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Effects of a Thermostable Phytase on the Growth Performance and Bone Mineralization of Broilers

Abstract. ***An experiment was conducted to assess the effects of a novel thermostable phytase in male broiler ("Ross-308") chicks fed available phosphorus (AP) deficient diets on growth performance and bone mineralization. The treatments consisted of 8 experimental diets: 1 positive control diet containing an adequate level of AP, 1 negative control diet deficient in AP, and 6 diets with the same level of AP as that in negative control but supplemented with different levels of phytase (250, 500, 750, 1,000, 1,250, and 1,500 phytase units (FTU/kg diet). The addition of phytase improved significantly (P < 0.05) weight gain, feed intake, FCR, toe ash, tibia ash and tibia P of broilers compared with those in negative control. No significant differences (P > 0.05) were found in FCR and bone mineralization among the broilers fed different levels of phytase and those fed the positive control. The results indicated normal growth performance and bone mineralization could be maintained in broilers fed AP-deficient diets supplemented with the thermostable phytase.***

Key words: *phytase, broiler, growth performance, bone mineralization*

It has been estimated that the total global harvest of crop seeds and fruits, which is considered as an antinutritional factor (*Lott et al., 2000; Peter et al., 2007*). Phytate in plants also couples with various nutrients, such as protein, starch, and minerals, and negatively affects the utilization of these nutrients by monogastric animals (Lantzsch et al. 1998; Ravindran et al., 1999). The partial availability of phytate-P to poultry assumes importance as the global rock phosphate reserves are not renewable, which could lead to a P supply crisis in the future (*Abelson, 1999*). Excessive P excretion is also the most common cause of eutrophication of rivers, lakes and reservoirs (*Correll et al., 1999*). Therefore, protection of environment and preservation of global P reserves would be facilitated by more efficient phytate-P utilization.

Phytate-degrading enzymes, via step-wise dephosphorylation of phytate, have the capacity to liberate phytate-P, thus enhance P absorption and reduce P excretion, which have both nutritionally and ecologically beneficial consequences (*Mullaney*

et al., 2000). Since the first research showed that exogenous phytase enhanced phytate-P utilization and bone mineralization in broiler chicks in 1962 (*Warden, Schaibe, 1962*), the inclusion of phytase in poultry diets has been far more widely accepted. Feeds used for broilers are often pelleted during the production, however, the ability of phytase to withstand this heat treatment is questioned (*Bustany, 1996*). Controversy exits on the extent of the loss in phytase activity and on the feed properties that can diminish these losses. Pelleting losses are affected by the pelleting conditions and properties of the enzyme, including its resistance to denaturation and its capacity to re-nature into an active form during cooling (*Ribeiro et al., 2003*).

Recently, we have developed a new recombinant phytase product that has shown good thermostability (*Fu, 2009*). The phytase properties, especially about withstanding heat, were further optimized. However, its thermostability during pelleting process and effects on bird performance and nutrient metabolism have not been assessed. Therefore, the present study was designed to evaluate the effects of this novel phytase product on the growth performance and bone mineralization of broilers fed pelleting diets deficient in available (AP).

1. Ingredient composition and nutrient contents of the experimental diets

Significative	Treatments	
	Positive control	Negative control
Ingredient, %		
Maize	60.00	60.00
Soybean meal	32.23	32.23
Soybean oil	3.00	3.00
Monocalcium phosphate	1.80	0.80
Limestone	1.18	1.10
Sand	0.00	1.08
Lysine.HCl	0.11	0.11
DL-methionine	0.38	0.38
Salt	0.30	0.30
Premix1	1.00	1.00
Nutrient content, calculated		
ME, kcal/kg	2.99	2.99
CP, %	20.68	20.68
Ca, %	0.96	0.70
Total P, %	0.69	0.50
Available P, %	0.45	0.26
Phytic P, %	0.24	0.24
Ca: total P	1.39	1.39
Lysine, %	1.07	1.07
Methionine, %	0.65	0.65
Nutrient content, analyzed		
CP, %	20.84	20.82
Ca, %	0.98	0.73
Total P, %	0.71	0.48

[1]Premix supplied the following amounts of vitamin and minerals per kilogram of diet for the age of 1 to 21 d: vitamin A, 8,000 IU; vitamin D_3, 2,400 IU; vitamin E, 20 IU; vitamin K_3, 0.5 mg; thiamine, 2 mg; riboflavin, 8 mg; vitamin B_6, 3.5 mg; vitamin B_{12}, 0.01 mg; pantothenic acid, 20 mg; niacin, 35 mg; biotin, 0.26 mg; folic acid, 0.75 mg; Mn, 150 mg; Fe, 90 mg; Zn, 120 mg; Cu, 11 mg; I, 0.35 mg; and Se, 0.20 mg.

