Dietary L-arginine supplementation ameliorates inflammatory response and alters gut microbiota composition in broiler chickens infected with *Salmonella enterica* serovar Typhimurium

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ABSTRACT This study was conducted to investigate the effects of dietary arginine (**Arg**) supplementation on the inflammatory response and gut microbiota of broiler chickens subjected to *Salmonella enterica* serovar Typhimurium. One hundred and forty 1-day-old Arbor Acres male birds were randomly assigned to a 2 × 2 factorial arrangement including diet treatment (with or without 0.3% Arg supplementation) and immunological stress (with or without *S. typhimurium* challenge). Samples were obtained at 7 D after infection (day 23). Results showed that *S. typhimurium* challenge caused histopathological and morphological damages, but Arg addition greatly reduced these intestinal injuries. *S. typhimurium* challenge elevated the levels of serum inflammatory parameters, including diamine oxidase, C-reactive protein, procalcitonin, IL-1β, IL-8, and lipopolysaccharide-induced tumor necrosis factor-alpha factor (**LITNF**) homolog. However, Arg supplementation decreased the serum procalcitonin, IL-1β, IL-8, and LITNF concentration. *S. typhimurium* challenge significantly increased jejunal *IL-1β*, *IL-8*, *IL-10*, and *IL-17* mRNA expression and tended to upregulate *IL-22* mRNA expression, but Arg supplementation remarkably reduced *IL-8* mRNA expression, tended to downregulate *IL-22* mRNA expression, and dramatically elevated *IFN-γ* and *IL-10* mRNA expression. In addition, sequencing data of 16S rDNA indicated that the population of Proteobacteria phylum; Enterobacteriaceae family; *Escherichia–Shigella,* and *Nitrosomonas* genera; and *Escherichia coli* and *Ochrobactrum intermedium* species were more abundant, but the population of Rhodocyclaceae and Clostridiaceae_1 families and *Candidatus Arthromitus* genus were less abundant in the ileal digesta of birds with only *S. typhimurium* infection when compared with the controls. Treatment with Arg in birds subjected to *S. typhimurium* challenge increased the abundances of Firmicutes phylum, Clostridiaceae_*1* family, *Methylobacterium* and *Candidatus Arthromitus* genera but decreased the abundance of *Nitrosomonas* genus and *Rhizobium cellulosilyticum* and *Rubrobacter xylanophilus* species as compared with the only *S. typhimurium*–challenged birds. In conclusion, Arg supplementation can alleviate intestinal mucosal impairment by ameliorating inflammatory response and modulating gut microbiota in broiler chickens challenged with *S. typhimurium*.

Key words: arginine, *Salmonella enterica* serovar Typhimurium, broiler chicken, inflammatory response, gut microbiota

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INTRODUCTION

Salmonella enterica serotype Typhimurium is a gram-negative intracellular pathogen and one of the most prevalent serovars of *Salmonella* bacteria, causing food-borne salmonellosis in humans (Eng et al., 2015; Antunes et al., 2016). Contaminated poultry meat and eggs are important carriers of *S. typhimurium*, threatening human health (Antunes et al., 2016; Dar et al., 2017). *S. typhimurium* infection in chicken can occur at any age, but the infection mainly causes systemic disease with high mortality in day-old chicks as they are more susceptible to *Salmonella* infection. However, *S. typhimurium* infection in older birds usually causes subclinical disease with gut inflammation, intestinal barrier damage, poor growth rate, and reduced egg production (Shao et al., 2013; Bai et al., 2014; Dar et al., 2017). Previously subtherapeutic antibiotics have long been used in poultry feed to control gut pathogenic bacteria load and promote

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animal growth. However, with the rise of multidrug-resistant bacteria, the European Union began to ban the use of in-feed antibiotics for growth promotion since 2006 (Anadón, 2006). In the postantibiotic era, various alternative control measures for *Salmonella* infection have been proposed and investigated in animal feed. These approaches include but are not limited to the use of organic acids (Saleem et al., 2016), functional amino acids (Chen et al., 2012a,b), plant extracts (Varmuzova et al., 2019), or probiotics (Nakphaichit et al., 2019).

Owing to the inimitable effects on modulating immune system and controlling the pathogen challenge (Gogoi et al., 2016), the application of arginine (**Arg**) has been increasing in attention. L-Arginine is an essential amino acid for chickens and an important precursor for the synthesis of nitric oxide and polyamines, which have prominent antimicrobial and anti-inflammatory properties, respectively (Gogoi et al., 2016). Arg supplementation is capable of promoting the proliferation of lymphocytes and enterocytes, maintaining intestinal barrier function, regulating cytokines and hormones, and so on (Field et al., 2000, 2002; Li et al., 2007). Furthermore, Arg supplementation has been reported to ameliorate inflammatory responses in various stress models. Tan et al. (2014) demonstrated that dietary Arg supplementation attenuated lipopolysaccharide-induced inflammatory response probably through the suppression of the TLR4 pathway and $CD14^+$ cell percentage in broiler chickens. Arg pretreatment attenuated the gene expression of inflammatory cytokines *IL-1β* and *TNF-α* in high-fat diet–induced obese mice with limb ischemia (Kuo et al., 2018). Arg ameliorated intestinal permeability enhancement, inflammatory infiltration, and lipid peroxidation of dextran sulfate sodium–induced colitis in mice (Andrade et al., 2019).

Changes in the gut microbiota community are considered to be closely related to the occurrence of certain diseases, including inflammation, obesity, and metabolic disease (Boulangé et al., 2016). The gut microbiota effectively influences host in the regulation of homeostasis, organ development, metabolic processes, and immune response (Tremaroli and Backhed, 2012). Recent findings suggest that Arg positively regulates gut microbiota composition. Dietary Arg addition of mice has been reported to change the intestinal microbiota, contributing to the activation of intestinal innate immunity through nuclear factor-κB, mitogen-activated protein kinase, and phosphatidylinositol 3-kinase Akt signaling pathways (Ren et al., 2014). Hu et al. (2019) demonstrated that dietary supplementation with Arg in combination with leucine decreased body fat weight and increased colonic butyrate and propionate concentrations, which were related to the alteration of gut microbiota composition in finishing pigs. Our previous results showed that dietary Arg supplementation normalized the ileal microbiota composition of *Clostridium perfringens*–challenged chickens similar to that of unchallenged controls (Zhang et al., 2018).

However, little is known about the effects of Arg on the inflammatory response and gut microbiota composition in *S. typhimurium*–challenged broiler chickens. The present study was conducted to evaluate the effects of Arg addition on the inflammatory response and intestinal histopathology, morphology, and microbiota composition in the *S. typhimurium*–challenged broiler chickens.

