The effects of L-Arginine supplementation on growth performance and intestinal health of broiler chickens challenged with *Eimeria* spp.

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ABSTRACT This study evaluated the effects of varying levels of L-arginine (**Arg**) on performance and intestinal health of broilers challenged with *Eimeria*. Cobb 500 male chicks ($n = 720$) were randomly distributed in a 5×2 factorial arrangement (6 replicates/12 birds). The main factors were Arg levels (1.04, 1.14, 1.24, 1.34, 1.44%) and challenge or non-challenge with *Eimeria*. At day 12, in the challenge group, each bird received orally 12,500 *Eimeria maxima*, 12,500 *Eimeria tenella*, and 62,500 *Eimeria acervulina* sporulated oocysts. At 5 d postinfection (**dpi**), intestinal permeability was measured. At 6 and 14 dpi, performance, intestinal histomorphology, nutrient digestibility, tight junction protein (**TJP**) gene expression, and antioxidant markers were evaluated. Few interactions were found, and when significant, the supplementation of Arg did not counteract the negative effects of *Eimeria* challenge. Challenge, regardless of Arg level, increased intestinal permeability, although the expression of Claudin-1, a TJP, was upregulated. At 6 dpi, the antioxidant system was impaired by the challenge. Moreover, growth performance, intestinal histomorphology, and nutrient digestibility were negatively affected by challenge at 6 and 14 dpi. Regardless of challenge, from 0 to 14 dpi, birds fed 1.44% showed higher weight gain than 1.04% of Arg, and birds fed 1.34% showed lower feed conversion than 1.04% of Arg. At 5 dpi, intestinal permeability was improved in birds fed 1.34% than 1.04% of Arg. Moreover, 1.34% of Arg upregulated the expression of the TJP Zonula occludens-1 (**ZO-1**) as compared with 1.24 and 1.44% of Arg at 6 dpi. At 14 dpi, 1.44% of Arg upregulated the expression of ZO-1 and ZO-2 compared with 1.24 and 1.34% of Arg. The nutrient digestibility was quadratically influenced by Arg, whereas the antioxidant markers were unaffected. Thus, the challenge with *Eimeria* had a negative impact on growth and intestinal health. The dietary supplementation of levels ranging from 1.24 to 1.44% of Arg showed promising results, improving overall growth, intestinal integrity, and morphology in broilers subjected or not to *Eimeria* challenge.

Key words: L-arginine, broiler, *Eimeria*, intestinal health

INTRODUCTION

Coccidiosis is the most prevalent parasitic disease in poultry production, and it is caused by protozoa of the genus *Eimeria* (Williams, 2005). It has been estimated that the annual cost with coccidiosis surpasses £38 million in the United Kingdom, which includes losses associated to prophylaxis, treatment, and subclinical effects on the performance (Williams, 1999). Moreover, in the United States, preventative medication was reported to cost, approximately, US$ 127 million to the poultry industry annually (Chapman, 2009). Even though coccidiosis has been controlled for decades with the use of anticoccidial drugs (Peek and Landman, 2011), there has been an increase in drug resistance and consumer concerns about the use of chemotherapeutic agents in the animal feed (Williams, 1998; Peek and Landman, 2011). Therefore, the manipulation of the intestinal health through nutrition could be a potential strategy to reduce the impact of coccidial infection in birds.

Historically, studies with amino acids (**AA**) have focused on the protein synthesis and accretion, whereas the secondary functions of AA in the metabolism have been neglected, especially when formulating diets and

determining requirements (Wu et al., 2009; Wu, 2010). According to Wu (2010, 2013), the secondary functions of some AA are related to the their role in regulating specific signaling pathways, leading to changes in gene expression, protein turnover, modulating oxidative defense, and improving health and growth of animals. More specifically, arginine (**Arg**), which is an essential AA for poultry, was shown to directly or indirectly, through derivative molecules, alleviate oxidative stress, improve antioxidant capacity, and attenuate the intestinal mucosa disruption.

Arginine supplementation was demonstrated to improve the total antioxidant capacity in quails and broiler breeders (Atakisi et al., 2009; Duan et al., 2015). Moreover, Arg is a strong superoxide and hydroxyl radical scavenger, and its supplementation increased the activity and expression of important molecules in the antioxidant system, such as glutathione and superoxide dismutase (**SOD**) in rats (Liang et al., 2018). Nitric oxide, which is derived from Arg, was shown to be an important vasodilator and immune-modulator and to promote metabolic regulation by increasing hormone sensitive lipase and downregulating genes associated with lipogenesis and gluconeogenesis (Moncada et al., 1991; Jobgen et al., 2006; Morris, 2006; Khajali and Wideman, 2010), as well as to have direct toxic effect on other protozoa parasites (Vespa et al., 1994; Alvarez et al., 2011).

Furthermore, Arg is, indirectly, the precursor of putrescine, spermidine, and spermine through L-ornithine and agmatine. Putrescine can be formed virtually in all tissues, including intestinal cells of poultry, via Arg-ornithine-putrescine pathway that is catalyzed by the enzymes arginase and ornithine decarboxylase (Tabor and Tabor, 1984; Fischer da Silva et al., 2007). Alternatively, putrescine can be synthesized in the Arg–agmatine—putrescine pathway, which includes the enzymes arginine decarboxylase and agmatinase (Horyn et al., 2005). Polyamines have been recognized as important molecules in modulating gene expression, protein translation, cell growth, proliferation, and apoptosis, and providing defense against oxidative stress (Seiler, 1996; Miller-Fleming et al., 2015). Therefore, Arg supplementation could be beneficial during a coccidia infection. For this reason, the aim of this study was to evaluate the effect of Arg as a functional AA in partially alleviating the detrimental effects of an *Eimeria* challenge on performance, nutrient digestibility, intestinal health, and antioxidant system in broilers during the challenge and recovery phases.

MATERIALS AND METHODS

General Procedures

The experiment was conducted under the approval of the Institutional Animal Care and Use Committee of University of Georgia (Athens, GA). A total of 720 off-sex Cobb 500 1-day-old male broiler chicks were distributed in a completely randomized design with a factorial arrangement 5×2, with 6 replicates of 12 birds each. The main effects were the diets (increasing arginine levels) and pathogen exposure (challenged with or without a pool of *Eimeria acervulina, Eimeria maxima, and Eimeria tenella*). The chicks were allocated to 60 identical metabolic cages equipped with a feeder and drinker, providing free access to water and feed from 1 to 26 d of age. Temperature and lighting program followed the recommendation of Cobb 500 management guide (Cobb Vantress, 2018a).

The diets were based on corn and soybean meal and formulated to meet or exceed the nutritional levels from the starter phase (Cobb Vantress, 2018b) for all ingredients, except for Arg (Table 1). It is believed that changes in feed formulation, for example feed ingredients and nutritional levels, could be a source of inflammation in the gastrointestinal tract (Kogut et al., 2018; Cardoso Dal Pont et al., 2020). To avoid a potential influence of the basal diet on the intestinal health status, the same nutritional level was used from 1 to 26 d. The dietary treatments were determined by formulating an Arg deficient diet, without Arg supplementation (1.04%), and adding Arg as a replacement of the inert component (sand) to reach equidistant calculated levels below and above the Cobb 500 recommendation (1.24%). The treatments were as follows: 1.04, 1.14, 1.24, 1.34, and 1.44% of digestible Arg. The diets were kept isocaloric and isonitrogenous using glycine to balance the addition of Arg. The diets included 0.3% of chromium oxide (Cr_2O_3, Sigma Aldrich, St. Louis, MO) as an indigestible marker for determination of apparent ileal digestibility of nutrients. Diets were sent to the Agricultural Experiment Station Chemical Laboratories at the University of Missouri-Columbia, and the analyzed Arg levels were 1.10, 1.15, 1.23, 1.35, and 1.41%.

