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Research article

Safety and efficacy profile of a phytase produced by fermentation and used as a feed additive

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ABSTRACT

Enzymes can aid in optimal feed stock utilization when used as feed additives. A range of toxicological studies were performed to evaluate the safety profile of a novel phytase (phytase HM) from *Citrobacter braakii* produced in *Aspergillus oryzae*. Phytase HM was found to be non-mutagenic and non-clastogenic in *in vitro* tests. Further, the phytase HM preparation did not exhibit irritative potential to the eye and skin when applied in *in vitro* models. A 13-week subchronic toxicity study with oral administration of phytase HM to rats did not show any adverse effects. Efficacy studies showed that the dietary supplementation of this phytase significantly improved growth performance and bone mineralization in broiler chickens and piglets fed P-deficient diets, and increased retention of phosphorus (P) and calcium (Ca), and phytate-P degradation in excreta of broiler chickens in a dose-dependent manner.

In conclusion, there are no safety concerns using phytase HM as a feed additive and the phytase is well tolerated by broiler chickens and pigs. Further, phytase HM improves with high efficacy the growth performance in both broiler chickens and pigs.

1. Introduction

Feed to monogastric animals is routinely supplemented with enzymes to increase nutrient digestibility and optimize the nutritional value of the diet (Adeola and Cowieson, 2011). Many different enzymes are used to optimize nutrient utilization from feed ingredients; e.g. use of amylases, glucanases, xylanases, hemi-cellulases, muramidases and proteases (Adeola and Cowieson, 2011; Aureli et al., 2018; Dersjant-Li et al., 2015; Lichtenberg et al., 2017 Cowieson and Roos, 2016) enhances the bioavailability of nutrients for the animal and/or for its intestinal microbiota (Jozefiak et al., 2010; Bedford and Cowieson, 2012; Kiarie et al., 2013). Enzymes can reduce the cost of feed by optimizing the feed utilization, as this will lead to a reduced consumption of feed. Further, the increased efficiency of feed utilization makes enzymes essential to improve the sustainability of meat and egg production (Bundgaard et al., 2014; Leinonen and Kyriazakis, 2016).

The use of phytases in animal feed for monogastric animals has a long history and the advantages are well characterized (Dersjant-Li et al., 2015). The purpose of adding phytase to animal feed is to utilize a phosphorus source already present in the feed, counter the anti-nutritional effect of phytate, increase the availability of myo-inositol and to reduce the phosphate emissions to environment. When more phosphate in the feed is available to the animal, addition of inorganic phosphate can be avoided or strongly reduced (Selle and Ravindran, 2007).

In addition, nutrients and energy could be utilized with greater efficiency in the presence of phytases (Cowieson et al., 2009) and thereby the pressure on the natural resources by farm animals can be reduced.

Commercially available phytase feed additives have been derived from a handful of microorganisms e.g. *Aspergillus* spp., *Buttiauxella* sp., *Citrobacter braakii*, *Escherichia coli*, *Hafnia* sp., and *Peniophora lycii* and produced in various production hosts. The phytase preparation (a modified *C. braakii* phytase expressed in *A. oryzae*) described here is denoted as Phytase HM and will be commercially available as HiPhorius™ (DSM Nutritional Products, Switzerland). The Phytase HM has increased intrinsic temperature and pH stability compared to the wild type phytase, both achieved by protein engineering. One of the concepts used for temperature stability is the introduction of additional disulfide

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bridges which leads to higher unfolding temperatures and slower thermal deactivation kinetics (Sanchez-Romero et al., 2013).

Aspergillus oryzae is a well-known production host and is currently used to produce commercial enzymes used in animal feed as well as other industries. The *A. oryzae* production strain used in this study derives from A1560 (synonym IFO 4177) and is genetically modified to produce a modified phytase from *Citrobacter braakii (ATCC 51113)*. The strain has been genetically modified to remove the genes homologous to the *Aspergillus flavus* aflatoxin gene cluster and the genes involved in cyclopiazonic acid synthesis. Moreover, the background strain has a decreased potential for production of kojic acid. These modifications make the strain suitable for production of enzymes to be used in animal feed.

Phytases are considered readily biodegradable (REACH registration, European Community number (EC number) 629-845-9, IUBMB 3.1.3.26) and, consequently, the potential environmental toxicity of phytases is minimal. In commercial phytase products the main components are formulation agents (e.g. salts, cellulose, dextrins and vegetable oils for solid products, and water and polyols for liquid products) where the phytase content is typically below 10%. All formulation chemicals used for HiPhorius™ comply with the recommended purity specifications for food-grade enzymes given by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemical Codex (FCC) (http://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/enzymes/en/). Therefore an evaluation of the safety of the formulation agents is not the aim of this study.

Efficacy studies were conducted in young broiler chickens and weaned piglets in compliance with the European Food Safety Authority (EFSA) guidance on the assessment of the efficacy of feed additives (EFSA 2018). In these studies, Phytase HM was supplemented to the animals fed phosphorus (P) deficient diets and its efficacy was demonstrated by measuring the animal's response in growth performance, bone mineralization and mineral utilization.

Toxicological studies were performed to evaluate the safety of this new Phytase HM following the guidance by Pariza and his colleagues (Pariza and Johnson, 2001; Pariza and Cook, 2010) and consistent with the safety assessment of enzymes performed by Nováková, Lichtenberg, and Aureli (Nováková et al., 2021; Lichtenberg et al., 2011, 2017; Aureli et al., 2018). The studies reported here comply with the requirements of the European Union Regulation N° 1831/2003 on additives for use in animal nutrition and the corresponding guidelines (EFSA, 2017a; 2017b). Specifically, the enzyme preparation was subjected to genetic toxicity testing (Ames test and *in vitro* micronucleus test), test for irritation potential (*in vitro* eye irritation and *in vitro* skin irritation), and a subchronic 90 days oral toxicity study in rats.

The safety information on the production strain and the results of the toxicological studies as well as the efficacy studies in broiler chickens and pigs demonstrates the safety and advantages of Phytase HM as a feed additive, as guided by the European Food Safety Authority (EFSA, 2011) and prescribed by EC Regulation 1831/2003.

2. Materials and methods

2.1. Construction of the production strain

The wildtype phytase gene from *Citrobacter braakii* (ATCC51113) was modified to create the Phytase HM product. The phytase expression cassette was made using standard vectors with strictly defined and well-characterized DNA sequences and does not encode or express any harmful or toxic substances. The phytase expression cassette was introduced through recombination into the *A. oryzae* recipient strain derived from A1560 (synonym IFO 4177) using standard transformation procedures. The transformants were subsequently evaluated by gene sequencing to assess incorporation of the expression cassette and to ensure that no unintended sequences were incorporated into the genome of the selected production strain. The phytase protein expressed from the introduced genes in the final production strain was verified by mass spectroscopy to match the expected protein sequence.

2.2. Preparation of the phytase test substance

The phytase preparation evaluated in the present study is denoted as Phytase HM and was made in an industrial setting certified to ISO 9001 and in accordance with the procedures used for the manufacturing of commercial enzyme products. In brief, the *A. oryzae* production strain described in Section 2.1, was cultivated in a bioreactor with pH adjusted and sterilized food-grade ingredients. After fermentation, the production organism was separated from the fermentation broth through a series of filtration and concentration steps. A filtered and concentrated liquid fermentation broth was used for the toxicological studies. A solid granulation form of the product was made by well-established industrial production practices. This thermostable granulated form of phytase HM was used for animal efficacy studies.

