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Experimental infection of commercial layers with wild or attenuated Salmonella Gallinarum mutant strains: anatomic pathology, total blood cell count and serum protein levels

■Author(s)

Garcia KO[1*]
Berchieri Jr. A[1]
Santana AM[2]
Alarcon MFF[3]
Freitas Neto OC[1]
Fagliari JJ[2]

[1] Department of Veterinary Pathology;
[2] Department of Veterinary Clinical Sciences;
[3] Department of Animal Physiology; Sao Paulo State University, Campus of Jaboticabal (College of Agricultural and Veterinarian Sciences); Via de Acesso Prof. Paulo Donato Castellane, s/n; 14884-900 – Jaboticabal - SP – Brazil.

■Mail Adress

*Corresponding author e-mail address
Tel: 55 17 3121 9323 or 17 9745 7309.
E-mail: klebergarcia7@hotmail.com

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ABSTRACT

The aim of the present study was to comparatively evaluate hemogram, blood serum components and anatomopathologic alterations in commercial layers experimentally challenged with an attenuated vaccine candidate strain (SGΔ*cob*SΔ*cbi*A) and other two pathogenic strains (SGΔ*cob*S and SGNal^r) of Gallinarum (SG). In total, 280 commercial layers were randomly divided into 4 groups (G1, G2, G3 and G4). At five days of age, birds from groups G1 received approximately 10^7 colony forming units (CFU) of SGΔ*cob*S; meanwhile birds from group G2 and G3 received the same dose of SGNal^r and SGΔ*cob*SΔ*cbi*A, respectively. Birds from G4 were not infected. At 24 hours before (DBI) and 24 hours after (1 DAI), and three (3 DAI), five (5 DAI), seven (7 DAI) ten (10 DAI), and fifteen (15 DAI) days after the infection, 10 birds of each group were humanely killed and blood samples collected to hematological and serum tests. Samples of liver, spleen, thymus, bursa of Fabricius, kidney and heart were also collected for the histological examination. Birds inoculated with SGΔ*cob*S and SGNal^r showed similar alterations in hemogram, blood serum components and anatomopathologic exams. On the other hand, the exams of birds inoculated with SGΔ*cob*SΔ*cbi*A strain were similar to those of the uninfected birds. However, changes could be noticed in levels of uric acid and cholesterol during the course of the infection of birds from G3. Decrease in levels of light IgG 3 DAI was also observed in birds from this group. Pyknosis in kidney cells was a microscopic alteration found in birds from G3. Further studies must be done to verify if these alterations will not interfere in the performance of the vaccinate birds with SGΔ*cob*SΔ*cbi*A strain.

INTRODUCTION

The concern about avian biosecurity on the world stage has been remarkable, which demands a stringent control for the prevention of the diseases and, among the diseases, salmonellosis is a concern, being essential its control. One of the main salmonellosis is fowl typhoid, caused by *Salmonella* Gallinarum (SG), which is a severe systemic disease that affects commercial poultry and other galliform species. When it infects a poultry farm, SG causes relevant economic losses due to high morbidity and mortality rates, as well as drops in egg production. In the affected poultry farms, the mortality may be between 10 and 80% (Berchieri Júnior & Freitas Neto, 2009).

Due to the importance of fowl typhoid to the avian production, understanding of pathobiology and improvements in control measures of this disease are needed. As a result, in the past 15 to 20 years, there has been an explosion of genetic and immunological information on the biology of the fowl typhoid (Barrow & Freitas Neto, 2011).

This study was elaborated to evaluate the clinical signs and macroscopical, histopathological and hematological parameters as well as metabolic alterations of the blood serum including acute-phase protein serum concentrations in birds experimentally infected by nalidixic-acid resistant *Salmonella* Gallinarum strain (SGNal^r) and mutant strains containing a *cob*S and *cob*S*cbi*A deleted genes. These genes are part of that necessary to *Salmonella* in an anaerobic environment. According to Paiva *et al*. (2009), when the simultaneous deletion of *cob*S and *cbi*A genes was carried out, *Salmonella* Gallinarum double mutant was unable to provoke mortality in susceptible chickens, however the singular deletion in *cob*S gene did not interfere with the pothogenicity of *Salmonella* Gallinarum; it provoked even higher mortality than the wild strain of SG.

Penha Filho *et al*. (2010) showed that SGΔ*cob*SΔ*cbi*A strain has a potential to be used as vaccine against *Salmonella* Gallinarum and *and Salmonella Enteriditis.* An ideal vaccine should induce protection with a minimum of side effects in the host. SGΔ*cob*SΔ*cbi*A does not cause mortality in chickens and apparently no clinical signs of fowl typhoid. However, evaluations of hemogram, blood serum components and microscopic alterations which could point out changes provoked by subclinical infection of this strain have not been assessed so far. For this reason the present study was carried out.

MATERIAL AND METHODS

Birds and housing

A number of 280 commercial semi-heavy (brown) layers, considered susceptible to the infection by *Salmonella* Gallinarum were used. Blood samples were collected at arrival at the experimental facilities for the serological test (fast serum agglutination test using a commercial *Salmonella* Pullorum antigen). Fecal swabs were taken from the transport crate for the bacteriological detection of *Salmonella*, in order to ensure that birds were free from pathogens that might compromise the experiment (Zancan *et al*., 2000). The results showed that all birds were negative for *Salmonella* Gallinarum. Birds were then divided into four groups (G1, G2, G3 and G4) containing seventy animals each, and housed in battery cages, located at the isolation units of the Laboratory of Veterinary Pathology of the Department of Veterinary Pathology, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista (FCAVJ-UNESP), Brazil. Birds received water and feed *ad libitum*.

Preparation of the mutants and SGNal^r strains

The construction of the mutants, conjugation and transduction followed Molecular Cloning protocols (Sambrook & Russel, 2001). The double deletion (*cob*S and *cbi*A) and single deletion (*cob*S) SG mutant strains were previously prepared by Paiva *et al*., (2009).

A nalidixic-acid resistant *Salmonella* Gallinarum strain (SGNal^r) is pathogenic isolate previously used by Berchieri Júnior *et al*. (2001). This strain was prepared and kept at the Department of Veterinary Pathology (FCAVJUNESP).

Preparation of the inoculum

The bacterium strains were cultivated in nutrient Luria Bertani (LB) broth (Invitrogen N^o 12780-052), incubated in a shaking incubator (100 strokes/min) at 37°C, for 24h. Samples of the 0.1mL of each culture were diluted in 0.9mL phosphate-buffered saline, pH 7.4 (PBS) in the 1:10 proportion. After that, cultures contained approximately $3.3x10^8$ CFU.

Experimental procedures

Birds from G1, G2 and G3 received 0.2mL of inoculum containing $3.3x10^8$ CFU/mL of SGΔ*cob*S, SGNal^r and SGΔ*cob*SΔ*cbi*A, respectively, directly into the crop using a metal gavage neddle. Birds from G4 did not receive any inoculum (control group). All birds were challenged at five days of age and humanely killed to obtain blood samples. For the sample collection, euthanasia was carried out (ten birds at a time in each group) 24 hours before (DBI) and after (first DAI) infection, and 3 (third DAI), 5 (fifth DAI), 7 (seventh DAI), 10 (tenth DAI) and 15 (fifteenth DAI) days after infection.

This study was approved by the animal welfare commission (protocol number CEBEA-02666-08) due to be necessary the inoculation of harmful strain it causes damage for the avian health as well as sacrificed them on the days of the sample collection.

Clinical evaluation

Birds were submitted to a physical examination twice a day (in the morning and afternoon) and, in the case of death, mortality was recorded.

Laboratory analysis

The laboratorial exams were undertaken in the Laboratory for Research Support of the Clinics and Surgery Department and the Laboratory of Ornitopathology from the Veterinary Pathology Department of FCAV/UNESP – Jaboticabal campus.

