A novel consensus bacterial 6-phytase variant added to phytate-rich diets totally replaced inorganic phosphate in broilers

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Received: 6 September 2021 / Accepted: 31 January 2022

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RESEARCH ARTICLE

POULTRY

Abstract

The capacity of a novel bacterial 6-phytase variant (PhyG) to totally replace dietary supplemental inorganic phosphate (Pi) in broilers aged one day old was evaluated using a phased dosing strategy and reduction in dietary phytate. A total of 1,248 one-day-old Ross 308 broilers were assigned to 24 pens (52 birds/pen, 12 pens/diet, six each males and females) in a complete randomised block design with a 2×2 factorial arrangement (two diets, two genders). Diets comprised: (1) a positive control (PC) based on maize, wheat and soybean meal, containing Pi from monocalcium phosphate and 2,000 XU/kg xylanase, and; (2) an inorganic phosphate-free (IPF) diet equivalent to PC but with reduced Ca (-2.0 g/kg) and supplemented with PhyG at 3,000, 2,000 and 1000 FTU/kg from d 0 to 11 (3.4 g/kg phytate-P (PP)), d 11 to 22 (3.3 g/kg PP) and d 22 to 42 (2.8 to 2.9 g/kg PP), respectively. A negative control was not included for animal welfare reasons. Tibias were collected on d 11, 22 and 42 from two birds/pen. Treatment IPF maintained or improved average daily gain (ADG), average daily feed intake and feed conversion ratio (FCR) vs PC during all phases; Body weight was higher in the IPF treatment than the PC at d 42 (+3.97%, $P<0.01$) and ADG during d 0 to 42 (+4.10%, $P<0.01$). Overall (d 0 to 42), treatment IPF improved FCR in males (-5 points vs PC, $P<0.05$) but not females. Tibia ash was equivalent in IPF and PC throughout, with no gender differences. In conclusion, when applied as a phased dosing strategy to diets with graded reduction in PP content, PhyG totally replaced supplemental Pi during all growth phases in males and females.

Keywords: phosphorus, phytase, phytate, poultry

1. Introduction

To ensure phosphorus (P) requirements are met, inorganic phosphate (Pi) is routinely added to poultry feed, often in the form of mono- or di-calcium phosphate (MCP or DCP) which are manufactured from naturally occurring phosphate rock. However, phosphate rock is a finite resource and there is high global demand for inorganic phosphates. As a result, costs remain high (Markets and Markets, 2018). If dietary P is in excess of bird requirements, it is excreted, which can have negative environmental consequences (Sharply *et al.*, 1994). There is therefore a need to develop more sustainable production practices that do not require Pi to be added to the diet.

Microbial phytase supplementation is one potential approach to achieving this. Phytase hydrolyses phytate (the salt of phytic acid, myo-inositol hexaphosphate; IP₆) in plant-based ingredients (Selle and Ravindran, 2007), releasing P in the form of phosphate that can be utilised by the bird, thereby reducing the amount of undigested P that is excreted. The efficacy of microbial phytase for increasing the bioavailability of P in broiler diets is well recognised (Lei *et al.*, 2013; Selle and Ravindran, 2007) and it is commonly used in commercial broiler diets.

Matrix values prescribing the nutrient reductions that may be applied to the diet in conjunction with the use of a given

phytase have enabled a reduction in the amount of Pi (and other nutrients) that needs to be added to broiler diets, but total Pi-replacement by phytase is a more novel concept. A limited number of previous studies (Ribiero *et al.*, 2019; Scholey *et al.*, 2018) have reported total replacement of Pi by a phytase in broiler diets during grower and/or finisher phases, but not during the starter phase. Total replacement of Pi during this phase is a particular challenge because of the high requirement of young birds (available P (AvP) at 4.8 g/kg of the diet during 0 to 10 d of age, Aviagen Inc., 2019a) which is necessary to support a period of rapid growth and bone mineralisation. Meanwhile, when diets are formulated without Pi, the available P in all vegetable-based diets with high phytate content (3.3 to 3.8 g/kg phytate-P (PP), formulated values) is about 1.5 to 1.6 g/kg (Marchal *et al.*, 2021). This means that in theory, a phytase needs to release up to ~3.2–3.3 g/kg AvP from phytate in order to totally replace Pi during the starter phase.

Recently, 1000 FTU/kg of a novel consensus bacterial 6-phytase variant with high *in vitro* and *in vivo* capacity to degrade phytate (Christensen *et al.*, 2020) was successful in replacing an estimated 2.07 g MCP-derived P per kilogram of diet in broilers (Dersjant-Li *et al.*, 2020a). The phytase maintained performance and tibia ash equivalent to a nutritionally adequate diet during all growth phases (Dersjant-Li *et al.*, 2020a). A further analysis of two separate trials showed that the same phytase applied with different dosing strategies maintained or improved growth performance and maintained tibia ash when used as a total replacement for Pi (Marchal *et al.*, 2021). One of the treatments applied the phytase to nutrient-reduced diets at 1000 FTU/kg throughout all phases whereas the other employed a phased dosing-regimen which was high in starter phase (3,000 FTU/kg) and lower in finisher phase (1000 FTU/kg). In both trials, although a consistent dose of 1000 FTU/kg during all phases maintained performance compared to a positive control (PC), the phased dosing strategy improved growth performance compared to the PC (Marchal *et al.*, 2021). In these trials, the PC was formulated without other enzymes and the basal diets were high in PP during all phases in order to ensure sufficient substrate for the phytase in diets without Pi. However, whilst high phytate is thought to be critical for phytase efficacy in the starter phase to provide sufficient substrate to meet higher digestibility needs, the lower P requirement during later growth stages means that it may be possible to reduce the PP content in later phases. This is preferable on sustainability grounds, because it would allow more flexibility in the choice of feed ingredients. In addition, the two trials reported by Marchal *et al.* (2021) were carried out with 24 to 26 birds per pen and were carried out in males or in mixed sexes housed together. The concept of dietary Pi-replacement by phytase needs to be evaluated in large-scale studies and in males and females separately, as the different growth rates and nutrient requirements

of the sexes may lead to different responses to phytase. In addition, the PC diets in the Marchal *et al.* (2021) trials were formulated without carbohydrase. When diets contain increased levels of phytate rich ingredients such as rapeseed meal and rice bran, xylanase is frequently added to such diets to improve the digestion of non-starch polysaccharides (NSP) (Choct and Annison, 1992).