2.3. Characterization of the phytase test substance

Phytase activity is expressed in phytase units (here abbreviated FYT). One FYT is defined as the amount of enzyme which liberates 1 μmol inorganic phosphate per minute from a 0.0051 M sodium–phytate solution at pH 5.5 and 37 °C (Engelen et al., 1994), as these are standard conditions for determining feed phytase activity. In brief, phytase samples were mixed with sodium–phytate and incubated at 37 °C for 30 min. Stop reagent (20.2 mM ammonium hepta-molybdate tetrahydrate; 0.06% ammonium vanadate; 11% nitric acid) was added, and the absorbance at 405 nm was measured and compared to a standard curve. The Phytase HM preparation was also analyzed for chemical and microbial levels using standard methods (Table 1). Total organic solids (TOS) from the fermentation consists mainly of protein and carbohydrate components and was calculated as follows: TOS (%) = 100 - water (%) - ash (%). The TOS content of phytase was 10.5% w/w.

The test item used for the toxicological studies had an enzyme activity of 34000 FYT/g. In addition, a high activity batch with 65000 FYT/g were also tested in the skin and eye irritation studies. In the efficacy studies with broiler chickens and piglets a granulated form of phytase HM with an activity of 13850 FYT/g was used.

2.4. Toxicological evaluation of phytase HM

The toxicological studies were performed under GLP and in accordance with current guidelines from the Organisation for Economic Cooperation and Development (OECD), and the *in vivo* studies were moreover performed in accordance with the EU's legislation on the protection of animals used for scientific purposes, Directive 2010/63/EU (EU, 2010).

2.4.1. Subchronic oral toxicity of phytase in rat

The subchronic toxic potential of Phytase HM was evaluated by daily oral administration to rats over 13 weeks. The study was performed according to OECD TG No. 408 (OECD 2018), which includes endocrine-sensitive endpoints intended to improve detection of potential endocrine activity of test chemicals. The protocol was approved by the Animal Welfare Ethical Review Body at Covance Laboratories Ltd. (UK) and the Home Office Inspectorate (UK). In the study 80 Han Wistar rats (RccHan™; WIST) obtained from Covance RMS Ltd were randomly divided into four groups, each comprising of ten males and ten females. They were fed an expanded rodent diet (Teklad, 2014C, pelleted diet) and offered potable water *ad libitum*. The animals were housed in groups of five of the same gender. Wood shavings were used as bedding, and aspen chew blocks and plastic shelters were provided as environmental enrichment in each cage. The room temperature was maintained within the range 20–24

°C, the relative humidity within the range 40–70% and with a 12-hour light and 12-hour dark cycle. The animals were allowed to acclimatize for 15 days and they were 5–6 weeks old at commencement of treatment.

Phytase HM was prepared by dilution with reverse osmosis water for each dose level group to 0%, 10%, 33% and 100% of the test item. These dose levels were equivalent to 0, 110.3, 363.8 and 1102.5 mg TOS/kg body weight/day and 0, 35700, 117810 and 357000 FYT/kg body weight/day. Animals received vehicle (reverse osmosis water) or the test item formulations orally by gavage at a total dose volume of 10 mL/kg body weight. Content checks of the dose formulations and 24-hour stability at 21 °C were confirmed by measurement of total nitrogen content from samples obtained at weeks 1, 6 and 13.

Clinical signs were recorded daily. Body weights and food consumption were recorded once weekly. Water consumption was monitored daily by visual inspection.

Furthermore, during the study, detailed physical examination and arena observations, sensory reactivity observations, grip strength, motor activity, ophthalmic examination, hematology (peripheral blood), blood chemistry, thyroid hormone, estrous cycle, organ weight, pathology and histopathology investigations were undertaken. All observations were performed blinded and at the same time of day on each occasion.

The blood samples were taken in week 13 after overnight food deprivation in three different types of tubes containing either EDTA anticoagulant, citrate anticoagulant or lithium heparin anticoagulant and analyzed for a wide range of biochemical and hematological parameters. The estrous cycles were evaluated by taking wet smears from the vagina of all females using pipette lavage for four days before scheduled necropsy.

After completion of the treatment period, the rats were euthanized by carbon dioxide inhalation followed by exsanguination. All animals were dissected, examined macroscopically, a wide range of tissues were collected, organ weights were recorded and histopathological examination of organs from the high dose animals and the control animals was performed. Sperm motility was analyzed. A full macroscopic examination of the tissues was performed for all groups whereas histopathological examination was only performed for the high dose and control group.

Statistical analyses: A parametric analysis was performed if Bartlett's test for variance homogeneity (Bartlett, 1937) was not significant at the 1% level. If a F1 approximate test for monotonicity of dose-response was not significant at the 1% level, Williams' test for a monotonic trend was applied (Williams, 1971, 1972). If the F1 approximate test was significant, suggesting that the dose response was not monotone, Dunnett's test (Dunnett, 1955, 1964) was performed instead. For organ weights, analysis of covariance was performed using terminal body weight as covariate (Angervall and Carlstrom, 1963) unless non-parametric methods were applied. The treatment comparisons were made on adjusted group means to allow for differences in body weight which might influence the organ weights. Significant differences between the groups compared were expressed at the 5% ($p < 0.05$) or 1% ($p < 0.01$) level.

2.4.2. *In vitro eye irritation test*

The eye irritation potential of Phytase HM was evaluated in the Bovine Corneal Opacity Permeability (BCOP) test in accordance with OECD TG 437 (OECD, 2020). The study was carried out at Covance Laboratories Ltd. (UK). Two liquid Phytase HM preparations were tested with activities of 34000 FYT/g and 65000 FYT/g. In addition, the test included a negative and positive control (sodium chloride 0.9% and 100% ethanol, respectively). Eyes from adult cattle were obtained from an abattoir as a by-product from freshly slaughtered animals. The cornea from each eye was removed, and after exposure, the two test method endpoints, opacity and permeability, were recorded and combined in the empirically derived formula to generate an *In Vitro* Irritancy Score (IVIS):

$$\text{In Vitro Irritancy Score} = \text{mean opacity value} + (15 \times \text{mean permeability } OD_{492} \text{ value})$$

2.4.3. *In vitro skin irritation test*

In vitro skin irritation potential of Phytase HM was assessed using the EPISKIN™ Reconstructed Human Epidermis Model. The study was carried out in accordance with OECD guideline 439 (OECD, 2015) and was performed at Covance Laboratories Ltd. (UK). Two liquid Phytase HM preparations were tested with activities of 34000 FYT/g and 65000 FYT/g. The test method endpoint, optical density (OD), was measured at 570 nm (OD_{570}). Results were presented as percentage viability (MTT reduction in the test item treated tissues relative to negative control tissues).

Table 1. Composition analyzes of the Phytase HM preparation for the toxicological studies.

Composition	Limits	Unit	
Enzyme activity	-	FYT/g	34000
Water (Karl Fisher)	-	% w/w	88.0
Total Organic Solids	-	% w/w	10.5
Dry Matter	-	% w/w	12
Ash (600C)	-	% w/w	1.5
N-total	-	% w/w	1.18
Carbohydrate (anthron)	-	g/kg	24.2
Carbohydrate (tryptophan)	-	g/kg	36.4
Water at 105 °C	-	% w/w	88
Heavy Metals[(1)]	≤30 mg/kg	mg/kg	4.3
Coliforms	≤30	/g	<4
Escherichia coli	ND	/25g	ND
Bacillus cereus	$\leq 10^2$	/g	<10
Sulphite red. *clostridia*	≤30	/g	<10
Staphylococcus aureus	ND	/g	ND
Salmonella sp.	ND	/25g	ND
Antimicrobial activity	ND	-	ND
Toxins[(2)]	<LOD	mg/kg	<LOD
Production organism (*Aspergillus oryzae*)	ND	/g	ND

ND = not detected.
(1) Heavy Metals = ΣDL_T of Ag, As, Bi, Cd, Co, Cu, Hg, Mo, Ni, Pb, Sb, Sn.
(2) Aflatoxin B1, Cyclopiazonic acid, Beta-nitropropion acid and Kojic acid.

