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Original Research Article

Over-processed meat and bone meal and phytase effects on broilers challenged with subclinical necrotic enteritis: Part 2. Inositol phosphate esters hydrolysis, intestinal permeability, hematology, jejunal gene expression and intestinal morphology

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A B S T R A C T

This study investigated the hypothesis that feeding broilers over-processed meat and bone meal (MBM) would impair gut health in the absence of phytase and in turn, affect inositol phosphate (inositol x-phosphate, IPx: IP3, IP4, IP5 and IP6) ester hydrolysis, intestinal permeability, hematology, jejunal gene expression and intestinal morphology during necrotic enteritis (NE). Ross 308 male broilers (n = 768) were assigned to one of 8 dietary treatments in a 2 × 2 × 2 factorial arrangement, with 6 replicate pens per diet and 16 birds per pen in a completely randomized design. Factors were: NE challenge (no or yes), phytase level (500 or 5,000 FTU/kg) and MBM processing (as-received or over-processed). For the NE challenge, half of the birds were challenged with field strains of *Eimeria* spp. on d 9 and 10^8 CFU/mL of *Clostridium perfringens* strain EHE-NE18 on d 14 and 15. A 3-way challenge, phytase and MBM processing interaction was detected for IP5 (P < 0.05) and IP6 (P < 0.05) levels in the ileum. Birds fed low phytase had increased IP5 and IP6 in unchallenged birds only when diets contained over-processed MBM. Challenge with NE increased intestinal permeability as measured by serum fluorescein isothiocyanate dextran (FITC-d; P < 0.001), increased white blood cells (WBC; P < 0.001), decreased mean corpuscular volume (MCV; P < 0.001) and mean corpuscular hemoglobin (MCH; P < 0.05), and decreased crypt-to-villi ratio (P < 0.05). The over-processed MBM reduced the villi-to-crypt ratio (P < 0.05). A 3-way challenge × phytase × MBM processing interaction was detected for mucin 2 (*MUC-2*) expression (P < 0.05) where only in unchallenged birds fed over-processed MBM did high phytase reduce *MUC-2* expression. A lower expression of aminopeptidase N (*APN*; P < 0.001) and vitamin D receptor (*VDR*; P < 0.001) were recorded in NE challenged birds. In conclusion, NE has a negative impact on the gut and hematology of broilers, but its effect on phytate hydrolysis is minimal.

1. Introduction

The gastrointestinal tract or gut plays a key role in the performance and health of chickens. The gut is constantly exposed to a variety of harmful factors including dietary factors that have a negative impact on intestinal health and function. Compromised gut health leads to inefficiencies in the digestion and absorption of nutrients. In such a situation, nutrients are partitioned to maintain gut health through the repair of enterocytes, secretion of mucus and immune response rather than on growth (Celi et al., 2017). One of the major threats to the gut in present-day poultry production is necrotic

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enteritis (NE). Because of its considerable impact on intestinal health, NE is known to be of economic importance. *Clostridium perfringens* is the causative bacteria and the infection is triggered by the presence of cocci and dietary factors such as pentosans in wheat and rye, hexosans in barley, or diets high in antigenic or poorly digested protein sources. Therefore, there has been considerable research on gut health necessitated by the withdrawal of in-feed antibiotics, antimicrobials and anticoccidials in poultry worldwide.

The onset of NE does not start unless there are predisposing factors (Palliyeguru et al., 2010; Guo et al., 2013; Knight et al., 2016). One suggested precursor is undigested proteins in meat and bone meal (MBM) (M'Sadeq et al., 2015). The fermentation of these undigested proteins by *C. perfringens* leads to the production of biogenic amines, total volatile nitrogen (TVN) and ammonia (Qaisrani et al., 2014, 2015; Ma et al., 2017). These metabolites increase the pH in the gut and favor the activity of *C. perfringens.* A recent study has confirmed that MBM increases the proliferation of *C. perfringens* and decreases the performance of broilers fed high phytase without antibiotics (Zanu et al., 2020a). The use of exogenous phytase at higher levels than normally practiced can facilitate the reduction or removal of MBM because phytase releases P and increases amino acid (AA) digestibility of plant proteins. This reduces the need for animal protein supplements such as MBM. Phytase supplementation dephosphorylates phytic acid and decreases the negative impact of this antinutritional factor on the digestive tract. Phytase may also improve the general health and immune status of broilers (Liu et al., 2008; Bedford and Cowieson, 2012; Kiarie et al., 2013; Ptak et al., 2015).

The objective of this work is to provide a mode of action for how processing of MBM affects the onset of NE. The use of high levels of dietary phytase might alleviate the negative impact of NE through improved protein and amino acid digestion and absorption in birds fed diets containing over-processed MBM. Phytate degradation is rarely reported in research papers and so was also the focus of this research. These effects might be measured as changes in intestinal permeability, hematology, intestinal morphology and jejunal gene expression in broilers during NE.

2. Materials and methods

2.1. Birds management and dietary treatments

Details of the management and dietary treatment offered to the birds are described (Zanu et al., 2020b). Briefly, experimental procedures were approved by the University of New England's Animal Ethics Committee. A total of 768 day-old Ross 308 male broiler chicks were used in this study. Eight diets were formulated in accordance with Ross 308 standard specifications. Treatments were arranged in a 2 × 2 × 2 factorial design. Factors were NE challenge (no or yes), phytase (500 or 5,000 FTU/kg) (Quantum Blue, AB Vista, Malborough, UK) and MBM processing (as received or over-processed). The phytase matrix values for 500 FTU/kg were applied in both the 500 and 5,000 FTU/kg phytase groups. The diets were offered ad libitum throughout the starter phase (d 0 to 14) in crumbled form, and in the grower (d 14 to 28) and finisher (d 28 to 42) in pelleted form. MBM was autoclaved at a temperature of 128 °C at 2 bars for 90 min in an autoclave (Hirayama manufacturing corporation, Saitama, Japan) and designated as over-processed MBM. The unautoclaved MBM was designated as as-received.

