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Original study

Effect of different phytases on the performance, nutrient retention and tibia composition in broiler chickens

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Abstract

The effect of different phytases on the performance response, nutrient utilization and tibia characteristics of chickens was investigated. The five pelleted diets were the following: positive control (PC) with added monocalcium phosphate; negative control (NC) formulated with equivalency values of phytase for Ca and digestible P; and three further diets where different phytases were individually added to the NC diet at 500 FTU/kg. The phytases were derived either from *Aspergillus* (phytase I), or *E. coli* (phytases II and III). Compared to PC, the performance parameters, as well as apparent metabolizable energy (AMEn), mineral retention, bone breaking force and tibia mineral content were suppressed by the reduction of dietary Ca and digestible P. All phytases enhanced the overall body weight gains and feed conversion ratio in comparison with NC, but none outperformed PC. Only phytase II improved AMEn compared to NC and PC group. However only phytase I outperformed NC group in terms of mineral retention and P retention was higher than phytase II and III. No significant differences were observed in fat digestibility and N retention. Bone strength among phytases did not differ and all improved this parameter compared to the NC diet. However, even though all phytases enhanced tibia minerals content, the improvement was less pronounced with phytase III. Moreover, the differences in all analysed tibia minerals between phytase III and II were significant suggesting that even among 6-phytases derived from and expressed in the same organism, different efficacy or mode of action can occur.

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Abbreviations: AMEn: apparent metabolizable energy, BWG: body weight gain, FCR: feed conversion ratio, NC: negative control, PC: positive control

Introduction

Exogenous phytase preparations are probably one of the most commonly used feed additives in the feed industry and many phytase products are commercially available. Moreover, each year novel generations of the enzyme come onto the market but there is a wide variation in the recommended dosages of enzyme necessary for similar P release from feeds (Jones *et al.* 2010). Thus, potential users can be confused about the efficacy as well as about a proper application of a particular enzyme. Furthermore, it is well documented that benefits of phytase action are not restricted only to Ca and P release, but also include a better absorption of trace minerals. Pintar *et al.* (2005) demonstrated that phytase improved Fe and Mg levels in broiler tibias but had no effect on Ca, P and Zn contents. In contrast, Shelton & Southern (2006) demonstrated that the concentration of Zn in tibia was increased while Fe and Mn levels were not affected by a dietary phytase. In other studies, Yi *et al.* (1996) and Świątkiewicz *et al.* (2001) observed that phytase improved Zn utilization in broilers. Viveros *et al.* (2002) also observed that phytase supplementation increased ($P<0.0001$) Ca, P, Mg and Zn retention in broilers at three and six weeks of age. These inconsistent effects on the deposition of trace minerals in bones were probably linked to a different diet composition and diverse concentrations of phytates in feeds. However, the type of the phytase could have also played an important role. The usage of the phytase is also connected to better energy utilization of birds. In broiler chickens, metabolizable energy level results from a sum of many well available nutrients as well as from phytate presence which can impair digestibility of fat, protein or starch. Thus, the overall phytase effect on the apparent metabolizable energy (AMEn) gain, based on several studies, is estimated to be approximately 2.8 %. However, there are very few information available on how different supplementary phytases can affect AMEn levels in the same diet.

The objectives of this experiment were, therefore, to determine the effects of three different phytases on growth performance, AMEn values, nutrients digestibility, bone-breaking strength and tibia mineral content in broilers fed to market weight under commercial conditions.

Material and methods

Birds and housing

The experiment was carried out in floor pens (1×1 m) arranged by blocks in the centre of a commercial chicken house. In order to simulate commercial production conditions, the experimental pens were surrounded by a commercial broiler flock comprising birds of the same origin and the same age as those used in the experiments. Five hundred 1-d old Ross 308 broiler male chickens, obtained from commercial hatchery and with an average initial

weight of 41 g, were selected and allotted randomly to the five experimental groups, using 10 replicate pens per treatment and 10 birds per pen. The birds were given 23 h of light and 1 h of dark during the first week and then 19 h of light and 5 h of dark from d 7 to 21. From 22 to 42 d of age, there was 23 h of light and 1 h of dark. The experiment complied with the guidelines of the Local Ethics Commission with respect to animal experimentation and care of animals under study.