2.4.4. Bacterial reverse mutation assay

To assess mutagenic activity of the Phytase HM enzyme preparation, an Ames bacterial reverse mutation test was performed at Covance Laboratories Ltd. (UK).

The Phytase HM enzyme preparation may contain histidine and tryptophan which may increase the apparent number of revertants in the test. To avoid artefacts due to growth facilitated by exogenous histidine and tryptophan, a 'treat and wash' protocol was applied (Mahon et al., 1989; Thompson et al., 2005) as described by Pedersen and Broadmeadow (2000). The study was conducted in accordance with the general recommendations in OECD Guideline 471 (OECD, 1997).

The study utilized four *Salmonella typhimurium* strains and one *Escherichia coli strain* capable of detecting induced frame-shift mutations (*S. typhimurium* TA1537 and TA98) or base pair substitutions (*S. typhimurium* TA1535, TA100 and *E. coli* WP2uvrApKM101). The genotypes of the bacterial test strains were confirmed by standard procedures (Maron and Ames, 1983; Green, 1984). Two independent experiments were conducted. The tests were carried out in parallel with and without S9 Metabolizing System (Molecular Toxicology Incorporated, USA).

For both experiments, up to 5000 μg TOS/mL was tested. 5000 mg TOS/mL is the maximum recommended concentration (EFSA, 2014). Further, the bacterial strains were exposed to vehicle (reverse osmosis water), and suitable positive controls (Sodium azide, 9-Aminoacridine, 2-Nitrofluorene, 4-Nitroquinoline-1-oxide, 2-Aminoanthracene or Benzo[a]pyrene as detailed in Table 8).

2.4.5. In vitro micronucleus test in cultured human lymphocytes

The Phytase HM enzyme preparation was tested in an *in vitro* micronucleus assay, to assess the potential of Phytase HM enzyme preparation to induce an increase of micronuclei in cultured human lymphocytes *in vitro*, at Covance Laboratories Ltd. (UK). The study was conducted according to OECD guideline 487 (OECD, 2016). Phytase HM was formulated in purified water, and the maximum concentration for micronucleus analysis was 5000 μg TOS/mL based on a preliminary toxicity test where concentrations up to 5000 μg TOS/mL were tested. 5000 μg TOS/mL is the maximum recommended concentration in the current OECD Guideline 487 (OECD, 2016). Treatments were performed both with and without S9 Metabolizing System (Molecular Toxicology Incorporated, Boone, USA).

2.5. Efficacy studies in broiler chickens and weaned piglets

The efficacy studies were conducted at DSM (China) Animal Nutrition Research Center Co. Ltd. (Bazhou, P. R. China). The protocols were approved by the Animal Welfare Committee of DSM (China) Animal Nutrition Research Center (AWCCAN). The studies complied with the guidelines in European Union council directive 2010/63/EU for animal experiments.

In the broiler study, 768 birds (Cobb 500, male) were housed in wire-floored battery cages in an environmentally controlled room. From hatch to day 8 of age, all the birds were fed with a corn-soybean meal based common starter diet, which was formulated to be deficient only in total P (0.46%). At the start of trial (day 8 of age), birds were sorted by weight and divided into replicate groups, each comprising 8 birds. The birds with similar cage weight were randomly allocated to one of the different treatments. Each treatment was replicated with 12 cages. There were 8 dietary treatments consisting of a negative control (NC) and the NC supplemented with 7 levels of the Phytase HM: 187.5, 375, 750, 1125, 1500, 1875 or 2250 FYT/kg. A basal diet was prepared with corn and soybean meal as the main ingredients, and was formulated to be deficient only in total P (0.46%). The Phytase HM was pre-mixed with a small amount of the basal diet before the complete mixing of the experimental diets to ensure uniformity of mixing. The feed was pelleted at 75 °C. The analyzed Phytase HM activities of the dietary treatments were 182, 328, 640, 1060, 1347, 1560 and 1931 FYT/kg.

The experimental diets were supplied to birds from day 8–18 of age. Feed and water were supplied *ad libitum* through the whole trial. At day 8 and 17 of age, feed consumption and body weight (BW) by cage were recorded to calculate the body weight gain (WG), feed intake (FI) and the feed conversion ratio (FCR). Excreta were collected on day 14 through day 17. During this period, the excreta from 12 cages of each treatment were quantitatively collected once per day, and the excreta per cage from the 4 days were pooled together, and frozen immediately at -20 °C after collection. After thawing, the total excreta of each cage were homogenized, and the representative sub-samples were taken and freeze-dried for the determination of dry matter (DM), P, Ca and phytate-P. The total amount of feed consumption during the excreta collection period was recorded as well. At day 18 of age, the right tibia was taken from 2 birds randomly chosen from each of the 12 replicate cages. Tibias were defleshed, and cartilaginous caps were removed after collection. They were kept frozen in plastic bags at -20 °C to maintain wetness until analysis of ash, Ca and total P content.

In the piglet study, 140 castrated male piglets (Redon x Large White) were used. The piglets were weaned at 28 days of age and had an average body weight of 7.5 ± 1.1 kg (mean ± standard deviation) at the start of trial. The piglets were housed in 35 flat-deck cages with 4 animals per cage in an environmentally controlled room. Each cage had a plastic-coated welded wire floor and was equipped with two water nipples and two stainless-steel feeders. The experimental diets were fed for 42 days which were divided into a starter phase of 14 days and a grower phase of 28 days. Water and feed were supplied *ad libitum*. The feed was offered in mash form.

There were 7 dietary treatments consisting of a positive control (PC), a NC and the NC supplemented with 187.5, 375, 750, 1500 or 3000 FYT test phytase/kg feed (on analysis: 266, 383, 771, 1445, and 2914 FYT/kg in starter diets; 219, 395, 884, 1408, and 2730 FYT/kg in grower diets). The PC diets for the starter and grower phases met the pig's requirement for energy and nutrients prescribed by NRC (2012) for the body weight range of 7–11 kg and 11–25 kg, respectively, and were formulated with corn, soybean meal and rapeseed meal as the main ingredients. The NC diets were established by withdrawing the dicalcium phosphate from the PC diets resulting in P deficient diets (0.43% and 0.41% total P for starter and grower, respectively) with adequate Ca. The ingredient and nutrition compositions of diets are shown in Table 2.

The pigs were weighed individually, and feed consumption was recorded for each pen to calculate average daily gain (ADG), average daily feed intake (ADFI) and FCR. At the end of the trial, 2 pigs of each pen with body weight closest to the average body weight of their pen were slaughtered for collection of femurs. The right femurs were separated and removed of the soft tissue. A diaphysis section (~3.5 cm in length) of each femur was obtained by sawing and then subjected to compression to determine the force in Newton to break the bone. The broken bones were used for the determination of ash, Ca and P content.

The samples of diets and excreta collected from broiler chicken and pig trials were ground to pass through a 0.5-mm screen before analysis. All samples were analyzed in duplicate. The samples were dried at 105 °C in an oven for 4 h for dry matter determination (method 934.01; AOAC International, 2006). Ca and P were determined by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES; 5100 Dual View, Agilent, Santa Clara, CA, USA; method 985.01; AOAC International, 2006) after sulfuric acid mineralization. Phytate P was calculated as the difference between total P and free P. Total P was determined after treating the dietary and excreta samples with a megadose of phytase to release the P bound by phytate (and its degradation products). The free P, not bound by phytate (and its degradation products), was determined after overnight extraction in 0.66 M HCl. Phytase activity was measured by a colorimetric method and expressed in phytase units (FYT), as defined in section 2.3.

In both broiler chicken and pig trials, the data were analyzed by one-way ANOVA using GLM procedure of SAS (version 9.0). Orthogonal contrasts were constructed to test the linear and quadratic effects of

Table 2. Ingredient and nutrition composition of experimental diets in broiler chickens and piglet trials, %.

