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Supplementation of reduced protein diets with L-arginine and L-citrulline for broilers challenged with subclinical necrotic enteritis. 2. Intestinal permeability, microbiota, and short-chain fatty acid production

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ABSTRACT

Context. Necrotic enteritis (NE) has been considered a major threat to broiler gut health and growth performance. **Aims.** This study aimed at investigating the effects of L-arginine (Arg) or L-citrulline (Cit) supplementation on intestinal morphology, short-chain fatty acid (SCFA), microbiota count, gut permeability, and pH in broilers fed reduced-protein diets 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 NE challenge (SP−), or with NE challenge (SP+); reduced protein (two percentage points lower crude protein) without NE challenge (RP−), or with NE challenge (RP+); RP+ plus added Arg (103% of Ross 308 requirement, RPA+) and RPC+ where supplemental Arg in RPA+ was replaced with Cit. A 2 × 2 factorial arrangement was employed for the first four treatments. Factors were NE (− or +) and protein concentration (SP or RP). Treatments SP+, RP+, RPA+, and RPC+ were analysed by one-way ANOVA. **Key results.** Necrotic enteritis × protein interactions were detected for serum fluorescein isothiocyanate dextran (FITC-d) level, *C. perfringens* ($P < 0.05$) count in the caeca ($P < 0.01$), and acetic acid ($P < 0.01$) and total SCFA concentrations in the ileum on Day 16 ($P < 0.001$). Feeding the RP diet reduced serum FITC-d concentration, number of *C. perfringens* in the caeca, and increased acetic acid and total SCFA concentrations in the ileum compared with the SP group only in birds challenged with NE. Birds in the RPC+ treatment had greater jejunal villus height ($P < 0.001$), and lower caecal *C. perfringens* and Enterobacteriaceae count than did those in the SP+ treatment ($P \leq 0.001$). **Conclusions.** The results indicated a benefit to gut health of broilers during NE challenge when replacing crystalline Arg with Cit in RP diets. **Implications.** In part, replacement of Arg by Cit in the RP diets is of great potential to increase gut health, reduce growth loss, thus, minimising negative effects of NE in broilers.

Keywords: arginine, citrulline, gut health, low protein, meat chicken, microbiota, morphology, necrotic enteritis.

Introduction

The gastrointestinal tract (GIT) plays a crucial role in the growth performance and health of chickens. An optimal morphological architecture is crucial for the digestion, absorption, and transportation of nutrients in birds (Swatson *et al.* 2002; Zanu *et al.* 2020*a*). Inside the GIT, the microbiota are known to regulate the rates of mucin production, epithelial cell proliferation, and short-chain fatty acid (SCFA) composition (Deplancke and Gaskins 2001; Hörmann *et al.* 2014) that are important to fight against negative effects from the environment (Den Besten *et al.* 2013; Apajalahti and Vienola 2016). Necrotic enteritis (NE), a disease caused by *Clostridium perfringens*, has been considered a major

threat to broiler gut health as gut integrity is impaired with major shifts in the microbiota observed (Wilson *et al.* 2005; Stanley *et al.* 2012, 2014; Latorre *et al.* 2018). While the inclusion of antibiotics in broiler diets can effectively control NE (Widyaratne 2012), a ban on in-feed antibiotic growth promoters in several countries has intensified the search for alternatives to control NE.

Reduction in dietary protein level has been reported to increase intestinal morphology and SCFA level (Gu and Li 2004; Opapeju *et al.* 2008; Apajalahti and Vienola 2016). This effect is probably associated with the decrease in the population of pathogenic bacteria as a result of there being less undigested protein in the hindgut (Belloir *et al.* 2017; Hilliar *et al.* 2019, 2020*a*). Reducing the dietary protein level may decrease ammonia production in the hindgut as there are lower concentrations of fermentable nitrogenous substrates (He *et al.* 2015; Sharma *et al.* 2017). Ammonia production has been reported to increase intestinal pH and facilitate the growth of *C. perfringens* (Paiva and McElroy 2014; Qaisrani *et al.* 2015). Hence, reduced dietary protein may benefit the gut by reducing *C. perfringens* counts and, in turn, help alleviate the adverse effects of subclinical NE on growth performance.

As a precursor of nitric oxide and polyamines, arginine (Arg) plays an important role in mucosal development, villus physiology (Murakami *et al.* 2012), and proliferation and migration of intestinal epithelial cells in the gut (Rhoads and Wu 2009). Dietary Arg supplementation has been reported to preserve gut morphology in *Eimeria*- and/or *C. perfringens*-challenged birds by decreasing intestinal lesion scores, increasing villus height, villus height to crypt depth ratio, and villus surface area (Tan *et al.* 2014; Laika and Jahanian 2017; Zhang *et al.* 2018). Citrulline (Cit), a metabolite of Arg, has been demonstrated to have Arg-sparing effects in chickens (Tamir and Ratner 1963; Su and Austic 1999). The effects of Arg and Cit supplementation in reduced protein diets on growth performance, carcass traits, internal organ weights, intestinal lesion score, and serum uric acid concentration during NE were reported in the first part of this series (Dao *et al.* 2022). The findings of that report showed that supplementation of Cit to the reduced-protein diets promoted growth performance and recovery in NE-challenged birds compared with standard protein diets or reduced-protein diets supplemented with Arg. The objective of the present work was to provide further insight into the effects of Arg and Cit supplementation in reduced-protein diets on intestinal morphology, microbiota composition, SCFA, gut permeability, and pH in broilers subjected to the 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 practice 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* using nipple drinkers and tube feeders 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 as follows: standard protein diet without NE challenge (SP−), or with NE challenge (SP+); reduced-protein diet balanced with crystalline amino acids without NE challenge (RP−), or with NE challenge (RP+); RP diet supplemented with additional Arg to 103% of requirement (equal to 15% additional supplemental crystalline Arg) with NE challenge (RPA+); and RP with Cit replacing all supplemental Arg in previous treatment with NE challenge (RPC+). A 2 × 2 factorial arrangement was employed for the first four treatments. Factors were NE (− or +) and protein level (SP or RP). In addition, all six treatments were analysed by one-way ANOVA. There was a two percentage point difference in crude protein level between SP and RP diets for all feeding phases. The concentrations of essential amino acids in the RP diet were equivalent to those in the SP diet and in accordance with Ross 308 broiler nutrition specifications (Aviagen 2014*b*). Concentrations of added crystalline Arg in the RP treatments in starter, grower, and finisher phases were 0.217%, 0.213%, and 0.212% respectively. Concentrations 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 Arg concentration 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 diet at the expense of wheat. Feeds were provided as crumbles for starter (Days 0–10), and pellets for grower (Days 10–24), and finisher (Days 24–35) phases. Details on feed analysis were provided in the first part of this series (Dao *et al.* 2022).

