Preventive Antibiotic Treatment Increases Susceptibility of Neonatal chicks to *Salmonella* Infection via Disrupting Gut Microbiota and Linoleic Acid Metabolism

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Abstract

Background: Antibiotics are widely employed in animal husbandry to prevent and treat diseases. Increasing evidence suggests they may alter the animals' natural microbiota and increase their susceptibility to pathogen. However, the mechanisms linking the gut microbiota and pathogen colonization in poultry have not yet been fully elucidated. Herein, we used metagenomic and metabonomic approaches to investigate the effects of florfenicol (FFC) pre-treatment on *Salmonella enterica* serovar Enteritidis (*S. Enteritidis*) colonization in the intestines of neonatal chicks in terms of host response, microbiota composition and metabolism.

Results: We determined that FFC pre-treatment significantly alters the cecal microbiota and metabolome, and also increases the intestinal permeability and promotes a pro-inflammatory gene expression profile in the host. Host physiological changes were concordant with significantly increased susceptibility to *S. Enteritidis* infection in chicks with FFC pre-treatment relative to without pre-treatment chicks. Prior to *Salmonella* infection, FFC pre-treatment significantly reduced the abundance of *Lactobacillus*, and significantly affected linoleic acid metabolism, including significantly reducing the levels of conjugated linoleic acid (CLA), and significantly elevating the abundance of 12,13-EpOME and 12,13-diHOME in cecum. After infection with *S. Enteritidis*, the abundance of *Proteobacteria* were significantly increased and host inflammatory responses and intestinal permeability were significantly aggravated relative to without FFC pre-treatment chicks, suggestive of a profound exacerbating of the host response influenced by infection in the context of FFC pre-treatment. The linoleic acid metabolism was still significantly different pathway after *Salmonella* infection, and we screened CLA and 12,13-diHOME as the target metabolites using a multi-omics technique. Supplementation with CLA maintained intestinal integrity, reduced intestinal inflammation, and accelerated *Salmonella* clearance from the gut and remission of enteropathy. Whereas, treatment with 12,13-diHOME promoted intestinal inflammation and disrupted the intestinal barrier function to sustain *Salmonella* infection. Therefore, florfenicol reduces production of CLA by inhibiting *Lactobacillus* growth, increases 12,13-diHOME level of intestine, thereby reducing colonization resistance of neonatal chicks to *Salmonella* infection.

Conclusion: This study reveals the potential health impact of antibiotics on gut microbiota and linoleic acid metabolism and contributing factors influencing *Salmonella* colonization in neonatal chicks, and provides mechanistic understanding into the role of the antibiotics promote the colonization of pathogens.

Background

The global population will reach 8.5 billion by 2030, leading to a high demand for livestock products [1]. Chickens are the most abundant livestock in the world, with more than 60 billion produced annually, and predicted to increase further in the next 20 years [2, 3]. Effective control and management of diseases are essential to ensure adequate health of chickens and the safety of poultry products. Outbreaks attributed to *Salmonella enterica* are a major medical and economic problem worldwide, causing significant

morbidity and mortality in poultry [4, 5]. *Salmonella* infections in chickens are of particular importance, as consumption of contaminated products is a significant source of infection for humans and is the leading cause of foodborne disease outbreaks worldwide [6–9].

Microbiota-mediated mechanisms of colonization resistance are critical to prevent pathogen invasion in certain niches within animal hosts [10]. In particular, the intestine is a pertinent site to characterize the mechanisms of colonization resistance because the intestinal lumen contains trillions of commensal microbes that interact closely with their hosts as well as other microbes via intricate networks [11, 12]. The intestinal microbiota of chicken is highly diverse with more than 1000 species of bacteria and a population density of up to 10^{11} cells/g intestinal contents [13, 14]. The gut microbiota confers protection against colonization by zoonotic pathogens; however, this can be weakened by administration of antibiotics that disturb the gut bacterial community and metabolism [15]. Large-scale intensive rearing systems, usually containing flocks of 20,000 birds or more, depend on antibiotics to prevent and treat animal diseases [16]. As per a study in China, veterinary antibiotics account for 84.3% (pig: 52.2%, chicken: 19.6%, and other animals: 12.5%) of the total usage of 36 target antibiotics, whereas human antibiotics only comprise 15.6% [17]. It is estimated that the global application of antibiotics for chickens, pigs, and cattle will increase by 67%, from 63,151 tons in 2010 to 105,596 tons in 2030 [18]. Consequently, antibiotic-mediated disruptions of the microbiota composition lead to disorders in the ecological balance between microbes and host, dramatically reducing colonization resistance, and enhancing the susceptibility to infection by multiple pathogens [19–21]. In murine models, antibiotic pretreatment by oral administration allows efficient colonization of the cecum and colon by *S. enterica* serovar Typhimurium, and enhances *Salmonella* expansion and fecal shedding [22, 23].

Furthermore, neonatal chicks exhibit high susceptibility to infections with *Salmonella* serovars because of their immature gut microbiota; the complexity of which gradually increases from day 1 to day 19 of life [24, 25]. Nonetheless, chickens are coprophagic and the transfer of cecal microbiota from adult chickens to neonatal chicks increases resistance to *Salmonella* infection [26, 27]. Unlike other farm animals, neonatal chicks are hatched in the clean environment of a hatchery, without any contact with adult chickens and their colonization resistance is only dependent on the environment [28]. If a pathogen is present in the environment, the immature gut microbiota of a newly hatched chick essentially enables its unrestricted multiplication. Therefore, large-scale intensive rearing systems typically depend on antibiotics to prevent and control disease outbreaks, which disrupt the gut microbiota of chicks and facilitate higher susceptibility to *Salmonella* infections.

We hypothesize that pre-treatment of newly hatched chicks with antibiotics is accompanied by changes in their gut microbiota and metabolic profile, which result in increased susceptibility to *Salmonella* infections. Therefore, a greater understanding of how antibiotics affect the mechanism of colonization resistance is required. The objectives of the present study were to investigate the influence of a 7-day treatment course of florfenicol, a commonly used broad-spectrum antibiotic in poultry in many countries [17, 18, 29], on the composition of the microbial community and metabolic profile of neonatal chicks, and determine the potential factors that facilitate the growth of *S. Enteritidis* in the cecum.

Methods

Bacterial strains

Chicks were challenged with the *S. Enteritidis* (ATCC 13076) *floR* mutant strain. The *S. Enteritidis floR* mutant was constructed using a plasmid-based homologous recombination integration method as previously described [30]. Briefly, the *floR* gene (NG_047860.1) was synthesized and cloned into an *E. coli* cloning vector (pCVD442). The upstream and downstream homologous recombinant arms were amplified by PCR from the *S. Enteritidis* genome using ultra-fidelity DNA polymerase, and *floR* sequence was amplified by PCR from the template vector. The fragments were assembled by fusion PCR to construct the gene targeting fragment. The pCVD442 suicide plasmid was digested with restriction endonuclease (SmaI) to construct the gene targeting plasmid pCVD442-*floR*, which was transformed into *E. coli* SY327 λ pir. The plasmid pCVD442-*floR* was conjugated into *S. Enteritidis* using *E. coli* SY327 λ pir as a donor strain and plated on FFC-resistant chromogenic XLT4 agar for selection positive clones that integrated the suicide plasmid into their genome. Finally, FFC-resistant colonies were verified by PCR and gene sequencing. Prior to inoculation, *S. Enteritidis* was grown overnight in Luria-Bertani broth at 37 °C with shaking at 200 rpm.

Florfenicol intervention and *S. Enteritidis* infection

Leghorn layer chicks (1-day-old) were hatched from the same batch eggs of SPF birds (Beijing Boehringer Ingelheim Vital Biotechnology Co., Ltd., China), and each assigned group was reared in an individual GJ-1 SPF isolator (Suzhou Fengshi Laboratory Animal Equipment Co., Ltd., China). Animals received non-medicated chick feed and water *ad libitum*; they were raised under controlled environmental conditions with a 16-h lighting cycle and a temperature of 32°C at day 1 which was gradually reduced and maintained at 24°C from day 10.

