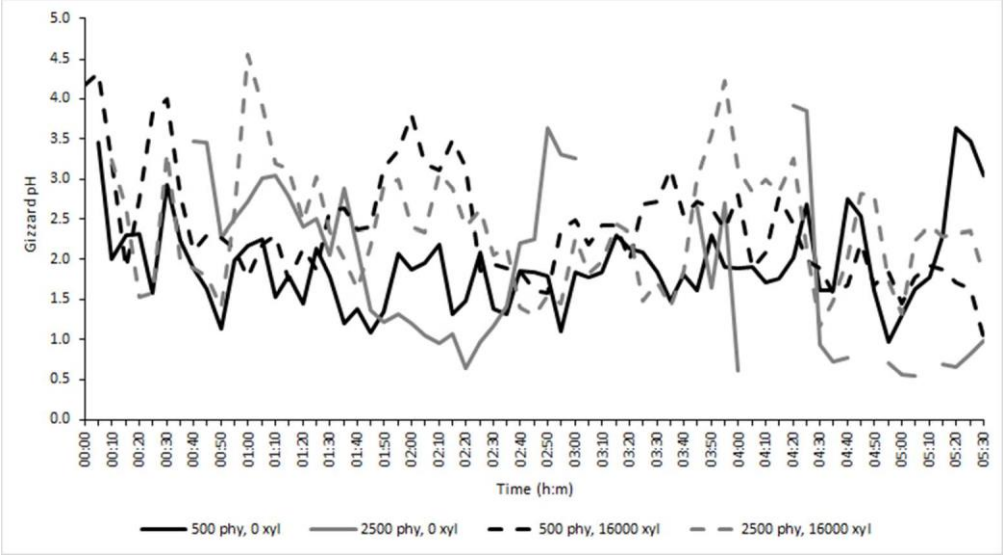

Figure 1

200x111mm (96 x 96 DPI)

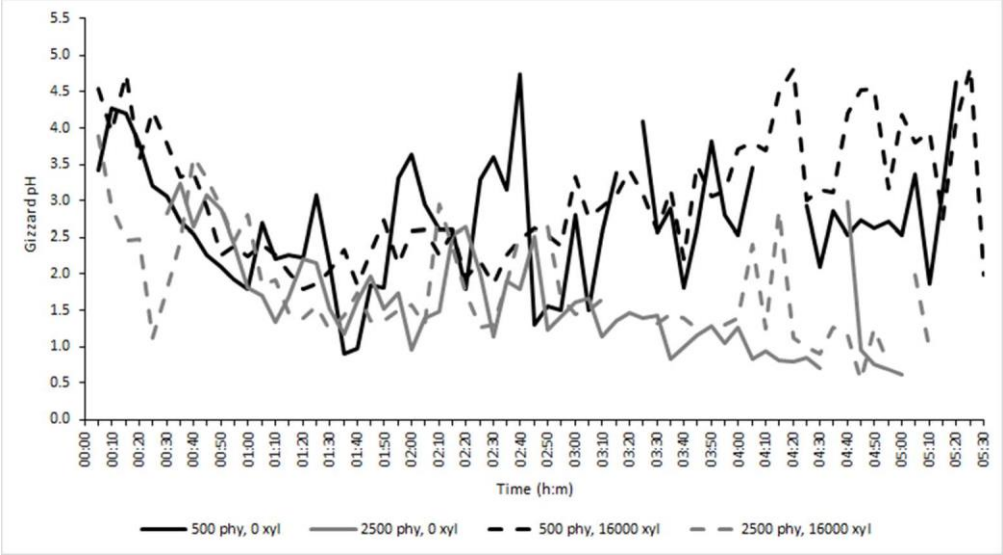

Figure 2

200x111mm (96 x 96 DPI)

