The effects of total sulfur amino acids on the intestinal health status of broilers challenged with *Eimeria* spp.

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ABSTRACT This study evaluated the effects of total sulfur amino acid (**TSAA**) levels on the performance and intestinal health of broilers challenged with *Eimeria* spp. A total of 432 one-day-old off-sex Cobb 500 male chicks were randomly assigned to a 3 × 2 factorial arrangement (6 replicates/12 birds), with diets and *Eimeria* challenge as the main factors. The diets were as follows: 70% (no methionine [**Met**] supplementation), 85, and 100% TSAA, supplemented with L-Met. At day 14, the challenged birds ($n = 216$) were orally gavaged with a pool of *Eimeria acervulina*, *Eimeria maxima*, and *Eimeria tenella* sporulated oocysts, and the unchallenged birds ($n = 216$) received water. At 6 and 12 D post inoculation (**dpi**), performance and intestinal health were evaluated. The challenge, regardless of diets, significantly impaired the performance, intestinal villi height, villus-to-crypt ratio, and ileal digestibility of dry matter, energy, and crude protein (**CP**) and modulated the tight junction protein (**TJP**) expression throughout the experiment. Moreover, the superoxide dismutase activity was increased, whereas the reduced glutathione (**GSH**)-to-oxidized glutathione (**GSSG**) ratio was decreased by the challenge at 6 dpi. Regardless of the challenge, the 70% TSAA diet reduced the body weight and feed intake in all phases, whereas the ileal digestibility of CP was higher in birds fed with the 70% TSAA diet than in those fed with the 100% TSAA diet at 6 dpi. No major differences were observed among the diets with regard to the intestinal histomorphology and TJP expression, and birds fed with the 100% TSAA diet had the highest GSH concentration at 12 dpi. Few interactions were observed, and the Met supplementation counteracted the negative effects of the *Eimeria* challenge on GSH concentration when 85 and 100% of TSAA levels were reached. Overall, the *Eimeria* challenge had a negative impact on growth and intestinal health. Moreover, the supplementation of L-Met until either 85 or 100% of TSAA levels were reached was enough to assure good performance and intestinal health in birds challenged or not challenged with *Eimeria* spp.

Key words: broiler chicken, *Eimeria* spp., intestinal health, total sulfur amino acid

2020 Poultry Science 99:5027–5036
https://doi.org/10.1016/j.psj.2020.06.055

INTRODUCTION

Methionine (**Met**) is a nutritionally essential amino acid (**AA**) because its endogenous synthesis cannot support normal growth in birds. Methionine can be irreversibly converted to cysteine (**Cys**) in the organism, and the requirement for Cys can be fulfilled by supplementing Met in the diet (Stipanuk, 2004). Therefore, Cys is commonly considered a semiessential AA (Bin et al., 2017), and both AA are referred to as the total sulfur AA (**TSAA**). Several key metabolic functions are attributed to the TSAA, and according to Wu (2010), they are considered functional AA. Studies have shown the importance of TSAA in the epigenetic gene regulation and DNA methylation (Waterland, 2006); in the synthesis of polyamines, which are associated with cellular repair and turnover (Timmons, 2013); and participation in the antioxidant system, either by being a free radical scavenger or through reduced glutathione (**GSH**) and taurine synthesis (Levine et al., 2001; Tesseraud et al., 2008; Bin et al., 2017).

Several studies have suggested that the gastrointestinal tract (**GIT**) may have its own requirement for TSAA. Approximately 52% of the dietary intake of Met was sequestered in first-pass utilization by the gut and metabolized in pigs, whereas the appearance of Cys in portal blood was close to zero when given in the diet (Stoll et al., 1998). In addition, the Met requirement for piglets fed enterally was 30% higher than for those

Received March 11, 2020.
Accepted June 17, 2020.

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fed parenterally (Shoveller et al., 2003). A direct effect of the Met metabolism on the GIT was provided by Burrin and Stoll (2007), who observed that approximately 20% of the dietary Met intake was metabolized in the GIT and released either as Cys or completely oxidized to CO_2 in infant pigs. These findings reinforce the importance of the TSAA in GIT function, and although these values have not been yet determined for poultry, it is reasonable to assume similar importance of these AA in maintaining the intestinal health in birds.

The most prevalent intestinal disease in poultry is caused by a protozoon of the genus *Eimeira*. The *Eimeria* spp. infection in poultry is associated with intestinal epithelial cell damage, diarrhea, oxidative stress, and, consequently, malabsorption of nutrients and decrease in growth performance (Yegani and Korver, 2008; Perez-Carbajal et al., 2010; McDougald et al., 2013). Thus, the supplementation with TSAA might be necessary to improve the intestinal health during and after an *Eimeria* infection. For this reason, the objective of this study was to evaluate the effects of different TSAA levels, obtained by the supplementation of L-Met, on the growth performance, intestinal health, and antioxidant system during an *Eimeria* spp. infection.

MATERIALS AND METHODS

General Procedures

The experiment was approved by the Institutional Animal Care and Use Committee of the University of Georgia (Athens, Georgia). A total of 432 off-sex Cobb 500 1-day-old male broiler chicks were distributed in a completely randomized design with a factorial arrangement of 3 by 2, with 6 replicates of 12 birds. The diets (70, 85, and 100% TSAA) and presence or absence of the *Eimeria* spp. challenge were considered the main factors. The chicks were allocated to 60 metabolic cages equipped with a feeder and a drinker, providing *ad libitum* water and feed from 1 to 26 D of age. The temperature and lighting programs were set as per Cobb 500 management guide (Cobb Vantress, 2018a).

The corn–soybean meal–based diets were formulated to reach or exceed the Cobb 500 nutrient specifications (Cobb Vantress, 2018b) of the starter phase for all ingredients, except for Met and TSAA, and this diet was used from 1 to 26 D of age (Table 1). The treatments were given as a percentage of the TSAA Cobb 500 recommended level (100% TSAA), and the dietary treatments were obtained by adding L-Met to the basal diet (70% TSAA without Met supplementation), as a replacement of an inert component (sand), until 85 and 100% were reached. The calculated digestible TSAA and Met levels in the diets were as follows: 0.58% of TSAA and 0.30% of L-Met for the 70% TSAA diet, 0.73% of TSAA and 0.45% of L-Met for the 85% TSAA diet, and 0.88% of TSAA and 0.61% of L-Met for the 100% TSAA diet. The diets were kept isocaloric and isonitrogenous using glycine (**Gly**) to balance the addition of Met. For nutrient ileal digestibility determination, 0.3% of chromium oxide ($\mathbf{Cr_2O_3}$; Sigma-Aldrich, St. Louis, MO) was added as an indigestible marker.

