Modeling improvements in ileal digestible amino acids by a novel consensus bacterial 6-phytase variant in broilers

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ABSTRACT Data from 13 datasets from 4 trials on the effect of a novel consensus bacterial 6-phytase variant (**PhyG**) on the apparent ileal digestibility (**AID**) of amino acids (**AA**) in broilers were used to model AID AA responses. The datasets were obtained from 3 trial locations (New Zealand, Australia and United States) and collectively incorporated variations in diet composition (feedstuff composition, phytate-P (**PP**) level, limestone solubility), feed form (mash or pellet), bird genetics (strain), and age at sampling (11−35 d of age). In total, 384 observations were analyzed. First, the relationships between AID of AA (as coefficients) and increasing phytase dose level from 0 to 4,000 FTU/kg were evaluated across all datasets using exponential curve fitting. Second, the percentage unit change in AID of AA at each phytase dose level from baseline (basal diet [**BD**] without phytase) was calculated separately for each dataset and the data then modeled together using exponential curve fitting. The model-predicted mean coefficient of AID of total AA in basal diets was 0.76 (range 0.56 [Cys] to 0.83 [Glu]), which was increased by PhyG to 0.80 and 0.81 at 2,000 and 4,000 FTU/kg, respectively. Exponential increases in the percentage unit improvement in AID of 18 individual and of total AA with increasing phytase dose level were evident ($P < 0.05$). Improvements (vs. BD) at 2,000 FTU/kg and 4,000 FTU/kg, respectively, were greatest for Cys (+9.2 and +11.0% units), Met (after deduction of synthetic Met, +8.4 and +9.0% units), and Thr (after deduction of synthetic Thr, +6.2 and +7.3% units). The data demonstrated consistent improvements in the AID of AA by the phytase. The modeling results generated from data gathered from birds sampled at different ages and from different dietary settings with correction of synthetic AA for Lys, Met, Thr, and Trp, enabled a more accurate prediction of the digestible AA contribution from the diet by this novel phytase. This will allow diet-specific AA matrix recommendations to be made in commercial feed formulations.

Key words: amino acids, broiler, digestibility, phytate, nutrient matrix

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INTRODUCTION

Since the original study of an *Aspergillus niger* phytase by Simons et al. (1990), numerous studies have demonstrated the ability of exogenous microbial phytase to improve phosphorus (**P**) digestibility and utilization in broilers (reviewed in Selle and Ravindran, 2007; Lei et al., 2013). As a result of these benefits, and associated reductions in the excretion of undigested P into the environment, the addition of phytase to broiler feeds has become common practice. It is also recognized that phytase supplementation improves the digestibility of calcium (**Ca**) and other minerals, principally by reducing the availability of phytate to form complexes with mineral cations which precipitate above pH 5 and thus are resistant to digestion (Selle et al., 2009a). Further beneficial effects on the digestibility of other nutrients have been reported for energy (Ravindran et al., 2006; Selle et al. 2009b), sodium (Liu et al., 2015; Truong et al., 2015) and amino acids (**AA**) (Sebastian et al., 1997; Ravindran et al., 1999; Selle et al., 2012).

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However, these 'extra-phosphoric' effects of phytase are variable across studies (Adeola and Sands, 2003; Dersjant-Li and Kwakernaak, 2019) and the underlying mechanisms are not completely understood.

The degradation of dietary phytate by exogenous phytase improves AA digestibility by a number of modes of action: 1) prevention of binary protein-phytate complex formation in the acidic upper gastrointestinal tract (**GIT**) at less than the isoelectric point (iP) of protein and ternary complex formation in the more neutral pH of the lower GIT at more than the isoelectric point of protein, 2) reduction in mucin-associated endogenous AA flows, and 3) enhanced intestinal uptakes of amino acids by Na+-dependent transporters via facilitated Na+,K+-ATPase pump activity. The likelihood is that the refractory nature of phytate-bound protein to pepsin digestion prompts compensatory hypersecretions of pepsin and HCl which in turn triggers protective hypersecretions of mucin and sodium bicarbonate. Hence elevated endogenous amino acid flows and compromised Na+, K+-ATPase pump activity. Increasing the level of phytate in the diet has been shown to decrease the activity of Na+,K+-ATPase whereas an *E. coli* phytase increased Na+,K+-ATPase pump activity in broiler chickens (Liu et al., 2008) and although the precise mechanism is not clear it may be related to increased sodium concentrations within enterocytes (Selle et al., 2012).

These modes of action dictate that the critical site for phytase to improve AA digestibility is the upper GIT (proventriculus and gizzard) in which pH may be as low as 2.5 to 3.0 (Selle et al., 2009a). The isoelectric point (**iP**) of protein is, on average, 5.5; however, the iP of feed grain proteins exceed this value and those of protein meals are less (Csonka et al., 1926). It is possible that the differential between the isoelectric point of proteins and the pH of digesta in the proventriculus and gizzard governs the intensity of binary protein-phytate complex formation which is pivotal to the 'protein effect' of exogenous phytase. The analysis of commercial phytase activities is standardized at pH 5.5 but activity varies substantially at lower pH (Menezes-Blackburn et al., 2015) which affects the in vivo activity of the enzyme. Hence, the degree of improvement in AA digestibility mediated via the degradation of phytate by phytase especially in the upper GIT differs among individual phytases (Dersjant-Li and Kwakernaak, 2019).

Perhaps due to the inconsistency in AA responses to phytase reported in the literature, the assignment of AA matrix values for commercial phytases is variable (Dersjant-Li et al., 2019). Matrix values prescribe the degree of reduction in the digestible AA content of the diet that may be applied to account for the expected AA-contribution of the phytase when used at a given dose level. The derivation of accurate AA matrix values is of interest both to phytase manufacturers and feed producers. If an optimal proportion of dietary protein is utilized by the bird, the amount of undigested protein that reaches the hind gut will be reduced, lowering nitrogen emissions into the environment and providing less substrate for the growth of potentially harmful bacteria which can lead to disease and the need for antibiotic intervention. In addition, lowering the usage of expensive protein ingredients by optimal use of supplemental phytase may enable a reduction in feed costs. However, defining accurate, globally representative and scientifically sound matrix values is not straightforward, as recently discussed by Bedford and Cowieson, 2020. It involves performing multiple digestibility trials to model the nature and extent of improvements delivered by the phytase at different dose levels and in different settings. Many dietary factors can alter AA digestibility responses to phytase, such as the AA and protein source and content of the diet (Ravindran et al., 1999; Krieg et al., 2020), Ca content and solubility (Sebastian et al., 1997; Amerah et al., 2014; Li et al., 2015; Majeed et al., 2020), feedstuff composition (Selle et al., 2009a; Truong et al., 2015; Liu et al., 2016) and phytate level (Ravindran et al., 2006; Babatunde et al., 2021a). Factors related to the birds, such as genetics (strain) and age affect both AA requirements and responses to phytase (Li et al., 2015) and must also be considered. The derivation of a matrix value that will be globally applicable across the majority of situations requires a large number of studies incorporating variation in these factors in commercially relevant diets.

The analysis described herein combined data on AA digestibility responses to increasing phytase dose level from 4 digestibility trials comprising 13 datasets of basal diets containing different phytate-P (**PP**) levels, variation in feedstuff composition, digesta sampling timepoint and broiler strain. All used the same commercial phytase. The aim was to generate a model based on these data that could be used for generating accurate and reliable AA matrix values that, once validated, could confidently be applied to diets supplemented with this phytase in a variety of broiler production settings. The model was subsequently used to illustrate how a digestible AA matrix for the phytase could be derived for a diet of known AA content. Information on how nutrient matrices are derived by phytase manufacturers is currently lacking in the published literature and it is hoped that the present analysis will address this issue and build confidence in the use of scientifically determined AA matrix values for microbial phytase.

MATERIALS AND METHODS

Overview of the Trials

Data from 4 digestibility trials conducted in 3 research locations were included. An overview of the key features of the 4 trials is presented in Table 1. In total, 13 separate datasets were generated from the 4 trials, with dataset being defined as the subset of data points obtained from any given trial that relate to basal diets of the same basal composition (for example, at a given phytate P level or limestone solubility), as shown in Table 1. Across all datasets, there were a total of 384 datapoints (observations, cages).

Table 1. Overview of the four trials[1].