Materials and methods. The novel phytase product used in this study was derived from Yersinia frederiksenii and was produced in the Key Laboratory for Feed Biotechnology of the Ministry of Agriculture (Chinese Academy of Agricultural Sciences, Beijing, P. R. China). The phytase gene of Y. frederiksenii was amplified from genomic DNA by PCR and heterologously expressed in Escherichia coli. Based on sequence alignment and molecular modeling of the phytase and related phytases, the phytase has only one divergent residue, Ser51, in close proximity to the catalytic site.Mutant S51T showed higher specific activity, greater activity over pH 2.0–5.5, and increased thermal and acid stability compared with wild-type the phytase (*Fu, 2009*).

Experimental diets were based on maize and soybean meal. The basal diets, which were a little deficient in CP, energy and amino acid, were formulated for broiler starters, according to Ross Nutrition Specification (2007). The NC is also deficient in Ca in addition to the intended deficiency of P (Table 1). The eight dietary treatments were then randomly assigned to six replicate cages. In the positive control (PC) treatment, birds were fed a standard diet which meets the requirements for AP and Ca with the supplementation of monocalcium phosphate and limestone. In the negative control (NC) diet, the levels of AP and total P were reduced by 0.19%, while the level of Ca was reduced by 0.26% to maintain the Ca: total P ratio at 1.39:1. The other 6 dietary treatments were the diets of negative control supplemented with graded levels of phytase (250, 500, 750, 1,000, 1,250, and 1,500 phytase units (FTU) /kg diet) (Pecozyme 5000, Challenge Group, Beijing, China).

The diets were pelleted at 90°C and ring die compression ratio (1:10) for 15 s and the graded levels of phytase products (granular form) were applied respectively during feed mixing.

Male broiler chicks ("Ross-308", one-day-old) were obtained from a commercial hatchery. The chicks were individually weighed and randomly allocated to 48 pens (8 chicks per cage) in an environmentally controlled room with constant lighting. The temperature was maintained at 31°C on the d 1 and then gradually reduced to 22°C by d 21. On d 12, the birds were transferred to grower cages and maintained in these cages until the termination of the trial on d 21. Diets were provided for ad libitum consumption, and the birds had free access to water. All birds used in this study were cared for in accordance with local ethical guidelines.

Phytase activity used in diets was assayed after pelleting by the Test Standard of Feed Grade Phytase of P.R. China (2009). Free inorganic phosphate was assayed in the culture supernatant based on the concentration of phosphate released after hydrolysis of sodium phytate by phytase. Precisely weigh 5.0±0.1 g formula feed in a beaker, and dissolve with 0.25 M acetate buffers (pH 5.5) in a 100 mL volumetric flask. 100 μL of enzyme solution were mixed with 900 μL of 0.25 M acetate buffers (pH 5.5) supplemented with 2.0 mL of 7.5 mM sodium phytate, and incubated at 37°C for 30 min. 2.0 mL of the stop/color mixture were added to stop reaction and generate phosphomolybdate. The concentration of inorganic orthophosphate was

determined colorimetrically by measuring the absorbance of the solution at 415 nm using an ultraviolet spectrophotometer (T6. Pgenral. Beijing, China). The stop/color mixture was prepared fresh by mixing two volumes nitric acid (30%), 1 volume of 100 g/L ammonium molybdate solution supplemented with 1.0% (v/v) ammonium hydroxide (25%) and 1 volume of 2.35 g/L ammonium metavanadate solution supplemented with 2.0% (v/v) nitric acid (30%). The results were compared to a standard curve prepared using K_2HPO_4 as a source of inorganic phosphate at concentrations ranging from 5.0 to 25.0 mM. One unit of phytase is the amount of enzyme that releases 1 µmol of inorganic P/min at pH 5.5 and 37°C.

Body weights and feed intake were recorded on a cage basis at weekly intervals. Mortality was recorded daily. Any bird that died was weighed to adjust the FCR, and FCR were calculated by dividing total feed intake by weight gain of live plus dead birds.

On d 21, 4 birds (closest to the mean weight of pen) were selected from each pen, and killed by cervical dislocation. Toe samples were obtained, by severing the middle toe through the joint between the 2nd and 3rd tarsal bones from the distal end, for toe ash measurements. The left and right middle toes of the birds were pooled, respectively, to yield samples of toes per pen.