Table 1. Diet formulation according to the treatments (1–26 D, as-fed basis; % diet).

Ingredients	70% TSAA	85% TSAA	100% TSAA
Corn	62.34	62.34	62.34
Soybean meal	31.19	31.19	31.19
Soybean oil	1.01	1.01	1.01
Salt	0.34	0.34	0.34
Limestone	1.19	1.19	1.19
Dicalcium phosphate	1.61	1.61	1.61
Vitamin premix[1]	0.25	0.25	0.25
Mineral premix[2]	0.08	0.08	0.08
Methionine	0.00	0.15	0.31
L-Lysine	0.27	0.27	0.27
L-Glutamine	0.45	0.45	0.45
L-Threonine	0.07	0.07	0.07
L-Leucine	0.40	0.40	0.40
Glycine[3]	0.20	0.13	0.05
Sand	0.30	0.22	0.14
Chromium oxide	0.30	0.30	0.30
Total	100	100	100
ME (kcal/kg)	3,000	3,000	3,000
CP (%)	21.0	21.0	21.0
Lysine (%)	1.18	1.18	1.18
Methionine (%)	0.30	0.45	0.61
Cys (%)	0.28	0.28	0.28
Met–Cys (%)	0.58 (0.72)[4]	0.73 (0.82)	0.88 (0.89)
Ca (%)	0.90	0.90	0.90
Available *P* (%)	0.45	0.45	0.45

Abbreviations: CP, crude protein; Cys, cysteine; Met, methionine; TSAA, total sulfur amino acid.

[1]Provided per kilogram of DSM Vitamin premix: vitamin A, 2,204,586 IU; vitamin D_3, 200,000 ICU; vitamin E, 2,000 IU; vitamin B12, 2 mg; biotin, 20 mg; menadione, 200 mg; thiamine, 400 mg; riboflavin, 800 mg; d-pantothenic acid, 2,000 mg; vitamin B6, 400 mg; niacin, 8,000 mg; folic acid, 100 mg; and choline, 34,720 mg.

[2]Provided per kilogram 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; and Se, 9.07 g.

[3]Analyzed total glycine levels (%) were as follows: 1.04 (70% of TSAA), 0.97 (85% of TSAA), and 0.89 (100% of TSAA).

[4]Analyzed total TSAA levels (%).

At day 14, half of the birds ($n = 216$) were challenged through oral gavage with approximately 12,500 *Eimeria maxima*, 12,500 *Eimeria tenella*, and 62,500 *Eimeria acervulina* sporulated oocysts (challenged group), whereas the other half ($n = 216$) were gavaged with water (unchallenged group). A previous study conducted by our research group determined these challenge levels to cause a mild coccidiosis infection (Teng et al., 2020).

For growth performance assessment, the birds and feed were weighed, by replicate, at day 14, 20, and 26 of the experiment. The body weight gain (**BWG**), feed intake (**FI**), and feed conversion ratio (**FCR**) were determined before the challenge (1–14 D of age), during the challenge (14–20 D of age, 0–6 D post inoculation [**dpi**]), and at recovery (20–26 D of age, 6–12 dpi) phases. Mortality was recorded daily and used to correct the FCR.

Analysis Performed

Nutrient Digestibility At 12 dpi (26 D of age) 4 birds per replicate were selected and euthanized by cervical dislocation to collect the ileal digesta from 2 cm below Meckel's diverticulum until 2 cm from the ileocecal junction. The samples from different birds within the same

replicate were pooled and dried in a ventilated oven at 75°C for 48 h and grinded. Chromium oxide concentration was measured according to the method of Dansky and Hill (1952), and gross energy was evaluated using a bomb calorimeter (IKA Calorimeter C1; IKA Works Inc., Wilmington, NC) at the University of Georgia. Crude protein (**CP**) was analyzed in the Agricultural Experiment Station Chemical Laboratories at the University of Missouri, Columbia, through combustion analysis (AOAC Official method 990.03; AOAC International, 2006) using a LECO instrument (LECO, St. Joseph, MI) (N x 6.25). The apparent ileal digestibility (**AID**) of CP (**AIDCP**), AID of energy (**AIDE**), and AID of dry matter (**DM**; **AIDDM**) were calculated as per the following equation:

$$AID, \% = \left\{ \left[(nutrient/Cr_2O_3)_{Diet} - (nutrient/Cr_2O_3)_{digesta} \right] \Big/ (nutrient/Cr_2O_3)_{diet} \right\} \times 100$$

where (nutrient/Cr_2O_3) is the ratio of DM, CP, and energy to Cr_2O_3 in the diet or ileal digesta.

Intestinal Morphology At 6 and 12 dpi, 2-cm portions of the duodenal loop, middle jejunum, and ileum were collected from one bird per replicate. The samples were gently flushed with 1× PBS (National Diagnostics, Atlanta, GA) and stored in 10% neutral-buffered formalin until processed. The slide preparation process consisted of tissue dehydration in increasing concentrations of ethanol, diaphanization in xylol, and paraffin fixation. Subsequently, serial cuts of 4-µm thickness were stained with hematoxylin and eosin and analyzed under a light microscope (10× eyepiece and 1.6× magnification) (Leica DC500 camera; Leica Microsystems Inc., Buffalo Grove, IL). The pictures were taken and analyzed using ImageJ (Image Processing and Analysis in Java–ImageJ 1.50i; National Institutes of Health, Arlington, VA) to measure crypt depth and villus height of 4 villi and crypts per slide.

