Journal of Microbiology (2014) Vol. 52, No. 12, pp. 1002–1011
DOI 10.1007/s12275-014-4347-y
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Effect of Zinc on Growth Performance, Gut Morphometry, and Cecal Microbial Community in Broilers Challenged with *Salmonella enterica* serovar Typhimurium

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(Received Jun 16, 2014 / Revised Sep 24, 2014 / Accepted Oct 14, 2014)

To evaluate the effects of supplemental zinc on growth performance, gut morphometry, and the cecal microbial community in broilers challenged with *Salmonella typhimurium*, 180, 1-day-old male Cobb 500 broiler chicks were randomly assigned to 3 treatments with ten replicates for a 42 day experiment. The 3 treatments were: unchallenged, *S. typhimurium*-challenged, and *S. typhimurium*-challenged with 120 mg/kg of zinc supplementation in the diet. *Salmonella* infection caused a reduction in body-weight gain and feed intake, disrupted the intestinal structure by decreasing the villus-height/crypt-depth ratio of the ileum and increasing the apoptotic index of ileal epithelial cells. Moreover, the cecal microbial community was altered by *Salmonella* infection, as demonstrated by a reduced number of *Lactobacillus* and total bacteria. Dietary zinc supplementation improved growth performance by increasing the body-weight gain and feed intake in the challenged broilers. In addition, zinc repaired intestinal injury by reducing the apoptotic index of ileal epithelial cells, enhancing villus height and the villus-height/crypt-depth ratio of the ileum, and the proliferation index of ileal epithelial cells. Finally, zinc regulated the cecal microbial community by increasing the number of total bacteria and beneficial *Lactobacillus* bacteria, and reducing the number of *Salmonella*. The results indicated that dietary zinc supplementation improved growth performance, intestinal morphology, and intestinal microbiota in *S. typhimurium*-challenged broilers.

***Keywords*:** zinc, broiler, growth, gut morphometry, microbiota, *S. typhimurium*

Introduction

Salmonella enterica serovar Typhimurium is one of the most prevalent serovars responsible for food-borne salmonellosis in humans (McClelland *et al*., 2001; Lim *et al*., 2003) and is commonly isolated from contaminated poultry and poultry products, such as broiler chicken meat and eggs (Lynch *et al*., 2004; Pezoa *et al*., 2013). Furthermore, *S. typhimurium* infection can result in systemic disease with high mortality in day-old chicks and cause enteric or subclinical disease with reduction of growth performance, intestinal injury and inflammation, and intestinal barrier dysfunction in older birds (Barrow *et al*., 1987; Barrow *et al*., 1988; Fasina *et al*., 2010). Therefore, it is necessary and important to prevent intestinal infection of chickens by *Salmonellas* to ensure poultry food safety.

Gut commensal microorganisms comprise a diverse collection of microbial species and perform an important role in nutrient digestion, production of digestive enzymes, gut mucosal morphology, epithelial cell growth and differentiation, and protection against pathogenic bacteria (Amit-Romach *et al*., 2004; Lan *et al*., 2005; Chambers and Gong, 2011). At the initiation of intestinal infection of broiler chickens, *S. typhimurium* must outcompete the resident microbiota of the distal ileum and cecum and penetrate the mucosal epithelium (Berndt *et al*., 2007). After overcoming colonization resistance, *S. typhimurium* disrupts the intestinal ecosystem in mammals and chickens, leading to infection of the gastrointestinal tract (Stecher *et al*., 2007; Barman *et al*., 2008; Juricova *et al*., 2013; Videnska *et al*., 2013). Furthermore, *Salmonella* infections can cause infected epithelial cell shedding from the villus surface, resulting in villus blunting and loss of absorptive surfaces (Wallis and Galyov, 2000). Similarly, *in vitro* models of *S. typhimurium* infection can induce an increase in apoptosis of human colon epithelial cells (Kim *et al*., 1998). Therefore, it is essential to manipulate the intestinal microbiota to protect the intestine from pathogen infection and improve the health and performance of the chickens.

Zinc is an essential trace element that is involved in growth, healing, and multiple cellular functions, such as enzymatic reactions, DNA synthesis, and gene expression (Brandão-Neto *et al*., 1995; Powell, 2000). The importance of zinc to intestinal development and function has been demonstrated in many studies. It has effects such as increasing intestinal crypt-cell proliferation, improving epithelial cell turnover and repair, and maintaining the structure and function of the intestinal barrier (Hu *et al*., 2013; Mocchegiani *et al*., 2013). Most studies have focused mainly on the effect of zinc on intestinal development and function without evaluating the effect of zinc on the gut microflora. The possibility of a relationship between zinc and intestinal microbiota was noted in an earlier study

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showing that germfree animals had a lower dietary zinc requirement than conventional animals (Smith *et al*., 1972). Moreover, recent studies using weaned piglets indicated that dietary zinc supplementation affected the composition and metabolic activity of the intestinal microbiota (Hojberg *et al*., 2005; Starke *et al*., 2013). However, the direct interaction of zinc with the gut microbial community has not been investigated in broiler chickens.

The purpose of the present experiment was to evaluate the effects of supplemental zinc on intestinal microbiota, as well as intestinal histomorphology, and proliferation and apoptosis of the intestinal epithelium in *S. typhimurium*-challenged broiler chickens.