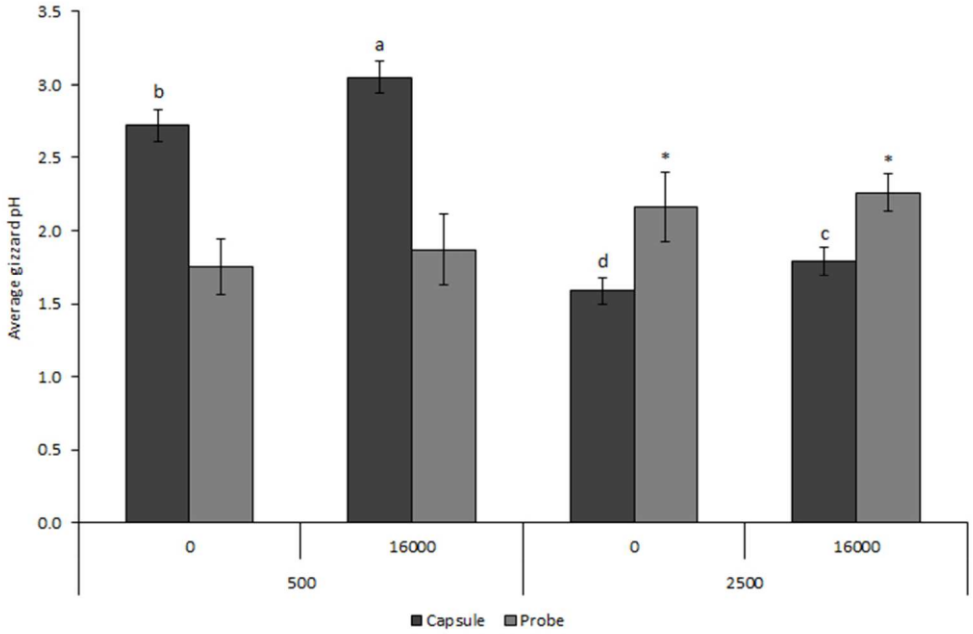

Figure 3

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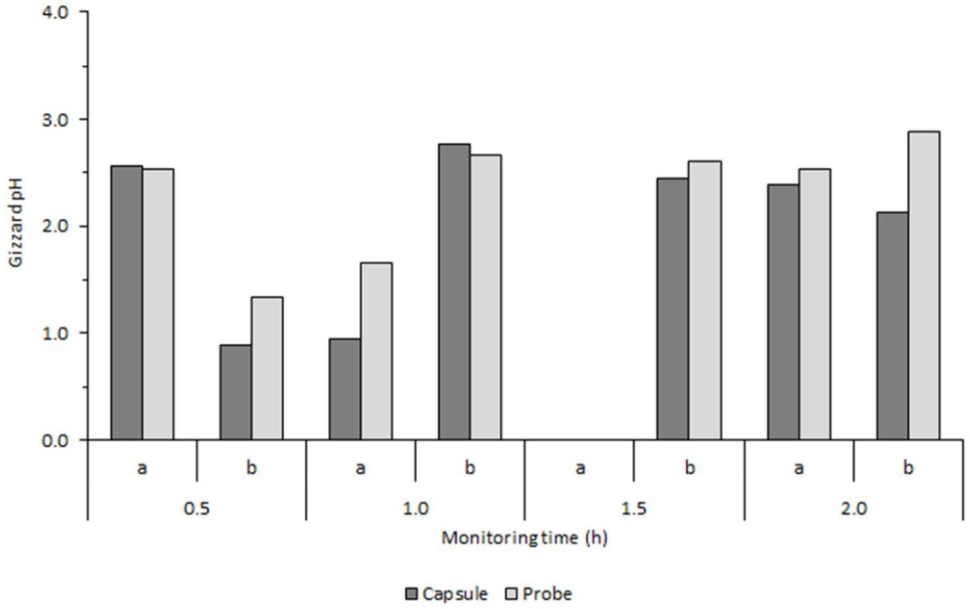

Figure 4

169x107mm (96 x 96 DPI)

Exogenous phytase and xylanase exhibit opposing effects on real-time gizzard pH in broiler chickens

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Abstract

1. The current study was conducted to evaluate the influence of high phytase doses and xylanase, individually and in combination, on performance, blood inositol and real-time gastric pH in broilers fed wheat-based diets.

2. In a 42 d experiment, a total of 576 male Ross 308 broiler chicks were allocated to four dietary treatments. Treatments consisted of a 2 × 2 factorial arrangement, with 500 or 2500 FTU/kg phytase and 0 or 16000 BXU/kg xylanase, fed in two phases (starter 0–21; grower 21–42 d). Heidelberg pH capsules were administered to eight birds from each treatment group, pre and post diet phase change, with readings captured over a 5.5 h period.

3. At 21 and 42 d, birds fed 500 FTU/kg phytase without xylanase had on average 127g and 223 g lower weight gain than all other treatments, respectively ($P<0.05$). At 21 d, FCR was reduced ($P<0.01$) by 2500 FTU/kg phytase or xylanase, however, 42 d FCR was unaffected by enzyme treatment. Inositol content of plasma was twice that of the erythrocyte ($P<0.001$), with 2500 FTU/kg phytase tending to increase ($P=0.07$) inositol content in both blood fractions.

4. Across all treatments, capsule readings ranged from pH 0.54 to 4.84 in the gizzard of broilers. Addition of 2500 FTU/kg phytase to the grower diet reduced ($P<0.05$) average gizzard pH from 2.89 to 1.69, whilst feeding xylanase increased ($P<0.001$) gizzard pH from 2.04 to 2.40. In contrast, digital probe measurements showed no effect of xylanase on gizzard pH, while addition of 2500 FTU/kg phytase increased ($P=0.05$) pH compared to 500 FTU/kg phytase with or without xylanase.

5. These findings suggested that xylanase and high phytase doses have opposite effects on real-time gastric pH, while similarly improving performance of broilers.

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Keywords: Gizzard; pH; Capsule; Phytase; Xylanase

Introduction

The use of exogenous enzymes in feed is common practice in today's poultry farming. Plant feedstuffs contain a variety of anti-nutritional factors (ANF), including non-starch polysaccharides (NSP) and phytate, which hinder diet utilisation and encourage the use of enzymes that reduce the impact of ANF. The predominant enzyme in poultry diets is phytase, which is added to increase phytate hydrolysis and release phosphorus (P), thereby lowering the requirement for expensive inorganic phosphorus and reducing P excretion (Nelson *et al.*, 1971; Ravindran *et al.*, 1995). The physiological importance of P is primarily associated to bone mineralisation (Bailey *et al.*, 1986), and to a lesser extent growth performance (Waldroup *et al.*, 2000; Yan *et al.*, 2001). Recent developments have led to the application of higher phytase inclusion rates, referred to as superdosing (Walk *et al.*, 2013), to exploit the 'extra-phosphoric effects' of phytase by reducing the anti-nutritive influence of phytate on protein and mineral digestion and retention. Higher phytase doses have been shown to improve weight gain, FCR, meat yield, bone ash, phytate-P disappearance and inositol provision in poultry (Cowieson *et al.*, 2011).

Arabinoxylans, the major NSP fraction in wheat, are largely indigestible and reduce nutrient digestibility of the diet through increased digesta viscosity and reduced enzyme access to nutrients (Choct and Annison, 1992a; Choct and Annison, 1992b). Exogenous xylanases have been widely used in wheat-based diets to reduce digesta viscosity and improve nutrient utilisation and growth performance of poultry (Adeola and Bedford, 2004; Choct *et al.*, 2004, Gao *et al.*, 2008; Kiarie *et al.*, 2014). Reports have indicated a link between increased gizzard weight and feed retention and xylanase supplementation (Masey O'Neill *et al.*, 2014; Singh *et al.*, 2012). Svihus (2014) speculated that a greater gizzard volume and retention time may

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elevate HCl secretion and thus lower gizzard pH. However, previous reports have found no effect of xylanase on gizzard pH in broiler chickens (Engberg *et al.*, 2004; Lee *et al.*, 2017c). Although substrate specificity of these enzymes is different, a number of studies have reported synergistic responses to phytase and xylanase (Kühn *et al.*, 2013; Schramm *et al.*, 2017; Selle *et al.*, 2003; Selle *et al.*, 2009), and hence the use of more than one enzyme is becoming routine in commercial practice. When used in combination, xylanase may enhance the availability of phytate within the food-matrix to phytase (Adeola and Cowieson, 2011), thereby improving precaecal nutrient and mineral digestibility. By manipulating the digestive process, it is possible that these enzymes can influence the digestive environment. In previous studies (Lee *et al.*, 2017a; Lee *et al.*, 2018), the ability of phytase to alter gastric pH using real-time pH capsule technology has been demonstrated. However, pH response to xylanase over time has not yet been evaluated. Consequently, the objective of the current study was to investigate the effect of high phytase inclusion rates and xylanase supplementation on growth performance and real-time gastric pH measurements in broiler chickens.

