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SIMONE SOMMERFELD

Exploring Inflammation of Infection of Chicken Embryos before 12 days of embryonic development: A Promising Experimental Model

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Orientador: Belchiolina Beatriz Fonseca

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“Sozinho vamos mais rápido. Juntos vamos mais longe.”

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RESUMO

O embrião de galinha (EG) tem se destacado como um valioso modelo in vivo para investigar o desenvolvimento do sistema imune, infecções e inflamação. Esta revisão descreve as vantagens biológicas e práticas do modelo de EG, enfatizando sua proximidade filogenética com os mamíferos, o desenvolvimento bem caracterizado e a acessibilidade para manipulações experimentais. A ontogenia do sistema imunológico é discutida, incluindo a formação dos órgãos linfoides primários e secundários e o surgimento precoce das respostas imune inata e adaptativa. Atenção especial é dada à adequação do EG para modelar respostas inflamatórias, especialmente em estudos que utilizam patógenos vivos como a *Salmonella* Pullorum. Diversos estudos utilizaram com sucesso o EG para avaliar a virulência bacteriana e as interações patógeno-hospedeiro, demonstrando sua relevância para a biologia das infecções. Além disso, destacamos considerações éticas relacionadas ao bem-estar animal, observando que o uso de embriões antes do desenvolvimento da nocicepção está alinhado aos princípios dos 3Rs. Em conjunto, esses achados sustentam o EG como um modelo robusto e eticamente responsável para o estudo das respostas iniciais do hospedeiro à infecção e à inflamação.

Palavras-chave: Modelo alternativo, princípio dos 3Rs, *Salmonella*

ABSTRACT

The chicken embryo (CE) has emerged as a valuable in vivo model for investigating immune development, infection, and inflammation. This review outlines the biological and practical advantages of the CE model, emphasizing its phylogenetic proximity to mammals, well-characterized development, and accessibility for experimental manipulation. The ontogeny of the immune system is discussed, including the formation of primary and secondary lymphoid organs and the early appearance of innate and adaptive immune responses. Special attention is given to the suitability of the CE for modeling inflammatory responses, particularly in studies using live pathogens such as *Salmonella* Pullorum. Several studies have successfully employed the CE to assess bacterial virulence and host–pathogen interactions, demonstrating its relevance for infection biology. Furthermore, we highlight ethical considerations related to animal welfare, noting that the use of embryos before the development of nociception aligns with the principles of the 3Rs. Together, these findings support the CE as a robust and ethically responsible model for studying early host responses to infection and inflammation.

Keywords: Alternative model, 3Rs principle, *Salmonella*

CHAPTER 1
LITERATURE REVIEW

INTRODUCTION

Animal models are selected based on their functional and genetic similarities to humans or other species, enabling the study of physiological and pathological processes. From 1901 to 2020, two-thirds of Nobel Prize-winning studies used animal models to explore diseases and develop treatments. Most species employed were mammals (especially rodents, due to their physiological similarity to humans) although interest in non-mammalian species is increasing. The number and types of animals reported vary by country, depending on legal regulations and reporting practices (Domínguez-Oliva et al., 2023).

The use of chicken embryos (CE) as an *in vivo* model is well-established and is becoming increasingly popular in research. Although this model has limitations and cannot fully replace traditional preclinical systems, it serves as a valuable intermediate step between *in vitro* studies and more complex mammalian models, aligning with the principles of the 3Rs (Garcia et al., 2021).

LPS is commonly used to induce inflammation in animal models, including chickens, where it triggers heterophil influx and increases pro-inflammatory mediators (H. Li et al., 2022; Yu et al., 2023). Although LPS is a well-established agent for inducing inflammation, using live bacteria more accurately replicates the complexity of natural infections. Live pathogens trigger broader immune responses beyond TLR4, offering a more physiologically relevant model of inflammation (Anastasiadou & Michailidis, 2016).

The CE has also been widely used to study infections caused by viruses (Yan et al., 2025; H. Yin et al., 2022) and bacteria (Zou et al., 2021), including *Salmonella* (Zhu et al., 2024), offering a valuable system to investigate host–pathogen interactions and immune responses during early development.

We have previously published relevant findings in a study proposing the use of chicken embryos infected with *Salmonella* Pullorum (SP) as a model of infection and inflammation for analyzing various peptides expressed by M13 through phage display selection (de Souza et al., 2024). However, in that study, we used embryos older than 13 days of embryonic development. We now aim to investigate whether younger embryos

can also serve as a suitable model for studying *Salmonella*-induced infection and inflammation.

The Chicken Embryo Experimental Model

The CE is a desirable animal model due to several advantages. It is phylogenetically closer to mammals than other alternatives like zebrafish or nematodes. It has well-developed central nervous, cardiovascular, and respiratory systems. The embryo is also easy to handle because of its convenient size, short incubation period, and accessibility both *in ovo* and *ex ovo*. Its development can be easily monitored, and each egg is self-sustaining, growing independently at 37–39°C and 45–55% relative humidity, without the need for complex or costly infrastructure (Kaplan-Arabaci et al., 2025).

In 1951, Viktor Hamburger and Howard Hamilton published a comprehensive study detailing all stages of chick development from the first cell divisions to hatching. This developmental process spans approximately 21 days of embryonic development (DED) and is still classified using the Hamburger and Hamilton (HH) staging system (Hamburger & Hamilton, 1992). The stages are generally divided into early (1–7 DED), intermediate (8–14 DED), and late (15–21 DED) phases. During the early and intermediate periods, critical processes such as organogenesis and the maturation of physiological systems occur, particularly between 12 and 14 DED(Bellairs & Osmond, 2005). Between 12 and 18 DED, the kidney gradually enhances its filtration capacity, and at 16 DED, the chicken embryo developed a functional liver and an effective blood coagulation system (Ribeiro et al., 2022).

