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Article

Efficacy of three heat-stable microbial phytases on growth performance and bone development and strength of broilers fed diets deficient in available phosphorus

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Abstract

A total of 2340 as-hatched Cobb500 chicks were allocated to 9 treatments, each with 13 replicate pens to evaluate the effects of either three phytase enzymes in a P-deficient diet. Starter and finisher diets consisted of a positive control (PC) and negative control 1 and 2 (NC1 and NC2, respectively). The PC, NC1, and NC2 diets had Ca:avP (available phosphorus) ratios of 0.50, 0.33, and 0.43 in the starter feed, and 0.46, 0.22, and 0.35 in the finisher feed, respectively; NC1 diets were then supplemented with Phytaverse, Quantum Blue, and Axtra-PHY at 500 and 1000 FTU/kg. Enzyme type had significant effects on body weight gain, feed conversion ratio, and production efficiency factor during the 0–7-day period. Interactions between enzyme type and inclusion levels had a significant effect on feed intake (FI) at 1–21-day ($P = 0.02$) and 1–35-day ($P = 0.031$) age. While FI decreased as Axtra-PHY inclusion levels increased from 500 to 1000 FTU/kg feed, FI increased in birds supplemented with Quantum Blue, but with no effects on Phytaverse-supplemented birds. Increasing the enzyme dose to 1000 FTU/kg feed improved bone-breaking strength but did not affect growth performance, tibia ash, Ca, or P concentration of the birds.

Key words: broiler chicks, body weight gain, available phosphorus, microbial phytases, bone mineralization

Résumé

Un total de 2340 poussins Cobb500 éclos ont été alloués à neuf traitements, chacun avec 13 enclos répliqués pour évaluer la réponse des poulets à griller à trois enzymes phytases dans une diète déficiente en P. Les diètes de démarrage et de finition consistaient d'un témoin positif (PC — « positive control »), et témoins négatifs 1 et 2 (NC — « negative control »; NC1, NC2). Les diètes PC, NC1 et NC2 avaient des rapports Ca:P disponible (Ca:avP — « calcium:available P ») de 0,50, 0,33 et 0,43 dans la diète de démarrage; et 0,46, 0,22, 0,35 dans la diète de finition; les diètes NC1 étaient alors supplémentées de Phytaverse, Quantum Blue et Axtra-PHY à raison de 500 et 1000 FTU/kg. Le type d'enzyme a eu un effet significatif sur le gain de poids corporel, le taux de conversion alimentaire (FCR — « feed conversion ratio ») et le facteur d'efficacité de production (PEF — « production efficiency factor ») durant la période de 0 à 7 jours. Les interactions entre le type d'enzyme et les niveaux d'inclusion avaient des effets significatifs sur la consommation (FI — « feed intake ») aux jours 1 à 21 ($P = 0,02$) et jours 1 à 35 ($P = 0,031$) d'âge. Tandis que le FI diminuait avec l'augmentation des niveaux d'inclusion d'Axtra-PHY de 500 à 1000 FTU/kg d'aliments, le FI a augmenté chez les poulets ayant reçu des suppléments de Quantum Blue, et il n'y a pas eu d'effet chez les poulets ayant reçu les suppléments de Phytaverse. Augmenter la dose d'enzyme à 1000 FTU/kg d'aliments a amélioré la résistance aux fractures, mais n'a pas eu d'effet sur la performance de croissance, ni les cendres du tibia, ni les concentrations de Ca ou P des poulets. [Traduit par la Rédaction]

Mots-clés : poussins de chair à griller, gain de poids corporel, phosphore disponible, phytases microbiennes, minéralisation osseuse

Introduction

The intense genetic selection for rapid growth in broiler chickens has resulted in the inadequate supply of minerals and metabolic imbalances, leading to a series of unintended negative effects, including skeletal deformities (Julian 1998; Plumstead et al. 2008; Leeson and Summers 2009; Shim et al. 2012). Distress and skeletal disorders are a constant concern, not only for production efficiency and profit for producers, but also for the welfare and health of the birds (Julian 1998; Shim et al. 2012). Changes in conformation and ever-increasing body weight on a physically less-mature skeleton induce the greatest skeletal anomalies in modern broilers (Applegate and Lilburn 2002). Together with Ca, available phosphorus (avP) is responsible for bone rigidity and compressive strength (Proszkowiec-Weglarz and Angel 2013). Moreover, phosphorus (P) is necessary for the production of

ATP, RNA and deoxyribonucleic acid, enzymes, and phosphoglycerides, and plays an integral role in maintaining acid–base balance and immune function (Campbell and Farrell 2012; Shim et al. 2012).

P is also an important cause of eutrophication, often increasing the amount of plant and algae growth in lakes and reservoirs (Yan et al. 2004; Jeon et al. 2015). The amount of dietary P and the efficiency of its utilization in the chick's gut determine the amount of P in the excreta. Approximately 50%–85% of P in plants is in the phytate structure that birds cannot utilize, thereby reducing its absorption efficiency (Plumstead et al. 2008). Phytate (*myo-inositol hexakisphosphate*, IP_6) or phytic acid is the major storage form of P, comprising 1%–5% by weight in cereals, legumes, oilseeds, and nuts (Vats and Banerjee 2004). The bioavailability of phytate-P for broilers ranges from 0% to 80% depending on diet composition, bird age, and metabolic adaptation (Van der Klis and Versteegh 1999). The chemical characteristics of IP_6 influence exogenous and intestinal phytase efficacy in releasing both phytic-P and any minerals it has complexed with (Angel et al. 2002; Lu et al. 2013). In the gut, phytate forms insoluble complexes with other dietary nutrients, limiting micro-flora and endogenous phytase activity, and resulting in up to 70% dietary P excretion (Lei and Stahl 2001; Gupta et al. 2015). Phytate–protein complexes at both acidic and alkaline pH affect changes in protein structure, resulting in reduced enzymatic activity, protein solubility, and proteolytic digestibility (Adeola and Cowieson 2011).

