NUTRITION

Supplementation of an all-plant-based inorganic phosphate-free diet with a novel phytase maintained tibia ash and performance in broilers under a commercial production setting

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Primary Audience: Poultry Nutritionists, Poultry Producers.

SUMMARY

Removal of inorganic phosphate (**Pi**) from broiler diets could improve sustainability in poultry production. Small-scale studies of total Pi replacement by a novel consensus bacterial 6-phytase variant have been promising but larger scale studies are needed. In this study, effects of 4 diets on growth performance and bone quality were evaluated: PC1, nutritionally adequate and containing Pi without enzyme supplementation; PC2, as PC1 but containing xylanase (2,000 units [XU]/kg) and 75 kcal/kg reduction in ME; IPF1 and IPF2, as PC1 and PC2, respectively, formulated without Pi and 1.5 g/kg reduction in Ca, containing phytase at 3,000, 2,000, and 1,000 phytase units (FTU)/kg during starter (1−10 d), grower (10−22 d), and finisher (22−37 d) phases. A randomized complete block design used 820 Ross 308 mixed-sex birds/pen and 8 pens/diet. The phytase in IPF diets maintained all growth performance parameters, per phase and cumulatively, equivalent to the respective PC. Contrast analysis of pooled PC vs. pooled IPF data showed that phytase in IPF diets reduced or maintained FCR during all phases and cumulatively (−5.0 points during 22−37 d and −3.0 points during 1−37 d; $P < 0.05$). Tibia ash at d 21 and 36 was not different in birds fed IPF and PC diets, but tibia breaking strength was higher in birds fed IPF diets (+6.6% and +1.3%, respectively; $P < 0.05$). Overall (0−37 d), total feed costs per kilogram BW gain were reduced ($P < 0.05$) in the phytase supplemented IPF diets vs. PC diets under commercial broiler production conditions.

Key words: broiler, bacterial 6-phytase, growth performance, inorganic phosphate, tibia ash

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DESCRIPTION OF THE PROBLEM

Phosphorus (**P**) is an essential component of broiler diets that is critical for normal growth and bone development, egg formation, nutrient metabolism, and protein synthesis (Li et al., 2016). Inorganic phosphate (**Pi**) is routinely added to commercial broiler diets to ensure phosphorus requirements are met. However, as a natural resource, the availability of Pi is finite.

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Global supply is decreasing year on year, was expected to fall below 7 million tons by 2020 (FAO, 2017) and may be used up within the next 100 yr. Current commercial diets containing phytase supplemented at 1,000 phytase units (FTU)/kg may contain around 0.3 to 0.4% of monocalcium phosphate (**MCP**) (average across all feeding phases). This equates to an annual usage of MCP of around 1 million tons based on a total estimated broiler feed production of 307.3 million ton globally in 2019 (Alltech Global Feed Survey; Alltech, 2020). Carbon dioxide emissions per ton of MCP have been estimated to range from 569 (Feedprint NL, Wageningen UR, 2020) to as much as 1,202 kg/ton (Mosnier et al. 2011). With a growing interest in improving the sustainability and reducing the environmental impact of broiler diets, there is a need to develop strategies that remove the need to add Pi to the feed.

Certain plant-derived feed ingredients are particularly rich in P, for example oilseed meals and cereal brans such as rice bran, wheat middlings, and rapeseed meal (P content: 1.72, 1.08, and 0.97%, respectively) (Selle and Ravindran, 2007; Li et al., 2016). Replacement of part of the soybean meal, corn and/or wheat base of the diet with high P-containing ingredients such as these presents an opportunity to increase the overall P content of the diet. It can also widen the variety of potential source ingredients (which could be advantageous from a feed cost perspective) that may be sourced more locally (which may improve sustainability). However, the availability of the P in feedstuffs must also be considered. The majority of P in plant-based ingredients is in the form of phytate (salt of phytic acid, myo-inositol hexaphosphate; IP_6) (Selle and Ravindran, 2007), which is poorly hydrolyzed without addition of exogenous microbial phytase to the diet (Leske and Coon, 1999). The efficacy of microbial phytase for degrading plant-derived phytate, thereby improving P-availability and utilization and reducing unwanted P-excretion into the environment is well demonstrated (Lei et al., 2013; Humer et al., 2015). Phytase has enabled some reduction in the amount of Pi that must be added to the feed due to the expected P-contribution of the phytase. However, total replacement of dietary Pi by phytase from day one to market age has seldom been studied in broilers. Early studies such as those by Temperton and Cassidy (1964a,b,c) with White Leghorn × Buff Rock pullets, provided valuable information in determining inherent phytate P (**PP**) utilization and overall P physiological requirements in poultry, and birds fed Pi-free diets in these studies grew normally. However, the breed used in these early studies was considerably slower growing and later maturing than current commercial breeds. As such, the responses are unlikely to be representative of modern commercial breeds (such as Ross 308) fed modern dietary formulations. More recently, a single study involving a commercial broiler breed showed that microbial phytase can totally replace Pi from the grower phase (21 d of age onward; Ribeiro et al., 2019), but only the most recent studies by Marchal et al. (2021) and Dersjant-Li et al. (2022) have demonstrated total Pi replacement by phytase during an entire growth cycle that includes a starter phase. Total Pi removal is a particular challenge during the starter phase because P requirements are high (available P dietary requirements recommended by Aviagen Inc. for Ross 308 broilers are 0.48% during d 0−10), reflecting a period of rapid bone growth and development.

