managemental practices. The experiment was conducted in accordance with the ethical guidelines of the Institutional Animal Ethics Committee at KVAFSU, Bidar, Karnataka, under approval number.

Marek's disease vaccine (HVT strain), ND vaccine (Live B1 strain), and Infectious bursal disease (IBD) of Intermediate strain were sourced from Ventri Biologicals, Bengaluru. The study used phytase (Zymo-phytase), an enzyme produced by the fungus *Aspergillus niger*, which provides 5,000 Fungal Thermal Units (FTU) per kilogram. This enzyme was sourced from STS Biotech Pvt Ltd in Mysuru.

Based on the BIS-2007 guidelines, standard rations for broiler pre-starter, starter, and finisher phases were developed using common feed components. The control group (T_1) received a diet with 0.45% available phosphorus to ensure adequate nutrient supply. The treatment groups (T_2, T_3, and T_4) were fed diets with reduced phosphorus levels and varying amounts of phytase enzyme. Group T_2 diet contained 0.35% available phosphorus with an addition of 0.01% Phytase (5,000 FTU). Group T_3 received 0.30% available phosphorus plus 0.02% Phytase (5,000 FTU). Group T_4 was given a diet with 0.25% available phosphorus and 0.03% Phytase (5,000 FTU). The purpose of these specific dietary configurations was to examine how varying phosphorus content, along with the use of phytase enzyme, influences broiler performance and phosphorus utilization. This approach allowed for the evaluation of the potential to reduce phosphorus excretion while maintaining optimal growth and health in broilers.

2.1 Immunological response

2.1.1 Antibody titters against Newcastle and Infectious bursal disease

On last day of experiment, blood samples were collected from two birds in each replicate. Serum was extracted from blood drawn from the wing vein, then analysed to determine antibody titters against Newcastle disease virus and Infectious bursal disease virus. These evaluations involved Hemagglutination and Hemagglutination Inhibition (Allan and Gough, 1974) [2], along with an indirect ELISA kit, to measure antibody levels in the treatment groups.

a) Newcastle disease

Antibody titters against Newcastle disease were measured using Hemagglutination followed by Hemagglutination Inhibition (HA-HI) testing. The micro-test method described by Allan and Gough (1974) [2] was used to determine HI titters in serum samples, with the aim of exploring how they correlated with different dietary treatments. The HI test was conducted manually using U-bottom microplates, with diluters, droppers, and 4 HA units of ND viral antigen. Serum samples were diluted in a two-fold serial manner with normal saline, and 25 µl of 4 HA units of antigen was added to each well. After 45 minutes of incubation at room temperature, 50 µl of a 0.8% chicken erythrocyte suspension was added to each well. The plates were then incubated for one hour before results were assessed. Titters were recorded as log10 values, representing the reciprocal of the highest serum dilution that demonstrated Hemagglutination Inhibition or the formation of a button pattern in the well.

b) Infectious bursal disease

Serum antibodies against IBDV were quantified using the Poultry Diagnostic and Research Centre (PDRC) with indirect ELISA Kit, following the manufacturer's instructions. Each well of the antigen pre - coated plate from the kit was utilized for the test. Duplicate additions of 100 µl each of positive and negative control serum were made to their respective control wells. Additionally, 100 µl of each test serum sample diluted in the sample buffer was added in duplicates to the corresponding wells (excluding control wells) and incubated at 37° C for one hour. After washing the plate with the provided buffer, 100 µl of mouse anti-chicken IgG conjugated with Horse Radish Peroxidase (HRP) in wash buffer was added to each well and incubated for one hour at 37° C. Following another wash, 100 µl of freshly prepared chromogen-substrate solution containing OPD and 3% H_2O_2 was added to each well and the plate was left at room temperature for 15 minutes. Finally, 50 µl of 2.5 N HCl was introduced to each well to halt the enzyme-substrate reaction and absorbance values were read using an ELISA reader (Bio Rad) at 402 nm. Readings were recorded after blanking the wells with only substrate - chromogen and HCl to zero at 492 nm.

2.1.2 Lymphoid organ weight measurement

At the end of the experiment, two birds from each replication within the various treatment groups were euthanized. The weights of lymphoid organs, including the spleen, thymus and bursa of Fabricius were documented and expressed as a percentage of the pre-slaughter weight (% of live weight). Lymphoid organ weight as a percentage was calculated by

taking the weight of the lymphoid organ in grams, multiplying it by 100, and then dividing by the pre-slaughter live weight in grams.

2.2 Statistical evaluation
The study employed a completely randomized design (CRD) with a one-way analysis approach. Data for different parameters from the biological trial were analysed according to the standard methods detailed by Snedecor and Cochran (1994) [16]. Statistical analysis was performed using SPSS 20 software. Differences among treatment groups were evaluated using Tukey's Range Test, with a significance level of $p \leq 0.05$.