2.2. NE 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 *Eimeria maxima* and 2,500 oocytes of *Eimeria brunetti* (Eimeria Pty Ltd., Australia) in 1 mL of 1% (wt/vol) sterile saline at d 9, and 10^8 CFU of *C. perfringens* Strain EHE-NE18 (known to express NetB toxin, Commonwealth Scientific and Industrial Research Organization, Geelong, Australia) at d 14 and again on d 15. Non-challenged birds received buffer instead of *Eimeria* and sterile broth instead of *C. perfringens.*

2.3. Phytate esters determination

The determination of phytate and its intermediate derivatives in the experimental diets and digesta at d 29 was carried out according to the procedures of Walk et al. (2018). Briefly, freeze-dried gizzard and ileal digesta samples were extracted with 10 mL of 0.5 mol/L HCl for 1 h at 20 °C by ultrasonication. The extracts were then centrifuged for 10 min at 2,200 × *g*, and 5 mL of the supernatant was evaporated to dryness in a vacuum centrifuge. The samples were then re-dissolved in 1 mL of distilled, deionized water by ultrasonication for 1 h at 20 °C and centrifuged for 15 min at 18,000 × *g*. The resulting supernatant was filtered through a 13-mm syringe filter with a 0.45-μm membrane (GH Polypro Acrodisc, Pall Corporation, Ann Arbor, MI) and placed in a 30-kDa centrifugal filter (Microcon Ultracel YM30, Millipore Corporation, Bedford, MA) and finally centrifuged for 30 min at 9,000 × *g*. Quantification of inositol phosphates (IP3 to IP6) was performed using high-performance ion chromatography and UV detection at 290 nm after post-column derivatization. Myoinositol was determined using high-performance liquid chromatography with pulsed amperometric detection.

2.4. Gut permeability with fluorescein isothiocyanate dextran (FITC-d)

Two birds per pen were gavaged with 4.17 mg/kg solution per body weight of fluorescein isothiocyanate dextran solution (FITC-d; Sigma–Aldrich, Sweden; average molar weight of 4,000 and FITC-d: glucose of 1:250) at 4.17 mg/kg body weight on d 16. After exactly 150 min, blood samples were taken from 2 birds per pen after electrical stunning and decapitation. The blood samples were centrifuged at 3,000 × *g* for 15 min, and the serum samples were collected and stored at −20 °C until used. The serum samples (10 mL) were diluted in phosphate buffer saline (10 mL) in the ratio of 1:1. The 20-mL serum and phosphate buffer saline mixture were placed into a 96-well black plate. Fluorescence was measured with excitation at 485 nm and emission at 528 nm using SpectraMax M2e Microplate Reader, Molecular Devices, USA. Levels of fluorescence in the samples were converted to the respective FITC-d microgram per milliliter of serum based on a calculated standard curve.

2.5. Blood collection and hematology

On d 16 post-hatch, blood samples were collected from the jugular veins of the 2 birds used for FITC-d measurement into a plastic blood tube (spray-coated with K_2EDTA) for the determination of full blood count. The blood sample tubes were immediately rolled for 4 min and analyzed for complete blood cell counts using a CELL-DYN 3700 automated blood analyzer (Abbott Laboratories, Abbott Park, USA). The hematologic variables measured included leukocytes, neutrophils, lymphocytes, monocytes, eosinophils, basophils, red blood cells (RBC), hemoglobin (Hgb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and thrombocytes.

2.6. Intestinal morphology

Jejunal samples were collected from 2 birds from each pen on d 16 and fixed in 10% buffered formalin until processing. The tissue samples were processed in a Leica TP 1020 45 processor (GMI Inc., Ramsey, MN, USA) according to the program as follows: 30% ethanol for 2 h, 50% ethanol for 2 h, 70% ethanol for 2 h, 80% alcohol for 2 h, 95% ethanol for 1 h, absolute alcohol for 1 h, absolute ethanol for 1 h, 50:50 (vol/vol) ethanol:xylol for 1 h, xylol for 1 h, xylol for 1 h, paraffin for 2 h, and paraplast + vacuum for 2 h. Tissue blocks were prepared and sectioned. The tissue blocks were cut into 6-μm cross-section thickness and mounted on glass slides. The slides were then stained using Harris's hematoxylin and eosin staining method. The cross-sections were viewed by light microscopy (Olympus CX41 microscope) using a 10× objective and color images captured with the software Analysis 5.0 (Olympus Soft Imaging Solutions GmbH, Münster, Germany). Five muscularis layers, villi height, total height (muscularis layer height + villi height), crypt depth, basal width and apical width per section and 4 sections per sample were analyzed. The apparent villi surface area was calculated as: (basal width + apical width)/2 × villus length.

2.7. RNA extraction and cDNA synthesis

For RNA extraction, the jejunal tissues were rinsed with chilled phosphate-buffered saline and immediately placed in RNAlater (Invitrogen, Carlsbad, CA), stored at 4 °C for 3 to 5 h and then stored at −20 °C until further use. Approximately 80 mg of the sample tissue was homogenized in 1-mL TRIsure (Bioline, Sydney, Australia) using an IKA T10 basic Homogenizer (Wilmington, NC, USA) and the total RNA was extracted as per the manufacturer's instructions. Total RNA was then purified using a ISOLATE II RNA Mini kit (Bioline, Sydney, Australia) as per the manufacturer's instructions and a DNA digestion step using DNase I was included to eliminate genomic DNA. A NanoDrop ND-8000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Waldbronn, Germany) were used to determine the concentration and RNA integrity (RNA integrity number, RIN) respectively. Purified RNA from each sample was reverse-transcribed to cDNA with the SensiFAST cDNA synthesis kit (Bioline, Sydney, Australia) as per the manufacturer's instructions. A Rotorgene 6000 real-time PCR instrument (Corbett, Sydney, Australia) was used to convert the RNA into cDNA. Synthesized cDNA samples were diluted 1:10 with nuclease-free water and stored at −20 °C until used.

2.8. Primer sources

Primers for real-time quantitative PCR (qPCR) were either sourced from published papers or designed with NCBI primer tool (https://www.ncbi.nlm.nih.gov). Table 1 presents the primers that were used in this study. An Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Germany) was employed to determine the primer specificity for each pair using an Agilent DNA 1000 Kit (Agilent Technologies, Inc., Germany). Only the specific primer pairs with high efficiency were used in this study.