MATERIALS AND METHODS

Animal Care and Diets

The experimental protocol was approved by the Animal Care and Use Committee of China Agricultural University. A total of 140 one-day-old male Arbor Acres broiler chickens were obtained from a local hatchery and were used in a 2 × 2 factorial arrangement including diet treatment (with or without 0.3% Arg supplementation) and immunological stress (with or without *S. typhimurium* challenge). Briefly, the chickens were randomly assigned to 4 treatments: **CTL**, control group, birds received basal diet plus mock challenge; **ARG**, birds received basal diet supplemented with 0.3% Arg plus mock challenge; **ST**, birds received basal diet plus *S. typhimurium* challenge; **ARGST**, birds received basal diet supplemented with 0.3% Arg plus *S. typhimurium* challenge. Each treatment (n = 35) was raised in a rearing isolator (1.6 m × 0.7 m × 0.7 m) equipped with a feeder and 2 nipple drinkers to allow chickens to access feed and water ad libitum. The initial body weights were similar across all the treatments. The composition of diets is presented in Table 1. The concentration of amino acids in the diets was measured by high-performance liquid chromatography. The chickens were inoculated with combined Newcastle disease virus and infectious bronchitis virus at day 7 and day 21 of age. The lighting program was 23-h light and 1-h dark per day. The room temperature was controlled at 35°C for the first wk and gradually reduced from 35°C to 24°C on day 21 and then kept constant thereafter.

Salmonella typhimurium *Challenge*

The *S. typhimurium* strain CMCC50115 was provided by the China Institute of Veterinary Drug Control (Beijing, China). The frozen culture was inoculated in Luria-Bertani medium with orbital shaking at 37°C, 200 r/min, for 24 h. After a 1:100 dilution using fresh Luria-Bertani medium, the bacterial cells were cultured for 16 h at 37°C with orbital shaking at a speed of 200 r/min to reach the exponential phase. From day 8 to 10 and day 13 to 16, birds in the ST group and ARGST group were inoculated orally with 1.0 mL of culture media containing 1×10^9 colony-forming unit *S. typhimurium* each day and birds in the CON group and ARG group were administered with an equal amount of sterile Luria-Bertani medium.

Table 1. Ingredient and nutrient composition of diets.

Item (%, unless otherwise indicated)	Basal diet	L-Arginine–supplemented diet
Ingredient		
Maize	58.28	58.28
Soybean meal (44% CP)	29.11	29.11
Maize gluten meal	5.00	5.00
Soybean oil	2.50	2.50
Dicalcium phosphate	1.92	1.92
Limestone	0.89	0.89
Sodium chloride	0.30	0.30
Choline chloride (50%)	0.25	0.25
Mineral premix[1]	0.20	0.20
DL-Methionine (98%)	0.26	0.26
L-Lys-HCl (98%)	0.24	0.24
Vitamin premix[2]	0.03	0.03
Ethoxyquin (33%)	0.02	0.02
L-Ala	1.00	0.60
L-Arg	0.00	0.40
Nutrient level[3]		
ME (Mcal/kg)	2.98	2.98
CP	21.94	21.94
Ca	1.00	1.00
Nonphytate phosphorus	0.45	0.45
Lys (measured value)	1.36	1.39
Met	0.59	0.59
Thr (measured value)	0.86	0.88
Arg (measured value)	1.42	1.72

[1]Supplied per kilogram of complete feed: Mn, 100 mg; Fe, 80 mg; Zn, 75 mg; Cu, 8 mg; I, 0.35 mg; and Se, 0.15 mg.
[2]Supplied per kilogram of complete feed: vitamin A, 12,500 IU; vitamin D_3, 2,500 IU; vitamin E, 30 IU; vitamin K_3, 2.65 mg; vitamin B_1, 2 mg; vitamin B_2, 6 mg; vitamin B_5, 12 mg; vitamin B_{12}, 0.025 mg; niacin, 50 mg; folic acid, 1.25 mg and biotin, 0.0325 mg.
[3]Calculated composition, unless otherwise indicated.

Sample Collection

At 7 D after challenge (day 23 of age), after 8 hr of starvation, 8 birds per group were randomly selected and then blood was aseptically collected from the wing vein. After centrifugation at 3,000 r/min for 10 min at 4°C, the serum samples were isolated and preserved at −20°C for assays. To obtain enough ileal digesta and to determine gut microbiota composition in the fed state, all birds were refed under ad libitum conditions for 3 h. Then, the blood-drawn birds were slaughtered by intravenous injection of pentobarbital sodium (30 mg/kg body weight) and jugular exsanguination. Two fragments from the mid-jejunum were immediately removed and gently washed with sodium chloride. One was immediately frozen in liquid nitrogen and stored at -80°C until mRNA assay. The other was placed in 4% paraformaldehyde solution for hematoxylin and eosin staining. The contents in the middle of the ileum were collected in sterile tubes and immediately stored at −80°C for further processing.

Intestinal Histopathological and Morphological Evaluation

Paraformaldehyde-fixed jejunum samples were embedded by paraffin, and then 5-μm sections were stained with hematoxylin and eosin. Ten nonoverlapping fields spanning the submucosa to epithelial layer were randomly selected in each slice using a light microscope (Leica model DMi8, Leica, Wetzlar, Germany) with an image analysis software (version 4.2, Leica application suite, Leica, Wetzlar, Germany) at magnification of × 200. The histopathological score was measured based on the parameters of epithelial cell defects, lymphohistiocytic infiltration, villus fusion, capillary dilation, capillary hemorrhages, and submucosal edema, as described previously (Splichalova et al., 2019) with some modifications. Each scoring criterion was scored using a scale from 0 to 3 (0, none; 1, mild; 2, moderate; and 3, severe), and the total score of 0 to 18 points was obtained for each image. Then, mean scores were attached to each sample. All observations and measurements were performed in a blinded manner. In the morphological analysis, the villus height was measured from the villus tip to the villus–crypt junction. The crypt depth was defined as the vertical distance from the base of the crypt up to the villus–crypt junction, and the villus height-to-crypt depth ratio (**VCR**) was subsequently calculated. Morphological evaluation was performed on 8 intact villi and crypts for each slice at 100 × magnification.

Measurement of Serum Inflammatory Parameters

Serum diamine oxidase (**DAO**) activity was analyzed following the instruction of a colorimetric kit (HY-60106, Huaying Biotechnology Research, Beijing, China). Serum C-reactive protein (**CRP**) level was quantified using a CRP immunoturbidimetric assay kit (Shanghai Kehua Bio-engineering Co., Ltd., Shanghai, China) in accordance with the manufacturer's protocols.

Table 2. Primer sequences of target and reference genes.

Gene name[1]	Accession number	Primer sequence[2] (5′ to 3′)	Product size (bp)
IFN-γ	NM_205149.1	F: AGCTGACGGTGGACCTATTATT R: GGCTTTGCGCTGGATTC	259
LITNF	XM_015294124.1	F: GAGCGTTGACTTGGCTGTC R: AAGCAACAACCAGCTATGCAC	64
IL-1β	XM_015297469.1	F: ACTGGGCATCAAGGGCTA R: GGTAGAAGATGAAGCGGGTC	131
IL-8	XM_015301388.1	F: ATGAACGGCAAGCTTGGAGCTG R: TCCAAGCACACCTCTCTTCCATCC	233
IL-10	NM_001004414.2	F: CGCTGTCACCGCTTCTTCA R: TCCCGTTCTCATCCATCTTCTC	88
IL-17	NM_204460.1	F: CTCCGATCCCTTATTCTCCTC R: AAGCGGTTGTGGTCCTCAT	292
IL-22	NM_001199614.1	F: GGTTGTCTTCTGCTGTTGTTGCTG R: GCCAAGGTGTAGGTGCGATTCC	156
GAPDH	NM_204305.1	F: TGCTGCCCAGAACATCATCC R: ACGGCAGGTCAGGTCAACAA	142

[1]*IFN-γ* = interferon-γ; *LITNF* = lipopolysaccharide-induced tumor necrosis factor-alpha factor homolog; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase.
[2]F = forward; R = reverse.