Additive supplementation of microbial phytases (*myo-inositol hexakisphosphate phosphohydrolase*) in commercial feed manufacturing has become standard to catalyse the breakdown of phytic acid (IP_6) into a series of lower phosphate esters of myo-inositol and inorganic orthophosphate (Dersjant-Li et al. 2015). With advances in biotechnology, it has become possible to improve the phytase enzyme structure by genetic manipulations in coding sequence of genes (Onyango et al. 2005). Furthermore, extra-phosphoric effects from phytase supplementation beyond the standard doses have been reported to enhance nutrient digestibility when marginally deficient diets are fed, thereby improving the performance of broilers (Lei and Stahl 2001; Campasino et al. 2014). Yan et al. (2004) showed that broilers may be grown effectively on a modified feeding program with sufficient P levels with or without phytase supplementation in the starter period, followed by markedly reduced levels during the grower and finisher period. However, the ideal phytase should be active in the stomach, be catalytically efficient, heat stable during feed processing and storage, and proteolysis resistant, and be inexpensive (Lei and Stahl 2001). It is hypothesized that enzyme type will affect weight gain, feed intake (FI), and bone mineralization in broilers fed P-deficient diets supplemented with phytase enzymes at different doses. Therefore, the objective of this study was to evaluate the efficacy of three microbial phytases, namely a modified *Escherichia coli* phytase, Phytaverse; an enhanced *E. coli* phytase, Quantum Blue; and a *Buttiauxella* species bacterium-sourced phytase, Axtra-PHY (AxP) in diets deficient in avP on the growth performance, bone development, and strength of broilers.

Materials and methods

Management of birds and housing

The Agricultural Research Council Ethics Committee approved the use of broiler chicks for this experiment (reference number: APIEC17/18). All procedures complied with the South African Poultry Association Code of Practice that provides defined minimum standards for the well-being of poultry in commercial operations, research, and educational facilities. Cleaning and disinfection of the house were done 2 weeks prior to the start of the trial. Bedding material consisted of mixed pine wood shavings, distributed evenly on the floor at a depth of 5 cm in a pen size of 1 m^2 with a maximum stocking density of 40 kg/m^2.

Two thousand three hundred and forty as-hatched 1-day-old Cobb 500 broiler chicks were purchased from a commercial hatchery. At the hatchery, prior to delivery at the research facility, chicks were vaccinated against Newcastle Disease, Marek's Disease, Infectious Bursal Disease, and Infectious Bronchitis. Chicks were weighed at placement to obtain the mean initial body weight (BW) and were randomly assigned to nine treatments in a randomized completely blocked design with 13 replicates per treatment and 20 birds per pen (2 m^2) corresponding to 10 birds per square metre in an environmentally controlled house with tunnel ventilation. Before placement, the house temperature was set at 32 °C, and then it was reduced by 3 °C every week until it reached 22 °C where it was maintained until the end of the trial period. All chicks received 24 h of light on the first day, 23L:1 D from day 1 to 6 and 16L:8 D from day 7 to 35. Feed and water were offered ad libitum and were checked for availability twice a day during the entire 35-day trial period. On days 7, 14, 21, 28, and 35, birds were weighed to record body weight gain (BWG) per pen and cumulative FI per pen was calculated as the difference between the amount of feed allocated and the amount remaining in the feeders. Feed conversion ratio (FCR) was calculated as daily FI over the average daily weight gain per bird. Mortalities and culls were recorded daily to accurately calculate FI and FCR. Production efficiency factor (PEF) was calculated as: [(BW (kg) × 100-cumulative mortality)/(7 days × cumulative FCR)/1000].

Experimental diets

Birds were fed in two dietary phases, the starter (0–21 days) and the finisher (22–35 days), to reduce diet-induced variations. The ingredient and chemical composition of experimental diets is shown in Table 1. The description of the nine dietary treatments is shown in Table 2 with three control diets that contained no phytase enzymes. The control starter diets (T1–T3) were formulated as follows: T1 – positive control (PC), the recommended breed standard with 0.43% avP; T2 – negative control 1 (NC1), with 0.23% avP; and T3 – negative control 2 (NC2), the industry standard with 0.33% avP. Treatments T4 to T9 were arranged with enzyme supplementation of the NC1 diet in a 3 × 2 factorial with three phytase enzymes (Fig. 1) added at two different levels, 500 and 1000 FTU/kg (1 FTU of phytase is defined as the quantity of enzyme that liberates 1 μmol of inorganic phosphate per minute from

Table 1. Composition of broiler starter and finisher diets on as fed basis.

Feed ingredients (%)	Starter diets (0–21 days)			Finisher diets (21–35 days)		
	PC[a]	NC1[a]	NC2[a]	PC	NC1	NC2
Maize (finely milled)	57.15	59.02	58.10	64.57	65.61	65.09
Soya oilcake	23.06	26.01	24.54	22.61	22.47	22.54
Sunflower oilcake	5.01	5.00	5.01	7.19	7.15	7.17
Fullfat soya	7.52	3.50	5.51	0.00	0.00	0.00
Soya oil	3.01	3.00	3.01	2.06	2.04	2.05
Limestone	0.75	1.20	0.95	0.82	1.28	1.08
Mono-calcium phosphate	1.80	0.60	1.20	1.29	0.00	0.62
Salt	0.35	0.35	0.35	0.31	0.31	0.31
Lysine	0.23	0.23	0.23	0.26	0.26	0.26
DL-Methionine	0.27	0.26	0.26	0.24	0.23	0.24
Threonine	0.03	0.03	0.03	0.04	0.04	0.04
Vitamin–mineral premix[b]	0.30	0.30	0.30	0.20	0.20	0.20
Zinc bacitracin	0.05	0.05	0.05	0.05	0.05	0.05
Ancoban	0.05	0.05	0.05	0.05	0.05	0.05
Sodium bicarbonate	0.20	0.20	0.20	0.10	0.10	0.10
Mould binder	0.20	0.20	0.20	0.20	0.20	0.20
Analyzed chemical composition						
ME (MJ/kg), *formulated*	11.91	11.90	11.91	12.42	12.39	12.41
Crude protein (%)	20.27	21.17	21.13	19.29	18.34	18.72
Crude fibre (%)	4.14	4.18	4.17	3.62	3.65	3.57
Fat, AH (%)	8.19	8.40	8.10	6.67	6.77	7.13
Ash (%)	5.32	5.16	5.13	4.81	4.67	4.65
Calcium (%)	0.92	0.87	0.91	0.79	0.78	0.85
Total phosphorus (%)	0.82	0.58	0.68	0.72	0.44	0.56
Ca:avP (%)	2.14	3.71	2.76	2.44	6.38	3.88

