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Original Research Article

Negative consequences of reduced protein diets supplemented with synthetic amino acids for performance, intestinal barrier function, and caecal microbiota composition of broiler chickens

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The consequences of feeding broiler chickens with reduced protein (RP) diets for gut health and barrier function are not well understood. This study was performed to elucidate the effect of reducing dietary protein and source of protein on gut health and performance parameters. Four experimental diets included 2 control diets with standard protein levels either containing meat and bone meal (CMBM) or an all-vegetable diet (CVEG), a medium RP diet (17.5% in growers and 16.5% in finisher), and a severe RP diet (15.6% in grower and 14.6% in finisher). Off-sex Ross 308 birds were assigned to each of the 4 diets and performance measurements were taken from d 7 to 42 post-hatch. Each diet was replicated 8 times (10 birds per replicate). A challenge study was conducted on additional 96 broilers (24 birds per diet) from d 13 to 21. Half of the birds in each dietary treatment were challenged by dexamethasone (DEX) to induce a leaky gut. Feeding birds with RP diets decreased weight gain ($P < 0.0001$) and increased feed conversion ratio ($P < 0.0001$) from d 7 to 42 compared with control diets. There was no difference between CVEG and CMBM control diets for any parameter. The diet containing 15.6% protein increased ($P < 0.05$) intestinal permeability independent of the DEX challenge. Gene expression of claudin-3 was downregulated ($P < 0.05$) in birds fed 15.6% protein. There was a significant interaction between diet and DEX ($P < 0.05$) and both RP diets (17.5% and 15.6%) downregulated claudin-2 expression in DEX-challenged birds. The overall composition of the caecal microbiota was affected in birds fed 15.6% protein having a significantly lower richness of microbiota in both sham and DEX-injected birds. Proteobacteria was the main phylum driving the differences in birds fed 15.6% protein. At the family level, Bifidobacteriaceae, Unclassified Bifidobacteriales, Enterococcaceae, Enterobacteriaceae, and Lachnospiraceae were the main taxa in birds fed 15.6% protein. Despite supplementation of synthetic amino acids, severe reduction of dietary protein compromised performance and intestinal health parameters in broilers, evidenced by differential mRNA expression of tight junction proteins, higher permeability, and changes in caecal microbiota composition.

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1. Introduction

There is increasing interest to feed broiler chickens with reduced protein (RP) diets to minimise nitrogen and ammonia excretion, reduce reliance on quality protein sources, save cost, and decrease the incidences of wet litter and associated welfare issues such as hock burn and foot pad dermatitis (Lemme et al., 2019). The implications of dietary protein reduction and amino

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acid supplementation in broiler chickens have been the subject of several investigations and reviews (Hofmann et al., 2019; Liu et al., 2021b). These studies have mostly investigated nutrient digestion and absorption dynamics to provide explanations for the often-impaired performance of broiler chickens fed RP diets as well as nutritional strategies to compensate for any negative impact on birds' performance. However, little is known about the implication of adopting RP diets supplemented with synthetic amino acids for gastrointestinal tract functionality, health, and microbiota composition. It is known that ileal bypass protein can be fermented by putrefactive bacteria leading to the production of toxic compounds such as amines, indoles, phenols, cresol, and ammonia which can in turn negatively influence health and performance of the host (Apajalahti and Vienola, 2016). Therefore, potentially, reducing dietary protein can reduce the ileal bypass protein and subsequently lower the concentration of toxic compounds rendering a potential benefit for gut health. However, when diets are balanced to compensate for protein reduction by supplementing amino acids and adjusting the energy, the other components of the diet such as fibre content, starch and lipid are subsequently changed, which may differentially impact intestinal functions.

Barekatain et al. (2019b) found that broilers fed a diet containing 17% protein supplemented with the required synthetic essential amino acids had a significantly higher intestinal permeability only compared with a diet having 10% extra essential amino acids and 22% protein but not when compared with a standard diet. This observation coupled with differential effects on gene expression of tight junction proteins pointed to a possible negative effect on gut integrity by reducing dietary protein (Barekatain et al., 2019b). However, in that study, the lack of difference between the RP diet and a standard protein diet raises the question of whether the concentration of amino acids may have contributed in addition to the reduction of protein in observing the difference in gut integrity of broilers. Additionally, when dietary protein is reduced, and synthetic amino acids are supplemented, the concentration of high-protein ingredients is reduced. Therefore, in an experimental setting, RP diets are compared with control treatments that may differ in their composition further complicating the source of variation in observed differences in intestinal functions. For instance, when a RP diet is formulated, meat and bone meal (MBM) as a rich source of amino acids including glycine is omitted in favour of supplementation of synthetic amino acids to reduce the level of dietary protein. It has been recently shown that the inclusion of MBM may increase gut permeability and compromise intestinal health in broiler chickens (Zanu et al., 2020).

There is also evidence that birds fed RP diets often have poor performance under challenging conditions, showing that feeding birds with RP failed to improve the performance of broiler chickens subjected to a necrotic enteritis model (Hilliar et al., 2020) or leaky gut induced by dexamethasone (DEX) (Barekatain et al., 2019b). The mechanisms underpinning responses to RP diets at the intestinal level are still poorly understood and have not been comprehensively studied and validated, particularly for the effects of such diets on microbiota composition and intestinal barrier function.

Thus, the study reported here aimed to investigate the intestinal health and integrity of broiler chickens fed RP diets compared to birds fed standard diets that contained either all vegetable-based protein sources or included MBM as an animal protein source.

2. Materials and methods

2.1. Animal ethics statement

The Animal Ethics Committee of the Department of Primary Industries and Regions, South Australia approved the experimental procedures of the study (PIRSA AEC 27/20).

2.2. Study design and dietary treatments

The study comprised 2 parts, one addressed the general growth performance of group-housed birds that received experimental diets and the other involved a leaky gut model that used challenge with DEX, as a stress factor, to induce a leaky gut in individually housed broilers.

Four dietary treatments were used; control diets with standard levels of protein (20.36% in grower diets and 18.33% in finisher diets), one that was wholly vegetable based (CVEG) and the other contained MBM (CMBM) as an animal source of protein, and 2 treatments were RP diets at medium and low levels, supplemented with synthetic amino acids to meet or exceed the nutrient specifications of Ross 308 broilers (Ross, 2019). The low-level protein group had 15.6% in the grower diet and 14.6% in the finisher diet. The medium protein group had 17.5% and 16.5% protein in grower and finisher diets, respectively. The lowest level of protein was selected based on the feasible solution given by formulation software (Concept 5) after importing the nutrient content of the ingredients measured by near-infrared reflectance (NIR) analysis. All the essential nutrients and glycine equivalent of the RP diets were either matched exactly with control diets or met the Ross 308 specification (Ross, 2019) when practically not feasible by the software. Tables 1 and 2 show the composition of experimental grower and finisher diets, respectively.

For the performance aspect of the study, a total of 320 male off-sex Ross 308 one-day-old broiler chickens from a commercial hatchery (Aviagen, Goulburn, NSW) were transferred to the poultry facilities of the South Australian Research and Development Institute at Roseworthy, South Australia. A fully ventilated and temperature-controlled broiler shed was used for this study. Upon arrival, birds were weighed and assigned to 32 raised-floor pens with each pen accommodating 10 birds. For the first 7 d, birds were on paper after which wood shavings were provided as bedding material. From d 0 to 7, all birds received the same commercial starter diet. On d 7, birds were assigned to the 4 experimental diets by allocating 8 replicates (pens) to each diet in a completely randomised design. Feed and water were provided ad libitum for the entire period of study. The temperature was maintained at 32 °C for the first 2 d and then gradually decreased until a temperature of 23 °C was reached on d 21, and then kept constant for the remainder of the study. Birds were given 24 h light for the first 2 d followed by 16 h light and 8 h dark until the end of the study. Birds were weighed on d 7, 21 and 42. Feed intake was recorded, and the feed conversion ratio (FCR) was then calculated and adjusted for mortality, for each pen.

Concurrent with the performance experiment, an additional 96 male off-sex Ross broiler chickens from the same hatchery were raised on 4 separate floor pens until d 13 of age. As with the other birds, these birds were also given the same diet for the first 7 d. On d 8, the birds were given the experimental diets which were from the same mixing batch of feed used in the performance trial. On d 13, birds were transferred to individual cages, with 24 birds assigned to each dietary treatment. Each cage had a separate feeding trough and birds had access to nipple drinkers. Feed and

Table 1
Ingredient and nutrient composition of experimental grower diets (%, as-is basis unless noted).

