Animal Feed Science and Technology 253 (2019) 166–180

Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci

Comparative effects of two phytases versus increasing the inorganic phosphorus content of the diet, on nutrient and amino acid digestibility in boilers

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ARTICLE INFO

Keywords:
Amino acids digestibility
Broilers
Growth performance
Phosphorus digestibility
Phytase

ABSTRACT

Comparative effects of graded doses of two phytases on growth performance, nutrient and amino acid (AA) digestibility were investigated in corn-soybean meal-based broiler diets. A negative control diet (NC) with low phosphorus (P) content (1.8, 4.4 and 2.5 g/kg retainable, total and phytate P respectively) was supplemented with four doses of a *Buttiauxella* sp phytase (analyzed activity of 303 to 1046 FTU/kg) or an *E. coli* phytase (analyzed activity of 442 to 1811 FTU/kg), and tested against three positive control diets comprising the NC + 0.6, 1.2 or 1.8 g P from monocalcium phosphate (MCP)/kg feed and increased Ca content (+0.8 g/kg). A total of 1152 male Ross 308 broilers at 5 days of age were assigned to 72 cages with 16 birds/cage and 6 cages/treatment were given free access to pelleted diets until 21 days of age. Feed intake (FI), body weight and mortality were recorded and used to calculate body weight gain (BWG) and feed conversion ratio (FCR) during 5–20 days of age. Excreta was collected on day 18–20 to determine total tract retention of nutrients. Tibias (from 4 birds pooled per cage) and ileal digesta (from all birds and pooled per cage) were sampled at 21 days of age to determine tibia ash, ileal digestibility of nutrients and AA. Compared to NC, increasing phytase dose and MCP levels produced a stepwise increase in FI, BWG and reduction in FCR. Increasing phytase dose produced curvilinear increases in BWG, ileal P digestibility, retention and tibia ash ($P < 0.01$) but effects were greater for *Buttiauxella vs. E. coli* phytase. In contrast, only *Buttiauxella* phytase increased the ileal digestibility of protein and total AA (+3.7% on average *vs.* NC at 1046 FTU/kg, $P < 0.05$). Linear increases in ileal digestibility of all individual AA were seen with increasing dose of *Buttiauxella* ($P < 0.05$ in all cases) and were greatest for cysteine (+ 7.9% vs. NC at 1046 FTU/kg), whereas only 2 individual AA showed linear increases in digestibility in response to increasing dose of the *E. coli* phytase (cysteine and proline, $P < 0.05$). Ileal digestibility of Na (%) was markedly increased (less negative) by phytase, especially for the *Buttiauxella* phytase which produced a 96% increase in ileal Na digestibility at 1046 FTU/kg *vs.* NC. Across treatments, ileal digestibility of AA and Na were positively correlated ($P < 0.001$). These results provide insight into the 'extra-phosphoric' effects in broilers fed corn-soybean meal-based diets and suggest that the *Buttiauxella* and *E. coli* phytase had markedly different degrees of 'extra-phosphoric' effects on AA digestibility per standard FTU. The data showed that the AA digestibility response to

Abbreviations: AA, amino acid; AID, apparent ileal digestibility; ATTD, apparent total tract digestibility; AME, apparent metabolizable energy; AMEn, apparent metabolizable energy corrected for N retention; BWG, body weight gain; Ca, Calcium; CP, crude protein; DM, dry matter; GE, gross energy; GIT, gastrointestinal tract; FCR, feed conversion ratio; FI, feed intake; MCP, monocalcium phosphate; NC, negative control; P, phosphorus; PC, positive control

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https://doi.org/10.1016/j.anifeedsci.2019.05.018
Received 14 December 2018; Received in revised form 17 May 2019; Accepted 31 May 2019

increasing phytase dose do not follow the same response curve as for digestible P, and that the response curves are specific for different phytases.

1. Introduction

Broiler diets are based on cereals and oilseeds in which up to 70–80% of the phosphorus content is bound in the form of phytate (Taylor and Coleman, 1979; Selle and Ravindran, 2007). Phytate is poorly digested by non-ruminants and therefore largely unavailable as a source of P, except in extreme low Ca diets (Tamim et al., 2004). The negative effects of phytate in limiting the availability of P have been extensively studied and characterized in broilers (Sebastian et al., 1996; Angel et al., 2002). To mitigate these effects and reduce the excretion of unwanted P into the environment, phytase enzymes have been developed from a variety of fungal and microbial sources and are now routinely added to poultry diets. Phytase efficacy in releasing phytate-bound P and improving P digestibility and utilization is well accepted (reviewed by Selle and Ravindran, 2007; Dersjant-Li et al., 2015), but its capacity to also affect the digestibility and utilization of other nutrients, so called "extra-phosphoric" effects, remains a major topic of research. It is well known that phytate can bind to minerals (such as Ca) and form insoluble complexes at pH above 4, that are resistant to phytase activity (Shafey and McDonald, 1991; Selle et al., 2009). In addition, phytate readily binds with protein at pH levels above or below their isoelectric point, forming binary or ternary protein-phytate aggregates that are less susceptible to digestion by proteolytic enzymes such as pepsin (Katayama, 1997; Ravindran et al., 1995; Yu et al., 2012). This can result in increased excretion of total endogenous amino acids (AA) in high-phytate content diets (Cowieson et al., 2004). Addition of microbial phytase to broiler diets has been shown to improve the digestion of AA (Ravindran et al., 1999; Selle et al., 2000; Ravindran et al., 2006; Amerah et al., 2014; Truong et al., 2015). In addition, beneficial effects on energy and starch utilization have also recently been reported (Ravindran et al., 2006; Truong et al., 2014, 2015). However, the magnitude of these effects on nutrients other than phosphorus has varied significantly between studies, and the degree of influence of factors such as the source of the phytase, dose, and dietary context, is not yet fully understood. The phytase source is likely to have a particular influence because different phytases exhibit different biochemical properties and operate at different pH optima. Whilst commercial phytase activity is standardized at pH 5.5, activity at pH levels below this (e.g. in the gizzard and proventriculus) varies significantly (Menezes-Blackburn et al., 2015). Furthermore, it is thought that phytase hydrolyses less than 35% of dietary phytate in the ileum, where the pH is broadly neutral (Zyla et al., 1999), with the majority of degradation occurring in more acidic regions such as the crop, gizzard and proventriculus, which is where binary protein-phytate complexes are most likely to occur (Selle et al., 2000, 2012). It is therefore hypothesized that a phytase which is active at a more acidic pH (such as pH 2.5–3.5) would be more efficient at reducing the anti-nutritional effects of phytate on the digestibility of proteins, AA, and other nutrients, than a phytase active in higher pH environments. It is also of interest to consider effects on Na digestibility in this context, since it is now known that phytate adversely affects (increases) both intestinal secretion and excretion of Na (Cowieson et al., 2004; Ravindran et al., 2006), and that phytase can markedly improve the digestibility of Na in the small intestine (Ravindran et al., 2006; Truong et al., 2014). Changes in Na secretion and absorption are important because they have the capacity to affect the absorption of other nutrients including AA, a process largely dependent on the sodium-potassium pump (Na+, K+-ATPase) transport system.

The objective of this study was to compare the effects of a *Buttiauxella* sp. phytase known to have a high activity at relatively low pH (max. activity at pH 3, Menezes-Blackburn et al., 2015), with an *E. coli* phytase which is less active at low pH levels (Christensen

Table 1
Dietary treatments and analyzed phytase activity in the test diets.

Treatment no.	Diets	*Buttiauxella* phytase (FTU/kg)		*E. coli* phytase (FTU/kg)		MCP[b]
		target values	analyzed values[a]	*target values*	analyzed values[a]	g/kg
1	NC[c]					
2	NC+	250	303			
3	NC+	500	530			
4	NC+	750	887			
5	NC+	1000	1046			
6	NC+			250	442	
7	NC+			500	961	
8	NC+			750	1505	
9	NC+			1000	1811	
10	NC+[d]					0.6
11	NC+[d]					1.2
12	NC+[d]					1.8

[a] Figures represent dosed phytase in the final feed based on analyzed FTU levels in the premix and dosed amounts to the phytase treatment diets. The phytase activity was determined by an independent laboratory (LUFA, Nord West, Oldenburg, Germany).
[b] MCP: Monocalcium phosphate.
[c] NC: negative control diet.
[d] Treatments 10–12 served as positive control diets.

et al., 2017), on growth performance, tibia ash and digestibility of protein, AA, Na and energy, in broilers fed corn-soybean meal based diets without added inorganic P. Diets containing graded levels of added inorganic P were also tested, to provide a reference for the phosphorus effect.

Table 2
Ingredient and calculated nutritional composition of control diets.