Blood collection

Birds were rendered unconscious, and then submitted to euthanasia. Blood samples were collected from the cervical vein. This procedure was made in all days of the sample collection. For the hematological analysis blood samples were collected in tubes containing 10% EDTA. Blood samples for the biochemical exams and serum protein profile were collected in tubes without anticoagulant; after coagulation and retraction of the clot, serum samples were obtained for analysis procedure.

Hemogram

The red and white blood cells counts were carried out in a Neubauer hemocytometer (Natt & Herrick, 1952). Blood smears were prepared for differential leukocyte count, and stained by the Rosenfeld method (Lucas & Jamroz, 1961). The hematocrit was determined using microhematocrit method (Jain, 1983).

Biochemical exams of blood serum

Albumin (bromocresol green method), triglycerides (Trinder method), cholesterol (Trinder method) and uric acid (Trinder method), as well as the activities of *gamma-glutamyltransferase* (GGT) (Szasz modified method) and *aspartate-aminotransferase* (AST) (Reitman-Frankel method) were accomplished with the use of commercial kits (Labtest). Sample readings were made in semiautomatic spectrophotometer (LABQUEST).

Serum protein concentrations

Serum protein electrophoresis was carried out in sodium dodecyl sulfate acrylamide gel (SDS-PAGE), as proposed by Weber & Osborn (1969). The gel was stained for 10 minutes in 0.25% Coomassie blue solution and then destained in 7% acetic acid, until protein fractions were clear. The concentrations of those proteins were determined in a scanning densitometer (Shimadzu CS9301, Tokyo). A marker solution with different molecular weights (Sigma S8445), as well as, purified ceruloplasmin, haptoglobin, antitrypsin, transferrin, and IgG proteins were used as references.

Gross and histopathological exams

After blood collection and posterior euthanasia, the aspect, size and color of the organs were examined, using the control group as reference. In each moment of sample collection, fragments of liver, spleen, thymus, bursa of Fabricius, heart and kidneys were obtained for histopathological examination. Fragments of organs were fixed in 10% phosphate buffered formalin, pH 7.2, at a ratio of 20:1 (buffer:organ fragment) and processed for paraffin embedding according to Behmer *et al*. (1976). Sections of 5µm thickness were stained with hematoxilin-eosin and analyzed under a light microscope equipped with a digital camera (NICKON ECLIPSE E200 + COOLPIX 5400).

Statistical analysis

A completely randomized experimental design with in a 4x7 factorial arrangement (infection levels and pre and post-infection days) was used for statistical analysis. Data were submitted to analysis of variance using SAS statistical program, and means were compared by Tukey's test (P <0.05).

RESULTS AND DISCUSSION

Clinical Evaluation

Starting at fourth day after infection 11 birds from G1 and 15 birds from G2 showed typical clinical signs of fowl typhoid such as apathy, prostration, dropped wings, anorexia, dehydration and yellow-greenish bloody diarrhea. This clinical picture is similar to the one observed by Freitas Neto *et al*. (2007) and Berchieri Júnior & Freitas Neto (2009). On the fifth and sixth day after infection (DAI) four birds from group G1 and 12 birds from G2 had already died. At the end of the experiment, 15 birds from G1 and 19 birds from G2 died.

Salmonella infection stimulates the induction and release of proinflammatory mediators (Wyant *et al*., 1999). Lipopolysaccharide (LPS), a bacterial endotoxin, constituent of the outer membrane of Gram-negative bacteria, can initiate systemic inflammatory response by activating a variety of monocytic cells and other leukocytes, stimulating the release of proinflammatory cytokines. Systemic release of LPS into blood circulation causes endotoxemia, and can lead to organ dysfunction and death (Kwong-Fai Wong *et al*. 2007). In our study, it is likely that the typical clinical signs of fowl typhoid and posterior deaths of the birds from G1 and G2 are due to an intense inflammatory process, since these groups of birds were inoculated with highly pathogenic strains.

On the other hand, no bird from G3 died or showed typical clinical signs of fowl typhoid, behaving similarly to G4. These results corroborate with those observed by Paiva *et al*. (2009) and Penha Filho *et al*. (2010) studying birds with SGΔ*cob*SΔ*cbi*A strains, and indicate that deletion of the two genes produces an attenuated mutant strain.

Hemogram

The red blood cell counts (RBC) results are presented in Table 1. The major profile changes were observed in erythrocytes of G1 and G2 birds that showed anemia in the most advanced phases of the infection, with decrease in the erythrocytes and hematocrit values, typical results of a bacterial infection like fowl typhoid, firstly due to a anemia of the inflammatory disease (Alencar *et al*., 2002) and then after the hemorrhagic anemia (Stockham, 2000). The avian anemia should be caused by hemorrhage, erythrocytes destruction or by the erythrocytes reduction production. In the salmonellosis case, some studies shows, in the most of the cases, erythrocytes reduction production (Campbell & Dein, 1984) and when the disease is specifically fowl typhoid, the anemia might be occur in the acute phase of infection. The anemia status in birds from G1 and G2 was observed in the most advanced phases of infection as shows in the table 1, with RBC and hematocrit decrease. Similar results were verified in other studies (Allan & Duffus, 1971; Christensen *et al*., 1996; Cardoso *et al*., 2003; Freitas Neto *et al*., 2007). Birds from G3 showed no relevant alterations as well as birds from G4 (control group).

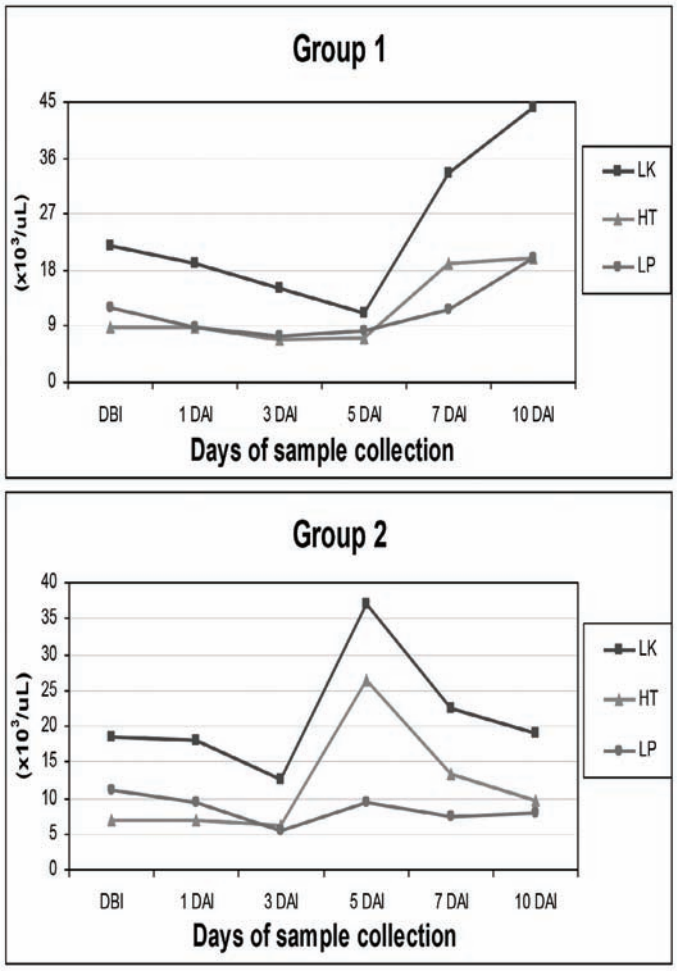

Figure 1 - Mean leukocyte (LK) , heterophil (HT) and lymphocyte (LP) counts of brown layers experimentally infected with SGΔcobS and SGNal^r 24 hours before (dbi) inoculation and 24 hours (1 dpi), and three days (3 dpi), five days (5 dpi), seven days (7 dpi) and ten days (10 dpi) post inoculation.

