Research Article

Protective Effects of Cinnamaldehyde on the Oxidative Stress, Inflammatory Response, and Apoptosis in the Hepatocytes of Salmonella Gallinarum-Challenged Young Chicks

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The development of novel therapeutics to treat multidrug-resistant pathogenic infections like Salmonella gallinarum is the need of the hour. Salmonella infection causes typhoid fever, jaundice, and Salmonella hepatitis resulting in severe liver injury. Natural compounds have been proved beneficial for the treatment of these bacterial infections. The beneficial roles of cinnamaldehyde due to its antibacterial, anti-inflammatory, and antioxidative properties have been determined by many researchers. However, alleviation of liver damage caused by S. gallinarum infection to young chicks by cinnamaldehyde remains largely unknown. Therefore, this study was performed to identify the effects of cinnamaldehyde on ameliorating liver damage in young chicks. Young chicks were intraperitoneally infected with S. gallinarum and treated with cinnamaldehyde orally. Liver and serum parameters were investigated by qRT-PCR, ELISA kits, biochemistry kits, flow cytometry, JC-1 dye experiment, and transcriptome analysis. We found that ROS, cytochrome c, mitochondrial membrane potential (Ψm), caspase-3 activity, ATP production, hepatic CFU, ALT, and AST, which were initially increased by Salmonella infection, significantly (P<0.05) decreased by cinnamaldehyde treatment at 1, 3, and 5 days postinfection (DPI). In addition, S. gallinarum infection significantly increased proinflammatory gene expression (IL-1β, IL-6, IL-12, NF-κB, TNF-α, and MyD-88) and decreased the expression of anti-inflammatory genes (IL-8, IL-10, and iNOS); however, cinnamaldehyde reverted these effects at 1, 3, and 5 DPI. Transcriptome analysis showed that S. gallinarum modulates certain genes of the AMPK-mTOR pathway for its survival and replication, and these pathway modulations were reversed by cinnamaldehyde treatment. We concluded that cinnamaldehyde ameliorates inflammation and apoptosis by suppressing NF-κB/caspase-3 pathway and reverts the metabolic changes caused by S. gallinarum infection via modulating the AMPK-mTOR pathway. Furthermore, cinnamaldehyde has antibacterial, anti-inflammatory, antioxidative, and antiapoptotic properties against S. gallinarum-challenged young chicks and can be a candidate novel drug to treat salmonellosis in poultry production.

1. Introduction

Family Enterobacteriaceae contains a genus named Salmonella, consisting of non-spore-forming facultative anaerobic bacilli [1]. This genus has two species, i.e., Salmonella enterica (S. enterica) and S. bongori. Five subspecies are placed under the category of S. enterica [2]. Humans and animals are mostly affected by S. enterica subspecies Enterica, which further contains typhoidal and nontyphoidal serovars [3]. More than 2600 serovars are present in S. enterica subspecies Enterica [4]. Annually, 93.8 million cases of nontyphoidal gastroenteritis have been reported worldwide, with 155,000 deaths and an average incidence of 1.14 episodes/100 person-years, making this disease a major public health concern [5]. 10–20 million cases of typhoid and about 100,000–200,000 deaths per annum have been reported recently [6]. Salmonellosis is not only a public health concern but also a major threat to the poultry industry as two nonmotile
serovars of Salmonella, i.e., S. enterica serovar pullorum (causing pullorum disease) and S. enterica serovar gallinarum (causing fowl typhoid), are specific to birds and pose a serious impact on the business of poultry producers [7]. Salmonella gallinarum and Salmonella pullorum cause the systemic infection of poultry resulting in sepsis, independent of the age of birds [8].

Salmonella, a primary food-borne pathogen [9], makes its way to the intestine after being ingested with contaminated food. In the intestine, Salmonella causes intestinal dysbiosis by damaging the intestinal barrier function [9]. There is always the induction of a strong immune response involving cytokines and chemokines secretion in S. typhimurium infection, resulting in inflammatory damage [10]. Moreover, Salmonella also modulates the host metabolism for its benefit, evidenced by the upregulated glycolysis in the macrophages infected with S. typhimurium [11]. Complicated gut-liver interactions through the gut-liver axis act as a ladder in the liver injury during Salmonella infection, manifested by inflammatory cell infiltration, oxidative stress, hepatic apoptosis, and intense congestion [12].

Detoxification, nutrient metabolism, and immune response are some of the important physiological activities of the liver [13]. In humans, many factors like infection, drug exposure, and alcohol abuse contribute to the etiology of liver damage [14]. Liver injury due to Salmonella infection is an example of infection-associated liver damage [15]. Moreover, enteric or typhoid fever is a protracted febrile condition that is caused by S. typhi infection and characterized by abnormal levels of liver enzymes in the serum [16]. Kalia et al. also found increased serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels during S. typhimurium infection to mice [17]. A severe liver abnormality with jaundice is observed in the patients of typhoid fever, and this condition is termed Salmonella hepatitis [16]. In poultry, persistent infection of S. gallinarum and S. Pullorum has been observed, causing localization in the ovaries and resulting in vertical transmission by producing contaminated eggs [18]. Hepatomegaly, friable liver consistency and yellow-greenish color of the liver along with multifocal hepatic necrosis by the histopathology of birds infected with S. gallinarum have also been found [19].

