Effects of low-crude protein diets supplemented with arginine, glutamine, threonine, and methionine on regulating nutrient absorption, intestinal health, and growth performance of *Eimeria*-infected chickens

Po-Yun Teng, Janghan Choi, Sudhir Yadav, Y. H. Tompkins, and Woo Kyun Kim[1]

Department of Poultry Science, University of Georgia, Athens, GA, USA

ABSTRACT The study was conducted to evaluate the effects of low crude protein diets supplemented with arginine, glutamine, methionine, and/or threonine on apparent ileal digestibility of amino acids, intestinal morphology, intestinal permeability, gene expression of nutrient transporters, and tight junction proteins of broiler chickens challenged with mixed *Eimeria* spp. A total of five hundred seventy-six, 12-day-old male broiler chickens were allocated into 8 treatments, and 6 replicate cages of 12 chickens per cage. This experiment included a nonchallenged control (**NC**) fed regular corn-soybean meal-based diet (Regular diet, 19% crude protein), an *Eimeria*-challenged control (**CC**) fed Regular diet, an *Eimeria* challenge group fed low-crude protein diet (**LCP**, 16% crude protein), 4 *Eimeria* challenge groups fed low-crude protein diet supplemented with 0.75% arginine, glutamine, methionine, and threonine, respectively (**ARG, GLN, MET,** and **THR**), and an *Eimeria* challenge group fed low-crude protein diet with 0.75% supplemented arginine, glutamine, methionine, and threonine collectively as a combination group (**COMB**). On d 14, birds in the challenge groups were gavaged with a mixed *Eimeria* spp. solution containing 12,500 oocysts of *E. maxima*, 12,500 oocysts of *E. tenella*, and 62,500 oocysts of *E. acervulina*. The results showed that the *Eimeria* challenge reduced overall growth performance, but the LCP had no adverse impacts on intestinal health and growth of *Eimeria*-infected birds compared to the CC. Additionally, supplementation of crystalline arginine, glutamine, methionine, and threonine improved the apparent ileal digestibility of these specific amino acids on 6 dpi. Moreover, the THR treatment increased villus height in the duodenum. The ARG treatment decreased intestinal permeability and gene expression of amino acid transporters, whereas the GLN and THR treatments both reversed adverse effects of coccidiosis on gene expression of tight junction protein (claudin 1). However, the MET and COMB treatments exacerbated infection severity of coccidiosis. In summary, adding 0.75% of arginine, glutamine, or threonine in a low crude protein diet can improve the intestinal health of birds challenged with a mild coccidia infection.

Key words: low crude protein diets, coccidiosis, arginine, glutamine, threonine, methionine

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INTRODUCTION

Attempts to reduce crude protein levels in diets have successfully offered several advantages in the poultry industry, such as reducing feed cost, enhancing nutrient utilization, and improving animal welfare. The commercially available crystalline amino acids improve the cost-effective formulation to meet the nutrient requirements for broiler chickens. It has been reported that decreasing dietary crude protein contents up to 3% would not affect the growth performance and meat quality of chickens (Belloir et al., 2017; Badawi et al., 2019). Furthermore, reduced crude protein diets could enhance animal welfare by promoting litter quality and limiting ammonia production (Greenhalgh et al., 2020). Dietary crude protein cutback decreases water intakes of chickens coupled with a reduction in litter moisture (Alleman and Leclercq, 1997). Better control of litter quality further reduces the incidence of foot-pad dermatitis, thereby improving animal welfare (Lemme et al., 2019; van Harn et al., 2019).

In addition, the reduced dietary crude protein also enhances nitrogen utilization of chickens (Aletor et al., 2000; Belloir et al., 2017). Because of the limited nutrients in the lower intestine, proliferation of pathogens, such as *C. perfringens,* would be suppressed in the birds fed low crude protein diets (**Low-CP diet**) (Drew et al., 2004). Furthermore, a previous study

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[1]Corresponding author: wkkim@uga.edu

indicated that reducing dietary crude protein levels from 24 to 16% suppressed mortality from 40 to 27.5% (Sharma et al., 1973); Overall, the Low-CP diet not only enhances nutrient digestibility but also reduces the incidence of coccidiosis and necrotic enteritis (Drew et al., 2004).

Coccidiosis, a common poultry disease infected by genus *Eimeria*, causes intestinal inflammation, sloughing cells, hemorrhage, maldigestion, morbidity, and even high mortality in some severe cases (Allen and Fetterer, 2002; Chapman, 2014). It has been estimated that the annual economic loss due to coccidiosis is up to 12.5 billion in the global poultry industry (Blake et al., 2020).

Nutritional strategies, such as formulating Low-CP diet and the addition of crystalline amino acids, are promising to improve the antioxidant capacity and immunity of host against coccidiosis in the modern "Antibiotic-Free" and "No Antibiotics Ever" production systems. Supplementing specific amino acids, such as arginine, glutamine, methionine, and threonine, in diets has shown profound benefits in regulating the intestinal health of animals (Bortoluzzi et al., 2018). Arginine, an essential amino acid for chickens, has been shown to be involved in protein synthesis, nitrite oxide production, and maintenance of oxidative capacity (Castro and Kim, 2020). Increasing arginine levels in the broiler diets could regulate immune responses and attenuate adverse effects of *Eimeria* on growth performance and intestinal morphology (Tan et al., 2014; Laika and Jahanian, 2017).

Being one of the main sources of energy for enterocytes, glutamine has been considered a curial amino acid to maintain intestinal cell linings (Rao and Samak, 2012). Furthermore, glutamine is the precursor for the synthesis of glutathione, a powerful antioxidant that attenuates oxidative stress by combating free radicals (Yu et al., 1999); thus, glutamine plays an essential role in maintaining the intestinal health in broiler chickens. Previous studies have reported that the application of glutamine enhanced growth performance, regulated immune responses, altered tight junction protein expression, and reduced intestinal permeability of animals (Luquetti et al., 2016; Oxford and Selvaraj, 2019).

Serving as precursors for synthesizing muscle and feather protein, methionine is the first limiting essential amino acid in corn-soybean meal-based diets for broiler chickens (Vieira et al., 2004). Increasing dietary methionine supplementation has shown positive outcomes on growth performance, antioxidant parameters, and intestinal immunity of birds challenged with *Eimeria* (Lai et al., 2018; Ren et al., 2020). On the other hand, threonine, another limiting amino acid, is a crucial nutrient that regulates mucosal integrity (Mao et al., 2011; Bortoluzzi et al., 2018). A previous study suggested to formulate feed with a higher ratio of threonine to lysine for improving growth performance and intestinal health against pathogen infections (Star et al., 2012). However, the feasibility of adding amino acids in a Low-CP diet to ameliorate intestinal injury of *Eimeria*-infected chickens has not been revealed in previous researches.

We hypothesized that the birds challenged with *Eimeria* does not require a high concentration of dietary crude protein but needs specific amino acids for accelerating tissue recovery during the acute infection (0−6 dpi) and the early recovery (6−9 dpi) phases. In other words, the requirement of crude protein for birds susceptible to coccidiosis could be reduced if specific amino acids were supplemented in the diet. We further hypothesized that stronger intestinal ecosystem constructed during the acute infection and early recovery phases could subsequently enhance the compensatory growth, even though treatment diets are replaced by the regular grower diet during the late recovery phase from 9 to 14 dpi. To confirm the hypothesis, the current study investigated the effects of reduced crude protein diets with supplementing arginine, glutamine, methionine, and threonine, separately or collectively, on nutrient digestibility, gene expressions of tight junction proteins, and nutrient transporters of chickens infected with mixed *Eimeria* spp.

MATERIALS AND METHODS

The current study was conducted under the approval of the Institutional Animal Care and Use Committee of the University of Georgia, Athens, GA.

