

Influence of meat and bone meal, phytase, and antibiotics on broiler chickens challenged with subclinical necrotic enteritis: 1. growth performance, intestinal pH, apparent ileal digestibility, cecal microbiota, and tibial mineralization

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ABSTRACT This study investigated the influence of meat and bone meal (MBM), phytase, and antibiotics (AB) on the performance, intestinal pH, ileal digestibility, cecal microbiota, and tibial mineralization in Ross 308 broilers challenged with necrotic enteritis (NE). A total of 672-day-old male Ross 308 chicks were allocated to 8 treatments with 6 replicate pens, with 14 birds each. The study employed a 2 × 2 × 2 factorial arrangement of treatments: MBM (no or yes), AB (no or yes, zinc bacitracin + salinomycin), and phytase level (500 or 1,500 FTU/kg; both using 500 matrix recommendations). Diets were based on wheat–soybean meal–canola meal. All birds were challenged with *Eimeria* spp on day 9 and *Clostridium perfringens* (*C. perfringens*) strain EHE-NE18 on day 14 and day 15. On day 21 (postchallenge), birds fed MBM had reduced weight gain (WG; $P < 0.05$) relative to without MBM. A 2-way phytase × AB interaction for WG on

day 14 ($P < 0.001$) and day 21 ($P < 0.001$) and feed conversion ratio on day 21 ($P < 0.001$) and day 42 ($P < 0.01$) indicated positive effects of high phytase on bird performance in the presence of AB. On day 42, a 3-way MBM × phytase × AB interaction ($P < 0.01$) was observed for WG, showing high phytase increased WG with AB, relative to the birds without AB in the presence of MBM. A 2-way MBM × phytase interaction ($P < 0.01$) was observed for apparent ileal digestibility of Ca and P on day 16, whereby there was a notable reduction in Ca and P digestibility in birds fed MBM-free diets and a low phytase level, but with the high phytase level, Ca and P digestibility was not influenced by MBM. In conclusion, in NE challenged birds, high phytase has a beneficial effect on leg health and mineral utilization to the extent that it can replace MBM and has beneficial effects on bird performance in the presence of AB.

Key words: antibiotics, growth performance, indigestible protein, necrotic enteritis, bone mineralization

2020 Poultry Science 99:1540–1550

<https://doi.org/10.1016/j.psj.2019.11.021>

INTRODUCTION

Enteric diseases, such as necrotic enteritis (NE), are a major challenge faced by the poultry industry globally. The subclinical form of NE is more financially devastating than the acute form because of a lack of obvious symptoms, resulting in delayed manifestation and effective treatment, loss in flock performance, and reduced feed efficiency (Lovland and Kaldhusdal, 2001). The primary measure used to combat subclinical NE is the use of in-feed antibiotics (AB). However, in many countries,

for example, those within the European Union, the routine feeding of AB is banned (Castanon, 2007), and in other countries, voluntary withdraw of AB from animal feed has been in action. Therefore, occurrences of NE cases in the field have been increased.

Meat and bone meal (MBM) provides a valuable source of protein, calcium, and available phosphorus for broiler diets (Drewyor and Waldroup, 2000; Sulabo and Stein, 2013; Anwar et al., 2016) with levels above 50 g/kg, minimizing the need to incorporate inorganic P into diets, thus reducing feed cost. However, feeding high levels of MBM to poultry has been suggested to trigger the onset of NE (Wilkie et al., 2005). The usefulness of MBM in diets devoid of AB needs to be examined. It is possible that a proportion of the protein in MBM is not readily digested, as MBM contains structural proteins such as keratin, elastin, and collagen that are easily denatured during heat processing (Onifade et al., 1998).

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Received September 18, 2019.

Accepted November 15, 2019.

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The presence of undigested protein in the hindgut may instigate NE, by fueling pathogenic bacteria, with increased production of nitrogenous bacterial metabolites, including amines and ammonia, with increased pH favoring the proliferation of *Clostridium perfringens* (*C. perfringens*) as examined by Sharma et al. (2017).

Another limiting factor of MBM utilization in poultry diets is its high Ca content that may increase gastrointestinal pH and thus interfere with amino acid and mineral utilization (Paiva et al., 2014). High concentrations of dietary Ca increase the chance of amino acids and P remaining chemically bound to phytic acid thus reducing their digestibility (Shirley and Parsons, 2001; Yan et al., 2005; Plumstead et al., 2008). Further, high concentrations of Ca have adverse effects on digestibility of other nutrients such as fat and energy (Paiva et al., 2013). Also, Ca has a high acid-binding capacity and increases digesta pH which reduces the solubility and degradation of phytate (Kim et al., 2018) in the gastric environment and decreases pepsin efficiency which leads to increased levels of undigested protein in the distal gut (Walk, 2016). High dietary Ca has also been directly implicated in the pathogenesis of NE, as it is required for α -toxin activity (Williams, 2005).

The use of dietary exogenous phytase, especially at high doses, may reduce the nutritional need or economic advantage of MBM as a source of protein, P, and Ca. Phytase reduces the amount of inorganic phosphorus and calcium required in poultry diets and improves feed conversion ratio (FCR) and amino acid digestibility. By virtue of allowing for reduced Ca levels to be fed, protein utilization is enhanced in the gastrointestinal tract (Walk et al., 2012; Gautier et al., 2017; Leyva-Jimenez et al., 2018; Fan et al., 2019). Improved weight gain (WG) following supplementation of phytase to birds fed low Ca and P diets as compared with those fed higher Ca and P has been reported recently (Delezie et al., 2015). The objective of this study was to investigate the hypothesis that MBM predisposes broilers to NE and that superdosing phytase (using a higher dosage above standard commercial usage) enhances growth performance, apparent ileal nutrient digestibility (AID), and tibial mineralization, partially through manipulating intestinal pH and ceca microbiota in NE-challenged birds fed MBM-free diets compared with those fed diets containing MBM.