Diets and feeding program

The compositions and nutritive values of the diets are shown in Table 1. The experimental diets were prepared from the same raw materials, which were stored before feed processing. All diets, with exception of finisher were produced with one batch. The finisher feeds were produced with two batches, with and without inert marker (TiO2). From day 21-35 and 40-42 birds were fed diets with TiO2 and from 35-40 d without. The negative control diets were formulated to meet or exceed NRC (1994) requirements for all nutrients except P and Ca. The positive control diet was formulated to meet or exceed the NRC (1994) requirement for all nutrients. The dietary treatments consisted of the control diet, negative control and the P and Ca deficient diets supplemented with different phytase products at 500 FTU/kg. All diets were supplemented with the ionophore coccidiostat salinomycin (60 mg/kg) from d 0 to 42 (Sacox, Huvepharma NV, Belgium). Diets were pelleted at 78 °C and were fed *ad libitum* throughout the starter (1-6 d), grower (7-20 d), and finisher (21-42 d) phases of the experiment. Both, positive and negative control diets were prepared without addition of any phytase. The three further diets were the NC diets supplemented with 500 FTU/kg of either *Aspergillus ficuum* 3-phytase, EC 3.2.1.8 expressed in *Aspergillus niger* CBS 114.94 – Natuphos 5000 (phytase I), or *Escherichia coli* 6-phytase, EC 3.2.1.26, overproduced in *Pichia pastoris* DSM 15 927 – Quantum 2500 (phytase II), or *Escherichia coli* 6-phytase, expressed in *Pichia pastoris DSM 23036 – Hostazym P 5000* (phytase III).

Measurements

The chickens' body weight and feed intake were recorded at seven, 21 and 42 d of age. The body weight gain (BWG) and feed conversion ratio (FCR) were calculated for first (1-6 d), second (7-20), third (21-42 d) and the entire feeding period (1-42 d of age). Mortality and weights of dead birds were recorded twice daily.

For the evaluation of the AMEn nitrogen retention and total tract fat digestibility, 100 g of the excreta from each pen were collected at 40 d (n=10). To avoid excreta contamination, 1 h before collection in each pen plastic cover was placed directly on the litter. The excreta samples were immediately frozen, freeze-dried and ground before further analyses. Five days (35-40 d) before sample collection birds were fed diets in which 0.3 % of the wheat was replaced by titanium oxide as an internal marker for the calculation of nutrient digestibility.

At the termination of the experiment (42 d) from each experimental group, 10 randomly picked chickens (one bird/pen) were killed by cervical dislocation. The right tibias from 10 birds per each treatment (one bird/pen) were removed and frozen (–20 °C) until analysis. Measurement of the bones' breaking force was taken by means as described by Świątkiewicz *et al.* (2011).

Table 1
Composition and nutrient content of the experimental diets

Item	1-6 d		7-20 d		21-42 d	
	Positive control	Negative control	Positive control	Negative control	Positive control	Negative control
Ingredients, %						
Wheat	59.63	63.49	63.92	67.76	65.80	68.99
Rapeseed expeller	5.00	5.00	10.00	10.00	10.00	10.00
Soybean meal	27.43	26.21	17.57	16.37	15.68	15.11
Soybean oil	4.20	2.37	5.39	3.56	5.69	3.91
Vitamin-mineral premix1	0.30	0.30	0.30	0.30	0.30	0.30
Monocalcium phosphate	1.35	0.74	0.95	0.34	0.71	0.08
Limestone	1.13	0.93	0.91	0.70	0.89	0.69
NaCl	0.33	0.33	0.33	0.30	0.28	0.28
Na2CO3	0.05	0.05	0.03	0.05	0.05	0.05
L-Lizyna HCl	0.28	0.29	0.33	0.34	0.33	0.32
Methionine hydroxy analogue	0.28	0.28	0.25	0.24	0.24	0.24
L-threonine	0.04	0.04	0.05	0.06	0.06	0.05
Nutrient content, calculated						
Crude protein, %	22.2	22.2	20.00	20.00	19.5	19.5
Dig. Lys, %	1.16	1.16	1.04	1.04	1.00	1.00
Dig. Met, %	0.53	0.53	0.48	0.48	0.47	0.47
Phosphorus-total, %	0.72	0.59	0.64	0.50	0.57	0.44
P-digestible, g/kg	4.20	3.04	3.40	2.24	2.90	1.74
P-digestible – phytase equivalency values, g/kg	0	1.16	0	1.16	0	1.16
Calcium-total, %	0.85	0.67	0.70	0.52	0.65	0.47
Calcium-total – phytase equivalency values, %	0	0.18	0	0.18	0	0.18
AMEn, kcal/kg	2 980	2 980	3 110	3 110	3 150	3 150
Analysed phytase activity, FTU/kg[2,3]						
no phytase addition	76	LQ	LQ	LQ	LQ	106
phytase I (3-phytase)	-	863	-	800	-	970
phytase II (6-phytase)	-	443	-	529	-	620
phytase III (6-phytase)	-	638	-	582	-	794

