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Dietary Supplementation of Endoxylanases and Phospholipase for Turkeys Fed Wheat-based Rations

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Abstract: The adverse effects of non-starch polysaccharides (NSP) on turkeys fed wheat-based diets may be alleviated by dietary supplementation of endoxylanase (to reduce the adverse effects of digesta viscosity) or phospholipase (to improve the digestibility of fat). BUTA toms were fed wheat-based diets containing one of 5 enzyme treatments: unsupplemented control, Natugrain Blend® (≥ 5,500 EXU/kg diet; NB), Lyxasan®-50 (≥ 2,250 EXU/kg diet; LX50), Lyxasan®-100 (≥ 5,500 EXU/kg diet; LX100), and Phospholipase (> 500 PLU/kg diet; PL) (BASF, Germany). Each treatment group was assigned to 8 pens containing 12 birds to evaluate growth performance (1-128 d), and 2 pens of 12 birds (excluding LX50) for the apparent metabolizable energy N-corrected (AMEn) and ileum viscosity determination (56-128 d). All enzyme treatments improved growth performance. In comparison to the control, dietary enzyme increased ($P < 0.05$) BW and decreased 1-128 d feed/gain (2.45 vs 2.37, $P < 0.005$). PL was most effective in reducing feed/gain during the starting phase and LX100 during the finishing phase, while NB had intermediate benefits throughout the experiment. PL increased AMEn from 9 to 12 wk, while NB and LX-100 resulted in the highest AMEn during the later finishing period. Viscosity was significantly higher for PL than the other treatments (13.5 vs 7.07 cP, $P < 0.001$). Growth performance and energy utilization of turkeys fed wheat-based diets can be significantly enhanced by phospholipase supplementation of starter feeds and endoxylanase supplementation of growing and finishing feeds. However, enzyme blends may provide a positive response regardless of turkey age.

Key words: Non-starch polysaccharides, wheat, enzymes, turkey, growth performance

Introduction

Cereals are important foodstuffs for man and livestock, but some contain high levels of antinutrients that limit their use. Cereals and their co-products comprise a significant proportion of livestock diets (Evers *et al*., 1999). Wheat is the most widely cultivated crop in the world, accounting for almost 30% (600.7 million metric tons in average of the years 1997 to 1999 - FAO, 2002) of total cereal production, and it is one of the major energy contributors to the diets of turkeys and broilers (Steenfeldt *et al*., 1998). Wheat contributes up to 80% of finishing poultry diets in the United Kingdom (Longstaff and McNab, 1986; Wiseman and Inborr, 1990). However, the use of wheat in commercial turkey and broiler diets has traditionally been limited by their low and variable energy values.

The apparent metabolizable energy (AME) of wheat can be lower and more variable than expected among different cultivars because of the amount of non-starch polysaccharides (NSP) contained in the kernel. The AME and soluble NSP are negatively correlated in wheat (Choct and Annison, 1990; Choct *et al*., 1999a), and various sources of purified NSP depress AME (Choct and Annison, 1990). Moreover, NSP isolated from wheat depress AME in a dosage dependent manner (Choct and Annison, 1990; Hughes and Choct, 1999). In contrast, degradation of cell wall NSP by glycanases increases AME (Annison, 1992).

Arabinoxylan is the major NSP in wheat that produce the antinutritive effects of wheat. Arabinoxylan is a linear polymer of variable length and consists of a long backbone chain of β-(1,4) anhydro-D-xylopyranosyl to which are attached single α-L-arabinofuranosyl residues at the two- or three-position (Lineback and Rasper, 1988). The principal antinutritive factor of arabinoxylans is their capacity to bind water and increase digesta viscosity (Knudsen, 2001). In the digestive tract, viscous solutions interfere with the digestion and/or absorption of nutrients (Marquardt *et al*., 1979) directly by affecting the rate of diffusion and indirectly by stimulating the growth of anaerobic microflora (Vukic Vranjes and Wenk, 1996). The viscous intestinal environment slows digestion and digesta passage rate, thus increasing the amount of undigested materials available for microflora proliferation and microbial fermentation in the small intestine (Preston *et al*., 2001). The microflora competes with the host for nutrients by converting nutrients into microbial protein and impeding digestion and absorption. An increase in microbial activity in the small intestine increases deconjugation of bile acids (Langhout *et al*., 2000). This reduction in conjugated bile acids reduces the formation of micelles, limiting digestion of fat and fat-soluble vitamins that decrease the AME of wheat (Hoffman and

Small, 1967).

Accompanying the dramatic effect of NSP on the performance and nutrient digestibility, birds often exhibit symptoms of considerable gastrointestinal stress. Choct and Annison (1992a) reported that birds fed high NSP diets excreted excessive amounts of fluid in their feces. Ward (1995) stated that wheat is often associated with wet litter for poultry, especially when fed at levels exceeding about 20% of the diet. Osmotic diarrhea caused by high levels of NSP in the diet of poultry is associated with a high concentration of dietary components that are not completely digested and/or absorbed in the small intestine, giving rise to an increased amount of osmotically active compounds in the gut (Choct and Annison, 1992a). Moisture content of the excreta has been shown to be 10% higher in birds fed NSP-rich diets than in birds fed a corn-soy diet that contains little NSP (Choct *et al.*, 1996). This increased diarrhea can adversely affect the health of birds by increasing the susceptibility of poultry to enteric disease and leg problems (Grimes and Crouch, 1997), and increase carcass downgrading (Hughes *et al.*, 2000). Moreover, excessive wet litter causes problems with litter handling and disposal (Pawlik *et al.*, 1990) and nuisance complaints from neighbors about flies and odor.

Dietary supplementation of fungal and microbial enzymes has been studied as a means to avoid the adverse effects of wheat NSP. Dietary enzyme supplementation has been shown to improve the feeding value of wheat by disrupting the water holding capacity of the NSP, improving nutrient digestion, and reducing microflora fermentation in the small intestine (Choct *et al.*, 1999b). Supplementing wheat-based diets with an enzyme preparation that is capable of hydrolyzing the long complex of xylan into smaller units has been shown to increase performance and nutrient digestibility (Annison and Choct, 1991; Steenfeldt *et al.*, 1998; Preston *et al.*, 2001). Furthermore, dietary enzyme supplementation has been shown to alleviate osmotic diarrhea by improving nutrient digestion via reduction in the concentration of osmotically active compounds in the gut (Fischer and Classen, 2000). Thus, appropriate enzyme supplementation to wheat-based diets not only improves growth performance and AME, but it also reduces disease and management problems associated with poor and wet litter conditions.

