Transcriptional responses of cytokines, immunoglobulin A, and nitric oxide genes in 1-day-old chicks post *Salmonella typhimurium* infection: An experimental study

Yasmine I. Mosa[1†*], Ahlam A. Gharib[†1], Sara Y. Abd-El Galil1, Amira. M. Ali[2] and Etab M. Abo Remela[3,4]

[1]*Microbiology Department, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Sharkia, Egypt*
[2]*The Veterinary Hospital, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Sharkia, Egypt*
[3]*Department of Bacteriology, Mycology and Immunology, Faculty of Veterinary Medicine, Kafrelsheikh University, Kafrelsheikh, Egypt*
[4]*Department of Biology, College of Science, Taibah University, Medina, Saudi Arabia*
[†]*These authors contributed equally to this work*

Abstract

Background: *Salmonella* has become one of the hazards prevalent foodborne pathogens causing different diseases in chickens. However, *Salmonella typhimurium* (ST), a nonhost-specific serovar, is a major avian agent that causes severe disturbance in young chicken wellness.

Aim: The occurrence of *Salmonella* in chickens and their antimicrobial resistance were explored in this study. In addition, the immune response of 1-day-old broiler chicks, against multidrug resistant (MDR) ST infection, was also assessed at 4 and 24 hours post infection (pi) in the cecum and spleen, representing their mucosal and systemic immune responses, respectively.

Methods: A total of 375 samples from 130 diseased and apparently healthy broiler and layer chickens were randomly collected for *Salmonella* isolation, identification, and resistance profile evaluation, from farms and different clinical laboratories. The immune response of 1-day-old broiler chicks, Ross 308, against *in-vivo* ST infection was ascertained through the evaluation of heterophile phagocytosis and s expression of cytokines, immunoglobulin A and other immune-regulating genes in the cecum and spleen. Twenty-four, 1-day-old nonvaccinated broiler chicks were used and divided into two groups. The chicks in the infected group were orally inoculated with 0.5 ml of 2×10^8 colony forming units (CFU)/ml of MDR ST suspension, while those in the control group were taken nutrient broth.

Results: Seven out of 130 (5.38%) examined chickens were positive for *Salmonella*. All isolates (100%) were resistant to amoxicillin–clavulanic acid (AMC), cefazolin (CZ), cefoxitin (FOX), ciprofloxacin (CIP), nalidixic acid (NA), tetracycline (TE), fosfomycin (FOS), and colistin (CT) with multiple antimicrobial resistances (MARs) index range of 0.72–0.83, where none of them was resistant to meropenem (MEM). The results of immune response revealed that chicks infected with ST showed significantly different phagocytosis percentages and index values compared to controls. According to the real-time quantitative polymerase chain reaction (RT-qPCR) results, the transcription of *IL-8, iNOS, IL-18, IgA,* and *IFN-γ* for chicks infected by ST showed a significantly increased trend ($p < 0.01$) with increasing chicken age and was higher in the cecum than spleen compared to controls ($p < 0.05$) during 24 hours after infection.

Conclusion: The findings indicated a strong mucosal immune response in the chicks after the ST challenge, which reflects humoral and cellular responses. Our insight recommended the occurrence of a natural immune response stimulator at 1 day age to face the infection, and this can prevent the resistance transfer, with efficient control measures.

Keywords: *Salmonella typhimurium*, Cytokines, Challenge, Phagocytosis, Real-time PCR.

Introduction

Salmonella is a common microorganism that can live in the intestines of animals, including birds. It can be passed to humans through contaminated water or food causing a variety of illnesses, from mild diarrhea to severe food poisoning. *Salmonella* infections are a serious problem for community health, animal welfare, and food safety around the world (Jajere, 2019; Hassan *et al.*, 2021). Salmonellosis is a broad term that encompasses all infections caused by *Salmonella* bacteria. These infections can range from mild food

poisoning to more serious invasive infections. Invasive infections can systematically reach many host parts, such as the bloodstream. Food poisoning caused by *Salmonella* is usually less serious, but it can still cause symptoms such as diarrhea, vomiting, and fever (Fàbrega and Vila, 2013). There are over 2,500 different types of *Salmonella* bacteria, and about 2,000 of them have been found in chickens. This means that chickens are the main cause of human salmonellosis (Ramatla *et al*., 2020). Avian salmonellosis is an infectious disease that has two forms: acute and chronic. Many types of birds may be infected by highly specialized serotypes such as *Salmonella* Gallinarum, *S.* Pullorum, and *S.* Arizona, or by zoonotic species such as *Salmonella typhimurium* (ST), *Salmonella enterica* (SE), and further paratyphoid *Salmonella* serotypes (Abd EL-Ghany *et al*., 2012; Wibisono *et al*., 2020).

A genetic relationship was found between *Salmonella* of chicken origin and that of infected humans with *Salmonella* food poisoning. Chickens receive *Salmonella* serotypes infection either by cross-contamination with feces, water, tools, and workers or from infected birds (Meteab and Abed, 2018; Shen *et al*., 2023). The chicken industry has been found to be responsible for up to 50% of salmonellosis outbreaks (Antunes *et al*., 2016). Infection with SE in chickens can happen at every age, although it is most common in young chicks because they are more susceptible to infection (Berndt *et al*., 2007). Adult birds are more resilient to salmonellosis than young age because they have stronger immune systems that are able to fight off the infection and with well-developed humoral and cell-mediated immune responses (Beal *et al*., 2004). The serovar of ST can cause serious illness in both chickens and humans. In chickens, it can cause severe intestinal inflammation and diarrhea, leading to death in young birds. In humans, it can cause acute gastrointestinal disease, with symptoms such as diarrhea, vomiting, and fever. This bacterial agent is the most important challenge for the poultry economy, as it can lead to the loss of chickens and the contamination of poultry products (Dar *et al*., 2019).

The intestinal mucosal immune system is the host's major line of defense against *Salmonella* infection. This system includes antibodies called IgA, as well as lymphocytes and leukocytes that are found in the lining of the intestine. These cells work together to prevent *Salmonella* from entering the body and causing infection (Sheela *et al*., 2003). Phagocytosis is an innate immune response that is primarily a response to body infection (Sornplang *et al*., 2015). It is a process by which the cells engulf and destroy foreign particles, including pathogens and dead cells. It is a vital immune response part that plays a significant role in maintaining host health. Phagocytes, such as neutrophils and macrophages, are able to recognize and engulf foreign particles through a process called receptor-mediated endocytosis (Rosales and Uribe,

2017). Chicken heterophils, a type of white blood cell, are able to engulf and destroy all types of pathogens. After a pathogen is engulfed, the heterophil releases granules that contain enzymes and other chemicals. These chemicals kill the pathogen and also produce an oxidative burst, which is a release of free radicals that can also damage the pathogen (Genovese *et al*., 2013). After *Salmonella* bacteria invaded the gut mucosa and localized there, immune cells were detected in the cecum by immunohistochemistry from 4 hours to 9 days post infection (pi). The number of TCR1+ cells, granulocytes, CD8+ cells, and the gene expression fold change for *IL-18, TNF-α, IL-12*, and *iNOS* in the cecum increased with the tissue invasiveness of *Salmonella* (Berndt *et al*., 2007).