Item	CVEG	CMBM	CP 17.5	CP 15.6
Ingredients				
Wheat	64.456	69.341	79.014	86.136
Soybean meal	27.229	20.402	11.952	1.960
Meat and bone meal	–	4.090	–	–
Canola oil	4.535	3.105	2.233	1.389
Limestone	1.135	0.817	1.200	1.236
Dicalcium phosphate	0.738	–	0.776	0.816
Xylanase	0.005	0.005	0.005	0.005
Phytase	0.010	0.010	0.010	0.010
Sodium chloride	0.213	0.150	0.075	–
Sodium bicarbonate	0.298	0.292	0.488	0.593
Sand	–	–	–	1.330
Vitamin and mineral premix[1]	0.200	0.200	0.200	0.200
Choline chloride (60%)	0.100	0.100	0.100	0.100
L-Lysine HCl (78.4%)	0.354	0.443	0.786	1.077
DL-Methionine	0.301	0.325	0.413	0.495
L-Threonine	0.180	0.220	0.369	0.500
L-Tryptophan	–	–	0.014	0.061
L-Leucine	–	0.072	0.369	0.628
L-Phenylalanine	–	–	0.074	0.247
L-Histidine	–	–	0.063	0.153
Glycine	–	–	0.404	0.655
L-Arginine HCl	0.070	0.151	0.554	0.884
L-Valine	0.125	0.165	0.356	0.519
L-Isoleucine	0.051	0.112	0.290	0.457
Potassium carbonate	–	–	0.257	0.551
Nutrient composition				
Dry matter	89.89	89.84	89.88	90.13
AMEn, kcal/kg	3,100	3,100	3,100	3,100
Crude protein	20.36	20.36	17.50	15.60
Crude fat	6.37	5.36	4.06	3.15
Crude fiber	2.17	2.06	1.88	1.65
Dig. Arg	1.21	1.21	1.21	1.21
Dig. Lys	1.15	1.15	1.15	1.15
Dig. Met	0.56	0.58	0.61	0.64
Dig. Cys	0.29	0.27	0.25	0.21
Dig. Met + Cys	0.85	0.85	0.85	0.85
Dig. Trp	0.23	0.21	0.18	0.18
Dig. His	0.44	0.42	0.38	0.38
Dig. Phe	0.87	0.81	0.70	0.70
Dig. Leu	1.27	1.27	1.27	1.27
Dig. Ile	0.78	0.78	0.78	0.78
Dig. Thr	0.77	0.77	0.77	0.77
Dig. Val	0.93	0.93	0.93	0.93
Dig. Gly	0.70	0.79	0.91	1.03
Dig. Ser	0.85	0.79	0.62	0.45
Dig. Gly equivalent	1.31	1.36	1.35	1.35
Starch	36.74	39.45	44.78	48.69
Calcium	0.87	0.87	0.87	0.87
Available phosphorus	0.43	0.43	0.43	0.43
Sodium	0.20	0.20	0.20	0.20
Potassium	0.84	0.74	0.74	0.74
Chloride	0.26	0.26	0.26	0.27

CVEG = Control diet containing all-vegetable ingredients and standard level of protein; CMBM = Control diet containing meat and bone meal and standard level of protein; AMEn = Nitrogen corrected apparent metabolizable energy; Dig. = Digestible.
[1] Supplied per kilogram of diet: vitamin A, 12,000 IU; vitamin D_3, 3,000 IU; vitamin E, 25 mg, vitamin K_3, 3 mg; vitamin B_1, 2 mg; vitamin B_2, 6 mg; niacin, 45 mg; pantothenate, 15 mg; pyridoxine, 5 mg; folate, 1 mg; cyanocobalamin, 16 μg; biotin, 150 μg; Cu (sulfate), 10 mg; Fe (sulfate), 60 mg; I (iodide), 1 mg; Se, 0.3 mg; Mn (sulfate and oxide), 120 mg; Zn, 70 mg; antioxidant, 20 mg.

Table 2
Ingredient and nutrient composition of experimental finisher diets (%, as-is basis unless noted).

Item	CVEG	CMBM	CP 16.5	CP 14.6
Ingredients				
Wheat	69.886	72.921	79.258	88.644
Soybean meal	21.422	16.553	11.596	1.000
Meat and bone meal	–	3.000	–	–
Canola oil	5.195	4.300	3.679	2.204
Limestone	1.069	0.834	1.110	1.153
Dicalcium phosphate	0.539	–	0.563	0.594
Xylanase	0.005	0.005	0.005	0.005
Phytase	0.010	0.010	0.010	0.010
Sodium chloride	0.226	0.181	0.137	0.041
Sodium bicarbonate	0.280	0.275	0.402	0.536
Sand	–	0.260	–	0.265
Vitamin and mineral premix[1]	0.200	0.200	0.200	0.200
Choline Cl 60%	0.100	0.100	0.100	0.100
L-Lysine HCl 78.4	0.367	0.430	0.645	0.947
DL-Methionine	0.289	0.308	0.361	0.441
L-Threonine	0.168	0.197	0.289	0.422
L-Tryptophan	–	–	–	0.043
L-Leucine	–	0.052	0.238	0.499
L-Phenylalanine	–	–	0.110	0.285
L-Histidine	–	–	0.026	0.117
Glycine	–	–	0.264	0.517
L-Arginine HCl	0.125	0.182	0.436	0.775
L-Valine	0.056	0.085	0.204	0.368
L-Isoleucine	0.064	0.108	0.218	0.386
Potassium carbonate	–	–	0.149	0.446
Nutrient composition				
Dry matter	89.90	89.91	89.89	89.96
AMEn, kcal/kg	3,200	3,200	3,200	3,200
Crude protein	18.33	18.33	16.50	14.60
Crude fat	7.02	6.42	5.49	4.00
Crude fiber	2.06	1.97	1.87	1.66
Dig. Arg	1.10	1.10	1.10	1.10
Dig. Lys	1.03	1.03	1.03	1.03
Dig. Met	0.52	0.54	0.55	0.59
Dig. Cys	0.28	0.26	0.25	0.21
Dig. Met + Cys	0.80	0.80	0.80	0.80
Dig. Trp	0.21	0.19	0.16	0.16
Dig. His	0.40	0.38	0.34	0.34
Dig. Phe	0.78	0.73	0.73	0.73
Dig. Leu	1.13	1.13	1.13	1.13
Dig. Ile	0.71	0.71	0.71	0.71
Dig. Thr	0.69	0.69	0.69	0.69
Dig. Val	0.78	0.78	0.78	0.78
Dig. Gly	0.63	0.69	0.77	0.89
Dig. Ser	0.76	0.72	0.61	0.45
Dig. Gly equivalent	1.17	1.21	1.21	1.21
Starch	39.74	41.42	44.92	50.10
Calcium	0.79	0.79	0.79	0.79
Available phosphorus	0.40	0.40	0.40	0.40
Sodium	0.20	0.20	0.20	0.20
Potassium	0.74	0.68	0.68	0.68
Chloride	0.27	0.27	0.27	0.27

CVEG = Control diet containing all-vegetable ingredients and standard level of protein; CMBM = Control diet containing meat and bone meal and standard level of protein; AMEn = Nitrogen corrected apparent metabolizable energy; Dig. = Digestible.
[1] Supplied per kilogram of diet: vitamin A, 12,000 IU; vitamin D_3, 3,000 IU; vitamin E, 25 mg, vitamin K_3, 3 mg; vitamin B_1, 2 mg; vitamin B_2, 6 mg; niacin, 45 mg; pantothenate, 15 mg; pyridoxine, 5 mg; folate, 1 mg; cyanocobalamin, 16 μg; biotin, 150 μg; Cu (sulfate), 10 mg; Fe (sulfate), 60 mg; I (iodide), 1 mg; Se, 0.3 mg; Mn (sulfate and oxide), 120 mg; Zn, 70 mg; antioxidant, 20 mg.

water were provided ad libitum throughout the experiment. On d 14, 16, and 20, half of the birds in each dietary treatment (12 birds) were given 0.35 mg of DEX (Sigma–Aldrich) per kilogram body weight (BW) injections in breast muscle to induce a leaky gut. The unchallenged birds were given a sham injection of saline solution (0.9%). The stock and working solutions of DEX were prepared following the instruction of Wideman and Pevzner (2012).

2.3. Fluorescein isothiocyanate-dextran assay and sampling

To measure the intestinal permeability, on d 21, each bird was given 4.16 mg/kg BW of fluorescein isothiocyanate dextran (FITC-d; Sigma–Aldrich, average 4,000 Da) by oral gavage of the prepared solution into the crop. The process of preparing the solution was according to a previous study (Barekatain et al., 2021). A blood

sample was taken from the wing of the live bird after precisely 150 min. Blood samples were left to clot at room temperature for at least 3 h before being centrifuged at 1,500 × g for 15 min and then the serum was stored at −20 °C. The spectrophotometric analysis of FITC-d was performed in duplicate, including a blank sample from a previously stored serum sample from a bird without FITC-d. Excitation was with 485 nm light and emission at 530 nm was measured in a Synergy MX plate reader (Biotek Instruments).

All birds were subsequently euthanised by cervical dislocation to collect ileal tissues and caecal content used in gene expression and microbiota analysis, respectively.

2.4. Gene expression assays

The procedures for gene expression analysis, including RNA extraction, cDNA synthesis, and quantitative PCR assays were as previously detailed by Barekatain et al. (2021). Briefly, RNA was extracted from ileal tissue samples using a RNeasy Plus Universal Mini Kit (Qiagen) after homogenising approximately 100 mg of tissue in Qiazol (Qiagen). The concentration of RNA was measured using a NanoDrop One spectrophotometer (ThermoFisher Scientific) and the RNA integrity of 12 randomly selected samples was assessed using a Tape Station (Agilent Technologies). Samples had RNA integrity numbers above 8. A high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific) was used to synthesise the cDNA. The qPCR conditions included an initial 10 min denaturation at 95 °C followed by 95 °C for 15 s, 40 cycles of 60 °C for 20 s, and 72 °C for 40 s. Table 3 contains sequences of all the primers used in the gene expression assays. Two reference genes of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and Tata-Box binding protein (*TBP*) were included. The final values used for statistical analysis were the quantified values expressed relative to *TBP* which was the most stable gene used as a reference gene.

2.5. Caecal sample processing and DNA extraction

A DNeasy PowerSoil Pro Kit (Qiagen) was used to extract the DNA of caecal samples collected from the challenge experiment, following the manufacturer's instructions. A Qiagen Tissue Lyser II was used to homogenise approximately 50 mg of caecal samples for 5 min at maximum speed. Subsequently, homogenised suspensions were heated for 10 min at 90 °C.