Against this background, the following study tested the hypothesis that the novel consensus 6-phytase variant previously used in trials could allow total replacement for dietary Pi containing a graded reduction in PP by phase, in both male and female broilers.

2. Materials and methods

The study was conducted at the Institute of Agrifood Research and Technology (IRTA) Mas Bové Research Facility in Constantí, Spain. All experimental procedures were conducted in compliance with European Directive 2010/63/EU (European Council, 2010) and the Spanish guidelines for the care and use of animals in research (B.O.E. number 252, Real Decreto 2010/2005).

Experimental design and diets

A randomised complete block design was used with a 2×2 factorial arrangement including two treatment diets and two genders. Diets were based on maize, soybean meal, wheat, rapeseed meal, rice bran, soy oil and wheat bran, and were formulated by phase. This included a PC formulated to supply adequate levels of all nutrients in accordance with breeder recommendations issued in 2014 (these were identical to the 2019 recommendations (Aviagen Inc. NL, 2019a) except for avP in starter phase), but with reduced ME due to the expected contribution of 71 kcal/kg ME from the xylanase included. The xylanase was a commercial product from *Trichoderma reesei* (Danisco Animal Nutrition and Health, IFF, Oegstgeest, the Netherlands) and dosed at 2,000 XU/kg in all phases. The PP content of the diet was reduced by phase to 3.4 g/kg (d 0 to 11, starter), 3.3 g/kg (d 11 to 22, grower), 2.9 g/kg (d 22 to 35, finisher 1) and 2.8 g/kg (d 35 to 42, finisher 2). Oat hulls were included to stimulate gizzard development (Sacranie *et al.*, 2017) and soy hulls were used as a replacement material. The experimental diet (inorganic phosphate-free; IPF) was based on the PC but formulated with the complete absence of added Pi, containing 1.5, 1.5, 1.4 and 1.4 g/kg digestible P in starter, grower, finisher 1 and finisher 2 diets, respectively, excluding the expected P-contribution of the phytase. The IPF diets were formulated with a 2 g/kg reduction in calcium (Ca) vs PC. The IPF diet was supplemented with a novel consensus bacterial 6-phytase variant expressed in *T. reesei* (PhyG; Danisco Animal Nutrition and Health, IFF) at a dose-level of 3,000 FTU/kg from d 0 to 11, 2,000 FTU/kg from d 11 to 22 and 1000 FTU/kg from d 22 to 42. A negative control

was not included, as the very low levels of P would have led to health and welfare issues (such as leg deformities) in the

birds. The ingredient and calculated nutrient composition of the treatment diets is presented in Table 1.

Table 1. Ingredient and calculated nutrient content (g/kg, as fed basis) of the treatment diets, by phase.¹

	Starter (d 0 to 11)		Grower (d 11 to 22)		Finisher 1 (d 22 to 35)		Finisher 2 (d 35 to 42)	
	PC	IPF	PC	IPF	PC	IPF	PC	IPF
Ingredients g/kg								
Maize	240.1	244	284.1	283.8	341.6	340.4	371.1	369.8
Soybean meal	299.9	299.5	298.8	298.1	280.4	279.5	260.3	259.9
Wheat	200	200	200	200	200	200	200	200
Rapeseed meal	70	70	56.3	57.4	27.2	28.4	26.6	27.2
Rice bran	60	60	60	60	50	50	45	45
Soy oil	51.9	50.5	40.1	40.3	44	44.4	42.6	43
Wheat bran	20	20	10	10	10	10	10	10
Soy hulls	5.0	16.9	3.4	15.2	3.2	14.8	3.2	14.5
Monocalcium phosphate	15.4	-	11.6	-	10.2	-	9.4	-
Limestone	12.8	14.2	12.7	12.4	11.4	10.5	11	9.7
Oat hulls	10	10	10	10	10	10	10	10
Vitamin-mineral premix ²	4	4	4	4	4	4	4	4
Salt	3.9	3.9	3.9	3.9	3.7	3.7	3.7	3.7
DL-methionine	3.1	3.1	2.5	2.5	2.2	2.2	1.7	1.7
L-lysine HCL	3.0	3.0	1.8	1.8	1.6	1.6	1.3	1.3
L-threonine	0.8	0.8	0.6	0.6	0.5	0.5	0.2	0.2
Phytase, FTU/kg	-	3,000	-	2,000	-	1000	-	1000
Xylanase, XU/kg	2,000	2,000	2,000	2,000	2,000	2,000	2,000	2,000
Calculated nutrients (g/kg)								
ME, kcal/kg	2,850	2,850	2,928	2,928	3,029	3,029	3,049	3,049
Dry matter	895.1	893.5	892.4	891.2	890.9	889.8	889.7	888.7
Crude protein	223.6	224.8	217.8	219	200.9	202	192.2	193.3
Crude fat	71.1	70.1	60.2	60.5	64.9	65.5	64.1	64.7
Fibre	46.7	50.9	44.6	48.8	40.0	44.1	38.8	42.7
Ash	55.0	56.8	53.8	54.0	48.6	48.2	46.6	45.8
Calcium	9.2	7.2	8.4	6.4	7.4	5.4	7.0	5.0
Digestible P ^{3,4}	4.5	1.5	3.7	1.5	3.4	1.4	3.2	1.4
Non-phytate P	4.9	1.4	3.7	1.1	3.4	1.1	3.2	1.1
Total P	8.3	4.8	7.0	4.4	6.3	4.0	6.0	3.9
Ca:P ⁵	1.1	1.5	1.2	1.5	1.2	1.4	1.2	1.3
Phytate-P	3.4	3.4	3.3	3.3	2.9	2.9	2.8	2.8
Sodium	1.9	1.9	1.9	1.9	1.8	1.8	1.8	1.8
Chloride	2.7	2.7	2.7	2.7	2.6	2.6	2.6	2.6
Dig. Lys	12.2	12.2	11.0	11.0	10.0	10.0	9.2	9.2
Dig. Met + Cys	9.1	9.1	8.4	8.4	7.6	7.6	7.0	7.0
Dig. Met	6.0	6.0	5.4	5.4	4.8	4.8	4.3	4.3
Dig. Thr	7.6	7.6	7.3	7.3	6.6	6.6	6.1	6.1
Dig. Trp	2.2	2.2	2.2	2.2	2.0	2.0	1.9	1.9
Dig. Arg	13.0	13.0	12.8	12.8	11.7	11.7	11.2	11.2
Dig. Ile	7.8	7.8	7.7	7.7	7.1	7.1	6.8	6.8
Dig. Val	8.6	8.6	8.4	8.4	7.8	7.8	7.4	7.4

