RESEARCH ARTICLE

ANALYSIS OF SOME INTERLEUKINS AND LEUKOGRAM IN ISA BROWN COCKS EXPERIMENTALLY INFECTED WITH *SALMONELLA GALLINARUM*

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ABSTRACT

This research aimed to investigate the profile of IL- 1β, INFγ, and leukogram in cocks experimentally infected with *Salmonella* gallinarum. A total of 40 cocks, infected and control, 20 birds each were used for this study. Each bird in the infected group was administered orally 1ml inoculum 9.0 x 108 CFU/ml *Salmonella* gallinarum. Blood samples were collec ted and analyzed for leukogram, and the harvested serum samples were analyzed for IL-Iβ and INF γ. There were significant differences ($p < 0.05$) in the mean absolute TWBC, heterophil and lymphocyte counts in the infected and the control cocks almost at levels. The highest values of TWBC (14.5 ± 0.60 x 109/1), heterophil (4.4 ± 0.31 x 109/1) and lymphocyte (10.4±0.55 x 109/1) counts were observed in the infected group on Day 21 (p.i.). There were significant differences ($p < 0.05$) in the mean serum IL-1β and IFN-γ concentrations in the infected and the control cocks on Day 4 (185±46.0 pg/ml) and Day 7 p.i. (2634± 342 pg/ml), respectively. The highest and lowest values for mean serum IL-1β concentration kwere observed to be 695±1.0 pg/ml and 185±46.0 pg/ml in the infected group on Days 14 and 7 p.i., while that of IFN γ was 4134±217 pg/ml and 2634 ± 342 pg/ml in the infected group on Days15 p.i. and cocks may play a vital role in driving higher immune responses during *Salmonella* gallinarum infection.

Keywords: Cockerels, inoculum, ISA Brown, *Salmonella*

INTRODUCTION

Among poultry diseases, fowl typhoid, caused by *Salmonella g*allinarum, is one of the most important bacterial diseases that poses serious challenges to poultry production, worldwide (Saidu et al., 1990; Saidu et al., 1994; Majid et al., 2010). This is aside the fact that it also constitutes a source of food-borne and zoonotic transmission of the disease to humans (Hafez *et al*., 2020). Fowl typhoid is an acute disease, which affects primarily chickens and turkeys, but pheasants, quails, and guinea-fowl are also susceptible (Shivaprasad, 2000; Casagrande et *al*., 2014). Fowl typhoid is considered one of the most important septicaemic bacterial diseases of chickens with consequent huge economic losses (Evans, 2011). These losses are represented by variable morbidity and high mortality, with a severe septicemic disease, occurring primarily in adult birds (Gemechu et al., 2020). The most significant sources of the infection in the poultry industry are food, environment, or contaminated eggs from infected or carrier birds (Shivaprasad, 2000; Celis-Estupiñan et al., 2017, NVRI, 2020).

Infection with *Salmonella* has been reported to not only affect poultry but is also an emerging pandemic and threat to public health (Zeinab et al., 2020). *Salmonella* gallinarum is a host-specific pathogen causing systemic infection in poultry, which leads to significant economic losses due to high mortality (Ojima et al., 2021) and decreased egg production in layers (Haque et al., 2021).

The gross and histopathological changes induced by *Salmonella* gallinarum infection are majorly observed in liver, spleen, kidneys, heart, intestines and other organs and is characterised by depletion of lymphocytes, vascular congestion in various organs, especially liver, spleen and kidney; there is multifocal necrosis of hepatocytes with accumulation of fibrin and infiltration of heterophils mixed with a few lymphocytes and plasma cells in the liver (Kokosharov et al., 1997; Hossain et al., 2006; Dutta et al 2015., Anny et al., 2017).