On day 12, the birds in challenged group (360 birds) were orally gavaged with 1 mL of a solution containing distilled water and approximately 12,500 sporulated *E. maxima*, 12,500 *E. tenella* oocysts, and 62,500 sporulated *E. acervulina* oocysts, whereas the other half was gavaged with distilled water. The challenge dose was determined by a previous study conducted at our lab and was intended to cause mild coccidiosis infection (Teng et al., 2020).

The birds and feed were weighed, by cage, at 12, 18, and 26 d of the experiment. Mortality was recorded daily. The body weight gain (**BWG**), and feed intake (**FI**) were determined from 12 to 18 d (0–6 d postinoculation–***dpi***, challenge phase) and from 19 to 26 d (6–14 dpi, recovery phase). Feed conversion rate (**FCR**) was calculated and corrected for mortality.

Sample Collection and Analysis Performed

Intestinal Permeability On day 17 (5 ***dpi***), 1 bird per cage (6 birds/treatment) was orally gavaged with 1 mL of fluorescein isothiocyanate dextran (2.2 mg/mL, **FITC-d**, 100 mg, MW 4,000; Sigma-Aldrich, Canada). These birds were kept, without feed, for 2 h-post oral gavage, followed by blood collection by cardiac

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

	Arginine levels (%)				
Ingredients	1.04	1.14	1.24	1.34	1.44
Corn	67.85	67.85	67.85	67.85	67.85
Soybean meal	24.14	24.14	24.14	24.14	24.14
Soybean oil	0.50	0.50	0.50	0.50	0.50
Salt	0.35	0.35	0.35	0.35	0.35
Limestone	1.22	1.22	1.22	1.22	1.22
Dicalcium phosphate	1.66	1.66	1.66	1.66	1.66
Vitamin premix[1]	0.25	0.25	0.25	0.25	0.25
Mineral premix[2]	0.08	0.08	0.08	0.08	0.08
L-Methionine	0.38	0.38	0.38	0.38	0.38
L-Lysine	0.52	0.52	0.52	0.52	0.52
L-Glutamine	0.94	0.94	0.94	0.94	0.94
L-Threonine	0.18	0.18	0.18	0.18	0.18
L-Arginine	0.00	0.10	0.20	0.30	0.40
L-Valine	0.05	0.05	0.05	0.05	0.05
L-Isoleucine	0.10	0.10	0.10	0.10	0.10
Glycine	1.00	0.83	0.66	0.49	0.31
Sand	0.48	0.55	0.62	0.69	0.77
Chromium oxide	0.30	0.30	0.30	0.30	0.30
Calculated composition					
ME (kcal/kg)	3,010	3,010	3,010	3,010	3,010
CP (%)	20.0 (20.14)[3]	20.0 (19.46)	20.0 (19.94)	20.0 (19.98)	20.0 (19.87)
Dig. Lysine (%)	1.18	1.18	1.18	1.18	1.18
Dig. Methionine (%)	0.64	0.64	0.64	0.64	0.64
Dig. Met-Cys (%)	0.88	0.88	0.88	0.88	0.88
Dig. Arginine (%)	1.04 (1.10)[4]	1.14 (1.15)	1.24 (1.23)	1.34 (1.35)	1.44 (1.41)
Arg:Lys ratio	0.88	0.96	1.05	1.13	1.22
Ca (%)	0.90	0.90	0.90	0.90	0.90
Available P (%)	0.45	0.45	0.45	0.45	0.45

[1]Provided per kg of DSM Vitamin premix: Vit. A 2,204,586 IU, Vit. D$_3$ 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]Analyzed crude protein levels.
[4]Analyzed arginine levels.

puncture immediately after euthanasia by cervical dislocation. The blood was centrifuged at 3,000 rpm for 12 min (Eppendorf Centrifuge 5430R, Eppendorf, Hamburg, Germany), and 100 μL of serum was used to determine the FITC-d concentration. The samples were placed, in duplicate, in a dark 96-well microplate (Ref. 655077, Greiner Bio-one, Monroe, NC) and read in a spectrophotometer (SpectraMax ABS Plus, Softmax Pro 7 software, Molecular devices, San Jose, CA) at excitation wavelength of 485 nm and emission wavelength of 528 nm. The samples were compared with a standard curve with known FITC-d concentration, prepared using the serum from 10 extra birds that were not part of the experiment. The entire procedure was performed in a dark room, and the FITC-d solution,

the Meckel's diverticulum until 2 cm from the ileocecal junction. The samples from the same replicate were pooled, dried in a ventilated oven at 75°C for 48 h, and finely ground using a Kitchen aid coffee grinder. Gross energy was measured using a bomb calorimeter (IKA Calorimeter C1, IKA Works Inc., Wilmington, NC), and the chromium oxide concentration was measured following the methodology described by Dansky and Hill (1952) at the University of Georgia. The crude protein (**CP**) was analyzed in the Agricultural Experiment Station Chemical Laboratories at the University of Missouri-Columbia (N × 6.25, LECO). The apparent ileal digestibility of CP, energy, and dry matter were calculated according to the following equation:

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

blood, and serum samples were protected from direct light exposure.

Nutrient Digestibility On 6 and 14 **dpi** (18 and 26 d, respectively), 4 birds/replicate were randomly selected, euthanized by cervical dislocation, and digesta were collected from an ileal section starting from 2 cm below

where (nutrient/Cr$_2$O$_3$) is the ratio of dry matter, CP, and energy to Cr$_2$O$_3$ in the diet or ileal digesta.

Intestinal Morphology On 6 and 14 **dpi**, 1 bird/replicate was euthanized by cervical dislocation, and portions of approximately 2 cm of the duodenal loop, middle jejunum, and ileum were collected, flushed with 1x

PBS (National Diagnostics, Atlanta, GA), and stored in 10% neutral-buffered formalin until processed. For the preparation of the histology slides, the tissue samples were dehydrated in increasing concentrations of ethanol, diaphanized in xylol, and embedded in paraffin. Serial cuts of 4 μm were stained with hematoxylin-eosin and analyzed in a light microscope (1.6 × 10 × 1.6 ×) (Leica DC500 camera, Leica Mucrosystems Inc., Buffalo Groove, IL). Pictures were taken and analyzed using ImageJ (Image Processing and Analysis in Java–ImageJ 1.50i, National Institutes of Health) to measure crypts depth and villi height of 4 villi and 4 crypts per slide. The ratio of villi height to crypts depth was calculated from each sample.

Superoxide Dismutase and Glutathione For the SOD activity analysis and quantification of reduced glutathione (**GSH**) and oxidized glutathione (**GSSG**), liver samples were collected from 1 bird/replicate at 6 and 14 **dpi**, snap-frozen in liquid nitrogen, and kept in −80°C. Samples were analyzed within 24 h of collection. For SOD, approximately 75 mg of liver was homogenized in 1 mL of cold buffer (20 mmol/L HEPES buffer, pH 7.2, 2 mmol/L EGTA, 10 mmol/L mannitol, and 70 mmol/L sucrose per gram of tissue). Homogenized samples were centrifuged at 1,500 × g for 5 min at 4°C, and the supernatant was removed. Subsequently, the samples were diluted 1:1,000 using the sample buffer, and the analysis was performed using a superoxide dismutase assay kit (Cayman chemical, Superoxide dismutase assay kit, item No. 706002, AnnArbor, MI) following the instructions provided by the manufacturer. For GSH and GSSG quantification, approximately 100 mg of liver was homogenized in 800 μL of solution containing cold PBS and 10 mmol/L of diethylenetriamine pentaacetate, for no longer than 25 s. After homogenized, 500 μL of the tissue solution was transferred to a tube containing 500 μL of 10% PCA, which were kept in −80°C until analyzed. The samples were submitted to be analyzed by high performance liquid chromatography (Dionex UltiMate 3000, Thermo Scientific, Waltham, MA) coupled with electrochemical detection. The results were given as a ratio between GSH and GSSG (**GSH/GSSG**) concentrations.