Materials and Methods

Birds, diets, and experimental design

A total of 180, 1-day-old, male, Cobb 500 broiler chicks were randomly assigned to three treatments, each involving ten replicate cages (six birds per cage). The groups were the unchallenged group (CON), *S. typhimurium*-challenged group (STC), and *S. typhimurium*-challenged group receiving dietary supplementation with 120 mg/kg zinc (zinc resource: $ZnSO_4 \cdot H_2O$) (ZnT). Chickens were raised in a temperature-controlled room with 24 h of constant light. The temperature was maintained at 35°C during the first 3 days, between 28 and 30°C during the subsequent two weeks, and at 25°C during the last 3 weeks of the study. All birds were provided with feed and water *ad libitum* throughout the entire experiment. The basal diets were corn-soybean-meal based, and formulated to meet or exceed the nutrient requirements for broilers as recommended by the NRC (1994). The dietary phases consisted of starter (0 to 21 days) and grower (22 to 42 days) as shown in Table 1. Experimental procedures were approved and conducted under the guidelines of the China Agricultural University Animal Care and Use Committee.

S. typhimurium challenge

The *S. typhimurium* strain CVCC541 was obtained from the China Institute of Veterinary Drug Control (China). The organism was cultured in lactose broth (CM 228, Land Bridge Technology Ltd, China) for 18 h at 37°C. To determine the number of colony-forming units (CFU), the inoculum was diluted and plated on XLD agar (Beijing Aoboxing Bio-tech Co. Ltd., China) for 24 h at 37°C.

At 5 days of age, all birds in challenged groups were orally infected with *S. typhimurium* (1.2×10^9 CFU/ml, 0.8 ml/bird) suspended in sterile buffered peptone water, and the non-challenged chicks were mock-challenged with 0.8 ml sterile, buffered-peptone water. The infection protocol was based on results obtained from previous trials in our laboratory, which showed consistent and uniform *Salmonella* colonization in chicks.

Performance parameters

Chicks and feed were weighed by cage (replicate) at day of hatch, 21 days and 42 days. Feed intake (FI) was measured, and the body weight gain (BWG) and feed conversion ratio (FCR) were calculated for each period and throughout the entire experimental period. Mortalities and health status were visually observed and recorded daily throughout the experiment.

Sample collection

On day 14, ten birds (one bird per cage) of each treatment was randomly selected and killed by intracardial administration of sodium pentobarbital (30 mg/kg body weight) and jugular exsanguination. Samples (~4 cm) of ileum were taken from midway between Meckel's diverticulum and the ileocecal junction and flushed with 0.9% physiological saline before obtaining the mucosa of the intestinal segment and then preserving it at -20°C for sucrase activity determination. Samples (~2 cm) of the ileum were taken proximal to the ileocecal junction and washed in PBS (flushed with 0.05 M PBS, pH 7.2), fixed in 2.5% (vol/vol) polyoxymethylene-glutaraldehyde solution and kept at 4°C for histological and immunohistochemical examination. The contents of the cecum were collected aseptically, snap-frozen in liquid nitrogen, and stored at -80°C for DNA extraction. Each pool was composed of an equal amount of cecal contents from ten birds (one bird per cage) of each treatment and three replicates for each pool were used for DNA extraction and PCR-DGGE to minimize natural individual variation (Zhou *et al*., 2007). DNA extracted from individual birds was used as template for qPCR assays.

Table 1. **Ingredients and nutrient composition of basal diets (g/kg as fed unless noted)**

Item	Starter (1 to 21days)	Grower (22 to 42 days)
Ingredients		
Maize	537.1	600.0
Soybean meal	393.2	325.4
Soybean oil	29.4	33.2
Dicalcium phosphate	20.0	18.1
Limestone	10.3	13.0
Sodium Chloride	3.5	3.4
D,L-Methionine (98%)	2.3	1.6
L-Lysine (98%)	0.2	0.8
Vitamin premix[a]	0.2	0.2
Mineral premix[b]	2.0	2.0
Choline chloride (50%)	1.6	2.0
Ethoxyquinoline (33%)	0.2	0. 3
Chemical composition, analyzed		
Metabolizable energy (Mcal/kg)	2.95	3.00
Crude protein	215.0	191.0
Lysine	11.6	10.0
Methionine	5.3	4.6
Calcium	9.1	9.5
Available phosphorus	4.5	4.6
Zinc (mg/kg)[c]	33.26	32.30

[a] Supplied per kg of diet: vitamin A, 12,500 IU; cholecalciferol, 2,500 IU; vitamin E, 30 IU; vitamin K3, 2.65 mg; thiamin, 2 mg; riboflavin, 6 mg; pantothenic acid, 12 mg; cobalamin, 0.025 mg; niacin, 50 mg; biotin, 0.0325 mg; and folic acid, 1.25 mg
[b] Supplied per kg of diet: Cu, 8 mg ($CuSO_4 \cdot 5H_2O$); Fe, 80 mg ($FeSO_4$); Mn, 100 mg ($MnSO_4 \cdot H_2O$); Se, 0.15 mg (Na_2SeO_3); I, 0.35 mg (KI);
[c] Zinc concentration in the basal diet was analyzed by atomic absorption spectrophotometer

Biochemically assessed sucrase activity

Sucrase activity was measured in the ileum as an indication of intestinal damage. Sucrase activity was assayed colorimetrically according to Lamb-Rosteski *et al.* (2008) using sucrose as a substrate and expressed as units/g protein. The concentration of protein in the sample was determined using a BCA (bicinchoninic acid) protein assay kit (Pierce Chemical, USA).

