Dietary supplementation with vitamin C ameliorates the adverse effects of *Salmonella* Enteritidis-challenge in broilers by shaping intestinal microbiota

Liping Gan, Hao Fan, Tahir Mahmood, and Yuming Guo[1]

State Key Laboratory of Animal Nutrition, College of Animal Science and Technology, China Agricultural University, Beijing, 100193, People's Republic of China

ABSTRACT *Salmonella* Enteritidis (**SE**) infection is not only a leading cause of poor production performance and compromised animal welfare in broilers but also a potential threat to public health. Two experiments were conducted to evaluate the effects of dietary supplemental vitamin C (**VC**) on SE challenged-broilers. In experiment 1, one hundred eighty 1-day-old Arbor Acre broilers were randomly allocated into 3 treatments, with 0, 500, or 1,000 mg/kg VC included in the diet. In experiment 2, dietary VC at 0 or 500 mg/kg, with or without SE challenge was applied in a 2 × 2 factorial arrangement in 6 randomized complete blocks. In experiment 1, addition with 500 mg/kg VC increased BW and infectious bursal disease (**IBD**) titer of broilers on 35 D ($P < 0.05$), whereas 1,000 mg/kg VC had no effects on the IBD titer ($P > 0.05$) compared with the control group. In experiment 2, SE challenge depressed BW on 11 and 21 D ($P < 0.05$ and $P = 0.088$, respectively), whereas increased mortality and hepatic bacterial translocation ($P < 0.05$) on 21 D. Further, SE challenge resulted in lower villus height in jejunum, lower microbial richness, and diversity, whereas higher abundance of *Enterobacteriaceae* in cecum ($P < 0.05$). Importantly, supplementation with VC increased BW on both 21 and 35 D ($P < 0.05$ and $P = 0.088$, respectively) and enhanced the intestinal health by improving villus morphology and microbial structure as indicated by higher cecal microbial richness and *Firmicutes* to *Bacteroidetes* ratio, while lower abundance of *Enterobacteriaceae* ($P < 0.05$). In addition, birds fed with 500 mg/kg VC in the diet had significantly increased jejunal secretory immunoglobulin A levels, T lymphocytes stimulation index, and serum total antioxidant capability compared with groups without VC ($P < 0.05$). In conclusion, SE challenge induced lower production performance and higher mortality in broilers. However, dietary supplementation with VC ameliorated SE-caused damage in broilers by improving the intestinal health, partly mediated by shaping the structure of cecal microbiota.

Key words: broiler, *Salmonella* Enteritidis, microbiota, vitamin C, immunity

INTRODUCTION

Salmonellosis is a major cause of bacterial enteric illness in both humans and animals. Approximately 93.8 million cases of gastroenteritis due to *Salmonella* species occurred globally each year, of which 80.3 million cases were foodborne (F. W. Brenner et al., 2000; Majowicz et al., 2010). Owing to infection of broilers and contamination of eggs, poultry-derived products have been identified as a major source of *Salmonella* for human beings (Mahmoud, 2012). Clearly, *Salmonella* species colonization in broilers affects birds' health status and growth performance, thereby significantly reducing the economic returns from poultry flocks (Remus et al., 2014). Thus, reducing *Salmonella* infection in broilers can benefit the poultry industry and minimize the public health risk. Among all the serotypes, *Salmonella enterica* subsp. *enterica* serovar Enteritidis (**SE**) is the most frequently isolated serovar from broiler flocks (EFSA, 2007). Notably, chickens infected with SE are usually serve as asymptotic carrier, whereas, in humans, it is an enteric pathogen which incurs diarrheal disease (Awad et al., 2012). Chickens harboring SE asymptomatically make the identification of infected chickens much more challenging and could further result

in persistent infections, contamination of poultry products, and propagation of the pathogen (Mon et al., 2015; Shanmugasundaram et al., 2015).

Gut microbiota plays an instrumental role in shaping host's immune response, nutrient uptake, and production of essential metabolites for the host (Chambers and Gong, 2011; Pan and Yu, 2014). The symbiosis of microbiota is essential for reducing the risks of pathogen colonization and proliferation of already existing pathogens (Li et al., 2017). It has been observed that SE infection can disturb the gut microbiota balance in broilers by promoting pathogenic bacterial population (Bearson et al., 2013; Mon et al., 2015). Predominance of enteric pathogens can cause intestinal inflammation, thereby damaging the intestinal epithelium integrity and promoting electrolyte efflux which ultimately can transpire into diarrhea (Petra Videnska et al., 2013). Diarrhea can further aggravate the health status by reducing feed digestion and nutrient absorption in broilers (Remus et al., 2014).

Vitamin C (VC) is a key circulating antioxidant with anti-inflammatory and immune-modulatory properties. Moreover, it acts as a cofactor for many important mono-oxygenase and dioxygenase enzymes (Spoelstra-de Man et al., 2018). Interestingly, VC has also been suggested to alleviate the side-effects of multiple stressors in animals as well as cell culture experiments (J. S. Mckee and Harrison, 1995; Nosrati et al., 2017; Cheng et al., 2018; Yin et al., 2018). Chickens possess the ability to synthesize VC in kidneys through the glucuronatexylulose-xylulose cycle in the presence of L-gulonolactone oxidase ([EC 1.1.3.8], GLO). Several researches were reported that VC alone or with other components could decrease SE (Hernandez-Patlan et al., 2018) or other Salmonella species (Tamoghna Ghosh et al., 2019) in vitro. Hernandez-Patlan et al. (2019) also found 0.1% of dietary VC could serve as prophylactic or therapeutic ways to control SE infection in broilers. Still, it is unclear if VC could enhance the production performance of SE challenged broilers. In addition, there are limited studies reporting the correlation of VC and microbiota in broilers. Therefore, the main objectives of this study were to investigate the effects of dietary supplementation with VC on the production performance and gut microbiota of Salmonella-challenged broilers, as well decipher the underlying mechanisms.

MATERIALS AND METHODS

Animals, Experimental Design, and Feeding Management

Two trials were carried out to ascertain (1) the appropriate dose of VC used in the experiment and (2) the effects of VC on SE challenged broilers. The experimental animal protocols for the study was approved by and conducted in accordance with the Animal Care and Use Committee of China Agricultural University. In experiment 1, a total of one hundred eighty 1-day-old male Arbor Acre broilers were randomly allocated into 3 treatment groups, with 6 replicates of 10 birds in each replicate in battery cages. During the experimental period, the birds were fed with the pelleted basal diet with varying levels of VC, which was 0, 500, and 1,000 mg/kg in V 0, V 500, and V 1,000 groups. All the birds had free access to feed and fresh water. Body weight and feed intake were recorded for each replicate at 35 D. On the same day, 1 bird with average weight from each replicate was selected for blood collection.

