Effects of dietary methionine plus cysteine levels on growth performance and intestinal antibody production in broilers during *Eimeria* challenge

Zhouzheng Ren,[*,1] Daniel E. Bütz,[†] Rose Whelan,[‡] Victor Naranjo,[‡] Maria K. Arendt,[†] Mitchell D. Ramuta,[†] Xiaojun Yang,[*] Thomas D. Crenshaw,[†] and Mark E. Cook[†]

[*]*College of Animal Science and Technology, Northwest A&F University, 22 Xinong Road, Yangling, Shaanxi 712100, China;* [†]*Department of Animal Sciences, University of Wisconsin-Madison, 1675 Observatory Drive, Madison, WI 53706, USA; and* [‡]*Evonik Nutrition & Care GmbH, 4 Rodenbacher Chaussee, Hanau-Wolfgang 63457, Germany*

ABSTRACT Research has shown that methionine+cysteine (M+C) requirements may be higher when chickens are infected with *Eimeria* app. In a 4 × 2 factorial design, broilers (11 to 21 D) were fed one of 4 corn–soybean meal-based diets containing either 0.6, 0.8, 0.9, or 1.0% standardized ileal digestible (SID) M+C; on day 14, broilers from each diet were gavaged with either phosphate-buffered saline (PBS) or a commercial coccidiosis vaccine (at 100 × vaccine dose) which provide a mixture of live *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella* oocysts. Growth performance was recorded from day 11 to 21. Plasma and intestinal luminal samples were collected on days 14 and 21. Intestine lesion scores and fecal oocyst counts were conducted on day 21. Regardless of dietary SID M+C levels, compared to PBS gavaged broilers, the *Eimeria*-challenged broilers had (1) decreased ($P < 0.05$) body weight gain (BWG), feed intake (FI), and gain-to-feed ratio (G:F); (2) increased ($P < 0.05$) intestinal lesion scores and fecal oocyst counts; (3) increased ($P < 0.05$) plasma anti-*Eimeria* IgG, and intestinal luminal total IgA and anti-*Eimeria* IgA concentrations; and (4) increased ($P < 0.05$) levels of duodenum luminal gamma interferon (IFN-γ) and interleukin-10 (IL-10), as well as jejunum and cecum luminal IFN-γ concentrations. Regardless of *Eimeria* challenge, when compared to 0.6% SID M+C, broilers fed ≥0.8% SID M+C had (1) increased ($P < 0.05$) BWG, FI, and G:F and (2) increased ($P < 0.05$) levels of jejunum luminal total IgA. After *Eimeria* challenge, broilers fed 0.8% SID M+C had increased ($P < 0.05$) levels of jejunum luminal anti-*Eimeria* IgA compared to broilers fed diets containing 0.6 and 1.0% SID M+C. Collectively, in 11- to 21-D broilers, the growth suppression caused by *Eimeria* infection could not be mitigated by further increasing dietary M+C alone ≥0.8%. Further research should investigate interactions between dietary M+C and other nutrients for support of immune function and growth in pathogen-challenged broilers.

Key words: methionine, sulfur amino acid, broiler, *Eimeria,* intestinal IgA

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INTRODUCTION

Coccidiosis, a parasitic intestinal disease caused by *Eimeria* infection, is one of the main challenges for the antibiotic-free poultry industry to overcome (Kadykalo et al., 2018). Previous work has shown that there is possibly an interaction between *Eimeria* infection and dietary sulfur amino acid levels in chickens. Murillo et al. (1976) reported that the growth performance was more seriously suppressed by *Eimeria* infection in Cobb broilers (1 to 28 D) fed a methionine+cysteine (**M+C**)-deficient diet (total M+C = 0.73%) compared to broilers fed a M+C-sufficient diet (total M+C = 0.83%). In a subsequent study, Southern and Baker (1982) observed that supplementation of 0.50% DL-methionine to a conventional corn–soybean meal based diet, to achieve a total M+C of 0.91%, partially alleviated the decrease in growth performance in *Eimeria* infected, cobalt-fed male chicks from 8 to 21 D of age. Lai et al. (2018) found that increasing dietary total methionine to at least 125% of the requirements (specified by the Ministry of Agricultural of the People's Republic of China, 2004) could improve not only growth performance of male Partridge Shank broilers (from 22 to 42 D) in an *Eimeria* challenge, but also intestinal luminal levels of sIgA, and cecal tonsil mRNA expressions of tumor necrosis factor alpha (**TNFα**), interleukin-2 (**IL-2**), and gamma interferon (**IFN-γ**) in response to *Eimeria* challenge; however, the effects of increased dietary methionine were mostly observed for broilers reared with diets containing narasin (60 mg/kg),

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[1]Corresponding author: poultryren@nwafu.edu.cn

rather than those conversely protected with a live anticoccidial vaccine administrated at day 3. These studies support a hypothesis that the sulfur amino acid requirement may be increased in *Eimeria*-challenged broilers compared to uninfected broilers. The requirements for M+C differ among the widespread nutrition specifications. For 11- to 21-D broilers, the Ministry of Agricultural of the People's Republic of China (2004) recommends a total M+C of 0.91%, while the major broiler breeding companies recommend 0.80 and 0.87% digestible M+C for Cobb 500 or Ross 308/708 broilers, respectively. Amino acid providers may also offer recommendations; for example, AMINOChick 2.0 (Evonik Nutrition and Care GmbH, Hanau, Germany) recommends standardized ileal digestible (SID) M+C of 0.83% for 11- to 21-D male broilers. Regardless of the recommendations used to formulate diets, it remains to be investigated whether or not the current sulfur amino acid recommendations are adequate for broilers undergoing a health challenge, like an *Eimeria* infection.