Necrotic enteritis challenge

Subclinical NE was established following procedures previously described by Rodgers *et al.* (2015). Birds in the challenged groups (SP+, RP+, RPA+, and RPC+) 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

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

Item	Starter		Grower		Finisher	
	SP	RP	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[A]	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[B]	0.01	0.01	0.01	0.01	0.01	0.01
Phytase[C]	0.01	0.01	0.01	0.01	0.01	0.01
Vitamin premix[D]	0.09	0.09	0.08	0.08	0.08	0.08
Mineral premix[E]	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 fiber	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[F]	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

(*Continued on next page*)

Table 1. (*Continued*).

Item	Starter		Grower		Finisher	
	SP	RP	SP	RP	SP	RP
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

Diet contained standard protein at a concentration of 23.2%, 21.4% and 19.4% crude protein for starter, grower and finisher phases respectively.
Diet had 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 was 0.25%, 0.25% and 0.24% respectively. The RPC diet was created by replacing all supplemental L-arginine in RPA diet by L-citrulline.
[A]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.
[B]Econase XT, 25 (AB Vista, 16 000 BXU/kg of diet).
[C]Quantum Blue, 5G (AB Vista, 500 FTU/kg of diet).
[D]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.
[E]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.
[F]Methionine + cysteine.
AMEn, apparent metabolisable energy corrected to zero N retention; Dig, standard ileal digestible amino acid coefficients as determined by near-infra red spectroscopy (Foss NIR 6500, Denmark) standardised with Evonik AMINONIR® Advanced calibration.

oocysts of *Eimeria brunetti* (Eimeria Pty Ltd, Melbourne, Vic., 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, Australia) in a starch thioglycollate broth on Day 14. Birds in the unchanged control groups (SP− and RP−) 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. The results of the first part of this series showed that subclinical NE was successfully established in the current study as shown by increased intestinal lesion scores on Day 16, decreased feed intake and bodyweight gain and increased feed conversion ratio in the grower, finisher, and overall growth periods in NE-challenged birds.

Morphometric measurements of jejunum

On Day 16, three birds per pen were randomly selected, weighed, electrically stunned (MEFE CAT 44N, Mitchell Engineering Food Equipment, Clontarf, Qld, Australia), and euthanised by decapitation for sample collection. Jejunal samples were obtained and flushed with PBS and approximately 2 cm of jejunal tissue (mid-point between the end of duodenum and the Meckel's diverticulum) was collected for morphometric measurements. The samples were fixed with 10% buffered formalin in 50-mL containers until processing. The jejunal samples were sectioned and processed using standard haematoxylin and eosin assay as described by Golder *et al.* (2011). The histological slides were scanned by NanoZoomer 2.0-RS (Model C10730-12, Hamamatsu Photonics K.K., Hamamatsu, Japan) and then readings were performed on the scanned slides using NDP.scan 2.5.8 software supplied by the same company. Five villi per section were randomly selected for measurements of villus height, crypt depth, basal width, middle width, and apical width, resulting in 10 measurements per sample and 30 measurements per pen. The apparent villus surface area was calculated as (basal width + apical width)/2 × villus height, following Zanu *et al.* (2020*b*).

Caecal DNA extraction and quantification of bacteria

To quantify microbiota populations, caecal digesta from three birds per pen on Day 16 were pooled in 50-mL containers. Then, subsamples of the pooled caecal digesta were aliquoted into 2-mL Eppendorf tubes and snap-frozen with liquid nitrogen during the sampling. All the samples were stored at −20°C until analysis. The DNA of caecal content was extracted, then the relative amounts of *Bacillus* spp., *Bacteroides* spp., *Bifidobacterium* spp., *C. perfringens*, Enterobacteriaceae, *Lactobacillus* spp., *Ruminococcus* spp., and total bacteria, expressed as $\log_{10}$ genomic DNA copies per gram of caecal digesta, were quantified as described by Kheravii *et al.* (2017). The quantitative real-time PCR (Rotorgene 6000 real-time PCR machine, Corbett, Sydney, NSW, Australia) was employed to determine the bacterial populations. The specific *16S* rRNA primers used for the quantification of different bacterial populations are presented in Table 3.

Determination of succinic acid, lactic acid, and SCFA

Pooled ileal and caecal digesta from three birds per pen on Day 16 were used for the analysis of succinic acid, lactic

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 are total amino acids. The RPA diet was created by adding L-arginine on top of the RP diet at a 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.
SP, diet containing standard protein at a concentration of 23.2%, 21.4% and 19.4% crude protein for starter, grower and finisher phases respectively; RP, diet with reduced protein concentration, with two percentage points lower crude protein than in SP diets in all feeding phases.

acid, and SCFA. The samples were stored at −20°C until analysis. Concentrations of succinic acid, lactic acid, and SCFA in the samples were determined in duplicate, following procedures described by Wu *et al.* (2010).