2.6. 16S rRNA gene amplification and analysis

Amplicon sequencing across the V3–V4 region of 16S rRNA genes was performed to assess microbiota composition. Amplicons were produced using Q5 DNA Polymerase (New England BioLabs) and custom-designed barcoded primers, ACTCCTACGGGAGGCAGCAG (forward) and GGACTACHVGGGTWTCTAAT (reverse). As per Fadrosh et al. (2014), primers also contained barcodes, spacer sequences, and Illumina sequencing linkers. Amplicon sequencing was performed on an Illumina MiSeq Sequencer using a 2 × 300 bp kit. The sequence data were processed in QIIME v1.9.1 (Caporaso et al., 2010) for quality control, joining, assessment of chimeras, grouping into operational taxonomic units (OTUs), and assignment of taxonomy against the Greengenes database. De novo OTU picking using uclast within QIIME was used.

Trimming and removal of rare sequences was undertaken to leave OTUs that represented at least 0.01% of the sequences present, the data set consists of 3,617,073 sequences, an average of 38,480 sequences per sample, with 16,776 the lowest representation and 64,155 the highest. A total of 687 OTUs were found. A Biome table was exported to Calypso (Zakrzewski et al., 2017) for further analysis and data visualisation.

2.7. Statistical analysis

The growth performance data of the first part of the study were analysed by one-way ANOVA of General Linear Model of SAS 9.4. Each pen was considered an experimental unit. For the challenge part, all data, including gene expression, were subjected to two-way ANOVA to assess the effects of diet, DEX and their interaction. Accordingly, for the challenge study, an experimental unit constituted an individual bird or its representative sample. Fisher's least square differences test was used to separate means when a significant effect was detected. The level of significance was set as $P < 0.05$ and the tendency as $0.05 \leq P \leq 0.10$.

Table 3
Primer sequences of target and reference genes used in PCR assays.

Gene	Forward (5′→3′)	Reverse (5′→3′)	Reference
Claudin-1	AAGGTGTACGACTCGCTGCT	CAGCAACAAACACACCAACC	Gilani et al. (2018)
Claudin-2	CCTACATTGGTTCAAGCATCGTGA	GATGTCGGGAGGCAGGTTGA	Gong et al. (2020)
Claudin-3	GCCAAGATCACCATCGTCTC	CACCAGCGGGTTGTAGAAAT	Gilani et al. (2018)
ZO-1	CCGCAGTCGTTCACGATCT	GGAGAATGTCTGGAATGGTCTGA	Chen et al. (2015)
ZO-2	GCCCAGCAGATGGATTACTT	TGGCCACTTTTCCACTTTTC	Gilani et al. (2018)
MUC-2	ATTGAAGCCAGCAATGGTGT	TTGTTGGCCTTGTCATCAAA	Gilani et al. (2018)
NFκB	GAAGGAATCGTACCGGGAACA	CTCAGAGGGCCTTGTGACAGTAA	Lee et al. (2018)
IFNγ	ACACTGACAAGTCAAAGCCGC	AGTCGTTCATCGGGAGCTTG	Brisbin et al. (2010)
GLP-2	CGTGCCACAGCCATTCTTA	AGCGGCTCTGCAAATGATTA	Gilani et al. (2018)
Nrf2	GGAAGAAGGTGCTTTTCGGAGC	GGGCAAGGCAGATCTCTTCCAA	Lee et al. (2018)
IL-10	ATGAACTTAACATCCAACTGCTC	TGTTGCCCAGGTCGCCCAT	Xiao et al. (2018)
IL1-β	CAGCCCGTGGGCATCA	CTTAGCTTGTAGGTGGCGATGTT	Chen et al. (2015)
PepT-1	ACACGTTTGTTGCTCTGTGC	GACTGCCTGCCCAATTGTAT	Barekatain et al. (2019b)
SGLT-1	TGCCGGAGTATCTGAGGAAG	CCCCATGGCCAACTGTATAA	Gilani et al. (2018)
FN	GGTGTGAACTACAAGATTGG	TCCTTCTGCCACTGCTCTCC	Bennett (2006)
OVO	TTGTTCAGCATTCCACCGTT	ACAGCAACTCAAAGTCATCC	Bennett (2006)
GPx-1	TCCCCTGCAACCAATTCG	AGCGCAGGATCTCCTCGTT	Greene et al. (2021)
SOD-1	TGGCTTCCATGTGCATGAAT	ACGACCTGCGCTGGTACAC	Greene et al. (2021)
TBP	GTCCACGGTGAATCTTGGTT	GCGCAGTAGTACGTGGTTCTC	Gilani et al. (2018)
GAPDH	CAACCCCCAATGTCTCTGTT	TCAGCAGCAGCCTTCACTAC	Gilani et al. (2018)

ZO-1 = Zonula occludens 1; *ZO-2* = Zonula occludens 2; *IL1-β* = Interleukin 1-β; *IL-10* = Interleukin 10; *NFκB* = Nuclear factor kappa B; *MUC-2* = Mucin 2; *IFNγ* = Interferon-gamma; *GLP-2* = Glucagon-like peptide-2; *Nrf2* = Nuclear factor erythroid 2-related factor 2; *GPx-1* = Glutathione peroxidase 1; *PepT-1* = Peptide transporter 1; *SGLT-1* = Sodium dependent glucose transporter 1; *FN* = Fibronectin; *OVO* = Ovotransferrin; *SOD-1* = Superoxide dismutase 1; *TBP* = Tata-Box binding protein; *GAPDH* = Glyceraldehyde 3-phosphate dehydrogenase.

Normalisation of data for bacterial composition was performed using cumulative-sum scaling and subsequently a $\log_2$ transformation prior to statistical analysis. The Calypso software (http://cgenome.net/wiki/index.php/Calypso) was used for statistical analysis of the microbiota data. The alpha diversity measures were 'richness' and 'evenness' as implemented in the Calypso analysis package. The visualisation of the differences between the treatments for bacterial communities was facilitated by principle-coordinate analysis (PCoA) using a Bray–Curtis dissimilarity matrix. The analysis of similarity (ANOSIM) was performed to compare the overall composition of bacterial community structures between the experimental treatments. *R* values associated with ANOSIM closer to zero indicate no variation between bacterial communities while higher variation is associated with values closer to 1. The differences in the abundance of the bacterial taxa between dietary treatments were tested by linear discriminant analysis effect size (LEfSe).

3. Results

3.1. Growth performance

Table 4 shows the growth performance of broiler chickens raised in group-housed pens. Feed consumption of birds remained unaffected by the experimental diets at any stage of the study. There was no difference between the 2 control diets for BW, body weight gain (BWG), or FCR for the growth performance part of the study. Birds fed medium and low-level protein diets gained less weight than both standard control diets from d 7 to 21 of age with the least BWG associated with the lowest level of dietary protein ($P < 0.0001$). From d 21 to 42 of age, the BWG was reduced only when broilers were fed the lowest level of protein ($P < 0.001$). When assessed from d 7 to 42, RP at both levels, decreased ($P < 0.0001$) BWG compared with the all-vegetable-based control diet whilst the difference between medium protein and MBM-containing diet was not significant. The BW results for d 21 and 42 had the same trend as BWG from d 21 to 24 and d 7 to 42, respectively. FCR increased as the level of protein in the diet decreased, at all stages of the study ($P < 0.0001$).

3.2. Growth performance of the challenge experiment

The feed intake, BWG and FCR of individually housed birds are shown in Table 5. There was no interaction between DEX and diet for any of the performance parameters. Dietary treatments had no effect on feed intake. DEX independently reduced feed consumption ($P < 0.0001$) and BWG ($P < 0.0001$). BWG was reduced ($P < 0.05$) when birds were fed both RP diets only compared with control diet containing MBM. Feeding birds with dietary protein of 17.5% and 15.6% similarly increased ($P < 0.001$) the FCR compared with both control diets.

Table 5
Indicative growth performance parameters of broilers fed experimental diets under DEX challenge or Sham injections.[1]

Item	Feed intake, g/bird	Weight gain, g/bird	FCR
Main effect			
Diets			
CVEG	826	585^{ab}	1.458^{b}
CMBM	885	609^{a}	1.501^{b}
CP17.5	826	543^{b}	1.588^{a}
CP15.6	876	562^{b}	1.597^{a}
DEX			
Sham	944^{a}	706^{a}	1.340^{b}
Injected	762^{b}	443^{b}	1.732^{a}
SEM	12.9	8.1	0.0131
P-values			
DEX	<0.0001	<0.0001	<0.0001
Diet	0.207	0.029	<0.001
Diet × DEX	0.762	0.561	0.182

CVEG = Control diet containing all-vegetable ingredients and standard level of protein; CMBM = Control diet containing meat and bone meal and standard level of protein; CP17.5 = Reduced protein diet containing 17.5% crude protein supplemented with synthetic amino acids to match the control diets; CP15.6 = Reduced protein diet containing 15.6% crude protein supplemented with synthetic amino acids to match the control diets. DEX = Dexamethasone; SEM = Pooled standard error of the mean.
[1] Each value for each treatment represents the mean of 12 replicates. Values within the same main effect not sharing a superscripts letter differ significantly at the *P*-level shown.

3.3. Concentration of FITC-d in serum

As shown in Fig. 1, there was no interaction between DEX and diet for FITC-d concentration in blood samples on d 21 of age. Reducing dietary protein down to 15.6% increased ($P < 0.05$) FITC-d level compared with control diets. DEX also increased ($P < 0.001$) FITC-d passage from the intestine into blood in challenged birds.

