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Original Research Article

Over-processed meat and bone meal and phytase effects on broilers challenged with subclinical necrotic enteritis: Part 1. Performance, intestinal lesions and pH, bacterial counts and apparent ileal digestibility

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ABSTRACT

This feeding study investigated the hypothesis that over-processing of meat and bone meal (MBM) would impair the performance, gut health and ileal digestibility of nutrients in birds challenged with necrotic enteritis (NE). The effect of phytase (500 vs. 5,000 FTU/kg) was also examined using manufacturers recommended matrix values for 500 FTU for both levels. Ross 308 male broilers ($n = 768$) were assigned to 8 diets, with 6 replicate pens per diet and 16 birds per replicate pen using a randomized design with a factorial arrangement of treatments. Factors were NE challenge (no or yes), MBM (as received or over-processed), and phytase level (500 or 5,000 FTU/kg). Half of the birds were challenged with 5,000 oocysts of field strains of *Eimeria acervulina* and *Eimeria brunetti*, and 2,500 oocysts of *Eimeria maxima* on d 9 and 10^8 CFU/mL of *Clostridium perfringens* strain EHE-NE18 on d 14 and 15 post-hatch. Challenge \times MBM interactions were detected for weight gain (WG), feed conversion ratio (FCR) and feed intake (FI) at d 14, 21 and 28, showing that challenged birds fed over-processed MBM had decreased WG ($P < 0.05$) and FI ($P < 0.05$) at d 14, increased FCR ($P < 0.05$) at d 21 and decreased WG ($P < 0.05$) and FI ($P > 0.05$) at d 28. Birds fed low phytase had increased livability ($P < 0.05$) at d 42. The challenge increased the prevalence and severity of NE induced lesions in the jejunum ($P < 0.05$) and ileum ($P < 0.05$). The birds fed over-processed MBM had decreased pH in the jejunum ($P < 0.05$) and ileum ($P < 0.05$) at d 16. High phytase increased apparent ileal digestibility (AID) of Ca ($P < 0.05$) and P ($P < 0.05$), and over-processed MBM increased AID of carbon (C; $P < 0.05$) and Ca ($P < 0.05$) at d 29. The challenge increased the caecal counts of *Lactobacillus* spp. ($P < 0.05$) and *C. perfringens* ($P < 0.05$) at d 16. The results indicated that supplementation of diets with high phytase reduces the negative impact on performance from over-processed MBM during NE as a result of increased nutrient digestibility.

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1. Introduction

Necrotic enteritis (NE) has received much research attention in the last few years due to its economic significance in the poultry industry. The heightened interest in this area is partly due to the ongoing removal of in-feed antibiotics as the conventional means for preventing NE in poultry (Branton et al., 1997; Barekatin et al., 2013; M'Sadeq et al., 2015; Yang et al., 2016; Kheravii et al., 2017). Digestion inefficiency, presence of viscous cereal grains (barley, rye, oats and wheat) and poorly digested animal protein sources (meat

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and bone meal [MBM]; fishmeal) are predisposing factors that can increase susceptibility of broilers to NE (Hoerr, 1998; Palliyeguru et al., 2010; Guo et al., 2013; Knight et al., 2016). The poor digestibility leads to a build-up of undigested nutrients in the hindgut that serve as substrates for opportunistic pathogenic bacteria, including *Clostridium perfringens*.

Over-processing of MBM during heat processing causes crosslinking of the free epsilon amino group in lysine (Fernandez and Parsons, 1996; Fontaine et al., 2007; Almeida et al., 2014). Such crosslinks including isopeptides, disulfide bridges, aldol condensation, malonaldehyde links, polyphenol complexes that reduce the overall digestibility of both lysine and protein (Hammann and Schmid, 2014; Zhang et al., 2018). The amount of available lysine in MBM has been used as an indicator amino acid (AA) digestibility and optimum processing (Pahm et al., 2008; Rutherford, 2015). Lipid oxidation (e. g. malonaldehyde) increases with heat and may form enzyme-resistant cross-links resulting in racemization that decreases hydrolysis of peptide bonds involving D- AA (Dai et al., 2019). Keratin proteins present in wool, feathers, and hair are not digestible by pepsin unless the rendering process includes a pressure cycle or enzymes capable of hydrolyzing keratin. Hence, MBM that contains a high proportion of such proteins has low pepsin digestibility (Hegedus et al., 1989). Apajalahti and Vienola (2016) suggested that the indigestible proteins in MBM that escape gastric digestion might be used as a substrate by *C. perfringens*, meaning that high inclusion of MBM in poultry diets is a potential precursor for the onset of NE. Fermentation of AA in the terminal part of the gut produces metabolites including biogenic amines, total volatile nitrogen (TVN) and ammonia (Qaisrani et al., 2014, 2015a). These metabolites increase the pH of the lower gut, providing a favorable environment for the proliferation of pathogen bacteria leading to changes in microbial population and species diversity (Corrier et al., 1990; Walugembe et al., 2015; Ilhan et al., 2017).

Exogenous phytase has been used over the years to reduce the amount of inorganic phosphorus (P) and calcium (Ca) required in chicken diets and improve feed conversion ratio (FCR) and AA digestibility. The effect of phytase supplementation on improving growth and AA digestibility in broilers fed diets deficient in lysine has been well documented (Ravindran et al., 2001). Therefore, exogenous phytase supplementation, particularly at high inclusion level, might be used as a potential substitute for MBM as a source of protein, Ca and P in chicken diets. It was hypothesized that over-processed MBM, that is, excessive heating beyond what should be commercially practiced in rendering plants in Australia (110 to 115 °C without pressure), would exacerbate the onset of NE, due to the increased presence of undigested protein. It was also hypothesized that this effect may be countered using a super dose of phytase (5,000 FTU), above what is routinely included in most commercial poultry feed globally, to replace MBM. The objective of this study was to determine the effect of over-processed MBM and phytase supplementation on the performance, intestinal lesions and pH, bacterial counts and apparent ileal digestibility of nutrients in broilers under or not a NE challenge protocol.

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 of the chicks were weighed and randomly allocated to 48-floor pens (0.85 m²), with softwood shavings (8 cm) as bedding materials, using 6 replicate pens per treatment and 16 birds per pen. The chicks were reared and housed in an environmentally

controlled room with ad libitum access to feed and water. Each pen was fitted with a single tube feeder (diameter = 32 cm) and 4 nipple drinkers. The lighting and temperature program for the experimental period followed the Ross 308 recommendations (Aviagen, 2014). Mortality was recorded daily and cumulative pen weight and feed consumption were recorded on d 7, 14, 21, 28, 35 and 42. For brevity, only d 14, 21, 28 and 42 results are reported.

