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Original Research Article

Over-processed meat and bone meal and phytase effects on broilers challenged with subclinical necrotic enteritis: Part 3. Bone mineralization and litter quality

Holy K. Zanu [a], Sarbast K. Kheravii [a], Natalie K. Morgan [a], Michael R. Bedford [b], Robert A. Swick [a, *]

[a] School of Environmental and Rural Science, University of New England, Armidale, NSW, 2351, Australia
[b] AB Vista, Marlborough, Wiltshire, SN8 4AN, United Kingdom

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ABSTRACT

This study was conducted to determine the effect of necrotic enteritis (NE), phytase level and meat and bone meal (MBM) processing on bone mineralization of broilers and litter quality. Ross 308 male broiler chicks ($n = 768$) were allotted to 48 pens with 16 birds each. There were 8 dietary treatments in a 2 × 2 × 2 factorial arrangement. Factors were NE challenge (no or yes), phytase level (500 or 5,000 FTU/kg), and MBM (as-received or over-processed). Half of the birds were challenged with field strains of *Eimeria* spp. at d 9 and 10^8 CFU per mL of *Clostridium perfringens* strain EHE-NE18 on d 14 and 15. The middle toe, tibia and femur of 2 birds per pen were excised at d 16 and 29 for determination of ash, breaking strength (BS) and bone mineralization. At d 42, all were assessed for hock burns and litter was scored and assessed for dry matter (DM). At d 16, challenged birds had lower toe ash ($P < 0.01$), femur ash ($P < 0.001$), tibia ash ($P < 0.001$) and tibial BS ($P < 0.001$) than unchallenged birds. At d 16, challenged birds fed high phytase and over-processed MBM had higher toe Mn than those fed low phytase and as-received MBM. At d 29 unchallenged birds fed high phytase and as-received MBM had a higher toe Mn than those fed over-processed MBM. At d 16, a phytase × MBM interaction was detected for femur Zn concentration ($P < 0.05$), where a higher level of Zn was observed in the high phytase group fed over-processed MBM. At d 16, tibial Ca ($P < 0.05$) and P ($P < 0.05$) were lower in the challenged whereas the femur K ($P < 0.001$), Mn ($P < 0.01$) and Na ($P < 0.001$) were higher in the challenged at d 16. At d 42, challenged birds had higher litter DM ($P = 0.058$) and fewer hock burns than those unchallenged ($P < 0.05$). In conclusion, NE impaired bone traits but high phytase and over-processed MBM increased bone mineral contents. Cases of hock burns may be lower under NE incidences due to lower livability of birds reducing litter wetness.

1. Introduction

The bone is an important protective and supportive framework for animals. Modern-day broilers have been genetically selected for fast growth but not necessarily for bone strength. It is important that birds are fed to allow maximum bone growth to support the weight of muscle (Robison et al., 2015; Kittelsen et al., 2016). The bone is a store of minerals and lipids providing nutrients to the body and produces blood cells that protect the host from infections (Liu et al., 2003; Shim et al., 2012; Koppenol et al., 2019). The major inorganic component of the bone is calcium phosphate, $Ca_3(PO_4)_2$. The calcium phosphate interacts with calcium hydroxide, $Ca(OH)_2$, to form hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] which confers rigidity to the bone (Rath et al., 2000). Therefore, the feeding of mineral-adequate diets, especially Ca and P, is crucial in the development of healthy bones in chickens.

The meat and bone meal (MBM) is an excellent source of Ca and available P and may reduce the need for supplementation with

* Corresponding author.
E-mail address: rswick@une.edu.au (R.A. Swick).
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inorganic P. However, calcium levels in MBM are reported to be variable ranging from 40 to 150 g/kg (Sulabo and Stein, 2013). Waldroup and Adams (1994) reported that P from MBM is as equally available as those from mono-dicalcium phosphate. Apart from its use as a source of Ca and P, MBM also serves as a source of protein and energy in the form of fat. However, MBM proteins are mostly structural (keratin, elastin and collagen) and can easily be denatured during the required heat processing to reduce pathogens (Onifade et al., 1998). Denatured protein is less digestible, and a portion may by-pass into the hindgut where it is metabolized by the microbiota into bacterial metabolites, including amines and ammonia (Qaisrani et al., 2015). These metabolites, in turn, increase the pH in the hindgut and favor the proliferation of *Clostridium perfringens* (Sharma et al., 2017) which causes the onset of necrotic enteritis (NE). But, the effect of NE on bone mineralization is rarely reported in the literature.

It has been well documented that the exogenous phytase improves bone mineralization in chickens and might allow for reductions in Ca and P rich ingredients such as MBM (Qian et al., 1996; Rutherfurd et al., 2012; Kim et al., 2018; Leyva-Jimenez et al., 2018). A substantial part of the poultry skeletal system is made up of P (Ebeledike et al., 2010). However, 55% to 60% of the total P found in soybeans, for instance, is in the form of phytate (myo-inosotol hexakisdihydrogen phosphate [IP6]) (Ravindran et al., 1995). The availability of the P fraction depends on the hydrolysis of IP6. Consequently, phytases (myo-inositol hexaphosphate hydrolases) have been routinely added to feed to catalyze the hydrolysis of IP6 to less-phosphorylated inositol phosphates (IP5, IP4, IP3, IP2, IP1) in addition to myo-inositol and orthophosphates (Selle and Ravindran, 2007; Walk et al., 2014). Hydrolysis of IP6 to inositol radically improves the digestibility of many minerals other than Ca and P due to the removal of the chelating capacity of IP6 and the lower esters. This is of interest since several minerals are involved in bone formation. The role of Mn in collagen and cartilage formation (Pepa and Brandi, 2016), as well as the regulation of cell activity by Zn in the formation of osteoclasts and osteoblasts (Yamaguchi, 2010), might be enhanced through phytase supplementation.

A study was conducted to test the hypothesis that over-processing of MBM beyond those of commercial rendering plants and under pressure would denature proteins to the point of reducing digestibility and thus increase the incidence of NE in NE-challenged birds (confirmed and reported in Part 1 in this series; Zanu et al., 2020a). In addition, it was hypothesized that over-processing would reduce mineral availability needed for bone mineralization and whether the use of phytase at a higher than commercial dose would ameliorate the negative effects of over-processing of MBM. The effects of NE challenge, processing of MBM and phytase level on the litter quality, hock burns and bone mineralization of broilers were examined.