[a]PC, positive control; NC1, negative control 1; NC2, negative control 2; and AvP, available phosphorus.
[b]Provided per kilogram of diets: 12 000 IU vitamin A (retinol acetate); 5000 IU cholecalciferol; 60 IU vitamin E (α-tocopherol acetate); 2 mg vitamin K_3; 2 mg thiamin; 5 mg riboflavin; 50 mg niacin; 12 mg vitamin B_5 (calcium panthonate); 3 mg vitamin B_6; 2 mg folic acid; 0.010 mg vitamin B_{12}; 0.10 mg biotin; 125 mg antioxidant; 110 mg manganese; 40 mg iron; 100 mg zinc; 10 mg copper; 0.50 mg cobalt; 2 mg iodine; 0.30 mg selenium; and 350 mg choline (choline chloride).

Table 2. Description of the nine dietary treatments.

Dietary treatment (T)	Description
T1, PC (positive control)	Starter diet with 0.43% avP* and a Ca:avP of 0.50
	Finisher diet with 0.32% avP and a Ca:avP of 0.46
T2, NC1 (negative control 1)	Starter diet with 0.23% avP and a Ca:avP of 0.33
	Finisher diet with 0.12% avP with a Ca:avP of 0.22
T3, NC2 (negative control 2)	Starter diet with 0.33% avP and a Ca:avP of 0.43
	Finisher diet with 0.22% avP and a Ca:avP of 0.35
T4	NC1 with Phytaverse† at 500 FTU/kg diet
T5	NC1 with Phytaverse† at 1000 FTU/kg diet
T6	NC1 with Quantum Blue† at 500 FTU/kg diet
T7	NC1 with Quantum Blue† at 1000 FTU/kg diet
T8	NC1 with Axtra-PHY† at 500 FTU/kg diet
T9	NC1 with Axtra-PHY† at 1000 FTU/kg diet

*avP = available phosphorus.
†Phytaverse (PTV), a modified *E. coli* phytase; Quantum Blue (QB), an enhanced *E. coli* phytase; and Axtra-PHY (AxP), a *Buttiauxella* species bacterium-sourced phytase.

sodium phytate at pH 5.5 and 37 °C). The three phytases include Phytaverse (Novus International, St. Charles, MO, USA), Quantum Blue (AB Vista, Marlborough, UK), and AxP (DuPont, Wilmington, DE, USA). For each control, the finisher diets had a 0.11% reduction in avP as compared to the starter diet, and then treatments 4 to 9 consisted of NC1 with Phytaverse, Quantum Blue, or AxP at 500 or 1000 FTU/kg. The diets were formulated on Format Feed Formulation software (Format Solutions Ltd., Hopkins, USA) using the CVB (Dutch Net Energy) system and were mixed at Simple Grow Agricultural Services. Both diets were pelleted at temperatures set and monitored not to exceed 85 °C.

Fig. 1. Phytase enzymes that were used in the study. T4 and T5, Phytaverse (PTV), a modified *E. coli* phytase at 500/1000 FTU; T6 and T7, Quantum Blue (QB), an enhanced *E. coli* phytase at 500 and 1000 FTU; and T8 and T9, Axtra-PHY (AxP), a *Buttiauxella* species bacterium-sourced phytase at 500 and 1000 FTU. [Colour online.]

Bone mineralization measurements

At 21 and 35 days, two birds from each pen (468 in total) were randomly selected and euthanized by cervical dislocation. All adhering flesh, the kneecap, and the fibula were removed from the left and right drumsticks. The tibias were then kept in Ziploc bags marked according to treatments and frozen at −20 °C. At 24 h before the start of the analysis, both the left and right tibias were removed from the freezer and allowed to thaw at room temperature. Using a computer-controlled material testing machine with a load cell of 5 kN (Model LRX-plus; Lloyd Instruments Ltd., Fareham, UK), the left tibias were used to determine bone-breaking strength (BBS) at the Civil Engineering Department, University of Pretoria. The speed was set at 50 mm/min for a maximum displacement of 10 mm and a three-point configuration of 40 mm distance between the first and third supports. The right tibias were used for the analysis of tibia ash, as well as Ca and P contents at Lab-world Pty, South Africa. Before analysis, each tibia was dried at 50 °C for 72 h, wrapped in cheesecloth, and placed in the Soxhlet extractor for 12 h to remove all fats. Thereafter, the tibias were allowed to dry at room temperature for an additional 24 h, weighed and ashed at 650 °C for 12 h. Ash was determined on a fat-free dry matter basis using the AOAC method described by the AOAC (2000). Calcium and P contents were determined using the Official Methods of Analysis (AOAC 2000, Method 976.06). Tibia ash percentage was calculated as (weight ash ÷ weight dry bones) × 100.