Animal protocol 1: effect of florfenicol pre-treatment on intestinal *Salmonella* colonization. Eighty-eight newly hatched chicks were assigned at random to four groups. Each group included 22 chicks for three time points (n = 7 to 8 chicks in each time point) and were treated with 30 mg/kg b. w. of FFC for 7 days or infected with approximately 10⁸ CFU of the challenge strain *S. Enteritidis* by oral gavage. The groups were: (1) NT, control group neither FFC-treated nor *S. Enteritidis*-infected; (2) FT, FFC-treated group; (3) ST, *S. Enteritidis*-infected group; (4) and FST, FFC-pre-treated and *S. Enteritidis*-infected group. On days 11, 18, and 25, chicks were euthanized for analysis.

Animal protocol 2: effect of conjugated linoleic acid and 12,13-diHOME on intestinal *Salmonella* colonization. Forty newly hatched chicks were assigned at random to four groups (n = 10 chicks per group) and were treated with the CLA or 12,13-diHOME by gastric gavage. The CLA (purity: >99%, Nu-Chek Prep, Elysian, MN, USA) is a mixture of 65.5% c9, t11-CLA, and 34.5% t10 and c12-CLA isomers by LC-MS detection (data not shown). The 12,13-diHOME (purity: ≥98%, Cayman Chemical, Ann Arbor, Michigan, USA) solution was prepared as described in a previous report [31]. The doses of CLA and 12,13-diHOME corresponded to the amount of consumed diet supplemented with 1% CLA and 12,13-diHOME (1% [10

mg/g diet] $\times$ 3 - 9 [gram of diet coconsumed on average by chicks at day 1-7]). Chicks were divided into four groups: (1) NT, control group; (2) ST, *S. Enteritidis*-infected group; (3) CLA, CLA-pre-treated and *S. Enteritidis*-infected group; and 4) 12,13-diHOME, 12,13-diHOME-pre-treated and *S. Enteritidis*-infected group. On days 11, the chicks were euthanized for analysis.

After chicks were euthanized, the cecal contents and internal organs were aseptically collected and homogenized in PBS. For enumerating *Salmonella* loads, an aliquot (100 μ l) of appropriate dilutions was spread onto XLT4 agar plates (50 ug/ml florfenicol); *Salmonella* appeared as typical black colonies after incubation at 37 °C for 24 h.

Histopathology and microscopic analysis of the intestine

Parts of ileal tissue were perfusion-fixed with formalin for 24 h. After gradient dehydration with ethanol, specimens were embedded in paraffin. Subsequently, 5 μ m sections were rehydrated and stained with Alcian blue. Representative images were obtained with a BA400 digital microscope (Motic Group CO., LTD., China). Mean staining densities were calculated from the integral optical densities and areas of positive Alcian blue staining using the Image-Pro[®] Plus v6.0 analysis system (Media Cybernetics, USA). Statistical significance was determined by one-way ANOVA. A *P*-value of less than 0.05 was considered statistically significant. To determine the degree of lesion, the pathological score was monitored as previously described [32]. SEM (InspectTM, FEI Ltd., USA) of the intestinal villi was performed as previously described [33].

DNA extraction, 16S rRNA gene sequencing, and data analysis

Seven or eight chicks per treatment were randomly chosen at three time points: 11, 18, and 25 days of age, and euthanized by carotid artery bleeding. The cecal contents were collected within 5 min of euthanasia, immediately placed in pre-cooling cryogenic vials, and stored at -80 °C until DNA extraction. Total genomic DNA was extracted from cecal contents using the QIAamp DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer's protocols, and stored at -20 °C until analysis. The concentration and quality of extracted DNA samples were measured by Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, United States) and agarose gel electrophoresis, respectively.

Using the isolated genomic DNA as template, the V3-V4 hypervariable regions of the bacterial 16S rRNA genes were PCR-amplified with primers 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') following previously described method [34]. Amplicons were then sequenced on the Illumina MiSeq platform (Illumina Inc., USA) using 2 $\times$ 250 bp cycles. These sequence data were deposited in the NCBI database (Bioproject PRJNA655362) under the accession number SRP277009. QIIME was employed to process the sequencing data. Briefly, raw sequencing reads with exact matches to the barcodes were assigned to respective samples and identified as valid sequences. The low-quality sequences were filtered through the following criteria [35, 36]: sequences that had a length of <150 bp, sequences with an average Phred scores of <20, sequences that contained ambiguous bases, and sequences that contained mononucleotide repeats of >8 bp. Paired-end reads were assembled

using FLASH [37]. After chimera detection, the remaining high-quality sequences were clustered into operational taxonomic units (OTU) at 97% sequence identity by UCLUST [38]. A representative sequence was selected from each OTU using default parameters. OTU taxonomic classification was conducted by BLAST searches of the representative sequences set against the Greengenes Database [39] and the best hit was used for further analysis [40]. An OTU table was generated to record the abundance of each OTU in each sample and the taxonomy of these OTUs. OTUs containing less than 0.001% of total sequences across all samples were discarded. To minimize differences in sequencing depth across samples, an averaged, rounded rarefied OTU table was generated by averaging 100 evenly resampled OTU subsets under 90% of the minimum sequencing depth for further analysis.

Sequence data were analyzed using the QIIME and R software packages (v3.2.0) [41]. OTU-level alpha diversity indices, including Shannon indices, species abundance and Pielou indices were calculated using the OTU table in QIIME. Beta diversity analysis was performed to investigate the structural variation of microbial communities across samples using Bray-Curtis distances metrics and visualized via principal coordinate analysis (PCoA). Taxonomic compositions and relatives abundances were visualized using MEGAN [42] and GraPhlAn [43]. LEfSe was performed to detect differentially abundant taxa across groups using default parameters [44].

Quantitative PCR for microbiota analysis

Bacterial composition of the microbiota was measured by qPCR as previously described [45-48]. All qPCR reactions were performed using the Bio-Rad real-time PCR detection system (Bio-Rad CFX Maestro 1.1, 3.0, USA) and SsoFast EvaGreen Supermix (Bio-Rad Inc., USA) according to the manufacturers' instructions. Genomic DNA from cecal samples was used as a template for qPCR using the main group-specific primers (Supplementary Table S1): *all eubacteria*, *Lactobacillus*, *Bacteroidetes*, *Enterobacteriaceae*, *Clostridium butyricum* and *Faecalibacterium prausnitzii*. Serial dilutions of plasmids containing the target gene cloned into the pMD-19 T cloning vector (TaKaRa, Dalian, China) were analyzed to generate standard curves and calculate absolute counts of target genes.

Metabolomics for chicken cecal content

Untargeted metabolomics. Chickens were sacrificed, and the cecum was resected. The cecal contents were obtained and stored at -80 °C until analysis. Sample preparation for LC/MS was performed as previously described [49]. Briefly, 50 mg freeze-dried sample, 800 µl methanol, and 5 µl DL-o-chlorophenylalanine (internal standard) were added to a 1.5 mL Eppendorf tube. All samples were ground to fine powder using a grinding mill at 65 HZ for 90 s, vortexed for 30 s, and centrifuged at 12,000 rpm at 4°C for 15 min. Then, 200µL of supernatant was transferred to a new vial for LC-MS. A total of 10 µl of the sample solution at 4 °C was injected into the LC-MS system (Thermo, Ultimate 3000LC, Exactive Orbitrap) with an Agilent C18 column (Hypergod C18, 100 x 2.1 mm 1.9 µm) with the column temperature maintained at 40 °C. The mobile phase consisted of solutions A and B: A was 0.1% formic acid/5% acetonitrile/water (v/v/v) and B was 0.1% formic acid/acetonitrile (v/v). The flow rate was 350 µl/min. The gradient was set as: 0% B at 0 min, 20% B at 1.5 min, 100% B at 9.5 min to 14.5min, and 0% B at 14.6

min to 18min. Samples were analyzed in positive and negative ion modes using 300 °C heater temperature, 350 °C capillary temperature, and 3.0 KV spray voltage. The flow rates of sheath gas, auxiliary gas, and sweep gas were 45, 15, and 1 arb, respectively. Peaks were aligned according to m/z values and normalized migration time. Peak areas were calculated by normalizing against the internal standards. Metabolites were identified by searches against the database based on m/z values and normalized migration time. Compound Discoverer Software (Thermo) was used to process the Thermo RAW files. The data after editing were subjected to multivariate analysis using SIMCA-P 14.0 software (Umetrics AB, Umea, Sweden). Metabolites selected as biomarker candidates were identified on the basis of a VIP threshold of 1 from the sevenfold cross-validated OPLS-DA model, which was validated at a univariate level with adjusted $P < 0.05$. MetaboAnalyst (version 3.0) was used for the identification of metabolic pathways [50].