[1]The premix provided per kg of diet: vitamin A, 11 000 IU; vitamin D_3, 2 500 IU; vitamin E, 50 mg; vitamin K3, 2.50 mg; vitamin B1, 2.0 mg; vitamin B2, 7.0 mg; vitamin B5, 12.5 mg; vitamin B6, 4.0 mg; vitamin B12, 0.02 mg; niacin, 40 mg; folic acid, 1.0 mg; biotin 0.2 mg; choline chloride 300 mg; Mn, 70 mg; Zn, 55 mg; Fe, 45 mg; Cu, 20 mg; I, 0.60 mg; and Se, 0.35 mg; Co, 0.25 mg. [2]One FTU the amount of phytase that catalyses the release of one micromole of inorganic phosphorus per minute from 5.1 millimoles of sodium phytate in pH 5.5 buffer at 37 °C. [3]Addition of each phytase enzyme to the basal negative control diet was made at an activity level of 500 FTU/kg feed. LQ: limit of quantification

Chemical analyses

Feed samples were analysed in duplicate for crude protein, crude fat, crude fibre total P and phytase activity using AOAC (2005) methods 976.05, 920.39, 2002.04, 965.17 and 2000.12, respectively. The enzyme activities were measured in all diets and all periods. For finisher (21-42 d) feeds the phytase activities in table 1 are mean values of two batches (with and without TiO2) of this diet. For all chemical analyses, samples were ground to pass through a 0.5 mm sieve. The concentration of titanium dioxide was determined according to the method described by Short *et al.* (1996) and the samples were prepared according to the

procedure reported by Myers *et al.* (2004). Gross energy, nitrogen and fat content were determined as described in details by Józefiak *et al.* (2010). Calcium, P, Na, K, Mg, Fe and Zn concentrations were measured in the right tibia and Ca and P in excreta. The tibias were cleaned from adherent tissue and ashed (550 °C for 14 h). Ash weight was calculated relative to tibial dry weight. The resultant ash was solubilized on a sand heater (300VC 15 min) in 10 ml 6 N HCl and 30 ml demineralized water. The solution was transferred after filtration (ashless filters) into a 100 ml volumetric flask. The Ca, P, Na, K, Mg, Fe and Zn concentrations were measured by Atomic Absorption Spectrophotometry (VARIAN Techtron AA 475, Pty. Ltd. Springvale, Australia) as described by Revy *et al.* (2004).

Digestibility calculations

The apparent digestibility coefficients and AMEn contents of the experimental diets were analysed as described in detail by Józefiak *et al.* (2010).

Statistical analysis

Statistical analysis was conducted using the GLM procedure of SAS software (SAS 1990, SAS Institute Inc., Cary, NC, USA). All the data were analysed using one-way ANOVA. Means were separated using a Duncan's multiple range test. All statements of significance are based on $P<0.05$.