¹ IPF = inorganic phosphate free; PC = positive control.

² Supplied per kilogram of diet: retinyl acetate: 344 mg; cholecalciferol: 0.12 mg; all-rac-alpha-tocophery-acetate-50%: 45 mg; menadione sodium bisulfite-50%: 3 mg; thiamine mononitrate-98%: 3 mg; riboflavin-80%: 9 mg; pyridoxine monochloride-99%: 4.5 mg; cyanocobalamin-1 g/kg: 40 mg; folic acid-88%: 1.8 mg; biotin-2%: 150 mg; calcium pantothenate-98%: 16.5 mg; nicotinic acid-98%: 51 mg; Mn (as MnSO₄·H₂O): 90 mg; Zn (as ZnO): 66 mg; I (as KI): 1.2 mg; Fe (as FeSO₄·H₂O): 54 mg; Cu (as CuSO₄·5H₂O): 12 mg; Se (as NaSeO₃): 0.18 mg; butylhydroxytoluene: 25 mg; calcium formate, 5 mg; silicic acid, dry and precipitated, 25 mg; calcium stearate, 25 mg; calcium carbonate to 4 g.

³ Excluding the contribution from added phytase in the IPF diets.

⁴ Calculated based on the P digestibility coefficient values of different feed ingredients published in the CVB Feed Table 2018 (CVB, 2018).

⁵ Calculated as total Ca to total P rather than Ca:digP because the value for digestible P excludes the contribution of the phytase.

Feeds were prepared in bulk by mixing all ingredients except amino acids, vitamin and mineral premix and enzymes. The amino acids, vitamin and mineral and enzymes were premixed in 10 kg of basal feed ingredients prior to addition to the feed mixer to ensure homogenous distribution. Diets were provided as phased diets in crumble form during starter phase (d 0 to 11) and pellet form during grower (d 11 to 22) and finisher 1 and 2 (d 22 to 35 and d 35 to 42) phases. Pelleting was conducted at 80 °C with a conditioning time of between 13 and 18 seconds (dependent on the production rate, which was between 1,500 and 600 kg/h). Diets and water were provided *ad libitum* for the duration of the study.

Birds and housing

A total of 1,248 Ross 308 broiler hatchlings were obtained from a commercial hatchery where they had been feather-sexed, and were randomly assigned to 24 floor pens (52 birds/pen, 12 pens/treatment diet, six containing males and six containing females, with a stocking density at 42 days of age of 33 kg/m²). Pens were bedded with fresh wood shavings that had been disinfected with glutaraldehyde and were situated in an environmentally controlled broiler house with a lighting regime of LD24:0 h during d 0 to 2, LD18:6 h during d 3 to 7, and LD14:10 h thereafter. Temperature was initially maintained at 32 to 34 °C and then gradually reduced to 19 to 21 °C from d 3 to 28 and maintained at that temperature thereafter. During the first week of the study, individual birds were selected at random in the required numbers for later sampling and labelled by feather spraying. This was done to prevent later bias that may have been introduced if selection is done visually (due to the potential tendency to select larger or smaller birds).

Sampling and measurements

Birds and feed were weighed on a per pen basis on d 0, 11, 22, 35 and 42 and were used to calculate body weight (BW), average daily feed intake (ADFI) and average daily gain (ADG). On d 11, 22 and 42, all birds were weighed individually to assess uniformity. Pens were monitored daily for bird mortality which was recorded and used to calculate mortality-corrected feed conversion ratio (FCR). In addition, for the overall period (d 0 to 42), BW-corrected FCR (FCR_c) was calculated by correcting values by 3 points per 100 g of BW difference from the respective PC.

On d 11, 22 and 42, two selected (colour sprayed) birds from each pen were euthanised by cervical dislocation, and their right tibia bones collected for the determination of ash content. Tibias were frozen at -20 °C before analysis.

Chemical analysis

Diet samples were analysed for dry matter (DM), crude protein, ash, P and Ca. Phytase activity was measured by IRTA and xylanase was analysed by Danisco Animal Nutrition Research Centre (Brabrand, Denmark).

Tibias were thawed prior to the manual removal of fibula, muscle and connective tissue. The cleaned bones were then oven dried and ashed according to the method previously described by Dersjant-Li *et al.* (2020b). Dry matter, CP and ash were analysed according to AOAC methods 925.09, 942.05 and 968.06, respectively (AOAC, 2000). Phosphorus in feed was analysed according to AOAC method 965.17 (AOAC, 1990). Calcium in feed was analysed by inductively coupled plasma mass spectrometry (ICP-MS; Agilent Technologies model 7700X, Agilent Technologies Inc., Santa Clara, CA, USA) at Office Laboratory Castellbisbal (Barcelona, Spain) according to the method described by Pacquette and Thompson (2018). Phytate phosphorus, as inositol hexa-phosphate (IP₆) was determined at Danisco Animal Nutrition Research Centre using a modified version of the HPLC method described by Skoglund *et al.* (1997). Phytase activity was determined according to a modified version of the 2000.12 AOAC method (Engelen *et al.*, 2001). One phytase unit (FTU) was defined as the amount of enzyme that liberated 1 µmol of inorganic phosphate per minute from a sodium phytate substrate at a pH of 5.5 and a temperature of 37 °C. Xylanase activity was determined according to the method of Romero *et al.* (2014), where one 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. All feed analysis was conducted in duplicate.