2.2. Dietary treatment

The crude protein (CP), AA, crude fiber (CF), and crude fat component of homogenized samples of MBM, canola meal, soybean, and wheat were analyzed using a near-infrared spectroscopy (NIRS, Evonik AminoProx, Frankfurt, Germany) prior to feed formulation. Diets were then formulated in accordance with Ross 308 standard ileal digestibility AA 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. Treatments were arranged in a 2 × 2 × 2 factorial design. Factors were NE challenge (no or yes), MBM (as received or over-processed), phytase (500 or 5,000 FTU/kg) (Quantum Blue, AB Vista, Malborough, UK). The phytase matrix values for 500 FTU/kg were applied in both 500 and 5,000 FTU/kg phytase groups. 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. The starter diets were fed to the birds in crumbled form and the grower and finisher were fed in pelleted form.

2.3. 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 *Eimeria acervulina* and 5,000 oocysts of *Eimeria maxima* and 2,500 oocytes of *Eimeria brunetti* (*Eimeria* Pty Ltd) on d 9 and 10⁸ CFU/mL of *C. perfringens* strain EHE-NE18 (known to express NetB toxin, Commonwealth Scientific and Industrial Research Organization, Geelong, Australia) on d 14 and 15 while the remaining were gavaged with sterile buffer or broth.

2.4. Meat and bone meal processing

About 10 kg each of commercial MBM (Northern Cooperative Meat Company Limited, Casino, NSW, Australia; lot number 48855) were either kept as received or autoclaved at a temperature of 128 °C at 2 bars for 90 min in an autoclave (Hirayama manufacturing corporation, Saitama, Japan). The resulting over-processed MBM was crushed and milled through 0.5-mm sieves. The analysis of nutrient composition and pepsin digestibility of both as-received and over-processed MBM were carried out in triplicates and the averages are shown in Table 2. Pepsin digestibility was measured using 0.2% pepsin using the method 971.09 (AOAC, 2006). Amino acids were measured using method 982.30 (AOAC, 2006) and the Carpenter method (Carpenter, 1960) was used to measure available lysine.

2.5. Data collection

2.5.1. Performance

The birds and feed were weighed on a pen basis weekly. Feed intake (FI), weight gain (WG) and feed conversion ratio (FCR, feed:gain ratio) were calculated weekly. The pens were monitored for mortality twice daily and post-mortem examinations were conducted on dead birds throughout the study period. The FCR was

Table 1
Ingredients and nutrient composition of basal diets (% as-fed).

Item	Starter				Grower				Finisher			
	AR + low phytase	AR + high phytase	OP + low phytase	OP + high phytase	AR + low phytase	AR + high phytase	OP + low phytase	OP + high phytase	AR + low phytase	AR + high phytase	OP + low phytase	OP + high phytase
Ingredients												
Wheat	64.17	64.17	64.17	64.17	70.82	70.82	70.82	70.82	71.41	71.41	71.41	71.41
Soybean meal	26.56	26.56	26.56	26.56	18.98	18.98	18.98	18.98	17.33	17.33	17.33	17.33
Canola expeller cold	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
MBM (AR)	4.03	4.03	—	—	4.00	4.00	—	—	4.00	4.00	—	—
MBM (OP)	—	—	4.03	4.03	—	—	4.00	4.00	—	—	4.00	4.00
Canola oil	0.538	0.538	0.538	0.538	1.269	1.269	1.269	1.269	2.581	2.581	2.581	2.581
Limestone	0.529	0.529	0.529	0.529	0.423	0.423	0.423	0.423	0.223	0.223	0.223	0.223
Phytase ¹	0.1	1.0	0.1	1.0	0.1	1.0	0.1	1.0	0.1	1.0	0.1	1.0
Xylanase ²	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Salt	0.104	0.104	0.104	0.104	0.076	0.076	0.076	0.076	0.077	0.077	0.077	0.077
Na bicarb	0.150	0.150	0.150	0.150	0.150	0.150	0.150	0.150	0.150	0.150	0.150	0.150
TiO ₂	0.000	0.000	0.000	0.000	0.500	0.500	0.500	0.500	0.500	0.500	0.500	0.500
Vitamins ³	0.075	0.075	0.075	0.075	0.060	0.060	0.060	0.060	0.060	0.060	0.060	0.060
Trace minerals ⁴	0.100	0.100	0.100	0.100	0.080	0.080	0.080	0.080	0.080	0.080	0.080	0.080
Choline Cl (60%)	0.061	0.061	0.061	0.061	0.062	0.062	0.062	0.062	0.058	0.058	0.058	0.058
L-Lysine HCl	0.268	0.268	0.268	0.268	0.250	0.250	0.250	0.250	0.226	0.226	0.226	0.226
DL-Methionine	0.233	0.233	0.233	0.233	0.164	0.164	0.164	0.164	0.149	0.149	0.149	0.149
L-Threonine	0.066	0.066	0.066	0.066	0.055	0.055	0.055	0.055	0.049	0.049	0.049	0.049
Calculated nutrients												
ME poultry, kcal/kg	3,000	3,000	3,000	3,000	3,100	3,100	3,100	3,100	3,200	3,200	3,200	3,200
Crude protein	24.46	24.46	24.46	24.46	21.57	21.57	21.57	21.57	20.82	20.82	20.82	20.82
Ash	4.68	4.68	4.68	4.68	4.75	4.75	4.75	4.75	4.66	4.66	4.66	4.66
Standardized ileal digestibility												
Arginine	1.40	1.40	1.40	1.40	1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.20
Lysine	1.24	1.24	1.24	1.24	1.05	1.05	1.05	1.05	0.99	0.99	0.99	0.99
Methionine	0.539	0.539	0.539	0.539	0.443	0.443	0.443	0.443	0.42	0.42	0.42	0.42
Methionine + Cystine	0.9	0.9	0.9	0.9	0.79	0.79	0.79	0.79	0.762	0.762	0.762	0.762
Tryptophan	0.264	0.264	0.264	0.264	0.229	0.229	0.229	0.229	0.221	0.221	0.221	0.221
Isoleucine	0.853	0.853	0.853	0.853	0.73	0.73	0.73	0.73	0.7	0.7	0.7	0.7
Threonine	0.79	0.79	0.79	0.79	0.68	0.68	0.68	0.68	0.65	0.65	0.65	0.65
Valine	0.98	0.98	0.98	0.98	0.857	0.857	0.857	0.857	0.826	0.826	0.826	0.826
Calcium	0.9	0.9	0.9	0.9	0.84	0.84	0.84	0.84	0.76	0.76	0.76	0.76
Available phosphorus	0.45	0.45	0.45	0.45	0.439	0.439	0.439	0.439	0.437	0.437	0.437	0.437
Sodium	0.18	0.18	0.18	0.18	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17
Choline, mg/kg	1,699	1,699	1,699	1,699	1,600	1,600	1,600	1,600	1,550	1,550	1,550	1,550
Analyzed												
Crude protein	25.46	26.45	25.79	26.32	23.66	24.31	23.55	23.63	22.64	23.34	21.90	21.64
Calcium	1.36	1.26	1.31	1.37	1.21	1.15	1.22	1.24	1.21	1.18	1.09	1.18
Total phosphorus	0.88	0.84	0.85	0.88	0.79	0.79	0.82	0.82	0.83	0.80	0.76	0.79

AR = as-received; OP = over-processed; MBM = meat and bone meal.