	Broiler trial	Piglet trial			
	8–18 day of age	Starter		Grower	
Ingredient	NC	NC	PC	NC	PC
Corn	60.14	54.35	53.60	59.45	58.75
Soybean meal	33.50	24.00	24.00	20.00	20.00
Rapeseed meal	–	12.00	12.00	12.00	12.00
Soybean oil	2.50	4.50	4.70	3.70	3.90
NaCl	0.41	0.20	0.20	0.05	0.05
NaHCO3	0.14	0.35	0.35	0.35	0.35
DL-Met	0.58	0.10	0.10	0.05	0.05
L-Lys HCl	0.37	0.25	0.25	0.25	0.25
L-Thr	0.15	0.10	0.10	0.05	0.05
L-Val	–	0.15	0.15	0.10	0.10
Limestone	1.06	1.00	0.00	0.70	0.00
Dicalcium phosphate	0.55	0.00	1.55	0.00	1.20
Vit-Min Premix[(1)]	0.50	3.00	3.00	3.00	3.00
TiO_2	0.10	0.00	0.00	0.30	0.30
Total	100.00	100.00	100.00	100.00	100.00
CP, %	21.5	20.0	19.9	18.6	18.5
ME, kcal/kg	3066	3404	3396	3374	3367
Dig Lys, %	1.25	1.35	1.35	1.26	1.26
Dig Met, %	0.85	0.43	0.43	0.37	0.37
Dig Thr, %	0.83	0.83	0.83	0.74	0.74
Digestible P, %	–	0.17	0.40	0.16	0.34
Total P, %	0.46	0.43	0.72	0.41	0.64
Phytate P, %	0.24	0.28	0.28	0.27	0.27
Calcium, %	0.70	0.83	0.84	0.71	0.74

[(1)] Broiler premix supplied per kg of diet: vitamin A, 10,000 IU; vitamin D_3, 2,240 IU; 25-OH-D_3, 69 μg; vitamin E, 50 IU; vitamin K_3, 3 mg; vitamin B_1, 2 mg; vitamin B_2, 7 mg; vitamin B_6, 4 mg; vitamin B_{12}, 20 μg; biotin, 250 μg; folic acid, 2 mg; niacin, 60 mg; D-pantothenic acid, 12 mg; Fe, 40 mg; Cu, 15 mg; Mn, 110 mg; Zn, 90 mg; I, 0.5 mg; Se, 0.25 mg; and choline, 400 mg. Piglet premix supplied per kg of diet: vitamin A, 15,000 IU; vitamin D_3, 1,998 IU; vitamin E, 100 IU; vitamin K_3, 20 mg; vitamin B_1, 3.0 mg; vitamin B_2, 10 mg; vitamin B_6, 6 mg; vitamin B_{12}, 40 μg; biotin, 200 μg; D-pantothenic acid, 25 mg; folic acid, 1.5 mg; niacin, 35 mg; vitamin C, 100 mg; Cu, 160 mg; I, 2.0 mg; Fe, 200 mg; Mn, 60 mg; Zn, 100 mg; Se, 400 μg; choline, 375 mg; sodium, 1.5 g; chlorine, 3.2 g; Ca, 2.8 g; lysine, 2.9 g; methionine, 0.5 g; threonine, 1.4 g; tryptophan, 0.3 g; and valine, 0.2 g.

Phytase HM supplementation to the NC diet. Tukey's multiple comparison test was also applied to the broiler chicken trial. The least square means are presented.

3. Results & discussion

3.1. Subchronic oral toxicity in rat

The systemic toxic potential of Phytase HM at doses of 0, 110.3, 363.8 and 1102.5 mg TOS/kg body weight/day equivalent to 0, 35700, 117810 and 357000 FYT/kg body weight/day administered orally by gavage to Wistar rats was assessed over a period of 13 weeks. The Total Nitrogen content (N-Total %) values were as expected in the formulations prepared for administration in Week 1, 6 and 13 at all dose levels, confirming acceptable formulation.

The general appearance and behavior of the animals were unaffected by treatment, and there were no unscheduled deaths. Sensory reactivity responses, grip strength and motor activity were unaffected by treatment. Food consumption and bodyweight gain was unaffected by treatment and there were no treatment-related ophthalmoscopic findings.

The hematological examination during Week 13 did not identify any test item related differences from controls (data is presented in Tables 3 and 4). There was a slightly but statistically significant low mean hematocrit (Hct) and erythrocyte count (RBC) in males but no similar trend in females. All individual erythrocyte counts were within the historical control range (98-percentile range, 7.95×10^{12} to 9.57×10^{12}/L; n = 104), whilst for hematocrit, 2/7, 2/10, 0/10 and 2/10 males receiving vehicle, low, mid or high dose, respectively, had individual values that were above the historical control range (98-percentile range, 0.409–0.501 L/L, n = 104), but no values were lower than this range. Moreover, there was observed a small but statistically significant increased activated partial thromboplastin times (APTT), when compared to the controls, at all doses in females. This was also considered of no toxicological significance, as none of the individual values was above the historical control range (98-percentile range, 11.6–25.6 s; n = 148), there were no similar findings in males, and there was no change in prothrombin time.

The biochemical examination of the blood plasma after 13 weeks of treatment did not identify any differences from controls that were attributable to treatment (data presented in Tables 5 and 6) as intergroup differences from control were minor, occurred in one sex only or were without dose-relationship. This includes bile acid (BiAc) concentrations, which were slightly low in high dose males and females. The individual values for all high dose females were within the historical control range (98-percentile range: 3.96–104.90 μmol/L; n = 48), whereas one control female had a value above the historical control range. For males, two high dose males had individual values that were below the historical control range (98-percentile range: 8.92–100.28 μmol/L; n = 47) but one low dose male also had a value below the range. Further, as bile acids concentrations tend to be highly variable, and as the majority of individual values were within normal

Table 3. Hematology results of male (M) rats. Group mean values and standard deviation (SD).

Hematology	Units	Groups of male rats (dose level in mg TOS/kg bw/day)							
		1M (0)		2M (110.3)		3M (363.8)		4M (1102.5)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Hct	L/L	0.496	(0.0191)	0.480	(0.0214)	0.476	(0.0185)	0.473*	(0.0267)
Hb	g/dL	16.6	(0.45)	16.4	(0.52)	16.4	(0.73)	16.2	(0.67)
RBC	$\times 10^{12}$/L	9.18	(0.331)	8.81	(0.435)	8.80	(0.395)	8.72*	(0.492)
Retic	$\times 10^{12}$/L	0.140	(0.0280)	0.153	(0.0165)	0.143	(0.0166)	0.161	(0.0213)
MCH	Pg	18.1	(0.38)	18.6	(0.92)	18.6	(0.67)	18.6	(0.84)
MCHC	g/dL	33.4	(0.82)	34.2	(0.94)	34.5	(0.92)	34.2	(1.10)
MCV	fL	54.1	(0.54)	54.6	(2.12)	54.1	(1.12)	54.3	(1.32)
RDW	%	11.5	(0.43)	11.4	(0.36)	11.5	(0.58)	11.6	(0.61)
WBC	$\times 10^9$/L	7.11	(1.718)	6.20	(1.536)	7.21	(0.882)	5.81	(1.234)
Neut	$\times 10^9$/L	1.31	(0.418)	1.34	(0.483)	1.35	(0.388)	0.91	(0.324)
Lym	$\times 10^9$/L	5.49	(1.328)	4.60	(1.519)	5.55	(0.778)	4.65	(1.030)
Eos	$\times 10^9$/L	0.09	(0.043)	0.09	(0.032)	0.08	(0.037)	0.09	(0.021)
Baso	$\times 10^9$/L	0.02	(0.015)	0.02	(0.009)	0.03	(0.012)	0.02	(0.011)
Mono	$\times 10^9$/L	0.14	(0.037)	0.11	(0.049)	0.15	(0.055)	0.10	(0.039)
LUC	$\times 10^9$/L	0.06	(0.017)	0.05	(0.021)	0.06	(0.021)	0.04	(0.016)
Plat	$\times 10^9$/L	670	(82.7)	634	(54.6)	642	(82.6)	650	(80.7)
PT	sec	19.6	(1.10)	19.3	(1.55)	19.5	(1.38)	18.8	(0.75)
APTT	sec	16.7	(1.77)	17.7	(3.46)	18.4	(1.41)	18.0	(2.32)

Significantly different from the controls: *: $P < 0.05$; **: $P < 0.01$.
Hct: Hematocrit; Hb: Hemoglobin; RBC: Total Red Blood Cell; Retic: Absolute Reticulocytes Count; MCH: Mean Cell Hemoglobin; MCHC: Mean Cell Hemoglobin Concentration; MCV: Mean Cell Volume; RDW: Red Cell distribution Width; WBC: Total Leukocyte Count; Neut: Neutrophils; Lym: Lymphocytes; Eos: Eosinophils; Baso: Basophils; Mono: Monocytes; LUC: Large unstained cels; Plat: Platelet Count; PT: Prothrombin Time; APTT: Activated partial thromboplastin time.