For experiment 2, a total of two hundred forty 1-day-old male Arbor Acre broilers were randomly divided into 4 treatment groups with 6 replicates of 10 birds in each replicate. A cloaca swab was conducted to make sure all the birds were Salmonella-free. Birds were fed a pelleted basal diet with or without 500 mg/kg VC, together with or without SE challenge throughout the trial period. They were allotted into 2 dietary groups: VC0, basal diet with no VC supplementation and VC500, basal diet supplemented with 500 mg/kg VC. The basal diets were formulated to meet or exceed National Research Council (1994) requirements, of which the ingredient and nutrient composition is shown in Table 1. All the birds were kept on wire-floored cages in a 3-tier battery cage system. Birds were vaccinated against Newcastle disease virus and infectious bronchitis virus at 9 D of age, and infectious bursal disease (IBD) virus at 21 D of age via intranasal and intraocular administration. Feed and fresh water were available ad libitum. A 20 h light to 4 h dark cycle was followed throughout the trial period. Temperature was kept at 35°C on day 1 and then was gradually reduced by 5°C every wk till it reached at 24°C on 21 D.

Oral Challenge and Sampling

The Salmonella serotype Enteritidis CVCC3379 was obtained from China Veterinary Culture Collection Center, the China Institute of Veterinary Drug Control (Beijing, China). The freeze-dried cultures of SE were rehydrated in 10 mL sterile tryptone soy broth (CM201, TSB, Land Bridge Technology Ltd., Beijing, China). 5 μL of the suspension were plated 2 times successively on Xylose Lysine Desoxycholate Agar base (XLD, CM 219, Land Bridge Technology Ltd.) for 24 h at 37°C. Then 30 mL of preculture was prepared by picking a single colony into sterile prewarmed tryptone soy broth and incubated at 37°C with orbital shaking for 24 h. Subsequently, 10 mL of SE preculture was transferred into 300 mL of sterile tryptone soy broth and incubated with orbital shaking at 37°C for 16 h. Determination of the number of colony-forming units (CFU) through decimal dilution series was performed in sterile buffered 0.9% peptone water with pH 7.2. The stock culture was adjusted to 10^{10} CFU/mL by centrifuging at 1,600 g, 4°C, 10 min. The SE solution was freshly prepared before oral challenging.

At 8 to 10 D and 17 to 19 D of age, birds in challenged groups were orally gavaged 1 mL bacterial suspension containing approximately 10^{10} CFU Salmonella.

Table 1. Ingredients and chemical composition of basal diet (experiments 1 and 2).

	Growing period, g/kg	
Ingredients, %	Starter period (1–21 D)	Grower-finisher period (22–35 D)
Corn	53.82	59.76
Soybean meal	38.54	33.22
Soy oil	3.52	3.50
Dicalcium phosphate	1.76	1.21
Calcium carbonate	1.26	1.39
Sodium chloride	0.35	0.30
Choline chloride (50%)	0.20	0.20
L-Lys × HCl	0.20	0.16
DL-Met	0.15	0.10
Trace mineral premix[1]	0.18	0.13
Vitamin premix[2]	0.02	0.02
Total	100.00	100.00
Analyzed/calculated chemical composition		
ME, MJ/kg	12.36	12.60
CP, %	22.02	19.72
Ca, %	1.00	0.90
available P, %	0.45	0.35
Lys, %	1.19	1.04
Met, %	0.5	0.43
Met + Cys, %	0.85	0.75

[1]Provided per kilogram of diet: Cu, 8 mg; Zn, 75 mg; Fe, 80 mg; Mn, 100 mg; Se, 0.15 mg; I, 0.35 mg.
[2]Provided per kilogram of diet: vitamin A, 10,000 IU; vitamin D_3, 2,400 IU; vitamin E, 40IU; vitamin K_3, 2 mg; vitamin B_1, 2 mg; vitamin B_2, 6.4 mg; vitamin B_6, 3 mg; vitamin B_{12}, 0.02 mg; folic acid, 1 mg; niacin, 30 mg; Ca-pantothenate acid, 10 mg.

Unchallenged group received the same amount of sterile tryptone soy broth without *Salmonella* at the same time. Feed was withdrawn from all the challenged and unchallenged birds 10 h before challenge and returned immediately after the challenge. At 21 D, 2 D after infection, 6 birds from each group were randomly selected for sample collection. Blood samples were collected aseptically from the wing vein. Part of the fresh blood were used to separate peripheral blood mononuclear cells (**PBMC**), and the rest were centrifuged at 1,600 g for 10 min at 4°C for serum collection. After blood collection, the birds were sacrificed by intravenously injecting with 30 mg pentasorbital sodium per kg weight immediately. The midpoint of jejunum (about 1 cm) was cut off and fixed in 4% paraformaldehyde solution (Kiernan, 2000; Du et al., 2016), and the mucosa was scraped from 10 cm of the jejunum (proximal to Meckel's diverticulum), whereas the cecal contents were collected by gently squeezing. The liver was collected to detect the bacteria translocation. The kidney and spleen were immediately snap-frozen in liquid nitrogen and put into –80°C until analysis. The timeline of SE challenging and sampling is present in Figure 1.

Production Performance

In experiment 1, body weight and feed intake of broilers were recorded for each replicate, and feed conversion ratio (**FCR**) were calculated at 35 D of age. While in experiment 2, body weight and feed intake of broilers were recorded for each replicate at 11, 21, and 35 D of age. Feed conversion ratio and mortality during the overall period (1–35 D) were calculated.

Intestinal Morphological Observation and Bacterial Translocation

The jejunum segments fixed in 4% paraformaldehyde were embedded in paraffin. Tissue rings were cut to a thickness of 5 μm and stained by hematoxylin and eosin. The slides were photographed by a Leica microscope (Wetzlar, Germany, Model DMi8).

For detection of bacterial translocation in liver, approximately 0.4 g of sample were aseptically placed into preweighed 5-mL sterile plastic tubes (Corning Inc., Beijing, China), weighed, and diluted with sterile buffered peptone water (CM201, Land Bridge
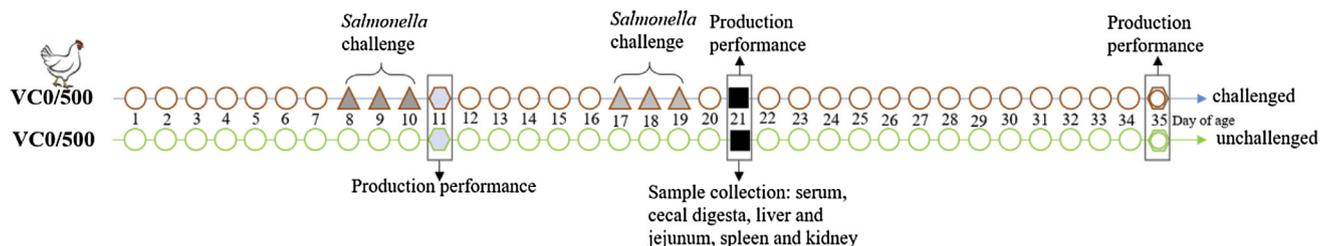

Figure 1. Animal experiment design in experiment 2. VC0/500, dietary supplementation with either 0 or 500 mg/kg vitamin C.