Understanding the interplay of host factors in immune responses during the pathogenesis of fowl typhoid is pivotal not only in diagnosis of the disease but also in development of treatment protocol and in vaccines development. Among the host's inflammatory response factors aimed at surmounting diseases are cytokines. Cytokines are proteins or peptides that are secreted by cells that play a significant role in immune and inflammatory responses through the activation and regulation of other cell types and tissues. Cytokines are effective elements of the avian immune system that are capable of eliminating foreign antigens (Al-Khalaifah et al., 2018).

In mammals, the roles of cytokines are well known with a vast number of publications describing the structure of cytokines and their roles in health and disease (Wigley et al, 2003). IL-1β is produced by a range of cells following stimulation by microbes or microbial products (Dinarello, 1998), and its biological activity is highly inflammatory, with its main function being to activate the immune system in an acute phase response. IL-1β activates a range of cells such as macrophages and lymphocytes that may thus lead to production of other cytokines and chemokines. The production of IL-1β should be expected in many avian infections where a proinflammatory response occurs, as is the case with mammalian models of infection. The IFN-γ is known to play a vital role in immune response, and more involved in the inflammatory response of chickens, it is also known to play a role in macrophage activation (Horiuch et al., 2001). Interferons (IFN) are a group of cytokines that are produced by leukocytes and viral-infected cells because of stimulation of the immune system by viral infection and inflammation reactions.

This present study evaluated the profile of Interleukin 1 beta (IL 1β), Interferon gamma (INF γ) and leukogram in commercial ISA Brown cocks experimentally infected with *Salmonella* gallinarum.

MATERIAL AND METHODS

Ethical Clearance

Ethical approval for the experimental protocol was sought from Ahmadu Bello University Committee on animal use and care (ABUCAUC) with approval number ABUCAUC/2023/155.

Experimental Birds

A total of 40 Isa brown cocks weighing between 3-4kg were used for this study. They were unvaccinated against fowl typhoid, but vaccinated against other infectious diseases, such as Newcastle disease, infectious bursal disease and fowl pox and were purchased from a reputable farm in Jos and brooded for four weeks and reared to 18 weeks of age. They were then housed on the deep litter system and managed intensively. Throughout the experiment, standard commercial feed and water were provided to the birds *ad libitum*. The birds were acclimatized for a period of four weeks to get used to all the handling conditions and environment.

Experimental Design

The *Salmonella enterica* serovar gallinarum that was used for the research was obtained from the Department of Microbiology, National Veterinary Research Institute (NVRI), Vom, Nigeria. After the cocks attained reproductive age (23 weeks), the cocks were randomly allocated into two groups, infected and control of 20 birds each. Before infecting the experimental birds, cloacal swab was collected from individual bird and was then immersed in buffered peptone water, followed by plating them in MacConkey agar (MCA). Both cloacal swab and plates were incubated in a bacteriological incubator at 370C for 24 hours according to the standard laboratory methods (Wigley et al., 2001; Parmer and Davies, 2007). *Salmonella* gallinarum from the previously prepared slant was reactivated by inoculating it onto MacConkey agar (MCA). The colonies formed were then examined for their characteristic features, color and morphology and tested for the Gram stain reaction (Gram-negative). McFarland turbidity standards were made in the laboratory by preparing a 1% solution of anhydrous barium chloride and 1% solution of sulfuric acid, and they were mixed to obtain a barium precipitate. The volumes of the two reagents were adjusted to prepare standards of different turbidities that represent different concentrations of bacteria. The standards were used to visually compare the turbidity of a suspension of bacteria (McFarland, 1907). All the cocks in the infected group were inoculated orally at a dose of 1.0 ml inoculum containing 9x108 cfu/ml of *Salmonella* gallinarum, while cocks in control group were not challenged with the bacterium, but were given each 1 ml of distilled water only.

Clinical Examination

Following inoculation of the birds with the *Salmonella* Gallinarum, the infected group was observed daily for clinical signs of fowl typhoid and findings were recorded. All observations made during the six-week experiment were recorded, accordingly.