Cytokines are proteins that are released by the immune cells and show a vital role in the immune system. They are the envoys that initiate as well as manipulate immune responses, helping to destroy microbial infections. Cytokines are also involved in inflammation, which is the body's response to injury or infection (Charles *et al*., 2001). Chemokines are a kind of cytokine that has chemoattractant properties and regulates the migration of immune cells (Kaiser and Staheli, 2008; Kaiser *et al*., 2022). In chickens, the gut immune response begins to interact with the commensal microbiota shortly after hatching. This interaction results in inflammation with low levels, which is related to up-regulation of the cytokine *IL-8* gene expression. IL-8 is a protein that helps to recruit immune cells to the gut, which helps to protect the chicken from infection (Bar-Shira and Friedman, 2006). In chickens, IL18 is a protein that is released by intestinal epithelial cells. It induces a vital share in defending the chicken against infection by attracting immune cells to the gut, helping to kill pathogens, and stimulating the adaptive immune response. IL18 and iNOS are also released by macrophages, where they help to kill pathogens by creating nitric oxide and reactive oxygen species. IFNγ is a protein that is released from γδ T cells and natural killer (NK) cells. It helps to destroy the infected cells, stimulate the immune response, and attract other immune cells to the infection site (Ijaz *et al*., 2021).

Understanding the mechanisms of *Salmonella* infection, intestinal colonization, persistence, and excretion in feces was previously discussed and proved critical (Andino and Hanning, 2015). The evaluation of chicken mucosal or systemic immune response, at 1 day old, is important to face *Salmonella* infection in poultry. This knowledge is essential for developing effective measures to exaggerate immune response and/or to decrease *Salmonella* infection in poultry farms and then salmonellosis risk in humans.

 Herein, we investigated the occurrence of *Salmonella* in Egyptian chickens and their antimicrobial susceptibilities to various antimicrobial agents. Furthermore, the immune response of 1-day-old broiler chicks was assessed against *in-vivo* ST infection by

evaluation of heterophil phagocytosis and mRNA expression of certain cytokines, IgA, and other immune regulating genes in both cecum and spleen.

Materials and Methods

Sampling

This study was conducted during the period from April to September 2022. A total of 375 chicken organs including 130 livers, 130 cecum, and 115 spleens of 130 diseased and apparently healthy broiler (n = 75) and layer (n = 55) chickens were included in the study. The chickens (Ages ranged from 1 to 30 days) were randomly collected from chicken farms and different clinical laboratories (private laboratories and Animal Health Research Institute, Zagazig branch) in Sharkia Province, Egypt. The samples were put in sterile vessels and transferred as early as possible to the Bacteriological Laboratory, Microbiology Department, Faculty of Veterinary Medicine, Zagazig University, where they were subjected to routine bacteriological examination for *Salmonella* isolation. The investigation was recommended and approved by the Zagazig University Institutional Animal Care and Use Committee (ZU-IACUC/2/F/76/2022).

Salmonella isolation and identification

Salmonella isolation from the samples was carried out based on the protocol of ISO, the International Organization for Standardization (ISO, 2017). First, one gram of each organ sample was suspended in 9 ml of buffered peptone water (BPW) (Oxoid, UK) and incubated at 37°C for 18 ± 2 hours. After the pre-enrichment, 0.1 ml of culture was inoculated into 10 ml broth of Rappaport Vassiliadis (RV) (Oxoid, United Kingdom) and incubated at 42°C for 24 hours. The plates were cultivated on xylose lysine deoxycholate agar (XLD) (Oxoid, UK) and salmonella shigella agar (SS) (Oxoid, UK) then they were incubated at 37°C/24-hours. For the purpose of biotyping, colonies from each fresh pure culture were examined for urease production and for their growth on triple sugar iron (Oxoid, United Kingdom) media. All isolates were serotyped using commercial antisera (Denka Seiken Company Ltd., UK) (Kauffmann, 1957) via slide agglutination tests at the serology unit, Animal Health Research Institute, Dokki, Giza.

Molecular identification of *Salmonella* isolates was confirmed by polymerase chain reaction (PCR) using the genus (*inv*A) and species (ST *fli*C)—specific primers at the Biotechnology unit, Animal Health Research Institute, Zagazig branch. The amplification for *invA* gene was conducted using the primer set of S139-F: 5′-GTGAAATTATCGCCACGTTCGGGCAA-3′and S141-R: 5′-TCATCGCACCGTCAAAGGAACC-3′, with thermocycling conditions consisting of an early step at 94°C for 5 minutes followed by 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, then a last extension step at 72°C for 7 minutes (Oliveira *et al*., 2003). The amplification for

ST *fliC* gene was conducted using the primer pair of *Fli15*-F: 5′-CGGTGTTGCCCAGGTTGGTAAT-3′ and Tym-R: 5′-ACTGGTAAAGATGGCT-3′, with thermocycling conditions consisting of an early step at 94°C for 3 minutes, followed by 30 cycles of at 94°C for 1 minutes, 55°C for 1 minutes, 72°C for 1.5 minutes, then a last extension step at 72°C for 10 minutes (Halimi *et al*., 2014). SE subspecies enterica serovar Typhimurium derived from ATCC® 14028 was used for quality control.

Antimicrobial sensitivity testing of Salmonella isolates

The recovered *Salmonella* isolates were examined for their susceptibilities against 18 commonly used antimicrobial agents (representing ten antimicrobial groups) by the disc diffusion method using Mueller–Hinton agar (Oxoid, UK) media. The examined antimicrobial agents including: ampicillin (AMP, 10 μg), amoxycillin-clavulanic acid (AMC, 20/10 μg), cefazolin (CZ, 30 μg), cefepime (FEP, 30 μg), ceftriaxone (CRO, 30 μg), cefoxitin (FOX, 30 μg), imipenem (IPM, 10 μg), meropenem (MEM, 10 μg), tobramycin (TOB, 10 μg), gentamicin (CN, 10 μg), amikacin (AK, 30 μg), ciprofloxacin (CIP, 5 μg), nalidixic acid (NA, 30 μg), tetracycline (TE, 30 μg), fosfomycin (FOS, 50 μg), chloramphenicol (C, 30 μg), sulfamethoxazole-trimethoprim (SXT, 23.75/1.25 μg), and colistin (CT, 10 μg). The minimum inhibitory concentration (MIC) for CT was determined by the broth microdilution assay (Rankin, 2005). The inhibition zone size around the antimicrobial discs and interpretation for MICs were deduced based on the guidelines of CLSI, the Clinical and Laboratory Standards Institute (CLSI, 2020). Multidrug-resistant (MDR) *Salmonella* isolates were ascertained when the isolates were resistant to ≥3 different antimicrobial classes. The multiple antimicrobial resistance (MAR) index of each antimicrobial was calculated by the following formula: Number of resistance recorded/ (Total number of examined antimicrobials × Total number of ST isolates), whereas MAR index for each ST isolate = Number of antimicrobials to which the isolate showed resistance/Number of antimicrobials to which the isolate had been examined (Tambekar *et al*., 2006). ST ATCC® 14028 was used for quality control.

