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Evaluation of different levels of *Bacillus* sp. (HCYL03) phytase in broiler chickens fed maize-soyabean meal based diets with a low non-phytate phosphorus content

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ABSTRACT

The objective of the present study was to evaluate the impact of various levels of phytase derived from *Bacillus* sp. (HCYL03) in corn-soy diets fed to broilers. Experimental treatments included a positive control (PC) with a calculated non-phytate phosphorus (nPP) level of 4.0g/kg for the 35 days of trial. The negative control (NC) diet included a reduction in nPP to 3.0g/kg during the experiment, and commercially available phytase (@500FTU/kg), as well as new bacterial phytase added to the NC diet in increasing amounts of 500, 800, and 1100FTU/kg. Treatment effects on growth performance, the apparent digestibility of P, tibia mineralization, and Ca and P status in blood plasma were evaluated on day 35. The NC diet decreased feed intake ($P<0.05$), body weight gain (BWG) ($P<0.05$), and improved feed conversion ratio (FCR) ($P<0.05$) compared to the PC. Phytase addition improved all growth parameters. Birds fed the NC diet displayed lower ($P<0.05$) digestibility of P, reduced ($P<0.05$) tibial mineralization, and decreased ($P<0.05$) P and Ca concentrations in blood plasma compared to birds fed the PC diet. Improvements in digestibility of P, tibia mineralization, and mineral contents in blood plasma were observed with phytase addition. High level inclusion of phytase (1100FTU/kg) yielded the greatest improvement in bird performance, nutrient digestibility, and bone mineralization in the NC group and low levels of phytase treatments. It may be concluded that inorganic P incorporated in the normal-nPP diet of chickens could be effectively replaced by a *Bacillus* sp. (HCYL03) phytase diet without any adverse effect on the performance and nutrient use of broilers.

Key words: *Bacillus* sp. (HCYL03); bacterial phytase; non-phytate phosphorus; growth performance; digestibility

Introduction

Phosphorus (P) is a critical nutrient in poultry nutrition that influences efficient growth performance and skeletal health. This mineral is a key molecule in the formation of phospholipid bilayers, generates the high-energy bonds for adenosine triphosphate, DNA, and RNA, and along

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with Ca, provides structure for skeletal integrity (MULLER et al., 2019). The main components of diets for poultry are cereal grains, legume seeds, fat-rich seeds and the by-products of plant food processing. Most P (from 50 to 80%) is present in complexes with phytate (myo-inositol-1,2,3,4,5,6-hexakisphosphate) (MAROLT and KOLAR, 2021). However, phytate is considered as an antinutritive factor: it can ionically chelate with nutritionally important minerals, such as Ca^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+} and Fe^{2+}, thus reducing their bioavailability (SAMTIYA et al., 2021).

P is available to broilers only if the enzyme phytase (*myo*-inositol hexakisphosphate phosphohydrolase) is present in their digestive tract. Broiler chickens produce very small amounts of phytase, and phytase production by microorganisms in the gastrointestinal tract is also limited (ENNIS et al., 2020). Phytase is an enzyme that has been in existence since the 1970s, and it was first used by NELSON et al. (1968), who postulated that phytase has the ability to hydrolyze phytate in broiler chickens, thus releasing inorganic P for use by the birds. Maize is a widely used ingredient in poultry diets. Phytate complexes present in this feed ingredient can be reduced with the use of exogenous phytases, i.e. the new 6-phytase derived from *Escherichia coli*. The utilization of the P contained in maize may be increased if diets are supplemented with microbial *E. coli* phytase (ATTIA et al., 2021).

Owing to their potential for industrial applications, phytases have triggered great interest in the last few decades. Several bacteria (*Bacillus subtilis*, *Escherichia coli*, *Klebsiella aerogenes Corynebactrium bovis*, *Pseudomonas* sps.), fungi (*Aspergillus niger*, *Aspergillus ficcum*, *Aspergillus oryzae*), yeasts (*Saccharomyces cerevisiae*, *Schwanniomyces castellii*) and certain soil organisms have been reported to produce phytases (SINGH, 2008). In fact, the commercial production of phytases for the animal feed industry has so far focused primarily on aspergilli and yeasts (KAMMOUN et al., 2012). Recently, phytases from the *Bacillus* species have been reported to have the advantage of being naturally thermostable and being strictly specific for phytates (BORGI et al., 2015; ROCKY-SALIMI et al., 2016; PURNAMASARI and MISWAR, 2018; NEZHAD et al., 2020). However, due to inefficient enzyme production methods, *Bacillus* phytases have not been applied on a large scale (KAMMOUN et al., 2012). Due to their heat stability, *Bacillus* phytases could be commercialized for use by the poultry and swine feed industry. ELKHALILY et al., (2007) compared bacterial phytases derived from *Bacillus* and *Aspergillus niger in-vitro* and *in-vivo*. The *in-vitro* results revealed that *Aspergillus* phytase exhibited their optimum activity in an acid pH range, while *Bacillus* phytase did so in neutral pH, and *Bacillus* phytase also showed more resistance to heat treatments. *In vivo* phytases derived from both *Aspergillu and Bacillus* equally improved the utilization of P balance substantially to 0·54 compared to 0·42 in the negative control.

