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Supplementation of reduced protein diets with L-arginine and L-citrulline for broilers challenged with subclinical necrotic enteritis. 3. Immunological parameters and gene expression

Hiep Thi Dao[A,B], Nishchal K. Sharma[A], Sarbast K. Kheravii[A], Emma J. Bradbury[C], Shu-Biao Wu[A] and Robert A. Swick[A,*]

For full list of author affiliations and declarations see end of paper

***Correspondence to:**
Robert A. Swick
School of Environmental and Rural Science, Faculty of Science, Agriculture, Business and Law, University of New England, Armidale, NSW 2351, Australia
Email: rswick@une.edu.au

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ABSTRACT

Context. The impact of necrotic enteritis (NE) on acute-phase proteins, interleukins, blood mineral profiles, and gene expression have not been well documented. **Aims.** This study aimed to determine the effects of L-arginine (Arg) or L-citrulline (Cit) supplementation on serum immunological parameters, serum mineral composition and gene expression in broilers fed reduced-protein diets (RP) during subclinical NE challenge. **Methods.** Ross 308 cockerels (n = 720) were randomly assigned to six experimental treatments, with eight replicates of 15 birds per pen. The treatments were standard protein without and with NE challenge (SP–, SP+); reduced protein (2% points lower crude protein) without and with NE challenge (RP–, RP+), RP plus added Arg (103% of Ross 308 requirement) with NE challenge (RPA+) and RPC+ where supplemental Arg in RPA+ was replaced with Cit. A 2 × 2 factorial arrangement was employed for the first four treatments. Additionally, treatments SP+, RP+, RPA+, and RPC+ were analysed by one-way ANOVA. **Key results.** The NE × protein interactions indicated that serum calcium concentration decreased in birds fed the RP diets only when challenged with NE ($P < 0.05$). The NE × protein interactions showed that the NE challenge downregulated the mRNA expression of jejunal y+ L amino acid transporter-2, and mucin 2 only in birds fed the RP diets ($P < 0.05$). Feeding the RP decreased expression of catenin alpha 1, but increased expression of claudin 5 and tight junction protein genes compared with the SP ($P < 0.05$). Birds in the RPC+ treatment had increased gene expression of tight junction protein and claudin 5 compared with the SP+ treatment ($P < 0.05$). **Conclusions.** Dietary protein level and infection with NE both have an impact on immune response and expression of genes involved in immunity and nutrient digestibility. In part replacement of Arg with Cit in the RPC diet may have beneficial effects on gene expression in NE-challenged birds. **Implications.** Feeding RP diets may alleviate a decline in growth during subclinical NE by increasing gene expression of tight junction proteins compared with the SP diets.

Keywords: alpha-1 acid glycoprotein, arginine, calcium, citrulline, interleukins, low protein, minerals, ovotransferrin.

Introduction

Necrotic enteritis (NE), caused by *Clostridium* (*C.*) *perfringens* has been estimated to cause losses of USD6 billion annually to the global broiler industry (Wade and Keyburn 2015). The negative effects of NE on growth, feed conversion, intestinal morphology and permeability, production of short-chain fatty acids, and microbiota population have been extensively studied (Wu *et al.* 2016; Latorre *et al.* 2018; Gharib-Naseri *et al.* 2019; Hilliar *et al.* 2020). Besides, challenge with NE and infection with Newcastle disease have been shown to reduce serum calcium (Ca) and phosphorus (P); however, the effects on serum levels of other minerals such as sodium (Na), potassium (K), and zinc

(Zn) have not been reported (Fernandez *et al.* 1994; Igwe *et al.* 2018; Zanu *et al.* 2020*a*). The impact of NE on blood immunological parameters such as acute-phase proteins and interleukins and blood mineral profiles have not been well documented. Acute-phase proteins including alpha-1 acid glycoprotein and ovotransferrin are primarily synthesised in the liver as part of the acute-phase response and serve as a key part of the innate immune response to external stresses such as tissue injury and infection (O'Reilly and Eckersall 2014; Zulkifli *et al.* 2014). Alpha-1 acid glycoprotein acts as an immune-regulator influencing T-cell function, while ovotransferrin modulates heterophil and macrophage function and possesses antimicrobial properties *via* sequestration of iron (Murata *et al.* 2004). Interleukins (IL) such as IL-1 and IL-6 are pro-inflammatory agents and influence the production of acute-phase proteins (Marinkovic *et al.* 1989; Zulkifli *et al.* 2014). A recent study by Xue *et al.* (2017) showed that NE challenge increased serum IL-1 and immunoglobulin G (IgG) concentrations.

There is evidence that feeding reduced-protein diets (17% and 15% crude protein in grower and finisher phases respectively) downregulates gene expression of tight junction proteins such as zonula occludens-2 and upregulate expression of Na^+-dependent glucose transporter 1 in the ileum of broiler chickens (Barekatain *et al.* 2019*a*). Furthermore, supplementation of arginine (Arg) to reduced-protein diets (19.4% and 17.7% crude protein in grower and finisher phases respectively) has been reported to upregulate expression of the claudin-1 gene but did not affect the expression of the claudin-3 gene in broilers subjected to leaky-gut model, compared with those offered the reduced-protein diets alone or reduced-protein diets supplemented with either L-glutamine or glycine (Barekatain *et al.* 2019*b*). Claudins are important tight junction proteins associated with intestinal permeability and are considered as one of the most relevant immune-histochemical markers to evaluate the tight junction function (Guo *et al.* 2018). Dietary supplementation of Arg has been reported to increase the expression of antioxidant genes and reduce the expression of pro-inflammatory genes in the small intestine and adipose tissue in rats (Fu *et al.* 2005; Jobgen *et al.* 2009). In broiler chickens, Tan *et al.* (2014) found that Arg supplementation decreased mucosal secretory IgG concentrations and gene expression of jejunal pro-inflammatory interleukin (IL-1b) during a coccidial vaccine challenge. As a metabolite of Arg, citrulline (Cit) has been demonstrated to have Arg-sparing effects in chickens and even more effective than Arg in increasing blood Arg concentrations (Su and Austic 1999; Lassala *et al.* 2009; Dao *et al.* 2021*a*, 2021*b*). The objective of the current study was to determine the effects of Arg and Cit supplementation in reduced-protein diets on serum acute-phase protein, IL-6, immunoglobulins, mineral composition, and expression of nutrient-related, tight junction protein, mucin, and inflammatory-related genes in broilers during NE challenge.

Materials and methods

Experimental design and diets

The study was implemented at the Centre of Animal Research and Teaching at the University of New England, Armidale, New South Wales, Australia, approved by its Animal Ethics Committee (Approval number AEC19-119), and met the requirements of the Australian code of practise to care and use of animals for scientific purposes (NHMRC 2013). Day-old Ross 308 cockerels (n = 720) were allocated to 48 equal-sized floor pens (120 × 80 cm) with 15 birds per pens. Starting pen weights were similar across treatments. Birds were grown to mimic commercial conditions with hardwood shavings as bedding material in environmentally controlled rooms. Feed and water were provided *ad libitum* throughout the 35-day feeding study. The temperature, lighting, and ventilation conditions followed Ross 308 recommendations (Aviagen 2014*a*). Six treatments were used in this study, with eight replicate pens per treatment. The treatments were standard protein diet without NE challenge (SP−), SP treatment with NE challenge (SP+), reduced-protein diet balanced with crystalline amino acids without NE challenge (RP−), RP− with NE challenge (RP+), RP+ with additional Arg to 103% of requirement (equal to 15% additional supplemental crystalline Arg, RPA+), RP+ with Cit replacing all supplemental Arg in RPA+ (RPC+). A 2 × 2 factorial arrangement was employed for the first four treatments. Factors were NE (− or +) and protein level (SP or RP). All six treatments were analysed by one-way ANOVA. Crude protein was 2% points lower in RP than in SP diets for all feeding phases. The concentrations of essential amino acids in the RP diet were equivalent to the SP diet and in accordance with Ross 308 broiler nutrition specifications (Aviagen 2014*b*). Feeds were provided as crumbles for starter (Days 0–10), and pellets for grower (Days 10–24), and finisher (Days 24–35). Supplementation levels of added crystalline Arg in the RP treatments in starter, grower, and finisher phases were 0.217%, 0.213%, and 0.212% respectively. Levels of added crystalline Arg in the RPA+ treatment in starter, grower, and finisher phases were 0.249%, 0.245%, and 0.244% respectively. Concentrations of Cit in the RPC+ treatment were equivalent to the supplemental Arg level in the RPA+ treatment. Details on diet composition and nutrient contents are presented in Tables 1 and 2. Arginine and Cit were supplemented to the RP diets at the expense of wheat. More detailed information on the feed analysis was provided in the first part of this series (Dao *et al.* 2022*a*).