Trial No.[2]	Strain	Duration of feeding on experimental diet (d)	Age at ileal digesta sampling (d)	No. datasets[3]	Dataset ID	No. datapoints (cages)[4]	No. treatments	Phytase dose levels FTU/kg	Dietary phytate-P (PP) levels (g/kg)[5]	Ca level (g/kg)	Feed form	Main ingredients[6]
Trial 1	Ross 308	9	15	1	4	24	3	0, 500, 1,000	3.33	6.4	mash	Corn/SBM/RM/RB
Trial 2	Cobb 500	10	11	3	1	30	15	0, 500, 1,000,	2.3	7.2	mash	Corn/SBM/PR/
					2	30		2,000, 4,000	2.8	7.2		RB/M&B
					3	30			3.3	7.2		
Trial 3	Cobb 500	11	23	3	8	30	15		2.3	6.4		
					9	30			2.8	6.4		
					10	30			3.3	6.4		
Trial 4	Ross 308	21	21	3	5	30	15		2.45	6.4	Pellet (crumble	Wheat/corn/
					6	30			2.95	6.4	during d 0 to 10)	SBM/BR/
					7	30			3.45	6.4		RB/RM
	Ross 308	35	35	3	11	30			2.45	5.5		
					12	30			2.95	5.5		
					13	30			3.45	5.5		

[1]In total 13 datasets and 384 observations, all male broilers.
[2]Trial 1: Massey University, Palmerston North, New Zealand; Trial 2 & 3: Purdue University, West Lafayette, IN, USA; Trial 4: University of Sydney, Sydney, Australia.
[3]Dataset = A subset of the data points obtained from a trial that relate to basal diets of the same basal composition (e.g. the same phytate P level).
[4]Trial 1: 8 birds/cage (8 cages/treatment); Trial 2: 12 birds/cage (6 cages/treatment); Trial 3: 8 birds/cage (6 cages/treatment); Trial 4: 20 birds/cage (6 cages/treatment).
[5]Formulated values.
[6]SBM, soybean meal; RM, rapeseed meal; PR, polished rice; RB, rice bran; BR, broken rice, M&B, meat and bone meal. Limestone solubility at 5 min was in the range of 45 to 92% across datasets.

All trials employed a randomized complete block design to test the effect of a novel consensus bacterial 6-phytase variant on nutrient digestibility, growth performance and bone mineralization in male broilers when added to basal diets that were deficient in digestible P, Ca, and Na (see Tables 2–4). From each trial, data on the apparent ileal digestibility (**AID**) of AA were generated as the focus of the current paper.

Ethical Considerations and Animal Husbandry

Trial 1 was conducted in accordance with the New Zealand Revised Code of Ethical Conduct for the Use of Animals for Research, Testing and Teaching. Experimental protocols were approved by the Massey University Animal Ethics Committee. Protocols of all experiments in trials 2 and 3 were reviewed and approved by the Purdue University Animal Care and Use Committee. Trial 4 was conducted in compliance with the New South Wales Animal Research Act 1985 and its associated Regulations, the Australian code for the care and use of animals for scientific purposes 8th edition 2013 and the Australian Code for the Responsible Conduct of Research, 2018. In all trials, birds were reared in environmentally controlled houses where the temperature and lighting regimes were controlled and monitored in accordance with local practice.

Phytase Enzyme and Diets

The phytase enzyme used in all trials was a novel consensus bacterial 6-phytase variant, PhyG (Danisco Animal Nutrition, International Flavors & Fragrances (**IFF**) Inc., NY, United States) produced in *Trichoderma reesei*. The biochemical and enzymatic characteristics of this phytase have been described previously (Christensen et al., 2020). In Trial 1, the phytase was premixed with a portion of the final diet (10 kg) prior to adding to the main batch. In trials 2, 3, and 4, the phytase was prepared as a premix with ground corn before being added to the experimental diets. All diets were thoroughly mixed to ensure a homogeneous distribution of phytase and other ingredients. Diets and water were provided ad libitum for the duration of each trial.

Common Chemical Analysis

Phytate phosphorus concentrations in diets were determined at Danisco Animal Nutrition Research Centre (Brabrand, Denmark) using a modified version of the HPLC method described by Skoglund et al. (1998). The in vitro solubility of limestones used in each trial was determined according to the method described by Kim et al. (2019). Phytase activities in the diets were analyzed by Danisco Animal Nutrition Research Centre according to a modified version of the 2000.12 AOAC method (Engelen et al., 2001. One phytase unit (**FTU**) was defined as the quantity of enzyme

Table 2. Ingredient composition and calculated nutrient content of the basal experimental diet excluding phytase, Trial 1.

Item	d 5-15[1]
Ingredient, g/kg (as-fed basis)	
Corn	628.7
Soybean Meal, 480 g/kg CP	244.8
Rapeseed meal	50.0
Rice bran	47.7
L-lysine HCl	2.3
DL-methionine	2.8
L-threonine	1.2
Salt	2.4
Sodium bicarbonate	0.10
Limestone	13.3
Dicalcium phosphate	0.00
Vitamin-mineral premix[2]	1.7
Titanium dioxide	5.0
Total	1,000
Calculated nutrients and energy, g/kg	
ME, kcal/kg	2,907
Crude protein	194.0
Calcium	6.4
Digestible phosphorus	1.5
Phytate-P	3.33
Sodium	1.4
Digestible lysine	10.2
Digestible methionine	5.3
Digestible methionine + cysteine	7.8
Digestible threonine	7.0
Digestible isoleucine	6.7
Digestible leucine	14.9
Digestible tryptophan	1.8
Digestible arginine	11.1

[1]During d 1 to 5, birds were fed a pre-starter diet based on corn, soybean meal, rapeseed meal, and rice bran (9.6 g/kg Ca, 4.20 g/kg dig P). The basal diet was supplemented with phytase (PhyG) at each of three dose levels: 0, 500, 1,000 phytase units (FTU)/kg.

[2]Supplied per kilogram of diet: vitamin A (E 672), 10,000 IU; vitamin D_3 (E 671), 2,000 IU; vitamin E (a-tocopherol), 30.0 mg; vitamin K_3, 2.0 mg; vitamin B1, 1.0 mg; vitamin B_2, 5.0 mg; vitamin B_3, 40.0 mg; vitamin B_5, 10.0 mg; vitamin B_6, 3.0 mg; vitamin B_{12}, 12.0 μg; Folic acid, 1.0 mg; biotin, 0.1 mg; choline chloride, 400 mg; iodine as IK, 2.0 mg; manganese as MnO, 70.0 mg; iron as $FeCO_3$, 60.0 mg; zinc as ZnO, 35.0 mg; copper as $CuSO_4 \bullet 5H_2O$, 8.0 mg; selenium as Na_2SeO_3, 0.15 mg; butylated hydroxytoluene, 4 mg; citric acid, 13,8 mg; sodium citrate, 0.4 mg; sepiolite, 0.4 g; calcium carbonate, 2.34 g.

that released 1 μmol of inorganic phosphate per minute from a sodium phytate substrate at pH 5.5 at 37°C. Titanium dioxide in diets and digesta (except Trial 4, where acid insoluble ash [**AIA**] was used as the marker) was analyzed according to the method of Short et al. (1996).

Trial 1 Methods

In Trial 1 (Massey University, Palmerston North, New Zealand), Ross 308 male broiler chickens were obtained on the day of hatch and fed a pre-starter diet based on corn, soybean meal, rapeseed meal and rice bran. At 5 d of age, 192 individually-weighed birds were assigned to group cages (8 birds/cage) so that each cage contained birds of similar average bird weight. From 6 to 15 d of age, birds were fed the experimental diets (basal diet [BD], BD+PhyG at 500 or BD+PhyG at 1,000 FTU per kilogram of feed) according to treatment. The BD was based on corn, soybean meal, rapeseed meal and rice bran and contained 6.4 g/kg Ca, 1.5 g/kg dig P, 3.33 g/kg PP, 10.2 g/kg dig Lys and 2,907 kcal/kg metabolizable energy (**ME**) (Table 2). Titanium dioxide was added to all diets at 5 g/kg as an inert marker. Diets were provided in mash form.

Birds were housed in an environmentally controlled animal house in which the temperature was initially maintained at 32°C until d 7 and then gradually reduced to 27°C by d 14. The lighting regime was LD 20:4 h (dark hour between 11 pm and 3 am).

At 15 d of age, birds were euthanized by intravenous injection of sodium pentobarbitone. The timing of euthanasia was 5 to 6 h after the lights came on. Ileal digesta was collected from the distal half of the ileum, with ileum being defined as the length of the small intestine from Meckel's diverticulum to 4-cm anterior of the ileocecal junction. Digesta samples were pooled per cage, frozen at −20°C and stored until freeze drying. Freeze-dried digesta and diet samples were ground to pass through a 0.5-mm sieve and stored at 4°C until laboratory analyses.

Calcium and P in diets were determined by colorimetric methods after ashing at 550°C and acid digestion in 6.0 M HCl, in accordance AOAC Method 968.08D (AOAC, 2005). Amino acids in feed ingredients, diets and digesta samples were determined by HPLC with post-column derivatization and fluorometric detection of AA using 0-phthaldialdehyde, according to the detailed procedures described by Ravindran et al. (2009).

Trial 2 and 3 Methods

Trials 2 and 3 were conducted at Purdue University, West Lafayette, IN, using 1,080 and 720 Cobb 500 male broiler chickens, respectively, housed in battery cages. In Trial 2, the birds were fed the experimental diets for the duration of the study (1−11 d of age). In Trial 3, birds were initially (1−11 d of age) fed a commercial starter diet formulated to meet nutritional requirements (NRC, 1994) and thereafter (12−23 d of age) the experimental diets. At 1 d of age in Trial 2 and 12 d of age in Trial 3, birds were individually weighed and assigned to cages (Trial 2, 12 birds/cage; Trial 3, 8 birds/cage) so that each cage contained birds of similar average bird weight. The experimental diets in both trials were based on corn, soybean meal, polished rice and rice bran, with added meat and bone meal. They comprised of a BD (Trial 2: 2.0 g/kg available P, 7.2 g/kg Ca, 1.2 g/kg Na, 11.4 g/kg dig Lys and 2,862 kcal/kg ME; Trial 3: 1.8 g/kg available P, 6.4 g/kg Ca, 1.2 g/kg Na, 10.5 g/kg dig Lys and 2,962 kcal/kg ME) formulated to contain three levels of PP (2.3, 2.8 or 3.3 g/kg) and supplemented with PhyG at each of five dose levels (0, 500, 1,000, 2,000 or 4,000 FTU/kg), giving a total of 15 treatments in each trial (Table 3). The PP levels in the diets were modified by adjusting the level of inclusion of rice bran and polished rice. Soy hulls were used as filler. Titanium dioxide was added to all diets at 5 g/kg as an inert marker. Diets were provided in mash form.