3.4. Gene expression assays

Table 6 represents the data for gene expression of selected tight junction proteins in ileal tissues. Gene expression of claudin-1 and zonula occludens 1 (*ZO-1*) was not affected by DEX or diet. There were interactions between diet and DEX for the expression of claudin-2 ($P < 0.05$) and zonula occludens 2 (*ZO-2*) ($P < 0.05$). DEX

Table 4
Growth performance of broiler chickens fed reduced and low protein diets compared with 2 control diets either fully vegetable-based or containing meat and bone meal.

Treatments	Feed intake, g/bird			Body weight, g/bird			Body weight gain, g/bird			Feed conversion ratio		
	d 7–21	d 21–42	d 7–42	d 7	d 21	d 42	d 7–21	d 21–42	d 7–42	d 7–21	d 21–42	d 7–42
CVEG	1,112	3,627	4,739	231	1,154^{a}	3,544^{a}	923^{a}	2,390^{a}	3,313^{a}	1.204^{c}	1.519^{c}	1.431^{c}
CMBM	1,130	3,565	4,695	235	1,151^{a}	3,513^{ab}	916^{a}	2,362^{a}	3,278^{ab}	1.234^{c}	1.510^{c}	1.433^{c}
Reduced protein – medium [1]	1,108	3,698	4,806	234	1,084^{b}	3,406^{b}	850^{b}	2,322^{a}	3,172^{b}	1.304^{b}	1.593^{b}	1.515^{b}
Reduced protein – low [2]	1,111	3,778	4,888	233	970^{c}	3,162^{c}	738^{c}	2,192^{b}	2,929^{c}	1.507^{a}	1.725^{a}	1.670^{a}
SEM	9.4	28.8	33.6	1.3	6.1	19.3	5.6	16.8	19.1	0.0098	0.0100	0.0085
P-value	0.839	0.078	0.219	0.752	<0.0001	<0.0001	<0.0001	0.0014	<0.0001	<0.0001	<0.0001	<0.0001

CVEG = Control diet containing all-vegetable ingredients and standard level of protein; CMBM = Control diet containing meat and bone meal and standard level of protein; SEM = Pooled standard error of the mean.
[a–c] Means within a same column not sharing a same superscript are statistically different at *P*-level shown ($n = 32$).
[1] Medium reduced protein diets contained 17.5% and 16.5% protein for grower and finisher diets respectively and were supplemented with required synthetic amino acids to match the specifications of control diets.
[2] Low reduced protein diets contained 15.6% and 14.6% protein for grower and finisher diets respectively and were supplemented with required synthetic amino acids to match the specifications of control diets.

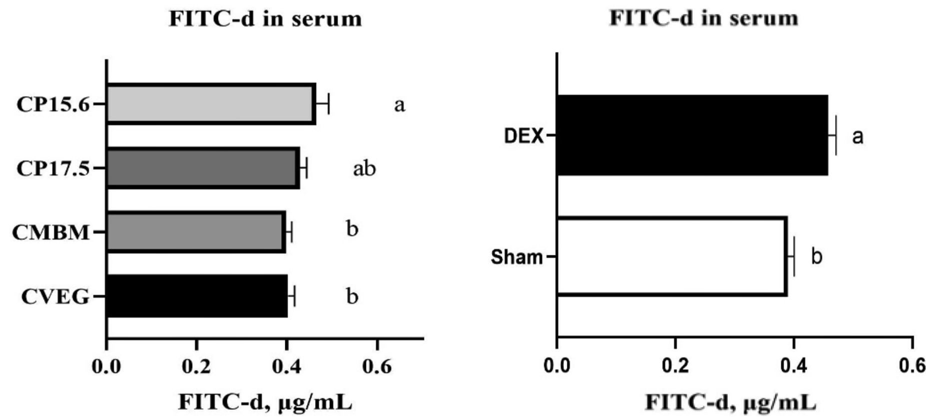

Fig. 1. Concentration of fluorescein isothiocyanate dextran (FITC-d) in serum of broiler chickens at d 21 of age. The error bars represent standard error of the mean. Bars not sharing a same letter are statistically different ($P < 0.01$). CVEG = Control diet containing all-vegetable ingredients and standard level of protein; CMBM = Control diet containing meat and bone meal and standard level of protein; CP17.5 = Reduced protein diet containing 17.5% crude protein; CP15.6 = Reduced protein diet containing 15.6% crude protein. DEX = Dexamethasone.

Table 6
Relative mRNA expression of selected genes encoding tight junction proteins in ileal tissues of broilers fed different levels of protein compared with control diets under DEX challenge or Sham injections.

Item	DEX	Claudin-1	Claudin-2	Claudin-3	*ZO-1*	*ZO-2*
Treatments [1]						
CVEG	−	0.740	0.354[abc]	0.447	1.160	0.823[bc]
CVEG	+	0.694	0.287[c]	0.498	1.057	0.951[ab]
CMBM	−	0.779	0.280[c]	0.451	1.132	0.852[bc]
CMBM	+	0.680	0.321[bc]	0.492	1.118	0.934[abc]
CP17.5	−	0.774	0.426[ab]	0.493	1.095	0.832[bc]
CP17.5	+	0.727	0.271[c]	0.477	1.121	1.009[a]
CP15.6	−	0.766	0.441[a]	0.344	0.999	0.909[abc]
CP15.6	+	0.635	0.276[c]	0.427	0.957	0.789[c]
SEM		0.0320	0.0144	0.0121	0.0270	0.0187
Main effect						
Diets						
CVEG		0.717	0.321	0.472[a]	1.109	0.887
CMBM		0.730	0.301	0.471[a]	1.125	0.893
CP17.5		0.750	0.348	0.485[a]	1.108	0.920
CP15.6		0.701	0.358	0.386[b]	0.978	0.849
DEX						
Sham		0.764	0.375[a]	0.433	1.096	0.853
Injected		0.684	0.289[b]	0.473	1.063	0.921
P-values						
DEX		0.215	0.005	0.108	0.545	0.079
Diet		0.955	0.486	0.022	0.205	0.601
Diet × DEX		0.956	0.045	0.536	0.856	0.039

ZO1 = Zonula occludens 1; *ZO2* = Zonula occludens 2; DEX = Dexamethasone; SEM = Pooled standard error of the mean.
[a–c] Mean values that do not share a same superscript letter differ significantly at the *P*-level shown for the main effects or interaction ($n = 48$).
[1] CVEG = Control diet containing all-vegetable ingredients and standard level of protein; CMBM = Control diet containing meat and bone meal and standard level of protein; CP17.5 = Reduced protein diet containing 17.5% crude protein supplemented with synthetic amino acids to match the control diets; CP15.6 = Reduced protein diet containing 15.6% crude protein supplemented with synthetic amino acids to match the control diets.

Table 7
Relative mRNA expression of selected genes involved in inflammation and mucosal immunity in ileal tissues of broilers fed different level of protein compared with control diets under DEX challenge or Sham injections.

Item	DEX	*MUC-2*	*IFNγ*	*IL1-β*	*NFκB*	*Nrf2*	*GLP-2*	*IL-10*
Treatments [1]								
CVEG	−	0.493	1.698	0.367	1.168	1.197	1.360	1.328
CVEG	+	0.422	2.147	0.462	1.046	1.036	1.186	1.557
CMBM	−	0.437	2.119	0.419	0.993	1.148	1.377	1.166
CMBM	+	0.440	2.559	0.643	1.075	1.142	1.359	1.917
CP17.5	−	0.511	1.278	0.536	1.114	0.973	1.321	1.504
CP17.5	+	0.486	1.668	0.453	1.133	1.183	1.169	0.954
CP15.6	−	0.468	2.723	0.367	0.965	0.929	1.218	1.102
CP15.6	+	0.538	1.327	0.411	0.985	1.020	1.137	1.362
SEM		0.0156	0.2023	0.0287	0.0241	0.0284	0.0338	0.0909
Main effect								
Diets								
CVEG		0.458	1.922	0.414	1.107	1.117	1.273	1.442
CMBM		0.438	2.339	0.531	1.034	1.145	1.368	1.541
CP17.5		0.499	1.473	0.495	1.123	1.078	1.245	1.229
CP15.6		0.503	2.025	0.389	0.975	0.975	1.177	1.232
DEX								
Sham		0.477	1.954	0.422	1.060	1.062	1.318	1.275
Injected		0.471	1.925	0.492	1.059	1.095	1.212	1.447
P-values								
DEX		0.861	0.943	0.231	0.995	0.561	0.124	0.348
Diet		0.391	0.510	0.272	0.125	0.177	0.266	0.535
Diet × DEX		0.453	0.298	0.314	0.497	0.144	0.846	0.104

MUC-2 = Mucin 2; *IFNγ* = Interferon-gamma; *IL1-β* = Interleukin 1-β; *NFκB* = Nuclear factor kappa B; *Nrf2* = Nuclear factor erythroid 2-related factor 2; *GLP-2* = Glucagon-like peptide-2; *IL-10* = Interleukin 10; SEM = Pooled standard error of the mean.
[a–c] Mean values that do not share a same superscript letter differ significantly at the *P*-level shown for the main effects or interaction ($n = 48$).
[1] CVEG = Control diet containing all-vegetable ingredients and standard level of protein; CMBM = Control diet containing meat and bone meal and standard level of protein; CP17.5 = Reduced protein diet containing 17.5% crude protein supplemented with synthetic amino acids to match the control diets; CP15.6 = Reduced protein diet containing 15.6% crude protein supplemented with synthetic amino acids to match the control diets.

decreased the expression of claudin-2 in birds that received both RP diets, but not for the birds that received the control diets. Unchallenged birds fed 15.6% CP had the highest mRNA expression of claudin-2. With no interaction, the expression of claudin-3 was reduced ($P < 0.05$) when birds were fed the lowest level of dietary protein (15.6%).