Gut permeability with fluorescein isothiocyanate dextran in blood serum

The fluorescein isothiocyanate dextran (FITC-d, Sigma Aldrich, NSW, Australia; average mole weight of 4000 and FITC-d:glucose of 1:250) was used as a marker to measure the gut permeability on Day 16. The FITC-d solution was prepared, kept at 4°C, and wrapped in aluminium foil to avoid light exposure. Three birds per pen were selected, marked, and inoculated with a 1 mL dose of FITC-d (4.17 mg/kg body weight dissolved in water). Vacutainers (Becton, Dickinson UK Ltd, Plymouth, UK) that contained spray-coated silica and a polymer gel were used to collect the blood from sampled birds between 2 and 2.5 h after the inoculation. Then, blood samples were centrifuged at 3000*g* at 4°C for 10 min to separate the serum. Serum samples were stored at −20°C until further analysis. To determine FITC-d concentration, a standard curve was prepared following the method previously described by Prado-Rebolledo *et al.* (2017). Concentrations of FITC-d in diluted serum (1:1 PBS), blank, and standard samples were determined at an excitation wavelength of 480 nm and an emission wavelength of 520 nm by using a Synergy MX plate reader (Biotek Instruments, Bedfordshire, UK).

Measurements of ileal and caecal pH

Immediately after dissecting, the intact ileum and caeca were removed from three birds per pen on Day 16. The pH was measured using a digital pH metre (Mettler-Toledo, UK) with a spear tip piercing pH electrode (Sensorex, Garden Grove, CA, USA) by directly inserting the pH metre probe into the digesta of the caeca and distal ileum. Three

Table 3. Sequence of primers used for the qPCR analysis of selected caecal microbial populations.

Target group or organism	Primer sequence (5′–3′)	Annealing temperature (°C)	References
Bacillus spp.	F-GCA ACG AGC GCA ACC CTT GA R-TCA TCC CCA CCT TCC TCC GGT	63	Zhang *et al.* (2015)
Bacteroides spp.	F-GAG AGG AAG GTC CCC CAC R-CGC TAC TTG GCT GGT TCA G	63	Layton *et al.* (2006)
Bifidobacterium spp.	F-GCG TCC GCT GTG GGC R-CTT CTC CGG CAT GGT GTT G	63	Requena *et al.* (2002)
Clostridium spp.	F-ATG CAA GTC GAG CGA KG R-TAT GCG GTA TTA ATC TYC CTT T	60	Rinttilä *et al.* (2004)
Enterobacteriaceae	F-CAT TGA CGT TAC CCG CAG AAG AAG C R-CTC TAC GAG ACT CAA GCT TGC	63	Bartosch *et al.* (2004)
Lactobacillus spp.	F-CAC CGC TAC ACA TGG AG R-AGC AGT AGG GAA TCT TCC A	63	Wise and Siragusa (2007)
Ruminococcus spp.	F-GGC GGC YTR CTG GGC TTT R-CCA GGT GGA TWA CTT ATT GTG TTA A	63	Ramirez-Farias *et al.* (2009)
Total bacteria	F-CGG YCC AGA CTC CTA CGG G R-TTA CCG CGG CTG CTG GCA C	63	Lee *et al.* (1996)

readings were taken for each sample. The probe was washed with ultra-pure water (ICW 3000 water purifier for ion chromatography; Millipore, Burlington, MA, USA) between the samples to avoid cross-contamination.

Data analyses

R Commander (version 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 *posthoc* test was used to identify pairwise differences between the treatments from significant ANOVA results. The *P*-value of <0.05 was considered significant.

Results

Jejunal morphology

Results on jejunal morphology on Day 16 are presented in Table 4 and Fig. 1. No NE × protein interaction was detected for jejunal morphology (Table 4). Necrotic enteritis challenge as the main effect reduced villus height ($P < 0.001$), villus height to crypt depth ratio ($P < 0.001$), and apparent villus area ($P < 0.001$), while increasing crypt depth ($P < 0.001$), and apical width ($P < 0.001$), and tended to increase middle width ($P = 0.065$) on Day 16. Protein level as the main effect did not affect morphological parameters in the jejunum on Day 16. The addition of Cit to the RP+ treatment increased villus height ($P < 0.01$) and villus height to crypt depth ratio ($P < 0.05$) compared with the RP+ treatment, and increased apparent villus area ($P < 0.05$) compared with the SP+ treatment on Day 16 (Table 4).

Caecal microbiota

Necrotic enteritis × protein interactions were detected for caecal *C. perfringens* ($P < 0.05$) and total bacteria counts ($P < 0.01$). Feeding the RP diet reduced *C. perfringens* and total bacteria counts in the caeca compared with the SP group only in birds challenged with NE (Table 5). Similarly, a tendency for a NE × protein interaction was observed for caecal Enterobacteriaceae count ($P = 0.06$). The number of Enterobacteriaceae tended to decrease in birds fed the RP diet compared with the SP diet only when challenged with NE. Necrotic enteritis challenge as the main effect decreased numbers of *Ruminococcus* spp. ($P < 0.001$) and *Bacillus* spp. ($P < 0.001$) in the caeca on Day 16 (Table 5). Numbers of caecal *Lactobacillus* spp., *Bacteroides* spp., and *Bifidobacterium* spp. were not influenced by the NE challenge. Feeding the RP diet reduced the number of *Lactobacillus* spp. ($P < 0.001$) and tended to reduce the number of *Bacillus* spp. in the caeca on Day 16 ($P = 0.078$) compared with those of the SP fed birds as shown by the main effect of protein level in Table 5. Supplementation of Cit to the RP+ treatment reduced *C. perfringens* and Enterobacteriaceae counts in the caeca compared with those of the SP+ treatment on Day 16 ($P < 0.05$; Table 5).

Table 4. Jejunal morphological measurements of broiler chickens on Day 16.