2.9. qPCR

Real-time quantitative PCR was performed using a Rotorgene 6000 real-time PCR machine (Corbett Research, Sydney, Australia). The qBase + version 3.0 (Biogazelle, Zwijnbeke, Belgium) software was employed to determine the 2 most stable genes among 7 (18S, β-actin [*ACTB*], glyceraldehyde-3-phosphate dehydrogenase [*GAPDH*], hypoxanthine phosphoribosyltransferase 1 [*HPRT1*], hydroxymethylbilane synthase [*HMBS*], TATA-box binding protein [*TBP*] and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation [*YWHAZ*]) different reference genes. Based on the expression stability *HPRT1* and *TBP* were used to normalize the target genes in the jejunum. The target genes (Table 1) determined were: claudin 1 (*CLDN1*), tight junction protein 1 (*TJP1*), junctional adhesion 2 (*JAM2*), occludin (*OCLD*), mucin 2 (*MUC-2*), mucin 5 (*MUC5AC*), aminopeptidase N (*APN*), calbindin 1 (*CALB1*), ATPase Na^+/K^+ transporting subunit alpha 1 (*ATP1A1*), ATP synthase subunit alpha (*ATP5A1W*), calcium-sensing receptor (*CaSR*), calcium channel, voltage-dependent, P/Q type alpha 1A subunit (*CACNA1A*), Na-dependent Pi cotransporters, type IIb (*NaPi-IIb*) and vitamin D receptor (*VDR*). The relative expression of genes using the arithmetic mean method in qBase+ was exported to SAS 9.3 package for further analysis.

2.10. Statistical analysis of data

The data were analyzed as a 2 × 2 × 2 factorial arrangement of treatments using Minitab 19 statistical software to assess the main effects and 2 or 3-way interactions, with the 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.

3. Results

3.1. Phytate esters degradation in the ileum

Table 2 shows a 3-way challenge, phytase and MBM processing interaction for IP5 ($P < 0.05$) and IP6 ($P < 0.05$) hydrolysis. Birds fed low phytase greatly increased IP5 and IP6 in unchallenged birds only when diets contained over-processed MBM. A challenge × phytase interaction was detected for IP3 ($P < 0.05$). There was higher IP3 detected in unchallenged birds fed high phytase and lower IP3 concentration in challenged birds fed high phytase. The IP3 degradation in birds fed low phytase was not affected by the challenge. As a main effect, both challenge and high phytase dose decreased the concentration of IP4 ($P < 0.001$) and both increased the concentration of inositol ($P < 0.001$).

3.2. Gut permeability with fluorescein isothiocyanate dextran (FITC-d) and leucocytes, d 16

The challenge as a main effect increased the concentration of serum FITC-d ($P < 0.001$) as shown in Table 3. High phytase tended to increase serum FITC-d concentration ($P = 0.067$). The challenge as a main effect increased the concentration of white blood cells (WBC) ($P < 0.001$), Heterophils ($P < 0.001$), monocytes ($P < 0.05$), eosinophils ($P < 0.001$) and basophils ($P < 0.001$), but it decreased the concentration of lymphocytes ($P < 0.05$) (Table 3).

3.3. Erythrocytes, d 16

The challenge as a main effect decreased the concentration of MCV ($P < 0.001$) and MCH ($P < 0.05$), with no observed interactions. The challenge tended to increase the concentration of RBC ($P = 0.079$) (Table 4).

3.4. Jejunal morphology

Table 5 shows that the challenge as a main effect decreased the total height (muscularis layer height + villi height) of the

Table 1
Sequences of primers used for real-time quantitative PCR.

Gene symbol	Group	Primer sequence (5′-3′)	Tm, °C	Amplicon size, bp	Reference	Function
CLDN1	Tight junction protein	F-CTTCATCATTGCAGGTCTGTCAG R-AAATCTGGTGTTAACGGGTGTG	60	103	Self-designed	Maintenance of intestinal barrier function
JAM2	Tight junction protein	F-AGACAGGAACAGGCAGTGCTAG R-ATCCAATCCCATTTGAGGCTAC	60	135	Self-designed	Maintenance of intestinal barrier function
TJP1	Tight junction protein	F-GGATGTTTATTTGGGCGGC R-GTCACCGTGTGTTGTTCCCAT	60	187	Self-designed	Maintenance of intestinal barrier function
OCLD	Tight junction protein	F-ACGGCAGCACCTACCTCAA R-GGGCGAAGAAGCAGATGAG	60	123	Du et al. (2016)	Maintenance of intestinal barrier function
NaPi-IIb	Phosphorus transporter	F-CGGTCCGTTCACTCTGTTGC R-GAAGCCACGTTGCCTTTGTG	60	166	Self-designed	Mediation of intestinal phosphate uptake
VDR	Vitamin D transporter	F-ACGCAGACATGGACACCGT R-GGACAGGTGAACATCGCTTTC	60	193	Self-designed	Provides instructions for making vitamin D receptor
CALB1	Calcium transporter	F-GGCAGGCTTGGACTTAACACC R-GTCGGCAACACCTGAGCAAG	60	105	Self-designed	Transport protein of calcium
ATP1A1	Calcium transporter	F-GTCAACCCGAGGGATGCTAA R-ACTGCTACAATGGCACCCTG	60	179	Kheravii et al. (2018)	provides instructions for making Na^+/K^+ ATPase
ATP5A1W	Calcium transporter	F-GGCAATGAAACAGGTGGCAG R-GGGCTCCAGCTTGTCTAAGTGA	60	232	Self-designed	Synthesis of ATP
APN		F-AATACGCGCTCGAGAAAACC R-AGCGGGTACGCCGTGTT	60	70	Gilbert et al. (2007)	Final digestion of peptides by N terminus cleavage
CaSR	Calcium transporter	F-CTGCGTGATTTGGCTCTACA R-GGCAAAGAAGAAGCAGATGG	60	160	Lee and Kim (2018)	Regulator of parathyroid hormone synthesis and secretion and systemic calcium homeostasis.
CACNA1A	Calcium channel	F-GCAGCGGGTCTATAAGCAGT R-GCGATGATGGTGGCTAAAAT	60	198	Lee and Kim (2018)	Provides instructions for making calcium channels
MUC-2	Inflammatory genes	F-CCCTGGAAGTAGAGGTGACTG R-TGACAAGCCATTGAAGGACA	143	60	Fan et al. (2015)	The physical and biological barrier protecting mucous epithelia
MUC5AC	Inflammatory genes	F-AAGACGGCATTTATTTCTCCAC R-TCATTACCAACAAGCCAGTGA	244	60	Fan et al. (2015)	The physical and biological barrier protecting mucous epithelia
HPRT1	Housekeeping genes	F-ACTGGCTGCTTCTTGTG R-GGTTGGGTTGTGCTGTT	63	245	Yang et al. (2013)	purine synthesis in salvage pathway
TBP	Housekeeping genes	F-TAGCCCGATGATGCCGTAT R-GTTCCCTGTGTCGCTTGC	62	147	Li et al. (2005)	is a general transcription factor that binds specifically to a DNA sequence called the TATA box