MATERIALS AND METHODS

Birds and Management

All experimental procedures were reviewed and approved by the University of New England's Animal Ethics Committee (AEC17-009). A total of 672 one-day-old Ross 308 male broiler chicks were obtained from Darwalla poultry hatchery (Mt. Cotton Queensland 4,169, Australia). On arrival, chicks of similar weight (average weight of 45 g) were weighed and randomly allocated to 8 treatments with each replicated with 6 pens (0.85 m²), housing 14 birds. The chicks were

reared and housed in an environmentally controlled room bedded with fresh softwood shavings (8 cm deep), with *ad libitum* access to feed and water. Each pen was fitted with a single tube feeder (32 cm diameter) and 4 nipple drinkers. The lighting and temperature program for the experimental period followed the breeder's recommendations (Aviagen, 2014). Mortality was recorded daily, and cumulative pen weight and feed intake (FI) were recorded on day 7, day 14, day 21, day 28, day 35, and day 42. However, day 14 and day 35 are not reported in this study.

Dietary Treatment

The crude protein, amino acid, crude fiber, and crude fat component of homogenized samples of MBM, canola meal, soybean, and wheat were analyzed using NIRS (Evonik AminoProx, Frankfurt, Germany) before feed formulation. Eight diets were then formulated in accordance to Ross 308 standard ileal digestibility amino acid and energy specifications (Table 1). The diets were mixed and pelleted using a Palmer PP300SW Pellet Press (Palmer Milling Engineers Plt Ltd., Altin Street, Griffith, NSW, Australia) at 65°C. A 2 × 2 × 2 factorial arrangement of treatments was employed using a completely randomized design. Factors were MBM (no or yes, 6% in starter (S) and 5% in grower (G) and finisher (F)), AB (no or yes, zinc bacitracin, 100 mg/kg in S and 50 mg/kg in G and F, and salinomycin, 60 mg/kg in all phases), phytase (500 or 1,500 FTU/kg) (Quantum Blue; AB Vista, Malborough, UK). The recommended phytase matrix values (AB Vista) for 500 FTU/kg were applied in both the 500 and 1,500 FTU/kg phytase groups. The diets were offered as S (day 0 to day 14), G (day 14 to day 28), and F (day 28 to day 42) phases. The S diets were fed in crumbled form, whereas the G and F were pelleted. Custom-formulated broiler premixes as well as salinomycin (Sacox 120 Huvepharma) were purchased from BEC Feed Solutions P/L, (Brisbane, QLD, Australia). Zinc bacitracin (Albac 150 Zoetis) was purchased from Ridley AgriProducts (Tamworth, NSW, Australia) and phytase (Quantum Blue; AB Vista) was sourced from RCI Industries (Sydney, Australia).

Necrotic Enteritis Challenge

The NE challenge was performed in accordance with reported procedures (Stanley et al., 2014; Wu et al. 2014; Rodgers et al., 2015). As a predisposing factor to increase colonization of *C. perfringens*, all birds were challenged with field strains of *Eimeria acervulina* (5,000 sporulated oocysts) and *Eimeria maxima* (2,500 sporulated oocysts) and *Eimeria brunetti* (2,500 sporulated oocysts) supplied by Eimeria Pty Ltd. (Ringwood, Vic, Australia) in 1 mL of 1% (w/v) sterile saline on day 9 and approximately 10⁸ CFU of *C. perfringens* Strain EHE-NE18 (known to express NetB toxin, (Commonwealth Scientific and Industrial

Table 1. Ingredient and nutrient composition of basal diets (g/kg), as-fed.

Item	Starter (day 0–14)		Grower (day 14–28)		Finisher (day 28–42)	
	Non-MBM-based	MBM-based	Non-MBM-based	MBM-based	Non-MBM-based	MBM-based
Ingredients						
Wheat	612	620	656	657	707	700
Soybean meal	293	257	226	191	181	140
Canola expeller cold press	50	50	75	75	80	80
Meat and bone meal	0	60	0	50	0	50
Canola oil	17	10	16	11	14	13
Limestone	11	0	9	0	8	7
Mono-dicalcium phosphate 21	4.3	0	2.1	0	0	0
Salt	3.0	2.5	1.5	0.9	1	0.4
Sodium bicarbonate	2	2	2	2	2	2
L-lysine HCL	1.9	2	2.2	2.3	2.1	2.4
D,L-methionine	2.1	2.2	1.9	2	1.7	1.8
L-threonine	0.4	0.5	0.7	0.7	0.8	1
Choline chloride 60	0.9	0.9	0.3	0.3	0.1	0.1
Vitamins ¹	0.75	0.75	0.75	0.75	0.75	0.75
Trace minerals ²	1	1	1	1	1	1
Econase XP 25.	0.1	0.1	0.1	0.1	0.1	0.1
TiO ₂	-	-	5	5	-	-
Nutrients						
ME, kcal/kg	3,000	3,000	3,050	3,050	3,100	3,100
Protein, %	22.4	23	20.5	21.50	19.20	19.70
SID, %						
Lysine	1.20	1.20	1.10	1.10	1.00	1.00
Methionine	0.44	0.44	0.42	0.42	0.40	0.40
M + C	0.84	0.84	0.8	0.8	0.76	0.76
Tryptophan	0.19	0.19	0.19	0.19	0.18	0.18
Threonine	0.77	0.77	0.73	0.73	0.70	0.70
Arginine	1.26	1.26	1.19	1.19	1.10	1.10
Isoleucine	0.78	0.78	0.74	0.74	0.69	0.69
Valine	0.92	0.92	0.85	0.85	0.76	0.76
Calcium, %	0.80	0.80	0.70	0.70	0.60	0.60
Available P, %	0.40	0.40	0.35	0.35	0.30	0.30
Sodium, %	0.24	0.24	0.18	0.18	0.16	0.16
Choline, ppm	1,800	1,800	1,450	1,450	1,250	1,250

For each of the columns above, phytase (Quantum Blue 5G) was added at either 0.1 or 0.3 g/kg and AB (zinc bacitracin plus salinomycin) was added at either 0 or 100 mg/kg in S and 0 or 50 mg/kg in G and F for zinc bacitracin and salinomycin was added at either 0 or 60 mg/kg for all the phases. Abbreviation: MBM, meat and bone meal; SID, standardized ileal digestible.