Necrotic-enteritis challenge

Subclinical NE was introduced following procedures previously described by Rodgers *et al.* (2015). Birds in the RPA+ and RPC+ treatments and half of the birds in the SP

Table 1. Diet composition for standard and reduced protein diets (as-fed basis).

Item	Starter		Grower		Finisher	
	SP[A]	RP[B]	SP	RP	SP	RP
Ingredient (%)						
Wheat	39.85	47.84	35.22	43.07	40.19	47.96
Sorghum	20.00	20.00	30.00	30.00	30.00	30.00
Soybean meal	34.15	26.32	29.20	21.49	24.12	16.46
Canola oil	2.45	1.37	2.51	1.51	2.96	1.98
Calcium carbonate	1.31	1.33	1.21	1.22	1.13	1.15
Dicalcium phosphate	0.89	0.93	0.67	0.72	0.49	0.54
Sodium chloride	0.25	0.16	0.21	0.15	0.21	0.10
Sodium bicarbonate	0.11	0.23	0.10	0.18	0.10	0.25
Choline chloride 70%	0.04	0.06	0.04	0.07	0.04	0.06
L-lysine HCl[C]	0.23	0.46	0.22	0.45	0.20	0.43
D,L-methionine	0.36	0.41	0.31	0.36	0.28	0.33
L-threonine	0.15	0.25	0.12	0.21	0.09	0.19
Xylanase[D]	0.01	0.01	0.01	0.01	0.01	0.01
Phytase[E]	0.01	0.01	0.01	0.01	0.01	0.01
Vitamin premix[F]	0.09	0.09	0.08	0.08	0.08	0.08
Mineral premix[G]	0.11	0.11	0.10	0.10	0.10	0.10
L-valine	–	0.11	–	0.09	–	0.07
L-arginine	–	0.22	–	0.21	–	0.21
L-isoleucine	–	0.10	–	0.08	–	0.08
Calculated composition (%)						
AMEn (kcal/kg)	3000	3000	3075	3075	3150	3150
Crude protein	23.20	21.20	21.38	19.38	19.44	17.44
Crude fat	4.47	3.46	4.65	3.71	5.13	4.22
Crude fibre	2.91	2.74	2.80	2.63	2.68	2.52
Dig arginine	1.37	1.37	1.23	1.23	1.09	1.09
Dig lysine	1.28	1.28	1.15	1.15	1.02	1.02
Dig methionine	0.65	0.67	0.59	0.61	0.53	0.55
Dig cysteine	0.30	0.28	0.29	0.26	0.27	0.25
Dig M + C	0.95	0.95	0.87	0.87	0.80	0.80
Dig tryptophan	0.28	0.24	0.26	0.22	0.23	0.20
Dig histidine	0.51	0.44	0.47	0.40	0.42	0.35
Dig phenylalanine	1.00	0.87	0.93	0.79	0.84	0.71
Dig leucine	1.67	1.47	1.62	1.43	1.49	1.30
Dig isoleucine	0.88	0.86	0.82	0.78	0.74	0.70
Dig threonine	0.86	0.86	0.77	0.77	0.68	0.68
Dig valine	0.97	0.96	0.91	0.87	0.83	0.78
Dig glycine	0.77	0.67	0.70	0.60	0.63	0.53
Calcium	0.96	0.96	0.86	0.86	0.78	0.78
Available phosphorus	0.48	0.48	0.43	0.43	0.39	0.39
Sodium	0.20	0.20	0.18	0.18	0.18	0.18
Potassium	1.01	0.88	0.92	0.79	0.84	0.70
Chloride	0.25	0.25	0.23	0.24	0.22	0.21
Linoleic acid	1.56	1.29	1.63	1.38	1.74	1.49

[A]Diet contained standard protein concentrations at 23.2%, 21.4% and 19.4% crude protein for starter, grower and finisher phases respectively.

[B]Diet contained reduced protein concentrations with two percentage points lower crude protein than in SP diets in all feeding phases. The RPA diet was created by adding L-arginine on top of the RP diet at the level of 0.03% in all feeding phases. Concentration of calculated supplemental L-arginine in the RPA diet in starter, grower and finisher phases was 0.25%, 0.25% and 0.24% respectively. The RPC diet was created by replacing all supplemental L-arginine in the RPA diet by L-citrulline.

[C]The supplemental amino acids contained the following energy (AME), crude protein (CP), and amino acid: L-lysine HCl: 4063 kcal/kg AME, 95% CP, 78% digestible lysine; D,L-methionine: 4635 kcal/kg AME, 58.7% CP, 99% digestible methionine; L-threonine: 3560 kcal/kg AME, 73.5% CP, 98% digestible threonine; L-valine: 5255 kcal/kg AME, 72.1% CP, 96.5% digestible valine; L-arginine: 2940 kcal/kg AME, 201% CP, 99% digestible arginine; L-isoleucine: 5617 kcal/kg AME, 66.0% CP, 99% digestible isoleucine.

[D]Econase XT, 25 (AB Vista, 16 000 BXU/kg of diet).

[E]Quantum Blue, 5G (AB Vista, 500 FTU/kg of diet).

[F]Vitamin premix per kg diet (UNE VM, Rabar Pty Ltd): vitamin A, 12 MIU; vitamin D, 5 MIU; vitamin E, 75 mg; vitamin K, 3 mg; nicotinic acid, 55 mg; pantothenic acid, 13 mg; folic acid, 2 mg; riboflavin, 8 mg; cyanocobalamin, 0.016 mg; biotin, 0.25 mg; pyridoxine, 5 mg; thiamine, 3 mg; antioxidant, 50 mg.

[G]Mineral premix per kg diet (UNE TM, Rabar Pty Ltd): Cu, 16 mg as copper sulfate; Mn, 60 mg as manganese sulfate; Mn, 60 mg as manganous oxide; I, 0.125 mg as potassium iodide; Se, 0.3 mg; Fe, 40 mg, as iron sulfate; Zn, 50 mg as zinc oxide; Zn, 50 mg as zinc sulfate.

AMEn, apparent metabolisable energy corrected to zero N retention; Dig, standard ileal digestible amino acid coefficients as determined by near-infrared spectroscopy (Foss NIR 6500, Denmark) standardised with Evonik AMINONIR® Advanced calibration; M + C, methionine + cysteine.

and RP treatments (challenged) were orally inoculated with 1 mL of sterile phosphate-buffered solution (PBS) containing a vaccine strain of *Eimeria*, with 5000 sporulated oocysts of *Eimeria acervulina*, 5000 sporulated oocysts of *Eimeria maxima*, and 2500 sporulated oocysts of *Eimeria brunetti* (Eimeria Pty Ltd, Ringwood, Victoria, Australia) on Day 9 and 1 mL of *C. perfringens* with an approximate concentration of 10^8 CFU (EHE-NE18 strain, Commonwealth Scientific and Industrial Research Organization, Geelong, Victoria, Australia) in a starch thioglycollate broth on Day 14. The remaining birds in the SP and RP treatments were given 1 mL of sterile PBS on Day 9 and 1 mL of sterile thioglycollate broth media as a sham treatment on Day 14 (unchallenged control groups).