Technology Ltd.) to an initial 10^{-1} dilution. The solutions were subsequently homogenized separately using a Heidolph Diax 600 homogenizer (Heidolph, Schwabach, Germany) for 30 s. Then 100 μL of each solution was plated on XLD agar. After aerobic incubation at 37°C for 24 h, the liver *Salmonella* was evaluated by counting *Salmonella* colonies on XLD agar.

Immunoglobulin G and IBD Titer in the Serum Contents in Serum, Secretory Immunoglobulin A of Jejunum

The IBD titer of the serum in experiment 1 was determined using IBD virus antibody test kit (IDEXX Laboratories, Inc., Westbrook, ME) following the protocols provided by the manufacturer.

The immunoglobulin G in plasma concentrations were determined using an ELISA kit from Bethyl Laboratories (Montgomery, TX) according to the manufacturer's protocol. In the present study, the suitable serum dilution was found to be 1: 200,000. The immunoglobulin G levels were determined using a standard curve and were expressed in ng/mL serum. The secretory immunoglobulin A (**sIgA**) concentration in jejunum was detected following the method described by Du et al. (2016). Briefly, the mucosa samples were thawed at room temperature and homogenized in 4 volumes of ice-cold PBS. The homogenate was centrifuged at 2,800 g for 20 min at 4°C, and the supernatant was analyzed for sIgA and protein content by ELISA kit (Bethyl Laboratories Inc.) and BCA protein assay kit (Pierce Biotechnology, Rockford, IL) according to the manufacture's protocol, respectively. The final sIgA concentrations were expressed as mg/g protein.

Total Antioxidant Ability

Total antioxidant capacity (**T-AOC**) of the serum was detected by a biochemical method following the instructions provided with the reagent kits (A015), purchased from Nanjing Jiancheng Bioengineering Institute of China.

Measurement of GLO Activity in Kidneys and VC Concentrations in Spleens

Kidney and spleen samples collected on 21-day-old broilers were put in −80°C before analyzing. L-gulonolactone oxidase enzyme activity was measured by determining the rate of total VC synthesized in kidney tissues by adding the substrate L-gulonolactone. The detailed procedures for the activity of GLO were described before (Gan et al., 2018).

Vitamin C was measured using HPLC detection method as described by Gan et al. (2018). The spleen samples were homogenized in 5.4% metaphosphoric acid and centrifuged at 16,000 × g for 15 min at 4°C. For the measurement of total VC, 5 mmol/L Tris (2-carboxyethyl) phosphine hydrochloride in water (pH 2)

was used. The retention time was 3.89 min. A 7-min delay was provided between injections to produce a smooth baseline. For both methods, a standard curve was developed by using a peak area linear regression equation from 6 VC standards made in metaphosphoric acid and EDTA ranging in concentration from 2 to 50 mg/L.

The Proliferation of T Lymphocyte

It was reported that stimulated T lymphocyte is responsible for protecting the chicks from SE invasion (E. D. Mcgruder et al., 1993). To monitor T lymphocytes proliferation, the PBMC was isolated from the blood collected from brachial vein on 21-day-old, using a method that described before (Gan et al., 2018). The procedures for measuring the proliferation of T lymphocytes were based on the method described by Mosmann (1983) with some modification. The isolated PBMC were split in 96-well plate and grown in RPMI 1640 supplemented with 50 μM 2-mercaptoethanol and 10% fetal bovine serum, in a 5% CO_2 atmosphere. After incubating with 25 μg/mL of concanavalin A (Sigma, catalog No. L2128) for 68 h, the stock 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma catalog NO. M2128) solution was added to get a final concentration of 5 mg/mL, and the plate was incubated at 37°C for another 4 h, following by acid-isopropanol to dissolve the dark blue crystals. After keeping at room temperature a few minutes to ensure that all crystals were dissolved, the plates were read on a Dynatech MR580 Microelisa reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm. The results of T lymphocytes stimulation were expressed as stimulation index.

Pyrosequencing Cecal Microbiota

DNA samples were extracted from cecal digesta using QIAamp DNA Stool Mini Kits (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions. The concentration and purity of the DNA samples were checked with gel electrophoresis. The microbial 16S rRNA sequences were amplified with universal primers 515 F (5′-GTG CCA GCM GCC GCG GTA A-3′) and 806 R (5′-GGA CTA CHV GGG TWT CTA AT-3′) targeting the V3-V4 region according to the PCR methods described by Wang et al. (2016). The PCR products were detected by 2% gel electrophoresis and purified with QIAquick Gel Extraction Kit (Qiagen Inc.). A library was constructed using Ion Plus Fragment Library Kit 48 rxns (Thermo Scientific Inc., Waltham, MA) and quantified by Qubit 2.0 Fluorometer (Thermo Fisher Scientific) to pool at equal concentrations. Pyrosequencing for 16S rRNA was carried out on the Illumina HiSeq2500 PE250 platform (Illumina, San Diego, CA). All of the procedures were conducted by Novogene Bioinformatics Technology Co. Ltd. (Beijing, China). The clean reads were obtained after the quality filtering by Cutadapt (V1.9.1, http://cutadapt.readthedocs.io/en/stable/) and Chimera removal by UCHIME algorithm

(UCHIME Algorithm, http://www.drive5.com/usearch/manual/uchime_algo.html). Then the clean reads were clustered into operational taxonomic units (**OTU**) using Uparse in QIIME software (Uparse v7.0.1001, http://drive5.com/uparse/) with a similarity threshold of 97%. The OTU were obtained from Mothur and were sorted from most to least abundant. The 16S rRNA gene sequences were aligned using the multiple sequence alignment method MUSCLE (Version 3.8.31 , http://www.drive5.com/muscle/). And the OTU were further subjected to the taxonomy-based analysis by the RDP algorithm using the Greengenes database (http://greengenes.lbl.gov). Alpha diversity metrics analysis describing community richness including observed species, abundance-based coverage estimators (**ACE**) and chao1, and indices describing microbial diversity including Shannon and Simpson index were present. Beta diversity (weighted UniFrac, principal coordinate analysis) were analysed using QIIME (Version 1.9.1) to characterize the microbial population diversity.

Statistical Analysis

In experiment 1, one-way analysis of variance was conducted followed by Duncan's multiple comparisons to obtain the significance of differences between experimental groups (SPSS for windows, version 22; IBM). Linear and quadratic polynomial contrasts were used to evaluate the effects of the different dietary levels of VC. Data from experiment 2 were analyzed using the General Linear Model procedure in SPSS (version 22, IBM) and subjected to two-way ANOVA in a 2 x 2 factorial arrangement to analyze the main effects of dietary treatment and SE challenge and their interaction. One-way ANOVA was used to analyze the results if interaction was significant. For the Pearson's correlation analysis, the t test was used to establish if the correlation coefficient is statistically significant. Statistical differences were considered significant at $P \leq 0.05$, and $0.05 < P \leq 0.10$ was viewed as a trend toward significance.