In addition, left tibias were stripped of all soft tissues and stored at -20°C for subsequent analysis. The oven-dry weight of each bone was recorded. Following this, the bones were crushed and defatted by refluxing petroleum ether over the crushed samples in a Soxlet apparatus for 20 hours. Defatted samples were oven-dried at 100°C for 24 hours and then burned in a muffle furnace at 600°C for 24 hours. Tibia ash was expressed as percentage of fat-free dry weight. Tibia ash was also analyzed for Ca and P using standard AOAC (2007) procedures (AOAC International, 2007). The Ca content was precipitated by ammonium oxalate and then analyzed by the potassium permanganate method. P was analyzed by the vanadate colorimetric method with an ultraviolet spectrophotometer (T6. Pgenral. Beijing, China). Tibia Ca and P concentrations were expressed as percentage of fat-free dry tibia weight.

The data were subjected to ANOVA using the General Linear Models procedure of SAS (2001). Differences were determined by Duncan's multiple range test. In all analyses, significance was declared at P<0.05.

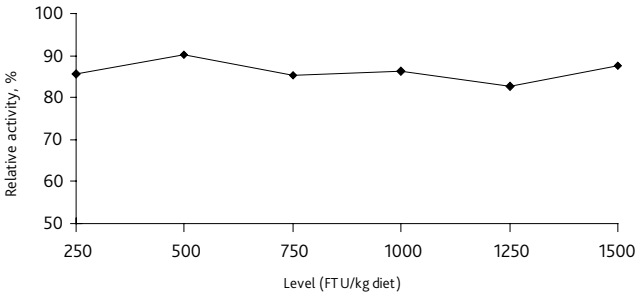


Figure 1. Course for the relative activities of the phytase after pelleting at different graded levels. (Relative activity of 100% refers to the initial activity of pre-pelleting. Each relative activity represents the mean of 3 samples. FTU = phytase units. Pelleted at 90°C and ring die compression ratio (1:10) for 15 s.)

Results and discussion. The relative activities of the phytase after pelleting at graded levels from 250 to 1500 FTU/kg diet were showed in Fig. 1. Residual activities of the phytase at different graded levels changed from 82.7% to 90.3%, and yet all of the activities remained over 80% of its initial activity after pelleting for 15 s at 90°C. The result indicated that the thermostability of this phytase was high and it had the ability to withstand pelleting process.

The growth performance of birds in different treatments was summarized in Table 2. The weight gain and FCR of broilers fed the PC treatment (containing 0.45% AP) were better (P<0.05) than those fed the NC (containing 0.26% AP). The PC and NC did not differ in intake. The weight gain, feed intake and FCR of broilers were improved (P<0.05) by supplementing different levels (250 to 1,500 FTU/kg diet) of phytase in the NC diet. Broilers fed diets containing 750, 1,000, 1,250 and 1,500 FTU/kg phytase grew faster (P<0.05) than those fed the PC diet. Feed intake of broilers fed diets containing phytase, except the treatment of 250 FTU/kg diet, were higher (P<0.05) than that of chicks fed the PC diet. However, there were no significant differences (P>0.05) in FCR among the broilers fed diets containing phytase and those fed the PC diet. There were no significant differences (P>0.05) in the weight gain, feed intake and FCR of broilers fed diets containing different levels of phytase, but the FCR of broilers in the treatment of 1000 FTU/kg diet were significantly better (P<0.05) than those of broilers in the treatment of 250 FTU/kg diet. The best weight gain, feed intake and FCR were obtained at 1,000 FTU/kg diet. The body weight of all broilers at 21 days was lower than commercial standards of Ross Performance Objectives due to the low nutrient levels in the diets (2007). Mortality during the trial was low (2.2%), and was not related to any dietary treatments.

P is an essential nutrient for all animals, and crucial for skeletal integrity and growth performance. It is important to maintain adequate P during the starter period of the bird, as a continuous submarginal level of P from the onset of feeding inhibits growth and results in carcass defects (*Moran, Todd, 1994*). Earliest symptoms of P deficiency will induce decreased appetite, lowered blood P, reduced rate of gain. If severe deficiency occurs, there will be skeletal problems (*Kessler, 1999*). This study showed that AP-deficient diets result in performance reduction, which is in general agreement with previous reports (*Rama et al., 1999; Venalainen et al., 2006*). Deficiency of P is the most widespread of all the mineral deficiencies affecting poultry.