White blood cell count

The findings about white blood cell counts (WBC) are presented in Table 2. WBC initially decreased in the third DAI in birds from G1 and G2 due to a reduction in lymphocyte and heterophil counts when compared with DBI (Figure 1). Garcia *et al*. (2009), studying commercial layers experimentally infected with *Salmonella* Gallinarum strain observed WBC decrease in the first DAI. In the present study, the initial WBC decrease may have been caused by the cytopathic effect of bacterial LPS on poultry leukocytes due to a first contact between SG and blood cells, causing cell lysis. In studies using a mixture of heterophils and *Salmonella* Typhimurium, Lam & Munn (2002) detected changes in heterophil morphology and fast disappearance of that cell type, suggesting that the contact with LPS caused heterophil degranulation. This first contact between bacterium and host host was enough to cause, in the fourth DAI, typical clinical signs of fowl typhoid in birds of both groups (11 birds from G1 and 15 birds from G2). After an initial decrease in the third DAI, there was a recovery in the number of circulating leukocytes in birds from G1, that presented evident leukocytosis at seventh DAI (1.5 times higher than DBI and 2.2 times higher than fifth DAI), characterized by an increase in lymphocyte and heterophil counts, especially heterophil counts (2.3 times higher than 1 DAI and 2.7 times higher than 5 DAI), leading to an inversion of the heterophil:lymphocyte ratio (Figure 1). In G2 the recovery in the number of circulating leukocyte started earlier and leukocytosis was observed at fifth DAI (2 times comparing to DBI and control group), also due to an increase of lymphocyte and heterophil counts, especially heterophil (3.8 times comparing to 1 DAI and 3.5 times comparing to control group), leading, as in G1, to an inversion of the heterophil:lymphocyte ratio. In G2, after a high leukocyte count at fifth DAI, there was a reduction at the final course of the study (seventh DAI and tenth DAI), due to a decrease in heterophil and lymphocyte counts, especially heterophil, which returned leukocytes to basal levels (Figure 1).

The ability of some lineage birds to inhibit infection caused by SG is related, among others, to two mechanisms: inhibition of multiplication inside macrophages and capacity to inhibit the release of

Table 1 - Erythrocyte and hematocrit means in brown layers experimentally infected with *Salmonella* Gallinarum Nal^r, Δ*cob*S, or Δ*cob*SΔ*cbi*A.

Parameters	Days of sample collection	Groups				CV
		1	2	3	4	%
Reference values	1 dbi	2.04 a	2.02 a	1.95	2.14	12.4
	1 dpi	2.20 a	2.01 a	2.17	2.10	21.0
	3 dpi	1.81 ab	1.68 ab	2.12	2.14	15.9
Erythrocytes/μL	5 dpi	1.47 bcB	1.50 abB	2.30 A	2.22 A	10.5
(x106)	7 dpi	1.28 cB	1.89 abA	2.22 A	2.05 A	14.2
	10 dpi	1.27 cB	1.28 bB	2.10 A	2.01 A	10.4
	15 dpi	-	-	1.97	2.05	27.7
CV (%)		15.2	15.9	15.5	12.3	
Reference values	1 dbi	31.4 a	29.6 a	31.0	29.8	13.4
	1 dpi	27.2 ab	28.7 ab	26.4	26.0	17.3
	3 dpi	19.6 bcB	22.4 abB	28.6 A	29.5A	12.8
Hematocrit	5 dpi	19.4 cB	19.8 abB	29.0 A	26.8A	15.9
(%)	7 dpi	22.0 bcAB	17.8 abB	27.4 AB	28.8A	15.8
	10 dpi	19.0 cB	18.0 bB	28.0 AB	29.4A	15.4
	15 dpi	*	*	29.0	28.0	7.20
CV (%)		11.9	16.0	17.6	16.8	

Means followed by different small letters in the same column or by different capital letters in the same row are significantly different by Tukey's test (P<0,05)
Group 1= oral inoculation with SGNalrΔ*cobS*; Group 2= oral inoculation with SGNalr; Group 3= oral inoculation with SGNalrΔ*cob*SΔ*cbi*A; Group 4= not inoculated (control group).
*= Groups 1 and 2 – all birds had already died; CV= Coefficient of variation; dbi= day before inoculation; dpi= day post-inoculation.

Table 2 - Leukocyte, lymphocyte and heterophil mean counts of brown layers experimentally infected with *Salmonella* Gallinarum Nalr, Δ*cob*S, or Δ*cob*SΔ*cbi*A strains

Parameters	Days of sample collection	Groups				CV
		1	2	3	4	%
Reference values	1 dbi	21.9 ^{c}	18.6 ^{b}	18.7	18.8	14.4
	1 dpi	19.3 ^{dc}	17.9 ^{b}	17.6	20.1	20.4
	3 dpi	15.0 ^{dcBC}	12.6 ^{cC}	20.4 ^{AB}	24.2 ^{A}	21.4
Leukocytes/μL	5 dpi	11.0 ^{dC}	37.1 ^{aA}	19.8 ^{B}	17.9 ^{BC}	25.2
(x 10^3)	7 dpi	33.6 ^{bA}	22.3 ^{abB}	20.7 ^{B}	19.9 ^{B}	18.0
	10 dpi	44.1 ^{aA}	19.0 ^{bB}	21.8 ^{B}	21.3 ^{B}	18.9
	15 dpi	-	-	18.4	18.8	14.3
CV (%)	%	17.8	20.0	17.9	17.7	
Reference values	1 dbi	11.9 ^{b}	11.0 ^{a}	10.33	9.91	16.9
	1 dpi	8.72 ^{b}	9.36 ^{a}	9.36	10.30	19.0
	3 dpi	7.36 ^{cB}	5.47B ^{bB}	9.80 ^{AB}	13.13 ^{A}	17.9
Lymphocytes/μL	5 dpi	8.24 ^{bcB}	9.44 ^{aA}	9.97 ^{A}	9.26 ^{A}	26.0
(x 10^3)	7 dpi	11.8 ^{b}	7.37 ^{b}	10.15	11.66	23.7
	10 dpi	19.8 ^{aA}	7.98 ^{abC}	12.59 ^{B}	11.75 ^{B}	17.1
	15 dpi	*	*	10.19	10.53	19.4
CV (%)		16.1	20.5	18.3	24.4	
Reference values	1 dbi	8.80^{b}	6.91^{b}	6.97	7.62	19.7
	1 dpi	8.90^{b}	6.92^{b}	7.35	8.32	23.3
	3 dpi	6.78^{bAB}	6.11^{bB}	9.85^{A}	9.87^{A}	18.6
Heterophils/μL	5 dpi	7.03^{bB}	26.4^{aA}	9.63^{B}	7.62^{B}	25.6
(x 10^3)	7 dpi	19.1^{aA}	13.3^{bB}	9.19^{BC}	7.15^{C}	22.4
	10 dpi	19.9^{aA}	9.69^{bB}	8.14^{B}	8.58^{B}	19.9
	15 dpi	*	*	7.59	7.25	22.6
CV (%)		17.2	24.2	16.8	22.0	

Means followed by different small letters in the same column or by different capital letters in the same row are significantly different by Tukey's test ($p < 0.05$).
Group 1= oral inoculation with SGNalrΔ*cobS*; Group 2= oral inoculation with SGNalr; Group 3= oral inoculation with SGNalrΔ*cob*SΔ*cbi*A; Group 4= not inoculated (control group).
*= Groups 1 and 2 – all birds had already died; CV= Coefficient of variation; dbi= day before inoculation; dpi= day post-inoculation;

the agent in the blood (Bumstead & Barrow, 1993; Wigley *et al*., 2002). Therefore, the light lineages are considered more resistant, while the semi-heavy and heavy are considered more susceptible to the disease (Freitas Neto *et al*., 2007). In this study, the utilization of more susceptible lineages suggests, so as Berchieri Júnior (2000), that there is leukocytosis because, during the acute-phase of fowl typhoid, there is a fast multiplication of *Salmonella* Gallinarum inside the phagocytes, causing cell lysis and release of the bacterium into the extracellular compartment. This stimulates a strong immune response and induces a type of antigen-antibody reaction that causes an endotoxemia that can be responsible for stronger clinical signs and death of the birds. This can be a possible explanation in this study, considering that in G1 and G2, leukocytosis was followed by worsening of symptoms and death of the birds. At the same time, the possible presence of the bacterium in target organs, such as the liver, spleen, kidneys, thymus, and heart, may stimulate the production and release of leukocytes into the blood stream.