Two small-scale trials of a novel consensus bacterial 6-phytase variant (**PhyG**) added to Pi-free diets that were rich in phytate (3.4−3.6 g/kg PP), reported improved or maintained growth performance, bone mineralization and − strength compared with a nutritionally adequate diet without phytase from 1 to 42 d of age (Marchal et al. 2021). These results were obtained when the phytase was added at a dose level of 3,000, 2,000, and 1,000 FTU/kg during starter, grower and finisher phases, respectively, in the presence or absence of xylanase to aid digestion of fiber. In a follow-up study by Dersjant-Li et al. (2022), in which the number of birds per pen was increased (from < 30 to 52) and PP levels in the finisher 1 and 2 phases were reduced to 2.9 and 2.8 g/kg, it was shown that PhyG totally replaced dietary Pi in both male and female birds (i.e., there was no sex effect). The objective of the current study was to evaluate the effect of PhyG in diets containing no added Pi fed to birds reared under commercial broiler production settings. In such settings, the stocking density is higher, environmental

conditions are more variable, and birds are exposed to a higher horizontal spread of health-challenging issues than in typical small-scale research settings. These factors can negatively affect bird health, growth performance, feed utilization, and animal welfare (Dawkins et al., 2004; Buijs et al., 2009; Houshmand et al., 2012; Abudabos et al., 2013; Wang et al., 2019) and may also impact on phytase efficacy. As in the studies by Marchal et al. (2021) and Dersjant-Li et al (2022), xylanase was included in the study design to increase ME digestibility by increasing fiber degradation and by decreasing viscosity and water holding capacity of the intestinal digesta (Liu et al., 2011; Kiarie et al., 2014), and to enable feed cost savings (Dersjant-Li et al., 2022). A phased phytase dosing strategy was employed in parallel with a high dietary PP level during the starter and grower phases in accordance with Marchal et al. (2021) and Dersjant-Li et al. (2022), but with the adjustment of a lower PP level in the finisher diet (2.6 g/kg PP, formulated value). This was done to better reflect current commercial practice and may help to further reduce P excretion into the environment.

MATERIALS AND METHODS

All experimental protocols and animal care procedures were approved by the Institutional Animal Care and Use Committee of Schothorst Feed Research B.V. (Lelystad, the Netherlands) and conformed to the European Union Guidelines on the protection of animals used for scientific purposes (Directive 2010/63/EU; European Council, 2010).

Birds, Housing and Experimental Design

A total of 26,240 Ross 308 one-day-old broilers (mixed male and female) were randomly assigned to 32 floor pens with 820 birds per pen and 8 pens per treatment in a randomized complete block design with four dietary treatments. Stocking density at 37 days (d) of age was 37.7 kg/m^2. Fresh wood shavings were used as litter. Pens were housed in a broiler house in which environmental conditions were closely maintained according to primary breeder recommendations (Aviagen Inc, 2018). The lighting regime was light-dark (LD) 24:0 h for the first 24 h followed by LD 22:2 h. From 3 d of age onward, the light-dark (**LD**) cycle was LDLD 8:4:10:2 h. Temperature was initially maintained at 34.5°C and gradually reduced to 20°C by 37 d of age. Humidity was controlled by mechanical ventilation. Diets and water were provided ad libitum for the duration of the study (37 d). Water was provided via 72 nipple drinkers per pen.

Experimental Diets

Experimental diets were mixed-grain diets based on corn, wheat, soybean meal, and sunflower meal, with added soy oil and wheat middlings. Oat hulls were included to stimulate gizzard development. The full composition of the diets is given in Table 2.

The positive control diets (PC1 and PC2) were formulated by phase to supply adequate levels of all nutrients (Centraal Veevoederbureau, 2018) containing Pi from MCP and a graded reduction in PP content by phase (PP: 3.3 g/kg in starter [1 to 10 d of age], 3.1 g/kg in grower [10−22 d of age], and 2.6 g/kg in finisher phase [22−37 d of age]; retainable P: 3.8 g/kg in starter, 3.0 g/kg in grower and 2.6 g/kg in finisher phase). Retainable P levels in the PC diets were marginal compared to breeder recommendations to ensure that they were nutritionally adequate but not in excess relative to the phase-specific physiological

Table 1. Details of the experimental treatments.

	PhyG, FTU/kg			Xylanase, XU/kg	Nutrient reductions vs. respective PC
Treatment	1 to 10 d of age	10 to 22 d of age	22 to 37 d of age	All phases	All phases
PC1		None		None	none
PC2		None		2,000	ME -75 kcal/kg
IPF1	3,000	2,000	1,000	None	Pi-free, Ca -1.5 g/kg
IPF2	3,000	2,000	1,000	2,000	Pi-free, Ca -1.5 g/kg, ME -75 kcal/kg

Abbreviations: IPF, inorganic phosphate-free; Pi, inorganic phosphate.

Table 2. Ingredient (g/kg) and calculated nutrient composition (%) of the experimental diets (as fed basis).