Measurements of serum immunological parameters and minerals

On Day 16, two birds per pen were randomly collected, weighed, electrically stunned (MEFE CAT 44N, Mitchell Engineering Food Equipment, Clontarf, Queensland, Australia), and euthanised by decapitation for sample collection. Blood

Table 2. Analysed nutrient values of experimental diets (as-fed basis).

Nutrient composition (%)	Starter				Grower				Finisher			
	SP	RP	RPA	RPC	SP	RP	RPA	RPC	SP	RP	RPA	RPC
Dry matter	87.2	87.6	87.6	87.9	87.7	87.4	87.7	87.5	86.7	87.7	87.2	87.1
Gross energy (kcal/kg)	3979	3956	3938	3958	4004	3940	3939	3934	3972	3959	3948	3952
Crude protein	24.30	23.12	23.35	23.44	20.35	19.03	18.51	18.58	18.95	16.70	16.41	16.57
Crude fibre	2.86	2.64	2.89	2.94	2.71	2.80	2.52	2.48	3.19	2.74	3.21	2.68
Ash	4.98	4.90	4.85	4.85	4.42	4.61	4.39	4.26	4.15	4.04	3.77	3.82
Arginine	1.41	1.44	1.48	1.23	1.19	1.19	1.22	0.96	1.09	1.06	1.09	0.85
Citrulline	–	–	–	0.23	–	–	–	0.26	–	–	–	0.24
Lysine	1.32	1.33	1.32	1.34	1.12	1.13	1.14	1.14	1.05	0.95	0.95	1.01
Methionine	0.55	0.65	0.63	0.48	0.48	0.57	0.54	0.45	0.44	0.47	0.47	0.47
Histidine	0.61	0.55	0.55	0.55	0.51	0.44	0.45	0.44	0.48	0.38	0.39	0.39
Phenylalanine	1.19	1.07	1.09	1.10	1.01	0.86	0.87	0.87	0.94	0.74	0.75	0.78
Leucine	1.88	1.69	1.72	1.76	1.68	1.44	1.46	1.49	1.57	1.25	1.29	1.34
Isoleucine	1.00	0.97	0.97	0.99	0.85	0.79	0.80	0.80	0.79	0.67	0.68	0.71
Threonine	0.99	0.97	0.98	0.99	0.83	0.82	0.81	0.82	0.78	0.69	0.70	0.73
Valine	1.12	1.11	1.12	1.13	0.97	0.91	0.91	0.91	0.90	0.77	0.79	0.81
Glycine	0.98	0.89	0.88	0.91	0.82	0.70	0.71	0.72	0.77	0.60	0.62	0.65
Serine	1.17	1.06	1.06	1.09	0.98	0.83	0.84	0.85	0.91	0.71	0.73	0.77
Glutamic acid	5.13	4.91	5.02	5.02	4.13	3.63	3.72	3.70	3.88	3.22	3.33	3.45
Proline	1.66	1.61	1.64	1.64	1.33	1.21	1.23	1.22	1.25	1.09	1.12	1.15
Alanine	1.03	0.93	0.94	0.95	0.96	0.83	0.84	0.84	0.90	0.72	0.74	0.76
Tyrosine	0.61	0.56	0.55	0.46	0.51	0.45	0.46	0.34	0.48	0.38	0.40	0.34
Aspartic acid	2.14	1.86	1.85	1.89	1.85	1.47	1.51	1.51	1.70	1.22	1.23	1.28

Values of all amino acids presented were total amino acids. SP, diet contained standard protein concentrations at 23.2%, 21.4% and 19.4% crude protein for starter, grower and finisher phases respectively. RP, diet contained reduced protein concentrations with two percentage points lower crude protein than in SP diets in all feeding phases. The RPA diet was created by adding L-arginine on top of the RP diet at the concentration of 0.03% in all feeding phases. Concentration of calculated supplemental L-arginine in the RPA diet in starter, grower and finisher phases was 0.25%, 0.25% and 0.24% respectively. The RPC diet was created by replacing all supplemental L-arginine in the RPA diet by L-citrulline.

samples (from a jugular vein) were collected in vacutainers (Becton, Dickinson UK Ltd, Plymouth, UK) that contained spray-coated silica and a polymer gel, and centrifuged at 3000*g* at 4°C for 10 min to separate the serum. Serum samples were stored at −20°C until further analysis. Concentrations of alpha-1 acid glycoprotein, ovotransferrin, IL-6, IgA, IgM, and IgG in serum were quantified using an indirect enzyme-linked immunosorbent assay (ELISA) commercial kits according to manufacturer's instructions (catalogue numbers ab157690 (Abcam, Cambridge, MA, USA), ab157694 (Abcam), ELG-IL6 (RayBiotech, Norcross, GA, USA), ab157691 (Abcam), ab157692 (Abcam) and KT-619 (Kamiya Biomedical Company, Seattle, WA, USA) respectively). The concentrations of K, Na, Ca, and P in the serum were determined using the commercial kits in a Thermo Scientific™ Indiko™ Plus clinical chemistry analyser (Thermo Fisher Scientific Inc., Waltham, MA, USA) following the manufacturer's instruction. The kits used were as follows: sodium (Na), Enzymatic Colorimetric Test, catalogue number NA 3851 (Randox Laboratories Ltd, County Antrim, UK); potassium (K), U.V. Test, catalogue number PT 3852 (Randox Laboratories Ltd); calcium, REF number 981772 (Thermo Fisher Scientific Inc., Waltham, MA, USA); and phosphorus, REF number 981890 (Thermo Fisher Scientific Inc.). The blood serum Zn concentration was determined using a Zinc (Zn) kit, catalogue number ZN 2341 (Randox Laboratories Ltd), following the manufacturer's instruction, and the results were read on a SpectraMax M2e plate reader (Molecular Devices, California, USA).

RNA extraction and cDNA synthesis

Jejunal tissues from two birds per pen were collected for gene expression on Day 16. Approximately 2 cm of the proximal jejunal tissues were excised, carefully flushed with chilled sterile phosphate-buffered saline (PBS), then placed in 2-mL Eppendorf tubes containing RNAlater™ Solution (Invitrogen by Thermo Fisher Scientific). The jejunal tissues were stored at 4°C for 4 h, then at −20°C until further analysis. For RNA

extraction, approximately 25 mg of the tissue sample was weighed in a 2-mL Eppendorf tube containing a 3-mm bead. Then, 350 μL of lysis buffer RLY-β-ME was added into the tubes, and samples were homogenised for 5 min by using a Tissuelyser II. Total RNA of the tissue sample was extracted using ISOLATE II RNA Mini Kit (Bioline, Sydney, NSW, Australia), as per the manufacturer's instructions, with the inclusion of a DNase I digestion step to eliminate the genomic DNA. After extraction, the RNA concentration and purity were checked using a NanoDrop ND-8000 spectrophotometer (Thermo Fisher Scientific). The integrity of RNA samples was checked on the Agilent 2100 Bioanalyser (Agilent Technologies Inc., Waldbronn, Germany) by using an RNA 6000 Nano kit (Agilent Technologies Inc., Palo Alto, CA, USA), following the manufacturer's instructions. The RIN values of the samples were ranged between 8 and 9.8 in the present study. The total RNA samples were reverse-transcribed to cDNA by using the SensiFAST cDNA Synthesis Kit (Bioline, Sydney, NSW, Australia) in a Rotorgene 6000 real-time (RT) polymerase chain reaction instrument (Corbett, Sydney, NSW, Australia), as per the manufacturer's instructions. Synthesised cDNA samples were diluted 10 times with nuclease-free water and kept at −20°C until further analysis.