Leukocytes blood levels showed no relevant alterations in G3, remaining in basal values during all the experiment, so as the control group. WBC in G3 differed from those in G1 on the seventh DAI (2.1 times less heterophils than G1) and in G2 on the fifth DAI (2.7 times less heterophils than G2), moments of leukocytosis in G1 and G2. These results in G3 reinforces that deletion of the two genes produces an attenuated mutant strain.

Serum Biochemical Profile

The serum biochemical parameters evaluation has a great importance about vital metabolic activity that occurs in the corporal fluids of sick animals. According to Kaneko *et al*. (2009), the blood serum biochemical exams evaluations in animals allow precocious diagnosis of the subclinical symptomatology. As shows in Table 3, G1 and G2 groups presented higher alterations when compared to G3 and G4, however, some alterations was also found in G3.

Albumin, which is synthesized only in the liver, is the main responsible for maintain the oncotic blood pressure; it may occur hypoalbuminemia in cases of hepatic insufficiency, malnutrition and gastrointestinal disturbances within other conditions (Meyer *et al*., 1995). In the seventh DAI starts serum albumin profile reduction at G1 and G2, where the serum level reduces at a half when compared with DBI. Similar results were reported by Garcia *et al*., (2010) who observed serum albumin reduction in susceptible birds infected with SG and they connected the results with the incapacity of albumin production by the liver due to the harm caused by *Salmonella*. This reduction coincided with the day which the histopathological exam revealed hepatic degeneration, started at fifth DAI in G1 and G2 groups as can be observed at Figures 3 and 4. Although albumin half-life is superior to the period studied, the decrease in the levels of this protein might be due to hepatic disturbances observed in these groups, or even because it is considered a negative acute phase protein (Kaneko *et al*., 2009).

As the main product of nitrogen metabolism, uric acid is responsible for excretion through the urine, about 60 to 80% of nitrogen excreted by birds (Schimidt *et al*., 2007). The most relevant uric acid alterations were observed in G1 and G3 with a mild reduction during the course of the infection. According to Schimidt *et al*. (2007), uric acid is synthesized in both liver and kidney, so the hypothesis is that for the G1, the reduction might be related with the harm in the liver and for G3, as the reduction starting at tenth DAI, it can be related with renal disturbances also in the tenth DAI, described in the histopathological exam.

The evaluation of the triglycerides and cholesterol serum concentrations has a great importance to determinate the function of many metabolic activities. The significant alterations observed in the cholesterol serum level shows decrease in G1 and G2 as well as oscillations in G3 during the course of the infection. With regard to triglycerides serum level, the alterations were observed just in G1 and G2, with initial decrease in the third DAI, with a concentration four times lower in the tenth DAI when compared with DBI and tenth DAI of the G3 and G4 as shows in Table 3. Similar results were found by Garcia *et al*. (2010), who observed decrease in the triglycerides serum level in the similar way at cholesterol reduction in the infected birds with SG. These results are pertinent, as besides less feed ingestion by the birds, the lipid metabolism is compromised due to the hepatic insufficiency showed by the macroscopical and histopathological exams, as the liver has a main importance in the lipid metabolism.

The determination of serum enzymes activity allows to evaluate many organs and tissues function. About liver function, GGT and AST are good indicators. The GGT and AST alterations are restricted to G1 and G2. The alterations of GGT and AST serum activities might be related with hepatic disturbances, however, it activities are not hepatic-specific, thus can be altered

Table 3 - Albumin, uric acid, cholesterol, and triglyceride serum levels, and GGT and AST serum activities of brown layers experimentally infected with *Salmonella* Gallinarum Nalr, Δ*cob*S, or Δ*cob*SΔ*cbi*A strains.

Parameters	Days of sample collection	Groups				CV
		1	2	3	4	%
Reference values	**1 dbi**	1.76 ^{a}	1.72 ^{ab}	1.51	1.96	29.2
	1 dpi	1.87 ^{a}	2.09 ^{a}	1.72	1.95	21.3
	3 dpi	1.94 ^{ab}	2.21 ^{abc}	1.70	1.70	25.7
Albumin	**5 dpi**	2.37 ^{aA}	1.31 ^{abcB}	1.60 ^{AB}	1.73 ^{AB}	23.2
(g/dL)	**7 dpi**	1.18 ^{abB}	1.11 ^{bcB}	1.54 ^{AB}	1.65 ^{A}	25.5
	10 dpi	0.91 ^{bBC}	0.74 ^{cC}	1.51 ^{AB}	1.72 ^{A}	18.9
	15 dpi	-	-	1.80	1.76	21.8
CV (%)		20.9	22.7	29.2	17.5	
Reference values	**1 dbi**	8.70 ^{a}	6.74 ^{a}	8.20 ^{a}	6.55	23.9
	1 dpi	7.42 ^{ab}	7.19 ^{a}	7.53 ^{a}	6.56	24.6
	3 dpi	6.16 ^{ab}	7.43 ^{a}	6.30 ^{abc}	7.25	23.4
Uric Acid	**5 dpi**	5.95 ^{abAB}	4.96 ^{bB}	7.02 ^{abA}	6.50 ^{B}	21.0
(mg/dL)	**7 dpi**	6.65 ^{ab}	5.94 ^{b}	6.69 ^{ab}	6.88	30.3
	10 dpi	4.85 ^{bB}	6.61 ^{abA}	4.50 ^{cB}	7.72 ^{A}	24.1
	15 dpi	*	*	5.45 ^{bcA}	6.48 ^{B}	16.4
CV (%)		28.8	34.6	23.1	28.9	
Reference values	**1 dbi**	348 ^{a}	360 ^{a}	366 ^{ab}	360	20.2
	1 dpi	296 ^{aA}	230 ^{abB}	328 ^{abcA}	302 ^{A}	18.1
	3 dpi	178 ^{bB}	173 ^{bB}	280 ^{bcA}	307 ^{A}	23.8
Cholesterol	**5 dpi**	172 ^{bB}	241 ^{abAB}	274 ^{bcA}	321 ^{A}	32.4
(mg/dL)	**7 dpi**	145 ^{bB}	150 ^{bB}	401 ^{aA}	316 ^{A}	26.1
	10 dpi	145 ^{bB}	164 ^{bB}	233 ^{cAB}	320 ^{A}	20.8
	15 dpi	*	*	286 ^{bc}	289	18.1
CV (%)	%	28.2	30.8	25.5	19.5	
Reference values	**1 dbi**	179 ^{a}	169 ^{ab}	188	149 ^{ab}	17.8
	1 dpi	138 ^{abB}	194 ^{aA}	196 ^{A}	169 ^{abAB}	25.6
	3 dpi	96.8 ^{bcB}	91.7 ^{bcB}	170 ^{A}	189 ^{aA}	20.1
Triglycerides	**5 dpi**	71.2 ^{cB}	72 ^{cB}	141 ^{A}	146 ^{bA}	12.8
(mg/dL)	**7 dpi**	64.6 ^{cB}	52.4 ^{cB}	139 ^{A}	153 ^{abA}	26.6
	10 dpi	46.8 ^{cB}	40.8 ^{cB}	153 ^{A}	163 ^{abA}	20.4
	15 dpi	-	-	177	131 ^{b}	17.7
CV (%)		28.6	16.6	12.9	18.3	
Reference values	**1 dbi**	8.18 ^{b}	7.65 ^{b}	8.50	7.37	26.5
	1 dpi	7.65 ^{bB}	8.41 ^{bAB}	6.88 ^{B}	9.18 ^{A}	33.1
	3 dpi	13.0 ^{abA}	9.18 ^{bB}	7.65 ^{B}	9.94 ^{B}	24.1
GGT	**5 dpi**	13.0 ^{abAB}	16.1 ^{abA}	8.88 ^{B}	7.58 ^{B}	28.8
(U/L)	**7 dpi**	13.8 ^{abA}	13.0 ^{abA}	10.7 ^{B}	9.94 ^{B}	25.9
	10 dpi	18.4 ^{aAB}	22.9 ^{aA}	9.88 ^{C}	9.94 ^{C}	32.9
	15 dpi	*	*	9.94	7.65	30.6
CV (%)		17.3	23.9	27.5	30.8	
Reference values	**1 dbi**	214 ^{c}	197 ^{c}	209	218	33.8
	1 dpi	177 ^{cB}	193 ^{c}	222 ^{AB}	282 ^{A}	27.8
	3 dpi	281 ^{b}	224 ^{bc}	224	232	18.7
AST	**5 dpi**	302 ^{b}	272 ^{bB}	247	261	18.6
(U/L)	**7 dpi**	466 ^{aA}	388 ^{aB}	205 ^{C}	280 ^{C}	23.0
	10 dpi	252 ^{bcB}	399 ^{aA}	236 ^{B}	256 ^{B}	18.3
	15 dpi	*	*	221	209	27.6
CV (%)		18.7	25.1	25.1	23.3	