Statistical analysis

Pen was defined as the experimental unit. Data were analysed by two-way ANOVA to evaluate main effects of treatment and gender, and their interaction, on performance and bone ash measures. Where a significant interaction was identified, Tukey's test was used to separate the means. Analyses were conducted using the Fit Model platform of JMP v14.0 (SAS Institute Inc., Cary, NC, USA) with treatment and gender included as fixed effects and block as a random effect. Differences were considered significant at $P < 0.05$. Where $0.05 < P < 0.1$, this was considered a tendency.

3. Results

Analysed levels of nutrients and enzyme activities in the diets are presented in Table 2. Analysed Ca was higher than formulated (on average, by 29.5%). Analysed total P was consistently within 10% of formulated values. Phytase activities were close to target levels in all treatments and phases. Xylanase activities were more variable, varying

by a maximum of 14% below target (finisher 2 phase IPF) to a maximum of 34% above target (finisher 2 phase PC).

Growth performance and mortality

No interactions between treatment and gender were observed for growth performance parameters in each phase. During all growth phases, the ADG and ADFI of birds fed the IPF diet were equivalent to or greater than, and the FCR was equivalent to or less than, the respective value achieved by the PC (Table 3). From d 0 to 11, ADG, ADFI and FCR in the IPF-fed birds were equivalent to the corresponding values in those fed PC. From d 11 to 22, ADG and ADFI in birds fed IPF were higher than in the PC groups ($P<0.001$ in all cases). From d 22 to 35, ADG in birds fed IPF was greater than ($P<0.01$), whereas ADFI and FCR did not differ from the PC groups, and from d 35 to 42, ADG tended to be higher in birds fed IPF ($P=0.063$) and FCR tended to be lower ($P<0.10$) compared to PC groups (Table 3). The positive effect of treatment IPF vs PC on growth performance was greatest from d 11 to 22 (ADG +6.02%, ADFI +4.57%, vs PC, $P<0.001$, Table 3).

On a cumulative basis, compared to birds fed PC, those given IPF had higher BW, ADG, and ADFI during the periods d 0 to 22 and d 0 to 35 ($P<0.05$ in all cases). From d 0 to 35 birds fed IPF had lower FCR and FCRc (-1.5 and 3.7 points respectively vs PC, $P<0.05$) (Table 4). For the overall period (d 0 to 42), compared to PC, birds in treatment IPF exhibited higher BW at 42 days of age (+3.97%, $P<0.01$), ADG (+4.10%, $P<0.01$), lower FCR (-3 points) and FCRc (-6.3 points), whereas ADFI tended to be greater (+1.94%, $P=0.054$; Table 4). Mortality levels were at or below 3% across treatments and growth phases and did not differ by gender or treatment during any phase (Table 4). During d 0 to 35 and d 0 to 42, the European performance efficiency factor (EPPF) was greater in IPF than PC birds and in males than females ($P<0.05$, Table 4).

Independent of treatment, beyond the starter phase (d 11 onwards) male birds consistently exhibited higher BW, ADG and ADFI than females, both during individual growth phases (d 11 to 22, 22 to 35 and 35 to 42) and cumulatively (Table 4; $P<0.05$). The FCR of males was lower than that of females during finisher 1 phase only (d 22 to 35, -2.2 points, $P=0.05$).

No interactions between treatment and gender were observed for ADG or ADFI. For FCR, there was an interaction between these factors from d 0 to 22 ($P<0.01$) and d 0 to 42 ($P<0.05$) (Table 4). There was a tendency towards an interaction between diet and gender from d 11 to 22 ($P=0.051$) (Table 3) and during the overall period for FCRc (d 0 to 42, $P=0.052$; Table 4). In all of these cases, the effect of the IPF treatment in reducing FCR vs PC was greater in males than females (Table 4). For the overall period (d 0 to 42), feeding IPF reduced FCR vs PC by 5.1 points in males ($P<0.05$) and by 1.1 points in females ($P>0.05$).

The uniformity of bird BW by treatment and by gender was assessed (data not shown). Body weight CVs were, on average, 11.8, 11.9 and 10.2% at d 11, 22 and 42, respectively. There were no main effects or interaction between diet and gender on BW CV, except for d 22, where there was a tendency ($P<0.10$) for lower CV in BW in the IPF group (11.3%) vs PC (12.6%), and at d 42 the CV of BW was higher in males (11.1%) than females (9.4%; $P<0.05$).

Tibia ash

Tibia ash, dry weight relative to live weight and ash weight relative to live weight were not different at any sampling time points (Table 5). There was no effect of gender on these response measures, except at d 42 when tibia dry weight and ash weight relative to live weight were reduced in females vs males ($P<0.05$; Table 5). No interactions between treatment and gender were observed.

Table 2. Analysed nutrient content (g/kg as fed basis) and enzyme activities of the treatment diets, by phase.¹

Parameter	Starter (d 0 to 11)		Grower (d 11 to 22)		Finisher 1 (d 22 to 35)		Finisher 2 (d 35 to 42)	
	PC	IPF	PC	IPF	PC	IPF	PC	IPF
Dry matter	884.4	897.3	892	889	893.5	890.9	890.9	895.3
Crude protein	227	228.6	216.6	216.9	199.8	200.2	195.4	197.1
Ash	63.2	54.7	58.3	50.5	53.3	51.8	51	44
Calcium	11.5	9.2	10.2	7.9	9.4	7.4	8.9	7.1
Phosphorus	8.3	5.2	6.7	4.1	5.8	3.6	5.6	3.6
Phytate-P ²	3.32	3.42	3.19	3.29	2.87	2.97	2.8	2.9
Ca:P	1.39	1.77	1.52	1.93	1.62	2.06	1.59	1.97
Phytase, FTU/kg	82	3,084	67	2,255	94	1,028	47	917
Xylanase, XU/kg	2,125	2,207	1,742	2,524	2,657	1,984	2,684	1,728

¹ IPF = inorganic phosphate free; PC = positive control.