Quantitative Reverse Transcription Polymerase Chain Reaction The samples of the middle portion of the jejunum were collected and snap frozen in liquid nitrogen until processing. Total RNA was extracted using the QIAzol Lysis Reagent (Qiagen, Germantown, MD), and RNA quantity and purity were determined using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA). Subsequently, cDNA was obtained from total RNA using high-capacity cDNA reverse transcription kits (Thermo Fisher Scientific, Waltham, MA). The cDNA was diluted in the ratio of 1:5 (200 ng) for quantitative reverse-transcriptase polymerase chain reaction analysis (Applied Biosystems StepOnePlus; Thermo Fisher Scientific, Waltham, MA) using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). The expression of occludin (Liu et al., 2012), claudin-1 (**Cla-1**) (Shao et al., 2013), claudin-2 (Chen et al., 2017), zonula occludens-1 (**ZO-1**) (Metzler-Zebeli et al., 2018), and zonula occludens-2 (**ZO-2**) (Kim et al., 2017) was evaluated. The glyceraldehyde 3-phosphate dehydrogenase was used as an internal control gene. The forward and reverse primers are shown in Table 2. The running condition used for all genes was as follows: 95°C for 15 s, 58°C for 20 s, and 72°C for 15 s during 40 cycles. The samples were run in duplicate, and relative gene expression data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Scmittgen, 2001). The mean ΔCt of challenged 70% TSAA group was used to calculate the ΔΔCt value.

Antioxidant System For antioxidant system evaluation, superoxide dismutase (**SOD**) activity and GSH and oxidized glutathione (**GSSG**) quantification were determined in liver samples from one bird per replicate at 6 and 12 dpi. The liver samples were collected, immediately frozen in liquid nitrogen, and stored at −80°C until processing the following day. For SOD analysis, approximately 75 mg of the liver samples was homogenized in 1 mL of cold buffer (20 mmol/L HEPES buffer, pH 7.2, 2 mmol/L egtazic acid, 10 mmol/L mannitol, and 70 mmol/L sucrose per gram of tissue). After the homogenized samples were centrifuged at 1,500× *g* for 5 min at 4°C, the supernatants were removed. Subsequently, the samples were diluted at a ratio of 1:1,000 using the sample buffer, and the analysis was performed. A SOD assay kit (Superoxide Dismutase assay kit, item no. 706002; Cayman Chemical, Ann Arbor, MI) was used according to the manufacturer's instruction. The absorbance was measured at 440 nm in a microplate reader (SpectraMax ABS Plus; Molecular Devices, San Jose, CA). The GSH and GSSG quantification was performed by HPLC (Dionex UltiMate 3000; Thermo Scientific, Waltham, MA) coupled with electrochemical detection. Approximately 80 mg of the liver samples was homogenized in 800 µL of solution containing cold PBS and 10 mmol/L pentetic acid. Subsequently, 500 µL of the tissue solutions were transferred to tubes containing 500 µL of 10% perchloric acid and were stored at −80°C until analysis. The results were given as a ratio between GSH and GSSG concentrations (**GSH:GSSG**).

A protein quantification assay was also performed (Pierce BCA Protein Assay Kit, Ref. 23227; Thermo Scientific, Rockford, IL) to standardize the samples and obtain the SOD activity and GSH and GSSG concentrations relative to the protein concentration. The diluted tissue samples from SOD and GSH assays were used in this analysis, following the manufacturer's instruction. Bovine serum albumin (2 mg/mL) was used as a protein standard. The absorbance was measured at 562 nm using a microplate reader (SpectraMax ABS Plus; Molecular Devices, San Jose, CA).

Statistical Analysis

Data were tested for homogeneity of variances and normality of studentized residuals. The performance results from 1 to 14 D of age were subjected to one-way ANOVA, and in case of significant differences, the treatments were compared using Tukey's test. Data from the challenge (15–20 D) and recovery (21–26 D) phases were subjected to a 2-way ANOVA, with diets and challenge

Table 2. Primer pairs used for qRT-PCR analysis.

Gene	Gene bank identification	Primer sequence, sense/antisense	Product size (bp)
GAPDH	NM_204305.1	GCTAAGGCTGTGGGGAAAGT/ TCAGCAGCAGCCTTCACTAC	161
Cla-1	NM_001013611.2	TGGAGGATGACCAGGTGAAGA/ CGAGCCACTCTGTTGCCATA	115
Cla-2	NM_001277622.1	CCTGCTCACCCTCATTGGAG/ GCTGAACTCACTCTTGGGCT	145
ZO-1	XM_015278981.2	CAACTGGTGTGGGTTTCTGAA/ TCACTACCAGGAGCTGAGAGGTAA	101
ZO-2	XM_025144669.1	ATCCAAGAAGGCACCTCAGC/ CATCCTCCCGAACAATGC	100
Occludin	XM_026041453.1	ACGGCAGCACCTACCTCAA/ GGCGAAGAAGCAGATGAG	122

Abbreviations: Cla-1, claudin-1; Cla-2, claudin-2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; ZO-1, zonula occludens-1; ZO-2, zonula occludens-2.

being considered the main factors. In case of significant differences, Tukey's test was used for *post hoc* comparison. All statistical procedures were performed using SAS University Edition (3.8, basic edition, 2018, SAS Institute Inc., Cary, NC), considering significant values at $P < 0.05$.

RESULTS

Growth Performance

The BWG, FI, and FCR before the challenge (1–14 D of age) were influenced by the TSAA levels (Table 3). Birds fed with the 70% TSAA diet had the lowest BWG ($P < 0.001$) and FI ($P < 0.001$) among the treatments. The FCR was the highest for birds fed with the 70% TSAA diet, followed by birds fed with 85% TSAA diet, whereas birds with the 100% TSAA diet had the lowest values ($P < 0.001$).

During the challenge phase (0–6 dpi), an interaction between the factors was found for FCR ($P = 0.032$) (Table 4). The unchallenged birds showed a lower FCR than the challenged ones. In addition, the challenged birds fed with the 100% TSAA diet had no statistical difference in FCR compared with birds in the unchallenged groups (70 and 85% TSAA diets). No interactions were observed between FI and BWG, which were influenced independently by the challenge or diets. The unchallenged birds had higher FI and BWG than the challenged ones ($P < 0.001$). The birds fed with the 70% TSAA diet had the lowest FI, and birds fed with the 100% TSAA diet had a lower FI than birds fed with the 85% TSAA diet ($P < 0.001$). Moreover, the birds fed with the 70% TSAA diet had the lowest BWG among the dietary treatments ($P < 0.001$).