To standardize the samples and obtain the SOD activity and GSH/GSSG quantification relative to the protein concentration in the sample, a protein quantification assay was also performed (Pierce BCA Protein Assay Kit, Ref. 23,227, Thermo Scientific, Rockford, IL), following the manufacturer's instructions. Briefly, 25 μL of the diluted sample used in the SOD assay as well as standards with known protein concentrations (Bovine Serum Albumin - 2 mg/mL) were transferred to a 96-well microplate. Subsequently, 200 μL of the working reagent (reagent A: reagent B, 50:1) was added and mixed thoroughly using a plate shaker for 30 s. The plate was incubated at 37°C for 30 min using an incubator (VWR 1525 Digital Incubator, Sheldon Manufacturing Inc., Cornelius, OR), and the absorbance at 562 nm was measured on a plate reader (SpectraMax ABS Plus, Molecular Devices, San Jose, CA). The SOD and GSH/GSSG results were divided by the BCA results to obtain the corrected values.

Nitric Oxide Assay On 6 and 14 **dpi**, blood from 1 bird/replicate was rapidly collected by heart puncture immediately after euthanasia by cervical dislocation, without causing hemolysis (Bryan and Grisham, 2007), using blood collection tubes containing EDTA (Vacuette tube 9 mL K3E E3EDTA, Greiner Bio-One, Monroe, NC). The blood was centrifuged at 550 × g for 10 min at 4°C, and plasma was separated from the red blood cells and stored at −80°C until analyzed. The plasma samples were first ultrafiltered using centrifugal filter units (Amicon Ultra-0.5 centrifugal filter unit, 10 kDa, Millipore Sigma, Burlington, MA) to remove the background absorbance due to the presence of hemoglobin. Subsequently, total NO production, given as the sum of nitrite (NO_2^-) and nitrate (NO_3^-), was determined using a Nitrate/Nitrite colorimetric assay kit (Cayman chemical) according to the manufacturer's instructions.

Gene Expression Analysis Jejunum samples were collected from 2 inches above the middle of jejunum, flushed with 1× PBS, and snap-frozen in liquid nitrogen. This region was chosen because it can be co-infected by *E. acervulina* and *E. maxima*. Samples were kept in −80°C until processed. Total RNA was extracted using QIAzol Lysis Reagent (Qiagen, Germantown, MD), and RNA quantity and purity were determined (Nanodrop 1000 spectophotometer, Thermo Fisher Scientific, Pittsburgh, PA). Subsequently, cDNA was synthesized from total RNA using high-capacity cDNA reverse transcription kits (Thermo Fisher Scientific) and diluted 1:5 for quantitative reverse-transcriptase polymerase chain reaction analysis. The quantitative reverse-transcriptase polymerase chain reaction was performed on an Applied Biosystems StepOnePlus (Thermo Fisher Scientific) with iTaq Universal SYBR Green Supermix (BioRad, Hercules, CA). Inducible nitric oxide synthase (**iNOS**) (Li et al., 2008), 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) gene expressions were evaluated, and glyceraldehyde-3-phosphate dehydrogenase was used as the reference gene. The forward and reverse primers are shown in Table 2. The running condition used for all genes was: 95°C for 15 s, 58°C for 20 s, and 72°C for 15 s during 40 cycles. Samples were run in duplicate, and relative gene expression data were analyzed using the $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). The mean ΔCt of challenged 1.04% of Arg was used to calculate the ΔΔCt value (Table 5).

Statistical Analysis

Data were first tested for homogeneity of variances and normality of studentized residuals. Performance results from 1 to 12 d of age were subjected to one-way

Table 2. Primer pairs used for quantitative reverse-transcriptase polymerase chain reaction analysis.

Gene[1]	Gene bank identification	Primer sequence, sense/antisense	Product size (bp)[2]
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
Ocln	XM_026041453.1	ACGGCAGCACCTACCTCAA/ GGCGAAGAAGCAGATGAG	122
iNOS	NM_204961.1	CAGCTGATTGGGTGTGGAT/ TTTCTTTGGCCTACGGGT	158

[1]GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Cla-1, Claudin-1; Cla-2, Claudin-2; ZO-1, Zonula Occludens-1; ZO-2, Zonula Occludens-2; Ocln, Occludin; iNOS, nitric oxide synthase.
[2]bp, Base pairs.

ANOVA, and in case of significant differences, the treatments were compared by Tukey's test. All other data were subjected to 2-way ANOVA, obtaining results for each factor (Arg dietary levels and challenge) as well as their interaction. In case of significant differences, the treatments were compared by Tukey's test. Moreover, the analysis was extended to include polynomial contrasts to test linear and quadratic components of Arg levels and interaction components, such as linear and quadratic effects of Arg at each level of challenge (challenged and unchallenged). All statistical procedures were performed using SAS University Edition (version 9.4, SAS Institute, Cary, NC) following the methodology and codes described by Shim et al. (2014) and Billard et al. (2014). Statements of significance were based on $P < 0.05$.

Table 3. Body weight gain (BWG, kg), feed intake (FI, kg), and feed conversion ratio (FCR) from 0 to 6, 7 to 14, and 0 to 14 d postinoculation (dpi) according to dietary digestible Arg levels (1.04, 1.14, 1.24, 1.34, and 1.44%) in broilers challenged (Cha) or unchallenged (Unch) with a mixed *Eimeria* spp. infection.

Challenge	Arg	0–6 dpi BWG	FI	FCR	7–14 dpi BWG	FI	FCR	0–14 dpi BWG	FI	FCR
Cha	1.04	0.22	0.39	1.75	0.46	0.78	1.69	0.68	1.17	1.71
	1.14	0.22	0.38	1.75	0.51	0.79	1.56	0.73	1.18	1.62
	1.24	0.22	0.39	1.73	0.52	0.84	1.64	0.74	1.23	1.67
	1.34	0.22	0.39	1.79	0.51	0.81	1.58	0.75	1.23	1.64
	1.44	0.24	0.40	1.67	0.51	0.83	1.63	0.74	1.22	1.68
Unch	1.04	0.29	0.41	1.44	0.54	0.86	1.61	0.82	1.27	1.55
	1.14	0.29	0.44	1.42	0.54	0.85	1.56	0.83	1.28	1.50
	1.24	0.30	0.42	1.47	0.56	0.85	1.52	0.86	1.29	1.50
	1.34	0.29	0.42	1.43	0.54	0.85	1.50	0.83	1.29	1.47
	1.44	0.30	0.44	1.45	0.57	0.87	1.52	0.87	1.32	1.50
Challenge	Cha	0.22[b]	0.39[b]	1.74[a]	0.50[b]	0.81[b]	1.62[a]	0.73[b]	1.20[b]	1.66[a]
	Unch	0.29[a]	0.42[a]	1.44[b]	0.55[a]	0.86[a]	1.54[b]	0.84[a]	1.29[a]	1.50[b]
Arg	1.04	0.25	0.40	1.60	0.50	0.82	1.65[a]	0.75[b]	1.22	1.63[a]
	1.14	0.26	0.41	1.58	0.53	0.82	1.56[a,b]	0.78[a,b]	1.23	1.56[b]
	1.24	0.26	0.40	1.60	0.54	0.85	1.58[a,b]	0.80[a,b]	1.26	1.58[a,b]
	1.34	0.26	0.40	1.61	0.52	0.83	1.54[b]	0.79[a,b]	1.26	1.55[b]
	1.44	0.27	0.42	1.56	0.54	0.85	1.57[a,b]	0.81[a]	1.27	1.59[a,b]
SEM	Challenge	0.004	0.005	0.026	0.011	0.012	0.024	0.013	0.015	0.017
	Arg	0.002	0.003	0.015	0.006	0.007	0.014	0.007	0.008	0.010
	Interaction	0.002	0.002	0.010	0.004	0.005	0.009	0.005	0.006	0.007
P-value	Challenge	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	Arg	0.192	0.122	0.671	0.089	0.303	0.018	0.039	0.113	0.022
	L[1]	0.046	0.039	0.482	0.026	0.084	0.026	0.005	0.011	0.124
	Q[1]	0.250	0.875	0.441	0.256	0.986	0.040	0.287	0.502	0.080
	Interaction	0.822	0.287	0.306	0.531	0.325	0.421	0.535	0.861	0.810
	L × Unch[2]	0.290	0.101	0.822	0.166	0.805	0.020	0.058	0.183	0.082
	L × Cha[2]	0.075	0.188	0.223	0.074	0.029	0.373	0.034	0.022	0.655
	Q × Unch[3]	0.964	0.657	0.932	0.762	0.298	0.150	0.993	0.968	0.158
	Q × Cha[3]	0.116	0.523	0.257	0.060	0.306	0.134	0.145	0.370	0.078

[a,b]Means followed by superscript letters are different by Tukey's test ($P < 0.05$) within the column.
[1]L, Linear effect; Q, Quadratic effect.
[2]L at Unch—Linear effect of Arg on unchallenged birds; L at Cha–Linear effect of Arg on challenged birds.
[3]Q at Unch—Quadratic effect of Arg on unchallenged birds; Q at Cha–Quadratic effect of Arg on challenged birds.