Table 7 shows data for genes involved in inflammation and mucosal immunity. The gene expression of mucin 2 (*MUC-2*), interferon-gamma (*IFN-γ*), interleukin 1-β (*IL1-β*), nuclear factor kappa B (*NFκB*), nuclear factor erythroid 2-related factor 2 (*Nrf2*), glucagon-like peptide-2 (*GLP-2*) and interleukin 10 (*IL-10*) was not influenced by the experimental treatments.

The data of relative expression of genes encoding peptide transporter 1 (*PepT-1*), sodium dependent glucose transporter 1 (*SGLT-1*), fibronectin (*FN*), ovotransferrin (*OVO*), glutathione peroxidase 1 (*GPx-1*) and superoxide dismutase 1 (*SOD-1*) are shown in Table 8. There was no interaction between DEX and diet for the expression of *PepT-1*, *SGLT-1*, *FN*, *OVO* and *GPx-1*. All-

Table 8
The mRNA expression of selected mechanistic genes in ileal tissues of broilers fed different level of protein compared with control diets under DEX challenge or Sham injections.

Item	DEX	*PepT-1*	*SGLT-1*	*FN*	*OVO*	*GPx-1*	*SOD-1*
Treatments [1]							
CVEG	−	0.708	0.739	1.147	2.611	0.438	1.514[ab]
CVEG	+	0.580	0.524	0.818	2.526	0.424	1.240[c]
CMBM	−	0.527	0.673	1.102	2.494	0.395	1.482[abc]
CMBM	+	0.505	0.581	0.928	2.507	0.421	1.257[c]
CP17.5	−	0.438	0.927	0.913	2.495	0.420	1.415[abc]
CP17.5	+	0.387	0.877	0.882	2.910	0.473	1.590[a]
CP15.6	−	0.346	0.858	0.964	2.286	0.424	1.301[bc]
CP15.6	+	0.392	0.809	0.914	2.506	0.433	1.314[bc]
SEM		0.0230	0.0241	0.0251	0.1365	0.0120	0.0299
Main effect							
Diets							
CVEG		0.644[a]	0.631[b]	0.982	2.568	0.431	1.377
CMBM		0.516[ab]	0.627[b]	1.015	2.500	0.407	1.369
CP17.5		0.413[bc]	0.902[a]	0.897	2.702	0.446	1.438
CP15.6		0.369[c]	0.834[a]	0.939	2.396	0.428	1.307
DEX							
Sham		0.504	0.799[a]	1.031[a]	2.471	0.419	1.428
Injected		0.466	0.698[b]	0.885[b]	2.612	0.437	1.318
P-values							
DEX		0.404	0.041	0.006	0.608	0.440	0.201
Diet		0.0006	0.0002	0.387	0.881	0.726	0.151
Diet × DEX		0.614	0.578	0.150	0.918	0.790	0.038

PepT-1 = Peptide transporter 1; *SGLT-1* = Sodium dependent glucose transporter 1; *FN* = Fibronectin; *OVO* = Ovotransferrin; *SOD-1* = Superoxide dismutase 1, *GPx-1* = Glutathione peroxidase 1; SEM = Pooled standard error of the mean.
[a-c] Mean values that do not share a same superscript letter differ significantly at the *P*-level shown for the main effects or interaction ($n = 48$).
[1] CVEG = Control diet containing all-vegetable ingredients and standard level of protein; CMBM = Control diet containing meat and bone meal and standard level of protein; CP17.5 = Reduced protein diet containing 17.5% crude protein supplemented with synthetic amino acids to match the control diets; CP15.6 = Reduced protein diet containing 15.6% crude protein supplemented with synthetic amino acids to match the control diets.

vegetable based control diet had the highest ($P = 0.0006$) relative expression of *PepT-1* which was significantly different compared with birds fed diets containing 17.5% and 15.4%. Feeding birds with both RP diets upregulated ($P = 0.0002$) gene expression of *SGLT-1* in ileum tissues. DEX injections downregulated ($P = 0.041$) mRNA expression of *SGLT-1* in the ileum.

With no effect of diet, DEX independently downregulated ($P = 0.006$) the ileal gene expression of *FN*. The expression of *OVO* and *GPx-1* was not influenced by experimental factors. However, DEX and diet interacted ($P = 0.038$) significantly for the expression of *SOD-1*. Accordingly, only DEX-injected birds showed a response to dietary treatments where birds fed diets with medium reduction of protein (17.5%) had higher expression of *SOD-1* compared with other 3 dietary treatments.

3.5. Caecal microbiota composition

Birds fed the lowest protein level (CP15.6) irrespective of whether they received DEX challenge or were Sham injected had a lower microbiota complexity with lower alpha diversity metric, richness ($P < 0.01$; Fig. 2A and B). This was the same for the alpha diversity metric, evenness; however, only for Sham injected birds ($P = 0.022$), while no significant difference between diets was observed in evenness for DEX challenged birds ($P = 0.12$; Fig. 2C). Principal coordinate analysis (PCoA) plot also showed variation between birds fed different diets, irrespective of whether they received DEX challenge or were Sham injected (Fig. 3). Greater microbial variation was observed between birds fed the lowest protein level (CP15.6) as depicted by a greater dispersion in points on the plot, while the birds on all other diets clustered more closely together, indicating a greater similarity.

Multivariate analysis showed significant differences in microbiota structure in the caecal contents of broilers fed diets of

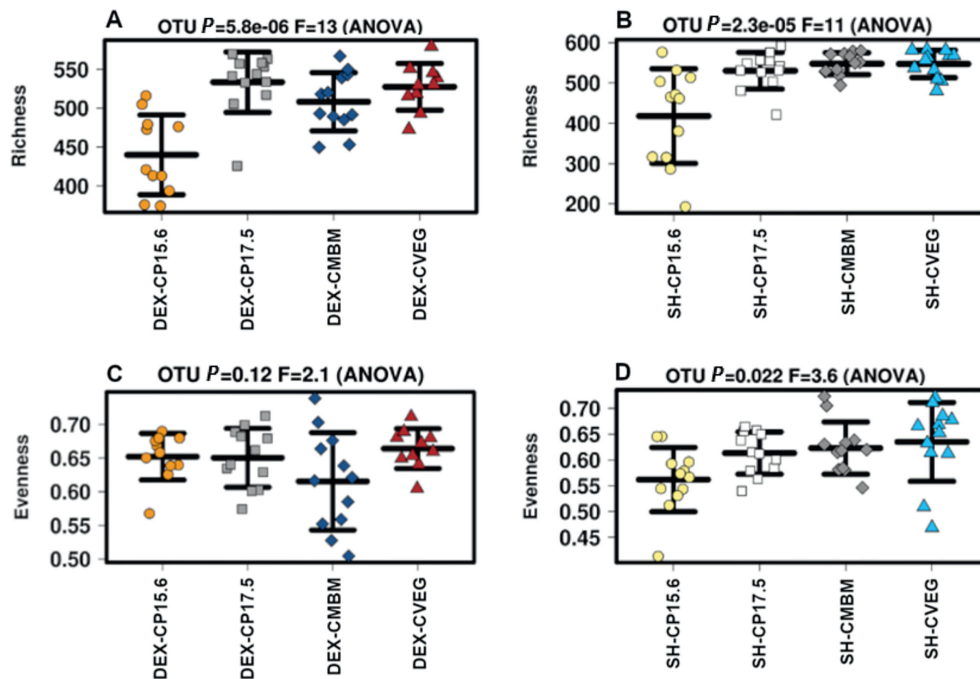

Fig. 2. Alpha diversity assessed by richness (A and B) and evenness (C and D) in the caecal contents of broilers fed different levels of protein (CP15.6 and CP17.5) compared with control diets (CMBM and CVEG) under dexamethasone (DEX) challenge or Sham injections (SH). CVEG = Control diet containing all-vegetable ingredients and standard level of protein; CMBM = Control diet containing meat and bone meal and standard level of protein; CP17.5 = Reduced protein diet containing 17.5% crude protein; CP15.6 = Reduced protein diet containing 15.6% crude protein.

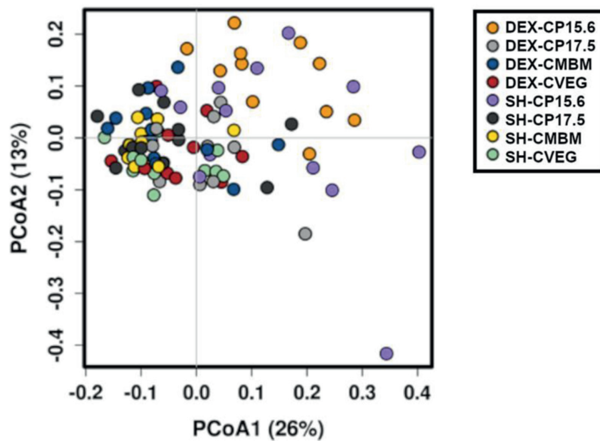

Fig. 3. Principal coordinate analysis (PCoA) plot using Bray–Curtis dissimilarity showing variation in the bacterial community structure in the caecal contents of broilers fed different levels of protein (CP15.6 and CP17.5) compared with control diets (CMBM and CVEG) under dexamethasone (DEX) challenge or Sham injections (SH). CVEG = Control diet containing all-vegetable ingredients and standard level of protein; CMBM = Control diet containing meat and bone meal and standard level of protein; CP17.5 = Reduced protein diet containing 17.5% crude protein; CP15.6 = Reduced protein diet containing 15.6% crude protein.

differing protein levels irrespective of receiving the DEX challenge or being Sham injected for both redundancy analysis ($P < 0.001$) and Adonis permutational analysis based on the Bray–Curtis distance matrix ($R^2 = 0.184$, $P < 0.001$). When the caecal microbiota composition of DEX challenged and Sham injected birds for each diet individually were assessed, there was no significant difference in overall microbiota composition for all diets except for the lowest protein diet (CP15.6), whereby both redundancy analysis ($P < 0.01$) and Adonis permutational analysis based on the Bray–Curtis distance matrix were significantly different ($R^2 = 0.115$, $P < 0.01$).