RESULTS

Production Performance

As described in Table 2, in experiment 1, inclusion of 500 mg/kg or 1,000 mg/kg VC in the diet of the broilers significantly increased the body weight on 35-day-old age compared with V 0 group ($P < 0.05$). However, FCR was not influenced by the treatments ($P > 0.05$). Supplementation with 500 mg/kg VC in the diet significantly increased the IBD titer ($P < 0.05$) in the serum of 35-day-old broilers, whereas no influence was observed between the groups V 0 and V 1,000 ($P > 0.05$). Besides, no difference on body weight was noted between V 500 and V 1,000 groups ($P > 0.05$). Therefore, the dose of 500 mg/kg VC was used in experiment 2 to explore the effects of VC on SE challenged broilers.

In experiment 2, the broilers challenged with SE had higher mortality rate ($P < 0.05$) during the whole rearing period while lower body weight on day 11 and 21 ($P < 0.05$ and $P = 0.088$, respectively) compared with unchallenged birds. However, on 35-day-old, the body weight of the broilers was not influenced by SE challenge ($P > 0.05$, Table 3). Dietary supplementation with VC significantly increased the body weight of birds on day 21 and 35 ($P < 0.05$ and $P = 0.067$, respectively). The increased mortality of birds because of SE challenge was not influenced ($P > 0.05$) by dietary treatment. No interactions on the production parameters were found between dietary treatments and SE challenge on days 11, 21, and 35 ($P > 0.05$).

Hepatic Salmonella Translocation and Intestinal Morphology (Experiment 2)

On day 21, birds in SE challenged groups had higher hepatic *Salmonella* concentrations compared with unchallenged groups ($P < 0.05$, Figures 2A and 2B). No *Salmonella* translocation was found in unchallenged groups in the liver of broilers. Compared with VC0, supplementary with VC at 500 mg/kg decreased the frequency of SE translocation rate to liver (66.7 vs. 16.7%).

Because most digested nutrients are absorbed in upper portion of the small intestine (duodenum and jejunum) (Yamauchi et al., 2010), the jejunal histology was detected in the present study. As shown in Figures 2C and 2D, SE challenge caused atrophy of the jejunal villi, which resulted in reduction in jejunal villus height (**VH**, $P < 0.05$) and villus height: crypt depth ratio (**VH:CD**, $P = 0.06$). However, inclusion of VC in the diet of broilers significantly decreased the jejunal CD, whereas increased jejunal VH:CD ($P < 0.05$) on 21-day-old.

Table 2. Supplementation of vitamin C on production performance of broilers (experiment 1).

	Treatments				P-value		
Items	V 0	V 500	V 1,000	SEM[1]	Main effect	Linear	Quadratic
BW, kg	1.859[b]	1.927[a]	1.946[a]	0.073	0.012	0.008	0.290
FCR, g:g	1.672	1.658	1.681	0.042	0.449	0.632	0.226
IBD titer	1,892[b]	2,737[a]	2,098[a,b]	141.4	0.037	0.523	0.013

[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$.
Data represent the mean of 6 cages (10 broilers per cage) per treatment.
Abbreviations: BW, body weight; FCR, feed conversion ratio; IBD, infectious bursal disease; V 0, diet without vitamin C supplementation; V 500, diet with 500 mg VC/kg diet; V 1,000, diet with 1,000 mg VC/kg diet.
[1]The SEM values represent the overall standard error of means in each line.

Table 3. Production performance of broilers challenged with *Salmonella* (experiment 2).

Items	SE (-)		SE (+)		SEM[1]	*P*-values		
	VC0	VC500	VC0	VC500		Challenge	Diet	Interaction
BW, g								
11 D	257.7	254	245.2	251.5	1.84	0.028	0.356	0.612
21 D	659.7	702.3	646.8	673.8	5.75	0.088	0.007	0.501
35 D	1,736	1,871	1,810	1,867	19.06	0.494	0.067	0.446
FCR, g:g								
1–11 D	1.37	1.35	1.39	1.36	0.009	0.396	0.173	0.824
12–21 D	1.59	1.53	1.56	1.54	0.016	0.325	0.504	0.233
0–35 D	1.66	1.63	1.62	1.63	0.012	0.482	0.599	0.441
mortality rate, %	1.67	1.67	13.33	10	1.214	0.001	0.468	0.468

Data represent the mean of 6 cages (10 broilers per cage) per treatment.

Abbreviations: BW, body weight; SE, *Salmonella* Enteritidis; VC0, diet without vitamin C supplementation; VC500, diet with 500 mg VC/kg diet.

[1]The SEM values represent the overall standard error of means in each line.

The jejunal VH of the broilers tended to be increased ($P = 0.088$) in VC500 group.

Immune and Antioxidant Ability (Experiment 2)

Challenge with SE showed no effects on the tested immune indicators nor serum total antioxidant capability on day 21 ($P > 0.05$). However, dietary addition with 500 mg/kg VC significantly increased jejunal sIgA concentration and serum antioxidant capability ($P < 0.05$, Figures 3A and 3D). Supplementation with VC also enhanced the proliferation of T-lymphocytes after stimulating with concanavalin A (Figure 3B, $P < 0.05$). There was no interaction between SE challenge and dietary treatments on the immunity and antioxidant capability of broilers on day 21 ($P > 0.05$). Besides, supplemented with exogenous VC significantly decreased GLO activity in kidney, whereas significantly increased VC concentration in spleen on 21 D of the broilers ($P < 0.05$, Figures 3E and 3F).

Cecal Microbiota (Experiment 2)

The cecal microbiota of the broilers was analyzed by sequencing the bacterial 16S rRNA V3+V4 region. High-throughput pyrosequencing of the samples (n = 3) generated a total of 763,262 raw reads in cecal content. After removing the low-quality sequences, 723,267 clean reads were acquired from the 12 cecum
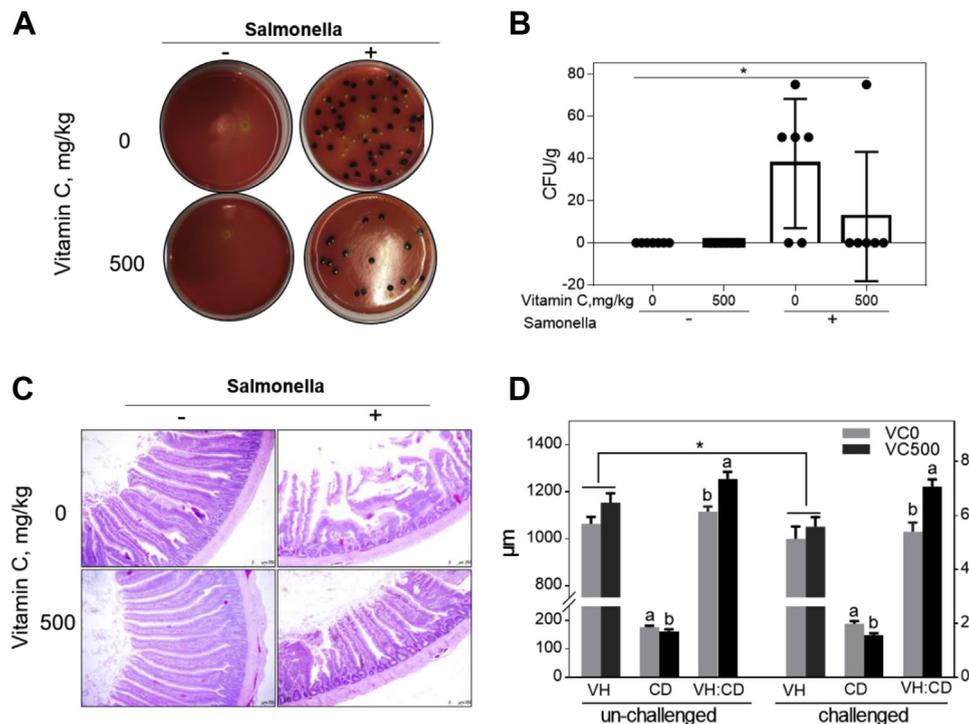