Effects		Villus height (μm)	Crypt depth (μm)	Villus height: crypt depth	Basal width (μm)	Middle width (μm)	Apical width (μm)	Apparent villus area ($\mu m^2 \times 10^3$)
Two-way ANOVA results (2 × 2 factorial arrangement of treatments)								
Treatment	SP–	1236	124	10.23	252	220	191	275
	RP–	1335	132	10.34	259	228	196	305
	SP+	846	233	3.77	248	237	225	199
	RP+	842	241	3.64	281	262	249	221
NE	No	1285b	128a	10.28b	255	224	193a	290b
	Yes	844a	237b	3.70a	265	250	237b	210a
Protein	SP	1041	179	7.00	250	228	208	237
	RP	1088	186	6.99	270	245	222	263
s.e.m.		44	10	0.61	7	7	7	11
P-value	NE	<0.001	<0.001	<0.001	0.542	0.065	<0.001	<0.001
	Protein	0.599	0.738	0.994	0.180	0.386	0.366	0.122
	NE × protein	0.180	0.996	0.716	0.393	0.550	0.378	0.818
One-way ANOVA results (four NE-challenged treatments)								
Treatment	SP+	846a	233	3.77ab	248	237	225	199a
	RP+	842a	241	3.64a	281	262	249	221ab
	RPA+	921ab	22	4.23ab	278	256	229	235ab
	RPC+	1077b	242	4.65b	274	250	229	271b
s.e.m.		27	4	0.14	7	6	4	8
P-value		0.003	0.552	0.027	0.335	0.501	0.230	0.016

Symbols –/+ indicate the absence or presence of necrotic enteritis challenge in the treatments. 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 presents 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).
Different letters within a column indicate significant differences between the means.
SP, diet containing standard protein at a concentration of 23.2%, 21.4% and 19.4% crude protein for starter, grower and finisher phases respectively; RP, diet with reduced protein concentrations, with two percentage points lower crude protein than for SP diets in all feeding phases.

Ileal and caecal succinic acid, lactic acid, and SCFA

The results on the concentrations of ileal and caecal succinic acid, lactic acid, and SCFA on Day 16 are shown in Tables 6 and 7. Interactions for NE × protein were observed for acetic acid ($P < 0.01$), succinic acid ($P < 0.01$), and total SCFA ($P < 0.001$) concentrations in the ileum on Day 16. Concentrations of ileal acetic acid and total SCFA were increased in birds fed the RP diets compared with the SP-fed birds only when challenged with NE ($P < 0.01$), whereas the concentration of ileal succinic acid was increased in birds fed the SP diets only when without NE challenge ($P < 0.01$, Table 6). A tendency for interaction between NE and protein level was detected for caecal butyric acid concentration on Day 16 ($P = 0.059$). The butyric acid concentration was increased in caecal contents in birds fed the SP diet compared with the RP diet only when challenged with NE (Table 7).

Necrotic enteritis challenge increased ileal lactic acid ($P < 0.001$) but decreased propionic acid ($P < 0.001$) concentrations compared with unchallenged birds, as shown in Table 6. In the caeca, NE challenge increased butyric ($P < 0.01$), isobutyric ($P < 0.05$), and isovaleric acid concentrations ($P < 0.05$) on Day 16 compared with unchallenged birds. Feeding the RP diets increased acetic acid ($P < 0.001$), lactic acid ($P < 0.05$) and total SCFA ($P < 0.05$) but decreased propionic acid ($P < 0.05$) and butyric acid ($P < 0.05$) in the caeca on Day 16, when compared with SP diets, irrespective of the NE challenge (Table 7).

Supplementation of Arg and Cit to the RP+ treatment did not affect concentrations of succinic acid, lactic acid, and SCFA in the ileum on Day 16 ($P > 0.05$, Table 6). Supplementation of Cit to the RP+ treatment decreased concentration of butyric acid ($P < 0.001$) compared with the RP+ treatment and decreased concentration of valeric acid ($P < 0.05$) compared with the SP+ treatment in the caeca on Day 16 (Table 7).