No interactions were found between BWG, FI, and FCR during the recovery phase (7–12 dpi) and in the overall period (0–12 dpi) ($P > 0.170$). From 7 to 12 dpi, the FI and BWG were influenced by the challenge or diets. The unchallenged birds had higher FI and BWG than the challenged ones ($P < 0.001$). Moreover, the birds fed with the 70% TSAA diet had lower FI than birds fed with the 85% TSAA diet ($P = 0.007$), whereas birds fed with the 70% TSAA diet had the lowest BWG ($P < 0.001$). No differences were observed for FCR ($P > 0.050$). From 0 to 12 dpi, the unchallenged birds showed higher FI and BWG than the challenged ones ($P < 0.001$), and birds fed with the 70% TSAA diet had the lowest FI and BWG ($P < 0.001$). The mortality results did not differ statistically among the treatments ($P > 0.05$).

Table 3. Body weight gain (BWG; kg), feed intake (FI; kg), and feed conversion ratio (FCR) from 1 to 14 D of age according to the treatments.

Diets	BWG	FI	FCR
70% TSAA	0.231[b]	0.310[b]	1.346[a]
85% TSAA	0.309[a]	0.386[a]	1.251[b]
100% TSAA	0.321[a]	0.387[a]	1.206[c]
P-value	<0.0001	<0.0001	<0.0001
SE	0.0053	0.0047	0.0087

[a,b,c]Means followed by superscript letters are different as per Tukey's test ($P < 0.05$).
N = 6.
Abbreviations: TSAA, total sulfur amino acid.

Intestinal Health

Intestinal Histomorphology and Nutrient Ileal Digestibility No interactions were found among the main factors for the morphometric intestinal characteristics ($P > 0.060$) (Tables 5 and 6) and for the nutrient ileal digestibility ($P > 0.066$) (Table 7) at 6 and 12 dpi. At 6 dpi, the duodenum and jejunum of the unchallenged birds had higher villus height ($P < 0.001$), lower crypt depth ($P < 0.038$), and a higher villus-to-crypt ratio ($P < 0.001$) than those of the challenged birds. The ileal crypt depth was lower, and the villus-to-crypt ratio was higher for the unchallenged birds than for the challenged ones ($P < 0.001$). At 12 dpi, the duodenum and jejunum of the unchallenged birds showed higher villus height ($P < 0.020$) and a higher villus-to-crypt ratio ($P < 0.010$) than those of the challenged birds. In addition, the ileum of the unchallenged birds had lower crypt depth than that of the challenged ones ($P = 0.038$).

The AIDDM and AIDE, at 6 and 12 dpi, were affected by the challenge, and the unchallenged birds had higher values for both traits than the

Table 4. Body weight gain (BWG; kg), feed intake (FI; kg), and feed conversion ratio (FCR) from 0 to 6, 7 to 12, and 0 to 12 dpi according to the treatments.

		0–6 dpi			7–12 dpi			0–12 dpi		
Challenge	Diets	FI	BWG	FCR	FI	BWG	FCR	FI	BWG	FCR
+	70% TSAA	0.340	0.204	1.681^{b}	0.485	0.264	1.704	0.825	0.465	1.688
	85% TSAA	0.454	0.238	1.853^{a}	0.646	0.383	1.600	1.105	0.630	1.692
	100% TSAA	0.417	0.249	1.602^{b,c}	0.536	0.369	1.444	0.953	0.618	1.539
−	70% TSAA	0.384	0.265	1.452^{c,d}	0.613	0.308	1.784	0.997	0.596	1.690
	85% TSAA	0.482	0.336	1.482^{c,d}	0.690	0.420	1.638	1.225	0.715	1.676
	100% TSAA	0.466	0.328	1.423^{d}	0.722	0.458	1.628	1.188	0.785	1.587
Challenge	+	0.404^{b}	0.230^{b}	1.712	0.555^{b}	0.338^{b}	1.582	0.961^{b}	0.571^{b}	1.639
	−	0.444^{a}	0.309^{a}	1.452	0.675^{a}	0.396^{a}	1.683	1.136^{a}	0.698^{a}	1.651
Diets	70% TSAA	0.362^{c}	0.234^{b}	1.567	0.549^{b}	0.286^{b}	1.744	0.911^{b}	0.530^{b}	1.689
	85% TSAA	0.468^{a}	0.287^{a}	1.668	0.668^{a}	0.402^{a}	1.619	1.165^{a}	0.673^{a}	1.684
	100% TSAA	0.441^{b}	0.289^{a}	1.513	0.629^{a,b}	0.414^{a}	1.536	1.070^{a}	0.702^{a}	1.563
P-value	Challenge	<0.0001	<0.0001	<0.0001	0.0003	0.0002	0.2625	<0.0001	<0.0001	0.8665
	Diets	<0.0001	<0.0001	0.0007	0.0075	<0.0001	0.1633	<0.0001	<0.0001	0.2167
	Challenge × diets	0.4433	0.1845	0.0328	0.1700	0.2174	0.7879	0.3662	0.1981	0.9179
SE		0.0090	0.0088	0.0293	0.0200	0.0129	0.0445	0.0288	0.0192	0.0320

[a,b,c,d]Means followed by superscript letters are different as per Tukey's test ($P < 0.05$).
N = 6.
Abbreviations: dpi, D post inoculation; TSAA, total sulfur amino acid.

challenged group ($P < 0.039$). In addition, the AIDCP at 6 dpi was affected by both challenge and diets. The unchallenged birds showed higher AIDCP values ($P < 0.001$), and the birds fed with the 70% TSAA diet had higher AIDCP values than birds fed with the 100% TSAA diet ($P = 0.035$).