Statistical analyses

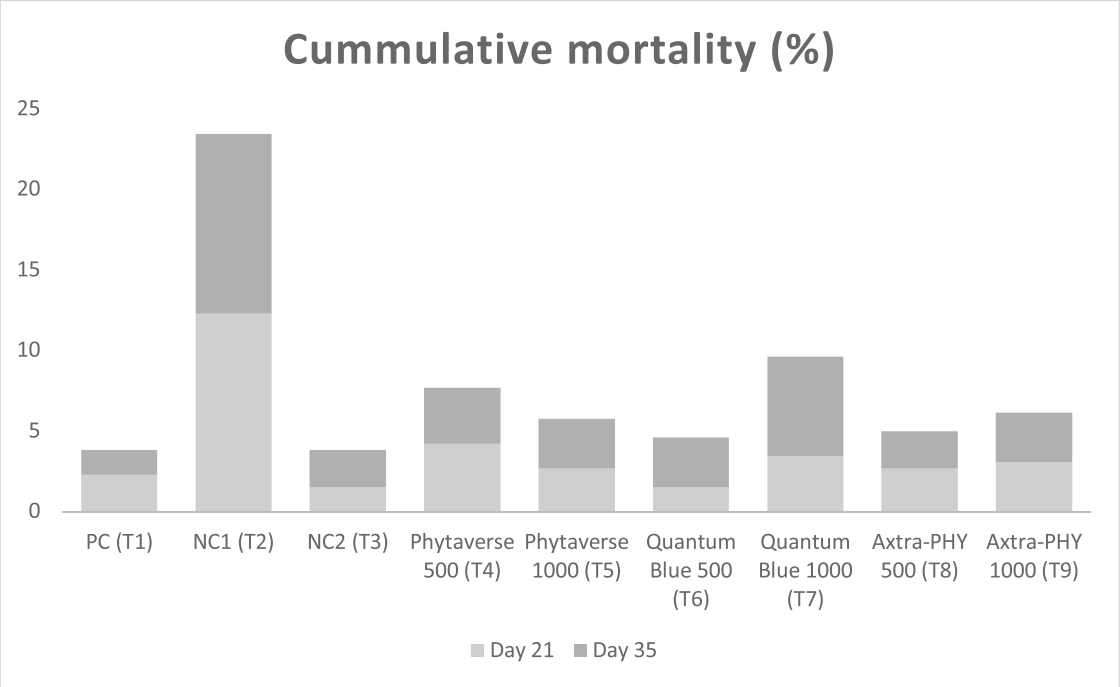
The data obtained for BW, FI, FCR, and PEF, as well as bone mineralization parameters, were analysed using the GLM procedure of SAS version 9.4 (SAS Institute Inc. 2017). The model includes the pen location as a blocking factor. The effects of enzyme type and inclusion levels were compared as a 3 × 2 factorial and where significant interaction was observed, the slice function was used to compare inclusion levels within each enzyme type. Furthermore, single degree of freedom contrasts was used to compare (*i*) between the PC versus average of enzyme-supplemented treatments, (*ii*) PC versus Phytaverse treatments, (*iii*) PC versus Quantum Blue treatments, (*iv*) PC versus AxP treatments, and (*v*) PC versus negative control treatments. Significant differences were declared at $P < 0.05$.

Results

Growth performance

The response of broiler birds fed avP-deficient, enzyme-supplemented diets is shown in Table 3. There was no interaction effect between phytase enzyme type and their inclusion levels, on BWG of birds at days 7 (d7), 21 (d21), and 35

Fig. 2. Cumulative mortality (%) of broilers consuming diets deficient in available phosphorus with or without supplementation of heat-stable microbial phytases. PC, positive control (starter diet with 0.43% available phosphorus, avP, finisher with 0.32% avP); NC1, negative control 1 (starter diet with 0.23% avP, finisher diet with 0.12% avP); NC2, negative control 2 (starter diet with 0.33% avP, finisher diet with 0.22% avP); and T4 to T9, NC1 with Phytaverse, Quantum Blue, or Axtra-PHY enzymes at 500 and 1000 FTU/kg.



(d35). Furthermore, the simple effects of enzyme inclusion level did not affect the weight gain of the birds across the growth periods. However, while enzyme type affected BWG at d7 ($P = 0.032$), this effect was not observed at d21 and d35. The cumulative mortality of the birds showed an average flock mortality of 7.78% with a very high mortality occurring in birds consuming the NC1 diet with 12% mortality at d21 and 23% by d35 (Fig. 2).

Across the periods, the NC1 birds (without enzyme supplementation) seemed to have the lowest response in terms of BWG and FI. Furthermore, birds on the negative control treatments (NC1 and NC2) showed lower weight gain when compared with birds on the PC treatment ($P < 0.05$). When compared with birds on the PC diet, the average of the enzyme-supplemented birds had lower BWG at d7 ($P = 0.002$) but such difference was not significant at d21 and d35. Phytaverse supplemented birds did not differ from birds on the PC diet in terms of BWG but birds fed Quantum Blue recorded lower weight gain at d7 while birds on AxP diet had lower weight gain at d7 and d35 ($P < 0.05$) as compared to the PC. While FI differed between birds on the negative control (NC1 and NC2) diets and birds on the PC ($P < 0.05$), no differences in FI were observed between the PC and the average of the enzyme-supplemented birds or between PC and birds on the respective enzyme diets.

The interaction effect of enzyme type and inclusion level did not affect FI at d7, while the simple effects of enzyme type, as well as inclusion levels, also did not affect FI at d7. In contrast, the interaction effect of enzyme type and level was observed at d21 ($P = 0.002$) and d35 ($P = 0.031$), respectively. At d21, while FI decreased as the inclusion level of AxP increased from 500 to 1000 FTU/kg feed, no such effect was observed in birds supplemented with Phytaverse. However, birds supplemented with Quantum Blue showed a significant increase in FI as inclusion levels increased from 500 to 1000 FTU/kg feed at d21 and d35 ($P < 0.05$). At d35, inclusion level did not affect FI of birds fed Phytaverse and AxP.