Table 3. Ingredient composition and calculated nutrient content of the basal experimental diet excluding phytase, trials 2 and 3.

	Trial 2 (d 1-11)			Trial 3 (d 12-23)		
Phytate-P level	Low	Medium	High	Low	Medium	High
Ingredient, g/kg (as-fed basis)[1]						
Corn	508.1	504.8	503.6	549.0	546.0	543.0
Soybean meal, 480 g/kg CP	308.0	302.7	297.2	276.2	272.8	269.7
Soybean oil	5.0	5.0	5.0	9.1	10.0	8.0
Rice, Polished	80.8	49.4	15.9	80.4	45.0	18.1
Rice bran	3.1	42.5	82.0	7.2	46.4	84.5
Soy hulls	46.1	47.7	49.1	38.8	41.5	39.4
Meat and bone meal	22.4	21.2	20.2	12.2	11.0	9.8
Limestone	8.8	9.1	9.3	9.6	9.9	10.2
Monocalcium phosphate	0.0	0.0	0.0	0.0	0.0	0.0
Salt	2.5	2.5	2.5	2.7	2.7	2.7
Vitamin-mineral premix[2]	3.0	3.0	3.0	3.0	3.0	3.0
DL-methionine	3.0	3.1	3.1	2.9	2.9	3.0
L-lysine.HCl	2.7	2.7	2.7	2.8	2.8	2.7
Threonine	1.3	1.3	1.2	0.9	0.9	0.8
L-tryptophan	0.1	0.1	0.1	0.2	0.1	0.1
Titanium dioxide	5.0	5.0	5.0	5.0	5.0	5.0
Total	1,000	1,000	1,000	1,000	1,000	1,000
Calculated nutrients and energy, g/kg						
ME, kcal/kg	2,862	2,862	2,862	2,962	2,962	2,962
Crude protein	209.7	209.7	209.7	192.4	193.0	193.6
Calcium	7.2	7.2	7.2	6.4	6.4	6.4
Phosphorus	4.6	5.1	5.6	4.1	4.6	5.1
Phytate-P	2.3	2.8	3.3	2.3	2.8	3.3
Non-phytate P	2.3	2.3	2.3	1.8	1.8	1.8
Sodium	1.2	1.2	1.2	1.2	1.2	1.2
Digestible lysine	11.4	11.4	11.4	10.5	10.5	10.5
Digestible methionine	5.7	5.8	5.8	5.4	5.5	5.5
Digestible methionine + cysteine	8.4	8.4	8.4	7.9	7.9	7.9
Digestible threonine	7.6	7.6	7.6	6.7	6.7	6.7
Digestible isoleucine	7.2	7.2	7.2	6.8	6.8	6.8
Digestible leucine	14.4	14.4	14.4	13.9	13.9	13.9
Digestible tryptophan	2.1	2.1	2.1	1.9	1.9	1.9
Digestible arginine	12.4	12.3	12.3	11.3	11.2	11.3

[1]Each basal diet was supplemented with phytase (PhyG) at each of five dose levels: 0, 500, 1,000, 2,000, and 4,000 phytase units (FTU)/kg.
[2]Supplied per kilogram of diet: vitamin A, 5,484 IU; vitamin D_3, 2,643 ICU; vitamin E, 11 IU; vitamin K_3, 4.38 mg; vitamin B_1, 2.2 mg; vitamin B_2, 5.49 mg; vitamin B_3, 44.1 mg; vitamin B_5, vitamin B_6, 3.3 mg; 11 mg; vitamin B_{12}, 13.2 μg; folic acid, 990 μg; biotin, 55.2 μg; choline chloride, 771 mg; iodine, 1.11 mg; manganese, 66.06 mg; copper, 4.44 mg; iron, 44.1 mg; zinc, 44.1 mg; selenium, 300 μg.

Birds were housed in environmentally controlled animal houses. In Trial 2, temperature was maintained at 35°C during d 0 to 7 and 31°C during d 7 to 11. In Trial 3, temperature was maintained at 35°C from d 0 to 7, 31°C during d 7 to 14 and 27°C during d 14 to 23. The lighting regime was LD 23:1 h in both trials (dark hour between 12 am and 1 am).

On d 11 (Trial 2) and d 23 (Trial 3), all birds were euthanized by carbon dioxide asphyxiation. The timing of euthanasia was 08:00 h (7 h after the lights came on). Digesta was collected from the distal ileum as described for Trial 1. Digesta samples were pooled per cage and stored and processed as described for Trial 1.

Nutrient analysis of final feed and digesta samples was carried out at the University of Missouri Experiment Station Chemical Laboratories (Columbia, MO). Amino acids in final feed and digesta were determined using AOAC method 982.30 E (AOAC, 2006). Determination of P and Ca in feed was performed after wet ash digestion with nitric and hydrochloric acids as described by Babatunde et al. (2019).

Trial 4 Methods

In Trial 4 (University of Sydney, Camden, NSW, Australia), a total of 1,800 Ross 308 male broilers were obtained on day-of-hatch and randomly allocated to 90 cages with 20 birds/cage and 6 cages/treatment. Birds were fed the experimental diets according to treatment in 3 phases: starter (d 0−10), grower (d 11−21) and finisher (d 22−35). The experimental diets were based on wheat, soybean meal, corn, broken rice, rice bran, and rapeseed meal. They comprised a BD containing 2.6 g/kg digestible P, 7.6 g/kg Ca, 1.20 g/kg Na, 11.38 g/kg dig Lys and 2,860 kcal/kg ME during starter phase, 2.1 g/kg digestible P, 6.4 g/kg Ca, 1.22 g/kg Na, 10.54 dig Lys and 2,970 kcal/kg ME during grower phase, and 1.6 g/kg digestible P, 5.5 g/kg Ca, 1.22 g/kg Na, 9.76 g/kg dig Lys and 3,050 kcal/kg ME during finisher phase. The BDs were formulated to contain 3 levels of PP (2.45, 2.95 or 3.45 g/kg) and were supplemented with PhyG at each of 5 dose levels (0, 250, 1,000, 2,000 or 4,000 FTU/kg) giving a total of 15 treatments. The PP content of the diets was modified by adjusting the inclusion of rice bran, broken rice and canola meal in the

Table 4. Ingredient and calculated nutrient content of the basal experimental diets excluding phytase, Trial 4.

	Starter (d 0-10)			Grower (d 10-21)			Finisher (d 21-35)		
Phytate-P level	Low PP	Med PP	High PP	Low PP	Med PP	High PP	Low PP	Med PP	High PP
Ingredients, g/kg as-fed basis[1]									
Wheat	450.0	450.0	450.0	500.0	500.0	489.0	550.0	550.0	514.0
Soybean meal, 475 g/kg CP	293.0	274.6	266.9	280.9	236.5	229.5	218.0	199.9	194.9
Corn	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Broken rice	96.6	50.5	14.9	50.2	26.6	0.00	51.4	6.1	0.00
Celite	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Limestone	13.3	13.0	12.9	12.0	11.7	11.5	11.4	11.1	10.9
Vegetable oil	8.8	9.5	7.8	23.1	20.4	19.0	25.5	26.3	25.6
Dicalcium phosphate	7.7	7.5	7.3	4.8	4.7	4.6	2.3	2.0	2.0
DL-methionine	2.7	2.5	2.5	2.3	2.3	2.2	2.0	1.8	1.7
L-lysine	2.3	0.21	0.20	0.15	0.22	0.20	0.23	0.22	0.20
Vitamin-mineral premix[2]	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Salt	1.5	1.5	1.5	1.8	1.6	1.6	1.6	1.6	1.6
Sodium bicarbonate	0.90	0.7	0.5	0.5	0.6	0.5	0.8	0.6	0.4
L-threonine	0.90	0.8	0.8	0.4	0.7	0.7	0.7	0.7	0.6
Choline chloride	0.60	0.06	0.06	0.05	0.05	0.05	0.05	0.05	0.05
Rapeseed meal	-	30.0	40.0	-	30.0	40.0	-	30.0	40.0
Rice bran	-	34.6	70.3	-	40.3	76.8	11.8	45.4	83.7
Total	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000	1,000
Calculated nutrients, g/kg									
ME (kcal/kg)	2,860	2,860	2,860	2,970	2,970	2,970	3,050	3,050	3,050
Crude protein	215.0	219.0	221.0	211.0	206.0	208.0	190.0	193.0	195.0
Digestible lysine	11.4	11.4	11.4	10.5	10.5	10.5	9.8	9.8	9.8
Digestible methionine	5.4	5.4	5.2	4.9	4.9	4.8	4.4	4.3	4.2
Digestible methionine + cysteine	8.4	8.4	8.4	7.9	7.9	7.9	7.2	7.2	7.2
Digestible threonine	7.3	7.3	7.3	6.8	6.8	6.8	6.3	6.3	6.3
Digestible isoleucine	7.7	7.7	7.7	7.6	7.2	7.2	6.6	6.6	6.6
Digestible leucine	14.0	13.8	13.5	13.8	12.8	12.6	12.1	11.9	11.6
Digestible tryptophan	2.4	2.3	2.3	2.3	2.1	2.1	2.0	2.0	1.9
Digestible arginine	12.7	12.5	12.2	12.3	11.4	11.2	10.6	10.4	10.2
Total phosphorus	4.8	5.4	6.0	4.3	4.9	5.5	3.9	4.4	5.0
Phytate phosphorus	2.45	2.95	3.45	2.45	2.95	3.45	2.45	2.95	3.45
Digestible phosphorus	2.6	2.6	2.6	2.1	2.1	2.1	1.6	1.6	1.6
Calcium	7.6	7.6	7.6	6.4	6.4	6.4	5.5	5.5	5.5