Quantitative polymerase chain reaction (RT-qPCR)

Quantitative polymerase chain reaction (qPCR) was performed using an SYBR Green kit SensiFAST SYBR No-ROX (Bioline, Sydney, NSW, Australia) on a Rotorgene 6000 real-time PCR machine (Corbett Research, Sydney, NSW, Australia). The PCR reaction was performed in a volume of 10 μL containing 5 μL of 2 × SensiFAST, 400 mmol/L of each primer, 2.2 μL of nuclease-free water, and 2 μL of 10 × diluted cDNA sample. To determine two suitable reference genes for the analysis, the geNorm module in qbase+ software ver. 3.0 (Biogazelle, Zwijnbeke, Belgium) was used to calculate the gene-expression stability (geNorm M) from 10 widely used house-keeping genes (hydroxymethylbilane synthase (*HMBS*), succinate dehydrogenase complex flavoprotein subunit A (*SDHA*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), hypoxanthine-guanine phosphoribosyltransferase (*HPRT1*), thyroxine-binding protein (*TBP*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*), β-actin (*ACTB*), ribosomal protein L4 (*RPL4*), nuclear ribosomal RNA small subunit (*18S*), and albumin (*ALB*)). On the basis of the expression stability of the reference genes, *HPRT1* and *TBP* were chosen to normalise the target genes in the jejunum because they had the lowest *M* values compared with the other reference genes ($M = 0.311$ for both genes). The candidate genes analysed were aminopeptidase N (*APN*), solute carrier family 7, member 9 ($b^{0,+}AT$), cationic amino acid transporter-1 (*CAT1*), cationic amino acid transporter-2 (*CAT2*), claudin 1 (*CLDN1*), claudin 5 (*CLDN5*), excitatory amino acid transporter 3 (*EEAT3*), junctional adhesion 2 (*JAM2*), mucin 2 (*MUC2*), peptide transporter-1 (*PepT1*), peptide transporter-2 (*PepT2*), tight junction protein (*TJP1*), y+ L amino acid transporter-2 (y^+LAT2), E-cadherin (*CDH1*), interferon-gamma (*IFN-γ*), nitric oxide synthase 2 (*NOS2*), and protein kinase AMP-activated non-catalytic subunit gamma 2 (*PRKAG2*). The relative quantification of genes using the arithmetic mean method in the qBase+ software was exported to SAS 9.3 package (SAS Institute, Inc., Cary, NC, USA) for further analysis.

Primers for qPCR were either sourced from published papers or designed using the NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), as shown in Table 3. All primers used in this study were checked for specificity by using an Agilent DNA 1000 Kit (Agilent Technologies, Inc., Waldron, Germany) on an Agilent 2100 Bioanalyser (Agilent Technologies, Inc.).

Data analyses

R Commander (ver. 3.3.1, R Foundation for Statistical Computing, Vienna, Austria) was used to analyse data. All data were tested for normality and variance homogeneity before analysis. First, a two-way ANOVA was used to test the interaction between NE challenge (no or yes) and protein level (SP or RP), excluding RPA+ and RPC+ treatments (2 × 2 factorial arrangement of treatments). Then, one-way ANOVA was used to test statistical differences among the four NE-challenged treatments (SP+, RP+, RPA+, and RPC+). Tukey's *post hoc* test was used to identify pairwise differences between the treatments from significant ANOVA results. The *P*-value of <0.05 was considered significant.

Results

Serum immunological parameters

Results on concentrations of immunological parameters, including IL-6, IgA, IgM, IgG, alpha-1 acid glycoprotein, and ovotransferrin in the blood serum on Day 16 are presented in Table 4. No NE × protein interactions were detected for any of the above immunological parameters. Necrotic-enteritis challenge as the main effect increased the concentrations of serum alpha-1 acid glycoprotein ($P < 0.05$) and IgA ($P < 0.05$) on Day 16. Feeding the RP diets decreased both alpha-1 acid glycoprotein ($P < 0.001$) and ovotransferrin ($P < 0.001$) concentrations in the blood serum on Day 16, as shown by the main effect of protein supplementation level in Table 4. Concentrations of serum IL-6, IgM, and IgG were not affected by either NE challenge or protein supplementation level (Table 4). Supplementation of either Arg or Cit to the RP+ treatment did not affect concentrations of serum IL-6, IgA, IgM, IgG, alpha-1 acid

Table 3. Sequences of primers used for quantitative real-time PCR.

Gene symbol	Gene title	Primer sequence (5′–3′)	Ta °C	Amplicon size (bp)	Reference
Nutrient-related genes					
APN	Aminopeptidase N	F-AATACGCGCTCGAGAAAACC	60	70	Gilbert *et al.* (2007)
		R-AGCGGGTACGCCGTGTT			
$b^{0,+}AT$	Solute carrier family 7, member 9	F-CAGTAGTGAATTCTCTGAGTG TGAAGCT	60	88	Gilbert *et al.* (2007)
		R-GCAATGATTGCCACAACTACCA			
PepT1	Peptide transporter-1	F-TACGCATACTGTCACCATCA	60	205	Guo *et al.* (2014)
		R-TCCTGAGAACGGACTGTAAT			
PepT2	Peptide transporter-2	F-TGACTGGGCATCGGAACAA	60	63	Paris and Wong (2013)
		R-ACCCGTGTCACCATTTTAACCT			
CAT1	Cationic amino acid transporter-1	F-CAAGAGGAAAACTCCAGTAATTGCA	60	75	Gilbert *et al.* (2007)
		R-AAGTCGAAGAGGAAGGCCATAA			
CAT2	Cationic amino acid transporter-2	F-TGCTCGCGTTCCCAAGA	60	67	Gilbert *et al.* (2007)
		R-GGCCCACAGTTCACCAACAG			
EEAT3	Excitatory amino acid transporter 3	F-TGCTGCTTTGGATTCCAGTGT	60	79	Su *et al.* (2014)
		R-AGCAATGACTGTAGTGCAGAAGTA ATATATG			
y+ LAT2	y+ L amino acid transporter-2	F-GCCCTGTCAGTAAATCAGACAAGA	60	82	Gilbert *et al.* (2007)
		R-TTCAGTTGCATTGTGTTTTGGTT			
PRKAG2	Protein kinase AMP-activated non-catalytic subunit gamma 2	F-ACGCTGGAATTACAAACCTGC	60	73	Nafari (2019)
		R-ACTTGGTTGTGGTCTTGGTGG			
Tight junction proteins, mucin and inflammatory-related genes					
CLDN1	Claudin 1	F-CTTCATCATTGCAGGTCTGTCAG	60	103	Zanu *et al.* (2020*b*)
		R-AAATCTGGTGTTAACGGGTGTG			
CLDN5	Claudin 5	F-GCAGGTCGCCAGAGATACAG	61	162	Zanu *et al.* (2020*b*)
		R-CCACGAAGCCTCTCATAGCC			
JAM2	Junctional adhesion 2	F-AGACAGGAACAGGCAGTGCTAG	60	135	Zanu *et al.* (2020*b*)
		R-ATCCAATCCCATTTGAGGCTAC			
TJP1	Tight junction protein	F-GGATGTTTATTTGGGCGGC	60	187	Zanu *et al.* (2020*b*)
		R-GTCACCGTGTGTTGTTCCCAT			
MUC2	Mucin 2	F-CCCTGGAAGTAGAGGTGACTG	60	143	Fan *et al.* (2015)
		R-TGACAAGCCATTGAAGGACA			
CDH1	E-cadherin	F-GCAAGCCGTTTACCACATCA	61	178	This study
		R-GGTGGGGAGAAGGGTTGAG			
IFN-γ	Interferon gamma	F-GTGAAGAAGGTGAAAGATATCATGGA	60	71	Hangalapura *et al.* (2006)
		R-GCTTTGCGCTGGATTCTCA			
NOS2	Nitric oxide synthase 2	F-CAGCTAAAGAGCCAAAAGCGA	60	107	This study
		R-GTTCATGCCCGGACCAATG			
Housekeeping genes					
HPRT1	Hypoxanthine guanine phosphoribosyl transferase 1	F-ACTGGCTGCTTCTTGTG	63	245	Yang *et al.* (2013)
		R-GGTTGGGTTGTGCTGTT			
TBP	TATA-Box binding protein	F-TAGCCCGATGATGCCGTAT	62	147	Li *et al.* (2005)
		R-GTTCCCTGTGTCGCTTGC			

Table 4. Serum immunological parameters of experimental treatments on Day 16.