Intestinal histomorphology

Formalin-fixed ileum samples were prepared using paraffin embedding techniques. Consecutive sections (5 μm) were stained using haematoxylin and eosin and observed for histomorphology. The villus height (from the tip of the villus to the crypt opening) and crypt depth (from the base of the crypt to the level of the crypt opening) were measured from 15 randomly selected villi and associated crypts with one section per chicken at 40× magnification. The villus height to crypt depth ratio was then calculated from these measurements by dividing the villus height with the crypt depth. All examinations and measurements were performed with an Olympus optical microscope using ProgRes CapturePro software, version 2.7 (Jenoptik, Germany).

Proliferation assay

Formalin-fixed ileum samples were prepared using paraffin embedding techniques. Consecutive sections (4 μm) were stained using mouse anti-PCNA at a 1:100 dilution (bscx-biotech, China) as the primary antibody. The concrete staining procedure of proliferating cell nuclear antigen (PCNA) was described previously (Foley *et al.*, 1993). Proliferating and total cells were quantified at 400× magnification using images of 10 separate fields. The proliferation index was defined as the ratio of proliferating cells, expressed as PCNA stained cells, to the total cell number within the crypt-villus axis, per section.

Apoptosis assay

Formalin-fixed ileum samples were prepared using paraffin embedding techniques. Consecutive sections (4 μm) were stained using the Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL) technique for quantification of apoptosis *in situ*. The FITC/propidium iodide (PI) apoptosis detection kit (DeadEnd Fluorometric TUNEL System, Promega, USA) was used and the staining procedure was performed according to the manufacturer's protocol. Apoptotic and total cells were quantified at 400× magnification using images of 10 separate fields. The apoptotic index was defined as the ratio of apoptotic cells, expressed as apoptotic bodies, to the total cell number within the crypt-villus axis per section.

Genomic DNA extraction and PCR-DGGE analysis

The QIAamp DNA Stool Mini Kit (Qiagen, Germany) was used to lyse bacterial cells and extract chromosomal DNA from cell lysates following the manufacturer's instructions. DNA extracts were quantified by Bio-Photometer (Eppendorf AG22331, Germany) at 260 nm. The V3 region of the bacterial 16S rRNA gene was amplified using primers HDA1-GC (5′-**CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G**AC TCC TAC GGG AGG CAG CAG T-3′; the 40-bp GC-clamp is in boldface) and HDA2 (5′-GTA TTA CCG CGG CTG CTG GCA C-3′) for DGGE analysis as described previously (Walter *et al.*, 2000). The PCR amplification program was: preheating at 95°C for 5 min; 30 cycles of denaturing (94°C, 30 sec), annealing (56°C, 30 sec) and extension (72°C, 2 min); finally 72°C for 10 min. The 200-bp PCR products were confirmed by electrophoresis on a 2% agarose gel followed by ethidium bromide staining. DGGE was performed with the Bio-Rad D-code system using 16 cm by 16 cm by 1 mm gels as described previously (Johansen *et al.*, 2007). The 10% polyacrylamide gels (acrylamide/bisacrylamide 37.5:1) contained a 35 to 60% gradient of urea and formamide increasing in the direction of electrophoresis, which were conducted in 0.5× TAE buffer at 200 V for 10 min and subsequently at 85 V for 16 h at 60°C. The gels were stained with Gelred (1:10,000 dilution) (Biotium, USA) and photographed by UVP Bioimaging System (USA).

Estimates of microbial richness and diversity

Quantity One software, version 4.6.2 (Bio-Rad Laboratory, USA) was used to analyze the DGGE gel. After bands were assigned to the gel tracks, the corresponding bands in independent tracks were matched. The Sørensen-Dice index (aka Dice coefficient) (S_D) and the un-weighted pair group method using arithmetic averages (UPGMA) were used for clustering analysis. Dice similarity coefficients were calculated as in Ben and Ampe (2000). Three parameters were used to assess the richness, diversity and concentration dominance

Table 2. **Primers used for the quantification of the predominant bacterial divisions' expression by real-time PCR**

Target	Amplicon length (bp)	Sequence (5′→3′)	References
S. typhimurium	133	F：TAACACCTGCTGCTGTCAATGCGG R：ACTCTTGCTGGCGGTGCGACTT	Hadjinicolaou *et al.* (2009)
Lactobacillus group	341	F： AGCAGTAGGGAATCTTCCA R： CACCGCTACACATGGAG	Walter *et al.* (2001)
Enterococcus genus	144	F： CCCTTATTGTTAGTTGCCATCATT R： ACTCGTTGTACTTCCCATTGT	Rinttila *et al.* (2004)
Enterobacteriaceae family	195	F：CATTGACGTTACCCGCAGAAGAAGC R： CTCTACGAGACTCAAGCTTGC	Bartosch *et al.* (2004)
Domain Bacteria	200	F： ACTCCTAC GGGAGG CAG CAGT R： GTATTACCGCGG CTGCTGGCAC	Walter *et al.* (2000)