Some literature indicates that the performance of broilers, fed with different inclusion levels of phytase and low levels of non-phytate phosphorus (nPP) in their diets, can improve with the addition of the enzyme (WALTERS et al., 2019; Al-HARTHY et al., 2020; METWALLY et al., 2020; TAHERI and ABBASI, 2020). However, other researchers did not find any beneficial effect on broiler performance with the addition of phytase in their diet (LIMA et al., 2002; LELIS et al., 2012). Similarly, SATO et al. (2017) reported that in the first phase of broiler growth (1-21 days of age), performance was not improved by phytase addition. The currently available feed-grade phytase products have scope for improvement in efficacy. The search for novel phytase preparations, with relatively greater potency in intestinal phytate hydrolysis and better heat stability, is an ongoing process. In line with this goal, the objective of this study was to evaluate the efficacy of *Bacillus* sp. (HCYL03) phytase on growth performance, P digestibility, tibial mineralization and blood profile, when fed to broiler chicks in a maize-soyabean based diet with low nPP.

Materials and methods

Enzyme production. The phytase was produced from Bacillus sp. bacteria (HCYL03) through 72

hrs fermentation at 45°C and pH 5 on wheat bran-based medium. After 72 hrs fermentation, the broth was subjected to a series of filtrations by using cotton cloth and, finally, filter paper, until a clear filtrate was obtained. This filtrate was then used in liquid form as the crude phytase enzyme. The filtrate was stored at 4°C for not more than 7 days.

Assay of enzyme. The crude enzyme was extracted by mixing the contents of each flask with 50 mL distilled water. The mixture was placed in WIs-20R shaking incubator at 120 rpm and 37°C for 1 h. The crude enzyme was filtered through filter paper and subjected to centrifugation in a Centurion Scientific K241R centrifuge at 1000 rpm for 15 min at 4oC to remove impurities. The supernatant obtained was used for phytase assay. Enzyme activity was calculated by a calibration curve over the range of 5-25 µg/mL "orthophosphate". Activity was expressed in units per mililitre (U/mL). According to HEINONEN and LAHTI (1981), one unit of phytase is defined as the amount of enzyme releasing 1 µmol of inorganic phosphorus per ml per minute under the assay conditions.

Enzyme treatment of substrate. The conditions, viz. enzyme: substrate ratio (1:1), incubation time of 8 h for maize, and 10 h for soyabean meal at 5 pH and 45°C, were determined for the optimum release of free phosphate. The maize and soyabean meal were treated by spraying with a predetermined level of crude phytase enzyme for the enzymatic degradation of phytate P. After enzyme treatment, these ingredients were dried at 60°C in the hot air oven for about 12 h and used in broiler diets (Table 1).

Table 1. Composition of the experimental diets (g/kg)

Ingredients	PC[1]	NC[2]	NC+ comm Phyt[3]	NC + Lab phyt (FTU/kg)[4]		
				500	800	1100
Maize	656.1	656.1	656.1	656.1	656.1	656.1
Soybean meal (440g crude protein/kg)	234.9	234.9	234.9	234.9	234.9	234.9
Fish meal	50	50	50	50	50	50
Poultry fat	13	13	13	13	13	13
Marble chips	8.7	8.0	8.0	8.0	8.0	8.0
Monocalcium P (225g P/kg)	6.2	4.0	4.0	4.0	4.0	4.0
Na Cl	2.1	2.1	2.1	2.1	2.1	2.1
$NaHCO_3$	2.3	2.3	2.3	2.3	2.3	2.3
Lysine sulphate	2.7	2.7	2.7	2.7	2.7	2.7
DL-Methionine	2.3	2.3	2.3	2.3	2.3	2.3
L-Threonine	0.7	0.7	0.7	0.7	0.7	0.7
Cholone Chloride	1.0	1.0	1.0	1.0	1.0	1.0
Vitamin and mineral Premix[5]	20	20	20	20	20	20
Commercial phytase (FTU/kg)	-	-	500	-	-	-
Lab prepared phytase (FTU/kg)	-	-	-	500	800	1100
Calculated analysis						
Dry matter	896.5	896.5	896.5	896.5	896.5	896.5
Crude protein	229.9	229.5	229.8	229.9	229.8	229.9
Metabolizable energy (MJ/kg)	12.56	12.55	12.56	12.56	12.56	12.55
Crude fiber	25.4	30.0	25.5	30.0	30.0	30.0
Calcium	7.9	7.9	7.9	7.9	7.9	7.9
non-phytate phosphorus	4.0	3.0	3.0	3.0	3.0	3.0