Effect		Alpha-1 acid glycoprotein (μg/mL)	Ovotransferrin (μg/mL)	IgA (μg/mL)	IgM (μg/mL)	IgG (μg/mL)	Interleukin-6 (ng/mL)
Two-way ANOVA results (2 × 2 factorial arrangement of treatments)							
Treatment	SP–	196.9	1319	138.7	143.2	1117	0.188
	RP–	119.9	1024	131.1	137.8	947.3	0.226
	SP+	241.3	1269	174.4	172.6	1071	0.222
	RP+	144.3	1024	174.8	125.0	1024	0.177
NE	No	158.4a	1162	134.9a	140.3	1027	0.207
	Yes	192.8b	1138	174.7b	148.8	1048	0.202
Protein	SP	219.1b	1294b	154.0	157.9	1094	0.205
	RP	132.1a	1024a	153.0	131.8	983.1	0.204
s.e.m.		10.7	33	10.0	9.5	40	0.019
P-value	NE	0.020	0.727	0.046	0.663	0.800	0.895
	Protein	<0.001	<0.001	0.868	0.174	0.175	0.979
	NE × protein	0.479	0.574	0.841	0.273	0.462	0.310
One-way ANOVA results (four NE-challenged treatments)							
Treatment	SP+	241.3b	1269	174.4	172.6	1071	0.222
	RP+	144.3a	1024	174.8	125.0	1024	0.177
	RPA+	126.5a	1150	162.2	173.1	965.9	0.160
	RPC+	114.3a	1062	156.8	164.3	1018	0.183
s.e.m.		10.6	46	9.1	8.7	41	0.019
P-value		<0.001	0.164	0.882	0.173	0.861	0.706

Different letters within a column indicate significant differences between the means. Symbols –/+ indicate the absence or presence of necrotic enteritis challenge in the treatments. SP, diet contained standard protein concentrations at 23.2%, 21.4% and 19.4% crude protein for starter, grower and finisher phases respectively. RP, diet contained reduced protein concentrations with two percentage points lower crude protein than in SP diets in all feeding phases. The RPA diet was created by adding L-arginine on top of the RP diet at the level of 0.03% in all feeding phases. Concentration of calculated supplemental L-arginine in the RPA diet in starter, grower and finisher phases was 0.25%, 0.25% and 0.24% respectively. The RPC diet was created by replacing all supplemental L-arginine in the RPA diet by L-citrulline. Two-way ANOVA presented results of the 2 × 2 factorial arrangement of treatments, with the main factors being necrotic enteritis challenge (NE, yes or no) and protein level (SP or RP).

glycoprotein, and ovotransferrin in the respective groups ($P > 0.05$; Table 4).

Serum mineral composition

Results for serum mineral concentrations of Ca, K, Na, P, and Zn on Day 16 are shown in Table 5. NE × protein interactions were obtained for Ca ($P < 0.05$), K ($P < 0.01$) and Na ($P < 0.001$) concentration. The results indicated that the NE challenge decreased serum Ca concentration only in birds fed the RP diets. Whereas the NE challenge increased serum K concentration and decreased serum Na concentration only in birds fed the SP diets (Table 5). Necrotic-enteritis challenge as the main effect increased Zn concentration ($P < 0.05$) but decreased P concentration ($P < 0.05$) in the blood serum. Supplementation of either Arg or Cit to the RP+ treatment did not affect serum Ca, K, Na, P, and Zn concentrations in the respective groups ($P > 0.05$; Table 5). A negative correlation was found between serum Na and K ($P < 0.001$) concentration, while serum Na concentration was positively correlated with serum *P* concentration ($P < 0.001$; Fig. 1).

Expression of jejunal nutrient-related genes

Relative mRNA expression of nutrient-related genes in the jejunum on Day 16, including *APN*, $b^{0,+}AT$, *PepT1*, *PepT2*, *CAT1*, *CAT2*, *EEAT3*, *y+ LAT2*, and *PRKAG2* are shown in Table 6. NE × protein interactions were observed for $b^{0,+}AT$ ($P < 0.05$) and *y+ LAT2* ($P < 0.05$). The NE challenge downregulated the mRNA expression of $b^{0,+}AT$ in both SP- and RP-fed birds, but greater downregulation was observed in RP-fed birds. The NE challenge downregulated the mRNA expression of *y+ LAT2* only in birds fed the RP diets (Table 6). Necrotic-enteritis challenge as the main effect decreased the expression of *APN* ($P < 0.001$), *PepT1* ($P < 0.001$), *EEAT3* ($P < 0.001$), and *PRKAG2* ($P < 0.01$) and increased the expression of *PepT2* ($P < 0.05$) and *CAT1* ($P < 0.01$; Table 6). Additional supplementation of Arg to

Table 5. Serum mineral composition of experimental treatments on Day 16 (mg/dL).

Effect		Ca	K	Na	P	Zn
Two-way ANOVA results (2 × 2 factorial arrangement of treatments)						
Treatment	SP–	8.27ab	34.41a	374.8b	6.64	0.117
	RP–	8.67b	30.55a	373.4b	6.29	0.109
	SP+	8.32ab	44.22b	342.0a	5.55	0.136
	RP+	7.43a	30.72a	366.6b	5.99	0.128
NE	No	8.47	32.48a	374.1b	6.47b	0.113a
	Yes	7.87	37.47b	354.3a	5.77a	0.132b
Protein	SP	8.29	39.32b	358.4a	6.09	0.127
	RP	8.05	30.63a	370.0b	6.14	0.118
s.e.m.		0.16	1.25	2.9	0.15	0.004
P-value	NE	0.066	0.043	<0.001	0.011	0.015
	Protein	0.463	<0.001	0.034	0.868	0.271
	NE × protein	0.039	0.005	<0.001	0.130	0.989
One-way ANOVA results (four NE-challenged treatments)						
Treatment	SP+	8.32	44.22b	342.0a	5.55	0.136
	RP+	7.43	30.72a	366.6b	5.99	0.128
	RPA+	7.86	32.41a	369.5b	6.03	0.125
	RPC+	7.42	31.12a	369.2b	6.18	0.122
s.e.m.		0.17	1.20	2.5	0.09	0.005
P-value		0.195	<0.001	<0.001	0.053	0.869

Different letters within a column indicate significant differences between the means. Symbols –/+ indicate the absence or presence of necrotic enteritis challenge in the treatments. SP, diet contained standard protein concentrations at 23.2%, 21.4% and 19.4% crude protein for starter, grower and finisher phases respectively. RP, diet contained reduced protein concentrations with two percentage points lower crude protein than in SP diets in all feeding phases. The RPA diet was created by adding L-arginine on top of the RP diet at the level of 0.03% in all feeding phases. Concentration of calculated supplemental L-arginine in the RPA diet in starter, grower and finisher phases was 0.25%, 0.25% and 0.24% respectively. The RPC diet was created by replacing all supplemental L-arginine in the RPA diet by L-citrulline. Two-way ANOVA presented results of the 2 × 2 factorial arrangement of treatments, with the main factors being necrotic enteritis challenge (NE, yes or no) and protein level (SP or RP).

the RP+ treatment decreased *PepT2* expression compared with the RP+ treatment ($P < 0.01$), and increased *CAT1* expression compared with the SP+ treatment ($P < 0.001$; Table 6). Supplementation of Cit to the RP+ treatment increased *PRKAG2* expression compared with the SP+ treatment ($P < 0.05$; Table 6).

