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Effects of astaxanthin on growth performance, intestinal integrity, and microbiota in *Salmonella* Enteritidis-infected chickens

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ABSTRACT

This study investigated the effects of astaxanthin (**AST**) supplementation in drinking water on the growth performance, intestinal barrier function, and cecal microbiota of broilers challenged with *Salmonella* Enteritidis. During the 20-day experiment, two hundred and forty 1-day-old male Arbor Acres birds were randomly assigned into a 2 × 2 factorial design with four groups: a non-challenged control (**CON**), an *S.* Enteritidis-challenged group (SA), a group receiving AST treatment (**AST**), and an *S.* Enteritidis-challenged group receiving AST treatment (**SA+AST**). Each treatment comprised six replicate groups, and challenged groups were inoculated with *S. Enteritidis* from day 2 to day 4. The results indicated that *S.* Enteritidis infection significantly reduced the average daily feed intake (**ADFI**) in broilers and adversely affected average daily gain (**ADG**) and feed conversion ratio (**FCR**) by day 20. AST supplementation significantly improved FCR. While *S.* Enteritidis infection did not significantly affect ileal mucosa antioxidation, it significantly decreased villus height and the villus height-to-crypt depth ratio (VCR), and significantly downregulated mRNA expression of *ZO-1* and *Occludin*. However, AST supplementation significantly enhanced antioxidant capacity (T-AOC), increased villus height and VCR in the ileum, and notably upregulated *ZO-1* and *MUC2* expression levels, particularly mitigating the adverse effects of *S.* Enteritidis infection on ileal crypt depth. Furthermore, *S.* Enteritidis infection significantly affected both the α- and β-diversity of cecal microbiota. Infection with *S.* Enteritidis was associated with changes at the phylum level, including significant increases in *Alistipes, unclassified_f_Lachnospiraceae*, and bacteria of the *Clostridia* UCG-014 grouping, alongside notable decreases in *Bacteroides, Akkermansia, Blautia*, and *Butyricicoccus*. AST supplementation significantly decreased the abundance of *norank_f_Ruminococcaceae* and increased the abundance of *Lachnoclostridium* and *unclassified_f_Lachnospiraceae* in the challenged group. In conclusion, AST supplementation in drinking water could improve growth performance and intestinal health in broilers challenged with *S.* Enteritidis.

Introduction

Salmonella, a Gram-negative facultative intracellular bacterium, poses significant health risks in poultry and causes food-borne salmonellosis in humans, thus resulting in substantial economic losses worldwide (Hu et al., 2023). This pathogen is classified into approximately 2,659 recognized serovars based on the O antigen and H antigen, with occasional reference to the capsular antigen, includes *Salmonella*

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enterica serovar Enteritidis (***S.* Enteritidis**) as one of the most common serovars in poultry infection (Hu et al., 2023; Ferrari et al., 2019). In chickens, *S*. Enteritidis infection can damage intestinal mucosal barrier integrity, influenced by factors such as inflammation, oxidative stress, tight junction disruptions, and imbalance of gut microbiota. When *S*. Enteritidis breaches the intestinal mucosal barrier, it can colonize internal organs, like the spleen and liver, resulting in bacteremia and even mortality in chickens (Xiao et al., 2018). Traditionally, antibiotics have been the primary method for controlling salmonellosis in poultry (Erinle et al., 2022). However, the abuse of antibiotics has led to the rise of *S*. Enteritidis multidrug resistance, resulting in not only imbalances in the intestinal microbiota but also in reducing antibiotic efficacy (Elsayed et al., 2024). Consequently, alternative strategies, including probiotics, antimicrobial peptides, organic acids, essential oils, and bioactive compounds, have gained attention for their effectiveness in promoting animal growth and preventing diseases in poultry without the downsides, and have been widely applied in the animal husbandry industry (El-Saadony et al., 2022; Gomez et al., 2022).

Astaxanthin (**AST**), chemically known as 3,3′-dihydroxy-b, b-carotene-4,40-dione, is a lipid-soluble red-orange xanthophyll carotenoid found extensively in marine seafood and can also be extracted from the yeast *Phaffia rhodozyma* (Tolba et al., 2020). Due to its distinctive chemical structure, the presence of hydroxyl and keto groups on each ionone ring, AST has an antioxidant capacity approximately ten times greater than that of other carotenoids (Tolba et al., 2020; Hosseindoust et al., 2020). Previous research on AST's biological functions has highlighted its potential as a feed additive with antioxidative, anti-inflammatory, and immunomodulatory activity along with promotion for animal growth (Kim and Kim., 2018). Several studies have demonstrated that adding AST to feed can significantly enhance the growth performance of broilers and reduce disease incidence (Pertiwi et al., 2022). In models of heat stress, feed AST supplementation modulates stress, inflammation, and lipid metabolism in broilers and laying hens (Tolba et al., 2020). Additionally, AST administration has been shown to alleviate gastric inflammation and enhance the immune response by promoting the secretion of IFN-γ, IL-10, and IL-2 in splenocytes from *Helicobacter* pylori-infected mice (Davinelli et al., 2019). However, the potential of AST as an additive for the prevention and treatment of *S*. Enteritidis infection has not yet been explored.

Maintaining a balanced gut microbiota is essential for gastrointestinal health in animals, as it enhances digestion and nutrient absorption, regulates energy metabolism, prevents mucosal infections, and modulates immune responses, thereby promoting host homeostasis (Li et al., 2020). Numerous studies have demonstrated that *S*. Enteritidis infection disrupts the intestinal microbiota balance in hosts (Hu et al., 2023; Gan et al., 2020). Previous research indicated that AST supplementation can elicit beneficial modifications in gut microbiota upon entering the host's gastrointestinal tract, thanks to its potent oxygen radical scavenging capabilities. Consequently, these effects highlight AST's potential in modulating metabolic syndromes and alleviating enteritis or autoimmune diseases (Yasuda et al., 2023; Zhang et al., 2022). However, research examining AST's specific effects on the intestinal flora of broilers, particularly under *Salmonella* infection, is still lacking.

Hence, this study aims to explore the effects of AST supplementation on growth performance, antioxidant properties of intestinal mucosa, morphological alterations in intestinal epithelial, gene expression of tight junction proteins, and the intestinal microbiota in broilers infected with *S*. Enteritis. These findings could provide new insights into non-antibiotic approaches for reducing and preventing *S*. Enteritidis infections.

Materials and methods

Animal ethics statement

All animal experiments were approved by the Animal Care and Ethics Committee of Jiangsu Academy of Agricultural Sciences (SYXK2020-0023), and every effort was taken to ensure animal welfare and minimize suffering.