[1] Positive control diet with normal non-phytate P (nPP = 4.0g/kg).
[2] Negative control diet with low nPP (nPP= 3.0g/kg).
[3]Negative control diet+commercial phytase named Axtra® PHY-DuPont Nutrition & Biosciences @ 500 FTU/kg.
[4] Negative control diet + Laboratory phytase @ 500, 800 and 1100 FTU/kg.
[5]Supplied per kg of diet: vitamin A, 1300 IU; vitamin D_3, 200 ICU; vitamin E, 10 IU; vitamin K, 0.3 mg; thiamine, 1.8 mg; riboflavin, 3.6 mg; pyridoxine, 3.0 mg; vitamin B12, 0.009 mg; pantothenic acid, 10 mg; niacin, 27 mg; choline, 300 mg; biotin, 0.13 mg; folic acid, 0.33 mg; m[TA1]anganese, 60 mg; zinc, 40 mg; copper, 8 mg; iron, 80 mg; and antioxidant (Santoquin(r)), 123 mg.

Experimental diets. Six isocaloric and isonitrogenous diets were formulated according to the National Research Council (NRC, 1994) requirements. Six experimental diets were designated as the positive control (PC) diet with normal P content (nPP=4.0g/kg), the negative control (NC) diet with low P content (nPP=3.0g/kg) without phytase enzyme, NC plus commercially available phytase (i.e. Axtra® PHY, a *Buttiauxella sp*. phytase expressed in *Trichoderma reesei*, Danisco Animal Nutrition, DuPont Industrial Biosciences, Marlborough, UK which incorporated @ 500 FTU/kg of diet), and three diets were prepared with the addition of 3 different levels (500, 800 and 1100 FTU/kg of feed, respectively) of lab-prepared phytase enzyme in the NC diet. The experimental diets are described in Table 1. Samples of basal diet were analyzed for dry matter, crude protein, crude fat, total ash, P and Ca (AOAC, 2011). The feed ingredients and nutrient analysis of the basal diets are also presented in Table 1.

Experimental birds. Two hundred and seventy day-old Hubbard broiler chickens were used in the 5 week study. On the first day, after wing banding, they were randomly divided into eighteen experimental units of 15 chickens each. The chickens of each experimental unit were kept in separate pens with standard management conditions following the accepted guidelines (FASS, 2010). Six experimental diets were randomly allotted to these experimental units in such a way that each diet was given to 3 experimental units of 15 chickens each. Feed and water were offered ad libitum during the entire experimental period.

Measurement of performance traits. Feed intake by the birds was recorded on a pen basis at weekly intervals until the last week at the age of 35 days. Birds in each pen were weighed at weekly intervals. The average body weight, on a pen basis, was thus used for the statistical evaluation of treatments. Feed conversion ratio (FCR) was calculated on Days 7, 14, 21, 28 and 35 by dividing feed intake by body weight gain (BWG). Mortality, if any, was recorded.

Collection of excreta. At the end of the fourth week, wire mesh of 2.3 cm size was placed in each pen at a height of about 13 cm from the floor. Plastic trays were placed under the wire mesh during the last 3 days of the fifth week (i.e. days 33, 34 and 35), for collection of total excreta. Excreta were collected separately from each pen. During the collection period, the birds were fed *ad libitum*. At the end of the collection period, excreta of each pen were thoroughly mixed and weighed. Representative samples of the excreta were dried in an oven at 60°C and ground to pass through a 1-mm sieve. These ground excreta samples were used for the analysis of P (AOAC, 2011). All calculations were expressed on a DM basis. Feed intake by the birds was recorded on a pen basis at weekly intervals and daily for the last 3 days of week 5 (i.e. days 33-35). Samples of each diet were analyzed for DM, and total P (AOAC, 2011).

Sampling and measurement of tibial bone. Tibia samples of one bird (selected at random) from each experimental unit (3 birds/diet) were collected after slaughtering the birds at the end of week 5. The left tibia (with cartilage caps) of each killed bird was excised and cleaned of adhering tissues. Tibia samples were dried to a constant weight at 100°C in an oven, and then ashed at 600°C for 4 h in a muffle furnace for determination of bone ash, which was expressed as the dry weight of the tibia (SCHEIDELER, 1993). The ash from the tibia was solubilized with a nitric and perchloric acid mixture (3 : 3, vol/vol) in 100 ml conical flasks, and the volume was made to 100 ml with distilled water (YI et al., 1996). Determination of P and Ca content from this sample was performed as in the samples from feed and faeces.