of the microbial community, respectively, including the number of bands, the Shannon-Weaver index of general diversity (H'), and the Simpson index of dominance concentration (D). The Shannon-Weaver index of general diversity (H') was calculated on the basis of the bands in the gel tracks by using the intensity of the bands as judged by peak heights in the densitometric curves. Each band in the gel tracks forms a peak, and the peak height in the densitometric curve was measured using Quantity One software version 4.6.2. H' was calculated as follows: $H' = -\sum(P_i * \ln P_i)$; $P_i = n_i/N$, where n_i is the height of a peak and N is the sum of all peak heights in the densitometric curve. The Simpson index of dominance concentration (D) (Simpson, 1949) was calculated with the following equation: $D = \sum(P_i^2)$. The band intensity was used as an estimate of the relative population size of each species (Ben and Ampe, 2000; Ricca *et al*., 2010).

Cloning and sequencing DGGE fragments

Bands of interest were excised aseptically from the DGGE gels into 1×TAE buffer, incubated overnight at 4°C, and then used as templates for the following PCR reamplification with the primers HDA1 without GC-clamp and HDA2 under the conditions described above. The reamplified 16S rDNA was ligated into the pCR™2.1 Vector (TA Cloning® Kit, Invitrogen, USA), and then the plasmids were introduced into *E. coli* DH5α competent cells (TaKaRa, China). Sequencing of insert DNA was carried out by Sangon Biotech (China) using M13 forward and reverse sequencing primers. The sequencing data were analyzed using a Blast search of the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov) to find closely related sequences. The accession numbers for 16S rDNA partial sequences retrieved from DGGE bands are given in Table 5.

Analysis by real-time qPCR

The total bacteria, *Lactobacillus* group, *S. typhimurium*, *Enterococcus* genus, and *Enterobacteriaceae* family in cecal contents were analyzed by qPCR. Genomic DNA from cecal samples was used as templates for PCR amplification using SYBR Green PCR technology (TaKaRa) and an ABI 7500 Real-Time PCR machine (Applied Biosystems, USA). The targeted groups and species, primer sequences, amplicon sizes, and literature references are given in Table 2. All primer sets were highly specific and gave positive results only for the corresponding target bacteria with the expected product size. Protocols for the qPCR analysis were performed according to (Wise and Siragusa, 2007). Absolute quantification was done by using standard curves made from known concentrations of plasmid DNA containing the respective amplicon for each set of primers. To construct standard curves, the respective amplicon was cloned into pCR4-TOPO using the TOPO-TA Cloning Kit (Invitrogen) following the manufacturer's procedure. The insert-containing plasmids were purified and quantified by spectrophotometer. The number of target gene copies was calculated by the following equation: the number of target gene copies = $(A\times 6.022\times10^{23})(660\times B)^{-1}$, where A is the purified plasmid concentration and B is the length of the product insert. Tenfold serial dilutions ranging from 1×10^6 to 1×10^1 gene copies were included on each 96-well plate. The Ct was determined for the unknowns and compared with the standard curves. Results were expressed as the number of 16S rRNA gene targets per gram (wet weight) intestinal material. For statistical analysis, the number of gene copies was $\log_{10}$ transformed to achieve normal distribution.

Nucleotide sequence accession numbers

Representative sequences have been deposited in the European Nucleotide Archive database (http://www.ebi.ac.uk/ena/data/view/HG967641-HG967644) and are available under accession numbers HG967641 to HG967644.

Statistical analysis

Data were analyzed by one-way ANOVA with the post hoc Duncan multiple comparison tests, using SPSS statistical software (ver.17.0 for Windows, SPSS Inc., USA). Assumptions for ANOVA are: normal distribution, homogeneity of variances and randomization. The DGGE patterns were compared using Quantity One software version 4.6.2 software package (Bio-Rad Laboratory, USA). The means and standard error of the mean (SEM) are presented. Differences were considered significant at $P < 0.05$, and tendencies were indicated while the P value was between 0.05 and 0.10.

Results

Growth performance

Infection with *S. typhimurium* caused significant adverse (P<0.05) effects on body weight gain (BWG) in both dietary

Table 3. Effect of Supplemental zinc on growth performance of broiler chickens infected with *S. typhimurium*

Item	CON	STC	ZnT	*P*-value
Days 1 to 21				
BWG (g)	705±18.4a	656±11.5b	721±13.8a	0.013
FI (g)	989±26.3a	904±24.4b	966±26.6ab	0.073
FCR, feed (g) / gain (g)	1.39±0.03a	1.40±0.02a	1.34±0.01b	0.040
Days 1 to 42				
BWG (g)	1986±24.7a	1825±37.1b	2018±37.5a	0.001
FI (g)	3463±68.1ab	3187±59.4b	3545±123.9a	0.023
FCR, feed(g)/gain(g)	1.74±0.03	1.75±0.01	1.72±0.02	0.663

Values represent means ± SEM (n = 10 Cages with 6 chicks per cage) and values within a row followed by different letters differ significantly (P<0.05)
CON, nonchallenge control; STC, *S. typhimurium*-challenged control; ZnT, *S. typhimurium*-challenged group with dietary 120 mg/kg of supplementation $ZnSO_4 \cdot H_2O$
BWG, body weight gain; FI, feed intake; FCR, feed conversion ratio