[1]Each basal diet was supplemented with phytase (PhyG) at each of 5 dose levels: 0, 500, 1,000, 2,000, and 4,000 phytase units (FTU)/kg.
[2]Supplied per kilogram of diet: vitamin A, 12,000 IU; vitamin D_3, 5,000 IU; vitamin E, 75 mg; vitamin K_3, 3.5 mg; vitamin B_1, 3 mg; vitamin B_2, 9 mg; vitamin B_3, 55 mg; vitamin B_5, 18 mg; vitamin B_6, 5 mg; vitamin B_{12}, 25 μg; folate, 2 mg; biotin, 200 μg; cereal-based carrier, 149 mg; mineral oil, 2.5 mg; iodine (iodide), 1.25 mg; Manganese (sulphate and oxide), 80 mg; iron (sulphate), 40 mg; Zinc (sulphate and oxide), 100 mg; copper (sulphate), 16 mg; selenium (selenate), 0.3 mg.

formulation. Celite, a source of AIA, was added to all diets at 20 g/kg as an inert marker. The detailed composition of the diets is given in Table 4. Diets were provided to birds in crumbled form during starter phase and in pelleted form (pelleting temperature 80°C) during grower and finisher phases.

Birds were housed in an environmentally controlled animal house in which the temperature was initially maintained at 33°C until d 3 after which it was gradually reduced by 1.0°C every 2 d to reach 23.0°C and maintained at that level thereafter. The lighting regime was LD 23:1 h during the first 2 d and then gradually decreased to LD18:6 h (dark hour between 8 pm and 2 am).

At 21 and 35 d of age, 6 birds per cage were euthanized by injection of sodium pentobarbitone. The timing of euthanasia was between 08:00 and 09:00 h (6−7 h after the lights came on). Digesta was collected from the distal ileum and samples were pooled per cage, stored and processed as described for Trial 1. Amino acids in diets and distal ileal digesta were determined via 24 h liquid hydrolysis at 110°C in 6 M HCl followed by analysis of 16 AA using the Waters AccQTag Ultra chemistry on a Waters Acquity UPLC, as described by Cohen (2001). Acid insoluble ash was determined according to the methods described by Siriwan et al. (1993). Phosphorus and Ca were determined by inductively coupled plasma-optical emission spectrometry (**ICP-OES**).

Calculations

AID coefficients were calculated according to the following equation:

$$\text{Ileal digestibility coefficient} = \frac{(\text{AA/Inert marker})_{\text{diet}} - (\text{AA/Inert marker})_{\text{digesta}}}{(\text{AA/Inert marker})_{\text{diet}}}$$

Where $(\text{AA/Inert marker})_{\text{diet}}$ is the ratio of the AA and inert marker (titanium dioxide or AIA) in the experimental diet, and $(\text{AA/Inert marker})_{\text{digesta}}$ is the ratio of the AA and inert marker in the ileal digesta.

On the assumption that the digestibility of synthetic AA is considered to be close to 100% and therefore minimally affected by exogenous phytase (Izquierdo et al., 1988; Selle et al., 2020), it was considered that the supplemental phytase would improve the digestibility of

AA from the basal plant-based ingredients and not from the supplemental synthetic AA in the diets. Therefore, calculation of the digestibility coefficients for Lys, Met, Thr, and Trp was performed after deduction of the added synthetic AA content from the total content of the AA in the diet.

Statistical Modeling

Cage served as the experimental unit for all analyses, unless otherwise stated. Relationships between AID of AA (individual and total) and increasing phytase dose level (based on analyzed values) were initially evaluated separately for each dataset by linear regression and exponential curve fitting. Following this, the combined data were modeled in 2 ways: In the first approach, the actual AID of AA values (as coefficients) were modeled against increasing phytase dose level (based on analyzed values), using nonlinear exponential functions. This approach did not take account of variations between trials but was undertaken to provide an indication of overarching trends in response within the entire master dataset that included all potential influencing factors. In the second approach, the percentage unit increase in AID of AA at each tested phytase dose level compared with that in the respective BD without phytase (percentage unit improvement above the average value of BD) was calculated separately for each dataset. These data were then tested by separate two-way ANOVA to determine if there were any interactions between phytase dose level and: 1) bird age at digesta sampling, or 2) dietary analyzed PP level. As no interactions were identified ($P > 0.05$), the data from all 13 datasets were combined and modeled together, using nonlinear exponential functions. This second approach accounted for the variation between datasets in the original AID of AA (i.e., that in the BD without phytase). All statistical analyses were conducted in JMP14.0 (JMP, 2019; SAS Institute Inc., Cary, NC). Differences or effects were considered significant at $P < 0.05$ and $0.05 < P < 0.1$ was considered a tendency.

RESULTS

Analyzed Nutrient Levels and Phytase Activities

Analyzed levels of nutrients (averaged across BD and phytase treatment diets for each dataset) are presented in Table 5. Phytate-P levels varied modestly from formulated levels, by a minimum of 6% and a maximum of 24%. Phytase activities in PhyG-supplemented treatments were generally within 30% of target levels after accounting for endogenous activity in the basal diets and adequate separation between adjacent dose levels was maintained throughout. Modeling of AA responses was based on analyzed phytase activities to improve the accuracy of model predictions. Analyzed levels of P and Ca were consistently within 20% of formulated values. The in vitro solubility of the limestone at 5 min varied from 45 to 92% across trials.

Effect of Increasing Phytase Dose Level on Apparent Ileal Total AA Digestibility, Within Each of the 13 Datasets

A total of 18 individual AA were determined in trials 2 and 3 (Ala, Arg, Asp, Cys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val), 17 AA were determined in Trial 1 (as for trials 2 and 3 but excluding Trp), and 16 AA in Trial 4 (as for trials 2 and 3 but excluding Cys and Trp).

Changes in the coefficient of AID of total AA (based on the measurement of 17, 18, 18, and 16 individual AA in Trial 1, 2, 3, and 4, respectively) with increasing phytase dose level are separately shown for each dataset in Figure 1. A general pattern of increasing AID AA with increasing phytase dose level is evident, but there was considerable variation in the initial AID coefficients and also some variation in the magnitude and pattern of response to increasing phytase dose levels among individual datasets. Significant ($P < 0.05$) linear relationships were identified for all 13 datasets, but for some datasets there was also a significant ($P < 0.05$; datasets 9 and 10) or near significant ($0.05 < P < 0.1$; datasets 12 and 13) exponential relationship between the two variables (Figure 1).

Coefficient of Apparent Ileal Digestibility of Amino Acids in Response to Increasing Phytase Dose Level Based on Exponential Curve Fitting of the Combined Data From All 13 Datasets

Table 6 shows the AID of individual and total AA (as coefficients) in response to increasing analyzed phytase dose level, as predicted by fitting an exponential curve to the combined data from all 13 datasets. The associated parameters of the fitted exponential response curves are given in Table 7. It should be noted that the calculated values (coefficients) of AID for Lys, Met, Thr, and Trp that were used as the basis for the exponential modeling were based on exclusion of the synthetic components from the AID calculation. In the BD without phytase, the model-predicted AID coefficient of individual AA ranged from a minimum of 0.561 (Cys) to a maximum of 0.826 (Glu) (equivalent to 56.1 and 82.6%, respectively) and the mean coefficient AID of total AA was 0.758. The AID coefficient of total AA and of all individual AA except Lys and Trp increased exponentially ($P < 0.05$) with increasing phytase dose level within the range 0 to 4,000 FTU/kg. Both Lys and Trp showed near significant ($0.5 < P < 0.1$) exponential increases. The model-predicted AID of AA coefficients at 4,000 FTU/kg were very close to the respective asymptote value of the respective response curve for all individual AA (>99.5% when compared with the AID of AA coefficient at 4,000 FTU/kg to the asymptote except for Trp, which was 98.4%; Table 7), suggesting that the AID AA response reached a plateau

Table 5. Analyzed nutrient composition (average of basal diets and phytase-supplemented diets, g/kg as is, otherwise indicated) and phytase activity of the experimental diets in trials 1 to 4 (13 datasets).

