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The Efficacy of a Novel Microbial 6-Phytase Expressed in *Aspergillus oryzae* on the Performance and Phosphorus Utilization in Broiler Chickens

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Abstract: The efficacy and safety of a novel microbial 6-phytase expressed via the use of synthetic genes in *Aspergillus oryzae* was investigated from d 8 to 22 of age using 480 Ross PM3 broiler chickens. Five treatments were tested. A diet containing 5.6 g/kg of Phosphorus (P) was fed to the control treatment. Another diet containing 4.1 g/kg P was fed to another treatment as negative control. This diet was fed in 3 other treatments with the addition of phytase (500, 1000, or 2000 U/kg). Feed intake, body weight, tibia ash (%) and strength (N) and P and Ca utilization (% of intake) and excretion (g/kg DM) were measured. Enzyme safety was determined by genotoxicity and sub-chronic oral toxicity studies. Lower feed intake and higher weight gain was obtained with the treatment containing 2000 U/kg phytase compared to the two control treatments and the treatment containing 500 U/kg phytase, leading to a significant improvement in FCR with the 2000 U/kg phytase. Tibia strength and ash were improved with the latter and were dose-dependent described by an exponential function. Safety test using a concentrated preparation of the novel 6-phytase enzyme did not reveal any toxicological significant findings. The enzyme did not induce mutagenic activity in the Ames test and did not increase the frequency of micronucleated binucleated cells in the micronucleus assay. In conclusion, this novel microbial 6-phytase improved broiler performance and reduces the need for phosphate fortification of feed. In addition, it can be Generally Recognized as Safe (GRAS) feed ingredient according to the safety test carried out.

Key words: Broiler, phytic acid, microbial phytase, microbial 6-phytase, efficacy, phytate, safety

INTRODUCTION

Plants are the main source of dietary Phosphorus (P). However, approximately two-thirds of the total P in plants is in the form of phytate (Erdman, 1979; Reddy *et al*., 1982). Phytates are salts of myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate present in feedstuffs of plant origin where they serve as the principal storage form of P (Reddy *et al*., 1982). They play an essential role in the germination of seeds and the growth of the plant. Phytate is an ester of cyclic alcohol inositol with six phosphates. Therefore, it is only logical that the hydrolysis of phytate can be a good source of P to the animal. Phytase is a naturally occurring enzyme that can degrade phytate to yield inositol and P (Liu *et al*., 1998). However, monogastric animals do not synthesize phytase in sufficient quantities for an efficient utilization of this rich source of P (Nelson, 1967). Thus, much of the phytate P present in the diet is passed out in the excreta. This is a source of environmental pollution, especially when environmental microbes (capable of degrading phytate to release P) act on the excreta. Furthermore, phytic acid has a strong chelating potential in the gut and can make up complexes with minerals, starch and protein (Kies *et al*., 2001), thereby reducing their bioavailability. Singh and Krikorian (1982) also suggested that phytate may inhibit proteolysis by altering the protein configuration. Phytate is also known to inhibit a number of digestive enzymes such as pepsin, alpha-amylase (Deshpande and Cheryan, 1984) and trypsin (Caldwell, 1992).

The inability of monogastric animals to efficiently utilize the phytate P necessitates the addition of inorganic phosphates (e.g. dicalcium phosphate) to the diets in order to meet their P requirement. The supplementation of monogastric diets with exogenous microbial phytase to enhance phytate P utilization is today a widely accepted practice (Waldroup, 1999). Supplementation with microbial phytase allowed complete, safe and economic replacement of dicalcium phosphate and reduced feed cost per unit of weight gain (Singh and Khatta, 2003). Microbial phytases have an optimum pH of 2.5-7.5 and preferred temperatures of 35-36°C (Wodzinski and Ullah, 1996; Simon and Igbasan, 2002) and are more effective within the gastrointestinal environment compared to plant phytase. On the basis of the site of initial breakdown of the phytate P, microbial

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phytases are divided into two classes. The 3-phytases such as those from *Aspergillus niger* initiate phytate degradation at the 3rd carbon position and 6-phytases such as those from *Peniophora lycii* initiate phytate degradation from the 6th carbon position.