Expression of jejunal tight junction protein, mucin, and inflammatory-related genes

Results on relative mRNA expressions of tight junction protein and inflammatory-related genes in the jejunum on Day 16, including *CLDN1*, *CLDN5*, *JAM2*, *TJP1*, *CDH1*, *NOS2*, *IFN-γ*, and *MUC2* are presented in Table 7. A NE × protein interaction was detected for *MUC2* ($P < 0.05$), where NE challenge downregulated *MUC2* expression only in birds fed the RP diets (Table 7). Necrotic-enteritis challenge as the main effect decreased the expression of *CLDN5* ($P < 0.05$) and *TJP1* ($P < 0.001$) and increased the expression of *NOS2* ($P < 0.001$) and *IFN-γ* on Day 16 ($P < 0.001$; Table 7). Feeding the RP diet increased the expression of *CLDN5* ($P < 0.01$) and *TJP1* ($P < 0.01$), and decreased the expression of *CDH1* ($P = 0.05$), regardless of the NE challenge on Day 16 (Table 7). Supplementation of both Arg and Cit to the RP+ treatment increased *TJP1* expression and Cit supplementation increased *CLDN5* expression compared with the SP+ treatment ($P < 0.05$; Table 7).

Discussion

Interleukin-6 is known as a primary initiator for the production of acute-phase proteins (Marinkovic *et al.* 1989; Le Floc'h *et al.* 2004). Increasing concentrations of pro-inflammatory cytokines, including IL-1 and IL-6 following an infection, act as a signal for the liver to increase the production of acute-phase proteins such as alpha-1 acid glycoprotein and ovotransferrin to prepare the host to fight against the infection, resulting in the increased concentrations of these proteins in the blood stream (Tosi 2005). In the current study, although serum IL-6 concentration was not altered following the NE challenge, the increased serum alpha-1 acid glycoprotein and IgA concentrations in NE-challenged birds indicated that there was an inflammatory response. The absence of treatment effects on IL-6 concentration in the current study may be attributed to the following factors: (1) collection time of the serum sample because it can be rapidly cleared from circulation after the stimulus (O'Reilly and Eckersall 2014); (2) the upregulation of jejunal *IFN-γ* gene in NE-challenged birds might decrease the synthesis of IL-6 (Schroder *et al.* 2004).

Acute-phase proteins have been used as disease biomarkers in humans and veterinary medicine (O'Reilly and Eckersall 2014). The results of the current study and Saleem (2013) suggest that serum alpha-1 acid glycoprotein concentration may be more sensitive than is ovotransferrin to evaluate disease status in birds. Likewise, determination of serum IgA concentration may be more effective than is determination of IgM or IgG to assess immune response in NE-challenged birds, and that might be the reason for it being widely used in diagnosing *Eimeria* infection (Yun *et al.* 2000). Noticebly, reduced serum Ca and P concentrations have been reported in birds infected with NE and Newcastle disease, and have been considered as an indicator for the disease infection (Fernandez *et al.* 1994; Igwe *et al.* 2018; Zanu *et al.* 2020*a*). In the current study, the decreased serum Ca and P concentrations in NE-challenged birds might be attributed to reductions in feed intake, villus height, and apparent

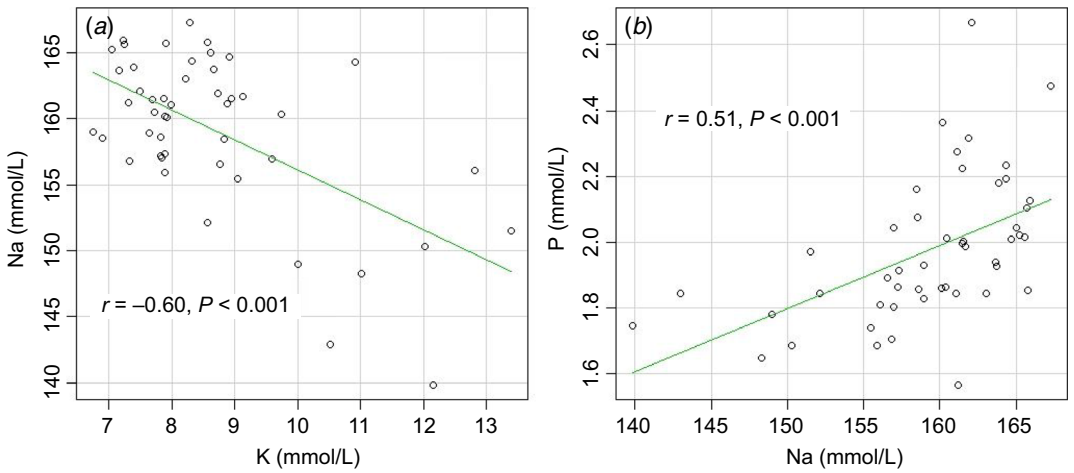

Fig. 1. Associations among concentrations of the minerals in the blood serum. The lines represent the lines of best fit. (*a*) Na and K; (*b*) P and Na.

Table 6. Expression of jejunal nutrient-related genes.

Effect		*APN*	*b*$^{0,+}$*AT*	*PepT1*	*PepT2*	*CAT1*	*CAT2*	*EEAT3*	*y+ LAT2*	*PRKAG2*
Two-way ANOVA results (2 × 2 factorial arrangement of treatments)										
Treatment	SP–	1.741	1.464a	1.541	0.695	0.733	1.010	1.857	1.172bc	0.969
	RP–	1.643	1.715a	1.583	0.666	0.855	0.875	1.952	1.297c	1.219
	SP+	0.890	0.924b	0.979	1.267	0.956	0.746	0.727	1.000ab	0.712
	RP+	0.735	0.771b	0.834	1.316	1.329	0.831	0.699	0.861a	0.868
NE	No	1.692b	1.589b	1.562b	0.679a	0.794a	0.943	1.904b	1.235b	1.102b
	Yes	0.812a	0.848a	0.907a	1.295b	1.143b	0.791	0.713a	0.931a	0.790a
Protein	SP	1.316	1.194	1.260	0.981	0.844	0.878	1.292	1.086	0.832
	RP	1.189	1.243	1.208	0.991	1.092	0.852	1.325	1.079	1.044
s.e.m.		0.102	0.082	0.078	0.120	0.068	0.040	0.124	0.039	0.058
P-value	NE	<0.001	<0.001	<0.001	0.017	0.008	0.056	<0.001	<0.001	0.005
	Protein	0.545	0.770	0.746	0.969	0.067	0.749	0.896	0.930	0.069
	NE × protein	0.833	0.037	0.380	0.864	0.440	0.737	0.641	0.022	0.644
One-way ANOVA results (four NE-challenged treatments)										
Treatment	SP+	0.890	0.924	0.979	1.267ab	0.956a	0.746	0.727	1.000	0.712a
	RP+	0.735	0.771	0.834	1.316b	1.329ab	0.831	0.699	0.861	0.868ab
	RPA+	0.851	0.810	0.807	0.399a	1.580b	0.908	0.846	0.900	0.974ab
	RPC+	0.859	0.848	0.911	1.438b	1.133ab	0.840	0.874	0.934	1.060b
s.e.m.		0.045	0.040	0.058	0.142	0.079	0.035	0.051	0.033	0.042
P-value		0.655	0.606	0.737	0.018	0.025	0.465	0.632	0.474	0.014

Different letters within a column indicate significant differences between the means. Symbols –/+ indicate the absence or presence of necrotic enteritis challenge in the treatments. SP, diet contained standard protein concentrations at 23.2%, 21.4% and 19.4% crude protein for starter, grower and finisher phases respectively. RP, diet contained reduced protein concentrations with two percentage points lower crude protein than in SP diets in all feeding phases. The RPA diet was created by adding L-arginine on top of the RP diet at the concentration of 0.03% in all feeding phases. Concentration of calculated supplemental L-arginine in the RPA diet in starter, grower and finisher phases was 0.25%, 0.25% and 0.24% respectively. The RPC diet was created by replacing all supplemental L-arginine in the RPA diet by L-citrulline. Two-way ANOVA presented results of the 2 × 2 factorial arrangement of treatments, with the main factors being necrotic enteritis challenge (NE, yes or no) and protein level (SP or RP).