Unsuccessful vaccination and antibiotic resistance against salmonellosis demand the development of novel therapeutics [20]. In this regard, bacteriostatic and anti-inflammatory activities of natural products have attracted researchers’ attention [21]. Cinnamaldehyde is the main component of Chinese traditional medicine cassia twig and genus Cinnamon containing several hundred species. Isolates from cinnamon plant are more than 89 while cinnamaldehyde is the major constituent [22, 23]. The beneficial roles of cinnamaldehyde due to its antibacterial, anti-inflammatory, and antioxidative properties have been determined. For example, immune response triggered by lipopolysaccharides in macrophages has been reduced by cinnamaldehyde treatment in vitro [24]. In the human dental pulp cells, cinnamaldehyde causes the alleviation of H$_2$O$_2$-induced oxidative stress by nuclear factor erythroid 2 (NF-E2) p45-related factor 2 (NRF2) and shows the heme oxygenase-1 (HO-1)-dependent antioxidant response [25]. Intestinal inflammatory response against Cronobacter sakazakii has been mitigated by cinnamaldehyde in the mice model, as it suppresses the IL-1β, IL-6, and TNF-α expression. It also causes the inhibition of the NF-kB activation pathway and caspase-3 activity that leads to the reduction in enterocytic apoptosis [26]. A recent study indicated that the type-three secretion system (T3SS) of Salmonella enterica serovar Typhimurium had been inhibited by cinnamaldehyde as it affects the expression of key effector proteins in the mice model [21]. Despite these researches, evidence of mitigation of liver injury in the chicken models is largely unknown. Therefore, this study was performed to investigate the effects of cinnamaldehyde treatment on the morphological, physiological, pathological, biochemical, and metabolic changes caused by S. gallinarum infection to chicken hepatocytes at 1, 3, and 5 days postinfection (DPI). Furthermore, transcriptome analysis of 5 DPI hepatic tissue from control, challenge, and treatment groups was performed to get a deeper insight into the molecular mechanisms of cinnamaldehyde and gene expression changes at mRNA level in the S. gallinarum-challenged young chicks.

2. Materials and Methods

2.1. Animal Model, Sampling, and Ethical Issues. A total of 90 specific pathogen-free (SPF) day-old chicks (Lohman broiler chicks, negative for Salmonella, bought from Chengdu Muxing poultry industry Co., Ltd.), weighing 50-56 g, were used in this study. After three days of acclimatization, the flock was divided into three groups, each with 30 chicks, i.e., control group (CON), challenge group (SG), i.e., infected with S. gallinarum (isolated from diseased chickens), and treatment group (SG+CA), i.e., infected with S. gallinarum and treated with cinnamaldehyde. 150 mg/kg body weight cinnamaldehyde in the SG+CA group (orally), 0.3 ml of 3 x 10$^7$ CFU/ml S. gallinarum in SG group (intraperitoneally), and the same volume of normal saline (orally) in the CON group were injected. At 1, 3, and 5 DPI, blood samples were taken from 10 chicks (in each group). Chicks were euthanized (by cervical dislocation method) on the respective days. Livers were aseptically collected and cut into smaller pieces. Half of the samples were placed in an Eppendorf tube containing 4% formaldehyde, and the other half were immersed in liquid nitrogen for 10 minutes and stored in a -80°C freezer (see Figure 1). All experiments were conducted following the approved guidelines and experimental protocols by the Animal Care and Use Committee of Sichuan Agricultural University, China (permit number DKY-B2019603001).

2.2. Bacterial Colonization. The individual liver sample was weighed and subsequently transferred in sterilized mortar and pestle for maceration. The macerated liver contents were serially diluted (6-fold) in a sterilized 1% PBS solution. Later, tissue contents were plated on Salmonella-Shigella (SS) agar. Colonies typical for Salmonella were counted as Log$_{10}$ CFU/g.
2.3. Estimation of ALT and AST. Blood samples were obtained and centrifuged to collect the serum. Serum ALT and AST levels were measured spectrophotometrically at an absorbance wavelength (505 nm) using commercial kits purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, China, and expressed as international units per litre (IU/l).

2.4. Quantitative Real-Time PCR. Total RNA was extracted following the manufacturer’s instructions using a specialized kit (Aibimeng Biotechnology Co., Ltd. Zhenjiang, China). Precisely, 1 ml of TRIZol was added for 50 mg of liver tissue. Following lysis and centrifugation, the supernatant was transferred to an RNase-free centrifuge tube for subsequent purification steps. RNA was then eluted in 50 μl of RNase-free water and quantified by the ratio of absorbance (260 nm/280 nm) using a Nanodrop spectrophotometer (Thermo Fisher Scientific, MA, USA). The eluted RNA was then stored at −70°C until use. Total RNA was transformed into cDNA using 5X All-In-One Master Mix (Applied Biological Materials, Vancouver, BC, Canada) following the manufacturer’s instructions. After reverse transcription, the cDNA was collected and stored at -20°C. The qRT-PCR reaction was carried out by using abm® EvaGreen 2X qPCR Master Mix kit (Applied Biological Materials, Canada) on a CFX Connect™ Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA) with specific primers (see Table 1) designed using the Primer Premier 6.0 software. Relative gene expression was calculated by the ratio of the target gene to reference gene (β-actin) expression. $2^{-ΔΔCt}$ method [27] was used for the analysis of results.

2.5. Histopathological Examination. 1 cm² liver samples were taken from each group. 10% neutral-buffered formalin was used for tissue fixation. Tissue processing, trimming, and embedding in paraffin were performed subsequently. 5 μm thick tissue sections were selected and stained with hematox- ylin and eosin (H&E). Afterwards, the tissue sections were observed by a light microscope (Olympus, Tokyo, Japan).

2.6. Transmission Electron Microscopy (TEM) of Hepatic Tissue. At least 2 h fixation of liver samples was performed in 2.5% glutaraldehyde with a subsequent 2 h fixation in 1% osmium tetroxide. Later, a series of ethanol washes for gradient dehydration were applied on the samples and embedded in Epon 812 epoxy resin. Ultrathin sections (about 70 nm) were obtained by an ultramicrotome (EM UC6, Leica). Uranyl acetate and citrimalic acid lead were applied for double-staining. Samples were observed by TEM (Philips CM100) at 80 kV.