The concentration of serum procalcitonin (**PCT**) was measured using a commercial ELISA double-antibody sandwich kit (JH-00512, Jinhai Keyu Biotech Development Co., Ltd., Beijing, China). Serum levels of IL-1β, IL-8, and lipopolysaccharide-induced tumor necrosis factor-alpha factor (**LITNF**) homolog were determined using chicken cytokine ELISA kits (Kete Biological Technology Co., Ltd., Yancheng, China). Briefly, 50 μl of standard solutions or serum samples were added to 96-well plates (coated with purified chicken IL-1β, IL-8, and LITNF antibody). Then, the horseradish peroxidase–labeled second antibody was added to the wells, and the plates were incubated for 60 min at 37°C. The wells were washed 5 times, and chromogen solutions were added and preserved in dark for 15 min at 37°C. Finally, the absorbance was measured at 450 nm using a microplate reader (ELX 800, BioTek, Winooski, VT) after addition of the stop solution.

RNA Isolation and mRNA Expression Analysis

Total RNA isolation, reverse transcription, and real-time PCR were carried out as previously described (Zhang et al., 2017). The primer sequences are presented in Table 2. The R^2 of all the standard curves of target genes were greater than 0.98, and the amplification efficiency values were between 90 and 110%. The glyceraldehyde-3-phosphate dehydrogenase (***GAPDH***) was used as the housekeeping gene, and its cycle threshold value did not show a significant difference among the groups. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used to analyze the relative mRNA expression of intestinal genes.

16S rDNA Sequencing of Ileal Microbiota

Five samples of ileal digesta per group were randomly selected to proceed to 16S rDNA sequencing. The total bacterial DNA was extracted from 0.2 g of ileal contents using a QIAamp DNA Stool Mini Kit (51,604, Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. The V4 hypervariable region of the 16S rRNA gene was amplified using the primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACVSGGG TATCTAAT-3′). The 16S rRNA gene was sequenced on the Illumina HiSeq 2500 sequencing platform at Novogene Bioinformatics Technology Co. Ltd. (Beijing, China). Raw sequences were quality-filtered using the QIIME (version 1.7.0; http://qiime.org/scripts/split_libraries_fastq.html). The chimera sequences were identified and removed using UCHIME algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html; Edgar et al., 2011). Then, the effective reads were finally obtained. Effective reads with more than 97% similarity were clustered into the same operational taxonomic units (**OTU**) using Uparse software (version 7.1; http://drive5.com/uparse/). The taxonomy of OTU sequences was annotated from phylum to genus level using RDP classifier (http://rdp.cme.msu.edu/; Wang et al., 2007). Alpha diversity (Observed species, Chao1, Shannon, Simpson, ACE and Good's coverage) and beta diversity (principal coordinates analysis [**PCoA**] and unweighted pair-group method with arithmetic means [**UPGMA**]) were calculated by QIIME. Differentially abundant taxa among the groups were identified using linear discriminant analysis (**LDA**) effect size (**LEfSe**) analysis ($\alpha = 0.05$, LDA score > 4).

Statistical Analysis

The general linear model procedure of SPSS, version 18.0 (SPSS Inc., Chicago, IL) was used to evaluate the main effects of Arg supplementation, *S. typhimurium* challenge, and the associated interaction between Arg supplementation and *S. typhimurium* challenge. When a significant interaction effect was found, one-way ANOVA and Duncan's multiple comparison were performed to compare the differences among all groups. The individual bird was considered as the experimental

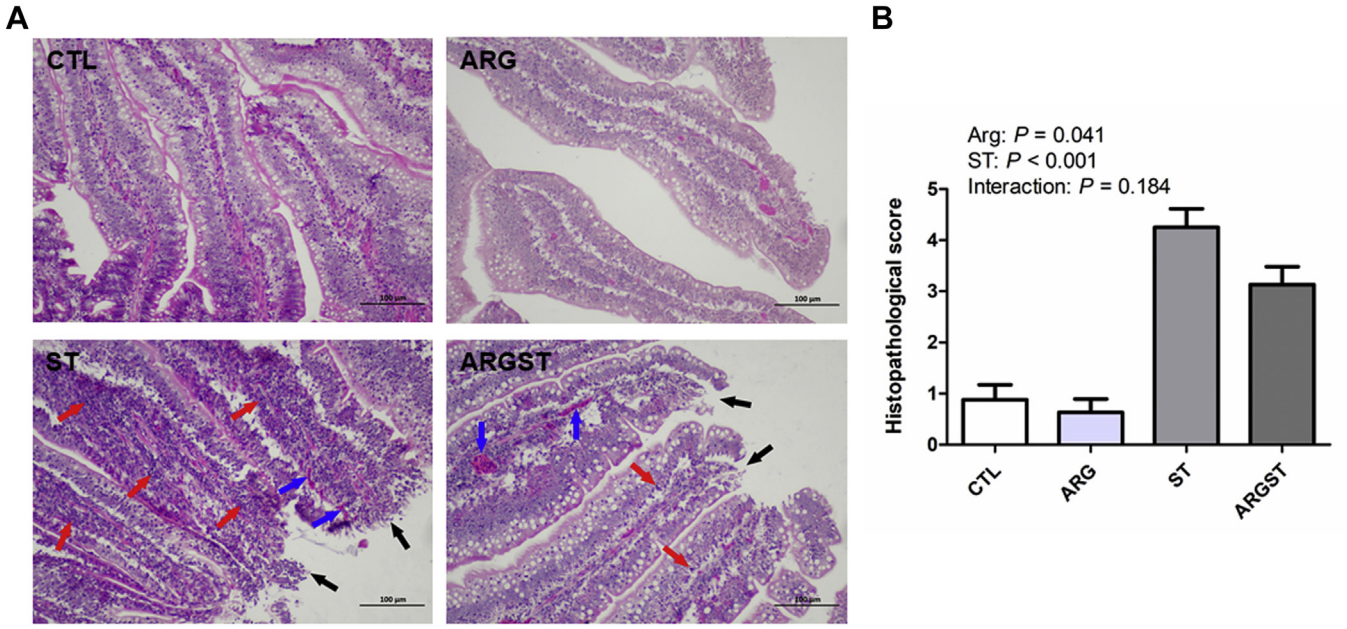

Figure 1. Jejunal histopathology of broiler chickens (hematoxylin and eosin staining; n = 8). (A) Representative histopathological pictures; (B) histopathological injury score. CTL = chickens received a basal diet without *Salmonella typhimurium* challenge; ARG = chickens received a basal diet supplemented with 0.3% arginine, but no *S. typhimurium* challenge; ST = chickens received a basal diet plus *S. typhimurium* challenge; ARGST = chickens received a basal diet supplemented with 0.3% arginine plus *S. typhimurium* challenge. Data are shown as the mean ± SEM. Original magnification, 200 ×. Scale bar = 100 μm. Red arrow, lymphocytes infiltration; blue arrow, hemorrhage; black arrow, defects of epithelium at the tip of villus.

unit. Significance was set at $P \leq 0.05$, and a trend towards significance at $P \leq 0.10$ was seen. Data in the tables were expressed as means and pooled SEM.