Item	NC	NC + inorganic P from MCP, g/kg		
		+0.6	+1.2	+1.8
Ingredients (g/kg, as is), fixed part				
Corn	560.3	560.3	560.3	560.3
Soybean meal	300.5	300.5	300.5	300.5
Animal blended fat	20.0	20.0	20.0	20.0
Rapeseed meal	38.0	38.0	38.0	38.0
Corn gluten meal	14.8	14.8	14.8	14.8
Soya oil	21.1	21.1	21.1	21.1
Sodium chloride	2.4	2.4	2.4	2.4
Sodium hydrogen carbonate	1.7	1.7	1.7	1.7
Vitamin-mineral premix[a]	5.0	5.0	5.0	5.0
Titanium dioxide	2.5	2.5	2.5	2.5
Corn starch	2.5	2.5	2.5	2.5
Lysine-hydrochloride (79%)	1.7	1.7	1.7	1.7
DL-Methionine (99%)	2.2	2.2	2.2	2.2
L-Threonine (98%)	0.4	0.4	0.4	0.4
Limestone	12.5	12.5	12.5	12.5
MCP	1.0	1.0	1.0	1.0
Ingredients (g/kg, as is), variable part				
MCP	0.00	2.67	5.33	8.00
Calcium carbonate	1.21	2.16	1.08	0.00
Diamol[b]	12.2	8.6	7.0	5.4
Calculated nutrients (g/kg)				
AMEn (kcal/kg)	2900	2900	2900	2900
Calcium	6.5	7.3	7.3	7.3
Phosphorus	4.4	5.0	5.6	6.2
retainable Phosphorus	1.8	2.3	2.8	3.3
Phytate-P	2.5	2.5	2.5	2.5
Sodium	1.6	1.6	1.6	1.6
Potassium	9.1	9.1	9.1	9.1
Chlorine	2.3	2.3	2.3	2.3
Crude protein	210	210	210	210
Crude fat	75	75	75	75
Crude fibre	27	27	27	27
dig. Lysine	10.5	10.5	10.5	10.5
dig. Methionine + Cysteine	8.0	8.0	8.0	8.0
dig. Threonine	6.9	6.9	6.9	6.9
dig. Tryptophan	2.1	2.1	2.1	2.1
Analyzed nutrients (g/kg)[c]				
Moisture	102	102	101	104
Calcium	6.4	7.1	7.3	7.1
Phosphorus	4.3	4.9	5.5	6.1
Chlorine	2.3	2.1	2.4	2.3
Potassium	9.2	9.1	9.3	9.0
Sodium	1.5	1.5	1.6	1.5
Crude protein	226	–	–	–
Crude fiber	25	–	–	–
Crude fat	74	–	–	–
Gross Energy (kcal/kg)	4187	–	–	–

MCP: Monocalcium phosphate.
- = not analyzed.
[a] Supplied per kilogram of diet: Vitamin A, 12,000 IU; Vitamin D3, 2400 IU; Vitamin E, 30 mg; Vitamin K3, 1.5 mg; Vitamin B1, 2.0 mg; Vitamin B2, 7.5 mg; pantothenic acid, 10 mg; niacin, 35 mg; biotin, 200 µg; Vitamin B12, 20 µg; Folic acid, 1.0 mg; Vitamin B6, 3.5 mg; Choline chloride, 460 mg; Iron, 80 mg (as $FeSO_4.H_2O$); Copper, 12 mg (as $CuSO_4.5H_2O$); Zinc, 60 mg (as $ZnSO_4.H_2O$); Manganese, 85 mg (as MnO); Iodine, 0.8 mg (as KI); Selenium, 0.1 (as Na_2SeO_3); Cobalt, 0.4 mg (as $CoSO_4.7H_2O$); Anti-oxidant, 125 mg (Oxytrap PXN).
[b] Diamol was added as a filler ingredient to maintain dietary composition at 100%.
[c] Analyzed by Schothorst Feed Research B. V., Lelystad, The Netherlands.

2. Materials and methods

2.1. Enzymes

Two microbial phytases were utilized in the study: a *Buttiauxella* sp. phytase expressed in *Trichoderma reesei*, and an *E. coli* phytase expressed in *Pichia pastoris*. Enzymes were included at four target dose levels: 250, 500, 750 and 1000 FTU/kg feed, where FTU referred to phytase units and was defined as the amount of enzyme required to hydrolyze 1 μmol inorganic P per minute from 0.0051 mol/L sodium phytate at pH 5.5 and a temperature of 37 °C (AOAC, 2000).

2.2. Diets and experimental design

The study was a completely randomized block design incorporating twelve dietary treatments to evaluate the effects of the *Buttiauxella* and *E. coli* phytase at four dose levels (as detailed in Section 2.1) against a negative control diet containing a low retainable P content (1.8 g/kg) and three positive control (PC) diets containing graded increases in inorganic phosphorus content (NC + 0.6, NC + 1.2 and NC + 1.8 g/kg P from monocalcium phosphate (MCP)) (Table 1). The ingredient and nutrient compositions of the NC and PC diets are presented in Table 2. The basal control (NC) diet was formulated to meet the nutritional requirements of the birds (CVB, 2016) except phosphorus and calcium. One single batch of basal diet was made, comprised of a corn-soybean meal base with added vitamins and minerals, and contained 2.5 g/kg titanium dioxide as an indigestible marker. No other enzymes or coccidiostat were added to the diets. The PC diets were formulated from sub-batches of the NC by the addition of MCP and limestone. Dietary Ca was increased by 0.8 g/kg in the PC diets in order to maintain an adequate Ca to P ratio. Phytase was added to sub-batches of the NC diet according to treatment. Diamol was used as a filler to maintain the total composition of the diets across treatments at 100%. All diets were pelleted; pellet temperature measured directly after leaving the press was ca. 66 °C.

2.3. Birds and housing

All animal care procedures were approved by the Institutional Animal Ethics Committee. The experiment was conducted in a trial setting that avoided unnecessary discomfort of the animals and conformed to European Union Guidelines on animal treatment, management, housing husbandry and slaughtering conditions (Council Directive 2007/43/CE as amended).

Ross 308 day-old male broilers were obtained from a commercial hatchery, vaccinated against infectious bronchitis and housed in a single large floor pen for a five-day pre-experimental period during which they were given free access to water and a nutritionally adequate pelleted broiler starter diet. At 5 days of age, 1152 healthy broilers were allocated to cages based on body weight (BW), so that cages contained birds with approximately equal average bird weight. There were 72 two-tier balance cages with 16 birds/cage and 6 cages per dietary treatment. Experimental diets were fed to the birds *ad libitum*, from 5 to 21 days of age. Water was freely available. Cages were located in an environmentally controlled broiler house where the temperature was gradually decreased from 32 °C on day 5 to 24 °C by day 21. During the experimental period an intermittent lighting schedule of 2L:1D was applied to stimulate frequent mobility of birds housed on wire floor. For the last four days the lighting regime was adjusted to 23L:1D to enable excreta collection and ileal sampling.

2.4. Sampling and measurements

Feed intake (FI) was measured from 5 to 20 days of age. Bird weight was recorded on day 5 and day 20 per cage and body weight gain (BWG) was calculated from 5 to 20 days of age. Mortality was recorded daily and used to calculate mortality corrected feed conversion ratio (FCR). FCR was calculated as total feed consumed per cage (g) / total BWG (g) including the BWG of dead birds. Excreta were collected per cage on three consecutive days (day 18, 19 and 20) for a 7 h period on each day. Samples were freeze-dried, ground to pass through a 0.5 mm sieve and analyzed for dry matter (DM), gross energy (GE), titanium, P, Ca, and Na. All birds were euthanized on day 21 by intracardial injection and the contents of the terminal ileum extracted by gentle flushing with distilled water. Digesta samples were pooled per cage, freeze-dried, ground to pass through a 1.0 mm sieve and analyzed for DM, ash, titanium, P, Ca, Na, N and AA. The left tibia bones of 4 euthanized birds per cage were collected, autoclaved, cleaned, defatted and ashed to determine the ash content in fat-free dry matter (pooled from 4 birds per cage). Samples of the NC and PC diets were analyzed for moisture, Ca, P and crude protein (CP) by Schothorst Feed Research B. V. (Lelystad, The Netherlands), and for phytase content by LUFA (Nord West, Oldenburg, Germany).

2.5. Chemical analysis

The moisture content of feed samples was determined gravimetrically according to ISO method 6496 (ISO, 1999). Calcium and phosphorus in feed, digesta and excreta samples were analyzed, respectively, by the AAS (dry ashing) method (ISO 6869, 2001) and by UV-VIS spectrophotometry (ISO 6491, 1999). Nitrogen was determined according to the Dumas combustion method (ISO/CD 15670) and CP was then calculated (N*6.25). Titanium was measured by colorimetry at 407 nm based on the procedure described by Short et al. (1996). Crude fat was determined after acid hydrolysis, in accordance with ISO method 6865 (ISO, 2001). Ash was determined gravimetrically after ashing the samples in a muffler furnace for 3 h at 550 °C. Gross energy was determined by an adiabatic bomb calorimeter according to ISO method 9831 (ISO, 1998). Sodium was analyzed by the AAS (dry ashing) method (NEN/

ISO 6869, 2001). Amino acids in ileal digesta and feed samples were quantified following oxidation and/or hydrolysis according to ISO method 13903 (2005) by an independent laboratory (Masterlab Analytical, Putten, The Netherlands). The phytase activity of feed samples was analyzed by an independent laboratory (LUFA, Nord West, Oldenburg, Germany).