Table 4. Effect of supplemental zinc on epithelium cell apoptosis and proliferation index, histomorphological parameters and sucrase activity in the ileum of 14-day-old broiler chickens infected with *S. typhimurium*

Item	CON	STC	ZnT	*P*-value
Villus height (μm)	416±21.7b	382±21.3b	515±20.9a	0.006
Crypt depth (μm)	84±6.7	105±11.8	101±4.7	0.168
V/C	5.04±0.354a	3.69±0.254b	5.13±0.324a	0.033
Proliferation index (%)	42.03±1.812b	42.02±2.832b	54.22±3.779a	0.027
Apoptotic index (%)	3.51±0.446b	7.80±0.888a	3.66±0.353b	0.001
Sucrase activity (units/g protein)	2.54±0.204a	1.71±0.154b	2.42±0.261ab	0.062

Values represent means ± SEM (n =10 chicks per treatment) and values within a row followed by different letters differ significantly (P<0.05)
CON, nonchallenge control; STC, *S. typhimurium*-challenged control; ZnT, *S. typhimurium*-challenged group with dietary 120 mg/kg of supplementation $ZnSO_4 \cdot H_2O$.
V/C, villus height to crypt depth ratio

phases, but no significant effect on feed intake (FI) and feed conversion ratio (FCR) of broilers was observed in either phase in comparison with uninfected birds (Table 3). Birds fed a diet with supplemental zinc had significantly improved (P<0.05) BWG in both phases, increased (P<0.05) FI during days 1 to 42, and decreased (P<0.05) FCR during days 1 to 21 when compared with the *S. typhimurium*-infected group (STC).

Ileum morphology and sucrase activity

A comparison of ileum morphology and sucrase activity are shown in Table 4. *S. typhimurium* infection significantly decreased (P<0.05) the villus-height/crypt-depth ratio in the ileum and tended (P=0.062) to reduce the activity of ileal mucosal sucrase, while the villus height and crypt depth in the ileum were not affected by *Salmonella* infection. However, birds fed a diet with supplemental zinc had significant increases (P<0.05) in villus height and in the villus-height/crypt-depth ratio of the ileum, but had no significant change on crypt depth or in the activity of ileal mucosal sucrase as compared with the STC group.

Proliferation and apoptosis of the intestinal epithelial cells

The data concerning the proliferation and apoptotic index of epithelial cells in the ileum are shown in Table 4. The proliferation index was not affected in the *Salmonella*-challenged treatment. Conversely, the apoptotic index was increased (P<0.05) markedly in the *Salmonella*-challenged birds compared with that in the CON group. However, birds fed a diet with supplemental zinc had a significant increase (P< 0.05) in the proliferation index and a decrease (P<0.05) in the apoptotic index compared with the STC group.

Community structure of the cecal microbiota

The similarity between the DGGE patterns of the bacterial communities and the fingerprints of the bacterial communities are shown in Fig. 1, and the sequence homologies of bands A, B, C, and D are presented in Table 5. The DGGE patterns of the three groups were clustered into two branches. The *S. typhimurium*-challenged group was in a single cluster. The unchallenged group and the *S. typhimurium*-challenged group getting supplemental zinc were in a second cluster. The similarity index between the two branches was shown to be only approximately 50%. The within-group-similarity index of the *S. typhimurium*-challenged, the unchallenged and the *S. typhimurium*-challenged group receiving supplemental zinc group was 88%, 74%, 83% respectively. Sequence analysis of band A showed similarity to *Clostridium populeti* (98%). It was present in groups CON and STC but not in the ZnT group. Sequence analysis of band B indicated 99% similarity for *Alistipes finegoldii.* It appeared in all gels regardless of treatment. The average intensities of the bands in the groups CON, STC, and ZnT were 39.5±6.88, 119.6±3.67, and 40.2±2.32 respectively, with the STC group having the most intense (P<0.001) bands. Band C was closely related to *Robinsoniella peoriensis* with a similarity of 100%, and was

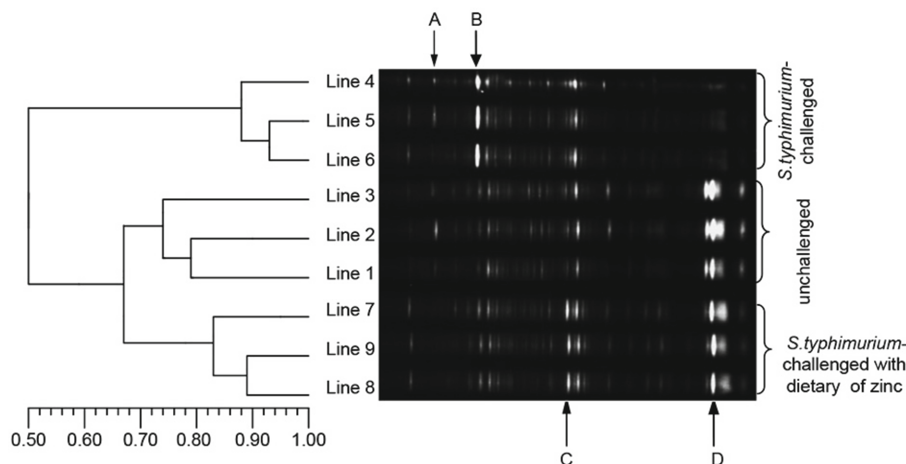

Fig. 1. UPGMA dendrogram of DGGE profiles based on cecal samples from 14-day-old chickens. The scale bar represents a unitary scale of similarity, and arrows indicate positions on the profile where bands particular to a specific lane or group of lanes were analyzed. Band A, B, C, D were compared to 16S rRNA gene sequences in GenBank in Table