The effect of feeding diets deficient in avP with or without microbial phytase supplementation on FCR and PEF is presented in Table 4. There was no interaction effect of enzyme type and inclusion level on FCR and PEF at d7, d21, and d35. While the simple effect of enzyme type showed differences in FCR ($P = 0.014$) and PEF ($P = 0.003$) of the birds at d7, the differences were not observed at d21 and d35. Furthermore, the simple effect of enzyme inclusion levels did not affect both FCR and PEF.

The average of birds on the negative control diets (NC1 and NC2) was not different from birds on the PC diet in terms of FCR, but PC birds showed superior PEF at d21 ($P = 0.01$) and d35 ($P < 0.01$). Birds fed the PC diet had lower and thus, better FCR, compared to the average of birds receiving avP-deficient, enzyme-supplemented diets at d7 ($P = 0.005$) but the FCR did not differ at d21 and d35. Furthermore, when compared to the PC treatment, PEF was lower at d7 and d35 in birds receiving the avP-deficient, enzyme-supplemented diets. Phytaverse-supplemented birds did not differ from the

Table 3. Body weight gain and feed intake of broilers fed diets deficient in available phosphorus with or without supplementation of heat-stable microbial phytases.

		Period (days)					
		Body weight gain (g/bird)			Feed intake (g/bird)		
Parameter[a]		0–7 days	0–21 days	0–35 days	0–7 days	0–21 days	0–35 days
PC (T1)		186.5	917.5	2173.1	181.8	1239.2	3263.7
NC1 (T2)		171.3	842.5	1813.9	170.9	1100.8	2788.8
NC2 (T3)		185.7	873.9	2092.9	178.7	1162.6	3206.7
Phytaverse (FTU)	500 (T4)	179.0	898.3	2196.0	191.6	1230.3	3304.8
	1000 (T5)	184.3	888.6	2120.8	198.6	1215.2	3254.3
Quantum Blue (FTU)	500 (T6)	174.0	857.5	2014.0	198.1	1179.7	3120.1
	1000 (T7)	174.5	883.8	2154.3	240.3	1259.7	3302.2
Axtra-PHY (FTU)	500 (T8)	177.5	884.9	2016.2	200.4	1231.2	3211.3
	1000 (T9)	176.1	871.5	2045.4	200.4	1199.3	3245.4
SEM		0.95	6.91	20.12	4.24	7.38	20.98
P values							
Enzyme type and level[b]	E	0.032	0.385	0.068	0.175	0.902	0.269
	L	0.522	0.939	0.481	0.137	0.415	0.125
	E∗L	0.469	0.427	0.147	0.247	0.002	0.031
Contrasts[c]	PC versus enzyme	0.002	0.103	0.162	0.078	0.328	0.649
	PC versus Phytaverse	0.127	0.344	0.824	0.368	0.477	0.789
	PC versus Quantum Blue	<0.01	0.067	0.181	0.013	0.399	0.379
	PC versus Axtra-PHY	0.003	0.123	0.033	0.210	0.301	0.554
	PC versus NC	0.013	0.021	0.001	0.636	<0.01	<0.01

[a]PC, positive control (starter diet with 0.43% available phosphorus, avP, finisher with 0.32% avP); NC1, negative control 1 (starter diet with 0.23% avP, finisher diet with 0.12% avP); NC2, negative control 2 (starter diet with 0.33% avP, finisher diet with 0.22% avP); and T4 to T9, NC1 with Phytaverse, Quantum Blue, or Axtra-PHY enzymes at 500 and 1000 FTU/kg.
[b]*P* values, E∗L, interaction effect of enzymes type (E) and inclusion level (L).
[c]Contrast, single degree of freedom contrast (*i*) between positive control (PC) versus average of enzyme-supplemented treatments, (*ii*) PC versus phytaverse treatments, (*iii*) PC versus Quantum Blue treatments, (*iv*) PC versus Axtra-PHY treatments, and (*v*) PC versus negative control treatments.

PC birds in terms of FCR and PEF except at d7 when PEF was lower ($P = 0.03$). In contrast, birds on Quantum Blue and AxP treatments had lower PEF across the periods ($P < 0.05$), while their FCR was higher and inferior at d7.

Bone mineralization

As shown in Table 5, there was no significant interaction effect of enzyme type and enzyme inclusion level on the BBS, tibia ash, and tibia Ca and P concentrations of the birds receiving avP-deficient diet supplemented with phytase enzymes. Furthermore, enzyme type did not affect tibia BBS and tibia ash but tibia Ca and P concentrations were affected by enzyme type. In contrast, while the simple effect of enzyme inclusion level showed differences in tibia BBS at only d35 ($P = 0.04$) and tibia ash concentration at only d21 ($P = 0.019$), tibia Ca and P concentrations were not affected by enzyme inclusion levels.

Tibia BBS was not different between the PC birds and the average of birds on AvP-deficient, enzyme-supplemented diets, and between PC and the individual enzyme-supplemented birds. In contrast, birds on the negative control diets (NC1 and NC2) had lower BBS compared to the PC birds at d21. Tibia ash concentration was not different when each of the enzyme-supplemented birds or their average is compared with birds on the PC diet except at d35 where tibia ash was higher in Phytaverse-supplemented birds compared to the PC birds.

Tibia Ca and P concentrations were generally lower in birds fed avP-deficient, enzyme-supplemented diet compared to the PC birds. Similarly, birds on Quantum Blue treatment had lower Ca and P compared to the PC birds, while Ca concentration at d35 was lower in Phytaverse supplemented compared to the PC birds. Furthermore, birds on the AxP enzyme recorded lower tibia Ca at d21 ($P < 0.01$) and d35 ($P = 0.011$), but their P concentrations at both periods were not different from the PC birds.