Table 4. Hematology results of female (F) rats. Group mean values and standard deviation (SD).

Hematology	Units	Groups of female rats (dose level in mg TOS/kg bw/day)							
		1F (0)		2F (110.3)		3F (363.8)		4F (1102.5)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Hct	L/L	0.443	(0.0163)	0.442	(0.0166)	0.441	(0.0182)	0.442	(0.0116)
Hb	g/dL	14.9	(0.42)	14.9	(0.54)	14.9	(0.65)	15.0	(0.28)
RBC	$\times 10^{12}$/L	7.90	(0.371)	7.85	(0.359)	7.83	(0.265)	7.79	(0.350)
Retic	$\times 10^{12}$/L	0.145	(0.0208)	0.143	(0.0254)	0.143	(0.0284)	0.156	(0.0215)
MCH	pg	18.9	(0.53)	19.0	(0.63)	19.0	(0.36)	19.3	(0.61)
MCHC	g/dL	33.6	(0.62)	33.8	(0.43)	33.7	(0.38)	33.9	(0.39)
MCV	fL	56.0	(0.92)	56.3	(1.45)	56.3	(0.85)	56.8	(1.25)
RDW	%	9.7	(0.28)	10.0	(0.37)	9.9	(0.23)	9.9	(0.29)
WBC	$\times 10^9$/L	4.05	(0.840)	4.76	(0.950)	4.19	(1.338)	3.82	(0.693)
Neut	$\times 10^9$/L	0.51	(0.237)	0.59	(0.204)	0.49	(0.177)	0.54	(0.222)
Lym	$\times 10^9$/L	3.38	(0.750)	3.97	(0.926)	3.55	(1.204)	3.14	(0.659)
Eos	$\times 10^9$/L	0.06	(0.034)	0.07	(0.059)	0.04	(0.018)	0.04	(0.011)
Baso	$\times 10^9$/L	0.01	(0.007)	0.02	(0.014)	0.02	(0.018)	0.01	(0.005)
Mono	$\times 10^9$/L	0.06	(0.029)	0.07	(0.020)	0.06	(0.023)	0.06	(0.015)
LUC	$\times 10^9$/L	0.04	(0.018)	0.04	(0.019)	0.03	(0.013)	0.03	(0.011)
Plat	$\times 10^9$/L	808	(124.7)	771	(160.7)	767	(93.9)	843	(121.9)
PT	sec	20.5	(0.88)	20.2	(0.84)	20.1	(0.68)	20.6	(0.72)
APTT	sec	12.3	(1.55)	13.7*	(1.46)	14.5**	(1.08)	14.8**	(0.79)

Significantly different from the controls: *: $P < 0.05$; **: $P < 0.01$.
Hct: Hematocrit; Hb: Hemoglobin; RBC: Total Red Blood Cell; Retic: Absolute Reticulocytes Count; MCH: Mean Cell Hemoglobin; MCHC: Mean Cell Hemoglobin Concentration; MCV: Mean Cell Volume; RDW: Red Cell distribution Width; WBC: Total Leukocyte Count; Neut: Neutrophils; Lym: Lymphocytes; Eos: Eosinophils; Baso: Basophils; Mono: Monocytes; LUC: Large unstained cels; Plat: Platelet Count; PT: Prothrombin Time; APTT: Activated partial thromboplastin time.

Table 5. Clinical chemistry results of male (M) rats. Group mean values and standard deviation (SD).

Clinical chemistry	Units	Groups of male rats (dose level in mg TOS/kg bw/day)							
		1M (0)		2M (110.3)		3M (363.8)		4M (1102.5)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
ALP	U/L	84	(13.5)	83	(20.1)	77	(13.6)	73	(13.6)
ALT	U/L	56	(14.2)	62	(25.6)	49	(9.3)	50	(6.9)
AST	U/L	88	(34.4)	103	(57.8)	78	(21.8)	89	(27.6)
Bi Ac	µmol/L	35.0	(8.65)	26.6	(12.16)	29.6	(8.80)	19.6**	(8.67)
Urea	mmol/L	6.66	(1.044)	7.23	(0.859)	6.40	(0.883)	6.57	(1.292)
BUN	mmol/L	6.66	(1.044)	7.23	(0.859)	6.40	(0.883)	6.57	(1.292)
Creat	µmol/L	24	(2.8)	26	(3.0)	25	(2.8)	26	(2.3)
Gluc	mmol/L	7.69	(1.588)	9.10	(1.339)	8.37	(1.948)	7.81	(1.327)
Chol	mmol/L	2.29	(0.444)	2.39	(0.255)	2.27	(0.397)	2.35	(0.348)
HDL	mmol/L	1.72	(0.330)	1.76	(0.214)	1.68	(0.294)	1.73	(0.229)
LDL	mmol/L	0.25	(0.084)	0.28	(0.088)	0.28	(0.086)	0.25	(0.072)
Na	mmol/L	142	(1.0)	142	(1.5)	142	(1.2)	142	(0.8)
K	mmol/L	4.06	(0.193)	4.09	(0.384)	4.00	(0.306)	3.98	(0.237)
Total Prot	g/L	68	(2.3)	69	(2.9)	69	(3.9)	70	(3.2)
Alb	g/L	40	(1.1)	40	(1.4)	40	(1.3)	40	(1.0)
A/G	Ratio	1.45	(0.137)	1.40	(0.137)	1.35	(0.172)	1.35	(0.096)

Significantly different from the controls: *: $P < 0.05$; **: $P < 0.01$.
ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Bi Ac: Bile acids – total; BUN: Blood urea nitrogen; Crea: Creatinine; Gluc: Glucose; Chol: Total cholesterol; HDL: High density lipoprotein; LDL: High density lipoprotein; Na: Sodium; K: Potassium; Total Prot: Total protein; Alb: Albumin and A/G: Albumin/globulin ratio.

historical control ranges and as there was no dose-relationship, these variations were not attributed to treatment. Plasma glucose (Gluc) and sodium (Na) concentrations were slightly but statistically significant higher in mid or high dose females. Total protein (Total Prot) concentration was slightly but statistically significantly high for high dose females but all individual values (apart from one Na concentration in one high dose female) were within the historical control range (98-percentile range: 4.98–10.73 mmol/L for glucose, 136–145 mmol/L for sodium; and 61–79 g/L for total protein, n = 104) and, consequently, these variations were also not attributed to treatment.

Table 6. Clinical chemistry results of female (F) rats. Group mean values and standard deviation (SD).