2.6. Calculations

The apparent ileal digestibility (AID) and total tract digestibility (ATTD) of nutrients and the apparent metabolizable energy content, corrected for N retention (AMEn), were calculated as a percentage of intake according to the following formulas, based on the determined concentration of titanium in the diet and ileal digesta or excreta:

$$\text{AID or ATTD} = 1 - [(Ti_d/Ti_i) \times (N_i/N_d)]$$

Where Ti_d is the titanium concentration in the diet, Ti_i is the titanium concentration in the ileal digesta or excreta, N_i is the nutrient concentration in the ileal digesta or excreta and N_d is the nutrient concentration in the diet, and;

$$\text{AME} = [GE_{diet} - (Ti_d/Ti_{total\ tract\ excreta}) \times GE_{total\ tract\ excreta}]$$

$$\text{AMEn} = \text{AME} - [(N_{diet} - (Ti_d/Ti_{total\ tract\ excreta}) \times N_{total\ tract\ excreta}) \times 8.73],$$

where GE refers to gross energy (kcal/kg DM) and N to nitrogen (in g/kg DM).

2.7. Statistical analysis

Data were based on cage as the experimental unit. Data were analyzed as a randomized block design by analysis of variance (ANOVA) using GenStat® statistical package for Windows (14th edition) (VSN International, Hemel Hempstead, UK). Outliers were identified and excluded from the dataset prior to statistical analyses if the residual (fitted – observed value) was more than 2.5 x the standard error of the residuals of the data set (ANOVA). Where an identified outlier related to one of the performance measures (FI, BWG or FCR), the complete record was removed from the dataset.

Differences were considered statistically significant at $P \leq 0.05$, whereas $0.05 < P \leq 0.10$ was considered to be a near-significant trend. Statistics were based on a two-sided test. Dose-response relationships were determined using a linear model or a non-linear regression model, where:

$$Y = A + B * R^X + \text{residual error},$$

in which: Y = response variable; A = upper asymptote value; B = response compared to upper asymptote without phytase supplementation; R = nonlinear slope parameter; X = dose variable (analyzed phytase activity); Error = error term. If a reversed curve was observed than linear curve fitting was performed.

3. Results

3.1. Diet analysis

Analyzed activity of the *E. coli* phytase premix was higher than expected, therefore analyzed values of *E. coli* and *Buttiauxella* phytase were used for the dose-response analyses rather than target values (Table 1). The reason for a higher analyzed *E coli* phytase vs targeted dose may be due to overage of the activity in the product. The analyzed nutrient content of the treatment diets is shown in

Table 3
Analyzed nutrient content (g/kg) of the experimental diets[a].

Treatment no.	Dry matter	Ash	Ca	P	Titanium[b]
1	903	56.9	6.40	4.31	1.50
2	897	58.7	7.00	4.32	1.54
3	904	58.6	6.90	4.33	1.52
4	897	58.0	6.80	4.28	1.55
5	907	58.2	6.70	4.35	1.57
6	905	57.5	6.50	4.32	1.55
7	901	57.3	6.40	4.32	1.56
8	901	57.7	6.50	4.36	1.55
9	895	57.6	6.50	4.33	1.54
10	901	57.1	7.10	4.91	1.63
11	910	57.9	7.30	5.51	1.52
12	895	56.6	7.10	6.05	1.49

[a] Analyses were carried out by Schothorst Feed Research B. V., Lelystad, The Netherlands.
[b] Average values of titanium across treatments were used for nutrient digestibility calculations.

Table 3. The analyzed P content of the control diets (treatments 10, 11 and 12 in Table 3) was close to the expected values based on the addition of inorganic P (from MCP) to these diets. The analyzed AA content in NC diet is presented in Table 4.

3.2. Growth performance

Both BWG and FCR were improved by dietary supplementation with either the two phytases or MCP-derived P, at all dose levels, compared with the NC ($P < 0.05$) (Table 5). Although feed intake also was increased in all treatments compared with the NC, FCR was still significantly improved compared to NC. A curve-linear response in BWG was seen with increasing phytase dose for both *Buttiauxella* and *E. coli* phytase ($P < 0.001$) (Table 10). However, the slope of the relationship was steeper for *Buttiauxella* phytase, meaning that, at an equivalent dose, the magnitude of the positive effect on these measures was greater for the *Buttiauxella* phytase compared with the *E. coli* phytase. Thus, the highest BWG (+27% *vs.* NC) was produced by a lower dose of *Buttiauxella* phytase (1046 FTU/kg) compared with that produced by *E. coli* phytase (+22% *vs.* NC at a dose of 1505 FTU/kg) ($P < 0.05$). This increase in BWG by *Buttiauxella* phytase was similar in magnitude to that produced by the highest level of MCP-derived P supplementation (NC + 1.8 g P/kg). The greatest reduction in FCR (−6.6% vs. NC) was also produced by a lower dose of *Buttiauxella* (1046 FTU/kg) compared with that produced by *E. coli* phytase (−3.7% vs. NC at a dose of 1505 FTU/kg) ($P < 0.05$), and was greater than that produced by the highest level of MCP-derived P supplementation ($P < 0.05$). Average mortality was low (0.9%) and was not significantly different among treatments.

3.3. Ileal digestibility of nutrient and amino acid

The effect of phytase supplementation and addition of P from MCP on ileal digestibility of CP and 17 AA is summarized in Table 6. There was no effect (*vs.* NC) of the addition of P from MCP on the digestibility of crude protein or AA regardless of the P levels (Table 6). The two phytases had different effects on CP and AA digestibility. *Buttiauxella* phytase improved significantly the ileal digestibility of total AA and CP *vs.* the NC at the top two dose levels (887 FTU/kg and 1046 FTU/kg), by 2.9 and 3.7% (total AA), and by 2.8% and 3.6% (CP), respectively ($P < 0.05$), lower phytase doses numerically improved dig AA but did not reach significant level. The *E. coli* phytase had no significant effect on the ileal digestibility of CP, total AA, or individual AA *vs.* the NC, with the exception of cysteine and tyrosine whose digestibility was increased at the top *E. coli* dose level (1811 FTU/kg) (+5.1% and +4.1 vs. NC, respectively ($P < 0.05$). In contrast, *Buttiauxella* at 887 FTU/kg and/or 1046 FTU/kg improved digestion of all individual AA except glycine, methionine and threonine. The greatest effect was on digestibility of cysteine (+7.9% vs. NC), which was the AA with the lowest digestibility in the NC. A positive linear response (Table 11) was observed with increasing *Buttiauxella* phytase dose from 0 (NC) to 1046 FTU/kg on the digestibility of all individual AA ($P < 0.05$). However, for the *E. coli* phytase, a linear dose-response was seen only for cysteine and proline ($P < 0.05$). Fig. 1 shows the linear relationship between phytase dose and ileal digestibility of total AA in which the steeper slope of the line for *Buttiauxella* vs. *E. coli* is evident. At the two highest dose levels (1505 and 1811 FTU/kg) the *E. coli* phytase improved total ileal AA digestibility above the level produced by the lowest dose of P supplementation (0.6 g P/kg), whilst the *Buttiauxella* phytase at 887 and 1046 FTU/kg achieved a markedly greater effect which was above that produced by all three P supplementation levels ($P < 0.05$, Fig. 1).

Phytase supplementation and dietary addition of P from MCP also affected ileal digestibility (as a percentage of intake) of P, Ca and Na (Table 7). Even at the lowest level of supplementation, *Buttiauxella* and *E. coli* phytase both increased P digestibility *vs*. NC.

Table 4
Analyzed amino acid (AA) content of the negative control (NC) diet (treatment 1)[a].

Item	g/kg diet	g/kg DM[b]
Alanine	11.9	13.2
Arginine	14.3	15.9
Aspartic acid	22.9	25.4
Cysteine	3.3	3.7
Glutamic acid	40.3	44.7
Glycine	9.1	10.1
Histidine	6.1	6.8
Isoleucine	9.8	10.9
Leucine	20.8	23.1
Lysine	13.1	14.5
Methionine	5.6	6.2
Phenylalanine	11.8	13.1
Proline	14.9	16.5
Serine	11.3	12.5
Threonine	8.9	9.9
Tyrosine	7.4	8.2
Valine	11.3	12.5
Total	223.0	247.4

[a] Analyses were conducted by Masterlab Analytical, Putten, The Netherlands.
[b] Values used to calculate AA digestibility coefficients.

Table 5
Effects of supplementation of two phytases or P from monocalcium phosphate to a low P NC diet on feed intake (FI), body weight gain (BWG) and mortality corrected feed conversion ratio (FCR) of broilers between 5 and 20 days of age.

Treatment no.	Treatment name	FI (g/bird)	BWG (g/bird)	FCR (g/g)
1	NC	944^{g}	692^{f}	1.36^{a}
2	NC + 303 FTU/kg *B*[1]	1012^{f}	766^{d}	1.32^{b}
3	NC + 530 FTU/kg *B*[1]	1057de	832^{b}	1.27^{g}
4	NC + 887 FTU/kg *B*[1]	1093^{bc}	848^{b}	1.29^{efg}
5	NC + 1046 FTU/kg *B*[1]	1113^{ab}	876^{a}	1.27^{fg}
6	NC + 442 FTU/kg *E*[1]	1026^{ef}	787^{d}	1.3^{cde}
7	NC + 961 FTU/kg *E*[1]	1066^{cd}	816^{c}	1.31^{bcde}
8	NC + 1505 FTU/kg *E*[1]	1111^{ab}	848^{b}	1.31^{bcd}
9	NC + 1811 FTU/kg *E*[1]	1089^{bcd}	844^{b}	1.29^{def}
10	NC +0.6 g P/kg	1004^{f}	763^{e}	1.32^{bc}
11	NC +1.2 g P/kg	1089^{bcd}	839^{b}	1.3^{cde}
12	NC + 1.8 g P/kg	1134^{a}	878^{a}	1.29^{de}
LSD		33	23	0.019
P value		< 0.001	< 0.001	< 0.001

$^{a-c}$ Mean values without a common superscript letter within a column are significantly different ($P < 0.05$).
[1] Analysed values (in final feed): *B: Buttiauxella* sp. phytase expressed in *Trichoderma reesei, E: E. coli* phytase expressed in *Pichia pastoris*.