Discussion

Earlier studies have associated P deficiency with the loss of appetite, rickets, and growth failure (Scott et al. 1982). As expected, the NC treatments with avP-deficient diets resulted in lower performance in the broilers compared to the PC diet formulated based on the breed standard across the growth period and this agrees with Sharma et al. (2016), who reported lower FI and BW in birds fed the NC diet without phytase, as compared to birds consuming the diet with the recommended P concentration. The lower growth performance of the NC diets showed that the diets were indeed nutrient deficient. Enzyme supplementation becomes less sensitive above the nutrient requirement of the animals (Wang et al. 2020)

Table 4. Feed conversion ratio and performance efficiency factor of broilers fed diets deficient in available phosphorus with or without supplementation of heat-stable microbial phytases.

Parameter[a]		Period (days)					
		Feed conversion ratio (g: g)			Protein efficiency factor (%)		
		0–7 days	0–21 days	0–35 days	0–7 days	0–21 days	0–35 days
PC (T1)		1.30	1.43	1.54	209.7	301.1	388.8
NCI (T2)		1.36	1.41	1.59	182.8	241.6	249.2
NC2 (T3)		1.27	1.41	1.57	211.9	293.5	367.2
Phytaverse (FTU)	500 (T4)	1.43	1.45	1.55	181.9	283.9	377.8
	1000 (T5)	1.43	1.45	1.58	188.0	286.2	363.8
Quantum Blue (FTU)	500 (T6)	1.54	1.46	1.59	167.8	277.7	347.3
	1000 (T7)	1.85	1.51	1.57	137.8	272.2	356.3
Axtra-PHY (FTU)	500 (T8)	1.50	1.47	1.64	169.8	280.8	338.3
	1000 (T9)	1.53	1.45	1.64	168.2	277.1	338.1
SEM		0.03	0.01	0.01	3.56	3.65	6.00
P values							
Enzyme type and level[b]	E	0.014	0.341	0.069	0.003	0.573	0.105
	L	0.122	0.573	0.973	0.261	0.772	0.890
	E∗L	0.153	0.369	0.748	0.124	0.912	0.749
Contrasts[c]	PC versus enzyme	0.005	0.319	0.130	<0.01	0.059	0.032
	PC versus Phytaverse	0.178	0.678	0.611	0.030	0.210	0.330
	PC versus Quantum Blue	<0.01	0.173	0.302	<0.01	0.042	0.047
	PC versus Axtra-PHY	0.029	0.393	0.014	<0.01	0.084	0.007
	PC versus NC	0.862	0.579	0.344	0.274	0.01	<0.01

[a]PC, positive control (starter diet with 0.43% available phosphorus, avP, finisher with 0.32% avP); NC1, negative control 1 (starter diet with 0.23% avP, finisher diet with 0.12% avP); NC2, negative control 2 (starter diet with 0.33% avP, finisher diet with 0.22% avP); and T4 to T9, NC1 with Phytaverse, Quantum Blue, or Axtra-PHY enzymes at 500 and 1000 FTU/kg.
[b]*P* values, E∗L, interaction effect of enzymes type (E) and inclusion level (L).
[c]Contrast, single degree of freedom contrast (*i*) between positive control (PC) versus average of enzyme-supplemented treatments, (*ii*) PC versus phytaverse treatments, (*iii*) PC versus Quantum Blue treatments, (*iv*) PC versus Axtra-PHY treatments, and (*v*) PC versus negative control treatments.

and this justifies the supplementation of the NC diet with the phytase enzyme.

The weight gain in enzyme-supplemented birds consuming avP-deficient diets validates previous studies that adding higher levels of phytase to a low-P diet promotes improved feed efficiency. Julian (1998) showed that supplementation of a low-P diet with the evolved *E. coli*-derived phytase improved weight gain, FI, and tibia ash of chicks. Exogenous phytases have extra-phosphoric effects such as improvement in Ca and amino acid digestibility (Adeola and Cowieson 2011), thus the associative effect on tissue accretion and BWG. Walk et al. (2013) proposed that phytase helps to reduce the extra-phosphoric antinutrient effects of phytate, rather than the liberation of excess P. Nevertheless, Cowieson et al. (2006) suggested that higher phytase doses can be beneficial in retaining P, mediated through an improved phytate P digestibility.

Within the period 1–7 days of age, PC birds had higher weight gains than birds fed an avP-deficient diet and supplemented with enzymes, but this effect was not observable across 1–21 and 1–35 days of age periods. The improved growth in NC + enzyme groups after the 7-day period could be associated with the reduced negative effects of phytic acid on the digestibility of protein. Phytic acid binds to protein in a pH-dependent manner and impeded protein and Ca digestion, and amino acid absorption, and this may be ameliorated by phytase (). Furthermore, the P requirement of broiler chickens continuously changes during growth (NRC 1994). Nevertheless, Lu et al. (2013) reported a significant increase in ileal P digestibility and overall P utilization with phytase enzyme.

The level of P deficiency may interact with the age at which P becomes limiting, and the extent of such interaction will influence overall broiler performance. Older birds have an increasing ability to utilize P in P-deficient diets compared to younger birds (Wang et al. 2020) and this may account for the higher tibia ash concentration across the growth phase. Besides, broilers are more sensitive to the level of Ca and avP in a diet than laying hens due to differences in their abilities to efficiently absorb and retain dietary Ca and P. Thus, bone disorders and impaired performance in broilers persist even when the optimal Ca:avP ratio is subsequently maintained (Driver et al. 2005).