Means followed by different small letters in the same column or by different capital letters in the same row are significantly different by the Tukey's test ($p < 0.05$)
Group 1= oral inoculation with SGNalrΔ*cob*S; Group 2= oral inoculation with SGNalr; Group 3= oral inoculation with SGNalrΔ*cob*SΔ*cbi*A; Group 4= not inoculated (control group).
*= Groups 1 and 2 – all birds had already died; CV= Coefficient of variation; dbi= day before inoculation; dpi= day post-inoculation.

with another organs like kidneys. About GGT serum activities, significant increase was verified at birds from G1 and G2 during the infection, as verified by Garcia *et al*. (2010), with the values doubled and trebled respectively when compared with DBI, as show in Table 3. About AST serum activities, the highlight was the G2, which had the enzyme serum active doubled when compared with DBI, analogous results were found by Freitas Neto *et al*. (2007) which also verified AST serum active increase in birds inoculated with SG during the course of the infection. Meyer *et al*. (1995) observed that GGT is a membrane enzyme; therefore its serum activity can be elevated from the beginning of hepatic lesion. On the other hand, the AST serum active relevant increase suggests harmful hepatic lesion (Kaneko *et al*., 2009). Therefore, according to this information, it is observed that G2 has acquired more pronounced liver damage when compared to G1, because G2 presents higher GGT serum activities and AST serum active alterations also were more relevant in this group.

Serum protein concentrations

The SDS-PAGE technique allowed the fraction of twenty-eight serum proteins, however nine of them are relevant to this study: IgA, transferrin, albumin, heavy chain IgG, haptoglobin, light chain IgG, and proteins with 99 Kd, 90Kd and 23 Kd of molecular weight (Table 4).

The initial phase of the inflammatory reaction induced by infection involves the release of acute-phase proteins (Gruys *et al*., 1994). In G1 was verified significant increase in the total protein serum concentration at fifth DAI. In this moment, seven of nine studied proteins were higher than the DBI, and six of it presented peak of concentration at this day, with highlight to 90Kd, 99Kd and transferrin (increase of thirty, five and four times respectively), besides albumin. For G2 significant decrease of total protein serum level was observed on tenth DAI, related with significant albumin decrease (twice less than the DBI) and light chain IgG (fifteen times less than the DBI).

In the birds from G1 and G2, the alterations of protein fraction considered acute-phase proteins were evidenced to decrease of albumin and 23 Kd molecular weight protein serum levels and for increase of transferrin, haptoglobin serum levels and particularly of 99 and 90 Kd molecular weight proteins (Figure 2).

The haptoglobin serum concentration (positive acute-phase protein), in G1, reached peak concentration in the fifth DAI, with subsequent decrease at seventh and tenth DAI. This decrease should be related with the anaemia observed in G1 birds. About G2, the haptoglobin serum levels increase was gradated and constant, reaching the peak at tenth DAI. Similar results were found by Garcia *et al*. (2009) in experimentally infected birds with SG.

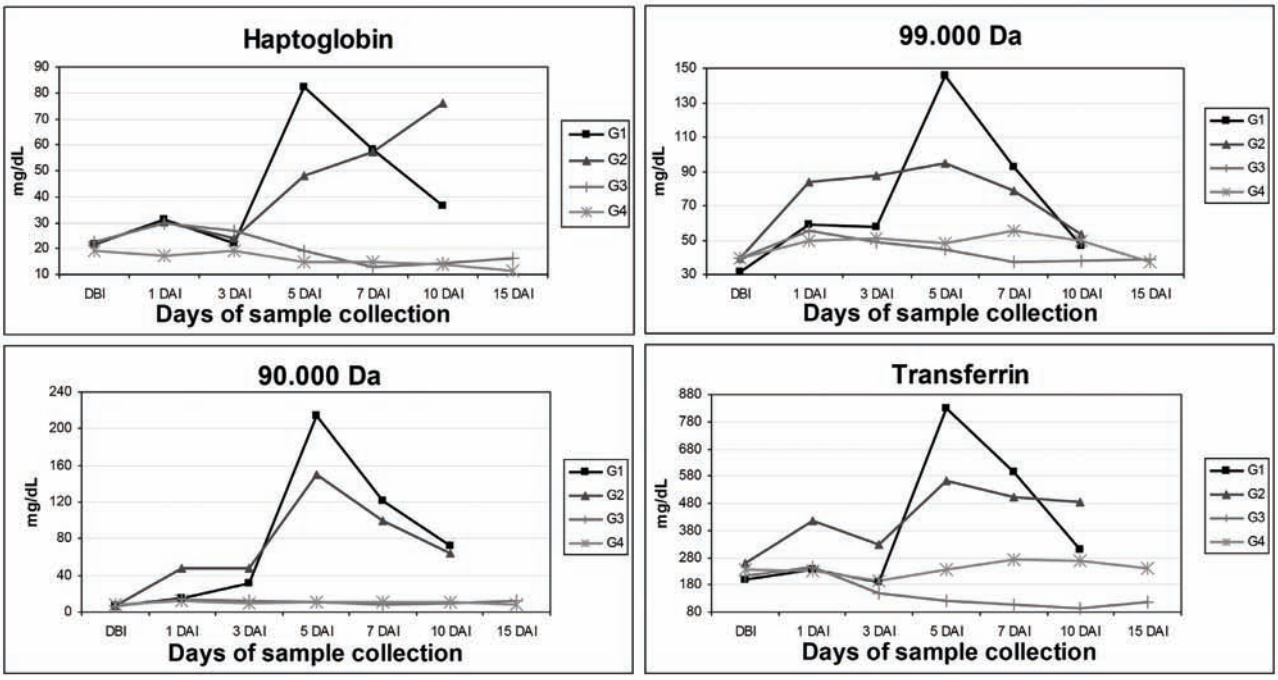

Figure 2 - Means of haptoglobin, 99.000 Da protein, 90.000 Da protein and transferrin serum concentrations from brown layers experimentally infected by SGΔ*cob*S, SGNal^r and SGΔ*cob*SΔ*cbi*A and control group 24 hours before (dbi) inoculation and 24 hours (1 dpi), and three days (3 dpi), five days (5 dpi), seven days (7 dpi) and ten days (10 dpi) post inoculation.

Table 4 - Means and standard deviation of the total protein serum levels (g/dL) and protein fractions (mg/dL) obtained by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in brown variety laying birds experimentally infected by S. Gallinarum Nalr, ΔcobS e ΔcobSΔcbiA.