Results

The results of the experiment are shown in tables 2-4. In the first period of the trial (1-6 d) the reduction of P and Ca levels as well as the supplementation of different kinds of phytase products had no effect on the performance (Table 2). However, during the grower phase (7-20 d) the NC diets with a low P and Ca concentration negatively affected BWG and FCR compared to PC diet ($P<0.05$). Moreover, only phytase II significantly enhanced body weight gains from 7 to 20 d compared to the NC group. No differences among groups in terms of feed intake were noted in grower phase. Also in this period all tested phytase products improved FCR in comparison with the NC treatment, however, only the group supplemented with phytase III produced FCR values that did not differ from the positive control. In the last stage of broiler growth (21-42 d), the lowest BWG was found in the NC treatment, and it did not differ only from the group supplemented with phytase I. Furthermore, the reduction of P and Ca levels resulted in a significantly poorer FCR than that recorded in the rest of experimental groups. However, none of supplemental phytase products improved FCR compared to the PC group. Additionally, the highest feed intake was noted in birds fed phytase II followed by those that received phytase III and then those fed the NC diet. This was also evident when the parameter was calculated for the entire experimental period (1-42 d).

No significant differences among experimental treatments were observed in fat digestibility and in N retention. However, feed supplementation with phytase II increased AMEn. Moreover, the phytase addition produced the highest AMEn value among all experimental groups, including the PC. The reduction of P and Ca levels had a negative effect on the calcium retention in broilers and only phytase I supplementation removed the effect.

Also, compared to the NC treatment, the phosphorus retention was increased only in broilers fed the diet supplemented with phytase I.

The lowest bone breaking force was reported in the NC group ($P<0.05$). Furthermore, the NC group had also *significantly lower* levels of *mineral content* in *tibia* bones than found in other treatments. The only exception was the level of Fe, for which the differences between the NC treatment and the treatment with supplemental phytase III were not statistically significant. In terms of K, Mg and Fe levels in tibiae, the addition of phytase III did not show any improvement compared to the PC diet. Moreover, only broilers fed the diets with added phytase I had tibia Na and Zn contents that were significantly higher than in birds fed the PC diet.

Table 2
The effect of different sources of phytase supplementation on the growth performance of broilers

Treatment[1]	Positive Control	Negative Control	NC + phytase I	NC + phytase II	NC + phytase III	Pooled SEM	Model P
Body weight gain[2], g							
1-6 d	145	139	145	145	140	0.940	0.178
7-20 d	821^{ab}	775^{c}	790^{bc}	833^{a}	808^{abc}	5.261	0.006
21-42 d	2134^{ab}	2049^{c}	2095^{cb}	2183^{a}	2146^{ab}	10.953	0.004
1-42 d	3101^{ab}	2963^{c}	3029^{bc}	3161^{a}	3094^{ab}	13.967	0.0002
Feed intake[2], g							
1-6 d	145	145	148	149	145	0.634	0.246
7-20 d	1 124	1 142	1 125	1 168	1 128	5 671	0.125
21-42 d	3847^{c}	3946^{abc}	3882^{bc}	4035^{a}	3977^{ab}	17.573	0.013
1-42 d	5116^{b}	5233^{ab}	5155^{b}	5351^{a}	5249^{ab}	20.868	0.009
Feed conversion ratio[2], g/g							
1-6 d	1.00	1.04	1.02	1.03	1.03	0.004	0.055
7-20 d	1.37^{d}	1.47^{a}	1.43^{b}	1.40^{bc}	1.40^{cd}	0.006	<.0001
21-42 d	1.80^{c}	1.93^{a}	1.86^{b}	1.85^{b}	1.85^{b}	0.007	<.0001
1-42 d	1.65^{c}	1.77^{a}	1.70^{b}	1.69^{b}	1.70^{b}	0.006	<.0001

^{a,b}Means in the rows with different letters are significantly different at $P<0.05$., SEM: standard error of the mean, Model P: model probability, [1]Positive control - diet optimized with monocalcium phosphate; negative control - diet formulated with phytase matrix which reduced Ca level by 0.18 % and digestible P by 0.12 %; phytase I – NC diet supplemented with of *Aspergillus ficuum* 3-phytase produced by *Aspergillus niger* CBS 114.94 (500 FTU/kg feed); phytase II – NC diet supplemented with of *Escherichia coli* 6-phytase produced by *Pichia pastoris* DSM 15 927 (500 FTU/kg feed); phytase III – NC diet supplemented with of *Escherichia coli* 6-phytase produced by *Pichia pastoris DSM 23 036* (500 FTU/kg feed). [2]Values are means, n=10.