It was demonstrated that microbial phytase supplementation to low-phosphorus diets increases body weight gain, feed intake and feed efficiency in broiler chickens (Simons *et al*., 1990; Broz *et al*., 1994; Denbow *et al*., 1995; Sebastian *et al*., 1996; Singh and Khatta, 2003). In an extensive review, Singh (2008) summarized results on the influence of phytase on growth performance of broiler chickens. Nelson *et al*. (1971) were the first to supplement 0.4% crude phytase in corn and soybean meal-based broiler diets containing 0.24% natural phytate phosphorus and recorded a 33.3% improvement in body weight gain. Simons *et al*. (1990) reported that addition of microbial phytase to low phosphorus broiler diets significantly improved the body weight gain, feed intake and feed efficiency and that this improvement was dependent on the level of phytase added. Broz *et al*. (1994) reported that graded levels of supplemental phytase (125, 250 and 500 phytase units/kg diet) improved the growth performances of broilers by 4.6, 6.4 and 8.5%, respectively. One unit (U) of phytase is defined as the activity that release 1 μmol inorganic phosphate from 5.0 mM phytate per minute at pH 5.5 and 37°C. The improvement observed in growth was connected with a proportional increase in food intake and according to these authors an increased bioavailability of phosphorus by phytase. Denbow *et al*. (1995) tested different levels of supplemental phytase (0, 200, 400, 600, 800, 1000 and 1200 phytase units/kg diet) in semi purified diets of broilers containing 0.20, 0.27 or 0.34% non-Phytate Phosphorus (nPP). They observed improved body weight gain and feed intake ($p<0.01$) at all NPP levels, but the magnitude of response was maximum at low NPP level. Feed efficiency was, however, unaffected by phytase addition. Rama Rao *et al*. (1999) reported significant ($p<0.05$) improvements in body weight gain, feed intake and feed efficiency in broiler chicks with phytase supplementation (500 phytase unit/kg diet) in a corn-soybean based low NPP diet. The improvements in feed intake and feed efficiency were attributed to phytate hydrolysis leading to improved overall utilization of the diet. Johnston and Southern (2000) used different levels of phytase supplementation (0, 200, 400, 600, 800, 1000 and 1200 phytase units/kg diet) in low phosphorus broiler diet and reported improvements in body weight gain and that increased levels of phytase supplementation had little effect on growth performance ($p<0.01$).

It was generally accepted that diets supplemented with phytase increases the retention of phosphorus (Simons *et al*., 1990; Broz *et al*., 1994), Ca (Johnston and Southern, 2000) and nitrogen (Sebastian *et al*., 1996). Phytase supplementation was further associated with increased tibia ash content and better bone strength compared to the control diets. Improvements in growth performance reported in chickens fed diets supplemented with microbial phytase may be due to an increase in feed intake and feed efficiency, potentially caused by the release and utilization of phosphorus from the phytate-mineral complex (Sebastian *et al*., 1996), utilization of inositol (Simons *et al*., 1990) increased starch digestibility (Knuckles and Betschart, 1987), increased utilization of protein and amino acids (Ravindran *et al*., 2000) or overall utilization of nutrients (Miles and Nelson, 1974). However, as a result of simultaneous increases in body weight gain and feed intake, several reports have indicated that microbial phytase supplementation had no always significant effect on feed to body weight gain ratio in broiler chickens (Simons *et al*., 1990; Broz *et al*., 1994; Denbow *et al*., 1995; Sebastian *et al*., 1996).

Research efforts in recent years have focused on the isolation and development of new, heat-stable microbial phytases from various microbial sources (Selle and Ravindran, 2007; Broz and Ward, 2007; Sands *et al*., 2008). The objective of the present work was therefore, to test the efficacy of a novel microbial 6-phytase preparation expressed via the use of synthetic genes in *Aspergillus oryzae* known commercially as Ronozyme HiPhos. Toxicological and tolerance studies were carried out prior to the efficacy study to evaluate the safety of the novel enzyme in living animals in line with the current European Union Regulation (EFSA, 2003) on the additives used for animal nutrition.

MATERIALS AND METHODS

Safety testing: The phytase preparation used in all the described studies was a microbial 6-phytase expressed via the use of synthetic genes in *Aspergillus oryzae* known commercially as Ronozyme HiPhos. Prior to the commencement of the efficacy study described further below, tolerance and toxicological studies were carried out in an attempt to evaluate the safety of the novel enzyme to meet the EU directives (EFSA, 2003) on additives for use in animal nutrition.