Table 6
Effects of supplementation of two phytases or P from monocalcium phosphate to a low-P NC diet on apparent ileal digestibility (%) of crude protein (CP) and amino acids (AA) in broilers (21 days of age).

Treatment no.	Treatment name	CP	Alanine	Arginine	Aspartic acid	Cysteine	Glutamine	Glycine	Histidine	Isoleucine	Leucine
1	NC	78.5^{cd}	80.0^{cd}	87.0^{bcd}	77.7^{cde}	63.5^{c}	83.5^{cd}	74.0	77.7^{cd}	81.1^{cd}	82.1^{de}
2	NC + 303 FTU/kg *B*[1]	78.1^{cd}	79.7^{cd}	87.1^{abcd}	77.7^{cde}	64.4^{bc}	83.4^{cd}	74.4	77.8^{bcd}	81.3^{cd}	82.7^{cde}
3	NC + 530 FTU/kg *B*[1]	79.8^{abc}	81.9^{abc}	87.6^{abc}	79.3^{ab}	66.9^{ab}	84.7^{abc}	75.6	79.2^{abc}	82.8^{abc}	84.1^{abcd}
4	NC + 887 FTU/kg *B*[1]	80.7^{ab}	82.9^{ab}	88.0^{ab}	80.2^{abc}	67.0^{ab}	85.6^{ab}	76.4	79.7^{ab}	83.9^{ab}	85.4^{ab}
5	NC + 1046 FTU/kg *B*[1]	81.3^{a}	83.6^{a}	88.2^{a}	80.4^{a}	68.5^{a}	86.1^{a}	76.9	80.5^{a}	84.5^{a}	86.3a
6	NC + 442 FTU/kg *E*[1]	79.2^{bcd}	81.3^{abc}	87.0^{bcd}	78.3^{bcd}	64.8^{bc}	84.0^{bcd}	74.7	78.5^{bcd}	82.1^{bc}	83.5^{bcde}
7	NC + 961 FTU/kg *E*[1]	79.0^{bcd}	80.8^{bcd}	87.1^{abcd}	78.2^{cd}	64.7^{bc}	84.0^{bcd}	74.6	77.8^{bcd}	82.1^{bc}	83.8^{bcd}
8	NC + 1505 FTU/kg *E*[1]	79.4^{abc}	81.1^{bcd}	86.9^{bcd}	78.5^{bcd}	65.8^{abc}	84.0^{bcd}	74.9	78.6^{abcd}	82.3^{bc}	83.8^{bcd}
9	NC + 1811 FTU/kg *E*[1]	79.7^{abc}	82.0^{abc}	87.2^{abc}	78.7^{abcd}	66.8^{ab}	84.4^{abc}	75.6	79.2^{abc}	82.6^{abc}	84.3^{abc}
10	NC +0.6 g P/kg	77.5^{d}	79.0^{d}	86.0^{d}	76.1^{e}	63.7^{c}	82.4^{d}	73.1	76.9^{d}	80.0^{d}	81.6^{e}
11	NC +1.2 g P/kg	78.7^{cd}	80.7^{bcd}	86.5^{cd}	77.3^{de}	65.5^{bc}	83.6^{cd}	74.3	78.6^{abcd}	81.6^{cd}	83.5^{bcde}
12	NC + 1.8 g P/kg	78.3^{cd}	80.4^{cd}	86.5^{cd}	77.0^{de}	66.2^{abc}	83.2^{cd}	74.3	78.3^{bcd}	81.2^{cd}	83.0^{cde}
LSD		1.89	2.34	1.21	1.93	2.81	1.77	2.15	1.97	2.04	2.18
P ANOVA value		0.009	0.009	0.04	0.001	0.02	0.01	0.056	0.05	0.003	0.005

Treatment no.	Treatment name	Lysine	Methionine	Phenylalanine	Proline	Serine	Threonine	Tyrosine	Valine	Total AA
1	NC	84.3^{bcde}	89.6	83.8^{de}	77.1^{cde}	77.3^{cd}	72.1	81.8^{d}	78.7^{cd}	80.6^{cd}
2	NC + 303 FTU/kg *B*[1]	83.9^{cde}	90.1	85.1^{bcde}	76.6^{de}	77.4^{cd}	72.4	82.3^{cd}	78.6^{cd}	80.7^{cd}
3	NC + 530 FTU/kg *B*[1]	85.1^{abc}	91.1	86.1^{abc}	78.9^{abc}	79.2^{abc}	73.8	84.3^{bc}	80.4^{abc}	82.2^{abc}
4	NC + 887 FTU/kg *B*[1]	85.7^{ab}	91.4	86.9^{ab}	79.5^{ab}	80.1^{ab}	74.4	85.2^{ab}	81.3^{ab}	83.0^{ab}
5	NC + 1046 FTU/kg *B*[1]	85.9^{a}	92.2	87.6^{a}	80.0^{a}	80.7^{a}	75.0	86.6^{a}	82.0^{a}	83.6^{a}
6	NC + 442 FTU/kg *E*[1]	84.6^{abcd}	90.8	85.2^{bcde}	76.5^{de}	78.1^{bcd}	73.0	83.4^{bcd}	79.6^{bc}	81.3^{bcd}
7	NC + 961 FTU/kg *E*[1]	84.4^{bcd}	90.4	85.9^{abcde}	77.4^{bcde}	78.2^{bcd}	72.7	83.8^{bcd}	79.3^{bcd}	81.3^{bcd}
8	NC + 1505 FTU/kg *E*[1]	84.5^{abcd}	90.6	85.6^{abcde}	78.2^{abcd}	78.7^{abc}	73.3	83.7^{bcd}	79.8^{bc}	81.6^{bc}
9	NC + 1811 FTU/kg *E*[1]	84.8^{abcd}	90.9	85.9^{abcd}	79.1^{abc}	79.2^{abc}	73.5	85.2^{ab}	80.2^{abc}	82.0^{bc}
10	NC +0.6 g P/kg	82.9^{e}	89.7	83.8^{e}	75.6^{e}	76.2^{d}	71.0	81.8^{d}	77.4^{d}	79.6^{d}
11	NC +1.2 g P/kg	83.8^{cde}	90.6	84.6^{cde}	78.6^{abcd}	77.9^{cd}	72.8	83.7^{bcd}	79.2^{bcd}	81.1^{cd}
12	NC + 1.8 g P/kg	83.6^{de}	90.7	84.8^{cde}	77.9^{abcd}	77.7^{cd}	72.5	82.3^{cd}	79.0^{cd}	80.8^{cd}
LSD		1.42	1.60	2.09	2.22	2.11	2.43	2.28	2.11	1.87
P value		0.004	0.12	0.02	0.004	0.005	0.16	0.001	0.007	0.007

$^{a-e}$ Mean values without a common superscript letter within a column are significantly different ($P < 0.05$).
[1] Analyzed values (in final feed): *B:* Buttiauxella sp. phytase expressed in *Trichoderma reesei, E: E. coli* phytase expressed in *Pichia pastoris*.

The P digestibility as a percentage of intake was lower in NC + P from MCP diets *vs.* NC ($P < 0.05$). On a grams of digestible P per kilogram feed basis, the lowest addition level of MCP (NC+0.6 g P/kg feed) resulted in a similar level of P digestibility *vs.* the NC (2.54 g dig. P/kg (NC); 2.58 g dig. P/kg (NC + 0.6 g P/kg)) whilst the two higher levels of inorganic P addition resulted in a higher digestibility of dietary P *vs.* NC (2.90 g digestible P/kg (NC + 1.2 g P/kg); 3.40 g digestible P/kg (NC + 1.8 g P/kg) (Table 9). Increasing *Buttiauxella* or *E. coli* phytase dose produced a curvilinear increase in ileal P digestibility (fitted exponential curve $P < 0.001$ in both cases, Table 10) with a maximum increase being produced by 1046 FTU/kg *Buttiauxella* phytase (+25% *vs.* NC) which was significantly greater than the effect produced by the *E. coli* phytase at the top dose level (1811 FTU/kg, +16% vs. NC) ($P <$