Determination of phosphorus and calcium in blood plasma. On the last day of the experiment, heparinized blood was obtained by cardiac puncture from two randomly selected chickens in each replicate. Plasma was separated immediately by centrifugation of blood at 2,000 rpm for 10 min. Plasma samples were analyzed for P and Ca. Inorganic P was determined by the method described by FISKE and SUBBAROW (1925), and Ca was analyzed by atomic absorption spectrophotometry.

Statistical analysis. All data were determined using SPSS version 9.5 (SPSS, Cary, NC, USA) statistical analysis program. A P-value of <0.05 was considered a significant difference between

groups, and mean values were compared using the Duncan's Multiple Range Test (STEEL and TORRIE, 1980).

Results

Growth performance. There was no negative impact from any of the bacterial phytase products on the health and general well-being of the broiler chickens. The growth performance responses of broiler chickens to phytase from day 0 to 21 and day 22 to 35 posthatching are presented in Table 2. Reducing nPP levels in the NC diet at both 21 and 35 days of age depressed BWG as compared to the PC, NC + commercial phytase and NC + different levels of laboratory phytase. Maximum (P<0.05) BWG levels were recorded in broilers fed the diet containing NC + commercial phytase and the diet containing NC + lab phytase with 1100 FTU/kg at both 21 and 35 days of age. Adding lab phytase at the level of 1100 FTU/kg improved BWG by 33 g and 50 g at 21 days (P<0.05) and 35 days of age (P<0.05), respectively, in comparison to birds on the PC. Low dose inclusion of lab phytase (500 or 800FTU/kg) yielded better (P<0.05) improvement in the birds' performance compared to the NC, but did not exhibit better performance with the treatments with a high level of lab phytase (1000FTU/kg), commercial phytase and PC. Reducing nPP levels in the NC diet decreased (P<0.05) feed intake by approximately 6% at 21 days of age (1234g vs. 1313g), and 8% at 35 days of age (2962g vs. 3220 g) compared to birds fed the PC (Table 2). This decrease in feed intake resulted in a reduction (P<0.05) in BWG by 118 and 383g, respectively, on days 21 and 35, when compared to the PC. Moreover, birds fed the NC displayed the highest FCR (P< 0.05) of the treatments during both phases. The inclusion of increasing levels of lab phytase numerically improved feed intake and BWG throughout the study. The feed intake of broilers fed the NC diet supplemented with lab phytase at 1100 FTU/kg was similar (P>0.05) to those fed the PC diet at 21 and 35 days of age. The highest (P<0.05) feed intake was observed in birds fed the diet containing NC + commercial phytase. The P reduction in the NC treatment increased (P<0.05) FCR at both 21 and 35 days of age compared to the other treatments (Table 2). In this case, in addition to a decline in their growth, the birds became less efficient in utilizing their diet. Phytase supplementation to the NC diet at the level of 1100 FTU/kg improved (P<0.05) the FCR of broilers at both 21 and 35 days of age, as compared to the NC diet and NC + low levels of phytase, but on par with PC and NC+ commercial phytase (Table 2). With the addition of phytase, there was a considerable improvement in FCR, recovering some of the lost performance. Therefore, the *Bacillus* sp. (HCYL03) phytase was effective in releasing nutrients and improving performance. It was observed that increasing levels of phytase (500, 800 or1100 FTU/kg) tended to improve FCR

Table 2. The effect of phytase supplementation on growth performance parameters during starting and finishing stages

Treatments	Body weight gain (g)		Feed intake (g)		Feed conversion ratio	
	21 days	35 days	21 days	35 days	21 days	35 days
PC[1]	832[b]	2023[b]	1313[b]	3220[b]	1.578[c]	1.590[c]
NC[2]	714[d]	1640[d]	1234[c]	2962[c]	1.750[a]	1.806[a]
NC + comm phyt[3]	867[a]	2083[a]	1345[a]	3250[a]	1.551[c]	1.560[c]
NC+Lab phyt (FTU/kg)[4]						
500	789[c]	1820[c]	1296[c]	3178[c]	1.643[b]	1.746[b]
800	798[c]	1833[c]	1323[bc]	3182[c]	1.657[b]	1.735[b]
1100	865[a]	2073[a]	1330[b]	3217[b]	1.537[c]	1.551[c]

[a-f]Mean values in the same column bearing different superscript were significantly (P<0.05) different

[1] Positive control diet with normal non-phytate P (nPP = 4.0g/kg).

[2] Negative control diet with low nPP (nPP= 3.0g/kg).

[3]Negative control diet + commercial phytase named Axtra® PHY-DuPont Nutrition & Biosciences @ 500 FTU/kg.