	Starter (1 to 10 d of age)				Grower (10 to 22 d of age)				Finisher (22 to 37 d of age)			
	PC1	PC2	IPF1	IPF2	PC1	PC2	IPF1	IPF2	PC1	PC2	IPF1	IPF2
Ingredient, g/kg, as fed												
Corn	308	308	308	308	303	303	303	303	399	399	399	399
Wheat	200	200	200	200	250	250	250	250	250	250	250	250
Soybean meal (48% CP)	246	246	246	246	227	227	227	227	220	220	220	220
Sunflower meal (37% CP)	115	115	115	115	90.3	90.3	90.3	90.3	41.9	41.9	41.9	41.9
Wheat middlings	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0	10.0	10.0	10.0	10.0
Soybean oil	43.8	34.8	43.8	34.8	44.9	35.9	44.9	35.9	35.2	26.2	35.2	26.2
Palm oil	0.00	0.00	0.00	0.00	1.92	1.92	1.92	1.92	0.00	0.00	0.00	0.00
Oat hulls	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Soy hulls	0.15	9.15	10.60	19.60	0.15	9.15	8.56	17.56	0.15	9.15	7.89	16.89
Limestone	12.24	12.24	13.60	13.60	12.14	12.14	11.79	11.79	11.43	11.43	10.52	10.52
Monocalcium phosphate	11.81	11.81	0.00	0.00	8.06	8.06	0.00	0.00	6.83	6.83	0.00	0.00
L-Lysine HCL	3.83	3.83	3.83	3.83	3.59	3.59	3.59	3.59	3.10	3.10	3.10	3.10
L/DL-Methionine	2.84	2.84	2.84	2.84	2.54	2.54	2.54	2.54	2.31	2.31	2.31	2.31
L-Threonine	1.25	1.25	1.25	1.25	1.17	1.17	1.17	1.17	1.04	1.04	1.04	1.04
L-Valine	0.81	0.81	0.81	0.81	0.74	0.74	0.74	0.74	0.71	0.71	0.71	0.71
Salt	3.17	3.17	3.17	3.17	2.88	2.88	2.88	2.88	2.16	2.16	2.16	2.16
Sodium bicarbonate	0.94	0.94	0.94	0.94	1.00	1.00	1.00	1.00	2.11	2.11	2.11	2.11
Vitamin-mineral premix[1]	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	4.00	4.00	4.00	4.00
Premix Sacox[2]	5.82	5.82	5.82	5.82	5.80	5.80	5.80	5.80	5.80	5.80	5.80	5.80
Calculated nutrients, g/kg												
ME, kcal/kg	2,800	2,725	2,800	2,725	2,875	2,800	2,875	2,800	2,950	2,875	2,950	2,875
Crude protein	220	220	220	220	206	206	206	206	187	187	187	187
Crude fat	71.5	62.6	71.5	62.6	74.2	65.3	74.2	65.3	63.7	54.7	63.7	54.7
Calcium	8.00	8.00	6.50	6.50	7.20	7.20	5.70	5.70	6.50	6.50	5.00	5.00
Phosphorus (P)	7.17	7.17	4.49	4.49	6.05	6.05	4.22	4.22	5.16	5.16	3.61	3.61
Retainable P	3.80	3.80	1.52	1.52	3.00	3.00	1.44	1.44	2.60	2.60	1.28	1.28
Phytate-P	3.30	3.30	3.30	3.30	3.10	3.10	3.10	3.10	2.60	2.60	2.60	2.60
Dig Lysine	11.8	11.8	11.8	11.8	11.0	11.0	11.0	11.0	10.0	10.0	10.0	10.0
Dig Methionine+Cysteine	8.65	8.65	8.66	8.66	8.03	8.03	8.04	8.04	7.30	7.30	7.31	7.31
Dig Threonine	7.67	7.67	7.68	7.68	7.15	7.15	7.16	7.16	6.50	6.50	6.50	6.50
Dig Tryptophan	2.25	2.25	2.25	2.25	2.11	2.11	2.11	2.11	1.89	1.89	1.89	1.89
Dig Valine	9.44	9.44	9.45	9.45	8.80	8.80	8.81	8.81	8.00	8.00	8.00	8.00
Dig Arginine	13.1	13.1	13.1	13.1	12.0	12.0	12.0	12.0	10.6	10.6	10.6	10.6
Dig Isoleucine	7.79	7.79	7.79	7.79	7.26	7.26	7.26	7.26	6.60	6.60	6.60	6.60

Abbreviation: IPF, inorganic phosphate-free.

[1]Vitamin and mineral premix supplied per kg diet (based on a 0.5% dose): Vitamin A 10,000 IU; vitamin D3 3,333 IU; vitamin E 50 mg; vitamin K3 2.5 mg; vitamin B1 2.5 mg; vitamin B2 7.5 mg; vitamin B6 5 mg; vitamin B12 25 mcg; Niacin 50 mg; D-pantothenic acid 15 mg; Choline chloride 500 mg; Folic acid 1.5 mg; Biotin 0.25 mg; Fe 50 mg (as $FeSO_4.H_2O$); Cu 12.5 mg (as $CuSO_4.5H_2O$); Mn 75 mg (as MnO); Zn 70 mg (as $ZnSO_4.H_2O$); I 2 mg (as $Ca(IO_3)_2$); Se 0.25 mg (as Na_2SeO_3); antioxidants (Luctanox EF 5 mg; BHT 2.01 mg, propyl gallate 0.17 mg).

[2]Premix Sacox contained: 12,000 mg salinomycin sodium (Huvepharma NV), delivering 70 mg/kg in feed with a 0.582% inclusion rate.

requirements of the birds. The PC2 diet was equivalent to PC1, except that it was supplemented with 2,000 XU/kg of a commercial xylanase produced in *Trichoderma reesei* (Danisco xylanase, Danisco Animal Nutrition & Health, International Flavors & Fragrances ([**IFF**] Inc., New York, NY) and formulated with a 75 kcal/kg reduction in ME during all phases to account for the expected energy contribution of the enzyme. Two experimental diets (IPF1 and IPF2) based on PC1 and PC2, respectively, were reformulated as inorganic phosphate free (**IPF**) with a reduction in Ca content (−1.5 g/kg vs. PC) and supplemented with PhyG at 3,000, 2,000, and 1,000 FTU/kg in starter, grower and finisher phases, respectively. The PhyG phytase was a novel consensus bacterial-6 phytase variant expressed in

Trichoderma reesei (Axtra PHY GOLD, Danisco Animal Nutrition & Health, International Flavors & Fragrances (IFF) Inc.). A negative control (**NC**) diet was not tested as a standalone diet, because the nutrient limitations in such a diet would have had a detrimental effect on the health and welfare of the birds and would not have been in compliance with the European Guidelines on the protection of animals used for scientific purposes (EU Directive 2010/63/EU; European Council, 2010). Diets were provided as phased diets in crumbled form during the starter and in pelleted form (3.0 mm pellet) during the grower and finisher phases. The experimental diets were prepared by a commercial feed mill (ABZ Diervoeding, Nijkerk, the Netherlands). Enzymes were premixed with corn to ensure a homogenous distribution within the diet. Following mixing, the diets were pelleted at a maximum pelleting temperature of 84˚C.