Table 3
The digestibility results at 40d based on titanium oxide retention in broiler chickens fed diets with different sources of the exogenous phytase

Treatment[1]	Positive Control	Negative Control	NC + phytase I	NC + phytase II	NC + phytase III	Pooled SEM	Model P
AMEn[2], kcal/kg	2988^{bc}	2958^{c}	3021^{bc}	3138^{a}	3031^{bc}	19.758	0.046
Fat digestibilty[2], %	84.86	84.36	86.59	86.25	87.09	0.461	0.376
N retention[2], %	57.78	54.52	56.81	58.38	57.65	0.505	0.122
Ca retention[2], %	74.36^{a}	68.27^{b}	74.31^{a}	70.97^{ab}	68.95^{b}	0.749	0.020
P retention[2], %	65.59^{ab}	60.73^{b}	68.45^{a}	60.34^{b}	62.21^{b}	0.920	0.020

^{a,b}Means in the rows with different letters are significantly different at $P<0.05$. SEM: standard error of the mean, Model P: Model probability, [1]Positive control - diet optimized with monocalcium phosphate; negative control - diet formulated with phytase matrix which reduced Ca level by 0.18 % and digestible P by 0.12 %; phytase I – NC diet supplemented with of *Aspergillus ficuum* 3-phytase produced by *Aspergillus niger* CBS 114.94 (500 FTU/kg feed); phytase II – NC diet supplemented with of *Escherichia coli* 6-phytase produced by *Pichia pastoris* DSM 15 927 (500 FTU/kg feed); phytase III – NC diet supplemented with of *Escherichia coli* 6-phytase produced by *Pichia pastoris* DSM 23 036 (500 FTU/kg feed), [2]Values are means, n=10.

Table 4
Tibia minerals and *tibial* bone breaking force in broiler chickens fed diets with different sources of the exogenous phytase (41 d)

Treatment[1]	Positive Control	Negative Control	NC + phytase I	NC + phytase II	NC + phytase III	Pooled SEM	Model P
Bone breaking force[2], N	392[a]	329[b]	360[a]	372[a]	378[a]	4.833	0.001
Ca[2], %	37.25[ab]	30.76[c]	37.57[a]	38.17[a]	35.94[b]	0.387	<.0001
P[2], %	20.22[ab]	16.45[c]	20.42[ab]	20.46[a]	19.60[b]	0.215	<.0001
Na[2], %	1.259[bc]	1.093[d]	1.325[a]	1.275[ab]	1.205[c]	0.012	<.0001
K[2], %	0.764[a]	0.586[c]	0.735[a]	0.753[a]	0.664[b]	0.011	<.0001
Mg[2], %	0.863[a]	0.657[c]	0.883[a]	0.863[a]	0.769[b]	0.012	<.0001
Fe[2], mg	375[a]	286[b]	394[a]	417[a]	261[b]	13.004	0.0002
Zn[2], mg	484[bc]	287[d]	535[a]	508[ab]	450[c]	12.110	<.0001

[a,b]means in the rows with different letters are significantly different at $P<0.05$, SEM: standard error of the mean, Model P: Model probability, 1Positive control - diet optimized with monocalcium phosphate; negative control - diet formulated with phytase matrix which reduced Ca level by 0.18 % and digestible P by 0.12 %; phytase I – NC diet supplemented with of *Aspergillus ficuum* 3-phytase produced by *Aspergillus niger* CBS 114.94 (500 FTU/kg feed); phytase II – NC diet supplemented with of *Escherichia coli* 6-phytase produced by *Pichia pastoris* DSM 15 927 (500 FTU/kg feed); phytase III – NC diet supplemented with of *Escherichia coli* 6-phytase produced by *Pichia pastoris* DSM 23 036 (500 FTU/kg feed), [2]Values are means, n=10.

Discussion

The commercial preparations of phytases used in poultry nutrition differ in origin, mode of action and form. Phytases are phosphatases capable of hydrolysing one or more phosphate groups. Depending on the position of the phosphate group on the myo-inositol ring which they hydrolyse first, they belongs to one of two sub-classes: 3-phytase and 6-phytase. The 3-phytase initiates phytate hydrolysis by removing phosphate residue from position three of the myo-inositol ring, and 6-phytase from position six or four of the phytic acid molecule (Żyła *et al.* 2004). Phytases differ also in terms of *pH*-activity *profile,* thermo stability and in resistance to digestive enzymatic degradation in the animal intestine (Lei & Stahl 2001, Onyango *et al.* 2004, Bedford & Cowieson 2009). As a result, different commercial phytase products may generate different efficacies under practical conditions.