[4] Negative control diet + Laboratory phytase @ 500, 800 and 1100 FTU/kg.

(1.643, 1.657 or 1.537, respectively) at 21 days of age as compared to the NC diet (1.750). A similar trend was observed on day 35, with increasing levels of phytase showing improvement of FCR (1.746, 1.735 or 1.551, respectively) as compared to the NC diet (1.806).

Phosphorus digestibility. The average values of P intake, outgo in excreta, and apparent digestibility, during the last 3 days (i.e. days 33–35) of the trial are presented in Table 3. The results indicate that P excretion with the NC diet decreased ($P < 0.05$) with the addition of phytase (0.14, 0.12 or 0.09g/d for 500, 800 or 1100FTU/kg, respectively) and this might have increased the availability of P because it is a part of the same complex and is released by the phytase enzyme at the same time. The increase in the availability of P decreases the amount of P in the droppings owing to the better balance of the P. The reduction in P losses in the excreta can reduce the environmental pollution caused by P. As expected, reducing the nPP content of the NC diet negatively impacted P digestibility throughout the study with a 48% reduction being observed on day 35. Increasing supplemental doses of phytase increased ($P<0.05$) P digestibility throughout the trial. It is worth mentioning that phytase at a high concentration (1100 FTU/kg) yielded the highest P digestibility of the treatments throughout the study, with further improvements of 17.39 and 13.04% detected, compared to the 500 or 800FTU/kg treatments, respectively, at the end of the trial.

Table 3. Effect of dietary phytase supplementation on phosphorus (P) digestibility in broiler chickens at 35d of age

Treatments	P intake (g/d)	P excretion (g/d)	P digestibility (g/100g)
PC[1]	0.40[a]	0.14[a]	65.0[a]
NC[2]	0.29[b]	0.15[a]	48.0[c]
NC + comm phyt[3]	0.30[b]	0.10[c]	67.0[a]
NC+Lab phyt (FTU/kg)[4]			
500	0.29[b]	0.14[a]	57.0[b]
800	0.30[b]	0.12[b]	60.0[b]
1100	0.30[b]	0.09[c]	69.0[a]

[a-c] Mean values within a classification in the same column followed by different letters are significantly different ($P<0.05$)
[1] Positive control diet with normal non-phytate P (nPP = 4.0g/kg).
[2] Negative control diet with low nPP (nPP= 3.0g/kg).
[3] Negative control diet + commercial phytase named Axtra® PHY-DuPont Nutrition & Biosciences @ 500 FTU/kg.
[4] Negative control diet + Laboratory phytase @ 500, 800 and 1100 FTU/kg.

Tibia mineralization. The effect of phytase supplementation on the tibia bone ash contents, as well as tibia bone P and Ca contents in 35 day-old broilers are shown in Table 4. The lower level of nPP in the NC reduced ($P<0.05$) the tibia ash percentage by 15.14% compared to the PC on day 35 (Table 4). Furthermore, birds fed the NC displayed the lowest ($P<0.05$) P and Ca contents amongst the dietary treatments. Bone mineralization, as designated by tibia ash percentage, improved ($P<0.05$) with increasing levels of supplemental phytase. Incorporating increasing doses of phytase improved tibia P throughout the duration of the trial. The addition of phytase, regardless of dose, increased ($P<0.05$) tibia ash percentage and P content when compared to the NC diet. The inclusion of phytase at 1100 FTU/kg yielded P and tibia ash percentage values similar to that of the PC and NC+ commercial phytase group. Almost the same trend was observed for Ca content. Calcium content was adversely affected ($P<0.05$) by feeding with the low-nPP diet compared to the PC. Supplementing phytase ($P<0.05$) improved tibia Ca content with a dose of 1100 FTU/kg and on par with Ca levels similar to that of the PC. This highest level of phytase (1100FTU/kg) increased tibia ash by 4.3%, improved tibia P content by 25.6%, and improved tibia Ca content by 12% compared to the phytase level at 500FTU/kg.

Status of minerals in blood plasma. The effects of bacterial phytase supplementation on plasma P and Ca levels are shown in Table 4. Chickens

fed the PC diet and the NC diet with commercial phytase or different levels of lab phytase had substantially higher (P<0.05) plasma P and Ca concentrations than those fed the solely NC diet. Supplementing lab phytase in the NC diet at the highest level (1100FTU/kg) increased the plasma P and Ca concentrations and they were greater (P<0.05) than those obtained with the low and medium level diets, and on par with the PC or NC diet with commercial phytase.