The mode of action by which increased M+C has reportedly protected broiler chickens from coccidiosis related growth suppression is not defined. Two mechanisms have been proposed: (1) *Eimeria* infection decreases the digestibility and/or absorption of M+C and therefore increased supplementation is required (Ruff and Wilkins, 1984; Adedokun et al., 2012, 2016); and (2) M+C is additionally required to support immune response to pathogenic *Eimeria* spp. and therefore additional supplementation helps confer resistance to *Eimeria*-infected chickens (Grimble and Grimble, 1998; Maroufyan et al., 2013; Wu et al., 2013). The current study will focus on immune-related functions. Previous research from our laboratory demonstrated that plasma total IgG concentrations, in response to sheep red blood cells, were linearly increased in 3-wk-old broilers when dietary total M+C was increased from 0.72 to 0.97% (Tsiagbe et al., 1987b). In that study, the dietary total M+C required for maximum broiler growth was approximately 0.78%, but ≥0.97% total M+C was required to maximize humoral immunity. Similarly, Swain and Johri (2000) reported that broilers fed 0.69% total methionine had a greater plasma humoral immune response to Newcastle Disease Virus when compared to broilers fed 0.37% total methionine (dietary cysteine level was not given in this paper). These findings were confirmed by Rama Rao et al. (2003). Hence, we hypothesized that dietary sulfur amino acid supplementation is crucial for broilers to develop humoral immunity against *Eimeria*. Secretory IgA, the main class of antibody responsible for arresting external pathogens at epithelial borders, plays a critical role in maintaining intestinal health (Mantis and Forbes, 2010; Chairatana and Nolan, 2017; Lycke and Bemark, 2017). To date, very limited information is available regarding the effects of dietary M+C on intestinal IgA production in *Eimeria*-challenged broilers. In the current study, broilers (with or without *Eimeria* challenge) were fed diets with different levels of dietary SID M+C. Broiler growth performance, coccidiosis pathology, and immune response were evaluated. We hypothesize that the optimal amino acid levels and ratios may shift during the immune response to a challenge as requirements for functional amino acids, like M+C, increase to support immune function in addition to growth. More specifically, we hypothesize that the current recommended SID M+C levels are not adequate for *Eimeria*-infected broilers, and increased dietary supplementation of methionine may improve *Eimeria* spp. resistance and growth performance during a challenge.

Table 1. Ingredient and nutrient composition of basal diets for starter and grower phases.

Item (%, unless noted)	Starter (1 to 10 D)	Grower (11 to 21 D)
Corn	57.26	60.06
Soybean meal, 48% CP	35.00	31.36
Soybean oil	3.14	4.09
Dicalcium phosphate, 22%	1.76	1.63
Calcium carbonate	0.74	0.71
Sodium chloride	0.37	0.37
Choline chloride, 60%	0.10	0.10
DL-Methionine, 99%	0.31	0.06
L-Lysine sulfate, 54.6%	0.24	0.17
L-Threonine, 98.5%	0.08	0.05
Premix[1]	1.00	1.00
Sand	–	0.40
In total	100.00	100.00
Nutritional composition (calculated)[2]		
AMEn (kcal/kg)	3,008	3,086
Crude protein, %	21.74	20.00
SID Methionine, %	0.59	0.33
SID Cysteine, %	0.29	0.27
SID Methionine + Cysteine, %	0.88	0.60
SID Lysine, %	1.18	1.05
SID Threonine, %	0.77	0.69

[1]Supplied per kilogram of diet: copper, 15 mg; iron, 40 mg; zinc, 100 mg; manganese, 100 mg; selenium, 0.35 mg; iodine, 1 mg; vitamin A, 10,000 IU; vitamin D_3, 5,000 IU; vitamin E, 80 IU; vitamin K, 3 mg; vitamin B_1, 3 mg; vitamin B_2, 9 mg; vitamin B_6, 4 mg; vitamin B_{12}, 0.02 mg; nicotinic acid, 60 mg; pantothenic acid, 15 mg; biotin, 0.15 mg; folic acid, 2 mg.

[2]AMEn, nitrogen-corrected apparent metabolizable energy; SID, standardized ileal digestible. SID levels were calculated based on poultry specific digestibility coefficients provided by Evonik Nutrition and Care GmbH (Hanau, Germany).

MATERIALS AND METHODS

All animal protocols conducted were approved by the College of Agricultural and Life Sciences Animal Care and Use Committee at the University of Wisconsin-Madison.

Broilers and Diets

One-day-old male broilers (n = 720; Welp Hatchery, Bancroft, IA) were randomly allotted to 80 battery cages with 9 broilers per cage. Broilers were fed with the same nutritionally adequate corn–soybean meal-based starter diet containing 0.88% SID M+C from 1 to 10 D (Table 1). On day 11, the 80 cages were equally assigned to one of 4 grower diets formulated to meet SID requirements (AMINOChick 2.0) except for M+C. The 4 diets contained 0.6, 0.8, 0.9, and 1.0% SID M+C, which was accomplished by adjusting the additions of subliminal

Table 2. Analyzed nutrient levels of experimental diets (as-is basis).

	Starter (1 to 10 D)		Grower (11 to 21 D)				
				Analyzed[1]			
Item (%)	Calculated	Analyzed	Calculated	0.6% SID M+C	0.8% SID M+C	0.9% SID M+C	1.0% SID M+C
Crude protein	21.74	21.27	20.00	18.80	19.54	20.16	18.87
Methionine	0.62	0.52	0.35/0.55/0.65/0.75	0.33	0.50	0.61	0.72
Cysteine	0.34	0.34	0.33	0.31	0.32	0.32	0.30
Methionine + Cysteine	0.96	0.86	0.68/0.88/0.98/1.08	0.64	0.81	0.93	1.02
Lysine	1.29	1.26	1.15	1.11	1.14	1.14	1.07
Threonine	0.89	0.85	0.80	0.75	0.77	0.77	0.75
Arginine	1.44	1.38	1.32	1.24	1.27	1.26	1.19
Isoleucine	0.91	0.90	0.84	0.81	0.83	0.82	0.76
Leucine	1.82	1.77	1.72	1.61	1.63	1.63	1.54
Valine	1.00	0.98	0.93	0.89	0.91	0.90	0.86
Histidine	0.57	0.55	0.53	0.49	0.51	0.50	0.48
Phenylalanine	1.07	1.08	0.99	0.98	0.99	0.99	0.92
Glycine	0.88	0.85	0.82	0.77	0.78	0.78	0.73
Serine	1.06	1.03	0.98	0.94	0.95	0.95	0.91
Proline	1.24	1.27	1.17	1.17	1.13	1.14	1.05
Alanine	1.06	1.01	1.00	0.93	0.94	0.94	0.92
Aspartate aminotransferase	2.22	2.16	2.04	1.94	1.99	1.98	1.88
Glutamic acid	3.86	3.68	3.58	3.34	3.40	3.38	3.21

[1]Amino acid content of treatment diets were analysed by ion-exchange chromatography (AA analyser LC 3000, Biotronic, Maintal, Germany). SID M+C, standardized ileal digestible methionine + cysteine.