ST challenge in 1-day-old chicks

Chicks and experimental design

Genetically distant chicks originated from the specific-pathogen-free Ross 308 line were used. Totally, 24 broiler chicks of 1 day age received no vaccination and tested to be free from *Salmonella*, were separated into two groups (infected and control), and kept under intense hygienic circumstances in the experimental unit of the Faculty of Veterinary Medicine, Zagazig University. The chicks in the infected group were separately and orally challenged with 0.5 ml of 2×10^8 CFU/ml ST suspension through crop instillation by a syringe, while the control group chicks were neither immunized nor

infected as shown in a previous protocol (Berndt *et al.*, 2007) with some modifications.

Bacterial isolate for the challenge

One pathogenic MDR ST isolate recovered during the study was used for the challenge. It was identified phenotypically then serotyped and confirmed by PCR. Before the experimental trial, it was subjected to virulence exaltation via chicken inoculation (Berndt *et al.*, 2007) with some modifications. The bacterial suspension for inoculation was prepared from a fresh colony inoculated into the nutrient broth (Oxoid, UK) and then incubated with shaking for 18 hours/37°C.

Sample collection after challenge

After two-timing intervals of 4 and 24 hours pi, blood was sampled from the wing veins of each chick (inoculated and controlled) for phagocytosis activity according to the method described previously (Lowry *et al.*, 2005). Moreover, tissue samples (one cecum and spleen) were collected from each euthanized bird (Berndt *et al.*, 2007). The samples were stored in RNA-later until they were used for real-time quantitative PCR (RT-qPCR).

Measurement of phagocytic activity

Preparation of *Candida glabrata (C. glabrata)*

Heat-inactivated *C. glabrata* (obtained from the Microbiology Department, Mycology unit, Faculty of Veterinary Medicine, Zagazig University) was used to evaluate the phagocytic activity of chick's heterophils. *Candida glabrata* was cultivated on Sabouraud's dextrose agar (Oxoid, UK) for 24 hours at 37°C. The grown yeast cells were harvested in sterile PBS, washed, and pelleted at 1,500 rpm for 10 minutes. The pellet was washed and adjusted to a concentration of 4 X 10⁶ CFU / ml The yeast cells were inactivated in a water bath at 100 °C/30 minutes (Sheena and Arthur, 1978).

Separation and preparation of chick's leukocytes (heterophil isolation)

Heparinized blood samples were used for leukocyte separation according to the method described by Lowry *et al.* (2005) with some modifications. Collected blood samples were diluted 1:2 in sterile heparinized PBS under sterile procedures, and the diluted blood was carefully layered over an equal volume of Ficoll–Hupaque solution (1.077 density gradient) (Sigma, USA) in sterile plastic centrifuge tubes. The over-layered blood was pelleted at 1,000 rpm/30 minutes. Thereafter, leukocytes in the white opaque band were aspirated using a sterile Pasteur pipette at the Histopaque plasma interface and placed in sterile Hank's Balanced Salt Solution (HBSS) (Wadsworth Center Media Core). The separated cells were washed three times in sterile HBSS, by centrifugation for 10 minutes at 2,500, 1,500 rpm then 1,000 rpm, separately. The pelleted cells were put in 2 ml media of RPMI 1640 (Oxoid, UK) with 5% chick serum and then counted using a hemocytometer (Weber Scientific comp., Hamilton). The cells were examined for viability and their concentration was adjusted to 1×10^6 cell/ml.

Estimation of phagocytic percentage and phagocytic index (PI) in the experimental groups

The phagocytosis activity was done as described previously (Sornplang *et al.*, 2015; Lowry *et al.*, 2005), with modifications. An equal amount of leukocyte suspension along with heat-inactivated *C. glabrata* was mixed and then incubated at 27°C/30 minutes in a humidified 5% carbon dioxide incubator. After incubation, they were centrifuged at 2,500 rpm/5 minutes. A small amount of the sediment was re-suspended and smeared onto a slide. The slide was then stained and viewed under an ordinary microscope. Heterophils were counted in ten microscopic fields, and the yeast cells were also recorded in each phagocytic cell. Phagocytosis and PI values were calculated from 200 heterophils in different fields. The phagocytic activity was assessed based on these equations (Lowry *et al.*, 2005): *P*% = Number of phagocytes with engulfed yeast cells/Total number of phagocytes × 100, PI= Total number of engulfed yeast cells/Total number of phagocytes with engulfed yeast.

Tissue cytokines and other immune gene expressions by RT-qPCR

The extraction of RNA was carried out using the QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH) according to the manufacturer's instructions. For SYBR green RT-qPCR, the specificity of amplified products was confirmed by generating melting curves, primer sets (Metabion, Germany) used for the amplification of *IFNγ*, *IL-8*, *IL-18*, *iNOS*, *IgA*, and *β-Actin* genes and their cyclic conditions were described in Table 1. The relative expression was performed in MX3005P real-time PCR thermal cycler (Agilent, La Jolla, CA, United States) using QuantiTect SYBR Green real-time PCR Kit (Qiagen, Germany) according to the manufacturer's recommendations. The *β-Actin* gene was used as a normalizer. The CT value of each sample was then compared to the value of the positive control using the method previously described (Yuan *et al.*, 2006) and the fold change values were estimated using the $2^{-\Delta\Delta ct}$ method.

Statistical analysis

Microsoft Office Excel (Microsoft Corporation, Redmond, WA) was used for data amending. Normality and homogeneity of variance were conducted by the Levene and Shapiro–Wilk tests (Razali and Wah, 2011). The significant effects of treatment, time, and their interaction on chicken phagocytic activity after *Salmonella* challenge were examined by conducting A two-way ANOVA (Proc ANOVA) (SAS, 2012). The mixed model of the statistical analysis system (SAS, 2012) (version 8, Cary, NC) was used for assessing gene transcription of *IL-8, iNOS, IL-18, IgA,* and *IFN-γ* for chickens infected by *Salmonella* spp. The statistical model includes the effect of treated time, organs, and their interactions as fixed factors, while chicks'

Table 1. Primer sequences, target genes, and cycling conditions for SYBR green Rt-qPCR.