Targeted metabolomics. A 50 mg sample of dried cecal content and 800 µl methanol were added to a 1.5 mL Eppendorf tube. The sample was ground to a fine powder using a grinding mill at 65 HZ for 90 s followed by being vortexed for 30 sec, and centrifuged at 12,000 rpm at 4 °C for 15 min. Next, 200 µl of supernatant was used for detection.

For quantitative detection of linoleic acid and CLA, 1 µl of each sample was injected into a DB-5 column (60 m x 0.25 mm 0.25 µm) using a Thermo Trace 1300 GC (Thermo Fisher Scientific, USA) system online with mass spectrometer (ISQ7000, Thermo Fisher Scientific, USA) (GC-MS). The temperature program was as: initial oven temperature of 140 °C held for 5 min, increased at with 10 °C /min to 180 °C, at 4 °C/min to 210 °C, and finally reached 260 °C at the rate of 10 °C /min, then held for 20 min. Helium (99.999% purity) was used as carrier gas with a flow rate of 1.5 ml/min. The MS inlet line and the ion source temperatures were maintained at 260 and 230 °C, respectively, and the MS ionization energy was 70 eV. A full scan mode set from 5 min to 20 min, monitoring m/z range from 33 to 550 Da, was used for the identification of possible interferences from the matrix extract.

For quantitative detection of 12,13-EpOME and 12,13-diHOME, 4 µl of each sample was injected into an Acquity UPLC BEH C18 column (100 mm x 2.1 mm x 1.7 µm) using an Acquity UPLC (Waters Corporation, USA) coupled with triple quadrupole mass spectrometry (API5500, AB SCIEX LLC., USA) (UPLC-QqQ-MS). The mobile phase consisted of solution A (water) and B (acetonitrile) and the flow rate was 300 µl/min. The gradient was set as: 10% B at 0 min, 10% B at 1.0 min, 90% B at 1.5 min, 90% B at 5.0 min, 10% B at 6.0 min, 10% B at 7.0 min. Samples were analyzed in negative ion modes using 550 °C atomizing temperature, -4.5 KV spray voltage, and MRM reaction monitoring of scanning method. The flow rates of curtain gas, collision gas, GS1 (atomizing gas) and GS2 (auxiliary gas) were 35, 9, 55, and 55 arb, respectively.

RNA isolation and RT-qPCR

Total RNA from the ileum and liver tissue was extracted using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The quality and concentration of RNA were measured using a Nanodrop 2000 spectrophotometer. One microgram of total RNA from

each sample was reverse transcribed into cDNA using a SuperScript II kit (Invitrogen Life Technologies, Carlsbad, USA) using oligo (dT) primer and random hexamer primers. The qPCR reaction was performed with the SsoFast EvaGreen Supermix using a Bio-Rad CFX real-time PCR detection system following the manufacturer's protocols. Primers listed in Supplementary Table S1 [51, 52]. Relative mRNA expression levels of each target gene were calculated using the log 2 of the fold change method. Triplicate parallel reactions were run for all samples.

ELISA detection

Concentrations of chicken IL-1 β , IL-6, IL-8, IL-10, INF- γ , TNF- α in the ileum tissue, serum IgG, LPS, DAO, D-lactate and intestinal mucosal SIgA were determined using the mlbio ELISA kit (Shanghai Enzyme-linked Biotechnology Co., Ltd., China) according to the manufacturer's instructions. Concentrations were calculated from the standard curves.

Data and statistical analysis

The heatmap of the interrelationship between the differential flora and the metabolites was generated using the R (3.6.1) pheatmap package. The calculated correlation coefficient ($R < 0.5$) was used to exclude metabolites and flora with weak correlation and no correlation. Cytoscape (3.7.1) software was used to draw the correlation network diagram; the flora and metabolites were used to form points and line segments represent the correlation size. The distributions of bacterial communities and their potential correlations with differential metabolites were determined using CCA, using the R (3.6.1) vegan package. Statistical analyses were conducted with SPSS 20.0 (SPSS Inc., USA). Data collected are presented as geometric medians or means $\pm$ standard deviation. Statistical significance was determined by Mann-Whitney tests or one-way ANOVA. The Mann-Whitney test was used for comparing two groups. One-way ANOVA with a Dunnett's multiple comparison test was used for pair-wise comparison of means from more than two groups in relation to the control group. The p values of less than 0.05 were considered statistically significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Results

Florfenicol exposure increases susceptibility to *S. Enteritidis* infection

We established a study design (Fig. 1a) in which SPF chicks were divided into four groups: orally administered florfenicol (FT); *S. Enteritidis*-infected by oral gavage (ST); simultaneously of FFC-treated and *S. Enteritidis* infected (FST); and an untreated control group (NT). Under the SPF environment, all chicks cultured negative for *Salmonella* spp. until experimental infection with *S. Enteritidis*. The control group remained culture negative for *Salmonella* spp. throughout the study. The colonization and translocation of *S. Enteritidis* in the intestinal tract of chicks directly determines its survival and pathogenicity. Therefore, we quantified *S. Enteritidis* levels in the caecum, spleen, and liver. The number of *S. Enteritidis* (log₁₀ CFU/g tissue) significantly increased by 25.49% (cecal contents, $P < 0.01$), 23.04% (spleen, $P < 0.01$) and 21.33% (liver, $P < 0.01$), respectively, in the FST group relative to that of in the ST

group at 3 days post-infection (dpi). Similar results were observed at days 18 (10 dpi) and 25 (17 dpi), although the *Salmonella* loads were less than those observed at 3 dpi (Fig. 1b).

Florfenicol administration aggravates *S. Enteritidis*-induced intestinal morphology and barrier injury

As FFC intervention made the chicks more susceptible to *Salmonella* infection, it is possible that antibiotics disrupted the immature intestinal barrier homeostasis of chicks, altered intestinal permeability, and facilitated greater translocation of *Salmonella* to their internal organs. Therefore, we investigated the effects of FFC administration on *S. Enteritidis*-induced changes in intestinal morphology. Hematoxylin and eosin (H & E) staining showed that the chicks in the NT group exhibited an intact ileal mucosa, neat intestinal villi, deep crypts, and a clear and complete gland structure, similar to that observed for the FT group (Fig. S1a and b). The ST group displayed an incomplete structure of the ileal mucosa, villi had a shorter length and sparse distribution, and crypts were shallow (Fig. S1c). However, the FST group morphology included loss of mucosal structures, atrophic crypts, and lamina propria bowel edema (Fig. S1d). Histological injury (Fig. S1e) was scored based on H & E-stained images; the scores indicated tissue damage. The score for the ST group (7.13 ± 0.44) was significantly higher than control (0.63 ± 0.18). Relative to the ST group, FFC pre-administration significantly increased the ileum injury score (10.75 ± 0.45). Scanning electron microscopy (SEM) showed that the NT group had complete ileal villi, forming full and closely arranged structures (Fig. 2e), whereas the FT group also had intact ileal villi, but their arrangement was relatively loose (Fig. 2f). As expected, the ileal villi of the ST group were damaged (Fig. 2g) and those of the FST group showed more severe damage (Fig. 2h). These results suggested that although FFC has less effect on intestinal morphology, intestinal injury was aggravated by *Salmonella* invasion.