The benefit of NSP-enzymes in wheat-based diets is mainly attributed to endoxylanase, but a blend including other enzymes may be more effective than a single endoxylanase preparation. Endoxylanase degrades the xylan backbone of arabinoxylan into smaller units, which decreases gut viscosity, and increases productive performance and nutrient digestibility (Odetallah, 2000). A blend of different enzymes has been shown to improve performance to a greater extent than single enzyme preparations (Ravindran *et al.*, 1999; Odetallah *et al.*, 2002). Odetallah *et al.* (2002) studied the effect of three different enzyme products. One of the products was exclusively endoxylanase produced by a genetically modified organism, while the other enzyme preparation had major activities of β-glucanase and endoxylanase and minor activities of hemicellulase, cellulase and protease. The third enzyme preparation evaluated was a blend of the first two products. These researchers reported that a blend of enzyme that contained high endoxylanase activities resulted in the best growth performance in turkeys fed wheat-based diets. Furthermore, the effect of enzyme supplementation on the performance and nutrient digestion increased as the enzyme level increased. Odetallah (2000) reported that the total amount of enzyme activity supplemented to the diet is crucial to its effect on the performance of turkey.

Endoxylanase and enzyme complex products decrease gut viscosity and improve fat digestibility by preserving the integrity and function of bile salts, and by allowing the formation of fat micelles. Another approach to enhance fat digestion in diets with high inclusion levels of wheat was studied using lipase (Martin and Farrell, 1998). However, dietary supplementation of lipase did not improve the performance of broilers. Al-Marzooqi and Leeson (1999) attributed the lack of lipase response to the contamination of the enzyme product with cholecystokinin by the microorganism responsible to produce the lipase. Cholecystokinin is a hormone that reduces feed intake. In contrast, dietary supplementation of exogenous phospholipase may significantly improve fat digestibility (Carey *et al.*, 1983). It is widely accepted that endoxylanase improves fat digestibility of birds fed wheat-based diets by decreasing viscosity and microbial fermentation in the gut. In this study we investigated the direct improvement of fat digestion by supplementation of phospholipase. Endogenous phospholipase A_2 (PLA) catalyzes the hydrolysis of the ester bond at sn-2 position of glycerophospholipids (GPL), producing fatty acids and lysophospholipids (e.g. Lyso-phosphatidylcholine or Lyso-PC). The fatty acids are then absorbed from the lumen as part of the fat micelle. Lyso-PC, the predominant GPL product in the luminal content, is essential for the emulsification of water-insoluble lipids (Homan and Jain, 2001). Lipid emulsification is the first stage of lipid digestibility. Lyso-PC is an important amphiphile molecule, which acts to stabilize microdroplets of triglycerides, cholesterol, and other nonpolar dietary lipids that are otherwise insoluble in the aqueous environment of the intestinal contents (Carey *et al.*, 1983). Also, PLA influences the capacity of the enterocyte to transport absorbed lipids into the circulation, since its capacity depends on cellular phosphatidylcholine synthesis controlled by the hydrolysis of phosphatidylcholine in the lumenal contents (Carey *et al.*, 1983). Additionally, PLA may

possess an intrinsic secretin-releasing activity that stimulates the release of pancreatic secretion and bicarbonate in the duodenum, which enhances the digestion and absorption of others macro nutrients (Chang *et al.*, 1999). Exogenous phospholipase may act in a similar manner to endogenous intestinal phospholipase A_2, and thus, it could alleviate the adverse effects of NSP by facilitating the formation of micelles of triglyceride, cholesterol, and other nonpolar dietary lipids. However, supplementation of exogenous phospholipase in wheat-based diets for poultry has not been investigated.

The general objective of this study was to evaluate the efficacy of supplemental enzymes with different enzyme activities on growth performance and energy utilization of turkeys fed an inferior-quality of wheat. The enzymes used in this study included (a) Natugrain Blend (an enzyme blend with high endoxylanase activity), (b) Lyxasan (endoxylanase only) at two application rates, and (C) phospholipase only. The specific objectives of this study were:

1) to determine the effect of dietary endoxylanase supplementation level on growth performance;
2) to compare the efficacy of endoxylanase from an organism that produces high endoxylanase activity with one that produces endoxylanase along with several other enzymes; and
3) to evaluate the effect of endoxylanase and phospholipase on growth performance and dietary energy utilization.

Materials and Methods

The study was divided into two experiments to achieve the desired objectives. Experiment 1 included performance and caked litter analysis. Experiment 2 included intestinal digesta viscosity and energy digestibility (AMEn) evaluation.

Enzymes: The same enzymes were used in experiment 1 and 2. The enzyme activity in the product and feed, and application rate used in the experimental diets are shown in Table 1 (G. K. Holkenborg, 2001, BASF, Aktiengesellschaft, Ludwigshafen, Germany, personal communication). Natugrain Blend® 66% is a commercial liquid enzyme preparation obtained from fungal fermentation of *Trichoderma longibrachiatum*. It contained standardized activities of at least 9,000 units of β-glucanase activity (BGU) per gram of product and at least 36,600 endoxylanase units (EXU) per gram of product. One BGU is defined as the activity required to liberate 0.278 µmol reducing sugar (measured as glucose equivalents) per minute at pH 3.5 and 40 °C, at a substrate concentration of 0.5% β-glucan from barley. One EXU is defined as the enzyme activity required to liberate 1 µmol of reducing sugar (measured as glucose equivalents) per minute from a 1% xylan solution at pH 3.5 and 40 °C. Natugrain Blend® also contained some hemicellulase (e.g. pectinase), cellulase and protease activities (BASF, 1997). Lyxasan Forte® is a commercial liquid preparation obtained from a genetically modified *Aspergillus niger* that produces endoxylanase exclusively. Lyxasan Forte® had an endoxylanase activity of at least 56,000 EXU/g of product. Phospholipase is an experimental liquid enzyme preparation obtained from a microbial source and it contained activities of at least 5,000 units of phospholipase A_2 per gram of product. All of the enzyme preparations were kindly supplied by BASF[1].

The Natugrain Blend® was supplemented to the diet to achieve the same level of endoxylanase activity in the feed as was supplied by Lyxasan Forte® when supplemented at 100 g/tonne of feed. This treatment design allowed us to evaluate the effect of endoxylanase as a single enzyme preparation or along with several other enzymes. Lyxasan Forte® was applied at two-application rates (100 and 50 g/tonne) so we could investigate the dose-dependent responses for dietary endoxylanase supplementation. Phospholipase was used to test our hypothesis that its dietary supplementation could alleviate the adverse effects of NSP by facilitating lipid digestibility.