² Analysed phytate-P in feed was based on the analysed phytate-P level in the ingredients and their inclusion rate in the diet (all ingredients containing phytate P were analysed).

Table 3. Effect of treatment and gender on broiler growth performance measures, by phase.^{1,2,3}

	Gender	No. of observations	ADG (g/bird)	ADFI (g/bird)	FCR (g/g)
Starter (d 0 to 11)					
Treatment means					
PC	Female	6	20.7	23.1	1.112
PC	Male	6	21.2	23.5	1.111
IPF ²	Female	6	20.7	22.9	1.106
IPF	Male	6	21.3	23.4	1.100
SEM			0.310	0.253	0.007
Main effect means					
Treatment	PC	12	21.0	23.3	1.112
	IPF	12	21.0	23.2	1.103
Gender	Female	12	20.7	23.0 ^b	1.109
	Male	12	21.2	23.5 ^a	1.106
SEM			0.259	0.208	0.006
P-value, treatment			0.905	0.476	0.146
P-value, gender			0.065	0.039	0.504
P-value, treatment × gender interaction			0.869	0.995	0.694
Grower (d 11 to 22)					
Treatment means					
PC	Female	6	54.9	74.7	1.361
PC	Male	6	61.2	82.7	1.350
IPF	Female	6	58.3	78.9	1.352
IPF	Male	6	64.9	85.7	1.320
SEM			0.625	0.755	0.003
Main effect means					
Treatment	PC	12	58.1 ^b	78.7 ^b	1.356 ^a
	IPF	12	61.6 ^a	82.3 ^a	1.336 ^b
Gender	Female	12	56.6 ^b	76.8 ^b	1.357 ^a
	Male	12	63.1 ^a	84.2 ^a	1.335 ^b
SEM			0.509	0.911	0.005
P-value, treatment			<0.001	<0.001	0.001
P-value, gender			<0.001	<0.001	0.001
P-value, treatment × gender interaction			0.820	0.446	0.051
Finisher 1 (d 22 to 35)					
Treatment means					
PC	Female	6	86.7	136.0	1.569
PC	Male	6	102.7	160.0	1.558
IPF	Female	6	89.0	139.4	1.566
IPF	Male	6	105.6	161.9	1.532
SEM			0.895	1.625	0.008
Main effect means					
Treatment	PC	12	94.7 ^b	148.0	1.563
	IPF	12	97.3 ^a	150.6	1.549
Gender	Female	12	87.9 ^b	137.7 ^b	1.567
	Male	12	104.2 ^a	160.9 ^a	1.545
SEM			0.623	1.131	0.011
P-value, treatment			0.011	0.129	0.199
P-value, gender			<0.001	<0.001	0.052
P-value, treatment × gender interaction			0.758	0.648	0.301
Finisher 2 (d 35 to 42)					
Treatment means					
PC	Female	6	74.9	169.1	2.258
PC	Male	6	86.1	205.0	2.396
IPF	Female	6	78.3	174.1	2.230
IPF	Male	6	92.8	203.2	2.196
SEM			2.349	2.085	0.060
Main effect means					
Treatment	PC	12	80.5	187.0	2.327
	IPF	12	85.5	188.6	2.213
Gender	Female	12	76.6 ^b	171.6 ^b	2.244
	Male	12	89.4 ^a	204.1 ^a	2.296
SEM			1.542	1.490	0.039
P-value, treatment			0.063	0.454	0.095
P-value, gender			<0.001	<0.001	0.425
P-value, treatment × gender interaction			0.519	0.124	0.197

¹ ADG = average daily gain; ADFI = average daily feed intake; FCR = feed conversion ratio; IPF = inorganic phosphate free; PC = positive control.² The statistical analysis was a 2×2 factorial Analysis of Variance (2-way ANOVA) with 2 treatments and 2 genders. All growth performance parameters (ADG, ADFI and FCR) are corrected for mortality.³ Main effect means with no common superscripts are significantly different at a $P < 0.05$.

Table 4. Effect of treatment and gender on broiler growth performance measures, cumulative of 0-22, 0-35 and 0-42 days of age.^{1,2,3}