¹Vitamin premix per kg diet: vitamin A, 12 MIU; vitamin D, 5 MIU; vitamin E, 75 mg; vitamin K, 3 mg; nicotinic acid, 55 mg; pantothenic acid, 13 mg; folic acid, 2 mg; riboflavin, 8 mg; cyanocobalamin, 0.016 mg; biotin, 0.25 mg; pyridoxine, 5 mg; thiamine, 3 mg; antioxidant, 50 mg.

²Trace mineral concentrate supplied per kilogram of diet: Cu (sulfate), 16 mg; Fe (sulfate), 40 mg; I (iodide), 1.25 mg; Se (selenate), 0.3 mg; Mn (sulfate and oxide), 120 mg; Zn (sulfate and oxide), 100 mg; cereal-based carrier, 128 mg; mineral oil, 3.75 mg.

Research Organization, Geelong, Australia) on day 14 and day 15.

Performance

The birds and feed were weighed on a pen basis weekly, allowing for the determination of weekly FI, WG, and FCR (feed:gain). The pens were monitored for mortality twice daily, and postmortem examinations were conducted on dead birds throughout the study period. Feed intake and FCR (FI/WG) were corrected for livability by adding any dead bird weight to the pen weight for each period. Livability was calculated weekly as the number of live birds/the number of birds starting \times 100.

Gastrointestinal pH

Immediately posteuthanasia on the day 16, the gizzard, ileum, and ceca were removed intact from 2 birds per pen. A digital pH meter (Mettler-Toledo, UK) with a spear tip piercing pH electrode (Sensorex,

Garden Grove, CA) was directly inserted into the digesta in the lumen of the proximal gizzard (proventricular opening), ileum, and ceca of the same bird, while avoiding direct contact of the pH electrode with the gut wall. The pH was measured and recorded in duplicate. Once all the readings were taken, the probe was rinsed with ultra-pure water (ICW 3000 water purifier for ion chromatograph; Millipore, Burlington, MA). The mean of the 2 readings per site of the tract was then calculated and recorded. The digesta of the ileum and ceca from the 2 birds were sampled to determine the digestibility of nutrients and bacterial quantification, respectively.

Chemical and Energy Analyses

The diets and ileal digesta samples (from 2 birds on day 16 and 28) were analyzed for nitrogen, carbon, gross energy, and mineral contents. The gross energy content of the diets and digesta samples were determined on a 0.5 g sample using an adiabatic bomb calorimeter (IKA Werke, C7000, GMBH and Co., Staufen, Germany)

with benzoic acid as standard. The nitrogen and carbon content of the diets and digesta samples were determined on a 0.25 g sample with a combustion analyzer (Leco model FP- 2000 N analyzer; Leco Corp., St. Joseph, MI) using EDTA as a calibration standard, with crude protein calculated by multiplying percentage N by a correction factor (6.25). Mineral content was determined using an inductively coupled plasma–optical emission spectroscopy following digestion in concentrated HNO₃.

Titanium Dioxide Analysis

The spectrophotometric method described by Short et al. (1996) was followed to measure TiO₂ concentration in the diet and ileal digesta samples. Titanium dioxide concentrations were determined in triplicate for diets and duplicate for digesta samples. Approximately 0.1 g of the freeze-dried digesta and 0.2 g diet samples were weighed in porcelain crucibles and ashed at 580°C for 13 h. Upon cooling, 5 mL of 7.4 mol H₂SO₄ was added to the samples and then boiled on a hotplate at 200°C for 30 min and another 30 min at 250°C to dissolve completely. The solutions were cooled at room temperature, and 5 mL of Milli-Q H₂O was added before filtering (Whatman 541, hardened, ashless, 90 mm, Whatman International Ltd. Maidstone, UK) into 50 mL volumetric flasks. Then 10 mL H₂O₂ (30% v/v) was added to each flask and the mixture adjusted to 50 mL with Milli-Q H₂O and mixed thoroughly. The absorbance of the solutions and of prepared standards were determined at 410 nm using a Hitachi 150-20 UV spectrophotometer (Hitachi Science Systems Ltd., Ibaraki, Japan). The TiO₂ content was calculated from the standard curve.

Digestibility Calculation

The apparent ileal digestibility (day 16) of energy, crude protein, carbon, and minerals (Ca and P) was calculated based on the indigestible marker using the following formula:

$$\text{Apparent ileal digestibility(AID)(\%)} \\ = \{1 - [TiO_2 \text{ diet}(\%) / TiO_2 \text{ digesta}(\%)] \\ \times [\text{digesta nutrient}(\%) / \text{diet nutrient}(\%)]\} \times 100$$

Cecal DNA Extraction and Quantification of Cecal Bacteria

The DNA of cecal content collected on day 16 was extracted using the method described by Kheravii et al. (2017). The quantitative real-time PCR was used to quantify relative amounts of bacterial groups present in cecal contents expressed as log₁₀ genomic DNA copies per gram of cecal contents of *Bifidobacterium* spp., *Lactobacillus* spp., *Bacillus* spp., *Ruminococcus* spp., *Bacteroides* spp., total anaerobic

bacteria, and *C. perfringens* following the procedures of Kheravii et al. (2017). However only *Bacillus* spp and *C. perfringens* have been reported in this study as the rest were not significantly affected. The specific 16S rRNA primers used for the quantification of different groups of bacteria are illustrated in Table 2.