Experiment 1: Performance Trial: The facility used in this study was an industry-standard curtain-sided house containing ninety-six 9.3 square meter pens. Each pen was top-dressed with 4 cm of soft pine shavings at the start of the experiment. Ventilation was provided by natural air movement through appropriately adjusted curtain sides and air mixing fans located on the ceiling throughout the house. High and low ambient temperatures within the house were recorded at two places twice daily throughout the duration of the trial. The house temperatures was kept at 29-31 °C during the first week, and then gradually stepped down to the ambient outside temperature, which ranged from 6 °C (43 °F) to 34 °C (94 °F). The house was illuminated with incandescent lights for 23 hours per day for the first week and by natural daylight thereafter. A heat lamp unit[2] with one 125-watt bulb[3] provided supplemental heat for each pen. Feed and water were provided *ad libitum* throughout the duration of the study. Visual heath inspection of all birds within the study was performed daily, and the weights of all culled birds and the reasons why they were removed were recorded. Crippled or dead birds were removed and replaced up through day seven, following which mortalities were removed and recorded but not replaced. All mortalities were weighed soon after death and recorded so that the weights could be included for the calculation of feed conversion.

One-day-old commercial Large White BUTA[4] male turkeys were obtained from a commercial hatchery[5] and were then randomly assigned to the pens. They were

Table 1: Enzyme activity in the products[1] and in the feed, and rate of application used in the experimental diets[2]

Enzyme	Activity (units/g DM)[3]	Application rate (g/tonne feed)	Activity (units/kg feed)
Natugrain Blend® (66%)	≥ 36,600 EXU/g ≥ 9,000 BGU/g	150	≥ 5,500 EXU/kg ≥ 1,350 BGU/kg
Phospholipase	≥ 5,000 PLU/g	100	≥ 500 PLU/kg
Lyxasan Forte®	≥ 56,000 EXU/g	100 and 50[4]	≥ 5,500 EXU/kg[5] ≥ 2,250 EXU/kg[6]

[1]Products supplied by BASF (BASF AG, 67059 Ludwigshafen, Germany). [2]Data from G. K. Holkenborg, 2001, BASF, Ludwigshafen, Germany, personal communication. [3]EXU= endoxylanase units, BGU= β-glucanase units, PLU= phospholipase units. [4]Lyxasan Forte® was used at 2 application rate (Lyxasan-100 and Lyxasan-50 treatments). [5]Concentration in feed from Lyxasan Forte® at 100 g/tonne feed. [6]Concentration in feed from Lyxasan Forte® at 50 g/tonne feed.

used for both the growth performance trial (Experiment 1) and the digestability trial (Experiment 2).

The experimental diets are presented in Table 2. Five feed phases were used during the course of the experiment. All feeds were formulated using least-cost linear programming software, such that the diets contained about 95% of the NRC (1994) recommendations for amino acids and energy. The diets were formulated slightly below requirements so that any improvement in nutrient availability due to enzyme supplementation could be observed as an improvement in growth performance. All experimental diets consisted of the same wheat-soybean meal basal diet with different supplemental enzyme treatments using an inclusion level of 0.1%. The control diet was supplemented with 0.1% washed builder's sand, but no enzymes. All feed was pelleted and fed as crumbles to 28-days of age, and subsequently as a whole 5/16-pellet. Composite feed samples from each diet were taken immediately after manufacture and were then analyzed for crude protein, fat, ash, Ca, and P.

The enzymes were applied to the feed in a 500 kg capacity horizontal double ribbon mixer and then bagged into 20 kg bags. The three enzymes (Natugrain Blend®, Lyxasan Forte®, and Phospholipase) were applied as a fine spray onto the pelleted feed during mixing using a plant mister[6]. All enzymes were added to the diet in amounts recommended by the supplier. The enzymes were diluted in water to a volume of 1 liter, such that the dosage per tonne of feed were 150 g of Natugrain Blend®, 100 g and 50 g of Lyxasan Forte® (Lyxasan-100 and Lyxasan-50, respectively), and 100g of Phospholipase. The wheat sample used in this experiment was previously determined to be a low-AME wheat (2,216 kcal/kg) (Santos *et al.*, 2001) because wheat samples assaying less than 2,850 kcal/kg are arbitrarily classified as low-AME wheat (Mollah *et al.*, 1983). This wheat, from Western Canada, had been exposed to a damaging frost event during the grain filling stage (mid-milk to early-dough stage).

The purpose of the experiment was to evaluate the influence of dietary enzyme supplementation on growth performance (body weight, feed consumption, feed conversion ratio) and litter condition of turkeys fed wheat-based diets. To achieve this objective forty pens of the experimental house were assigned to this trial, such that the inside 20 pens were contained in blocks two and three and the outside 20 pens were contained in blocks one and four. Five dietary treatments were randomly assigned to pens within each of the four blocks so that position effects within the turkey house were removed statistically. The dietary treatments were randomly assigned to pens using the Proc-Plan procedure of SAS® (SAS, 1996). Each pen of 12 turkeys, the experimental unit, were subjected to one of five dietary enzyme treatments from 1 to 128 (0-18 wk) days of age, as follows: (1) unsupplemented control, (2) Natugrain Blend, (3) Lyxasan-50, (4) Lyxasan-100, and (5) Phospholipase. Each treatment combination was replicated in 8 pens (2 pens per block).

Feed consumption (kg) and body weights (kg) (by pen and by individual bird, respectively) were recorded at 0, 2, 4, 8, 12, 16, and 18 weeks of age. Caked litter from each pen was removed (11, 14, 16, and 18 wk of age) when required to maintain acceptable litter conditions, and the weight of the caked litter was recorded.

Experiment 2: Digestibility Trial: Experiment 2 used the same husbandry practices and animals and a similar house to what was used in Experiment 1, except that there were a few modifications to the experimental diets and pen facilities to accommodate the objectives of determining dietary energy utilization. The experimental diets for the digestibility trial were formulated to contain 0.8% Celite[7] (w/w), a source of acid-insoluble ash, as an indigestible marker (Table 2).