DL-methionine (MetAMINO 99%, Evonik Nutrition and Care GmbH, Hanau-Wolfgang, Germany) at levels of 0, 0.2, 0.3, and 0.4%, respectively, in place of sand (River Run Products Corp., Custer, WI) as an inert ingredient (Table 1). The composition and calculated nutrient levels of the experimental diets are listed in Table 1. Amino acid content of treatment diets were analyzed by ion-exchange chromatography (AA analyser LC 3000, Biotronic, Maintal, Germany). The analyzed nutrient levels of the experimental diets are listed in Table 2. On day 14, broilers fed each grower diet group were orally gavaged with either phosphate-buffered saline (**PBS**) or a commercial coccidiosis vaccine (100× vaccine dose; Advent, Lincoln, NE). The vaccine provided a mixture of live *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella* oocysts. Body weight gain (**BWG**), gain-to-feed ratio (**G:F**), and feed intake (**FI**) of the broilers were recorded/calculated at the following intervals: from 11 to 14 D, from 15 to 21 D, and from 11 to 21 D of age. On days 14 and 21, 1 bird per cage was randomly selected and euthanized for collection of plasma and intestinal (duodenum, jejunum, ileum, and cecum) lumen content samples. On day 21, the intestine of sampled birds were subjected to a lesion scoring system described in Johnson and Reid (1970), ranging from a score of 0 (no gross lesion) to 4 (serve gross lesions). On day 21, excreta of each cage were collected, and the *Eimeria* oocysts were quantified using the McMaster technique as previously described (Arendt et al., 2016).

Anti-Eimeria Antibody Enzyme-Linked Immunosorbent Assay (ELISA)

To create the plate coating antigen for ELISA, the Advent coccidiosis vaccine (10 mL; 10,000× vaccine dose) was diluted (1:2) with PBS and then centrifuged at 3,000× *g* for 10 min. The supernatant was discarded and the pellet was washed with 10 mL PBS (add PBS, suspend, centrifuge at 3,000× *g* for 10 min, and discard supernatant). The pellet was then diluted in 2 mL PBS and agitated with 4 mL glass beads on a vortex, until the pellet was thoroughly dispersed. The breakdown of oocysts was verified using a microscope (10×). The supernatant (broken oocysts) was pipetted off and the glass beads were washed (add PBS, vortex, and pipet supernatant off) 3 times using 8, 8, and 7 mL PBS, respectively. The supernatant (2+8+8+7 = 25 mL) was subjected to 2 freeze-thaw cycles to further ensure oocyst disruption. After that, the supernatant, which served as plate-coating *Eimeria* antigen in our anti-*Eimeria* antibody ELISA, was stored in 1 mL aliquots at −20°C until required for use. Anti-*Eimeria* antibody positive broiler plasma and intestinal lumen contents, obtained from another research project, were used as in-lab standards. Seven-point standard curves were obtained by using the 2-fold serial dilutions from the standard plasma (diluted in a protein-free blocking buffer from Pierce, Thermo Scientific, Rockford, IL; from 1:25 to 1:1600) and the standard intestinal lumen content (diluted in 1% milk powder; from 1:2 to 1:128). For plasma anti-*Eimeria* antibody ELISA, samples were diluted to 1:200, and the secondary detection antibody (goat anti-chicken IgG [IgY]–horseradish peroxidase [**HRP**] conjugated; Bethyl Laboratories, Inc., Montgomery, TX) was diluted to 1:4000, using the aforementioned protein-free blocking buffer. For intestinal lumen content anti-*Eimeria* antibody ELISA, duodenum, jejunum, ileum, and cecum luminal samples were diluted to 8, 3, 5, and 7 mg/mL, respectively, and the second antibody (goat anti-chicken IgA–HRP conjugated; Bethyl Laboratories, Inc., Montgomery, TX)

was diluted to 1:4000, using 1% milk powder. Protein concentrations of intestinal luminal samples were determined using Pierce BCA Protein Assay Kit (Thermo Scientific).

At the time of ELISA, the *Eimeria* antigen (1 mL) was diluted in 10 mL of carbonate coating buffer (pH = 9.6) and coated (100 μL/well, 4°C, overnight) to a Costar EIA/RIA 96-well plate (Corning Inc., Corning, NY). The plate was washed twice with PBS Tween 20 (Thermo Fisher Scientific, Rockford, IL) and then blocked (room temperature, ~2 h) with the aforementioned protein-free blocking buffer (200 μL/well). The standards, blanks, and samples were then added into the wells at 100 μL/well. The plates were incubated (1 h) on an orbital shaker at room temperature. The plate was then washed 4 times, the secondary detection antibody was diluted, applied to each well (100 μL/well), and incubated (1 h) on a shaker at room temperature. Plates were then washed 6 times, and a Pierce 1-Step Ultra TMB-ELISA Substrate Solution (Thermo Scientific) was applied at 100 μL/well. The colorimetric reaction was stopped by addition of 50 μL/well of a 0.5 M H_2SO_4 solution. The plates were read at 450 nm wavelength using an EL800 plate reader (BioTek, Winooski, VT). The log 2 anti-*Eimeria* antibody titer was calculated by comparing samples with the in-lab standard. The antibody titer was defined as the highest dilution of sample with an optical density equal to the standard plasma diluted 1:1600 (cutoff value, two times of the background) or the standard intestinal lumen content diluted 1:128 (cutoff value, two times of the background).

Intestinal Luminal Total IgA ELISA

Intestinal luminal concentrations of total IgA were determined using a commercial Chicken IgA ELISA Quantitation Set (E30-103) according to the manufacturer's specifications (Bethyl Laboratories, Inc., Montgomery, TX). Briefly, affinity purified goat anti-chicken IgA antibody (A30-103A-19) was coated to the 96-well plates. The plates were blocked after washing. The standards (chicken reference serum RS10-102-3), blanks, and samples (duodenum, jejunum, ileum, and cecum luminal samples were diluted to 1:1000, 1:1500, 1:1000, and 1:500, respectively, prior to analysis) were then added into the wells. After incubation, the plates were washed and HRP-conjugated goat anti-chicken IgA detection antibody (A30-103P-39, diluted to 1:75,000) was added. 3,3′,5,5′-Tetramethylbenzidin (**TMB**) and 0.5 M H_2SO_4 solution were then applied to induce and stop the colorimetric reaction. The optical density values were recorded at 450 nm wavelength. Data were expressed as microgram total IgA per milligram protein.