RESULTS

Jejunal Histopathological and Morphological Analyses

Histopathological results showed that the jejuna of chickens in the CTL group and the ARG group were almost normal, except for the presence of a few lymphocytes (Figure 1A). *S. typhimurium* challenge caused a significant histopathological damage, as indicated by a mass of lymphocytes infiltration, the obvious loss of villus tip, distinct villus fusion, and conspicuous capillary hemorrhages or even defect of villus structure. Dietary Arg supplementation could alleviate intestinal histopathological damage to a great extent. As shown in Figure 1B, the histopathological score was significantly increased by *S. typhimurium* challenge ($P < 0.05$) but was remarkably suppressed by Arg addition ($P < 0.05$). Morphological evaluation (Figure 2) showed that jejunal villus height was significantly decreased by *S. typhimurium* challenge ($P < 0.05$), but Arg supplementation had no influence on jejunal villus height ($P > 0.05$). In addition, a significant decrease was noted in jejunal crypt depth of broilers after Arg addition ($P < 0.05$). The VCR in the jejunum tended to be reduced by *S. typhimurium* challenge ($P < 0.10$). However, dietary Arg supplementation tended to increase jejunal VCR ($P < 0.10$). No significant interaction was observed on jejunal VCR ($P > 0.05$). In addition, no chicken died of *S. typhimurium* challenge in this study.

Serum Inflammatory Parameters

The effect of dietary Arg supplementation on serum inflammatory parameters is summarized in Table 3. *S. typhimurium* challenge significantly increased serum DAO activity and CRP concentration ($P < 0.05$). However, the effects of Arg supplementation and Arg × ST interaction did not influence the serum DAO activity, as well as CRP concentration ($P > 0.05$). Besides, *S. typhimurium* challenge significantly increased serum PCT level ($P < 0.05$), which was reduced by dietary Arg supplementation ($P < 0.05$). There was an Arg × ST interaction on the levels of serum proinflammatory cytokines IL-1β, IL-8, and LITNF ($P < 0.05$), wherein the chickens with only *S. typhimurium* challenge had the highest levels of serum proinflammatory cytokines IL-1β, IL-8, and LITNF, and dietary Arg supplementation significantly reduced the levels of these 3 proinflammatory cytokines in the serum of birds challenged with *S. typhimurium* ($P < 0.05$).

Gene Expression of Cytokines in the Jejunum

As shown in Table 4, Arg administration dramatically increased the mRNA expression of interferon-γ (***IFN-γ***) ($P < 0.05$). No change was observed in the mRNA expression of *LITNF* in response to Arg addition, *S. typhimurium* challenge, and their interaction ($P > 0.05$). *S. typhimurium* challenge significantly

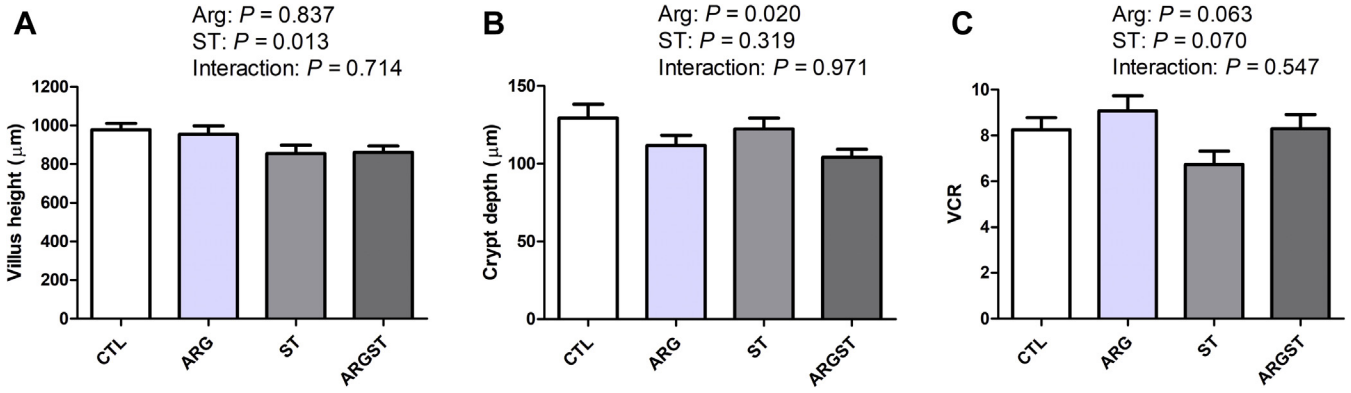

Figure 2. Jejunal morphology of broiler chickens (hematoxylin and eosin staining; n = 8). (A) Villus height; (B) crypt depth; (C) the villus height-to-crypt depth ratio. CTL = chickens received a basal diet without *Salmonella typhimurium* challenge; ARG = chickens received a basal diet supplemented with 0.3% arginine, but no *S. typhimurium* challenge; ST = chickens received a basal diet plus *S. typhimurium* challenge; ARGST = chickens received a basal diet supplemented with 0.3% arginine plus *S. typhimurium* challenge. Data are shown as the mean ± SEM. Abbreviation: VCR, the villus height-to-crypt depth ratio.

elevated the mRNA abundances of *IL-1β* and *IL-17* ($P < 0.05$). A significant interaction on the *IL-8* mRNA expression was observed in the jejunum of broiler chickens ($P = 0.05$). Birds in the ST group had a higher mRNA abundance of *IL-8* in the jejunum than those in the CTL, ARG, and ARGST groups ($P = 0{\cdot}05$). Both *S. typhimurium* challenge and Arg supplementation significantly elevated the mRNA abundance of *IL-10* ($P < 0.05$). A trend for Arg × ST interaction was displayed on *IL-22* mRNA expression ($P < 0.10$).

Gut Microbiome

A total of 1,171,782 reads were generated from 20 ileal samples (5 samples per group) of broiler chickens, and these reads were assigned to 8,734 OTU (Supplemental Table 1). Each simple had 58,589 ± 1,305 (mean ± standard error) reads and 437 ± 29 (mean ± standard error) OTU on average. The Good's coverage indices were greater than 99.7% in all the ileal samples (Supplemental Table 1) and rarefaction curves based on the observed OTU reached a plateau (Supplemental Figure 1), both indicating that sufficient sequencing coverage was achieved to represent all OTU present in the samples (Supplemental Figure 1).