The objective of the digestability trial was to evaluate the influence of enzyme additions on energy utilization (AMEn) and digesta viscosity of turkeys fed wheat-based diets. Twenty-five turkeys were maintained in each of eight conventional floor-pens, and were randomly assigned to one of four dietary treatments: (1) unsupplemented control; (2) Natugrain Blend; (3) Lyxasan-100; and (4) Phospholipase. At 56 days of age

Table 2: Composition and calculated nutrient content of the experimental diets fed to turkey toms from 1 to 128 days of age on the growth performance trial (Experiment 1) and digestibility trial (Experiment 2)[6]

	Treat. 1 and 2		Treatment 1 only			Treatment 2 only		
Ingredients	Feed 1	Feed 2	Feed 3	Feed 4	Feed 5	Feed 3	Feed 4	Feed 5
	(%)							
Wheat	46.82	61.18	66.90	75.09	76.45	65.66	73.54	76.45
Soybean meal (48% CP)	42.83	23.25	19.92	10.35	11.44	19.58	10.63	11.04
Poultry meal (60% CP)	0.00	5.00	1.77	2.62	0.00	2.30	2.75	0.00
Poultry Fat[3]	0.85	4.62	6.21	7.45	8.00	1.16	0.89	0.88
Dicalcium phosphate (18.5% P)	2.42	3.13	1.22	0.89	0.88	6.54	7.83	7.60
Limestone	1.66	0.17	1.28	1.00	1.01	1.25	0.99	1.01
Crude soy oil	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Salt	0.35	0.19	0.36	0.28	0.31	0.35	0.28	0.31
Mineral premix[1]	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Choline Cl (60%)	0.18	0.11	0.07	0.03	0.00	0.07	0.01	0.00
DL-Methionine	0.16	0.16	0.11	0.09	0.05	0.11	0.09	0.05
L-Threonine	0.00	0.14	0.11	0.19	0.00	0.11	0.19	0.00
L-Lysine HCL	0.08	0.41	0.41	0.36	0.20	0.41	0.35	0.20
Vitamin premix[2]	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Celite[3]	0.00	0.00	0.00	0.00	0.00	0.80	0.80	0.80
Sand or enzyme[4]	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Selenium premix[5]	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Calculated analysis								
ME, kcal/kg	2,700	2,800	2,900	3,000	3,050	2,900	3,000	3,050
Crude protein, %	27.0	23.0	20.0	17.0	15.8	20.0	17.0	15.8
Lysine, %	1.55	1.40	1.23	0.95	0.80	1.23	0.95	0.80
Methionine + Cysteine, %	1.00	0.90	0.76	0.67	0.61	0.76	0.67	0.61
Threonine, %	0.97	0.90	0.76	0.71	0.50	0.76	0.71	0.50
Calcium, %	1.25	1.00	0.90	0.75	0.65	0.90	0.75	0.65
Non-phytate phosphorus, %	0.60	0.85	0.42	0.38	0.32	0.42	0.38	0.32
Sodium, %	0.18	0.15	0.18	0.15	0.15	0.18	0.15	0.15

[1]Supplied the following per kilogram of feed: 120 mg Zn as $ZnSO_4.H_2O$; 120 mg MN as $MnSO_4.H_2O$; 80 mg Fe as $FeSO_4.H_2O$; 10 mg Cu as $CuSO_4$; 2.5 mg I as $Ca(IO_3)_2$; 1.0 mg Co as $CoSO_4$. [2]Supplied the following per kilogram of feed: vitamin A, 26,400 IU; cholecalciferol, 8,000 IU; niacin, 220 mg; pantothenic acid, 44 mg; riboflavin, 26.4mg; pyridoxine, 15.8 mg; menadione, 8 mg; folic acid, 4.4 mg; thiamin, 8 mg; biotin, 0.506 mg; vitamin B_{12}, 0.08 mg;ethoxyquin, 200 mg. The vitamin E premix provided the necessary amount of vitamin E as DL-α-tocopheryl acetate. [3]Celite (CeliteTM, Celite Corp., Lompar, CA 93436), a source of acid-insoluble ash, were used as an indigestible marker. [4]Enzyme treatments were supplemented with enzyme products that accounted as a dry ingredient (0.1%) and an equivalent amount of sand was applied to the unsuplemented control treatment. [5]Selenium premix provided 0.3 ppm Se from sodium selenate. [6]All birds were fed the same diets (feed 1 and 2) until 56 d of age, however feed 3, 4, and 5 were different between the two trials. The feeding period were as follow: feed 1 (1-28 d), feed 2 (29-56 d), feed 3 (57-84 d), feed 4 (85-112 d), feed 5 (113-128 d).

(8 wk), eight birds per pen were banded[8] and sampled for digesta viscosity. The remaining twelve birds per pen were weighed and then transferred to 8 digestibility pens for the AMEn assay. The digestibility pens were modified floor-pens with plastic slats secured upon two by four lumber frames. The 2.5 cm slats were spaced 2.5 cm apart, which allowed the excreta of the birds to fall onto a plastic sheet under the pen. The plastic sheet could be pulled from under the slatted floor to facilitate excreta sampling. New plastic sheets were utilized after each excreta collection time.

The birds used for the digesta viscosity measurements were fasted over night (8 h) and then given *ad libitum* access to feed for 3 h before sampling. Eight birds from each pen were then weighed and euthanized with carbon dioxide. About 2 grams of digesta was then gently expressed from the terminal part of the ileum (midway between the Meckel's diverticulum and the ileo-caecal junction to 1 cm above the ileo-caecal junction), placed into micro-centrifuge tubes, centrifuged[9] at 3,000 rpm for 2 min, and approximately 500 µl of the supernatant was then collected. The viscosity of the supernatant was determined using Brookfield Digital Viscometer LVDVII+CP[10] at 15 °C according to the method described by the Brookfield Digital Viscometer Operating Instructions Manual[11]. The measurement from each bird was considered as the experimental unit for the statistical analysis of digesta viscosity.

The digestibility experiment was conducted with tom turkeys from 9 to 18 wk of age. Excreta were collected twice per week from several locations on the plastic sheets from each pen, and care was taken to avoid

Table 3: Effects of different exogenous enzyme supplementation on body weight of turkey toms fed wheat-based diets from 0 to 18 weeks of age

Treatment	2 wk	4 wk	8 wk	12 wk	16 wk	18 wk
	(kg)					
Control[1]	0.288[b]	0.922[c]	4.051[b]	7.958[b]	12.565[b]	15.290
Natugrain Blend[2]	0.327[a]	1.007[ab]	4.294[a]	8.420[a]	13.305[a]	15.953
Lyxasan-50[3]	0.336[a]	1.020[a]	4.280[a]	8.386[a]	13.131[a]	15.528
Lyxasan-100[4]	0.322[a]	0.981[b]	4.186[ab]	8.403[a]	13.291[a]	15.953
Phospholipase[5]	0.321[a]	1.004[ab]	4.204[a]	8.282[a]	13.107[a]	15.643
SEM(32)[6]	0.0056	0.0128	0.0518	0.1096	0.1603	0.1801
P-value	0.0001	0.0001	0.0174	0.0286	0.0168	0.0617

[a-c] Means with different superscripts within a column differ significantly (P < 0.05). There were no significant differences in poults starting weights at 1 d of age (60g). [1]Unsupplemented wheat/SBM basal diet. [2]150g of Natugrain Blend/tonne of basal diet provided at least 5,500 EXU/kg feed and 1,350 BGU/kg feed. [3]50g of Lyxasan Forte/tonne of basal diet provided at least 2,250 EXU/kg feed. [4]100g of Lyxasan Forte/tonne of basal diet provided at least 5,500 EXU/kg feed. [5]100g of Phospholipase/tonne of basal diet provided 500 PLU/kg feed. [6]SEM(32) = Standard Error of the mean with 32 degrees of freedom.