2.7. Caspase-3 Activity. The activity of caspase-3 in all the groups was measured using a commercial caspase-3 activity assay kit (Biyuntian Biotechnology Co., Ltd, Shanghai, China). 100 μl of tissue lysing fluid (from the kit) was added to 3-10 mg of liver tissue in a sterilized Dounce homogenizer and homogenized. Homogenate was transferred to a 1.5 ml Eppendorf tube and placed on ice for 5 minutes. Centrifugation of the homogenate was done at 16000-20000 g for 10-15 minutes, and the supernatant was used for further experimentation following kit instructions.

2.8. Reactive Oxygen Species (ROS) Content in Chicken Hepatocytes. 2,7-Dichloro-dihydro-fluorescein diacetate (DCFH-DA) was used to measure the ROS content in the hepatocytes following the kit instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Approximately 1 mm² liver tissue sample was taken and washed with PBS for subsequent digestion with trypsin enzyme at 37°C. The solution was screened using a nylon mesh, washed twice with PBS, and centrifuged to get a single-cell suspension. Later, DCFH-DA was added with dimethyl sulfoxide (DMSO) as solvent. The sample was incubated at 37°C for...
1 hour in the dark, and fluorescence was observed at an optical excitation wavelength of 485 nm and an optical emission wavelength of 510 nm using a spectrophotometer (Thermo Fisher Scientific, Vantaa, Finland).

2.9. Estimation of Mitochondrial Membrane Potential (Ψm) by JC-1 Experiment. Liver tissue was cut into 2-4 mm pieces with surgical blades. After gently blowing the dispersed cells with a pipette, remove the cells and debris with a filter and collect the cell suspension into a centrifuge tube for centrifugation and collection of the sediment. 10 mM of carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was added to the positive group cells at the ratio of 1 : 1000, and the cells were treated for 20 minutes. 0.5 ml JC-1 staining solution was added, mixed, and incubated at 37°C for 20 minutes. Prepare proper amount of JC-1 dyeing buffer (1X) according to the ratio of JC-1 dyeing buffer (5X): distilled water (1:4) and placed on ice. After incubation, centrifuge at 600 g for 3-4 minutes, precipitate cells, and discard the supernatant. Wash cells with JC-1 staining buffer (1X), add 1 ml of JC-1 staining buffer (1X) to resuspend cells, and centrifuge at 600 g for 3-4 minutes, precipitate cells, and discard the supernatant. JC-1 staining solution addition and incubation were done again at the same conditions and concentration. The cells were suspended with JC-1 staining buffer (1X) and analyzed by Beckman-Coulter Gallios flow cytometer (United States).

2.10. Cytochrome c Release Assay. Enzyme-linked immunosorbent assay (ELISA) was performed to measure cytochrome c concentration using a commercial ELISA kit (Nanjing Camilo Bioengineering Co., Ltd. Nanjing, China). 5-10 ml of tissue extraction reagent (from the kit) was added in 1 g of tissue and homogenized using sterilized mortar and

### Table 1: All primers for quantitative real-time PCR used in the study.

| Target       | Sequence (5′→3′) | Gene bank accession number |
|--------------|-----------------|---------------------------|
| TNF-α-F      | TGTCCGTCAGCCGCCTCTC | MF000729.1               |
| TNF-α-R      | TGGTGGTCTCCAACCTGTC |                       |
| IL-1β-F      | GGTCAACATGGCGACCTACA |                       |
| IL-1β-R      | CATACGAGATGGAAACAGCAA | HM01529469.2           |
| IL-6-F       | AAATCCCTCTGCGGCCATCT | HM0179640.1           |
| IL-6-R       | CCCCTGCCGTTTCTCTCAAA | HM0179639.1           |
| IL-8-F       | CCATCTTCCACCTTTCAACA |                       |
| IL-8-R       | ATCCACAGCAGCAGCAT |                       |
| IL-10-F      | CGGGAGCTGAGGGGTAA |                       |
| IL-10-R      | GTGAAAGACGCGACAGC |                       |
| IL-12-F      | TGAGGTCAGAAGTGAAATCT | DQ029328.1           |
| IL-12-R      | TCTTGGCAAATGGACAGTAG |                       |
| NF-κB-F      | TCAAAGGAGCAGATTAAGCAT |                       |
| NF-κB-R      | GCAGATAGCAGGCTAGGTAG |                       |
| MyD88-F      | CTGGCATTTCTGAGTAG |                       |
| MyD88-R      | TCCCTTATAGTCTCGGCTCTC |                       |
| iNOS-F       | CTCGGAGGTCGTAGGAAGAGT |                       |
| iNOS-R       | CCTGGGTTTCAGAAAGT |                       |
| Wnt1-F       | GATCGTTAACAGGGGTTGCC |                       |
| Wnt1-R       | GGTAACTGCGAGTGAACGAT |                       |
| G6Pase-F     | CACCGTCAGCCTGGTTTC |                       |
| G6Pase-R     | CCCAGGTTGCTGGTTTCTG |                       |
| Rhee-F       | ATCCACAGCAGGTGATGTT |                       |
| Rhee-R       | ACTTCCTTGTGAGCCTGTC |                       |
| Lipin 1-F    | AGCTGGTGTAGGAAATACTCT |                       |
| Lipin 1-R    | CTGGGCTTTGAGCTGTC |                       |
| PEPCK-F      | GTTACCTGGGACATTTGGCTGTC |                       |
| PEPCK-R      | CAGGTGCCAATCCCCTTCTT |                       |
| SREBP1-F     | CTACCGCTCATCCTCAACAG |                       |
| SREBP1-R     | CTGCTCCAGGTTTCTGTTGTC |                       |
| β-Actin-F    | GGTATGCGAGGAAGACAGAC |                       |
| β-Actin-R    | CTCCACGGCGCTACTCTT |                       |

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pestle. Homogenate was centrifuged at 5000-10000 rpm for 10 minutes. The supernatant was collected to perform the next steps of the experiment following kit instructions.