¹ Phytase (Quantum Blue 5G, AB Vista feed ingredient, UK). Both 500 (0.1% phytase) and 5,000 (1.0% phytase) FTU/kg phytase used the matrix values for Ca, P, Na, arginine, lysine, methionine, methionine + cystine, tryptophan, isoleucine, threonine and valine at 1.65, 1.50, 0.35, 0.130, 0.170, 0.039, 0.390, 0.190, 0.255, 0.330 and 0.230 g/kg respectively.

² Xylanase (Econase XP 25, AB Vista feed ingredient, UK).

³ Vitamin premix provided the following per kilogram 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.

⁴ Mineral premix provided the following per kilogram diets: Cu, 16 mg as copper sulphate; Mn, 60 mg as manganese sulphate; Mn, 60 mg as manganous oxide; I, 0.125 mg as potassium iodide; Se, 0.3 mg; Fe, 40 mg, as iron sulphate; Zn, 50 mg as zinc oxide; Zn, 50 mg as zinc sulphate.

calculated as the total feed consumed per pen for each period divided by total pen gain (live and dead). This gives the FCR of surviving live birds. Feed intake was calculated as $FCR \times WG$. Livability was calculated weekly as the number of live birds/the number of starting birds $\times 100$.

2.5.2. Gastrointestinal pH

Immediately post euthanasia (d 16 and 29), the crop, gizzard, ileum, and caeca were removed intact from 2 birds per pen. A digital pH meter (Mettler-Toledo, UK) with a spear tip piercing pH electrode (Sensorex, California, USA) was directly inserted into the digesta in the lumen of the proximal gizzard, ileum and caeca of the same bird while ensuring the pH electrode did not touch the walls. The pH was measured and recorded in duplicate. The probe was rinsed with ultra-pure water (ICW 3000 water purifier for ion chromatography, Millipore) between each measurement. The mean of the 2 readings per site of the tract was

calculated and recorded. The digesta of the ileum and caeca from the 2 birds per pen used for measuring the pH and an additional bird from each pen totaling 3, were sampled to determine the digestibility of nutrients and bacterial quantification, respectively.

2.5.3. Intestinal lesions

The entire length of the small intestine (duodenum, jejunum, and ileum) of the sampled birds were scored for lesions as described by Keyburn et al. (2006) as follows: 0, no lesions; 1, thin-walled and friable intestines; 2, focal necrosis or ulceration (1 to 5 foci); 3, focal necrosis (6 to 15 foci); 4, focal necrosis (16 or more foci); 5, patches of necrosis 2 to 3 cm long; and 6, diffuse necrosis typical of acute field cases. Two trained personnel were involved in the scoring. The average of the scores was computed and the pen was the experimental unit for lesion scoring.

Table 2
Percentage DM composition and pepsin digestibility of meat and bone meal (MBM).

Item	MBM	
	AR	OP
Crude protein	49.9	48.9
Ether extract	8.53	8.08
Ash	33.8	33.3
Pepsin digestibility	86.8	85.0
Hydroxyproline	3.58	3.77
Aspartic acid	3.54	3.56
Threonine	1.51	1.52
Serine	1.83	1.93
Glutamic acid	5.83	5.99
Proline	4.51	4.56
Glycine	7.13	7.28
Alanine	3.92	3.99
Cysteine	0.43	0.36
Valine	1.99	2.00
Methionine	0.57	0.57
Isoleucine	1.33	1.33
Leucine	2.81	2.83
Tyrosine	1.02	1.03
Phenylalanine	1.70	1.64
Hydroxylysine	0.38	0.38
Lysine	2.57	2.49
Histidine	0.78	0.76
Arginine	3.79	3.80
Tryptophan	0.23	0.23
Available lysine ¹	2.16	2.09

AR = as-received; OP = over-processed.

¹ Analyzed using the Carpenter method (Carpenter, 1960).

2.5.4. Chemical analyses

The diets (in duplicate) and pooled ileal digesta samples (from 3 birds on d 16 and 29) were analyzed for nitrogen, carbon (C), Ca and P contents. Data on chemical analyses are presented in Table 2. The nitrogen and C 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 ethylene diamine tetraacetic acid (EDTA) as a calibration standard, with CP calculated by multiplying percentage nitrogen by a correction factor (6.25). Mineral contents were determined using an inductively coupled plasma–optical emission spectroscopy following digestion in concentrated HNO₃.

2.5.5. 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. The TiO₂ concentrations were determined in triplicate and duplicate for diets and digesta samples, respectively. Approximately 0.1 g of the freeze-dried digesta and 0.2 g of diet samples were weighed in porcelain crucibles and ashed at 580 °C for 13 h. Upon cooling, 5 mL of H₂SO₄ (7.4 mol/L) was added to the samples, and then the samples were 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% vol/vol) 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 a standard curve.

2.5.6. Digestibility calculation

The apparent ileal digestibility (d 16 and 29) of CP, C and minerals (Ca, P, and Mg) were calculated using the indigestible marker using the following formula:

$$\text{Apparent ileal digestibility (\%)} = \{1 - [\text{TiO}_2\text{diet (\%)/TiO}_2\text{digesta (\%)}] \times [\text{Digesta nutrient (\%)/Diet nutrient (\%)}] \times 100.$$

2.5.7. Extraction of caecal bacterial DNA

The DNA of pooled caecal content collected on d 16 was extracted using PowerFecal QIACube HT Kit, (Qiagen, Inc., Doncaster, VIC, Australia), with slight modification. In brief, approximately 100 mg of frozen caecal contents were weighed in a 2-mL Eppendorf tube containing 300 mg of glass beads (0.1 mm). Then 500-μL pre-warmed PW1 was pipetted to samples prior to disrupting the cells by Tissuelyser II for 5 min at frequency 30 times/s. The samples were then incubated at 90 °C for 15 min prior to centrifugation at 20,000 × g for 1 min. An aliquot of 400-μL supernatant was mixed with 150 μL of Buffer C3. The mixture was incubated for 5 min at 4 °C prior to centrifuge at 20,000 × g for 1 min. The supernatant was transferred into a loading block (S-block) containing 20-μL Proteinase K and incubated for 10 min at room temperature and the extraction followed the manufacture's instruction using QIACube HT instrument. The extracted caecal DNA was diluted 20 times with nuclease-free water and stored at –20 °C until required.