Clinical chemistry	Units	Groups of female rats (dose level in mg TOS/kg bw/day)							
		1F (0)		2F (110.3)		3F (363.8)		4F (1102.5)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
ALP	U/L	39	(7.9)	36	(9.5)	44	(10.4)	37	(8.0)
ALT	U/L	46	(8.4)	45	(10.6)	43	(5.8)	47	(7.6)
AST	U/L	84	(30.5)	73	(12.6)	78	(16.1)	84	(18.2)
Bi Ac	µmol/L	53.7	(29.10)	33.8	(13.49)	37.4	(24.95)	33.0*	(16.21)
Urea	mmol/L	6.79	(0.729)	6.86	(0.665)	6.36	(0.865)	6.72	(1.406)
BUN	mmol/L	6.79	(0.729)	6.86	(0.665)	6.36	(0.865)	6.72	(1.406)
Creat	µmol/L	30	(3.2)	31	(2.5)	30	(4.3)	30	(3.2)
Gluc	mmol/L	6.68	(0.777)	7.44	(0.855)	7.66*	(0.591)	7.89*	(1.534)
Chol	mmol/L	2.04	(0.397)	2.30	(0.569)	2.26	(0.385)	2.06	(0.256)
HDL	mmol/L	1.69	(0.313)	1.87	(0.402)	1.90	(0.307)	1.72	(0.212)
LDL	mmol/L	0.12	(0.031)	0.16	(0.067)	0.16	(0.057)	0.12	(0.035)
Na	mmol/L	141	(1.1)	142	(1.4)	142*	(1.3)	142*	(1.6)
K	mmol/L	3.85	(0.107)	3.95	(0.318)	3.92	(0.165)	3.74	(0.213)
Total Prot	g/L	72	(3.5)	73	(1.8)	74	(2.4)	75*	(3.0)
Alb	g/L	43	(1.8)	44	(1.6)	44	(1.5)	44	(1.8)
A/G	Ratio	1.46	(0.099)	1.47	(0.113)	1.47	(0.074)	1.41	(0.063)

Significantly different from the controls: *: $P < 0.05$; **: $P < 0.01$.
ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Bi Ac: Bile acids – total; BUN: Blood urea nitrogen; Crea: Creatinine; Gluc: Glucose; Chol: Total cholesterol; HDL: High density lipoprotein; LDL: High density lipoprotein; Na: Sodium; K: Potassium; Total Prot: Total protein; Alb: Albumin and A/G: Albumin/globulin ratio.

Table 7. *In Vitro* Irritancy Scores of the two test items and of the controls.

In Vitro Irritancy Score (IVIS)	UN GHS
≤3	No Category
>3; ≤55	No prediction can be made
>55	Category 1
Scoring key for category assignment based on IVIS	
Treatment	*In Vitro* Irritancy Score (IVIS)
Phytase HM (34000 FYT/g)	0.1
Phytase HM (65000 FYT/g)	0.1
Negative control	0.1
Positive control	44.0

IVIS results from treatment and controls. Phytase HM is classified as 'No Category'.

The serum concentrations of triiodothyronine (T3), thyroxine (T4) and thyroid stimulating hormone (TSH) concentrations were considered to have been unaffected by treatment.

There was no effect of treatment on estrous cycle at the end of the treatment period, and examination of the testes revealed normal progression of the spermatogenic cycle, and the expected cell associations and proportions in the various stages of spermatogenesis were present.

Lastly, there were no test item related findings on the organ weights, or during the macroscopic or microscopic observations of the organs.

It was concluded that oral administration of Phytase HM to Han Wistar rats at doses up to 100% of the test batch for 13 weeks was well-tolerated, with no evidence of any adverse finding at any of the administered doses. Consequently, the no-observed-adverse-effect level (NOAEL) was considered to be 1102.5 mg TOS/kg bw/day (equivalent to 357000 FYT/kg body weight/day).

According to the EFSA Guidance on the safety for the target species, the maximum safe concentration of an enzyme in feed can be derived from the NOAEL from toxicological studies in laboratory animals (EFSA, 2017a). The default feed intake (g DM/kg body weight) for the relevant target species, weaned piglets and chickens for fattening, is 44 and 79 g DM/kg BW/day, respectively. In the present case, where the NOEAL is 357000 FYT/kg BW/day, calculations point at a safe dose of 71400 FYT/kg feed in weaned piglets and 39767 FYT/kg feed in broiler chickens.

3.2. Skin and eye irritation

3.2.1. In vitro Bovine Corneal Opacity and Permeability Test

The purpose of the test was to identify if Phytase HM could induce serious eye damage and to determine if it should be classified according to the United Nations (UN) Globally Harmonized System of Classification and Labelling of Items (GHS). Interpretation of data follows the below prediction model for classification, and results are presented in Table 7. Based on the *In Vitro* Irritancy Score (IVIS) obtained and according to the UN GHS Classification, it was concluded that Phytase HM is given No Category classification under the conditions of the test.

3.2.2. In vitro skin irritation test using EpiSkin™ reconstructed human epidermis model

The purpose of this test was to evaluate the skin irritation potential of two Phytase HM formulations, Phytase HM (34000 FYT/g) and Phytase HM (65000 FYT/g), using the EPISKIN™ reconstructed human epidermis model. After the exposure period of 15 min followed by a post-exposure incubation period of 42 h, the relative mean viabilities of the test item treated tissues were calculated to be 104.3% and 109.2%, respectively. A test item is considered to be non-irritant if the relative mean viability is above 50%, thus it was concluded that Phytase HM under the conditions applied in this test was not classified for irritation (UN GHS No Category).

3.3. Bacterial reverse mutation assay

The results of the bacterial reverse mutation assay (Ames test) are presented in Table 8.

Table 8. Bacterial reverse mutation assay.

Compound	μg/plate	S9	TA98	TA100	TA1535	TA1537	WP2 *uvrA* pKM101
Purified water	-	-	53.3	158.3	25	13.3	150.7
Phytase HM	50	-	37.0 (0.7)	179.7 (1.1)	25.3 (1.0)	13.7 (1.0)	119.3 (0.8)
Phytase HM	150	-	55.0 (1.0)	153.3 (1.0)	22.0 (0.9)	10.3 (0.8)	128.0 (0.8)
Phytase HM	500	-	51.3 (1.0)	167.0 (1.1)	30.0 (1.2)	12.7 (0.9)	124.3 (0.8)
Phytase HM	1500	-	44.3 (0.8)	179.7 (1.1)	28.0 (1.1)	11.7 (0.9)	129.7 (0.9)
Phytase HM	5000	-	55.7 (1.0)	207.0 (1.3)	19.7 (0.8)	14.0 (1.0)	142.3 (0.9)
2NF	1.25	-	174.3 (3.3)				
NQO	0.3	-		1278.7 (8.1)			
NaN3	1.9	-			85.0 (3.4)		
AAC	31.3	-				71.0 (5.3)	
NQO	0.3	-					1028.3 (6.8)
Purified water	-	+	51	178.3	25	10	153.7
Phytase HM	50	+	57.0 (1.1)	147.0 (0.8)	24.0 (1.0)	8.3 (0.8)	141.3 (0.9)
Phytase HM	150	+	51.3 (1.0)	146.3 (0.8)	24.7 (1.0)	10.3 (1.0)	158.0 (1.0)
Phytase HM	500	+	55.3 (1.1)	154.7 (0.9)	25.3 (1.0)	10.3 (1.0)	159.0 (1.0)
Phytase HM	1500	+	41.7 (0.8)	163.3 (0.9)	23.7 (0.9)	9.0 (0.9)	170.7 (1.1)
Phytase HM	5000	+	55.3 (1.1)	164.7 (0.9)	24.3 (1.0)	7.3 (0.7)	164.3 (1.1)
B[a]P	9.4	+	152.3 (3.0)				
AAN	6.3	+		1337.0 (7.5)	148.0 (5.9)		
AAN	9.4	+				120.7 (12.1)	425.0 (2.8)

Mean number of revertant colonies per plate. Fold increase in revertants in treated cells relative to vehicle is reported in the parentheses. The fold increase ranges from 0.7 to 1.2 demonstrating no mutagenic potential of Phytase HM. Abbreviations; TOS: Total Organic Solids, NaN3: Sodium azide, 2-NF: 2-Nitrofluorene, NQO: 4-Nitroquinoline-1-oxide, AAC: 9-Aminoacridine, B[a]P: Benzo[a]pyrene, AAN: 2-Aminoanthracene.