An effect of FFC on intestinal barrier function in the ileum after *S. Enteritidis* infection was also observed exacerbating. The FFC pre-treatment can exacerbate the *S. Enteritidis*-induced increase in permeability of the ileum (Fig. 3a-d). The serum diamine oxidase (DAO) and lipopolysaccharide (LPS) levels of the FT group were significantly higher ($P < 0.001$ and 0.05 respectively) than those of the NT group (Fig. 3c and d). In the case of *Salmonella* infection, serum D-lactate, DAO, and LPS levels in both the ST and FST groups were significantly increased ($P < 0.001$) relative to those of the NT group. However, FFC treatment significantly increased ($P < 0.001$) serum D-lactate, DAO, and LPS levels in chicks exposed to *Salmonella* (Fig. 3b-d). Alcian blue staining indicated that FFC significantly decreased ($P < 0.05$) the acidic mucin of ileum relative to the ileum of chicks in the NT group, as evident by the quantitative evaluation of positive Alcian blue staining (Fig. 2a and b) using integral optical density measurement (Fig. 3a). Similarly, FFC treatment also significantly decreased ($P < 0.01$) the mean density of acidic mucin (Fig. 2d and Fig. 3A) after exposure to *Salmonella*. Transcriptional analysis of a range of relevant intestinal barrier genes (Fig. 5) showed that FFC treatment significantly altered the transcription of (Claudin 1, IL-17A, and IFN- α) in the FT group. However, in the presence of *Salmonella*, FFC significantly reduced the expression of ZO-1, Occludin, Claudin 1, MUC2, and TFF2, and significantly increased the expression of IL-17A, IL-22, and IFN- α . Furthermore, treatment with FFC significantly decreased ($P < 0.01$) secretory immune globulin A (SIgA) secretion, but had no effect on serum IgG (Fig. 3e and f). Nevertheless, FFC treatment reduced SIgA

secretion to a higher degree ($P < 0.001$) after *Salmonella* infection (Fig. 3f). Thus, FFC intervention, to some extent, increased the permeability of the intestinal mucosal of chicks, reduced mucosal immunity, and substantially increased the extent of damage to the intestinal mucosal barrier after *Salmonella* infection.

The level of *Salmonella* colonization is strongly associated with the triggering of intestinal inflammation [53]. We therefore hypothesized that increased colonization may be linked to increased mucosal inflammation. Indeed, FFC-treated chicks exhibited higher levels of gut inflammation after *Salmonella* infection (Fig. 4). This was verified by quantification of pro- and anti-inflammatory cytokines in ileal tissues. *Salmonella* infection after FFC-pretreatment significantly increased levels of cytokines IL-1 β (Fig. 4a), IL-6 (Fig. 4b), IL-8 (Fig. 4c), TNF- α (Fig. 4e), and IFN- γ (Fig. 4f), whereas the level of IL-10 (Fig. 4d) was significantly decreased. Moreover, levels of inflammatory cytokines (IL-1 β , IL-6, TNF- α and IFN- γ) were significantly increased in the FT group. These results indicated that FFC-pretreatment exacerbated the *Salmonella*-induced inflammatory response.

Florfenicol administration alters the gut microbiota

The composition and density of the gut microbiota play an important role in combating *Salmonella* invasion. Oral pretreatment with antibiotics decreases colonization resistance and leads to an post-treatment expansion of *Salmonella* loading in the gut [53]. Thus, we hypothesized that the higher *Salmonella* population observed in the chicks of the FFC-treated group may be linked to the disruption of microbiota composition and density. To this end, the microbiota compositions of the cecal content of chicks at 3, 10, and 17 dpi were determined by 16S rRNA gene sequencing. Fig. S2 illustrates microbiota diversity, represented as boxplots of measures of α -diversity, Shannon index, Observed Species and Pielou index. The α -diversity at 3 dpi was neither affected by FFC treatment nor by *S. Enteritidis* infection (Fig. S2a). However, a significant decrease in α -diversity was observed in the FST group at 10 dpi (Fig. S2b). Fig. S2c shows that the Shannon and Pielou index of the cecal microbial communities were significantly increased in the FST group at 17 dpi. These results indicated that the α -diversity of gut microbiota from chicks is not significant affected by a single FFC treatment or *Salmonella* challenge. However, the combination of both treatments significantly disturbed cecal α -diversity.

Phylum and genus distributions of microbial compositions are shown in Fig. S3. *Firmicutes* (67.85 – 99.62%) dominated the microbiota in all four groups at three different stages of infection (Fig. S3a). At 3, 10, and 17 dpi, the FST chicks had the highest relative abundance of *Proteobacteria* (2.68%, 1.30%, and 1.75%, respectively) relative to the other three groups (Fig. S3a). At 17 dpi, the FFC (9.23%) chicks had significantly reduced relative abundance of *Bacteroidetes* relative to the NT group (31.26%). *Salmonella* infection (23.87%) had negligible effect on *Bacteroidetes* abundance. However, infection with *Salmonella* after pretreatment with FFC almost eliminated the growth of *Bacteroidetes* (0.01%) (Fig. S3). We applied the LEfSe (linear discriminant analysis effect size) method to identify abundant bacterial taxa among these groups; only those taxa that obtained a log LDA (linear discriminant analysis) score > 3 were ultimately considered. A cladogram from phylum to genus level abundance is shown in Fig. 6. In total, 21,

21, and 28 differentially abundant taxa were identified at 3, 10, and 17 dpi, respectively (Fig. 6). In the untreated control chicks, LEfSe highlighted the greater differential abundance of *Lactobacillus* at 3 and 10 dpi, and *Bacteroides* at 17 dpi. Notably, the relative abundance of *Enterobacteriaceae* was significantly higher in the FST group than in the other three groups at all three time points. However, the other taxa were altered irregularly at different times in different groups. The relative abundance of these biomarkers is shown in Fig. S4, wherein consistent results were obtained. We also established taxonomic cladograms at 11 day (3 dpi), with the relative abundance of the taxa node of each group shown as a pie chart; only those taxa with relative abundance > 0.1% were considered (Fig. 7a). Similarly, the abundance ratio of *Lactobacillus* was considerably higher in the control group than in the other three groups. Additionally, the abundance ratio of *Enterobacteriaceae* in the FST group dominated among all four groups. Furthermore, *Salmonella* was only found in the challenged groups (ST and FST) at the genus level, with the abundance ratio of *Salmonella* in the FFC pretreatment group significantly higher than those of the untreated groups (Fig. 7a). We measured the cecal loads of these biomarkers and two intestinal protective bacteria by quantitative real-time PCR (qPCR) (Fig. 7b). At day 11 (3 dpi), FFC pre-treatment significantly reduced the densities of total bacteria, *Lactobacillus*, *Clostridium butyricum* and *Faecalibacterium prausnitzii*. Although *Salmonella* infection had no effect on cecal bacterial densities, chicks with *Salmonella* infection after pretreatment with FFC harbored much higher densities of *Enterobacteriaceae*, and lower densities of *Lactobacillus*, *Bacteroides*, *C. butyricum* and *F. prausnitzii* relative to the control group. At day 25 (17 dpi), *C. butyricum* and *F. prausnitzii* were present at equivalent densities in the cecal content of all four groups. However, significant differences in the bacterial densities of total bacteria, *Lactobacillus*, *Bacteroides*, and *Enterobacteriaceae* were still apparent between the NT and FST groups or between the ST and FST groups (Fig. 7b). *Lactobacillus* and *Bacteroides* are generally considered as beneficial bacteria that provide protection for the gut, whereas *Enterobacteriaceae* are potential pathogens in poultry and humans. These observations suggested that FFC exposure significantly decreased the abundance of *Lactobacillus* in chicks, and this inhibitory effect may provide a growth advantage for *Enterobacteriaceae*, especially *Salmonella*.