2.5.8. Quantification of caecal bacteria

The quantitative real-time polymerase chain reaction (PCR) of *Bifidobacterium* spp., *Lactobacillus* spp., *Bacillus* spp., *Ruminococcus* spp., *Bacteroides* spp., total anaerobic bacteria, and *C. perfringens* was achieved by using quantitative PCR (qPCR) assay. The Rotorgene 6000 real-time PCR machine (Corbett, Sydney, Australia) was employed for qPCR assay of the desired bacteria from the extracted caecal DNA. The PCR was performed in duplicate for each sample in 10 μL of reaction where PCR was repeated when the difference between the threshold cycle (CT) values of the duplicates was >0.5. For PCR, a SYBR Green-containing Mix (SensiMix SYBR No-Rox, Biorline, Sydney, Australia) was applied. The reaction in a volume of 10 μL contained 5 μL of 2 × SensiMix, 300 mmol/L of each primer, and 2 μL of diluted DNA template. Table 3 shows the specific 16 S rRNA primers used for the quantification of different groups of bacteria. The PCR was performed in a Rotorgene 6500 real-time PCR machine and a threshold cycle average from the duplicate samples was used for data analysis. Serial dilutions of linearized plasmid DNA (pCR 4-TOPO Vector, Life Technologies, Carlsbad, CA) inserted with respective bacterial amplicons were used to construct a standard curve. The concentrations of the plasmid DNA were measured using NanoDrop ND-8000 (Thermo Fisher Scientific, Waltham, MA) prior to the serial dilutions. The number of target DNA copies was calculated from the mass of DNA taking into account the size of the amplicon insert in the plasmid. Bacteria numbers were expressed as log₁₀ (genomic DNA copy number)/g digesta.

2.6. Statistical analyses

The data were analyzed as a 2 × 2 × 2 factorial arrangement of treatments using the Minitab 19 statistical software to assess the main effects and 2- or 3-way interactions, with the factors NE challenge (no or yes), MBM (as-received or over-processed) and Phytase (500 or 5,000 FTU/kg). Tukey's mean separation test was

Table 3

The sequence of primers used for the quantitative PCR analysis of selected microbial populations in caecal digesta samples, d 16.

Target group or organism	Primer sequence (5'-3')	Annealing temperature, °C	Reference
<i>Bacillus</i> spp.	F-GCA ACG AGC GCA ACC CTTGA R-TCA TCC CCA CCT TCC TCC GGT	63	Zhang et al. (2015)
<i>Bacteroides</i> spp.	F-GAG AGG AAG GTC CCC CAC R-CGC TAC TTG GCT GGT TCA G	63	Layton et al. (2006)
<i>Bifidobacterium</i> spp.	F-GCG TCC GCT GTG GGC R-CIT CTC CGG CAT GGT GTT G	63	Requena et al. (2002)
<i>Lactobacillus</i> spp.	F-CAC CGC TAC ACA TGG AG R-AGC AGT AGG GAA TCT TCC A	63	Wise and Siragusa (2007)
<i>Ruminococcus</i> spp.	F-GGC GGC YTR CTG GGC TTT R-CCA GGT GGA TWA CTT ATT GTG TTA A	63	Ramirez-Farias et al. (2008)
Total bacteria	F- CCG YCC AGA CTC CTA CGG G R- TTA CCG CGG CTG CTG GCA C	63	Lee et al. (1996)
<i>Clostridium perfringens</i>	F- ATG CAA GTC GAG CGA KG R- TAT GCG GTA TTA ATC TYC CTT T Probe-FAM-TCA TCA TTC AAC CAA AGG AGC AAT CC-TAMRA	58	Rinttila et al. (2004)

used to make pairwise comparisons between treatment means ($P < 0.05$). The Box-Cos transformation of the Minitab 19 statistical software was used test for normality of data. Data deemed to be not-normally distributed (including percentages) and were tested for significance using this non-parametric procedure.

3. Results

3.1. Meat and bone meal processing

The analytical results for MBM processing are shown in Table 2. The results show that total lysine and available lysine were both reduced as a result of autoclaving for 90 min. The ratio of available lysine to lysine was unchanged. Cysteine was reduced as a result of autoclaving. Pepsin digestibility in 0.2% pepsin was lowered from 86.8 to 85.0 as a result of autoclaving. The other essential AA expressed on a DM bases were largely unchanged.

3.2. Performance

The results on growth performance are shown in Tables 4–6. No treatment or main effect was detected for any production indices at d 7 (pre-NE challenge; data not shown). Challenge (with *Eimeria* but not *C. perfringens*) \times MBM interactions were detected on d 14

Table 4

Effect of necrotic enteritis (NE), phytase (Phy) and meat and bone meal (MBM) on the performance of broilers, d 14.

Item	Factors			WG, g	FCR	FI, g	Livability, %
	NE	Phy	MBM				
Two-way interaction							
NE \times MBM ¹	–		AR	453 ^a	1.154 ^b	480 ^{ab}	97
	–		OP	460 ^a	1.146 ^b	484 ^a	99
	+		AR	400 ^b	1.268 ^a	459 ^b	97
	+		OP	374 ^c	1.299 ^a	435 ^c	95
<i>P</i> -value							
NE				0.001	0.001	0.001	0.182
Phy				0.787	0.450	0.932	0.182
MBM				0.135	0.247	0.132	0.847
NE \times Phy				0.760	0.445	0.423	0.182
NE \times MBM				0.011	0.048	0.028	0.089
Phy \times MBM				0.678	0.154	0.845	0.338
NE \times Phy \times MBM				0.646	0.083	0.684	0.338

WG = weight gain; FCR = feed conversion ratio; FI = feed intake; AR = as-received; OP = over-processed.

^{a, b, c} In the same column within 2-way interaction, means with different super-scripts are different ($P < 0.05$).

¹ Two-way interaction was separated by Tukey's mean separation test.

(Table 4) indicating over-processing of MBM reduced FI ($P < 0.05$), WG ($P < 0.05$) and increased FCR ($P > 0.05$) only in challenged birds. Challenge \times phytase interactions were detected for WG ($P < 0.05$) and FI ($P < 0.05$) on d 21 (Table 5), indicating the super dose of phytase increased WG and FI in unchallenged birds but decreased in challenged birds. Additionally, challenge \times MBM interactions were detected for WG ($P < 0.05$) and FCR ($P < 0.05$) on d 21, where only in the NE challenged birds did over-processed MBM reduce WG and increase FCR relative to counterparts fed as-received MBM. The challenge decreased livability at d 21 ($P < 0.05$). A challenge \times MBM interaction was detected for WG ($P < 0.05$) and FI ($P < 0.05$) on d 28 (Table 5). In the challenged birds, over-processed MBM decreased WG ($P < 0.05$) and FI ($P < 0.05$) compared to birds fed as-received MBM on d 28. The challenge increased FCR ($P < 0.05$) on d 28. The over-processed MBM increased FCR ($P < 0.05$) at d 28. The challenge as a main effect decreased WG ($P < 0.05$), increased FCR ($P < 0.05$) and decreased FI ($P < 0.05$) at d 35 (not shown). Similarly, feeding over-processed MBM decreased WG ($P < 0.05$), increased FCR ($P < 0.05$) and decreased FI ($P < 0.05$) at d 35. Birds fed the low level of phytase tended ($P = 0.053$) to have increased livability and over-processed MBM tended ($P = 0.089$) to increase livability at d 35. The challenge decreased WG ($P < 0.05$), increased FCR ($P < 0.05$) and decreased FI ($P < 0.05$) at d 42 (Table 6). Livability was increased in birds fed the low level of phytase ($P < 0.05$) and over-processed MBM ($P < 0.05$) on d 42.