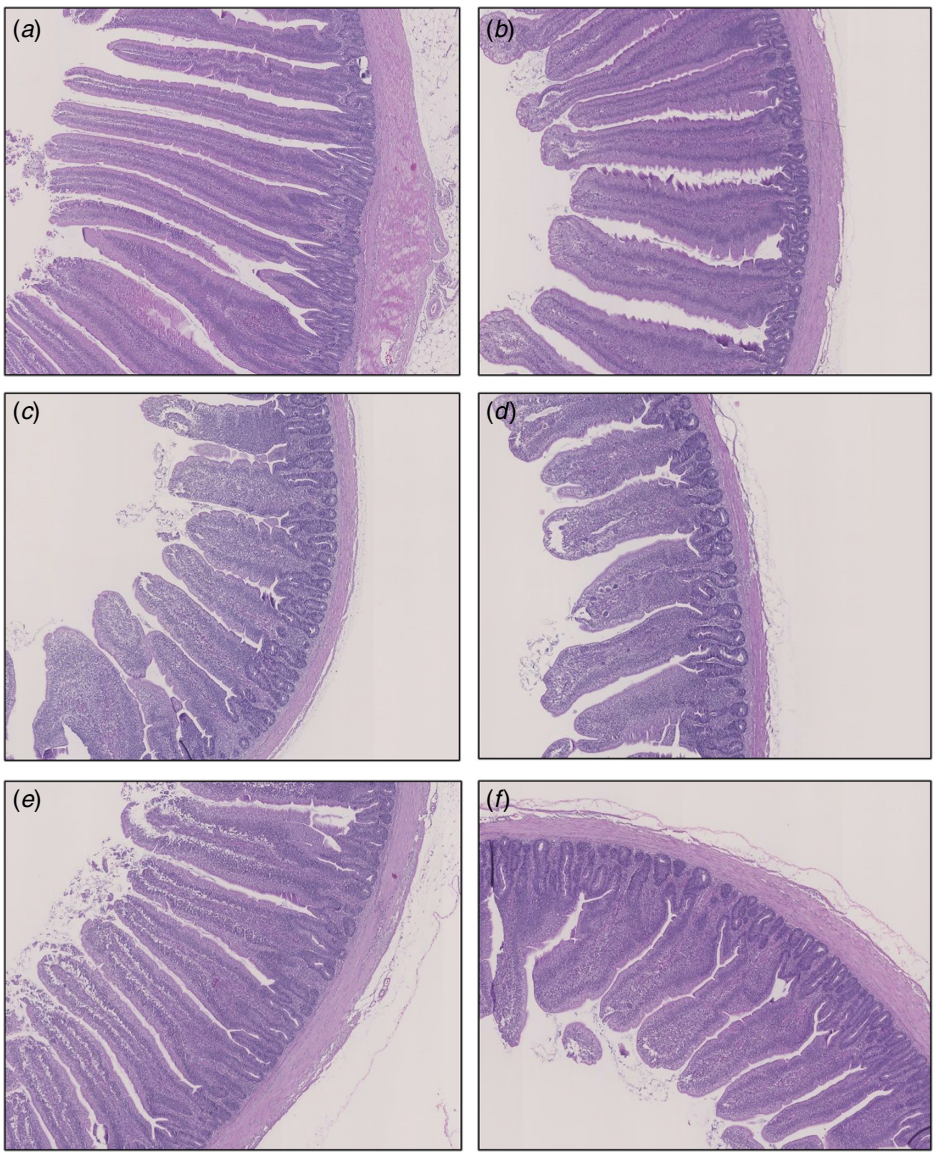

Fig. 1. Effects of necrotic enteritis challenge and dietary treatments on jejunal morphology on Day 16. All slides were scanned by NanoZoomer 2.0-RS (Model C10730-12, Hamamatsu Photonics K.K., Hamamatsu, Japan) and viewed at the same objective (×5.04). The colour images were captured by NDP.scan 2.5.8 software (Hamamatsu Photonics K.K., Hamamatsu, Japan). (*a*) Standard protein diet without necrotic enteritis challenge; (*b*) reduced-protein diet without necrotic enteritis challenge; (*c*) standard protein diet with necrotic enteritis challenge; (*d*) reduced-protein diet with necrotic enteritis challenge; (e) reduced-protein diet with additional L-arginine added and with necrotic enteritis challenge; (*f*) reduced-protein diet with L-citrulline added and with necrotic enteritis challenge.

Serum FITC-d concentration, ileal and caecal pH

A NE × protein interaction was observed for gut leakage as evidenced by Day 16 serum FITC-d concentrations ($P < 0.001$, Table 8). Serum FITC-d concentation was decreased in birds fed the RP diet compared with those offered the SP diet only when challenged with NE. The one-way ANOVA results showed that supplementation of either Arg or Cit to the RP+ treatment did not affect serum FITC-d concentration on Day 16 when compared with the RP control treatment ($P > 0.05$), as shown in Table 8.

No NE by protein interactions were detected for ileal or caecal pH on Day 16 ($P > 0.05$), as shown in Table 8. Necrotic enteritis challenge decreased ileal pH ($P < 0.001$) but did not affect caecal pH on Day 16. Birds fed the RP diet had a higher caecal pH ($P < 0.01$) than, but a similar

Table 5. Caecal microbiota of broiler chickens on Day 16 (log_{10} [genomic DNA copies/g of caecal contents]).

Effects		*Clostridium perfringens*	*Lactobacillus* spp.	*Ruminococcus* spp.	*Bacteroides* spp.	*Bacillus* spp.	*Bifidobacterium* spp.	Enterobacteriaceae	Total bacteria
Two-way ANOVA results (2 × 2 factorial arrangement of treatments)									
Treatment	SP–	0.00a	9.20	9.90	5.68	8.69	7.88	7.06	11.27b
	RP–	0.62a	8.84	9.88	5.61	8.10	7.90	7.25	11.19b
	SP+	7.61b	9.19	9.55	5.68	7.75	7.80	8.56	11.26b
	RP+	3.58a	8.53	9.39	5.45	7.50	7.73	7.91	10.69a
NE	No	0.31a	9.02	9.89b	5.64	8.39b	7.89	7.15a	11.23
	Yes	5.59b	8.86	9.47a	5.56	7.62a	7.77	8.23b	10.97
Protein	SP	3.81	9.20b	9.73	5.68	8.22	7.84	7.81	11.26b
	RP	2.10	8.69a	9.64	5.53	7.80	7.82	7.58	10.94a
s.e.m.		0.74	0.07	0.05	0.05	0.12	0.04	0.15	0.06
P-value	NE	<0.001	0.646	<0.001	0.560	<0.001	0.148	<0.001	0.081
	Protein	0.253	<0.001	0.362	0.121	0.078	0.781	0.460	0.004
	NE × protein	0.023	0.173	0.243	0.385	0.338	0.609	0.060	0.003
One-way ANOVA results (four NE-challenged treatments)									
Treatment	SP+	7.61b	9.19b	9.55	5.68b	7.75	7.80	8.56b	11.26b
	RP+	3.58ab	8.53a	9.39	5.45a	7.50	7.73	7.91ab	10.69a
	RPA+	5.15ab	8.86ab	9.56	5.50a	7.98	7.62	7.73ab	10.90a
	RPC+	1.83a	8.55a	9.48	5.43a	8.17	7.71	7.45a	10.81a
s.e.m		0.74	0.08	0.04	0.02	0.12	0.04	0.14	0.06
P-value		0.031	0.002	0.331	<0.001	0.249	0.340	0.020	0.001

Symbols –/+ indicate the absence or presence of necrotic enteritis challenge in the treatments. The RPA diet was created by adding L-arginine on top of the RP diet at a concentration of 0.03% in all feeding phases. Concentration of calculated supplemental L-arginine in 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 presents 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).

Different letters within a column indicate significant differences between the means.

SP, diet containing standard protein levels at 23.2%, 21.4% and 19.4% crude protein for starter, grower and finisher phases respectively; RP, diet with reduced protein concentrations, with two percentage points lower crude protein than in SP diets in all feeding phases.

ileal pH to those offered the SP diet on Day 16, as shown by the main effect of protein level in Table 8. Supplementation of either Arg or Cit to the RP+ treatment did not affect ileal pH. Birds in the RPC+ treatment had a higher caecal pH than did those in the SP+ treatment ($P < 0.05$), whereas the caecal pH values of RP+, RPA+, and SP+ treatments were not different (Table 8).