Table 4. Effect of dietary phytase supplementation on tibia mineralization and plasma phosphorus, calcium in 35-d-old broiler chickens

Treatments	Tibia ash (g/100g)	Tibia P (g/100g ash)	Tibia Ca (g/100g ash)	Plasma P (mg/dl)	Plasma Ca (mg/dl)
PC[1]	50.63[a]	16.0[a]	35.0[a]	12.9[a]	15.4[a]
NC[2]	42.96[c]	8.9[c]	28.8[c]	10.2[c]	09.8[c]
NC + comm phyt[3]	49.97[a]	15.5[a]	36.0[a]	12.7[a]	15.5[a]
NC+Lab phyt (FTU/kg)[4]					
500	48.21[b]	11.9[b]	31.8[b]	11.1[b]	12.8[b]
800	48.85[b]	12.5[b]	32.0[b]	11.4[b]	12.9[b]
1100	50.40[a]	16.0[a]	36.0[a]	12.8[a]	15.3[a]

[a-c] Mean values within a classification in the same column followed by different letters are significantly different (P<0.05)
[1] Positive control diet with normal non-phytate P (nPP = 4.0g/kg).
[2] Negative control diet with low nPP (nPP= 3.0g/kg).
[3] Negative control diet + commercial phytase named Axtra® PHY-DuPont Nutrition & Biosciences @ 500 FTU/kg.
[4] Negative control diet + Laboratory phytase @ 500, 800 and 1100 FTU/kg.

Discussion

Microbial phytase is one of the most commonly used feed additives in the poultry industry. To test the efficiency of phytase used in diets with nutrient reduction on broiler performance, it is first necessary to show that dietary reductions affect bird performance. Subsequently, the phytase included should be assessed to determine the response to the deficient diet and a diet that meets the requirement of the broilers. The nutrient reductions, such as nPP in the NC diet, were considerable for impairing broiler performance. Hence, it was possible to monitor the responses to the use of *Bacillus* sp. (HCYL03) phytase on broiler performance. The results showed that decreasing nPP levels in the NC diet showed a decrease in BWG due to the deficiency of P in the broilers fed a lower level of P, lower than the recommended levels for broilers during the starter and finisher periods (NRC, 1994). This effect of P deficiency was also reported in other broiler studies as well (BOZKURT, et al., 2006; MONDAL et al., 2007; ANJUM et al., 2018; MAJEED et al., 2020). The literature showed that when broilers are fed P-deficient diets, their serum concentrations of growth hormone decreased (DESSIMONI et al., 2019). In this sense, low levels of growth hormone in P-deficient diets may be directly related to the development of the bird, since a bird that exhibits lower levels of this hormone will have decreased BWG, which explains the lower feed intake. Tested phytase supplementation at the high level of 1100 FTU/kg at 21 and 35 days of age solved the problem of P deficiency, and resulted in birds with a higher average BWG than those of the PC and lower levels (500 or 800FTU/kg) of phytase, but on par with the commercial phytase diet. Some reports show that the inclusion of higher levels of phytase (1000 FTU/kg and above) can further improve performance compared with 500 FTU/kg (WALK et al., 2014; MANOBHAVAN et al., 2016; LIU et al., 2020; METWALLY et al., 2020). Moreover, levels of phytase lower than 1000 FTU/kg had no substantial effect on improving broilers BWG when compared to the PC (DERSJANT-LI et al., 2021). A possible mode of action that may explain the response in this study is that a higher concentration of phytase (1100 FTU/kg) has the ability to hydrolyze phytate molecules quickly, reducing the formation of complexes between