Proteins	1 DBI	1 DAI	3 DAI	5 DAI	7 DAI	10 DAI	15 DAI
Total protein	R. Val.						
G1	3.2±0.5 ^{b}	3.8±0.5 ^{b}	3.5±0.9 ^{b}	5.2±1.3 ^{aA}	3.0±0.9 ^{b}	3.9±1.0 ^{abA}	*
G2	3.6±0.7 ^{ab}	4.3±0.7 ^{a}	4.0±0.9 ^{a}	2.8±1.2 ^{abB}	3.1±0.8 ^{ab}	1.9±0.7 ^{bB}	*
G3	3.6±0.5 ^{ab}	3.8±0.5 ^{a}	3.4±0.6 ^{ab}	3.2±1.05 ^{abB}	2.9±0.7 ^{b}	3.0±0.4 ^{abA}	3.2±0.8 ^{ab}
G4	3.6±0.2 ^{ab}	3.9±0.3 ^{a}	3.9±0.7 ^{a}	4.0±0.8 ^{aAB}	3.7±0.8 ^{abc}	3.1±0.7 ^{bA}	3.0±0.5 ^{b}
IgA	R. Val.						
G1	51.3±9.60 ^{b}	56.5±15.3 ^{abB}	61.4±18.9 ^{abAB}	64.2±14.9 ^{aA}	39.8±16.8 ^{cB}	31.6±17.5 ^{cB}	*
G2	49.2±10.2 ^{b}	63.5±12.5 ^{aA}	58.2±14.9 ^{abA}	42.7±17.9 ^{bB}	48.9±18.0 ^{bB}	26.1±9.3 ^{cC}	*
G3	49.6±9.96	59.9±14.6 ^{AB}	52.4±15.5 ^{B}	52.8±19.9 ^{AB}	45.9±14.2 ^{B}	54.0±12.3 ^{AB}	51.1±18.5
G4	60.2±13.1	61.2±17.9 ^{A}	68.2±24.6 ^{A}	55.4±16.2 ^{B}	49.2±19.1 ^{A}	63.3±16.6 ^{A}	62.1±11.6
99 Kd	R. Val.						
G1	31.8±14.8 ^{d}	59.3±20.5 ^{bcB}	57.6±22.4 ^{cdAB}	146±49.3 ^{aA}	92.3±41.6 ^{bA}	46.5±18.3 ^{cdAB}	*
G2	39.1±15.1 ^{c}	83.5±30.0 ^{aA}	87.3±28.8 ^{aA}	94.6±32.8 ^{aB}	78.4±22.6 ^{abA}	53.3±17.8 ^{bcA}	*
G3	39.6±15.7 ^{b}	55.1±23.3 ^{aB}	48.7±19.6 ^{abB}	44.6±17.9 ^{abC}	37.4±12.1 ^{bB}	37.8±12.6 ^{bB}	38.4±14.5 ^{b}
G4	39.1±13.7	49.9±18.4^{B}	50.8±25.8 ^{B}	48.2±18.9 ^{C}	55.5±20.4 ^{B}	49.7±20.1 ^{AB}	37.4±8.43
90 Kd	R. Val.						
G1	6.76±1.08 ^{e}	14.4±7.5 ^{eB}	31.4±15.2 ^{dA}	214±42.3 ^{aA}	122±35.8 ^{bA}	72.3±18.8 ^{cA}	*
G2	7.32±2.41 ^{c}	47.6±17.3 ^{bcA}	47.3±17.7 ^{bcA}	150±38.3 ^{aB}	100±32.7 ^{abA}	64.0±22.6 ^{bA}	*
G3	7.45±0.59	13.3±8.4^{B}	12.9±5.7^{B}	11.5±7.7 ^{C}	8.48±4.24 ^{B}	9.27±0.65 ^{B}	11.7±5.1
G4	8.03±3.86	11.6±6.3^{B}	9.80±3.5^{B}	10.8±8.54 ^{C}	11.2±5.77 ^{B}	10.7±5.36 ^{B}	8.29±2.76
Transferrin	R. Val.						
G1	198±25.2 ^{d}	235±38.9 ^{cdB}	192±28.4 ^{dB}	829±130.4 ^{aA}	593±119.8 ^{bA}	308±54.0 ^{cAB}	*
G2	259±35.0 ^{b}	417±70.7 ^{abA}	330±63.1 ^{abA}	562±80.1 ^{aB}	504±94.8 ^{aA}	486±42.6 ^{abA}	*
G3	212±49.1 ^{a}	246±48.5 ^{aB}	149±29.7 ^{bB}	121±28.2 ^{bC}	108±20.5 ^{bC}	94.2±16.7 ^{bC}	117±19.9 ^{bB}
G4	238±30.1	232±29.1 ^{B}	193±33.1 ^{B}	235±40.5 ^{C}	273±38.9 ^{BC}	269±44.1 ^{BC}	240±26.8^{A}
Albumin	R. Val.						
G1	1.7±0.3 ^{bc}	1.9±0.3 ^{abAB}	1.9±0.6 ^{abAB}	2.5±0.7 ^{aA}	1.2±0.4 ^{bcB}	0.85±0.3 ^{cC}	*
G2	1.8±0.4 ^{ab}	2.3±0.4 ^{aA}	2.3±0.5 ^{aA}	1.2±0.5 ^{cB}	1.5±0.4 ^{bcB}	0.77±0.7 ^{cB}	*
G3	1.9±0.3	1.8±0.2 ^{B}	1.8±0.4^{B}	1.9±0.6 ^{AB}	2.0±0.4 ^{A}	1.8±0.2 ^{A}	1.8±0.5
G4	2.0±0.2	2.1±0.2 ^{AB}	2.3±0.4^{A}	2.4±0.5 ^{A}	2.3±0.5 ^{A}	2.0±0.4 ^{A}	1.9±0.4
Haptoglobin	R. Val.						
G1	21.7±7.08 ^{c}	31.0±9.07 ^{bc}	22.2±8.66 ^{c}	82.2±19.5 ^{aA}	58.0±21.2 ^{abA}	36.7±19.7 ^{bcB}	*
G2	22.1±10.7 ^{c}	30.2±8.87 ^{bc}	24.0±7.60 ^{c}	48.2±16.6 ^{bB}	57.1±15.7 ^{bA}	76.0±12.6 ^{aA}	*
G3	22.7±8.77 ^{a}	29.8±8.43 ^{a}	26.8±9.8 ^{ab}	19.0±8.15 ^{abC}	12.9±4.52 ^{bB}	14.1±6.53 ^{bC}	16.1±9.73 ^{ab}
G4	19.1±11.2	17.1±7.67	19.2±8.39	14.8±7.81 ^{C}	14.7±6.26 ^{B}	13.7±5.7 ^{C}	11.6±9.25
LIgG	R. Val.						
G1	85.7±23.4 ^{a}	52.7±20.0 ^{abB}	31.1±16.3 ^{bcB}	42.3±17.6 ^{bcB}	28.0±8.6 ^{cB}	37.7±7.1 ^{bcB}	*
G2	93.6±28.4 ^{a}	53.8±24.2 ^{abB}	48.5±12.7 ^{bcB}	16.9±9.1 ^{cdC}	30.5±9.0 ^{bcB}	5.98±2.2 ^{dC}	*
G3	84.6±18.6 ^{a}	45.7±18.4 ^{bB}	31.4±13.5 ^{bB}	53.8±16.6 ^{abB}	88.8±11.7 ^{aA}	91.9±7.74 ^{aA}	85.0±6.59 ^{a}
G4	93.7±22.4	108±27.2^{A}	95.8±20.2^{A}	82.7±9.8 ^{A}	104±14.3^{A}	96.7±8.65^{A}	86.5±7.1
23 Kd	R. Val.						
G1	887±67.9 ^{aA}	835±83.0 ^{aA}	762±77.3 ^{aA}	744±67.4 ^{aA}	464±76.7 ^{bB}	260±43.1 ^{cB}	*
G2	722±68.2 ^{aB}	655±94.5 ^{aB}	590±51.2 ^{aB}	244±46.1 ^{cB}	371±53.2 ^{bB}	230±42.6 ^{cB}	*
G3	791±105 ^{aA}	759±78.1 ^{aA}	775±75.6 ^{abA}	708±104 ^{abA}	619±55.8 ^{bA}	690±69.4 ^{bA}	691±78.5 ^{b}
G4	786±51.4 ^{B}	729±80.7 ^{AB}	613±63.4 ^{B}	701±92.7 ^{A}	694±87.5 ^{A}	674±40.1 ^{A}	649±71.3

Means followed by different small letters in the same column or by different capital letters in the same row are significantly different by the Tukey's test ($p < 0.05$).