Discussion

Necrotic enteritis challenge decreased villus height, villus height to crypt depth ratio and apparent villus area, and increased crypt depth, villus apical width, and middle width on Day 16 in the current study. Similar findings have been previously reported in broilers challenged with *Eimeria* spp. and/or *C. perfringens* (Onrust *et al.* 2018; Wu *et al.* 2018; Xue *et al.* 2018). The decreased villus height and thereby the smaller absorptive area reduce digestion and absorption capacity (Gao *et al.* 2008). Also, a deeper crypt may not be preferred as it indicates increased tissue turnover, and thus a higher maintenance requirement that might consequently reduce feed conversion efficiency and growth (Gao *et al.* 2008; Zanu *et al.* 2020*c*). This was illustrated by the reduced bodyweight gain and feed efficiency in NE-challenged birds, as reported in the first part of this series (Dao *et al.* 2022). The increased crypt depth may also suggest inflammation and toxin secretions by enteric pathogens (Gao *et al.* 2008), and the wider villus may indicate a reduction in nutrient absorptive area and possibly an increase of gut-associated immune tissues (Chen *et al.* 2015). Furthermore, alterations in the GIT microbiota population following the NE challenge have been reported in birds (Stanley *et al.* 2012, 2014; Latorre *et al.* 2018). The increase in the numbers of *C. perfringens* in NE-challenged birds was expected and may confirm the success of the NE challenge model established in the current study. The NE challenge increased the number of Enterobacteriaceae, and decreased numbers of *Bacillus* spp. and *Ruminococcus* spp. in the caeca, which is similar to the results of other reports

Table 6. Ileal succinic acid, lactic acid, and short-chain fatty acids (SCFA) of broiler chickens on Day 16 (μmol/g).

Effects		Formic	Acetic	Lactic	Propionic	Butyric	Succinic	Total SCFA
Two-way ANOVA results (2 × 2 factorial arrangement of treatments)								
Treatment	SP−	0.68	4.32ab	35.13	0.09	0.00	0.72b	4.28a
	RP−	0.19	3.74ab	29.67	0.12	0.00	0.25a	4.06a
	SP+	0.28	2.58a	78.77	0.03	0.02	0.23a	2.92a
	RP+	0.56	5.64b	78.47	0.03	0.06	0.57ab	6.38b
NE	No	0.40	4.03	32.40a	0.10b	0.00	0.48	4.15
	Yes	0.43	4.11	78.61b	0.03a	0.04	0.41	4.65
Protein	SP	0.47	3.51	55.50	0.06	0.01	0.49	3.55a
	RP	0.38	4.63	54.07	0.08	0.03	0.41	5.14b
s.e.m.		0.13	0.32	5.28	0.01	0.02	0.07	0.33
P-value	NE	0.282	0.909	<0.001	<0.001	0.196	0.618	0.818
	Protein	0.744	0.075	0.896	0.542	0.544	0.577	0.025
	NE × protein	0.147	0.002	0.150	0.293	0.559	0.002	<0.001
One-way ANOVA results (four NE-challenged treatments)								
Treatment	SP+	0.28	2.58a	78.77	0.03	0.02	0.23a	2.92a
	RP+	0.56	5.64b	78.47	0.03	0.06	0.57b	6.38b
	RPA+	0.62	5.92b	73.96	0.06	0.00	0.59b	6.59b
	RPC+	0.32	5.48b	76.02	0.05	0.05	0.44ab	5.90b
s.e.m.		0.09	0.38	3.97	0.01	0.02	0.05	0.41
P-value		0.501	0.001	0.974	0.646	0.542	0.023	<0.001

Symbols −/+ indicate the absence or presence of necrotic enteritis challenge in the treatments. The RPA diet was created by adding L-arginine on top of the RP diet at a concentration of 0.03% in all feeding phases. Level 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 RPA diet by L-citrulline. Two-way ANOVA presens 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).

Different letters within a column indicate significant differences between the means.

SP, diet containing standard protein at a concentration of 23.2%, 21.4% and 19.4% crude protein for starter, grower and finisher phases respectively; RP, diet with reduced protein concentrations, with two percentage points lower crude protein than in SP diets in all feeding phases.

(Keerqin *et al.* 2017; Gharib-Naseri *et al.* 2019; Zanu *et al.* 2020*d*). *Bacillus* spp. are Gram-positive, aerobic bacteria, that have been illustrated to have beneficial effects on gut health through the production of extracellular enzymes, and thus have been used as a source of industrial enzymes (Hong *et al.* 2009; Latorre *et al.* 2015, 2016). Whereas *Ruminococcus* spp. plays various important roles in digestion, and production of bacteriocins and butyric acid that might influence the proliferation of intestinal epithelial cells and the development of *C. perfringens* in the GIT (Crost *et al.* 2011; Rinttilä and Apajalahti 2013). The decrease of *Bacillus* spp. and *Ruminococcus* spp. may indicate digestion disruption and impaired immune functions leading to reduced performance in NE challenged birds in the current study.

Short-chain fatty acids are required to maintain redox equivalent production within the anaerobic microbial community in the gut (Den Besten *et al.* 2013). As neutral or slightly alkaline is a favourable environment for the majority of gut pathogenic bacteria (Rodrigues and Choct 2018), the high level of SCFA can lower gut pH and inhibit the development of many pathogens (Ricke 2003; Huyghebaert *et al.* 2011). The results of the current study showed that the NE challenge increased lactic acid concentration, decreased propionic acid concentration in the ileum, and increased the concentrations of butyric, isobutyric, and isovaleric acids in the caeca of respective groups. The alterations in the ileal and caecal SCFA profile might indicate the changes in the gut microbiome in NE-challenged birds. Similar results were reported by Hilliar *et al.* (2020*b*). Meanwhile, no difference in the concentrations of caecal propionic, butyric, isobutyric, valeric, isovaleric, and lactic acids between NE-challenged and unchallenged birds were observed by Kheravii *et al.* (2018). It is generally accepted that higher concentrations of lactic acid, succinic acid, and SCFA in the gut indicate a healthier gut (Wu *et al.* 2016). However, NE disease is typically associated with the small intestine rather than the caeca *per se* (Timbermont *et al.* 2011; Shojadoost *et al.* 2012). Hence, higher concentrations of lactic acid, succinic acid, and SCFA in the caeca do not mean that birds will be protected from NE infection (Wu *et al.* 2016). Besides, increases in butyric acid

Table 7. Caecal succinic acid, lactic acid, and short-chain fatty acids (SCFA) of broiler chickens on Day 16 (μmol/g).