Table 5. **Identities of bands obtained from DGGE profiles**

marker of band[a]	Accession number[b]	Closest relatives (NCBI database)	Taxonomic group	%Identity[c]
A	HG967641	*Clostridium populeti* strain 743A 16S ribosomal RNA, partial sequence	*Firmicutes*	98%
B	HG967642	*Alistipes finegoldii* DSM 17242 strain DSM 17242 16S ribosomal RNA, complete sequence	*Bacteroidetes*	99%
C	HG967643	*Robinsoniella peoriensis* strain PPC31 16S ribosomal RNA, complete sequence	*Firmicutes*	100%
D	HG967644	*Faecalibacterium prausnitzii* strain ATCC 27768 16S ribosomal RNA, partial sequence	*Firmicutes*	96%

[a] Bands were extracted from the DGGE gel shown in Fig. 1
[b] Accession numbers were provided by the European Nucleotide Archive database
[c] Percentage of identical nucleotides in the sequence retrieved from the DGGE gel and the sequence of the closest relative found in the GenBank database

present in all the groups. The average intensities of the bands in the groups CON, STC, and ZnT were 29.2±5.10, 29.0±0.71, and 72.6±7.32 respectively, with the ZnT group having the most intense (P<0.001) bands. Band D appeared in all gels regardless of treatment and was determined by sequence analysis to have similarity to *Faecalibacterium prausnitzii* (96%). The average intensities of the bands in the groups CON, STC, and ZnT were 182.0±42.81, 17.9±2.63, and 159.4 ±14.91 respectively, with the STC group having the faintest (P=0.001) bands. Among the above bands, band B fell into the taxonomic group *Bacteroidetes* and the other bands (A, C, D) were *Firmicutes*.

The analysis of bacterial diversity of the cecum in broiler chickens is shown in Table 6. Compared with the CON group, *S. typhimurium* infection reduced (P<0.05) the number of bands in the DGGE profiles and Shannon index of diversity. However, the bacterial diversity was not significantly affected by supplemental zinc in the diet. There was no significant difference in the Simpson index of dominance concentration among the groups of broilers.

Quantification of *S. typhimurium*, *Lactobacillus* group, and domain Bacteria in cecal contents

Quantitative real-time PCR was used to determine which major microbial groups were affected (Table 7). The uninfected birds remained free of *Salmonella* throughout the experiment. The *Salmonella* concentration in the cecal contents of challenged group was 3.24 $\log_{10}$ (16S rDNA gene copies/g wet cecal contents), whereas the number of *S. typhimurium* in the group ZnT was reduced by approximately 53.2% compared with the STC group. *S. typhimurium* infection significantly decreased (P<0.05) the number of bacteria in the *Lactobacillus* group, *Enterobacteriaceae* family, and total bacteria in comparison with uninfected birds. Zinc supplementation significantly increased (P<0.05) the number of *Lactobacillus* group, and total bacteria, when compared with the STC group but had no significant effect on the *Enterobacteriaceae*. No differences were observed in the cell counts of *Enterococcus* among the groups.

Discussion

Infection with *Salmonellas* leads to a significant negative effect on growth performance of poultry (Vandeplas *et al*., 2009; Marcq *et al*., 2011; Wang *et al*., 2012), which was confirmed in this study. In the present study, there was a significant reduction in BWG after *S. typhimurium* challenge. However, zinc supplementation improved BWG, FI, and FCR and reduced the number of *S. typhimurium* in the ce-

Table 6. **Effect of supplemental zinc on richness, Shannon-Weaver index and Simpson index of cecal microflora from 14-day-old chickens infected with *S. typhimurium* based on PCR-DGGE banding patterns**

Item	CON	STC	ZnT	P-value
The number of bands[a]	25.33±0.333a	23.67±0.333b	24.67±0.333ab	0.033
Shannon-Weaver index[b]	1.38±0.008a	1.35±0.006b	1.37±0.007ab	0.041
Simpson index[c]	0.044±0.0012	0.047±0.0009	0.045±0.0007	0.119

Values represent means ± SEM (n=3 replicates of each pool and ten chicks per pool) and values within a row followed by different letters differ significantly (P<0.05).
[a] The number of bands on each lane of the DGGE gel tracks refers to the richness of microbial community.
[b] The Shannon-Weaver index (H') refers to the diversity of microbial community.
[c] The Simpson index (D) refers to the concentration dominance of microbial community.
CON, nonchallenge control; STC, *S. typhimurium*-challenged control; ZnT, *S. typhimurium*-challenged group with dietary 120 mg/kg of supplementation $ZnSO_4 \cdot H_2O$.