RESULTS

Growth Performance

During the prechallenge period (1–12 d), the BWG, FI, and FCR did not differ among the treatments ($P > 0.05$) (data not shown). During the challenge phase (0–6 **dpi**), birds that received the challenge had inferior BWG, FI, and worse FCR ($P < 0.001$) when compared with the non-challenged birds (Table 3). Moreover, BWG ($P < 0.05$) and FI ($P < 0.05$) linearly increased with increasing levels of Arg. On recovery phase (19–26 d, 7–14 **dpi**), the BWG and FI were inferior for birds challenged with *Eimeria* compared with the unchallenged ones ($P < 0.001$). Additionally, the FCR was affected by both diets ($P < 0.001$) and challenge ($P < 0.05$). The challenged birds had worse FCR than the unchallenged ones, and birds fed 1.34% of Arg had improved FCR than the ones fed 1.04% of Arg. Additionally, BWG ($P < 0.05$) and FCR ($P < 0.05$) linearly increased and decreased, respectively, with addition of Arg to the diet. In the interaction term, the FI ($P < 0.05$) of challenged birds linearly increased with increasing levels of Arg.

From 12 to 26 d of age (0–14 **dpi**), the FI was affected only by challenge, whereas the BWG and FCR were affected by both challenge and Arg levels in the diet. Birds challenged with *Eimeria* had reduced BWG, FI, and worse FCR ($P < 0.001$) compared with the unchallenged birds. Furthermore, birds fed 1.44% of Arg had improved BWG than those fed 1.04% of Arg ($P < 0.05$), whereas birds fed 1.34 and 1.14% of Arg showed better FCR than those fed 1.04% of Arg ($P < 0.05$). Furthermore, as the interaction component, the BWG ($P < 0.05$) and FI ($P < 0.05$) in the challenged birds linearly increased as Arg levels increased.

Intestinal Health

Intestinal Permeability Effects of challenge ($P < 0.001$) and Arg levels ($P < 0.05$) were observed for intestinal permeability (Table 4). Birds challenged with *Eimeria* showed higher concentration of FITC-d in serum compared with the unchallenged birds, indicating worsened gut permeability. Moreover, birds fed 1.34% of Arg showed significantly lower values (improved gut permeability) than birds fed 1.04% of Arg. Moreover, in the interaction term, a positive quadratic effect ($P < 0.01$) was found in the challenge birds, and the lowest FITC-d concentration level was obtained when 1.34% of Arg was used.

Table 4. Fluorescein isothiocyanate dextran concentration (FITC-d, µg/mL) at 5 d postinoculation (dpi), and claudin-1 (Cla-1), claudin-2 (Cla-2), zonula occludens-1 (ZO-1), zonula occludens-2 (ZO-2), and occludin (Ocln) gene expression at 6 and 14 dpi according to dietary Arg levels (1.04, 1.14, 1.24, 1.34, and 1.44%) in broilers challenged (Cha) or unchallenged (Unch) with a mixed *Eimeria* spp. infection.

Challenge	Arg	5 dpi FITC-d	6 dpi Cla-1	Cla-2	ZO-1	ZO-2	Ocln	14 dpi Cla-1	Cla-2	ZO-1	ZO-2	Ocln
Cha	1.04	0.38	1.26	1.06	1.19	1.09	1.03	1.04	0.88	1.03	1.00	1.12
	1.14	0.34	2.07	1.15	1.21	1.09	1.15	0.98	1.09	1.15	1.11	1.18
	1.24	0.18	1.24	0.97	0.93	0.91	1.04	0.87	0.85	0.98	0.96	1.06
	1.34	0.07	1.32	1.08	1.43	1.16	1.23	0.95	0.89	0.94	0.89	1.08
	1.44	0.41	0.88	0.93	1.07	0.97	1.15	1.15	0.97	1.27	1.16	1.29
Unch	1.04	0.16	0.77	1.02	1.35	1.08	0.88	1.01	0.78	1.07	1.00	1.14
	1.14	0.03	0.50	0.84	1.01	0.94	1.11	1.19	0.87	1.13	1.12	1.29
	1.24	0.13	0.50	0.95	1.03	1.00	1.15	1.20	0.89	0.88	0.81	1.09
	1.34	0.05	0.40	1.13	1.47	1.13	1.05	0.89	0.82	0.87	0.93	1.14
	1.44	0.09	0.47	0.89	0.88	0.91	1.13	0.80	0.87	1.24	1.19	1.13
Challenge	Cha	0.28[a]	1.35[a]	1.04	1.17	1.04	1.12	1.00	0.94	1.07	1.02	1.15
	Unch	0.09[b]	0.53[b]	0.97	1.15	1.01	1.06	1.02	0.85	1.04	1.01	1.16
Arg	1.04	0.27[a]	1.01	1.04	1.27[a,b]	1.08	0.96	1.02	0.83	1.05[a,b]	1.00[a,b]	1.13
	1.14	0.18[a,b]	1.28	0.99	1.11[a,b]	1.01	1.13	1.08	0.98	1.14[a,b]	1.12[a,b]	1.24
	1.24	0.16[a,b]	0.87	0.96	0.98[b]	0.95	1.10	1.04	0.87	0.93[b]	0.88[b]	1.07
	1.34	0.06[b]	0.86	1.11	1.45[a]	1.15	1.14	0.92	0.86	0.90[b]	0.91[a,b]	1.11
	1.44	0.25[a,b]	0.67	0.91	0.97[b]	0.94	1.14	0.98	0.92	1.25[a]	1.17[a]	1.21
SEM	Challenge	0.044	0.175	0.098	0.116	0.069	0.075	0.145	0.068	0.091	0.071	0.068
	Arg	0.025	0.101	0.057	0.067	0.039	0.043	0.083	0.039	0.052	0.041	0.039
	Interaction	0.018	0.071	0.040	0.047	0.028	0.031	0.059	0.028	0.037	0.029	0.028
P-value	Challenge	<0.001	<0.001	0.409	0.843	0.562	0.408	0.897	0.152	0.668	0.844	0.840
	Arg	0.019	0.258	0.650	0.012	0.129	0.397	0.970	0.588	0.038	0.012	0.368
	L[1]	0.269	0.079	0.636	0.463	0.461	0.122	0.636	0.806	0.550	0.524	0.896
	Q[1]	0.008	0.480	0.757	0.922	0.931	0.345	0.913	0.784	0.040	0.033	0.438
	Interaction	0.060	0.237	0.703	0.661	0.783	0.667	0.641	0.769	0.983	0.863	0.704
	L × Unch[2]	0.586	0.420	0.945	0.326	0.615	0.207	0.343	0.681	0.863	0.540	0.588
	L × Cha[2]	0.310	0.091	0.461	0.958	0.588	0.353	0.772	0.948	0.501	0.772	0.453
	Q × Unch[3]	0.552	0.585	0.944	0.867	0.765	0.300	0.344	0.659	0.072	0.052	0.880
	Q × Cha[3]	0.002	0.128	0.712	0.976	0.859	0.771	0.423	0.957	0.257	0.271	0.208

[a,b]Means followed by superscript letters are different by Tukey's test ($P < 0.05$) within the column.
[1]L, Linear effect; Q, Quadratic effect.
[2]L at Unch—Linear effect of Arg on unchallenged birds; L at Cha–Linear effect of Arg on challenged birds.
[3]Q at Unch—Quadratic effect of Arg on unchallenged birds; Q at Cha–Quadratic effect of Arg on challenged birds.