3.3. Intestinal lesions, d 16

The challenge increased the severity of NE lesions in the jejunum ($P < 0.05$) and ileum ($P < 0.05$) as shown in Fig. 1. The challenge tended to increase lesions in the duodenum ($P = 0.082$).

3.4. Intestinal pH, d 16

A phytase \times MBM interaction was detected for pH in the caeca where, in birds fed high phytase (Table 7), over-processed MBM reduced the pH ($P < 0.05$). Challenged birds had lower pH in the crop ($P < 0.05$), jejunum ($P < 0.05$), ileum ($P < 0.05$) and caeca ($P < 0.05$), but higher pH in the gizzard ($P < 0.05$). The over-processed MBM decreased the pH in the jejunum ($P < 0.05$) and ileum ($P < 0.05$).

3.5. Intestinal pH, d 29

The challenge decreased pH in the crop ($P < 0.05$), jejunum ($P < 0.05$) and ileum ($P < 0.05$) (Table 8).

Table 5
Effect of necrotic enteritis (NE), phytase (Phy) and meat and bone meal (MBM) on the performance of broilers.

Item	Factors			Day 21				Day 28			
	NE	Phy	MBM	WG, g	FCR	FI, g	Livability, %	WG, g	FCR	FI, g	Livability, %
Two-way interactions¹											
NE × Phy	–	500		904 ^a	1.273	1,102 ^a	98	1,551	1.325	2,004	97
	–	5,000		924 ^a	1.267	1,122 ^a	98	1,548	1.333	2,013	96
NE × MBM	+	500		680 ^b	1.476	945 ^b	95	1,210	1.512	1,744	95
	+	5,000		656 ^b	1.466	904 ^b	93	1,253	1.409	1,700	92
	–		AR	925 ^a	1.256 ^c	1,114	97	1,557 ^a	1.323	2,009 ^a	95
	–		OP	903 ^a	1.285 ^c	1,111	99	1,542 ^a	1.335	2,009 ^a	97
Main effect	+		AR	700 ^b	1.431 ^b	948	95	1,318 ^b	1.399	1,776 ^b	93
	+		OP	635 ^c	1.511 ^a	901	93	1,145 ^c	1.523	1,668 ^c	93
	–			913	1.270	1,112	98 ^a	1,549	1.329 ^b	2,009	96
	+			668	1.471	924	94 ^b	1,231	1.461 ^a	1,721	93
MBM			AR	813	1.343	1031	96	1,437	1.361 ^b	1,892	94
			OP	769	1.398	1006	96	1,344	1.429 ^a	1,838	95
P-value											
NE				0.001	0.001	0.001	0.015	0.001	0.001	0.001	0.078
Phy				0.839	0.357	0.445	0.613	0.510	0.122	0.405	0.235
MBM				0.001	0.001	0.082	0.866	0.003	0.030	0.015	0.550
NE × Phy				0.048	0.827	0.034	0.400	0.450	0.075	0.212	0.550
NE × MBM				0.050	0.007	0.119	0.134	0.012	0.073	0.015	0.550
Phy × MBM				0.992	0.138	0.530	0.400	0.518	0.196	0.649	0.550
NE × Phy × MBM				0.534	0.239	0.911	0.241	0.815	0.634	0.739	0.550

WG = weight gain; FCR = feed conversion ratio; FI = feed intake; AR = as-received; OP = over-processed.

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

¹ Two-way interaction was separated by Tukey's mean separation test.

Table 6
Effect of necrotic enteritis (NE), phytase (Phy) and meat and bone meal (MBM) on the performance of broilers, d 42.

Item	Factors			WG, g	FCR	FI, g	Livability, %
	NE	Phy	MBM				
Main effects							
NE	–			3,134 ^a	1.437 ^b	4,446 ^a	91
	+			2,779 ^b	1.474 ^a	4,041 ^b	90
Phy		500		2,960	1.461	4,263	93 ^a
		5,000		2,953	1.450	4,224	88 ^b
MBM			AR	2,998	1.453	4,295	88 ^b
			OP	2,916	1.458	4,192	93 ^a
P-value							
NE				0.001	0.004	0.001	0.490
Phy				0.885	0.402	0.569	0.025
MBM				0.095	0.653	0.139	0.043
NE × Phy				0.702	0.930	0.672	1.000
NE × MBM				0.211	0.749	0.131	0.817
Phy × MBM				0.543	0.182	0.196	1.000
NE × Phy × MBM				0.734	0.678	0.532	0.644

WG = weight gain; FCR = feed conversion ratio; FI = feed intake; AR = as-received; OP = over-processed.

^{a, b} In the same column within the main effect, means with different superscripts are different ($P < 0.05$).

3.6. Apparent ileal digestibility, d 16

The challenge decreased AID of C ($P < 0.05$) but it increased the AID of Ca ($P < 0.05$) and P ($P < 0.05$) (Table 9). High phytase as a main effect increased AID of P ($P < 0.05$). Birds fed over-processed MBM had lower AID of CP ($P < 0.05$) and C ($P < 0.05$) but higher AID of Ca ($P < 0.05$).

3.7. Apparent ileal digestibility, d 29

A 3-way challenge × phytase × MBM was detected for AID of CP ($P < 0.05$) indicating that the AID was highest in the unchallenged birds fed high phytase and AR MBM compared with their

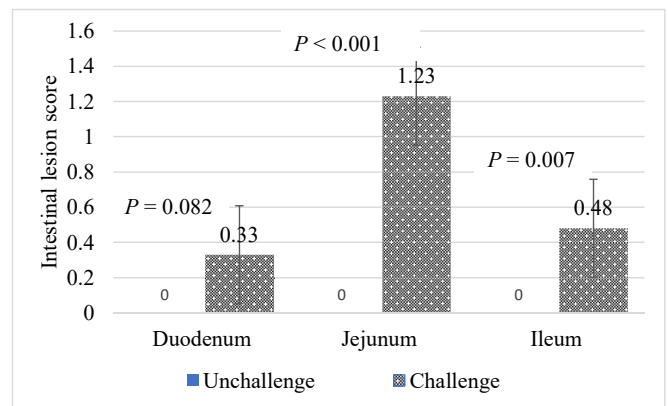


Fig. 1. Effect of necrotic enteritis (NE), phytase (Phy) and meat and bone meal (MBM) on lesion score, d 16.

counterparts fed the same diet in the challenged group (Table 9). A higher AID of CP ($P < 0.05$) was observed in the unchallenged birds fed low phytase and over-processed MBM compared to those in the challenged group fed the same diet. High phytase as a main effect increased C ($P < 0.05$), Ca ($P < 0.05$) and P ($P < 0.05$). The over-processed MBM as a main effect tended to increase the AID of C ($P = 0.056$).