Tight Junction Protein Gene Expression No interactions were found among the main factors for tight junction protein (**TJP**) expression at 6 dpi ($P > 0.073$) (Table 8). The gene expression of Cla-1, ZO-1, and ZO-2 was influenced by the challenge, and these genes were upregulated in the challenged birds compared with the unchallenged ones ($P < 0.001$). At 12 dpi, a significant interaction among factors was found for ZO-2 expression ($P = 0.008$). The unchallenged birds fed with the 100% TSAA diet and the challenged birds fed with the 85% TSAA diet showed higher expression of this gene than the challenged birds fed with the 70% TSAA diet. Moreover, occludin expression was affected independently by both the challenge or diets. The expression in the unchallenged birds was upregulated ($P = 0.001$), and birds fed with the 70% TSAA diet had higher expression than birds fed with the 85% TSAA diet ($P = 0.033$).

Antioxidant System At 6 dpi, an interaction between the *Eimeria* challenge and diets was found for GSH concentration ($P = 0.016$) (Table 9). The challenged birds fed with the 70% TSAA diet had the lowest values among all the treatments in the challenged and unchallenged groups. No interactions were found for the other antioxidant markers at 6 and 12 dpi ($P > 0.109$). At 6 dpi, the SOD activity was increased in the challenged birds compared with the unchallenged ones ($P = 0.006$). Furthermore, the GSH:GSSG was higher in the unchallenged birds than in the challenged birds ($P < 0.001$). At 12 dpi, the GSH concentration was

Table 5. Villus height (μm), crypt depth (μm), and villus-to-crypt ratio in the duodenum, jejunum, and ileum of birds at 6 dpi according to the treatments.

		Duodenum			Jejunum			Ileum		
Challenge	Diets	Villi	Crypt	Villus-to-crypt	Villi	Crypt	Villus-to-crypt	Villi	Crypt	Villus-to-crypt
+	70% TSAA	1.979	0.287	7.039	1.090	0.236	4.795	0.988	0.308	3.345
	85% TSAA	1.842	0.270	5.721	1.032	0.239	4.524	0.890	0.290	3.130
	100% TSAA	2.090	0.367	5.871	1.130	0.278	4.222	1.024	0.301	3.436
−	70% TSAA	2.334	0.193	12.51	1.255	0.210	6.057	0.966	0.183	5.755
	85% TSAA	2.404	0.217	11.302	1.332	0.214	6.497	0.928	0.204	4.796
	100% TSAA	2.421	0.217	11.335	1.413	0.222	6.536	0.869	0.164	5.423
Challenge	+	1.970^{b}	0.327^{a}	6.210^{b}	1.083^{b}	0.251^{a}	4.513^{b}	0.968	0.299^{a}	3.333^{b}
	−	2.401^{a}	0.212^{b}	11.592^{a}	1.321^{a}	0.211^{b}	6.429^{a}	0.916	0.184^{b}	5.304^{a}
Diets	70% TSAA	2.156	0.240	9.775	1.172	0.223	5.426	0.977	0.245	4.595
	85% TSAA	2.123	0.272	8.512	1.182	0.226	5.511	0.909	0.247	3.963
	100% TSAA	2.255	0.292	8.603	1.272	0.250	5.379	0.947	0.232	4.429
P-value	Challenge	<0.0001	<0.0001	<0.0001	0.0002	0.0384	<0.0001	0.1830	<0.0001	<0.0001
	Diets	0.5383	0.0818	0.0922	0.2188	0.3598	0.8742	0.2868	0.7988	0.3842
	Challenge × diets	0.3700	0.4336	0.9768	0.6402	0.7825	0.4504	0.1488	0.5398	0.7683
SE		0.0566	0.0138	0.5469	0.0341	0.0093	0.2425	19.9713	13.8258	0.2580

[a,b]Means followed by superscript letters are different as per Tukey's test ($P < 0.05$).
N = 6.
Abbreviations: dpi, D post inoculation; TSAA, total sulfur amino acid.

Table 6. Villus height (μm), crypt depth (μm), and villus-to-crypt ratio in the duodenum, jejunum, and ileum of birds at 12 dpi according to the treatments.

		Duodenum			Jejunum			Ileum		
Challenge	Diets	Villi	Crypt	Villus-to-crypt	Villi	Crypt	Villus-to-crypt	Villi	Crypt	Villus-to-crypt
+	70% TSAA	2.516	0.273	9.345	1.498	0.261	5.951	1.204	0.258	5.180
	85% TSAA	2.497	0.257	9.814	1.440	0.231	6.352	1.302	0.281	4.670
	100% TSAA	2.391	0.226	10.484	1.458	0.245	6.108	1.215	0.268	5.533
−	70% TSAA	2.654	0.245	11.017	1.674	0.210	8.331	1.144	0.233	4.949
	85% TSAA	2.828	0.256	11.303	1.686	0.189	9.071	1.185	0.179	6.852
	100% TSAA	2.722	0.247	11.333	1.594	0.228	7.133	1.170	0.217	5.419
Challenge	+	2.468[b]	0.252	9.887[b]	1.465[b]	0.245	6.136[b]	1.240	0.268[a]	5.127
	−	2.747[a]	0.246	11.377[a]	1.667[a]	0.211	8.169[a]	1.173	0.209[b]	5.792
Diets	70% TSAA	2.585	0.258	10.181	1.586	0.235	7.141	1.174	0.245	5.064
	85% TSAA	2.662	0.256	10.558	1.563	0.210	7.711	1.243	0.229	5.761
	100% TSAA	2.557	0.236	10.908	1.526	0.237	6.620	1.192	0.242	5.475
P-value	Challenge	0.0181	0.6722	0.0100	0.0203	0.0552	0.0003	0.2769	0.0383	0.1760
	Diets	0.6426	0.4183	0.4958	0.7873	0.4817	0.2290	0.5260	0.8802	0.4052
	Challenge × diets	0.6579	0.3692	0.6928	0.7196	0.7318	0.3602	0.9365	0.4900	0.0604
SE		0.0574	0.0068	0.2893	0.0422	0.0087	0.3003	28.8987	13.6165	0.2531

[a,b]Means followed by superscript letters are different as per Tukey's test ($P < 0.05$).
N = 6.
Abbreviations: dpi, D post inoculation; TSAA, total sulfur amino acid.

influenced by diets, and birds fed with the 100% TSAA diet had higher values than the birds fed with the 85 and 70% TSAA diets ($P = 0.018$).