2. Materials and methods

2.1. Birds and management

All experimental procedures were reviewed and approved by the University of New England's Animal Ethics Committee. A total of 768 Ross 308 cockerels were weighed and randomly allocated into 48 floor pens (0.85 m^2) containing 16 birds per pen. Softwood shavings were used as bedding material of about 8 cm deep in each pen. There were 8 treatments with 6 replicates pens per treatment. Each pen was fitted with a single tube feeder (32 cm diameter) and 4 nipple drinkers. The lighting and temperature program during the experimental period followed the Ross 308 guidelines (Aviagen, 2014).

2.2. Diet composition

Eight diets were formulated in accordance with Ross 308 nutrient specifications. Treatments were arranged in a 2 × 2 × 2 factorial arrangement. Factors were NE challenge (no or yes), phytase (500 or 5,000 FTU/kg) (Quantum Blue, AB Vista, Malborough, UK) and MBM (as-received or over-processed). The diets were offered ad libitum throughout the starter (d 0 to 14), grower (d 14 to 28) and finisher (d 28 to 42) phases. All diets were pelleted at 65 °C and the starter diet was crumbled. The diet compositions are reported in Part 1 in this series (Zanu et al., 2020a).

2.3. Meat and bone meal processing

Commercial MBM (Northern Cooperative Meat Company Limited, Casino, NSW, Australia; lot number 48855) was used as received (as-received MBM) or autoclaved at a temperature of 128 °C at 2 bars (1 bar = 101.325 kPa) for 90 min in an autoclave (Hirayama manufacturing corporation, Saitama, Japan). The resulting caked MBM was crushed and milled through a 0.2-mm sieve and designated as over-processed MBM.

2.4. Challenge

The NE challenge was performed in accordance with reported procedures Stanley et al. (2014); Rodgers et al. (2015) Half of the birds (384) were challenged with 5,000 oocysts of field strains of *E. acervulina* and *E. maxima* and 2,500 oocytes of *E. brunetti* (Eimeria Pty Ltd, Australia) on d 9, and 10^8 CFU per mL of *C. perfringens* Strain EHE-NE18 (known to express necrotic enteritis B-like toxin [NetB] toxin, Commonwealth Scientific and Industrial Research Organization, Geelong, Australia) on d 14 and 15. The remaining birds were given buffer instead of *Eimeria* and sterile broth instead of *C. perfringens*.

2.5. Bone dimensions and breaking strength (BS)

At d 16 and 29 post–hatch, 2 birds per pen of average body weight were euthanized by rapid cervical dislocation. Toe (middle), tibia and femur were excised from the right leg of each bird. The tibia and femur were cleaned by hand using a scalpel and the length (mm) (from the tip of the proximal end to the tip of the distal end) and width (mm) (at the medial region of the bone) were measured with a Vernier caliper. The tibia and femur were then subjected to testing for BS (N) on a Lloyd LRX material testing machine (Ametek Lloyd Instrument, Sussex, UK) set up with a 50 N load cell and 3-point fixture bed at a test speed of 10 points of data per second. The Blue Hill 3 software was used to record the data. The force was applied to the midpoint of each tibia and femur with a 2-cm distance between the 2 fixed points supporting the bone.

2.6. Bone ash contents

The toe and remnant of the femur and tibia were dried at 100 °C in a forced-air oven (Watson Victor Ltd Sydney, Australia) for 24 h, weighed, and ashed at 600 °C overnight in a muffle furnace (Carbolite, Sheffield, England) for determination of ash (%).

2.7. Bone mineral contents

For the determination of minerals Ca (%), P (%), K (%), Cu (%), Mg (%), Fe (mg/kg), Mn (mg/kg), Na (mg/kg) and Zn (mg/kg) in the tibia, femur, and toe, approximately 1 g of ash were homogenized and digested in a Milestone Ultrawave Microwave (Milestone Srl, Sorisole, Italy) with nitric acid (HNO_3). Minerals were measured on

inductively coupled plasma emission spectrometer (ICP-OES) (Agilent, Victoria, Australia).

2.8. Litter score and hock burn

The litter in each experimental pen was scored by visual appraisal at d 42 according to the methods of Dersjant-Li et al. (2015). The scoring was done on a scale of 0 to 10, where a score of 0 denotes wet litter and 10 denotes fresh litter as shown below:

0: The whole floor surface is caked from bottom to top of the layer.
1: The whole floor surface is caked. Some parts are caked from bottom to top; some parts are only caked on the upper layer.
2: The whole floor space is caked but the lower layer is friable.
3: >75% of the floor surface is caked.
4: >50% of the floor surface is caked.
5: Litter under the waterline is caked from top to bottom.
6: Upper layer of the litter under the waterline is caked.
7: Litter under the waterline is wet but still friable.
8: Litter under the waterline is damp.
9: The litter is not fresh anymore but still friable and dry everywhere in the pen.
10: Fresh litter.

The hock burns of both feet of all birds in each pen were scored according to the methods of (Welfare Quality, 2009). The average score per bird per pen was calculated. The score was conducted on a scale from 0 to 4 as shown below. The hock burns per pen were calculated as the number of feet with a score/total number of feet × 100.

0: No evidence of hock burn.
1 & 2: Minimal evidence of hock burn.
3 & 4: Evidence of hock burn.

2.9. Litter dry matter (DM)

At d 42, litter samples from 6 different locations in each replicate pen of approximately 1 kg were collected into plastic bags. Each sample was weighed before and after drying in an oven (Carbolite, Sheffield, England) at 105 °C for 168 h (after reaching constant weight) to determine DM content.

2.10. Statistical analyses

The study used a completely randomized design and data were analyzed as a 2 × 2 × 2 factorial arrangement of treatments using Minitab 19 statistical software. Main effects and 2- or 3-way interactions were assessed, with the factors (all fixed factors) as NE (no or yes), phytase (500 or 5,000 FTU/kg) and MBM (as-received or over-processed). Tukey's mean separation test was used to make pairwise comparisons between treatment means ($P < 0.05$). The Box-Cos transformation of the Minitab 19 statistical software was used to test and confirm normality of all the data before analysis. The statistical unit was the average of the pen.