Target gene	Primer sequences (5 → 3′)	RT-qPCR cyclic conditions	Reference
B-actin	F: CAACACAGTGCTGTCTGGTGG R: ATCGTACTCCTGCTTGCTGAT	Primary denaturation at 94°C for 15 minutes, followed by 40 cycles at 94°C for 15 seconds, 55°C for 30 seconds, and final extension at 72°C for 30 seconds.	Abdul-Careem *et al*. (2008)
IL-8	F: ATGTGAAGCTGACGCCAAG R: GGCCATAAGTGCCTTTACGA	Primary denaturation at 95°C for 5 minutes, followed by 40 cycles at 95°C for 15 seconds, 60°C for 30 seconds, and final extension at 72°C for 30 seconds.	Miska *et al*. (2022)
iNos	F: AGGCCAAACATCCTGGAGGTC R: TCATAGAGACGCTGCTGCCAG	Primary denaturation at 94°C for 10 minutes, followed by 40 cycles at 94°C for 50 seconds, 63°C for 60 seconds, and final extension at 72°C for 30 seconds.	Hassanpour *et al*. (2009)
IL-18	F: TGAAATCTGGCAGTGGAATG R: CAACCATTTTCCCATGCTCT	Primary denaturation at 95°C for 5 minutes, followed by 40 cycles at 95°C for 15 seconds, 60°C for 30 seconds, and final extension at 72°C for 30 seconds.	Miska *et al*. (2022)
IFN-γ	F: GCCGCACATCAAACACATATCT R: TGAGACTGGCTCCTTTTCCTT	Primary denaturation at 95°C for 5 minutes, followed by 40 cycles at 95°C for 15 seconds, 60°C for 30 seconds, and final extension at 72°C for 30 seconds.	Miska *et al*. (2022)
IgA	F: TCCGCACTCCATCCTCTTGC R: ACCGATGGTCTCCTTCACATC	Primary denaturation at 95°C for 10 minutes, followed by 40 cycles at 94°C for 15 seconds, 59°C for 30 seconds, and final extension at 72°C for 36 seconds.	Lammers *et al*. (2010)

F: Forward; R: Reverse.

individuality is a random factor. Duncan's Multiple Range Test (DMRT) (Steel and Torrie, 1980) was used for the detection of the multiple comparisons among means. Resulted data were expressed as means ± SE. Statistical significance was accepted at a probability <0.05 ($p \leq 0.05$).

Results

Occurrence of Salmonella in chicken samples
Seven positive *Salmonella* isolates were recovered from 130 chickens (7/130, 5.38% as 7/375, 1.86% of total samples); four of them were from diseased chicken samples, and three were from apparent healthy ones, mostly they were recovered from the cecum (Table 2). Traditional phenotypic methods identified the isolates as *Salmonella* based on their characteristic appearance on XLD and SS agar media. On XLD agar, the isolates appeared as pink colonies usually with black centers, but on SS agar, they gave white colonies with black centers. Biochemically, the isolates were negative for the urease test and produced a yellow butt and red slant with black coloration on TSI agar. The isolates were consistently serotyped as *Salmonella*, three of them were recorded as ST (2.3%; 3/130) showing 1, 4, 5, 12:i, formula, and the other four recovered isolates showing 1, 9, 12:-:- formula (suspected to be *Salmonella pullorum* in young chicks). ST isolates were further genotypically confirmed at the genus level (284 bp for the *invA* gene), as well as at the species level (613 bp *fli*C gene) to be used for the experimental design (Suppl. Fig. 1).

The antimicrobial sensitivity of Salmonella isolates
The susceptibility result of seven recovered *Salmonella* isolates to 18 antimicrobial drugs presented that they were resistant to amoxicillin–clavulanic acid, CZ, FOX, CIP, NA, TE, FOS, and CT (100%), where none of them was resistant to MEM. A high resistance rate was observed to CRO, FEP, CN, C, and sulfamethoxazole–trimethoprim (85.7%, each), while 71.4% of the isolates were resistant to AMP and TOB. A low resistance rate was observed for AK and IPM with percentages of 28.6% and 14.3%, respectively (Table 3). The broth microdilution results indicated that the MIC value for CT ranged from 32 to 128 µg/ml. *Salmonella* isolates showed different MAR patterns and the MAR index of *Salmonella* isolates (*n* = 7) ranged from 0.72 to 0.83 (Table 3) with an average of 0.78, while that of each antimicrobial agent ranged from 0.008 to 0.05. There is a significant difference ($p < 0.05$) in the MAR index between the different antimicrobial agents (Table 4).

ST challenge:
Salmonella challenge agent
One pathogenic MDR ST isolates (code no. DBc30) was selected for the challenge. It was serotyped by the formula: 1, 4, 5, 12: i, and molecularly confirmed. It showed antimicrobial resistance to 15 antimicrobial agents with a MAR index of 0.83 (Table 3).

Clinical signs and postmortem lesions in the experimental groups
After ST inoculation, the challenged chicks showed noticeable clinical symptoms such as weakness, depression, dullness, ruffled feathers, anorexia, whitish diarrhea, *Salmonella* arch, and chicks collected together

Table 2. *Salmonella*-positive isolates from chicken samples.

Chicken type (No.)	Health state (No.)	Organ samples (No.)	Positive isolates (%)*	p-value
Broiler (75)	Diseased (45)	L (45)	-	
		C (45)	1	
		S (40)	-	
	Total	130	1/130 (0.8)	
	Apparently healthy (30)	L (30)	1	
		C (30)	-	
		S (25)	-	
	Total	85	1/85 (1.2)	
Layer (55)	Diseased (35)	L (35)	-	0.17
		C (35)	3	
		S (35)	-	
	Total	105	3/105 (2.86)	
	Apparently healthy (20)	L (20)	1	
		C (20)	1	
		S (15)	-	
	Total	55	2/55 (3.63)	
Sum (130)		375	7/130 (5.38), 7/375 (1.86)	

L: liver; C: cecum; S: spleen; *Number (%) of positive isolates relative to each source. *p-values* > 0.05 are statistically nonsignificant.

Table 3. Antibiogram of *Salmonella* isolates (*n* = 7) recovered chicken organs.