Tibial Ash, Weight (% Body Weight), Breaking Strength, and Mineral Concentration

On day 42 posthatch, 2 birds per pen of average body weight were euthanized, and the tibiae collected from the right leg of each bird. Samples were stored at -20°C pending analysis. For the determination of breaking strength (BS), the bones were defleshed by hand using a scalpel. The resulting tibiae were then subjected to a universal texture analyzer (LX 300 Instron, Norwood, MA) set up with a 30 KN load cell and 3-point fixture bed at a test speed of 10 points of data per second to determine BS. The force was applied to the midpoint of each tibia, with a 2 cm distance between fixed points supporting either end of the bone. The tibiae were then dried at 100°C for 24 h, weighed, and ashed at 600°C overnight for the determination of ash content. For the determination of mineral concentration, approximately 1 g of the ashed and homogenized tibial samples were microwave digested using an Ultrawave Microwave Digestion system (Milestone, Italy) with nitric acid and analyzed for Ca and P using inductively coupled plasma optical emission spectrometry (Agilent ICP-OES, Sydney, Australia).

Statistical Analysis of Data

The data were analyzed as a 2 × 2 × 2 factorial arrangement of treatments using the PROC General Linear Models (GLM) procedure of SAS 9.3 package (SAS Institute Inc., 2010) to assess the main effects and 2-way or 3-way interactions, with the factors as MBM (no or yes) phytase (500 or 1,500 FTU/kg) and AB (no or yes). Tukey's mean separation test was used to make pairwise comparisons between treatment means (*P* < 0.05). The SAS statistical package (PROC NPAR1WAY WILCOXON) was used to test and confirm normality for livability, tibia (% BW) before analysis.

RESULTS

Performance

On day 7 (data not shown), birds fed MBM had greater WG compared with those not fed MBM (*P* < 0.05). Growth performance results for 0 to 14 D and 0 to 21 D, 0 to 28 D and 0 to 42 D are presented in Tables 3, 4, and 5, respectively. From 0 to 14 D (Table 3), 2-way phytase × AB interactions were detected for WG (*P* < 0.001) and FI (*P* < 0.01). In the high phytase groups, WG increased in birds fed AB compared with those without AB, whereas with low phytase, the

Table 2. The sequence of primers used for the qPCR analysis of selected microbial populations in cecal digesta samples at day 16.

Target group or organism	Primer sequence (5'-3')	Annealing T ⁰ (C°)	Reference
<i>Bacillus</i> spp.	F-GCAACGAGCGCAACCCTTGA R-TCATCCCCACCTTCCGGT	63	Zhang, et al. (2015)
<i>C. perfringens</i>	F-ATGCAAGTCGAGCGAKG R-TATGCGGTATTAATCTYCCTTT Probe-5'-FAM-TCATCATTCAACCAA- AGGAGCAATCC-TAMRA-3	60	Rinttila, et al. (2004)

presence of AB did not affect WG or FI. Similarly, only in the birds offered high phytase, did presences of AB increase FI. As a main effect, the inclusion of AB resulted in lower FCR ($P < 0.05$). Table 4 shows 0 to 21 D results. Two way phytase \times AB interactions were detected for WG ($P < 0.001$), FCR ($P < 0.001$), and FI ($P < 0.05$). Birds fed diets without AB had higher WG when fed the low level of phytase compared with the high level of phytase, but with AB, WG was unchanged by phytase level. Only in birds fed the high phytase level did AB decrease FCR. In the high level phytase group, AB supplementation decreased intake, whereas in low level phytase group, presence of AB increased intake. As a main effect, MBM decreased WG ($P < 0.05$). From 0 to 28 D and 0 to 42 D, 3-way interactions were observed for WG ($P < 0.01$ and $P < 0.01$), respectively. The lowest WG was only observed in birds fed diets with MBM, high level of phytase, and without AB. Three-way interactions were observed for FCR from 0 to 28 D ($P < 0.05$). Similarly, the highest FCR was only observed in the birds

fed diets with MBM, high level of phytase, and without AB. Finally, from 0 to 42 D (Table 5), 2-way phytase \times AB interactions were detected for FCR ($P < 0.01$) and FI ($P < 0.01$). Only in birds fed high phytase was lower FCR observed as a result of AB inclusion. Only in birds fed high phytase was lower FI observed in birds fed without the inclusion of AB. Livability was increased as a result of AB inclusion (main effect) from 0 to 21 D ($P < 0.001$), 0 to 28 D ($P < 0.001$), and 0 to 42 D ($P < 0.01$).

Nutrient Digestibility, Day 16 and 28 Posthatch

Table 6 shows that interactions between MBM and phytase were observed for AID of Ca ($P < 0.01$) and P ($P < 0.05$) on day 16 (samples collected after challenge). On day 16, birds fed the lower level of phytase had higher Ca and P AID only when MBM was included in the diet. On day 28, birds fed the high level of phytase had lower

Table 3. Effect of meat and bone meal, phytase, and antibiotics levels on the performance of broilers.

Effects	MBM	Phy	AB	0–14 D				0–21 D			
				WG g	FCR	FI g	Livability %	WG g	FCR	FI g	Livability %
2-way interactions											
Phy*AB											
		500	-	417 ^b	1.224	510 ^a	98	624 ^b	1.609 ^a	1,003 ^c	88
		500	+	421 ^b	1.212	508 ^a	99	687 ^a	1.663 ^a	1,138 ^a	99
		1,500	-	371 ^c	1.248	463 ^b	99	538 ^c	2.018 ^c	1,083 ^b	88
		1,500	+	441 ^a	1.157	510 ^a	98	698 ^a	1.489 ^a	1,035 ^a	97
Main effects											
MBM											
	-			407	1.210	491	98.2	651 ^a	1.690	1,086	93
	+			418	1.211	505	99.1	624 ^b	1.700	1,044	93
Phy											
		500		419	1.218	509	98.8	656	1.636	1,071	93
		1,500		406	1.203	486	98.5	618	1.754	1,059	92
AB											
			-	394	1.236 ^a	486	99	581	1.814	1,043	88 ^b
			+	431	1.185 ^b	509	99	693	1.576	1,087	98 ^a
SEM											
P > f											
MBM											
				0.095	0.975	0.100	0.396	0.041	0.889	0.325	0.898
Phy											
				0.049	0.521	0.010	0.776	0.001	0.104	0.787	0.701
AB											
				<0.001	0.034	0.010	0.776	<0.001	0.002	0.306	0.001
MBM \times Phy											
				0.718	0.735	0.936	0.396	0.120	0.429	0.808	0.372
MBM \times AB											
				0.487	0.987	0.576	0.160	0.054	0.867	0.417	0.372
Phy \times AB											
				<0.001	0.096	0.006	0.396	0.001	0.001	0.036	0.372
MBM \times Phy \times AB											
				0.068	0.433	0.441	0.776	0.803	0.571	0.614	0.898