2.11. Determination of ATP Concentration in Hepatocytes. Hepatocytic ATP concentration was assayed by spectrophotometry using a commercial kit purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, China. 30-50 mg of liver sample was taken and ground in 270 μl double distilled water in a sterilized mortar and pestle. Eppendorf tubes were used for centrifugation at 3500 rpm for 10 minutes, and the supernatant was collected for further experiment. Absorbance was recorded at 636 nm, and ATP concentration was expressed as millimoles per gram protein (mmol/g protein).

2.12. Transcriptome Analysis. Total RNA was extracted from the tissue using TRIzol® reagent, following the manufacturer’s instructions (Invitrogen), and genomic DNA was removed by DNase I (TaKaRa). Then, RNA quality was determined by a 2100 Bioanalyzer (Agilent) and quantified using the ND-2000 (NanoDrop Technologies). Only high-quality RNA sample (OD 260/280 = 1.8 ~ 2.2, OD 260/230 ≥ 2.0, RIN ≥ 6.5, 28S : 18S ≥ 1.0, >1 μg) was used to construct sequencing library. RNA-seq transcriptome library was prepared following the TruSeqTM RNA sample preparation kit from Illumina (San Diego, CA) using 1 μg of total RNA. Shortly, mRNA was isolated according to the polyA selection method by oligo (dT) beads and then fragmented by fragmentation buffer firstly. Secondly, double-stranded cDNA was synthesized using a superscript double-stranded cDNA synthesis kit (Invitrogen, CA) with random hexamer primers (Illumina).

Then, the synthesized cDNA was subjected to end-repair, phosphorylation, and “A” base addition according to Illumina’s library construction protocol. Libraries were size selected for cDNA target fragments of 300 bp on 2% Low Range Ultra Agarose followed by PCR, amplified using Phusion DNA polymerase (NEB) for 15 PCR cycles. After being quantified by TBS380, the paired-end RNA-seq sequencing library was sequenced with the Illumina HiSeq X Ten/NovaSeq 6000 sequencer (2 × 150 bp read length). The raw paired-end reads were trimmed, and quality was controlled by SeqPrep (https://github.com/jstjohn/SeqPrep) and Sickle (https://github.com/najoshi/sickle) with default parameters. Then, clean reads were separately aligned to reference genome with orientation mode using HISAT2 software. The mapped reads of each sample were assembled by StringTie in a reference-based approach [28]. To identify differentially expressed genes (DEGs) between two different samples, the expression level of each transcript was calculated according to the transcripts per million reads (TPM) method. RSEM [29] was used to quantify gene abundances. Essentially, differential expression analysis was performed using the DESeq2 [30]/DEGseq [31]/EdgeR [32] with P ≤ 0.05; DEGs with log2FC > 1 and P ≤ 0.05 (DESeq2 or EdgeR)/P ≤ 0.001 (DEGseq) were considered to be DEGs. In addition, functional-enrichment analyses including GO (gene ontology) and KEGG (Kyoto encyclopedia of genes and genomes) were performed to identify which DEGs were significantly enriched in GO terms and metabolic pathways at Bonferroni-corrected P ≤ 0.05 compared with the whole-transcriptome background. GO functional enrichment and KEGG pathway analysis were carried out by Goatools and KOBAS [33].

2.13. Statistical Analysis. All experiments were repeated at least thrice. Data are presented as the mean ± standard error of the mean (SEM). The statistical significance of differences was analyzed by unpaired two-tailed Student’s t-test using GraphPad Prism 9 software. Differences with P < 0.05, 0.01, and 0.001 were considered statistically significant (+), highly significant (***), and extremely significant (***) respectively.

3. Results

3.1. Cinnamaldehyde Decreases the Colony Counts of S. gallinarum in the Chicken Hepatocytes. To determine the effect of cinnamaldehyde on the colonization of S. gallinarum in the chicken hepatic tissue, liver samples from all groups were taken and plated on SS agar to measure the colony counts at 1, 3, and 5 DPI. Data showed that there were significantly (P < 0.05) higher colony counts of Salmonella in the hepatic tissue from the SG group than that of the CON group at 1, 3, and 5 DPI (see Figure 2). However, the colony counts of Salmonella in the liver samples from the SG+CA group were significantly (P < 0.05) lower than that of the SG group at 1, 3, and 5 DPI. Hence, these data
presented that cinnamaldehyde treatment decreased the Salmonella colonization in the chicken hepatocytes.

3.2. Cinnamaldehyde Decreases Serum AST and ALT Levels of S. gallinarum-Challenged Young Chicks. A study demonstrated an increased serum AST and ALT concentrations after S. typhimurium infection in mice [17]. To find the effect of cinnamaldehyde treatment on the serum AST and ALT concentrations of S. gallinarum-challenged young chicks, serum AST and ALT levels were assayed. The concentrations of AST and ALT were significantly \((P < 0.05)\) higher in the SG group at 1, 3, and 5 DPI than that of the CON group (see Figure 3). However, compared to the challenge (SG) group, the cinnamaldehyde treatment (SG+CA) group significantly \((P < 0.05)\) decreased the serum AST and ALT concentrations at 1, 3, and 5 DPI. Hence, cinnamaldehyde treatment decreases serum AST and ALT levels of S. gallinarum-challenged young chicks.