The alpha diversity indexes, observed species, Simpson, Chao1, and ACE were not influenced by the effects of Arg supplementation, *S. typhimurium* challenge, or their interaction ($P > 0.05$, Supplemental Table 2). There was a significant interaction between Arg addition and *S. typhimurium* challenge on the Shannon index ($P < 0.05$). Beta diversity analysis was illustrated by PCoA and UPGMA in Figure 3. PCoA of OTU based on weighted UniFrac distances indicated there was no distinct separation of ileal microbiota between CTL, ARG, and ARGST groups, but the CTL and ST group

Table 3. The concentration of serum inflammatory parameters[1] (n = 8).

Items[2]	DAO (U/L)	CRP (ug/mL)	PCT (pg/mL)	IL-1β (pg/mL)	IL-8 (pg/mL)	LITNF (pg/mL)
CTL	2.67	1.33	199.36	29.00[a]	80.18[a]	102.34[a]
ARG	3.13	1.68	154.99	32.26[a]	81.35[a]	95.34[a]
ST	3.78	2.10	249.07	49.85[b]	172.32[b]	294.85[b]
ARGST	3.49	2.57	215.67	25.88[a]	88.42[a]	85.98[a]
SEM	0.153	0.203	10.801	2.023	10.009	25.900
Main effect						
Arg						
−	3.26	1.71	222.30	39.42	119.67	198.60
+	3.32	2.09	185.33	29.07	85.12	90.35
ST						
−	2.90	1.52	175.70	30.63	80.73	99.07
+	3.63	2.33	229.98	37.87	124.37	190.42
P value						
Arg	0.756	0.297	0.048	<0.001	0.014	0.018
ST	0.014	0.045	0.007	0.006	0.004	0.042
Interaction	0.191	0.876	0.771	<0.001	0.011	0.026

[a,b]Means with no common superscript in the same column are significantly different ($P < 0.05$).

[1]DAO = diamine oxidase; CRP = C-reactive protein; PCT = procalcitonin; LITNF = lipopolysaccharide-induced tumor necrosis factor-alpha factor homolog.

[2]CTL = chickens received a basal diet without *Salmonella typhimurium* challenge; ARG = chickens received a basal diet supplemented with 0.3% arginine, but no *S. typhimurium* challenge; ST = chickens received a basal diet plus *S. typhimurium* challenge. ARGST = chickens received a basal diet supplemented with 0.3% arginine plus *S. typhimurium* challenge.

Table 4. The gene expression of cytokines[1] in the jejunum (n = 8).

Items[2]	*IFN-γ*	*LITNF*	*IL-1β*	*IL-8*	*IL-10*	*IL-17*	*IL-22*
CTL	1.01	1.04	1.01	1.07[a]	1.12	1.04	1.11
ARG	1.31	1.11	1.12	1.22[a]	2.34	0.96	0.97
ST	0.87	1.01	1.61	2.47[b]	2.22	1.18	5.53
ARGST	1.28	0.91	1.43	1.16[a]	4.71	1.54	1.11
SEM	0.081	0.050	0.105	0.205	0.469	0.089	0.682
Main effect							
Arg							
−	0.94	1.03	1.31	1.77	1.67	1.11	3.12
+	1.29	1.01	1.27	1.19	3.52	1.25	1.04
ST							
−	1.14	1.08	1.07	1.14	1.66	1.00	1.05
+	1.07	0.96	1.52	1.76	3.32	1.36	3.12
P value							
Arg	0.032	0.875	0.845	0.114	0.032	0.385	0.070
ST	0.589	0.283	0.034	0.072	0.043	0.040	0.071
Interaction	0.728	0.417	0.461	0.050	0.431	0.187	0.088

[a,b]Means with no common superscript in the same column are significantly different ($P < 0.05$).

[1]IFN-γ = interferon-γ; LITNF = lipopolysaccharide-induced tumor necrosis factor-alpha factor homolog.

[2]CTL = chickens received a basal diet without *Salmonella typhimurium* challenge; ARG = chickens received a basal diet supplemented with 0.3% arginine, but no *S. typhimurium* challenge; ST = chickens received a basal diet plus *S. typhimurium* challenge. ARGST = chickens received a basal diet supplemented with 0.3% arginine plus *S. typhimurium* challenge.

and ARGST and ST group occupied different positions. The UPGMA analysis based on weighted UniFrac distance matrix showed that gut microbiota derived from the ST group displays little similarity with the other 3 groups.

As shown in Table 5, Arg addition significantly increased the relative abundance of ileal Firmicutes ($P < 0.05$) and tended to decrease the relative abundance of ileal Bacteroidetes ($P < 0.10$) regardless of *S. typhimurium* challenge. There was a significant interaction between Arg supplementation and *S. typhimurium* challenge on the relative abundance of Proteobacteria ($P < 0.05$). Birds with only *S. typhimurium* challenge had the highest relative abundance of Proteobacteria in the ileum, but it did not differ significantly among the other 3 groups ($P > 0.05$).

According to Table 6, there was a significant interaction effect between Arg addition and *S. typhimurium* challenge on the relative abundance of *Candidatus Arthromitus* genus ($P < 0.05$). Challenged birds fed Arg-supplemented diet exerted a notable increase in the relative abundance of ileal *Candidatus Arthromitus* genus compared with that in the challenged birds fed control diet. The relative abundance of *Escherichia–Shigella* genus was significantly influenced by Arg supplementation, *S. typhimurium* challenge, and their interaction ($P < 0.05$). The relative abundance of ileal *Escherichia–Shigella* genus was significantly increased in the ST group compared with that in the other 3 groups. A dramatic interaction effect on the relative abundance of *Lactobacillus* genus was observed between Arg addition and *S. typhimurium* infection ($P < 0.05$). Uninfected birds fed diets supplemented with Arg had the highest relative abundance of *Lactobacillus* genus in the ileum among the 4 groups.

To identify differentially abundant biomarkers in experimental groups, we performed LefSe analysis (Figure 4). The results show that the species *Escherichia coli* and *Ochrobactrum intermedium* and the genus *Nitrosomonas* were significantly more abundant in the ST group than in the CTL group, whereas the genus *Candidatus Arthromitus* and the families Rhodocyclaceae were more enriched in the CTL group than in the ST group ($P < 0.05$) (Figure 4A). The species *Rhizobium cellulosilyticum* and *Rubrobacter xylanophilus* and the genus *Nitrosomonas* exhibited relatively higher abundances in the ST samples than in the ARGST samples, but the genus *Methylobacterium* and *Candidatus Arthromitus* were over-represented in the ARGST group (Figure 4B). The species *Lactobacillus salivarius* and *Streptococcus suis* were significantly more abundant in the ARG group than in the CTL group ($P < 0.05$); however, no bacterial taxon was over-represented in the CTL group (Figure 4C).