Given that the largest variation was observed in the caecal microbiota of broilers fed the lowest protein diet (CP15.6), further taxonomic analysis was undertaken on this group of animals and compared to its control (CVEG). When assessing the lowest protein diet (CP15.6) with the matched control (CVEG) using PCoA, the samples grouped together according to diet, but not by challenge status (Fig. 4). This indicates that any differences observed in overall microbiota composition between CVEG and CP15.6 were driven by the influence of the diet and not the challenge status. Therefore, all direct comparisons of the microbiota community differences observed between CVEG and CP15.6 were further assessed with challenge status combined.

As illustrated in Fig. 5, 3 phyla were identified as the dominant drivers for the variation observed in the caecal contents of broilers fed the control diet made from vegetable materials (CVEG) and those fed the low protein diet (CP15.6) using the linear discriminant analysis effect size method (LEfSe). Proteobacteria was characterised as the main phyla driving the difference in broilers fed CP15.6, while Firmicutes and Tenericutes were identified as the main phyla driving the differences observed in broilers fed CVEG ($P < 0.01$). The primary taxa contributing to the differences observed at the family level using LEfSe were Bifidobacteriaceae, Enterococcaceae and Enterobacteriaceae for birds fed the low protein diet (CP15.6) whereas Plantococcaceae, Bacillacaeae, Lachnospiraceae, Unclassified RF39 and Unclassified Clostridiales were the primary taxa contributing to the differences observed in the caecal microbiota of birds fed CVEG ($P < 0.001$, Fig. 6).

Differences in caecal microbial community structure between broilers fed CVEG and CP15.6 were evident at the genus level, with 18 genera identified as significantly contributing to the differences between diets using LEfSe (Fig. 7). The main genera driving these differences for broilers fed CP15.6 were *Bifidobacterium, Enterococcus,* Unclassified *Bifidobacteriales,*Unclassified *Clostridiaceae* , Unclassified *Enterobacteriaceae, Pediococcus, Ruminococcus, Proteus,* Unclassified *Enterococcaceae* and *Coprobacillus*, while the main genera driving these differences for broilers fed CVEG were Unclassified *Plantococcaceae, Bacillus, Sediminibacillus,* Unclassified *RF39,* Unclassified *Erysipelotrichacaea, Antaerotruncus,* Unclassified *Clostridiales,* and *Eggerthella.*

When comparing the caecal microbial taxa between broilers fed CVEG and those fed the slightly higher protein diet, CP17.5, less compositional differences were observed. No taxa were identified to be different at the phyla level, while LEfSe analysis detected Erysipelotrichaceae and Bifidocateriaceae as dominant drivers in CP17.5 at the family level and *Bifidobacterium* at the genus level. While CVEG had dominant genera Unclassified *Erysipelotrichaceae* and *Eggerthella* as determined by LEfSe ($P < 0.01$).

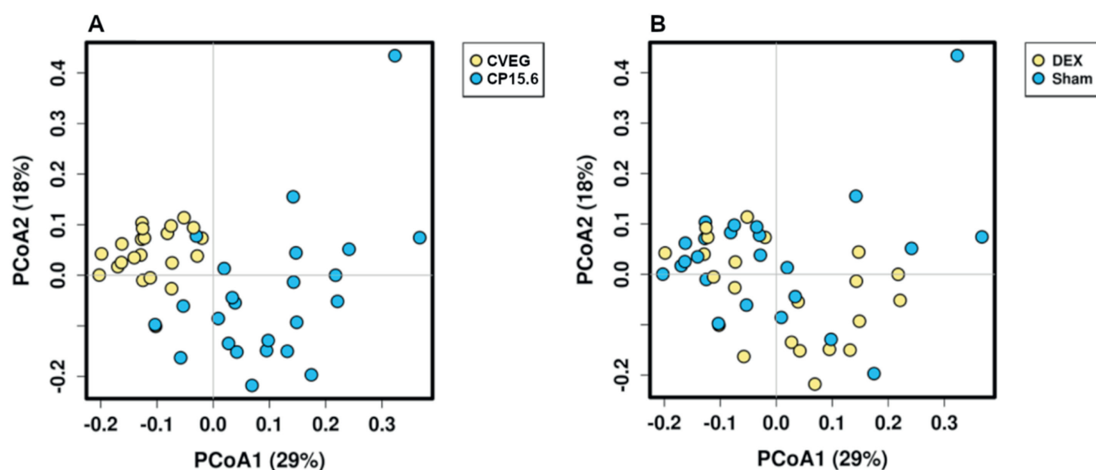

Fig. 4. Principal coordinate analysis (PCoA) plot using Bray–Curtis dissimilarity showing variation in the caecal bacterial community structure when broilers are grouped by (A) treatments CVEG and CP15.6 or (B) dexamethasone (DEX) challenge or Sham injection. CVEG = Control diet containing all-vegetable ingredients and standard level of protein; CMBM = Control diet containing meat and bone meal and standard level of protein; CP15.6 = Reduced protein diet containing 15.6% crude protein.

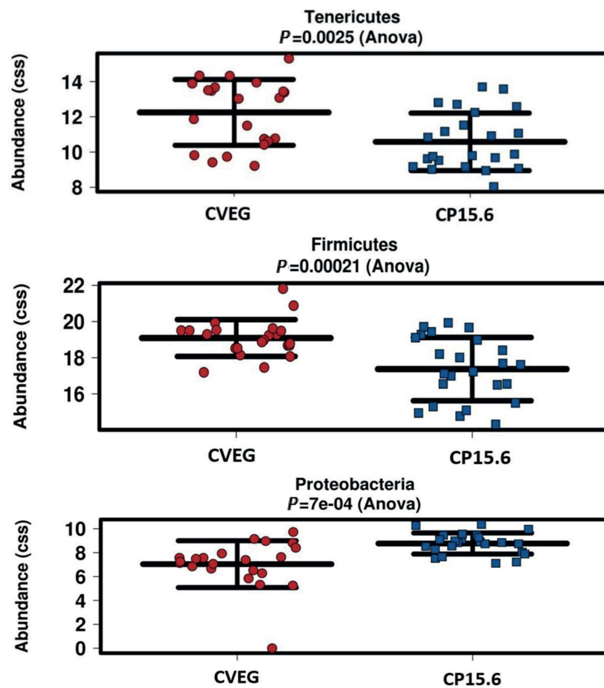

Fig. 5. Bacterial phyla identified using LEfSe as driving the differences observed in the caecum of broilers fed 2 different diets: control diet (CVEG) and low protein diet (CP15.6). CVEG = Control diet containing all-vegetable ingredients and standard level of protein; CP15.6 = Reduced protein diet containing 15.6% crude protein.

4. Discussion

In the present study, dietary protein of the RP diets was formulated to 2 levels that could be considered extreme to allow detection of possible changes induced on parameters related to intestinal functionality and health, compared with control diets. In the performance study, birds fed control diets exceeded the performance objectives for BW (Aviagen, 2019). Despite formulating the required amino acids of the RP diets to match the 2 control diets, the growth performance of broilers fed 2 RP diets were inferior compared with control diets. These results were consistent with previous studies (Barekatain et al., 2019b; Hofmann et al., 2019). The excess level of grains, starch content, changes in digestive dynamics and absorption of amino acids and glucose as well as a low content of dietary structural components may be amongst the main reasons for poor performance of birds fed RP diets (Greenhalgh et al., 2022). Birds fed both RP diets consumed a similar amount of feed compared with control diets, which may indicate compensatory feed consumption driven by deficiency or imbalance of nutrient(s) while exhibiting lower BW and higher FCR. The lack of significant differences between CVEG and CMBM rejects the hypothesis that assessing the growth performance of broilers fed RP diets may be dependent on the presence of MBM in control diets to which RP diets are compared. However, given the diets were balanced for all the essential amino acids and energy, we hypothesised further that the poor performance of broiler chicken fed RP diets may have been associated with compromised gut health assessed by parameters of intestinal barrier function and microbiota composition.

In the challenge study, the response to dietary treatments for most parameters was independent of the DEX challenge applied to the birds. Unlike previous studies, a low dosage of DEX was administered to restrict the magnitude of growth performance retardation expected by the DEX challenge. A significant and independent impact of DEX on BW and feed consumption of broilers confirms that DEX as a model of leaky gut is more suitable for assessing dietary interventions at the intestinal and cellular level as proposed in this study, and it may be unlikely that an interaction of diet and DEX could be observed for growth performance of the birds regardless of the applied dosage of DEX.