3.3. Cinnamaldehyde Has Anti-inflammatory Properties in S. gallinarum-Challenged Chicken Hepatocytes. To assess the anti-inflammatory properties of cinnamaldehyde against S. gallinarum infection, relative mRNA expressions of certain proinflammatory and anti-inflammatory genes in hepatocytes of each group at 1, 3, and 5 DPI were determined using the qRT-PCR technique. Results indicated that the expression of proinflammatory genes \((IL-1\beta, IL-6, IL-12, NF-kB, TNF-\alpha, and MyD-88)\) were significantly \((P < 0.05)\) higher in the SG group than that of SG+CA group at 1, 3, and 5 DPI (see Figure 4). However, the expression of anti-inflammatory genes \((IL-8, IL-10, and iNOS)\) were significantly \((P < 0.05)\) higher in the SG+CA group than that in the SG group. Hence, these data illustrated the anti-inflammatory properties of cinnamaldehyde in the hepatic tissue of S. gallinarum-challenged young chicks.

3.4. Cinnamaldehyde Protects against the Hepatic Morphological Injury Caused by S. gallinarum. H&E staining (see Figure 5) and TEM (see Figure 6) of liver tissue indicated that intraperitoneal infection of S. gallinarum to young chicks resulted in hepatic colonization and disrupted normal hepatocytic architecture. Disruption of hepatic tissue was manifested by congestion, hemorrhages, and deformation of subcellular structures. However, cinnamaldehyde treatment reverted these morphological changes to a relatively normal state. Hence, cinnamaldehyde protects against the hepatic morphological injury caused by S. gallinarum infection.

3.5. Cinnamaldehyde Has Antiapoptotic Properties and Decreases ATP Concentration of S. gallinarum-Challenged Chicken Hepatocytes. Host-pathogen interactions result in ROS production [34]. ROS production has an important role in regulating many cellular pathways, determining cell fate [35], oxidative stress, impairment of cellular metabolism, and apoptosis or necrosis [36]. To find the effect of cinnamaldehyde on the hepatocytic ROS production in the S. gallinarum-challenged young chicks, hepatocytic ROS content
Figure 4: Continued.
was measured in all groups at 1, 3, and 5 DPI. ROS content was significantly \( P < 0.05 \) higher in the SG group than that of the CON group at 1, 3, and 5 DPI (see Figure 7(a)). However, we observed a significantly \( P < 0.05 \) lower ROS production in the SG+CA group than that of the SG group at 1, 3, and 5 DPI. Hence, cinnamaldehyde treatment decreases ROS production in the \textit{S. gallinarum}-challenged young chicks.

Increased ROS production causes a decrease in \( \Psi_m \) [37]. To assess the mitochondrial membrane permeability transition due to \textit{S. gallinarum} infection and treatment effect of cinnamaldehyde on it, the JC-1 experiment using flow cytometry technique was performed. We found a significant \( P < 0.05 \) decrease in \( \Psi_m \) in the SG group than that of the CON group at 1, 3, and 5 DPI. However, in the SG + CA group, \( \Psi_m \) was significantly \( P < 0.05 \) higher than that of the SG group at 1, 3, and 5 DPI (see Figures 7(e) and 7(f)). Therefore, cinnamaldehyde treatment protects the \textit{S. gallinarum}-challenged chicken hepatocytes from the decrease in \( \Psi_m \).

During mitochondrial membrane permeabilization, cytochrome c is released from mitochondria to cytosol and activates caspases (like caspase-3) to initiate a cascade of events for apoptosis [38, 39]. To find the effect of cinnamaldehyde on the cytochrome c release from mitochondria of \textit{S. gallinarum}-challenged chicken hepatocytes, ELISA was performed on the tissue sample from all groups at 1, 3, and 5 DPI. We found that in the SG group, the release of cytochrome c from mitochondria was significantly \( P < 0.05 \) higher than that of the CON group at 1, 3, and 5 DPI (see Figure 7(d)). At the same time, cytochrome c release from mitochondria was significantly \( P < 0.05 \) lower in the SG + CA group than that of the SG group at 1, 3, and 5 DPI. Hence, cinnamaldehyde treatment protects the chicken hepatocytes from cytochrome c release in the cytosol.

Enterocytic apoptosis is reduced by cinnamaldehyde treatment as it inhibits the caspase-3 activity [26]. Caspase-3 is the key enzyme for apoptosis [40]. As cytochrome c released from mitochondria activates caspases and apoptotic pathways, we performed the caspase-3 activity assay on the
Figure 5: Histological findings in chicken hepatocytes challenged with *S. gallinarum* and treated with cinnamaldehyde in comparison with the control group at 1, 3, and 5 DPI. (a–c) represent the control (CON) groups, showing normal architecture of hepatic tissue (100X). (d) represents the challenge group (SG) at 1 DPI. Arrows indicate erythrocytes and congestion (white arrow) and lipid droplet formation (black arrow). (e) represents the challenge group (SG) at 3 DPI. Arrows indicate congestion (black arrow) and hepatic sinusoid (white arrow). (f) represents the challenge group (SG) at 5 DPI. Arrows indicate the necrotic foci (black arrow) and erythrocytes (white arrow). (g–i) show histology of hepatic tissue from the treatment (SG+CA) group at 1, 3, and 5 DPI. The black arrow in (g) indicates lipid droplet formation (100x).