DISCUSSION

S. typhimurium infection causes gut inflammation, intestinal barrier damage, a decline of growth performance, or even higher mortality (Barrow et al., 1987, 1988; Fasina et al., 2010). Intestinal histopathology, morphology, and microbiota balance are important indicators of intestinal health. In the present study, *S. typhimurium* challenge damaged intestinal mucosa, as observed by higher histopathological injury score (Figure 1B), less villus height (Figure 2A), and decreased VCR (Figure 2C), which was similar to the observations of the previous studies (Rajani et al., 2016; Jazi et al., 2018). However, the addition of Arg attenuated intestinal histopathological (Figure 1) and morphological damage (Figure 2). In line with our results, dietary Arg addition has been reported to maintain intestinal health in weaned pigs challenged by *E. coli* lipopolysaccharide (Liu et al., 2008) and in weaned

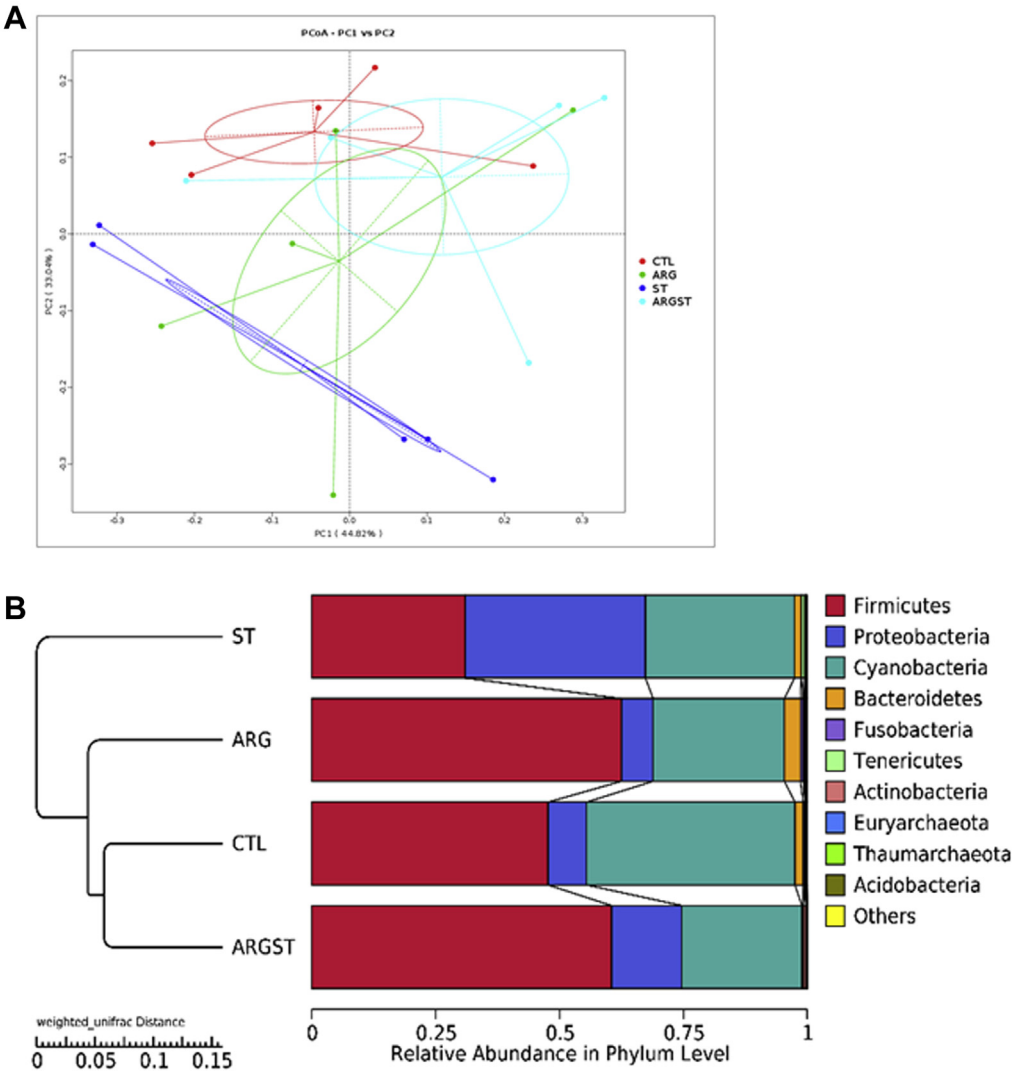

Figure 3. Beta diversity analysis of ileal microbiota based on weighted UniFrac distances (n = 5). (A) Principal coordinates analysis (PCoA); (B) unweighted pair-group method with arithmetic means (UPGMA) clustering tree structure. CTL = chickens received a basal diet without *Salmonella typhimurium* challenge; ARG = chickens received a basal diet supplemented with 0.3% arginine, but no *S. typhimurium* challenge; ST = chickens received a basal diet plus *S. typhimurium* challenge; ARGST = chickens received a basal diet supplemented with 0.3% arginine plus *S. typhimurium* challenge.

pigs under oxidative stress induced by diquat (Zheng et al., 2017). These results suggested that Arg addition had protective effects on the intestine of broiler chickens subjected to *S. typhimurium* challenge.

DAO is an intracellular enzyme particularly abundant in the small intestinal epithelia and released into the peripheral circulation as a result of intestinal villi damage, so the level of serum DAO could reflect the severity of intestinal mucosal injury (Fukudome et al., 2014). In this study, the serum DAO levels of *S. typhimurium*–challenged groups were significantly increased compared with those of unchallenged groups (Table 3), suggesting the idea of an impairment of small intestinal mucosal integrity after *S. typhimurium* challenge. CRP is a classic acute phase protein and has been used as a sensitive indicator of inflammation (Simon et al., 2004). Our results demonstrated that *S. typhimurium* infection significantly increased the serum CRP level of broiler chickens (Table 3). Chen et al. (2012b) found that Arg addition reduced the serum CRP level which was enhanced by *Salmonella choleraesuis* challenge in weaned piglets. However, in our study, dietary Arg supplementation failed to reduce serum CRP level (Table 3). PCT, a diagnostic marker of bacterial infection and sepsis (Simon et al., 2004), was increased by *S. typhimurium* challenge, but pretreatment with Arg remarkably decreased the serum PCT level (Table 3), reflecting that the severity of *S. typhimurium* infection in broiler chickens was ameliorated by Arg addition.

Proinflammatory cytokines are essential in initiating immune responses and eliminating pathogens from the host. However, their exaggerated or prolonged secretion may be detrimental to the host (Smith and Humphries, 2009). In our study, *S. typhimurium* challenge significantly promoted serum IL-1β, IL-8, and LITNF levels (Table 3) and jejunal *IL-1β* and *IL-8* mRNA expression

Table 5. The top 4 most abundant bacteria at the phylum level (n = 5).

Items[1]	Firmicutes	Proteobacteria	Cyanobacteria	Bacteroidetes
CTL	47.666	7.780[a]	42.083	1.640
ARG	62.528	6.393[a]	26.485	0.332
ST	30.942	36.385[b]	30.090	1.391
ARGST	60.508	5.706[a]	24.251	0.249
SEM	4.9962	4.2604	4.5127	0.3230
Main effects				
Arg				
-	39.304	22.082	36.087	1.160
+	61.518	6.088	25.368	0.286
ST				
-	55.097	7.087	34.284	1.059
+	45.725	22.750	27.170	0.820
P value				
Arg	0.024	0.025	0.262	0.072
ST	0.307	0.047	0.451	0.797
Interaction	0.420	0.039	0.604	0.897

[a,b]Means with no common superscript in the same column are significantly different ($P < 0.05$).