The increase in leakage of FITC-d from the intestine into the blood of birds fed CP15.6 indicated an increase in paracellular permeability and impairment of intestinal barrier function. In a previous study, an increase in intestinal permeability was only documented compared with a diet containing higher amino acids and protein content (Barekatain et al., 2019b). In the present study, given that the concentration of digestible amino acids was largely similar between the control diets and RP treatments, one can conclude that the severe reduction of dietary protein indeed reduced gut integrity. Changes in intestinal permeability resulting from stress induced by DEX can be mediated by profound impact on glucose mobilisation through type II glucocorticoid receptors, mast cells, cholinergic and adrenergic nerves, and corticotropin-releasing hormone (Barekatain et al., 2019b, Ünsal and Balkaya, 2012).

Tight junctions (TJ) are fundamentally important in controlling paracellular permeability by preventing the passage of unwanted microorganisms, toxins, and luminal antigens, whilst allowing the passage of ions and solutes. These proteins are dynamic, complex, and highly regulated. Claudins constitute the backbone of TJ and can be both barrier- and pore-forming proteins. In the present study, the gene expressions of claudin-1 and 3, as barrier forming, and claudin-2, as pore-forming, were undertaken. In most cases, an increase in the expression of barrier-forming claudins can be interpreted as a positive response for strengthening the intestinal barrier function and integrity. The absence of an effect of DEX injection on mRNA expression of claudin-1 and 3 was somewhat unexpected and contrary to previous studies in which DEX was used as a leaky gut model (Barekatain et al., 2019a, 2019b). This discrepancy may be explained by the lower dosage of DEX used in the present study. The downregulation of claudin-3 in birds fed the CP15.6 diet supports the higher intestinal permeability in the same birds, an indication of reduced gut integrity. Redistribution or downregulation of claudins 1 and 3 are widely demonstrated in gastrointestinal diseases such as inflammatory bowel diseases in humans (Kim et al., 2019). The higher expression of claudin-2, a pore-forming claudin, is shown to be associated with leaky epithelia (Venugopal et al., 2019). In the present study, birds fed RP diets and challenged with DEX downregulated claudin 2 in the ileal tissues. Indeed, the exact mechanisms of the downregulation of claudin-2 are poorly understood (Venugopal et al., 2019). However, given that DEX-challenged birds and particularly those fed RP diet at the lowest level had higher permeability, downregulation of claudin-2 may have been attributed to a protective response in birds to resuscitate intestinal integrity by preventing further increase in paracellular permeability and limiting the passage of ions and water into intercellular space. By a less straightforward interaction, ZO2 overexpression observed in birds fed CP17.5 may have been another indication of changes at the molecular level of tight junction expression contributing to compromised integrity and permeability of the intestine of birds fed RP diets.

Genes encoding mucin and a number of cytokines were investigated to identify possible changes in inflammatory responses associated with dietary protein and the DEX challenge. Inflammatory cytokines, *IFN-γ* and *IL1-β*, and *IL-10* as an anti-inflammatory cytokine, as well as *MUC-2*, were not altered in expression level across the different treatments in this study. A similar lack of effect was observed for *NFκB*, a potent regulator of innate immunity, the activation of which has been associated with intestinal

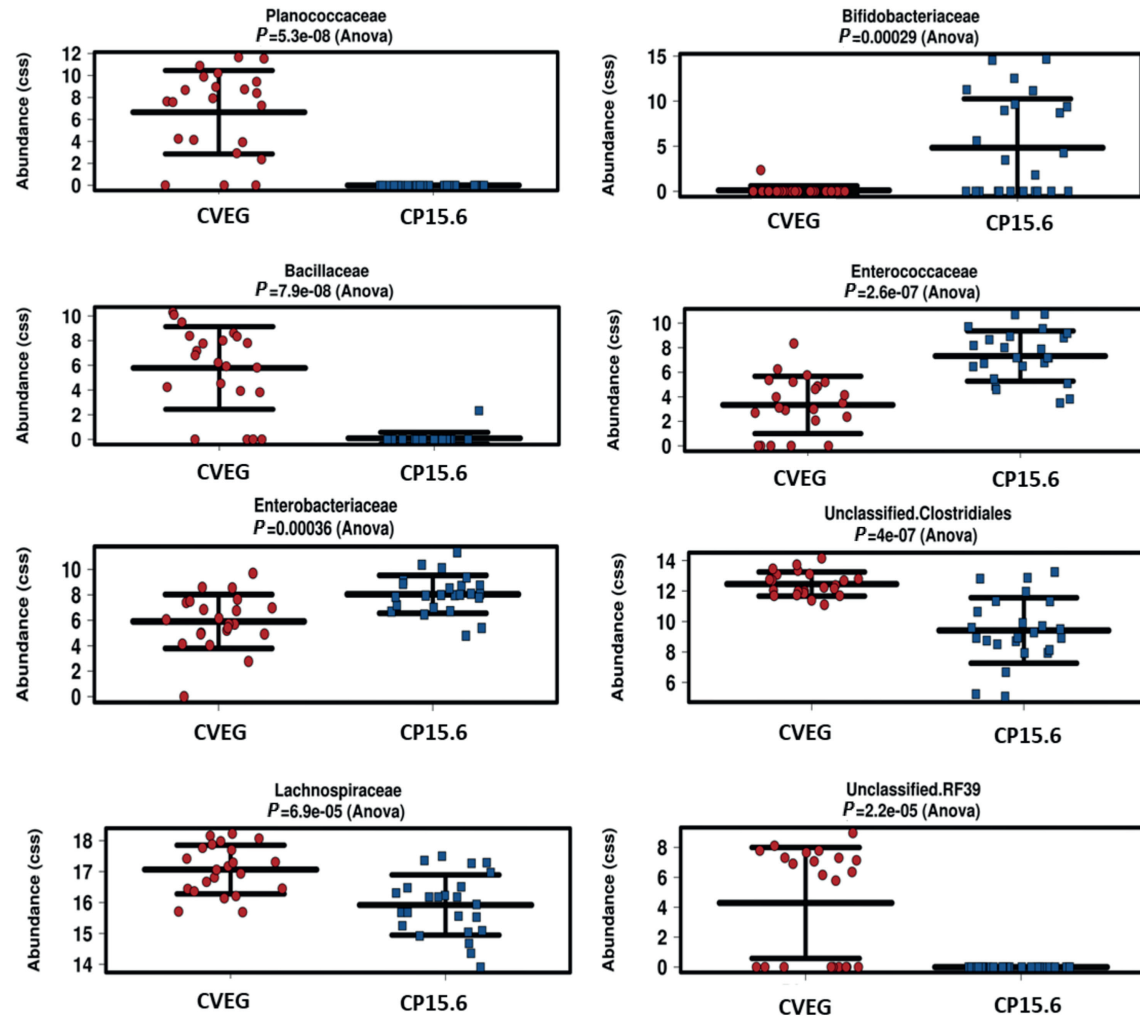

Fig. 6. Bacterial families identified using LEfSe as driving the differences observed in the caecum of broilers fed 2 different diets: control diet (CVEG) and low protein diet (CP15.6). CVEG = Control diet containing all-vegetable ingredients and standard level of protein; CP15.6 = Reduced protein diet containing 15.6% crude protein.

inflammation (Neurath et al., 1998). *Nrf2* is a cytoprotective transcription factor that is involved in anti-inflammatory cellular responses and activates antioxidant protective mechanisms. This gene was studied with the hypothesis that its expression may be affected by the possible detrimental effect of DEX on oxidative stress and increased reactive oxygen species (Bjelaković et al., 2007) which could be further influenced by dietary factors such as protein due to the nutrient-interacting nature of *Nrf2* (Stefanson and Bakovic, 2014). Nevertheless, *Nrf2* also remained unaffected by the experimental treatments. Likewise, *GLP-2*, with known impacts for improving intestinal barrier function, tissue healing and repair after injury, nutrient absorption, and energy metabolism (Connor et al., 2016) remained unaltered. The absence of changes in the selected genes related to inflammation and gut functions was unexpected, particularly for birds exposed to DEX that is known to impact immune response and various intestinal inflammation pathways (Barekatain et al., 2019b). A couple of probable reasons may be provided to explain such a lack of differences. First, there may have been other pathways involved that were not specifically studied. Second, there is a need to quantify the protein rather than mRNA to provide a better understanding of the role of these inflammatory-regulated genes.

PepT-1 as one of the proton-coupled oligopeptide transporters was downregulated in birds fed RP diet, particularly compared with CVEG. This observation may be related to lower amounts of protein-binding amino acids in those diets. The abundance of *PepT-1* has been shown to be altered by dietary protein with less protein associated with the downregulation of *PepT-1* (Chen et al., 2005), and it is known that *PepT-1* significantly contributes to the absorption of amino acids as di- and tri-peptides into enterocytes. The expression of *PepT-1* may also have implications for intestinal inflammation and fat deposition (Spanier, 2014) which can be impacted by feeding RP diets to poultry.