Effects		Acetic	Lactic	Propionic	Butyric	Isobutyric	Valeric	Isovaleric	Succinic	Total SCFA
Two-way ANOVA results (2 × 2 factorial arrangement of treatments)										
Treatment	SP−	78.35	0.59	4.65	15.94	0.66	0.78	0.21	6.32	100.6
	RP−	107.2	2.72	3.66	12.17	0.63	0.61	0.14	11.00	126.0
	SP+	69.70	0.46	5.93	30.34	0.84	1.01	0.26	11.21	111.9
	RP+	93.56	1.28	3.93	17.46	0.88	0.88	0.18	10.69	116.2
NE	No	91.82	1.59	4.19	14.18a	0.65a	0.70a	0.17	8.33	111.5
	Yes	81.63	0.94	4.93	23.90b	0.86b	0.94b	0.22	10.95	114.0
Protein	SP	74.64a	0.54a	5.20b	22.11b	0.74	0.88	0.23	8.42	104.9a
	RP	100.9b	2.00b	3.78a	14.61a	0.75	0.73	0.15	10.84	121.5b
s.e.m.		4.02	0.28	0.33	1.68	0.05	0.05	0.03	1.04	4.1
P-value	NE	0.214	0.251	0.270	0.002	0.041	0.023	0.183	0.214	0.769
	Protein	<0.001	0.014	0.028	0.023	0.935	0.183	0.083	0.251	0.040
	NE × protein	0.689	0.165	0.415	0.059	0.734	0.821	0.903	0.214	0.187
One-way ANOVA results (four NE-challenged treatments)										
Treatment	SP+	69.70	0.46	5.93b	30.34c	0.84	1.01b	0.26	11.21	111.9
	RP+	93.56	1.28	3.93a	17.46b	0.88	0.88ab	0.18	10.69	116.2
	RPA+	94.71	0.57	3.75a	13.56ab	0.92	0.88ab	0.28	8.31	114.1
	RPC+	88.15	0.40	3.60a	7.72a	0.82	0.49a	0.17	9.29	104.7
s.e.m.		4.05	0.20	0.29	1.98	0.05	0.07	0.03	1.20	4.4
P-value		0.114	0.372	0.010	<0.001	0.910	0.020	0.483	0.842	0.831

Symbols −/+ indicate the absence or presence of necrotic enteritis challenge in the treatments. The RPA diet was created by adding L-arginine on top of the RP diet at a concentration of 0.03% in all feeding phases. Concentration of calculated supplemental L-arginine in 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 presens 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).

Different letters within a column indicate significant differences between the means.

SP, diet containing standard protein at a concentration of 23.2%, 21.4% and 19.4% crude protein for starter, grower and finisher phases respectively; RP, diet with reduced protein concentrations, with two percentage points lower crude protein than in SP diets in all feeding phases.

concentration in the caeca might indicate the response of birds to the increase of *C. perfringens* as a result of NE challenge that can occur via the influence of the immune system (Meijer *et al.* 2010) or stimulation of intestinal mucin gene expression (Gaudier *et al.* 2004).

The intestinal pH is crucial for nutrient solubility and the microorganism population as the microbes rely on the pH of the environment where they reside (Zanu *et al.* 2020*c*). In the current study, the NE challenge decreased distal ileal pH but did not affect caecal pH on Day 16. These findings were consistent with the results on concentrations of succinic acid, lactic acid, and SCFA in the ileum and caeca on Day 16 in the current study. In particular, the NE challenge resulted in a marked increase in the concentration of lactic acid (2.4 times), which was the most abundant acid in the ileum, accounting for 94% and 88% of total examined ileal acids in NE-challenged and unchallenged birds respectively. Meanwhile, the total SCFA that accounted for 91–92% of examined acids in the caeca were not different between the challenged and unchallenged groups. The decreased ileal pH in NE-challenged birds has been previously reported and is believed to be a result of the increase in the numbers of lactic- and butyric acid-producing bacteria (Hilliar *et al.* 2020*b*).

Dietary protein concentration did not influence jejunal morphology in the current study. Similar results were found by Buwjoom *et al.* (2010) and Laudadio *et al.* (2012). Conversely, decreased jejunal villus height and villus height to crypt depth ratio in birds fed RP diets compared with those offered SP diets have been reported by others (Dehghani and Jahanian 2012; Houshmand *et al.* 2012). The differences in dietary protein level, diet composition, and bird age might be the reasons for discrepancies among the studies. Meanwhile, the decrease in gut permeability as shown by the serum FITC-d concentration on Day 16 as a result of reducing dietary protein compared with the SP diet when challenged with NE might be associated with less undigested feed materials and lower NE incidence than for the SP diet, as reported in the first part of this series (Dao *et al.* 2022). Noticeably, feeding the RP diets reduced numbers of caecal *Lactobacillus* spp. and total bacteria compared with those of the SP diets in the current study.

Table 8. Serum fluorescein isothiocyanate dextran (FITC-d) concentration, and ileal and caecal pH of broiler chickens on Day 16.

Effects		FITC-d level (μg/mL)	Ileal pH	Caecal pH
Two-way ANOVA results (2 × 2 factorial arrangement of treatments)				
Treatment	SP–	0.074a	6.49	6.43
	RP–	0.071a	6.50	6.64
	SP+	0.161c	5.58	6.39
	RP+	0.109b	5.58	6.57
NE	No	0.072a	6.49b	6.54
	Yes	0.135b	5.58a	6.48
Protein	SP	0.117	6.03	6.41a
	RP	0.090	6.04	6.60b
s.e.m.		0.007	0.10	0.03
P-value	NE	<0.001	<0.001	0.407
	Protein	0.053	0.960	0.002
	NE × protein	<0.001	0.980	0.825
One-way ANOVA results (four NE-challenged treatments)				
Treatment	SP+	0.161b	5.58	6.39a
	RP+	0.109a	5.58	6.57ab
	RPA+	0.114a	5.53	6.60ab
	RPC+	0.110a	5.64	6.66b
s.e.m.		0.004	0.04	0.03
P-value		<0.001	0.821	0.018

To determine the FITC-d concentration, blood samples were collected between 2 and 2.5 h after inoculating birds with a 1 mL dose of FITC-d (4.17 mg/kg bodyweight dissolved in water). Symbols –/+ indicate the absence or presence of necrotic enteritis challenge in the treatments. The RPA diet was created by adding L-arginine on top of the RP diet at a 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 presents 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).