Materials and methods

Animal trials were presented and accepted by the Drayton Animal Health Welfare and Ethical Review Body and conducted according to the Animals (Scientific Procedures) Act 1986.

Animal and housing

A total of 576 male Ross 308 broiler chicks were supplied from a commercial hatchery (P D Hook Hatcheries Ltd, UK) in a 42-day experiment. Chicks were vaccinated against infectious bronchitis at the hatchery before arriving at the experimental housing unit in two batches, one week apart. Birds were raised in separate rooms to allow for sufficient pH capsule monitoring to be performed. On day 1, chicks were randomly allocated to one of four dietary treatments,

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whereby each treatment group had eight replicate floor pens (1.5 x 1.3m) bedded on wood shavings, each containing 18 chicks. Light was provided for 23 h for 1 d.o. birds, 20 h for 2 d.o. and 3 d.o. birds, and 16 h for 4-42 d.o. birds. Light intensity was provided at approximately 40 lux on d 1, reducing to a target of 20 lux over the following 10 d. The temperature of the housing unit was set to 31°C at d 1, and gradually decreased to 20°C over the rearing period. Each pen of birds was weighed on days 0, 21 and 42 of the study. Any birds withdrawn from study or died during the study were weighed manually when removed.

Dietary treatments

Treatments consisted of a 2 × 2 factorial arrangement, with 500 or 2500 FTU/kg phytase (modified *E. coli*-derived 6-phytase; Quantum Blue, AB Vista, Marlborough, UK) and 0 or 16000 BXU/kg xylanase (family 11 xylanase derived from *Nonomurea flexuosa;* Econase XT25, AB Vista, Marlborough, UK). Treatment diets were wheat-soy based (Table 1), and formulated to meet or exceed the NRC (1994) nutritional requirements of broilers.

Table 1 Composition of starter and grower broiler diets

Ingredient, g/kg	Starter (0-21 d)	Grower (21-42 d)
Wheat	633.0	735.7
Soybean meal 48	308.5	205.2
Soy oil	27.1	35.9
Salt	3.9	3.9
DL Methionine	1.8	0.8
Lysine HCl	2.1	2.1
Threonine	0.2	0.0
Limestone	12.8	9.7
Mono Ca Phosphorus	6.0	2.1
Premix[1]	4.0	4.0
Monteban G100	0.6	0.6
Quantum Blue[2]	0.1	0.1
Nutrient composition, %		
Crude protein	21.85	17.90
ME, MJ/kg	12.45	12.97
Calcium	0.98	0.78
Phosphorus	0.71	0.59

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Phytate Phosphorus	0.23	0.21
Available Phosphorus	0.46	0.37
Fat	4.12	5.04
Crude fibre	2.60	2.50
Methionine	0.50	0.34
Methionine + Cysteine	0.88	0.67
Lysine	1.28	1.00
Tryptophan	0.27	0.22
Threonine	0.80	0.62
Sodium	0.19	0.19
Chloride	0.33	0.33

[1] Starter premix- supplied per kg of diet: manganese, 100 mg; zinc, 80 mg; iron (ferrous sulphate), 20 mg; copper, 10 mg; iodine, 1.0 mg; molybdenum, 0.50 mg; selenium, 0.25 mg; retinol (vitamin A), 13.5 mg; cholecalciferol (vitamin D_3), 5 mg; tocopherol (vitamin E), 100 mg; thiamine (vitamin B_1), 3 mg; riboflavin (vitamin B_2), 10 mg; pyridoxine (vitamin B_6), 3.0 mg; cobalamin (vitamin B_{12}), 30 mg; hetra, 5.0 mg; nicotinic acid, 60 mg; pantothenic acid, 15 mg; folic acid, 1.5 mg; and biotin 251 mg. choline chloride, 250 mg. Grower premix- same as starter, except retinol (vitamin A), 10.0 mg.

[2]Quantum Blue was included at 100g/t, with an expected activity of 500FTU/kg, into all diets. Phytase matrix applied: 0.15% available phosphorus, 0.165% calcium, 0.035% sodium.

Phytase was included at 100 g/t (expected activity of 500 FTU/kg) in all diets, and assigned a matrix value of 0.15% available phosphorus, 0.165% calcium, 0.035% sodium. No matrices were used for the subsequent addition of enzymes. For treatments with 2500 FTU/kg phytase, a further 400 g/t (2000 FTU/kg) phytase was added to the basal diet. Diets were fed in two phases; starter crumb (0–21d) and grower pellet (21–42 d) and were provided *ad libitum* along with water throughout the study. Analysed nutrients in starter and grower feed are shown in Table 2.

TABLE 2 HERE

Capsule administration and data collection

Four pens per treatment were selected for capsule dosing, with eight birds per treatment (four birds from each batch, two birds per pen) being randomly selected for capsule administration

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on either day 19 or 20 (pre-diet phase change). The same eight birds were dosed again on either d 22 or 23 (post diet phase change). The Heidelberg pH Diagnostic System (fifth generation) from Heidelberg Medical, including a pH capsule and transceiver, was used to capture pH readings. Capsules were administered to birds as previously described (Lee *et al.*, 2017a). Capsuled birds were isolated into individual pens placed within the original treatment pen. This allowed the transceiver to remain in close proximity to the bird, thereby optimising data collection. Individual pens had separate feeders and drinkers with diets and water provided *ad libitum* during the monitoring period.

Capsule readings were collected every second, over a 5.5 h period, and aggregated into 5 min averages prior to analysis. Readings of pH 0, owing to lost signal between the capsule and the transceiver, were removed from the data set as these were not considered 'true' values. Data anomalies were removed from the data set prior to statistical analysis, as determined by values residing outside 3 x root mean square error (RMSE).