Dataset ID	Age at sampling (d)	Ca (total)	P (total)	Ca:P ratio (total)	Phytate-P	Limestone solubility[1]	Analyzed phytase, FTU/kg				
							BD	BD+PhyG 500	BD+PhyG 1,000	BD+PhyG 2,000	BD+PhyG 4,000
1	11	8.49	4.60	1.85	2.02	62.3	200	848	1,331	2,348	4,493
2	11	8.51	5.09	1.67	2.96	62.3	184	879	1,245	2,005	4,517
3	11	8.48	5.60	1.51	3.71	62.3	202	715	1,070	3,093	5,143
4	15	7.08	3.74	1.89	3.30	45.2	45	586	1,036	-	-
5	21	7.08	4.81	1.47	2.98	91.7	355	844	1,396	2,547	4,407
6	21	7.02	5.16	1.36	3.45	91.7	344	958	1,646	2,521	4,327
7	21	6.63	5.49	1.21	3.89	91.7	416	828	1,245	2,312	4,820
8	23	7.28	4.10	1.78	2.00	62.3	181	817	1,420	2,393	4,170
9	23	7.28	4.61	1.58	3.10	62.3	200	623	1,219	1,807	5,060
10	23	7.24	5.13	1.41	3.60	62.3	210	684	1,403	2,486	4,641
11	35	7.00	3.88	1.80	2.61	91.7	482	993	1,713	2,395	4,598
12	35	5.90	3.95	1.49	3.20	91.7	541	1,038	1,470	2,461	4,464
13	35	5.80	4.45	1.30	3.75	91.7	421	939	1,292	2,265	4,475

Dataset ID	Age at sampling (d)	Lys (total)	Lys(excl. synthetic)	Met (total)	Met (excl. synthetic)	Cys (total)	Thr (total)	Thr (excl. synthetic)	Trp (total)	Trp (excl. synthetic)	Total AA
1	11	13.52	11.41	5.90	2.92	3.24	8.70	7.42	2.62	2.53	208.3
2	11	13.56	11.45	5.68	2.65	3.22	8.56	7.30	2.64	2.56	205.4
3	11	13.28	11.16	5.62	2.53	3.14	8.64	7.41	2.74	2.67	203.9
4	15	12.71	10.91	5.91	3.14	3.28	8.70	7.52	-	-	230.5
5	21	11.47	10.30	3.84	1.56	-	8.05	7.65	-	-	209.5
6	21	11.52	9.81	3.62	1.34	-	8.16	7.46	-	-	209.1
7	21	11.55	9.99	3.54	1.36	-	8.18	7.48	-	-	212.3
8	23	11.84	9.63	5.32	2.45	2.98	7.44	6.51	2.42	2.22	185.5
9	23	12.98	10.80	5.78	2.87	3.14	7.74	6.86	2.32	2.22	193.9
10	23	12.22	10.11	5.64	2.71	3.08	7.60	6.78	2.44	2.34	191.4
11	35	10.53	8.74	3.58	1.60	-	7.30	6.60	-	-	175.1
12	35	10.68	8.96	3.45	1.67	-	7.54	6.84	-	-	180.5
13	35	10.77	9.21	3.43	1.74	-	7.61	7.01	-	-	182.9

[1]Determined after 5-min incubation at pH 3.0, according to the method of Kim et al. (2019).

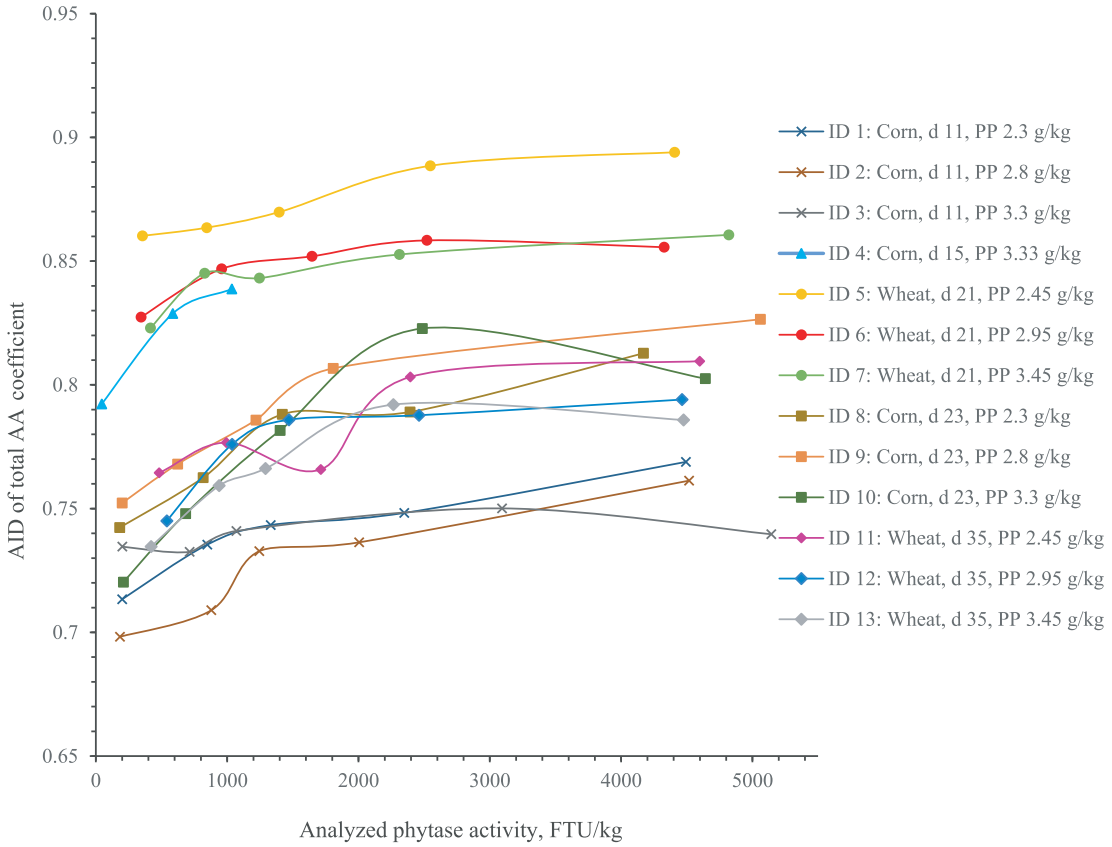

Figure 1. Apparent ileal total amino acid digestibility coefficients plotted against increasing phytase dose level (analyzed values, above basal diet), for each of the 13 datasets.

	Trial ID												
	1	2	3	4	5	6	7	8	9	10	11	12	13
P - value 'linear'	0.010	0.006	0.551	< 0.001	< 0.001	0.022	0.005	< 0.001	< 0.001	< 0.001	0.003	0.001	0.008
P - value 'exponential'	0.429	0.442	0.750	0.184	0.546	0.241	0.192	0.211	0.009	0.035	0.349	0.075	0.083

Table 6. Estimated apparent ileal amino acid digestibility coefficients in response to increasing analyzed phytase activity[1], modeled across all 13 datasets by exponential curve fitting.

	Phytase (PhyG) dose level, FTU/kg							
Item	0	250	500	1,000	2,000	3,000	4,000	Exponential curve *P*-value
Amino acid								
Alanine	0.723	0.733	0.742	0.753	0.764	0.769	0.770	0.0212
Arginine	0.820	0.828	0.834	0.843	0.853	0.858	0.860	0.0058
Aspartic acid	0.720	0.732	0.741	0.754	0.769	0.775	0.778	0.0007
Cysteine	0.561	0.581	0.597	0.620	0.645	0.655	0.660	0.0030
Glutamic acid	0.826	0.834	0.839	0.848	0.857	0.860	0.862	0.0245
Glycine	0.671	0.685	0.696	0.711	0.724	0.729	0.730	0.0021
Histidine	0.763	0.773	0.781	0.791	0.802	0.805	0.807	0.0082
Isoleucine	0.739	0.750	0.759	0.772	0.785	0.790	0.792	0.0034
Leucine	0.753	0.765	0.773	0.785	0.796	0.800	0.802	0.0063
Lysine[2]	0.739	0.749	0.756	0.768	0.779	0.783	0.785	0.0603
Methionine[2]	0.691	0.722	0.740	0.757	0.764	0.764	0.765	0.0071
Phenylalanine	0.768	0.778	0.787	0.799	0.812	0.817	0.818	0.0041
Proline	0.760	0.771	0.779	0.790	0.801	0.805	0.807	0.0150
Serine	0.711	0.725	0.737	0.753	0.769	0.776	0.778	0.0005
Threonine[2]	0.624	0.638	0.650	0.667	0.685	0.693	0.697	0.0026
Tryptophan[2]	0.761	0.768	0.774	0.785	0.800	0.810	0.817	0.0711
Tyrosine	0.728	0.742	0.752	0.767	0.782	0.788	0.790	0.0002
Valine	0.709	0.720	0.728	0.740	0.752	0.756	0.758	0.0245
Total AA	0.758	0.768	0.776	0.787	0.799	0.803	0.805	0.0072

[1]Above the analyzed activity in the basal diet without added phytase.
[2]The calculated AID coefficient for these AA excludes the content of supplemental synthetic AA.