Table 4: Effects of different exogenous enzyme supplementation on cumulative feed consumption of turkey toms fed wheat-based diets from 0 to 18 weeks of age

Treatment	0 to 2 wk	0 to 4 wk	0 to 8 wk	0 to 12 wk	0 to 16 wk	0 to 18 wk
	(kg)					
Control[1]	0.725	1.766	7.012	16.254	28.630	36.775
Natugrain Blend[2]	0.716	1.774	7.198	16.712	29.680	37.657
Lyxasan-50[3]	0.815	1.873	7.138	16.444	29.015	36.822
Lyxasan-100[4]	0.749	1.798	7.027	16.343	29.052	36.706
Phospholipase[5]	0.678	1.731	7.278	16.628	29.496	37.220
SEM(32)[6]	0.0319	0.0403	0.0756	0.2012	0.3663	0.4147
P-value	0.0601	0.1639	0.0867	0.4718	0.2923	0.4562

[a,b]Means with different superscripts within a column differ significantly (P < 0.05). [1]Unsupplemented wheat/SBM basal diet. [2]150g of Natugrain Blend/tonne of basal diet provided at least 5,500 EXU/kg feed and 1,350 BGU/kg feed. [3]50g of Lyxasan Forte/tonne of basal diet provided at least 2,250 EXU/kg feed. [4]100g of Lyxasan Forte/tonne of basal diet provided at least 5,500 EXU/kg feed. [5]100g of Phospholipase/tonne of basal diet provided 500 PLU/kg feed. [6]SEM(32)= Standard Error of the mean with 32 degrees of freedom.

contamination of feathers, scales and other debris. After each collection, the excreta samples were sealed in moisture-impermeable sample bags[12] and stored at minus 20 °C until they were processed for nutrient analysis. Before analysis, the frozen-excreta were placed on the laboratory table to thaw overnight at room temperature. Then, the two-200 g samples of excreta collected from each pen each week were blended[13] together to a slurry following the addition of approximately 100 ml of distilled water to form a pooled sample. The pH of the fecal slurry was then adjusted to 5.4 by the addition of sulfuric acid (0.1 N) to minimize microbial fermentation of the fecal nitrogen during overnight drying at 70 °C in a forced-air convection oven[14]. The dried samples were ground in a blender,[13] and then stored at minus 20 °C before analysis to determine AMEn. Gross energy content of feed and dried-excreta samples were determined by combustion in an adiabatic oxygen bomb calorimeter[15]. Celite recovery was performed using the method described by Vogtmann *et al.* (1975). Moisture levels were analyzed by drying 3 to 5 g of the materials for 6 h in a forced-air convection oven[14] at 105 °C. Nitrogen content of all feed and dried-excreta samples were determined using a Kjeldahl automatic analyzer[16]. The AMEn values were determined using the equations shown on the caption of Fig. 1. The values of AMEn were calculated relative to the acid-insoluble ash marker, and were then corrected to zero N-retention by using a value of 8.22 kcal/g nitrogen retained (Hill and Anderson, 1958).

Statistical Analysis: All data were analyzed using the general linear models procedure for ANOVA (SAS, 1996). Pen means served as the experimental unit for statistical analysis, unless otherwise stated. Treatment means having a significant F-test were separated using the least-squares-means function of SAS® (SAS, 1996), and were considered to be significant at P < 0.05. All percentage data was transformed to arc sine before analysis.

Animal Ethics: The experiments reported herein were

conducted according to the guidelines of the Institutional Animal Care and Use Committee at North Carolina State University. All husbandry practices and euthanasia were done with full consideration of animal welfare.

Results

Experiment 1: Performance Trial: Performance. All enzyme supplementation treatments significantly ($P < 0.05$) increased the turkey body weights (BW) throughout the experiment, although their effect on 18-week BW was marginal (15.8 vs 15.3 kg, $P = 0.062$) (Table 3). In comparison to the unsupplemented control treatments, enzyme supplementation improved BW in a range from 3.1 to 28.1%. Although there was no significant change observed in feed consumption (Table 4), the enzyme treatments clearly improved feed conversion ratio (FCR) in comparison to the control (Table 5). The phospholipase supplementation had a greater effect on FCR during the starting phase (0-2 wk) than during the growing-finishing phase (3 to 18 wk). In contrast, Lyxasan-100 was most effective during the growing-finishing period. Natugrain Blend had an intermediate improvement on FCR throughout the entire period studied. The effect of endoxylanase supplementation on the FCR was dose-dependent, as indicated by the difference between the Lyxasan-50 and Lyxasan-100 treatments. In general, supplementation with all of the enzymes used increased the growth performance of turkeys consuming wheat-based diets.

Caked Litter. Data on the effect of different exogenous enzyme supplementations on the cumulative amount of caked litter measured are presented in Table 6. During the period of 0 to 11 weeks of age, the highest amount of cumulative caked litter measured was from the control treatment, and the lowest amount was from the Lyxasan-100 treatments ($P<0.05$). Dietary enzyme supplementation reduced the amount of caked litter by 40% in comparison to the control group ($P<0.10$). Generally, the Lyxasan-100 treatments had the lowest amounts of caked litter.

Experiment 2: Digestibility Trial: Ileum Digesta Viscosity. The ileum digesta viscosities of turkeys fed the wheat-based diets supplemented with phospholipase were 48% higher ($P < 0.001$) than those fed the other dietary treatments (Table 6). There were no significant differences between the control birds and turkeys fed diets supplemented with enzyme preparations containing endoxylanase (Lyxasan-100 and Natugrain Blend treatments) on the ileum digesta viscosity at 56 days of age.