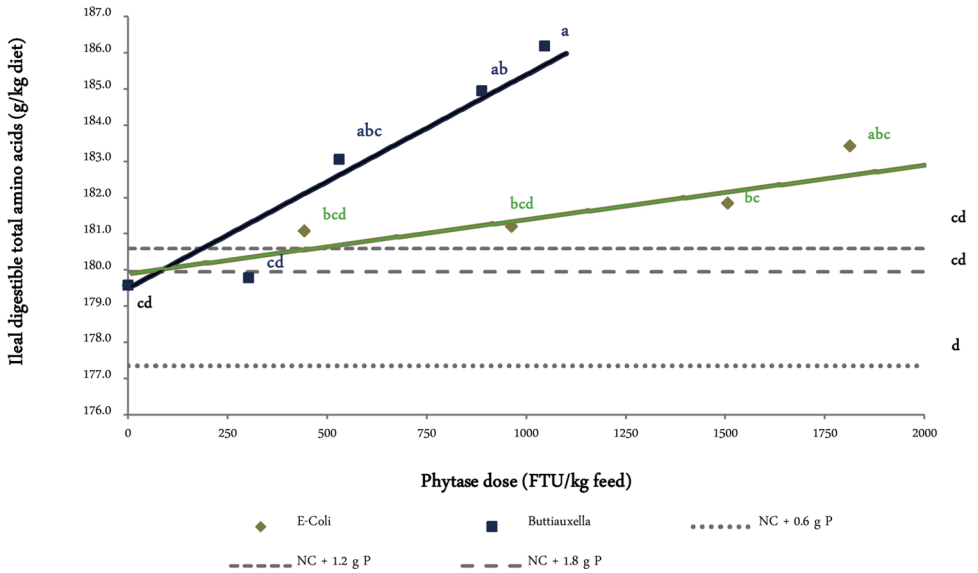

Fig. 1. Relationships between increasing phytase dose or dietary inorganic P content and ileal digestibility of total amino acids[1], in broilers at 21 days of age.
[1]n = 17 amino acids.
[a-d] Data points (or lines in the case of the three NC + P treatments) bearing different superscript letters are significantly different ($P < 0.05$). *P* linear '*Buttiauxella* phytase' = 0.0009; *P* linear '*E. coli* phytase' = 0.15; *P* linear MCP = 0.55; *P* quadratic MCP = 0.63.

Table 7
Effects of supplementation of two phytases or P from monocalcium phosphate to a low P NC diet on apparent ileal digestibility (AID, %) of P, Ca, crude ash (Ash) and sodium (Na) in broilers (21 days of age).

Treatment no.	Treatment name	P	Ca	Ash	Na
1	NC	59.1^{e}	68.1^{a}	44.8	-51.0^{ef}
2	NC + 303 FTU/kg *B*[1]	65.5^{d}	64.0^{abc}	45.4	-39.0^{cdef}
3	NC + 530 FTU/kg *B*[1]	67.8^{cd}	59.9^{cde}	44.6	-27.0^{bcd}
4	NC + 887 FTU/kg *B*[1]	71.5^{ab}	59.5^{de}	45.3	-26.0^{bc}
5	NC + 1046 FTU/kg *B*[1]	73.9^{a}	53.8^{f}	45.3	-2.0^{a}
6	NC + 442 FTU/kg *E*[1]	65.2^{d}	66.1^{ab}	45.3	-44.0^{def}
7	NC + 961 FTU/kg *E*[1]	68.8^{bc}	64.6^{ab}	46.5	-26.0^{bcd}
8	NC + 1505 FTU/kg *E*[1]	67.6^{cd}	60.0^{cde}	45.6	-28.0^{bcd}
9	NC + 1811 FTU/kg *E*[1]	68.6^{bc}	57.0^{ef}	45.7	-10.0^{ab}
10	NC +0.6 g P/kg	52.7^{g}	61.6^{bcd}	44.9	-56.0^{f}
11	NC +1.2 g P/kg	52.7^{g}	53.1^{f}	44.5	-36.0^{cde}
12	NC + 1.8 g P/kg	55.8^{f}	48.3^{g}	46.4	-40.0^{cdef}
LSD		2.92	4.48	2.00	18.0
P value		< 0.001	< 0.001	0.70	< 0.001

[a−f] Mean values within a column without a common superscript letter are significantly different ($P < 0.05$).
[1] Analyzed values (in final feed): *B: Buttiauxella* sp. phytase expressed in *Trichoderma reesei, E: E. coli* phytase expressed in *Pichia pastoris*.

0.05). In contrast, phytase addition (at any dose-level) did not improve the ileal digestibility of Ca (Table 7). In fact, on either a percentage intake or grams per kilogram feed basis, ileal Ca digestibility was reduced in birds supplemented with the top two doses of *E. coli* (1505 FTU/kg and 1811 FTU/kg) and in birds supplemented with the top three doses of *Buttiauxella* (530, 887 or 1046 FTU/kg) *vs.* the NC ($P < 0.05$). Dietary supplementation with P from MCP also reduced ileal Ca digestibility, in a dose-dependent manner ($P < 0.001$) (Table 7). Sodium digestibility values in the ileum were negative across all treatments, indicating a net secretion of sodium into the ileum rather than absorption. There were significant effects of dietary treatment on ileal digestibility of Na ($P < 0.001$); Na digestibilities in birds supplemented with 1046 FTU/kg *Buttiauxella* (-2.0%) or 1811 FTU/kg *E. coli* phytase (-10.0%) were markedly less negative than that of the NC (-51.0%). Adding phytase to the diet resulted in a stepwise increase in the digestibility (less negative) of Na *vs.* the NC, but dose-dependent degree of improvement was greater for *Buttiauxella* compared with *E. coli* phytase. No effect of treatment on ileal digestibility of ash was observed. A strong linear relationship ($P < 0.001$) was found between ileal Na digestibility and total AA digestibility in broilers at 21 days of age (Fig. 2).

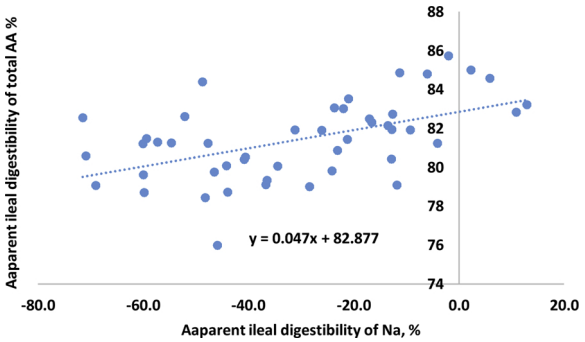

Fig. 2. Linear relationship ($P < 0.001$) between ileal Na digestibility and total amino acids[1] digestibility in broilers at 21 days of age.
[1]n = 17 amino acids.

3.4. Total tract retention of nutrients

Dietary treatment had a significant effect on total tract retention (as a percentage of intake) of P, Ca and AMEn ($P < 0.01$ in all cases), but not Na ($P = 0.11$) (Table 8, Fig. 3). As was seen for effects on ileal digestibility of P, supplementation with either *Buttiauxella* or *E. coli* phytase improved the total tract retention coefficient of P *vs.* NC ($P < 0.05$, Table 8), in a curvilinear, dose-dependent, manner (fitted exponential curve $P < 0.001$ in both cases; Table 10). The greatest effect was produced by 1046 FTU/kg *Buttiauxella* phytase, which was significantly greater than the effect produced by 1811 FTU/kg *E. coli* phytase ($P < 0.05$). On a grams of P retained per kilogram feed basis, there was a stepwise increase with increasing phytase dose for *Buttiauxella* and *E. coli*, however, *Buttiauxella* phytase at 1046 FTU showed greater P retention ($P < 0.05$) compared to *E. coli* phytase at 1505 and 1811 FTU (Table 9). On a percentage of intake basis, dietary addition of P from MCP reduced total tract retention of total P *vs.* NC, but on a grams of P retained per kilogram feed basis, P retention increased stepwise with increasing dietary level of P from MCP (Table 9). Effects of phytase on total tract retention of Ca were opposite to the effects on ileal digestibility; on either a percentage intake or grams per kilogram feed basis. Phytase supplementation improved total tract retention of Ca ($P < 0.001$) in a dose-dependent manner (Table 8; Table 9), with *Buttiauxella* producing a greater effect than *E. coli* phytase (+28.3% compared with +21.8% *vs.* NC for 1046 FTU/kg *Buttiauxella* and 1811 FTU/kg *E. coli*, respectively) (Table 9). Addition of P from MCP (and Ca) to the diet also improved total tract retention of Ca in a stepwise manner. AMEn was increased (*vs.* NC) in the top three phytase dose groups for *Buttiauxella* and the highest dose of *E. coli* ($P < 0.05$) (Fig. 3). Effects of phytase on AMEn were linear and dose-dependent for *Buttiauxella* ($P < 0.001$), but no significant linear dose-response relationship was evident for the *E. coli* phytase ($P = 0.25$) (Table 11, Fig. 3). Addition of P from MCP had no significant effect on AMEn *vs*. NC (Fig. 3).

3.5. Tibia ash

As expected, addition of P from MCP improved tibia ash concentration at day 21, in a dose-dependent manner ($P < 0.001$, Fig. 4). Phytase supplementation also improved tibia ash concentrations *vs.* NC ($P < 0.05$), with increasing phytase dose resulting in a curvilinear increase in tibia ash concentration ($P < 0.001$, Table 10. and Fig. 4). The gradient of the curve was steeper for

Table 8
Effects of supplementation of two phytases or P from monocalcium phosphate to a low P NC diet on total tract retention (%) of phosphorus (P), calcium (Ca) and sodium (Na), in broilers (18–20 days of age).