IL-10 ELISA

Details regarding the IL-10 capture ELISA had been carefully described in our previous publication (Arendt et al., 2016). In the current study, the plasma samples were diluted to 1:500 prior to analysis and the results were recorded as μg/mL. The duodenum, jejunum, ileum, and cecum luminal samples were diluted to 1:5, 1:4, 1:4, and 1:5, respectively, prior to analysis, and the results were recorded as ng/mg protein.

IFN-γ ELISA

Plasma and intestinal luminal levels of IFN-γ were determined using a Chicken IFN-γ Cytoset ELISA kit (Lot # 102803) according to the manufacturer's specifications (Invitrogen Corporation, Camarillo, CA). Briefly, anti-chicken IFN-γ antibody (Lot # 10F16/1) was coated to the 96-well plates. The plates were blocked after washing. The standards (recombinant chicken IFN-γ, Lot # 7C4/1), blanks, and samples (the plasma samples were diluted to 1:200, and the intestinal luminal samples were not diluted, prior to analysis) were then added into the wells. After washing, the detection antibody (anti-chicken IFN-γ biotin, Lot # 10F18/1) was added. After incubation, the plates were washed and streptavidin-HRP solution (Lot # 10A8/1) was added. The colorimetric reaction was induced and then stopped by TMB and 0.5 M H_2SO_4, respectively. The plates were read at a wavelength of 450 nm. Data were expressed as ng/mL (plasma) and pg/mg protein (intestinal luminal).

Statistics

Data were analyzed using General Linear Model (GLM) procedures (SPSS 23, IBM Corp., Chicago, IL) to detect differences among treatments based on a 4 × 2 factorial arrangement. The main effects were (1) dietary SID M+C levels (0.6, 0.8, 0.9, and 1.0%) and (2) *Eimeria* challenge (with or without). Post hoc analysis (Duncan's test) was conducted to detect differences among all experimental treatments or differences within each main effect. Intestinal lesion scores were transformed to square root of n+1 prior to analysis. Statistical significance was set at $P < 0.05$.

RESULTS

Growth Performance

Pooled across *Eimeria*-challenged treatments, which were not yet imposed, broilers fed 0.6% SID M+C over the 11 to 14 D interval had decreased ($P < 0.05$) BWG compared to broilers fed 0.8 and 1.0% SID M+C, and had decreased ($P < 0.05$) FI compared to broilers fed 0.8% SID M+C (Table 3). During the intervals of 15 to 21 D and 11 to 21 D, broilers fed 0.6% SID M+C (pooled across main effects of *Eimeria* challenge) had decreased ($P < 0.05$) BWG and G:F ratio compared to broilers fed with 0.8, 0.9, and 1.0% SID M+C. From 15 to 21 D, BWG, G:F ratio, and FI were decreased ($P < 0.05$) by 18, 12, and 8% respectively, in

Table 3. Growth performance of broilers fed varying levels of sulfur amino acids in control conditions or challenged with *Eimeria* spp.[1]

Challenge[2]	SID M+C (%) Day 11–21	BWG (g)			G:F (g:g)			FI (g)		
		Day 11–14	Day 15–21	Day 11–21	Day 11–14	Day 15–21	Day 11–21	Day 11–14	Day 15–21	Day 11–21
PBS	0.6	127	255	382	0.64	0.55	0.58	198	457	651
	0.8	155	330	485	0.68	0.67	0.67	231	498	729
	0.9	139	336	474	0.68	0.70	0.69	203	483	687
	1.0	154	341	495	0.69	0.67	0.68	222	509	730
Eimeria	0.6	124	218	342	0.66	0.46	0.53	196	421	621
	0.8	148	277	425	0.70	0.60	0.63	211	466	673
	0.9	137	271	407	0.69	0.60	0.63	203	452	652
	1.0	139	269	408	0.66	0.61	0.63	209	445	652
	SEM	3	9	10	0.01	0.02	0.01	3	10	12
Main effect										
	0.6	125[b]	237[b]	362[b]	0.65	0.50[b]	0.55[b]	197[b]	439	636
	0.8	152[a]	303[a]	455[a]	0.69	0.63[a]	0.65[a]	221[a]	482	701
	0.9	138[a,b]	303[a]	441[a]	0.68	0.65[a]	0.66[a]	203[a,b]	468	669
	1.0	147[a]	305[a]	452[a]	0.68	0.64[a]	0.65[a]	216[a,b]	477	691
PBS		137	315[a]	459[a]	0.68	0.65[a]	0.65[a]	205	487[a]	699[a]
Eimeria		144	259[b]	396[b]	0.67	0.57[b]	0.60[b]	214	446[b]	650[b]
						P-value				
SID M+C		0.001	0.004	0.001	0.610	0.002	<0.001	0.044	0.408	0.196
Challenge		0.143	<0.001	0.001	0.871	0.006	0.002	0.178	0.040	0.031
Interaction		0.760	0.855	0.819	0.852	0.983	0.940	0.668	0.920	0.867

[a,b]Means with different superscripts within a column were significantly different ($P < 0.05$).

[1]SID M+C, standardized ileal digestible methionine + cysteine; BWG, body weight gain; G:F, gain to feed ratio; FI, feed intake; PBS, phosphate-buffered saline.

[2]On day 14, broilers were orally gavaged with either PBS or the Advent Coccidiosis Vaccine (Lincoln, NE; 100× vaccine dose; consisting of a blend of live *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella* oocysts).

Table 4. Intestinal lesion score and fecal oocyst counts of 21-day-old broilers exposed to an *Eimeria* spp. challenge.