Apparent Metabolizable Energy Nitrogen-Corrected (AMEn). Dietary AMEn was increased by providing phospholipase from 9 to 11 wk of age. Supplementation with Natugrain Blend and Lyxasan-100 resulted in the highest dietary energy utilization after 12 wk of age (Fig. 1). There were no statistical significant differences between treatments from 15 to 17 weeks of age, but the dietary phospholipase treatment had lower AMEn than the other treatments at 18 weeks of age. Although not amenable to statistical analysis, there was a clear increasing trend in AMEn throughout the trial. Diets supplemented with Natugrain Blend and Lyxasan-100 produced a more consistent increase in AMEn than the control diet or the diet supplemented with phospholipase. Considerable fluctuation in the AMEn values was observed throughout the trial in the phospholipase and unsupplemented control treatment groups.

Discussion

The studies herein evaluated the effect of different sources of supplemental enzymes on growth performance and energy utilization of turkeys fed diets containing an inferior-quality of wheat. The wheat used in this experiment was previously determined to be a low-AME wheat (2,216 kg/kg) when fed to turkeys (Santos *et al.*, 2001), due to frost-damage during the grain filling (mid-milk to early-dough) stage of seed development. The frost damage reduced the nutrient content of the wheat, presumably due to an increased content of NSP to starch (Santos *et al.*, 2001). We chose this wheat because there is evidence that responses to enzymes are greatest with low-AME wheat due to its higher NSP content (Choct *et al.*, 1994).

As hypothesized, all the enzyme treatments improved growth performance and increased energy utilization throughout the experiment, but this effect was age dependent. Enzyme supplementation increased BW and decreased feed conversion ratio throughout the trial. No significant treatment effects were observed on feed consumption, although differences were noted in AMEn. Phospholipase treatment was most effective in reducing FCR and increasing AMEn during the early phase of growth, while Lyxasan-100 was most effective towards the later phases of growth, and Natugrain Blend had intermediate to best results throughout the study.

Endoxylanase activity provides the primary effect of Natugrain Blend and Lyxasan Forte enzymes. Evidently, degradation of the NSP in wheat by the added endoxylanase contributed to the improved growth performance of turkeys. Endoxylanase degrades the xylan backbone of arabinoxylan into smaller units, which disrupts the water holding capacity of the NSP (Scott and Boldaji, 1997) and reduces the viscosity of the digesta in the small intestine (Bedford and Schulze, 1998; Choct *et al.*, 1999b). Reduced digesta viscosity increases the diffusion rates of nutrients and endogenous enzymes, enabling the bird to digest and absorb more nutrients that lead to increased growth performance (Pawlik *et al.*, 1990). Also, endoxylanase inhibits the proliferation of the fermentative

Table 5: Effects of different exogenous enzyme supplementation on cumulative feed conversion ratio[7] of turkey toms fed wheat-based diets from 0 to 18 weeks of age

Treatment	0 to 2 wk	0 to 4 wk	0 to 8 wk	0 to 12 wk	0 to 16 wk	0 to 18 wk
	(g/g)					
Control[1]	3.201[a]	2.051	1.775[a]	2.079[a]	2.327[a]	2.451[a]
Natugrain Blend[2]	2.695[b]	1.904	1.706[c]	2.003[bc]	2.255[b]	2.384[b]
Lyxasan-50[3]	2.968[ab]	1.954	1.724[ab]	1.992[bc]	2.232[b]	2.399[ab]
Lyxasan-100[4]	2.883[ab]	1.956	1.713[c]	1.970[c]	2.212[b]	2.322[c]
Phospholipase[5]	2.603[b]	1.847	1.761[ab]	2.025[b]	2.261[b]	2.400[ab]
SEM(32)[6]	0.1425	0.0477	0.0139	0.0155	0.0172	0.0183
P-value	0.0466	0.0624	0.0039	0.0003	0.0008	0.0006

[a-c]Means with different superscripts within a column differ significantly (P < 0.05). [1]Unsupplemented wheat/SBM basal diet. [2]150g of Natugrain Blend/tonne of basal diet provided at least 5,500 EXU/kg feed and 1,350 BGU/kg feed. [3]50g of Lyxasan Forte/tonne of basal diet provided at least 2,250 EXU/kg feed. [4]100g of Lyxasan Forte/tonne of basal diet provided at least 5,500 EXU/kg feed. [5]100g of Phospholipase/tonne of basal diet provided 500 PLU/kg feed. [6]SEM(32)= Standard Error of the mean with 32 degrees of freedom. [7]Equation to determine feed conversion ratio (g/g) = total pen feed consumed/total weight gained including mortality

Table 6: Effects of different exogenous enzyme supplementation of wheat-based diets on the cumulative caked litter[7] and ileum digesta viscosity of turkey toms

	Cumulative Caked Litter per turkey				Ileum Viscosity
Treatment	0 to 11 wk	0 to 14 wk	0 to 16 wk	0 to 18 wk	56 d
	(kg)				cP[8]
Control[1]	1.823[a]	5.157	7.524	9.666	7.118[b]
Natugrain Blend[2]	1.205[ab]	3.248	4.516	5.576	6.705[b]
Lyxasan-50[3]	1.381[ab]	3.979	5.495	6.881	7.389[b]
Lyxasan-100[4]	0.847[b]	2.549	3.700	5.016	13.551[a]
Phospholipase[5]	1.440[ab]	2.984	4.153	5.513	7.118[b]
SEM[6]	0.2156	0.6786	1.0074	1.2224	1.2892
P-value	0.0467	0.0839	0.0830	0.0729	0.0009

[a,b]Means with different superscripts within a column differ significantly (P < 0.05). [1]Unsupplemented wheat/SBM basal diet. [2]150g of Natugrain Blend/tonne of basal diet provided at least 5,500 EXU/kg feed and 1,350 BGU/kg feed. [3]50g of Lyxasan Forte/tonne of basal diet provided at least 2,250 EXU/kg feed. [4]100g of Lyxasan Forte/tonne of basal diet provided at least 5,500 EXU/kg feed. [5]100g of Phospholipase/tonne of basal diet provided 500 PLU/kg feed. [6]SEM = Standard Error of the mean with 32 degrees of freedom on the cumulative caked litter analysis and 58 degrees of freedom on the Ileum viscosity analysis. [7]Equation to determine cumulative caked litter per bird (kg) = kilogram of caked litter removed from a pen/number of birds in the pen, adjusted for mortality. [8]Centipoise (cP). A centimeter-gram-second unit of dynamic viscosity equal to one dyne-second per square centimeter.

microorganisms in the small intestine by increasing the digesta passage rate and nutrient digestion (Choct *et al.*, 1999b). As the microflora characteristics are changed by enzyme supplementation, there is a decrease in the adverse effects of microbial fermentation, including the reduction in fat digestion by the deconjugation of bile salts (Langhout, 1999) and an increased competition between the host and the microflora for available nutrients (Bedford, 1995; Choct *et al.*, 1996; Langhout *et al.*, 2000). Therefore, endoxylanase improved growth performance and energy utilization by improving digestion and absorption of nutrients, and decreasing the fermentation of microorganisms in the intestinal tract.