	Gender	Treatment	BW (g/bird)	ADG (g/bird)	ADFI (g/bird)	FCR (g/g)	Mortality (%)	FCRc (g/g) ⁴	EPEF ⁵
d 0 to 22			d 22						
Treatment means ⁶									
PC	Female		872	37.8	48.8	1.291 ^a	1.28	-	-
PC	Male		947	41.2	53.0	1.287 ^a	0.32	-	-
IPF	Female		910	39.5	50.8	1.285 ^a	2.24	-	-
IPF	Male		984	42.9	54.2	1.262 ^b	1.92	-	-
SEM			9.18	0.415	0.539	0.003	0.842	-	-
Main effect means ⁷									
Treatment		PC	910 ^b	39.5 ^b	50.9 ^b	1.289	0.80	-	-
		IPF	947 ^a	41.2 ^a	52.5 ^a	1.274	2.08	-	-
Gender	Female		891 ^b	38.7 ^b	49.8 ^b	1.288	1.76	-	-
	Male		965 ^a	42.1 ^a	53.6 ^a	1.274	1.12	-	-
SEM			7.83	0.355	0.459	0.002	0.617	-	-
P-value, treatment			<0.001	<0.001	0.001	<0.001	0.135	-	-
P-value, gender			<0.001	<0.001	<0.001	<0.001	0.442	-	-
P-value, treatment × gender interaction			0.988	0.994	0.310	0.004	0.698	-	-
d 0 to 35			d 35						
Treatment means ⁶									
PC	Female		1,999	56.0	81.0	1.447	1.28	1.447	382
PC	Male		2,282	64.0	92.5	1.444	1.28	1.444	438
IPF	Female		2,068	57.9	83.5	1.441	2.88	1.421	390
IPF	Male		2,358	66.2	93.9	1.419	1.92	1.396	458
SEM			19.03	0.543	0.889	0.006	0.964	0.007	5.075
Main effect means ⁷									
Treatment		PC	2,141 ^b	60.0 ^b	86.7 ^b	1.445 ^a	1.28	1.445 ^a	410
		IPF	2,213 ^a	62.1 ^a	88.7 ^a	1.430 ^b	2.40	1.408 ^b	424
Gender	Female		2,033 ^b	56.9 ^b	82.2 ^b	1.444 ^a	2.08	1.434	386 ^b
	Male		2,320 ^a	65.1 ^a	93.2 ^a	1.431 ^b	1.60	1.420	448 ^a
SEM			14.53	0.414	0.665	0.004	0.728	0.005	3.588
P-value, treatment			0.001	0.001	0.031	0.019	0.229	<0.001	0.012
P-value, gender			<0.001	<0.001	<0.001	0.046	0.599	0.051	<0.001
P-value, treatment × gender interaction			0.830	0.832	0.530	0.104	0.599	0.108	0.254
d 0 to 42			d 42						
Treatment means ⁶									
PC	Female		2,524	59.1	95.5	1.615 ^a	1.92	1.615	359
PC	Male		2,884	67.7	110.5	1.632 ^a	2.88	1.632	403
IPF	Female		2,616	61.3	98.4	1.604 ^{ab}	2.88	1.577	371
IPF	Male		3,007	70.6	111.7	1.581 ^b	4.17	1.544	428
SEM			29.18	0.694	0.986	0.008	1.214	0.012	5.982
Main effect means ⁷									
Treatment		PC	2,704 ^b	63.4 ^b	103.0	1.623	2.40	1.623 ^a	381 ^b
		IPF	2,811 ^a	66.0 ^a	105.0	1.593	3.53	1.561 ^b	400 ^a
Gender	Female		2,570 ^b	60.2 ^b	96.9 ^b	1.609	2.40	1.596	365 ^b
	Male		2,946 ^a	69.2 ^a	111.1 ^a	1.607	3.53	1.588	416 ^a
SEM			20.55	0.489	0.713	0.006	0.947	0.009	4.156
P-value, treatment			0.002	0.002	0.052	0.002	0.313	<0.001	0.008
P-value, gender			<0.001	<0.001	<0.001	0.724	0.313	0.538	<0.001
P-value, treatment × gender interaction			0.607	0.608	0.385	0.021	0.884	0.0521	0.287

¹ ADG = average daily gain; ADFI = average daily feed intake; FCR = feed conversion ratio; IPF = inorganic phosphate free; PC = positive control.

² The statistical analysis was a 2×2 factorial Analysis of Variance (two-way ANOVA) with 2 treatments and 2 genders. All growth performance parameters (ADG, ADFI and FCR) are corrected for mortality.

³ Treatment or main effect means in the same column with no common superscripts are significantly different at a probability level of $P < 0.05$.

⁴ FCRc: body weight corrected FCR, calculated by correction of FCR values by 3 points per 100 g of BW difference from the respective PC.

⁵ EPEF, European performance efficiency factor (Aviagen Inc., 2018) = $\text{ADG (g)} \times (100 - \text{mortality (\%)}) / (10 \times \text{FCR})$.

⁶ Number of observations = 6.

⁷ Number of observations = 12.

Table 5. Effect of treatment and gender on tibia ash content, tibia dry weight relative to live weight, and tibia ash weight relative to live weight of the sampled birds at 11, 22 and 42 days of age.^{1,2,3}

	Gender	Tibia ash, % DM	Tibia dry weight/live weight, %	Tibia ash dry weight/live weight, %
d 11				
Treatment means ⁴				
PC	Female	48.3	0.177	0.086
PC	Male	48.1	0.172	0.083
IPF	Female	48.0	0.171	0.082
IPF	Male	47.6	0.167	0.079
SEM		0.686	0.005	0.003
Main effect means ⁵				
Treatment	PC	48.2	0.175	0.084
	IPF	47.8	0.169	0.081
Gender	Female	48.2	0.174	0.084
	Male	47.9	0.169	0.081
SEM		0.454	0.003	0.002
P-value, treatment		0.599	0.384	0.233
P-value, gender		0.691	0.411	0.297
P-value, treatment × gender interaction		0.925	0.940	0.978
d 21				
Treatment means ⁴				
PC	Female	49.0	0.204	0.100
PC	Male	49.8	0.209	0.104
IPF	Female	49.8	0.204	0.102
IPF	Male	49.4	0.208	0.103
SEM		0.644	0.004	0.002
Main effect means ⁵				
Treatment	PC	49.4	0.206	0.102
	IPF	49.6	0.206	0.102
Gender	Female	49.4	0.204	0.101
	Male	49.6	0.209	0.103
SEM		0.405	0.003	0.001
P-value, treatment		0.840	0.978	0.967
P-value, gender		0.755	0.267	0.279
P-value, treatment × gender interaction		0.414	0.930	0.573
d 42				
Treatment means ⁴				
PC	Female	43.2	0.214	0.092
PC	Male	43.3	0.233	0.100
IPF	Female	43.1	0.212	0.091
IPF	Male	41.7	0.237	0.099
SEM		0.790	0.005	0.002
Main effect means ⁵				
Treatment	PC	43.2	0.223	0.096
	IPF	42.4	0.225	0.095
Gender	Female	43.1	0.213 ^b	0.092 ^b
	Male	42.5	0.235 ^a	0.100 ^a
SEM		0.6	0.004	0.002
P-value, treatment		0.282	0.766	0.424
P-value, gender		0.369	<0.001	<0.001
P-value, treatment × gender interaction		0.296	0.511	0.821

¹ IPF = inorganic phosphate free; PC = positive control.² The statistical analysis was a 2×2 factorial Analysis of Variance (ANOVA) with 2 treatments and 2 genders.³ Main effect means with no common superscripts are significantly different at a probability level of $P < 0.05$.⁴ Number of observations = 6.⁵ Number of observations = 12.