Challenge[1]	SID M+C (%)[2] day 11–21	Lesion score[3,4]			Fecal oocyst (No./g)
		Duodenum	Jejunum	Cecum	
Eimeria	0.6	1.40	1.20	1.60	632,267
Eimeria	0.8	1.20	0.60	1.70	618,933
Eimeria	0.9	1.20	0.80	1.70	342,933
Eimeria	1.0	1.40	1.00	1.40	561,333
	SEM	0.12	0.12	0.23	57,440
			P-value		
		0.904	0.209	0.951	0.256

[1]On day 14, broilers were orally gavaged with either PBS or the Advent Coccidiosis Vaccine (Lincoln, NE; 100× vaccine dose; consisting of a blend of live *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella* oocysts).

[2]SID M+C, standardized ileal digestible methionine + cysteine; PBS, phosphate-buffered saline.

[3]Lesion scores were determined according to Johnson and Reid (1970). Lesion scores were transformed to square root of n+1 prior to statistical analysis; data are presented as the means of the non-transformed observed scores within treatment groups.

[4]No intestinal lesions were observed in PBS gavaged broilers. No lesions were observed in the ileum of *Eimeria*-challenged broilers.

Eimeria-challenged broilers. From 11 to 21 D, BWG, G:F ratio, and FI were decreased ($P < 0.05$) by 14, 8, and 7%, respectively, in *Eimeria*-challenged broilers. No interactions ($P > 0.05$) between the main effects of SID M+C levels and *Eimeria* challenge were detected for any of the growth performance traits (Table 3).

Intestinal Lesion Score, Fecal Oocyst Counts, and Plasma Anti-Eimeria IgG Titer

Intestine lesion scores and excreta *Eimeria* oocysts were detected in *Eimeria*-challenged broilers, but not PBS gavaged controls (Table 4). On day 21, *Eimeria*-challenged broilers had increased ($P < 0.05$) plasma anti-*Eimeria* IgG concentrations compared to PBS gavaged broilers (Table 5). Dietary SID M+C levels had no effect ($P > 0.05$) on intestinal lesion score (Table 4), fecal oocyst counts (Table 4), and plasma anti-*Eimeria* IgG concentrations (Table 5). No statistical differences ($P > 0.05$) were attributed to the interactions of dietary SID M+C × *Eimeria* challenge treatments, although the greatest anti-*Eimeria* IgG concentrations were detected in *Eimeria*-challenged broilers fed 0.9 and 1.0% SID M+C.

Table 5. Plasma anti-*Eimeria* IgG titer of broilers fed varying levels of sulfur amino acids in control conditions or challenged with *Eimeria* spp.[1]

Challenge[2]	SID M+C (%)[3] day 11–21	Day 14	Day 21
PBS	0.6	10.5	9.7
	0.8	10.5	9.4
	0.9	10.5	9.6
	1.0	10.4	9.8
Eimeria	0.6	10.5	10.5
	0.8	10.3	10.6
	0.9	10.5	11.0
	1.0	10.4	11.1
	SEM	0.1	0.1
Main effect			
	0.6	10.5	10.4
	0.8	10.4	10.3
	0.9	10.5	10.5
	1.0	10.4	10.7
PBS		10.5	9.8[b]
Eimeria		10.4	11.0[a]
		P-value	
SID M+C		0.896	0.158
Challenge		0.613	<0.001
Interaction		0.993	0.502

[a,b]Means with different superscripts within a column were significantly different ($P < 0.05$).

[1]Titer was defined as Log 2 of the highest dilution of sample with an optical density equal to the standard plasma diluted 1:1600 (cutoff, two times of the background). The average maternally derived anti-*Eimeria* IgG titer was 14.3 in the plasma of 1-day-old broilers (data not shown).

[2]On day 14, broilers were orally gavaged with either PBS or the Advent Coccidiosis Vaccine (Lincoln, NE; 100× vaccine dose; consisting of a blend of live *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella* oocysts).

[3]SID M+C, standardized ileal digestible methionine + cysteine; PBS, phosphate-buffered saline.

Intestinal Luminal Anti-Eimeria IgA and Total IgA Concentrations

On day 14 (before *Eimeria* challenge), no difference ($P > 0.05$) was detected in intestinal luminal anti-*Eimeria* IgA concentrations (Table 6) or in total IgA concentrations (Table 7) among treatments. *Eimeria* challenge increased ($P < 0.05$) duodenum, jejunum, ileum, and cecum luminal concentrations of anti-*Eimeria* IgA (Table 6), and increased ($P < 0.05$) duodenum, jejunum, and ileum luminal concentrations of total IgA (Table 7), on day 21. Broilers fed 0.6% SID M+C tended to have decreased ($P = 0.067$) concentrations of jejunum luminal total IgA on day 21 compared to broilers fed 0.8, 0.9 and 1.0% SID M+C, regardless of *Eimeria* challenge. An interaction tendency ($P = 0.067$) was detected on day 21 jejunum luminal anti-*Eimeria* IgA concentrations (Table 6). Briefly, dietary SID M+C levels had no effect on day 21 jejunum luminal anti-*Eimeria* IgA concentrations in PBS gavaged broilers; however, *Eimeria*-challenged broilers fed 0.8% SID M+C had increased jejunum luminal anti-*Eimeria* IgA concentrations at day 21, compared to broilers fed diets containing 0.6 and 1.0% SID M+C.

Plasma and Intestinal Luminal Levels of IL-10 and IFN-γ

Differences in plasma IL-10 and IFN-γ concentrations were not detected among dietary SID M+C or *Eimeria* challenge groups on day 21 (Table 8). On day 21, *Eimeria* challenge increased ($P < 0.05$) duodenum luminal concentrations of IL-10 and IFN-γ, and increased ($P < 0.05$) jejunum and cecum luminal levels IFN-γ (Table 9). Dietary SID M+C levels or the SID M+C × *Eimeria* interaction had no effect ($P > 0.05$) on intestinal luminal IL-10 and IFN-γ concentrations.