3. Results

3.1. Bone traits, d 16

The challenge as a main effect decreased toe ash ($P < 0.01$), femur length ($P < 0.001$), femur width ($P < 0.001$), femur ash ($P < 0.001$), tibial length ($P < 0.001$), tibial width ($P < 0.001$), tibial ash ($P < 0.001$) and tibial BS ($P < 0.001$) with no interactions (Table 1). The over-processed MBM decreased femur ash ($P < 0.05$) and tended to decrease tibial ash ($P = 0.055$).

There was no significant MBM effect detected for toe ash, femur length, femur width, tibial length, tibial width and tibial BS.

3.2. Bone traits, d 29

The challenge as a main effect decreased femur length ($P < 0.001$), femur width ($P < 0.001$), femur ash ($P < 0.05$), tibial length ($P < 0.001$), tibial width ($P < 0.001$) and tibial BS ($P < 0.05$) with no interactions (Table 2). The challenge increased femur ash ($P < 0.05$).

There is no significant challenge effect detected for toe ash, tibial ash and femur BS.

3.3. Toe mineral concentration, d 16

A 3-way challenge × phytase × MBM interaction was detected for toe Mn ($P < 0.05$). The Mn content was not different in unchallenged birds but in challenged birds fed high phytase with over-processed MBM had higher Mn concentration in toe than birds fed low phytase and as-received MBM (Table 3). A challenge × MBM interaction was detected for toe Fe ($P < 0.05$) where unchallenged birds fed as-received MBM had higher toe Fe concentration than challenged birds fed as-received MBM. There was a strong tendency ($P = 0.051$) for a challenge × MBM interaction for toe Na concentration where the level increased only in challenged birds fed over-processed MBM group. The challenge as a main effect decreased K ($P < 0.001$), Mg ($P < 0.001$), Na ($P < 0.001$) and Zn ($P < 0.01$) concentration in toe with no interactions. High phytase as a main effect decreased Cu content ($P < 0.05$) with no interactions detected ($P > 0.05$). High phytase tended to decrease K ($P = 0.071$) and Mn ($P = 0.065$) contents. The over-processed MBM increased the level of Na ($P < 0.05$).

There was no significant 3-way challenge × phytase × MBM interaction observed for toe Ca, P, K, Cu, Mg, Fe, Na and Zn. There was a no significant challenge × MBM effect detected for toe Ca, P, K, Cu, Mg, Mn, Na, and Zn. There was no significant challenge effect observed for toe Ca, P, Cu and Mn. There was no significant phytase effect observed for toe Ca, P, Mg, Fe, Na, and Zn. There was no significant MBM effect observed for toe Ca, P, K, Cu, Mg, Fe, Mn and Zn.

3.4. Toe mineral concentration, d 29

A 3-way challenge × phytase × MBM interaction was detected for Mn ($P < 0.05$) as shown in Table 4. The unchallenged birds fed high phytase had higher Mn compared to any other treatment when as-received MBM was fed, but lowest compared to any other treatment when over-processed MBM was fed. A challenge × MBM interaction was detected for Mg ($P < 0.05$) and Zn ($P < 0.05$). Challenge had no impact when over-processed MBM was fed, but when as-received MBM was fed Mg was higher in unchallenged birds. The Zn concentration was higher in challenged birds fed over-processed MBM and unchallenged birds fed as-received MBM compared to those on the other treatments. The challenge as a main effect decreased toe Ca ($P < 0.05$), P ($P < 0.01$) and Fe ($P < 0.05$).

There was no significant 3-way challenge × phytase × MBM interaction observed for toe Ca, P, Mg, Fe, and Zn. There was no significant challenge × MBM interaction detected for toe Ca, P, Fe and Mn. There was no significant challenge effect observed for toe Mg, Mn and Zn.

3.5. Tibial mineral concentration, d 16

A challenge × MBM interaction was detected for Mg ($P < 0.05$), in which the challenged birds had lower Mg compared to the

Table 1
Effect of necrotic enteritis (NE), phytase (Phy) and meat and bone meal (MBM) on broiler bone traits, d 16[1].

Item	NE	Phy[2]	MBM	Toe ash, %	Femur length, mm	Femur width, mm	Femur ash, %	Tibial length, mm	Tibial width, mm	Tibial ash, %	Tibial BS, N
Main effects											
NE	−			12.28[a]	42.76[a]	5.63[a]	52.43[a]	55.96[a]	4.53[a]	51.57[a]	135.1[a]
	+			11.32[b]	41.06[b]	5.17[b]	49.56[b]	53.57[b]	4.13[b]	49.21[b]	107.5[b]
MBM			AR	11.77	41.79	5.41	51.35[a]	54.69	4.35	50.68	123.4
			OP	11.84	42.02	5.39	50.65[b]	54.85	4.31	50.10	119.4
SEM				0.69	0.86	0.24	1.32	1.27	0.16	1.01	13.06
P-value											
NE				0.003	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Phy				0.276	0.417	0.693	0.162	0.815	0.131	0.647	0.813
MBM				0.824	0.480	0.834	0.033	0.717	0.570	0.055	0.315
NE × Phy				0.561	0.251	0.094	0.071	0.151	0.284	0.119	0.771
NE × MBM				0.720	0.224	0.493	0.066	0.180	0.152	0.688	0.137
Phy × MBM				0.218	0.254	0.107	0.993	0.188	0.666	0.751	0.545
NE × Phy × MBM				0.097	0.314	0.946	0.174	0.270	0.502	0.313	0.558

BS = breaking strength; AR = as-received; OP = over-processed.
[a, b] Means in the same column with different superscripts are significantly different ($P < 0.05$).
[1] 2- or 3-way interaction separated by Tukey's.
[2] Phy: Quantum Blue 5G.

Table 2
Effect of necrotic enteritis (NE), phytase (Phy) and meat and bone meal (MBM) on broiler bone traits, d 29[1].