Table 7. **Effect of supplemental zinc on the microbial populations of cecal samples from 14-day-old chickens infected with *S. typhimurium* [$\log_{10}$ 16S rRNA gene targets per gram (wet weight) intestinal material]**

Items	CON	STC	ZnT	P-value
S. typhimurium	BT	3.24±0.217	2.91±0.121	0.609
Lactobacillus group	5.66±0.327a	3.58±0.536b	5.47±0.343a	0.036
Enterococcus genus	5.31±0.189	4.77±0.248	5.26±0.305	0.402
Enterobacteriaceae family	7.12±0.376a	5.89±0.245b	6.53±0.277ab	0.048
Domain Bacteria	8.38±0.095a	7.62±0.129b	8.06±0.169a	0.003

Values represent means ± SEM (n= 10 chicks per treatment) and values within a row followed by different letters differ significantly (P<0.05)
CON, nonchallenge control; STC, *S. typhimurium*-challenged control; ZnT, *S. typhimurium*-challenged group with dietary 120 mg/kg of supplementation $ZnSO_4 \cdot H_2O$.
BT, below the detection threshold of qPCR

cum of the challenged chickens, indicating that the zinc might exert a protective role in controlling *Salmonella* infection. These results are in agreement with previous studies showing that there is a significant improvement in growth performance for *Salmonella*-challenged poultry fed a zinc-enriched diet (Hegazy and Adachi, 2000). Moreover, Roselli *et al*. (2003) demonstrated that zinc-bearing clinoptilolite could be used as a new antibacterial agent for improving the growth performance of broilers challenged with *S. pullorum*. Our previous study also showed that supplementation with zinc at the level of 120 mg/kg in the diet was very effective in improving growth performance and enhancing intestinal barrier function against *Salmonella* infection (Zhang *et al*., 2012). Therefore, supplemental zinc was used at the concentration of 120 mg/kg in the diets in this study. However, the mechanisms underlying these effects have not been fully illuminated. Further investigation into the mechanism underlying the protective effects of zinc is needed.

The small intestine is the major site for digestion and absorption of nutrients and the intestinal villus:crypt ratios and sucrase activity are the most widely established ways of investigating intestinal mucosal integrity and intestinal function (Lamb-Rosteski *et al*., 2008). A reduction of both the villus-height/crypt-depth ratio and the activity of the ileal sucrase induced by *S. typhimurium* challenge in the current study indicated an impairment of the mucosal barrier functional capacity by infection with *S. typhimurium*. Moreover, we also found that supplemental zinc resulted in increased villus height, villus height to crypt depth ratio and numerically enhanced the activity of sucrase in the ileum after *S. typhimurium* challenge, which may lead to improved digestive and absorptive function of the gastrointestinal tract. These results are consistent with previous results obtained by ourselves and others (Fasina *et al*., 2010; Zhang *et al*., 2012; Shao *et al*., 2013). The balance between enterocyte proliferation and apoptosis is very important for epithelial cell turnover and intestinal integrity (Yan *et al*., 2007). However, in the present study, the apoptotic index of ileum epithelial cells was considerably increased in *S. typhimurium* infection chicks, but the cell proliferation index was not affected by *S. typhimurium* infection. An increase in epithelial cell apoptosis is associated with a parallel increase of gut permeability (Watson *et al*., 2005). Moreover, Vetuschi *et al*. (2002) showed that the apoptotic index progressively increased in DSS colitis, leading to a disruption in the epithelial barrier function. Thus, the present results suggest that *S. typhimurium* may compromise epithelial integrity by inducing death of enterocytes. *In vitro* studies have been reported in which human colon epithelial cell apoptosis was markedly increased in response to infection with *Salmonella* (Kim *et al*., 1998; Paesold *et al*., 2002). Intestinal epithelial cell proliferation is required to replenish the reduced cell pool, which is crucial for maintaining a healthy epithelial barrier (Cario *et al*., 2000). We found that dietary supplemental zinc increased the proliferation index and decreased the apoptotic index of enterocytes. This indicates that zinc can repair the epithelial cell injury by stimulating proliferation and enhancing resistance to apoptosis. Eventually these changes are manifested as increased villus height and the villus height to crypt depth ratio in the ileum of chicks supplemented with zinc. A previous study demonstrated that zinc directly regulated DNA and protein synthesis, and inhibited cell apoptosis (Truong-Tran *et al*., 2001). It also appeared to be essential for IGF-I induction of cell proliferation (MacDonald, 2000).

A healthy and stable intestinal microbial community plays a beneficial role in maintaining the structural and functional characteristics of the mucosa, influencing the immune system as well as preventing the development of intestinal diseases, especially resisting colonization by pathogens in chickens (Southon *et al*., 1986; Chambers and Gong, 2011; Sommer and Backhed, 2013). A diverse microbiota is distributed along the entire gastrointestinal tract of broiler chickens, and is most extensive in the cecum, which contains as many as 10^{11} bacteria per gram (wet weight) of cecal content (Barnes *et al*., 1980). Moreover, Zhu *et al*. (2002) reported that the cecum has received most of the attention because the diverse microbial species in the cecum could form a strong barrier against pathogens. However, *S. typhimurium* can outcompete the resident microbial flora and localize in the cecal region, under certain conditions, after it is ingested by chickens (Revolledo *et al*., 2009). Therefore, in the present study, the microflora of the cecum rather than other parts of digestive tract was examined. PCR-DGGE profiling techniques were used to detect shifts in cecal microbial populations. The clustering analysis indicated that the unchallenged group and the *S. typhimurium*-infected group belonged to two different clusters, which showed that there had been shifts in the commensal intestinal bacterial populations after *S. typhimurium* infection. However, the unchallenged group and the *S. typhimurium*-challenged, zinc-supplemented group were clustered together in a single branch, implying that zinc promoted the restoration of the normal intestinal microbial community of the *S. typhimurium*-infected chicks. The mechanism of these beneficial effects of zinc on the microbial community needs further study.