Blood Sampling

Blood samples were collected via wing vein, using 23-gauge sterile hypodermic needles and syringes. 2 to 3 ml of blood from each cock were collected in the morning (8:00 AM) from the infected and control groups. Blood collection were on Days 0, 4, 7, 14, 21, 28, 35, 42 post infection (p.i.). The blood was divided into two parts, one part of blood was dispensed into a tube containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant and was used to determine total white blood cell count (WBC) and differential leukocyte count that were determined using methods described by Campbell and Ellis (2007). The second part of the blood was centrifuged for 10 minutes at approximately 1000×g. The harvested serum from each cock was then emptied into micro-vials and stored at -20oC, and the harvested serum was then assayed for serum IL-1β and IFN-γ using chicken-specific enzyme-linked immunosorbent assay (ELISA) kit (ELK Biotechnology CO., LTD), following the manufacturer's instructions.

Blood Serum IL-1 β and IFN- γ Concentrations

Cocks from each of the two groups were used at each time point post-infection Days 0, 4, 7, 14, 21, 28, 35 and 42 post infection (p.i.). The serum IL-1β and IFN-γ concentrations were measured using enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (ELK Biotechnology CO., LTD).

Statistical Analysis

Data obtained were subjected to statistical analysis using Graph Pad Prism Version 8.00 for Windows, GraphPad Software, San Diego, California, USA. Data from the two groups was compared using the student t-test, and values of $P<0.05$ were considered significant.

RESULTS

Clinical Manifestations of Fowl Typhoid in Infected Commercial ISA Brown Cocks

During the experimental period, neither morbidity nor mortalities were recorded in normal control ISA Brown cocks. However, in the infected group, starting from Day 8 post-infection up to Day 15 post-infection, the clinical signs observed in the infected ISA Brown cocks included: sudden death, depression, somnolence, decreased in feed and water consumption, greenish-yellow diarrhea, and some with blood stained, huddling, ruffled feathers, somnolence, and paleness of the comb and wattle. The morbidity rate recorded in the infected group was 50% (Table 1), while the mortality rate was 35% (Table 2).

Table 1 Morbidity rate in control and experimentally-infected ISA Brown cocks with Salmonella gallinarum during 42 days post infection

Days post- infection	Infected (N=20)	Control (N=20)
0	0	0
4	0	0
7	0	0
8-14	8	0
15-21	2	0
22-28	0	0
29-35	0	0
36-42	0	0
Total	10	0
MR	50%	0

Morbidity rate= Number of sick birds/ Number of birds inoculated x 100

Table 2 Mortality rate in control and experimentally-infected ISA Brown cocks with Salmonella gallinarum during 42 days post infection

Days post- infection	Infected (N=20)	Control (N=20)
0	0	0
4	0	0
7	0	0
8-14	5	0
15-21	2	0
22-28	0	0

Days post- infection	Infected (N=20)	Control (N=20)
29-35	0	0
36-42	0	0
Total	7	0
MR	35%	0

Mortality rate = Number of dead birds/ Number of birds inoculated x 100%

Effect of *Salmonella Enterica* Serovar Gallinarum Infection on Leukogram of ISA Brown Cocks

Absolute Mean White Blood Cell Count

The absolute mean white blood cell values of the *Salmonella enterica* serovar gallinarum infected and uninfected control groups are presented in Figure 1. The absolute mean WBC value in the infected (7.00 ± 0.28 x109/L) and control broiler cocks (6.90 ± 1.30 x109/L) was not significantly different (P>0.05) on Day 0 p.i.. A decrease in WBC value was observed in the infected group from Day 4 p.i. up to Day 7 p.i.. Thereafter, WBC value in the infected group was found to be significantly higher (p<0.05) in the infected cocks when compared to the control counterpart, and reaching its peak value on Day 14 p.i. (9.2±0.46 x109/L) than that of the control group (7.10 ± 1.20 x109/L). This was followed by a gradual decrease from Day 21 p.i. until termination of the experiment.