The similarity of microbial communities (β -diversity) between groups was visualized using principal-coordinate analysis (PCoA) of Bray-Curtis distances. PCoA plots for 3 dpi showed that microbial communities from *Salmonella* or FFC treated chicks are clearly different from those of the untreated chicks. The first axis of the PCoA plot shows 19.0% of variation in bacterial diversity while the second axis shows 13.0% (Fig. 7c). The first axis roughly distinguishes the antibiotic pre-treated chicks and non-pretreated chicks, and the second axis roughly distinguishes the infected and non-infected chicks. The PCoA at 10 dpi shows that the microbiota composition was very similar between the NT and FT groups, whereas the ST and FST groups are still obviously distinguish from the NT group (Fig. S5a). Intriguingly, at day 25 (17 dpi), the PCoA plot demonstrated that both the microbiota composition of ST and FT groups tends toward the NT group, whereas composition of the FST group is still strikingly divergent from the NT group (Fig. S5b). These findings suggested that a single FFC or *Salmonella* treatment alters the microbiota composition, with recovery two weeks after infection. Whereas FFC pretreatment hindered the recovery of microbiota composition of chicks after *Salmonella* infection.

Florfenicol administration alters the metabolic profiling

We hypothesized that differences in key metabolites may be crucial to the effect of *Salmonella* colonization on chicks. Therefore, we analyzed metabolomes by LC-MS to determine differential levels of metabolites on day 11 (3 dpi) in cecal contents. The PCA score plot shows that the metabolome of NT group and ST group was significantly separated among the four groups, whereas there was no clear distinction in cecal metabolites between the FT and FST groups (Fig. 8a). Orthogonal projections to latent structures discriminant analysis (OPLS-DA) and permutation test plot of OPLS-DA were performed. As shown in Fig. 8, cecal metabolites of the NT group were clearly distinguished from those of the FT group (Fig. 8b), ST group (Fig. 8d), and FST group (Fig. 8f). In addition, there was a clear separation between the FST group and ST group in cecal metabolites (Fig. 8h).

From the OPLS-DA models, we identified 72 differential metabolites between the NT and FT groups, 42 differential metabolites between the NT and ST groups, 69 differential metabolites between the NT and FST groups, and 57 differential metabolites between the FST and ST groups, using to the threshold $VIP > 1$ and $p < 0.05$ (Welch's t test). The differential metabolites are listed in Supplementary Table S2. Next, we performed pathway enrichment analysis based on these differential metabolites to better understand the effect of FFC on metabolism (Fig. 9). Linoleic acid metabolism, aminoacyl-tRNA biosynthesis, lysine biosynthesis, phenylalanine metabolism and lysine degradation were enriched after FFC treatment (Fig. 9a). Arginine and proline metabolism, lysine biosynthesis, lysine degradation and D-glutamine and D-glutamate metabolism were enriched by *Salmonella* infection (Fig. 9b). Linoleic acid metabolism, aminoacyl-tRNA biosynthesis, lysine biosynthesis, butanoate metabolism and phenylalanine metabolism were enriched in FFC pre-treated, *Salmonella* infected chicks (Fig. 9c). Linoleic acid metabolism was enriched between the FST and ST groups (Fig. 9d). These data indicated that linoleic acid metabolism is the most noteworthy metabolic pathway in the FFC-treated groups with or without *Salmonella* challenge. We mapped the metabolic pathway of linoleic acid based on the identified differential metabolites, as well as the relative amounts (means $\pm$ SD) of these metabolites in the four groups (Fig. 9e). The metabolites that affect the metabolic pathways of linoleic acid are primarily linoleic acid, 12,13-EpOME, and 12,13-diHOME; the relative amounts of these metabolites in the FT and FST groups were significantly higher than in the NT and ST groups. Notably, the relative levels of 12,13-EpOME and 12,13-diHOME were significantly higher in the FFC-pretreated group, but were negligible in the non-pretreated group (Fig. 9e).

Correlation between the differential gut microbiota and metabolites

After observing marked differences in metabolite content as well as the microbial composition after FFC pre-treatment, we tested for specific correlations between the microbial taxa and key metabolites. Spearman correlation analysis revealed an association between four bacterial genera and nine discriminant metabolites in FFC-pretreated chicks (Fig. 10a). *Enterobacteriaceae* was a taxon with strong correlation, particularly with linoleic acid, 12,13-EpOME, 12,13-diHOME and L-tyrosine (positive correlations), whereas only L-ascorbic acid was negatively correlated. Furthermore, *Clostridium* positively correlated with L-palmitoyl carnitine, linoleic acid, 12,13-diHOME and L-tyrosine, while the taxon

negatively correlated with L-ascorbic acid, anandamide, and 4-pyridoxic acid. The genus *Lactobacillus* negatively correlated with L-palmitoyl carnitine, linoleic acid, 12,13-EpOME, 12,13-diHOME, and L-tyrosine, and positively correlated with L-ascorbic acid. Lastly, a weaker positive correlation was detected between *Ruminococcus* and metabolites 4-pyridoxic acid and gamma-aminobutyric acid. Canonical correspondence analysis (CCA) showed that *Enterobacteriaceae* was the most important bacterial taxon influencing linoleic acid metabolism (including linoleic acid, 12,13-EpOME, and 12,13-diHOME) after FFC pre-treatment (Fig. 10b). The correlation network between differential bacterial taxa and metabolites consisted of 13 nodes and 22 edges. We found that the metabolic pathway of linoleic acid has a strong positive correlation with *Enterobacteriaceae*, whereas *Lactobacillus* has a negative correlation with it (Fig. 10c).

As linoleic acid can be converted into conjugated linoleic acid (CLA) by *Lactobacillus* [54], and a significantly negative correlation between linoleic acid and *Lactobacillus* was observed in our study, we hypothesized that the non-FFC pretreated chicks (more abundance of *Lactobacillus*) may have higher CLA levels. However, CLA is an isomer of linoleic acid, so the use of untargeted metabolomics cannot distinguish between these compounds. Therefore, we employed targeted LC-MS to detect compounds including linoleic acid, 9c,11t-CLA, 10t,11c-CLA, 12,13-EpOME, and 12,13-diHOME (Fig. 10d). In line with the results of metabolic profiling, linoleic acid, 12,13-EpOME, and 12,13-diHOME levels were higher in the FFC-pretreated groups. Moreover, we observed a higher CLA concentrations in the cecal contents of non-FFC pretreated chicks, with the levels of 9c,11t-CLA significantly higher than that of 10t,11c-CLA (Fig. 10d). Spearman correlation analysis showed a strong association between the abundance of *Lactobacillus* and CLA concentrations (Fig. S6). Collectively, these findings suggested that 12,13-EpOME and 12,13-diHOME may be the key metabolites for the propagation of gut colonization of *Salmonella*, whereas CLA may limit the *Salmonella* growth during infection.

Contrasting effects of conjugated linoleic acid and 12,13-diHOME on *S. Enteritidis* colonization

We pre-administered CLA and 12,13-diHOME to newly-hatched chicks before infecting them with *S. Enteritidis* (Fig. 11a). By 3 dpi, *Salmonella* loads in the caecum, spleen, and liver were significantly reduced in the chicks pretreated with CLA, whereas they were significantly increased by pre-treatment with 12,13-diHOME (Fig. 11b). Consistent with the fecal *Salmonella* loads, pre-treatment with CLA significantly reduced enteropathy at 3 dpi, whereas 12,13-diHOME significantly increased it (Fig. S8). Furthermore, CLA-pretreated chicks exhibited a decrease in intestinal permeability (serum D-lactate, DAO and LPS levels), and pro-inflammatory factors (IL-1 β , IL-6, IL-8, TNF- α and IFN- γ), as well as a significant increase in IL-10 levels. Notably, 12,13-diHOME-pretreated chicks exhibited contrasting results (Fig. 12). We also compared the effect of these two metabolites on the expression of genes related to intestinal barrier function after *Salmonella* infection (Fig. 13). We found that CLA significantly increased the expression of ZO-1 and Occludin, whereas the 12,13-diHOME significantly reduced the expression ZO-1, Occludin, Claudin1, and MUC2. CLA also significantly increased the expression of IL-17A (Fig. 13). To evaluate whether orally administered CLA and 12,13-diHOME reach the gut lumen, we quantified their concentrations in the cecal contents, and observed a significant increase in CLA and 12,13-diHOME

relative to non-treated chicks (data not shown). Together, these results demonstrated that pretreatment with CLA attenuates *Salmonella* colonization, whereas 12,13-diHOME promotes it (Fig. 14).