3.8. Caecal bacterial count, d 16

The challenge increased the counts of *Lactobacillus* spp. ($P < 0.05$), *Bacteroides* spp. ($P < 0.05$) and *Bifidobacteria* spp. ($P < 0.05$) and *C. perfringens* ($P < 0.05$) with no interactions (Table 10). High phytase tended to increase the count of *Bifidobacteria* spp. ($P = 0.065$).

Table 7

Effect of necrotic enteritis (NE), phytase (Phy) and meat and bone meal (MBM) on intestinal pH response of broilers, d 16.

Item	Factors			Crop	Gizzard	Duodenum	Jejunum	Ileum	Caeca
	NE	Phy	MBM						
Two-way interaction ¹									
Phy × MBM		500	AR	5.46	3.05	5.90	5.83	5.77	5.93 ^b
		500	OP	5.39	2.72	5.86	5.67	5.56	6.14 ^{ab}
		5,000	AR	5.47	2.64	5.86	5.87	5.91	6.43 ^a
		5,000	OP	5.23	2.84	6.02	5.69	5.66	6.03 ^b
Main effects									
NE	–			5.60 ^a	2.51 ^b	5.91	5.87 ^a	5.88 ^a	6.26 ^a
	+			5.18 ^b	3.11 ^a	5.91	5.65 ^b	5.56 ^b	6.01 ^b
MBM			AR	5.46	2.84	5.88	5.85 ^a	5.84 ^a	6.18
			OP	5.31	2.78	5.94	5.68 ^b	5.61 ^b	6.09
P-value									
NE				0.001	0.001	0.840	0.002	0.001	0.018
Phy				0.466	0.305	0.331	0.643	0.177	0.061
MBM				0.137	0.656	0.360	0.018	0.010	0.356
NE × Phy				0.533	0.200	0.814	0.073	0.785	0.168
NE × MBM				0.159	0.859	0.487	0.098	0.056	0.269
Phy × MBM				0.384	0.074	0.114	0.893	0.860	0.005
NE × Phy × MBM				0.469	0.838	0.876	0.272	0.383	0.592

AR = as-received; OP = over-processed.

^{a, b} In the same column within the main effect or 2-way interaction, means with different superscripts are different ($P < 0.05$).¹ Two-way interaction was separated by Tukey's mean separation test.**Table 8**

Effect of necrotic enteritis (NE), phytase (Phy) and meat and bone meal (MBM) on intestinal pH response of broilers, d 29.

Item	Factors			Crop	Gizzard	Duodenum	Jejunum	Ileum	Caeca
	NE	Phy	MBM						
Main effects									
NE	–			5.07 ^a	2.60	5.70	5.81 ^a	5.88 ^a	5.89
	+			4.46 ^b	2.66	5.69	5.67 ^b	5.33 ^b	5.89
P-value									
NE				0.001	0.563	0.932	0.042	0.001	0.974
Phy				0.327	0.184	0.874	0.176	0.857	0.103
MBM				0.949	0.683	0.454	0.458	0.930	0.309
NE × Phy				0.338	0.137	0.755	0.537	0.312	0.236
NE × MBM				0.995	0.133	0.122	0.957	0.434	0.506
Phy × MBM				0.194	0.686	0.689	0.769	0.787	0.847
NE × Phy × MBM				0.227	0.363	0.770	0.383	0.420	0.192

^{a, b} In the same column within the main effect, means with different superscripts are different ($P < 0.05$).

4. Discussion

It was established that MBM that predisposes chickens to NE reduces the performance of broilers while the presence of a high phytase dose and antibiotics in diets containing MBM improves performance (Zanu et al., 2020). The present study was designed to test the hypothesis that over-processed MBM by heating under pressure (autoclaving) would decrease the digestibility of the protein and further exacerbate the incidence of NE. A high level of phytase was examined to potentially compensate for nutrient damage likely to result from over-processed MBM.

The concentration and digestibility of AA in feed ingredients are reduced by exposure to excessive heat. Autoclave processing of MBM for 90 min reduced the levels of heat-labile AA cysteine, lysine and available lysine however the ratio of available to total lysine was unchanged. This indicates complete irreversible destruction of a portion of the lysine. Non-essential AA including hydroxyproline, serine, glutamic acid and glycine were increased as a result of autoclaving. The depressed WG, FCR, and FI in challenged birds due to over-processed MBM agree with the hypothesis of this study. The mechanism behind the poor performance only in challenged birds fed over-processed MBM is herein discussed. Foremost, heat processing under pressure caused nutritional damage to MBM and reduced its lysine availability and quality due to

conformational changes (Hendriks et al., 1999; Buckley et al., 2012). Lysine has a free amino group; therefore, it is often damaged following exposure to heat (Moughan and Rutherford, 1996; Mavromichalis and Baker, 2000; Brestenský et al., 2014). In addition, Shirley and Parsons (2000) determined the effect of pressure processing on AA digestibility of MBM and found that the true digestibility of most AA, especially cysteine and lysine significantly decreased with increasing pressure. The reduction in AA due to pressure heating might have been as a result to the racemization of AA or cross-linking between AA. Further, over-processing might have resulted in the isomerization of L-AA into D-AA and meso-isomers which are poorly digested and unavailable for absorption (Finot, 2005). Cross-linking of peptide bonds between a carbonyl group and amino group (Mallard reaction) during such severe heating processing is another possibility (Heck et al., 2013; Bellagamba et al., 2015).

The implications of poor AA digestibility in over-processed MBM on gut microbiota are clear, in that, bacteria in the gut depend on nutrients from dietary sources which have escaped digestion and absorption in the upper gut or on endogenous secretions (Apajalahti and Vienola, 2016). For instance, the metabolism of bypassed proteins produces total volatile nitrogen consisting of trimethylamine (TMA) and ammonia (NH₃) (He et al., 2015) due to nitrogenous putrefactive fermentation. Many caecal

Table 9
Effect of necrotic enteritis (NE), phytase (Phy) and meat and bone meal (MBM) on apparent ileal digestibility coefficients of CP, C, Ca and P of broilers, d 16 and 29.