Measurements and Sampling

Individual BW was measured for 180 birds selected at random on arrival at the experimental farm and used to calculate average BW per bird. Bird BW was continuously monitored per pen by automatic weighing scales. At 37 d of age all birds were collectively weighed per pen. Pens were checked daily for mortality and dead birds removed. Feed and water consumption were recorded per pen at the end of each phase (10, 22, and 37 d of age) and used to calculate average feed intake (**FI**), water intake (**WI**) and water-to-feed intake ratio (**WF**) per bird per feeding phase. Feed conversion ratio was calculated, corrected for mortality weight.

At 21 and 36 d of age, 4 male birds per pen were euthanized by CO_2 asphyxiation, and the left and right tibia bones were collected. Left tibias were used to determine tibia breaking strength. Right tibias were pooled per pen and analyzed for ash content. At 36 d of age, left humerus bones were collected in addition to tibias for breaking strength analysis. Collected bones were frozen at −20˚C before analysis. Breaking strength was analyzed following thawing for 4 h to a temperature of 20˚C and was determined according to a three-point bending set up, using the following procedure: The width and thickness of each bone was first measured (mm), then the bone was positioned onto 2 supports, spaced according to the bone type and sampling time-point (40 mm apart for tibias collected at 21 d of age, 45 mm for tibias collected at 36 d of age, and 40 mm for humeri collected at 36 d of age). An increasing force (measured in Newton) was applied to the middle of the bone until it fractured. Flexural strength was measured in terms of stress (unit: MPa) according to test method ISO178 for the determination of flexural properties of rigid plastics (ISO, 2019).

Chemical Analysis

Tibias were stripped of adjacent tissues and dried overnight initially at 40˚C and subsequently at 70˚C. Fat was extracted with 100% petroleum ether using a Soxhlet apparatus (Thermo Fisher Scientific, Roskilde, Denmark), in accordance with the methods described by Watson et al. (2006). De-fatted tibias were dried for 4 h at 103˚C and ashed for 24 h at 700˚C in a muffle furnace for the determination of ash content in defatted dry matter.

Representative samples of all diets were analyzed for dry matter (**DM**), crude protein (**CP**), crude fat, crude fiber, calcium (**Ca**), P, PP and phytase and xylanase activities. Nutritional analyses were performed by Schothorst Feed Research (Lelystad, The Netherlands). Phytate-P concentrations and phytase activities in the diets were determined by Danisco Animal Nutrition Research Centre (Brabrand, Denmark). Samples were analyzed in duplicate for all analyses. Nutrients were analyzed according to the following methods: moisture, NEN-ISO 6496 (ISO, 1999a); crude protein, NEN-EN-ISO 16634 (ISO, 2008); crude fat, NEN-ISO 6492 (ISO, 1999b); crude fiber, NEN-ISO 6865 (ISO, 2000); starch, NEN-ISO 15914 (ISO, 2004); phosphorus, NEN-ISO 6491 (ISO, 1999c); calcium, NEN-ISO 6869 (ISO, 2001) and; ash, NEN-ISO 5984 (ISO, 2002). Phytase activities were determined according to a modified version of the 2000.12 AOAC method (Engelen et al., 2001) where one phytase unit (FTU) was defined as the amount of enzyme that released 1 μmol of inorganic orthophosphate from a sodium phytate

Table 3. Analyzed nutrient concentrations (g/kg, as is basis, otherwise stated) and enzyme activities in the experimental diets[1].

	Starter (1 to 10 d of age)				Grower (10 to 22 d of age)				Finisher (22 to 37 d of age)			
	PC1	PC2	IPF1	IPF2	PC1	PC2	IPF1	IPF2	PC1	PC2	IPF1	IPF2
GE, kcal/kg	4,217	4,176	4,258	4,211	4,170	4,142	4,211	4,168	4,050	4,028	4,113	4,047
Dry matter	910	909	908	910	907	905	906	903	894	920	893	895
Crude protein	221	227	225	227	213	215	215	216	192	191	194	193
Crude fiber	52.0	52.0	52.0	54.2	40.4	45.3	44.4	47.0	33.0	37.1	36.5	40.0
Crude fat	71.2	63.1	70.5	64.2	71.8	64.1	70.0	62.0	61.1	53.6	62.8	53.6
Ash	55.7	56.9	49.5	49.5	53.4	54.2	47.4	49.4	53.4	47.8	41.2	47.3
Calcium	8.30	8.70	6.70	6.80	7.30	7.50	5.40	6.10	9.20	7.00	5.20	7.20
Total P	7.30	7.40	4.90	4.70	6.50	6.50	4.90	5.00	5.80	5.30	3.80	3.70
Phytate-P, calculated from analyzed ingredients[2]	3.26	3.27	3.27	3.27	3.10	3.11	3.11	3.11	2.67	2.68	2.68	2.68
Phytate-P, analyzed in final diet[3]	3.15	3.23	n/a	n/a	3.32	n/a	3.31	n/a	2.69	n/a	2.72	n/a
Phytase, FTU/kg	246	265	3,458	3,239	334	245	2,587	2,036	220	179	1,093	1,286
Xylanase, XU/kg	<100	1,849	<100	1,501	<100	1,534	<100	1,413	<100	772	<100	2,104

Abbreviation: IPF, inorganic phosphate-free.

[1]Each diet sample was analyzed in duplicate.

[2]Calculated from the sum of analyzed phytate-P in individual ingredients including soy hulls (as presented in Table 4) based on their inclusion level in the diet.