Upon completion of the initial capsule readings, birds were subsequently placed back into their respective original treatment pen. However, following the final capsule reading at 22 or 23 d, birds were humanely killed by electrical stunning and exsanguination. Immediately, the gizzard was located and a small incision made to allow a spear tip pH probe (Oakton, USA) to be inserted. Concurrent to pH readings taken by the probe, capsule readings were collected at the same time to assess method comparability. The spear-tip probe was calibrated using the same pH standards (pH 1.0 and 7.0) that were used to calibrate the Heidelberg capsules to maintain consistency between the two methods.

Capsule Benchmarking

At the end of the experiment (d 42) birds from the 500 FTU/kg phytase without xylanase treatment group were used in a benchmarking assessment to confirm the accuracy of the capsule readings when dosed for different periods of time. Eight birds were monitored in

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total, two from each group at the following time points: 0.5, 1.0, 1.5 and 2.0 h post dosing with the pH capsule. On completion of capsule dosing, birds were humanely euthanised and a spear-tip probe used to measure gizzard pH simultaneously to a capsule reading.

Foot pad and litter scores

External foot pad dermatitis (FPD) scores were recorded for all birds on day 21 and 42. Scores were assessed as follows: 1 = good condition, no lesions; 2 = mild superficial lesions are visible within a small area; 3 = moderate lesions, discolouration and thickening to the foot pad, not widespread; 4 = lesions over majority of the area, maybe inflamed; 5 = severe lesions over majority of the area, may have signs of ulcers and/or scabs, haemorrhages, bleeding and inflammation.

Litter quality, in terms of friability, was determined on day 21 and 42 for each pen. Throughout the experimental period, all pens received approximately equal quantities of shavings. Scores were determined using the following criteria: 1 = fully friable - no capping in any area; 2 = mostly friable - very slight capping (5-40%); 3 = friable litter area reduced (~50%); 4 = still small areas of friable litter - most of assessment area capped (60-75%); 5 = extensive capping over all of assessment area (>80%).

Blood inositol

Following euthanasia of capsulated birds, a terminal blood sample was collected into lithium heparin vacutainers. Erythrocytes were pelleted by centrifugation at 1,500 x *g* for 10 min and an aliquot was washed by mixing with 10 volumes of phosphate-buffered saline, followed by centrifugation at 1,500 x *g* for 10 min. Plasma samples were mixed with 2 volumes of ice-cold 1N-perchloric acid and held on ice for 20 min to allow precipitation of protein. Samples were centrifuged at 16,000 x *g* for 15 min at 4°C and the supernatant diluted 50-100-fold in 18.2 MOhm.cm water. Inositol was determined by HPLC pulsed amperometry (HPLC-PAD) on a Dionex DX-600 HPLC System fitted with two 6-port valves. Following this, 20 ml of

sample was injected onto a 4 mm x 50 mm CarboPac PA1 column (Dionex, UK) arranged in series with a 4 mm x 250 mm CarboPac MA1 column with 4 mm x 50 mm guard column of the same material.

Initial flow rate of the 150 mM NaOH eluent was 0.4 ml/min. Once inositol had eluted from the CarboPac PA1 column onto the CarboPac MA1 column, the flow through the CarboPac PA1 column was switched at 1.5 min to 750 mM NaOH, at 0.4 ml min^{-1}. Eluent (150 mM NaOH) from the CarboPac MA1 column was directed to an ED50 electrochemical detector (Dionex) configured with a gold electrode and operating a standard Dionex carbohydrate waveform. After 11.5 min, the CarboPac PA1 column was returned to the 150 mM NaOH flow, in series with the MA1 column, conditioning the columns for a further 8.5 min before the next injection. Inositol was eluted at approximately 10.5 min. For determination of inositol concentration, peaks derived from inositol standards (0.01-0.2 nM in 20 µl) were used to create a linear calibration curve (r^2>0.995) with a slope of approximately 100 nC.min/nmol.

Statistical analysis

The effect of phytase and xylanase on performance parameters and pH readings were compared statistically by Least Squares ANOVA using JMP Pro 13.0 (SAS Institute Inc., Cary, NC). When considering gastric pH changes, diet phase change was included in the model. When differences were significant, least square means were separated using Student's t-test. Mortality, footpad and litter scores were analysed using a non-parametric Wilcoxon Test. Significance was accepted at P≤0.05, with trends (P<0.10) discussed.

Results

In-feed phytase activities were measured by ELISA (performed by AB Vista Lab Services) and were as expected (Table 2).

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Performance

The effect of phytase and xylanase dose on performance parameters in broilers is shown in Table 3. At days 0-21, neither high phytase dose nor xylanase inclusion significantly influenced feed intake. However, during days 21-42, feed intake was affected by a phytase and xylanase interaction (P = 0.03), with birds fed 500 FTU/kg phytase without xylanase having lower feed consumption than all the other treatments. Considering the entire experimental period (d 0-42), dietary treatment had no significant effect on feed consumption in birds.

TABLE 3 HERE

An interaction between phytase and xylanase (P=0.04) was seen for BWG from d 0 to 21, whereby birds fed 500 FTU/kg phytase without xylanase gained less (127g on average) than all other treatments. From d 21-42, higher doses of phytase (2500 FTU/kg) improved (P=0.04) BWG of broilers by approximately 70g, compared to diets with 500 FTU/kg phytase. Addition of xylanase, however, had no effect on BWG from d 21-42. Over the entire experimental period, an interaction between phytase and xylanase (P=0.04) was evident, with birds fed 500 FTU/kg phytase without xylanase having, on average, 223 g lower weight gain than all other treatments.

From d 0-21, FCR was lowered (P<0.01) by five points with addition of 2500 FTU/kg phytase and seven points with xylanase, although no interaction between these enzymes was shown. However, from d 21-42 and over the entire experimental period, dietary treatment had no significant effect on FCR or body weight corrected FCR.