DISCUSSION

A well-known effect of *Eimeria* infection in broilers is the reduction in FI and BWG (Williams, 2005). In the present study, we observed that the decrease in BWG (25.6%) in the challenged birds was more pronounced than the reduction in FI (9.0%) during the challenge phase (0–6 dpi), whereas during the recovery phase (7–12 dpi), the FI and BWG decreased by 14.6 and 17.7%, respectively. A meta-analysis conducted by Kipper et al. (2013) has shown that the decrease in BWG happened before a reduction in FI was observed, suggesting that the negative effect of *Eimeria* on performance was not exclusively related to a decrease in FI. This decrease in BWG might be related to a redirection of nutrient utilization to support the immune system and the acute phase response rather than body growth (Klasing, 1998) and a decrease in nutrient digestion and absorption (Adams et al., 1996; Persia et al., 2006; Amerah and Ravindran, 2015; Rochell et al., 2016b).

In the present study, during the challenge (0–6 dpi) and recovery phases (7–12 dpi), we observed that the challenged birds showed lower AIDDM and AIDE values than the unchallenged ones. Adedokun et al. (2016) reported a reduction in AIDE and AIDDM in birds challenged with a coccidial vaccine during the challenge, but not during the recovery phase. Furthermore, in our study, when assessing the intestinal histomorphology, the *Eimeria*-challenged group showed lower villus height, higher crypt depth, and a lower villus-to-crypt ratio in at least one section of the small intestine during

Table 7. Apparent ileal digestibility of dry matter (AIDDM; %), apparent ileal digestibility of crude protein (AIDCP; %), and apparent ileal digestibility of energy (AIDE; %) at 12 dpi according to the treatments.

		6 dpi			12 dpi		
Challenge	Diets	AIDDM	AIDCP	AIDE	AIDDM	AIDCP	AIDE
+	70% TSAA	47.878	63.400	1725.330	66.847	78.865	2529.304
	85% TSAA	49.188	61.754	1801.965	69.458	79.928	2600.642
	100% TSAA	49.004	51.868	1492.378	68.007	76.797	2549.705
−	70% TSAA	68.545	80.305	2620.535	70.415	81.730	2685.588
	85% TSAA	69.060	79.122	2513.663	68.825	79.240	2555.490
	100% TSAA	68.532	77.707	2563.727	71.023	78.650	2714.612
Challenge	+	48.690[b]	59.007[b]	1673.224[b]	68.103[b]	78.529	2559.883[b]
	−	68.712[a]	79.044[a]	2565.975[a]	70.087[a]	79.873	2651.896[a]
Diets	70% TSAA	58.212	71.853[a]	2172.933	68.631	80.298	2607.446
	85% TSAA	59.124	70.438[a,b]	2157.814	69.142	79.584	2578.066
	100% TSAA	58.768	64.787[b]	2028.052	69.515	77.723	2632.158
P-value	Challenge	<0.0001	<0.0001	<0.0001	0.0397	0.1575	0.0224
	Diets	0.9551	0.0351	0.2696	0.7175	0.0685	0.5227
	Challenge × diets	0.9816	0.1937	0.2010	0.1666	0.3084	0.0660
SE		2.1218	2.0960	88.8435	0.4888	0.4966	21.5101

[a,b]Means followed by superscript letters are different as per Tukey's test ($P < 0.05$).
N = 6.
Abbreviations: dpi, D post inoculation; TSAA, total sulfur amino acid.

Table 8. Claudin-1 (Cla-1), claudin-2 (Cla-2), zonula occludens-1 (ZO-1), zonula occludens-2 (ZO-2), and occludin gene expression at 6 and 12 dpi according to the treatments.

		6 dpi					12 dpi				
Challenge	Diets	Cla-1	Cla-2	ZO-1	ZO-2	Occludin	Cla-1	Cla-2	ZO-1	ZO-2	Occludin
+	70% TSAA	0.979	1.016	1.130	1.006	1.091	1.041	1.041	1.012	0.949^{b}	1.004
	85% TSAA	1.337	1.060	1.022	1.054	1.014	1.089	1.089	1.137	1.216^{a}	0.857
	100% TSAA	0.681	1.162	1.016	1.004	1.048	1.071	1.071	1.107	1.116^{a,b}	1.125
−	70% TSAA	0.104	1.029	0.846	0.823	1.009	1.151	1.151	1.168	1.204^{a,b}	1.330
	85% TSAA	0.098	0.757	0.659	0.758	0.982	0.802	0.802	1.050	1.057^{a,b}	1.125
	100% TSAA	0.111	1.257	0.856	0.804	1.142	1.046	1.046	1.216	1.252^{a}	1.136
Challenge	+	0.998^{a}	1.079	1.055^{a}	1.020^{a}	1.051	1.066	1.066	1.085	1.093	0.995^{b}
	−	0.104^{b}	1.014	0.787^{b}	0.795^{b}	1.044	0.998	0.999	1.144	1.171	1.197^{a}
Diets	70% TSAA	0.541	1.023	0.988	0.914	1.050	1.096	1.096	1.090	1.076	1.167^{a}
	85% TSAA	0.717	0.908	0.841	0.906	0.998	0.946	0.946	1.094	1.136	0.991^{b}
	100% TSAA	0.396	1.210	0.936	0.904	1.095	1.059	1.059	1.161	1.184	1.131^{a,b}
P-value	Challenge	<0.0001	0.6593	<0.0001	0.0002	0.9684	0.6270	0.6270	0.2818	0.1328	0.0011
	Diets	0.0886	0.2580	0.1063	0.9836	0.8931	0.6490	0.6490	0.5084	0.2161	0.0332
	Challenge × diets	0.0738	0.5224	0.3607	0.6471	0.9052	0.4895	0.4895	0.1644	0.0080	0.0707
SE		0.0980	0.0723	0.0377	0.0307	0.0777	0.0652	0.0652	0.0276	0.0293	0.0358

[a,b]Means followed by superscript letters are different as per Tukey's test ($P < 0.05$).
N = 6.
Abbreviations: dpi, D post inoculation; TSAA, total sulfur amino acid.

the challenge and recovery phases. These results, in addition to the reduction in energy and DM digestibility, are an indication that intestinal damage persists throughout the recovery phase.