Group 1= oral inoculation of SGNalrΔcobS; Group 2= oral inoculation of SGNalr; Group 3= oral inoculation of SGNalrΔcobSΔcbiA; Group 4= did not receive inoculum (control group).

*= Groups 1 and 2 – all birds were dead at this day; DBI= day before inoculation; DAI= day after inoculation; R. Val. = Reference Values; LIgG = light chain IgG.

The 99 and 90 Kd molecular weight proteins showed extremely high values during the course of the infection in G1 and G2, with thirty times in G1 and twenty times in G2 in the fifth DAI when compared with DBI (Table 4). According to Serra (1997), this response is characteristic of some acute-phase protein, it can increase thousand times in some tissue injury.

The transferrin, considered negative acute-phase protein, had atypical behavior in G1 and G2, increasing and reaching the concentration peak at fifth DAI and decreasing at this day (Figure 2). This fact can be explained by the *Salmonella* characteristic which uses serum free iron for its replication, as the serum free iron reduction can cause transferrin increase. On the other hand, in G3 the transferrin concentration had a degrade decrease, as observed by Garcia *et al*. (2009) in the experimental infection with SG in commercial layers. The 23Kd molecular weight protein also had a negative acute-phase protein behavior to G1 and G2, which was observed gradated decrease during the infection for these groups.

Good protection against *Salmonella* requires as much immunity mediated by T cells as humoral immunity (Berchieri Jr., 2000). Therefore, in systemic *Salmonella* infections, the humoral response, characterized by high IgG levels, does not eliminate the bacterium from the body if there is no cellular immune response; however IgG is essential for the extracellular bacterial destruction process.

In the G1, G2 and G3 groups the light chain IgG (LIgG) presents decrease in the third DAI, and having as a possible explanation initial LIgG recruitment for the bacterium opsonization. In the G1 and G2 the immunoglobulin levels still lower until tenth DAI (2.3 and 15.7 times lower than DBI respectively), but G3 presents different results and the concentrations returning to basal values (Table 4). An explanation for the strong LIgG reduction in the end of the infection for G1 and G2 groups should be related with irregular production, maturation or differentiation of the B cells in the bursa of Fabricius because *Salmonella* can cause disturbances on this

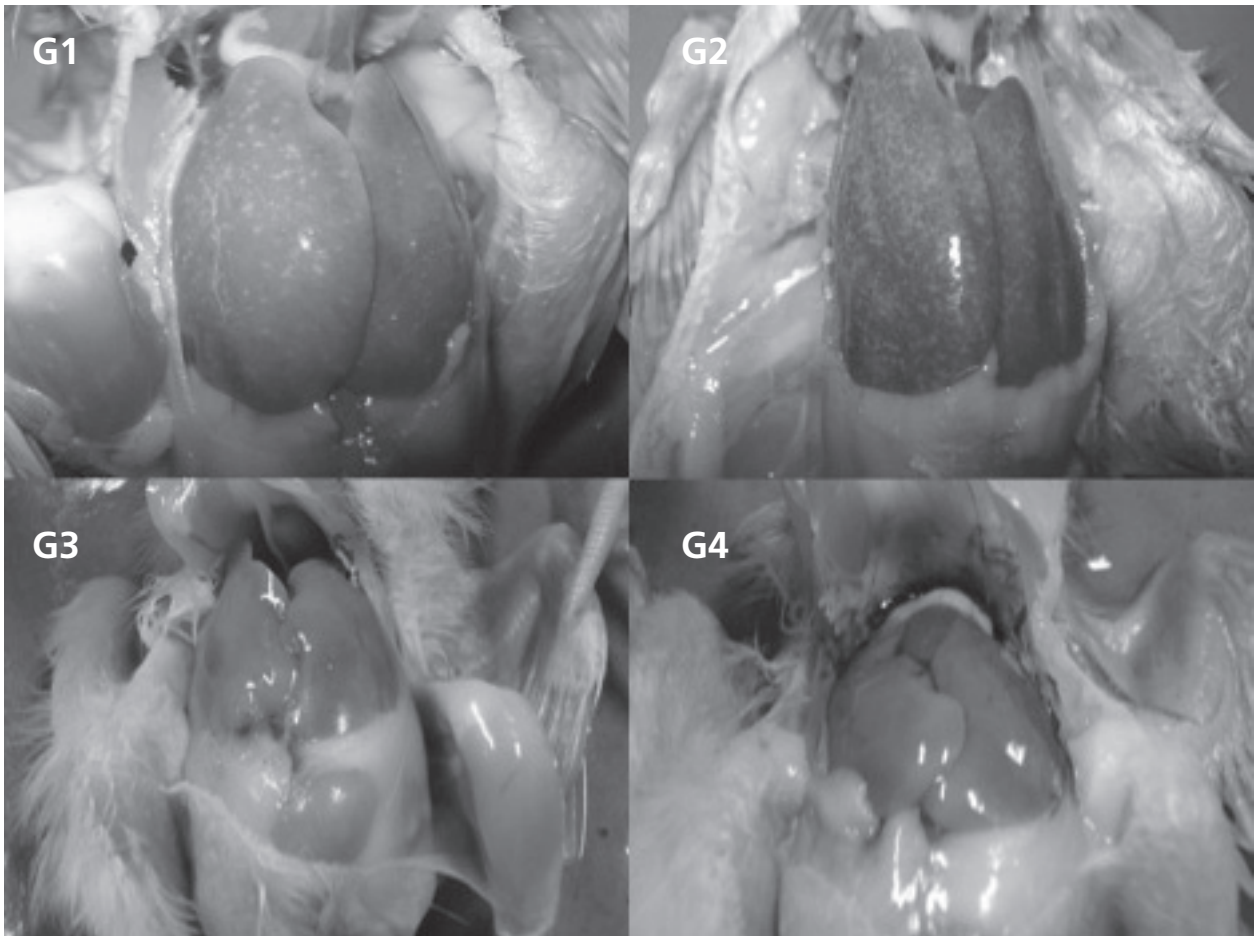

Figure 3 - Gross aspect of the liver of layers seven days after inoculation with different *Salmonella* Gallinarum strains. In G1 birds, the liver was enlarged, yellow-greenish, and presented disseminated white necrotic foci. In G2 birds, similar lesions were found, but the liver was brownish. The liver did not present any changes in G3 and G4 birds.

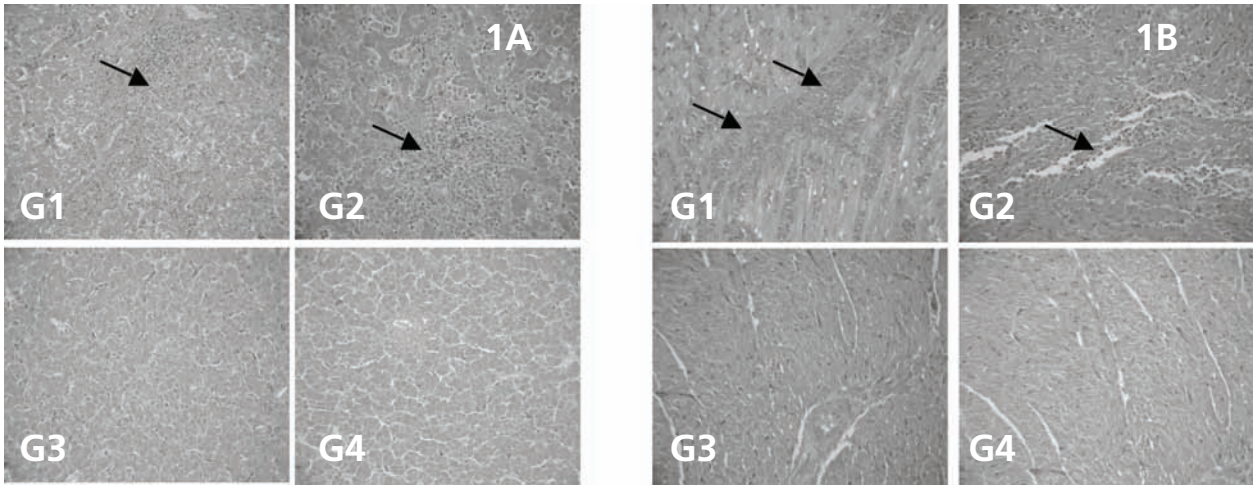

Figure 4 - Photomicrograph of the liver (1A) and heart (1B) sections of the experimental birds on 5 dpi with *Salmonella* Gallinarum. In G1 birds, the liver presents inflammatory infiltrate and hydropic degeneration and in G2 birds, there is multifocal necrosis with inflammatory infiltrate. The liver of G3 and G4 birds does not present any changes. The heart of G1 and G2 birds also presented mixed inflammatory infiltrate. No changes were observed in the heart of G3 and G4 layers (HE, 40x magnification).

organ. The recovery of LIgG concentration in the G3 birds since third DAI can mean which after the initial combat against microorganism, the birds immune response controlled the infection, likely due to the strain's avirulent characteristic.