Absolute Mean Heterophil Value

The absolute mean heterophil values of the *Salmonella enterica* serovar gallinarum–infected and uninfected control groups are presented in Figure 2. The absolute mean heterophil value followed similar pattern of changes as that of the WBC (x109/L) value in the two groups of experimental birds up till the end of the study. However, starting from Day 14 p.i., a significant (P<0.05) increase in heterophil value was recorded in the infected group, which reached its maximum value of 4.4±0.31 x109/L on Day 21 p.i., which then followed by gradual decrease from Day 28 p.i. till the termination of the study.

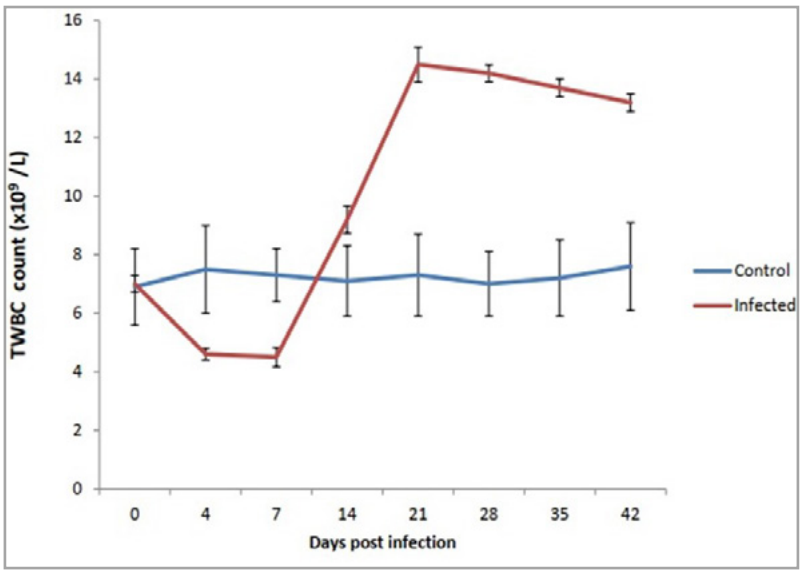

Figure 1

Absolute mean (± SEM) of white blood cell value of ISA Brown cocks experimentally-infected with *Salmonella enterica* serovar gallinarum, and control cocks

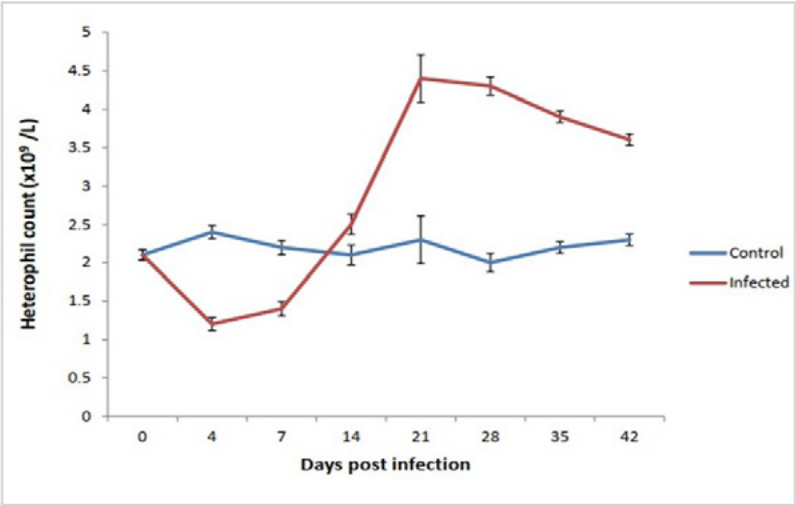

Figure 2

Absolute mean (± SEM) of heterophil value of ISA Brown cocks experimentally- infected with *Salmonella enterica* serovar gallinarum, and control cocks