Item	NE	Phy[2]	MBM	Toe ash, %	Femur length, mm	Femur width, mm	Femur ash, %	Tibial length, mm	Tibial width, mm	Tibial ash, %	Tibial BS, N	Femur BS, N
Main effect												
NE	−			11.34	61.35[a]	8.23[a]	47.61[b]	80.19[a]	6.49[a]	48.52	253[a]	271
	+			11.67	58.07[b]	7.79[b]	48.77[a]	76.18[b]	6.12[b]	49.05	231[b]	256
SEM				0.53	2.09	0.25	0.79	1.42	0.21	0.74	20.33	16.86
P-value												
NE				0.185	<0.001	<0.001	0.011	<0.001	<0.001	0.178	0.010	0.101
Phy				0.227	0.202	0.793	0.837	0.874	0.804	0.823	0.716	0.619
MBM				0.395	0.328	0.112	0.394	0.478	0.638	0.145	0.648	0.447
NE × Phy				0.431	0.962	0.144	0.357	0.525	0.581	0.078	0.844	0.504
NE × MBM				0.199	0.242	0.613	0.532	0.886	0.297	0.887	0.722	0.522
Phy × MBM				0.846	0.094	0.312	0.981	0.374	0.587	0.937	0.709	0.990
NE × Phy × MBM				0.882	0.897	0.264	0.200	0.437	0.526	0.764	0.738	0.594

BS = breaking strength.
[a, b] Means in the same column with different superscripts are significantly different ($P < 0.05$).
[1] 2- or 3-way interaction separated by Tukey's.
[2] Phy: Quantum Blue 5G.

unchallenged birds fed the over-processed MBM (Table 5). A challenge × MBM interaction was detected for Mn ($P < 0.01$) where the unchallenged birds had lower Mn compared to the challenged birds, and the challenged group Mn was comparatively higher in the birds fed over-processed MBM. The challenge as a main effect decreased the concentration of Ca ($P < 0.05$), P ($P < 0.05$), Na ($P < 0.05$) and Zn ($P < 0.01$) in the tibia. High phytase tended to increase Na ($P = 0.054$).

There was no significant 3-way challenge × phytase × MBM interaction observed for tibial Ca, P, Na and Zn. There was no significant challenge effect observed for tibial Mg and Mn. There was no significant phytase effect detected for tibial Ca, P, Mg, Mn and Zn. There was no significant MBM effect detected for tibial Ca, P, Mg, Na and Zn.

3.6. Tibial mineral concentration, d 29

A 3-way challenge × phytase × MBM interaction was detected for Mn where ($P < 0.05$) in the unchallenged, the highest level was observed in the group fed low phytase and over-processed MBM and the lowest was observed in those fed low phytase and as-received MBM (Table 6). In the challenged, the highest was observed in birds fed low phytase and over-processed MBM. A challenge × MBM was detected for P ($P < 0.05$) and Fe ($P < 0.05$) where concentrations increased in unchallenged birds fed over-processed MBM and challenged birds fed as-received MBM. The challenge as a main effect decreased K ($P < 0.05$) and Zn ($P < 0.01$) concentrations in the tibia. The over-processed MBM as a main effect increased Mg deposition ($P < 0.01$).

There was no significant 3-way challenge × phytase × MBM interaction observed for tibial Ca, P, K, Mg, Mn and Zn. There was no significant challenge × MBM interaction observed for tibial Ca, P, K, Mg, Mn and Zn. There was no significant challenge effect detected for tibial Ca, P, Mg, Fe and Mn. There was no significant MBM effect observed for tibial Ca, P, K, Fe, Mn and Zn.

3.7. Femur mineral concentration, d 16

A challenge × phytase interaction was observed for Fe ($P < 0.05$) where the concentration in challenged birds was similar to that in the unchallenged with low phytase, and there was no effect of phytase within the challenged birds (Table 7). A challenge × phytase interaction was observed for Zn ($P < 0.05$) where low phytase increased the level in unchallenged and challenged groups, and the concentration in birds fed high phytase was higher in the unchallenged than in challenged. A challenge × MBM

Table 3
Effect of necrotic enteritis (NE), phytase (Phy) and meat and bone meal (MBM) on toe mineral concentration, d 16[1].

Item	NE	Phy[2], FTU/kg	MBM	Ca, %	P, %	K, %	Cu, %	Mg, %	Fe, mg/kg	Mn, mg/kg	Na, mg/kg	Zn, mg/kg
Treatments												
1	−	500	AR	34.95	17.58	3.25	29.62	1.21	411	46.84^{ab}	1.01	816
2	−	5,000	AR	35.16	17.60	3.10	28.69	1.22	510	52.27^{ab}	1.05	784
3	−	500	OP	35.40	17.79	3.13	41.78	1.14	393	50.98^{ab}	1.04	729
4	−	5,000	OP	36.09	17.87	2.84	27.93	1.18	402	42.65^{b}	1.03	747
5	+	500	AR	34.76	17.50	2.72	38.07	1.02	309	40.30^{b}	1.17	623
6	+	5,000	AR	35.15	17.73	2.80	29.31	1.01	351	47.01^{ab}	1.10	657
7	+	500	OP	35.25	17.84	2.68	62.32	1.06	374	43.05^{ab}	1.44	641
8	+	5,000	OP	35.05	17.62	2.96	27.79	1.03	390	60.79^{a}	1.23	698
Two-way interaction												
NE × MBM	−		AR	35.05	17.67	3.18	29.15	1.21	461^{a}	49.56	1.03	800
	−		OP	35.75	17.54	2.99	34.85	1.16	397^{ab}	46.82	1.03	738
	+		AR	35.95	17.75	2.76	33.69	1.01	330^{b}	43.65	1.14	640
	+		OP	35.15	17.82	2.82	45.05	1.05	382^{ab}	51.92	1.34	670
Main effects												
NE	−			35.40	17.71	3.08^{a}	32.00	1.19^{a}	429^{a}	48.19	1.03^{b}	769^{a}
	+			35.05	17.68	2.79^{b}	39.37	1.03^{b}	356^{b}	47.79	1.24^{a}	655^{b}
Phy		500		35.09	17.68	3.01	42.95^{a}	1.11	372	48.19	1.17	702
		5,000		35.36	17.71	2.86	28.43^{b}	1.11	413	47.79	1.10	722
MBM			AR	35.00	17.60	2.97	31.42	1.11	395	46.61	1.08^{b}	720
			OP	35.45	17.78	2.90	39.95	1.10	390	49.37	1.19^{a}	703
SEM				0.74	0.40	0.19	20.8	0.08	55.38	6.63	0.07	66.94
P-value												
NE				0.284	0.794	<0.001	0.259	<0.001	0.007	0.889	<0.001	0.002
Phy				0.404	0.821	0.070	0.030	0.915	0.111	0.065	0.189	0.573
MBM				0.171	0.169	0.441	0.193	0.711	0.828	0.336	0.046	0.637
NE × Phy				0.576	0.871	0.466	0.275	0.406	0.629	0.021	0.116	0.440
NE × MBM				0.442	0.622	0.149	0.663	0.146	0.028	0.059	0.051	0.183
Phy × MBM				0.927	0.446	0.133	0.141	0.982	0.267	0.811	0.300	0.593
NE × Phy × MBM				0.411	0.329	0.526	0.621	0.671	0.545	0.035	0.668	0.848