The effect of dietary enzyme supplementation on the growth performance of turkeys appears to be dependent upon the dose of endoxylanase. It is possible that the level of endoxylanase in the Lyxasan-50 treatment ($\leq$ 2,250 EXU/kg feed) was insufficient to degrade enough the xylan backbone present in the diet with a high inclusion level of wheat, whereas the double dosage of endoxylanase used in Lyxasan-100 treatment ($\geq$ 5,500 EXU/kg feed) was able to produce a superior effect. Several researchers have shown dose-dependent responses for dietary supplementation of NSP enzymes (Hesselman *et al.*, 1982; Petterson and Aman, 1989; Bedford and Classen, 1992). Crouch *et al.* (1997) stated that one possible reason for the general lack of response with some enzymes might be the presence of a higher content of water-soluble pentosans (arabinoxylan) in some cultivars of wheat. Other authors, attributes it to the low level of enzyme (Odetallah, 2000),

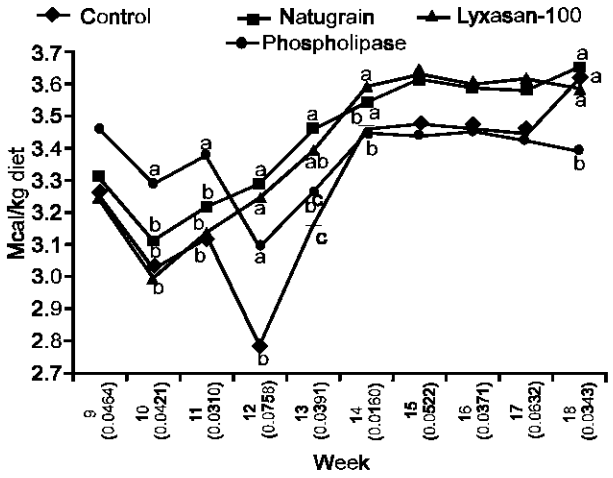

Fig. 1: Effects of different exogenous enzyme supplementation on apparent metabolizable energy nitrogen-corrected (AMEn) of turkeys fed wheat based-diets from 9 to 18 weeks of age. Different letters on each line-point within each week, signify a significant ($P < 0.05$) difference between mean values of AME (Mcal per kilogram diet). Mean values represent values from two pooled samples of excreta. Pooled samples represent two samples of excreta of each pen (approximately 200g from each) collected from each week mixed together. Values in parenthesis represent the Standard Error of the mean with 4 degrees of freedom for each week. The treatments were: unsupplemented wheat/SBM basal diet (control); 150g of Natugrain Blend/tonne of basal diet that provided at least 5,500 EXU/kg feed and 1,350 BGU/kg feed (Natugrain); 100g of Lyxasan Forte/tonne of basal diet that provided at least 5,500 EXU/kg feed (Lyxasan-100); and 100g of Phospholipase/tonne of basal diet that provided 500 PLU/kg feed (Phospholipase). Equation to determine AMEn (kcal/kg diet on dry matter basis) is shown below:

AMEn = Metabolizable energy per gram diet dry matter nitrogen corrected = Ediet – Eexcreta – 8.22 N, which

Ediet = kilocalories combustible energy per gram of diet dry matter (directly from bomb calorimeter).

Eexcreta = kilocalories combustible energy in excreta per gram of diet dry matter = kilocalories per gram excreta x (g celite per gram diet/g celite per gram excreta).

N = Nitrogen retention per gram of diet dry matter = N per gram diet - N per gram excreta x (g celite per gram diet/g celite per gram excreta).

and/or an inappropriate enzyme for the type of grain involved (Friesen *et al.*, 1992).

The beneficial effect of endoxylanase may be enhanced by synergy with other enzymes in a blended enzyme preparation. In comparison to the Lyxasan Forte and Phospholipase treatments, dietary supplementation of Natugrain Blend, which contained endoxylanase and other enzymes, improved performance and AMEn regardless of turkey age. Even though the level of endoxylanase activity in the feed (at least 5,500 EXU/kg feed) was the same for both the Lyxasan-100 and Natugrain Blend treatment groups, the presence of hemicellulase (e.g. pectinase), cellulase, and protease (BASF, 1997) in the Natugrain Blend mixture may have afforded this enzyme product the greatest degree of versatility for use in wheat-rich turkey diets. Similar results have been reported by Odetallah *et al.* (2002), who observed that enzyme mixtures with high endoxylanase activity, like Natugrain Blend, resulted in the best growth performance in turkeys fed wheat-based diets. Ravindran *et al.* (1999) stated that there is considerable synergy in activities among enzymes when they are supplemented as blended preparations.

To our knowledge, this study is the first to report on the dietary supplementation of phospholipase to poultry diets, considering that fat digestion are compromised in wheat-based diets (Choct and Annison, 1992a; Friesen *et al.*, 1992). We hypothesized that the exogenous source of phospholipase would act on a similar matter as endogenous PLA. Therefore, dietary phospholipase supplementation may alleviate the effects of dietary NSP by facilitating the formation of micelles of triglyceride, cholesterol, and other nonpolar dietary lipids, enhancing the capacity of the enterocytes to absorb lipids, and increasing the digestion of the other macronutrients. This hypothesis is consistent with the positive response observed in the growth performance and AMEn in turkeys up to 12 weeks of age. However, the beneficial effect of dietary phospholipase supplementation on growth performance diminished during the growing-finishing phases, which may be associated with the increased ability of the birds to digest lipid as the birds get older.