Broilers husbandry and experimental design

A total of 240 one-day-old male Arbor Acres broilers were obtained from Jinghai Group (Hai'an, China). Upon arrival, the broilers were weighted and randomly assigned to 4 treatment groups, with 6 replicates of 10 birds each. The experimental groups consisted of a non-challenged control (**CON**), a *S*. Enteritidis-challenged group (**SA**), a group receiving AST supplementation in drinking water (**AST**), and *S*. Enteritidis-challenged birds supplemented with AST in drinking water (**SA+AST**). At 4-6 days old, each bird in SA and SA+AST groups received an oral gavage of 1 mL PBS solution containing 1×10^6 cfu/mL *S*. Enteritidis, while chicks in the other groups were administered an equivalent volume of PBS. From day 7 until the end of the experiment, all chickens in the AST or SA+AST group were fed astaxanthin in their drinking water at a concentration of 500 mg/L. The astaxanthin (purity 1.0 %) used in this study was supplied by Zhejiang Kangmu Animal Health Co., Ltd (Zhejiang, China).

Before the experiment, the interior of the poultry house and all the equipment were thoroughly cleaned and disinfected. To prevent cross-contamination, *S*. Enteritidis-infected and non-infected broilers were housed separately in different houses, with each replicate maintained in an individual cage (100 cm × 80 cm × 61 cm). Each cage was equipped with a feed barrel and a vacuum drinker, both of which were regularly cleaned and disinfected. Management of each facility was assigned to a designated staff member. One-day-old broilers were assigned to experimental groups, following which cloacal swabs were collected from each bird. Selective culture media and molecular detection methods, as described by Erinle and Gong et al., were employed to detect and confirm the absence of *Salmonella* in the collected samples (Erinle et al., 2023; Gong et al., 2016). Upon completion of the challenge, the same strategy was used to verify the successful colonization of *Salmonella* and to confirm that the non-challenged group remained negative. The indoor air temperature was maintained at 31-32°C for the first 3 days and gradually decreased by 1°C every two days until a final indoor air temperature of 22 - 24°C. Lighting was adjusted to provide 24 h for 1-3 days, 16 h during days 4-14, and 12 h from days 15 - 20, respectively. Food and water were provided *ad libitum* throughout the entire experimental period. The ingredients and nutrient levels of the basal diet are shown in Table 1.

Preparation of S. enteritidis

S. Enteritidis ATCC 13076 was cultured overnight on Luria-Bertani

Table 1
Composition and Levels of Dietary Raw Materials and Nutrients (Day 1 - 20).

Ingredients	(%)	Nutrient levels	(%)
Corn	57.61	Metabolizable energy (MJ/kg)	12.60
Soybean meal	31.00	Crude protein	21.10
Corn gluten meal	3.29	Calcium	1.00
Soybean oil	3.11	Available phosphorus	0.46
Limestone	1.20	Lysine	1.20
CaHPO4	2.00	Methionine	0.50
Lysine	0.34	Methionine + Cystine	0.85
Methionine	0.15		
Sodium chloride	0.30		
Premix	1.00		
Total	100.00		

Provided per 1 kg of diet: vitamin A, 10000 IU; vitamin D_3, 3000 IU; vitamin E, 30 IU; vitamin K_3, 1.3 mg; vitamin B1, 2.2 mg; vitamin B_2, 8.0 mg; vitamin B_6, 4.0 mg; vitamin B_{12}, 0.013 mg; niacin, 40 mg; choline chloride, 400 mg; pantothenic acid, 10.0 mg; biotin, 0.04 mg; folic acid, 1.0 mg; iron, 80 mg; manganese, 100 mg; zinc, 60 mg; iodine, 1.1 mg; selenium, 0.3 mg.

(**LB**) agar at 37 °C. A single colony was then transferred into LB broth and incubated at 37 °C under shaking for 18 h. Afterward, the bacteria suspension was centrifuged at 6,000 rpm for 15 min, and the supernatant was discarded. The pellet was washed with 0.85 % physiological saline solution, and the concentration of viable *S*. Enteritidis was determined on *Salmonella Shigella* (**SS**) agar plates. Finally, the bacterial solution was serially diluted to a concentration of 1×10^6 cfu/mL for experiment use.

Growth performance

Individual body weight (**BW**) was measured on day 1 and day 20. Throughout the experiment, feed intake was tracked on a per-cage basis. These data were used to calculate average body weight (**ABW**), average daily gain (**ADG**), average daily feed intake (**ADFI**), and feed conversion ratios (**FCR**). Mortality was monitored daily for each cage.

Sample collection

All samples were collected at 20 days of age of broiler chickens. Eight broilers per treatment group, whose body weight was close to the mean body weight, were euthanized via cervical dislocation. Subsequently, approximately 1 cm segments from the median sections of the ileum were harvested and preserved in a 4 % paraformaldehyde solution for intestinal morphological measurements. The ileal mucosa was scraped off from the middle portion, transferred into sterile centrifuge tubes, and immediately snap-frozen in liquid nitrogen at −80°C for assessing antioxidant indices and barrier function. The cecal contents of each deceased bird were aseptically collected in sterile tubes and frozen at −80°C for gut microbial composition analysis.

Antioxidant properties of ileum mucosa

Briefly, 0.1 g of ileal mucosa was homogenized in ice-cold PBS and subsequently centrifuged at 10,000 × g at 4°C for 10 min. The supernatant was then collected for the determination of total superoxide dismutase (**T-SOD**), myeloperoxidase (**MPO**), and total antioxidant capacity (**T-AOC**) using commercial assay kits purchased from Jianchen Bioengineering Institute (Nanjing, China). Following the manufacturer's instructions, the absorbance values of each sample were measured at the specified wavelength using a spectrophotometer, and the concentrations of T-SOD, MPO, and T-AOC in the ileal mucosa were calculated.

Ileum morphology analysis

Ileum morphology analysis was performed as previously described (Livak and Schmittgen, 2001). Tissue samples were dehydrated, embedded in paraffin wax, and sectioned into 5-μm-thick slices. The sections were then stained with hematoxylin and eosin for histological examination. Subsequently, the processed sections were observed under a microscope, and representative fields were photographed. Villus height (**VH**) and crypt depth (**CD**) were measured using Image J software (v1.8.0, National Institutes of Health, Bethesda, MD, USA). The villus height to crypt depth ratio (**VCR**) was subsequently calculated to evaluate intestinal morphology.

Determination of gene expression in the ileum

Total RNA was extracted from the ileum mucosa using an RNA isolation kit (Beijing Transgene Biotech Ltd., Beijing, China) in accordance with the manufacturer's protocol. The purity and concentration of the extracted RNA were evaluated using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized by using a PrimeScript RT Reagent Kit with gDNA Eraser (Beijing Transgene Biotech Ltd., Beijing, China) in a 20 μL reaction volume. Quantitative real-time PCR (**qRT-PCR**) was conducted to measure the expression levels of *ZO-1, MUC2*, and *Occludin* using the SYBR fluorescence quantification kit on a LightCycler 480 (Roche, Basel, Switzerland), with *β-actin* serving as the internal control. Primer sequences are listed in Table 2. Relative gene expression of target genes was calculated using the 2ˆ-ΔΔCt method (Livak and Schmittgen, 2001).