Toxicological studies: These included investigations for genotoxicity and subchronic oral toxicity studies. For the genotoxicity studies, the microbial reverse mutation assay (Ames test) and the *in-vitro* micronucleus assay (induction of micronuclei in cultured human peripheral blood lymphocytes) were carried out. The objective of the Ames test was to evaluate the mutagenic potential of the product by examining its effect on amino acids "growth dependant" bacteria (reversion effect). The study was conducted in accordance with the Organization for Economic Co-operation and Development guidelines (OECD, 1997) and in compliance with current Good

Laboratory Practice (GLP) regulations. Mutagenic activity was examined in four histidine-dependent strains of *Salmonella typhimurium*, (TA98, TA100, TA1535 and TA1537), and the tryptophan-dependent strain *Escherichia coli* WP2uvrApKM101. In order to avoid a "feeding effect" due to the presence of free amino acids in the phytase preparation, bacteria were treated in liquid culture - "treat and plate" assay. The study was conducted in the presence and absence of an activating system (S9 mix) derived from rat liver. The test included solvent (purified water) and positive controls with and without S9 mix. All microbial strains were tested at concentration of the test article ranging from 156-5000 µg/mL (concentrations based on dry matter).

The objective of the *in vitro* micronucleus assay was to evaluate the clastogenic and aneugenic potential of the novel phytase by examining its effects on the frequency of micronuclei in cultured human peripheral blood lymphocytes treated in the absence and presence of S9 mixes (obtained from rats induced with Aroclor). The test methodology was based on the new OECD draft (OECD, 1997) guidelines and accepted regulatory principles for clastogenecity testing *in vitro*. The highest dose level tested was 5000 µg/mL (concentration based on test material weighed out as received). The highest dose level was the recommended maximum for *in-vitro* chromosome aberration studies according to the regulatory guidelines. The product was added at 48 hours following culture initiation and cultures were treated for 3 h in the presence and absence of S9 mix. In addition, a continuous 24 h treatment (equivalent to approximately 1.5 to 2 times the average generation time of cultured lymphocytes from the panel of donors used in the laboratory) in the absence of S9 mix was included. All cultures were sampled 24 h after the beginning of treatment (i.e 72 h after culture initiation). Appropriate negative (vehicle) control cultures were included in the test system under each treatment condition. The proportion of Micronucleated Binucleate Cells (MNBN) in these cultures fell within current vehicle control (normal) ranges. Mitomycin C (MMC) and Vinblastine (VIN) were employed as clastogenic and aneugenic positive control chemicals, respectively, in the absence of rat liver S-9. Cyclophosphamide (CPA) was employed as a clastogenic positive control chemical in the presence of rat liver S-9. Cells receiving these were sampled in the main experiment at 24 h after the start of treatment; all compounds induced statistically significant increases in the proportion of cells with micronuclei. The assay system was therefore considered as both sensitive and valid.

As mentioned above, a subchronic oral toxicity test in rats was carried out in addition to the genotoxicity tests. The objective was to assess the systemic toxic potential of the 6-phytase preparation when administered daily by oral gavage over a period of 13 weeks. A total of 80 rats (40 males and 40 females) were used in the study. The animals were allocated into 4 groups, each containing 10 males and 10 females, receiving the 6-phytase preparation at doses of 1.0, 3.3 or 10.0 mL/kg body weight per day. A control group received the vehicle (purified water) at the same dose volume. At the end of the study, all the animals were killed and a detailed necropsy was carried out. Parameters measured include the clinical condition, detailed physical and arena observations, sensory reactivity, grip strength, motor activity, body weight, food consumption, ophthalmic examination, haematology, blood chemistry, organ weight, macro pathology and histopathology investigations.

Tolerance study: To further evaluate the microbial 6-phytase, a tolerance study was carried out using broiler chickens. The objective of this study was to evaluate the tolerance of broiler chickens to the microbial 6-phytase at a maximum recommended dose (4 000 U/kg diet) and a ten times overdose (40 000 U/kg diet) when added to standard starter and grower diets for a period of 5 weeks.

For this study, a total of 192 day-old Ross PM3 broiler chickens obtained from a local hatchery were kept in a floor-pen facility and divided into 48 replicate groups of 4 birds each. The birds were fed starter and grower diets based on maize and soybean meal as the main feed ingredients, formulated to meet the National Research Council (NRC, 1994) nutrient recommendations except for total and non-Phytate Phosphorus. The composition of both basal diets, as well as the calculated and the analyzed nutrient contents are summarized in Table 1. The tested microbial 6-phytase was analyzed to have a phytase activity of 60 700 U/g product.

The experiment consisted of 3 dietary treatments as follows: A- negative control fed non-supplemented basal diets; B- treatment receiving 6-phytase at 4 000 U/kg diet; C- treatment receiving 6-phytase at 40 000 U/kg diet (overdose). Each dietary treatment was assigned to 16 replicates (8 replicates contained male birds and the other 8 contained female birds). Each replicate contained 4 birds.