The upregulation of *SGLT-1* in birds fed RP diets may indicate several possible mechanisms. First, the increase in starch availability in the RP diet may increase the competition between the absorption of glucose and amino acids which can manifest itself in higher expression of *SGLT-1* as one of the main transporters of glucose into the enterocytes. Second, intestinal cells are able to increase *SGLT-1* to protect intestinal barrier function, as shown in Caco2 cells (Linda et al., 2008). Additionally, elevated activity of *SGLT-1* increases intestinal permeability through activation of myosin light chain kinase (de Punder and Pruimboom, 2015). This explanation is plausible as in the current study the birds fed the lowest level of protein indeed exhibited higher intestinal permeability coupled with increased expression of *SGLT-1* in the intestine. In DEX-challenged birds, however, *SGLT-1* was downregulated independently. While this result was consistent with one other

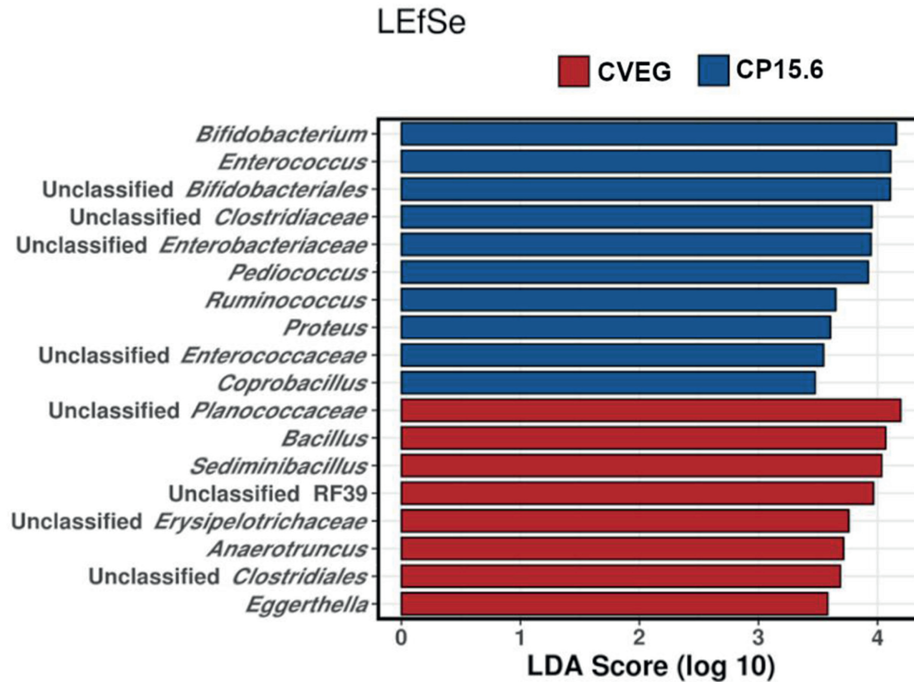

Fig. 7. Linear discriminant analysis (LDA) effect size method (LEfSe) showing the genera most likely to explain differences in broiler chicken caecal contents between control diet (CVEG) and low protein diet (CP15.6). CVEG = Control diet containing all-vegetable ingredients and standard level of protein; CP15.6 = Reduced protein diet containing 15.6% crude protein.

study (Li et al., 2009), it contradicts another previous study (Barekatain et al., 2019b). The experimental conditions as well as a lower dosage of DEX used in the present study may have contributed to this discrepancy.

Fibronectin, an extracellular matrix (ECM) protein is involved in tissue repair and is regarded as a biomarker of intestinal barrier function in chickens (Barekatain et al., 2020). The concentration of FN is expected to increase in response to intestinal inflammation or barrier dysfunction. Surprisingly, DEX decreased the mRNA expression level of *FN* independent of dietary treatments. The reason for such a reduction is not immediately known. It may be possible that exposure to DEX has led to the leakage of *FN* into the intestinal lumen content and the measured mRNA in the tissue could be a simple reflection of disturbance of ECM proteins in cell damage caused by DEX. Indeed, it was shown in a previous study that the concentration of FN can increase in the excreta of broilers in response to DEX as a leaky gut model (Barekatain et al., 2020).

The genes encoding *OVO* as a biomarker of intestinal integrity and *GPx-1* as oxidative stress, were not influenced by any of the experimental factors. The overexpression of *SOD-1* in challenged birds fed the CP17.5 diet could not be explained by data of the current study and needs further investigation.

Prolonged exposure to stressors is expected to shift the intestinal microbiota composition (Shi et al., 2019). Elevation of glucocorticoids in animal experiments has been shown to reduce gut microbiota diversity (Noguera et al., 2018; Petrullo et al., 2022). Data are scarce on the effect of DEX as a synthetic glucocorticoid on the composition of intestinal microbiota in broiler chickens. In the present study, the compositions of samples associated with sham and DEX-injected birds did not show any clear separation on the PCoA plot, which highlights that the overall composition of microbiota remained unaffected by the challenge applied to the birds. Using culture methods, Duff et al. (2019) found no shift in bacterial profiles in response to DEX in broilers. Our sequencing analysis supports the conclusion that the mode of action of DEX for impacting the intestinal barrier function does not relate to perturbations in the microbiota composition. Therefore, the discussion hereafter is limited to the effect of diet on the composition of caecal microbiota.

A lower richness of bacterial taxa was associated with the lowest dietary protein compared to the richness of microbiota in birds fed on the other diets. A complex and stable intestinal microbiota is highly desirable for supporting intestinal health and enhancing the growth performance of broiler chickens. A low richness of microbiota is often a marker of microbial imbalance or so-called "gut dysbiosis" that can result in impairment of intestinal barrier function and inflammation (Valdes et al., 2018), although a direct relationship between microbiota diversity and feed efficiency in poultry is not straightforward (Diaz Carrasco et al., 2019). At the phylum level, an increase in Proteobacteria was found to be the main driver of differences in birds fed the lowest level of protein. This observation further supports the occurrence of gut dysbiosis in RP fed birds as a sustained increase in the abundance of Proteobacteria has been shown to be a biomarker of gut dysbiosis (Shin et al., 2015). The association of the abundance of Proteobacteria with intestinal inflammation should also be noted (Shin et al., 2015). Data are scarce regarding the effect of RP diets supplemented with synthetic amino acids on gut microbiota of broilers. He et al. (2020) found that feeding broilers with a low protein and low energy diet increased the ileal abundance of Proteobacteria accompanied by lower diversity compared with a high protein and high energy diet.

The higher abundances of Firmicutes in the CVEG group compared with CP15.6 was expected as in healthy broilers Firmicutes can constitute between 50% and 96% of the bacteria at the phylum level in caeca (Elling-Staats et al., 2022). The families of Planococcaceae and Bacillacaea were strongly suppressed by reducing dietary protein. The family of Enterobacteriaceae was highly abundant in caeca of birds fed CP15.6 compared with control birds fed a vegetable-based diet. In the inflamed mucosa, Enterobacteriaceae appear to overgrow, as shown in mouse models of

inflammatory bowel disease (Håkansson et al., 2015), and they can also elevate intestinal permeability (Mirsepasi-Lauridsen et al., 2019). In chickens, a high abundance of Enterobacteriaceae has been correlated with poor growth performance (Eeckhaut et al., 2016). Therefore, this increased abundance of Enterobacteriaceae can be linked to a possible inflammation in the intestine of birds fed RP diet, in line with increased permeability and compromised intestinal health and performance.

Lachnospiraceae belong to the Firmicutes phylum. They are abundant in poultry gut and are involved in the hydrolysis of cellulose and indigestible polysaccharides (Liu et al., 2021a). In our study, birds fed CVEG, which had better feed efficiency, also had a higher caecal abundance of Lachnospiraceae compared with birds fed the least amount of dietary protein. A higher presence of Lachnospiraceae has been shown in pigs with a lower FCR, similar to the present study, which may be related to a possible higher fermentation in caeca and butyrate production, given that many Lachnospiraceae produce butyrate (Freetly et al., 2020; Quan et al., 2018).

Notably, the Bifidobacteriaceae family and in particular *Bifidobacterium* at the genus level were present in increased abundance in birds fed both RP diets (CP17.5 and CP15.6) in the present study, which concurs with a similar observation in growing pigs (Zhao et al., 2019). Higher fermentable carbohydrates resulted from the higher wheat level in the RP diet and lower acid-binding capacity of such diets may explain this observation because there is a relationship between intestinal *Bifidobacterium* and carbohydrate content in the diet (Mikkelsen and Jensen, 2004; Zhao et al., 2019). Further at the genus level, the higher *Ruminococcus* and *Pediococcus* in low-performing birds fed RP may be in contrast with some literature (Stanley et al., 2016) but it should be noted that probiotic properties are more specific to the selected strains rather than associated with an overall genus (Rodrigues et al., 2020), therefore, different species of the same genus or family may have different influence on intestinal functions and animal performance.

The present study provided objective and comprehensive evidence that despite supplementation of the required synthetic amino acids to match a standard diet, severe reduction of dietary protein negatively influences intestinal health, highlighted by the independent increase in intestinal permeability, differential expression of tight junction proteins, and reduction in microbiota diversity in the caecal contents. These comparisons were not impacted by the inclusion of MBM as an animal source of protein in control diets and to a large extent independent of the challenge applied to the birds. Specifically, Proteobacteria, a known microbiota biomarker of gut dysbiosis, were more abundantly present in the caeca of birds fed the least amount of dietary protein and this was accompanied by a wide range of compositional changes at family and genus levels, as already discussed. These novel data highlight potential opportunities for manipulation of the intestinal microbiota of broiler chickens fed RP diets to improve intestinal health and performance. Further, the strategies for managing excess grains, starch, and digestive dynamics for intestinal health and microbiota of birds fed RP diets need to be investigated for more sustainable adoption of RP diets in broiler production, particularly under enteric or environmental stress conditions.

Author contributions

R. Barekatain: Conceptualization, Funding acquisition, Investigation, Methodology, Formal analysis, Data curation, Resources, Project administration, Writing – original draft. **P.V. Chrystal**: Investigation, Methodology, Writing – review & editing. **T. Nowland**; Investigation, Methodology, Writing – review & editing. **A.F. Moss**; Investigation, Methodology, Writing – review & editing. **G.S. Howarth**: Investigation, Writing – review & editing. **T.T.H. Van**: Methodology, Writing – review & editing. **R.J. Moore**: Methodology, Formal analysis, Writing – review & editing.

Declaration of competing interest

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

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