16S rRNA gene sequencing and analysis

Total genomic DNA was extracted from cecal content samples using the E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's protocol. The purity and concentration of the extracted DNA were evaluated using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the DNA integrity was confirmed by 1 % agarose gel electrophoresis. The V3-V4 hypervariable regions of bacterial 16S rRNA gene were amplified via PCR with the primers (338F: 5′-ACTCCTACGGGAGGCAGCAG-3′; 806R: 5′-GACTACHVGGGTWTCTAAT-3′) in an ABI GeneAmp 9700 PCR thermal cycler (Applied Biosystem, CA, USA). The PCR products were analyzed through 2 % agarose gel electrophoresis and purified by using a PCR Cleanup Kit (YuHua Bioengineering, Shanghai, China). Quantification was performed with a Qubit 4.0 fluorometer (Thermo Fisher Scientific, MA, USA), and then the Miseq library was constructed. Purified amplicons were sequenced by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China) via an Illumina PE250 platform (Illumina, San Diego, USA) according to standard protocols. The resulting raw sequencing reads were deposited in the NCBI Sequence Read Archive (SRA) database. (Accession Number: PRJNA1193984).

Raw FASTQ files were de-multiplexed and quality-filtered by fastp (version 0.19.6) and paired-end reads were merged by FLASH (version 1.2.7). Subsequently, the optimized sequences were clustered into operational taxonomic units (**OTUs**) at a 97 % sequence similarity level using UPARSE (version 7.1) according to the previously described method (Edgar, 2013). The α diversity indices, such as the Chao index (for richness) and Shannon index (for richness and evenness) were calculated. The β diversity was evaluated using Principal coordinate analysis (**PCoA**) and non-metric multidimensional scaling analysis (**NMDS**) to examine the visualization of differences in microbial community composition among samples. The metagenomic functions were performed using PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) based on the representative sequences of Amplicon Sequence Variants (**ASVs**), and the pipeline followed the standard guidelines established for PICRUSt2.

Statistical analysis

Data were analyzed as a 2 × 2 factorial using PROC GLM (SAS Institute Inc., Cary, NC, USA) in SAS 9.4. The statistical model included the main effects of *Salmonella* infection, AST treatment, and their interaction. Least-squares means (**LSMEANS**) was employed for multiple comparisons, and the percentage was arcsine transformed before the normality test. Results were considered highly statistically significant at $P < 0.01$, statistically significant at $P < 0.05$, and having a significant trend when $0.05 < P < 0.10$. Pearson correlation analysis between the cecal microbiota and other variables was conducted using GraphPad

Table 2
Primer sequences for target genes.

Primer Name	Sequence (5′–3′)	Gene ID
β-actin	F: CAGCCAGCCATGGATGATGA R: ACCAACCATCACACCCTGAT	NM_205518.2
ZO-1	F: CCACTGCCTACACCACCATCTC R: CGTGTCACTGGGGTCCTTCAT	CP100.564.1
MUC2	F: TTCATGAGCCTGCTCTTGTG R: CCTGAGCCTTGGTACATTCTTGT	XM_421035
Occludin	F: ACGGCAGCACCTACCTCAA R: GGGCGAAGAAGCAGATGAG	D21837.1

Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA). Data in the tables were presented as means and pooled SEM.

Results

Growth performance

The effects of AST supplementation on the growth performance of broilers are displayed in Table 3. Compared to the non-challenged treatment groups, chickens challenged with *S*. Enteritidis exhibited significantly reduced ADFI ($P < 0.01$) alongside compromised ADG, ABW and FCR. However, AST supplementation significantly lowered the FCR ($P < 0.05$) and effectively mitigated the negative impact of *S*. Enteritidis infection on feed efficiency. Furthermore, no significant interaction effects between *S*. Enteritidis infection and AST supplementation on growth performance were observed ($P > 0.05$).

Antioxidation of Ileal mucosa

The effects of AST supplementation on the antioxidation of ileal mucosa in broilers are illustrated in Fig. 1. *S*. Enteritidis infection did not significantly alter the activities of MPO, T-SOD or T-AOC ($P > 0.05$). However, AST supplementation significantly enhanced T-AOC activity ($P < 0.05$) and showed a tendency to increase T-SOD activity ($P = 0.065$). A significant SA×AST interaction was also observed for the MPO concentration ($P < 0.01$).

Ileal morphology

The morphology of ileal tissue in broilers is presented in Table 4. *S*. Enteritidis infection significantly reduced villus height and VCR ($P < 0.05$) compared to the non-challenged groups. AST supplementation notably enhanced the height of the villus and significantly reduced the depth of the crypt ($P < 0.05$), showing a tendency to improve VCR ($P = 0.071$). Furthermore, a trend of the interaction of *S*. Enteritidis and AST (SA×AST) supplementation was observed for crypt depth ($P = 0.051$) and VCR ($P = 0.091$). Compared to the SA group, the SA+AST group exhibited improved villus height and VCR ($P > 0.05$) and a significantly decreased crypt depth ($P < 0.05$).

Gene expression of tight junction protein

Fig. 2 presents the results of ileal barrier-related gene expression in broilers. Broilers infected with *S. Enteritidis* exhibited significantly lower mRNA expression levels of *ZO-1* ($P = 0.001$) and *Occludin* ($P = 0.004$) but higher mRNA levels of *MUC2* ($P = 0.017$) compared to the non-challenged groups. AST supplementation significantly increased the mRNA expression of *ZO-1* ($P = 0.046$) and *MUC2* ($P = 0.009$) compared to those without ingesting AST. Additionally, a significant SA×AST interaction was observed for the mRNA expression of *MUC2* ($P = 0.012$).

Cecal microbiota

Fig. 3A shows the rarefaction curves of each group based on the OTU, which plateaued, indicating that the sequencing depth was sufficient to analyze the microbial richness and diversity comprehensively. As shown in Fig. 3B, a total of OTUs were identified based on 97 % sequence similarity, with 1201, 1744, 1199, and 1371 OTUs detected in the CON group, the SA group, the AST group, and the SA+AST group, respectively. All groups shared 465 OTUs. Furthermore, the unique OTUs identified in each group were recorded as follows: 223 in the CON group, 697 in the SA group, 237 in the AST group, and 350 in the SA+AST group.

As indicated in Table 5, *S*. Enteritidis infection significantly reduced the coverage and the Simpson index ($P < 0.05$) while increasing the remaining alpha diversity indices (Chao1, Ace, Sobs, and Shannon) ($P < 0.05$) compared to the non-challenged groups. There were no significant differences between the AST group and groups without AST supplementation ($P > 0.05$). Additionally, no significant SA×AST interaction for the α diversity was noted ($P > 0.05$). The principal coordinate analysis (PCoA) and non-metric multidimensional scaling analysis (NMDS) plots presented in Fig. 3C and 3D reveal that the CON group, the AST group, the SA group and the SA+AST group partially overlapped, respectively. Meanwhile, the CON and AST groups were completely separated from the SA and SA+AST groups, indicating distinct microbial community compositions.