Figure 2. Hepatic bacterial translocation (A, B) and jejunal intestinal morphology (C, D) in experiment 2. *Suggest significant main effects ($P < 0.05$) of SE challenge. [a,b]Treatments with unlike letters are significantly different ($P < 0.05$). Values are means (n = 6) and standard error represented by vertical bars. Abbreviations: VC0, broilers in this group received 0 mg/kg vitamin C in the diet; VC500, broilers in this group received 500 mg/kg vitamin C; VH, villus height; CD, crypt depth; VH:CD, villus height:crypt depth; SE, *Salmonella* Enteritidis.
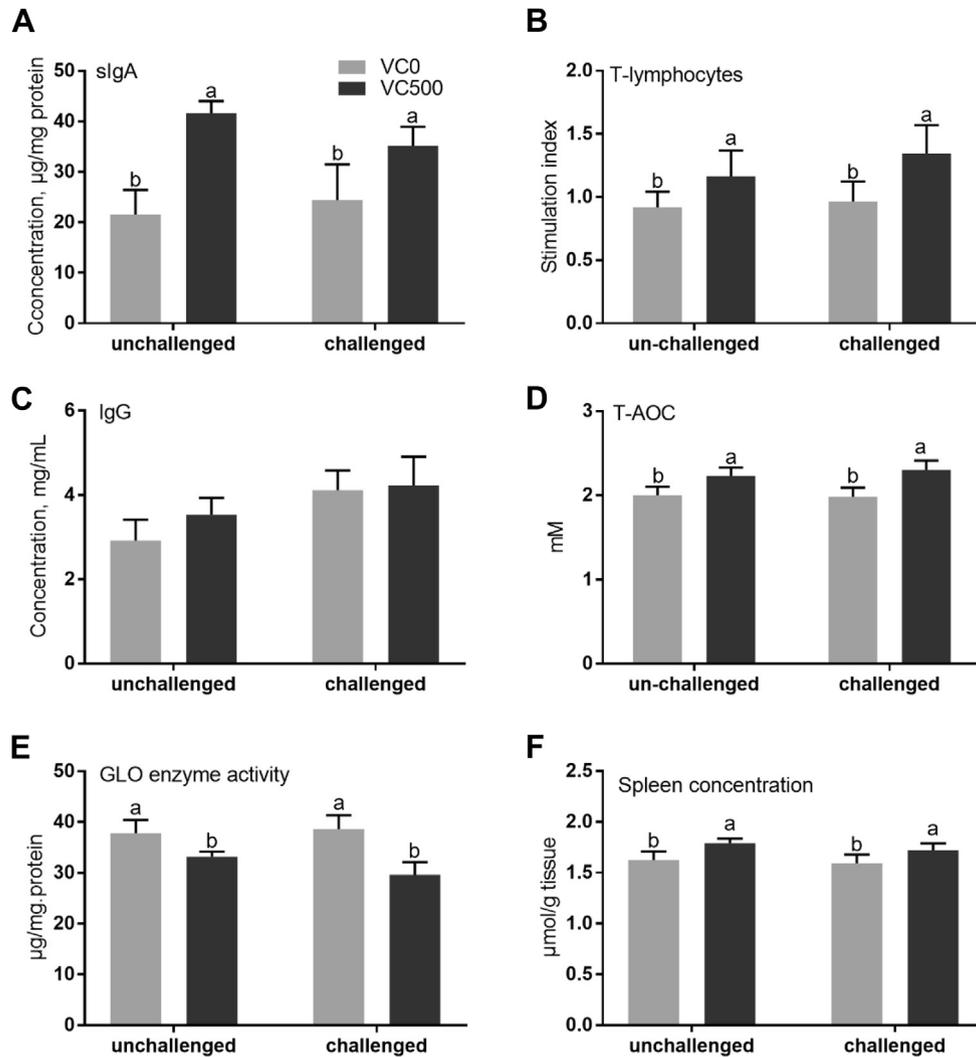
Figure 3. Effects of dietary vitamin C levels and SE challenge on serum sIgA levels (A). Transformation rate of T lymphocytes derived from peripheral blood in vitro (B), IgG concentrations (C), serum total antioxidant ability (D), renal GLO enzyme activity (E), and splenic VC concentration (F) of broilers on day 21 (experiment 2). Values are means (n = 6), with their standard errors represented by vertical bars. Unchallenged = broilers without SE infection; challenged = chickens with SE infection. [a,b]Mean values with unlike letters were significantly different ($P < 0.05$). Abbreviations: VC0, chickens received basal diet without vitamin C supplementation; VC500, chickens received basal diet with 500 mg/kg vitamin C included; sIgA, secretory immunoglobulin A; IgG, immunoglobulin G; T-AOC, total antioxidant capacity; GLO, L-gulonolactone oxidase; SE, *Salmonella* Enteritidis.

digesta samples through Illumina miSequencing analysis. Based on 97% sequence similarity, a total of 4,822 OTU were identified and clustered.

This sequencing depth was sufficient for the coverage (>99%) of all OTU present in cecal samples and almost reflected the total microbial species richness, as demonstrated by the Rarefaction and rank abundance (Supplementary Figures 1C and 1D). As shown in Figure 4, without SE challenge, broilers in VC500 group obtained 1,317 OTU, whereas VC0 group get 1,239 OTU in the cecum. Moreover, 1,242 OTU were obtained in VC500 group, whereas 1,024 OTU were found in the VC0 group under the condition of SE challenge. It indicated that challenging with SE decreased the richness of the microbiota in cecum, whereas dietary VC reversed the trend. In addition, SE challenge significantly decreased the microbial richness and diversity referring to decreased observed-species, chao1, ACE indexes ($P < 0.05$), and Shannon indices ($P = 0.067$). At the

same time, dietary VC supplementation significantly increased the observed-species and ACE indexes ($P < 0.05$, Figure 4C). Beta diversity analysis was depicted via principal component analysis and principal coordinate analysis (Supplementary Figures 1A and 1B) and showed that the 4 clusters were not clearly separable.