Performance parameters monitored during the experimental period included live weight, live weight gain, feed intake, feed conversion ratio and mortality. At the termination of the study (day 35), a 5 mL blood sample was taken from 1 bird per pen and haematological and biochemical examinations were conducted using blood samples from 16 birds per treatment.

Haematological examinations conducted involved the total number of Red Blood Cells (RBC), blood Haemoglobin concentration (HGB), Heamatocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin

Table 1: Composition and nutritive value of the experimental diets

	Efficacy study[1]		Tolerance study[2]	
Ingredient (g/kg)	Low-P diet	Medium-P diet	Starter	Grower
Maize	590.6	590.6	580.8	624.5
Soybean meal	368.0	368.0	349.7	320.0
Soybean oil	15.0	15.0	40.0	31.0
DL-Methionine	2.0	2.0	0.2	0.1
Dicalcium phosphate	3.0	11.0	-	-
Calcium carbonate	6.7	1.8	14.6	11.6
Sand	3.1	-	-	-
Sodium chloride	1.0	1.0	3.1	3.2
Premix	10.0	10.0	5.0	5.0
Avatec	0.6	0.6	-	-
L-Lysine	-	-	0.2	0.3
Monocalcium phosphate	-	-	6.4	4.3
Calculated composition (g/kg)				
ME_N (kcal/kg)	3010.0	3010.0	3047.0	3047.0
Crude protein	216.0	216.0	216.0	206.0
Calcium	6.0	6.0	7.5	6.0
Total P	4.1	5.6	5.6	5.0
Analyzed composition (g/kg)				
ME_N (kcal/kg)	3010.0	3010.0	-	-
Crude protein	222.0	222.0	201.0	198.0
Calcium	5.6	5.7	-	-
Total P	3.8	4.9	-	-
Phytate P	3.0	-	-	-
Non Phytate-P [3]	0.8	-	-	-

[1]Vitamin-mineral premix provided per kilogram of diet: Vitamin A: 10'000 I.U.; vitamin E: 40 I.U.; vitamin K3: 3.0 mg; vitamin C: 100 mg; vitamin B1: 2.50 mg; vitamin B2: 8.00 mg; vitamin B6: 5.00 mg; vitamin B12: 0.03 mg; niacin: 50.0 mg; pantothenate calcium: 12.0 mg; folic acid: 1.50 mg; biotin 0.15 mg; choline: 450 mg; ethoxyquine: 54 mg; Na: 1.17 g; Mg: 0.8 g; Mn: 80 mg; Fe: 60 mg; Cu: 30 mg; Zn: 54 mg; I: 1.24 mg; Co: 0.6 mg; Se: 0.3 mg.

[2]Vitamin-mineral premix provided per kilogram of diet: Vitamin A: 13'500 IU; vitamin D3: 5000 IU; vitamin E: 75 IU; vitamin K3: 4.0 mg; vitamin B1: 4.0 mg; vitamin B2: 8.0 mg; vitamin B6: 4.5 mg; vitamin B12: 0.02 mg; niacin: 60 mg; pantothenate calcium: 17.0 mg; folic acid: 2.0 mg; biotin: 0.2 mg; choline: 300 mg; antioxidants: 14 mg; Mn: 120 mg; Fe: 60 mg; Cu 17.5 mg; Zn: 90 mg; I: 1.0 mg; Co: 0.3 mg; Se: 0.3 mg; amino acids ~ 1.8 g Methionine.

[3]Calculated as the difference between total and phytate P per kg feed

Concentration (MCHC), number of blood Platelets (PTL) and total and differential number of White Blood Cells (WBC). In blood serum the following biochemical parameters were determined: chloride, Ca, P, total protein, albumin, glucose, Amylase (AMS), uric acid, creatinine, Alkaline Phosphatase (ALP), Alanine Transaminase (ALT), Aspartate Aminotransferase (AST), Creatine Phosphokinase (CPK) and total bilirubin. Routine laboratory methods were used for all the above mentioned parameters. In addition, the gross pathological examination was conducted in all birds after slaughter, which focused mainly on gastro-intestinal tract, lymph nodes, liver, kidney, lung, heart and spleen.