Absolute Mean Lymphocyte Value

The absolute mean lymphocyte values of the *Salmonella enterica* serovar gallinarum–infected and uninfected control groups are presented in Figure 3. The absolute mean lymphocyte value (x109/L) was not significantly different ($p>0.05$) between the infected (4.90 ± 0.24 x109/L) and control (4.70 ± 0.20 x109/L) groups on Day 0 p.i. until Day 4 and 7 p.i., where slight reduction in mean lymphocyte value was recorded in the infected cocks. Thereafter, a significant rise ($p<0.05$) in this index was recorded in the infected group from Day 14 p.i., reaching its maximum value on Day 21 p.i. (10.4±0.55 x109/L). Following this, a slight drop was recorded on Day 28 p.i. in the infected group (9.6±0.19 x109/L). The mean lymphocyte value in the infected group during this period was still significantly higher ($p<0.05$) than in the control group.

Effect of *Salmonella Enterica* Serovar Gallinarum Infection on Serum IL-1β and IFN-γ of ISA Brown Cocks

Mean Interleukin-1beta Concentration

The mean IL-1β concentration of the *Salmonella enterica* serovar gallinarum–infected and uninfected control groups are presented in Figure 4. The mean IL-1β concentrations in the infected (74.00 ± 65.00 pg/ml) and control broiler cocks (74.00 ± 13.00 pg/ml) were not significantly different ($P>0.05$) on Day 0 p.i.. However, on Day 4 p.i., the value in the infected group increased to 185.00 ± 64.00 pg/ml, reaching its peak on Day 14

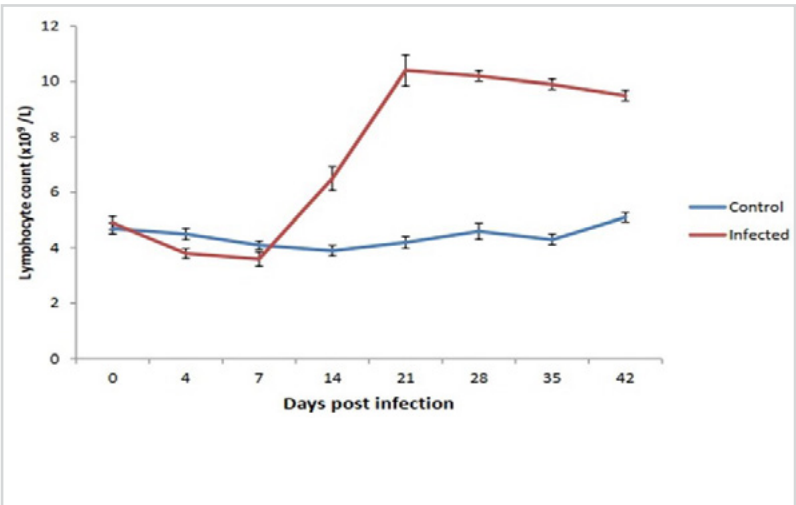

Figure 3

Absolute mean (± SEM) of lymphocyte value of ISA Brown cocks experimentally-infected with *Salmonella enterica* serovar gallinarum, and control cocks

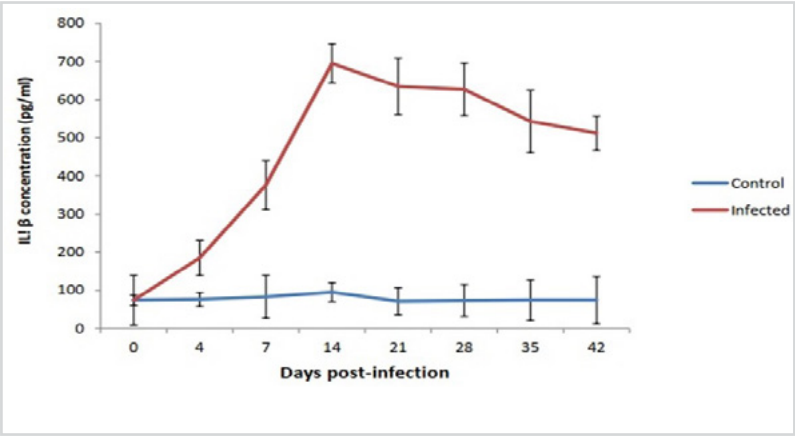

Figure 4

Mean (± SEM) of IL-1β concentration of ISA Brown cocks experimentally-infected with *Salmonella enterica* serovar gallinarum, and control cocks

p.i. and, thereafter, started declining on Day 21 p.i. up to the end of the experiment.