APN, aminopeptidase N; *b*$^{0,+}$*AT*, solute carrier family 7, member 9; *PepT1*, peptide transporter-1; *PepT2*, peptide transporter-2; *CAT1*, cationic amino acid transporter-1; *CAT2*, cationic amino acid transporter-2; *EEAT3*, excitatory amino acid transporter 3; *y*$^{+}$*LAT2*, y+ L amino acid transporter-2; *PRKAG2*, protein kinase AMP-activated non-catalytic subunit gamma 2.

Table 7. Expression of jejunal tight junction proteins, mucin, and inflammatory-related genes.

Effects		*CLDN1*	*CLDN5*	*JAM2*	*TJP1*	*MUC2*	*CDH1*	*NOS2*	*IFN-γ*
Two-way ANOVA results (2 × 2 factorial arrangement of treatments)									
Treatment	SP−	0.963	0.958	1.058	1.057	1.148ab	0.979	0.763	0.284
	RP−	0.843	1.249	1.157	1.513	1.638b	0.776	0.798	0.386
	SP+	0.867	0.773	1.035	0.696	0.946a	0.881	1.105	1.461
	RP+	0.987	1.016	0.982	0.982	0.801a	0.796	1.110	1.986
NE	No	0.899	1.113b	1.107	1.285b	1.377b	0.870	0.782a	0.338a
	Yes	0.927	0.895a	1.009	0.839a	0.873a	0.836	1.107b	1.724b
Protein	SP	0.912	0.859a	1.046	0.876a	1.047	0.926b	0.946	0.912
	RP	0.915	1.133b	1.069	1.247b	1.192	0.787a	0.954	1.186
s.e.m.		0.073	0.046	0.039	0.067	0.086	0.036	0.051	0.161
P-value	NE	0.813	0.015	0.209	<0.001	0.005	0.648	<0.001	<0.001
	Protein	0.984	0.002	0.772	0.004	0.453	0.050	0.935	0.405
	NE × protein	0.440	0.737	0.339	0.343	0.030	0.408	0.863	0.296
One-way ANOVA results (four NE-challenged treatments)									
Treatment	SP+	0.867	0.773a	1.035	0.696a	0.946	0.881	1.105	1.461
	RP+	0.987	1.016ab	0.982	0.982ab	0.801	0.796	1.110	1.986
	RPA+	1.152	1.012ab	1.010	1.063b	1.074	0.857	1.150	2.160
	RPC+	0.987	1.069b	0.942	1.012b	1.002	0.904	1.157	1.887
s.e.m.		0.070	0.038	0.042	0.045	0.064	0.040	0.062	0.141
P-value		0.560	0.021	0.887	0.012	0.294	0.817	0.988	0.342

Different letters within a column indicate significant differences between the means. Symbols −/+ indicate the absence or presence of necrotic enteritis challenge in the treatments. SP, diet contained standard protein concentrations at 23.2%, 21.4% and 19.4% crude protein for starter, grower and finisher phases respectively. RP, diet contained reduced protein concentrations with two percentage points lower crude protein than in SP diets in all feeding phases. The RPA diet was created by adding L-arginine on top of the RP diet at the concentration of 0.03% in all feeding phases. Concentration of calculated supplemental L-arginine in the RPA diet in starter, grower and finisher phases was 0.25%, 0.25% and 0.24% respectively. The RPC diet was created by replacing all supplemental L-arginine in the RPA diet by L-citrulline. Two-way ANOVA presented results of the 2 × 2 factorial arrangement of treatments, with the main factors being necrotic enteritis challenge (NE, yes or no) and protein level (SP or RP).

CLDN1, claudin 1; *CLDN5*, claudin 5; *JAM2*, junctional adhesion 2; *TJP1*, tight junction protein; *MUC2*, mucin 2; *CDH1*, E-cadherin; *NOS2*, nitric oxide synthase 2; *IFN-γ*, interferon gamma.

villus area, and increases in gut permeability compared with the unchallenged group, as reported in previous parts of this series (Dao *et al.* 2022*a*, 2022*b*). Reduced Ca and P digestibility was also reported in NE-infected birds compared with the uninfected birds by Paiva *et al.* (2014).

Intestinal tight junctions are a major defence against pathogenic bacteria but also regulate nutrient absorption and homeostasis in the gut (Gasbarrini and Montalto 1999). In the current study, the lower expression levels of tight junction genes, including *CLDN5* and *TJP1*, indicate the impairment of tight junction function and gut permeability in NE-challenged birds. This is in agreement with the observations reported in the previous studies (Park *et al.* 2008; Gharib-Naseri *et al.* 2020). Similarly, necrotic enteritis downregulated various nutrient-related genes examined in the current study including *PepT1*, *EEAT3*, and *PRKAG2*. This was consistent with the reduced feed efficiency and impaired jejunal morphology (reduced villus height, villus height to crypt depth ratio, and apparent villus area) observed in NE-challenged birds compared with unchallenged birds, as has been reported in previous parts of this series (Dao *et al.* 2022*a*, 2022*b*). Various nutrient transporters are located on the tip of the villi (Obst and Diamond 1992). The alteration in jejunal morphology, as shown by villi atrophy and sloughing of the brush border membrane of the enterocytes due to NE challenge (as reported in the second part of this series, Dao *et al.* 2022*b*), was likely to be the main reason for the lower expression levels of nutrient-related genes in NE-challenged birds in the current study. The digestive enzyme *APN* cleaves neutral and basic amino acids from the N-terminal end of peptides (Sanderink *et al.* 1988). The lower *APN* expression in NE-challenged birds indicates decreased amino acid digestion at the brush border membrane that may reduce substrates for the peptide and amino acid transport system and expression of related genes. In addition, the expression levels of amino acid transporter genes might be associated with feed intake. For instance, higher *PepT1* gene expression has been reported in feed-restricted chicks

(3–14 days old) raised under thermoneutral conditions, than in those offered the *ad libitum* feed access (Gilbert *et al.* 2008). In the current study, *PepT1* was downregulated while *PepT2* and *CAT1* were upregulated in NE-challenged birds compared with the unchallenged group. The differences in the bird age and experimental design best explain the discrepancies between the studies.