DISCUSSION

The current study was conducted in order to investigate whether increasing levels of dietary sulfur amino acids may offer improved immune response, growth performance of broilers exposed to *Eimeria* spp. parasites. Methionine is not only the first limiting amino acid for broiler growth but has also been shown to play vital roles in immune function. Methionine is a precursor of polyamines, required for immune cell proliferation, induction/regulation of inflammation and pathogen recognition (Minois et al., 2011; Correa-Fiz et al., 2012). Sulfur amino acids are also required for the production of glutathione which has been shown to enhance immune responses to intracellular parasites, improve proliferation or adaptive immune cells and control oxidants produced during inflammation (Dröge et al., 1998; Morris et al., 2013; Hughes et al., 2017). Additionally, intestinal infections like those with *Eimeria* spp. pathogens have been shown to reduce the digestibility of M+C (Adedokun et al., 2016) as well as other functional amino acids like threonine, valine, isoleucine, arginine, and lysine (Persia et al., 2006). Specifically, malabsorption of L-methionine was observed during *Eimeria* spp challenge (Ruff and Wilkins, 1980). As the protective immune response to *Eimeria* spp. infections involves many of these immune processes, it was hypothesized that coccidiosis increases the broilers' M+C requirement in order to maintain growth performance while additionally providing essential substrates for immune function.

Regardless of *Eimeria* challenge, broilers fed 0.8, 0.9 and 1.0% SID M+C exhibited greater growth than those fed 0.6% SID M+C, while no difference was observed among broilers fed diets with ≥0.80% SID M+C. Under the current experimental conditions (from 11 to 21 D), a corn–soybean meal-based diet containing ≥0.80% SID M+C was adequate for maximum growth performance for both normal broilers and *Eimeria*-challenged broilers. Similarly, a dietary total M+C level of ≥0.87% was sufficient to maintain the antioxidant status (Wang et al., 2018) and meat quality (Wen et al., 2017) of 1- to 21-D broilers. These responses to dietary SID M+C are consistent with the current industry feeding practices for dietary M+C supplementation and support the reliability of M+C

Table 6. Intestinal luminal anti-*Eimeria* IgA titer of broilers fed varying levels of sulfur amino acids in control conditions or challenged with *Eimeria* spp.[1]

Challenge[2]	SID M+C (%)[3] Day 11–21	Day 14				Day 21			
		Duodenum	Jejunum	Ileum	Cecum[4]	Duodenum	Jejunum	Ileum	Cecum
PBS	0.6	2.50	3.34	1.67	–	2.80	4.23[c]	2.80	1.31
	0.8	2.88	3.44	2.26	–	2.79	4.12[c]	2.83	1.42
	0.9	2.56	3.42	1.99	–	3.02	4.08[c]	2.98	1.31
	1.0	2.91	3.61	2.11	–	3.36	4.16[c]	2.50	1.33
Eimeria	0.6	2.75	3.99	2.36	–	4.65	5.27[b]	4.17	1.99
	0.8	2.89	3.51	2.04	–	5.07	6.51[a]	4.92	1.71
	0.9	2.78	3.55	1.62	–	5.43	5.96[a,b]	4.66	1.75
	1.0	2.48	3.78	1.85	–	4.64	5.74[b]	4.35	2.11
	SEM	0.12	0.13	0.12	–	0.17	0.13	0.17	0.12
Main effect									
	0.6	2.63	3.66	2.01	–	3.73	4.76	3.49	1.65
	0.8	2.89	3.48	2.15	–	3.93	5.32	3.87	1.57
	0.9	2.67	3.49	1.80	–	4.23	5.02	3.82	1.53
	1.0	2.70	3.70	1.98	–	4.00	4.94	3.42	1.72
PBS		2.71	3.45	2.01	–	2.99[b]	4.15[b]	2.78[b]	1.34[b]
Eimeria		2.73	3.71	1.97	–	4.95[a]	5.87[a]	4.53[a]	1.89[a]
					P-value				
SID M+C		0.888	0.912	0.819	–	0.635	0.159	0.579	0.940
Challenge		0.960	0.360	0.876	–	<0.001	<0.001	<0.001	0.022
Interaction		0.755	0.882	0.372	–	0.434	0.067	0.843	0.874

[a-c]Means with different superscripts within a column were significantly different ($P < 0.05$).

[1]Titer was defined as Log 2 of the highest dilution of sample with an optical density equal to the standard intestinal luminal diluted 1:128 (cutoff, two times of the background).

[2]On day 14, broilers were orally gavaged with either PBS or the Advent Coccidiosis Vaccine (Lincoln, NE; 100× vaccine dose; consisting of a blend of live *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella* oocysts).

[3]SID M+C, standardized ileal digestible methionine + cysteine; PBS, phosphate-buffered saline.

[4]No anti-*Eimeria* IgA was detected in cecum luminal on day 14.

specifications from academic groups (e.g., Ministry of Agricultural of the People's Republic of China, 2004), the major broiler breeding companies (e.g., Cobb 500, Ross 308, and Arbor Acres), and amino acid providers (e.g., EVONIK Nutrition and Care GmbH). An *Eimeria* challenge suppressed the growth of broilers regardless of dietary SID M+C levels. After challenge, the day 15 to 21 BWG was decreased by 15, 16, 19, and 21% in broilers fed 0.6, 0.8, 0.9, and 1.0%, respectively. In *Eimeria*-challenged broilers, feeding diets formulated with ≥0.8% SID M+C significantly increased growth performance when compared to 0.6% SID M+C. This response reflects a simple SID M+C deficiency at 0.6%, but does not infer that the additional supplemental SID M+C functioned against *Eimeria*. In addition, linear and quadratic effects of dietary SID M+C levels on growth performance have been tested using unequal coefficient calculations according to Carmer and Seif (1963). However, the results show a clear "lack of fit", in both the *Eimeria*-challenged and non-challenged groups, if we force a linear or even a quadratic curve fit (data not shown). Hence, we report that *Eimeria*-induced growth suppression was not mitigated by simply increasing dietary SID M+C levels.