[3]n/a, not analyzed.

substrate per minute at pH 5.5 and 37°C. Xylanase activity was determined according to the method of Romero et al. (2014), where one xylanase unit (**XU**) was defined as the amount of enzyme that released 0.48 μmol of reducing sugar as xylose from wheat arabinoxylan per minute at pH 4.2 and 50°C. Phytate-P concentrations were determined by Danisco Animal Nutrition Research Centre using a modified version of the HPLC method described by Skoglund et al. (1997).

Calculations

Feed conversion ratio was calculated based on BW gain (**BWG**) corrected for mortality weight, and FI during 1 to 10, 10 to 22 and 22 to 37 d of age. Mortality weight was estimated as 80% of the average body weight (measured by automatic weighing scale) in the pen on the day when the bird was removed.

Statistical Analysis

Pen was the experimental unit in all analyses. Data were analyzed by one-way ANOVA and by contrast analysis to compare responses in PC and IPF diets. Block was included as a random effect. Data were analyzed using the Fit Model platform of JMP 16 (JMP, 2020; SAS Institute Inc., Cary, NC). Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Diet Analysis

Analyzed nutrients and phytase activities in the final diets are given in Table 3, while the analyzed phytate and PP content of individual feed ingredients is presented in Table 4.

Table 4. Analyzed phytate content (g/kg) of individual ingredients (as is basis).

	Phytate[1] (g/kg)	Phytate-P[2](g/kg)
Corn	7.2	2.0
Wheat	8.7	2.5
Soybean meal (48% CP)	13.3	3.7
Sunflower meal (37% CP)	28.9	8.2
Wheat middlings	28.9	8.1
Oat hulls	0.4	0.1
Soya hulls	2.3	0.6

[1]Determined using a modified version of the HPLC method described by Skoglund et al. (1997).

[2]Calculated as analyzed phytate*28.2% in accordance with published information on the P content of phytate (Angel et al., 2002).

Concentrations (g/kg basis) of all analyzed nutrients were close to formulated values, except Ca during the finisher phase, which was moderately higher than expected in PC1 and IPF2 diets (+2.7 g/kg and +2.2 g/kg vs. formulated values, respectively). In theory, higher than expected dietary Ca levels could, especially in conjunction with the lower P level in the IPF diets, lead to an imbalance with Ca and P, which could negatively affect P availability and utilization. Numerous studies have shown that high dietary Ca can negatively affect growth performance in young broilers, especially when P is deficient (Amerah et al., 2014; Li et al., 2015; Dersjant-Li et al., 2018). However, during the finisher phase the P requirement is lower and growth performance was similar between PC1 and PC2, as well as between IPF1 and IPF2, indicating that the high analyzed Ca levels did not have any overriding negative effect on growth performance in these treatments.

The PP content of the soy hulls that were used as a filler ingredient in the IPF diets was low relative to that of the main plant ingredients (corn, wheat, soybean meal, sunflower meal, and wheat middlings), and that of oat hulls was even lower (Table 4). This, along with their low level of inclusion in the overall diet contributed very little to the formulated total PP value of the diets (Table 2). Analyzed PP values (where available) and calculated values (based on the analyzed PP content of the plant-based ingredients and taking account of their inclusion level in the diet) for each diet are shown in Table 3. These data indicate that the PP content of the final diets was comparable (within 5%) of the expected (formulated) PP levels in the diets, meaning that the diets had approximately equivalent PP levels across all treatments within each phase.

After accounting for endogenous phytase activities in the PC diets, activities in the experimental diets (IPF1 and IPF2) confirmed that the target phytase dose levels had been approximately achieved (±20%).

Xylanase activities in treatments PC2 and IPF2 were more variable, varying from 772 (PC2) to 2,104 XU/kg (IPF2) across treatments (Table 3). Xylanase recoveries in the literature are similarly variable (Kiarie et al., 2014; Romero et al., 2014) and reflect that accurate xylanase analysis in feed may be more challenging than phytase analysis. Despite the variation in xylanase recovery between PC2 and IPF2 diets, there were no differences in any of the growth and feed efficiency parameters between these two treatments, hence it was considered that the integrity of the study design was upheld.

Growth Performance

The effects of dietary treatment on growth performance are presented by phase in Table 5. There was no effect of treatment on any response measure during any individual phase. Comparison of the pooled PC treatments vs. pooled IPF treatments revealed that all growth performance responses in IPF diets were equivalent to those in PC diets during the starter phase (1−10 d of age). Similarly, during the grower phase (10−22 d of age), BW, BWG, and FCR were equivalent between PC and IPF treatments, while FI and WI were reduced in IPF compared to PC treatments (−96 g/bird and −70 mL/bird, respectively; $P < 0.05$), whereas water-to-feed intake ratio (**WF**) was unaffected. In addition, BW and BWG were equivalent between PC and IPF treatments, while FCR was reduced in IPF compared to PC treatments (−5.0 points; $P < 0.05$) during the finisher phase (22−37 d of age). In this phase, FI and WI were also reduced in IPF treatments vs. PC treatments (−66 g/bird and −168 mL/bird, respectively, $P < 0.05$; Table 5, whereas WF was unaffected.

When analyzed on a cumulative basis (Table 6), the results showed similar trends. Again, there was no effect of treatment on any response measure. Comparison of pooled PC treatments vs. pooled IPF treatments showed that FI was reduced (−27 g/bird) in IPF treatments vs. PC (−27 g/bird vs. PC; $P < 0.05$), while all other responses showed no significant differences during 1 to 22 d of age. For the overall period (1−37 d of age), FI, FCR, and WI were all reduced in IPF compared to PC treatments (−93 g/bird, −3.0 points and −231 mL/bird, respectively; $P < 0.05$), while WF and overall mortality showed no significant differences between IPF and PC treatments.

Table 5. Effect of total replacement of inorganic phosphate by phytase on growth performance by phase, in diets with and without xylanase; one-way ANOVA and contrast analysis.