Fig.s 3E and 3F demonstrate the composition of microbiota in the cecum of broilers. The predominant phyla in the cecum contents of broilers were *Firmicutes, Bacteroidota, Verrucomicrobiota*, and *Proteobacteria*, among which *Firmicutes* and *Bacteroides* had the highest relative abundance. At the genus level, *Bacteroides, Alistipes, norank_f_Ruminococcaceae*, and *Lactobacillus* were commonly dominant across all groups, while the composition of other dominant bacterial genera varied between treatments. As detailed in Table 6, in contrast to the control treatment, *S*. Enteritidis infection significantly augmented the *Firmicutes, Proteobacteria, unclassified_k_norank_d_Bacteria*, and *Desulfobacterota* ($P < 0.05$), while significantly reducing *Verrucomicrobiota* at the phylum level ($P < 0.05$). Additionally, AST supplementation significantly increased the relative abundances of *Desulfobacterota* ($P < 0.05$), and a significant SA×AST interaction was also observed for the relative abundances of *Campilobacterota* ($P < 0.05$). At the genus level, broilers infected with *S*. Enteritidis exhibited significantly higher relative abundances of *Alistipes, unclassified_f_Lachnospiraceae*, and *norank_f_norank_o_Clostridia_UCG-014* ($P < 0.05$), and had significantly lower abundances of *Bacteroides, Akkermansia, Blautia*, and *Butyricicoccus* compared to the non-challenged groups ($P < 0.05$). AST supplementation significantly influenced the relative abundance of *norank_f_Ruminococcaceae, Lachnoclostridium*, and *unclassified_f_Ruminococcaceae* ($P < 0.05$), and there was a significant SA×AST interaction for the relative abundances of *norank_f_Ruminococcaceae, unclassified_f_Lachnospiraceae*, and *Lachnoclostridium* ($P < 0.05$). Additionally, the relative abundances of *Akkermansia* and *Butyricicoccus* were conspicuously higher in the AST group compared to the SA group.

Table 3
Effect of astaxanthin supplementation on the growth performance of broilers infected with *S.* Enteritidis (1 - 20 days old).

Items	CON	SA	AST	SA+AST	SEM	*P-values*		
						SA	AST	SA×AST
ADFI, g/d	61.9[a]	59.8[ab]	61.3[ab]	59.1[b]	0.39	0.004	0.363	0.912
ADG, g/d	43.2	41.1	44.2	43.3	0.52	0.153	0.141	0.578
ABW, g	864.9	826.5	884.1	866.0	9.95	0.156	0.141	0.603
FCR	1.44[ab]	1.50[a]	1.40[ab]	1.37[b]	0.02	0.584	0.011	0.151
survival rate, %	100.0	96.7	96.7	96.7	1.38	0.570	0.570	0.570

Note: ADFI, average daily feed intake; ADG, average daily gain; ABW, average body weight; FCR, feed conversion ratio. Con, negative control group; SA, S. Enteritidis infection group; AST, astaxanthin treatment group; SA+AST, S. Enteritidis infection and astaxanthin treatment group. SEM, Standard error of the mean. Values are means and standard error of means. Labelled means in a row without a common letter differ, $P \leq 0.05$.

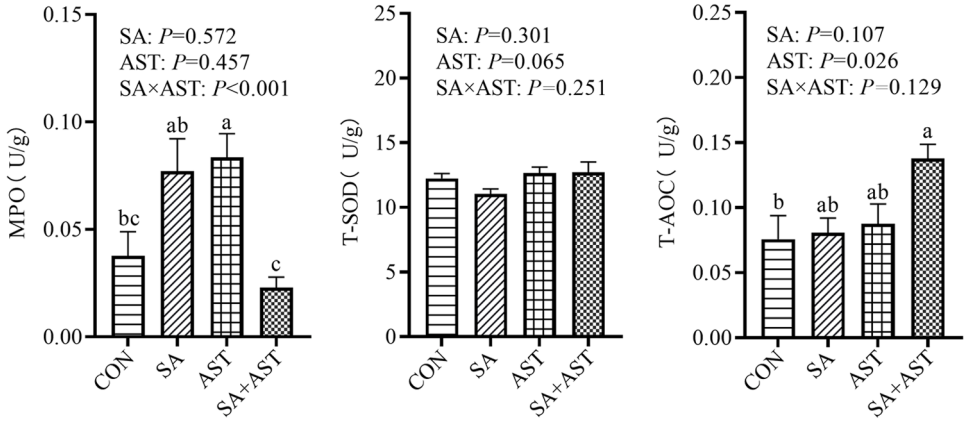

Fig. 1. Activities of MPO, T-SOD, and T-AOC in ileal mucosa. Different small letters indicate significant differences at $P < 0.05$, respectively.

Table 4
The effect of astaxanthin supplementation on the morphology of ileum tissue in broilers infected with *S.* Enteritidis.

Items	CON	SA	AST	SA+AST	SEM	*P-values*		
						SA	AST	SA×AST
Villus height, μm	1196.92^{b}	1153.33^{b}	1351.6^{a}	1223.33^{b}	16.79	0.006	0.001	0.173
Crypt depth, μm	208.09^{ab}	248.49^{a}	186.68^{b}	178.63^{b}	6.55	0.190	0.001	0.051
VCR	7.75^{a}	4.97^{b}	7.84^{a}	7.43^{ab}	0.36	0.024	0.071	0.091

Note: Standard error of the mean. Values are means and standard error of means. Labelled means in a row without a common letter differ, $P \leq 0.05$. VCR, Villus height to Crypt depth ratio.

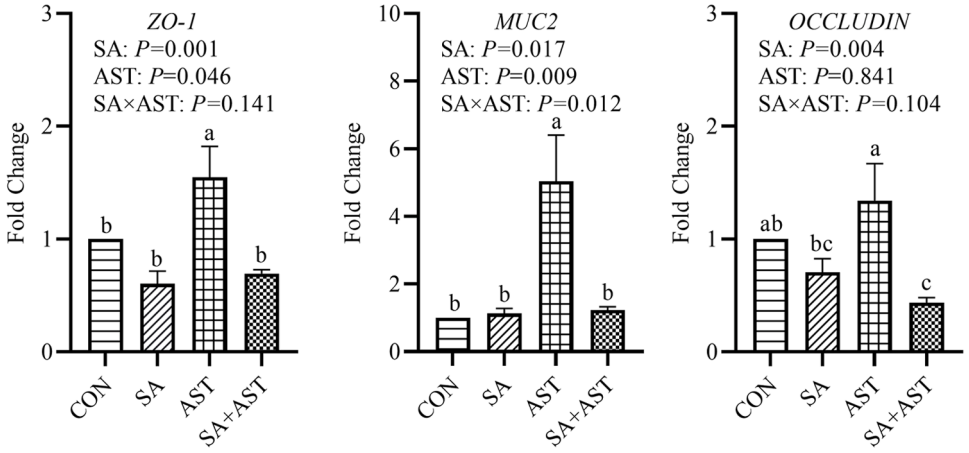

Fig. 2. The effect of astaxanthin on the expression of ileal barrier-related genes in broilers infected with *Salmonella* Enteritidis. Different small letters indicate significant differences at $P < 0.05$, respectively.

Notably, the SA+AST group significantly increased the abundances of *unclassified_f_Lachnospiraceae* and *Lachnoclostridium* compared to the SA group.