Tight Junction Proteins At 6 **dpi**, Cla-1 gene was upregulated in the challenged birds ($P < 0.001$) compared with the unchallenged ones (Table 4). The ZO-1 gene expression was upregulated when birds were fed 1.34% of Arg compared with 1.24 and 1.44% ($P < 0.05$). At 14 **dpi** (26 d), ZO-1 expression was upregulated when birds were fed 1.44% of Arg compared with 1.24 and 1.34% ($P < 0.05$), and ZO-2 expression was upregulated when birds were fed 1.44% compared with 1.24% of Arg ($P < 0.05$). Moreover, positive quadratic effects were found for ZO-1 ($P < 0.05$) and ZO-2 ($P < 0.05$), with minimum level obtained when 1.34 and 1.24% of Arg were used, respectively.

Intestinal Morphology At 6 **dpi**, the villi height and villi: crypt ratio were both improved in unchallenged than challenged birds in the duodenum ($P < 0.001$) (Table 5). No differences between treatments were observed for duodenal crypts depth ($P > 0.05$). Additionally, positive linear effects were found for villi height ($P \leq 0.05$) and in the challenged group, for duodenal crypt depth ($P < 0.05$). Both traits increased as Arg was supplemented in the diet. In the jejunum, an interaction between factors was found for crypts depth ($P < 0.05$) but not for villi height or villi: crypt ratio ($P > 0.05$). In the challenged group, birds fed 1.24% had greater crypts depth than birds fed 1.44% of Arg, whereas within the unchallenged group, all the dietary treatments were

statistically similar. Additionally, birds fed 1.24% of Arg and challenged with *Eimeria* had greater crypts depths than unchallenged birds fed 1.24% of Arg. In the interaction term, a negative quadratic effect was found for Arg levels when birds were challenged ($P < 0.05$), with peak at 1.34% of Arg. The jejunal villi: crypt ratio was superior for unchallenged birds ($P < 0.001$) than the challenged ones, and no differences were observed for villi height ($P > 0.05$). In the ileum, birds challenged with *Eimeria* showed improved villi height, increased crypts depth, and reduced villi: crypt ratio compared to the unchallenged ones ($P < 0.001$).

At 14 **dpi**, an interaction between factors was found for villi: crypt ratio in the duodenum ($P < 0.047$) (Table 6). In the unchallenged group, birds fed 1.24% of Arg showed greater ratio than birds fed 1.14 and 1.44% of Arg, whereas within the challenged group, all the dietary treatments were statistically similar. Additionally, unchallenged birds fed 1.24 and 1.34% of Arg had improved ratios than their counterparts in the challenged group. In the interaction component, a positive linear effect of Arg was found in the challenged group ($P < 0.05$). The duodenal crypts depth was influenced by challenge and Arg levels. Birds that were challenged had greater crypts depth ($P < 0.001$), and birds fed 1.14, 1.34, and 1.44% of Arg had greater crypts depths than birds fed 1.04 and 1.24% of Arg. No differences

Table 5. Villi height (V, mm), crypts depth (C, mm), and villi: crypt ratio (V:C) in the duodenum, jejunum, and ileum at 6 d postinoculation (dpi) according to dietary Arg levels (1.04, 1.14, 1.24, 1.34, and 1.44%) in broilers challenged (Cha) or unchallenged (Unch) with a mixed *Eimeria* spp. infection.

Challenge	Arg	Duodenum V	C	V:C	Jejunum V	C	V:C	Ileum V	C	V:C
Cha	1.04	1.96	0.22	8.94	1.06	0.23[a,b,c]	4.80	0.88	0.27	3.24
	1.14	1.82	0.21	8.65	1.22	0.28[a,b]	4.38	0.91	0.27	3.70
	1.24	2.03	0.24	9.23	1.21	0.31[a]	3.92	0.83	0.24	3.59
	1.34	2.26	0.26	9.09	1.17	0.26[a,b,c]	4.54	1.02	0.28	3.77
	1.44	2.04	0.29	7.13	1.15	0.23[b,c]	5.05	0.90	0.29	3.29
Unch	1.04	2.24	0.22	10.57	1.13	0.22[b,c]	5.19	0.82	0.17	4.79
	1.14	2.32	0.23	10.42	1.18	0.23[a,b,c]	5.23	0.66	0.14	4.69
	1.24	2.27	0.23	9.80	1.23	0.20[c]	6.33	0.74	0.16	4.80
	1.34	2.52	0.24	10.86	1.31	0.23[b,c]	5.83	0.86	0.18	4.89
	1.44	2.37	0.22	10.92	1.30	0.22[b,c]	5.87	0.80	0.16	5.70
Challenge	Cha	2.02[b]	0.24	8.61[b]	1.16	0.26	4.54[b]	0.91[a]	0.27[a]	3.52[b]
	Unch	2.34[a]	0.23	10.52[a]	1.23	0.22	5.69[a]	0.77[b]	0.16[b]	4.97[a]
Arg	1.04	2.10	0.22	9.76	1.09	0.23	4.99	0.85	0.22	4.03
	1.14	2.07	0.22	9.54	1.20	0.26	4.80	0.78	0.21	4.20
	1.24	2.15	0.24	9.52	1.22	0.25	5.13	0.79	0.20	4.19
	1.34	2.39	0.25	9.98	1.12	0.24	5.18	0.94	0.23	4.33
	1.44	2.20	0.25	9.03	1.22	0.22	5.46	0.85	0.22	4.49
SEM	Challenge	0.074	0.013	0.565	0.061	0.011	0.293	0.041	0.015	0.348
	Arg	0.046	0.007	0.326	0.035	0.006	0.169	0.023	0.008	0.201
	Interaction	0.033	0.005	0.230	0.025	0.004	0.120	0.016	0.006	0.142
P-value	Challenge	<0.001	0.204	<0.001	0.230	<0.001	<0.001	0.001	<0.001	<0.001
	Arg	0.074	0.404	0.840	0.503	0.202	0.699	0.077	0.663	0.923
	L[1]	0.049	0.055	0.593	0.164	0.662	0.217	0.256	0.637	0.361
	Q[1]	0.591	0.922	0.657	0.310	0.023	0.585	0.550	0.365	0.939
	Interaction	0.791	0.252	0.398	0.826	0.027	0.193	0.558	0.814	0.688
	L × Unch[2]	0.194	0.918	0.648	0.123	0.994	0.184	0.402	0.862	0.223
	L × Cha[2]	0.135	0.014	0.266	0.672	0.548	0.663	0.438	0.626	0.926
	Q × Unch[3]	0.687	0.382	0.480	0.754	0.737	0.342	0.318	0.794	0.361
	Q × Cha[3]	0.719	0.352	0.215	0.264	0.001	0.101	0.888	0.312	0.434

[1]L, Linear effect; Q, Quadratic effect.
[2]L at Unch—Linear effect of Arg on unchallenged birds; L at Cha–Linear effect of Arg on challenged birds.
[3]Q at Unch—Quadratic effect of Arg on unchallenged birds; Q at Cha–Quadratic effect of Arg on challenged birds.

Table 6. Villi height (V, μm), crypts depth (C, μm), and villi: crypt ratio (V:C) in the duodenum, jejunum, and ileum at 14 d postinoculation (dpi) according to dietary Arg levels (1.04, 1.14, 1.24, 1.34, and 1.44%) in broilers challenged (Cha) or unchallenged (Unch) with a mixed *Eimeria* spp. infection.