The expression of tight junction proteins can be influenced by the secretion of inflammatory cytokines such as *IFN-γ* (Sakaguchi *et al.* 2002). Also, there is evidence that expression of *NOS2* in macrophages and other cell types was induced by *IFN-γ* concentration and/or bacterial lipoproteins/exotoxins (Braun *et al.* 1999; Flak and Goldman 1999). In the current study, increased jejunal *IFN-γ* and *NOS2* expression levels were observed in NE-challenged birds compared with the unchallenged group, suggesting increased inflammatory responses in the NE group, similar to what has been reported by others (Collier *et al.* 2008; Lee *et al.* 2018; Emami *et al.* 2019). The increased production of *IFN-γ* is needed for the re-arrangement and redistribution of the intestinal actin cytoskeleton that may help increase paracellular permeability (Bruewer *et al.* 2005). Nitric oxide is involved in innate immunity as a toxic agent against pathogenic organisms. Equally, nitric oxide has been implicated as an anti-inflammatory or immunosuppressive agent through its inhibitory or apoptotic effects on cells (Coleman 2001). The beneficial effect of Arg on gut permeability is associated with the activity of *NOS2* (Meng *et al.* 2017; He *et al.* 2018). However, no difference in *NOS2* gene expression was observed in the current study when Arg or Cit was added to the RP diet for challenged birds. Higher Arg supplemental concentrations as well as effects of intestinal microorganisms on the Arg metabolism and interactions among Arg, cytokines, pro-inflammatory agents, and other amino acids during NE challenge should be investigated to clarify possible mechanisms.

There are few if any reports that have examined the effects of protein concentration, Arg/Cit supplementation, and/or NE challenge on the concentrations of serum alpha-1 acid glycoprotein, ovotransferrin and minerals. Feeding the RP diets decreased both serum alpha-1 acid glycoprotein and ovotransferrin concentrations compared with the SP diet in the current study. The lower concentrations of phenylalanine, tyrosine, and tryptophan in the RP diets than in the SP diets might partly explain the observations, as they are essential components of acute-phase proteins (Reeds *et al.* 1994). Also, the results of the current study illustrated that dietary protein concentrations could interact with the NE challenge and alter serum mineral concentrations in birds. This was shown by the decreased serum Ca concentration in RP-fed birds, and decreased serum Na and increased K concentrations in SP-fed birds during challenge with NE. This might be due to altered mineral metabolism during the NE challenge, and/or differences in mineral composition between the SP and RP diet.

In the current study, the NE challenge downregulated gene expression of $b^{0,+}AT$ in both SP- and RP-fed birds, but greater downregulation was observed in RP-fed birds. Also, feeding RP diets downregulated gene expression of *y+ LAT2* only during the NE challenge. It has been known that $b^{0,+}AT$ is responsible for transporting Na^+-independent cationic and zwitterionic amino acids at the brush border membrane, and *y+ LAT2* is a Na^+-independent cationic and Na^+-dependent neutral amino acid transporter at the basolateral membrane (Verrey *et al.* 2004; Gilbert *et al.* 2008). The lower feed intake in NE-challenged birds than in the unchallenged group and lower concentrations of amino acids such as histidine, alanine, serine, glycine, leucine, and phenylalanine in the RP diets than in the SP diets in the current study might reduce substrates for amino acid transport systems and, consequently, lead to the downregulation of these genes. Lower $b^{0,+}AT$ gene expression has been reported in birds fed a corn gluten meal-based diet than in those offered a soybean meal-based diet (Gilbert *et al.* 2008). In a similar manner, the lower inclusion of soybean meal and a higher inclusion of wheat in the RP diets than in the SP diets might influence the expression of jejunal $b^{0,+}AT$ gene in respective groups in the current study. Additionally, differential cell composition in the gut mucosa during NE challenge may also alter relative gene expression of nutrient transporters in this study.

Mucin-2 as a major component of intestinal mucus plays a crucial role in maintaining the thickness of the mucous layer and serves as a physical barrier preventing epithelial cells from direct contact with the intestinal microorganisms (Sovran *et al.* 2016). E-cadherin encoding by the *CDH1* gene is responsible for cell to cell adhesion, the organisation, and maintenance of epithelial cells, and plays essential roles in mediating intercellular cohesion and tissue development (Jiang 1996; Bhatt *et al.* 2013). In the current study, the jejunal *MUC2* gene was downregulated in birds fed the RP diets only when they were challenged with NE. Also, feeding the RP diets downregulated the expression of *CDH1* compared with the SP diets, regardless of the NE challenge. These results reflect unfavourable effects of the RP diets on the mucosal layer and intercellular strength during the NE challenge. However, feeding the RP diets resulted in a higher expression of *CLDN5* and *TJP1* than feeding the SP diets. This result was consistent with the increased growth performance in RP-fed birds compared with those offered the SP diet, as reported in the first part of this series (Dao *et al.* 2022*a*).

Supplementation of Arg to the RP diets for challenged birds decreased *PepT2* gene expression, and increased *CAT1* gene expression, whereas Cit supplementation to the RP diet for challenged birds did not affect *CAT1* gene expression in the current study. This differential effect of Arg and Cit might be attributed to the difference in transportation/absorption routes of Arg and Cit in the small intestine, and the lower

concentration of Arg in the RPC diet than the RPA diet in the current study. It is well known that *CAT1* mediates the transport of cationic amino acids such as lysine, Arg, and histidine with high affinity (Gilbert *et al.* 2007), whereas Cit is mainly transported and absorbed in the intestine by transporters belonging to the $B^{0,+}$, L, and $b^{0,+}$ systems, such as $b^{0,+}AT$, and *LAT1* (Bahri *et al.* 2013). The gene expression of the *CAT* family has been reported to depend on substrate availability (Zhang *et al.* 2019). However, Cit supplementation to the RP diet for challenged birds increased *PRKAG2* expression compared with the SP+ treatment in the current study. Expression of the *PRKAG2* gene has been reported to be closely associated with the feed intake and/or feed efficiency in chickens and cattle (Lindholm-Perry *et al.* 2014; Jin *et al.* 2016). Citrulline supplementation to the RP+ treatment also increased expression of *CLDN5* and *TJP1*, which are important tight junction proteins regulating nutrient absorption and homeostasis. Thus, the upregulation of *PRKAG2*, *CLDN5*, and *TJP1* genes might partly explain the higher feed efficiency in challenged birds fed the RPC diets than in those fed the SP diets, as reported in the first part of this series (Dao *et al.* 2022*a*). Additional supplementation of Arg and Cit to the RP diet for challenged birds did not affect jejunal *MUC2* expression in the present study. However, a higher dietary Arg supplemental level may increase *MUC2* expression and is a worthwhile subject for further studies.

Conclusions

The NE challenge reduced nutrient digestion and absorption and induced an immune response in infected birds by downregulating digestive enzyme, nutrient-related and tight junction protein genes, increasing serum alpha-1 acid glycoprotein concentration and upregulating gene expression of pro-inflammatory agents including *NOS2* and *IFN-γ*. Increased gene expressions of *CLDN5* and *TJP1* were observed in birds fed the RP diets compared with those fed the SP diets. The NE challenge downregulated the expression of $b^{0,+}AT$ in both SP- and RP-fed birds, but greater downregulation was observed in the RP-fed birds. These results were most likely due to the differences in the composition and amino acid concentrations of the RP diets compared with the SP diets. Supplementation of Arg to the RP diet for challenged birds decreased *PepT2* gene expression and increased *CAT1* and *TJP1* gene expression. Whereas Cit supplementation to the RP diet for challenged birds increased the expression of *PRKAG2*, *CLDN5*, and *TJP1*. Thus, in part replacement of Arg with Cit in the RPC diet may have beneficial effects on gene expression of broiler chickens during the NE challenge.

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Data availability. The data that support this study will be shared upon reasonable request to the corresponding author.

Conflicts of interest. The authors declare that there is no conflicts of interest.

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Author affiliations

[A]School of Environmental and Rural Science, Faculty of Science, Agriculture, Business and Law, University of New England, Armidale, NSW 2351, Australia.

[B]Faculty of Animal Science, Vietnam National University of Agriculture, Trau Quy Town, Gia Lam District, Hanoi 100000, Vietnam.

[C]Ridley AgriProducts, Melbourne, Vic. 3000, Australia.