Isolate code no.	Resistance profile	MAR* index
DLc 13	AMP, AMC, CZ, CRO, FEB, FOX, TOB, CN, CIP, NA, TE, FOS, C, CT	0.78
DBc 30	AMP, AMC, CZ, CRO, FEB, FOX, TOB, CN, CIP, NA, TE, FOS, C, SXT, CT	0.83
ALc 70	AMP, AMC, CZ, CRO, FEB, FOX, CN, AK, CIP, NA, TE, FOS, C, SXT, CT	0.83
DLc 85	AMC, CZ, CRO, FEB, FOX, TOB, CN, CIP, NA, TE, FOS, C, SXT, CT	0.78
DLc 110	AMP, AMC, CZ, FEB, FOX, IPM, TOB, CIP, NA, TE, FOS, SXT, CT	0.72
ABl 5	AMP, AMC, CZ, CRO, FEB, FOX, CN, AK, CIP, NA, TE, FOS, C, SXT, CT	0.83
ALl 60	AMC, CZ, CRO, FOX, TOB, CN, CIP, NA, TE, FOS, C, SXT, CT	0.72

D: diseased; A: apparently healthy; L: layer; B: broiler; c: cecum; l: liver; MAR: multiple antibiotic resistance. *MAR index of isolates. AMP: ampicillin; AMC: amoxicillin–clavulanic acid; CZ: cefazolin; CRO: ceftriaxone; FEP: cefepime; FOX: cefoxitin; IPM: imipenem; MEM: meropenem; TOB: tobramycin; CN: gentamicin; AK: amikacin; CIP: ciprofloxacin; NA: nalidixic acid; TE: tetracycline; FOS: fosfomycin; C: chloramphenicol; SXT: sulfamethoxazole–trimethoprim; CT: colistin.

at the room corner. While the postmortem lesions included hepatomegaly with hemorrhage and congestion in the livers. The spleen showed splenomegaly in addition to hemorrhage, and congestion. The cecum was enlarged with some congestion, hemorrhage, and inflammation.

Phagocytic activity of chicks after the ST challenge
The chicks group infected with ST showed significantly different phagocytosis and phagocytosis index values at two-time intervals of 4 and 24 hours pi matched with the unchallenged control chicks. The present outcomes clearly indicated that there was a significant reduction in phagocytic activity in the challenged group compared to the control one ($p = 0.0393$; Table 5). Regardless of the group effect, analysis of group-by-time interaction revealed that there were significant changes between the two considered groups (group of 4 hours pi and group of 24 hours pi) over time, maximizing in the control group after 24 hours pi, while the lowest phagocytosis percentage value was recorded in the diseased group during the same aforementioned time period ($p < 0.05$; Fig. 1). On contrary, there were no significant effects of treatment, time, or their interaction on the PI ($p = 0.3305, 0.2238$, and 0.9273, respectively; Table 5 and

Table 4. Frequency of antimicrobial resistance in *salmonella* isolates ($n = 7$) recovered from chicken organs.

Antimicrobial group	Antimicrobial agent	No. (%) of resistant *Salmonella*	MAR index*	*p*-value
Penicillin	AMP	5 (71.4)	0.039	
	AMC	7 (100)	0.056	
Cephalosporine	CZ	7 (100)	0.056	
	CRO	6 (85.7)	0.048	
	FEB	6 (85.7)	0.048	
	FOX	7 (100)	0.056	
Carbapenem	IPM	1 (14.3)	0.008	0.043
	MEM	0 (0)	0	
Aminoglycosides	TOB	5 (71.4)	0.039	
	CN	6 (85.7)	0.048	
	AK	2 (28.6)	0.016	
Quinolones	CIP	7 (100)	0.056	
	NA	7 (100)	0.056	
Tetracyclines	TE	7 (100)	0.056	
Phosphonics	FOS	7 (100)	0.056	
Phenicoles	C	6 (85.7)	0.048	
Sulphonamides	SXT	6 (85.7)	0.048	
Polymyxins	CT	7 (100)	0.056	

MAR: multiple antibiotic resistance. *MAR index of antimicrobial agents *p*-values > 0.05 are statistically non significant. AMP: ampicillin; AMC: amoxicillin–clavulanic acid; CZ: cefazolin; CRO: ceftriaxone; FEP: cefepime; FOX: cefoxitin; IPM: imipenem; MEM: meropenem; TOB: tobramycin; CN: gentamicin; AK: amikacin; CIP: ciprofloxacin; NA: nalidixic acid; TE: tetracycline; FOS: fosfomycin; C: chloramphenicol; SXT: sulfamethoxazole–trimethoprim; CT: colistin.

Table 5. Phagocytic activity of chicks after the *Salmonella* challenge.

Items	Treatment (T)		*p*–value		
	Control	Diseased	T	Time (Ti)	T × Ti
Phagocytic%	23.333 ± 0.33^a	20.500 ± 0.11^b	0.0393	0.7911	0.0447
PI	1.245 ± 0.02	1.208 ± 0.01	0.3305	0.2238	0.9273

Values bearing different superscripts (a,b) in the same row differed significantly ($p < 0.05$). Data were expressed as means± SE.

Suppl. Fig. 2). This result may be due to the better or instinctive immune response in unchallenged, unacclimatized young chicks than in challenged ones.

Results of tissue-specific mRNA gene expression by RT-qPCR

There were variations in the expression levels of all examined genes in infected chicks at two-time intervals compared to controls (Fig. 2). After 4 hours pi, the mRNA expression levels of *IL8, IL18, iNOs*, and *IgA* genes were higher in the cecum than the spleen; the highest mRNA expression levels in the cecum were noted for *iNOS*, followed by *IgA*, then *IL8* and *IL18* with fold changes of 2.9, 1.7, 1.6, and 1.4, respectively. While in the spleen, the mRNA peak expression level was recorded for *IL8* followed by *iNOS,* then *IL18*, with fold changes of 1.54, 1.5, and 1.2, respectively. After 24 hours post inoculation, similarly, the mRNA

expression ranks of all aforementioned genes in addition to *INF-Ɣ* were higher in the cecum than the spleen. The mRNA peak expression levels were presented for *IL18*, followed by *iNOS, IL8, INF-Ɣ,* and *IgA* in the cecum with fold changes of 28.2, 25.4, 21.7, 21.5, and 10.2, respectively. While in the spleen, the peak mRNA expression showed *IL18* followed by *INF-Ɣ, iNOs,* and *IL8,* with fold changes of 12.8, 12.3, 11.4, and 10.4, respectively. The transcription of *IL-8, iNOS, IL-18, IgA,* and *IFN-γ* for chicks infected by ST showed a significantly increased trend ($p < 0.01$; Table 6) with increasing chicken age.

Regardless of the effect of chicken age, the analysis of treatment by organ interactions revealed that the expressions of all aforementioned genes except *IgA* were significantly greater in the cecum than spleen ($p < 0.05$) during 24 hours after infection, whereas they

Table 6. *IL8, IL18, iNOS, IFN-ϓ and IgA* gene expression of chick organs at 4 and 24 hours post-*Salmonella* challenge.