Tm = annealing temperature; *CLDN1* = claudin 1; *JAM2* = junctional adhesion 2; *TJP1* = tight junction protein 1; *OCLD* = occludin; *NaPi-IIb* = Na-dependent Pi cotransporters, type IIb; *VDR* = vitamin D receptor; *CALB1* = calbindin 1; *ATP1A1* = ATPase Na^+/K^+ transporting subunit alpha 1; *ATP5A1W* = ATP synthase subunit alpha; *APN* = aminopeptidase N; *CaSR* = calcium-sensing receptor; *CACNA1A* = calcium channel, voltage-dependent, P/Q type alpha 1A subunit; *MUC-2* = mucin 2; *MUC5AC* = mucin 5AC; *HPRT1* = hypoxanthine phosphoribosyltransferase 1; *TBP* = TATA-box binding protein.

epithelium ($P < 0.001$) and villus ($P < 0.001$), as well as the villus height-to-crypt depth ratio ($P < 0.001$) and apparent villi area ($P < 0.05$). Crypt depth ($P < 0.001$) and apical width ($P < 0.001$) was however increased by the challenge. The challenge tended to increase basal width ($P = 0.077$). The over-processed MBM as a main effect decreased villus height-to-crypt depth ratio ($P < 0.05$). No effect of phytase was detected for any of the morphological parameters measured ($P > 0.05$).

3.5. Gene expression

Table 6 shows the mRNA expression of genes encoding tight junction proteins and mucins. A 3-way challenge, phytase and MBM interaction was detected for *MUC-2* ($P < 0.05$). In the unchallenged groups, *MUC-2* increased in birds fed high phytase without over-processed MBM, whereas in challenged birds *MUC-2* was lower in birds fed as-received MBM with high doses of phytase. In the challenged groups, *MUC-2* was not different between birds fed different levels of phytase or different processed MBM. The NE challenge decreased expression of *TJP1* ($P < 0.001$) and *OCLD* ($P < 0.001$) and tended to increase the expression of *CLDN1* ($P = 0.084$).

Table 7 shows mRNA expression of genes encoding for nutrient transporters, receptors and digestive enzymes. The challenge decreased the expression of *APN* ($P < 0.001$), *ATP1A1* ($P < 0.001$), *ATP5A1W* ($P < 0.001$), *CACNA1* ($P < 0.05$), *NaPi-IIb* ($P < 0.001$), and *VDR* ($P < 0.001$), and tended to downregulate *CALB1* ($P = 0.078$). A tendency for a challenge × phytase × MBM interaction was detected for *CaSR* ($P = 0.070$) where the expression of *CaSR* was highest in birds not challenged and fed low phytase and over-processed MBM. A tendency for a NE × MBM interaction was detected for *ATP1A1* ($P = 0.053$) and *NaPi-IIb* ($P = 0.097$) where the expression of both genes tended to decrease in challenged birds when fed over-processed MBM. A tendency of phytase × MBM interaction was detected for *NaPi-IIb* ($P = 0.056$) where birds fed low phytase and over-processed MBM exhibited a higher expression of *NaPi-IIb*. In birds fed high phytase, the expression was not different in birds fed either MBM.

4. Discussion

This study showed that application of 5,000 FTU/kg exogenous phytase, being well above the standard dose of 500 FTU/kg improved the degradation of phytate ester in both unchallenged and challenged birds. Also, it demonstrated that over-processed MBM impaired gut epithelia and that NE had a negative effect on epithelial tight junctions, immune system, blood counts, the expression of nutrient transporting genes and jejunal morphology.

Table 2
Effect of necrotic enteritis (NE), phytase (Phy) and meat and bone meal (MBM) on phytate esters and inositol concentration (μmol/g DM) in the ileal digesta of broilers, 29 d post-hatch[1].

Item	NE	Phy[2], FTU/kg	MBM	IP3	IP4	IP5	IP6	Inositol
Treatments								
1	–	500	AR	2.31	7.71	5.75[b]	17.80[b]	5.28
2	–	5,000	AR	2.96	5.85	0.33[c]	1.09[c]	14.91
3	–	500	OP	2.17	8.05	9.20[a]	27.38[a]	5.055
4	–	5,000	OP	3.11	6.90	0.50[c]	1.41[c]	13.50
5	+	500	AR	1.86	5.66	5.21[b]	16.09[b]	13.74
6	+	5,000	AR	0.86	1.28	0.12[c]	0.77[c]	22.79
7	+	500	OP	2.40	6.22	4.85[b]	14.51[b]	10.66
8	+	5,000	OP	1.85	3.26	0.35[c]	1.25[c]	21.41
Two-way interactions								
NE × Phy								
	–	500		2.24[ab]	7.88	7.48	22.61	5.17
	–	5,000		3.03[a]	6.37	0.42	1.25	14.20
	+	500		2.13[ab]	5.94	5.03	15.31	12.20
	+	5,000		1.36[b]	2.27	0.23	1.01	22.10
Main effects								
NE	–			2.64	7.13[a]	3.95	11.93	9.69[b]
	+			1.74	4.10[b]	2.63	8.16	17.15[a]
Phy		500		2.18	6.91[a]	6.25	18.96	8.69[b]
		5,000		2.20	4.32[b]	0.33	1.13	18.15[a]
P-values								
NE				0.002	0.001	0.003	0.010	0.001
Phy				0.963	0.001	0.001	0.001	0.001
MBM				0.171	0.146	0.039	0.121	0.137
NE × Phy				0.007	0.111	0.008	0.015	0.670
NE × MBM				0.174	0.668	0.026	0.055	0.485
Phy × MBM				0.506	0.424	0.106	0.202	0.898
NE × Phy × MBM				0.882	0.795	0.022	0.048	0.477