Item	Factors			CP		C		Ca		P	
	NE	Phy	MBM	Day 16	Day 29	Day 16	Day 29	Day 16	Day 29	Day 16	Day 29
Treatments ¹											
1	–	500	AR	0.76	0.74 ^{bc}	0.63	0.46	0.47	0.25	0.62	0.39
2	–	5,000	AR	0.80	0.80 ^a	0.66	0.62	0.43	0.32	0.71	0.56
3	–	500	OP	0.74	0.76 ^b	0.61	0.62	0.40	0.21	0.55	0.37
4	–	5,000	OP	0.72	0.77 ^{ab}	0.57	0.60	0.51	0.31	0.73	0.54
5	+	500	AR	0.75	0.72 ^c	0.46	0.56	0.52	0.30	0.67	0.47
6	+	5,000	AR	0.76	0.74 ^{bc}	0.50	0.59	0.59	0.44	0.76	0.66
7	+	500	OP	0.72	0.71 ^c	0.41	0.58	0.66	0.32	0.71	0.47
8	+	5,000	OP	0.72	0.74 ^{bc}	0.44	0.62	0.67	0.43	0.76	0.65
Main effects											
NE	–			0.76	0.77	0.61 ^a	0.58	0.45 ^b	0.27 ^b	0.65 ^b	0.46 ^b
	+			0.74	0.73	0.45 ^b	0.59	0.61 ^a	0.37 ^a	0.73 ^a	0.56 ^a
Phy		500		0.74	0.73	0.52	0.55 ^b	0.51	0.27 ^b	0.64 ^b	0.43 ^b
		5,000		0.75	0.76	0.54	0.61 ^a	0.55	0.37 ^a	0.74 ^a	0.60 ^a
MBM			AR	0.77 ^a	0.75	0.56 ^a	0.56	0.50 ^b	0.33	0.69	0.52
			OP	0.73 ^b	0.75	0.50 ^b	0.60	0.56 ^a	0.32	0.69	0.51
P-value											
NE				0.096	0.001	0.001	0.673	0.001	0.001	0.010	0.001
Phy				0.654	0.001	0.423	0.025	2.000	0.001	0.001	0.001
MBM				0.001	0.390	0.012	0.056	0.049	0.761	0.950	0.571
NE × Phy				0.852	0.835	0.358	0.502	0.916	0.438	0.256	0.741
NE × MBM				0.556	0.963	0.905	0.357	0.083	0.534	0.429	0.807
Phy × MBM				0.231	0.059	0.341	0.082	0.468	0.986	0.664	0.872
NE × Phy × MBM				0.262	0.005	0.437	0.053	0.080	0.583	0.215	0.933

CP = crude protein; C = carbon; Ca = calcium; P = phosphorus; AR = as-received; OP = over-processed.

a, b, c In the same column within the main effect or treatment, means with different superscripts are different ($P < 0.05$).

¹ Three-way interaction was separated by Tukey's mean separation test.

Table 10
Effect of necrotic enteritis (NE), phytase (Phy) and meat and bone meal (MBM) on bacterial quantification (\log_{10} [genomic DNA copies/g of caecal contents]) from broilers, d 16.

Item	Factors			<i>Lactobacillus</i> spp.	<i>Ruminococcus</i> spp.	<i>Bacteroides</i> spp.	<i>Bacillus</i> spp.	<i>Bifidobacteria</i> spp.	Total bacteria	<i>Clostridium perfringens</i>
	NE	Phy	MBM							
Main effects										
NE	–			9.35 ^b	8.86	7.52 ^b	8.15	9.51 ^b	10.47	0.00 ^b
	+			9.72 ^a	8.67	7.84 ^a	8.32	9.76 ^a	10.60	8.92 ^a
Phy		500		9.54	8.74	7.64	8.18	9.54	10.53	4.34
		5,000		9.53	8.80	7.72	8.29	9.72	10.55	4.58
P-value										
NE				0.002	0.189	0.001	0.133	0.015	0.366	0.001
Phy				0.932	0.675	0.375	0.357	0.065	0.880	0.241
MBM				0.155	0.383	0.783	0.408	0.603	0.913	0.769
NE × Phy				0.723	0.954	0.905	0.860	0.608	0.685	0.241
NE × MBM				0.862	0.566	0.786	0.314	0.637	0.664	0.769
Phy × MBM				0.311	0.549	0.597	0.132	0.926	0.286	0.920
NE × Phy × MBM				0.890	0.731	0.543	0.969	0.457	0.584	0.920

a, b In the same column within the main effect, means with different superscripts are different ($P < 0.05$).

bacteria such as *Bacteroides*, *Bifidobacteria*, and *Clostridia* possess putrefactive activity. Ammonia increases the pH of the intestinal contents and favors the growth of *C. perfringens* (Allison and Macfarlane, 1989; Paiva and McElroy, 2014; Qaisrani et al., 2015b). *C. perfringens* is reported to be mainly responsible for the build-up of NH₃ and other odorous metabolites in digesta of NE-challenged chickens (Sharma et al., 2017). The cascading effects of feeding over-processed MBM might explain the poor performance in the current study.

At d 42, high dietary phytase decreased livability. Though reports on the impact of high phytase on livability are rare, the mild decrease in WG and FI in birds fed 5,000 FTU/kg in the challenge group may indicate phytase promoting NE, perhaps through the release of Ca into the hindgut to fuel *C. perfringens* activity or a higher AA release due to the higher phytase activity reaching a maximum absorption and then making these AA more available to the bacteria in the hindgut.

Poor growth and impaired FCR in challenged birds were observed in the current study and this finding corroborates with those of other challenge studies (Perez et al., 2011; Rochell et al., 2016a, 2016b; Wang et al., 2018; Leung et al., 2019). Eimeria infection as part of the challenge model in this study has been reported to damage intestinal epithelial cells (Amerah and Ravindran, 2015; Kraieski et al., 2016). A compromised epithelial lining leads to plasma proteins leaking into the intestinal lumen and serve as substrates for *C. perfringens* (Collier et al., 2008; Amerah and Ravindran, 2015). Further, inflammation due to either Eimeria or *C. perfringens* potentiates T-cell-mediated inflammatory response that results in mucin production providing nutrients for *C. perfringens* (Kleessen et al., 2003; Kim et al., 2013). In addition, malabsorption of nutrients due to damaged intestinal lining might have added to the substrates for *C. perfringens* to proliferate hence the growth depression observed in the challenge birds (Guo et al., 2013; Amerah and Ravindran, 2015; Rochell et al., 2016c).

The effect of over-processed MBM in reducing WG, FCR, and FI on d 35 and 42 is in line with a report of a recent unchallenged study by Bryan et al. (2018). In that study, the indigestibility of protein decreased WG and FCR of broilers at d 32. Previous reports have also suggested a positive correlation between protein digestibility and broiler performance (Cowieson and Bedford, 2009; Cowieson and Roos, 2013), in that, when the diet is deficient in essential AA due to poor protein digestibility WG is reduced.

Gross examination of the duodenum, jejunum, and ileum revealed lesions of NE. The lesions were more severe in the jejunum. The duodenal lesion recorded in the present study was the lowest. It is probable that more severe lesions in the jejunum might be because this region has a median pH of 6.2 (Ravindran, 2013) and allowed *C. perfringens* to proliferate more freely. But the jejunal pH recorded in the study due to challenge was lower. Alternatively, the toxins of *C. perfringens* might have been inactivated by proteolytic enzymes (trypsinogen and chymotrypsinogen) from the pancreatic duct entering the duodenum (Talukdar et al., 2016). Before getting to the jejunum, these enzymes might have been inactivated thus leaving the toxins free to exert their debilitating effect in the jejunum. The jejunum has been reported to be the main site for the absorption of digested products like fat, starch, and protein with the ileum being the site for water and mineral absorption, although some uptake of fat, protein and starch takes place in the ileum (Svihus, 2014). Therefore, a dysfunctional small intestine would impair the digestibility and absorption of these nutrients.