	BW, g/bird	BWG, g/bird	FI, g/bird	FCR g/g	WI, mL/bird	WF, mL/g
Starter (1 to 10 d of age)	d 10					
ANOVA, treatment means						
PC1[1]	272	230	282	1.22	583	2.08
PC2[2]	279	237	288	1.22	600	2.08
IPF1[3]	276	234	285	1.22	592	2.08
IPF2[4]	278	236	283	1.21	607	2.14
SEM	3.46	3.46	3.92	0.020	10.15	0.042
P value	0.487	0.487	0.313	0.857	0.936	0.478
Contrast analysis						
PC	276	234	285	1.22	592	2.08
IPF	277	235	284	1.21	599	2.11
SEM	2.45	2.45	2.77	0.014	7.18	0.030
P value	0.816	0.816	0.826	0.692	0.475	0.489
Grower (10 to 22 d of age)	d 22					
ANOVA, Treatment means						
PC1	1,024	752	952	1.27	1,923	2.02
PC2	1,042	763	974	1.28	2,004	2.06
IPF1	1,024	748	942	1.26	1,896	2.01
IPF2	1,009	732	931	1.27	1,889	2.03
SEM	11.77	11.82	12.26	0.016	33.72	0.031
P value	0.178	0.252	0.190	0.883	0.203	0.729
Contrast analysis, PC vs. IPF						
PC	1,033	757	963[a]	1.27	1,963[a]	2.04
IPF	1,016	740	937[b]	1.27	1,893[b]	2.02
SEM	8.32	8.36	8.67	0.016	23.85	0.022
P value	0.178	0.160	0.040	0.636	0.045	0.486
Finisher (22 to 37 d of age)	d 37					
ANOVA, Treatment means						
PC1	2,297	1,274	2,025	1.59	3,749	1.85
PC2	2,307	1,265	2,042	1.62	3,756	1.84
IPF1	2,298	1,274	1,984	1.56	3,544	1.79
IPF2	2,284	1,275	1,952	1.53	3,626	1.86
SEM	20.73	18.83	22.29	0.021	71.36	0.033
P value	0.587	0.799	0.279	0.274	0.604	0.188
Contrast analysis, PC vs. IPF						
PC	2,302	1,269	2,034[a]	1.60[a]	3,753[a]	1.85
IPF	2,291	1,275	1,968[b]	1.55[b]	3,585[b]	1.82
SEM	14.66	13.31	15.76	0.015	50.46	0.023
P value	0.605	0.776	0.007	0.011	0.026	0.474

Abbreviations: IPF, inorganic phosphate-free; WI, water intake; WF, water-to-feed intake ratio.

[a,b]Pairs of means in the same column with no common superscripts are significantly different at a probability level of $P < 0.05$.

[1]Without enzyme supplementation.

[2]Containing xylanase dosed at 2,000 XU/kg of xylanase during all phases and 75 kcal/kg reduction in ME vs. PC1.

[3]Containing phytase at 3,000, 2,000 and 1,000 FTU/kg in starter, grower and finisher phases, respectively; Pi-free and with reduction in Ca of 1.5 g/kg vs. PC1.

[4]Containing xylanase at 2,000 XU/kg during all phases and phytase at 3,000, 2,000, and 1,000 FTU/kg in starter, grower and finisher phases, respectively; Pi-free and with reduction in ME of 75 kcal/kg vs. PC1 and reduction in Ca of 1.5 g/kg vs. PC2.

Collectively, the results of the contrast analysis suggest that the PhyG dosing strategy in Ca-reduced Pi-free diets, regardless of ME reduction and xylanase supplementation, maintained bird weight gain and improved or maintained FCR compared with P-adequate diets containing Pi from MCP. This was the case during each growth phase and over an entire growth cycle (1−37 d of age). As such, the findings complement those of the earlier

Table 6. Effect of total replacement of inorganic phosphate by phytase on cumulative growth performance, in diets with and without xylanase; one-way ANOVA and contrast analysis.

	BWG, g/bird	FI, g/bird	FCR g/g	WI, mL/bird	WF, mL/g	Mortality, %	Feed cost, €/kg BWG[5]
1 to 22 d of age							
ANOVA, treatment means							
PC1[1]	982	1,234	1.26	2,506	2.033	n/a	n/a
PC2[2]	1,000	1,262	1.26	2,605	2.064	n/a	n/a
IPF1[3]	982	1,227	1.25	2,487	2.027	n/a	n/a
IPF2[4]	967	1,214	1.26	2,496	2.053	n/a	n/a
SEM	11.77	12.69	0.011	40.63	0.029	n/a	n/a
P value, treatment	0.178	0.117	0.974	0.279	0.938	n/a	n/a
Contrast analysis, PC vs. IPF							
PC	991	1,248[a]	1.26	2,555	2.048	n/a	n/a
IPF	975	1,221[b]	1.25	2,491	2.040	n/a	n/a
SEM	8.32	8.98	0.008	28.73	0.021	n/a	n/a
P value	0.178	0.040	0.489	0.129	0.777	n/a	n/a
1 to 37 d of age							
ANOVA, treatment means							
PC1	2,256	3,259	1.45	6,255	1.921	4.7	0.36
PC2	2,265	3,305	1.46	6,361	1.925	5.1	0.36
IPF1	2,256	3,211	1.42	6,031	1.877	5.3	0.36
IPF2	2,245	3,167	1.41	6,122	1.934	4.9	0.35
SEM	20.73	30.82	0.011	89.82	0.026	0.42	0.003
P value, treatments	0.587	0.155	0.277	0.935	0.320	0.365	0.211
Contrast analysis, PC vs. IPF							
PC	2,260	3,282[a]	1.45[a]	6,308[a]	1.923	4.9	0.36[a]
IPF	2,249	3,189[b]	1.42[b]	6,077[b]	1.906	5.1	0.35[b]
SEM	14.66	21.80	0.008	63.51	0.019	0.30	0.002
P value	0.605	0.006	0.005	0.016	0.516	0.603	0.004

Abbreviations: IPF, inorganic phosphate-free; WI, water intake; WF, water-to-feed intake ratio.

n/a, Not measured.