Experimental Design

A total of 576, twelve-day-old male broiler chickens (Cobb 500, Cobb-Vantress, Cleveland, GA) were randomly allocated to 48 battery cages. The completely randomized design was used in the study, with 8 treatments and 6 replicate cages of 12 chickens per cage. The environment and temperature programs followed the Cobb 500 Broiler Management Guide (Cobb-Vantress, 2018). Chickens were provided ad libitum access to feed and water through the whole experiment.

The 8 treatments included a nonchallenged control (**NC**) fed regular corn-soybean meal-based diet (**Regular diet**) that meets the recommended nutritional levels from Cobb's guideline (Table 1, 19% crude protein), an *Eimeria*-challenged control (**CC**) fed Regular Diet, an *Eimeria* challenge group fed Low-CP diet (**LCP**) (Table 1, 16% crude protein), four *Eimeria* challenge groups fed Low-CP diet supplemented with 0.75% (0.75 g per 100 g of the diet) arginine, glutamine, methionine, and threonine, respectively (**ARG, GLN, MET,** and **THR**), and an *Eimeria* challenge group fed Low-CP diet with arginine, glutamine, methionine, and threonine collectively as a combination group (**COMB**). The minimum requirements of digestible amino acids for the LCP diet were set at 84% of the regular diet to reduce the total crude protein levels from 19 to 16 (84% reduction). Chickens were fed a regular starter diet (21% crude protein, 2,975 Kcal/kg) before the trial, and fed treatment diets (Table 1) from d 12 to d 23. On d 14, chickens, except the NC group, were challenged with mixed *Eimeria* spp. The challenge dose was

Table 1. Diet formulation according to the treatments (% diet).

Items	NC/CC[3]	LCP[3]	ARG[3]	GLN[3]	MET[3]	THR[3]	COMB[3]
Corn	62.38	66.94	66.94	66.94	66.94	66.94	66.94
Soybean Meal	31.90	24.81	24.81	24.81	24.81	24.81	24.81
Soybean oil	1.49	1.71	1.71	1.71	1.71	1.71	1.71
Limestone	0.71	0.81	0.81	0.81	0.81	0.81	0.81
Defluorinated phosphate	1.44	1.37	1.37	1.37	1.37	1.37	1.37
Sodium chloride	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Vitamin premix[1]	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Mineral premix[2]	0.08	0.08	0.08	0.08	0.08	0.08	0.08
Methionine	0.27	0.21	0.21	0.21	0.96	0.21	0.96
Lysine HCl	0.17	0.19	0.19	0.19	0.19	0.19	0.19
Threonine	0.02	0.02	0.02	0.02	0.02	0.77	0.77
Arginine	0.00	0.00	0.75	0.00	0.00	0.00	0.75
Glutamine	0.00	0.00	0.00	0.75	0.00	0.00	0.75
Sand	0.70	3.00	2.25	2.25	2.25	2.25	0.00
Titanium oxide	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Calculated value							
M.E. (kcal/kg)	3.03	3.03	3.05	3.05	3.05	3.05	3.13
CP (%)	19.00	16.00	17.51	16.9	16.43	16.54	19.37
Calcium (%)	0.84	0.84	0.84	0.84	0.84	0.84	0.84
Available P(%)	0.42	0.42	0.42	0.42	0.42	0.42	0.42
Dig Lys (%)	1.12	0.95	0.95	0.95	0.95	0.95	0.95
Dig Met (%)	0.57	0.48	0.48	0.48	1.23	0.48	1.23
Dig Thr (%)	0.74	0.62	0.62	0.62	0.62	1.37	1.37
Dig Arg (%)	1.31	1.07	1.82	1.07	1.07	1.07	1.82
Dig Gly (%)	0.86	0.73	0.73	0.73	0.73	0.73	0.73
Dig TSAA (%)	0.85	0.72	0.72	0.72	1.47	0.72	1.47
Dig Ser (%)	1.02	0.86	0.86	0.86	0.86	0.86	0.86
Dig Tyr (%)	0.75	0.64	0.64	0.64	0.64	0.64	0.64
Dig Val (%)	1.09	0.92	0.92	0.92	0.92	0.92	0.92
Dig Trp (%)	0.26	0.21	0.21	0.21	0.21	0.21	0.21
Analyzed value							
Total amino acids (%)	18.67	16.51	17.16	17.17	17.42	17.03	19.7
Arginine (%)	1.25	1.05	1.75	1.10	1.00	1.06	1.75
Glutamine (%)	3.38	2.96	2.93	3.56	2.89	2.90	3.65
Methionine (%)	0.58	0.46	0.46	0.42	1.49	0.45	1.56
Threonine (%)	0.72	0.63	0.59	0.72	0.52	1.27	1.41

[1]Provided per kg of DSM Vitamin premix: Vit. A 2,204,586 IU, Vit. D3 200,000 ICU, Vit. E 2,000 IU, Vit. B12 2 mg, Biotin 20 mg, Menadione 200 mg, Thiamine 400 mg, Riboflavin 800 mg, d-Pantothenic Acid 2,000 mg, Vit. B6 400 mg, Niacin 8,000 mg, Folic Acid 100 mg, Choline 34,720 mg.

[2]Provided per kg of Mineral premix: Ca 0.72 g, Mn 3.04 g, Zn 2.43 g, Mg 0.61 g, Fe 0.59 g, Cu 22.68 g, I 22.68 g, Se 9.07 g.

[3]NC, nonchallenged control; CC, *Eimeria*-challenged control; LCP, birds fed the low crude protein diet and received *Eimeria* challenge; ARG, birds fed the low crude protein diet adding 0.75% arginine and received *Eimeria* challenge; GLN, birds fed the low crude protein diet adding 0.75% glutamine and received *Eimeria* challenge; MET, birds fed the low crude protein diet adding 0.75% methionine and received *Eimeria* challenge; THR, birds fed low crude protein diet adding 0.75% threonine and received *Eimeria* challenge; COMB, birds fed the low crude protein diet adding arginine, glutamine, methionine, and threonine, and received *Eimeria* challenge.

selected based on our previous study, which would not cause significant mortality but reduce growth performance and damage intestinal epithelium as a mild coccidia infection (Teng et al., 2020). The birds were gavaged with a solution containing 12,500 sporulated oocysts of *E. maxima*, 12,500 sporulated oocysts of *E. tenella*, and 62,500 sporulated oocysts of *E. acervulina*. In contrast, the NC group orally received 1 mL water. After d 23, birds were fed the Regular diet to the end of the experiment (d 28). Growth performance was recorded and calculated for 3 periods, the acute infection phase (d 12−d 20; −2 to 6 dpi), the early recovery phase (d 20−d 23; 6−9 dpi), and the late recovery phase (d 23 −d 28; 9−14 dpi).

Sample Collections

On 6 days postinfection (**dpi**), 5 chickens per cage were euthanized by cervical dislocation. Duodenum, jejunum, and jejunal mucosa were removed from one of 5 sampled chickens. On 9 dpi, one more bird per cage was killed for collecting intestinal tissue. The intestinal tissue was rinsed with phosphate-buffered saline and fixed with 10% formalin immediately, whereas the mucosa was collected in a tube and snap-frozen in liquid nitrogen. The frozen samples were stored at −80°C until further analyses. On 6 dpi, ileal digesta samples collected from 5 birds per cage were flushed out from the ileum with deionized water. The samples were further pooled within the cage, stored in a container and frozen at −20°C.

Intestinal Morphology

The duodenum and jejunum tissues were removed from the 10% formalin, carefully cut into short sections, stored in cassettes, and finally embedded in paraffin. Afterward, the approximately 4-μm-sliced samples were stained with hematoxylin and eosin. The intestinal morphology of each sample was observed and captured under a light microscope with 2X magnification (BZ-X800, Keyence Inc, Itasca, IL). Five villi and crypts per sample were randomly picked to determine the length of

villus and crypt. The Image J (Image Processing and Analysis in Java−ImageJ 1.50i, National Institutes of Health, Bethesda, MD) was used to measure the villus height and crypt depth, whereas the ratio of villi height to crypt depth was further calculated from the averaged villi height and crypt depth of each sample (Teng et al., 2020).