Discussion

Administration of antibiotics perturbs the gut bacterial community, resulting in weakened resistance to gut colonization by pathogens [22, 45, 53, 55]. However, the mechanisms that promote *Salmonella* outgrowth after antibiotic pretreatment in chicks remain unclear. In this study, we investigated the effect of antibiotic (FFC) pre-administration on the intestinal *Salmonella* colonization of chicks and its mechanism through microbiome analysis and metabolomics. Consistent with reported studies [45, 55], our results indicated that FFC significantly increased *Salmonella* load in the gut and prolonged gut colonization. The abundance of *Salmonella* also significantly increased in livers and spleens exposed to FFC pretreatment. *Salmonella* employs two type III secretion systems, encoded by *Salmonella* pathogenicity island 1 (SPI-1) and *Salmonella* pathogenicity island 2 (SPI-2) to enter the intestine and adhere to the surfaces of intestinal epithelial cells. It subsequently enters the subepithelial tissue via a series of invasive pathological pathways [56]. In the current study, we found that FFC pre-treatment exacerbated *Salmonella*-induced defects in morphology, decreased intestinal barrier function, and increased intestinal barrier permeability. Enzyme-linked immunosorbent assays (ELISA) revealed that FFC directly decreased SIgA concentration, and mucous layer density, and increased the concentration of serum DAO and LPS. FFC significantly decreased the expression of mRNA encoding claudin 1, and increased the expression of mRNAs IL-17A and IFN- α . SIgA reflects the state of intestinal immunity, and stabilizes intestinal colonization by symbiotic microorganisms and confers resistance to future invasion by exogenous pathogens [57–59]. Studies have shown that the gut microbiota is the most important source of microbial stimulation of the immune response. The use of antibiotics disrupts the delicate ecosystem of the neonatal microbiome, which may impair stimulation of SIgA and a low IgA response, in which in turn leads to decreased mucosal barrier function [60–62]. Furthermore, FFC aggravates *Salmonella*-induced inflammation in the ileum; the secretion of IL-1 β , IL-6, IL-8, INF- γ , TNF- α is higher and IL-10 is lower. Previous studies have reported that intestinal inflammation provides a growth advantage for *Salmonella* [53, 63–65]. Taken together, these findings imply that FFC pretreatment impaired intestinal immunity, increased intestinal permeability and inflammation, and aggravated *Salmonella*-induced intestinal barrier damage. These changes collectively promoted *Salmonella* colonization in neonatal chicks.

As the gut microbiota plays an important role in combating *Salmonella* invasion and maintaining intestinal immunity [53, 66], we characterized the intestinal flora of neonatal chicks in the treated groups. The present study showed that *Firmicutes* dominated the gut microbiota of neonatal chicks at day 11 and 18, and the mature microbial communities of chickens (at day 25) were dominated by *Firmicutes* and *Bacteroidetes*. This finding was consistent with previous studies [67, 68]; however, FFC administration significantly decreased the abundance of *Lactobacillus* at day 11 and 18 (4 and 11 days post-treatment), and that of *Bacteroides* at day 25 (18 days post-treatment). *Lactobacillus* spp. are considered probiotic in nature and have been used in livestock feed processing for decades because of their beneficial effects on immunity, growth, and intestinal colonization resistance [69–71]. For example, the *L. rhamnosus* reduces

the colonization of pathogenic *Salmonella*, *Clostridium*, and *E. coli* strains in porcine intestinal mucus [72]. *L. acidophilus* binds to cultured human intestinal cell lines and inhibits cell invasion by enterovirulent bacteria including *Salmonella* Typhimurium [73]. Another study showed that *L. plantarum* exerts an antagonistic effect on pathogenic bacteria by increasing the content of SIgA [74]. In our study, FFC treatment significantly decreased the abundance of *Lactobacillus* in chicks, suggesting that this genus may be the main target bacteria of FFC. Similar observations have been found on intestinal microbiota upon FFC therapy in chickens [75]. This reduction may be responsible for the promotion of *Salmonella* colonization after FFC pre-treatment. *Bacteroidetes* is the dominant phylum in the mature microbiota of chickens [76], and may have some inhibitory effects on the gut colonization of *Salmonella*. Miki et al. reported that *Bacteroides* spp. accelerates the elimination of *S. Typhimurium* from the intestinal lumen of mice by producing vitamin B6 [45]. Another study demonstrated that *Bacteroides* species confer colonization resistance to *S. Typhimurium* infection by producing propionate, which directly limits *Salmonella* growth by disrupting intracellular pH homeostasis [77]. Our results showed that at day 25, FFC pretreatment significantly reduced the abundance of *Bacteroidetes* and the cecum contained higher *Salmonella* loads relative to non-pretreated controls, suggesting that FFC may have delayed the maturation of chicken intestinal flora and hindered the clearance of *Salmonella*. Furthermore, *Salmonella* infection after FFC pre-treatment chicks had the highest relative abundance of *Proteobacteria*, which is known to be potential pathogens of poultry and humans. A recent study showed that preventive treatment of calves with florfenicol resulted in a 10-fold increase in facultative anaerobic *Escherichia* spp, which is a signature of imbalanced microbiota [78]. Sáenz et al. reported that oral administration of florfenicol to fish resulted in a shift in the gut microbiome towards well-known putative pathogens such as *Salmonella*, *Plesiomonas*, and *Citrobacter* [79]. Combined with our results, we conclude that FFC administration changes the overall structure of gut microbiota and promotes the growth of *Proteobacteria*, especially *Salmonella*. Although the microbial community of chicken is complex and relatively stable, the restoration of microbiota after antibiotic withdrawal can be expected [80, 81]. Our results indicated that antibiotic administration at an early age in chickens may have a profound effect on microbial composition that hindered its restoration (Fig. S4). And the study also demonstrated that the maturation of intestinal microbiota is significantly retarded and eventually delayed by antibiotic intervention at early ages of chicks [82].

Next, we used metabolomics to determine how FFC affects *Salmonella* gut colonization. Our data suggested that linoleic acid metabolism is the most notable pathway affected by FFC pre-treatment. Linoleic acid, 12,13-EpOME and 12,13-diHOME are the most important compounds affected in the linoleic acid metabolic pathway, and concentrations of these metabolites are significantly higher after FFC pre-treatment. Notably, the concentration of 12,13-EpOME and 12,13-diHOME were significantly high in the FFC-pretreated group, but negligible in the non-pretreated group. Linoleic acid is firstly metabolized to 12,13-EpOME by cytochrome P450 (CYP) epoxigenase, followed by hydrolysis catalyzed by soluble epoxide hydrolases (sEHs) to form the diols 12,13-diHOME [83]. DiHOME compounds have multiple pathological features, such as decreasing post-ischemic cardiac recovery, participating in vascular cognitive impairment, increasing skeletal muscle fatty acid uptake, and impeding immune tolerance in

asthmatic children [31, 84–86]. The 12,13-diHOME produced by sEH hydrolysis of 12,13-EpOME showed stronger cytotoxicity [83, 87]. Our analysis of metabolic enzymes in this pathway revealed that FFC significantly increases the expression of CYP1A2, whereas it had no significant effect on sEH (Fig. S7). Besides liver, a variety of gut bacteria also produce sEH [31]. Correlation analysis results showed that the concentration of 12,13-diHOME positively correlated with *Enterobacteriaceae* and *Clostridium*, so we suspected that sEH may be produced by these bacteria in the gut. A recent study showed that the sEH and sEH-derived lipid metabolites induce intestinal barrier dysfunction, bacterial translocation, and colonic inflammation in mice [88]. Therefore, we propose that 12,13-diHOME promotes the intestinal colonization of *Salmonella*. Subsequently, we pretreated neonatal chicks with 12,13-diHOME and observed a significantly higher *Salmonella* colonization. Our results also showed that 12,13-diHOME pretreatment significantly increases *Salmonella*-induced expression of intestinal proinflammatory cytokines, exacerbates morphology, intestinal barrier injury, and increases the intestinal barrier permeability. Intestinal inflammation, particularly that due to proinflammatory cytokines, disrupts barrier function and lead to intestinal permeability, and promotes colonization by pathogens [63, 89, 90]. Previous studies showed that diHOMEs compounds exhibit pro-inflammatory effects on vascular endothelial cells [91], lung [31] and peripheral nervous tissue [92]. Our study indicated that 12,13-diHOME also exhibits a pro-inflammatory effect on intestinal epithelial cells. Moreover, the diHOMEs compounds also disrupt mitochondrial function, by altering mitochondrial permeability and inducing cellular apoptosis [93, 94], and this may be why 12,13-diHOME exacerbates intestinal barrier damage. Therefore, we suggest that 12,13-diHOME contributes to *Salmonella* colonization of chick intestine, by promoting intestinal inflammation and disrupting the intestinal barrier function.