The villus and brush border damage can negatively influence the activity of brush border digestive enzymes, decreasing not only the absorptive but also the digestive capacity of the intestinal cells (Adams et al., 1996). Thus, the shortening of villi and the reduced villus-to-crypt ratio have been associated with poor nutrient digestion and absorption and poor intestinal mucosa differentiation (Choct, 2009). These findings could partially explain the reduced growth performance observed in the *Eimeria*-challenged birds. In addition, the intestinal epithelium damage and unabsorbed nutrients are part of the pathophysiology of the disease, which could lead to diarrhea. According to Field (2003), the presence of low-molecular-weight and poorly absorbed solutes in the lumen causes, through an osmotic force, the movement of water and ions from the cells to the lumen. Furthermore, in an exudative manner, the loss of intestinal barrier function, caused by epithelium disruption, leads to increased hydrostatic pressure in blood vessels, causing the movement of water and other molecules to the lumen (Field, 2003).

To further investigate the effects of the *Eimeria* challenge on intestinal integrity, we evaluated the expression of TJP genes. During the challenge phase, we observed an upregulation of Cla-1, ZO-1, and ZO-2 in the *Eimeria*-challenged birds compared with the unchallenged ones. Moreover, during the recovery phase, occludin was downregulated in the challenged birds. The TJP are multiprotein complexes that are responsible for sealing and controlling, in a selective manner, the permeability of the paracellular space to water, ions, and small molecules (Robinson et al., 2015). The upregulation of these proteins is usually associated with increased integrity of the tight junction (Schneeberger and Lynch,

Table 9. Superoxide dismutase (SOD) activity (U/g of liver), GSH, GSSG, and GSH:GSSG (μmol/L/μmol/L) concentrations at 6 and 12 dpi according to the treatments.

		6 dpi				12 dpi			
Challenge	Diets	SOD	GSH	GSSG	GSH:GSSG	SOD	GSH	GSSG	GSH:GSSG
+	70% TSAA	11.148	0.274^{b}	0.0024	131.129	7.953	0.182	0.0006	256.845
	85% TSAA	7.802	0.326^{a}	0.0025	143.373	8.544	0.183	0.0008	216.933
	100% TSAA	8.424	0.376^{a}	0.0026	142.283	8.905	0.254	0.0006	250.467
−	70% TSAA	3.829	0.414^{a}	0.0025	211.269	6.504	0.173	0.0006	349.136
	85% TSAA	7.576	0.360^{a}	0.0022	174.849	7.648	0.203	0.0008	320.157
	100% TSAA	4.409	0.301^{a}	0.0013	199.733	6.846	0.319	0.0010	346.744
Challenge	+	8.798^{a}	0.324	0.0025	138.928^{b}	8.467	0.206	0.0006	241.415
	−	4.954^{b}	0.358	0.0020	195.253^{a}	6.999	0.231	0.0008	338.678
Diets	70% TSAA	7.488	0.344	0.0025	171.199	7.228	0.178^{b}	0.0006	302.991
	85% TSAA	7.689	0.343	0.0023	159.111	8.096	0.193^{b}	0.0008	268.545
	100% TSAA	6.416	0.338	0.0020	171.008	7.875	0.287^{a}	0.0008	298.605
P-value	Challenge	0.0066	0.2437	0.0615	0.0004	0.2157	0.4118	0.4834	0.1129
	Diets	0.5888	0.9838	0.3007	0.7047	0.8233	0.0188	0.5211	0.8710
	Challenge × diets	0.1514	0.0168	0.1092	0.3575	0.9168	0.6341	0.5431	0.9970
SE		0.7369	0.0152	0.0001	8.2725	0.5467	0.0165	0.0001	28.4287

[a,b]Means followed by superscript letters are different as per Tukey's test ($P < 0.05$).
N = 6.
Abbreviations: dpi, D post inoculation; GSH, reduced glutathione; GSSG, oxidized glutathione; TSAA, total sulfur amino acid.

2004). However, the damage and concurrent inflammation caused by the *Eimeria* infection might have triggered a signaling pathway that modulates the TJP expression to protect the intestinal tight junction. This regulatory mechanism could be mediated by anti-inflammatory cytokines, such as interleukin 10 (**IL-10**), and proinflammatory ones, such as interferon gamma (**IFN-γ**). IL-10 production in birds challenged with *Eimeria* has been reported (Han and Baker, 1993; Rothwell et al., 2004; Cornelissen et al., 2009), and IL-10 appears to have a positive effect on the TJP, including Cla-1 and ZO-1 in hepatocytes of mice (Mazzon et al., 2002). Furthermore, occludin downregulation and IFN-γ upregulation as a consequence of *Eimeria* infection have been previously demonstrated (Laurent et al., 2001; Khatlab et al., 2019), and IFN-γ has been shown to downregulate occludin expression *in vitro* in intestinal human cells (Mankertz et al., 2000).

The removal of reactive oxygen species (**ROS**) is achieved through a combined effect of enzymatic (SOD) and nonenzymatic processes (GSH) (Atmaca, 2004). The ROS are known to cause damage to the DNA, proteins, and lipids in the cells (Akbarian et al., 2016); therefore, it is important to keep an active antioxidant defense system in the cells. During the challenge phase, the SOD activity was increased in the *Eimeria*-challenged birds compared with the unchallenged ones. In our study, we evaluated the total SOD activity without distinction between manganese SOD and copper SOD, iron SOD, and zinc SOD. Because manganese SOD is an inducible enzyme, its activity might have been increased as an adaptive response to stress (Surai, 2016) in the challenged birds, contributing to a higher total SOD activity. Moreover, the ratio between GSH and GSSG is used as an indicator of oxidative stress in the cells (Townsend et al., 2003). In the present study, we observed a numerical decrease in GSH and increase in GSSG, resulting in a significantly lower GSH:GSSG in the challenged birds during the challenge phase. These findings are suggestive of an increased oxidative stress status due to the *Eimeria* challenge, which has been previously demonstrated by Koinarski et al. (2005) and Georgieva et al. (2006, 2011).