Enzyme safety analysis: Following the methodology of Pariza and Cook (2009), the safety of the enzyme was evaluated with respect to the potential for the introduction of potentially harmful metabolic by-products resulting from the transformation of the *Aspergillus oryzae* production strain by the introduction of two synthetic genes that mimic the coding for the phytase of the 'donor' organism. The safety history of the donor strain, the production strain and the enzyme modification techniques were compared to known safe strains and transformation practices for the risk of introducing a toxic compound.

Efficacy study

Experimental design and diets: The study was performed according to the official French guidelines for experiments with live animals. 480 male PM3 broiler chickens were housed in wire-floored battery cages in an environmentally controlled room. The experiment was carried out from day 8 to 22 of age. A total of 5 treatments, each containing 12 replicates with 8 birds per replicate, were tested. Birds were allocated to treatments based on BW, such that there was no difference in initial BW averaged distribution between replicates and treatments.

Two different diets were used and their detailed composition is shown on Table 1. The diet (medium-P) was a conventional broiler diet formulated to contain 5.6 g of total P. This diet was fed to a treatment as positive control (C). Another diet containing lower total P (4.1 g/kg) was fed to another treatment as negative control.

The other three treatments received this diet with the addition of either 500, 1000, or 2000 U per kg feed of the experimental microbial 6-phytase. The experimental phytase was used in a liquid form and contained 26000 U/g. One unit (U) of phytase is defined as the activity that release 1 µmol inorganic phosphate from 5.0 mM phytate per minute at pH 5.5 and 37°C. The added amount of the phytase product was based on the analyzed phytase activity. The determination of the phytase activity was performed by BIOPRACT GmbH, D-12489 Berlin (Germany). Appropriate amounts of the liquid phytase formulation were diluted with water and sprayed onto the pelleted feed to get the final concentrations in the feed corresponding to the different treatments. For procedural balance of all treatments, water was also sprayed onto the pellets of the negative and the positive control diets.

Measurements include the Body Weight (BW) and feed consumption on days 8, 15 and 22. Feed Conversion Ratio (FCR) was calculated respectively. Excreta samples were collected from day 14 to day 17 by the total collection method. Excreta from 4 randomly selected replicates per treatment were quantitatively collected once per day. The total excreta collected during the four collection days was pooled per replicate and were frozen (-20°C) immediately after collection. After thawing, the excreta was homogenized and representative samples were taken for the determination of dry matter, ash and concentration of P and Ca. The determination of P and Ca was done by Induction Coupled Plasma after mineralization with H_2SO_4/ Na_2SO_4.

On day 22, the chickens were euthanized by cervical dislocation and the right tibiae were taken from 4 chickens randomly chosen from each of the 12 replicates per treatment. Tibiae were defleshed and cartilaginous caps were removed before being frozen (-20°C) until further analysis of ash content and breaking strength. A segment of the central portion of the bone shaft (about 2 cm long) was prepared for determining bone strength using an LR10K compression machine with a XLC/10K/A1 force captor and a compression device TH23-196/AL (Lloyd Instruments, Fareham, UK). The force expressed in Newton (N) necessary to break the bone was determined. Broken bones were pooled per cage, defatted with ethanol and ether, dried and incinerated at 550°C and the ash content was determined.

Statistical analysis: A one-way Analysis of Variance (ANOVA) was used to evaluate the data concerning performance, mineral utilization and bone parameters using the software 'Stat Box Pro' version 5.0 (Grimmersoft, 1995). Results were considered different if $p<0.05$ and Newman-Keuls test was used to compare differences between treatment means. Nonlinear functions that best fitted the experimental data were derived for phytase levels with the nonlinear model:

$$y = a + b (1-\exp (-kx))$$

Where:

- x = Supplemented phytase (U/kg)
- y = Response (P, Ca utilization or tibia strength, tibia ash)
- a = Response (y-value) at zero phytase supplementation
- b = Maximum response to supplemented phytase (a+b = upper asymptote)
- k = parameter describing the steepness of the curve

RESULTS AND DISCUSSION

Safety testing

Genotoxicity studies: Results obtained from the Ames test showed that treatment of the *Salmonella typhimurium* and *E. coli* with the novel 6-phytase, either in the presence or absence of S9 mix, did not reveal any mutagenic activity. The results of the micronucleus assay showed that treatment of the cells with the novel 6-phytase preparation did not induce micronuclei in cultured human peripheral blood lymphocytes following treatment in the absence and presence of a rat liver metabolic activation system.