IP3 = inositol tri-phosphate; IP4 = inositol tetra-phosphate; IP5 = inositol penta-phosphate; IP6 = inositol hexa-phosphate; 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 3
Effect of necrotic enteritis (NE), phytase (Phy) and meat and bone meal (MBM) on FITC-D, leucocytes of broilers, d 16 post-hatch[1].

Item	NE	Phy[4]	MBM	FITC-D, μg/mL	WBC, 10^6/mL	Heterophils, 10^6/mL (3 to 17)[2]	Lymphocytes, 10^6/mL (10 to 30)[2]	Monocytes, 10^6/mL (0 to 5)[2]	Eosonophils, 10^6/mL (0 to 0.5)[2]	Basophils, 10^6/mL (0.3 to 2.5)[3]
Main effects										
NE	–			0.081[b]	44.62[b]	6.82[b]	27.28[a]	6.93[b]	0.01[b]	3.58[b]
	+			0.208[a]	82.72[a]	37.12[a]	20.02[b]	22.43[a]	2.57[a]	16.14[a]
P-values										
NE				<0.001	<0.001	<0.001	0.010	0.018	<0.001	<0.001
Phy				0.067	0.341	0.835	0.437	0.200	0.694	0.456
MBM				0.121	0.930	0.922	0.755	0.673	0.659	0.381
NE × Phy				0.442	0.842	0.773	0.967	0.504	0.691	0.835
NE × MBM				0.413	0.578	0.931	0.560	0.539	0.664	0.492
Phy × MBM				0.256	0.836	0.510	0.860	0.565	0.488	0.419
NE × Phy × MBM				0.615	0.663	0.618	0.760	0.476	0.486	0.606

FITC-D = fluorescein isothiocyanate dextran; WBC = white blood cells.
[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] Data in parentheses are a reference range reported by Frazer et al. (1991).
[3] Data in parentheses are a reference range reported by Douglas (2000).
[4] Phy: Quantum Blue 5G.

4.1. Phytate esters degradation

Supplementation of diets with exogenous phytase increases the degradation of phytate and phytate esters. In the current study, by increasing phytase from 500 to 5,000 FTU/kg the degradation of IP5 and IP6 were substantially increased along with a higher concentration of inositol, which agrees with the results of other studies (Sommerfeld et al., 2017, 2019; Ingelmann et al., 2018). This higher production of inositol is beneficial for cell survival and growth, lipid metabolism, and insulin sensitivity (Holub, 1986; Michell, 2008; Jia et al., 2019). Also, inositol, either in the free form or released as a result of phytase supplementation, has also been reported to improve growth performance in chickens (Cowieson et al., 2013; Walk et al., 2014).

Also worthy of note is the fact that the hydrolysis of IP5 and IP6 was more pronounced in the challenged birds, particularly when

Table 4
Effect of necrotic enteritis (NE), phytase (Phy) and meat and bone meal (MBM) on erythrocytes of broilers, d 16 post-hatch[1].

Item	NE	Phy[3]	MBM	RBC, 10^9/mL (2.5 to 3.5)[2]	Hgb, g/dL (7 to 13)[2]	PCV, % (22 to 52)[2]	MCV, fL (90 to 140)[2]	MCH, pg (33 to 47)[2]	MCHC, g/dL (26 to 35)[2]	Platelets, 10^6/mL
Main effects										
NE	–			2.35	12.91	27.14	115.47[a]	54.95[a]	47.60	1.40
	+			2.51	13.53	26.96	105.50[b]	51.78[b]	48.09	1.15
P-values										
NE				0.079	0.152	0.850	<0.001	0.049	0.731	0.525
Phy				0.354	0.121	0.289	0.454	0.923	0.984	0.269
MBM				0.302	0.585	0.320	0.359	0.881	0.886	0.870
NE × Phy				0.579	0.985	0.535	0.332	0.861	0.831	0.639
NE × MBM				0.444	0.897	0.622	0.591	0.797	0.972	0.518
Phy × MBM				0.262	0.155	0.179	0.205	0.092	0.153	0.371
NE × Phy × MBM				0.440	0.618	0.433	0.349	0.294	0.293	0.779

RBC = red blood cells; Hgb = hemoglobin; PCV = packed cell volume; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration.
[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] Data in parentheses are a reference range reported by Douglas (2000).
[3] Phy: Quantum Blue 5G.

Table 5
Effect of necrotic enteritis (NE), phytase (Phy) and meat and bone meal (MBM) on jejunal morphology, 16 post-hatch[1].