Figure 6: Effect of cinnamaldehyde on *S. gallinarum*-induced liver damage; evidence from transmission electron microscopy. (a–c) Liver of chicken from the control (CON) group showing the normal architecture of hepatocytes and smooth rounded nucleus with abundant normally distributed chromatin and prominent nucleolus, rough endoplasmic reticulum, and intact mitochondria. (d–f) Extensive liver damage in general and hepatocytic degeneration, in particular, have been found by intraperitoneal infection of *S. gallinarum* (SG group) at 1, 3, and 5 DPI by electron microscopy. However, (g–i) deleterious effects by intracellular activities of *Salmonella* were mitigated by cinnamaldehyde in the treatment (SG+CA) group, as evident by the figure showing relatively normal hepatocytic architecture compared to that of the challenge group (SG).
**Figure 7:** Continued.
Figure 7: Antiapoptotic properties of cinnamaldehyde in the chicken hepatocytes challenged with *S. gallinarum*. (a) Hepatocytic reactive oxygen species (ROS) content, (b) activity of caspase-3, (c) ATP concentration, (d) cytochrome c concentration released in the cytosol, (e) flow cytometry results of JC-1 analysis to estimate mitochondrial membrane potential ($\Psi_m$), and (f) percentage of JC-1 aggregates in the mitochondrial matrix of the chicken hepatocytes in the CON, SG, and SG+CA groups. Data are expressed as the mean ± SEM of three independent experiments. Significances were determined by nonparametric test (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$). Note: when the mitochondrial membrane potential is high, JC-1 aggregates in the mitochondrial matrix and forms a polymer, which can produce red fluorescence and is received by the FITC channel. When the mitochondrial membrane potential is low, JC-1 could not aggregate in the mitochondrial matrix. At this time, JC-1 exists as a monomer and produce green fluorescence, which is received by the PE channel.
Figure 8: Number of differentially expressed genes (DEGs), principal component analysis (PCA), and heat map of the CON, SG, and SG +CA groups. (a) Graph showing statistics of DEGs between challenge_vs_treatment, control_vs_treatment, and control_vs_challenge where red and green colors show the number of up- and downregulated genes, respectively. (b) Principal component analysis (PCA) shows 55.20% and 11.08% variations between (PC1) and within (PC2) the samples, respectively. (c) Heat map showing the DEGs with log 2FC > 1 at P < 0.05 in the CON, SG, and SG+CA groups.
hepatocytes of *S. gallinarum*-challenged young chicks. We observed a significantly (P < 0.05) higher caspase-3 activity in the hepatocytes of the SG group than that of the CON group at 1, 3, and 5 DPI (see Figure 7(b)). However, a significantly (P < 0.05) lower caspase-3 activity in the SG+CA group than that of SG group was observed at 1, 3, and 5 DPI. Therefore, cinnamaldehyde treatment decreases the caspase-3 activity in the *S. gallinarum*-challenged chicken hepatocytes.

To find the effect of cinnamaldehyde on the energy content of *S. gallinarum*-challenged chicken hepatocytes, the concentration of ATP was determined in the CON, SG, and SG+CA groups at 1, 3, and 5 DPI. We observed that at 1, 3, and 5 DPI, chicken hepatocytes infected with *S. gallinarum* (SG group) exhibited significantly higher (P < 0.05) ATP concentration than that of the CON group (see Figure 7(c)). However, the SG+CA group showed a significantly (P < 0.05) lower hepatocytic ATP concentration than that of the SG group at 1, 3, and 5 DPI. Hence, cinnamaldehyde treatment decreases the ATP content of *S. gallinarum*-challenged chicken hepatocytes.

3.6. Cinnamaldehyde Reverses the Modulations of AMPK-mTOR Pathway Caused by *S. gallinarum*. Transcriptome analysis of chicken hepatocytes infected with *S. gallinarum* using RNA sequencing Illumina Platform (HiSeq xten/Nova seq 6000 Sequencing) was performed to identify the DEGs and understand the pathways involved in mechanism of action of cinnamaldehyde against *S. gallinarum* infection. Three individual repeats were selected from the CON, SG, and SG+CA groups at 5 DPI. High-quality data with an adequate sequencing depth was generated by high-throughput RNA sequencing. 2314 DEGs (see Figure 8(a)) were found in the control_vs_challenge group (1139 upregulated and 1175 downregulated), the control_vs_treatment group showed 2402 DEGs (1214 upregulated and 1188 down-regulated), and the challenge_vs_treatment group exhibited 142 DEGs (56 upregulated and 86 downregulated). All raw transcriptome sequences were deposited in the National Center for Biotechnology Information (NCBI) sequence read archive (accession number: PRJNA784273). Principal component analysis (PCA) revealed 52.20% and 11.08% variance between the CON, SG, and SG+CA groups at PC1 and PC2, respectively (see Figure 8(b)). Moreover, the heat map showed a total of 2321 DEGs in the control_vs_treatment group, and their expressions were upregulated (in challenge_vs_treatment group) after the application of cinnamaldehyde. Moreover, two genes, ENSGALG00000016456 (Lipin-1) and ENSGALG000000041708 (Wnt4), which were downregulated in the control_vs_challenge group, were found upregulated in the challenge_vs_treatment group in the mTOR pathway. Upregulated genes by cinnamaldehyde in AMPK pathway, i.e., SREBP1, G6Pase, and PEPCK1, were involved in gluconeogenesis and fatty acid biosynthesis. At the same time, upregulated genes by medicine in the mTOR pathway, i.e., Lipin-1 and Wnt4, were involved in lipid and protein biosynthesis, respectively. Hence, cinnamaldehyde reverses the modulations of the AMPK-mTOR pathway caused by *S. gallinarum*. To validate the expression of DEGs obtained in RNA-seq data, we selected five genes among up- and downregulated DEGs and determined their relative expression by qRT-PCR. Consequently, qRT-PCR results were consistent with RNA-seq data (see Figure 9(c)).

4. Discussion

Components from traditional Chinese medicine (e.g., costunolide and cinnamaldehyde) have been proved effective in mitigating the liver damage [42]. In this study, we found that *S. gallinarum* infection to chicken resulted in many hepatic physiological and pathological changes which were abrogated by cinnamaldehyde treatment. We observed that mechanism of action of cinnamaldehyde in treating hepatic damage caused by *Salmonella gallinarum* infection to chicken hepatocytes was similar to Mao et al.’s study who demonstrated that costunolide is effective in treating acute liver injury by promoting antioxidative defense system, suppressing inflammatory response, and preventing hepatocytic apoptosis [43]. Lesions caused by infection and the protective efficiency of cinnamaldehyde were manifested by H&E staining and TEM. Previous research shows that intraperitoneal infection of *Salmonella* resulted in the heterophils aggregates in the parenchyma of chicken hepatocytes [44]. Similarly, oral infection of 1-week-old Rhode Red Island chickens with *S. typhimurium* resulted in bacterial colonization in the livers [45]. Our findings were consistent with these studies as we found bacterial colonization in liver of chickens infected with *S. gallinarum*. Results of our study showing the efficiency of cinnamaldehyde in reducing
Figure 9: Continued.
bacterial counts were also in accordance with Johny et al. [46], who found that trans-cinnamaldehyde reduces the plate count of *Salmonella* strains in cinnamaldehyde-treated water.