Challenge	Arg	Duodenum			Jejunum			Ileum		
		V	C	V:C	V	C	V:C	V	C	V:C
Cha	1.04	2.87	0.28	10.22[a,b,c]	1.52	0.27	5.84	1.03	0.24	4.59
	1.14	2.75	0.32	8.78[c]	1.45	0.26	5.57	1.02	0.26	4.15
	1.24	2.67	0.28	9.73[b,c]	1.35	0.26	5.22	1.13	0.29	4.18
	1.34	2.60	0.33	8.01[c]	1.62	0.24	7.22	1.29	0.24	4.68
	1.44	2.79	0.32	8.75[c]	1.50	0.23	6.95	1.20	0.26	4.82
Unch	1.04	2.63	0.23	11.58[a,b]	1.49	0.20	7.57	1.11	0.20	5.65
	1.14	2.47	0.28	9.05[b,c]	1.64	0.24	7.08	1.08	0.22	5.17
	1.24	2.61	0.22	12.36[a]	1.51	0.19	7.87	1.02	0.19	5.42
	1.34	2.95	0.26	11.32[a,b]	1.53	0.24	6.71	1.10	0.24	4.72
	1.44	2.76	0.28	9.90[b,c]	1.44	0.23	6.56	1.05	0.21	5.33
Challenge	Cha	2.74	0.31[a]	9.10	1.52	0.25	6.16[b]	1.10	0.26[a]	4.49[b]
	Unch	2.68	0.25[b]	10.84	1.49	0.22	7.16[a]	1.07	0.21[b]	5.26[a]
Arg	1.04	2.75	0.26[b]	10.90	1.50	0.24	6.70	1.07	0.22	5.12
	1.14	2.61	0.30[a]	8.91	1.54	0.25	6.33	1.05	0.24	4.66
	1.24	2.64	0.25[b]	11.05	1.43	0.22	6.55	1.08	0.24	4.77
	1.34	2.77	0.29[a]	9.66	1.58	0.24	6.97	1.12	0.24	4.70
	1.44	2.78	0.30[a]	9.32	1.47	0.23	6.76	1.13	0.24	5.08
SEM	Challenge	0.100	0.014	0.392	0.065	0.017	0.449	0.059	0.019	0.394
	Arg	0.063	0.008	0.226	0.037	0.010	0.259	0.034	0.011	0.227
	Interaction	0.045	0.006	0.160	0.026	0.007	0.183	0.024	0.008	0.160
P-value	Challenge	0.598	0.001	<0.001	0.632	0.060	0.029	0.585	0.011	0.035
	Arg	0.781	0.045	<0.001	0.697	0.899	0.932	0.863	0.959	0.886
	L[1]	0.550	0.064	0.048	0.907	0.621	0.622	0.326	0.767	0.974
	Q[1]	0.363	0.595	0.872	0.941	0.854	0.766	0.753	0.562	0.351
	Interaction	0.309	0.941	0.047	0.656	0.448	0.080	0.561	0.565	0.807
	L × Unch[2]	0.137	0.153	0.506	0.596	0.577	0.276	0.738	0.814	0.564
	L × Cha[2]	0.533	0.223	0.034	0.698	0.197	0.067	0.095	0.853	0.588
	Q × Unch[3]	0.794	0.511	0.280	0.440	0.998	0.615	0.766	0.825	0.576
	Q × Cha[3]	0.313	0.914	0.416	0.487	0.791	0.345	0.882	0.545	0.443

[a–c]Means followed by superscript letters are different by Tukey's test ($P < 0.05$) within the column.
[1]L, Linear effect; Q, Quadratic effect.
[2]L at Unch—Linear effect of Arg on unchallenged birds; L at Cha–Linear effect of Arg on challenged birds.
[3]Q at Unch—Quadratic effect of Arg on unchallenged birds; Q at Cha–Quadratic effect of Arg on challenged birds.

were found for duodenal villi height ($P > 0.05$). In the jejunum, only villi: crypt depth ratio was affected by treatments, and unchallenged birds showed improved ratio than the challenged ones ($P < 0.05$). In the ileum, the crypts depth and villi: crypt ratio were both affected by challenge, and unchallenged birds had reduced crypts depth and improved ratio than the challenged ones ($P < 0.05$). No other differences were found to be significant.

Nutrient Digestibility At 6 and 14 *dpi*, the challenged birds showed reduced digestibility of all the analyzed nutrients compared with the unchallenged ones ($P < 0.05$) (Table 7). Furthermore, a negative quadratic effect was observed for apparent ileal digestibility of dry matter, apparent ileal digestibility of CP, and apparent ileal digestibility of energy ($P < 0.05$), with maximum point at 1.24% of Arg.

Antioxidant System

At 6 *dpi*, an interaction was found for iNOS gene expression ($P < 0.05$) but not for the other traits. Challenged birds fed 1.14 and 1.34% of Arg had the expression of iNOS upregulated compared with unchallenged birds fed 1.14, 1.24, 1.34, and 1.44% of Arg. The SOD activity ($P < 0.01$) and GSH/GSSG ratio ($P < 0.01$) were reduced, and the NO concentration increased ($P < 0.01$) in the challenged than in the unchallenged birds. At 14 *dpi*, no differences were found between treatments for SOD activity and GSH/GSSG ($P > 0.05$). The iNOS expression ($P < 0.05$) and NO concentration ($P < 0.05$) were the greater in challenged than unchallenged birds. No differences were observed between the Arg levels for SOD, GSH/GSSG, and NO in any of the evaluated phases ($P > 0.05$).

DISCUSSION

In the current study, during all phases, the *Eimeria* challenge had negative effect on growth performance. On average, the BWG and FI were reduced by 13.7 and 6.5%, respectively, and the FCR was increased by 10.6% in the challenged birds from 1 to 14 *dpi*. This finding is in accordance with Rochell et al. (2017) who observed a reduction in BWG and FI by 9 and 4%, respectively, in *E. acervulina* challenged birds. The worsened growth performance observed during coccidiosis could be related to the reduction in intestinal integrity, and consequently intestinal nutrient digestion and

Table 7. Apparent ileal digestibility of dry matter (AIDDM, %), crude protein (AIDCP, %), and energy (AIDE, %) at 6 and 14 d postinoculation (dpi) according to dietary Arg levels (1.04, 1.14, 1.24, 1.34, and 1.44%) in broilers challenged (Cha) or unchallenged (Unch) with a mixed *Eimeria* spp. infection.

Challenge	Arg	6 dpi			14 dpi		
		AIDDM	AIDCP	AIDE	AIDDM	AIDCP	AIDE
Cha	1.04	63.22	75.11	2,305.20	69.45	79.12	2,701.00
	1.14	62.05	74.82	2,253.45	69.78	79.12	2,684.81
	1.24	64.72	78.59	2,376.93	71.00	81.31	2,764.18
	1.34	63.22	75.27	2,335.35	69.53	79.65	2,711.51
	1.44	65.87	76.88	2,413.15	68.71	77.040	2,679.66
Unch	1.04	70.96	81.60	2,709.45	72.07	82.22	2,841.8
	1.14	68.42	80.20	2,593.32	72.40	81.42	2,830.75
	1.24	70.53	81.30	2,699.75	74.60	83.88	2,922.61
	1.34	70.51	81.63	2,703.24	72.03	80.85	2,851.58
	1.44	69.73	81.02	2,648.25	68.86	80.65	2,699.11
Challenge	Cha	63.82[b]	76.13[b]	2,336.81[b]	69.70[b]	79.25[b]	2,708.23[b]
	Unch	70.03[a]	81.15[a]	2,670.80[a]	71.99[a]	81.80[a]	2,829.18[a]
Arg	1.04	67.09	78.36	2,507.33	70.76	80.67	2,771.43
	1.14	65.23	77.51	2,423.38	71.09	80.27	2,757.78
	1.24	67.63	79.95	2,538.34	72.80	82.59	2,843.39
	1.34	66.87	78.45	2,519.29	70.78	80.25	2,781.55
	1.44	67.80	78.95	2,530.70	68.79	78.84	2,689.38
SEM	Challenge	1.226	0.708	0.904	0.829	37.518	1.226
	Arg	0.708	0.447	0.522	0.478	21.661	0.708
	Interaction	0.500	0.316	0.369	0.338	15.316	0.500
P-value	Challenge	<0.001	<0.001	<0.001	0.007	0.002	<0.001
	Arg	0.665	0.333	0.570	0.058	0.077	0.075
	L[1]	0.467	0.407	0.408	0.146	0.203	0.231
	Q[1]	0.625	0.684	0.780	0.017	0.049	0.030
	Interaction	0.854	0.452	0.848	0.742	0.905	0.657
	L × Unch[2]	0.948	0.940	0.959	0.102	0.360	0.112
	L × Cha[2]	0.276	0.273	0.224	0.672	0.371	0.922
	Q × Unch[3]	0.843	0.853	0.946	0.180	0.371	0.250
	Q × Cha[3]	0.621	0.455	0.744	0.306	0.063	0.403