^{a,b,c}means in the same column within the main effect, 2-way interaction or means of treatment with different superscripts are different ($P < 0.05$).

AB = Salinomycin 60 ppm in S, G, F; Zn bacitracin 100 ppm in S, G and 50 ppm in F.

Phy = Phytase (Quantum Blue 5G).

2-way or 3-way interaction separated by Tukey.

Abbreviations: AB, antibiotics; FCR, feed conversion ratio; FI, feed intake; MBM, meat and bone meal; WG, weight gain.

Table 4. Effect of meat and bone meal, phytase, and antibiotics levels on the performance of broilers from 0 to 28 D.

Effects	MBM	Phy	AB	WG g	FCR	FI g	Livability %
Treatment							
1	–	500	–	1,398 ^a	1.461 ^b	2,042	89
2	–	1,500	–	1,190 ^{b,c}	1.610 ^{a,b}	1,916	84
3	–	500	+	1,430 ^a	1.470 ^b	2,099	97
4	–	1,500	+	1,398 ^a	1.448 ^b	2,020	92
5	+	500	–	1,381 ^a	1.445 ^b	1,993	79
6	+	1,500	–	1,051 ^c	1.780 ^a	1,867	83
7	+	500	+	1,319 ^{a,b}	1.581 ^{a,b}	2,079	97
8	+	1,500	+	1,442 ^a	1.358 ^b	1,955	97
Main effects							
AB			–	1,255	1.574	1,954	84 ^b
			+	1,397	1.464	2,038	96 ^a
SEM				21.8	0.026	28.00	1.66
<i>P</i> > <i>f</i>							
MBM				0.033	0.286	0.423	0.527
Phy				0.033	0.154	0.052	0.704
AB				<0.001	0.011	0.147	0.001
MBM × Phy				0.751	0.928	0.845	0.258
MBM × AB				0.377	0.418	0.960	0.168
Phy × AB				<0.001	<0.001	0.830	0.704
MBM × Phy × AB				0.009	0.023	0.845	0.704

^{a,b,c} means in the same column within a main effect, 2-way interaction or means of treatment with different superscripts are different (*P* < 0.05).

AB = Salinomycin 60 ppm in S, G, F; Zn bacitracin 100 ppm in S, G and 50 ppm in F.

Phy = Phytase (Quantum Blue 5G).

2-way or 3-way interaction separated by Tukey.

Abbreviations: AB, antibiotics; FCR, feed conversion ratio; FI, feed intake; MBM, meat and bone meal; WG, weight gain.

AID of carbon (*P* < 0.05) and energy (*P* < 0.05) but increased AID of P (*P* < 0.01) as compared with those fed the low level of phytase. On day 28, birds fed

MBM had decreased AID of Ca (*P* < 0.001), whereas those fed diets with AB had increased AID of Ca digestibility (*P* < 0.05).

Table 5. Effect of meat and bone meal, phytase, and antibiotics levels on the performance of broilers from 0 to 42 D.

Effects	MBM	Phy	AB	WG g	FCR	FI g	Livability %
Treatment							
1	–	500	–	2,762 ^a	1.559	4,306	85
2	–	1,500	–	2,451 ^b	1.588	3,894	85
3	–	500	+	2,744 ^a	1.550	4,254	96
4	–	1,500	+	2,713 ^a	1.527	4,142	88
5	+	500	–	2,769 ^a	1.517	4,200	76
6	+	1,500	–	2,186 ^c	1.657	3,619	80
7	+	500	+	2,665 ^{a,b}	1.555	4,138	92
8	+	1,500	+	2,816 ^a	1.512	4,260	89
2-way interactions							
Phy*AB							
		500	–	2,766	1.538 ^b	4,253 ^a	80
		500	+	2,704	1.553 ^{a,b}	4,196 ^a	94
		1,500	–	2,319	1.623 ^a	3,757 ^b	83
		1,500	+	2,765	1.519 ^b	4,201 ^a	89
Main effects							
AB			–	2,542	1.580	4,005	81 ^b
			+	2,734	1.536	4,198	91 ^b
SEM				33.6	0.012	46.4	1.65
<i>P</i> > <i>f</i>							
MBM				0.122	0.837	0.215	0.227
Phy				<0.001	0.233	0.002	0.626
AB				<0.001	0.042	0.014	0.003
MBM × Phy				0.552	0.285	0.827	0.466
MBM × AB				0.066	0.660	0.208	0.332
Phy × AB				<0.001	0.008	0.002	0.227
MBM × Phy × AB				0.004	0.129	0.187	1.000

^{a,b,c} means in the same column within a main effect, 2-way interaction or treatment means with different superscripts are different (*P* < 0.05).

AB = Salinomycin 60 ppm in S, G, F; Zn bacitracin 100 ppm in S, G and 50 ppm in F.

Phy = Phytase (Quantum Blue 5G).

2-way or 3-way interaction separated by Tukey.

Abbreviations: AB, antibiotics; FCR, feed conversion ratio; FI, feed intake; MBM, meat and bone meal; WG, weight gain.