phytate and the other dietary nutrients such as proteins (amino acids), carbohydrates, and minerals found in the diet (HUMER et al., 2015). Overall numerically, a trend was observed that increasing levels of phytase improved feed intake in this trial. The results of this study are supported by WALTERS et al. (2019) and BROCH et al. (2020), who reported that dietary phytase supplementation improved feed intake, whereas others reported that phytase did not affect feed intake (ANJUM et al., 2018; Al-HARTHY et al., 2020). The reasons for these contrasting results may be found in a number of factors, i.e. the source of phytase (type and phytate content), and dietary characteristics (processing, Ca: P ratio) etc. (ANJUM et al., 2018). SOMMERFELD et al. (2018) reported an increase in the disappearance of phytate and its isomers, and an increase in the appearance of *myo*-inositol in the crop and distal ileum with phytase supplementation. Therefore, with the increased time of retention of digesta in the crop and gizzard, phytase can initiate the process of hydrolyzing phytate and releasing nutrients for use by the birds. Moreover, improvements in BWG and FCR with the highest dose of phytase supplementation could be feed intake-based, as feed intake was substantially improved in broiler chickens receiving phytase at both 21 and 35 days of age. O'CONNOR-DENNIE and EMMERT (2012) found an improvement in FCR after adding phytase to P-deficient diets. Phytase supplementation is more effective in P-deficient diets than in P-sufficient diets for broilers (KARIMI et al., 2011; SANTOS et al., 2013). Most recent research also indicates that such improvements in the growth performance of chickens fed on phytase supplemented diets may be due to the release of minerals from the phytate mineral complex, and the utilization of inositol by the bird (KRIEG et al., 2021), or increased starch digestibility (MOSS et al., 2020). The same may also be due to the increased availability of proteins, because phytate also forms complexes with proteins, making them less available. Phytate-protein complexes are less subject to proteolytic digestion than without phytate. So it may be suggested that phytase liberates proteins from the complex, making them more available to the birds (MOSS et al., 2020). Evaluating digestibility is necessary to assess the efficiency of phytase on nutrient availability. The results showed that P excretion declined in the NC diet with the addition of phytase. Some researchers (BINGOL et al., 2009; SALEH et al., 2021) also reported a reduction of P excretion with phytase supplementation, which ultimately increased the availability of P. In this trial, the apparent digestibility of P in the NC was lower than with other treatments. These results corroborate those presented by DESSIMONI et al. (2019), who reported that the apparent digestibility of P in the NC (57.75%) was lower compared to the PC treatment (60.97%). These scientists also reported that the digestibility of P was 18.34% higher with the addition of *Escherichia coli* phytase, which elevated the bioavailability of P. Several studies exhibited improvements in P availability when supplementing phytase in low-nPP broiler diets (GAUTIER et al., 2018; WALTERS et al., 2019; BABATUNDE et al., 2020). HIRVONEN et al. (2019) suggested that the supplementation of phytase increases the hydrolysis of dietary phytate, resulting in improved P absorption and increased feed intake. In the current study, greater improvements in P digestibility were observed as the phytase concentration increased (1100FTU/kg), indicating that P digestibility may be attributed to the enhanced liberation of phytate-bound P via phytase inclusion (WOYENGO and NYACHOTI, 2011). These results are not surprising as it has been suggested that phytase at high dosage leads to greater destruction of the phytate structure, thus releasing additional phosphate molecules for absorption (COWIESON et al., 2011). Other authors detecting further increases in P digestibility beyond conventional doses have published similar results (MANOBHAVAN et al., 2016; WALTERS et al., 2019). The overall increase in the BWG observed in birds fed diets supplemented with phytase is because of a combination of factors, including the improvement in the apparent digestibility of P and other minerals during the entire lifecycle, and the increase in feed intake during both phases. The use of laboratory bacterial phytase in combination with a decreased dietary nPP

concentration is well known as an effective method of improving P utilization and decreasing P excretion. Tibia ash content generally reflects the absorption of P, Ca, and other minerals, which are also the main indicators of bone status. Tibia bone ash has been considered an ideal criterion for evaluating phytase efficacy in mineral utilization and deposition in broilers (WALTERS et al., 2019). In the present study, bone mineralization (ash, P and Ca) was badly affected in birds fed the NC diet, which may have resulted from the insufficient concentration of P available for bone development. Recently LI et al. (2020) reported similar results when feeding low-nPP diets (0.23% available P) to male broilers, with the reduction in dietary P decreasing tibia bone ash, P, and Ca content. Furthermore, ATTIA et al. (2020) noted a decline in tibia ash concentration (45.0 to 43.8%) as dietary nPP levels were reduced (0.5 to 0.4%) in 64 day-old colored broilers (Sasso strain). As a response to dietary supplementation, lab bacterial phytase increased the tibia ash content of broilers, in agreement with results of previous studies (OLUKOSI et al., 2013; GAUTIER et al., 2018). The improvement in the percentage of tibia ash bone mineralization exhibited was probably due to the availability of minerals liberated from the phytate mineral complex when lab phytase was added to the diets. As affirmed by WALTERS et al. (2019), it is suggested that the supplementation of phytase in the present study improved the availability of P in the GIT, resulting in better digestibility of P and higher concentrations of substrate for bone mineral accumulation, as noted in the tibia ash percentage and P content in this trial. Moreover, phytase fed at a high concentration (1100 FTU/kg) yielded the highest tibia ash, P and Ca content of all the treatments, with further improvements in ash and P content beyond that of the conventional doses (500 or 800FTU/kg) due to the potential liberation of greater proportions of minerals. In contrast, HUFF et al. (1998) did not find any improvements in tibia ash, P and Ca contents when phytase was added to low-P diets. A possible reason for the varied results could be the different ages and also the different sources of phytase used in the various studies. Concerning the P status of broilers, dietary P requirements vary depending on the outcome assessed. Phosphorus is quantifiably more imperative in bone mineralization than for soft tissue growth, as P is a main component of the bird's skeleton. Broilers may become more susceptible to mineral imbalances as nPP levels aer reduced, because bone mineralization requirements are met before growth requirements when P nutrition is the focus. Thus, tibia ash content is a more sensitive response measurement than growth response (GAUTIER et al., 2018). Briefly, broiler growth performance and tibia bone ash data substantiate the finding that addition of phytase was effective in enhancing utilization of phytate P when dietary nPP levels decreased. Plasma concentrations of P and Ca are the main indicators of poultry nutritional status in terms of P and Ca. In a low-P diet, the regulatory mechanism mobilizes the bone P and Ca to maintain normal P and Ca homeostasis. Various studies have reported about the effect of supplementing phytase in low-P diets on serum concentrations of P and Ca. HE et al. (2017) reported increased levels of serum P when microbial phytase was added to low-P diets in chickens. SEBASTIAN et al. (1994) reported that microbial phytase supplementation (500FTU/kg diet) in a corn-soyabean diet increased the plasma P by 15.7% more than with the NC diet in broilers, whereas the current study showed that a bacterial phytase (500 FTU/kg) diet raised plasma P by 8.11% in birds compared to the NC diet. ONYANGO et al. (2004) found that supplementation of three different types of phytase to a low-P diet resulted in higher serum P levels than a low-P diet. Likewise, a very recent study showed that P and Ca levels in blood plasma increased ($P<0.05$) in birds supplemented with bacterial and fungal phytases, and found that these levels were positively correlated with nutrient digestibility (SALEH et al., 2021). Furthermore, these increased serum P and Ca levels can be attributed to the ability of microbial phytases to dephosphorylate the phosphoester bonds of phytates and insoluble phytate salts, and release P (SALEH et al., 2021).