Apparent Ileal Digestibility of Amino Acids

To measure the apparent ileal digestibility (**AID**) of amino acids, 0.3% titanium oxide was formulated in the treatment diets as an indigestible marker. The concentrations of maker and amino acids in feed and digesta were used to calculate AID, as the formula shown below (Stein et al., 2007).

$$AID\ of\ amino\ acid(\%) = [1 - (Ti_i/Ti_o) \times N_o/N_i] \times 100$$

Where Ti_i means the determined concentration of titanium oxide in the diets; Ti_o represents the titanium oxide in the digesta samples; N_i indicates the value of crude protein or amino acids in the treatment diets; and N_o represents the concentration of the nutrients in the ileal digesta.

The frozen digesta samples were lyophilized and ground by a coffee grinder (KitchenAid, Benton Harbor, MI). Titanium oxide concentration in the diets and digesta samples were measured according to the description by Short et al. (1996). In addition, the amino acids levels were analyzed by the University of Missouri Agricultural Experiment Station Chemical Laboratory [method 982.30 E (a, b, c), AOAC, 2006].

Oocyst Shedding

Fresh excreta from 6 to 9 dpi were pooled thoroughly, and approximately 500 g of the fecal materials were collected from each cage in a sample bag. Five grams of sample were weighted and mixed with 45 mL of water and the homogenized solution was serially diluted to a countable level for determining oocysts shedding. Five milliliters of diluted samples were mixed with 45 mL of saturated salt solution in a centrifuge tube. After appropriate mixing, the samples were loaded in a Mcmaster chamber (Jorgensen Laboratories, Loveland, CO) and observed under a microscope (FEC Source, Grand Ronde, OR). The total oocyst shedding was counted and presented as oocysts per gram in the result.

Gastrointestinal Permeability

Gastrointestinal permeability was measured by fluorescein isothiocyanate dextran (**FITC-d**; 2.2 mg/mL**,** MW 4000; Sigma-Aldrich, Canada) on 5 dpi (Liu et al., 2021). One chicken randomly selected from each cage was gavaged with 1 mL of FITC-d. After 2 h postinoculation, chickens were killed by cervical dislocation, and blood was collected from the heart immediately. The clotted blood samples were centrifuged at 1,000 × *g* for 15 min to obtain at least 200 μL serum. About 100 μL serum was later transferred to a dark 96-well microplate with duplicates per sample. Standard solutions were prepared with the pool of serum from 10 additional chickens that were not used in the experiment but raised in the same house, following the recommendations described by Teng et al. (2020). The FITC-d concentration in the serum samples was determined by a microplate reader (Spectramax M5, Molecular Devices, San Jose, CA), where the excitation wavelength was set at 485 nm and the emission wavelength at 528. The transportation, processing, and analysis of samples were carefully carried in a container and performed under a dark environment to protect samples from light exposure.

Real-Time PCR

Total RNA of jejunal mucosa was extracted by QIAzol Lysis Reagents (Qiagen, Germantown, MD) and homogenized with a bead beater (MiniBeadBeater-16, Biospec Products Inc, Bartlesville, OK). The RNA quantity was determined by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Afterward, the total RNA was diluted to 200 ng/μL and reverse-transcribed to cDNA by the High Capacity cDNA synthesis kits (Applied BioSystems, Life Technologies, Waltham, CA). The cDNA samples were diluted 1:5 for real-time PCR analysis performed on the Step One thermo-cycler (Applied Biosystem, Foster City, CA). The cDNA was mixed with SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA) and reverse and forward primers for real-time PCR analysis. Samples were run in duplicates, and the $2^{-\Delta\Delta Ct}$ method was used to determine target gene expressions comparing to housekeeping genes (Livak and Schmittgen, 2001). The geometric average of 2 genes, glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**) and beta-actin, was used as the reference Ct value. The forward and reverse primer sequences of tight junction proteins and amino acid transporters are listed in Table 2. For tight junction proteins, claudin 1 (**CLDN1**), occludin (**OCLDN**), zonula occludens 1 (**ZO1**), and junctional adhesion molecule 2 (**JAM2**) were tested and for amino acid transporters, gene expression of Na^+-dependent amino acid transporter (**B^0AT**), Na^+-independent amino acid transporter (**B^{0+}AT**), excitatory amino acid transporter (**EAAT**), cationic amino acid transporter (**CAT1**), L-type amino acid transporter (**LAT1**), and Na^+-dependent neutral/cationic amino acid transporter (**LAT2**) was analyzed in the current study.

Statistical Analyses

All data were analyzed in the PROC GLM program of SAS software (SAS Institute Inc., Cary, NC). Duncan's multiple range test was used as the post hoc analysis to separate means with significance levels at $P \leq 0.05$ or a trend at $P \leq 0.1$. The experimental unit is cage in the current study.

Table 2. List of primers used for qPCR.

Gene symbol[1]	Accession number	Forward primer	Reverse primer
GAPDH[2]	NM_204305.1	CCTCTCTGGCAAAGTCCAAG	GGTCACGCTCCTGGAAGATA
Beta-actin[2]	NM_205518.1	CAACACAGTGCTGTCTGGTGGTA	ATCGTACTCCTGCTTGCTGATCC
CLDN1[3]	NM_001013611.2	TGGAGGATGACCAGGTGAAGA	CGAGCCACTCTGTTGCCATA
OCLDN[3]	NM_205128.1	ACGGCAGCACCTACCTCAA	GGCGAAGAAGCAGATGAG
ZO1[3]	XM_015278981.2	CAACTGGTGTGGGTTTCTGAA	TCACTACCAGGAGCTGAGAGGTAA
JAM2[3]	NM_001006257.1	AGCCTCAAATGGGATTGGATT	CATCAACTTGCATTCGCTTCA
B^0AT (SLC6A19)[4]	XM_419056.6	GGGTTTTGTGTTGGCTTAGGAA	TCCATGGCTCTGGCAGAGAT
$B^{0+}AT$ (SLC7A9)[4]	NM_001199133.1	CAGTAGTGAATTCTCTGAGTGTGAAGCT	GCAATGATTGCCACAACTACCA
EAAT (SLC1A1)[4]	XM_424930.6	TGCTGCTTTGGATTCCAGTGT	AGCAATGACTGTAGTGCAGAAGTAATATATG
CAT1 (SLC7A1)[4]	XM_015277945.2	CCAAGCACGCTGATAAAG	TACTCACAATAGGAAGAAGGG
LAT2 (SLC7A6)[4]	XM_025154295.1	TCAGCTTCAGTTACTGGTT	GCACAACCACGAGAAATAC
LAT1 (SLC7A5)[4]	NM_001030579.2	GATTGCAACGGGTGATGTGA	CCCCACACCCACTTTTGTTT

[1]GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CLDN1, Claudin 1 (Shao et al., 2013); OCLDN, Occludin (Liu et al., 2012); ZO1, Zonula occludens 1 (Metzler-Zebeli et al., 2018); JAM2, Junctional adhesion molecule 2 (Chen et al., 2015) B^0AT; Na^+-dependent amino acid transporter; $B^{0+}AT$, Na^+-independent amino acid transporter; EAAT, excitatory amino acid transporter; CAT1, Cationic amino acid transporter; LAT2, Na^+-dependent neutral/cationic amino acid transporter; LAT1, L-type amino acid transporter 1.
[2]Housekeeping gene.
[3]Tight junction proteins.
[4]Nutrient transporters (Gilbert et al., 2007).