Gastrointestinal pH and Cecal Microflora, Day 16 Posthatch

Dietary inclusion of MBM increased the ileal ($P < 0.01$) and cecal pH ($P < 0.05$) with no interactions as described in Table 7. A phytase \times AB interaction was detected for \log_{10} genomic DNA copies per g of cecal contents for *Bacillus* spp. ($P < 0.05$). Copy numbers were higher in birds fed high phytase with AB compared with those without AB, whereas the result was opposite in birds fed low phytase as shown in Table 7. Dietary inclusion of MBM had a tendency ($P = 0.059$) to increase copy numbers for *C. perfringens* in cecal contents.

Tibial Ash, Weight (% Body Weight), Breaking Strength, and Mineral Concentration, Day 42 Posthatch

Table 8 shows that inclusion of MBM (main effect) increased tibial ash concentration ($P < 0.01$), relative tibial weight ($P < 0.01$), and tibial BS ($P < 0.001$). A phytase \times AB interaction was detected for relative tibial weight showing lighter tibia in birds fed low phytase without AB compared with other treatments ($P < 0.05$). Table 8 indicates the presence of MBM \times phytase interactions for K ($P < 0.05$), Mn ($P < 0.01$), and Zn ($P < 0.01$) concentrations in tibia. Only in birds fed diets with MBM was tibial K concentration lower as a result of the high dose of phytase. Only in birds fed diets without MBM was tibial Mn and tibia Zn lower in birds fed the lower dose of phytase. Inclusion of meat and bone meal as a main effect

decreased tibial Fe concentrations ($P < 0.05$). Inclusion of AB as a main effect decreased tibial Mn uptake ($P < 0.05$).

DISCUSSION

Performance

The objective of this study was to determine the effect of MBM on the outcome of the NE challenge and to determine whether phytase supplementation would have benefits, especially in diets without MBM. Meat and bone meal has the potential to induce the onset of NE, but there is little reported research in this regard (Paiva et al., 2014). The results of this study confirmed that MBM exacerbates NE. While birds fed MBM had increased performance before challenge with NE, reduced growth performance was observed for those fed MBM and no AB after challenge. This agrees with reports of McDevitt et al. (2007) and M'Sadeq et al. (2015) suggesting that MBM may predispose chickens to NE. Some of the factors in MBM that might potentiate the onset of the disease could be indigestible proteins and high Ca levels both of which could increase pH in the hindgut and favor growth of pathogenic *C. perfringens* as was observed in the present study. Increasing dietary animal proteins or MBM have been reported to increase the pH in the upper part of the intestinal tract (Rinttilä and Apajalahti 2013; Stanley et al., 2014), as was also observed in this study. The fermentation of undigested protein in the hindgut produces by-products such as amines and ammonia that encourage the proliferation

Table 6. Effect of meat and bone meal, phytase, and antibiotics levels on apparent ileal digestibility coefficients of CP, carbon, Ca, P, and energy of broiler chickens, day 16 and day 28.

Effects	MBM	Phy	AB	CP day 16	CP day 28	C day 16	C day 28	Ca day 16	Ca day 28	P day 16	P day 28	Energy (MJ/g) day 28
2-way interactions												
MBM*Phy	-	500		0.65	0.81	0.40	0.69	0.55 ^b	0.65	0.61 ^b	0.68	0.69
	-	1,500		0.71	0.78	0.53	0.65	0.67 ^{a,b}	0.64	0.76 ^a	0.79	0.66
	+	500		0.65	0.81	0.47	0.70	0.76 ^a	0.57	0.79 ^a	0.67	0.71
	+	1,500		0.63	0.80	0.40	0.66	0.64 ^{a,b}	0.47	0.76 ^a	0.74	0.67
Main effects												
MBM	-			0.68	0.80	0.47	0.67	0.61 ^b	0.65 ^a	0.68	0.73	0.67
	+			0.64	0.80	0.43	0.68	0.70 ^a	0.52 ^b	0.77	0.70	0.69
Phy		500		0.65	0.81	0.44	0.69 ^a	0.65	0.61	0.70	0.67 ^b	0.70 ^a
		1,500		0.67	0.79	0.46	0.65 ^b	0.66	0.56	0.76	0.77 ^a	0.67 ^b
AB			-	0.62	0.79	0.45	0.67	0.69	0.55 ^b	0.74	0.70	0.69
			+	0.70	0.80	0.46	0.76	0.63	0.62 ^a	0.72	0.74	0.69
SEM				0.02	0.01	0.03	0.01	0.02	0.01	0.02	0.01	0.01
$P > f$												
MBM				0.343	0.598	0.620	0.665	0.012	0.000	0.010	0.246	0.326
Phy				0.521	0.135	0.689	0.029	0.925	0.132	0.062	0.000	0.040
AB				0.063	0.362	0.894	0.812	0.091	0.037	0.540	0.222	0.587
MBM \times Phy				0.315	0.365	0.123	0.862	0.002	0.193	0.011	0.373	0.982
MBM \times AB				0.932	0.064	0.552	0.394	0.196	0.148	0.668	0.300	0.270
Phy \times AB				0.924	0.374	0.737	0.953	0.657	0.945	0.256	0.929	0.790
MBM \times Phy \times AB				0.783	0.109	0.298	0.857	0.231	0.731	0.152	0.632	0.893

^{a,b} means in the same column within a main effect, 2-way interaction or treatment means with different superscripts are different ($P < 0.05$).

AB = Salinomycin 60 ppm in S, G, F; Zn bacitracin 100 ppm in S, G and 50 ppm in F.

Phy = Phytase (Quantum Blue 5G).

2 or 3-way interaction separated by Tukey.

Abbreviations: AB, antibiotics; MBM, meat and bone meal.

Table 7. Effect of meat and bone meal, phytase, and antibiotics levels on bacterial quantification as log₁₀ genomic DNA copies per gram of cecal contents from broilers on day 16.