Treatment no.	Treatment name	P (%)	Ca (%)	Na (%)
1	NC	56.7^{f}	44.9^{e}	37.3
2	NC + 303 FTU/kg *B*[1]	64.0^{de}	46.7^{de}	35.1
3	NC + 530 FTU/kg *B*[1]	68.3^{bc}	51.9^{c}	42.8
4	NC + 887 FTU/kg *B*[1]	69.4^{b}	53.4^{bc}	41.6
5	NC + 1046 FTU/kg *B*[1]	72.2^{a}	57.6^{a}	41.2
6	NC + 442 FTU/kg *E*[1]	62.0^{e}	48.1^{d}	40.7
7	NC + 961 FTU/kg *E*[1]	65.8^{cd}	53.6^{bc}	35.6
8	NC + 1505 FTU/kg *E*[1]	65.7^{cd}	54.0^{bc}	35.8
9	NC + 1811 FTU/kg *E*[1]	69.0^{b}	54.7^{b}	34.4
10	NC +0.6 g P/kg	54.7^{fg}	44.9^{e}	38.3
11	NC +1.2 g P/kg	53.7^{g}	52.0^{bc}	39.7
12	NC + 1.8 g P/kg	53.0^{g}	52.2^{bc}	41.0
LSD		2.78	2.81	6.40
P value		< 0.001	< 0.001	0.11

[a−g] Mean values within a column without a common superscript letter are significantly different ($P < 0.05$).
[1] Analyzed values (in final feed): *B: Buttiauxella* sp. phytase expressed in *Trichoderma reesei, E: E. coli* phytase expressed in *Pichia pastoris*.

Table 9
Effects of supplementation of two phytases or P from monocalcium phosphate to a low P NC diet on ileal absorption (at 21 days of age) and total tract retention of P and Ca in broilers (18–20 days of age) (g/kg diet basis).

Treatment no.	Treatment name	Ileal absorption (g/kg diet)		Total tract retention (g/kg diet)	
		P	Ca	P	Ca
1	NC	2.54^{f}	4.29^{ab}	2.44^{g}	2.83^{g}
2	NC + 303 FTU/kg *B*[1]	2.82^{e}	4.03^{bc}	2.75^{ef}	2.94^{fg}
3	NC + 530 FTU/kg *B*[1]	2.92^{de}	3.77^{cd}	2.94^{cd}	3.27^{cd}
4	NC + 887 FTU/kg *B*[1]	3.08^{bc}	3.75^{cd}	2.98^{bc}	3.36^{bcd}
5	NC + 1046 FTU/kg *B*[1]	3.18^{b}	3.39^{e}	3.11^{ab}	3.63^{a}
6	NC + 442 FTU/kg *E*[1]	2.80^{e}	4.16^{ab}	2.66^{f}	3.03^{ef}
7	NC + 961 FTU/kg *E*[1]	2.96^{cd}	4.07^{b}	2.83^{de}	3.38^{bc}
8	NC + 1505 FTU/kg *E*[1]	2.91^{de}	3.78^{cd}	2.82^{de}	3.40^{bc}
9	NC + 1811 FTU/kg *E*[1]	2.95^{cd}	3.59^{de}	2.96^{c}	3.45^{b}
10	NC + 0.6 g P/kg	2.58^{f}	4.38^{a}	2.68^{f}	3.19^{de}
11	NC + 1.2 g P/kg	2.90^{de}	3.77^{cd}	2.95^{cd}	3.69^{a}
12	NC + 1.8 g P/kg	3.40^{a}	3.43^{e}	3.23^{a}	3.70^{a}
	LSD	0.13	0.29	0.13	0.18
	P value	< 0.001	< 0.001	< 0.001	< 0.001

[a−g] Mean values within a column without a common superscript letter are significantly different ($P < 0.05$).
[1] Analyzed values (in final feed): *B: Buttiauxella* sp. phytase expressed in *Trichoderma reesei, E: E. coli* phytase expressed in *Pichia pastoris*.

Table 10
Fitted exponential response curves between analyzed phytase activity and BWG (5–20 days of age), tibia ash (21 day), ileal P absorption (at 21 days of age) and total tract P retention in broilers (18–20 days of age).

Y	Parameters exponential fitted curve[a]				*P*-value	PVA[b]
	A	B	R	X[c]		
Y = BWG (g)						
Buttiauxella phytase	931	−240	0.998652	Analyzed (FTU/kg)[d]	< 0.001	89
E. coli phytase	850	−158	0.998046	Analyzed (FTU/kg)[d]	< 0.001	93
Y = Tibia-ash (g/kg DM)						
Buttiauxella phytase	479	−99.5	0.998233	Analyzed (FTU/kg)[d]	< 0.001	86
E. coli phytase	466	−86.7	0.998894	Analyzed (FTU/kg)[d]	< 0.001	84
Y = ret. P (g/kg diet)						
Buttiauxella phytase	3.17	−0.73	0.998050	Analyzed (FTU/kg)[d]	< 0.001	60
E. coli phytase	3.06	−0.61	0.999055	Analyzed (FTU/kg)[d]	< 0.001	51
Y = (ileal) abs. P (g/kg diet)						
Buttiauxella phytase	3.51	−0.96	0.999044	Analyzed (FTU/kg)[d]	< 0.001	79
E. coli phytase	2.95	−0.41	0.997400	Analyzed (FTU/kg)[d]	0.006	64

[a] Fitted exponential model was $Y = A + B * R^X$.
[b] PVA = Percentage variance accounted (= adj. R^2).
[c] X = dose variable = analyzed phytase activity.
[d] Based on analyzed premix by LUFA, Nord West, Oldenburg, Germany.

Buttiauxella than for *E. coli* phytase, such that the highest concentration of tibia ash resulted from a lower dose of *Buttiauxella* than *E. coli* phytase (467 and 447 g/kg fat free DM for 1046 FTU/kg and 1505 FTU/kg, respectively, $P < 0.05$). The improvement in tibia ash concentration produced by the top dose of each phytase was equivalent to that produced by the addition of 1.8 g P/kg to the NC (Fig. 4).

4. Discussion

Growth performance represents the end production measure for evaluating beneficial effects of exogenous enzymes. In the present study, both phytases delivered relatively marked improvements in growth performance versus the NC, even at the lowest dose levels, and effects were magnified with increasing phytase dose up to the maxima applied in the study (1046 FTU/kg for *Buttiauxella* and 1811 FTU/kg for *E. coli* phytase). Relationships between phytase dose and BWG were curvilinear, meaning that a diminishing response would be expected with increasing inclusion levels. The *Buttiauxella* phytase was markedly more effective than the *E. coli* phytase at improving both BWG and FCR, on a dose equivalent basis, and at a dose of 1046 FTU/kg *Buttiauxella* achieved a greater reduction in FCR than a diet containing 3.7 g/kg non-phytate P (NC + 1.8 g inorganic P/kg). Published information on growth performance effects of the *E. coli* phytase used in this study is limited. However, the 27% increase in BWG and 7% reduction in FCR produced by 1046 FTU/kg *Buttiauxella* phytase are broadly consistent with reported effect sizes in comparable studies that have

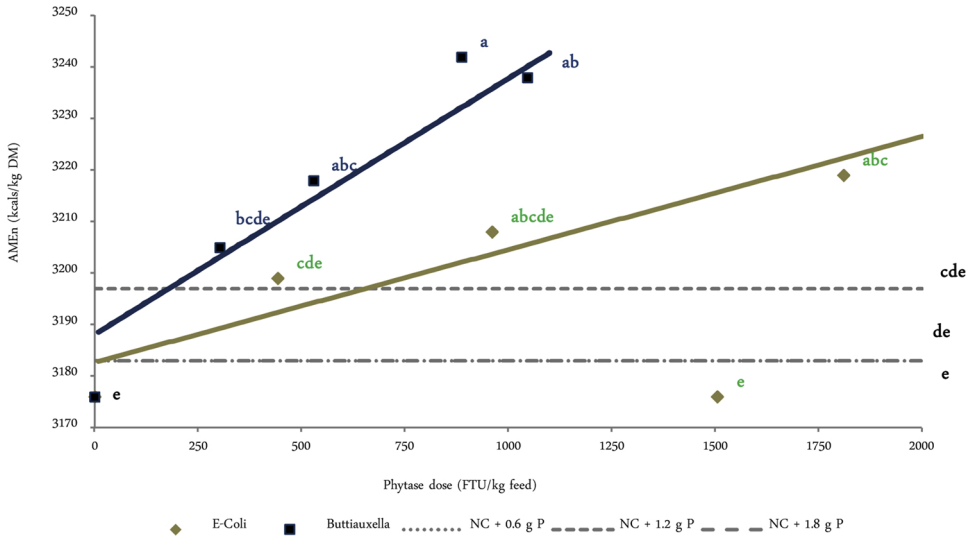

Fig. 3. Linear relationships between increasing phytase dose or dietary inorganic P content and AMEn in broilers at 21 days of age.
[a−e] Data points (or lines in the case of the three NC + P treatments) bearing different superscript letters are significantly different ($P < 0.05$). *P* linear '*Buttiauxella* phytase' = 0.0003; *P* linear '*E. coli* phytase' = 0.25; *P* linear 'MCP' = 0.54; *P* quadratic 'MCP' = 0.41.