In our experiment, birds fed AP-deficient diets supplemented with the thermostable phytase had same even better production performance than those fed diet with adequate P. The mechanism is that supplemental phytase can degrade phytate and phosphorus phytate in diet, and release inorganic phosphoric acid, inositol, mineral elements, protein, amino acid, starch and lipids, so as to improve the utilization rate of above substance. According to the present study, it is possible to gain a normal growth performance by reducing AP content from 0.45% to 0.26% with phytase supplementation. Similar improvements have been reported in numerous previous experiments. Santos et al. (2008) demonstrated that the reduction in P levels of diets resulted in lower nutrient utilization and feed consumption, and consequently reduced performance of start-

2. Influence of dietary treatments on the weight gain, feed intake and FCR of broilers[1]

Treatments	Weight gain, g/bird	Feed intake, g/bird	FCR, g feed/ g gain	Mortality, %
PC[2]	745.5[b]	964.4[bc]	1.322[bc]	2.08
NC[3]	687.3[c]	926.9[c]	1.372[a]	4.17
NC + 250 FTU/kg diet[4]	772.0[ab]	1,002.1[ab]	1.332[b]	2.08
NC + 500 FTU/kg diet	797.2[ab]	1,049.5[a]	1.325[bc]	0
NC + 750 FTU/kg diet	802.7[a]	1,035.3[a]	1.309[bc]	4.17
NC + 1000 FTU/kg diet	825.4[a]	1,065.7[a]	1.292[c]	2.08
NC + 1250 FTU/kg diet	819.4[a]	1,063.5[a]	1.311b[c]	2.08
NC + 1500 FTU/kg diet	815.7[a]	1,053.8[a]	1.315b[c]	0
Pooled SEM[5]	18.3	21.1	0.014	0.56

[a,b,c] Means in a column not sharing a common superscript are significantly different (P < 0.05).
[1] Each value represents the mean of 6 replicates.
[2] PC = positive control.
[3] NC = negative control.
[4] FTU = phytase units.
[5] Pooled standard error of mean.