[1]CTL = chickens received a basal diet without *Salmonella typhimurium* challenge; ARG = chickens received a basal diet supplemented with 0.3% arginine, but no *S. typhimurium* challenge; ST = chickens received a basal diet plus *S. typhimurium* challenge. ARGST = chickens received a basal diet supplemented with 0.3% arginine plus *S. typhimurium* challenge.

(Table 4). Similar studies showed that *S. typhimurium* challenge enhanced the levels of these proinflammatory cytokines in chickens (Withanage et al., 2004) and mice (Brown et al., 2013; Moreira et al., 2017). In the present study, Arg supplementation remarkably decreased LITNF, IL-1β, and IL-8 concentration in the serum (Table 3) and *IL-8* mRNA expression in the jejunum (Table 4) but increased jejunal *IFN-γ* mRNA expression (Table 4). Among the cytokines, IFN-γ was shown to play an important role in the clearance of systemic stages of primary *Salmonella* infection in chickens (Kogut et al., 2005; Withanage et al., 2005). The elevation of *IFN-γ* mRNA expression after Arg addition may contribute to the clearance of *S. typhimurium*. However, there are few studies about dietary Arg supplementation on the cytokine mRNA expression in animals infected with *S. typhimurium*. Previous studies have reported that Arg prevented the overproduction of proinflammatory cytokines (TNF-α, IL-1β, and IL-8) in many other stress models (Fu et al., 2005; Faddah et al., 2012; Wu et al., 2013). IL-10 is a pivotal anti-inflammatory cytokine to inhibit the production of proinflammatory mediators (Smith and Humphries, 2009). In this study, we observed elevated *IL-10* mRNA expression in the jejunum of birds after *S. typhimurium* challenge (Table 4). Similarly, Xu et al. (2018) reported that *S. typhimurium* challenge increased IL-10 secretion in the ileum, colon, and serum

Table 6. The top 10 most abundant bacteria[1] at the genus level (n = 5).

Items[2]	CA	ES	uCh	Lac	Tur	Bac	uMi	Str	Rom	Fae
CTL	40.576[a,b]	0.739[a]	42.074	3.210[a]	0.097	1.312	6.058	0.746	0.162	0.004
ARG	34.030[a,b]	1.897[a]	26.480	14.727[b]	0.607	2.670	3.443	3.905	0.308	0.012
ST	12.232[a]	37.094[b]	30.088	4.479[a]	5.297	0.927	3.612	0.788	1.263	0.015
ARGST	53.047[b]	5.906[a]	24.249	3.584[a]	1.173	0.036	2.585	0.401	0.658	0.001
SEM	6.0507	4.3536	4.5116	1.7285	1.0681	0.6863	0.6944	0.5621	0.2529	0.0028
Main effects										
Arg										
−	26.404	16.897	36.081	3.844	2.697	1.120	4.835	0.767	0.712	0.009
+	43.539	3.901	25.364	9.775	0.890	1.353	3.014	2.153	0.483	0.007
ST										
−	37.303	1.318	34.277	8.969	0.352	1.991	4.750	2.325	0.235	0.008
+	32.640	19.767	27.168	4.081	3.235	0.481	3.099	0.594	0.961	0.008
P value										
Arg	0.136	0.028	0.262	0.083	0.400	0.872	0.202	0.184	0.658	0.657
ST	0.675	0.005	0.452	0.104	0.186	0.303	0.245	0.102	0.173	0.974
Interaction	0.045	0.020	0.604	0.046	0.283	0.440	0.570	0.095	0.471	0.069

[a,b]Means with no common superscript in the same column are significantly different ($P < 0.05$).

[1]CA = *Candidatus Arthromitus*; ES = *Escherichia–Shigella*; uCh = unidentified chloroplast; Lac = *Lactobacillus*; Tur = *Turicibacter*; Bac = *Bacteroides*; uMi = unidentified mitochondria; Str = *Streptococcus*; Rom = *Romboutsia*; Fae = *Faecalibacterium*.

[2]CTL = chickens received a basal diet without *Salmonella typhimurium* challenge; ARG = chickens received a basal diet supplemented with 0.3% arginine, but no *S. typhimurium* challenge; ST = chickens received a basal diet plus *S. typhimurium* challenge. ARGST = chickens received a basal diet supplemented with 0.3% arginine plus *S. typhimurium* challenge.