The current *Eimeria* challenge model was an effective immune challenge as confirmed by intestine lesion scores and fecal oocyst count. No intestinal lesions were observed in the ileum of *Eimeria*-challenged broilers since *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella* are specific for duodenum, jejunum, and cecum, respectively. As expected, after *Eimeria* challenge, a significant humoral immune response was observed in broiler plasma and intestinal luminal samples, regardless of dietary SID M+C levels. Interestingly, broilers fed ≥0.8% SID M+C, irrespective of *Eimeria* infection, had increased levels of jejunum luminal total IgA concentrations when compared to broilers fed 0.6% SID M+C. Similar findings were reported in Rama Rao et al. (2003), where antibody response to sheep red blood cells and resistance to *Escherichia coli* were enhanced in broilers by increasing dietary total M+C levels from 0.72 to 0.88%. These results are supported by previous findings (Tsiagbe et al., 1987b) and are in good agreement with the general understanding that the nutritional status of sulfur amino acid is important to humoral immunity (Bouyeh, 2012; Jahanian and Khalifeh-Gholi, 2018; Lai et al., 2018). Of particular note, within the *Eimeria*-challenged groups, broilers fed 0.8% SID M+C had higher concentrations of jejunum luminal anti-*Eimeria* IgA when compared to broilers fed 0.6 and 1.0% SID M+C. Apparently, 0.6% SID M+C was not adequate for broilers to maximize intestinal anti-*Eimeria* IgA concentrations. However, further studies will need to confirm whether 1.0% SID M+C is too high and potentially cause adverse effects on intestinal anti-*Eimeria* IgA production in 11- to 21-D broilers. We previously reported that >0.97% dietary M+C depressed antibody responses (to sheep red blood cells) in 14- to 24-D broilers (Tsiagbe et al., 1987a). Bhargava et al. (1970) reported that the

Table 7. Intestinal luminal total IgA concentrations of broilers fed varying levels of sulfur amino acids in control conditions or challenged with *Eimeria* spp.

Challenge[1]	SID M+C (%)[2] Day 11–21	Day 14 (μg/mg protein)				Day 21 (μg/mg protein)			
		Duodenum	Jejunum	Ileum	Cecum	Duodenum	Jejunum	Ileum	Cecum
PBS	0.6	45.40	111.37	93.33	3.81	10.68	28.34	33.55	8.83
	0.8	48.34	81.32	92.49	1.94	13.03	34.81	37.36	9.22
	0.9	57.37	113.19	96.31	2.73	11.81	35.60	37.89	7.13
	1.0	40.07	79.81	94.62	3.66	11.59	35.48	37.65	8.18
Eimeria	0.6	36.48	78.87	61.83	2.04	13.44	35.40	47.93	9.39
	0.8	60.41	90.77	90.51	2.34	17.41	45.03	50.03	9.37
	0.9	54.30	110.02	88.01	2.91	20.53	43.03	53.63	9.18
	1.0	47.95	94.64	100.70	1.94	18.63	42.68	51.58	9.25
	SEM	3.03	4.40	4.19	0.30	1.16	1.25	1.64	0.58
Main effect									
	0.6	40.94	95.12	77.58	2.92	12.06	31.87[b]	40.74	9.11
	0.8	54.38	86.04	91.50	2.14	15.22	39.92[a]	43.70	9.30
	0.9	55.84	111.60	92.16	2.82	16.17	39.32[a]	45.76	8.16
	1.0	44.01	87.22	97.66	2.80	15.11	39.08[a]	44.62	8.72
PBS		47.80	96.42	94.19	3.04	11.78[b]	33.56[b]	36.61[b]	8.34
Eimeria		49.79	93.58	85.27	2.31	17.50[a]	41.54[a]	50.79[a]	9.30
					P-value				
SID M+C		0.230	0.135	0.379	0.790	0.634	0.067	0.684	0.910
Challenge		0.744	0.743	0.293	0.230	0.015	0.001	<0.001	0.427
Interaction		0.604	0.242	0.430	0.419	0.803	0.957	0.986	0.949

[a,b]Means with different superscripts within a column were significantly different ($P < 0.05$).

[1]On day 14, broilers were orally gavaged with either PBS or the Advent Coccidiosis Vaccine (Lincoln, NE; 100× vaccine dose; consisting of a blend of live *Eimeria acervulina, Eimeria maxima*, and *Eimeria tenella* oocysts).

[2]SID M+C, standardized ileal digestible methionine + cysteine; PBS, phosphate-buffered saline.

Table 8. Plasma concentrations of IL-10 and IFN-γ of 21-day-old broilers fed varying levels of sulfur amino acids in control conditions or challenged with *Eimeria* spp.

Challenge[1]	SID M+C (%)[2] day 11–21	IL-10 (μg/mL)	IFN-γ (ng/mL)
PBS	0.6	84.76	17.60
	0.8	74.12	15.93
	0.9	72.22	33.73
	1.0	82.40	18.34
Eimeria	0.6	93.28	16.29
	0.8	67.41	15.21
	0.9	86.07	16.54
	1.0	53.93	14.84
	SEM	4.48	1.96
Main effect			
	0.6	89.02	16.95
	0.8	70.77	15.57
	0.9	79.15	25.14
	1.0	68.16	16.59
PBS		78.38	21.40
Eimeria		75.17	15.72
		P-value	
SID M+C		0.354	0.278
Challenge		0.722	0.146
Interaction		0.347	0.394

[1]On day 14, broilers were orally gavaged with either PBS or the Advent Coccidiosis Vaccine (Lincoln, NE; 100× vaccine dose; consisting of a blend of live *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella* oocysts). IL-10, interleukin-10; IFN-γ, interferon-γ; PBS, phosphate-buffered saline.

[2]SID M+C, standardized ileal digestible methionine + cysteine.

antibody response (to Newcastle Disease Virus) was higher in broilers fed diets with 0.3 to 0.6% methionine than those fed diets with 0.7 to 1.1% methionine. Similarly, Bhargava et al. (1971) reported that antibody response (to Newcastle Disease Virus) was higher in broilers fed 0.4% methionine than in broilers fed 0.7 and 1.1% methionine. The experimental diets contend no cysteine in the aforementioned Bhargava et al. studies. In this sense, the marginal requirement for SID M+C is narrow in broilers when both growth performance and intestinal humoral immunity are considered (in the current study the range was ≥0.8 and <1.0%). One possible explanation for the lack of observed growth performance and immune response to increasing dietary SID M+C in *Eimeria*-infected birds could be related to ratios of essential amino acids. By increasing a single amino acid in the feed formulation, we may have inadvertently created a limitation in the another limiting amino acids required for an efficient anti-*Eimeria* immune response. A recent study looking at single amino acid reductions in *Eimeria*-infected broilers showed that not only did a reduction in total M+C detrimentally affect growth performance, but reducing threonine, isoleucine, arginine, phenalalyinen+tyrosine, and glycine+serine likewise impaired growth performance in challenged birds (Rochell et al., 2016). Follow-up studies by the same group reported arginine specifically may be vital to innate immune responses to *Eimeria* infection in broilers and be required in higher dietary concentrations in the diets of broilers with coccidiosis (Rochell et al., 2017). It would, therefore, be beneficial to conduct further research investigating the interactions of amino acids in the diets of *Eimeria*-challenged broilers to define not only the single amino acid requirements, but also the ideal ratios of amino acid in challenge conditions.