These data demonstrate that the effect of enzyme treatment is age-dependent. Phospholipase had a significantly better affect than the pentosanase enzyme treatment during the beginning of the trial, whereas the endoxylanase-containing enzyme products were more effective during later half end of the trial. These differences could be attributed to differences in gut maturity among young and older birds. The sensitivity of young birds to high dietary NSP is associated with their limited synthesis of lipase enzyme and inefficient recycling of bile salts (Sell *et al.*, 1986; Krogdahl and Sell, 1989; Martin and Farrel, 1998), and their incapability to replace lost bile as efficiently as older birds (Serafin and Nesheim, 1970). Thus, the antinutritive effects of dietary NSP from wheat in young birds were ameliorated

more effectively by the phospholipase supplementation via enhancement of lipid digestion than by the xylanase supplementation. In contrast, older birds have a more mature and stable gut ecosystem with greater fermentation capacity than younger birds and they are more tolerant to the effects of NSP (Choct and Annison, 1992b; Veldman and Vahl, 1994). The stability of the microflora in older birds may come from acclimatization of the digestive system to the diet through changes in the type and number of microorganism (Petersen *et al.*, 1999). A greater variability between birds is found in the numbers and types of microorganisms in young birds than in older birds (Annison, 1989). As discussed by Ferket (1991), the gut ecosystem becomes more resistant to change as the number of microbes increase. Thus, endoxylanase had a superior effect on older birds compared to phospholipase, because older birds have a more mature gut ecosystem with a greater capacity for lipid digestion than younger birds.

Another possible reason for the lower performance and AMEn shown by the birds fed phospholipase as compared to the ones supplemented with endoxylanase during the growing-finishing phase could be due to the effect of phospholipase on increasing gut viscosity. Phospholipase increases the stability of the emulsion, which is positively correlated to the viscosity of the oil (Jumaa and Muller, 1998; Chung *et al.*, 2001). In our study, we observed significantly higher ileum digesta viscosity of toms fed wheat-based diets supplemented with phospholipase than the other treatments at 8 weeks of age. However, no differences in the ileum digesta viscosity were observed between control and endoxylanase treatments. Similar results have been seen by other investigators who observed no decrease in digesta viscosity but an increase in performance and nutrient utilization from endoxylanase supplementation as compared to birds fed a NSP-rich diet without enzyme supplementation (Silva and Smithard, 2002; Veldman and Vahl, 1994).

Regardless of the dietary treatment, AMEn increased as the birds aged. These findings are in substantial agreement with many other authors that have observed higher dietary energy digestibility among older birds than younger ones (Salih *et al.*, 1991; Scott and Boldaji, 1997; Fuente *et al.*, 1998), indicating that older birds utilize cereal-based diets better. Fuente *et al.* (1998) reported that the AME of 30-day-old chickens was 4.6% higher than that of 10-day-old chicks. Salih *et al.* (1991) also showed that negative effects of high dietary levels of barley decreased as the broilers grew older. Jaroni *et al.* (1999) observed better fat utilization at 60 wk of age than at 50 wk of age in laying hens supplemented with wheat middlings. Brenes (1992) explained that younger birds are more sensitive to the negative effects of antinutritional factors in cereal and other raw material due to the immaturity of their digestive tract.

The increasing trend in AMEn as birds aged was most consistent among birds fed diets supplemented with Natugrain Blend and Lyxasan-100. The phospholipase-supplemented and control diets had highly variable AMEn values throughout the trial (Fig. 1). The AMEn observed in the phospholipase and control treatments deviated significantly from the other treatments at 12 weeks of age, which coincided with the change from diet 3 (65.66% of wheat) to diet 4 (73.54% of wheat). This observation demonstrated that dietary endoxylanase supplementation increased the bird's tolerance to high levels of wheat by hydrolyzing the xylan backbone of arabinoxylan. Similarly, Choct *et al.* (1995) reported that enzyme supplementation reduced the variability in nutrient utilization in diets with different levels of NSP. They observed that endoxylanase significantly improved the nutritive value of a diet containing a low-AME, high NSP wheat. Thus, endoxylanase addition not only improved energy utilization, but it also led to a more consistent and uniform nutrient utilization.

The dietary enzyme supplementation treatments used in this study reduced the amount of caked litter accumulation in the pens in comparison to the control treatment. This observation could be attributed to a reduction in osmotically active compounds in the gut because of improved digestion (Choct and Annison, 1992a; Choct *et al.*, 1996). This result agreed with the results of other investigators who reported improved litter quality by the use of dietary enzyme supplementation (Veldman and Vahl, 1994; Fischer and Classen, 2000), and reduced the incidence of health problems associated with poor litter quality. Such problems include pododermatitis, leg mobility abnormalities, and respiratory problems, which could lead to increased mortality rate.

In conclusion, the use of appropriate enzymes is an effective way to deal with grains with high NSP content in poultry diets. Growth performance and energy utilization of turkeys fed wheat-based diets can be significantly enhanced by phospholipase supplementation of starter feeds and endoxylanase (Lyxasan-100) supplementation of growing and finishing feeds; however, a natural blend of enzymes (Natugrain Blend) may provide a positive response regardless of turkey age. Evidently, phospholipase alleviated the adverse effect of dietary NSP by improving fat digestion and absorption in young turkeys, whereas endoxylanase is more effective in older birds that have greater digestive capacity and more mature gut microbial ecosystem. However, further studies are required to better elucidate the phospholipase mechanism of action. Future investigations with phospholipase in NSP-rich diets fed to broiler are also warranted.

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[1]BASF AG, 67059 Ludwigshafen, Germany.
[2]Heat Lamp, Model # 54411-Heave Gauge Aluminum Base, Hog Slats, Inc., Newton Grove, NC, 28366.
[3]125-watt bulb, SLI, China; Distributor: Hog Slats, Inc., Newton Grove, NC, 28366.
[4]British United Turkeys of America.
[5]Goldsboro Milling Co., Goldsboro, NC, USA.
[6]Pressure Sprayer 1 ½ quart, Delta Industries, North Hollywood, CA.
[7]Celite™, A diatomite product, Food Chemicals Codex Grade. Celite Corp., Lompar, CA 93436.
[8]National Wing Bands - Style 898, National Band & Tag Co., Newport, KY.
[9]Microcentrifuge Micro 13, Fisher Scientific, Pittsburgh, PA.
[10]Brookfield Engineering Laboratories Inc., Stoughton, MA.
[11]Brookfield Digital Viscosimeter, Model DV-II+ Version 2.0, Operating Instructions Manual No. M/92-161-F1193, Brookfield Engineering Laboratories Inc., Stoughton, MA.
[12]Ziploc Gallon Bags, Johnson & Son, Inc., Racine, WI.
[13]Waring Commercial Laboratory Blender, Model # 31BL91-7010, Torrington, CT.
[14]Blue-M, Model # DC-326F, Serial # DC-509, Blue M, Atlanta, GA.
[15]IKA Calorimeter System C5000 control, IKA® Werke Labortechnik, Staufen, Germany.
[16]KJELTEC Auto 1030 Analyzer, Tecator, Sweden.