IgA is the main immunoglobulin that acts in the intestinal epithelium, reducing bacterial colonization. In G1 and G2 was verified that IgA serum level decreased in the most advanced phases of the infection. According to Wyant *et al*. (1999) the weak initial intestinal immune response induced by *Salmonela* Gallinarum can probably be due to the absence of flagellum on the surface of this bacterium, as this structure is a powerful stimulus for cytokines production.

It can also be due to a mutation in *inv*H, one of the genes that controls the secretion of the type-III secretion system (Altmeyer *et al*., 1993). It must be noted that low IgA response in the mucosa in face of an infection may result in stronger pathogenicity, and consequently, in high mortality in field cases of fowl tiphoid, as also verified in the present study for G1 and G2. The damage to the barrier of the intestinal mucosa predisposes to systemic infection and consequently, to typical tissue lesions, especially in the liver, spleen, and heart (Berchieri Júnior & Freitas Neto, 2009).

However, besides IgA response absence, there was a decrease of it concentration to G1 and G2 since fifth DAI and third DAI respectively. This fact also can be explained by B cells irregular production as well as LIgG.

Gross and histopathological exams

Macroscopic and microscopic alterations in the organs which showed lesions are presented in Figures 3 to 5. The birds were submitted to macroscopic exam to verify possibly lesions. Since third DAI, the organs of G1 and G2 birds presented alterations mainly in the liver, which showed color alteration with white necrotic spots spread all over the organ besides hepatomegaly. The most evident alterations were found in the fifth and seventh DAI in the liver, spleen and bursa of Fabricius. These findings are typical from birds infected with SG and was reported by many other authors (Christensen *et al*., 1996; Shivaprasad, 2000; Berchieri Júnior *et al*., 2000; Freitas Neto *et al*., 2007; Berchieri Júnior & Freitas Neto, 2009; Garcia *et al*., 2010). G3 and G4 presented organs without lesions in all days of the study.

In the histopathological exams the alterations also started since third DAI in G1 and G2 birds and the liver was the most damaged organ, which since fifth DAI presented multifocal necrosis which is an irreversible pathologic alteration (Figure 4), as reported by Shivaprasad (2000) in the study of fowl typhoid and pullorum disease. In G2, also in the fifth DAI, were found necrotic spots on the spleen tissue (Figure 5). Heterophilic and lymphocytic infiltrate or both were presented in the other studied organs, with emphasis on G1 cardiac tissue in the fifth DAI, which presented diffuse mixed inflammatory infiltrate (Figure 4). Smith (1955) studying SG in birds found a proliferative cellular reaction in the heart with white nodular spots spread

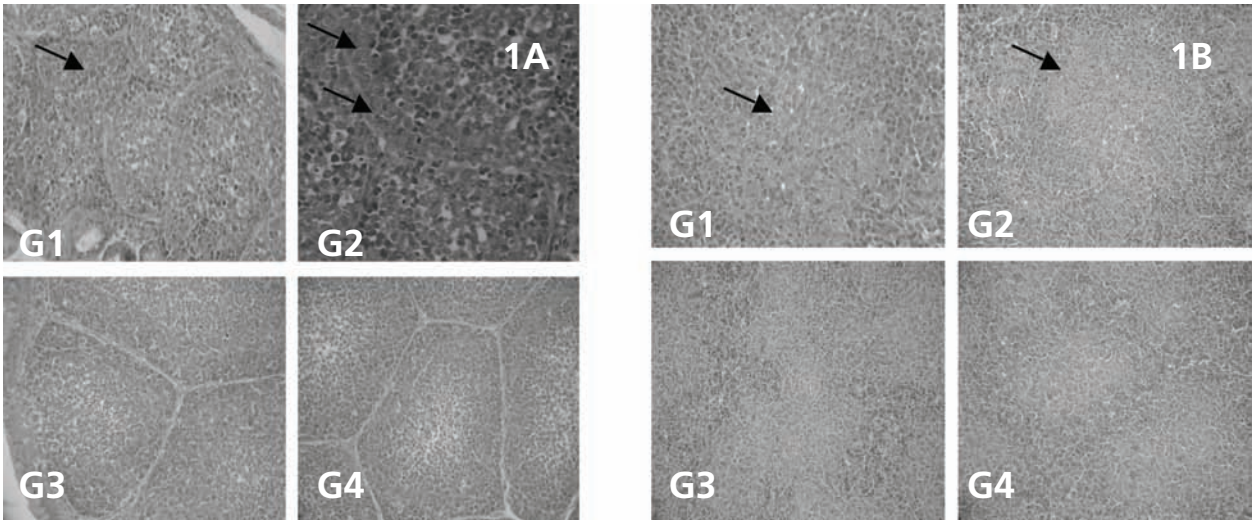

Figure 5 - Photomicrograph of the bursa of Fabricius (1A) and spleen (1B) sections of the experimental birds on 7 dpi with *Salmonella* Gallinarum.. G1 and G2 birds presented inflammatory infiltration in the bursa of Fabricius and multifocal necrosis in the spleen. Both organs showed no changes in G3 and G4 birds (HE, 40x magnification).

on the myocardium. In the other organs as bursa of Fabricius, kidneys and thymus, the inflammatory process were milder, highlighting slight pyknosis in the nucleus of renal cells at tenth DAI in G3, however this process is reversible, occurring in some initial case of stress and in the fifteenth DAI there were no more renal alterations in the birds of G3. It is important to highlight the significant decrease of uric acid also in the tenth DAI for this group (Table 3) which presents the higher decrease at this DAI, showing that there may be a renal disturbance at this time in G3, whereas, according to Schimidt *et al*., (2007), part of the uric acid is produced in the kidneys. These results corroborated with those reported by Freitas Neto *et al*. (2007) which observed microscopic alterations in the liver, heart and kidneys and Garcia *et al*. (2010) which found relevant histopathological lesions in the liver, spleen and kidneys in birds infected by SG. In both experiments, the liver also was the most harmed organ.

CONCLUSION

The G1 and G2 birds, which were inoculated with SGΔ*cob*S and SGNal^r strains respectively, presented, during the experiment, alterations consistent with infection by SG, with very similar macroscopic and histopathological alterations. However, the birds inoculated with the singular deletion (SGΔ*cob*S strain) presented some different response from those found in birds inoculated by SGNal^r strain, probably because the mutant strain might use alternative mechanisms of infection due to absence of the *cob*S gene. With respect to the G3 birds, which ones were inoculated with SGΔ*cob*SΔ*cbi*A strain, they behaved very similar to the control group (G4). However, mild alterations in biochemical parameters linked with liver, bursa of Fabricius and kidneys were found. These changes, even if mild, should be taken into account, since the main objective of this study was to evaluate possible changes caused by this double deletion strain.

Given what was reported, new studies in this line of research might be done for the strain in question, showing what would be tolerable for these changes with regard to performance and health of birds vaccinated with the SGΔ*cob*SΔ*cbi*A strain, because the market necessity are vaccines that interfere as little as possible on performance and health of poultry production.

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