Items	Times (T)			*p*-value		
	Control (0)	4 hours	24 hours	T	Organ (Oi)	*T×Oi*
IL-8	1.00 ± 0.00**b**	1.61 ± 0.10**b**	16.08 ± 3.13**a**	<0.0001	<0.0001	<0.0001
iNOS	1.00 ± 0.00**b**	2.24 ± 0.35**b**	18.42 ± 4.37**a**	0.0003	<0.0001	0.0002
IL-18	1.00 ± 0.00**b**	1.36 ± 0.04**b**	20.57 ± 4.26**a**	<0.0001	<0.0001	<0.0001
IgA	1.00 ± 0.00**b**	1.73 ± 0.30**b**	10.23 ± 2.31**a**	0.0051	<0.0001	0.0051
IFN-γ	1.00 ± 0.00**b**	Unmeasured	16.95 ± 4.57**a**	0.0058	<0.0001	0.0349

Values bearing different superscripts (a,b) in the same row differed significantly ($p < 0.05$).
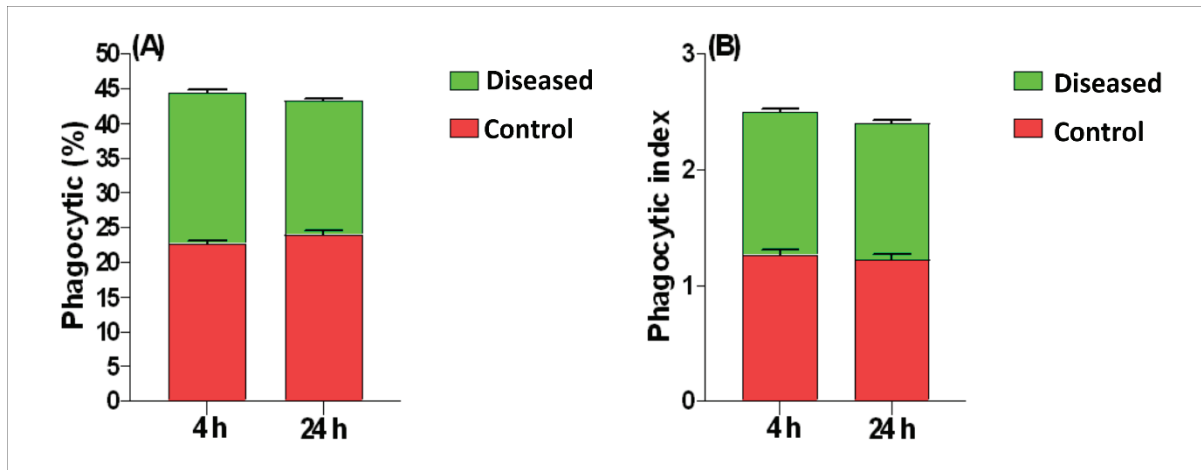
Fig. 1. Treatment by time interaction affecting chicks' phagocytic activity (A) and PI (B).

did not alter significantly during the 4 hours ($p > 0.05$; Fig. 2A–E). With respect to *IgA* gene expression, it was significantly up-regulated just in the cecum 24 pi compared to the control group ($p < 0.05$; Fig. 2E). Collectively, the present outcomes clearly indicated that the transcription of all previously detected genes (representing immune response's stages of innate and mucosal immunities, inflammation, and Th1 of phagocytosis) was significantly higher during the 24 hours after bacterial infection than their counterparts in the control chicks ($p < 0.05$); however, nonsignificant changes were noticed between the control chicks and challenged ones 4 hours pi.

Discussion

Poultry is a major reservoir of *Salmonella* bacteria, which can cause salmonellosis. International trade in poultry is a major factor that can facilitate the spread of *Salmonella*, as it can transport infected birds and their products between countries (EFSA and ECDC, 2019; Wibisono *et al*., 2020).

In our study, seven *Salmonella* isolates were found in 130 diseased and apparently healthy chickens, with an occurrence percentage of 5.38%. Our results were lower than those of other studies, such as Abd

El-Ghany *et al*. (2012), which found that *Salmonella* was recovered from 3.84% to 5.06% of the examined samples isolated from four chicken flocks located in El-Kalubia Governorate, Egypt; Helal *et al*. (2019), which found that 5.33% of the examined samples were *Salmonella* positive in Egypt; and Shen *et al*. (2023), which successfully isolated 108 (5.66%) *Salmonella* isolates from 1908 chicken samples in Anhui, China. However, these results were nearly similar for those of Menghistu and coauthors (Menghistu *et al*., 2011), who found that only 2.7% of the examined samples were *Salmonella* positive in India, and Medeiros team (Medeiros *et al*., 2011), who found that the prevalence of *Salmonella* in Brazil was 2.7%. However, the results of this study were lower than those of Andoh *et al*. (2016) (Ghana), lEl-Sharkawy *et al*. (2017) (Kafr El-Sheikh, Egypt), Ramatla *et al*. (2020) (South Africa), Ammar *et al*. (2016) (Sharkia Province, Egypt), and Abd El-Aziz *et al*. (2021) (Egypt), with recovery rates of 47%, 41%, 35%, 17%, and 11.54%, respectively.