Destruction of hepatic tissue leads to hepatocytic damage and an increase in the permeability of the hepatocytic plasma membrane, which is manifested by ALT and AST release into the blood from hepatocytes [47]. The level of these enzymes is proportional to the number of damaged hepatocytes [48]. Hence, hepatic injury can be predicted by measuring the level of these enzymes in the blood. Our results showed an increase in the ALT and AST levels after
Salmonella infection to the chicken. At the same time, the treatment group exhibited decreased ALT and AST levels than that of the challenge group. These findings were consistent with Wang et al.’s [49] work of cinnamaldehyde effect on S. typhimurium-challenged mouse hepatocytes.

Hepatic damage in animals results in the biosynthesis and secretion of proinflammatory cytokines and chemokines [14]. In our experiment, the expression of proinflammatory genes of the hepatocytes was analyzed to find their implication and beneficial effects of cinnamaldehyde on them. Wang et al. [49] also found the effects of cinnamaldehyde on the expression of proinflammatory genes of the hepatocytes in a mouse model. Our findings were consistent with their study as S. gallinarum significantly increased the expression of proinflammatory genes (IL-1β, IL-6, IL-12, NF-κB, TNF-α, and MyD-88). However, cinnamaldehyde treatment, as expected, significantly decreased proinflammatory gene expression and increased the expression of anti-inflammatory genes (IL-8, IL-10, and iNOS), than that of the challenge group. Furthermore, we speculated that S. gallinarum infection-induced inflammatory damage could be suppressed by cinnamaldehyde in MyD-88 dependent signaling. However, there is a need for further investigation in this regard.

The control group showed a complete and normal architecture of hepatocytes in light and electron microscope, as evident from Figures 5 and 6, respectively; hence, it was used to compare the challenge and treatment groups. The most common histological lesions observed included necrosis, degeneration, hemorrhages, and infiltration of inflammatory cells. We found less tissue damage and relatively normal hepatocytic architecture in the treatment group than that of the challenge group. Lower ALT and AST levels, inflammatory response, H&E changes, TEM changes, and hepatic CFU were also observed in the treatment group.

**Figure 10:** Summary of events of S. gallinarum infection to chicken hepatocytes and protective effects of cinnamaldehyde. S. gallinarum infection to chicken hepatocytes disrupts the electron transport chain resulting in high ROS production, decrease in ΔΨm, and cytochrome c release from mitochondria. Cytochrome c triggers caspase-3 activity, which eventually causes apoptosis. Bacterial effectors result in increased glycolysis and ATP production. At the same time, increased hepatic CFU, MyD88/NF-κB dependent inflammatory response, serum AST, and ALT levels were also found in S. gallinarum-infected chicken hepatocytes. In contrast, cinnamaldehyde treatment resulted in decreased ROS production and relatively normal mitochondrial membrane potential, and cytochrome c release was significantly lower than that of the infected group. Moreover, caspase-3 activity and extent of apoptosis were also significantly lower than that of the challenge group. Similarly, cinnamaldehyde protects the host from metabolic changes, indicated by significantly lower ATP concentration in the treatment group than that of the challenge group. Lower ALT and AST levels, inflammatory response, H&E changes, TEM changes, and hepatic CFU were also observed in the treatment group.
infected group of chicks. These findings were consistent with Yang et al. [52], who found that cinnamaldehyde, in vitro, inhibited ROS production in high-glucose induced dorsal root ganglion (DRG) neurons dose-dependently. Hence, we found that cinnamaldehyde treatment has anti-oxidative properties in the hepatocytes of the *S. gallinarum*-challenged young chicks.

An increased ROS production results in a decreased $\Psi_m$ [37]. A decrease in the $\Psi_m$ has been observed by the *S. typhi* plasmid infection to macrophages that result in apoptosis [53]. Lv et al. [54] found the antiapoptotic properties of cinnamaldehyde as it protected the PC12 cells from a glutamate-induced decrease in $\Psi_m$. Our results were consistent with these findings as *S. gallinarum*-infected hepatocytes showed decreased $\Psi_m$. In contrast, cinnamaldehyde protected this decrease in $\Psi_m$, exhibiting its antiapoptotic role during *S. gallinarum* infection to chickens.

Research shows that in *S. typhimurium*-infected macrophages (in vitro), cytochrome c is released from mitochondria [55]. The released cytochrome c activates a cascade of events involved in apoptosis [38]. Consistent with these studies, we found higher cytochrome c in the challenge group than that of the control group. At the same time, a significant decrease in cytochrome c in the treatment group than that of the challenge group was observed. These findings are consistent with the study of Lv et al., who found a dose-dependent decrease in the cytochrome c concentration in glutamate-induced PC12 cells treated with cinnamaldehyde [54].

Caspase-3 is the key enzyme for apoptosis [40]. After *Salmonella* infection, increased caspase-3 activity has been detected in monocyte-derived macrophages [55]. *S. typhimurium* infection to mice results in the caspase-3 dependent phagocytic apoptosis [56]. In our experiment, we found a significant increase in caspase-3 activity during *S. gallinarum* infection, while cinnamaldehyde treatment decreased caspase-3 activity significantly at 1, 3, and 5 DPI. These findings were similar to Yang et al.’s work which showed that cinnamaldehyde significantly suppressed overexpression of cleaved caspase-3 in high-glucose- (HG-) induced dorsal root ganglion (DRG) neurons *in vitro* [52]. As the decrease in $\Psi_m$ and increase in ROS production, cytochrome c release, and caspase activity are involved in apoptosis, and cinnamaldehyde treatment protects these phenomena (see Figure 10), we concluded that cinnamaldehyde has antiapoptotic properties against *S. gallinarum*-challenged chicken hepatocytes.