The relative abundance (>1%) of cecal microbiota was determined at the phylum, family, and genus levels (Figure 5). The dominant phyla in cecum were *Firmicutes* and *Bacteroidetes* accounting for 44.3, 57.03%, and 49.47, 29.52% in VC0 and VC500 groups without SE challenge, while accounting for 43.15, 53.72%, and 43.41, 32.90% in VC0 and VC500 groups, respectively, under SE challenges. In addition, supplementation with VC significantly increased the richness of *Firmicutes* whereas decreased the abundance of *Bacteroidetes* ($P < 0.05$, Figures 5A and 5D; Supplementary Table 1). At the same time, VC significantly increased the ratio of *Firmicutes* to *Bacteroidetes* (**F: B**, $P < 0.05$).
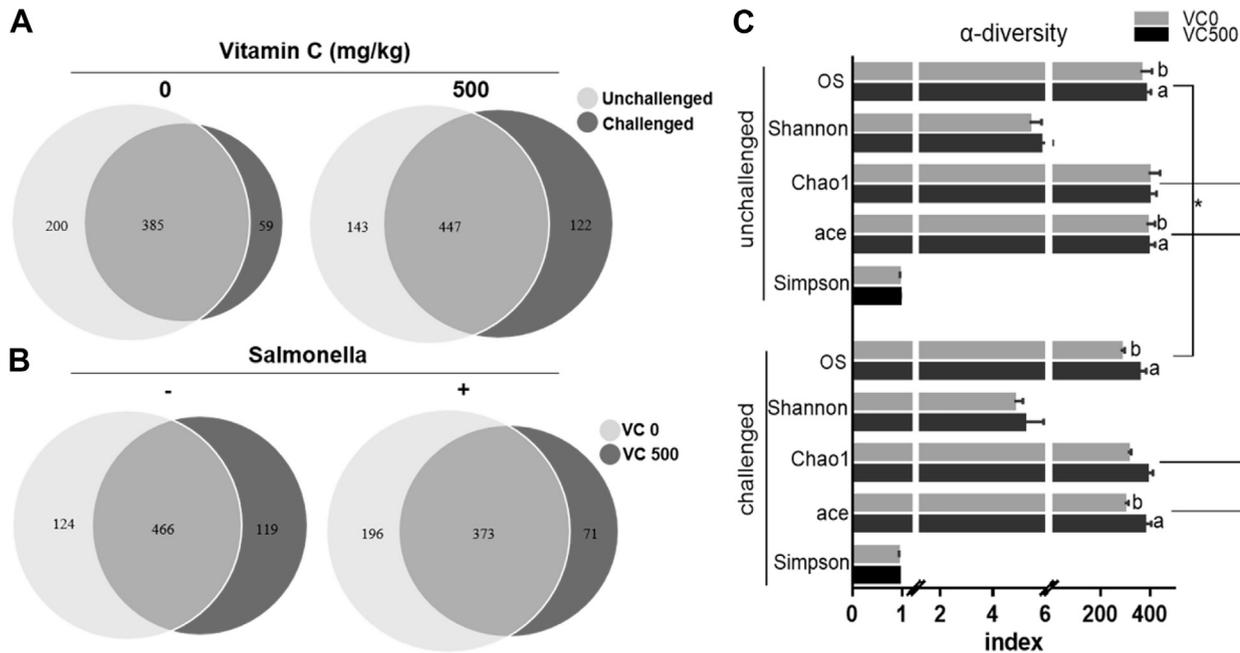
Figure 4. Effects of dietary vitamin C (A) or SE challenge (B) on cecal richness and diversity (C) in experiment 2. * Suggest significant main effects ($P < 0.05$) of SE challenge. [a,b]Treatments with unlike letters are significantly different ($P < 0.05$). Abbreviations: ACE, abundance-based coverage estimators; OS, observed species; SE, *Salmonella* Enteritidis; VC0, chickens received basal diet without vitamin C supplementation; VC500, chickens received basal diet with 500 mg/kg vitamin C included.

The main families belonging to cecum phyla *Bacteroidetes* and *Firmicutes* were the *Bacteroidaceae*, *Ruminococcaceae*, *Lachnospiraceae*, and *Rikenellaceae* (Figure 5B; Supplementary Table 1). Other phyla (*Proteobacteria and Cyanobacteria)* were present at low relative abundances, which accounts for around 9% and 1% of the abundance, respectively. Challenge with SE significantly increased the abundance of *Enterobacteriaceae*, which was reversed by adding 500 mg/kg VC in the diet ($P < 0.05$, Figure 5E). VC500 also significantly increased the richness of family *Bacteroidaceae* and *Ruminococcaceae* ($P < 0.05$) and tended to increase the abundance of family *Lachnospiraceae* ($P = 0.082$) compared to VC0 group.

The majority of genera of cecal microbiota phylotype in broilers belonged to the *Firmicutes*, *Bacteroides*, and *Proteobacteria* phyla, with *Faecalibacterium* and *Bacteroides* dominating the phylotype data (Figures 5C and 5F; Supplementary Table 1). Challenge with SE reduced the abundance of *Anaerostipes* and *Ruminiclostridium_9* ($P < 0.05$ and $P = 0.063$, respectively). Supplementation with VC significantly decreased the abundance of *Bacteroides* ($P < 0.05$) and tended to increase the abundance of *Anaerofilum* and *Parasutterella* ($P = 0.057$ and 0.079, respectively). At the same time, adding VC decreased the number of the *Escherichia-Shigella* under SE challenge.

Alterations in Intestinal Microbiota Composition Were Correlated With Health Parameters (Experiment 2)

The Pearson rank correlation analysis was performed to evaluate the potential link between alterations in intestinal microbiota composition and growth and health parameters of broilers under *Salmonella* challenge (Figure 6). Genus *RuminococcaceaeUCG005* and *Coprobacter* present negative correlation with body weight on 35 D-old broilers, whereas *Alistipes* positively correlated with body weight on 21 D. *Anaerofilum*, *unidentifiedRuminococcaceae*, *RuminococcaceaeUCG005*, and *Coprobacter* were associated with the decrease of FCR on 21 D, whereas *Bacteroides* were related to the increase of FCR on 21 D of the broilers ($P < 0.05$). The immunity was also associated with intestinal microbiota. *Faecalibacterium* positively correlated with T lymphocytes stimulation index, whereas *Bacteroides* displayed negative correlation with it. *Parasutterella* and *RuminococcaceaeUCG005* had positive correlation with IgG. At the same time, *Anaerofilum*, *Ruminiclostridium_9*, and *RuminococcaceaeUCG005* showed positive connection with T-AOC.