Table 9. 3-hour treatment in the absence of S9 mix.

Treatment/Concentration (μg/mL)^	Mean Cytostasis (%)	Binucleated cells containing micronuclei			
		per 1000 cells	Mean	Trend test	
				p-value[b]	*p*-value[c]
Vehicle[a]	0.0	8	7.8		
		6			
		8			
		9			
Phytase HM/1250	0.7	8	7.0	1.000	
		6			
Phytase HM/2500	0.0[d]	8	7.0	1.000	0.534
		6			
Phytase HM/5000	0.0[d]	10	9.0	0.422	0.363
		8			
MMC/0.3	33.4	39	38.5	<0.001***	
		38			
COL/0.06	32.4	31	28.5	<0.001***	
		26			

No statistically significant increases in the induction of micronuclei were seen after three hours exposure without S9 and no dose response relationship was found.
^ Test item concentrations are reported in terms of Total Organic Solids (TOS);
a. Vehicle control = Water (10% v/v);
b. *p*-values are for comparisons to control using Williams' test and *t*-tests;
c. Trend test *p*-values are for the linear contrast including the control group and lower concentrations of the same test item;
d. Cytostasis reported as 0.0 as the CBPI value is equal to or higher than the vehicle control value. ***$p < 0.001$.
MMC: Mitomycin C; COL: Colchicine.

The absence of colonies on sterility check plates confirmed the absence of microbial contamination of the S9 mix, buffer and test item formulation. The viability counts confirmed that the viable cell density of the cultures of the individual organisms exceeded 10^9 cells/mL in all cases, and therefore met the acceptance criteria.

The mean revertant colony counts for the vehicle controls were within range of the historical controls. Appropriate positive control chemicals (with S9 mix where required) induced substantial increases in revertant colony numbers with all strains in all reported tests, confirming sensitivity of the cultures and metabolizing activity of the S9 mix. No evidence of toxicity was obtained following exposure to the Phytase HM preparation. No precipitate was observed on any of the plates.

Increase in mean revertant colony numbers of at least two-fold (three times in the case of strains TA1535 and TA1537, which have relatively low spontaneous reversion rates) is considered indicative of mutagenic activity in this test system. Phytase HM did no induce substantial increases in revertant colony numbers over control counts in any of the tester strains at any concentration up to

Table 10. 3-hour treatment in the presence of S9 mix.

Treatment/Concentration (μg/mL)^	Mean Cytostasis (%)	Binucleated cells containing micronuclei			
		per 1000 cells[$]	Mean	*p*-value[b]	Trend test *p*-value[c]
Vehicle[a]	0.0	6, 8	6.8		
		8, 9			
		6, 4			
		7, 6			
Phytase HM/1250	1.2	5, 7	5.0	1.000	
		5, 3			
Phytase HM/2500	4.4	6, 9	7.8	0.551	0.295
		8, 8			
Phytase HM/5000	2.5	8, 5	7.3	0.551	0.160
		9, 7			
CPA/10	47.3	20, 17	18.5	<0.001***	
		18, 19			

No statistically significant increases in the induction of micronuclei were seen after three hours exposure with S9 and no dose response relationship was found.
^ Test item concentrations are reported in terms of Total Organic Solids (TOS);
[$]Values presented from individual slides; underlined values obtained from additional analysis;
a. Vehicle control = Water (10% v/v);
b. *p*-values are for comparisons to control using Williams' test and *t*-tests;
c. Trend test *p*-values are for the linear contrast including the control group and lower concentrations of the same test item.
***$p < 0.001$.
CPA: Cyclophosphamide.

Table 11. 20-hour treatment in the absence of S9 mix.

Treatment/Concentration (μg/mL)^	Mean Cytostasis (%)	Binucleated cells containing micronuclei			
		per 1000 cells	Mean	*p*-value[b]	Trend test *p*-value[c]
Vehicle[a]	0.0	7	5.8		
		4			
		5			
		7			
Phytase HM/1250	1.4	5	4.5	1.000	
		4			
Phytase HM/2500	5.7	6	5.5	1.000	0.853
		5			
Phytase HM/5000	14.7	6	6.5	0.562	0.303
		7			
MMC/0.1	21.1	20	19.5	<0.001***	
		19			
COL/0.015	58.1	17	18.5	<0.001***	
		20			

No statistically significant increases in the induction of micronuclei were seen after 20 h exposure without S9 and no dose response relationship was found.
^ Test item concentrations are reported in terms of Total Organic Solids (TOS);
a. Vehicle control = Water (10% v/v);
b. *p*-values are for comparisons to control using Williams' test and *t*-tests;
c. Trend test *p*-values are for the linear contrast including the control group and lower concentrations of the same test item.
***$p < 0.001$.
MMC: Mitomycin C; COL: Colchicine.

and including 5000 μg/plate in either the presence or absence of S9 mix. 5000 μg/plate is the recommended maximum concentration in the current OECD Guideline 471 (OECD, 1997). Based on the present data, it was concluded that Phytase HM is not mutagenic in the Ames test.

3.4. *In vitro micronucleus test in cultured human lymphocytes*

For medium dosed with the Phytase HM enzyme preparation at 5000 μg TOS/mL no increases in osmolality of the medium of more than 50 mOsmol/kg and no fluctuations in pH of more than 1.0 unit were observed compared with the vehicle control. Further, no precipitation problems were seen and the test item caused no significant reductions in the cytokinesis-block proliferative index (CBPI) at any concentration tested, which means that concentrations of 1250, 2500 and 5000 μg TOS/mL were analyzed for micronucleus frequency. No statistically significant increases in the induction of micronuclei were seen under the experimental conditions tested and no dose response relationship was found (Tables 9, 10, and 11).

3.5. *Efficacy studies in broiler chickens and weaned piglets*

In the broiler chicken study (Table 12), the body weight gain and feed consumption of birds in NC was 10–15% lower than Cobb 500

Table 12. Growth performance, retention of Ca and P, phytate-P degradation in excreta and bone parameters of broilers supplemented with phytase HM.

	Phytase HM treatments, FYT/kg feed								SEM	*P*-value	Linear	Quadratic
Items	NC	187.5	375	750	1125	1500	1875	2250				
Body weight, g												
D 8	193	192	192	192	192	192	192	193	0.3	0.08	0.68	0.11
D 17	652[a]	687[b]	691[b]	710 [bc]	716 [bc]	710 [bc]	724[c]	716 [bc]	7.3	<0.01	<0.01	<0.01
D8 to 17												
WG, g	460[a]	495[b]	499[b]	518 [bc]	524 [bc]	518 [bc]	532[c]	523 [bc]	7.3	<0.01	<0.01	<0.01
FI, g	572[a]	600 [ab]	609 [ab]	618[b]	621[b]	608 [ab]	640[b]	622[b]	10.0	<0.01	<0.01	0.05
FCR, g/g	1.244	1.215	1.220	1.195	1.187	1.174	1.205	1.190	0.017	0.10	0.01	0.06
Excreta												
Ca retention, %	55.08[a]	63.00[b]	66.03[b]	73.14[c]	74.64[c]	75.70[c]	73.43[c]	73.19[c]	0.84	<0.01	<0.01	<0.01
P retention, %	64.41[a]	71.46[b]	75.63[c]	80.52[d]	82.48[d]	83.31[d]	81.95[d]	81.87[d]	0.65	<0.01	<0.01	<0.01
Phytate-P degradation, %	64.01[a]	77.03[b]	82.99[c]	89.17[d]	92.88 [de]	94.38[e]	91.86 [de]	94.56[e]	0.97	<0.01	<0.01	<0.01
Bone												
Ash, %	46.80[a]	50.14[b]	51.22 [bc]	52.36 [cd]	52.93 [cde]	53.56 [de]	54.67[e]	53.74 [de]	0.46	<0.01	<0.01	<0.01
Ca, %	17.10[a]	18.26[b]	18.57 [bc]	19.07 [bcd]	19.22 [cd]	19.38 [cd]	19.96[d]	19.38 [cd]	0.20	<0.01	<0.01	<0.01
P, %	8.05[a]	8.61[b]	8.91 [bc]	9.22 [cd]	9.38 [de]	9.44 [de]	9.67[e]	9.39 [de]	0.09	<0.01	<0.01	<0.01

Means with different superscript within the same row differ significantly ($P < 0.05$).
* Phytate-P consisted of the P bound by phytate (and its degradation products).
The negative control (NC) was deficient in phosphorus (P). The phytase HM treatments were NC supplemented with phytase HM at the indicated FYT/kg feed.
SEM: standard error of mean; WG: weight gain; FI: feed intake; FCR: feed conversion ratio; Ca: calcium.