Subchronic toxicity study: No deaths were observed during the study and there were no treatment related findings observed during the routine weekly examinations, the arena observations, or during post dosing observations. Slightly higher BW were observed during the first two weeks of treatment for males receiving 10 mL/kg per day, while in females this increase in BW was only observed in the first week of treatment. Food consumption was not affected by treatment. No other effects were noted during the study on any of the studied parameters. Thus it was concluded that oral administration of the 6-phytase preparation to rats at doses up to 10.0 mL/kg per day for 13 weeks was well tolerated and did not cause any toxicologically significant changes.

Tolerance study in chickens: Results of the tolerance study are summarized in Table 2b. In general, dietary administration of this novel 6-phytase resulted in beneficial effects on chicken performance. This was because the final body weight increased with increased phytase concentration. Final body weight of the birds fed diets supplemented with 4 000 and 40 000 U/kg phytase was statistically significantly higher (1917 g and 1925 g, respectively) compared to the treatment without phytase supplementation (1720 g). Due to this markedly increased growth rate, the overall FCR (g feed/g gain) was improved with the treatments supplemented with 4 000 and 40 000 U/kg phytase (1.838 and 1.796, respectively) compared to the non phytase supplemented treatment (1.913), although this was not

statistically different. The results of this 5-week growth trial confirmed again that phytase addition to practical broiler diets deficient in available P markedly improves the performance of birds and this was earlier reported by many authors (Simons *et al*., 1990; Broz *et al*., 1994; Denbow *et al*., 1995). No clinical signs of any health problems were noted and there was no mortality during this study. Furthermore, no pathological changes were observed in birds during the post-mortem necropsy. The results of haematological and biochemical examinations did not reveal any significant changes due to dietary administration of this novel 6-phytase. However, significant increase in serum concentration of inorganic P was found in both phytase treated groups and this finding confirmed the efficacy of this phytase. This observation showed that the concentration of inorganic P in blood serum represents a valuable response parameter indicating the efficacy of supplemental phytase. Similar significant effects of microbial phytases on blood plasma level of inorganic P were described by Broz *et al*. (1994), Sebastian *et al*. (1996), Rama Rao *et al*. (1999) and Singh and Khatta (2003).

Table 2a: Analyzed phytase activity in samples of the experimental diets used in tolerance and efficacy studies

	Phytase (U/kg)[1]	
Treatment	Inclusion level	Measured activity
Tolerance study		
Starter period		
A	0	< LOQ*
B	4000	4133
C	40000	45400
Grower period		
A	0	< LOQ
B	4000	4750
C	40000	36460
Efficacy study		
Low-P diet	-	< LOQ
Low-P diet + phytase	500	500
Low-P diet + phytase	1000	983
Low-P diet + phytase	2000	2170
Medium-P diet	-	< LOQ

[1]One unit (U) of phytase is defined as the activity that release 1 μmol inorganic phosphate from 5.0 mM phytate per minute at pH 5.5 and 37°C. [2]LOQ: Limit of Quantification

Enzyme safety: The execution of the decision tree of Pariza and Cook did not reveal any risk of introducing a toxic compound. The decision tree and supporting documentation were sent to Dr. Pariza for confirmation and he concurred with the results of the analysis (Pariza, 2010).

Efficacy study in chickens: Proximate analyses in the negative control diet were close to the calculated values. P content was lower than expected (Table 1) and the difference between P content in the medium and the low P diet was 1.1 g total P per kg diet. As expected, the native phytase activity in the basal diet was under the Limit of Quantification (LOQ). The phytase activities measured in the experimental diets were in agreement with the targeted activities as shown in Table 2a.

Table 2b: Effects of the bacterial 6-phytase supplementation on broiler performance and selected haematological and blood biochemical parameters

Parameter	A (Negative control)	B (4 000 U/kg phytase)	C (40 000 U/kg phytase)
Performance parameters			
Final live weight (g), day 35	1.720[a]	1.917[b]	1.925[b]
Feed/gain ratio, day 0-35	1.913	1.838	1.796
Haematological parameters			
Red blood cells (x10^6/μL)	2.48	2.54	2.60
Haemoglobin (g/100 mL)	9.20	8.96	9.43
Haematocrit (%)	29.90	30.60	31.30
Mean corpuscular volume (fl)	120.50	120.30	120.60
Mean corpuscular haemoglobin (pg/L)	37.20[a]	35.30[b]	36.30[b]
White blood cells (x10^3/μL)	27.50	29.10	24.90
Blood biochemical parameters			
Calcium (mmol/L)	2.24	2.39	2.28
Phosphorus inorganic (mmol/L)	1.50[a]	1.95[b]	1.93[b]
Total protein (g/L)	33.20	32.00	31.90
Albumin (g/L)	14.30	13.40	13.20
Glucose (mmol/L)	12.90	12.50	13.00
Amylase (μkat/L)	22.10	21.50	21.50
Alkaline phosphatase (μkat/L)	31.40	30.80	31.20
Alanine transaminase (μkat/L)	0.02	0.01	0.02
Aspartate aminotransferase (μkat/L)	0.77[a]	0.36[b]	0.30[b]
Creatine phosphokinase (μkat/L)	86.00	77.10	87.20
Uric acid (mmol/L)	266.00[a]	208.60[b]	172.10[b]
Creatinine (μmol/L)	33.70	35.10	34.40