Interestingly, in the current study, all the *Salmonella* isolates ($n = 7$) were 100% resistant to amoxicillin–clavulanic acid, CZ, FOX, NA, CIP, TE, FOS, and CT where none of them was resistant to MEM. A high
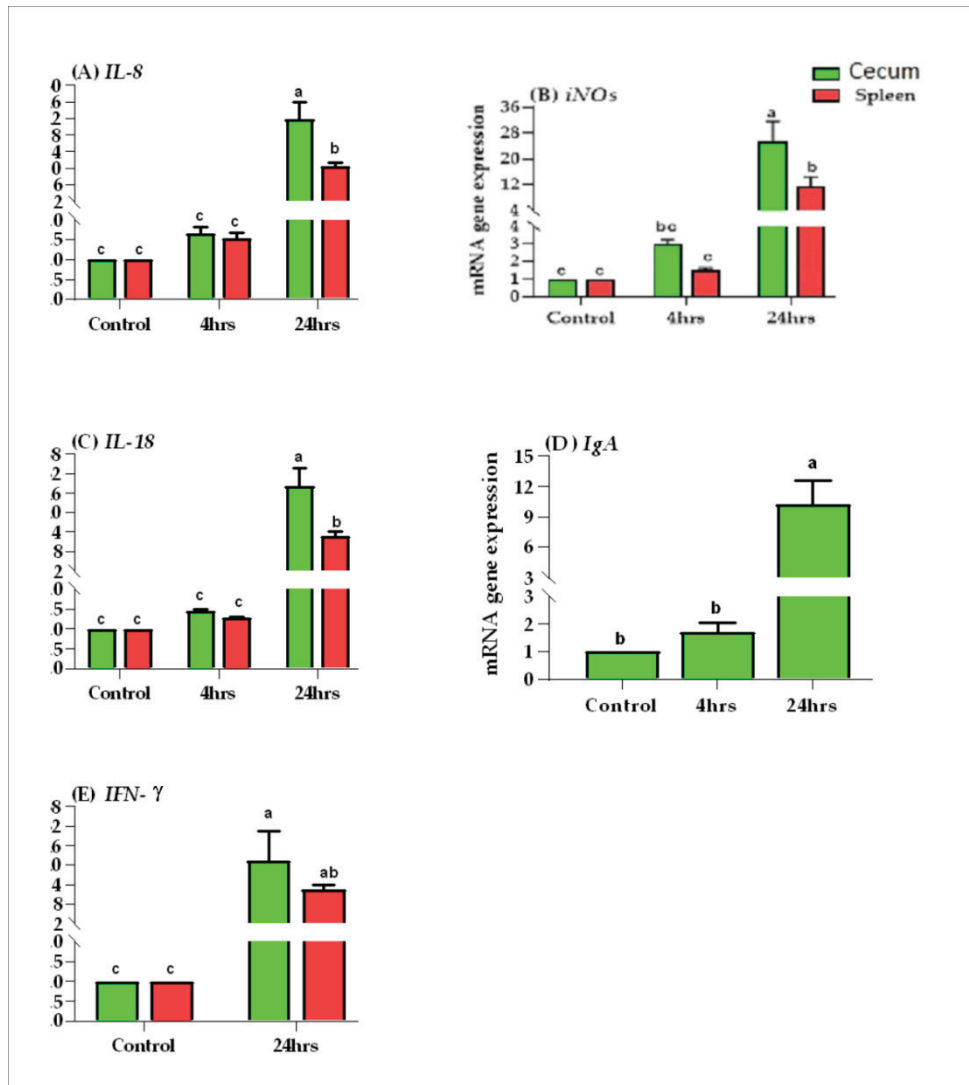
Fig. 2. Effects of time by organ interaction (means ± SEM) on fold change of (A) *IL-8*, (B) *iNOS*, (C) *IL-18*, (D) *IgA*, and (E) *IFN-γ* for *Salmonella* infected chicks.

resistance rate (85.7%) was observed to CRO, FEP, CN, C, and sulfamethoxazole–trimethoprim while resistance % to AMP and TOB was 71.4%. A low resistance rate was also observed for AK and IPM (28.6% and 14.3%, respectively). A similar resistance pattern against *Salmonella* isolated from different sources was observed previously (Abd El-Aziz *et al*., 2021). The detected highest MAR index ranged from 0.72–0.83 with an average of 0.78 was similar to that of Mir *et al*. (2022) who recorded that it ranged from 0.45 to 0.81 with an average of 0.63 in four *Salmonella* isolates that tested against 11 antimicrobial agents. The elevated MAR indices of each isolate and each antimicrobial agent pointed to the haphazard use of antibiotics.

In this study, the phagocytosis index and phagocytosis percentage of chicks challenged with ST were lower than those of control chicks after 4 and 24 hours pi.

The significantly dropped heterophil numbers might be due to stress-induced mechanisms or might be due to the death of intracellular bacteria and heterophils after *Salmonella* stimulation (Pieper *et al*., 2017). This result agreed with previous research (Sornplang *et al*., 2015), in which the chicks infected with SE showed lower phagocytic activity than controlled chicks at 24 and 48 hours, and another research (Sreekantapuram *et al*., 2021), in which the heterophils' count in the blood of white Leghorn chickens decreased significantly after being infected with *Salmonella*. The number of heterophils was considerably lower ($p \leq 0.05$) between 60 and 180 minutes in blood samples that were infected with SE and between 90 and 150 minutes in blood samples that were infected with Salmonella gallinarium. *Salmonella* bacteria initially colonize the digestive tract, especially the cecum, after being ingested orally. This
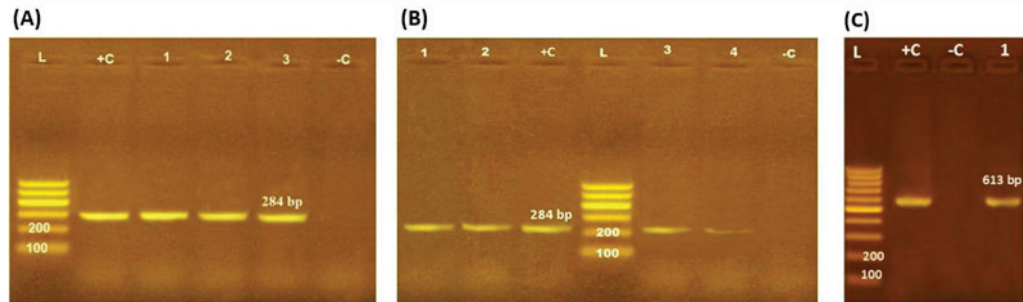
Supplementary Figure S1. Agarose gel electrophoresis pattern of PCR amplified products of *Salmonella* isolates genes.

M: DNA molecular size marker (100 bp), +c: control positive and – c: control negative

(A) and (B): showing PCR product of *inv*A gene of seven isolates {three isolates of Lanes 1–3 (A) and four isolates of Lanes 1–4 (B)}; (C): showing PCR product of *fli*C gene of one isolate confirmed *S. typhimurium* (Lanes 1 is positive).
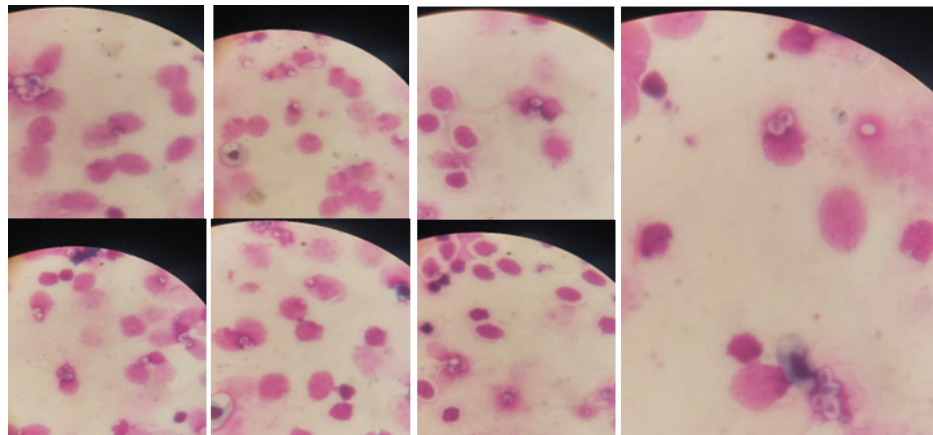
Supplementary Figure S2. Phagocytic activity of chicks after *Salmonella typhimurium* challenge.