In the current study, bacteria were identified from the DGGE banding profiles. It was observed that the intensity of band D (unknown *Faecalibacterium*) was reduced after *S. typhimurium* infection. However, the intensity of band B (unknown *Alistipes* sp.) was more prominent in the cecum of *S. typhimurium*-infected chickens. The unknown *Faecalibacterium* fell into the taxonomic group *Firmicutes* and the unknown *Alistipes* sp. was affiliated to the taxonomic group *Bacteroidetes*. These two kinds of bacteria were present as the most intensely staining fragments in the DGGE profiles. A decreased proportion of *Firmicutes* and an elevated population of *Bacteroidetes* have been related to weight loss (Ley *et al*., 2006; Guo *et al*., 2008). Therefore, the reduced intensity of bands of unknown *Faecalibacterium* and an increased intensity of bands of unknown *Alistipes* sp. may be partially responsible for a reduction of BWG in the *S. typhimurium*-challenged birds. Conversely, an elevated proportion of *Firmicutes* and a reduced population of *Bacteroides* were observed in the microbiota of obese humans and pigs (Ley *et al*., 2006; Turnbaugh *et al*., 2006; Guo *et al*., 2008). In this study, dietary zinc supplementation increased the intensity of band D (unknown *Faecalibacterium*), but decreased the intensity of band B (unknown *Alistipes* sp.). These changes may partially explain the significant improvement in growth performance when zinc-enriched diets were fed to a *Salmo-*

nella-challenged group. However, our results did not evaluate the total amount of *Firmicutes* and *Bacteroides* in the cecal microbiota, which should be further investigated.

Quantitative PCR revealed that the number of *Lactobacillus*, *Enterobacteriaceae* family and the total bacteria were reduced after *S. typhimurium* infection. These results indicate that *S. typhimurium* infection influenced the composition of the cecal microbiota in chickens. The changes in the composition of gut microbiota were also observed in animals infected with non-typhoid serovars of *Salmonella* (Barman *et al*., 2008; Juricova *et al*., 2013; Videnska *et al*., 2013), which were associated with inflammation (Stecher *et al*., 2007; Barman *et al*., 2008). Thus, these changes in the microbiotal composition induced by *S. typhimurium* infection, as observed in our study, may contribute to the processes of mucosal injury (Tamboli *et al*., 2004; Sokol *et al*., 2006), and support the observed results of a reduction in the villus-height/crypt-depth ratio of the ileum and an elevated apoptotic index of ileal epithelial cells in challenged broilers. Zinc has been shown to play an important role in cell replication and growth, including cell division, protein synthesis and carbohydrate metabolism, in several species of bacteria, as well as to be a cofactor for a wide range of enzymes (Vallee *et al*., 1981). An *in vitro* research project has shown that a zinc-limited culture condition can directly lead to a reduction in bacterial growth (Summers and Srinivasan, 1979). Studies using a mouse model indicated that the intestinal structure and function of the zinc-deficient rats were strikingly similar to those observed in germ-free rats (Southon *et al*., 1986), which indicated that there was an important relationship between zinc and the gut ecosystem. In this study, dietary zinc supplementation increased the number of bacteria in the *Lactobacillus* group and the total bacteria. *Lactobacillus* bacteria are known for their potential to prevent the colonization of pathogens and ameliorate barrier dysfunction by competing for epithelial binding sites as well as through the production of antimicrobial and anti-inflammatory factors (Jones and Versalovic, 2009; Fang, 2010). These results indicated that zinc supplementation in the diet may help maintaining the stability of the intestinal microflora, increasing the number of beneficial bacteria and reducing the chance of *S. typhimurium* colonization in the cecum, which is in line with the improved intestinal mucosal morphological structure and intestinal function (Sommer and Backhed, 2013). With regard to the main mechanism involved in the increased number of total bacteria, it can be speculated that the 120 mg zinc/kg feed was enough to provide the essential micronutrient metals needed for intestinal microbes, preventing calprotectin from sequestering needed zinc in an inflammation, which otherwise could limit the availability of zinc to microbes (Liu *et al*., 2012). However, further investigation is needed into the mechanism by which zinc restores the microbial communities to a near-normal composition.

In conclusion, dietary supplemental zinc improved intestinal mucosal morphology by promoting intestinal mucosal cell proliferation and inhibition of apoptosis, and regulated the cecal microbiota in *S. typhimurium*-challenged broilers by reducing the number of *Salmonella*, and increasing the number of the beneficial *Lactobacillus* bacteria.

Acknowledgements

The authors thank Shuangshuang Guo, Xu Zhao, Ting Lei and Yanfei Xu for their help with the experiments. This work was supported by the Chinese Universities Scientific Fund (No. 2013QJ068, 2014JD020), and the National Natural Science Foundation of China (No. 30901031).

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