[a,b]Means within the same row without a common letter are significantly different ($p<0.05$)

Table 3: Effects of the bacterial 6- phytase supplementation on broiler performance from day 8-22[1]

Treatment	Added phytase (U/kg)	Feed intake (g/bird)	Weight gain (g/bird)	FCR (g feed/g gain)
Low-P diet	-	600[d]	303[d]	1.988[a]
Low-P diet + phytase	500	870[c]	596[c]	1.461[b]
Low-P diet + phytase	1000	941[ab]	651[ab]	1.446[b]
Low-P diet + phytase	2000	973[a]	684[a]	1.426[b]
Medium-P diet	-	909[bc]	623[bc]	1.463[b]

[1]Twelve pens (eight birds per pen) per treatment mean, except Low-P diet, which had eleven pens.
[a,b,c,d]Means within the same column with common superscripts do not differ significantly ($p<0.05$)

Table 4: Effects of the bacterial 6-phytase supplementation on apparent utilization of P and Ca, on the excretion of P (measured in excreta) and on tibia parameters of broiler chicks

		Utilization[1] (% of intake)		Excretion[1] (g/kg DM)	Tibia[2]	
	Added phytase (U/kg)	P	Ca	P	Strength (N)	Ash (%)
Low-P diet	-	44.5[e]	32.8[e]	9.5[b]	73.9[d]	37.2[d]
Low-P diet + phytase	500	64.5[c]	56.0[c]	5.9[c]	155.5[c]	46.6[c]
Low-P diet + phytase	1000	70.7[b]	61.3[b]	4.8[d]	197.0[ab]	50.0[a]
Low-P diet + phytase	2000	77.9[a]	66.7[a]	3.7[e]	221.0[a]	50.8[a]
Medium-P diet	-	47.8[d]	50.3[d]	10.9[a]	171.5[bc]	48.3[b]

[1]Six pens (eight birds per pen) per treatment mean. [2]four pens (eight birds per pen) per treatment mean.
[a,b,c,d,e]Means within the same column with common superscripts do not differ significantly ($p<0.05$)

Results of the growth performance from day 8 to day 22 are presented in Table 3. The addition of DCP to the low P basal diet led to a significant improvement of the body weight gain and the FCR in the positive control and this clearly indicated that the negative control diet was P-deficient. Increased phytase supplementation from 500 to 2000 U/kg resulted in a significant improvement of the WG and the FCR compared to the negative control diet. The WG was improved by 97% and the FCR was improved by 26.5%. These beneficial effects are in fact very strong and higher than comparable effects reported in the literature as reviewed by Singh (2008). However, it is important to note that they were obtained under experimental conditions, in a short-term study involving birds fed a P deficient diet. Nevertheless, these effects on performance parameters indicate a strong potency for the novel 6-phytase.

Results of the apparent utilization of P and Ca are presented in Table 4. The apparent utilization of P and Ca was significantly improved with increasing dietary levels of the phytase. The effects were dose-dependent with significant differences between the dosages. Compared to the negative control diet, an improvement in a range of 45-78% (Fig. 1) was obtained for the apparent utilization of P. The results of this particular study clearly demonstrated that P utilization (retention) is a key response parameter when testing the efficacy of any microbial phytase as shown by many authors (Simons *et al*., 1990; Broz *et al*., 1994; Denbow *et al*., 1995; Sebastian *et al*., 1996; Rama Rao *et al*., 1999; Johnston and Southern, 2000). The effect of the 6-phytase supplementation on P utilization for all supplementary levels was further confirmed by a significant reduction in P excretion over the negative control diet. When compared to the positive control feed containing additional DCP, the phytase addition at 2000 U/kg diet reduced P excretion by 64%. This finding indicates a huge potential in terms of a reduction of