Item	NE	Phy	MBM	Total height, µm	Muscularis layer height, µm	Crypt depth, µm	Villi height, µm	Basal width, µm	Apical width, µm	Villi height: Crypt depth	Apparent villi area, × 10^3 µm^2
Main effects											
NE	–			1,478[a]	145.79	128.96[b]	1203[a]	59.39	31.93[b]	9.47[a]	54.84[a]
	+			1,211[b]	145.46	215.05[a]	850[b]	63.64	42.03[a]	4.11[b]	45.00[b]
MBM			AR	1,366	147.79	165.18	1053	61.37	36.66	7.20[a]	51.09
			OP	1,323	143.46	178.83	1001	61.66	37.30	6.38[b]	48.75
P-values											
NE				<0.001	0.964	<0.001	<0.001	0.077	<0.001	<0.001	0.005
Phy				0.747	0.656	0.858	0.726	0.960	0.575	0.792	0.679
MBM				0.421	0.548	0.146	0.330	0.901	0.729	0.041	0.479
NE × Phy				0.463	0.713	0.704	0.454	0.801	0.076	0.330	0.926
NE × MBM				0.581	0.889	0.792	0.627	0.324	0.201	0.223	0.717
Phy × MBM				0.233	0.233	0.153	0.435	0.801	0.796	0.888	0.766
NE × Phy × MBM				0.815	0.650	0.189	0.599	0.134	0.639	0.489	0.777

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. Total height = muscularis layer height + villi height.

low phytase was present. The higher degradation in the challenged birds might have been necessitated by either the lower pH recorded in the ileum at d 29 or lower feed (phytate) intake at d 28 as reported (Zanu et al., 2020a). The lower the pH, the less negatively charged IP6 becomes, and its ability to chelate positively charged ions is reduced (Bedford and Rousseau, 2017). This suggests that at a higher ileal pH as detected in the unchallenged birds in this study, potential chelators might have been precipitated by phytate and decreased hydrolysis by phytase. The higher destruction of the IP in the challenged birds also translated into a higher inositol production. However, a buildup of calcium and amino acids in the lower gut (during the severe stages of the NE infection) might feed pathogenic enteric bacteria such as *C. perfringens* and induce NE, unless birds are protected with antimicrobials (Zanu et al., 2020a). It is worth noting that the determination of phytate esters was conducted at d 29 (14 days post-challenge) when the challenge effect might have waned.

4.2. Intestinal permeability

The intestinal mucosa functions in the secretion of mucus and enzymes, absorption of nutrients, provision of barrier between the external and internal environments and maintenance of epithelial integrity in healthy chickens (Uni et al., 1998; Amat et al., 1999; Kamada et al., 2013). But inflammation due to NE disrupts the epithelial tight junctions and increases serum FITC-d (Vicuña et al., 2015a, 2015b) and possible bacterial translocation (Quinteiro-Filho et al., 2012; Tellez et al., 2014). Several reports support the fact that NE damages gut epithelium and increases mucosal permeability. The tendency of high phytase to increase the marker in the present study was unexpected as phytase has been reported to boost the immune system through increased lymphocyte numbers and mucosal antibodies (Liu et al., 2008). It is possible that high phytase might have released large amounts of Ca into the lower region of the intestine thus stimulating the growth of *C. perfringens*. Evaluation of phytase, Ca and P during a natural NE outbreak (Paiva et al., 2013, 2014) indicated that higher phytase increased mortality suggesting increasing the Ca supply to aid the activity of *C. perfringens*. Toxins secreted by *C. perfringens* have been reported to cause the death of enterocytes (Navarro et al., 2018).

4.3. Hematology

Hematological indices are helpful in detecting the effects of infections and stress on animals. Measurement of WBC is used as an indicator of immune response to an infection or parasites, or an indication of toxicant-induced immunosuppression (Huff et al., 2000; Genovese et al., 2007; Davis et al., 2008). In the current

Table 6
Effect of necrotic enteritis (NE), phytase (Phy) and meat and bone meal (MBM) on tight junction proteins and mucins secreting genes, d 16 post-hatch[1].

Item	NE	Phy[2], FTU/kg	MBM	*CLDN1*	*TJP1*	*JAM2*	*OCLD*	*MUC-2*	*MUC5AC*
Treatment means									
1	−	500	AR	0.683	1.091	0.809	1.355	1.449[ab]	1.159
2	−	5,000	AR	0.968	1.201	1.134	1.519	1.858[a]	1.194
3	−	500	OP	1.075	1.128	1.013	1.407	1.781[a]	1.159
4	−	5,000	OP	0.991	1.203	0.989	1.543	1.365[abc]	1.039
5	+	500	AR	1.017	0.828	1.004	0.988	0.802[bcd]	1.166
6	+	5,000	AR	1.346	0.869	0.968	0.680	0.522[d]	0.927
7	+	500	OP	1.262	0.822	0.913	0.601	0.578[cd]	0.916
8	+	5,000	OP	1.435	0.836	0.993	0.507	0.538[d]	0.974
Main effects									
NE	−			0.929	1.156[a]	0.986	1.456[a]	1.613	1.138
	+			1.265	0.839[b]	0.970	0.694[b]	0.610	0.996
P-values									
NE				0.084	<0.001	0.836	<0.001	<0.001	0.235
Phy				0.360	0.494	0.282	0.803	0.513	0.578
MBM				0.329	1.000	0.982	0.248	0.460	0.451
NE × Phy				0.693	0.708	0.422	0.096	0.530	0.839
NE × MBM				0.916	0.820	0.693	0.130	0.924	0.920
Phy × MBM				0.493	0.861	0.467	0.654	0.243	0.765
NE × Phy × MBM				0.780	0.982	0.152	0.564	0.038	0.344

CLDN1 = claudin 1; *TJP1* = tight junction protein 1; *JAM2* = Junctional adhesion 2; *OCLD* = occluding; *MUC-2* = mucin 2; *MUC5AC* = mucin 5; AR = as-received; OP = over processed.
[a,b,c,d] 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 7
Effect of necrotic enteritis (NE), phytase (Phy) and meat and bone meal (MBM) on nutrient transporting genes, d 16 post-hatch[1].