3. Results
3.1 Antibody titters against Newcastle disease and Infectious bursal disease
The antibody titters against Newcastle disease (log10 HI titter) in groups T_1, T_2, T_3 and T_4 were 1.405, 1.455, 1.355 and 1.355, respectively. The ANOVA revealed no significant ($p>0.05$) difference in antibody titters against Newcastle disease among the various treatment groups.
At the end of 42nd day, the antibody titters against Infectious bursal disease (ELISA) in groups T_1, T_2, T_3 and T_4 were 2231.67, 2234.0, 2226.67 and 2221.33, respectively. Statistical analysis revealed no significant ($p>0.05$) difference in antibody titters against Infectious bursal disease among treatment groups compared to control.

3.2 Lymphoid organ weights
The relative weight of spleen (% of live weight) on 42nd day of the experiment in groups T_1, T_2, T_3 and T_4 were 0.106, 0.102, 0.111 and 0.104 revealed no significant ($p>0.05$) difference between the treatments and control group.
The relative weight of bursa of Fabricius (% of live weight) on 42nd day of the experiment in groups T_1 (0.132), T_2 (0.143), T_3 (0.136) and T_4 (0.135) revealed no significant ($p>0.05$) difference between the treatments and control group.
The relative weight of thymus (% of live weight) on 42nd day of the experiment in groups T_1, T_2, T_3 and T_4 were 0.432, 0.422, 0.412 and 0.428 revealed no significant ($p>0.05$) difference among the treatments.

4. Discussion
The phytase supplemented groups had no significant difference ($p>0.05$) on immunological response against Newcastle disease and Infectious bursal disease compared to control group at the end of the experiment (42nd day). The findings of the present results were in agreement with Islam *et al*. (2017) [11] examined the humoral immune response, body weight and blood components of 180 male Cobb broilers given phytase supplements following immunisation against infectious bursal disease. Their results show that the serum IgM and IgG concentrations of broiler chickens receiving an IBD immunisation were unaffected by phytase levels (0, 500, 1,000 and 1,500 FTU /kg) with 0.19% non-phytate phosphorous and also the results of present study were in agreement with Hakami *et al*. (2022) [10] who investigated the immunological response of broilers given diets lacking calcium (0.61% and 0.71%) with added phytase (500 FTU / kg) and marine mineral complex (0.02% and 0.04%) on 300 Ross 308 broilers. Concluded that phytase and marine mineral complex supplemented diets containing 0.40% non-phytate phosphorous including control had no effect ($p>0.05$) on the titters of the Infectious bursal disease and Newcastle disease virus. Phytase supplementation may improve nutrient availability and absorption but it does not impact on the immune response elicited by NDV and IBDV vaccinations. The findings of the present results were in disagreement with Ghahri *et al*. (2012) [8] evaluated the impact of phytase on the immune system and overall performance of 240 Ross 308 broilers contains control diet with 0.85% calcium and 0.32% available phosphorous and other treatments with 0.59% calcium and 0.225% available phosphorous. They came to the conclusion that adding phytase (600, 800 and 1000 FTU / kg) to the feed improved ($p<0.01$) antibody production against the Newcastle disease virus in broiler chickens between the ages of 14 and 42 days. These findings showed phytase had a beneficial effect on the chicken immune system's reaction to vaccination.
The phytase supplemented groups had no significant difference ($p>0.05$) on per cent relative immune organ weights compared to control group at the end of the experiment (42nd day). The findings of the present results were in agreement with Al-Harthi M. A. (2017) [1] investigated the impact of olive cake on the lipid metabolism, lymphoid organs and carcass features of broiler chickens with or without phytase enzyme supplementation on 504 male Ross 308 broilers. Concluded that the immune organ ratios of the spleen and bursa to live body weight were unaffected by either raising the olive cake (5 or 10%) content with or without phytase enzyme (0.5 and 1 g / kg) contains 0.496% available phosphorus and also the results of present study were in agreement with Hakami *et al*. (2022) [10] who investigated the immunological response, bone strength, carcass and meat quality and growth performance of broilers fed low-calcium (0.61% and 0.71%) diets enhanced with 500 FTU / kg phytase and marine mineral complex (0.02% and 0.04%). Their findings show that the diets containing 0.40% non-phytate phosphorous including control had no significant difference ($p>0.05$) in the weight of bursa Fabricius and spleen. Phytase supplementation may improve digestibility of feed and absorption but it does not impact on immune organ weights. The findings of the present results were in disagreement with Metwally *et al*. (2020) [13] assessed the effect of different levels of optizyme (0, 250 and 500 mg / kg) and phytase enzymes (0, 1500 FTU / kg) and their interactions on the performance of 180 (IR) broiler chickens with diets containing 0.42% available phosphorous including control group. Revealed that the absolute and relative weight of the spleen was significantly improved compared to the control group when optizyme and phytase used.

5. Conclusion
The interpretation of the findings led to the following conclusion. Phytase enzyme supplementation at different level (0.01%, 0.02% and 0.03% of phytase 5,000 FTU) in low phosphorous diet could not affect the immune response against Infectious bursal disease and Newcastle disease and also immune organs weight like spleen, bursa Fabricius and thymus.

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