Correlation analysis of the cecal microbiota and variables

The correlations between variables related to growth performance, intestinal morphology, barrier function and the abundance of the main microbial genera were illustrated in Fig. 3G. The abundance of *Eisenbergiella* was significantly negatively correlated with ADFI ($P < 0.05$). The abundance of *Lactobacillus* had a significant positive correlation with VCR and a significant negative correlation with crypt depth ($P < 0.05$). In contrast, the abundance of *Akkermansia* showed a highly significant positive correlation with ADFI and the mRNA expression of *ZO-1* and *Occludin* ($P < 0.01$). The abundance of *Faecalibacterium* had a significant positive correlation with crypt depth and a significant negative correlation with ADFI, VCR, and the mRNA expression of *ZO-1* ($P < 0.05$). The abundance of *Lachnoclostridium* showed a very significant negative correlation with crypt depth ($P < 0.01$). The abundance of *Blautia* appeared to have a significant positive correlation with ADFI ($P < 0.05$) and a highly significant positive correlation with the mRNA expression of *ZO-1* and *Occludin* ($P < 0.01$). The abundance of *Ruminococcus_torques_group* had a significant positive correlation with ADG and ABW ($P < 0.05$), and a significant negative correlation with the mRNA expression of *Occludin* ($P < 0.05$), and a very significant negative correlation with FCR ($P < 0.01$). The abundance of *Intestinimonas* showed a significant positive correlation with ADFI and the mRNA expression of *ZO-1* ($P < 0.05$). Similarly, *Butyricicoccus* abundance had a significant positive correlation with ADG, ABW, and VCR ($P < 0.05$), and a very significant positive correlation with ADFI and the mRNA expression of *ZO-1* and *Occludin* ($P < 0.01$), while there was also a positive relation between the abundance of *Bacteroides* and the mRNA

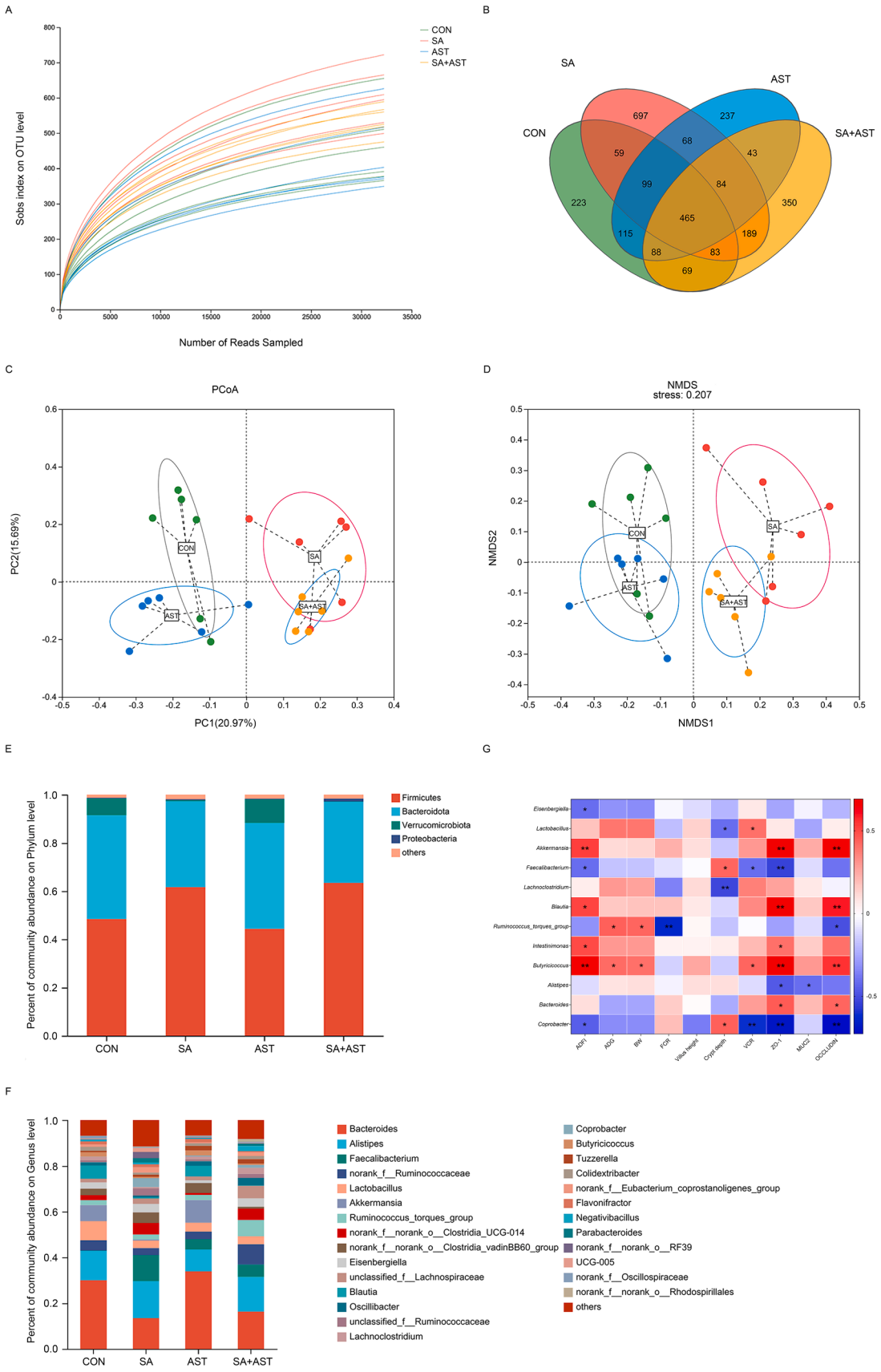

Fig. 3. The effect of astaxanthin on the cecal microbiota of broilers infected with *S.* Enteritidis. (A) Rarefaction curves based on OTUs in the cecal mucosa microbiota. (B) Veen diagram showing the shared and unique OTUs among groups. (C) Principal coordinates analysis (PCoA). (D) Non-metric multidimensional scaling (NMDS) (E) Phylum-level composition of the cecal microbiota in broilers. (F) Genus-level composition of the cecal microbiota in broilers. (G) Correlation analysis between variables related to growth performance, ileal morphology and its expression of tight junction protein genes and the abundance of the main microbial genera.

Table 5
α diversity analysis.

Items	CON	SA	AST	SA+AST	SEM	*P-values*		
						SA	AST	SA×AST
Coverage	0.996	0.995	0.996	0.996	0.000	0.040	0.697	0.223
Chao1	551.6	707.4	558.7	636.5	23.2	0.010	0.444	0.350
Ace	564.6	738.1	570.2	666.5	25.0	0.005	0.450	0.378
Sobs	460^{ab}	603^{a}	440^{b}	539^{ab}	22.0	0.004	0.267	0.552
Shannon	3.094^{b}	3.592^{ab}	3.178^{ab}	3.685^{a}	0.08	0.001	0.498	0.974
Simpson	0.123^{a}	0.088^{ab}	0.103^{ab}	0.066^{b}	0.007	0.012	0.111	0.944

Note: Labeled means in a row without a common letter differ, $P \leq 0.05$.

Table 6
Relative abundance of gut microbial at phylum level and genus level.