An arrow indicated engulfed yeast.

can happen as early as 3 hours after infection. However, it takes about 24 hours for the bacteria to colonize the internal organs through macrophage migration (Van-Immerseel *et al*., 2002).

In this research, the local and systemic immune response was evaluated after ST challenge of 1-day-old Egyptian line broiler chicks, the mRNA expression levels of *IL8, IL18, iNOS, INF-Υ*, and *IgA* genes were measured after 4 and 24 hours pi and showed significant up-regulation at 24 hours and was higher in cecum than spleen of challenged chicken compared with control unchallenged one. The mRNA of *IgA* was up-regulated only in the cecum at two time points, and it is not determined in the spleen. While *INF-Υ* was up-regulated only at 24 hours and data is not determined at 4 hours.

Similarly, in a previous study (Withanage *et al*., 2004), 1-day-old chicks, after hatching, exposed to ST showed an extra rapid inflammatory cytokine response in the intestine, including the cecal tonsils, more than in the spleen. However, Beal *et al*. (2006) found that the immune responses at young ages after infection with *Salmonella* serovar Typhimurium result in a dangerous systemic response after invasion and inflammation of systemic organs. This suggests that the spleen may also play a vital role in the chicks' immune response to *Salmonella* infection at young ages.

A previous study (Withanage *et al*., 2004) demonstrated that the immune responses in systemic sites such as the liver and spleen are slower than those in the gut. This is because it takes longer for bacteria to reach these organs after they are ingested. However, chemokine expression, which is a signal that entices immune cells to the infection site, was first detected in the spleen and liver 12 hours pi, even before the bacteria had arrived. This suggests that the immune system is already preparing to fight the infection even before the bacteria have reached these organs. In the gut, the IL-8 and K60, CXC chemokines, are formed in response to infection. These chemokines attract polymorphonuclear

heterophils, which are a type of white blood cells that play a role in combatting infection. The production of these chemokines and the subsequent heterophils influx into the intestine is responsible for the pathology and inflammation that is shown in the ceca and intestines.

Here, the transcription of *IL-8, iNOS, IL-18, IgA*, and *IFN-γ* genes for chicks infected by ST showed a significantly increased trend ($p < 0.01$; Table 6) with increasing chicken ages. Between 1- and 2-days pi, the highest levels of *iNOS* mRNA expression were found. Furthermore, *IFN-γ,* the greatest significant stimulator for macrophages, increased considerably 1-day pi, with the peak of *IL-18* mRNA at 2-days pi, then by the highest *IFN- γ* expression level at 4-days pi, and ST infected chicks produced more *IFN-γ* but less *IL-12* and *IL-18* than SE infected chicks (Berndt *et al*., 2007).

According to the data in this study, the expression of the inflammatory genes, including *IFN-γ* was also elevated than in control for two weeks before returning to baseline after activating peripheral chicken blood mononuclear cells with both SE (Matulova *et al*., 2013) at various time points and ST (Rani *et al*., 2012; Dar *et al*., 2019).

In addition, a study by Crhanova *et al*. (2011) found that in 1-day-old chicks infected with SE, the levels of cytokines peaked at 3 days pi but remained elevated for up to 10 days pi. *IFN-γ* was the only cytokine that remained up-regulated for this duration. The authors of the study proposed that the immune response of chickens infected with *Salmonella* shifts from a Th1 response, which is described by the creation of *iNOS* and *IFN-γ,* to a Th17 response, which is described by the creation of interleukin-17 (IL-17).

The present work clearly indicated that the transcription of all previously detected genes, which represent immune response stages of innate immunity, inflammation, and Th1 of phagocytosis including; *iNOS, IL8,* and *INF-Υ*; and *IL18*, respectively, were significantly higher during 24 hours after bacterial infection than their counterparts in the control group ($p < 0.05$); however, nonsignificant changes were noticed between 4 hours pi in relation to the control chicks and, similarly, the finding of immune response stages is shown in a previous study of SE (Kaiser *et al*., 2022).

A similar study (Dar *et al*., 2019) found that the *IFN-γ, IL-12*, and *IL-18* mRNA expression heights were significantly increased in the liver, spleen, and cecum of infected birds matched to the control group. The highest *IFN-γ* mRNA expression was detected in infected birds on day 1 after infection in the liver, and up to day 5 in the spleen and cecum. On day 5 pi, the spleen had the highest increase in *IFN-γ* mRNA expression, followed by the cecum and liver. The author concluded that during *Salmonella* infection, the mRNA expression levels of different genes (*IL-12, IFN-γ*, and *IL-18*) in the spleen, liver, and cecum increase depending on the timer.

Our study concentrated on the immune response first hours after the challenge in 1-day-old chick after hatching and found that the systemic immune response initiated in the spleen at 4 hours pi and increased after that till 24 hours pi; therefore, it is suggested using natural immune stimulator to face the risk of avian salmonellosis.

The ascertained research result for our study is the result recorded previously (Cheeseman *et al*., 2007) in which the mRNA expression levels of IL-18 and interferon-gamma were higher in the spleens of chicks exposed to *Salmonella*. This proposes that avian splenic cells may play a role in *Salmonella* infection resistance.

The most abundant immunoglobulin in the avian intestinal tract secretions is IgA. It protects the intestinal lining from invasion by pathogens (Tizard, 2002). In this article, the *IgA* mRNA expression level was measured in the cecum after 4 and 24 hours pi by ST and showed significant up-regulation. This is similar to the results of a previous study (Sheela *et al*., 2003), which found that oral SE inoculation of young chicks increased the number of IgA cells, the number of gut-associated T cells in the intestine, and the concentration of IgA in the intestinal mucus.

On the other hand, a previous study (Beal *et al*., 2006) found that humoral immunity is not essential for the lasting protection or even prevention of initial avian salmonellosis.

The obstacle in our study is due to limited funding resources. Therefore, the study identified the *Salmonella* occurrence in samples and evaluated the chick mucosal and systemic immune response at only two-time intervals using an efficient real-time PCR, but it requires various primer pairs targeting conserved genes to characterize various cytokines and other immune gene expression profiles at different age intervals after *in-vivo* ST infections. However, microarray is a microbiological implement used to determine the thousands of gene expressions together.

Conclusion

The findings indicated a strong mucosal immune response in the chicks after the ST challenge. Chicks infected with ST showed significantly different phagocytosis percentages and phagocytosis index values compared with controls. In addition to transcription of the most examined cytokines and immune genes, mainly *IL-8, iNOS, IL-18, IFN-γ*, and *IgA*, was indicated in chick's cecum and spleen at two-time intervals pi and was highly significant in cecum at 24 hours. Our insight recommended the occurrence of a natural immune response stimulator at 1 day age to face the infection and then prevent resistance transfer with efficient control measures.

Acknowledgments

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