Table 13. Growth performance and bone parameters of the piglets supplemented with phytase HM.

			Phytase HM treatments, FYT/kg feed					*P* - value		
Items	PC	NC	187.5	375	1500	3000	SEM	PC vs. NC	Linear	Quadratic
Body weight, kg										
D 0	7.4	7.5	7.5	7.4	7.4	7.5	0.05	0.23	0.81	0.34
D 14	10.9	10.1	10.6	10.0	10.8	10.5	11.1	0.33	0.13	0.05	0.94
D 42	25.9	19.6	22.5	22.6	25.3	25.7	27.0	0.70	<0.01	<0.01	<0.01
D 0 to 14										
ADG, g/d/pig	247	181	221	184	238	221	258	24	0.06	0.04	0.74
ADFI, g/d/pig	369	324	351	294	384	337	373	26	0.24	0.19	0.86
FCR, g/g	1.52	1.82	1.61	1.64	1.65	1.53	1.44	0.08	0.02	0.01	0.44
D 14 to 42										
ADG, g/d/pig	537	338	425	448	521	541	570	19	<0.01	<0.01	<0.01
ADFI, g/d/pig	721	432	564	578	707	627	717	25	<0.01	<0.01	<0.01
FCR, g/g	1.34	1.27	1.32	1.28	1.36	1.16	1.27	0.06	0.41	0.41	0.43
D 0 to 42										
ADG, g/d/pig	440	286	357	360	426	434	466	17	<0.01	<0.01	<0.01
ADFI, g/d/pig	603	396	493	483	599	530	602	21	<0.01	<0.01	<0.01
FCR, g/g	1.40	1.45	1.42	1.40	1.46	1.28	1.33	0.05	0.48	0.05	0.33
Bone, %										
Strength, N	366	95	171	229	275	344	417	28	<0.01	<0.01	<0.01
Ash	63.1	56.8	59.9	60.9	60.4	61.8	63.6	0.77	<0.01	<0.01	0.09
Ca	23.6	21.4	22.7	23.0	22.6	23.3	24.0	0.31	<0.01	<0.01	0.12
P	11.0	9.6	10.2	10.4	10.4	10.9	11.3	0.15	<0.01	<0.01	0.02

The positive control (PC) was adequate in all dietary nutrients and the negative control (NC) was deficient in phosphorus (P). The phytase HM treatments were NC supplemented with phytase HM at the indicated FYT/kg feed.
SEM: standard error of mean; ADG: average daily gain; ADFI: average daily feed intake; FCR: feed conversion ratio; Ca: calcium.

performance targets during day 8–17 of age, which was attributed to the P deficiency in NC diet. The increasing addition of the Phytase HM to the NC diet improved the 8–17 d body weight gain and feed intake of birds both linearly and quadratically ($p < 0.01$). The achievement of performance targets was observed in treatments with high doses of Phytase HM while no adverse effect was noted. Additionally, the data from excreta demonstrated that the P release from phytate-P degradation, and the corresponding retention of P and Ca were improved both linearly and quadratically ($p < 0.01$) with the increase of Phytase HM supplementation. Consistent with the performance and excreta results, Ca and P were increasingly deposited in the bone ash of broilers at 8–18 d of age in a dose-dependent manner with the increase of Phytase HM addition.

In the piglet study (Table 13), the growth performance of piglets was depressed by deficiency of P in feed as demonstrated by the significant reduction in ADG and ADFI of the NC piglets in comparison to PC during day 14–42 and the overall trial duration, which was gradually corrected by the increasing addition of the Phytase HM. The test phytase improved ADG and ADFI during day 14–42 and the overall trial duration both linearly and quadratically ($p < 0.01$) with the increasing dose of Phytase HM. This pattern was also observed with the average body weight of the piglets at the end of the trial. In keeping with the growth performance results, the significant improvement ($p < 0.01$) in bone strength and bone content of ash, Ca and P in association with added Phytase HM also showed a dose-response relationship. This concurs with the general trend that the release of phytate-bound P increases with increasing phytase dose and then reaches a plateau when phytate is depleted (Cowieson et al., 2016). Moreover, the growth performance and bone measurements achieved at 3000 FYT/kg feed, the highest dose of Phytase HM tested in the current study, exceeded the levels of the PC without causing any noticeable adverse effect during the trial, which agrees with the well-known extra-phosphoric effects of phytase (Lu et al., 2019). This finding implies that both the Ca and P supplied in the form of dicalcium phosphate in the PC diets could be completely replaced by Phytase HM when included at 3000 FYT/kg feed under the current experimental conditions. Research is warranted to guarantee the complete removal of inorganic P with phytase supplementation will not affect the growth and health performance of pigs from a whole life cycle perspective.

4. Conclusion

The efficacy studies in broiler chickens and piglets confirmed the efficacy of Phytase HM by salvaging the poor growth performance and bone imperfections caused by dietary P deficiency, and no safety concerns were revealed. The retention of Ca and P, and phytate-P degradation in excreta of broilers provided direct evidence of the Phytase HM on liberation and utilization of P from phytate. Similar results for both performance improvement and retention were obtained in piglets. Moreover, data from the piglet trial showed that 3000 FYT/kg of Phytase HM may enable replacement of all the inorganic P to meet the animal's requirement for P under current experimental conditions.

A toxicological evaluation of Phytase HM was carried out as prescribed by the European Food Safety Authority (EFSA, 2012). The studies were performed at the highest dose levels required by the current OECD guidelines. The toxicological studies included repeat dose oral toxicity in rats for 13 weeks, *in vitro* skin and eye irritation studies, and Ames and micronucleus genotox testing. All toxicological studies on Phytase HM were concluded to be favorable. Based on the efficacy trials in broiler chickens and pigs, the toxicological data and the fact that the *Aspergilus oryzae* production organism derives from a safe strain lineage, it is concluded that there are no reasons for safety concerns, when using Phytase HM as a feed additive.

In conclusion, Phytase HM is an advantageous additive to broiler chicken and pig feed. The positive safety evaluation of Phytase HM is in line with the fact that this type of enzyme is ubiquitous in nature. However, from a workers' safety perspective, Phytase HM should, like any other enzyme, be handled according to the standard safety guidelines for enzymes to avoid respiratory sensitization.

Declarations

Author contribution statement

Michael Thorsen; Heng-Xiao Zhai; Qian Zhang; Line Anker Nielsen: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Linda Wulf-Andersen; Lars Kobberøe Skov: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Most data is mentioned in the manuscript. Some data is confidential, but summary can be shared upon request.

Declaration of interests statement

The authors have affiliated with companies with commercial interest in animal feed but none of the authors has any conflicts of interest concerning this paper.

Additional information

No additional information is available for this paper.

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