Table 7. Fitted exponential response curve parameters[1] for the relationship between the apparent ileal digestibility of individual and total amino acid coefficients and increasing analyzed phytase activity[2].

	Parameters of the exponential fitted curve[1]			
y	Asymptote a	Scale b	Growth rate[2] c	Exponential curve *P*-value
Amino acid				
Alanine	0.771	−0.048	−0.0010	0.0212
Arginine	0.862	−0.042	−0.0008	0.0058
Aspartic acid	0.780	−0.060	−0.0008	0.0007
Cysteine	0.663	−0.102	−0.0009	0.0030
Glutamic acid	0.863	−0.037	−0.0009	0.0245
Glycine	0.731	−0.060	−0.0011	0.0021
Histidine	0.808	−0.044	−0.0010	0.0082
Isoleucine	0.794	−0.055	−0.0009	0.0034
Leucine	0.802	−0.049	−0.0011	0.0063
Lysine[3]	0.786	−0.048	−0.0009	0.0603
Methionine[3]	0.765	−0.074	−0.0022	0.0071
Phenylalanine	0.820	−0.052	−0.0009	0.0041
Proline	0.808	−0.048	−0.0010	0.0150
Serine	0.780	−0.069	−0.0009	0.0005
Threonine[3]	0.700	−0.076	−0.0008	0.0026
Tryptophan[3]	0.830	−0.069	−0.0004	0.0711
Tyrosine	0.792	−0.063	−0.0009	0.0002
Valine	0.759	−0.050	−0.0010	0.0245
Total AA	0.806	−0.048	−0.0010	0.0072

[1]The exponential equation is: $Y = a+b*e^{(c*\text{analyzed phytase})}$.
[2]Above the analyzed activity in the basal diet without added phytase.
[3]The calculated AID coefficient for these AA excludes the content of supplemental synthetic AA.

at 4,000 FTU/kg. The AID of total AA was predicted to increase from 0.758 to 0.799 or 0.805 with phytase at 2,000 or 4,000 FTU/kg, respectively. Greatest increases in AID coefficients were predicted for Cys (from 0.561 without phytase to 0.645 or 0.660 with phytase at 2,000 or 4,000 FTU/kg, respectively) followed by Met (from 0.691 without phytase to 0.764 or 0.765 with phytase at 2,000 or 4,000 FTU/kg, respectively). Least increments in AID coefficients were predicted for Glu (from 0.826 without phytase to 0.857 or 0.862 with phytase at 2,000 or 4,000 FTU/kg, respectively) and Arg (from 0.820 without phytase to 0.853 or 0.860 with phytase at 2,000 or 4,000 FTU/kg, respectively).

Percentage Unit Improvement (Above Basal Diet) in Ileal Digestibility of Amino Acids in Response to Increasing Phytase Dose Level, Based on Exponential Curve Fitting of the Combined Data From All 13 Datasets

Table 8 shows the modeled percentage unit increase (above the BD without phytase) in AID of AA in

Table 8. Estimated percentage unit increase[1] above the basal diet in the apparent ileal digestibility coefficients of individual and total amino acids in response to increasing phytase[2] based on exponential curve fitting.

	Phytase (PhyG) dose level, FTU/kg[2]						
Item	250	500	1,000	2,000	3,000	4,000	Exponential curve *P*-value
Amino acid							
Alanine	1.2	2.1	3.4	4.6	5.1	5.3	<0.0001
Arginine	0.8	1.4	2.4	3.4	3.9	4.1	<0.0001
Aspartic acid	1.1	2.1	3.5	5.0	5.6	5.8	<0.0001
Cysteine	2.0	3.8	6.3	9.2	10.5	11.0	<0.0001
Glutamic acid	0.7	1.3	2.2	3.2	3.6	3.8	<0.0001
Glycine	1.5	2.6	4.1	5.4	5.9	6.0	<0.0001
Histidine	1.0	1.8	3.0	4.1	4.6	4.7	<0.0001
Isoleucine	1.2	2.2	3.5	4.8	5.3	5.5	<0.0001
Leucine	1.2	2.2	3.5	4.7	5.1	5.3	<0.0001
Lysine[3]	1.1	1.9	3.2	4.5	5.1	5.3	<0.0001
Methionine[3]	2.8	4.5	6.7	8.4	8.9	9.0	<0.0001
Phenylalanine	1.1	2.0	3.3	4.6	5.2	5.4	<0.0001
Proline	1.1	1.9	3.1	4.2	4.6	4.7	<0.0001
Serine	1.5	2.6	4.3	5.8	6.4	6.7	<0.0001
Threonine[3]	1.5	2.7	4.4	6.2	7.0	7.3	<0.0001
Tryptophan[3]	0.5	1.2	2.4	3.9	4.9	5.4	0.0035
Tyrosine	1.5	2.6	4.1	5.6	6.1	6.3	<0.0001
Valine	1.1	2.0	3.3	4.6	5.1	5.3	<0.0001
Total AA	1.1	1.9	3.1	4.3	4.8	5.0	0.0035

[1]Percentage unit increase, calculated individually for each of the 13 datasets to account for the difference in digestible AA coefficient starting point (i.e. in the basal diet without phytase). The data were then fitted together to the exponential curve.
[2]Above the analyzed activity in the basal diet without phytase.
[3]The calculated percentage unit increase in AID coefficient for these AA excludes the content of supplemental synthetic AA.

response to increasing phytase dose level, based on fitting an exponential curve to the combined data from the 13 datasets. The associated parameters of the fitted exponential response curves are given in Table 9. Again, the improvements for Lys, Met, Thr, and Trp were based on exclusion of synthetic components from the AID calculation. According to the fitted response curves, the percentage unit improvement above BD in AID of all individual and total AA increased exponentially with increments of phytase dose level within the range 250 to 4,000 FTU/kg ($P < 0.01$ in all cases, Table 8). The AID of total AA was improved by 4.3 or 5.0% units with PhyG at 2,000 or 4,000 FTU/kg, respectively. Amongst the essential AA, the percentage unit increases in AID above BD with phytase at 2,000 or 4,000 FTU/kg were greatest for Met (+8.4 or +9.0% units, respectively) followed by Thr (+6.2 or +7.3% units, respectively), and smallest for His (+4.1 or +4.7% units, respectively). Amongst the non-essential AA, percentage unit increases in AID above BD with PhyG at 2,000 or 4,000 FTU/kg were greatest for Cys (+9.2 or +11.0% units, respectively) followed by Ser (+5.8 or 6.7% units, respectively), and smallest for Glu (+3.2 or +3.8% units, respectively).

DISCUSSION

Published information on the methodology to estimate digestible AA matrix values for phytase is scant. To the Authors' knowledge, this is the first study to report modeling of AA digestibility responses from a large number of datasets all involving the same phytase and to show how these data could be used to generate matrix values in commercially relevant diets. Given the multitude of factors that can affect phytase efficacy and AA responses (Selle and Ravindran 2007; Dersjant-Li et al., 2015), it is clearly impractical to derive different matrix values for each permutation of use. Instead, the intention here was to use these multiple datasets that collectively incorporated variation in broiler strain, age at ileal digesta sampling, ingredient composition and form, dietary PP content and limestone solubility, to generate accurate and globally representative AA matrix values for this particular phytase, given that AA responses can differ markedly between different phytases (Dersjant-Li and Kwakernaak, 2019) and therefore matrix values should ideally be generated on a phytase-specific basis.

Another novel feature of the current analysis, which should be noted prior to data interpretation, is the correction of AID of AA calculations for contribution from the synthetic forms of Lys, Met, Thr, and Trp added to the diet. It is not routine practice to correct AID values in this manner. However, it enables a more precise evaluation of the improvement of each of these digestible AA in the basal diet by the phytase, based on the assumption that the inherent digestibility of the synthetic forms of these AA is almost 100% (Izquierdo et al., 1988; Selle et al., 2020). It should be noted that whilst this correction of AID AA values is likely to improve accuracy and precision, it makes the values less comparable with those from other studies where such correction has not been applied.