Items	CON	SA	AST	SA+AST	SEM	*P-values*		
						SA	AST	SA×AST
Phylum level								
Firmicutes	48.45	61.65	44.34	63.37	2.94	0.005	0.912	0.549
Bacteroidota	43.03	35.66	43.92	33.76	2.55	0.101	0.913	0.831
Verrucomicrobiota	7.03^{ab}	0.51^{b}	9.85^{a}	0.03^{b}	1.33	0.001	0.590	0.449
Proteobacteria	0.22	0.28	0.26	1.24	0.15	0.047	0.059	0.079
Campilobacterota	0.13^{ab}	0.46^{ab}	0.88^{a}	0.06^{b}	0.11	0.208	0.369	0.007
*unclassified_k_norank_*d*_Bacteria*	0.24	0.34	0.24	0.34	0.02	0.007	0.959	0.996
Desulfobacterota	0.11	0.25	0.20	0.47	0.04	0.011	0.041	0.371
Actinobacteriota	0.13	0.11	0.21	0.03	0.03	0.087	0.914	0.192
Genus level								
Bacteroides	30.05	13.48	33.93	16.29	3.18	0.006	0.584	0.931
Alistipes	12.86	16.20	9.51	15.15	1.04	0.030	0.266	0.552
norank_f_Ruminococcaceae	4.54	3.05	3.35	8.86	0.70	0.077	0.044	0.004
Akkermansia	7.03^{ab}	0.51^{b}	9.85^{a}	0.03^{b}	1.33	0.001	0.590	0.449
unclassified_f_Lachnospiraceae	1.62^{b}	2.44^{b}	1.57^{b}	5.47^{a}	0.47	0.004	0.055	0.048
norank_f_norank_o_Clostridia_UCG-014	2.17	4.98	0.83	4.94	0.81	0.036	0.657	0.675
Lachnoclostridium	1.52^{ab}	0.65^{b}	1.31^{ab}	2.64^{a}	0.25	0.584	0.044	0.016
Blautia	5.76	0.45	4.74	0.15	0.91	0.006	0.682	0.820
unclassified_f_Ruminococcaceae	0.86	3.05	0.75	1.75	0.29	0.147	0.003	0.217
Butyricicoccus	1.89^{ab}	0.60^{b}	2.29^{a}	0.62^{ab}	0.25	0.002	0.612	0.643

Note: Labeled means in a row without a common letter differ, $P \leq 0.05$.

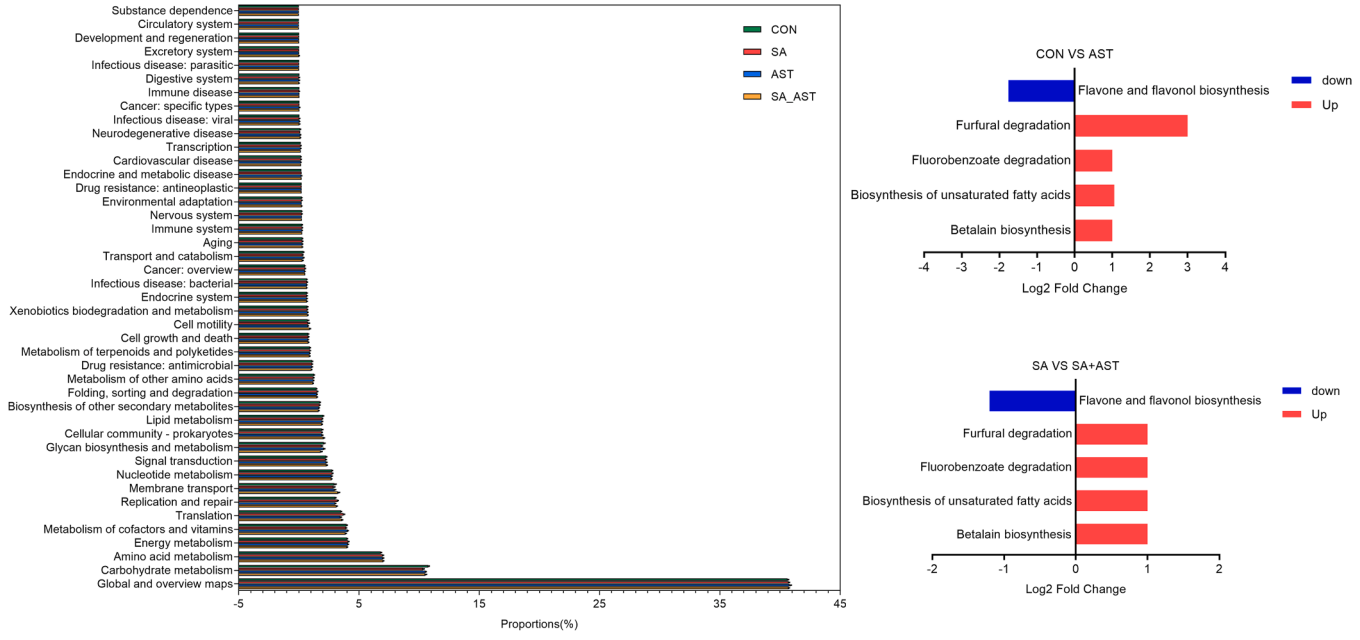

Fig. 4. Analyses of the Kyoto Encyclopedia of Genes and Genomes (KEGG) based on 16S rRNA sequencing data. (A) The functional abundances of KEGG pathways at level 2 across all samples. (B) Comparison of KEGG pathways at level 3 between the CON and AST groups and between the SA and SA+AST groups.

expression of *ZO-1* and *Occludin* ($P < 0.05$). In contrast, the abundance of *Alistipes* was significantly negatively correlated with the mRNA expression of *ZO-1* and *MUC2* ($P < 0.05$). Similarly, the abundance of *Coprobacter* showed a significant negative correlation with ADFI and a highly significant negative correlation with VCR, *ZO-1*, and *Occludin* mRNA expression ($P < 0.01$) but a significant positive correlation with crypt depth ($P < 0.05$).

PICRUSt was employed to predict the functional profiles of broiler cecal microbiota based on 16S rRNA sequencing data, as illustrated in Fig. 4. A comprehensive set of 43 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways level 2 was identified, with predominant pathways related to fundamental metabolic activities such as carbohydrate metabolism, amino acid metabolism, and energy metabolism, which are essential for microorganism survival. We conducted two pairwise comparisons (i.e., CON vs. AST, SA vs. SA+AST) to assess the upregulation and downregulation of the 293 functional pathways identified at KEGG level 3. Compared with the control group or the *Salmonella* infection group, the AST group or the SA+AST group significantly downregulated the flavone and flavonol biosynthesis pathways. It significantly upregulated the betalain biosynthesis, biosynthesis of unsaturated fatty acids, fluorobenzoate degradation, and furfural degradation pathways.