RESULTS

Growth Performance and Mortality

Growth performance results are present in Table 3. During d 12 to 20, *Eimeria* infection significantly reduced body weight (**BW**) and body weight gain of birds (**BWG**) compared to the NC group ($P < 0.05$). The nonchallenged chickens had the lowest feed conversion rate (**FCR**), whereas the MET group had the highest FCR among all treatments ($P < 0.05$). Reducing crude protein levels did not cause any significant differences in BW, BWG, FI, and FCR of *Eimeria*-challenged chickens. However, chickens in the MET and COMB treatments had less BW and BWG than the other treatments ($P < 0.05$). Feed intake (**FI**) in the COMB was significantly less than the other treatments, except the CC and MET treatments ($P < 0.05$).

During the early recovery phase (d 20−23; 6−9 dpi), the CC and ARG groups showed no significant difference in BWG compared to the NC treatment, but the MET and COMB groups had lower BWG and FI than the other treatments. Additionally, there were no significant differences in FI and FCR among the NC, CC, LCP, ARG, GLN, and THR from 6 to 9 dpi. During the late recovery phase (d 23−28; 9−14 dpi), the NC showed higher BW than the *Eimeria*-infected groups ($P < 0.05$). However, chickens fed the MET had less FI than those fed with GLN ($P < 0.05$).

Table 3. Effects of low crude protein diets supplemented with arginine, glutamine, methionine, and/or threonine on growth performance in chickens challenged with mixed *Eimeria* spp.

Items[1]	NC	CC	LCP	ARG	GLN	MET	THR	COMB	SEM	*P*-value
d 12−20										
BW (g)	721[a]	623[b]	624[b]	634[b]	625[b]	572[c]	633[b]	597[c]	6.35	<0.001
BWG (g)	447[a]	351[b]	352[b]	362[b]	352[b]	301[d]	362[b]	324[c]	6.26	<0.001
FI (g)	665[a]	612[bcd]	641[abc]	657[ab]	642[abc]	598[cd]	629[abc]	571[d]	6.79	0.003
FCR (g/g)	1.49[c]	1.75[b]	1.82[b]	1.82[b]	1.82[b]	2.00[a]	1.74[b]	1.77[b]	0.03	<0.001
d 20−23										
BW	941[a]	819[b]	823[b]	865[b]	842[b]	713[c]	844[b]	748[c]	10.94	<0.001
BWG	211[a]	199[b]	185[b]	201[ab]	183[b]	124[c]	188[b]	140[c]	4.75	<0.001
FI	336[a]	336[a]	332[a]	332[a]	310[a]	269[b]	321[a]	277[b]	5.12	<0.001
FCR	1.59[c]	1.69[c]	1.80[bc]	1.66[c]	1.71[c]	2.20[a]	1.72[c]	1.98[b]	0.03	<0.001
d 23−28										
BW	1,369[a]	1,210[bc]	1,221[b]	1,275[b]	1,279[b]	1,125[c]	1,239[b]	1,125[c]	14.65	<0.001
BWG	413	375	383	402	401	392	395	380	6.01	0.798
FI	659[ab]	635[ab]	653[ab]	665[ab]	695[a]	623[b]	678[ab]	640[ab]	7.33	0.243
FCR	1.61	1.70	1.72	1.66	1.74	1.60	1.73	1.69	0.02	0.417
d 12−28										
BWG	1071[a]	925[b]	921[b]	965[b]	936[b]	816[c]	945[b]	843[c]	12.67	<0.001
FI	1,660[a]	1,583[ab]	1,625[a]	1,653[a]	1,647[a]	1,490[b]	1,628[a]	1,488[ab]	15.02	0.002
FCR	1.55[c]	1.71[b]	1.77[ab]	1.71[b]	1.76[ab]	1.84[a]	1.72[ab]	1.77[ab]	0.02	0.001

[1]BW, body weight; BWG, body weight gain; FI, feed intake; FCR, feed conversion rate; NC, nonchallenged control; CC, *Eimeria*-challenged control; LCP, birds fed the low crude protein diet and received *Eimeria* challenge; ARG, birds fed the low crude protein diet adding 0.75% arginine and received *Eimeria* challenge; GLN, birds fed the low crude protein diet adding 0.75% glutamine and received *Eimeria* challenge; MET, birds fed the low crude protein diet adding 0.75% methionine and received *Eimeria* challenge; THR, birds fed low crude protein diet adding 0.75% threonine and received *Eimeria* challenge; COMB, birds fed the low crude protein diet adding arginine, glutamine, methionine, and threonine, and received *Eimeria* challenge.
[a-d]Means followed by superscript letters are different within the same row; $N = 6$.

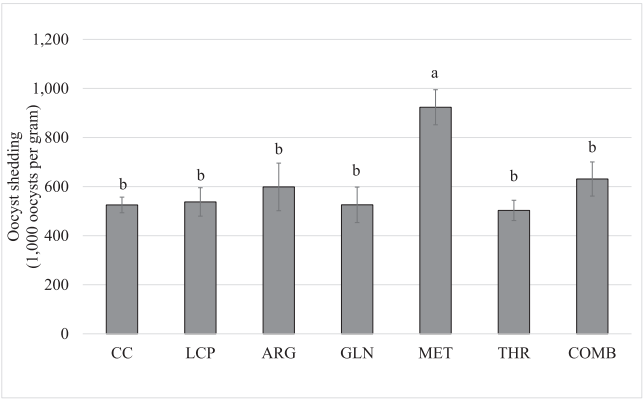

Figure 1. Effects of low crude protein diets supplemented with arginine, glutamine, methionine, and/or threonine on oocysts shedding in chickens challenged with mixed *Eimeria* spp. Birds were challenged with mixed *Eimeria* spp. on d 14. Excreta was collected from 6 to 9 dpi. Oocyst was not observed in the non-challenged control. CC, *Eimeria*-challenged control; LCP, birds fed the low crude protein diet and received *Eimeria* challenge; ARG, birds fed the low crude protein diet adding 0.75% arginine and received *Eimeria* challenge; GLN, birds fed the low crude protein diet adding 0.75% glutamine and received *Eimeria* challenge; MET, birds fed the low crude protein diet adding 0.75% methionine and received *Eimeria* challenge; THR, birds fed low crude protein diet adding 0.75% threonine and received *Eimeria* challenge; COMB, birds fed the low crude protein diet adding arginine, glutamine, methionine, and threonine, and received *Eimeria* challenge. [a,b]Means followed by superscript letters are different by Duncan's test. The error bars represent the SD values. $N = 6$.

The results of overall growth performance (d 12−28) indicated that mild *Eimeria* infection significantly reduced BWG and increased FCR ($P < 0.05$) compared with the NC treatment. Furthermore, supplementing 0.75% methionine resulted in the worse growth performance among all treatments ($P < 0.05$). The current results also demonstrated that LCP did not cause negative impacts on the growth performance of birds infected with coccidiosis. The *Eimeria*-challenged chickens fed the Low-CP diet had numerically higher BW on 6 and 9 dpi than those provided the Regular diet.

The NC, CC, GLN, MET, and COMB treatments had 0% mortality; the LCP and THR groups had 0.5% mortality; and the ARG treatment had 1% mortality. No significant difference in mortality among the treatments was found in the current study.

Total Oocyst Shedding and Gastrointestinal Permeability

Oocyst was not observed in the NC. The LCP treatment did not cause significant effects on total oocyst shedding compared to the CC control (Figure 1). However, chicken fed the MET diet had significantly higher oocysts counts in the excreta than the others (Figure 1, $P < 0.05$). Except for the MET, supplementing ARG, GLN or THR in Low-CP diet had no significant impacts on *Eimeria* oocysts shedding. On the other hand, the gastrointestinal permeability was significantly increased by coccidia infection (Figure 2, $P < 0.05$). The ARG had significantly lower gastrointestinal permeability than the LCP group (Figure 2, $P < 0.05$), but there was no significant difference between the CC and LCP treatments.