CLA is the second factor affecting *Salmonella* gut colonization in chicks pre-administered with FFC. Our correlation analysis combined with targeted metabolomics revealed that *Lactobacillus* and CLA showed a significant positive correlation, and FFC pretreatment reduced the abundance of both *Lactobacillus* and CLA in the gut lumen. CLA is formed from linoleic acid by *Lactobacillus* and can inhibit the growth of pathogenic bacteria [95]. Therefore, we assume that CLA may be additional factor affecting *Salmonella* colonization after FFC pretreatment. We pretreated neonatal chicks with CLA and observed that it effectively reduced *Salmonella* colonization, accompanied by an increased expression of tight junction proteins (ZO-1 and occludin). CLA also alleviated *Salmonella*-induced intestinal inflammation and intestinal barrier injury. We suggest that CLA reduces intestinal colonization by *Salmonella* by influencing several processes. Firstly, CLA treatment considerably upregulated the concentration of tight junction proteins (ZO-1, occludin, E-cadherin 1 and claudin-3) and ameliorated epithelial apoptosis [96–98], which protects intestinal cells from the impairment caused by *Salmonella* infection. Secondly, CLA modulates gut inflammation by attenuating the expression of proinflammatory cytokines (TNF- α , INF- γ , IL-1 β , and IL-6 and IL-17) and upregulating the level of anti-inflammatory cytokine IL-10 [96–101]. Thus, CLA exhibits anti-inflammatory effects that reduce gut colonization by *Salmonella*. Finally, studies also show that CLA directly inhibits the growth of pathogenic bacteria including *Salmonella*. Byeon et al. showed that CLA possesses antimicrobial activity against the growth of a variety of food-borne pathogens; 1.8 mM CLA completely inhibits the growth of *S. Typhimurium* [95]. Peng et al. indicated that CLA produced by

Lactobacillus competitively excludes *Salmonella* in a mixed-culture condition [102]. Additionally, Tabashsum et al. also showed that CLA produced by *Lactobacillus* inhibits the growth and survival of *Salmonella* by altering the relative expression of genes related to *Salmonella* virulence [103]. Thus, our results suggest that CLA maintains intestinal integrity, reduces intestinal inflammation, and inhibits *Salmonella* growth to effectively reduce gut colonization by *Salmonella* in chicks. Therefore, FFC may reduce production of CLA by inhibiting *Lactobacillus* growth, thereby reducing colonization resistance of neonatal chicks to *Salmonella* infection.

Conclusions

In conclusion, our study indicates that FFC pre-treatment significantly increases gut susceptibility to *S. Enteritidis*, in addition to enhancing *Salmonella*-induced inflammatory responses and intestinal barrier damage in neonatal chicks. Our findings suggest that FFC reduces production of CLA by inhibiting *Lactobacillus* growth, increases 12,13-diHOME level of intestine, thereby reducing colonization resistance of neonatal chicks to *Salmonella* infection. We provide a better understanding of the susceptibility of animal species to *Salmonella* after antibiotics intervention may help to elucidate infection mechanisms that are important in both animal and human health. And the observations also facilitate the more careful and rational use of antibiotics in poultry.

Abbreviations

FFC: Florfenicol; *S. Enteritidis*: *Salmonella enterica* serovar Enteritidis; XLT4: Xylose lysine tergitol 4; SPF: Specific-pathogen-free; SEM: Scanning electron microscope; OTUs: Operational taxonomic units; LEfSe: Linear discriminant analysis effect size; LDA: Linear discriminant analysis; VIP: Variable importance in the projection; DAO: Diamine oxidase; SIgA: Secretory immune globulin A; ELISA: Enzyme-linked immunosorbent assay; CLA: Conjugated linoleic acid; CCA: Canonical correspondence analysis; PCA: Principal-coordinate analysis; OPLS-DA: Orthogonal projections to latent structures-discriminate analysis.

Declarations

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Authors' contributions

HW and XM conceived the study and designed the experiments. XM, BM, XZ, LZ, YG and CZ collected the samples and performed experiments. HW, XM, BM and XZ analyzed the data. AZ, CL, YT and XY provide suggestions and help checking. HW, XM and BM wrote the manuscript. AZ, CL, YT, XY, YG and CZ help revise the manuscript. The final manuscript was read and approved by all authors.

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Availability of data and materials

The raw sequence data obtained in this study has been deposited in the Sequence Read Archive (SRA) database of the National Center for Biotechnology Information (NCBI) with an access number of SRP277009.

Ethics approval and consent to participate

The experimental protocols used in this experiment, including animal care and use, were reviewed and approved by the Animal Care and Use Ethics Committee of Sichuan University (Chengdu, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Effect of florfenicol pretreatment on *S. Enteritidis* infection in neonatal chicks. (a) Experimental design. Newly hatched chicks ($n = 7-8$ at each sampling point) were randomly divided into four groups (NT, FT, ST and FST). The chicks were either administered a 7-day treatment (30 mg/kg b. w.) of florfenicol or infected with $\sim 10^8$ CFU of the challenge strain *S. Enteritidis* by oral gavage. Sampling points for cecal microbiota analysis during infection are indicated. Animals were euthanized, and the *S. Enteritidis* loads in the cecal contents and organs were determined by the plate counting method. (b) At 3, 10, or 17 days post infection (dpi), chicks were sacrificed and *S. Enteritidis* loads in cecal content, spleen, and liver were determined. Bar indicates median. ns, not significant ($p \geq 0.05$); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; Mann-Whitney U test.

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Figure 2

Florfenicol exacerbates the development of *Salmonella*-induced ileal mucosal injury in neonatal chicks. Chicks were randomly divided into four groups: NT (Control), FT (FFC pretreated), ST (*S. Enteritidis* infected) and FST (FFC pretreated and *S. Enteritidis* infection). All the chicks were infected orally with ~10⁸ CFU of *S. Enteritidis* except the NT and FT groups. After 3 days of infection, the histopathology of the intestinal mucosa was analyzed by Alcian blue staining (a–d) and SEM (e–h). Scale bars, 50 μm (a–d); Scale bars, 300 μm (e–h).

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Florfenicol exacerbates the development of Salmonella-induced ileal mucosal injury in neonatal chicks. Chicks were randomly divided into four groups: NT (Control), FT (FFC pretreated), ST (*S. Enteritidis* infected) and FST (FFC pretreated and *S. Enteritidis* infection). All the chicks were infected orally with ~108 CFU of *S. Enteritidis* except the NT and FT groups. After 3 days of infection, the histopathology of the intestinal mucosa was analyzed by Alcian blue staining (a–d) and SEM (e–h). Scale bars, 50 μm (a–d); Scale bars, 300 μm (e–h).

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Figure 3

Effect of FFC on intestinal permeability and immune activity. Mean density of ileal tissue mucin (n = 8; a), serum D-lactate (n = 8; b), serum DAO (n = 8; c), serum LPS (n = 8; d), serum IgG (n = 8; e) and intestinal SIgA (n = 8; f) were compared at 3 dpi. Data are expressed as means ± standard deviation. Statistical significance was assessed by ANOVA and denoted as: *P < 0.05, **P < 0.01, ***P < 0.001, FT, ST and FST vs NT; #P < 0.05, ##P < 0.01, ###P < 0.001, FST vs ST; ns, not significant. NT: Control group. FT: FFC pretreated group. ST: S. Enteritidis infected group. FST: FFC pretreatment followed by S. Enteritidis infection group.