There is a great deal of evidence showing that the addition of exogenous phytases to the diets of poultry improves weight gains, mineral retention, AMEn, and amino acid digestibility (Ravindran *et al.* 1999, Newkirk & Classen 2001, Murai *et al.* 2002, Rutherfurd *et al.* 2002, Augspurger *et al.* 2003, Cowieson & Adeola 2005, Józefiak *et al.* 2010, Żyła *et al.* 2013). The results obtained in the present study also support the findings that phytase supplementation beneficially affects production parameters of broilers. The growth response of birds fed a phytase supplemented diet did not differ from that of the positive control treatment in any of the experimental periods. This would indicate that the adequate amounts of P were released from the phytate molecule to support the growth. However, it seems that the effect of addition of 6-phytase products, and particularly the phytase II on body weight gains, was more pronounced than that obtained by 3-phytase (phytase I) supplementation. This effect was not observed in relation to the FCR. Contrary to the expectation, the effect of reducing the Ca and dP concentration in the diet and supplementation of phytases on feed intake was small. The negative control diet was formulated to contain around 0.18 to 0.12 % less Ca and dP than the positive control diet, and it was expected that this would result in a decrease

in both BWG and feed intake. However, very low dietary Ca concentrations could influence the digestibility of phytate-P (and possibly nonphytate-P). Tamim *et al.* (2004) found out that phytate-P digestibility, in the absence of phytase, could be increased to almost 80 % if dietary Ca concentration was reduced to around 0.2 %. In the present study, dietary Ca concentrations were reduced by 0.18 %, and possibly changed the negative effect of the removal of the inorganic P.

Phytase supplementation also improved retention of minerals and AMEn level but did not affect fat digestibility and nitrogen retention. The lowest AMEn was measured in birds fed the NC diet while the highest was found in those that received feeds supplemented with phytase II or with phytase III ($P<0.05$). In a dose response trial, in which phytase was used at graded levels up to 12,000 FTU/kg^{-1}, a gradual increase in the AMEn and N retention was demonstrated (Shirley & Edwards 2003). In contrast to these findings, the addition of the enzyme at seven levels of activity in the range from 0 to 1 000 FTU kg^{-1} to broiler diets containing 7.5 and 3.0 g total P kg^{-1} slightly enhanced the AMEn values which reached a plateau at 750 FTU kg^{-1} (Ravindran *et al.* 2001). In the present trial supplementation of the broiler diets with phytase II significantly increased the AMEn level compared to PC and NC treatments. However, this was not reflected in the feed utilization, nitrogen retention and fat digestibility. Data from other studies indicate that the percentage responses in AMEn following phytase supplementation are negatively correlated ($r=-0.562$; $P<0.02$) to the energy density of the control diets (Selle & Ravindran 2007). In the present study, the diets with added phytase II (6-phytase) returned higher values of AMEn ($P<0.05$) than the diets supplemented with phytase I (3-phytase). These results are in partial agreement with the findings of Bedford & Cowieson (2009), who stated that for equivalent P release the 6-phytase would need to deplete a greater proportion of the phytate pool than 3-phytase. Therefore, compared to 3-phytases, the energy and amino acid matrices of a 6-phytase are usually larger per one unit of enzyme activity. However, results from the current study also demonstrate that even though both of the 6-phytase (II and III) are *Escherichia coli*-derived, both are overproduced in the same expression system of *Pichia pastoris* and both produced similar performances of the experimental birds, they give different responses in the AMEn values. This effect might have depended on the *Pichia pastoris* strain employed in both expression systems of the methylotrophic yeast which was different in phytase II than in phytase III. Furthermore, another discrepancy that evolved, when these three phytase products were compared, concerned mineral retention. In the current experiment, only birds fed phytase I supplemented diet outperformed ($P<0.05$) those on the deficient diet (NC) in both Ca and P retention, although similar Ca retention was characteristic for birds fed phytase II. Experimental data from research in which the sub-classes of phytase (3-phytase vs. 6-phytase) have been compared are contradictory. Augspurger *et al.* (2003) and Augspurger & Baker (2004) reported that at lower levels of phytase activity (i.e., 500 and 1,000 FTU/kg) *E. coli*-derived 6-phytase maintained a greater than a threefold advantage in P-releasing efficacy thanr fungal 3 and 6-phytases. In contrast, Tamim *et al.* (2004) demonstrated a higher ileal disappearance of phytate P and apparent absorption of P when 3-phytase was used compared with a 6-phytase. Also Żyła *et al.* (2004), in the *in vitro* model, provided evidence that 3-phytase released significantly more P than 6-phytase. A possible explanation for these varied results could be the different phytase origin (fungal or bacterial) and dissimilar diet ingredients used in the studies. Nevertheless, in the current study P retention did not differ