The pH of the gut is important for nutrient bioavailability and intestinal microbiota. In this study, birds fed over-processed MBM and challenged with NE recorded the lowest pH in the jejunum and ileum at d 16 post-challenge. This observation is contrary to what was detected in the preceding trial in the same facility in which normal MBM (5% inclusion) increased the pH in the ileum and the caeca in challenged birds (Zanu et al., 2020). It suggests that MBM as a Ca source has the potential to increase gut pH (Angel et al., 2002) and that over-processing of the MBM was most likely to have reduced the CaCO₃ content through the process of calcination (Barros et al., 2009; Yang et al., 2005; Silva et al., 2019). Again, it is most probable that overheating might have decreased the Ca solubility of over-processed MBM agreeing with report by Kim et al. (2018) that highly soluble Ca sources increase gut pH.

The higher caecal pH in birds fed high phytase and as-received MBM might also suggest that both phytase and as-received MBM released Ca to a certain concentration that elevated the pH. A similar elevation of pH in the ileum and caeca was observed in birds fed MBM (as-received) (5%) and high phytase (1,500 FTU/kg) (Zanu et al., 2020). Therefore, it is not surprising that a possible decrease in CaCO₃ as explained above would have led to a reduction in pH.

The challenge decreased the pH in the crop, jejunum, ileum, and caeca except for the gizzard where it increased the pH. A low gut pH was also reported in a previous NE challenge study (M'Sadeq et al., 2015). It has been argued that *C. perfringens* will grow over a wide pH range, varying from 5.5 to 8.5, though optimum pH for growth 6 to 7 (Setlow and Johnson, 2013). It is important to note that the gut environment, composition, and function of the bacterial community may vary from study to study due to differences in age of the birds, dietary components and facilities used (Kers et al., 2018). Feed withdrawal, for instance, had been reported to cause a significant increase in the pH of the crop contents of the broilers after 12 h of feed withdrawal (Hinton et al., 2000). The reason for the increased crop pH in that study was attributed to a reduction in the population of lactic acid bacteria. Conversely, in this study, the low FI due to the challenge led to an increased pH in the gizzard. The low pH in the jejunum and ileum by over-processing might be due to possible

degradation of CaCO₃ and reduction in solubility (Seiquer et al., 2010; Paiva et al., 2013).

The reduction in nutrient digestibility in the current study as a result of the challenge agrees with previous reports (Timbermont et al., 2011; Amerah and Ravindran, 2015). However, the higher digestibility observed for the minerals (Ca and P) at d 16 and 29 in challenged chickens was rather unexpected. Nonetheless, 2 other studies that preceded the current study had made a similar observation especially for high Ca digestibility in challenged birds. In one of the studies, feeding MBM (5% inclusion rate) led to a higher Ca digestibility in challenged birds (Zanu et al., 2020). Few studies have reported the digestibility of minerals during NE. The only report that was sighted for Ca digestibility in a NE challenged trial in chickens (naturally induced) did not show any trend (Paiva et al., 2014).

The increase in Ca and P digestibility in birds fed high phytase was consistent with previous reports (Walk et al., 2012; Farhadi et al., 2017; Kim et al., 2018). About 61% of the total P found in soybean meal and 72.5% in canola meal are in the form of phytate (Hanna et al., 2017). Therefore, exogenous phytases are routinely added to chicken diets to hydrolyze phytate to liberate Ca and P for absorption, reduce the antinutritional effects of phytate and reduce the cost of inorganic phosphate addition (Liu et al., 2008; Manangi and Coon, 2008; Cowieson et al., 2008; dos Santos et al., 2014; Hamdi et al., 2015).

The lower CP digestibility in the current study on d 16 confirmed the hypothesis of this study, that is, overheating of MBM would damage the AA and reduce their digestibility as also as confirmed in the lower lysine level of over-processed MBM in the present study. The lower AID of CP observed in the challenged birds fed high phytase and as-received MBM compared to those unchallenged but fed similar diets could have been more likely due to the damage incurred on the absorptive surface such that absorption is compromised (and hence AID reduced) but also endogenous losses increased which will result in further reductions in AID of CP. A corresponding lower AID of C was also observed in birds fed over-processed MBM perhaps because of the lower AID of CP since CP forms a significant fraction of the organic component of the diets. The higher digestibility of Ca recorded at d 16 in birds fed over-processed MBM might be due to a decrease in overall solubility of Ca from heating in the autoclave. Paiva et al. (2013) had observed increased AID of Ca in birds fed lower solubility limestone compared to those fed highly soluble calcified seaweed (HSC) at a standard industry level of Ca (0.9%). They explained that because HSC was more soluble than limestone, there was more Ca in the solution that bound to P and precipitated.

The higher counts of *C. perfringens* in the caeca in the present study were expected as the birds were challenged with the same bacteria. This confirmed that the challenge was successful. However, an increase in the count of *Bacteroides* in the challenged birds appeared to confirm a positive correlation between *C. perfringens* and *Bacteroides* spp. counts in previous studies (Zanu et al., 2019b). *Bacteroides* are normal intestinal flora but can evolve into a pathogenic form, and the amounts could increase when the gut is pathologically changed or impaired (Phong et al., 2010). Rather, the increased *Lactobacillus* spp. and *Bifidobacteria* spp. counts due to the challenge was unexpected. This observation contradicts consensus that favorable acid condition in the caeca leads to an increased population of *Lactobacillus* spp. and *Bifidobacteria* spp. thereby outnumbering the population of *C. perfringens* (Olnood et al., 2015; Li et al., 2018). The rather low pH in the challenge group, suggesting a possible lack of putrifaction of protein from endogenous sources entering the caeca, might have favored the growth of *Lactobacillus* spp. and *Bifidobacteria* spp. *Lactobacillus* spp. and *Bifidobacteria* spp. are short-chain fatty acid-producing

bacteria that secrete bacteriocins at an acidic pH to suppress the growth of pathogens in the gastrointestinal tract (La Ragione et al., 2004; Belenguer et al., 2007; Dec et al., 2014).

5. Conclusion

Concluding, this study demonstrated and confirmed the widely held theory that reduced protein digestibility as a result of over-processing could predispose chickens to NE and decrease production performance. Therefore, heat treatment of MBM in commercial rendering plants must be monitored so as not to prompt NE. Additionally, the use of the full matrix value of phytase is recommended when formulating diets when incidence of NE is expected so as not to increase nutrients to promote the activity of *C. perfringens* and reduce growth performance.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, 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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