AR = as-received; OP = over-processed.
[a, b] Means in the same column with different superscripts are significantly different ($P < 0.05$).
[1] 2- or 3-way interaction separated by Tukey's.
[2] Phy: Quantum Blue 5G.

Table 4
Effect of necrotic enteritis (NE), phytase (Phy) and meat and bone meal (MBM) on toe mineral concentration, d 29[1].

Item	NE	Phy[2], FTU/kg	MBM	Ca, %	P, %	Mg, %	Fe, mg/kg	Mn, mg/kg	Zn, mg/kg
Treatments									
1	−	500	AR	35.88	17.77	0.98	261	35.87^{b}	558
2	−	5,000	AR	34.54	17.14	0.96	296	50.94^{a}	557
3	−	500	OP	34.85	17.23	0.96	277	37.60^{b}	536
4	−	5,000	OP	34.68	17.13	0.95	244	31.56^{c}	509
5	+	500	AR	33.69	16.52	0.90	236	34.99^{b}	506
6	+	5,000	AR	33.83	16.73	0.92	231	33.25^{b}	519
7	+	500	OP	34.22	16.89	0.94	227	34.56^{b}	531
8	+	5,000	OP	34.39	16.92	0.95	241	42.27^{b}	565
Two-way interactions									
NE × MBM									
	−		AR	35.21	17.46	0.97^{a}	279	43.40	557^{a}
	−		OP	34.76	17.18	0.95^{ab}	261	34.58	522^{b}
	+		AR	33.76	16.63	0.91^{b}	234	34.12	512^{b}
	+		OP	34.30	16.91	0.95^{ab}	234	38.41	548^{a}
Main effects									
NE	−			34.99^{a}	17.32^{a}	0.96	270^{a}	38.99	540
	+			34.03^{b}	16.77^{b}	0.93	234^{b}	36.27	530
SEM				1.18	0.68	0.01	43.59	10.78	31.63
P-value									
NE				0.011	0.002	0.005	0.038	0.424	0.501
Phy				0.413	0.472	0.832	0.879	0.273	0.721
MBM				0.890	0.997	0.462	0.599	0.506	0.987
NE × Phy				0.212	0.161	0.215	0.940	0.821	0.194
NE × MBM				0.173	0.111	0.025	0.580	0.059	0.017
Phy × MBM				0.408	0.597	0.942	0.472	0.393	0.947
NE × Phy × MBM				0.432	0.302	0.955	0.200	0.029	0.414

AR = as-received; OP = over-processed.
[a, b, c] Means in the same column with different superscripts are significantly different ($P < 0.05$).
[1] 2- or 3-way interaction separated by Tukey's.
[2] Phy: Quantum Blue 5G.

Table 5
Effect of necrotic enteritis (NE), phytase (Phy) and meat and bone meal (MBM) on tibial mineral concentration, d 16[1].

Item	NE	Phy[2], FTU/kg	MBM	Ca, %	P, %	Mg, %	Mn, mg/kg	Na, mg/kg	Zn, mg/kg
Two-way interaction									
NE × MBM	−		AR	38.19	18.13	0.87[a]	13.32[c]	1.29	399
	−		OP	38.03	18.01	0.80[ab]	14.19[c]	1.32	344
	+		AR	37.38	17.68	0.74[b]	18.29[b]	1.27	290
	+		OP	37.59	17.82	0.76[b]	23.84[a]	1.25	306
Main effects									
NE	−			38.11[a]	18.07[a]	0.84	13.75	1.30[a]	371[a]
	+			37.48[b]	17.75[b]	0.75	21.06	1.26[b]	298[b]
Phy		500		37.73	17.92	0.80	16.86	1.26	324
		5,000		37.86	17.90	0.79	17.95	1.30	346
MBM			AR	37.79	17.91	0.80	15.80[b]	1.28	345
			OP	37.81	17.91	0.78	19.01[a]	1.28	325
SEM				0.78	0.35	0.06	3.63	0.03	44.33
P-value									
NE				0.034	0.022	<0.001	<0.001	0.038	0.002
Phy				0.660	0.830	0.605	0.334	0.054	0.311
MBM				0.948	0.947	0.215	0.006	0.800	0.379
NE × Phy				0.216	0.188	0.158	0.092	0.946	0.378
NE × MBM				0.518	0.352	0.038	0.042	0.223	0.110
Phy × MBM				0.859	0.962	0.795	0.972	0.266	0.591
NE × Phy × MBM				0.598	0.543	0.599	0.076	0.504	0.842

AR = as-received; OP = over-processed.
[a, b, c] Means in the same column with different superscripts are significantly different ($P < 0.05$).
[1] 2- or 3-way interaction separated by Tukey's.
[2] Phy: Quantum Blue 5G.

Table 6
Effect of necrotic enteritis (NE), phytase (Phy) and meat and bone meal (MBM) on tibial mineral concentration, d 29[1].