[1]Without enzyme supplementation.

[2]Containing xylanase dosed at 2,000 XU/kg of xylanase during all phases and 75 kcal/kg reduction in ME vs. PC1.

[3]Containing phytase at 3,000, 2,000 and 1,000 FTU/kg in starter, grower and finisher phases, respectively; Pi-free and with reduction in Ca of 1.5 g/kg vs. PC1.

[4]Containing xylanase at 2,000 XU/kg during all phases and phytase at 3,000, 2,000, and 1,000 FTU/kg in starter, grower and finisher phases, respectively; Pi-free and with reduction in ME of 75 kcal/kg vs. PC1 and reduction in Ca of 1.5 g/kg vs. PC2.

[5]Calculations based on Dutch market ingredient prices for feed ingredients in June 2020, including the cost of enzymes.

[a,b]Pairs of means in the same column with no common superscripts are significantly different at a probability level of $P < 0.05$.

studies by Marchal et al. (2021) and Dersjant-Li et al. (2022), who also evaluated the effect of total removal of dietary Pi from 1 d of age in combination with PhyG supplementation. However, their studies were conducted under small-scale research settings rather than a commercial broiler production setting. Marchal et al. (2021) observed a similar improvement in overall (1 −42 d of age) BW-corrected FCR (**FCRc**) beyond FCRc of a nutritionally adequate diet (−12 FCR points) with the same phased PhyG dosing-strategy as applied in the present study.

The high stocking density employed in the present study (820 birds/pen, equivalent to 37.7 kg/m^2 at 37 d of age) was expected to generate conditions more representative of commercial production practice. In the EU, a general stocking density limit of 33 kg/m^2 is mandated for commercial broiler production by animal welfare regulations (European Directive EC2007/43/EC [European Council, 2007]), rising to an absolute maximum of 42 kg/m^2 if a derogation is granted. Conditions in the present study were below, but close to, this limit and within the

range of stocking densities applied in other commercial-scale studies (Dawkins et al., 2004). It can, therefore, be expected that the present findings are indicative of the likely effects of the phytase when applied in a commercial production setting. Feed intake is closely linked to the P-requirement status of the birds, as P deficiency reduces feed intake. In literature it is commonly (but not always) reported that phytase supplementation to P-deficient diets increases feed intake (Dos Santos et al., 2013; Walk et al., 2014; Walk and Rao, 2020). Differences between studies may be due to differences in the P deficiency level in the respective basal diets, phytase dose level, and substrate availability in the diet. The present findings, in which feed intake during grower, finisher and overall phases was reduced with phytase, are not consistent with those of the earlier study of PhyG by Marchal et al. (2021). They applied the same phased phytase dosing strategy, but without xylanase, and feed intake was unaffected by phytase during the finisher phase but increased during the starter phase (compared to a nutritionally adequate diet). In diets containing low available PP, Walters et al. (2019) reported increased feed consumption during the starter-grower phase in birds fed low non-PP diets supplemented with a hybrid *Hafnia, Yersinia*, and *Buttiauxella* sp. phytase at 2,000 FTU/kg or 3,000 FTU/kg dropping to 1,000 FTU/kg in the grower phase. However, at lower phytase dose levels feed intake was reduced vs. a PC without phytase. It must be noted that available PP in the study of Walters et al. (2019) was not as low as in the present study (2.3−2.4 g/kg non-phytate-P compared to 1.3 to 1.6 g/kg retainable P). On the other hand, Dersjant-Li et al. (2020a) observed increased feed intake during the grower phase with a *Buttiauxella* sp. phytase dosed at 500 FTU/kg compared with a nutritionally adequate diet, but no effect at 1,000 FTU/kg. In the present study, the similar growth performance responses in the IPF and PC diets during the starter phase suggested that P requirements were met with the phytase supplementation in the IPF treatments without the need to increase feed intake during this phase, whereas feed intake was reduced during the grower and finisher phases. The latter effect could be linked to the energy requirement status of birds in the phytase treatments, since PhyG phytase can also improve the availability of energy in the diet (Dersjant-Li et al., 2020b), meaning that less feed would need to be consumed to meet the energy requirement. In line with the reduction in feed intake, water intake was also reduced in the IPF treatment that contained phytase, during the grower and finisher phases. Dersjant-Li et al. (2020b) recently noted a similar effect with a *Buttiauxella* sp. phytase dosed at 1,000 FTU/kg in nutrient-reduced diets. It may be relevant that the present study was carried out during a period of unusually hot weather, during which the temperature in the broiler house was on average 4.5˚C above target from d 17 onward. This may have elevated water intake in the PC treatments, while in the IPF treatments the reduced feed intake may have reduced the need for water consumption despite the high ambient temperature. Importantly, the water-to-feed intake ratio was unaffected by phytase in the IPF treatments, and this ratio remained close to the levels suggested by the breeder (Aviagen Inc, 2015) in all treatments during the overall period.

Feed Costs

A comparison of the total feed costs of the experimental diets, including the cost of the enzymes, is presented in Table 6. Reduction or, in this case, total removal of Pi from the diet formulation is of interest to producers on sustainability grounds, for four reasons: 1) reduction of the use of a natural, finite resource, 2) reduction of the overall carbon footprint of broiler feed production by a reduced need to transport large quantities of phosphates from the phosphate mine to the feed production site, coupled with the opportunity to replace Pi with high-phytate ingredients sourced more locally, 3) reduction of P excretion into the environment, and 4) reduction of overall feed costs. Based on market prices for feed ingredients in May−June 2020, including the cost of the enzymes, there was no significant difference in the estimated total feed costs per kg BWG of the individual treatments, but feed costs/kg BWG in the IPF treatments were 2.8% (i.e.,

0.01 €/kg BWG) lower than those in the PC treatments, over an entire growth cycle of 1 to 37 d (Table 6). This suggests that the use of PhyG at an appropriate dosing strategy in diets without added Pi can provide a production benefit compared to a P-adequate diet with or without xylanase.