P is associated with the modulation of FI and improved synthesis of growth hormones (Dos Santos et al. 2019). Unlike FCR, where lower values indicate greater flock performance efficiency, a higher PEF value reflects a greater production efficiency (). Overall results on the growth rate, FI, FCR, and PEF of birds consuming avP-deficient diets supplemented with 500 FTU microbial phytases improved close to the breed performance standards but increasing enzyme dose to 1000 FTU/kg feed may not result in added advantages on

Table 5. Tibia breaking strength, bone ash, calcium, and phosphorus of broilers fed diets deficient in available phosphorus with or without supplementation of heat-stable microbial phytases.

Parameter[a]		Bone mineralization parameters							
		Bone-breaking strength (N)		Ash		Ca (%)		P (%)	
		21 days	35 days	21 days	35 days	21 days	35 days	21 days	35 days
PC (T1)		165.1	272.1	47.9	47.3	16.6	17.4	9.37	9.43
NC1 (T2)		106.4	149.5	43.3	42.5	15.4	13.4	7.78	7.35
NC2 (T3)		171.8	310.9	49.6	47.1	16.1	16.3	9.26	9.28
Phytaverse (FTU)	500 (T4)	184.4	299.1	48.0	49.2	15.8	16.3	8.90	9.21
	1000 (T5)	182.9	320.8	49.0	49.8	16.6	16.3	9.19	9.61
Quantum Blue (FTU)	500 (T6)	170.8	271.0	46.7	47.1	15.1	15.7	8.37	8.88
	1000 (T7)	170.9	317.9	47.9	48.2	15.1	15.5	8.68	8.75
Axtra-PHY (FTU)	500 (T8)	177.7	278.1	47.1	47.7	15.3	17.2	9.04	9.43
	1000 (T9)	168.0	304.1	49.3	48.6	14.9	16.7	8.66	9.45
SEM		7.81	17.55	0.63	0.70	0.22	0.39	0.17	0.23
P value									
Enzyme type and level[b]	E	0.763	0.637	0.109	<0.01	<0.01	<0.01	0.007	<0.01
	L	0.633	0.040	0.019	0.427	0.553	0.831	0.650	0.613
	E∗L	0.931	0.339	0.213	0.568	0.477	0.309	0.184	0.438
Contrasts[c]	PC versus Enzyme	0.191	0.070	0.407	0.088	<0.01	<0.01	0.041	0.088
	PC versus Phytaverse	0.362	0.249	0.191	0.001	0.150	<0.01	0.520	0.441
	PC versus Quantum Blue	0.137	0.058	0.872	0.697	<0.01	0.01	0.001	<0.01
	PC versus Axtra-PHY	0.286	0.080	0.297	0.100	<0.01	0.011	0.131	0.853
	PC versus NC	0.009	0.166	0.618	0.065	0.011	<0.01	<0.01	<0.01

[a]PC, positive control (starter diet with 0.43% available phosphorus, avP, finisher with 0.32% avP); NC1, negative control 1 (starter diet with 0.23% avP, finisher diet with 0.12% avP); NC2, negative control 2 (starter diet with 0.33% avP, finisher diet with 0.22% avP); T4 to T9, NC1 with Phytaverse, Quantum Blue or Axtra-PHY enzymes at 500 and 1000 FTU/kg.
[b]*P* values, E∗L, interaction effect of enzymes type (E) and inclusion level (L).
[c]Contrast, single degree of freedom contrast (*i*) between positive control (PC) versus average of enzyme-supplemented treatments, (*ii*) PC versus phytaverse treatments, (*iii*) PC versus Quantum Blue treatments, (*iv*) PC versus Axtra-PHY treatments, and (*v*) PC versus negative control treatments.

growth performance. According to Walk et al. (2013), 500 FTU of phytase in corn-soy diet with approximately 0.24% phytate P hydrolyses approximately 62% of the phytate, liberating 0.15% phytate P. Although caution not to affect the mechanism through which intestinal Na-dependent phosphate uptake is stimulated, dietary phosphate deprivation is an important physiological regulator of intestinal phosphate absorption (Fang et al. 2012).

Furthermore, a higher Ca:P ratio of the diet may increase the formation of insoluble Ca–phytate complex in the small intestine (Dersjant-Li et al. 2015). The PC, NC1, and NC2 diets in the current study had Ca:avP ratios of 0.50, 0.33, and 0.43 in the starter feed and 0.46, 0.22, and 0.35 in the finisher feed, respectively. The NC diets were thus adequately balanced to allow phytase activity when supplemented with enzymes. All three enzyme products showed improved growth response in broilers, although birds supplemented with Quantum Blue had lower BWG and FI within the 1–7-day age period but this was short-lived as such differences were not observed at d21 and d35. The source of exogenous microbial phytase used may have a significant effect on the response by broilers in terms of growth as well as bone mineralization (Wang et al. 2020). According to Dersjant-Li et al. (2015), various enzyme products have different pH and temperature ranges within which phytase activity is optimized, with phosphoric effects on broiler performance.

The architecture of the bone structure influences bone strength. Consequently, if it is not maintained leg problems lead to poor animal welfare and become the biggest cause of economic losses in a poultry house due to condemned carcasses and (or) reduced meat grades (Almeida Paz and Bruno 2006; Buijs et al. 2012). BBS determines the amount of force the bones can withstand before fractures and breakage occurs, and is a function of its physical, architectural, and matrix characteristics (Proszkowiec-Weglarz and Angel 2013). Tibiae bone is one of the most mineralized bones in the poultry skeleton, and tibiae ash is the primary criterion for the determination of calcium and phosphorous requirements in most commercial poultry species (Skinner and Waldroup 1995; Angel 2007).