DISCUSSION

Intestinal histology is intimately associated with intestinal function, which enables the acquisition of nutrients, whereas prevents passage of harmful bacteria into the body (Sakamoto et al., 2004; Yamauchi et al., 2010; Broom, 2018). Increased VH suggests an increased surface area capable of greater absorption of available nutrients, while a shortening of the VH and deeper CD may lead to poor nutrient absorption and performance (Xu et al., 2003). In the present study, villus atrophy and mucosa damage caused by SE challenge could have contributed to the depressed production performance along with higher *Salmonella* translocation rate to the liver. The higher hepatic translocation rate
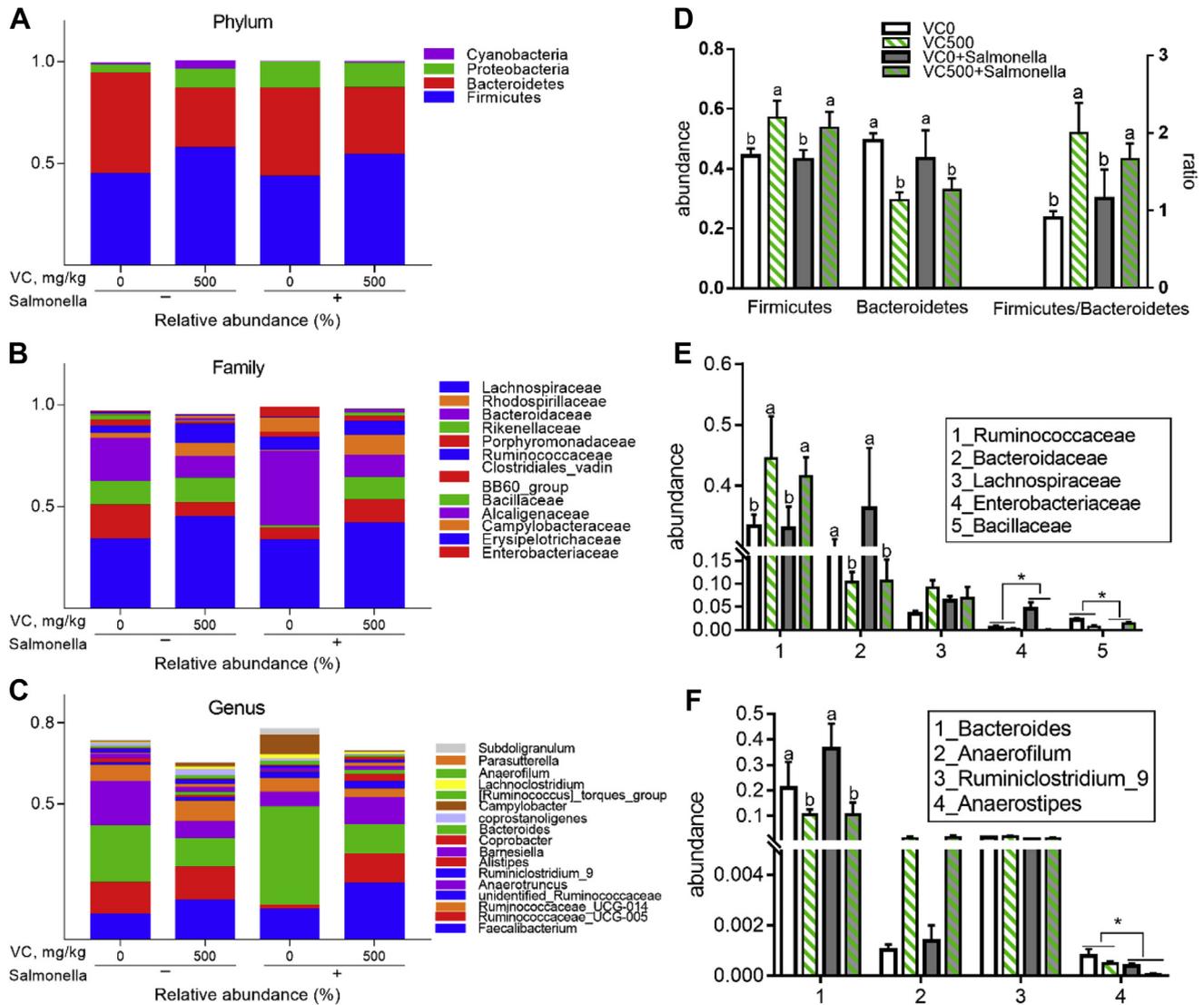
Figure 5. The effects of *Salmonella* challenge and dietary vitamin C supplementation on broiler intestinal bacterial phylotype in experiment 2. The relative abundance (>1%) and the changed microbiota of cecal intestine in phylum (A, D), family (B, E), and genus (C, F) are plotted (n = 3 for each group). * Suggest significant main effects ($P < 0.05$) of SE challenge. [a,b]Treatments with unlike letters are significantly different ($P < 0.05$). Abbreviations: SE, *Salmonella* Enteritidis; VC0, chickens received basal diet without vitamin C supplementation; VC500, chickens received basal diet with 500 mg/kg vitamin C included.

of SE may be further related to higher mortality of the SE challenged broilers. At the same time, the higher SE contents in the liver might pose serious health concern for consumers. However, dietary supplementation with VC decreased the frequency of SE translocation rate to the liver, which implies that VC can potentially inhibit the growth of *Salmonella* in broiler products. Meanwhile, the improved intestinal morphology by VC might be associated with the increased production performance of broilers.

The gut microbiota, as one of the components of gut barrier, have a profound influence on inhibiting the establishment of intestinal pathogens, while enhancing the production performance of the host (Zhu et al., 2002). Enteric pathogen invasion, like *Salmonella*, could induce the inflammation of the mucosa, which promote the overgrowth of *Enterobacteriaceae* (Lupp et al., 2007), while reduce microbial diversity (Borton et al.,

2017). Harboring high level of *Enterobacteriaceae* could potentially increase the susceptibility to infection by related enteric pathogens in broiler chickens (Mon et al., 2015). In addition, low microbial complexity in the intestine is considered detrimental for the intestinal health (Ding et al., 2019). *Anaerostipes* is shown to play an important role in changing some mucin degradation related gene expression of bacteria *Akkermansia muciniphila*, thus indirectly supporting the microbial community in the mucosal environment (Chia et al., 2018). The decreased richness of *Anaerostipes* might be responsible for the decreased microbial richness and diversity in SE challenged broilers. The enriched abundance of family *Ruminococcaceae* and *Lachnoclostridium* in VC treatment groups may contribute to the higher microbial diversity and richness, as the 2 family members are positively correlated with microbial diversity (Mon et al., 2015). Because high diversity is the hallmark of a
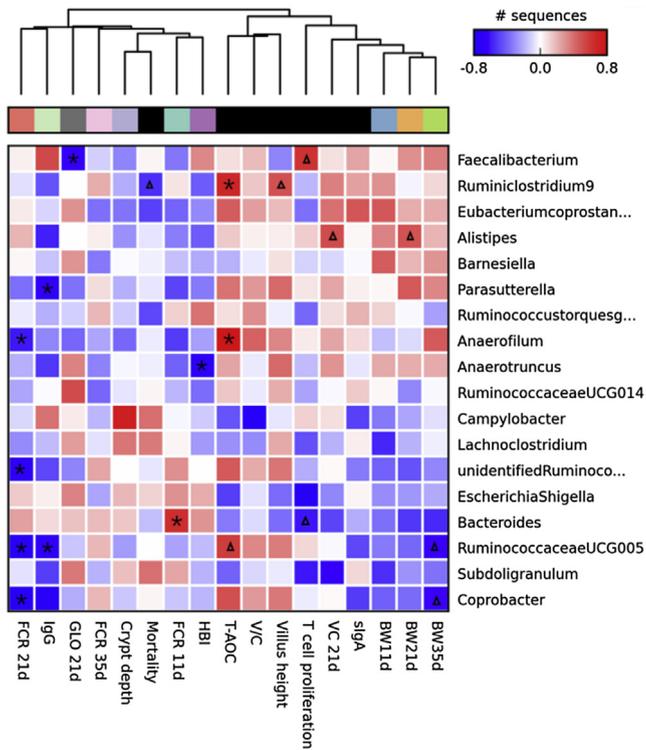
Figure 6. Heatmap of the Pearson rank correlations between the significantly modified microbiota and growth and health parameters in broilers (experiment 2). *means $P \leq 0.05$; Δmeans $0.05 < P \leq 0.10$. Abbreviations: BW, body weight; FCR, feed conversion ratio; GLO, L-gulonolaction oxidase; IgG, immunoglobulin G; sIgA, secretory immunoglobulin A; T-AOC: total antioxidant capacity; HBI, hepatic bacteria translocation rate; VC, vitamin C; V/C, villus height:crypt depth.