Effects	MBM	Phy	AB	Gizzard pH	Ileal pH	Cecal pH	<i>Bacillus</i> spp	<i>C. perfringens</i>
2-way interactions								
Phy*AB								
		500	–	2.87	5.87	6.41	8.34 ^a	11.73
		500	+	2.73	5.76	6.21	7.82 ^b	11.17
		1,500	–	2.83	5.69	6.40	7.55 ^b	11.57
		1,500	+	2.88	5.69	6.24	8.23 ^a	11.49
Main effects								
MBM								
	–			2.78	5.60 ^b	6.23 ^b	8.16	11.21 ^b
	+			2.87	5.91 ^a	6.40 ^a	7.81	11.77 ^a
SEM								
P > f								
MBM								
Phy								
AB								
MBM × Phy								
MBM × AB								
Phy × AB								
MBM × Phy × AB								

^{a,b} means in the same column within a main effect, 2-way interaction or treatment means with different superscripts are different ($P < 0.05$).

AB = Salinomycin 60 ppm in S, G, F; Zn bacitracin 100 ppm in S, G and 50 ppm in F.

Phy = Phytase (Quantum Blue 5G).

2-way or 3-way interaction separated by Tukey.

Abbreviations: AB, antibiotics; MBM, meat and bone meal.

of pathogenic bacteria (Qaisrani et al., 2015), as was also realized with high *C. perfringens* in this study.

In the weighing after challenge on day 21, there was reduced overall performance as a result of MBM inclusion in the diet with a strong tendency for a MBM × AB interaction showing MBM to have a negative effect on WG

without AB. Before challenge on day 7, MBM increased WG, and on day 14 (post-*Eimeria* and pre-*Clostridium* challenge), there was no effect of MBM on WG. After challenge and overall from 0 to 42 D, the 3-way interactions observed showed that MBM had a negative effect on WG without AB and with the high level of phytase.

Table 8. Effect of meat and bone meal, phytase, and antibiotics levels on the ash, weight (% body weight), breaking strength, K, Fe, Mn, and Zn of tibia, day 42.

Effects	MBM	Phy	AB	Tibial ash (%)	Tibial wt. % body weight	Tibial breaking strength (N)	K %	Fe mg/kg	Mn mg/kg	Zn mg/kg
2-way interactions										
MBM*Phy										
	–	500		44.41	0.48	346	0.79 ^{a,b}	358	15.1 ^b	443 ^b
	–	1,500		43.79	0.51	362	0.82 ^a	373	17.6 ^a	515 ^a
	+	500		45.43	0.53	406	0.87 ^a	349	11.1 ^c	399 ^{b,c}
	+	1,500		45.92	0.54	424	0.76 ^b	335	10.0 ^c	389 ^c
		500	–	45.33	0.47 ^b	370	0.81	358	15.1	443
		500	+	44.51	0.53 ^a	382	0.85	373	17.6	515
		1,500	–	45.19	0.52 ^{a,b}	371	0.77	349	11.1	399
		1,500	+	44.51	0.52 ^{a,b}	414	0.81	335	10.0	389
Main effects										
MBM										
	–			44.10 ^b	0.49 ^b	354 ^b	0.80	366 ^a	16.4	479
	+			45.68 ^a	0.53 ^a	415 ^a	0.82	342 ^b	10.5	394
Phy										
		500		44.92	0.50	376	0.83	353	13.1	421
		1,500		44.85	0.52	393	0.79	354	13.8	452
AB										
			–	45.26	0.50	371	0.79	358	14.2 ^a	429
			+	44.51	0.53	398	0.83	350	12.7 ^b	444
SEM										
P > f										
MBM										
Phy										
AB										
MBM × Phy										
MBM × AB										
Phy × AB										
MBM × Phy × AB										

^{a,b,c} means in the same column within a main effect, 2-way interaction or treatment means with different superscripts are different ($P < 0.05$).

AB = Salinomycin 60 ppm in S, G, F; Zn bacitracin 100 ppm in S, G and 50 ppm in F.

Phy = Phytase (Quantum Blue 5G).

2-way or 3-way interaction separated by Tukey.

Abbreviations: AB, antibiotics; MBM, meat and bone meal.

Thus, the impact of MBM on growth performance during NE and overall might have been aggravated by the addition of high phytase even though there have been many reports of positive effects of phytase on WG, FI, FCR, and mineral digestibility in chickens in unchallenged conditions similar to what was observed in the current study in the birds fed AB. Using high phytase in AB-free diets in clinically challenged birds must be guided as such inclusion might release additional nutrients such as protein, Ca, P, and inositol that may provide nutrients for the growth of pathogenic bacteria. High Ca diets have been implicated to decrease FI and WG (Paiva et al., 2013). Meat and bone meal improved WG prechallenge but had a negative effect in the postchallenge periods on day 21 and day 28 and tended to remain negative on day 35 and day 42. Birds fed the high phytase with AB had the highest WG on day 7 and day 14. However, the high phytase had a negative effect on WG in birds not fed AB both prechallenge and postchallenge, and inclusion of MBM exacerbated the situation. The finding suggests a negative effect of excess Ca reaching the hindgut. It should be noted that in the present study, additional nutrient matrix values were not used for the high level of phytase (1,500 FTU/kg) beyond those recommended for 500 FTU/kg. As such, the additional release of Ca and other nutrients were not accounted for as a result of the higher dose of phytase. These results indicate that the recommended incremental nutrient matrix values should be used when phytase is used at high (superdose) levels. In addition, the treatments with MBM had more Ca than expected. This suggests that more attention should be paid to Ca levels under commercial conditions to gain the greatest benefit from high phytase, especially when the gut challenge is expected.

Intestinal pH and Cecal Microbiota

The pH of the gut is important for nutrient solubility and intestinal microbiota. Destruction of the epithelium as a result of NE challenge is often mentioned in many review papers as a factor that reduces nutrient digestibility. But studies on NE rarely report this. Thus, these data were collected on day 16 and repeated on day 28, and show the receding effects of the challenge.