Intestinal Morphology

Results of intestinal morphology are shown in Table 4. On 6 dpi, the *Eimeria*-challenged chickens fed additional 0.75% threonine had a significant higher villus height of the duodenum than the CC, GLN, MET, and COMB groups ($P < 0.05$), whereas the LCP and ARG numerically increased the villus height from 1,912 μm (CC) to

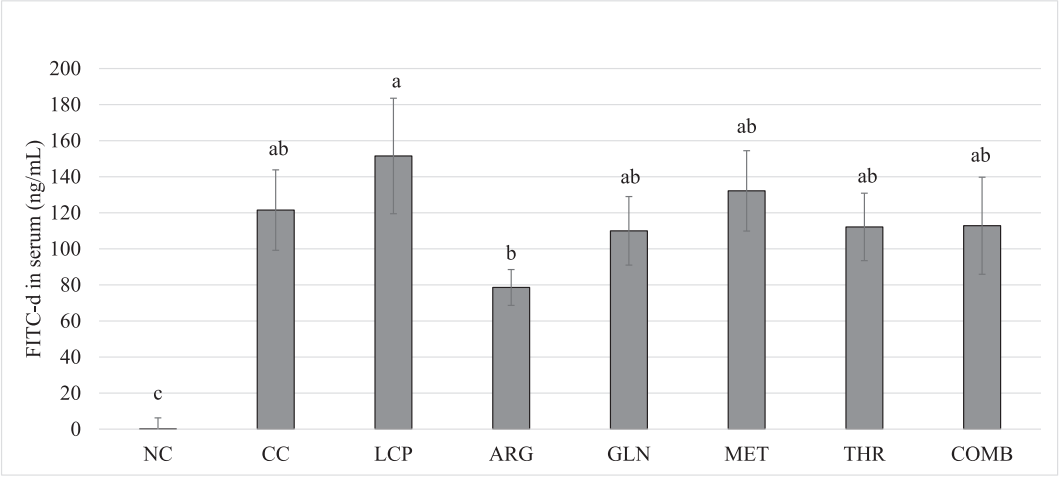

Figure 2. Effects of low crude protein diets supplemented with arginine, glutamine, methionine, and/or threonine on gastrointestinal permeability in chickens challenged with mixed *Eimeria* spp. Birds were challenged with mixed *Eimeria* spp. on d 14. Gastrointestinal permeability was determined by fluorescein isothiocyanate dextran on 5 dpi. NC, nonchallenged control; CC, *Eimeria*-challenged control; LCP, birds fed the low crude protein diet and received *Eimeria* challenge; ARG, birds fed the low crude protein diet adding 0.75% arginine and received *Eimeria* challenge; GLN, birds fed the low crude protein diet adding 0.75% glutamine and received *Eimeria* challenge; MET, birds fed the low crude protein diet adding 0.75% methionine and received *Eimeria* challenge; THR, birds fed low crude protein diet adding 0.75% threonine and received *Eimeria* challenge; COMB, birds fed the low crude protein diet adding arginine, glutamine, methionine, and threonine, and received *Eimeria* challenge. [a,b]Means followed by superscript letters are different by Duncan's test. The error bars represent the SD values. $N = 6$.

Table 4. Effects of low crude protein diets supplemented with arginine, glutamine, methionine, and/or threonine on intestinal morphology in chickens challenged with mixed *Eimeria* spp.

Items[1]	NC	CC	LCP	ARG	GLN	MET	THR	COMB	SEM	*P*-value
6 dpi										
Duodenum										
VH	2,255^{abc}	1,912^{bcd}	2,090^{abcd}	2,295^{ab}	1,901^{cd}	1,761^{d}	2,368^{a}	1,920^{bcd}	50	0.009
CD	119^{b}	183^{b}	165^{b}	179^{b}	175^{b}	179^{b}	173^{b}	249^{a}	8	0.013
Ratio	19.5^{a}	10.9^{bc}	13.1^{b}	14.1^{b}	11.3^{bc}	10.6^{bc}	14.3^{b}	8.0^{c}	0.67	<0.001
Jejunum										
VH	1,119	1,135	1,182	1,212	1,065	1,141	1,274	1,176	29	0.810
CD	146	156	161	145	149	150	158	146	4	0.939
Ratio	7.7	7.5	7.4	8.4	7.3	7.7	8.3	8.3	0.23	0.882
9 dpi										
Duodenum										
VH	2,425	2,350	2,509	2,461	2,467	2,217	2,431	2,203	45	0.522
CD	116	148	155	141	163	135	145	147	4	0.465
Ratio	21.04	16.01	16.46	17.51	16.00	17.25	17.26	15.29	0.52	0.418
Jejunum										
VH	1,310	1,403	1,365	1,368	1,405	1,216	1,473	1,166	33	0.295
CD	107	152	136	135	106	135	137	129	5	0.121
Ratio	12.15	9.69	10.14	10.39	11.18	9.14	10.80	9.06	0.29	0.258

[1]VH, villus height; CD, crypt depth; Ratio, villus height: crypt depth; NC, nonchallenged control; CC, *Eimeria*-challenged control; LCP, birds fed the low crude protein diet and received *Eimeria* challenge; ARG, birds fed the low crude protein diet adding 0.75% arginine and received *Eimeria* challenge; GLN, birds fed the low crude protein diet adding 0.75% glutamine and received *Eimeria* challenge; MET, birds fed the low crude protein diet adding 0.75% methionine and received *Eimeria* challenge; THR, birds fed low crude protein diet adding 0.75% threonine and received *Eimeria* challenge; COMB, birds fed the low crude protein diet adding arginine, glutamine, methionine, and threonine, and received *Eimeria* challenge.

[a-d]Means followed by superscript letters are different within the same row; Unit: μm; $N = 6$.

2,090 μm and 2,295 μm, respectively. Moreover, the nonchallenged birds had the highest ratio of villus height to crypt depth in the duodenum ($P < 0.05$), but there was no significant difference between the CC and LCP in the morphology results. The COMB treatment had the longest crypt depth and the lowest ratio of villus height to crypt depth compared to the other groups ($P < 0.05$).

On 9 dpi, although the ARG treatment showed positive outcomes in the duodenum morphology with numerically higher villus height to crypt depth ratio than the other *Eimeria*-challenged groups, there were no significant differences of the villus height, crypt depth, and the ratios in the duodenum among all treatments during the early recovery phase. It is noteworthy that the MET and COMB groups had numerically shorter villus height in the jejunum, whereas the non-challenged chickens had the numerically higher ratio than the challenged birds on 9 dpi.

Table 5. Effects of low crude protein diets supplemented with arginine, glutamine, methionine, and/or threonine on apparent ileal digestibility amino acids in chickens challenged with mixed *Eimeria* spp. (6 dpi).

Items[1]	NC	CC	LCP	ARG	GLN	MET	THR	COMB	SEM	*P*-value
Arginine	85^{b}	85^{b}	85^{b}	89^{a}	84^{bc}	81^{c}	84^{bc}	91^{a}	0.55	<0.001
Histidine	80	79	79	75	77	75	77	80	0.62	0.083
Isoleucine	75^{ab}	76^{ab}	79^{a}	74^{ab}	75^{ab}	73^{b}	75^{ab}	79^{a}	1.04	<0.001
Leucine	79	80	81	77	78	78	78	81	0.51	0.384
Lysine	80^{*a}	80^{*,ab}	81^{*a}	77^{*,ab}	79^{*,ab}	75^{*,b}	78^{*a,b}	82^{*a}	0.62	0.082
Methionine	91^{b}	91^{b}	90^{b}	88^{c}	88^{c}	96^{a}	91^{b}	97^{a}	0.51	<0.001
Phenylalanine	78	79	80	76	77	76	77	81	0.54	0.193
Threonine	69^{b}	69^{b}	68^{bc}	64^{bc}	67^{bc}	61^{c}	81^{a}	85^{a}	1.31	<0.001
Tryptophan	77	81	78	78	75	79	78	80	0.56	0.223
Valine	74	74	74	70	72	69	71	76	0.71	0.159
Alanine	78	77	79	74	76	74	75	79	0.62	0.367
Aspartic acid	76^{abc}	77^{ab}	78^{ab}	74^{bc}	76^{abc}	71^{c}	75^{abc}	80^{a}	0.59	0.014
Cysteine	63	63	68	62	61	61	66	69	0.88	0.108
Glutamine	84^{bcd}	85^{b,cd}	86^{abc}	83^{cd}	86^{ab}	82^{d}	84^{bcd}	89^{a}	0.45	0.001
Glycine	72^{*,ab}	73^{*,ab}	74^{*a}	69^{*,ab}	71^{*,ab}	67^{*,b}	71^{*a,b}	75^{*a}	0.70	0.082
Proline	78	79	79	76	77	76	77	80	0.52	0.607
Serine	76	76	75	73	74	70	73	77	0.66	0.197
Total	78^{b}	79^{b}	79^{ab}	77^{b}	78^{b}	76^{b}	78^{b}	83^{a}	0.56	0.048