Item	NE	Phy[2], FTU/kg	MBM	Ca, %	P, %	K, %	Mg, %	Fe, mg/kg	Mn, mg/kg	Zn, mg/kg
Treatments										
1	−	500	AR	37.41	17.56	0.80	0.67	279	9.20[c]	214
2	−	5,000	AR	37.88	17.55	0.79	0.67	265	11.36[b]	216
3	−	500	OP	38.04	17.75	0.78	0.71	278	12.55[a]	220
4	−	5,000	OP	38.34	17.94	0.82	0.71	311	10.29[b]	204
5	+	500	AR	38.30	17.77	0.85	0.67	306	10.96[b]	223
6	+	5,000	AR	38.34	17.71	0.82	0.68	282	11.44[b]	237
7	+	500	OP	37.76	17.61	0.85	0.69	274	13.87[a]	243
8	+	5,000	OP	38.19	17.70	0.81	0.70	277	10.46[b]	250
Two-way interactions										
NE × MBM	−		AR	37.64	17.55[b]	0.79	0.67	272[b]	10.28	215
	−		OP	38.19	17.84[a]	0.80	0.71	294[a]	11.42	212
	+		AR	38.32	17.74[a]	0.83	0.67	294[a]	11.20	230
	+		OP	37.97	17.65[b]	0.83	0.69	275[b]	12.17	246
NE	−			37.92	17.70	0.79[b]	0.69	283	10.85	214[b]
	+			38.15	17.70	0.83[a]	0.68	285	11.69	238[a]
MBM			AR	37.98	17.65	0.81	0.67[b]	283	10.74	222
			OP	38.08	17.75	0.82	0.70[a]	285	11.80	229
SEM				0.56	0.18	0.03	0.01	18.0	1.49	18.82
P-value										
NE				0.432	0.985	0.028	0.462	0.856	0.272	0.009
Phy				0.287	0.519	0.573	0.607	0.929	0.211	0.840
MBM				0.734	0.226	0.733	0.003	0.829	0.167	0.476
NE × Phy				0.795	0.647	0.271	0.563	0.326	0.190	0.326
NE × MBM				0.134	0.033	0.645	0.340	0.042	0.907	0.290
Phy × MBM				0.850	0.312	0.564	0.995	0.070	0.620	0.470
NE × Phy × MBM				0.632	0.906	0.307	0.863	0.615	0.018	0.753

AR = as-received; OP = over-processed.
[a, b, c] Means in the same column with different superscripts are significantly different ($P < 0.05$).
[1] 2- or 3-way interaction separated by Tukey's.
[2] Phy: Quantum Blue 5G.

interaction was observed for Fe ($P < 0.05$) showing that Fe was higher in the challenged birds, compared to the unchallenged birds fed as-received MBM. A phytase × MBM interaction was detected for Zn ($P < 0.05$) where as-received MBM increased the level of Zn in the group fed low phytase and in the group fed high phytase, the over-processed MBM increased the concentration. The challenge as a main effect increased K ($P < 0.001$), Mn ($P < 0.01$) and Na ($P < 0.001$) concentrations in the femur. The over-processed MBM as a main effect increased femur Mn concentration ($P < 0.05$).

There was no significant challenge × phytase interaction detected for femur Ca, P, K, Mn, Na and Zn. There was no significant challenge × MBM interaction observed for femur Ca, P, K, Mn, Na

Table 7
Effect of necrotic enteritis (NE), phytase (Phy) and meat and bone meal (MBM) on femur mineral concentration, d 16[1].

Item	NE	Phy[2], FTU/kg	MBM	Ca, %	P, %	K, %	Fe, mg/kg	Mn, mg/kg	Na, mg/kg	Zn, mg/kg
Two-way interactions										
NE × Phy	−	500		38.11	17.95	0.93	334^{ab}	23.65	1.13	565^{a}
	−	5,000		37.89	17.88	0.90	300^{b}	19.02	1.11	539^{b}
	+	500		38.04	17.85	1.00	359^{a}	27.38	1.19	553^{a}
	+	5,000		37.92	17.65	0.99	380^{a}	31.13	1.21	525^{c}
NE × MBM	−		AR	38.14	18.00	0.89	301^{b}	18.22	1.04	559
	−		OP	37.86	17.83	0.93	333^{ab}	24.45	1.14	546
	+		AR	38.06	17.75	0.99	380^{a}	26.18	1.18	533
	+		OP	37.89	17.74	1.00	358^{a}	32.33	1.21	544
Phy × MBM		500	AR	38.18	19.95	0.94	334	22.22	1.13	557^{a}
		500	OP	38.02	17.80	0.93	348	22.19	1.16	535^{b}
		5,000	AR	37.97	17.85	0.98	359	28.81	1.18	533^{b}
		5,000	OP	37.79	17.73	0.96	332	27.96	1.17	557^{a}
Main effects										
NE	−			38.00	17.91	0.91^{b}	317	21.33^{b}	1.12^{b}	552
	+			37.98	17.75	0.99^{a}	369	29.26^{a}	1.20^{a}	538
MBM			AR	38.10	17.88	0.94	341	22.20^{b}	1.14	546
			OP	37.88	17.79	0.97	346	28.39^{a}	1.17	545
SEM				0.18	0.34	0.05	41.63	8.95	0.04	21.71
P-value										
NE				0.929	0.203	<0.001	<0.001	0.005	<0.001	0.222
Phy				0.536	0.297	0.376	0.610	0.871	0.829	0.934
MBM				0.419	0.485	0.208	0.713	0.027	0.113	0.908
NE × Phy				0.850	0.622	0.747	0.042	0.128	0.385	0.019
NE × MBM				0.854	0.587	0.622	0.046	0.988	0.748	0.304
Phy × MBM				0.985	0.901	0.870	0.121	0.880	0.325	0.046
NE × Phy × MBM				0.657	0.756	0.740	0.623	0.162	0.513	0.447

AR = as-received; OP = over-processed.
[a, b, c] Means in the same column with different superscripts are significantly different ($P < 0.05$).
[1] 2- or 3-way interaction separated by Tukey's.
[2] Phy: Quantum Blue 5G.

and Zn. There was no significant phytase × MBM interaction observed for femur Ca, P, K, Fe, Mn and Na. There was no significant challenge effect observed for femur Ca, P, Fe and Zn. There was no significant MBM effect observed for femur Ca, P, K, Fe, Na and Zn.