Mortality was not significantly affected by treatment at any age (Table 4). However, mortality was clearly higher in the starter phase than in the grower. It was noted that, in the

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first batch of chicks, 74% of mortalities occurred within the first week. Mortality was 13.5% in the starter phase for the first batch compared to 5.1% in the second batch. Once these birds were removed, mortality was reduced during the grower phase to around 3% for both batches, which is within the expected level. Therefore, high mortality in this trial was attributed to poor chick quality in the first batch of chicks, and not dietary treatments.

Table 4 Influence of phytase and xylanase on broiler mortality[1]

		Mortality (%)		
Phytase (FTU/kg)	Xylanase (BXU/kg)	Days 0-21	Days 21-42	Days 0-42
500	0	5.48	3.42	8.82
2500	0	10.20	5.11	14.86
500	16000	9.58	1.44	10.94
2500	16000	12.04	1.39	13.36
	SEM	1.606	0.799	1.588
P-value				
Phytase		0.075	0.894	0.085
Xylanase		0.309	0.068	0.850

[1]Means represent the average response of 8 replicate pens (144 chicks) per treatment. SEM, standard error of the mean

Litter and FPD scores

The effects of treatment on footpad and litter scores were determined at 21 and 42 d of age (Table 5). Litter scores were unaffected by treatment and were given approximate scores of 2 and 3 for day 21 and 42, respectively, indicating that capping was not extensive in this trial. The majority of foot pad dermatitis (FPD) scores for all treatments ranged between 1 and 2, signifying overall good-to-mild footpad conditions in birds. At day 21, FPD scores were unaffected by treatment, however, at day 42 a xylanase effect was shown (P=0.01); feeding xylanase reduced the incidence of FPD scores of 5 (severe), compared to when no xylanase was supplemented.

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TABLE 5 HERE

Blood inositol content

Blood inositol content, measured in two separate blood fractions (erythrocyte and plasma), with results presented in Table 6. Xylanase in the diet had no effect on inositol levels, and no interaction between xylanase and phytase was shown. Addition of 2500 FTU/kg phytase tended to increase (P = 0.056) blood inositol level, compared to 500 FTU/kg phytase. The fraction of blood analysed had a considerable effect (P<0.001) on inositol levels, with samples taken from the plasma having higher inositol content than that from erythrocytes.

Table 6 Blood myo-inositol content in birds fed diets containing varying levels of phytase and xylanase[1]

Blood fraction	Phytase (FTU/kg)	Xylanase (BXU/kg)	Myo-inositol (nmol/mL)
Erythrocyte	500	0	98.1
	2500	0	106.8
	500	16000	95.2
	2500	16000	136.4
Plasma	500	0	246.3
	2500	0	281.8
	500	16000	246.2
	2500	16000	294.4
RMSE			49.90
Erythrocyte			109.1
Plasma			267.2
	500		171.4
	2500		204.9
		0	183.3
		16000	193.0
P-value			
Phytase			0.070
Xylanase			0.584
Blood fraction			<0.001
Phytase x Xylanase			0.528

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Phytase x Blood fraction	0.635
Xylanase x Blood fraction	0.842
Phytase x Xylanase x Blood fraction	0.780

[1] Means represent the average response of 4 birds per treatment
RMSE, root mean square error

Gizzard pH

Changes in gizzard pH over the 5.5 h period in response to supplementing phytase and xylanase to broiler starter (Figure 1) and grower (Figure 2) diets were recorded.

FIGS 1 AND 2 HERE

Capsule readings ranged from pH 0.54 to 4.84 in the gizzard of broilers across all treatments (Table 7). Following euthanasia, capsules were located in the gizzard of broilers, except for one bird in the 500 FTU/kg phytase with xylanase treatment group where the capsule was found in the crop. Data from this bird was kept in the analysis as pH readings were within the expected limits for gastric readings, and therefore it is possible that the capsule had moved out of the gizzard during euthanasia. A feed phase x phytase interaction ($P<0.001$) was seen for gizzard capsule pH, whereby increasing phytase dose from 500 to 2500 FTU/kg had no effect on average gizzard pH (2.16 *vs*. 2.15) in birds fed starter diets. However, in birds fed the grower diets, increasing phytase to 2500 FTU/kg reduced gizzard pH (1.69 *vs*. 2.89) compared to 500 FTU/kg phytase diet. Addition of xylanase to the diet increased ($P<0.001$) gizzard pH (2.40 *vs*. 2.04), irrespective of phytase dose or diet phase. There was no interaction between phytase and xylanase, indicating that these enzymes were working independently of one another.

Table 7 Influence of diet phase, QB and XT on gizzard pH as measured using pH capsule technology[1]

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Phase	QB (FTU/kg)	XT (BXU/kg)	Min	Max	Average gizzard pH
Starter	500	0	0.96	3.64	1.92
	2500	0	0.54	4.09	1.92
	500	16000	1.02	4.33	2.40
	2500	16000	1.17	4.56	2.37
Grower	500	0	0.91	4.74	2.72
	2500	0	0.61	3.24	1.59
	500	16000	1.79	4.84	3.05
	2500	16000	0.54	3.89	1.79
RMSE					0.782
Starter					2.15
Grower					2.29
	500				2.52
	2500				1.92
		0			2.04
		16000			2.40
P-value					
Phase					0.060
QB					<.0001
XT					<.0001
Phase x QB					<.0001
Phase x XT					0.175
QB x XT					0.572
Phase x QB x XT					0.720

[1] Means represent the average response of 8 birds per treatment

Capsule readings were compared to a standard method using a spear-tip pH probe to take gizzard pH readings following euthanasia (Figure 3). In contrast to the capsule readings, pH probe measurements showed no effect of feeding xylanase on gizzard pH (2.06 *vs*. 1.96), while 2500 FTU/kg phytase increased (P=0.05) gizzard pH (2.21 *vs* 1.81) compared to a 500 FTU/kg phytase diet, irrespective of xylanase inclusion. Simultaneous to probe measurements, capsule readings were taken to allow comparisons to be made between the methods. Out of the 32 birds sacrificed, 26 of the capsules had pH readings that plateaued at

0.50 at the time of simultaneous probe reading, indicating that the capsules had become unresponsive.