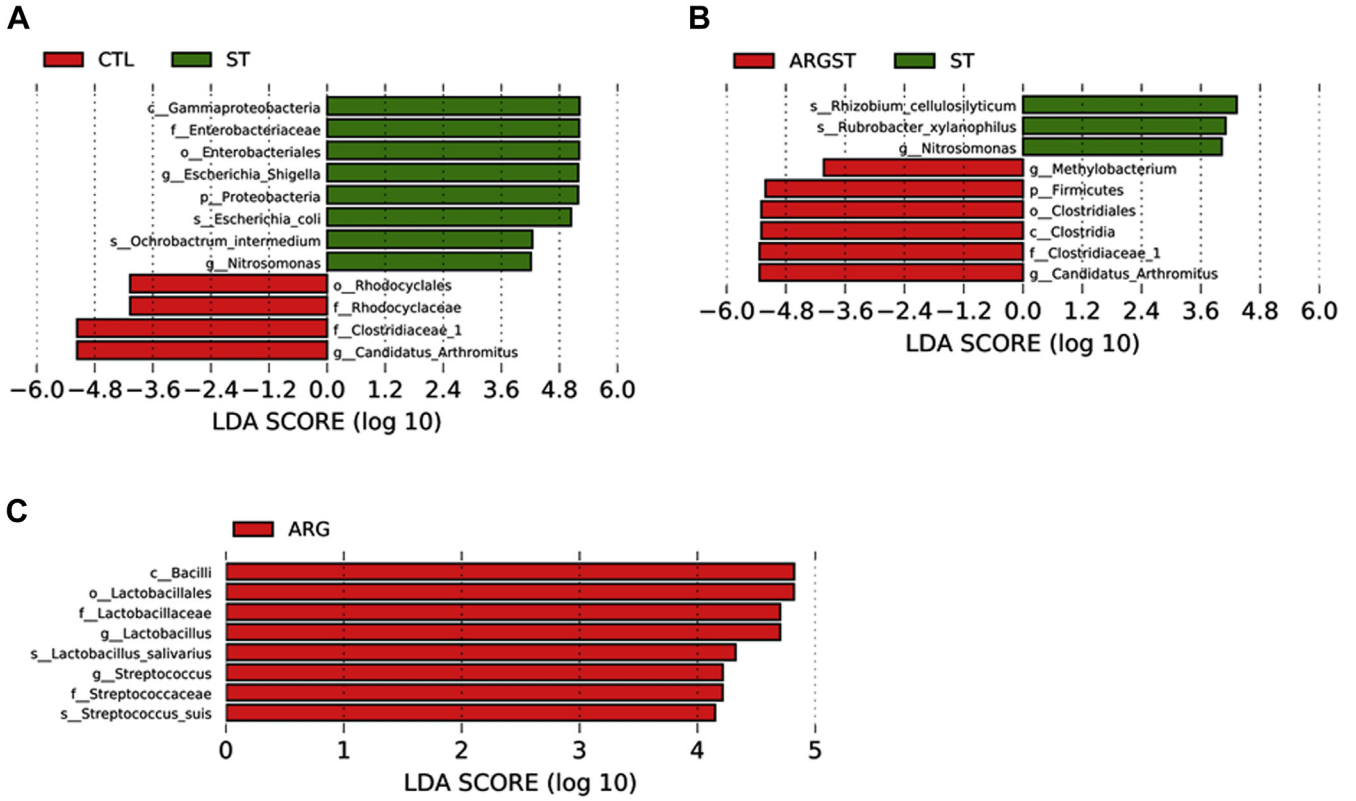

Figure 4. Differentially abundant bacterial taxa based on LEfSe analysis (n = 5). (A) Comparison between the CTL group and ST group; (B) comparison between the ARGST group and ST group; (C) comparison between the CTL group and ARG group. CTL = chickens received a basal diet without *Salmonella typhimurium* challenge; ARG = chickens received a basal diet supplemented with 0.3% arginine, but no *S. typhimurium* challenge; ST = chickens received a basal diet plus *S. typhimurium* challenge; ARGST = chickens received a basal diet supplemented with 0.3% arginine plus *S. typhimurium* challenge. LDA score >4 and $\alpha = 0.05$. Abbreviations: LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size.

of mice. In addition, Arg addition also promoted its mRNA expression (Table 4), which may be contributing to alleviating inflammation. Moreover, Powell et al. (2016) observed that IL-10 addition before or concurrent with infection dampened *S. typhimurium* replication in bone marrow–derived macrophages. IL-17 and IL-22 are key in regulating homeostasis and epithelial barrier function (Eyerich et al., 2010). Both can be protective against infections and also turn pathological in several inflammatory diseases (Eyerich et al., 2010). Behnsen et al. (2014) found that IL-22 induction can be exploited by pathogens such as *S. typhimurium* to suppress the growth of commensal bacteria, thereby promoting pathogen colonization. In the present study, *S. typhimurium* challenge significantly increased jejunal *IL-17* mRNA expression and tended to enhance *IL-22* mRNA expression in the jejunum of the broiler chickens, but Arg addition tended to reverse the increase of jejunal *IL-22* mRNA expression (Table 4). The changes of levels and mRNA expressions of these cytokines indicated that Arg attenuated systemic and intestinal inflammatory responses, which may be related to the improvement of gut health in broiler chickens after infection with *S. typhimurium*.

Gut microbiota is a critical bridge between diet and host health. The structure of gut microbial community is greatly influenced (estimated at 57%, compared with 12% for genetic factors) by the diet (Tomasello et al., 2014). The gut microbiota effectively influences host in the regulation of homeostasis, organ development, metabolic processes, and immune response (Tremaroli and Backhed, 2012). In this study, PCoA (Figure 3A) and UPGMA (Figure 3B) analyses revealed that the CTL, ARG, and ARGST groups shared a higher similarity in terms of the ileal microbiota composition than the ST group. These data suggested that Arg supplementation played a beneficial role in regulating the gut microbiota of broiler chickens challenged with *S. typhimurium*. In our previous study, Arg alleviated the gut injury of broiler chickens challenged with *C. perfringens* and regulated the gut microbiota of challenged chickens to resemble that of unchallenged controls (Zhang et al., 2018). The taxonomical composition analysis revealed that Arg addition enriched the relative abundance of the phylum Firmicutes and reduced the relative abundance of the phyla Proteobacteria and Bacteroidetes in the ileum (Table 5). Firmicutes accelerated the polysaccharide decomposition, improved the utilization of energy in the diet, and promoted intestinal health (Ley et al., 2006; Wang et al., 2013). The ratio of Firmicutes to Bacteroides was always positively associated with the growth performance of broilers (Singh et al., 2012). Gammaproteobacteria class belonging to Proteobacteria phylum contains the

majority of pathogens (Hernandez-Doria and Sperandio, 2013), such as *Salmonella* spp. and *Shigella* spp., which can induce severe disease under some conditions. Therefore, the enrichment in Firmicutes and the reduction in Bacteroides and Proteobacteria may contribute to the effect of Arg, promoting the intestinal health and growth performance of broiler chickens. At the genus level, Arg addition increased the relative abundance of *Candidatus Arthromitus* in the broiler chickens challenged with *S. typhimurium* (Table 6). *Candidatus Arthromitus*, a collective name for segmented, filamentous, nonculturable gram-positive bacteria, can specifically modulate host immune responses including the T helper (Th17) cell differentiation, the induction of gut IgA plasma cells, and intestinal IgA secretions (Robino et al., 2019). Recent studies found that *Shigella* strains are not clones of *E. coli* but members of the genus *Escherichia* (Zuo et al., 2013). *Shigella* species are a leading cause of bacterial diarrhea, which is a significant threat to the health of human and animals (Fischer Walker et al., 2010). Our study reflected that Arg supplementation may reduce the proportion of some harmful bacteria induced by *S. typhimurium* challenge.

LefSe analysis demonstrated that the family Enterobacteriaceae was more abundant in the ST group than in the CTL group (Figure 4A). Enterobacteriaceae, belonging to the class Gammaproteobacteria, encompasses gram-negative pathogens, such as *E. coli*, *Shigella* spp., and *Salmonella* spp. (Stecher et al., 2012). In this study, the family Clostridiaceae_1 exhibited higher abundance in the CTL group (Figure 4A) and ARGST group (Figure 4B) than in the ST group. Clostridiaceae contains genera that promote nutrient digestibility and pathogenic bacteria (Rajilic-Stojanovic and de Vos, 2014). Bermingham et al. (2017) revealed that Clostridiaceae is one of the 3 key families in the digestion of protein and energy in dogs. Our results showed that the abundance of *Lactobacillus* spp. was more dominant in the ARG group than in the CTL group (Figure 4C). Similar results were also found by Chen et al. (2012a) in the intestinal flora of fish. The *Lactobacillus* spp. is probiotic and can regulate host immune systems, enhance intestinal metabolic capacities, and maintain balance in the gut microbiota (Valeriano et al., 2017). In our study, the chickens in the ARG group contained a higher proportion of *Streptococcus* spp. than those of the CTL group (Figure 4C). The *Streptococcus* spp. includes commensals, pathogens, and opportunistic pathogens for humans and animals (Haenni et al., 2018). Similarly, Ren et al. (2014) demonstrated that Arg addition increased the abundance of *Streptococcus* in the ileum of mice.

In conclusion, Arg supplementation is effective in alleviating intestinal mucosal impairment in broiler chickens challenged with *S. typhimurium* by ameliorating inflammatory response and modulating gut microbiota. These results provide new evidence on the use of dietary Arg as an intervention strategy to control *S. typhimurium* infection in broiler production.

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SUPPLEMENTARY DATA

Supplementary data are available at *Poultry Science* online.

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