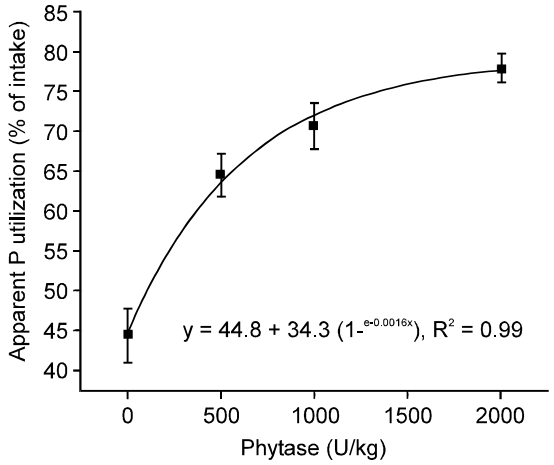

Fig. 1: Effect of phytase supplementation on the apparent P utilization (%) of broiler birds fed different levels of phytase

environmental pollution with non-utilized P in poultry nutrition, as discussed previously by Waldroup (1999), Selle and Ravindran (2007) and Singh (2008). The observed P utilization of the positive control group was lower than all the other groups. Results concerning the apparent utilization of Ca indicated that in addition to P release there is additional availability of calcium caused by the phytase. With increasing levels of phytase, an improvement ranging between 33% and 67% was obtained.

Supplementing phytase, irrespective of the dose, significantly improved tibia strength compared to the negative control diet (Table 4). Tibia strength values increased in a pattern corresponding to supplementation levels. The effects of phytase supplementation on tibia ash percentage, a parameter that indicates the extent of bone mineralization, were

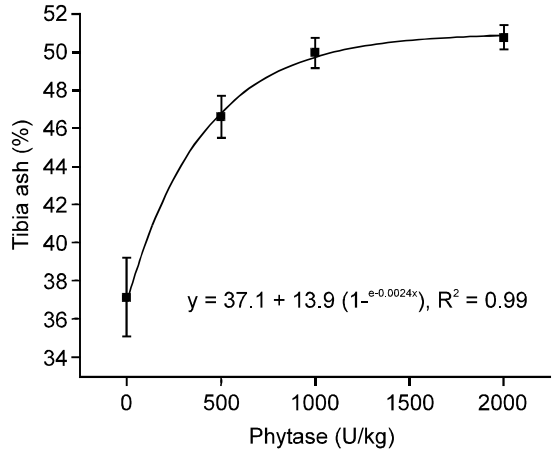

Fig. 2: Effect of phytase supplementation on tibia ash (%) of broiler birds fed different levels of phytase

significantly higher for all treatments compared to the negative control diet. With increasing levels of phytase, higher improvements ranging between 25-37% were noticed. An exponential dose-dependent relationship was found for tibia ash (Fig. 2), in which the slope curve rose very fast with increasing phytase level in the diet. The results of this study fully confirmed that tibia ash percentage is a classical response parameter to demonstrate the efficacy of microbial phytase, as reported previously by Broz *et al.* (1994), Denbow *et al.* (1995) and Sebastian *et al.* (1996).

Conclusions: It was concluded that oral administration of the 6-phytase preparation, Ronozyme HiPhos, a microbial phytase produced via synthetic genes by *Aspergillus oryzae*, to rats at dosages of up to 10.0 mL/kg BW per day for 13 weeks was well tolerated and did not produce any toxicologically significant changes. Consequently, this dose was considered to be the No-Observed-Adverse-Effect Level (NOAEL). Equally the phytase showed no mutagenic activities as was observed with the Ames test as well as with the in vitro micronucleus assay.

The 5-week tolerance study confirmed that this novel 6-phytase preparation expressed in *Aspergillus oryzae* is safe for broiler chickens when fed at the maximum recommended level, as well as at the 10 times higher level (4 000 and 40 000 U/kg diet, respectively). The results of the short-term efficacy study demonstrated that supplementation of the low P diet with this novel microbial 6-phytase in liquid form significantly improved the weight gain and the feed conversion ratio of male broiler chickens at 22 days of age. The apparent utilization of phosphorus was significantly increased and consequently the amount of P excreted in the faeces was reduced. Apparent P utilization was improved dependent on the dietary level of phytase and could be described by an exponential function. The novel microbial 6-phytase expressed in *Aspergillus oryzae* was effective in releasing phytate-P according to the effects obtained on tibia ash, a sensitive criterion for evaluating phosphorus bioavailability. In most of the measured parameters, even at low dosages, the treatments supplemented with the novel microbial 6-phytase performed equally or even outperformed that supplemented with mineral P (Positive control).

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