Commercially, phytase is supplemented at 500 FTU/kg diet; doses from 1000 FTU/kg diet upwards may have extra-phosphoric benefits due to a 30% additional increase in phytate dephosphorylation (Lei and Stahl 2001; Cowieson et al. 2006; Campasino et al. 2014). In the current study, enzyme type and interactions between enzyme type and levels had no significant effect on BBS at d21 and d35, respectively; however, enzyme level effect of $P = 0.040$ was obtained at d35. Phytaverse birds had higher BBS at 21 and 35 days, respectively, for both inclusion levels, while no differences were observed between PC and enzyme-supplemented birds. Morgan et al. (2016) showed that phytase supplementation at

500 FTU/kg increased BBS, regardless of phytate susceptibility to phytase, compared to a diet with no phytase. In turkey poults, Applegate et al. (2003) noted that 500 FTU/kg *E. coli* phytase supplementation provided 68.2% non-phytate P (nPP) sparing effects, with 0.49% of diet P retained towards the upper extreme of the linear curve of the reference diets at 0.23% nPP, and 0.47%–0.7% nPP, respectively, as compared with only 58.9% P being retained from the unsupplemented control diet.

The tibia has been noted as the primary storage site for P and tibia ash concentration is noted as a reliable indicator of P status in broilers (Wang et al. 2020). Applegate and Lilburn (2002) suggested that the femur may be a more reliable bone to measure breaking strength, rather than the tibia as it is a more precise indicator of the maximum load (weight of the broiler) at any specific age and it is more sensitive to overall skeletal mineralization, and Ca and P turnover. Vieira et al. (2015), however, reported that both the epiphysis of the tibia and femur showed a similar response in bone density (Seedor index) in birds fed P-deficient or enzyme-supplemented diets, although bone strength was generally higher in the tibia than the femur. Cowieson et al. (2006) reported that birds fed a P-deficient diet had significantly lower toe ash percentage compared to birds fed the diets with standard avP concentration, even though the P-deficient treatments had higher coefficients of P, Na, Ca, Cu, Fe, and Mn retention. In the current study, enzyme effect was highly significant at d35 ($P < 0.01$) for tibia ash, with significant differences between Phytaverse and PC. An increase in avP from NC1 to NC2 showed an increase in BBS and tibia ash at d21 and d35 and this can be associated with the 0.07% and 0.08% increase in Ca in the NC2 starter and finisher feeds, respectively. Dersjant-Li et al. (2015) noted that the addition of phytase to the NC diet at the levels of 1000 or 1500 FTU/kg significantly increased tibia ash weight, but with no effect on the PC (diet with adequate avP). It may be that the addition of higher concentrations of phytase allows complete dephosphorylation of phytate to occur more effectively under relatively constrictive conditions within the chicken gut (Cowieson et al. 2006).

As observed by Wilkinson et al. (2014), the addition of phytase to the NC1 diet eliminates the Ca–phytate complex, restoring the Ca to avP balance in the diet. The degree to which phytase can dephosphorylate phytate correlates to the Ca level and the ratio of Ca:avP in the diet may have a profound effect on bone mineralization (Dersjant-Li et al. 2015). As such, the actual Ca levels in the PC, NC1, and NC2 diets would be 0.85%, 0.70%, and 0.77% Ca in the starter, and 0.71%, 0.55%, and 0.63% Ca in the finisher diet, respectively. However, upon analysis of the final feed offered to birds, the total Ca concentrations were 0.93%, 0.87%, and 0.91% in the starter feed and 0.79%, 0.78%, and 0.85% in the finisher feed, for the PC, NC1, and NC2 treatments, respectively. Consequently, the Ca:avP ratio in the complete starter and finisher diets is expected to change. Although the actual avP content of the final feed was not analysed, we can speculate that the avP content would have increased in the NC2 diet such that the Ca:avP ratio in the starter feed could be in the range: PC (1.84–2.14); NC1 (3.11–3.78); and NC2 (2.46–2.76) and in the finisher feed: PC (1.93–2.47); NC1 (5.20–6.50); and NC2 finisher (3.15–3.86), respectively. The formulated Ca:avP ratio and Ca levels in this study were above the recommended Cobb500 (2018) nutrient specifications, but the avP level was less. In formulating broiler diets, the total Ca is typically included at 8.0 and 10.0 g/kg (Wilkinson et al. 2014). Similar studies on the Ross breed (female Ross 308 and male Ross 344 × 508) suggested a Ca:avP ratio of 2.3–2.5:1 for optimal performance and bone development (Plumstead et al. 2008; Han et al. 2016). According to Amerah et al. (2014), an increased Ca:avP ratio is likely to reduce phytate degradation and P digestibility.

Furthermore, microbial sources of phytases influence their catalytic activity and variations exist in the efficacy of the new generations of *E. coli* phytases to liberate inorganic phosphate (Dersjant-Li et al. 2015). As Morgan et al. (2016) suggested, the determination of phytase activity based on a standard procedure involving inorganic P release at a pH of 5.5 may lead to the underestimation of activity of phytase sources with low pH optima and overestimation of those with higher pH optima. Specifically, at pH 4, phytate binds to Ca^{2+} to form stable insoluble complexes, and upon hydrolysis by phytase, it releases P, which disrupts the Ca:avP ratio and increases the formation and excretion of Ca–P salts (Onyango et al. 2005; Dersjant-Li et al. 2015; Pieniazek et al. 2016). However, the use of limestone as a dietary Ca source increases the alkalinity of the diet, and thus digesta pH, inhibiting phytase activity (Pieniazek et al. 2016). The ideal phytase is the one that works over a wide range of pH values and is active in the stomach and upper intestine, in addition to being refractory to endogenous enzymes (Dersjant-Li et al. 2015). The analysis of dietary Ca and avP is necessary for ascertaining the optimal ratio for growth performance and bone development when diets marginal in available P are fed to broiler chickens, with phytase supplementation.

Conclusions

The supplementation of microbial phytases in diets deficient in avP resulted in improved growth performance similar to the PC that had optimal avP. For all three microbial phytases, a dose of 500 FTU/kg diet showed similar responses in broiler performance and 1000 FTU/kg dose only improved BBS and ash concentration, but not growth performance.

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