[1]NC, nonchallenged control; CC, *Eimeria*-challenged control; LCP, birds fed the low crude protein diet and received *Eimeria* challenge; ARG, birds fed the low crude protein diet adding 0.75% arginine and received *Eimeria* challenge; GLN, birds fed the low crude protein diet adding 0.75% glutamine and received *Eimeria* challenge; MET, birds fed the low crude protein diet adding 0.75% methionine and received *Eimeria* challenge; THR, birds fed low crude protein diet adding 0.75% threonine and received *Eimeria* challenge; COMB, birds fed the low crude protein diet adding arginine, glutamine, methionine, and threonine, and received *Eimeria* challenge.

[a-d]Means followed by superscript letters are different within the same row.

[*]Trend, $P < 0.1$; Unit: %; $N = 6$.

Apparent Ileal Digestibility of Amino Acids

The results of the AID of amino acids are present in Table 5. The COMB treatment significantly increased the AID of arginine, methionine, threonine, glutamine, and total amino acids compared to the CC group ($P < 0.05$). Supplementation of 0.75% threonine significantly increased the AID of threonine on 6 dpi ($P < 0.05$), whereas ARG treatment increased the AID of arginine from 85 to 90% but decreased the AID of methionine from 90% to 88% ($P < 0.05$). Moreover, GLN treatment also reduced the AID of methionine ($P < 0.05$). On the contrary, *Eimeria*-infected birds fed additional methionine significantly increased methionine digestibility but reduced the AID of isoleucine, arginine, aspartic acid, and glutamine ($P < 0.05$). Furthermore, there were trends showing that the AID of leucine and glycine were reduced by the MET treatment ($P < 0.1$).

Gene Expression of Tight Junction Proteins and Nutrient Transporters

The gene expression of tight junction proteins is present in Table 6. *Eimeria* challenge significantly increased gene expression of CLDN1 ($P < 0.05$). The challenge control and the LCP showed 4.33- and 4.38-fold increases of the CLDN1 on 6 dpi, respectively ($P < 0.05$). However, the GLN, THR, and COMB had significantly lower gene expression of CLDN than the LCP ($P < 0.05$), and there was no significant difference among the GLN, THR, COMB, and NC groups. The gene expression of the other tight junction proteins, including OCLDN, ZO1, and JAM2, was neither regulated by coccidia challenge nor dietary treatments on 6 dpi.

The gene expression of amino acid transporters is shown in Table 7. Coccidia infections resulted in a significant increase in LAT1 ($P < 0.05$). The challenge also caused numerical downregulation of b^{0+}at on 6 dpi ($P < 0.1$). Moreover, the ARG, MET, and COMB treatments upregulated expression of LAT1 compared to the NC ($P < 0.05$). Nevertheless, the B^0AT, EAAT, and LAT2 gene expression was not regulated by the *Eimeria* challenge and diet treatments on 6 dpi.

On 9 dpi, chickens fed the Low-CP diet with arginine supplementation had higher expression of LAT2 compared to the other challenged groups ($P < 0.05$). Furthermore, there was a trend showing that gene expression of LAT1 was higher in the COMB than the CC and LCP on 9 dpi ($P < 0.1$). However, no significant differences were observed for the other amino acid transporters during the early recovery phase.

DISCUSSION

In the current study, the mild *Eimeria* infection reduced BWG and increased FCR, but the Low-CP diet did not impact the overall growth performance of infected birds. Previous research on the investigation of different crude protein levels to alleviate adverse effects of coccidiosis has yielded inconsistent results. Some reports demonstrated that increasing dietary crude protein levels linearly enhanced growth performance during the acute phase of *Eimeria* infection (Sharma et al., 1973; Rochell et al., 2017). Sharma et al. (1973) also demonstrated that birds fed a 24% crude protein diet had superior compensatory growth during the late recovery phase (8−14 dpi). Nonetheless, the authors reported higher mortality rates and oocysts shedding of birds fed the 24% crude protein treatment (Sharma et al., 1973). It is possible that increasing crude protein levels simulates trypsin secretion of the host, facilitating excystation of parasites' sporozoites from the sporocysts and leading to severe coccidia infection (Britton et al., 1964; Chapman, 1978).

On the contrary, a recent study claimed that growth performance and oocysts shedding of the *Eimeria*-infected birds were not influenced by dietary crude protein levels (Arczewska-Włosek et al., 2017). Similar to the previous finding, *Eimeria*-infected birds fed the reduced crude protein diet had no adverse effects on

Table 6. Effects of low crude protein diets supplemented with arginine, glutamine, methionine, and/or threonine on gene expression of tight junction proteins in chickens challenged with mixed *Eimeria* spp.

Items[1]	NC	CC	LCP	ARG	GLN	MET	THR	COMB	SEM	*P*-value
6 dpi										
CLDN1	1.00^{d}	4.33^{ab}	4.38^{a}	4.10^{abc}	1.66^{cd}	3.10^{abcd}	1.72^{bcd}	1.75^{bcd}	0.33	0.015
OCLDN	1.00	1.56	1.23	1.23	1.32	1.38	1.30	1.34	0.05	0.197
ZO1	1.00	0.82	0.86	0.86	0.80	0.89	0.80	0.87	0.02	0.442
JAM2	1.00	1.19	1.08	1.43	0.96	1.46	0.89	1.24	0.07	0.419
9 dpi										
CLDN1	1.00	1.00	0.72	0.82	0.71	0.72	0.83	0.84	0.04	0.355
OCLDN	1.00	1.00	1.09	1.01	0.98	1.02	0.83	0.98	0.03	0.799
ZO1	1.00	0.85	0.88	1.10	0.85	0.94	0.94	1.01	0.02	0.150
JAM2	1.00^{*,a,b}	0.61^{*,b}	0.66^{*,b}	0.74^{*,ab}	0.46^{*,b}	0.73^{*,a,b}	1.22^{*,a}	0.86^{*,a,b}	0.06	0.078

[1]CLDN1, Claudin 1; OCLDN, Occludin; ZO1, Zonula occludens 1; JAM2, Junctional adhesion molecule 2; NC, nonchallenged control; CC, *Eimeria*-challenged control; LCP, birds fed the low crude protein diet and received *Eimeria* challenge; ARG, birds fed the low crude protein diet adding 0.75% arginine and received *Eimeria* challenge; GLN, birds fed the low crude protein diet adding 0.75% glutamine and received *Eimeria* challenge; MET, birds fed the low crude protein diet adding 0.75% methionine and received *Eimeria* challenge; THR, birds fed low crude protein diet adding 0.75% threonine and received *Eimeria* challenge; COMB, birds fed the low crude protein diet adding arginine, glutamine, methionine, and threonine, and received *Eimeria* challenge.