The tendency for higher pH recorded in the ileum and ceca was likely because of dietary MBM inclusion which likely promoted the growth of *C. perfringens*. Indigestible proteins in MBM entering the ceca might have contributed to this and is similar to that reported by Rinttilä and Apajalahti (2013). The detrimental performance effects observed in birds fed high levels of phytase without AB, as explained above, were also observed in *Bacillus* spp. counts in the ceca. A shift in the cecal microbiome during NE was expected to decrease the population of beneficial bacteria such as *Bacillus* spp. It appears that while high phytase decreased counts of *Bacillus* spp in the absence of AB by perhaps promoting the outgrowth of *C. perfringens* through the nutrient

supply (competitive exclusion), the presence of AB suppressed the counts of the latter. The impact that NE has on the reduction of beneficial bacteria in the gut is known (Yang et al., 2018). The outgrowth of *C. perfringens* in this study because of MBM seems to agree with the results of Ptak et al. (2015) who reported a lower *C. perfringens* population in diets having lower Ca and digestible P.

Nutrient Digestibility

The increased protein digestibility on 16 D in AB fed birds illustrates one of the beneficial actions of AB. This may be because of the thinning of the intestinal wall allowing greater assimilation of nutrients (Huyghebaert et al., 2011). Further, Sharifi et al. (2012) found that AB (flavomycin) significantly reduced the whole intestinal weights of broilers when compared with those fed probiotics. The authors observed an improvement in nutrient retention because of the thinning of the intestinal wall. Reduced intestinal weight increases the activity of digestive enzymes (Dibner et al., 2007). A thin-walled intestine may also spare energy from tissue maintenance that can be used by the host for either growth or improve the digestibility of nutrients (Miles et al., 2006).

The decline in carbon and energy digestibility in relation to high phytase inclusion on day 28 might be because of the damage caused by the exacerbated infection as a result of increased nutrient flow to the ceca with the higher phytase dose (Gehring et al., 2014). The increased Ca digestibility observed on 16 D in birds fed MBM and low phytase is noteworthy. Both MBM and phytase as sources of Ca and P and were expected to release more Ca and P. The increased Ca digestibility might be because of paracellular absorption following the compromise of the tight junction of the gut linings. Calcium absorption in chickens is primarily by a transcellular route at low concentrations of dietary Ca. At higher Ca concentrations as may be the case in this study, the paracellular route is used (Bronner, 2003). The P releasing ability of phytase supplementation at higher concentrations was only realized under the optimum condition on day 28 which is in agreement with several reports (Rutherford et al., 2012; Li et al., 2016; Scholey et al., 2018).

Tibial Mineralization

Bone deformities lead to downgrading or condemnation of carcasses during slaughter, yet studies on NE rarely report the effect of the disease on bones. Thus in the present study, the effect of the treatment on bone was examined on the last day (day 42).

In the present study, tibial ash was increased with the inclusion of MBM. The increase in the ash content, relative weight of tibia and BS of the tibia as a result of MBM inclusion was anticipated and agrees with the results of another study (Liu et al., 2016).

Tibial weight and bone mineralization have also been attributed to the level of Ca, with a low-Ca diet showing the lowest bone weight and ash content (Onyango et al., 2003). An increased bone BS and bone ash of chicks fed diets containing a higher level of Ca compared with lower concentration is also known (Abdulla et al., 2017). Similar to the observation in growth performance, phytase addition increased the weight of tibia, in the presence of AB. It can be argued that birds fed diets with AB (control group) were more healthy and active and as such had higher FI. Bone turnover is strongly associated with FI, being suppressed as a result of feed or nutrient deprivation (Erdal et al., 2012). The current results show that AB, either alone or in synergy with other nutrients, plays a role in the growth and development of bone as was also reported by Ziaie et al. (2011).

It appears that MBM either alone or in combination with phytase mostly decreased the retention of minerals, namely Fe, Mn, and Zn in the tibia. A recent study indicated that MBM at 6% dietary inclusion decreases mineral concentrations in broiler bones (Liu et al., 2016). For example, Mn is an essential cofactor involved in the biosynthesis of chondroitin sulfate (Alghadir et al., 2016). A deficiency in Mn would, therefore, cause a reduction in bone size. Zinc is essential for the conversion of procollagen into collagen, bone calcification, hydroxyapatite crystallization regulation, collagen synthesis, and cellular invasion of the cartilage matrix by the osteoblasts (Osorio et al., 2011). This increased retention of these minerals by AB could be because of increased plasma concentration as a result of a healthy gut for absorption. A progressive reduction in Ca and P digestibility was reported by Paiva et al. (2014) during NE challenge in broilers. They attributed the reduction in Ca and P retention to possible destruction of the intestinal lining during the challenge, which might have impaired nutrient digestibility. These authors had earlier reported the effect of malabsorption of nutrients in challenged birds (Paiva et al., 2013), and thus, in the current study, it appears AB played a role in ameliorating the effect of the challenge by improving nutrient utilization to enhance bone parameters in the presence of MBM.

In summary, MBM reduced WG in NE challenged birds; however, prechallenge MBM increased WG. This study shows that broilers fed diets with MBM under unfavorable health conditions (NE challenge) while superdosing with phytase without using a full matrix value is only beneficial when fed with AB. It can, therefore, be suggested that when NE is expected, AB and a full matrix value for the dose of phytase should be used to minimize excess nutrients in the hindgut that might interact with enteric disease challenge.

ACKNOWLEDGMENTS

The authors hereby acknowledge AB Vista Feed Ingredient, Malborough, UK for funding this research.

They also do acknowledge Brett Ruth of Ruth Consolidated Industries Pty. Ltd. for the input he gave in the design of this study. Also, the University of New England, Armidale (Australia) is acknowledged for providing the international postgraduate research award (IPRA) to the lead author.

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