4. Discussion

Details of the origin, development and characteristics of the novel consensus phytase variant (PhyG) have been published elsewhere (Christensen *et al.*, 2020; Ladics *et al.*, 2020). The first proof of concept evaluation of PhyG in Pi-free diets was recently reported by Marchal *et al.* (2021).

In that publication, a dose level of 1000 FTU/kg during all phases or a phased dosing strategy with high dose in starter phase (3,000 FTU/kg, or 2,000 FTU/kg in combination with xylanase) decreasing to a lower dose in finisher phases (1000 FTU/kg), maintained growth performance, bone strength and mineralisation, equivalent to a PC diet from 0 to 42 days of age, in two trials. There was some evidence from both

trials that the phased dosing strategy produced superior growth performance to 1000 FTU/kg in all phases, with higher ADG during d 0 to 10 (trial 1) or d 11 to 21 (trial 2) and lower overall FCRc (-6 points) in trial 1. Given this, and the known higher P requirement of birds in the starter phase, it was decided to implement the phased dosing strategy in the present study.

The control diets in the trials reported by Marchal *et al.* (2021) contained no exogenous enzymes and, by design, the diets in all phases contained high levels of phytate (≥ 3.3 g/kg PP) to ensure sufficient substrate for the phytase. The present study employed a more systematically graded reduction in PP by phase, with the aim of optimising the enzyme-to-substrate ratio. Xylanase was added to the control as a background enzyme because its supplementation of high fibre broiler diets is common practice to improve the digestibility of NSPs and energy utilisation. Other differences included a larger number of birds per pen and gender separation, which enabled the comparison of responses between males and females. As was the case in the studies by Marchal *et al.* (2021), a negative control diet without added Pi was not included as a stand-alone treatment. This was on ethical grounds, as the severe P deficiency in such a diet [having a non-phytate P (nPP) content of only 1.4 g/kg during starter phase, some 3.4 g/kg below than the nPP requirement stated by the breeder for the period d 0 to 10 (4.8 g/kg; Aviagen Inc., 2019a), would have been expected to cause leg weakness among the birds. Indeed, it has already been established that diets that are less P deficient than those in the present study can significantly impair normal bone formation, reduce tibia ash and strength and negatively affect growth performance in broilers (Dersjant-Li *et al.*, 2020a; Li *et al.*, 2015a, 2020; Qian *et al.*, 1996). The lack of a NC was not considered to have compromised the ability of the study design to address its primary aim, which was to determine whether or not a novel consensus 6-phytase variant applied in a graded dosing-strategy by feeding phase could totally replace dietary Pi in a high-fibre diet containing xylanase as a background enzyme and a graded reduction in PP by phase, in both males and females.

There was some variability in the analysed levels of nutrients in the diets compared with the formulated levels. Such variability can arise due to incomplete feed mixing, sampling errors or variation in actual ingredients compared to standard values. Across phases, analysed Ca levels in both the PC and IPF diets were ~30% higher than formulated levels (~20% higher than breeder recommendations (Aviagen Inc., 2019)). This was likely because limestone was used as a carrier for the vitamin-mineral premix and this additional Ca source was not taken account of during diet formulation. It is well recognised that high dietary Ca can negatively affect growth performance in broilers, especially when P is deficient (Amerah *et al.*, 2014; Dersjant-

Li *et al.*, 2018; Li *et al.*, 2015b). Thus, the high Ca level could have had a negative impact on P digestibility and performance in both PC and IPF diets, but especially for PC. However, previous research has shown clearly that, whilst increasing the dietary Ca level reduced performance in diets deficient in nPP (2 g/kg), it did not have this effect when nPP was adequate (4 g/kg; Li *et al.*, 2015b). The calculated values of nPP (analysed total P minus PP) in the PC diets indicated that they were adequate in AvP supply, for example, calculated nPP was 4.9 g/kg nPP in PC in starter phase, above the breeder requirement of 4.8 g/kg (Aviagen Inc., 2019a). Thus, a higher dietary Ca in the PC diet with adequate nPP level was not expected to have impacted negatively on performance in the present study. Final BW and overall (d 0 to 42) FCR values in both PC and IPF treatments were similar to the published breeder performance objectives (Aviagen Inc., 2019b) which did not suggest any overriding negative effects of the higher Ca content on performance.

The addition of PhyG according to the applied dosing strategy to the IPF diets resulted in performance measures that were equivalent to or better than results from birds fed PC, during all growth phases. The improvements above PC were evident in ADG, BW and FCR responses, whilst tibia ash remained equivalent to PC, which suggested an 'extra-phosphoric' effect of PhyG. This could include beneficial effects of phytase on digestion and/or retention of nutrients other than P, which could have contributed to the improved performance in birds fed the PhyG supplemented diet. A similar effect was observed by Marchal *et al.* (2021) for BW and FCR from d 0 to 21 of age, with IPF treatments containing the same phased PhyG dosing strategy in diets with higher PP content during the finisher phase. Dersjant-Li *et al.* (2020c) confirmed an extra-phosphoric effect of PhyG at 1000 FTU/kg in Pi-free broiler diets, citing improvements in the apparent ileal digestibility (AID) of dry matter, nitrogen, gross energy and amino acids in male broilers at 15 days of age.