The TSAA levels also influenced the performance results, regardless of the *Eimeria* challenge. The birds fed with the TSAA-deficient diet (70% TSAA diet) had the lowest FI and BWG throughout the experimental period (1–26 D of age). Similarly, Rochell et al. (2016a), Ren et al. (2019), and Fagundes et al. (2020) observed a reduction in BWG and the gain-to-feed ratio when birds were fed with Met- and TSAA-deficient diets. Moreover, during the challenge phase (0–6 dpi), a significant interaction among TSAA levels and the *Eimeria* challenge was found for FCR. The challenged birds fed with the 100% TSAA diet showed FCR values statistically similar to those of birds in the unchallenged group. This suggests that the use of the 100% TSAA diet, obtained by the addition of Met to the diet, could partially ameliorate the negative effect of the *Eimeria* challenge on feed efficiency during the challenge phase. Ren et al. (2019), however, did not find interactions between the *Eimeria* challenge and TSAA levels and concluded that the supplementation of TSAA at levels higher than 0.80%, on a standardized ileal digestibility basis, was not able to counteract the negative effects of a cocci infection.

Moreover, at 6 dpi, the AIDCP of birds fed with the 70% TSAA diet was higher than that of the birds fed with the 100% TSAA diet. We could observe a gradual numerical reduction in the AIDCP as we increased the TSAA levels, going from 71.8% in birds fed with the 70% TSAA diet to 70.4% in birds fed with the 85% TSAA diet and 64.8% in birds fed with the 100% TSAA diet. While the AA in the intact protein from feed ingredients need to be digested and are not released completely by the digestive enzymes in the lumen, the crystalline AA are fully available and rapidly absorbed in the intestine (Yen et al., 2004; Wu et al., 2014). Therefore, birds supplemented with higher levels of crystalline Met would have less need to break down the dietary protein to meet their TSAA requirement, leading to less digested CP in the ileum. Moreover, it has been shown that intestinal mRNA levels of chymotrypsin and duodenal enterokinase were upregulated in pigs fed with low-CP diet (He et al., 2016) and the expression of AA transporters was upregulated in low-Met diets in broilers (Fagundes et al., 2020). Therefore, protein digestion and absorption of birds fed with TSAA-deficient diet could be increased in a compensatory manner.

Few differences among TSAA levels were observed with regard to the intestinal histomorphology and TJP gene expression. In the present study, we attempted to keep the diets isocaloric and isonitrogenous by balancing the addition of Met with Gly, which would be the only varying components of the diets. However, Gly has secondary functions that could improve intestinal health. This AA is involved in bile salt formation, which is directly related to energy digestibility (Han and Baker, 1993), in synthesis of purine and pyrimidines, which is important for high rates of protein synthesis and cell proliferation in the intestine (Burrin and Reeds, 1997), and in the gene expression and methylation of proteins and DNA (Wu et al., 2014). According to Siegert et al. (2015), if the requirement for TSAA is fulfilled, the necessity for endogenous conversion of Met to Cys decreases, which reduces the demand for Gly. Therefore, birds fed with 70 and 85% TSAA diets had higher demand for Gly, which was possibly met by Gly supplementation, improving their GIT health in a similar manner to the 100% TSAA diet. Additional studies are needed to investigate the effects of Gly in combination with TSAA in improving the intestinal health of broilers and the use of other nonessential AA, such as alanine, to balance the total nitrogen levels in the diet.

As previously mentioned, GSH has a crucial role in the detoxication of ROS (Kretzschmar, 1996), and GSH deficiency puts the cell at risk of oxidative damage (Townsend et al., 2003). During the challenge phase, an interaction was found between the challenge and diets, and the supplementation of Met to reach 85 and 100% levels of TSAA was able to counteract the negative

effect of the *Eimeria* challenge on the GSH concentration. Moreover, during the recovery phase, birds fed with the 100% TSAA diet showed higher GSH concentration than birds fed with the 70 and 85% TSAA diets, regardless of the challenge. Similarly, Shen et al. (2015) observed that the supplementation of Met increased total GSH production in the liver. A decrease in GSH concentration in the 70 and 85% TSAA diet–fed birds might be a result of the decreased availability of substrates needed for GSH synthesis, especially Cys, which is considered to be one the most limiting AA in this process (Grimble et al., 1992). In vivo, rats fed with TSAA-deficient diets showed lower concentrations of GSH and Cys as well as a more oxidized redox state in plasma and the intestinal mucosa (Nkabyo et al., 2006). Del Vesco et al. (2015) observed that the supplementation with the recommended and excessive doses of DL-Met and TSAA led to increased expression of genes that are part of the antioxidant system of GSH in broilers.

Unexpectedly, we did not observe many interactions between the *Eimeria* challenge and TSAA treatments. This finding could be an indication that 1) there was no change in the TSAA requirement when broilers were infected with *Eimeria* (Murillo et al., 1976; Willis and Baker, 1981; Ren et al., 2019) and 2) the supplementation of Met or TSAA alone was not sufficient to mitigate the negative effects of an *Eimeria* infection on growth and intestinal health (Ren et al., 2019). Nevertheless, our overall results showed that the *Eimeria* challenge negatively affected growth performance, intestinal histomorphology, and nutrient digestion; impaired the antioxidant system; and modulated the expression of TJP genes throughout the experimental period. Regarding the TSAA levels, the use of the 70% TSAA diet (without Met supplementation) reduced growth performance throughout the experiment. However, no major differences were observed among the TSAA levels with respect to intestinal histomorphology and TJP gene expression, which could be related to the different Gly levels used in the diets. Furthermore, the Met supplementation up to 85 and 100% levels of TSAA was able to counteract the negative effect of the *Eimeria* challenge on GSH concentration, potentially improving the antioxidant system of birds under the challenge.

ACKNOWLEDGMENTS

This study was financed in part by a cooperative agreement 58-6040-8-034 from United States Department of Agriculture-Agricultural Research Service. The authors thank Dr. Lorraine Fuller from the Department of Poultry Science, University of Georgia, for providing the *Eimeria* oocysts used in this study.

Conflict of Interest Statement: The authors did not provide a conflict of interest statement.

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