Table 9. Intestinal luminal concentrations of IL-10 and IFN-γ of 21-day-old broilers fed varying levels of sulfur amino acids in control conditions or challenged with *Eimeria* spp.

Challenge[1]	SID M+C (%)[2] day 11–21	IL-10 (ng/mg protein)				IFN-γ (pg/mg protein)			
		Duodenum	Jejunum	Ileum	Cecum	Duodenum	Jejunum	Ileum	Cecum
PBS	0.6	116.25	251.40	54.62	31.13	3.79	9.56	1.95	2.17
	0.8	150.60	120.40	52.84	51.28	3.71	5.09	1.85	1.36
	0.9	124.58	146.82	75.34	73.01	5.27	2.17	2.86	2.68
	1.0	166.96	155.02	84.78	27.24	4.08	3.16	2.73	1.58
Eimeria	0.6	230.03	159.54	64.36	67.73	13.29	20.82	2.46	9.07
	0.8	258.64	208.50	42.28	49.70	18.46	24.23	1.59	1.46
	0.9	287.31	157.33	73.91	83.72	22.87	25.62	3.88	9.84
	1.0	317.40	148.25	55.99	96.49	15.37	20.49	2.35	10.66
	SEM	17.39	18.04	4.61	9.57	1.52	2.72	0.45	1.42
Main effect									
	0.6	173.14	205.47	59.49	49.43	8.54	15.19	2.21	5.62
	0.8	204.62	164.45	47.56	50.49	11.08	14.66	1.72	1.41
	0.9	205.95	152.08	74.63	78.37	14.07	13.90	3.37	6.26
	1.0	242.18	151.64	70.38	61.87	9.72	11.83	2.54	6.12
PBS		139.60[b]	168.41	66.90	45.67	4.21[b]	5.00[b]	2.35	1.95[b]
Eimeria		273.34[a]	168.41	59.14	74.41	17.50[a]	22.79[a]	2.57	7.76[a]
					P-value				
SID M+C		0.529	0.719	0.156	0.693	0.509	0.971	0.635	0.566
Challenge		<0.001	>0.999	0.400	0.142	<0.001	0.001	0.811	0.044
Interaction		0.913	0.411	0.510	0.571	0.722	0.880	0.942	0.688

[a,b]Means with different superscripts within a column were significantly different ($P < 0.05$).

[1]On day 14, broilers were orally gavaged with either PBS or the Advent Coccidiosis Vaccine (Lincoln, NE; 100× vaccine dose; consisting of a blend of live *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella* oocysts). IL-10, interleukin-10; IFN-γ, interferon-γ; PBS, phosphate-buffered saline.

[2]SID M+C, standardized ileal digestible methionine + cysteine.

In the current *Eimeria*-challenged broilers, when compared to broilers fed 0.8% SID M+C, diets with 1.0% SID M+C decreased intestinal anti-*Eimeria* IgA concentrations, but had no adverse effects on growth performance. Broilers fed diets with 0.8% SID M+C had enhanced growth performance and intestinal IgA production compared with broilers fed diets with 0.6% SID M+C, but no differences were observed in intestine lesion scores and excreta oocyst counts. The failure to detect differences in intestinal scores and excreta oocyst counts imply that these *Eimeria* infection indicators do not correlate well with growth performance or intestinal humoral immunity at least when these variables are measured at the same time point, consistent with earlier reports (Brake, 2002; Ding et al., 2004). Additionally, while the 1.0% SID M+C treatment did not show better growth performance than the treatment receiving 0.8% dietary SID M+C, the increase in anti-*Eimeria* antibody titers observed in this group may indicate protective effects and improved performance in a secondary infection. Further research should include studies investigating the effects of dietary sulfur amino acid levels in more than one cycle of coccidiosis.

Intestinal INF-γ is a cytokine which may inhibit the development of parasites (Fayer, 1971) and has been proposed as an important host immunological response during *Eimeria* infection (Yun et al., 2000). Lillehoj and Choi (1998) reported that protein administration of INF-γ protected against weight loss in *Eimeria*-challenged chickens. In the current study, dietary SID M+C levels had no effects on intestinal luminal INF-γ concentrations after *Eimeria* infection. These results suggest the currently observed effects of SID M+C on growth performance and jejunum anti-*Eimeria* IgA levels were not mediated by INF-γ responses. While the anti-parasitic effects of INF-γ may be explained in other aspects of immune function, based on the current results we can exclude humoral antibody production from INF-γ's mechanism of action. The current study also demonstrated dietary SID M+C levels had no effects on intestinal luminal levels of IL-10, an immune suppression cytokine that may thwart the development of host immunity against viruses, bacteria and helminths (Collier et al., 2008; Cyktor and Joanne, 2011). We have previously shown that an oral antibody to IL-10 can reduce the growth suppression caused by *Eimeria* infection (Arendt et al., 2016; Sand et al., 2016; Raabis et al., 2018). The current results indicate that IL-10 is not involved in the sulfur amino acids-derived effects on growth performance and intestinal anti-*Eimeria* IgA concentrations. Thus, a suitable level of SID M+C and supplementation of anti-IL-10 antibodies together may have additive effects in managing *Eimeria* infection in chickens, and need to be further studied.

In conclusion, when dietary SID M+C was ≥0.8%, the growth suppression caused by *Eimeria* infection could not be further reduced by increasing dietary SID M+C supplementation. For 11- to 21-D broilers, the margin for optimal SID M+C requirement was between 0.8 and 1.0% when considering both growth performance and intestinal humoral immunity. Dietary SID M+C levels had no effect on intestinal luminal production of INF-γ and IL-10. Further studies will investigate the requirement of SID M+C in diets enriched with

other essential amino acids to further define the ideal protein ratios for amino acids in coccidiosis conditions.

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