[a-d]Means followed by superscript letters are different within the same row;

[*]Trend, $P < 0.1$; Unit: fold change ($2^{-\Delta\Delta Ct}$); $N = 6$.

BWG compared to those infected birds fed the regular diet in the present study. Based on the unaffected performance of birds fed the Low-CP diet, it is unlikely that the mild *Eimeria*-infected birds required high dietary amino acid levels to optimize growth and feed efficiency. Nevertheless, adjusting amino acids levels in low crude protein diets could impact the growth performance of birds. Rochell et al. (2016) indicated a massive reduction of BWG in response to low crude protein diets with individual reduction of several amino acids. Similarly, but with an opposite experimental design, the current study evaluated the effects of the Low-CP diet with "addition" of individual amino acids on birds infected with coccidiosis. In the current study, treatment diets were tested from 2 d prior to *Eimeria* challenge through 9 dpi to evaluate the effects of specific amino acids supplementation in the low-crude protein diet on growth performance of birds. The results showed that the ARG and THR treatments had numerically higher BWG than CC groups during the acute infection phase (CC: 351 g; ARG: 362 g; THR: 362 g).

According to our previous findings, the intestinal tissue can be fully recovered from a severe *Eimeria* infection within 9 d (Teng et al., 2020). The intestinal epithelium is supposed to be healed and ready to digest and absorb as many nutrients as it could to support compensatory growth during the late recovery phase (9−14 dpi); thus, the current study was designed to replace treatment diets by a regular grower diet on 9 dpi. Even though the diet treatments were only applied in the first and second phases of the experiment (from 2 d prior *Eimeria* challenge to 9 dpi), numerically higher BWG were found in the ARG and THR groups compared to the CC treatment during the late recovery phase (CC: 375 g; ARG: 402 g; THR: 395 g). The current findings indicated that Low-CP diets adding 0.75% arginine or threonine are promising nutritional strategies to reduce the feed cost, enhance growth performance, and improve animal health against pathogens infection.

However, the MET and COMB exacerbated negative effects of coccidiosis on growth performance in the current study. Both groups had lower BWG and higher FCR than the CC and LCP treatments. Furthermore, the birds fed the diet with additional methionine had greater oocysts shedding than the other challenged groups. Although methionine is the first limiting amino acid in the corn-soybean meal-based diets, supplementation of methionine in excess has been reported to cause growth depression in poultry (Han and Baker, 1993; Xie et al., 2007). Increasing methionine intake elevates homocysteine, an intermediate produced from the transformation of methionine to cysteine, in the circulation of animals (Perna et al., 2003). The excess homocysteine accumulation will result in a metabolic disorder known as hyperhomocysteinemia, which is associated with several human diseases, such as cardiovascular disease, Parkinson's disease, osteoporosis, end-stage renal disease, and gastrointestinal disorders (Kumar et al., 2017). It has been reported that hyperhomocysteinemia could generate reactive oxygen species and cause considerable toxicity for endothelium (Perna et al., 2003). It is likely that supplementing 0.75% methionine in the Low-CP diets, whether combined with other amino acids or not, might have induced hyperhomocysteinemia, causing endothelial dysfunction and damaging intestinal integrity along with coccidiosis. In the current study, the MET treatment significantly reduced villi height in the duodenum, whereas the COMB treatment had the lowest ratio of villus height to crypt depth on 6 dpi. The intestinal destructions in these two treatments were even worse than those in the CC treatment. Apart from damaging the endothelium, homocysteine might

Table 7. Effects of low crude protein diets supplemented with arginine, glutamine, methionine, and/or threonine on gene expression of amino acid transporters in chickens challenged with mixed *Eimeria* spp.

Items[1]	NC	CC	LCP	ARG	GLN	MET	THR	COMB	SEM	*P*-value
6 dpi										
B^0AT	1.00	0.80	0.90	0.81	1.01	0.96	0.83	0.89	0.04	0.914
$B^{0+}AT$	1.00^{*a}	0.54^{*,b}	0.58^{*,b}	0.52^{*,b}	0.66^{*,ab}	0.64^{*,b}	0.66^{*,ab}	0.75^{*,ab}	0.04	0.091
EAAT	1.00	0.74	0.74	0.77	0.90	0.78	0.75	0.85	0.04	0.578
Cat1	1.00^{*,c}	1.86^{*,abc}	1.74^{*,abc}	2.19^{*a}	1.17^{*,bc}	1.73^{*,abc}	1.27^{*,abc}	1.99^{*,ab}	0.11	0.054
LAT1	1.00^{c}	9.94^{ab}	7.66^{bc}	16.65^{a}	5.45^{bc}	12.56^{ab}	6.29^{bc}	13.13^{ab}	1.11	0.004
LAT2	1.00	0.68	0.78	0.73	0.88	0.93	0.80	0.84	0.03	0.239
9 dpi										
B^0AT	1.00	1.31	1.48	1.68	1.41	1.38	1.03	1.26	0.06	0.130
$B^{0+}AT$	1.00	1.02	1.34	1.60	1.27	1.17	1.04	1.29	0.06	0.118
EAAT	1.00	0.83	1.20	1.12	1.10	0.96	0.91	1.04	0.04	0.333
Cat1	1.00	1.53	0.94	1.30	1.23	1.13	1.16	1.14	0.08	0.787
LAT1	1.00^{*,ab}	0.69^{*,b}	0.81^{*,b}	0.91^{*,ab}	0.67^{*,b}	1.06^{*,ab}	1.19^{*,ab}	1.46^{*a}	0.07	0.080
LAT2	1.00^{ab}	0.93^{b}	0.97^{b}	1.25^{a}	0.96^{b}	0.79^{b}	0.88^{b}	0.90^{b}	0.03	0.045

[1]B^0AT; Na+-dependent amino acid transporter; $B^{0+}AT$, Na+-independent amino acid transporter; EAAT, excitatory amino acid transporter; CAT1, cationic amino acid transporter; LAT2, Na+-dependent neutral/cationic amino acid transporter; LAT1, L-type amino acid transporter 1; NC, nonchallenged control; CC, *Eimeria*-challenged control; LCP, birds fed the low crude protein diet and received *Eimeria* challenge; ARG, birds fed the low crude protein diet adding 0.75% arginine and received *Eimeria* challenge; GLN, birds fed the low crude protein diet adding 0.75% glutamine and received *Eimeria* challenge; MET, birds fed the low crude protein diet adding 0.75% methionine and received *Eimeria* challenge; THR, birds fed low crude protein diet adding 0.75% threonine and received *Eimeria* challenge; COMB, birds fed the low crude protein diet adding arginine, glutamine, methionine, and threonine, and received *Eimeria* challenge.

[a-c]Means followed by superscript letters are different by Duncan's test.

[*]Trend, $P < 0.1$; Unit: fold change ($2^{-\Delta\Delta Ct}$); $N = 6$.

facilitate the parasite's development by inducing gametocytogenesis (Beri et al., 2017). This mode of action possibly contributes to the current finding of the increased oocysts shedding in the MET treatment. We speculated that the accumulation of homocysteine, due to the excess methionine supplementation, impaired intestinal epithelium of host and induced *Eimeria* gametocytogenesis, consequently leading to the increase of oocysts production and growth depression of birds.

Eimeria spp. impairs the intestinal integrity of the chicken in a short period of time. The coccidiosis-induced damage has been reported to escalate from 3 dpi and reach the peak of intestinal leakage and lesions on 6 dpi (Schneiders et al., 2019; Teng et al., 2020). In complement to previous findings, the current results showed the increased intestinal permeability and reduced ratio of villus height to crypt depth in the duodenum of *Eimeria*-challenged birds. Interestingly, for chickens fed the ARG treatment, a significant reduction of intestinal permeability was observed. Oral arginine supplementation has been known as one of nutritional approaches to preserve intestinal barrier integrity, ameliorate intestinal epithelium injury, and accelerate intestinal recovery in animals (Sukhotnik et al., 2005; Viana et al., 2010; Costa et al., 2014; Zhang et al., 2017). Castro et al. (2020) demonstrated that increasing dietary arginine levels from 1.04 to 1.34% improved the intestinal health of *Eimeria*-challenged chickens. As arginine is an important immune-modulator in response to *Eimeria* infection, increasing dietary arginine level has been shown to regulate the biosynthesis of nitric oxide and reduced oocysts shedding in *Eimeria*-infected chickens (Allen, 1999).