FIG 3 HERE

It would appear that the longer the monitoring period within the gizzard, the more likely the capsule was to become damaged, thereby prompting the 0.50 reading. This lead to a benchmarking experiment, that used eight 42 d birds from the 500 FTU/kg phytase without xylanase treatment group to dose capsules over 0.50 to 2.0 h prior to euthanasia, with pH recordings taken by both probe and capsule. Following euthanasia, all capsules were located in the gizzard of birds, except one bird dosed for 1.5 h where the capsule was located between the crop and gizzard. This bird gave a capsule reading of pH 2.62, however, data from this bird was removed from the dataset due to the capsule not being located in the gizzard. The range of difference between the capsule reading and the probe was -0.03 to +0.76, with the average difference across the eight birds being 0.30 (Figure 4). None of the capsules plateaued at pH 0.5, indicating that dosing up to 2 h in birds does not appear to cause damage to the capsules.

FIG 4 HERE

Discussion

Research implementing higher phytase inclusion rates in poultry feed has shown enhanced hydrolysis of lower inositol phosphate (IP) esters created from phytate degradation, thereby reducing anti-nutritive effects on protein and mineral digestibility (Beeson *et al.*, 2017, Yu *et al.*, 2012). As a result, increasing phytase dose above industry standards has been shown to improve performance of broilers (Lee *et al.*, 2017b, Shirley and Edwards, 2003, Walk *et al.*,

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2014, Walk *et al.*, 2013). Supplementation of xylanase to wheat-based diets has shown improvements in broiler performance (González-Ortiz *et al.*, 2016, Wu *et al.*, 2004). This response has been accredited to reductions in intestinal viscosity and enhanced AME of feed (Annison and Choct, 1991, Selle *et al.*, 2003, Wu *et al.*, 2004).

In the current study, day-old birds were 5 g lighter than expected (average weight 37g), although this did not appear to effect subsequent growth performance as suggested by dos Santos *et al.* (2010). Birds fed 2500 FTU/kg phytase, 16,000 xylanase or a combination of the two, gained significantly more weight than birds fed 500 FTU/kg phytase without xylanase, at 21 and 42 days. A study by dos Santos *et al.* (2017) reported a significant increase in weight gain with 1500 FTU/kg phytase, while 16,000 xylanase showed a tendency to improve gain in 42 day birds, compared to feeding a standard phytase dose (500 FTU/kg) alone. However, the combination of 1500 FTU/kg phytase and xylanase had no additional benefit on the body weight gain of broilers. A similar response was reported by Karimi *et al.* (2013), suggesting that phytase and xylanase exert non-additive effects in diets based on corn and sorghum based of performance parameters. However, Kühn *et al.* (2013) showed that a combination of 1500 FTU/kg phytase and 16,000 BXU/kg xylanase significantly increased weight gain in 35d wheat-fed broilers, compared to feeding these enzymes individually. This suggests that xylanase may give additional benefits alongside phytase in birds fed wheat-based diets.

This synergy may be explained by the morphology of the wheat grain. The primary storage site of phytate in wheat is in the aleurone layer (O'Dell and Boland, 1972), the cell walls of which are comprised essentially of b-glucans and arabinoxylans (Burton and Fincher, 2014). Xylanase may increase permeability of the aleurone layer by degradation of arabinoxylan in the cell walls (Parkkonen *et al.*, 1997), thereby enhancing availability of phytate for interaction with phytase (Karimi *et al.*, 2013).

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In the current study, FCR at 21 d was significantly reduced in birds fed 2500 FTU/kg phytase and 16000 BXU/kg xylanase, compared to birds fed 500 FTU/kg phytase alone. However, at d 42, FCR was not significantly affected by higher phytase dose or xylanase. This may be explained by the fact that growth performance of all birds was approximately 16% ahead of breed standards, and FCR was around 12% lower at this age. This makes it extremely challenging to observe any performance response to treatment when birds are already exceeding performance expectations. Even so, the combination of 16000 BXU/kg xylanase and 2500FTU/kg phytase gave a four point reduction in FCR (non-significant) compared to the 500 FTU/kg without xylanase diet. This is a considerable reduction in already well performing birds, and, although not statically significant, is highly commercially relevant.

Wet litter poses a major challenge for the poultry industry, with FPD among broilers being of increasing concern from both a welfare and economic standpoint. There is some evidence that exogenous phytase may reduce litter quality and increase faecal moisture (Debicki-Garnier and Hruby, 2003). Phytate and its lower esters have anti-nutritive effects on protein and mineral digestion and absorption (Beeson *et al.*, 2017, Yu *et al.*, 2012), leading to an imbalance that can increase water intake and thus wet litter. Increasing phytase dose promotes the near-destruction of phytase and its lower esters (Walk *et al.*, 2014, Walk *et al.*, 2013), thereby enhancing protein and mineral absorption and improving litter quality. In the current study, reasonable litter quality was observed for bird age and was unaffected by treatment. Consequently, incidence of FPD was relatively low in birds at 21 and 42 d of age. Exogenous xylanase has been widely acknowledged for its ability to resolve wet litter issues, particularly in birds fed wheat-based diets, through soluble NSP degradation and subsequent reduction in digesta viscosity and faecal moisture content. In the present study, feeding xylanase significantly reduced the incidence of severe FPD in 42 d.o. birds. Since litter quality was unaffected by xylanase, other factors such as altered health status and litter

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microbial population (Kim *et al.*, 2017, Shepherd and Fairchild, 2010) may explain these findings, or it may be that the measures of litter quality are not currently adequate.