Mean Interferon-γ Concentration

The mean INF—γ concentration of the *Salmonella enterica* serovar gallinarum–infected and uninfected control groups are presented in Figure 5. The mean INF--γ concentration followed the same pattern as mean IL-1β. The mean INF--γ concentration in the infected group started increasing from Day 7 p.i., reaching its peak on Day 14 p.i. (4134.00 ± 217.00 pg/ml), and in the uninfected control ISA Brown cocks (325.00 ± 210.00 pg/ml). Thereafter, the mean INF—γ concentration value of the infected group started declining from Day 21 p.i. up to the end of the experiment.

DISCUSSION AND CONCLUSION

The clinical findings observed in the present study indicate that the infected ISA Brown cocks developed severe fowl typhoid infection, which was marked by depression, ruffled feathers, huddling, reduction in body weight, emaciation, somnolence, loss of appetite, blood stained greenish-yellow diarrhoea, paleness of the comb and wattle. The clinical signs observed in the infected ISA Brown cocks in this present study were consistent with the findings of previous authors (Garcia et al., 2010; Soufy et al.,2016). The incubation period observed in the infected ISA Brown cocks in this present study was 8 days, which contrasts 3 days reported by Garcia et al. (2010) and 7 days by Chiroma et al. (2018). The difference in the incubation period could be due to several factors, which include differences in the infective dose of the bacteria, the pathogenicity of the bacteria, the nature of the host-pathogen interaction, virulence

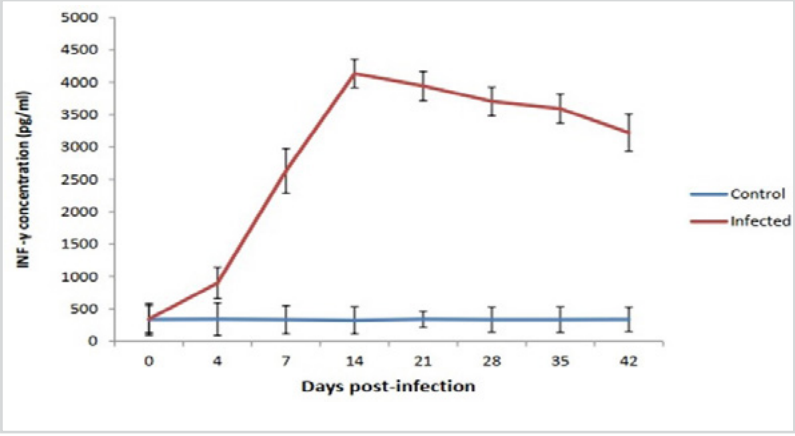

Figure 5

Mean (± SEM) of INF—γ concentration of ISA Brown cocks experimentally-infected with *Salmonella enterica* serovar gallinarum, and control cocks

of the organism, the capacity of the host to build adequate immune response against the pathogen (Lahiri et al., 2010; Sreekantapuram et al.,2021). The mortality rate recorded in the infected ISA Brown cocks in this study was 35%, which is in accordance with those of previous reports made by other authors (Paiva et al., 2009; Sannat et al., 2017; Chiroma et al., 2018). The mortality started 8 days p.i. with the range of 10-100%. Invasion of the organism via the gastrointestinal tract, thereby establishing systemic infection, might have accounted for the recorded mortalities. The ability of *Salmonella* to invade macrophages and probably dendritic cells and their translocation to the spleen and liverwhere multiplication occurs, is responsible for the clinical signs of the disease (Chappell et al., 2009).