The performance of birds fed the IPF diet containing phytase was in line with the breeders' performance objectives (Aviagen Inc., 2019b), where final BW of male and female birds were, respectively, 99.3% and 100.1% of the performance objective at 41 days of age. The FCR for birds fed IPF was in line with the breeder's objective (99.7 and 100.1%, respectively, for males and females). No evidence of leg weakness was observed during the study. These findings indicated that availability and balance of nutrients in the IPF diet, enhanced by the P-releasing capacity of the phytase, were sufficient to support normal growth performance, the equivalent to those fed the PC diet. At d 35 and 42 of age, BW of birds receiving IPF was higher compared to PC, and during 0 to 35 and 0 to 42 days of age FCR was improved. The calculated EPEF (Aviagen Inc., 2018; Table 4) was higher in birds who received the IPF diet

compared to PC (+4.9%) for the overall period (d 0 to 42). This suggested that the phytase was effective at replacing dietary Pi across the whole growth cycle, including in later growth stages when the diets had relatively low PP content (finisher; 2.8 to 2.9 g/kg PP), which was desirable from a sustainability perspective. This trial extended the findings of the study by Marchal *et al.* (2021) who reported total Pi-replacement with a higher phytate content in finisher phase (≥ 3.3 g/kg PP).

Bone mineralisation (tibia ash content on a dry matter basis, and tibia dry weight relative to live weight) in birds fed IPF was equivalent to the PC at all sampling timepoints. This suggested that the phased dosing strategy of the phytase to the IPF diet was effective in releasing enough P from phytate to maintain normal bone mineralisation in each growth phase, and that the diet was P-adequate. Tibia bone ash has previously been validated as an appropriate indirect response of P-release capacity of phytase in previous broiler studies. First, the dose response relationship between AvP in the diet and tibial ash in the absence of phytase was established using graded addition of an inorganic P source (such as MCP) to a highly P deficient diet. The linear regression slope was then used to estimate the AvP equivalence of the phytase when added at a given dose level to the diet in the same study (Adedokun *et al.* 2004; Dersjant-Li *et al.*, 2019; Li *et al.*, 2015a). Li *et al.* (2015a) concluded that tibia ash weight and ash percentage were equally sensitive to dietary non phytate P (nPP) concentration changes at 21 d of age. Thus, both can be used as response criteria for relative phytase efficacy estimation. In relation to PhyG, a linear relationship between ileal P digestibility and tibia ash has already been established, based on the data reported by Dersjant-Li *et al.* (2020a), which further supported the use of tibia ash as a good indicator of P status in broilers when a highly deficient diet was offered.

Other than the absence of added Pi and presence of supplemental phytase, other differences between the IPF and PC diets that could, in theory, have influenced the response to phytase were the soy hull content and the reduction in dietary Ca content. Soy hulls were used in both diets as a replacement ingredient to maintain a similar overall ingredient composition and PP level between treatments. The difference in their content was modest (~ 10 g/kg or 1% higher in IPF vs PC). Soy hulls inclusion at >20 g/kg can have a positive effect on nutrient digestibility and growth performance (Scapini *et al.*, 2018; Tejeda and Kim, 2020) but levels in the current study were below this and considered unlikely to have improved responses in the IPF vs PC diet. Similarly, any beneficial effect of dietary Ca reduction in the IPF diet was considered unlikely to have been a major contributor to the observed equivalence or

improvements in performance responses in the (phytase supplemented) IPF compared to PC diets. As discussed above, when P requirements are met, variation in dietary Ca levels are expected to have less influence on its utilisation (Li *et al.*, 2015b): In young broilers aged from 7 to 19 d, these authors observed that, in diets containing a low level of nPP (2 g/kg) and dietary Ca levels of 6.5, 8.0 and 9.5 g/kg, BWG was 452, 426 and 367 g, respectively, indicating a significant negative effect of increasing Ca level on performance. Whereas, in diets containing an adequate level of nPP (4 g/kg), BWG with increasing dietary Ca level was 496, 503 and 506 g, respectively, showing no effect of Ca (Li *et al.*, 2015b). This demonstrated clearly that increasing the dietary Ca level reduced performance in birds fed diets deficient in nPP but not those containing adequate nPP. As the PC was formulated with sufficient nPP in the current study, any effect of Ca level should have been minimal. Total removal of Pi in the IPF diet reduced the digestible P content (compared to PC) substantially, by 3.0, 2.2, 2.0 and 1.8 g/kg in starter, grower, finisher 1 and finisher 2 phases, respectively. The P-deficiency in these diets was expected to have been the major factor affecting performance. As tibia ash data were maintained at the same level between IPF and PC diets, which confirmed that P requirement was met in the phytase supplemented IPF treatment. The growth performance responses achieved by birds fed the IPF diet supplemented with PhyG were equivalent (or improved) compared to those achieved by the PC, which could be attributed to the P release and extra-phosphoric effect of the phytase.

Based on the feed intake in each phase and the calculated cost of the diets (based on market prices for ingredients in 2020 and inclusive of the costs of the enzymes), total feed costs per kilogram BWG were estimated as 0.416 €/kg BWG for the PC diet and 0.405 €/kg BWG for the IPF diet. On this basis, the PhyG supplemented diet represented a significant feed cost saving to producers.

5. Conclusion

The novel consensus bacterial 6-phytase variant was effective in facilitating total replacement of inorganic phosphate in broiler diets containing phytate rich ingredients during an entire growth cycle (0 to 42 d of age). The tiered dosing strategy by phase, in combination with a graded reduction in phytate-P content, such that finisher diets were relatively low in phytate-P (2.8 to 2.9 g/kg), maintained or improved broiler growth performance and tibial ash when compared with a nutritionally adequate control diet containing Pi from MCP. Commercial scale studies are needed to validate these findings in a production setting.

Acknowledgements

The authors would like to thank Dr Joelle Buck (Newbury, UK) for her assistance with the writing of this manuscript, which was sponsored by Danisco Animal Nutrition & Health, IFF, The Netherlands, in accordance with Good Publication Practice guidelines.

Conflict of interest

Y. Dersjant-Li, L. Marchal and A. Bello are employees of Danisco Animal Nutrition & Health, IFF, the Netherlands.

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