Discussion

During *S*. Enteritidis infection of chickens, the bacterium adheres to intestinal epithelial cells, induces intestinal inflammation, and compromises intestinal barrier integrity, often leading to diarrhea and impaired growth performance (Hu et al., 2023). This study evaluated the effects of adding 500 mg/L astaxanthin (AST) to drinking water on broilers challenged with *S.* Enteritidis. Our findings indicated that *S*. Enteritidis significantly reduced the average daily feed intake (ADFI) of broilers by day 20 of infection and led to the poorest outcomes in average daily gain (ADG), and feed conversion ratios (FCR). This observation aligns with previous research by Mountzouris et al., who reported that *S*. Enteritidis infection adversely impacts feed intake and body weight gain of Cobb broilers after a 21-day infection (Mountzouris et al., 2015). Similarly, Taiwo et al. reported that the *S*. Enteritidis lipopolysaccharides (LPS) significantly depressed AWG and FCR in Cobb-500 broilers between day 14 and day 21 of exposure (Erinle et al., 2022). Furthermore, our observations indicate that AST supplementation significantly improves the FCR of broilers and mitigates the growth performance decline induced by *S*. Enteritidis. However, research on the effects of AST supplementation on the growth performance of broilers *S.* Enteritidis is limited.

AST possesses antioxidative properties and can effectively mitigate oxidative stress generated under various pathological conditions by enhancing endogenous antioxidant enzyme activity, thereby contributing to maintaining host health (Kim et al., 2018). Zhao et al. indicated that fish fed the AST-supplemented diets showed higher superoxide dismutase (SOD) activity in the liver compared to those fed a diet without AST supplementation (Zhao et al., 2023). Similarly, Masoudi et al. demonstrated that AST supplementation led to significant increases in the concentrations of SOD, catalase (CAT) and glutathione peroxidase (GSH-PX) in spinal cord injury rat models (Masoudi et al., 2021). Furthermore, He et al. showed that dietary supplementation with AST increased total superoxide dismutase (T-SOD) activity in the serum and ovaries of aged laying hens (He et al., 2023). In our study, administering AST via drinking water demonstrated a tendency to elevate T-SOD concentrations, especially significantly enhanced T-AOC levels in broilers. This is important as total antioxidant capacity (T-AOC) levels reflect the total antioxidant defense of the organism. AST significantly increased T-AOC, demonstrating its potent ability to enhance the body's overall antioxidant defense mechanisms, which aligns with the well-known properties of AST (Wang et al., 2019).

The intestine is particularly vulnerable to oxidative damage, especially during stressful conditions such as infection, inflammation, and drastic environmental changes (Qiu et al., 2021). Intestinal morphology, intercellular and tight junction integrity and microbiota balance are important indicators of intestinal health (Hu et al., 2023; Bao et al., 2023). An increase in villus height, the ratio of villus height to crypt depth, and a decrease in crypt depth will lead to an expanded mucosal surface area in the small intestine, improving its digestive capacity (Bao et al., 2023). In this study, the challenge of *S*. Enteritidis brought about a significant reduction in the villus height and villus-to-crypt ratio (VCR), which was mainly in line with the results of previous studies (Hu et al., 2023; Zhang et al., 2022). Furthermore, in this experiment, the supplementation of AST functions to augment the height of intestinal villi and VCR and can strikingly reduce the crypt depth. Despite the challenge posed by *Salmonella*, AST still capable enhanced villus height and VCR, with a marked decrease in crypt depth. Consequently, we then examined the expression of tight junction proteins of the ileal. Our results demonstrated that the levels of *ZO-1* and *Occludin* were downregulated following *S*. Enteritidis infection. However, AST was able to upregulate the expression of *ZO-1* and *MUC2*. Intercellular tight junctions play a crucial role in establishing the intestinal physical barrier. They are made up of junctional proteins, including Claudin, Occludin, and Zonula Occludin, all of which are influenced by *Salmonella* infection, resulting in a reduction in the expression levels of associated genes (Hu et al., 2023; Suzuki, 2020; Lin et al., 2016). Meanwhile, Kuehu et al. found that AST supplementation might protect the integrity of broiler ileum epithelium to alleviate oxidative damage caused by heat stress, mainly through the upregulation of epithelial integrity genes *Lox, Cldn1*, and *MUC2*, and findings that are highly consistent with the present study (Kuehu et al., 2024). However, this study observed that *Salmonella* infection enhanced the expression of the *MUC2* gene, which might be ascribed to the relatively long sampling interval post-infection. Collectively, the addition of AST enhances the antioxidant capacity of the ileum, contributing to the improvement of ileal morphology and tight junction proteins, and subsequently optimizes the growth performance of broilers.

The cecum harbors a complex, diverse, and stable microbial community, and its dynamics amid competition play a crucial role in influencing the health and productivity of poultry (Erinle et al., 2022; Bao et al., 2023). In this study, *S*. Enteritidis infection resulted in alterations to α-diversity. At the same time, PCA analysis indicated obvious changes in the β-diversity of the cecal microbiota compared to the unchallenged broilers, which aligns with findings from previous studies (Hu et al., 2023; Erinle et al., 2022). Meanwhile, AST supplementation had no obvious effect on either α-diversity or β-diversity of the cecal microbiota in broilers. Additionally, the taxonomic profiles of the cecal microbiota revealed that *Firmicutes* and *Bacteroidota* were the two predominant phyla, followed by *Verrucomicrobiota* and *Proteobacteria*, which was consistent with previous research, while 27 major bacterial species, including *Bacteroides, Alistipes, unclassified_f_Ruminococcaceae*, and *Lactobacillus*, were identified and analyzed (Li et al., 2021; Toomer et al., 2024; Cai et al., 2024). Previous studies have demonstrated that *S*. Enteritidis infection tends to enhance the relative abundances of *Firmicutes* and *Proteobacteria* and increase the relative abundances of potentially pathogenic bacterial genera within *Proteobacteria* (Hu et al., 2023; Li et al., 2021; Toomer et al., 2024). In the present study, infection with *S*. Enteritidis significantly elevated the abundance of *Firmicutes, Proteobacteria, unclassified_k_norank_d_Bacteria*, and *Desulfobacterota* ($P < 0.05$), while concurrently reducing the levels of *Verrucomicrobiota* at the phylum level. Moreover, there was a significant increase in *Alistipes* and *unclassified_f_Lachnospiraceae, norank_f_norank_o_Clostridia_UCG-014* ($P < 0.05$), along with a marked decrease in *Bacteroides, Akkermansia, Blautia*, and *Butyricicoccus* when compared to the unchallenged groups ($P < 0.05$). The influence of *S*. Enteritidis infection on the cecal microbiota of broilers in this study was largely consistent with previous studies, and it had a greater variety of effects on the phylum level. One of the main reasons for such differences