The initial modeling of each of the 13 datasets revealed that the AID of AA was universally improved by phytase. Despite the variation in initial AID values (in the BD, without phytase), in the magnitude of AID improvement with increasing phytase dose levels and in the response at high dose levels (>2,000 FTU/kg), positive linear or exponential relationships were evident in all datasets. This indicates that the phytase was effective in improving AA digestibility in a dose-dependent manner across an array of diet types (wheat- or corn-based, different protein rich feedstuffs and all-vegetable or containing meat and bone meal) representative of those currently used globally in broiler production. The diets were characterized by a range of PP levels (2.00 −3.89 g/kg) and 5-min limestone solubilities (45−92%). Furthermore, the models generated used data collected at age ranges of 11 to 35 d post-hatch from 2 representative commercial broiler strains (Cobb 500 and Ross 308) across 3 different geographical locations (Australia, New Zealand and United States). Four of the datasets (datasets 3, 6, 10, and 13) showed a plateauing in the AID AA response at phytase dose levels higher than 2,000 FTU/kg. This effect could have occurred if AA requirements had already been met by lower dose levels in those particular settings. It is known that variation in AA digestibility measurements can be high, which is one of the reasons why a large number of studies is needed to generate globally representative digestible AA improvement estimates. In other datasets, there was no plateauing and AID AA responses continued to increase up to 4,000 FTU/kg. These findings are in contrast to a meta-analysis of 24 studies by Cowieson et al. (2017) in which phytase inclusion beyond 1,000 FTU/kg did not increase AID AA and suggests that, for PhyG phytase, high dose levels may deliver further AA digestibility responses under certain dietary settings. This may be linked to the mode of action of the phytase. As already discussed, phytase needs to be highly active in the upper GIT to efficiently improve AA digestibility. It is recognized that the high IP-esters, IP_6 and IP_5, exert the greatest negative effect on protease activity and protein digestion, however lower IP-esters such as IP_3 or IP_4 also exert some degree of negative effect (Yu et al., 2012). It is known that PhyG is able to break down $IP_{3\text{-}6}$ rapidly and extensively in vitro (Christensen et al., 2020), thus increasing the dose level could serve to further reduce the accumulation of lower IP esters in vivo (Dersjant-Li et al., 2021), resulting in the continued increase in the AID of digestible AA observed up to the highest dose level included in the trials (4,000 FTU/kg).

Among the datasets, the initial AID of AA in the BD without phytase appeared to be the more prominent source of difference in response rather than increasing phytase dose level. In the absence of phytase, the AID AA is likely to have been largely influenced by the inherent digestibility of the dietary feedstuffs (and their

relative concentrations); cereals and cereal-derived ingredients differ in their inherent AA digestibility (Ravindran et al., 1999; Ullah et al., 2016) and the diet compositions ranged from wheat-based to corn-based and contained different types and quantities of by-products such as rapeseed meal and rice bran. Bird age could also have influenced the baseline AID AA because the capacity to digest protein and AAs differs by age, being lower in young birds and increasing with age (Batal and Parsons, 2002; Li et al., 2015). Dietary PP level can also affect the inherent AA digestibility of the diet (Plumstead et al., 2013; Cowieson et al., 2017) and may well have contributed to the baseline AID. However, the individual data from trials 2 and 3 reported separately by Babatunde et al. (2021a,b,c) revealed no interactions between PP level and phytase dose level in respect of effects on AID of AA and no PP level effect on the magnitude of percentage unit change with phytase was evident for the majority of individual AA except for Met and Cys (Babatunde et al., 2021a,c). This suggests that the efficacy of the phytase to improve AID of AA (above BD) was largely unaffected by PP level, except for Met and Cys. Toghyani et al. (2021) similarly reported no interaction between PP level and phytase dose level in effects on AID of AA in Trial 4. The choice of indigestible marker included within the diet for determination of nutrient digestibility may also have influenced the AA digestibility values obtained from each trial. Several studies have reported that differences in AA response were related to the choice of inert marker (Selle et al., 2006; Selle and Ravindran, 2007), with AIA potentially producing a higher AA digestibility response to phytase than chromic oxide (Selle et al., 2006). In the trials reported herein, chromic oxide was not used. Datasets 5-7 and 11-13 used AIA, whilst the remainder used titanium dioxide. Whatever the reasons for the variation in the initial AID in BD among datasets, the subsequent modeling of phytase effects and derivation of matrix estimates based on the percentage unit change above BD rather than actual values was expected to enable the degree of improvement by phytase to be separated out from differences in the inherent digestibility of the diets between datasets.

Regardless of the sources of variation among datasets, the ANOVA performed on the percentage unit change in AID of individual and total AAs from the baseline (BD without phytase) in response to increasing dose levels of phytase revealed no interactions with bird age at sampling or dietary PP level except Met and Cys (for these 2 AA, the improvement above BD by phytase is greater in diets containing high level of phytate [Babatunde et al., 2021a,c]). This justified the modeling of all data together as the basis for generating AA matrix estimates (an adjustment could be made later for Met and Cys). Exponential curve fitting was the selected model as this has been used in several previous studies to describe and evaluate broiler digestibility responses to phytase (Bedford et al., 2016; Dersjant-Li and Kwakernaak, 2019). The model-predicted value for AID of total AA in basal diets was broadly similar to that reported in negative control diets in the meta-analysis by Cowieson et al. (2017) (0.76 vs. 0.80). As the composition of commercial diets is constantly evolving, the average AID of AA across the range of commercial diets is likely to change over time. In addition, it is worth mentioning that while most of the publications on AA digestibility response to phytase are with 21-day-old birds, the current datasets cut across different ages from 11 to 35 d of age. Other factors such as studies having been carried out in different years with different diet composition, nutrient specifications, phytase sources and levels make any comparisons across individual studies tenuous. It may also be expected that not all studies that have evaluated digestible AA responses to phytase will have been published and, as a result, any composite analyses from the literature will mainly be based on data showing positive responses.

Modeling of percentage unit changes in AID of AA from baseline (BD) with phytase (all datasets combined) revealed exponential increases in AID of all individual AA with increasing PhyG dose levels between 250 and 4,000 FTU/kg. At 1,000 FTU/kg, the model-predicted percentage unit improvement in AID of total AA by PhyG above BD was 3.11% unit, whereas at 2,000 and 4,000 FTU/kg it was 4.33 and 4.97% units, respectively. Digestibility improvements were generally greater in those AA that had low inherent digestibility in the absence of phytase, thus giving greater potential for improvement by phytase, as has been previously reported (Cowieson et al., 2017). Improvements in AID above BD were greatest for Cys (+9.18 and +11.04% unit vs. BD at 2,000 and 4,000 FTU/kg, respectively), in agreement with previous observations of Dersjant-Li and Kwakernaak (2019) for a *Buttiauxella* sp. phytase in corn-soybean meal-based diets and with the findings of the meta-analysis by Cowieson et al. (2017). Cys is important in cell metabolism (Brosnan and Brosnan, 2006) and along with Met (the other sulfur containing AA), is often the most limiting AA in poultry diets (Warnick and Andersen, 1968). Insufficiency causes reduced feed intake and impaired growth performance (Conde-Aguilera et al., 2013) and the Met:Cys ratio is important for optimal growth. Pronounced improvements in the digestibility of Thr, the third limiting essential AA after Met and Lys, were also observed in the present study (+6.23 and +7.27% unit vs. BD, at 2,000 and 4,000 FTU/kg, respectively), similar to those previous studies involving other phytases by Ravindran et al. (1999), Truong et al. (2015), and Cowieson et al. (2017). Threonine is a major constituent of mucin secretions in animals (Faure et al., 2005). Previous studies have implicated phytate in the upregulation of mucin and pepsin secretions (Cowieson and Ravindran, 2007; Cowieson et al., 2008) leading to increased endogenous AA losses. These data suggested that exogenous phytase has the most effect in improving the digestibility of those AA involved in endogenous protein losses via mucin secretion, and little or no effect on Met which is not a major constituent of mucin or pepsin (Cowieson and Ravindran, 2007; Pirgozliev et al., 2011).

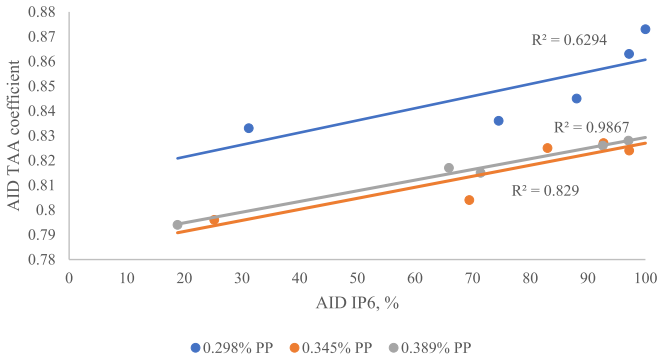

Figure 2. The relationship between apparent ileal digestibility (AID) of total amino acids (TAA) coefficients and AID IP_6 at 21 d of age in broilers fed wheat-based diets containing varied (analyzed) phytate-P levels; data from Trial 4. AID IP_6 data are based on analyzed values. Data were analyzed by linear regression.

In contrast, this was not wholly supported by the current work: Although Thr and Cys digestibilities were highly improved by PhyG phytase, the improvement in Met digestibility was higher than that of Thr and highest among all essential AA (+8.40 and +9.01% units vs. BD at 2,000 or 4,000 FTU/kg, respectively). As Met is the first limiting AA in SBM based broiler diets (see Tables 2–4), the present findings will be of particular interest in diet formulation. This marked improvement in Met digestibility by PhyG in the present analysis, that was not reported in other studies, may be linked to the correction that was applied herein during calculation of AID of Met values to deduct the contribution of synthetic Met, which comprises almost 50% of total Met in poultry diets, leading to a more accurate determination of the improvement in AID of Met by PhyG. As current matrix calculations are typically based on the total Met content of the diets, excluding the proportion of synthetic Met in this manner should generate a more accurate Met matrix value.