Table 11
P values for exponential response curves[1] or linear regression[2] between analyzed phytase activity and AMEn, and between analyzed phytase activity and digestibility of amino acids (AA), for the two phytases.

	Buttiauxella phytase		*E. coli* phytase	
	Exponential P-value	Linear P-value	Exponential P-value	Linear P-value
AMEn	< 0.001	< 0.001	0.35	0.25
dc Sum 17AA	Rc	< 0.001	Rc	0.15
dc ALA	Rc	< 0.001	Rc	0.17
dc ARG	Rc	0.037	Rc	0.65
dc ASP	Rc	0.001	Rc	0.35
dc CYS	0.007	0.002	Rc	0.022
dc GLU	Rc	0.002	Rc	0.33
dc GLY	Rc	0.006	Rc	0.18
dc HIS	Rc	0.009	Rc	0.15
dc ILE	Rc	< 0.001	0.43	0.14
dc LEU	Rc	< 0.001	0.22	0.10
dc LYS	Rc	0.008	Rc	0.54
dc MET	Rc	0.002	Rc	0.21
dc PHE	< 0.001	< 0.001	0.20	0.12
dc PRO	Rc	0.004	Rc	0.019
dc SER	Rc	0.001	Rc	0.08
dc THR	Rc	0.02	Rc	0.24
dc TYR	Rc	0.025	0.51	0.24
dc VAL	Rc	0.002	Rc	0.20

rc = reversed exponential fit.
AMEn in kcal/kg DM and apparent ileal dc (digestibility coefficient) AA in %.
[1] Fitted exponential model was $Y = A + B * R^X$, or linear regression using GenStat® statistical package for Windows (18th edition) (VSN International, Hemel Hempstead, UK).

applied the same *Buttiauxella* phytase at the same dose-level (Amerah et al., 2014; Liu et al., 2014).

Phytase is included in broiler diets with the principal aim of liberating phytate-bound P. As expected at the study outset, both phytases were effective at increasing P digestibility compared with the (P-deficient) NC diet, at both ileal and total tract levels. Again, effects were dose-dependent and the relationships were curvilinear, and at the highest phytase dose levels tested in this study (1046 FTU *Buttiauxella* phytase vs 1811FTU *E. coli* phytase), the *Buttiauxella* phytase was more effective than *E. coli* at improving P digestibility and retention. Thus, despite having similar (standardized) activity at pH 5.5, the two phytases exhibited differences in their ability to improve the digestibility of phytate-bound P at ileal and total tract levels *in vivo*. This is likely to be a reflection of the demonstrably higher activity of the *Buttiauxella* phytase at lower pH levels (and wider pH range) compared with the *E. coli* phytase

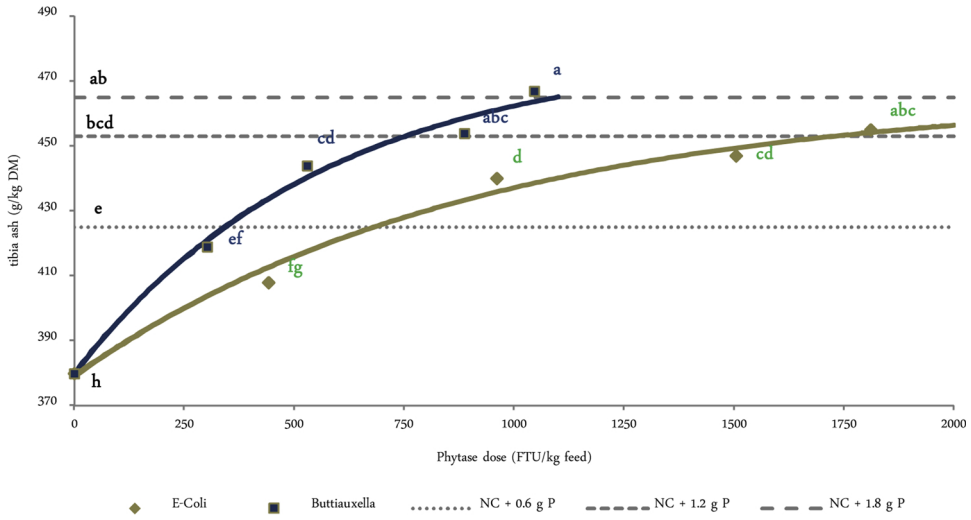

Fig. 4. Relationships between increasing phytase dose or dietary inorganic P content and tibia ash concentration, in broilers at 21 days of age. [a–h] Data points (or lines in the case of the three NC + P treatments) bearing different superscript letters are significantly different ($P < 0.05$). *P* exponential 'Buttiauxella phytase' < 0.001; *P* exponential 'E. coli phytase' < 0.001; *P* linear 'MCP' < 0.001. *P* quadratic 'MCP' = 0.001.

(Christensen et al., 2017) such that it is more able to liberate P from phytate in the upper regions of the digestive tract, before the phytate becomes complexed with minerals or other nutrients. Directly comparable studies involving P-deficient diets are scarce and there are no directly comparable data on P digestibility for the *E. coli* phytase used in this study. However, effects of the *Buttiauxella* phytase appear broadly consistent with the existing literature: The aforementioned study by Amerah et al. (2014), reported a similar increase in ileal P digestibility with 1000 FTU/kg of the same *Buttiauxella* phytase (+32% *vs.* NC compared with +25% *vs.* NC in the present study). Truong et al. (2015) reported apparent P digestibility coefficients in the distal ileum with and without 500 FTU/kg *Buttiauxella* phytase of 52.1% and 38.4%, respectively. The latter values are somewhat lower than dose-equivalent values obtained from the present study, but the percentage increase compared with the NC was higher in Truong et al. (2015) (26.3% *vs.* 14.7% in the present study). The differences could be associated with the higher digestible P content of the basal diet used by Truong et al. (2015) and/or the fact that measurements were in older birds (40 days of age). On a percentage intake basis, addition of P from MCP appeared to reduce ileal P digestibility in the present study. However, as the absolute P content in the MCP-supplemented treatments was higher than that of the NC, it is more meaningful to consider effects on a g/kg basis. By that analysis, a clear increase in P digestibility and retention with increasing dietary inorganic P content was evident, as expected. Comparison of the efficacy of the *Buttiauxella* phytase with that of raising the inorganic P content of the diet revealed that, at 1046 FTU/kg the *Buttiauxella* phytase produced comparable P retention values to a diet supplemented with 1.8 g MCP- P/kg diet. Effects of the phytases on tibia ash content largely mirrored those on P digestibility. At 1046 FTU/kg the increase in tibia ash produced by the *Buttiauxella* phytase (vs. NC) was very similar to that reported by Amerah et al. (2014).

It was unexpected that ileal digestibility of Ca was reduced by both phytase and MCP-derived P addition (either on a % intake or g/kg basis). Most previous studies have reported an increase in ileal digestibility of Ca in response to phytase supplementation (Selle et al., 2009; Amerah et al., 2014), although Truong et al. (2015) reported no significant response. The mechanism by which supplemental phytase reduced Ca digestibility in the present study is unknown, but may be to do with its effects on the balance of Ca:P. It is well known that maintaining an adequate Ca:P balance in the broiler diet and small intestine is a key factor in optimizing absorption of these minerals (Qian et al., 1997), and that an imbalance can lead to increased or decreased absorption of either mineral (Adedokun and Adeola, 2013; Proszkowiec-Weglarz and Angel, 2013). It is possible that the increased availability of free P in the small intestine as a result of the phytate degrading activities of the phytase, combined with a relatively low level of Ca in the basal diet (6.5 g/kg), altered the balance of Ca:P in favor of P absorption. However, based on the increased bone ash content we can conclude that the absorbed amount of Ca was still adequate to facilitate the bone mineralization. At the total tract level, MCP-derived P and phytase increased the retention of Ca, as expected, indicating a greater requirement for Ca of the bird in the presence of the increased available P. Conversely, in the NC, Ca retention was low, presumably due to the limited availability of P from the P-deficient diet which may have resulted in an excess of Ca that was not retained. The data indicated that increasing phytase dose restored Ca: P balance and resulted in improved Ca retention.