was the different bacterial strains and infection procedures used for the challenge. In contrast to the groups without AST, AST supplementation significantly increased the abundance of the *Desulfobacterota* phylum and influenced the abundances of *norank_f_Ruminococcaceae, Lachnoclostridium*, and *unclassified_f_Lachnospiraceae*. Moreover, in the SA + AST group, the abundances of *Lachnoclostridium* and *unclassified_f_Lachnospiraceae* were significantly higher compared with the SA group, and the abundances of *Akkermansia* and *Butyricicoccus* in the AST group were higher compared to the SA group. Some studies suggest that an increase in the relative abundance of *Desulfobacterota* in mouse models may release LPS into the intestine, causing an inflammatory response and disrupting energy metabolism in the intestine, though its role in poultry requires further exploration (Zhou et al., 2023; Ghezzi et al., 2024). *Bacteroides* and *Butyricicoccus* are significant bacteria known for their production of short-chain fatty acids (SCFAs) and are associated with anti-inflammatory effects as well as growth-promoting properties (Song et al., 2023; Zhang et al., 2022). *Alistipes*, which was the most relatively abundant taxon regardless of treatment, has been reported to be enriched in chickens exposed to elevated temperatures and was associated with inflammation, obesity and depressive inflammation, obesity and depressive disorders, while was found to be significantly negatively correlated with the mRNA expression of *ZO-1* and *MUC2* in this study (Toomer et al., 2024). *Akkermansia, Lachnoclostridium*, and *Blautia* are all genera of bacteria that are beneficial to the intestinal health of the host (Yasuda et al., 2024; Cai et al., 2024; Liu et al., 2021). Among them, *Lachnoclostridium* can generate butyric acid, which can facilitate the development of the host's intestinal tract, eliminate inflammation and promote weight gain (Cai et al., 2024; Jacquier et al., 2019; Vacca et al., 2020; Stanley et al., 2016). All members of the *Lachnospiraceae* family are anaerobic, fermentative, and chemoorganotrophic and can degrade non-starch polysaccharides and produce acetic acid and butyrate, which can also promote the growth of broilers (Vacca et al., 2020). *Norank_f_Ruminococcaceae, norank_f_norank_o_Clostridia_UCG-014*, and *Unclassified_f_Ruminococcaceae* have been relatively less studied. Among them, *norank_f_Ruminococcaceae* is implicated in pathogenicity and inflammation, and *Unclassified_f_Ruminococcaceae* is acknowledged as one of the most efficient bacterial genera for carbohydrate degradation and plays a pivotal role in metabolic processes (Zhou et al., 2024; Wu et al., 2024). In summary, *S.* Enteritidis infection leads to a reduction in the abundance of beneficial bacteria and an increase in the abundance of harmful bacteria in the ceca of broilers. Meanwhile, adding AST can significantly decrease the quantity of the harmful bacteria *norank_f_Ruminococcaceae* and alleviate the decrease of beneficial bacteria such as *Lachnoclostridium* resulting from *Salmonella* challenge.

Spearman's correlation analysis found that the abundances of both *Akkermansia* and *Blautia* exhibited a significant positive correlation with ADFI. Together with *Bacteroides*, they also demonstrated a significant positive correlation with the mRNA expression levels of *ZO-1* and *Occludin*. Chelakkot et al. demonstrated that *Akkermansia* can enhance tight junction expression by activating AMP-activated protein kinase (AMPK). At the same time, previous studies have reported a positive correlation between *Blautia* and *Bacteroides* with tight junction proteins such as ZO-1 and Occluding, all of which are in accordance with the results of this study (Chelakkot et al., 2018; Panasevich et al., 2015; Feng et al., 2024). The abundance of *Lachnoclostridium* showed a very significant negative correlation with crypt depth. At the same time, *Butyricicoccus* had a significant positive correlation with ADG, ABW, and VCR, as well as a very significant positive correlation with ADFI and the mRNA expression of *ZO-1* and *Occludin*. Previous research also indicates that *Lachnospiraceae* and *Butyricicoccus*, both of which are butyrate-producing bacteria, exhibit anti-inflammatory properties and are associated with improved performance (Jacquier et al., 2019; Vacca et al., 2020; Stanley et al., 2016; Keerqin et al., 2021). The abundance of *Alistipes* was significantly negatively correlated with the mRNA expression of *ZO-1* and *MUC2*, which was in line with the results of a previous study (Toomer et al., 2024). It can be stated that *Salmonella* infection can inflict damage to the intestinal tract of the host and limit growth by elevating harmful bacteria like *Alistipes* and suppressing beneficial ones such as *Bacteroides, Akkermansia, Blautia* and *Butyricicoccus*. Adding AST can mitigate the detrimental effects by increasing beneficial bacteria like *Lachnospiraceae*. The associated mechanisms await further investigation.

In this experiment, it was found that adding AST to the drinking water significantly downregulated the flavone and flavonol biosynthesis pathway in the cecal microorganisms of *Salmonella*-infected broilers and significantly upregulated the betalain biosynthesis, biosynthesis of unsaturated fatty acids, fluorobenzoate degradation, and furfural degradation pathways. Previous studies have shown that flavone and flavonol biosynthesis is inversely proportional to body weight, body mass index (BMI), and T-CHO levels in humans. However, there are few studies in the poultry field (Tsai et al., 2024). Betalain is a water-soluble nitrogenous pigment belonging to the class of biological alkaloids, renowned for its antioxidant properties and various other biological activities (Ninfali et al., 2017). Additionally, research has suggested that the biosynthesis pathways of unsaturated fatty acids are closely related to the lipid metabolism of the host and play a vital role in promoting both intestinal morphological development and growth in broilers (Chen et al., 2020; Li et al., 2022). In the category of xenobiotics biodegradation, some pathways are related to the severity of intestinal inflammation. Previous research has indicated that decreased fluorobenzoate degradation might alleviate *Clostridioides difficile* infection in the host's intestine (Liu et al., 2019). Within the category of xenobiotic biodegradation, specific metabolic pathways are associated with intestinal inflammation and microbial composition, as exemplified by the degradation processes of fluorobenzoate or furfural into less toxic or non-harmful substances, which may contribute to the enhancement of host health (Liu et al., 2019; Sun et al., 2022). Given that PICRUSt is merely a predictive tool, it is necessary to conduct metagenomic research in the future.

Conclusion

In conclusion, this study demonstrates that *S.* Enteritidis infection significantly suppresses the average daily feed intake (ADFI) of 20-day-old broilers, resulting in poor performance indicators such as average daily gain (ADG), average body weight (ABW), and feed conversion ratio (FCR). However, the supplementation of 500 mg/L astaxanthin (AST) in drinking water effectively mitigated the adverse effects of *S.* Enteritidis infection, particularly improving the FCR of broilers. AST supplementation also enhanced antioxidant activity by increasing total superoxide dismutase (T-SOD) levels and total antioxidant capacity (T-AOC) in the ileal mucosa. Additionally, it improved ileal morphology by significantly increasing villus height and crypt depth, with a trend toward elevated villus-to-crypt ratio (VCR) values. Notably, AST alleviated the infection-induced deepening of ileal crypts. The expression levels of tight junction proteins, such as *ZO-1* and *Occludin*, which were downregulated following *S.* Enteritidis infection, were restored with AST supplementation. Furthermore, AST promoted the expression of *ZO-1* and *MUC2* in the ileum, strengthening the intestinal barrier. *S.* Enteritidis infection was associated with a decrease in beneficial bacterial populations and an increase in harmful bacteria in the ceca, contributing to impaired intestinal health and performance. AST supplementation helped regulate the microbial community by promoting probiotic populations, such as *Lachnoclostridium*, thereby supporting better intestinal development and production performance. Overall, adding AST to drinking water effectively alleviates the detrimental impacts of *S.* Enteritidis infection on growth performance, intestinal development, and cecal microbiota composition in broilers

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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