If the observed improvements in AA digestibility by PhyG phytase are the direct result of the hydrolysis of phytate in the upper GIT and consequent reduction in its antinutritive effects (via aforementioned mechanisms), they would be expected to be related to phytate (IP_6) degradation. The data in Figure 2 (wheat-based diets, 21 d of age at sampling) lend some support to this hypothesis and confirm the positive linear relationship between the AID of total AA and AID IP_6 at analyzed dietary PP levels of 2.98, 3.45 and 3.89 g/kg (Dersjant-Li et al., 2021).

An example is given in Figure 3 of how the modeled relationship between increasing PhyG dose level and the percentage unit response in AID of individual AA can be used in practice to predict the expected digestible AA contribution (matrix value) of the phytase when added at a commercial dose level (1,000 FTU/kg) to a typical wheat- or corn-based commercial broiler diet. By this method, digestible AA matrix values can be calculated based on the percentage unit improvement in AID with PhyG at the prescribed dose level and the amino acid composition and synthetic AA inclusion level of the specific diet. During starter phase, when the AA

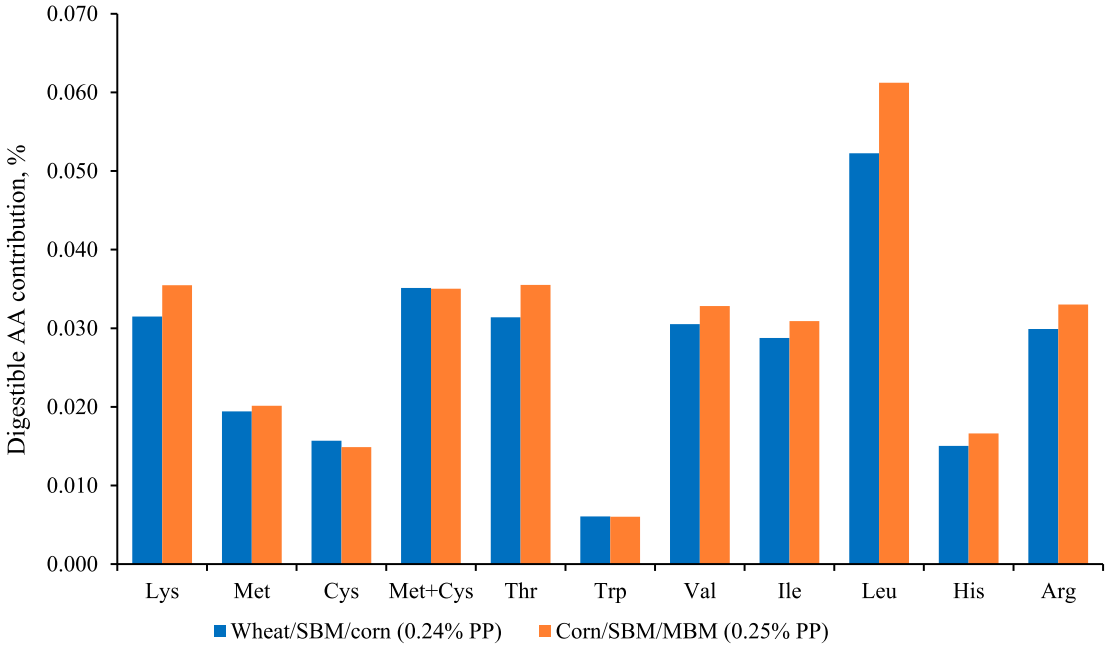

Figure 3. Example of estimated digestible amino acid (AA) contributions based on commercial wheat- or corn-based grower diets supplemented with 1,000 FTU/kg PhyG. PP, phytate-P content. Main ingredients of the wheat-based diet (as fed basis): wheat, 516 g/kg; soybean meal, 267 g/kg; corn 150 g/kg; soya oil, 34.0 g/kg; limestone, 10.3 g/kg; fishmeal, 5.0 g/kg, monocalcium phosphate, 3.20 g/kg. Main ingredients of the corn-based diet (as fed basis): corn, 633 g/kg; soybean meal, 322 g/kg, meat and bonemeal, 13.0 g/kg; limestone, 8.1 g/kg. The digestible AA contributions were calculated based on the total AA content and synthetic AA inclusion of the diet and the model-predicted improvement in AID of AA (percentage unit) by the phytase. As an example: for calculation of the expected contribution of Lys: in a diet containing 14.0 g/kg total Lys, with Lys HCL inclusion at 3.5 g/kg the actual content of synthetic Lys would be 3.5 g/kg × 78% (the purity of Lys) = 2.73 g/kg. Therefore, the content of Lys excluding the synthetic component would be 14.0 − 2.73 = 11.27 g/kg. According to the fitted exponential curve for the effect of PhyG on AID of Lys (based on the combined datasets, percentage unit improvement), the expected Lys contribution by PhyG at 1,000 FTU/kg is 3.2% of 11.27 = 0.36 g/kg (or 0.036%).

Table 9. Fitted exponential response curve[1] parameters for the relationship between the percentage unit increase above the basal diet in the apparent ileal digestibility of individual and total amino acids and increasing analyzed phytase activity[2].

	Parameters of the exponential fitted curve[1]			
y	Asymptote a	Scale b	Growth rate[2] c	Exponential curve *P*-value
Amino acid				
Alanine	0.054	−0.054	−0.0010	<0.0001
Arginine	0.043	−0.045	−0.0008	<0.0001
Aspartic acid	0.060	−0.061	−0.0009	<0.0001
Cysteine	0.115	−0.116	−0.0008	<0.0001
Glutamic acid	0.040	−0.040	−0.0008	<0.0001
Glycine	0.061	−0.061	−0.0011	<0.0001
Histidine	0.048	−0.049	−0.0010	<0.0001
Isoleucine	0.057	−0.057	−0.0010	<0.0001
Leucine	0.054	−0.054	−0.0010	<0.0001
Lysine[3]	0.055	−0.056	−0.0009	<0.0001
Methionine[3]	0.091	−0.087	−0.0013	<0.0001
Phenylalanine	0.056	−0.055	−0.0009	<0.0001
Proline	0.048	−0.048	−0.0010	<0.0001
Serine	0.068	−0.068	−0.0010	<0.0001
Threonine[3]	0.075	−0.075	−0.0009	<0.0001
Tryptophan[3]	0.062	−0.065	−0.0005	0.0035
Tyrosine	0.064	−0.064	−0.0010	<0.0001
Valine	0.054	−0.054	−0.0010	<0.0001
Total AA	0.051	−0.051	−0.0010	0.0035

[1]The exponential equation is: $Y = a+b*e^{(c*\text{analyzed phytase})}$.
[2]Above the analyzed activity in the basal diets.
[3]The calculated percentage unit increase in AID coefficient for these AA excludes the content of supplemental synthetic AA.

requirement and therefore total AA content of the diet is higher, the generated matrix values (predicted AA contributions by PhyG) would be higher than those in a finisher diet in which the AA requirement and therefore total AA content of the diet is lower. The matrix will also be impacted by the proportion of synthetic AA inclusion. Effects of dietary PP level on the AID of AA have been factored into the generated matrix values because the model incorporated data from diets with varied PP levels. It is not proposed that the derived matrix values need to be further adjusted for dietary PP content, because although it is clear dietary PP level can impact on the inherent AA digestibility of the BD (Babatunde et al. 2021a,b,c; Toghyani et al., 2021), our analysis of the data on digestibility of AA improvement above BD by PhyG does not support an adjustment of the AA matrix for dietary PP level for all individual AA except Met and Cys. A linear or exponential increase in digestible AA improvement with increasing PP level was observed only for Met and Cys (data not shown). In addition, a high PP level diet with phytate-rich ingredients may also correspond to a high total AA level (due to increased inclusion of industrial by-products with inherently low AA digestibility). Therefore, the matrix can also indirectly reflect the PP content of the diet. With this approach, an actual feed formulation-based digestible AA contribution is derived that will be more accurate than a 'one matrix fits all' approach. The digestible AA contribution values presented in Figure 3 are examples and these need to be validated in performance trials in commercial production settings as has been performed for a previous generation of phytase (Dersjant-Li et al., 2020). Quantifying the digestible AA contribution of phytase in diet formulations is beneficial for promoting greater use of alternative and local ingredients in diets.

In conclusion, the modeling of AA digestibility responses to supplementation of broiler diets with PhyG phytase based on 13 datasets obtained from 4 independent trials in 3 different geographical locations, incorporating variation in bird- and diet-related factors, has demonstrated consistent improvements in AA digestibility by the phytase in the dose range of 250 to 4,000 FTU/kg. Fitted exponential models predicted PhyG dose-related improvements in AID of total AA of +3.11, +4.33 and 4.97% units at 1,000, 2,000, and 3,000 FTU/kg, respectively. Improvements were greatest for Cys, Thr, and Met after correcting for added synthetic Thr and Met. For the first time, it was shown how matrix values can be generated for all 18 AA based on multiple in vivo studies across different bird ages with correction of synthetic AA for Lys, Met, Thr, and Trp, for a given phytase. Using the known AA content of the specific diet in which the phytase is to be used as the basis for generating the digestible AA matrix will enable more accurate prediction of the contribution of a given phytase dose in a given dietary setting and support the provision of diet-specific recommendations in commercial feed formulations.

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DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Y. Dersjant-Li, A. Bello, T. Stormink and L. Marchal are employees of Danisco Animal Nutrition (IFF).

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