[a,b]Means followed by superscript letters are different by Tukey's test (*P* < 0.05) within the column.
[1]L, Linear effect; Q, Quadratic effect.
[2]L at Unch—Linear effect of Arg on unchallenged birds; L at Cha–Linear effect of Arg on challenged birds.
[3]Q at Unch—Quadratic effect of Arg on unchallenged birds; Q at Cha–Quadratic effect of Arg on challenged birds.

absorption, commonly seen during the intestinal cycle of the parasite in the enterocytes (Adams et al., 1996; Persia et al., 2006; Amerah and Ravindran, 2015; Rochell et al., 2016).

The intestinal epithelia constitutes an important physical barrier against the passage of undesirable molecules, such as microorganisms and toxins, while being selectively permeable to nutrients and ions (Vancamelbeke and Vermeire, 2017). There are several techniques to evaluate the function of the intestinal barrier, including the use markers, assessment of tight junction proteins, as well as intestinal histomorphology (Vancamelbeke and Vermeire, 2017). A commonly used molecular marker in intestinal challenge models in poultry is FITC-d (Vicuña et al., 2015; Gilani et al., 2016; Bortoluzzi et al., 2019). Fluorescein isothiocyanate dextran-d molecular weight ranges between 3 and 5 kDa, which is impermeable across the intestinal barrier; therefore, high concentrations in the serum are indication of leaky gut (Kuttappan et al., 2015). Birds challenged with *Eimeria* had 3 times higher serum FITC-d concentration (0.09 vs. 0.28 µg/mL) than the unchallenged group at 5 **dpi**, indicating a greater loss in intestinal integrity. Moreover, the expression of tight

junction proteins was also evaluated. Cla-1 gene expression was upregulated in the challenged birds on 6 **dpi**, which is in agreement with Kim et al. (2010). The authors found an upregulation of this gene in the duodenum 3 to 4 **dpi** in the birds challenged with *E. acervulina*. Usually, the higher expression of Cla-1, a pore sealing tight junction protein, is associated with tight epithelia (Awad et al., 2017). However, during inflammatory processes, the expression of this tight junction protein (**TJP**) has been shown to increase (Garcia-Hernandez et al., 2017), possibly mediated by anti-inflammatory cytokines, such as interleukin-10 (Mazzon et al., 2002).

The intestinal epithelium formation consists of a dynamic process involving cell proliferation in the crypts, followed by cell maturation and apoptosis in the villi (Jeurissen et al., 2002). In general, shortening of the villi has been associated with reduced surface area for nutrient absorption, whereas a crypts enlargement has been associated with a rapid tissue turnover to support a high demand for new tissue (Choct, 2009). Thus, a high villi height and villi: crypt ratio are a common indicator of well-differentiated intestinal mucosa. In the current study, the *Eimeria* challenged group showed

Table 8. Superoxide dismutase (SOD, U/g liver), reduced:oxidized glutathione ratio (GSH/GSSG, μmol/L/ μmol/L, liver), nitric oxide synthase gene expression (iNOS, jejunum), and nitric oxide concentration (NO, μmol/L, plasma) at 6 and 14 d postinoculation (dpi) according to dietary Arg levels (1.04, 1.14, 1.24, 1.34, and 1.44%) in broilers challenged (Cha) or unchallenged (Unch) with a mixed *Eimeria* spp. infection.

Challenge	Arg	6 dpi				14 dpi			
		SOD	GSH/GSSG	iNOS	NO	SOD	GSH/GSSG	iNOS	NO
Cha	1.04	1.13	52.61	0.85[a,b,c,d]	22.22	0.43	73.93	1.10	27.48
	1.14	1.00	53.02	1.51[a]	33.79	0.34	93.87	1.11	23.86
	1.24	1.14	53.49	1.37[a,b,c]	27.03	0.43	77.02	1.77	22.95
	1.34	1.20	51.75	1.45[a,b]	24.13	0.45	72.76	0.97	25.44
	1.44	1.05	61.40	0.79[a,b,c,d]	22.66	0.45	78.18	1.50	28.58
Unch	1.04	1.84	68.70	0.68[a,b,c,d]	15.21	0.38	75.00	0.50	17.01
	1.14	1.55	74.81	0.54[c,d]	12.34	0.68	77.55	0.85	15.69
	1.24	1.98	54.50	0.53[c,d]	13.11	0.57	86.52	1.02	15.72
	1.34	2.05	83.89	0.49[d]	18.34	0.34	75.84	1.00	12.79
	1.44	1.66	77.31	0.62[c,d]	15.02	0.46	76.26	1.26	16.60
Challenge	Cha	1.10[b]	54.46[b]	1.19	25.97[a]	0.42	79.15	1.29[a]	25.66[a]
	Unch	1.66[a]	71.84[a]	0.57	14.80[b]	0.49	78.23	0.92[b]	15.56[b]
Arg	1.04	1.48	60.65	0.76	18.72	0.41	74.47	0.80	22.24
	1.14	1.27	63.92	1.03	23.06	0.51	85.71	0.98	19.78
	1.24	1.17	53.99	0.92	20.07	0.50	81.77	1.400	19.34
	1.34	1.63	67.82	0.97	21.24	0.39	74.30	0.98	19.12
	1.44	1.35	69.35	0.71	18.84	0.45	77.22	1.38	22.59
SEM	Challenge	0.113	5.779	0.128	2.572	0.077	5.982	0.196	2.970
	Arg	0.065	3.336	0.074	1.485	0.036	3.453	0.113	1.714
	Interaction	0.046	2.359	0.052	1.050	0.025	2.442	0.080	1.212
P-value	Challenge	<0.001	0.002	<0.001	<0.001	0.264	0.856	0.044	<0.001
	Arg	0.064	0.390	0.324	0.727	0.663	0.565	0.142	0.866
	L[1]	0.795	0.266	0.671	0.845	0.930	0.741	0.070	0.997
	Q[1]	0.322	0.369	0.070	0.332	0.468	0.341	0.598	0.282
	Interaction	0.187	0.478	0.047	0.168	0.140	0.604	0.652	0.959
	L × Unch[2]	0.779	0.331	0.765	0.620	0.537	0.974	0.066	0.781
	L × Cha[2]	0.932	0.545	0.762	0.455	0.640	0.619	0.462	0.777
	Q × Unch[3]	0.129	0.446	0.455	0.791	0.178	0.423	0.722	0.645
	Q × Cha[3]	0.839	0.610	0.100	0.110	0.802	0.583	0.696	0.287

[a–d]Means followed by superscript letters are different by Tukey's test ($P < 0.05$) within the column.
[1]L, Linear effect; Q, Quadratic effect.
[2]L at Unch—Linear effect of Arg on unchallenged birds; L at Cha–Linear effect of Arg on challenged birds.
[3]Q at Unch—Quadratic effect of Arg on unchallenged birds; Q at Cha–Quadratic effect of Arg on challenged birds.

reduced villi height and villi: crypt ratio in the duodenum and jejunum at 6 ***dpi***. Additionally, it was observed increased villi height and crypts depth in the ileum of challenged birds, whereas the villi: crypt ratio decreased in those birds. A compensatory increase in ileum villi was reported in the literature as an attempt to increase nutrient absorption when the other parts of the intestine were compromised (Yamauchi et al., 2010), which may explain the results. According to Fernando and McCraw (1973), the maximum intestinal epithelia damage can be seen 6 d after an *E. acervulina* challenge, followed by a rapid restoration of villi morphology. At 14 ***dpi***, no differences in villi height were observed; however, the crypts depth of the duodenum and ileum were increased in *Eimeria* challenged birds. These findings are suggestive of increased cell proliferation, as aforementioned, and intestinal epithelium recovery. As a consequence of the intestinal damage observed in our study, the ileal digestibility of dry matter, crude protein, and apparent metabolizable were also negatively affected, as previously reported (Persia et al., 2006; Rochell et al., 2016).