The significant reduction in heterophil, lymphocyte and total white blood cell count observed initially in the infected group when compared with the control counterparts may be a result of cytopathic effect of *Salmonella* gallinarum lipopolysaccharides (LPS) on leukocytes of the infected ISA Brown cocks, thereby inducing cell lysis. This finding is in accordance with the reports of Chiroma et al. *(*2017), as cited earlier by Lam and Munn (2002). The significant reduction in heterophil value in the *Salmonella* gallinarum-infected ISA Brown cocks may be due to interaction of heterophils with the bacteria (Genovese et al., 2013; Sreekantapuram et al.,2021). While the significant increase *(*$p<0.05$) in total white blood cell, heterophil and lymphocyte counts observed on Day 14 post infection in the infected group contributed to leukocytosis as earlier reported by Berchieri (2000), and may be a result of fast multiplication of *Salmonella* gallinarum inside the phagocytes, with subsequent cell lysis and release of the bacterium into the extracellular compartment, which evoked strong immune response, thereby inducing antigenantibody reaction that is responsible for the clinical signs of fowl typhoid. The findings in this current study support those of previous authors Brar et al., 2000; Morguli, 2002., Freitas Neto et al., 2007. Abou Zeid et al., 2020).

The significant increase in mean serum concentration of IL-1β and IFNγ in the infected gr oup, which occurred on Days 4 and 7 p.i., respectively, and reaching its maximum level on Day 14 post infection as observed in this study, coincided with increase in heterophils, lymphocytes and total white blood cell value on Day 14 post infection. The IL-1β responded significantly to the infection on Day 4 post infection which was then followed by IFN-γ, which responded on Day 7 p.i.. The increase in serum concentration of these proinflammatory cytokines in the infected group, when compared with the control group, may probably be due to the fact that these cytokines play an important role in the immune response to *Salmonella* gallinarum infection. The significant increase in the serum concentration of IL-1β and IFN-γ in the infected ISA Brown cocks observed in this study is in part similar to the findings of Ojima et al. (2021) who reported upregulation of IFN-γ in the spleen of chickens infected with *Salmonella* gallinarum between Days 4 and 6 p.i..

A similar suggestion was made in Newcastle disease in which an increase in both IL 1β and IFN-γ was observed in the testes of roosters experimentally-infected with Newcastle disease virus (Rehman et al., 2020). Thakur et al., (2020) also reported significant increase *(*$P<0.05$) in both IL-1β and IFN-γ, following an oral infection of chicken with oocysts of *Eimeriaadenoeides* on Day 7 p.i.. Additionally, Zhang et al. (2012) also recorded a significant increase in IL-1β and IFN-γ genes in the day-old chickens 3-hour post infection. Similar findings were also reported by Al-Idreesi et al. (2013) who observed a significantly higher level of IFN-γ in the serum samples of ISA Brown chicken infected with *Eimeriatenella*. However, these findings conflict with findings in the reports of Ying et al. (2020) who observed decrease expression of both IL-1β and IFN-γ in the caecal tonsil and spleen of chicken infected with *Salmonella* gallinarum when compared with the control group. In this study, the period of increase in the serum concentrations of IL-1β and IFN-γ coincides with the period of increase of heterophil and lymphocyte values in the infected