3. Influence of dietary treatments on toe ash, tibia ash, tibia Ca and P contents of broiler from 0-21 d old[1]

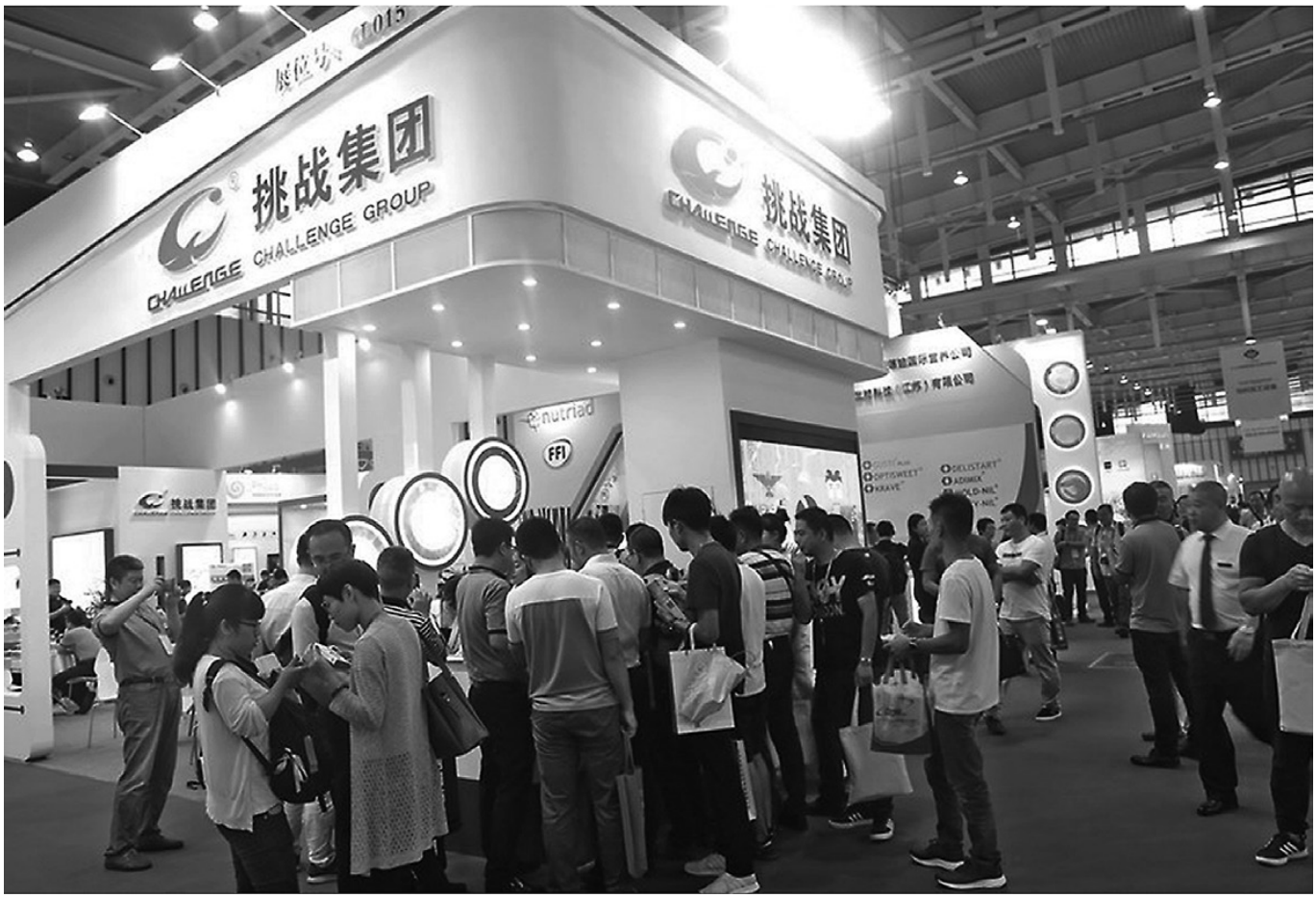
Treatments	Weight gain, g/bird	Feed intake, g/bird	FCR, g feed/ g gain	Mortality, %
PC[2]	12.2[a]	37.9[a]	37.1	14.6[a]
NC[3]	9.8[d]	28.8[c]	35.2	12.8[b]
NC + 250 FTU/kg diet[4]	10.7[c]	32.4[b]	35.3	14.1[a]
NC + 500 FTU/kg diet	12.1[ab]	36.8[a]	36.2	14.3[a]
NC + 750 FTU/kg diet	11.9[b]	36.6[a]	35.6	14.5[a]
NC + 1000 FTU/kg diet	12.2[ab]	36.5[a]	36.3	14.5[a]
NC + 1250 FTU/kg diet	12.1[ab]	36.8[a]	36.7	14.4[a]
NC + 1500 FTU/kg diet	12. 2[ab]	36.5[a]	36.7	14.4[a]
Pooled SEM[5]	0.097	0.51	1.11	0.196
P Value	0.012	0.004	0.195	0.018

[a,b,c] Means in a column not sharing a common superscript are significantly different (P < 0.05).
[1] Each value represents the mean of 6 replicates.
[2] PC = positive control.
[3] NC = negative control.
[4] FTU = phytase units.
[5] Pooled standard error of mean.

er broilers. Supplementing phytase in the NC diets increased feed intake and nutrient utilization, thus allowing better performance.

The results of the bone mineralization of birds in different treatments are showed in Table 3. Broilers fed the NC diet had lower (P < 0.05) bone mineralization (measured as toe ash, tibia ash and tibia P) than those fed the PC diet. Birds fed diets supplemented with 250 FTU/kg phytase had higher (P < 0.05) bone mineralization compared with birds fed the NC diet. But there were significant differences (P < 0.05) in the toe ash and tibia ash values between the broilers fed diets supplemented with 250 FTU/kg phytase and those fed the PC diet, indicating higher bone mineralization of the birds in the treatment of PC. No significant differences (P > 0.05) were found in tibia Ca values. Toe ash, tibia ash and tibia P values in birds fed 500 to 1,500 FTU/kg phytase were similar (P > 0.05) to those of chicks fed the PC diet, except that the toe ash in birds fed diets containing 750 FTU/kg phytase were lower (P < 0.05) than that of fed the PC diet. There were no significant differences (P > 0.05) in bone mineralization of broilers fed different levels of phytase (500 to 1,500 FTU/kg).

Although live performance is an important measure of any dietary change, bone mineralization was generally more representative index when P or Phytase were added. The content of bone ash Ca and P have been regarded as the indicators of bone mineralization. In this study, the dietary phytase contents had a significant effect on toe and tibia mineral contents, which were consistent with previous reports indicating the improvement in bone strength with the addition of phytase to AP-deficient diets (*Leeson et al., 2000*). The improvement was related to the increase in apparent metabolism of minerals from the phytate-mineral complex by phytase supplementa-



tion. Ribeiro et al. (2003) reported that the addition of 280 FTU/kg phytase in phosphorus deficient diet improved bone ash and breaking strength.