Blood inositol can be a useful indicator of complete dephosphorylation of dietary phytate by addition of exogenous phytase to the diet. In the body, inositol is involved in a number of signalling pathways that support the development and growth of animals (Lee and Bedford, 2016). Several studies have supported the benefits of inositol either by dietary supplementation or through high phytase inclusion rates (Cowieson *et al.*, 2015, Cowieson *et al.*, 2013, Lee *et al.*, 2017b, Sommerfeld *et al.*, 2017, Walk *et al.*, 2014), indicating that inositol may play an important role in animal growth response. Previously, inositol profile has been determined primarily using blood plasma samples (Cowieson *et al.*, 2015, Sommerfeld *et al.*, 2017). However, inositol has been detected in erythrocytes of day-old and 21 d chickens (Oshima *et al.*, 1964). In erythrocytes, myo-inositol appears to be a precursor for myo-inositol pentaphosphate (IP5), which interacts with haemoglobin to modulate affinity for oxygen (Isaacks *et al.*, 1982; Lutz, 1980). In the current study, the fraction of blood analysed had a considerable effect on inositol levels, with plasma inositol being more than twice the concentration than in erythrocytes. This is in contrast to Oshima *et al.* (1964), that found higher concentrations of free myo-inositol in erythrocytes than plasma. This discrepancy may be the result of differences in sensitivity between the previous and more current detection methods used. Nonetheless, increasing phytase dose to 2500 FTU/kg tended to increase inositol concentration in both blood fractions compared to the standard 500 FTU/kg phytase inclusion rate, suggesting more complete dephosphorylation of phytate. Addition of xylanase to the diet had no effect on blood inositol levels, as this enzyme would not be expected to directly affect phytate degradation.

It is clear from the current study and previous work (Lee *et al.*, 2017a, Lee *et al.*, 2018) that relatively large fluctuations in gastric pH can be detected using real-time capsule technology.

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The fact that pH is not kept at a consistent level illustrates that acid secretion is not static and questions the value of point-in-time measurements. Reports in both laying hens and broilers have shown no effect of adding xylanase to wheat- or corn-based diets on gizzard pH (Engberg *et al.*, 2004; Lee *et al.*, 2017c; Mirzaie *et al.*, 2012). Similarly, in the present study, digital pH probe measurements indicated that inclusion of xylanase into wheat-soy diets had no significant influence on gizzard pH in broilers. However, in contrast, pH capsule readings demonstrated that inclusion of xylanase into the diet significantly increased gizzard pH from 2.0 to 2.4, irrespective of phytase inclusion or diet phase. Morgan *et al.* (2017) reported a pH 2.5 optimum for xylanase degradation of wheat arabinoxylan to short-chain xylo-oligosaccharides. Conditions may therefore have been optimised in the current study in terms of xylanase efficacy.

Moreover, as measured by pH capsule technology, increasing phytase dose from 500 to 2500 FTU/kg significantly reduced gizzard pH in birds fed grower diets. A similar finding was evident in a previous trial (Lee *et al.*, 2018). It has been suggested that 500 FTU/kg phytase releases more Ca than P, while higher phytase doses increases P release beyond Ca, restoring this balance (Cowieson *et al.*, 2011). It may be this rebalancing of minerals lowers gastric pH with 2500 FTU/kg phytase, which accounts for the improved solubility and digestibility of dietary nutrients shown with high phytase inclusion rates (Manobhavan *et al.*, 2016; Pirgozliev *et al.*, 2012). However, capsule results were contradictory to pH probe measurements, showing an increase in gizzard pH with 2500 FTU/kg phytase compared to 500 FTU/kg phytase. Other studies adopting point-in-time pH measurements have reported a lack of effect of administering phytase doses up to 2500 FTU/kg on gastric pH (Lee *et al.*, 2018; Nourmohammadi *et al.*, 2011; Radcliffe *et al.*, 1998) while, application of much higher phytase inclusion rates of 5000 FTU/kg has been shown to increase gizzard pH in broilers (Walk *et al.*, 2012). Therefore, this may suggest that much higher enzyme doses are required

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to enable detection of a noticeable response to treatment using current methods. Even so, the direction of response, particularly for phytase, is conflicting between capsule and probe methods.

There are clear differences between the two methods used in this study to record gizzard pH, which may explain these opposing conclusions. For example, *in-situ* and *ex-situ* pH probe readings are taken at one point-in-time, once the animal has been sacrificed. Conversely, *in vivo* pH capsules take readings every second for several hours in the live animal, thereby providing a more representative outlook on real-time acid secretions in response to treated feed. It may be this ability to detect fluctuations in gastric pH that allows treatment responses to be realised, which would otherwise be missed using standard point-in-time methods. However, a limitation to the capsule technology is that only a restricted number of birds can be capsuled at the same time, due to the number of detection devices available. In order to determine the comparability between these two methods and the effect of euthanasia on gastric pH, capsule readings were taken simultaneous to probe measurements. However, the majority of capsules appeared to plateau at pH 0.50 at the point of probe measurement, suggesting potential damage to the capsule. In light of this, a benchmarking experiment was undertaken to confirm the accuracy of the capsule readings when dosed for different periods of time. The average pH difference between probe and the capsule readings was 0.30, with a range of -0.03 to +0.76. This suggested that digital probe measurements read higher than the capsule. This may be due to the positioning of the H^+ ion sensor within the food bolus when measurements are taken. The orientation of the capsule cannot be controlled, however, taking into account the size of the capsule (2cm in length) compared to the size of the gizzard, it could be assumed that the H^+ ion sensor would be located in the outer region of the food bolus, where exposure to gastric acid secretions is high. In contrast, the probe was inserted directly into the centre of the food bolus, the region less exposed to gastric secretions.

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Therefore, the method of choice may be dependent on the research question, as to whether a change in acid secretion is to be determined or the pH of the food bolus. Since none of the capsules plateaued at pH 0.5, this would suggest that dosing up to 2.0 h did not cause damage to the capsules, as indicated after a 5.5 h dosing period. However, capsule readings obtained over 5.5 h in the live bird did not suggest capsule damage, and therefore it is possible that this damage only becomes apparent once the bird has been killed. Further investigation is required for intermediate dosing periods to confirm the potential maximum period for capsule administration.

Conclusions

The current study demonstrated that body weight gain and FCR of broilers can be improved by addition of higher phytase doses and xylanase in wheat-based diets. Increasing phytase dose had the tendency to increase inositol in the blood, suggesting more complete phytase degradation with higher phytase inclusion rates. Addition of xylanase and higher phytase dose appeared to have opposite effects on real-time gastric pH, as measured by capsule technology. Supplementation of xylanase increased gizzard pH, while feeding high phytase in the grower diet led to a reduction in gizzard pH. However, these findings were not supported by probe measurements, indicating inconsistencies between the methods. The fact that xylanase and high phytase doses had opposing effects on real-time gastric pH, while giving similar performance responses, indicated that gastric conditions were not solely accountable for animal performance.