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Figure 4

Effect of FFC on the expression of cytokines in ileum of normal and *S. Enteritidis*-infected chicks. IL-1 β (n = 8; a), IL-6 (n = 8; b), IL-8 (n = 8; c), IL-10 (n = 8; d), TNF- α (n = 8; e) and IFN- γ (n = 8; f) levels were compared at 3 dpi. Data are expressed as means $\pm$ standard deviation. Statistical significance was assessed by ANOVA and denoted as: *P < 0.05, **P < 0.01, ***P < 0.001, FT, ST and FST vs NT; #P < 0.05, ##P < 0.01, ###P < 0.001, FST vs ST; ns, not significant. NT: Control group. FT: FFC pretreated group. ST: *S. Enteritidis* infected group. FST: FFC pretreatment followed by *S. Enteritidis* infection group.

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Figure 5

Gene expression profiles in response to FFC pretreatment or *S. Enteritidis* infection by qPCR at 3 dpi. Data are represented as log2 of the fold change between the treatment and control (NT) groups. Statistical analysis was conducted using one-way ANOVA and Dunnett's multiple comparison test and denoted as: *P < 0.05, **P < 0.01, ***P < 0.001; ns, not significant. NT: Control group. FT: FFC pretreated group. ST: *S. Enteritidis* infected group. FST: FFC pretreatment followed by *S. Enteritidis* infection group.

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Figure 7

Impact of FFC pretreatment on the cecal microbial communities in response to *S. Enteritidis* infection at 3 dpi. (a) Taxonomic cladogram showing the relative abundance of taxa nodes in each group (relative abundance > 0.1%). Larger sector area indicates higher abundance of taxa in the corresponding group. (b) Quantification of cecal microbiota at 3, 10, and 17 dpi by qPCR of 16S or 23S rRNA gene. $n = 7-8$. All bars represent means $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; ns, not significant; ANOVA and Dunnett's multiple comparison test. (c) PCoA for comparison of the changes in bacterial communities at 3 dpi generated using the Bray-Curtis distance method. NT: Control group. FT: FFC pretreated group. ST: *S. Enteritidis* infected group. FST: FFC pretreatment followed by *S. Enteritidis* infection group.

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Figure 8

PCA and OPLS-DA plot of cecal metabolomes. (a) PCA plot of cecal metabolomes. OPLS-DA scores plot of cecal metabolites based on LC-MS from the different groups at 3 dpi. OPLS-DA plots of four data sets: FT vs NT group (b), ST vs NT group (d), FST vs NT group (f), FST vs ST group (h). Validation plot of PLS-DA in these four group sets showed in c, e, g and i. NT: Control group. FT: FFC pretreated group. ST: *S. Enteritidis* infected group. FST: FFC pretreatment followed by *S. Enteritidis* infection group.

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Figure 9

Comparison of differential metabolites and key metabolic pathways at 3 dpi. Metabolic pathway analysis of biomarker metabolites. Plots showing over-represented metabolic pathways between the FT and NT group (a), ST and NT group (b), FST and NT group (c), FST and ST group (d). The x-axis represents pathway impact and the y-axis represents pathway enrichment. Larger sizes and darker colors represent higher pathway enrichment levels and higher pathway impact values, respectively. (e) Linoleic acid metabolomic pathway map. Bars represent relative amounts (mean $\pm$ SD) of metabolites. N.D., not detected. *P < 0.05, **P < 0.01, ***P < 0.001; ANOVA and Dunnett's multiple comparison test. NT: Control group. FT: FFC pretreated group. ST: S. Enteritidis infected group. FST: FFC pretreatment followed by S. Enteritidis infection group.

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Figure 10

Correlation between key bacterial taxa and differential metabolites, and their absolute abundance. (a) Spearman correlation between differential metabolites and bacterial taxon calculated for FT and NT groups at 3 dpi (*P < 0.05, **P < 0.01, ***P < 0.001). Positive correlation is labeled in red and negative correlation is labeled in blue. (b) CCA plot of differential metabolites and bacterial taxa for FT and NT groups at 3 dpi. CCA ordination plot shows correlations between bacterial community structures and metabolite factors. Correlations between metabolite factors and key bacterial taxon are represented by the lengths and angles of arrows. (c) Correlation network analysis of the key bacterial taxa and differential metabolites for FT and NT groups at 3 dpi. The hexagon indicates the metabolite and the circle indicates the bacterial taxon. Lines connecting each node represent Spearman correlation coefficient values. Red lines represent positive correlations, blue lines represent negative correlations, and the thickness of the edge represents strength of correlations. (d) Concentrations of key metabolites in cecal content at 3 dpi. n = 8. Bars indicate means $\pm$ SD. *P < 0.05, **P < 0.01, ***P < 0.001; ANOVA and Dunnett's multiple comparison test. NT: Control group. FT: FFC pretreated group. ST: S. Enteritidis infected group. FST: FFC pretreatment followed by S. Enteritidis infection group.

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Figure 11

Effects of CLA and 12,13-diHOME pretreatment on *S. Enteritidis* infection in neonatal chicks. (a) Experimental design. Newly hatched chicks (n = 10) were randomly divided into four groups (NT, ST, CLA and 12,13-diHOME), administered a 7-day treatment of CLA or 12,13-diHOME and infected with ~108 CFU of the challenge strain *S. Enteritidis* by oral gavage. Animals were euthanized and the *S. Enteritidis* loads in the cecal contents and organs were determined by plate counting. (b) At 3 dpi, chicks were sacrificed

and *S. Enteritidis* loads in the cecal content, spleen, and liver were determined. Bar indicates median. ns, not significant ($P \geq 0.05$); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; Mann-Whitney U test.

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Figure 12

Effect of CLA and 12,13-diHOME pretreatment on intestinal permeability and expression of cytokines in ileum of *S. Enteritidis*-infected chicks. Serum D-lactate (n = 10; a), serum DAO (n = 10; b), serum LPS (n = 10; c), IL-1 β (n = 10; d), serum IL-6 (n = 10; e), IL-8 (n = 10; f), IL-10 (n = 10; g), TNF- α (n = 10; h) and INF- γ (n = 10; i) were compared at 3 dpi. Data are expressed as means $\pm$ standard deviation. Statistical significance was assessed by ANOVA and denoted as: *P < 0.05, **P < 0.01, ***P < 0.001. NT: Control group. ST: *S. Enteritidis* infected group. CLA: CLA pretreated and following *S. Enteritidis* infection group. 12,13-diHOME: 12,13-diHOME pretreated and following *S. Enteritidis* infection group.

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Figure 13

Gene expression profiles in response to CLA and 12,13-diHOME pretreatment following *S. Enteritidis* infection at 3dpi. Represented as log2 of the fold change between the treatment group and the control group (NT). Statistical analysis was conducted using one-way ANOVA and Dunnett’s multiple comparison test and denoted as: *P < 0.05, **P < 0.01, ***P < 0.001; ns, not significant. NT: Control group. ST: *S. Enteritidis* infected group. CLA: CLA pretreated and following *S. Enteritidis* infection group. 12,13-diHOME: 12,13-diHOME pretreatment followed by *S. Enteritidis* infection group.

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Figure 14

Florfenicol increases susceptibility of neonatal chicks to Salmonella infection via disrupting gut microbiota and linoleic acid metabolism. (a) Florfenicol pre-treatment in neonatal chicks alters gut microbiota and linoleic acid metabolism, increasing susceptibility to Salmonella Enteritidis infection. (b) Pre-treatment with CLA maintained intestinal integrity, reduced Salmonella-induced intestinal inflammation to control Salmonella growth and colonization. Whereas, treatment with 12,13-diHOME promoted intestinal inflammation and disrupted the intestinal barrier function to sustain Salmonella infection.

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