Upregulated glycolysis has been observed in the macrophages infected with *S. typhimurium* [11]. Similarly, metabolic activities in the gut are induced and promoted by
Salmonella for its growth and survival [57]. Cinnamaldehyde treatment in mice causes gluconeogenesis, glucose storage, and inhibition of glycolysis [58]. Like S. typhimurium, Chlamydia species are also obligate intracellular pathogens that replicate only within the infected cells. Chlamydia infection results in increased ATP production, with a peak level occurring midway through the infection cycle where most metabolically active bacteria are proliferating [59]. Consistent with these studies, we found an increased ATP concentration in the hepatocytes infected with Salmonella, while a decrease in hepatocytic ATP content was found in the group treated with cinnamaldehyde. Hence, we assumed that cinnamaldehyde treatment protects chicken hepatocytes from going into a higher metabolic state for increased ATP production compelled by the S. gallinarum infection.

Immunometabolism (interface of the immune system and metabolism) is an emerging field of research [60]. Within poultry production, considered immunometabolic consequences are not valued, and the sole focus is on maximizing growth, which decreases immune potential. Distinct immune and metabolic pathways are changed between 2-4 days postinfection, resulting in the alterations of the local metabolic environment. AMPK and mTOR pathways are an important link between the extracellular environment and intracellular metabolism during Salmonella infection to young chickens [61]. As the first week is very crucial for the growth of broiler chickens because of higher metabolism and relatively weaker immune system than that of later stages [62], we selected the modulations caused by cinnamaldehyde in the AMPK and mTOR pathways at 5 DPI. DEGs obtained by the transcriptome analysis showed that genes of the AMPK-mTOR pathway, which were upregulated in the treatment group, were downregulated in the challenge group and vice versa. S. gallinarum infection activated the AMPK-mTOR pathway, eventually resulting in less gluconeogenesis, fatty acid, protein, and lipid biosynthesis. These results are consistent with Jiang et al. [11], who showed that decreased glucose levels in macrophages after S. typhimurium infection led to upregulated bacterial uptake of 2- and 3-phosphoglycerate and phosphoenolpyruvate (carbon source) while on the other hand, cinnamaldehyde treatment caused more gluconeogenesis, fatty acid, protein, and lipid biosynthesis (bring to a normal level). Gluconeogenesis, glucose storage, and inhibition of glycolysis are also observed by cinnamaldehyde treatment in mice [58]. In our study, downregulation of CREB, PEPCK, and G6Pase genes of the AMPK pathway in Salmonella infection eventually resulted in less glucose production (see Figure 11). However, downregulation of mTOR pathway genes, i.e., Lipin-1, Rheb, and SREBP1c genes in S. gallinarum infection, reflect less lipid, protein, and fatty acid biosynthesis, respectively. In contrast to this, cinnamaldehyde treatment upregulated PEPCK and G6Pase genes (AMPK pathway), which promote gluconeogenesis. Upregulation of Lipin-1 and Rheb genes (mTOR pathway) in the cinnamaldehyde treatment group indicates the promoted lipid biogenesis and protein synthesis, respectively. Hence, it is speculated that cinnamaldehyde reverses the AMPK-mTOR pathway changes caused by S. gallinarum infection.

5. Conclusion
Salmonellosis is a major concern to public health and poultry producers. Unsuccessful vaccination and antibiotic resistance against Salmonella infections demand researchers to develop novel therapeutics. Results of the current study revealed the protective effects of cinnamaldehyde on oxidative stress, inflammatory response, and apoptosis on the hepatocytes of Salmonella gallinarum-challenged young chicks. S. gallinarum induces inflammation and apoptosis via NF-κB/caspase-3-dependent pathway in the hepatocytes of young chicks. In addition, S. gallinarum infection may also result in metabolic changes via modulating the AMPK-mTOR pathway. However, cinnamaldehyde treatment ameliorated inflammation and apoptosis caused by Salmonella infection by suppressing NF-κB/caspase-3 pathway. Transcriptome analysis revealed that cinnamaldehyde treatment reverted the metabolic changes caused by S. gallinarum infection via the AMPK-mTOR pathway. Nonetheless, we only focused on investigating the effect of cinnamaldehyde on the hepatic tissue of S. gallinarum-challenged young chicks in the early days of life as this period is crucial for the growth of poultry birds because of higher metabolism and lower immunity than that of the later stages. In the future, long-term experimental studies and gene knockout systems can be used to validate the genes involved in the mechanism of action of cinnamaldehyde in the mitigation of liver injury caused by Salmonella gallinarum infection to chicken. We concluded that cinnamaldehyde has antibacterial, anti-inflammatory, antioxidative, and anti-apoptotic properties against S. gallinarum infection to chicken hepatocytes and it can be a candidate drug for the treatment of Salmonella infections in poultry production.

Data Availability
Sequencing data are available on NCBI (PRJNA784273). The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest
The authors declare no conflict of interest.

Authors’ Contributions
LY and SH designed and conducted the studies and obtained the data. SH, XS, HT, and PO did the graphical work of the manuscript and helped in manuscript preparation with assistance from other listed coauthors. CH, XL, FX, and ZY contributed through their intellectual inputs. GS, YZ, and HF corrected the contents. TT and YG assisted in conducting experiments. All authors contributed to the article and approved the submitted version. Lizi Yin and Sajjad Hussain contributed equally to this work.

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