3.8. Femur mineral concentration, d 29

A 3-way challenge × phytase × MBM interaction was detected for Mn ($P < 0.05$) where birds in the unchallenged group and fed low phytase and as-received MBM had a lower concentration compare those in the challenged group fed high phytase and over-processed MBM (Table 8). Challenge as a main effect increased the concentration of Zn ($P < 0.001$) in the femur.

There was no significant 3-way challenge × phytase × MBM interaction detected for femur Ca, P and Zn. There was no significant challenge effect observed for femur Ca, P and Mn.

3.9. Litter score, litter dry matter and hock burn, d 42

The challenge as a main effect tended to increase the litter DM ($P = 0.058$) but decrease hock burn ($P < 0.05$) (Table 9). The presence of over-processed MBM tended to increase litter score ($P = 0.052$).

There was no significant challenge effect observed for litter score. There was no significant MBM effect observed for litter DM and hock burn.

4. Discussion

The present study investigated the influence of MBM (as-received and over-processed) and phytase the on hock burns and bone traits in broilers, and litter quality during a NE challenge.

Nutrition and disease are important factors that influence skeletal health and integrity (Fleming et al., 2006). Although there is evidence that suggests that diseases do affect bone development in chickens (Rath et al., 2007; Oikeh et al., 2019), few studies have evaluated the effect of NE on bone development (Paiva et al., 2014). In the present study, the challenge decreased the bone ash, dimensions and BS at d 16. Also, the effects of the challenge on these traits at d 29 suggest that a loss in bone integrity during NE may linger on even during periods of optimal health condition. The decline in bone mineralization in the challenged-birds in the present study could be due to birds going off feed during the period of NE challenge as observed and reported in series 1 in the current study (Zanu et al., 2020) as well as in other studies (Williams, 2005; M'Sadeq et al., 2015). The inadequate feed and water intake lead to emaciation and weakness in bones. Bone turnover has been reported to be strongly associated with feed intake, with the turnover increasing during nutrient restriction and suppressed during periods of high nutrients density (Erdal et al., 2012). Furthermore, Newman and Leeson (1999) have reported a decrease in bone strength immediately after feed withdrawal and this was accompanied by a reduced percentage of ash. The reduction in bone strength and ash in that study might have also been due to bone mineral resorption. In fact, in studies involving laying hens, induction of molting using deliberate feed withdrawal has been reported to have adverse effect on mineralization and biochemical properties of bones (Biggs et al., 2003, 2004; Mazzuco and Hester, 2005) whereas a decrease in calcium absorption during feed deprivation was also suggested to contribute to bone resorption (Al-Batshan et al., 1994). Therefore, not only does low feed intake during disease incidence as was the case in the present study (Zanu et al., 2020a) contribute to poor bone development by itself, but more particularly the low intake of nutrients that are needed for bone growth. In the present study, the concentration of Ca, P, Mg, K,

Table 8
Effect of necrotic enteritis (NE), phytase (Phy) and meat and bone meal (MBM) on femur. mineral concentration, d 29[1].

Item	NE	Phy[2], FTU/kg	MBM	Ca, %	P, %	Mn, g/kg	Zn, mg/kg
Treatments							
1	−	500	AR	36.94	17.51	9.06[b]	223
2	−	5,000	AR	37.13	17.59	11.08[ab]	246
3	−	500	OP	37.18	17.59	11.89[ab]	225
4	−	5,000	OP	36.63	17.40	10.86[ab]	207
5	+	500	AR	37.07	17.47	11.64[ab]	255
6	+	5,000	AR	37.47	17.65	11.23[ab]	248
7	+	500	OP	37.05	17.62	11.59[ab]	262
8	+	5,000	OP	37.27	17.64	14.57[a]	269
Main effect							
NE	−			36.97	17.52	10.72	225[b]
	+			37.22	17.60	12.26	259[a]
SEM				0.56	0.15	1.49	18.78
P-value							
NE				0.238	0.317	0.056	<0.001
Phy				0.754	0.749	0.259	0.893
MBM				0.557	0.959	0.066	0.809
NE × Phy				0.243	0.281	0.618	0.854
NE × MBM				0.956	0.359	0.826	0.083
Phy × MBM				0.274	0.152	0.916	0.456
NE × Phy × MBM				0.493	0.715	0.045	0.139

AR = as-received; OP = over-processed.
[a, b] Means in the same column with different superscripts are significantly different ($P < 0.05$).
[1] 2- or 3-way interaction separated by Tukey's.
[2] Phy: Quantum Blue 5G.

Table 9
Effect of necrotic enteritis (NE), phytase (Phy) and meat and bone meal (MBM) on litter score, litter DM and hock burn, d 42[1].

Item	NE	Phy[2]	MBM	Litter score	Litter DM, %	Hock burn
Main effects						
NE	−			6.42	68.24	0.55[a]
	+			7.00	71.43	0.39[b]
MBM			AR	6.21	69.34	0.48
			OP	7.21	70.33	0.45
SEM				0.92	3.48	0.15
P-value						
NE				0.250	0.058	0.048
Phy				0.409	0.108	0.485
MBM				0.052	0.549	0.732
NE × Phy				0.141	0.512	0.896
NE × MBM				0.323	0.241	0.371
Phy × MBM				0.323	0.307	0.923
NE × Phy × MBM				0.189	0.490	0.839

AR = as-received; OP = over-processed.
[a, b] Means in the same column with different superscripts are significantly different ($P < 0.05$).
[1] 2- or 3-way interaction separated by Tukey's.
[2] Phy: Quantum Blue 5G.

Fe, Zn and Na in the bones was reduced in the challenged birds. Similarly, malabsorption of electrolytes Ca, F and Zn has been suggested as one of the possible causes of gastrointestinal disorders (Williams 2005; Caruso et al., 2013). The reduction of these minerals might explain the low BS recorded in the challenged group in the current study. The disturbance of the intestines as measured by lesions in the present study due to the challenge (Zanu et al., 2020a) might have also contributed to the resorption of minerals.