well-functioning, healthy gut ecosystem (Lozupone and Knight, 2008) and reduced microbial diversity has been found in certain kinds of diseases (Manichanh et al., 2006; Abrahamsson et al., 2014), and the microbial richness and diversity could possibly explain the changed production performance in SE challenged or VC treatment groups.

In addition to gut microbiota richness, the gut microbial composition also has profound influence on the host (Roytio et al., 2017). Several researches demonstrated that higher F: B were found in the microbiota of obese humans. Conversely, a decreased F: B has been directly related to weight loss (Ley et al., 2006; Guo et al., 2008; Mariat et al., 2009). Therefore, the higher F: B ratio in VC treatment group could be attributed to the increased body weight in both experiment 1 and experiment 2. Consistent with the shifts observed at phylum level, *Ruminococcaceae* was enriched, and the *Bacteroidaceae* was depleted after VC treatment. The same microbial composition was also observed in children who gain more weight (Riva et al., 2017). Meanwhile, the *Lachnospiraceae*, with the capacity of fermenting fibers and producing butyric acid (Biddle et al., 2013), has been shown to involve in the reduction of mucosal permeability, enhancing body weight and protecting from colon cancer in humans (Meehan and Beiko, 2014). Collectively, the

altered cecal microbiota richness and composition by SE challenge or VC treatment is highly correlated with the changed production performance of the broilers in the present study.

Moreover, some species of microbiota also showed close correlation with indexes describing health parameters in the present study. *Bacteroides*, decreased after VC treatment, was negatively correlated with T lymphocytes proliferation. In addition, the enhanced immunity in VC500 group may be the properties of enhancing the maturity of T lymphocytes (Manning et al., 2013) and promoting the differentiation of B cells (Ichiyama et al., 2009). It has been reported that SE can persist in the digestive tract of birds for months without triggering clinical signs except for the very young chicks (Sadeyen et al., 2004). The asymptomatic response after infection with SE accelerated the process of proliferation in intestine and other organs of the host, contaminating the neighboring naïve animals, thus threatening the public health for the consumption of contaminated products (Bearson et al., 2013; Kogut et al., 2016). At the same time, bacteria species, such as *Anaerofilum* and *Ruminiclostridium_9*, was positively correlated with T-AOC. Exogenous VC inhibited GLO enzyme activity in kidney, where the same amounts of byproduct H_2O_2 are produced in the process of VC synthesizing (Linster and Van Schaftingen, 2007). Lower GLO enzyme activity also means less H_2O_2 produced, which consumes less catalase, thereby contributing to the increased antioxidant ability in VC500 groups. At the same time, VC is known to have a broad spectrum of antioxidant activities because of its ability to react with numerous aqueous free radicals and reactive oxygen species (FREI, 1994). So, it is expected that VC500 had greater antioxidant ability than VC0.

Taken together, SE challenge could decrease the richness and diversity of cecal microbiota and negatively influence the production performance of the broilers. However, as shown in Figure 7, dietary supplementation with VC could enhance the intestinal health by improving the microbial structure and intestinal morphology, improving immunity and antioxidant ability, and thus enabling to improve the production performance of the broilers. Therefore, the detrimental effects of SE challenge in broilers can be partly ameliorated by dietary VC supplementation.
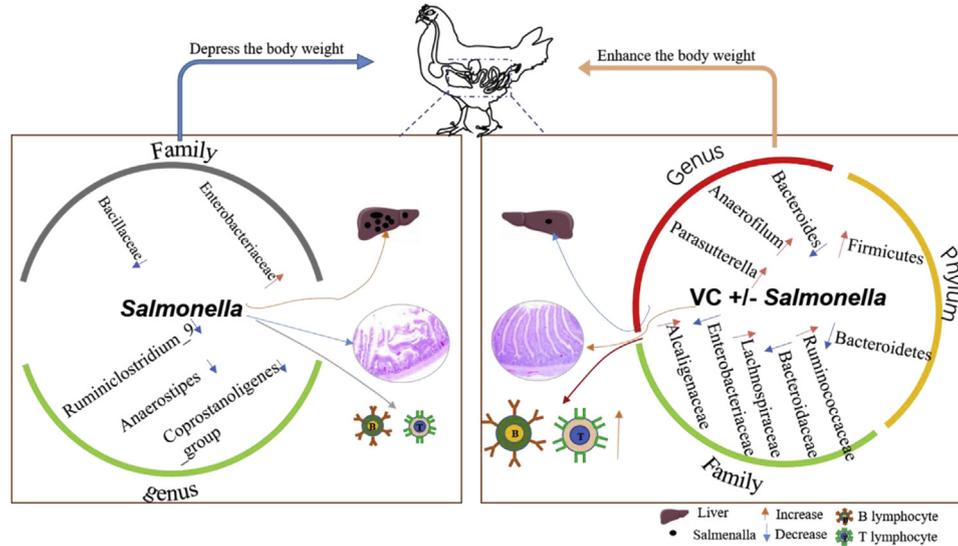
ACKNOWLEDGMENTS

Figure 7. The effects of *Salmonella* challenge and dietary vitamin C (VC) on the production performance of the broilers. *Salmonella* challenge decreased cecal microbial richness and diversity while increased the abundance of *Enterobacteriaceae*. The changed microbial composition and structure subsequently influenced the intestinal morphology and increased bacterial translocation to the liver, thus decreased the production performance. However, dietary supplementation with VC increased microbial richness and improved the composition of the microbiota. Meanwhile, VC improved intestinal morphology and immunity, which resulted in less frequency bacterial translocation rate while higher production performance.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at http://doi.org/10.1016/j.psj.2020.03.062.

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