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Figure 1. Effect of phytase and xylanase on gizzard pH in broilers fed starter diets. Phytase (phy) was supplemented at 500 or 2500 FTU/kg, and xylanase (xyl) at 0 or 16000 BXU/kg. Data points represent means of 8 birds per treatment.

Figure 2. Effect of phytase (phy) and xylanase (xyl) on gizzard pH in broilers fed grower diets. Phytase (phy) was supplemented at 500 or 2500 FTU/kg, and xylanase (xyl) at 0 or 16000 BXU/kg. Data points represent means of 8 birds per treatment.

Figure 3. Comparison of different methods, capsule or probe, on gizzard pH measurements in broilers. Broilers were fed diets supplemented with phytase at 500 or 2500 FTU/kg and xylanase at 0 or 16000 BXU/kg. Different letters denote significant difference for a specific method at $P<0.05$, with trends ($P<0.10$) indicated by an asterisk. Error bars indicate ± standard error of the mean. Capsule and probe data show means of 8 birds per treatment.

Figure 4. Benchmarking assessment comparing capsule and probe pH measurements taken 0.5, 1.0, 1.5 and 2.0 h post capsule application. Replicate birds per time point are indicated by letters ‘a’ and ‘b’. Data from one replicate bird following 1.5 h capsule dosing is missing due to the capsule being located between the crop and gizzard.

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Table 2 Expected and analysed diet composition for broilers

Phase	Phytase (FTU/kg)		Xylanase (BXU/kg)		Calcium (%)		Phosphorus (%)		Crude protein (%)		ME (MJ/kg)	
	Target	Analysed	Target	Analysed	Target	Analysed	Target	Analysed	Target	Analysed	Target	Analysed
Starter	500	722	0	-	0.98	0.99	0.71	0.61	21.85	20.3	12.45	11.9
	2500	2390	0	-	0.98	1.20	0.71	0.65	21.85	21.8	12.45	11.8
	500	868	16000	12300	0.98	1.22	0.71	0.63	21.85	22.3	12.45	11.7
	2500	2260	16000	11800	0.98	0.79	0.71	0.57	21.85	22.4	12.45	11.9
Grower	500	493	0	-	0.78	0.63	0.59	0.4	17.90	18.7	12.97	12.7
	2500	2500	0	-	0.78	0.66	0.59	0.44	17.90	18.6	12.97	12.4
	500	677	16000	14100	0.78	0.61	0.59	0.43	17.90	19.2	12.97	12.8
	2500	2670	16000	14100	0.78	0.67	0.59	0.43	17.90	19.3	12.97	12.5

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Table 3 Effect of phytase and xylanase on broiler performance[1]

			Feed intake (kg)			Weight gain (kg)			FCR			$_{bwc}$FCR
Phytase (FTU/kg)	Xylanase (BXU/kg)	Initial body weight (g)	Days 0-21	Days 21-42	Days 0-42	Days 0-21	Days 21-42	Days 0-42	Days 0-21	Days 21-42	Days 0-42	Days 0-42
500	0	36.6	1.28	3.79[b]	4.94	0.99[b]	2.25	3.24[b]	1.29	1.69	1.52	1.52
2500	0	36.4	1.35	4.04[a]	5.24	1.10[a]	2.37	3.47[a]	1.23	1.71	1.51	1.51
500	16000	36.5	1.34	3.99[a]	5.19	1.12[a]	2.32	3.43[a]	1.20	1.72	1.51	1.51
2500	16000	36.5	1.33	4.01[a]	5.18	1.13[a]	2.36	3.49[a]	1.18	1.70	1.48	1.48
	RMSE	0.00	0.098	0.135	0.280	0.063	0.100	0.115	0.033	0.059	0.060	0.060
500		3.7	1.31	3.89	5.06	1.05	2.29	3.34	1.25	1.70	1.52	1.52
2500		3.6	1.34	4.02	5.21	1.12	2.36	3.48	1.20	1.70	1.50	1.50
	0	3.6	1.31	3.91	5.09	1.04	2.31	3.35	1.26	1.70	1.52	1.52
	16000	3.6	1.34	4.00	5.18	1.12	2.34	3.46	1.19	1.71	1.50	1.50
P-value												
Phytase		0.621	0.322	0.012	0.149	0.006	0.041	0.002	0.001	0.987	0.384	0.383
Xylanase		0.981	0.540	0.089	0.364	0.001	0.415	0.011	<0.001	0.489	0.293	0.292
Phytase x Xylanase		0.836	0.286	0.025	0.124	0.044	0.287	0.044	0.063	0.317	0.724	0.725

Means of 8 replicate pens per treatment; main effects given as least square means

[a,b,c] Data in a column not sharing a common superscript letter significantly differ at $P<0.05$.

RMSE, root mean square error; FCR, feed conversion ratio (intake:gain) corrected for mortality and withdrawn birds; $_{bwc}$FCR, FCR corrected for body weight

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Table 5 Effect of phytase and xylanase on broiler litter and footpad dermatitis scores at 21 and 42 days[1]

		Footpad score										Litter score	
		Day 21					Day 42						
		Number of birds scored					Number of birds scored						
Phytase (FTU/kg)	Xylanase (BXU/kg)	Score 1	Score 2	Score 3	Score 4	Score 5	Score 1	Score 2	Score 3	Score 4	Score 5	Day 21	Day 42
500	0	71	25	4	0	0	4	46	33	14	4	2.0	3.1
2500	0	70	19	7	2	1	10	43	28	13	5	2.1	3.3
500	16000	63	29	4	3	0	13	46	28	12	1	2.0	3.1
2500	16000	63	31	4	2	1	20	54	20	4	1	2.0	3.0
P-value													
Phytase		0.705	0.663	0.447	0.342	0.151	0.143	0.860	0.110	0.186	0.735	0.317	1.000
Xylanase		0.264	0.437	0.983	0.922	1.000	0.053	0.338	0.238	0.110	0.009	0.317	0.651

[1]Means represent the average response of 8 replicate pens (144 chicks) per treatment.

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