cocks. In the chicken intestine, IL 1β and IFNγ are produced as a result of microorganism invasion-attracting heterophils to the site of infection (Ijaz et al., 2021). Upon contact with pathogens, heterophils are activated through the interaction of Toll-Like Receptors with bacterial ligands such as as lipopolysaccharide and peptidoglycan (Kogut et al., 2005). This activation of heterophils results in a sequence of events including phagocytosis, oxidative burst, degranulation, and IL-1β production. Both IL-1β and IFN-γ play a critical role in promoting immune responses, which are essential for promoting protective responses against invading pathogens (Eckmann et al., 2001). In the present study, the *Salmonella* gallinarum- infected cocks increased the IL-1β, and IFN-γ blood serum concentrations during the infective cycle. The increase in hetero- phil, lymphocyte and white blood cell value observed in the infected group on Day 14 p.i. in this present study may be possibly associated with the level of IL-1β and IFN-γ blood serum concentrations at Day 14 post infection. In general, the heterophil, lymphocyte and white blood cell value in the challanged cocks in this present study were negatively correlated with IL-1β and IFN-γ blood serum concentrations at Day 0 up to Day 7 p.i., but positively correlated with IL-1β and IFNγ blood serum concentrations at Day 14 p.i., indicating that chickens with high values of heterophils, lymphocytes and white blood cells have a more robust and appropriate inflammatory response to *Salmonella* gallinarum infection. It is possible to suggest that chickens with the low heterophil, lymphocyte and white blood cell value display a reduced heterophil cells number but with an enhanced function of this particular avian immune cell.

In conclusion, this study confirmed that the increase in the serum concentrations of IL-1β and IFN-γ in the cocks played a very critical role in protection against *Salmonella* gallinarum.

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COMPLIANCE WITH ETHICAL STANDARDS

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

CONTRIBUTION

Concept and Design- AS, OBS, and PHM; Supervison;-AS, OBS, and PHM; Funding- CMA; Materials and Data collection and or processing CMA, JJG and AH; Literature review and Analysis and or interpretation of the data- CMA, JJG and EI; Writing and Critical review- CMA, AS, OBS, and PHM.

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ANALIZA POJEDINIH INTERLEUKINA I LEUKOGRAMA KOD ISA BROWN PIJETLOVA EKSPERIMENTALNO ZARAŽENIH SA *SALMONELLOM GALLINARUM*

SAŽETAK

Cilj istraživanja jeste ispitati profile IL- 1β, INF-γ i leukogram kod pijetlova eksperimentalno zaraženih sa *Salmonellom* gallinarum. Za istraživanje je korišteno ukupno 40 pijetlova, 20 inficiranih i 20 kontrolnih. Svakom pijetlu iz inficirane grupe je oralno apliciran 1 ml inokuluma 9.0 x 108 CFU/ml *Salmonelle* gallinarum. Nakon što su prikupljeni krvni uzorci izvršena je analiza leukograma, a serumski uzorci su analizirani na IL-Iβ i INF γ. Postojale su statistički signifikantne razlike ($p < 0.05$) u srednjem apsolutnom broju leukocita, broju heterofila i broju limfocita između inficirane i kontrolne grupe. Najviše vrijednosti leukocita (14.5 ± 0.60 x 109/1), heterofila (4.4 ± 0.31 x 109/1) i limfocita (10.4±0.55 x 109/1) su zabilježene u inficiranoj grupi 21. dana (p.i.). Postojale su statistički signifikantne razlike ($p < 0.05$) u srednjim koncentracijama IL-1β i IFN-γ u serumu između inficirane i kontrolne grupe 4. dana (185±46.0 pg/ml) i 7. dana p.i. (2634± 342 pg/ml). Najviše i najniže zabilježene srednje vrijednosti koncentracije IL-1β u serumu su iznosile 695±1.0 pg/ml i 185±46.0 pg/ml u inficiranoj grupi 14. i 7. dana p.i., a IFN γ 4134±217 pg/ml i 2634 ± 342 pg/ml u inficiranoj grupi 15. i 7. dana p.i. U zaključku, povišene vrijednosti serumskog IL 1β i INF gamma kod inficiranih pijetlova može igrati odlučujuću ulogu u ispoljavanju pojačanog imunološkog odgovora kod infekcije *Salmonellom* gallinarum.

Ključne riječi: Inokulum, ISA Brown, pijetlovi, *Salmonella*