It appears that nutrient specification levels, phytate concentrations and phytase inclusion rates in broiler diets are critical, interactive variables. It is likely that high nutrient specification levels may accommodate the anti-nutritive properties of dietary phytate concentrations and negate responses to phytase supplementation. Consequently, one approach is to decrease nutrient specifications appropriately and counter potential reductions in growth performance with phytase supplementation, which has been shown to be economically viable (*Peter et al., 2007; Selle et al., 2003*). The benefits of the phytase were showed when the basal diets were formulated not to meet sufficiently requirements of Ross Nutrition Specification.

Conclusions

1. Broiler chickens fed AP-deficient (0.19% reduction) diet supplemented with the novel thermostable phytase had similar growth performance and tibia P to those of broilers fed the normal-AP diet (0.45%).

2. Increasing the phytase level to 1,000 FTU/kg offered further improvement ($P < 0.05$) only in FCR, as compared with phytase supplementation at a level of 250 FTU/kg. There were no significant advantages in terms of performance and bone mineralization by increasing the phytase addition beyond 1,000 FTU/kg.

3. Further research is needed to determine the effect of dietary phytase supplementation on plasma mineral content. ■

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Вплив термостабільної фітази на ефективність росту і мінералізацію кісток у бройлерів

Анотація. ***Був проведений експеримент щодо впливу нової термостабільної фітами на самців курчат-бройлерів ("Ross-308"), яких годували раціоном з нестачею доступного фосфору (ДФ) для росту і мінералізації кісток. Годівля складалась із 8 експериментальних раціонів: 1-й – позитивний контрольний раціон, що містив адекватний рівень ДФ, 2-й – негативний контрольний раціон з дефіцитом ДФ, і 6 раціонів з тим же рівнем ДФ, що і при негативному контролі, але з додаванням фітами з різною активністю (250, 500, 750, 1000, 1250 і 1500 фітазних одиниць (FTU/кг корму). Додавання фітази значно поліпшило***

(P<0,05) швидкість росту, споживання корму, конверсію, мінералізацію кісток пальців, мінералізацію великої гомілкової кістки і вміст фосфору у великій гомілковій кістці бройлерів порівняно з тими, які отримували раціон з дефіцитом ДФ (негативний контрольний раціон). Ніяких істотних відмінностей (P>0,05) не було виявлено у конверсії корму і мінералізації кісток серед бройлерів, які отримували різні рівні фітази і тими, яких годували раціоном з необхідним рівнем ДФ (позитивний контрольний раціон). Результати досліджень свідчать про задовільні показники росту і мінералізації кісток у бройлерів, яких годували раціонами з дефіцитом ДФ, доповненими термостабільною фітазою.

Ключові слова: фітаза, бройлер, показники росту, мінералізація кісток

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Влияние термостабильной фитазы на эффективность роста и минерализацию костей у бройлеров

Аннотация. ***Был проведен эксперимент по оценке влияния новой термостабильной фитазы на самцов цыплят-бройлеров ("Ross-308"), которых кормили рационом с нехваткой доступного фосфора (ДФ) для роста и минерализации костей. Кормление состояло из 8 экспериментальных рационов: 1-й – положительный контрольный рацион, содержащий адекватный уровень ДФ, 2-й – отрицательный контрольный рацион с дефицитом ДФ, и 6 рационов с тем же уровнем ДФ, что и при отрицательном контроле, но с добавлением фитазы с различной активностью (250, 500, 750, 1000, 1250 и 1500 фитазных единиц (FTU/кг корма). Добавление фитазы значительно улучшило (P<0,05) скорость роста, потребление корма, конверсию, минерализацию костей пальцев и большеберцовой кости и содержание фосфора в большеберцовой кости бройлеров по сравнению с теми, которые получали рацион с дефицитом ДФ (отрицательный контрольный рацион). Никаких существенных различий (P>0,05) не было обнаружено в конверсии корма и минерализации костей среди бройлеров, получавших различные уровни фитазы, и теми, которых кормили рационом, содержащим необходимый уровень ДФ (положительный контрольный рацион). Результаты исследований свидетельствуют о нормальных показателях ростах и минерализации костей у бройлеров, которых кормили рационами с дефицитом ДФ, дополненными термостабильной фитазой.***

Ключевые слова: фитаза, бройлер, показатели роста, минерализация костей

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