The effect of inadequate supply of especially Ca and P in broiler diets has been widely reported to cause weakness in bones (Driver et al., 2006). Calcium together with P form hydroxyapatite confers rigidity to the bone (Bonjour, 2011). However, malabsorption of these minerals can occur and reduce intestinal Ca absorption (Perry et al., 1991). This phenomenon might explain the low concentration of Ca observed in the toe at d 29 and tibia at d 16 in the challenged birds of the present study. Other minerals play an essential role in bone health and development as well. Trace elements such as Mg, Mn, and Zn have direct function in hydroxyapatite crystal formation and bone metabolism (Zofková et al., 2013). For example, about 50% to 60% of all magnesium in the body is found in bone, where it is a structural constituent, along with calcium and phosphate (Jahnen-Dechent and Ketteler, 2012). Again, Mg released by bone following resorption might help activate immune cells (Hu et al., 2018). In the current study, the challenged reduced the concentration of Mg in the toe and tibia. Therefore, it is most probable that Mg, the third major element stored in bone, was required to serve as a co-factor in the enzymatic reactions to generate the energy needed for immune cells as they were being activated during the challenge.

It is also worth noting that in other instances of the current study, the challenge led to a higher concentration of Na, Fe, K, Zn, and Mn. Although Ca has been generally accepted to be absorbed through the paracellular pathway, it is not known if other minerals are also absorbed in a similar manner. Assuming other minerals are equally absorbed through the paracellular pathway, an incidence of leaky gut, as was observed in the present study (Zanu et al., 2020a) would be expected to increase the uptake of minerals in the challenged birds.

In the current study, it appeared that when high phytase and over-processed MBM were fed to the challenged birds, the concentration of Mn in the toe (d 16) and femur (d 29) were higher compared to the other groups. It is most probable that high phytase and over-processed MBM aided the activity of *C. perfringens* by providing Ca and undigested proteins respectively, to cause the leakage of the gut. In a recent study, the absence of antibiotics increased NE infection in broilers fed high phytase (1,500 FTU/kg) and MBM (6% inclusion) (Zanu et al., 2020b). The over-processed MBM fed in the current study was hypothesized to increase the incidence. The high Mn in this instance might be beneficial as it plays a key role as a co-factor in the formation of bone cartilage and collagen (Pepa and Brandi, 2016). Under relatively favorable health conditions at d 29, high phytase increased the deposition of toe Mn in birds fed the as-received MBM in the unchallenged challenged.

The possible uptake of minerals through the paracellular was reaffirmed by the higher toe Zn (d 29) and tibial Mn observed in challenged birds fed over-processed MBM at d 16. At d 29 the challenged birds fed over-processed MBM had higher tibial P and Fe. A similar higher toe Fe was observed at d 16 in challenged birds fed over-processed MBM. A higher bone Fe is beneficial as it plays an important role in the synthesis of collagen (Toxqui and Vaquero, 2015). Iron-deficient rats, for instant, have shown to have poorly mineralized skeletons and defects in the microarchitecture of bone (Katsumata et al., 2008). Conversely, it also appeared that feeding as-received MBM at a lower inclusion rate of 4% in the present work did not disturb the gut as evidenced in the reduction toe Fe (d 16), Mg and Zn (d 29), tibial Mg and Mn (d 16). The higher femur Zn concentration at d 16 of the group fed over-processed MBM and high phytase might have been possibly due to less interaction of Ca with phytase activity thereby increasing the release of Zn. Over-processing of MBM might have reduced the level of $CaCO_3$ through the processes of calcination (Barros et al., 2009) and hence reduced the reactive Ca. In addition, the capability of over-processed MBM of reducing intestinal pH as previously reported (Zanu et al., 2020a) might have facilitated the activity of phytase to release Zn. High phytase activity is achieved at a lower pH (Li et al., 2017).

The occurrence of wet litter in broiler flocks is associated with concerns regarding flock health, bird welfare, and impairment in production efficiency. Wet litter has been associated with an increased incidence of leg diseases in broilers (Shepherd and Fairchild, 2010). When excreta are deposited on friable litter it gets coated with litter particles. The litter particles absorb some of the moisture from the excreta reducing the droppings from sticking to each other. As the birds move around, the droppings are further broken down into smaller particles increasing the surface area and moisture loss. However, as the moisture level of the litter increases due to the type of diets fed to the birds or increase in the stocking density relative to the size of the pen, as observed in the present study, it is unable to coat the excreta. In such an instance, the transfer of moisture to the litter beneath is reduced. The above phenomenon might explain the higher litter score (indicating friability of the litter) observed in the group fed over-processed MBM on d 42 in the present study. It is most probable that birds fed the over-processed MBM, having recorded lower feed intake on d 35 and 42 (Zanu et al., 2020a) might have deposited less excreta in their pens hence the high score. In addition, a compromise in the quantity of Ca in over-processed MBM might have contributed to the higher litter score. Previous reports have suggested that feeding high Ca-diets increases the wetness of litter (Bedford and Rousseau, 2017). Also, in the present study, the challenge increased DM of the litter while decreasing hock burns in the birds. High moisture content of the litter has been widely reported as the cause of foot diseases in various studies (Lay et al., 2011; Abd El-Wahab et al., 2012; de Jong et al., 2014). The higher the moisture in the litter the higher the microbiological risk. The present result might be driven by the fact the over-processed MBM and NE resulted in more deaths and smaller birds as reported in Part 1 in this series (Zanu et al., 2020a), so the total fecal output was radically reduced, hence improving the litter score. The present results thus corroborate those of other studies that lower the livability, the increase in litter DM (Shepherd and Fairchild, 2010; Guardia et al., 2011).

5. Conclusion

In conclusion, the toe, femur and tibia responded differently to the challenge, phytase and over-processed MBM. However, generally, the NE challenge had a detrimental effect on many bone parameters indicating that not only does NE reduce productive performance in broilers, but also it may compromise bone integrity. Therefore, diets that have the potential to cause the onset of NE should be fortified with antimicrobials to reduce bone deformities. The result also suggests that cases of hock burns in broilers might be minimal in poultry sheds during NE incidences due to low livability and corresponding low moisture content of the litter.

Author contributions

Holy K. Zanu: methodology, formal analysis, validation, writing original, review, statistics and editing; Micheal Bedford: conceptualization, review and editing; Sarbast Kheravaii: methodology assistance with molecular techniques, review; Natalie Morgan: review, editing and validation; Robert A. Swick: conceptualization, review and editing. supervision, project administration, resources.

Conflict of interest

We declare that we have no financial or personal relationships with other people or organizations that might inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper

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