between control treatments. The lack of a negative impact, and even a superior P retention in birds fed the deficient diet (NC) compared with the positive control die,t was also reported by Viveros *et al.* (2002) and Onyango *et al.* (2004). These authors attributed the effect to a greater ability of birds to retain P from diets with lower rather than higher nonphytate P content.

The skeletal integrity in poultry is affected by numerous factors, including nutritional regime, genetic factors, sex, age, management conditions and production system (Kleczek *et al.* 2012, Yildiz *et al.* 2009). In the present study, all of the phytases studied improved the tibial bone breaking force to the level found in the positive control group ($P<0.05$). Thus the minerals retention was not reflected in this parameter. Generally, this is in agreement with many other studies (Augspurger *et al.* 2003, Sands *et al.* 2003, Onyango *et al.* 2004, Payne *et al.* 2005) indicating therefore that irrespective of phytase sub-class or dosage, bone characteristics are positively affected by a dietary inclusion of the enzyme. However, some data do not show that phytase supplementation had a significant effect on bone mineralization in broilers (Perney *et al.* 1993, Żyła *et al.* 2000). In many studies, phytase supplementation affected macro and trace minerals content in tibia (Viveros *et al.* 2002, Pintar *et al.* 2005). Ravindran *et al.* (1995) demonstrated that bone mineralization criteria are more sensitive indicators of P status in birds than growth criteria. In our study, irrespective of the analysed element, the lowest concentrations were found in tibiae of broilers fed the NC treatment. In case of the Ca, P and Na, all of phytases improved their depositions to levels found in the positive control. For K, Mg, Fe and Zn contents in the tibiae, the lowest values were characteristic for birds fed the diet supplemented with phytase III compared to those that consumed phytase I or II. Pintar *et al.* (2005) observed a positive effect of phytase on Fe and Mg content in tibia, but, in contrast to the present work, no effect on Zn concentration. In yet another study (Shelton & Southern 2006), the removal of a trace mineral premix from a diet or phytase supplementation had no effect on Fe concentration in tibia. In the present study, similarly to many others (Mohanna & Nys 1999, Zanini & Sazzad 1999, Świątkiewicz *et al.* 2001, Viveros *et al.* 2002, Shelton & Southern 2006) phytase has been shown to increase the availability of Zn. In the previous study of Shelton & Southern (2006) bone strength was decreased in chicks fed diets with no supplemental Zn, while the removal of Mn and Cu from a diet did not affect bone strength. It could be suggested that the higher concentrations of Zn and other trace minerals in tibia, observed in the current trial in chickens fed diets supplemented with phytase I and II, was reflected in the strength of bones. However, this trend was less pronounced with phytase III.

In the present study the activity in phytase products were not measured, what may be one of the contributing factor of big differences between formulated and determined dietary phytase activities. However, large variation in assay results, and poor reproducibility often occurs when AOAC method is used to determine phytase activity especially when different phytase products are taken into consideration (Isaksen & Dalsgaard 2007, Gizzi *et al.* 2008, Kim & Lei 2005).

Phytase sub-class, the source as well as the microbial expression system may alter the biochemical and biophysical properties of the enzyme, which in turn affect the *in vivo* bio efficacy of phytases. Results from the present study also suggest that the strain of an organism used for overexpression of the enzyme may play a role in the efficacy of phytase what is in agreement with Onyango *et al.* (2004). All these factors, in addition to the catalytic activity alter the deposition of trace minerals in tibiae of broiler chickens.

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