Item	NE	Phy[2], FTU/kg	*MBM*	*APN*	*CALB1*	*ATP1A1*	*ATP5A1W*	*CaSR*	*CACNA1A*	*NaPi-IIb*	*VDR*
Two-way interactions											
NE × MBM	−		AR	1.749	1.217	1.193	1.540	0.964	1.311	1.117	1.644
	−		OP	1.901	1.470	1.350	1.639	1.213	1.466	1.390	1.554
	+		AR	0.671	1.067	0.796	0.933	0.961	0.926	0.918	0.833
	+		OP	0.466	0.882	0.692	0.751	0.899	0.929	0.796	0.564
Phy × MBM		500	AR	1.220	1.184	0.994	1.256	0.921	1.241	0.916	1.092
		500	OP	1.361	1.371	1.127	1.237	1.130	1.299	1.220	1.046
		5,000	AR	1.200	1.100	0.995	1.217	1.004	0.996	1.120	1.384
		5,000	OP	1.006	0.981	0.916	1.153	0.982	1.096	0.966	1.073
Main effects											
NE	−			1.825[a]	1.343	1.272[a]	1.590[a]	1.088	1.388[a]	1.253[a]	1.599[a]
	+			0.569[b]	0.975	0.744[b]	0.842[b]	0.930	0.927[b]	0.857[b]	0.699[b]
P-values											
NE				<0.001	0.078	<0.001	<0.001	0.069	0.011	<0.001	<0.001
Phy				0.219	0.251	0.116	0.561	0.706	0.206	0.828	0.414
MBM				0.861	0.869	0.689	0.696	0.279	0.652	0.519	0.358
NE × Phy				0.893	0.229	0.133	0.257	0.892	0.643	0.266	0.199
NE × MBM				0.241	0.289	0.053	0.188	0.075	0.664	0.097	0.647
Phy × MBM				0.273	0.456	0.113	0.830	0.182	0.903	0.056	0.496
NE × Phy × MBM				0.159	0.410	0.175	0.355	0.070	0.495	0.675	0.999

APN = aminopeptidase N; *CALB1* = calbindin 1; *ATP1A1* = ATPase Na^+/K^+ transporting subunit alpha 1; *ATP5A1W* = ATP synthase subunit alpha; *CaSR* = calcium-sensing receptor; *CACNA1* = calcium channel, voltage-dependent, P/Q type alpha 1A subunit; *NaPi-IIb* = Na-dependent Pi cotransporters, type IIb; *VDR* = vitamin D receptor; 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.

study, the challenge was associated with higher counts of WBC and its differentials (heterophils, monocytes, eosinophils and basophils), except for lymphocytes which were reduced in the challenged birds. An increased WBC count in the presence of elevated heterophils, as was observed in the present study, has been suggested as being an indication of inflammation (Swaggerty et al., 2004). Similarly, high basophil counts observed in the current study have also been reported to be associated with inflammatory response in other studies (Yuk et al., 2017). In other NE studies, higher RBC and PCV were detected (Ruhnke et al., 2017), but in the present study they were unresponsive to the challenge. A higher RBC and PCV are common features in dehydrated birds, especially during disease conditions. This may be due to RBC becoming more concentrated due to dehydration. Nonetheless, the MCV and MCH are components of the RBC and therefore lower counts as was the case in the present study might have been a result of anemia or reduced immune function (Shaw et al., 2009). The MCV is the average size (volume) of the red blood cells and MCH estimates the amount of Hgb in an average RBC. The low MCV (microcytic) recorded in the challenged birds in this study might be due to the inability to form Hgb. Also, a possible bacterial translocation of the necrotic enteritis B-like toxin (NetB) toxin could be the cause of reduced blood cells. The NetB toxin is reported to be a causative agent in pore formation in cellular membranes resulting in an influx of ions into the cytoplasm and eventually leading to osmotic lysis of cells (Keyburn et al., 2010). In addition, blood loss as a result of intestinal damage might have also put pressure on iron stores and led to anaemia.

4.4. Jejunal morphology

A sound intestinal morphology is essential in the absorption and activity of the digestive enzyme and the subsequent transportation of nutrients across the epithelia. Particularly, the villus height, crypt depth, and villus height-to-crypt ratio have been used as indicators for sound gut health and functions (Swatson et al., 2002). The higher the villus height, the higher the absorptive area thereby increasing the efficiency of digestion and absorption. A deeper crypt, on the other hand, is an indication of faster tissue turnover that allows the replenishment of the villi, perhaps suggesting a response mechanism by the chickens to sloughing or atrophy of the villi that might have resulted from inflammation and toxin secretions by enteric pathogens (Gao et al., 2008). The slower the turnover of tissues, the better for the growth of the chicken. This is because the maintenance requirement is reduced, thus improving the feed conversion ratio of the chicken as previously reported (Zanu et al., 2020a) in unchallenged birds. However, during incidences of enteric diseases such as NE the morphology of the intestine is impaired and the nutrient absorptive and enzyme secretory organs are reduced. In the current study, the challenge decreased the total length of the epithelium, villi height but increased the crypt depth. These observations appear to be the case in most *C. perfringens* and *Eimeria* spp. challenged studies (Jayaraman et al., 2013; Du et al., 2016; Kim et al., 2017; Leung et al., 2018, 2019; Wu et al., 2018; Xue et al., 2018). Also, the change in the architecture of the epithelium due to enteric disease compromises the tight junction barrier, nutrient digestion, absorption and transport (Persia et al., 2006; Rochell et al., 2016b).

The wider villus tip and base observed in the challenged group of the present study might be due to widened lamina propria which has been reported elsewhere (Zekarias et al., 2008). Chen et al. (2015) suggested that the widening of the villus indicated less nutrient absorption area and probably also a greater amount of gut-associated immune tissue proliferation and accumulation in the villus, which is also an indication of compromised gut health. A narrower crypt-to-villus ratio was observed in birds fed over-processed MBM. It is probable that the undigested proteins of over-processed MBM might have been fermented and led to a buildup of toxic products such as amine and ammonia and favored the proliferation of pathogenic bacteria including *C. perfringens*. Toxin secretions from this bacterium have been reported to impair the gut epithelium (Caly et al., 2015; Navarro et al., 2018).

4.5. Gene expression

The challenge downregulated almost all the target genes determined in the present study, agreeing with other studies that a damage to the intestinal epithelium by way of villi atrophy and sloughing of the brush border decreases the absorption capabilities of nutrient transporters (Guo et al., 2013; Fetterer et al., 2014). For instance, APN functions to cleave amino acids from the N terminus of peptides after proteins have been hydrolyzed to di- or tripeptides and amino acids. Therefore, the low expression of *APN* would reduce the absorption of amino acids.