Bone Ash and Strength

The effect of dietary treatment on bone ash and − strength is presented in Tables 7 and 8, respectively. Tibia ash content (g/kg) at 21 and 36 d of age did not differ among individual treatments or between PC and IPF treatments. This suggests that IPF diets, with or without xylanase, achieved a similar bone mineralization to that of the P adequate diets. At 21 and 36 d of age, tibia bone strength did not differAmong individual treatments but was higher in the pooled IPF treatments compared to the pooled PC treatments (+6.6% and +1.3%, respectively; $P < 0.05$; Table 8). In addition, humerus bone strength at 36 d of age tended to differ among treatments ($P = 0.097$) and was higher in the pooled IPF treatments than the pooled PC treatments (+1.7%; $P < 0.05$). This provides some limited evidence of increased

Table 7. Effect of total replacement of inorganic phosphate by phytase on tibia ash content (% dry matter), in diets with and without xylanase; one-way ANOVA and contrast analysis.

	Tibia ash	
	21 d of age	36 d of age[5]
ANOVA, treatment means		
PC1[1]	50.1	52.1
PC2[2]	49.8	52.2
IPF1[3]	49.6	52.1
IPF2[4]	50.1	51.4
SEM	0.37	0.37
P value	0.278	0.161
Contrast analysis, PC vs. IPF		
PC	49.9	52.1
IPF	49.8	51.8
Pooled SEM	0.26	0.19
P value	0.773	0.119

Abbreviation: IPF, inorganic phosphate-free.
[1]Without enzyme supplementation.
[2]Containing xylanase dosed at 2,000 XU/kg of xylanase during all phases and 75 kcal/kg reduction in ME vs. PC1.
[3]Containing phytase at 3,000, 2,000 and 1,000 FTU/kg in starter, grower and finisher phases, respectively; Pi-free and with reduction in Ca of 1.5 g/kg vs. PC1.
[4]Containing xylanase at 2,000 XU/kg during all phases and phytase at 3,000, 2,000, and 1,000 FTU/kg in starter, grower and finisher phases, respectively; Pi-free and with reduction in ME of 75 Kcal/kg vs. PC1 and reduction in Ca of 1.5 g/kg vs. PC2.
[5]Calculations based on Dutch market ingredient prices for feed ingredients in June 2020, including the cost of enzymes.

Table 8. Effect of total replacement of inorganic phosphate by phytase on bone strength, in diets with and without xylanase; one-way ANOVA and contrast analysis.

	Tibia bone strength, kgF		Humerus bone strength, kgF
	21 d of age	36 d of age	36 d of age
ANOVA, treatment means			
PC1[1]	47.4	45.4	51.8
PC2[2]	47.2	48.2	53.4
IPF1[3]	51.2	45.9	57.6
IPF2[4]	49.6	46.6	49.3
SEM	1.20	0.16	2.88
P value	0.546	0.376	0.097
Contrast analysis, PC vs. IPF			
PC	47.3[a]	46.8	52.6
IPF	50.4[b]	46.2	53.5
SEM	0.81	0.82	2.02
P value, phytase	0.011	0.624	0.765

Abbreviation: IPF, inorganic phosphate-free.
[1]Without enzyme supplementation.
[2]Containing xylanase dosed at 2,000 XU/kg of xylanase during all phases and 75 kcal/kg reduction in ME vs. PC1.
[3]Containing phytase at 3,000, 2,000, and 1,000 FTU/kg in starter, grower and finisher phases, respectively; Pi-free and with reduction in Ca of 1.5 g/kg vs. PC1.
[4]Containing xylanase at 2,000 XU/kg during all phases and phytase at 3,000, 2,000, and 1,000 FTU/kg in starter, grower and finisher phases, respectively; Pi-free and with reduction in ME of 75 kcal/kg vs. PC1 and reduction in Ca of 1.5 g/kg vs. PC2.
[ab]airs of means in the same column with no common superscripts are significantly different at a probability level of $P < 0.05$.

bone mineralization in the PhyG supplemented IPF treatments, at least during the grower and finisher phases, and complements the earlier growth performance results in further suggesting that P requirements during all growth phases were met by the nutrient-releasing capacity of the phytase (with or without the xylanase).

CONCLUSIONS AND APPLICATIONS

1. Under a commercial production setting, a novel consensus bacterial 6-phytase variant administered alone, or in combination with xylanase to diets containing no added inorganic phosphate can maintain or improve growth performance, bone mineralization and breaking strength compared to a nutritionally adequate diet containing Pi from MCP, during an entire growth cycle (1−37 d).
2. These results were observed using a phased dosing strategy of the phytase (3,000 FTU/kg in starter, 2,000 FTU/kg in grower and 1,000 FTU/kg in finisher phase) and phased reduction in dietary phytate content (Phytate-P 3.30 g/kg, 3.10 g/kg, and 2.60 g/kg during the starter, grower, and finisher phases, respectively).
3. Feed cost calculations indicated that useing the phytase with the applied dosing strategy in diets without added inorganic phosphate could reduce total feed costs per kg BWG.
4. These findings are of relevance to improving the sustainability of broiler diets by reducing the inclusion of inorganic phosphate and reducing overall feed costs in all-plant-based diets.

DISCLOSURES

This study was sponsored by Danisco Animal Nutrition, IFF, The Netherlands. Abiodun Bello, Yueming Dersjant-Li and Leon Marchal are employees of Danisco Animal Nutrition & Health, IFF. All authors declare that they have no financial or personal relationships with other people or organizations that could inappropriately influence their work. There is no professional or personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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