Although coccidiosis causes enormous intestinal necrosis within a week, the injury can be recuperated shortly due to the rapid turnover rate of intestinal epithelial cells (Cliffe et al., 2005; Teng et al., 2020). The acute inflammation caused by coccidiosis stimulates stem cell proliferation at the crypt base, which accelerates the cellular renewal in the intestine of chicken (Sun et al., 2016). The increase of enterocyte turnover has been considered as a mucosal defensive mechanism to expulse parasites from the intestinal epithelium of hosts (Cliffe et al., 2005). Previous studies have reported that the intestine recovery from coccidia infection is supposed to be less than 9 d (Schneiders et al., 2019; Teng et al., 2020). Similar to previous findings, a significant difference of intestinal morphology between challenged and nonchallenged birds was only observed on 6 dpi but not on 9 dpi in the current study, suggesting damaged intestinal cell linings had been rapidly recovered during the early recovery phase.

The tight junction complex, a protein structure located between enterocytes, serves as the first defensive barrier controlling paracellular translocation in the intestine of animals (Schneeberger and Lynch, 2004). The proteins constituting the tight junction complex can be categorized into 2 major groups: 1) the integral proteins, such as claudin, occludin, and JAM families, which attach on cell membrane forming a bridge that links 2 cells together and builds up protective barriers and 2) the plaque proteins, like the ZO family, which strengthen the construction of tight junction complex by connecting integral proteins to the cytoskeleton in the cytoplasm (Schneeberger and Lynch, 2004; Aijaz et al., 2006). Previous studies have reported that increasing the severity of *Eimeria* infection would linearly regulate gene expression of tight junction proteins (Teng et al., 2020, 2021). The current study also indicated an upregulation of CLDN1 in response to coccidia infection. Interestingly, the adverse effects of coccidiosis on tight junction protein were reversed by application of the THR and GLN treatments. Supplementation of glutamine and threonine reduced gene expression of CLDN1 from 4.38 (LCP) to 1.66- and 1.72-fold, respectively. A review article has emphasized the importance of threonine and glutamine in modulating intestinal health of chickens (Bortoluzzi et al., 2018). Glutamine is an essential amino acid for enterocytes proliferation, maintenance of intestinal barriers, and regulation of inflammatory pathways, whereas threonine plays important roles in mucin production and modulation of intestinal immune responses (Mao et al., 2011; Rao and Samak, 2012; Kim and Kim, 2017). Both glutamine and threonine can downregulate proinflammatory cytokines, such as interferon-γ (**IFN-γ**) and tumor necrosis factor-α (**TNF-α**) in broiler chickens (Chen et al., 2017; Chen et al., 2018; Oxford and Selvaraj, 2019). It has been reported that IFN-γ and TNF-α are strongly associated with CLDN1 expression during inflammatory intestine diseases (Utech et al., 2005; Poritz et al., 2011). It is likely that increase of IFN-γ and TNF-α production in birds infected with coccidiosis (Yun et al., 2000; Allen and Fetterer, 2002) induced CLDN1 gene expression in the current study, but dietary threonine or glutamine supplementation could reverse the expression of CLDN1 probably by attenuating the proinflammatory immune responses.

It is noteworthy that birds fed additional arginine, glutamine, methionine, and threonine showed increased AID of these amino acids, respectively, in the current study. Supplementation of highly digestible crystalline amino acids has been reported to improve nutrient digestibility of chickens (Hilliar et al., 2020). These findings suggested that supplementing a crystalline amino acid would facilitate the absorption of that particular one and strengthen its role in improving the intestinal health against pathogens infection. However, the inclusion of a particular amino acid in excess might interfere the digestibility of other amino acids. In the current study, the MET treatment enhanced the AID of methionine but suppressed absorption of isoleucine, threonine, aspartic acid, and glutamine. This difference in AID of methionine may be attributed to the high efficiency of methionine uptake in the jejunum and ileum, where brush border nutrient transporters, such as B^0AT, have a greater affinity for methionine over other amino acids (Broer and Fairweather, 2018).

Six amino acid transporters were determined in the current study. Three of them (B^0AT, $B^{0+}AT$, and

EAAT) are present on the brush border membrane, while the others (CAT1, LAT1, LAT2) attach to the basolateral membrane of the intestinal epithelial cells (Fetterer et al., 2014; Broer and Fairweather, 2018). The B^0AT is a Na^+- dependent nutrient transporter that accepts almost all neutral amino acids, including methionine, cysteine, glutamine, threonine, etc., whereas EAAT, another Na^+- dependent transporter, carries anionic amino acids, such as aspartate and glutamate, across the brush border membrane (Broer and Fairweather, 2018). Heteromeric amino acid exchangers, including rBAT and $B^{0+}AT$, are responsible for transporting cationic amino acids while exchanging neutral amino acids across the apical membrane of enterocytes. On the other side of the cell, CAT1 and LAT2 transport cationic amino acids, such as histidine, lysine, and arginine, across the basolateral membrane (Broer and Fairweather, 2018). Apart from moving cationic amino acids, LAT2, a Na^+- dependent transporter, also accepts neutral amino acids, whereas LAT1, a Na^+- independent transporter, is known for carrying large types of neutral amino acids, such as tryptophan, phenylalanine, leucine, methionine, and histidine (Broer and Fairweather, 2018). In the current study, *Eimeria* infection led to a significant increase of LAT1 and numerical reductions of B^0AT, $B^{0+}AT$, and EAAT. The trends showing upregulation of nutrient transporters located on basolateral membrane and downregulation of those on brush borders were in agreement with previous studies (Su et al., 2015; Teng et al., 2021). It has been proposed that decreased gene expression of B^0AT, $B^{0+}AT$, and EAAT, coupled with increased CAT1, LAT1, and LAT2 might stimulate apoptosis of enterocytes to accelerate cell renewals (Su et al., 2015). Interestingly, in the current study, the ARG treatment had the highest LAT1 and LAT2 expression among all treatments on 6 dpi and 9 dpi, respectively. Inclusion of arginine in the diet may have strengthened the action of apoptosis in the *Eimeria*-challenged chickens because arginine is the substrate for the formation of nitric oxide, which can induce apoptosis of epithelial cells directly (Allen, 1999; Chokshi et al., 2008). However, future research is still needed to understand how arginine modulates apoptosis and the expression of amino acid transporters in the intestinal epithelial cells of chickens infected with *Eimeria* spp.

In conclusion, application of the Low-CP diet showed no adverse impacts on intestinal health and growth of *Eimeria*-infected birds; thus, formulating low crude protein diets for birds infected with mild coccidiosis is a promising strategy to reduce feed cost and maintain performance. Additionally, supplementation of crystalline amino acids in the Low-CP diet could improve the apparent ileal digestibility of these target nutrients in the intestine of birds. The increase of absorption in threonine, arginine, and glutamine was able to ameliorate gut damage induced by coccidiosis; 1) threonine supplementation increased villus height in the duodenum; 2) the ARG treatment decreased intestinal permeability and gene expression of amino acid transporters; and 3) both the GLN and THR reversed adverse effects of coccidiosis on gene expression of tight junction protein. However, the MET and COMB treatments exacerbated infection severity of coccidiosis. Based on the current findings, adding 0.75% of arginine, glutamine, or threonine in a low crude protein diet can improve the intestinal health of birds infected with mixed *Eimeria* spp.

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DISCLOSURES

There is no conflict of interest.

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