In addition to the obvious effects on P digestibility and retention, dose-dependent effects of phytase on ileal digestibility of protein, AA, Na and AMEn were also demonstrated in the present study. The effects on protein and AA digestibility were markedly different between the two phytases. Increasing *Buttiauxella* phytase dose linearly enhanced ileal digestibility of crude protein, total AA and all of the measured individual AA. Whilst the *E. coli* phytase had no significant effect on crude protein or total AA digestibility and the linear effect was seen only for cysteine and proline. Cowieson et al. (2004), clearly demonstrated a negative effect of phytate on the digestibility of AA in broiler chickens and further showed that supplementation with 1000 FTU/kg of an *E. coli* phytase ameliorated this effect, although the phytase in that study was obtained from a different donor organism compared with the *E. coli*

phytase in the present study (*S. pombe* instead of *P. pastoris*). A number of further studies have since reported varying degrees of improvement in protein and AA digestibility in response to 500 to 1000 FTU/kg phytase from a range of sources, in both wheat and corn-based diets (Ravindran et al., 2006; Selle and Ravindran, 2007; Selle et al., 2009; Amerah et al., 2014; Truong et al., 2015). The 3.7% improvement, on average, in ileal digestibility of total AA obtained with 1046 FTU/kg *Buttiauxella* obtained in the present study is lower than figures reported by other studies that have utilized the same *Buttiauxella* phytase. Amerah et al. (2014) reported an average of 12.3% improvement (*vs.* NC) in total AA digestibility in response to an equivalent dose of the same phytase across diets with different Ca:P ratio, and Truong et al. (2015), reported 2.3–12% increases in the digestibility of 16 individual AA in the distal ileum with 500 FTU/kg of the same phytase. These differences could reflect a range of factors, including the age of the birds at which measurements were taken (21 days of age in the present study and Amerah et al., 2014; 40 days of age in Truong et al., 2015) as well as the aforementioned differences across the studies in their basal diet composition and dietary Ca levels. The analyzed Ca level was 6.4 g/kg in the current study, 6.2 g/kg in Truong et al. (2015) and in the range of 5.1 to 13 g/kg in Amerah et al. (2014). The latter study showed that increasing Ca: available P ratio from 1.43 to 3.57 linearly reduced AA digestibility and supplementation of *Buttiauxella* phytase at 1000 FTU/kg improved AA digestibility regardless of Ca: available P ratio. Similarly, Li et al. (2015) reported that increasing dietary Ca level from 6.5 to 9.5 g/kg (all diets had 2 g/kg non-phytate P) significantly reduced ileal AA digestibility by 3.3% (on average for 11 AA) in broilers fed test diets from 7 to 21 days of age. Addition of *Buttiauxella* phytase at 1000 FTU/kg improved ileal dig AA, on average, by 3.1, 5.7 and 6.9% in the diets with 6.5, 8 and 9.5 g/kg Ca respectively, demonstrating a clear effect of dietary ca. The study of Li et al. (2015) also indicated that addition of *Buttiauxella* phytase at 1000 FTU/kg alleviated the negative impact of high dietary Ca level. In all three above mentioned studies, birds were fed corn-SBM-based diets without any other by-products, and the diets for the current study contained rapeseed meal and corn gluten meal. Thus, the difference in ingredients composition (phytate level and source) may also contribute to the different responses. Interestingly, Truong et al. (2015) reported threonine as being the most responsive AA in terms of digestibility in the small intestine (increased by 12.3%), whilst both the Cowieson et al. review (2017) and the present study found cysteine to be the most responsive. Both Cowieson et al. (2017) and Selle and Ravindran (2007) further noted a pattern among the reviewed studies whereby effects of phytase on apparent ileal digestibility of threonine were more pronounced than effects on methionine and considered this to be indicative of a strong involvement of endogenous protein in phytase/phytate nutrition. This pattern was not replicated as such in the present study where effects of phytase on ileal digestibility of methionine and threonine were not statistically significant, although a linear response was evident. Nevertheless, overall the observed improvements in protein and AA digestibility with *Buttiauxella* phytase in the present study are broadly consistent with, and add to the existing evidence base. However, the absence of a significant response with the *E. coli* phytase under directly comparable experimental conditions, even though significant improvements in P digestibility were observed, suggest that the effects of phytases on AA digestibility differ for different phytases and do not follow the same response curve as for dig P. Based on the present study, we conclude that the AA digestibility capabilities of different phytases do not necessarily correlate with their P release capabilities and need to be determined individually for each phytase. Also, the data indicated that not all phytases can have the same effect on the improvement in the digestibility of AA, which could be related to the completeness and location of phytate degradation in the gastrointestinal tract (GIT). A rapid and more complete phytate degradation in the upper GIT will contribute to a greater dig AA response. The greatest improvement in digestible AA with *Buttiauxella* phytase at 1046 FTU/kg was associated with improved FCR, which was significantly lower (-3.2%) than the *E. coli* phytase dosed at 1505 FTU/kg.

The absence of an effect in the present study of addition of MCP-derived P to the diets on ileal AA digestibility, protein digestibility, or AMEn, indicates that the beneficial effects of phytase on these outcome measures were unrelated to increased P availability, i.e. were 'extra-phosphoric'. Although it may be argued that this also could be partially due to the negative effect of the increased Ca content in the PC diets, since the difference in analyzed Ca content between PC and NC diets was only 0.5 g/kg, it is expected that this small difference in Ca content would not have had a big impact on AA digestibility.

It is more likely that they were mediated via a reduction in the anti-nutritive effects of phytate, consistent with previous suggestions by Cowieson et al. (2017) and others. Selle et al. (2012) reviewed the available literature and put forward three possible mechanisms by which phytate could reduce protein and AA absorption in the broiler small intestine: 1) via binding to AA, forming binary- or ternary protein-phytate complexes at pH levels below or above their isoelectric points, that are resistant to pepsin digestion; 2) via an increase in the loss of endogenous AA contained in mucin, whose secretion may be increased in the presence of phytate in order to buffer an increased presence of pepsin (and HCl) that may occur as a compensatory mechanism to digest the protein-phytate complexes; phytate increases mucin excretion in broilers, and phytase reduces this effect (Cowieson et al., 2004), and; 3) via interference with starch, glucose and AA absorption from the gut lumen by compromising Na^+ dependent transport systems and the activity of the sodium-potassium (Na^+, K^+-ATPase) pump. In the present study, there was a marked increase in the apparent ileal digestibility (less negative) of Na in the presence of increasing doses of phytase. This was evident for the *E. coli* phytase and even more so for the *Buttiauxella* phytase which, at a dose of 1046 FTU/kg, increased Na digestibility by 96% *versus the* NC, almost restoring the intestinal levels to 'parity' (−2% vs. −51% in the NC). Truong et al. (2015) reported a lower, but still pronounced (36.1%) increase in Na digestibility in the distal ileum with 500 FTU/kg of the same *Buttiauxella* phytase. The different effect sizes between the studies may in part be a reflection of phytase dose, but in the study by Truong et al. (2015) it was noted that effects on Na digestibility were similar in magnitude to effects on P digestibility, whereas in the present study effects on Na digestibility were markedly higher (+96% vs. NC at 1046 FTU/kg) than those on P digestibility. In both studies, NC diets contained 0.15% Na. Studies by Ravindran et al. (2008) and Selle et al. (2009) also reported marked improvements in ileal digestibility of Na in response to 500 FTU/kg of an *E. coli* phytase expressed in *S. pombe*. Our results again suggest that the phytase source may have a significant bearing on the size of effects, as evidenced by the larger effect of the *Buttiauxella* phytase compared with the *E. coli* phytase at an equivalent dose level. Given the aforementioned role of Na in the intestinal uptake of AA, the implication of our results is that the improvements

in AA digestibility were mediated, at least in part, by improvements in Na digestibility. Truong et al. (2015) noted similar correlations between distal ileal Na digestibility and both protein and starch digestibility, implying that the improvements in both absorption of AA and of glucose that are observed with phytase supplementation may both be mediated via increased Na absorption leading to upregulation of 'sodium pump' activities. The substantial size of effect of the *Buttiauxella* phytase on ileal Na digestibility in both the present study and that by Truong et al. (2015), also suggest that it may be prudent to apply appropriate Na matrix values in dietary formulations containing phytase to account for the substantial amount of Na that phytase can liberate in the broiler small intestine. The ATTD Na was not impacted by dietary treatments which may be explained by that Na was reabsorbed after ileum.

As shown in Table 10 and 11, an exponential curve was fitted to the data to determine the dose response curve with increasing phytase dose. On comparison of the two phytases, when using tibia-ash as response parameter, the equivalence was estimated as: 260 FTU *Buttiauxella* phytase equals to 500 FTU *E coli* phytase. When using ileal P absorption as response parameter, the equivalence value was: 390 FTU *Buttiauxella* phytase equals to 500 FTU *E coli* phytase. Additionally, it can be estimated that for tibia-ash: 500 FTU Buttiauxella phytase equals to 1012 FTU *E coli* and for ileal P absorption: 500 FTU *Buttiauxella* phytase equals to 844 FTU *E coli* phytase. These calculations indicated that when using different methodologies and response parameters, the estimated equivalence value can differ significantly. In general, using tibia ash as a response parameter may result in a higher equivalence value compared to using ileal P absorption as a response parameter.

5. Conclusions

In conclusion, supplementation of phytase to a P-deficient corn-soybean meal based broiler diet could produce 'extra-phosphoric' effects on AA digestibility, Na digestibility and AMEn, but the effects were phytase and dose dependent. Compared to the NC diet, 1046 FTU/kg *Buttiauxella* phytase increased ileal digestibility of total (sum of 17) AA by 3.7% or 7.4 g/kg, and of Na by 96%, whilst the *E. coli* phytase had no significant effect on total AA digestibility and a dose level of 1811 FTU/kg improved ileal digestibility of Na by a moderate of 20%. These differences may be explained by the different pH optima of the phytases and suggest that, despite having standardized activity at pH 5.5, effects of different phytases on AA digestibility are different under *in vivo* condition. Both phytases improved BWG, P digestibility and tibia ash in a curvilinear manner with increasing phytase dose, however, the dose-equivalent effects of *Buttiauxella* were greater than those of *E. coli* phytase. The data showed that the AA digestibility response to increasing phytase dose do not follow the same response curve as for digestible P, and that the response curves are specific for different phytases.

Acknowledgements

The authors would like to thank Dr Joelle Buck (Reading, UK) for her assistance with the writing of this manuscript.

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