Different letters within a column indicate significant differences between the means.

SP, diet containing standard protein at a concentration of 23.2%, 21.4% and 19.4% crude protein for starter, grower and finisher phases respectively; RP, diet with reduced protein concentrations, with 2% lower crude protein than in the SP diets in all feeding phases.

Lactobacilli have been generally known as beneficial bacteria for the host due to their ability to use complex plant-derived carbohydrates, including non-starch polysaccharides, and produce SCFA (Biddle *et al.* 2013). The higher inclusion level of starch from wheat and less soy-derived non-starch polysaccharides in the RP diets than in the SP diets in the current study may reduce the substrates that are essential for the growth of *Lactobacillus* spp. and anaerobic bacteria, and thus reduce the abundance of these groups. In addition, it is possible that the increased nitrogen digestibility in the upper GIT in birds fed RP diets compared with those fed the SP diets (Hilliar *et al.* 2019; Dao *et al.* 2021*a*, 2021*b*) might reduce nutrient bypass to the caeca and, consequently, limit microbial populations reliant on nitrogenous substrates. The NE × protein interactions obtained in the current study indicate that feeding the RP diets might help reduce disease severity and promote recovery in NE-challenged birds by lowering *C. perfringens* and Enterobacteriaceae counts compared with the SP diets. However, it is noteworthy that feeding an RP diet may also reduce beneficial bacteria such as *Lactobacillus* spp. in the caeca.

Dietary protein concentration is associated with the development of the gut microbial population and SCFA profile (Apajalahti and Vienola 2016). The results of the current study showed a beneficial effect of the RP over the SP diet on some aspects of the gut health when birds were challenged with NE that were indicated by the increases of total SCFA and acetic acid concentrations in the ileum and the increases of acetic acid, lactic acid, and total SCFA in the caeca in the respective group. This effect of the RP diets may be related to the reduction of harmful bacteria, including *C. perfringens* and Enterobacteriaceae, in the caeca, as observed in the current study. Decreased concentrations of caecal propionic acid and butyric acid were also observed in the RP-fed birds compared with the SP-fed birds in the current study. The decreased caecal butyric acid concentration in birds fed the RP compared with the SP has been previously reported and is believed to attribute to lower non-starch polysaccharide content in the RP diets (Hilliar *et al.* 2020*b*). Studies on humans have also shown that the serum butyrate level is increased in protein-rich diets, whereas propionate is decreased in carbohydrate-rich diets (Mueller *et al.* 2020). The results of the current study reflect differential effects of the RP diets on individual SCFA in different segments of the gut, which are most likely attributed to the diet composition.

Arginine is associated with proliferation and migration of intestinal epithelial cells, intestinal mucosa development, and villus morphology (Rhoads and Wu 2009; Murakami *et al.* 2012). Dietary Arg supplementation has been shown to increase villus height, villus height to crypt depth ratio, villus surface area, and gut integrity in birds subjected to the *Eimeria* and/or *C. perfringens* challenge (Tan *et al.* 2014; Laika and Jahanian 2017; Zhang *et al.* 2018). Although Cit effects are rarely being reported in birds, studies on mice and rats have shown that Cit supplementation was effective in increasing intestinal barrier functions (Jegatheesan *et al.* 2016; Sellmann *et al.* 2017). Furthermore, dietary Arg supplementation can deplete Arg degradation pathways caused by pathogenic bacteria, including *C. perfringens*, and enhance T-cell proliferation and function, thus inhibiting the development of these bacteria (Stadelmann *et al.* 2013; Tan *et al.* 2015). In addition, combined supplementation of Arg, threonine, and glutamine has been reported to benefit gut microbiota and promote an immune response in birds subjected to

Eimeria and *E. coli* challenges (Gottardo *et al.* 2017; Bortoluzzi *et al.* 2018). In the current study, the addition of Cit to the RP diet increased villus height, villus height to crypt depth ratio, and apparent villus area, whereas Arg supplementation did not affect jejunal morphology in NE-challenged birds on Day 16. Similarly, supplementation of Arg to the RP diet did not affect caecal microbiota counts, whereas Cit supplementation reduced the numbers of caecal *C. perfringens* and Enterobacteriaceae in the NE-challenged birds in the current study. The advantages of Cit as a delivery form of Arg are that it is less toxic to the intestine (Grimble 2007) and can escape the degradation of arginase activity in the GIT compared with Arg (McCarty 2010). This may explain more beneficial effects of Cit than Arg in enhancing gut morphology and reducing numbers of caecal pathogenic bacteria in birds suffering from NE challenge in the current study. However, no beneficial effects of either Arg or Cit supplementation on gut permeability were observed in the current study. Supplementation of Arg and/or Cit to RP diets at higher levels than the levels used in the current study is worthwhile for further studies.

Conclusions

In conclusion, the NE challenge caused gut damage by impairing jejunal morphological architecture, increasing the number of *C. perfringens*, and decreasing numbers of *Bacillus* spp. and *Ruminococcus* spp. in the caeca. Feeding RP diets with approximately two percentage points lower crude protein supported gut health of NE-challenged birds by reducing *C. perfringens* in the caeca compared with the SP diets, as shown by the result of NE × protein interactions. Increased ileal acetic acid and total SCFA concentrations were also observed in birds fed the RP diets compared with those fed the SP diets. Supplementation of Cit to the RP diets further increased the beneficial effects of RP diets by increasing jejunal villus height, villus height to crypt depth ratio, and apparent villus area, and by reducing *C. perfringens* and Enterobacteriaceae counts in the caeca. Hence, it can be concluded that compared with RP or RPA diet, in part replacement of Arg by Cit in the RPC diet may have beneficial effects on gut health 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 are no conflicts of interest.

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