Oxidative stress in poultry because of *Eimeria* infection has been documented, and it occurs mainly because of the excessive free radical production and the reduction of antioxidant enzyme activities and nonenzymatic antioxidants (Koinarski et al., 2005; Georgieva et al., 2006, 2011; Khatlab et al., 2019). High levels of NO, a free radical produced from Arg and mediated by the enzyme nitric oxide synthase, have been reported during *Eimeria* infection in poultry (Lillehoj and Li, 2004; Pirali Kheirabadi et al., 2011; Dominguez et al., 2015; Khatlab et al., 2019). This free radical could be involved in the pathogenesis of coccidiosis in chickens (Pirali Kheirabadi et al., 2011), and it has been considered an inducer of oxidative stress and DNA damage (Watanabe et al., 2001). In the current study, challenged birds fed 1.14 and 1.34% of Arg had higher iNOS expression than the unchallenged Arg-supplemented ones, without differences between the Arg levels within the challenged and unchallenged groups. The iNOS expression on 14 ***dpi***, and NO plasmatic concentration at both phases were also significantly increased by challenge Additionally, both SOD and glutathione are considered important first level defense agents against oxidative stress. This enzyme and free radical scavenger act in preventing the free radical formation in the cells by removing free radical precursors, such as superoxide (Surai, 2016). Owing to the constant activity of glutathione reductase, which converts the GSSG to its

reduced form (GSH), the latter form is the one commonly found in the cells. Therefore, the ratio of GSH/GSSG within cells is often used as a marker of cellular toxicity (Chai et al., 1994; Carelli et al., 1997; Townsend et al., 2003). In the present study, the SOD activity and GSH/GSSG ratio in the liver of the challenged birds were decreased on 6 **dpi**. Thus, when combined, these findings suggest that the *Eimeria* infection led to oxidative stress in the birds; however, the Arg supplementation was not able to prevent it.

Arginine dietary levels also influenced the growth performance. Overall, increasing levels of Arg led to higher BWG, FI, and lower FCR in a linear fashion, in the challenged group or regardless of challenge. Moreover, from 0 to 14 dpi (12–26 d of age), birds fed a diet without Arg supplementation (1.04% of Arg) had a reduction of 7% in BWG compared with birds fed 1.44% of Arg, and an increase in FCR of 5% compared with birds fed 1.34% of Arg. The Arg deficiency has been shown to reduce growth in broilers (Gao et al., 2018; Castro et al., 2019), which might be related to its role as a potent secretagogue for insulin (Bolea et al., 1997), growth hormone (Collier et al., 2005), and, indirectly, insulin-like growth factor-1 (Houston and O'Neill, 1991). In poultry, the growth and feed efficiency modulation by insulin-like growth factor-1 could be partially because of its control on protein breakdown rate, stimulus of protein synthesis, and reduction in protein degradation (Tomas et al., 1998; Conlon and Kita, 2002). Therefore, Arg supplementation could increase overall muscle deposition and growth in both challenged and unchallenged birds.

Moreover, Arg has been shown to modulate the epithelial intestinal barrier by decreasing the intestinal permeability (Viana et al., 2010; Quirino et al., 2013; Costa et al., 2014) and improving epithelia proliferation and recovery (Sukhotnik et al., 2005; Tan et al., 2010) in animals. In the present study, the concentration of serum FITC-d was reduced when supplementing 1.34 vs. 1.04% of Arg, and in challenged birds, lower permeability could be obtained by supplementing Arg within the 1.24–1.34% range. Zhang et al. (2017) observed a reduction in ileal FITC-d passage when higher Arg level (1.87%) was fed to broilers, regardless of *Clostridium perfringens* challenge. Complementary to the FITC-d results, the expression of ZO-1 was upregulated in birds fed 1.34% compared with 1.24 and 1.44% of Arg on 6 **dpi**. The ZO are a group of tight junction proteins located at the cytoplasmatic surface of the cell membrane, serving as a link between other tight junction proteins and the actin cytoskeleton (Stevenson et al., 1986; Furuse et al., 1994; Awad et al., 2017). Therefore, they play an important role in keeping the integrity of the epithelial tight junction, and their higher expression might be an indicator of higher gut barrier integrity, corroborating with our findings. Furthermore, at 14 **dpi**, an upregulation of ZO-1 and ZO-2 was found when birds were fed 1.44% of Arg compared with 1.24 and 1.34% of Arg, which were also the levels with minimum expression of ZO-1 and ZO-2 in a quadratic

manner. The TJP can be assembled, disassembled, and maintained in a dynamic way upon different stimuli, such as dietary ingredients and pathogens (Ulluwishewa et al., 2011). Therefore, the overexpression in birds fed 1.44% of Arg could have been because of a compensatory mechanism to improve the tight junction after a challenge, as previously observed for other intestinal TJP (Barekatain et al., 2019).

The Arg levels had a moderate effect on the intestinal morphology. Overall, during challenge phase, the Arg supplementation linearly increased the duodenal villi height, regardless of challenge, and crypt depth, in challenged birds. Moreover, jejunum crypts depth increased in a quadratic manner when 1.24% of Arg was fed to challenged birds compared with their counterpart in the unchallenged group. In the recovery phase, the villi: crypt ratio in the duodenum decreased linearly with Arg supplementation in the challenged group. Intestinal crypt is characterized by the continuous enterocyte proliferation before they migrate up to the villi, where nutrients are digested and absorbed (Uni et al., 2001; Williams et al., 2015). Therefore, higher villi height and crypt depth with Arg supplementation indicates increased epithelial development and maturation, which will replenish the cells sloughed during the challenge.

Arginine is a known indirect precursor for polyamines, specially putrescine, spermidine, and spermine, through Arg-L-ornithine-putrescine and Arg–agmatine–putrescine pathways (Tabor and Tabor, 1984; Seiler, 1996; Horyn et al., 2005). These molecules are considered nutritionally important factors for cell growth, proliferation, and oxidative stress defense (Seiler, 1996; Miller-Fleming et al., 2015). The supplementation with Arg was shown to increase the intestinal concentration of polyamines and increased the cellular proliferation and intestinal repair after ischemia damage in rats (Raul et al., 1995). Furthermore, Yuan et al. (2015), in vitro, demonstrated that Arg increased the proliferation of intestinal crypt cells from chicken embryos. The authors showed that Arg supplementation in the media upregulated the target of rapamycin expression, which controls cellular physiology and protein synthesis. Therefore, Arg may be beneficial for the recovery of the intestine during and after a stress by stimulating the development of the intestine mucosa and accelerating the mitotic process in the intestinal epithelial cell. Moreover, during the recovery phase, the nutrient digestibility was quadratically affected by Arg levels, regardless of challenge, peaking when 1.24% of Arg was used. These finding indicates that the level recommended by the breeder guideline is enough to support the intestinal morphology and function.

Thus, we can conclude that a mixed *Eimeria* challenge acutely impaired the intestinal integrity and antioxidant system. The negative effects caused by *Eimeria* infection on the growth performance, intestinal histomorphology, and ileal digestibility persisted throughout the challenge and recovery phases. However, increasing levels of Arg were not able to counteract these effects. The dietary supplementation of levels ranging from 1.24 to 1.44%

of Arg showed promising results, improving overall growth, intestinal integrity, and morphology in broilers subjected or not to an *Eimeria* challenge.

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