The current consensus on the transcellular pathway transport of Ca^{2+} is that Ca^{2+} is carried across the epithelia into the cell through specific epithelial calcium channels, through the cytoplasm by calcium-binding protein 1 (CALB1) and extruded to the extracellular medium through the action of plasma membrane Ca^{2+} ATPase (PMCA) and Na^{+}/Ca^{2+} exchangers (NCX1). Contrary to the hypothesis of the present study, neither over-processing of MBM nor the use of high phytase influenced the expression of the Ca^{2+} transporters determined in the current study (*CALB1*, *ATP1A1*, *ATP5A1W* and *CACNA1*) and *VDR*. It was expected that over-processing of MBM would have compromised the Ca content and led to the upregulation of these genes. Rather, they were downregulated by the challenge. This observation in the challenge group might explain the lower nutrient absorption detected in this study (Zanu et al., 2020a) and in other studies (Guo et al., 2013; Rochell et al., 2016a). Nonetheless, over-processing of MBM tended to upregulate *CaSR* in unchallenged birds fed low phytase. The *CaSR* plays a key role in the regulation of calcium homeostasis in chickens. The Ca^{2+} ion is a multipurpose messenger that exerts its effects both inside and outside the cell. Through *CaSR*, the parathyroid gland maintains serum Ca^{2+} concentration within a very narrow physiological range by modulating the release of parathyroid hormone (PTH) into circulation. It is possible that such a diet was lower in digestible Ca due to over-processing of MBM, hence, the higher expression of this gene. Thus, the results of the current study confirmed that *CaSR* is indeed involved in nutrient sensing and that its expression is increased during periods of low Ca.

Additionally, Ca regulates *VDR* expression in the chicken's parathyroid glands, in that, at a higher dietary Ca, vitamin D-deficient chickens increases plasma Ca concentration (Meyer et al., 1992). Besides Ca being involved in the regulation of vitamin D, *VDR* is also an essential component of the immune system (Boodhoo et al., 2016; Rodriguez-Lecompte et al., 2016). A lower expression of *VDR* has been reported in many gut-related diseases in chickens and in other species (Wu et al., 2015; Garg et al., 2019). Those studies demonstrated that *VDR* in the intestinal tissues has a relationship with mucosal inflammation.

Furthermore, the jejunum is believed to be the main site for intestinal Pi (phosphate) absorption. The type IIb cotransporter of NaPi-IIb is the major Na-Pi cotransporter. It is generally accepted that the availability of P is the main regulator of the expression of *NaPi-IIb* gene and that a low P leads to an increased expression of the gene. But this gene was downregulated in the challenged birds. However, the higher expression of *NaPi-IIb* in the current study in birds fed low phytase and over-processed MBM was expected as the P content of over-processed MBM might have been also compromised following overheating.

Regarding intestinal tight junctions, claudins and occludins are the major components that define the barrier structure of the paracellular pathway (Gil-Cardoso et al., 2016). Therefore, the regulation of the expression of these proteins is crucial in minimizing gut disorders. In the present study, the challenge led to the upregulation of *CLDN1* which is believed to determine the permeability of the space between 2 adjacent cells. Disruption of the tight junction is thus a common observation during NE occurrence. It is believed that *C. perfringens* enterotoxins (CPE) bind to tight junction proteins which eventually leads to pore formation and increase paracellular permeability (Fanning et al., 1999). The downregulation of occludin as a result of the challenge has also been noted in other disease conditions (Wang et al., 2017; Li et al., 2018).

The expression of *MUC-2*, the main gel-forming mucin-producing gene in the small and large intestines, is secreted by the goblet cells that originate from the base of the crypt. In the present study, *MUC-2* was highly expressed in the unchallenged birds fed either high phytase and as-received MBM or low phytase and over-processed MBM diets. There are several potential explanations for this. Firstly, it was likely that the diet with high phytase and over-processed MBM fueled the NE infection, as was also observed in birds fed high phytase and MBM without antibiotics in a recent study (Zanu et al., 2020b). The present finding might have probably led to the sloughing of mucin or the mucin feeding *C. perfringens* (Collier et al., 2008; Forder et al., 2012). A decrease in mucin in such instance would, in turn, trigger the goblet cells to compensate for mucin losses by increasing mucin synthesis, hence the upregulation of *MUC-2* (Montagne et al., 2000). The benefits of phytase in the gastrointestinal health and immune competence as reported by Liu et al. (2008) might have been held in abeyance during the challenge period in the current study. Secondly, though low phytase was expected to reduce any negative impact on the gut during the infection, the presence of over-processed MBM appeared to have been enough to instigate the infection and cause a disturbance in the gut, as was hypothesized in the present study. Factors that reduce mucin in the gut trigger the upregulation of *MUC-2*. Furthermore, the lower expression of *MUC-2* in the challenged birds in this present study agrees with the findings of Kitessa et al. (2014) who observed a downregulation of *MUC-2* in birds exposed to Eimeria and *C. perfringens*. *MUC5AC* is expressed primarily in the proventriculus and therefore its unresponsiveness in the jejunum in the current study comes as no surprise.

Concluding, the efficacy of high phytase in degrading inositol phosphate esters was not affected by NE post-challenge. The hydrolysis of phytate yielded a higher concentration of inositol in the challenge birds but this might be potential ground for increasing the activity of pathogenic bacteria during a severe incidence. Additionally, this study demonstrated that over-processed MBM might have a detrimental effect on the gut epithelium and further confirms previous studies that NE is detrimental to chickens' gut and hematology. The decrease in expression of many genes encoding for nutrient transporters, receptors and digestive enzymes by the NE in the current study makes the call for a search for an antidote to curb NE in the post-antibiotic era more pressing.

Author contributions

Holy K. Zanu: methodology, formal analysis, validation, writing - original draft, writing - review & editing. Sarbast K. Kheravii: methodology, writing - review & editing. Natalie K. Morgan: writing - review & editing, validation. Michael R. Bedford: conceptualization, writing - original draft, writing - review & editing. Robert A. Swick: conceptualization, writing - review & editing, supervision, project administration, resources.

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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