Conclusions

The results from the current study confirm that supplementation of new microbial phytase derived from *Bacillus* sp. (HCYL03) to a corn-soybean diet containing low nPP substantially improved the growth performance of broiler chickens. Supplementation of laboratory bacterial phytase considerably increased the digestibility of P, and decreased excretion of P by broiler chickens. Bone mineralization (Tibia ash, P and Ca contents) was improved by the incorporation of bacterial phytase in the broilers' diets. Likewise, plasma P and Ca contents also improved with the addition of the tested phytase enzyme. Moreover, supplementing a high level of phytase (1100FTU/kg) in a P- deficient diet resulted in improvements in all parameters compared to low doses of phytase. Consequently, inorganic P supplemented in the normal-nPP diet of chickens could be effectively replaced by a *Bacillus* sp. (HCYL03) phytase diet without any adverse effect on the performance and nutrient use of broilers.

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SAŽETAK

Cilj istraživanja bio je procijeniti utjecaj različitih razina fitaze izdvojene iz *Bacillus* sp. (HCYL03) i dodane u obroke od kukuruza i soje kojima se hrane brojleri. Istraživanje je uključilo pozitivnu kontrolu (PC) s izračunatom razinom nefitatnog fosfora (nPP) od 4,0 g/kg tijekom 35 dana trajanja istraživanja. Prehrana brojlera u negativnoj kontroli (NC) uključila je smanjenje nPP-a na 3,0 g/kg tijekom trajanja pokusa, komercijalno dostupnu fitazu (@500FTU/kg), kao i novu bakterijsku fitazu dodanu NC prehrani, u količini koja se povećavala na 500, 800 i 1100 FTU/kg. Učinci na rast, probavljivost fosfora, mineralizaciju tibije i razinu kalcija i fosfora u krvnoj plazmi procijenjeni su 35. dan pokusa. U skupini NC smanjeni su unos hrane ($P<0,05$) i prirast tjelesne mase (BWG) ($P<0,05$), dok je stopa konverzije hrane povećana (FCR) ($P<0,05$) u usporedbi sa skupinom PC. Dodatak fitaze pozitivno je utjecao na sve pokazatelje rasta. Brojleri u skupini NC pokazali su manju probavljivost fosfora ($P<0,05$), smanjenu mineralizaciju tibije ($P<0,05$) te smanjenu količinu fosfora i kalcija ($P<0,05$) u krvnoj plazmi u usporedbi s brojlerima iz skupine PC. Utvrđeno je da dodatak fitaze poboljšava probavljivost fosfora, mineralizaciju tibije i sadržaj minerala u krvnoj plazmi. Dodatak veće količine fitaze (1100 FTU/kg) rezultirao je najvećim poboljšanjem u istraženim svojstvima brojlera, probavljivosti hrane i mineralizaciji kosti u skupini NC. Zaključeno je da bi se anorganski fosfor uključen u uobičajenu nPP prehranu pilića mogao učinkovito zamijeniti *Bacillus* sp. (HCYL03) fitazom, bez štetnih učinaka na prehranu i svojstva brojlera.

Ključne riječi: *Bacillus* sp. (HCYL03); bakterijska fitaza; nefitatni fosfor; prirast; probavljivost