Immune Development in the Chicken Embryo

The avian embryo offers several advantages for studying immune system development, including the clear separation between B and T cell lineages, which differentiate in distinct primary lymphoid organs, the bursa of Fabricius and thymus, respectively (Fellah et al., 2013). The availability of large numbers of embryos at well-defined developmental stages, along with genetic tools such as congenic and inbred chicken lines, markers, and monoclonal antibodies, has made the domestic chicken a preferred model. Additionally, quail–chick chimeras have provided valuable insights into

the emergence and migration of hematopoietic stem cells during embryogenesis (Martin et al., 1978).

The primary lymphoid organs include the thymus, which begins to develop at 3 days of embryonic development (DED) and is responsible for T cell development, and the bursa of Fabricius, a bird-specific organ that also starts developing at 3 DED, where B cell maturation and antibody repertoire formation occur (Garcia et al., 2021).

Secondary lymphoid organs in chickens develop at different stages: the spleen begins at 2 DED, the Harderian gland at 11 DED, Peyer's patches emerge around 13–18 DED, and Meckel's diverticulum (a remnant of the yolk stalk on the small intestine) acquires lymphoid tissue only post-hatch, becoming mature weeks later (Bellairs & Osmond, 2005; Fellah et al., 2013; Oláh et al., 1984). A key difference from mammals is that birds lack encapsulated lymph nodes; instead, they possess rudimentary mural lymphoid nodules, which have a more limited immunological role. To compensate, organs like the spleen and Harderian gland play a more prominent role in coordinating adaptive immune responses (Garcia et al., 2021).

Macrophages are key components of the innate immune system, including in the developing chicken embryo. As early immune sentinels, they are capable of phagocytosing cellular debris, pathogens, and apoptotic cells, while also contributing to tissue remodeling and homeostasis, processes that are particularly important during embryogenesis (Bellairs & Osmond, 2005). Macrophages can be broadly categorized into pro-inflammatory (M1) typically produce IL-1β, TNF-α, and IL-6 and are involved in host defense and inflammation, and anti-inflammatory or tissue-repairing (M2) phenotypes, which secrete IL-10, TGF-β, and VEGF (Garcia et al., 2021).

In chickens, T and B lymphocytes are central to the adaptive immune system. T cells develop in the thymus from hematopoietic progenitors and recognize antigens through T cell receptors (TCRs). As in mammals, chickens possess both αβ and γδ T cell lineages. $CD4^+$ αβ T cells act as helpers, while $CD8^+$ αβ T cells are cytotoxic. γδ T cells, more abundant in chickens than in humans, play key roles in immune surveillance and are enriched in peripheral tissues (Fellah et al., 2013). B cells mature in the bursa of Fabricius, where they generate a diverse antibody repertoire early in development. Although structurally distinct, chicken immunoglobulins such as IgY function similarly to mammalian IgG (Garcia et al., 2021).

The early onset of cytokine production during embryogenesis reinforces the suitability of the CE as a model for studying inflammatory responses. While certain

cytokines such as IL-6 have only been quantified at later stages like 18 DED, others (including IL-1β, IL-8, IL-12, and IL-18) have been detected as early as 3 DED. Additionally, the expression of IL-4, IL-10, and IFN-γ has been identified in the spleen by 12 DED (Abdul-Careem et al., 2007). Although maximal cytokine expression typically occurs post-hatch, these early developmental signals highlight the embryo's capacity to mount an immune response well before hatching (Alkie et al., 2019).

Salmonella Pullorum as an Infectious Agent in Embryos

Salmonella enterica serovar Pullorum (SP) is the etiological agent of Pullorum disease (PD) in chickens, typically causing host-specific acute septicemia characterized by white diarrhea. Although S. Pullorum is restricted to avian hosts, its pathogenic mechanisms remain incompletely understood (Q. Li et al., 2019).

One of the key mechanisms contributing to the persistence of SP infection in laying hens is its ability to survive within splenic macrophages, which facilitate vertical transmission to the eggs. Infection of eggs by SP has been linked to suppression of the host's cellular immune response. However, the mechanisms that allow SP to persist within the egg post-laying, as well as the factors enabling non-lethal colonization of developing embryos during incubation, remain poorly understood (Guo et al., 2017).

Using the CE as a model, Guo et al. (2017) investigated the O-polysaccharide component on the bacterial surface and found that it significantly increased both embryo lethality and post-oviposition bacterial colonization. Li et al. (2019) established an effective method for rapidly screening the virulence of SP strains by combining infectivity assays in avian cell lines with a CE infection model. Finally, Souza et al. (2024) standardized the use of CE as a model for systemic infection with SP up to 13 DED and demonstrated that the M13 phage (used without *E. coli*) is safe for in vivo applications.

Animal welfare in research using alternative models

The use of CE in scientific research is ethically permissible in many jurisdictions. Although regulations in regions like the European Union and the United States do not classify CE as live animals before 14–17 DED, allowing their use without ethical review

(IACUC, 2022; Ribatti & Annese, 2023), our decision to use this model is driven primarily by concern for animal welfare rather than regulatory convenience.

By conducting experiments during early embryonic stages (prior to the development of pain perception), we aim to generate meaningful scientific data while minimizing potential distress. This choice aligns with the principles of the 3Rs (Replacement, Reduction, and Refinement), prioritizing models that reduce harm to sentient animals whenever possible (Zosen et al., 2021).

In Brazil, current guidelines from the National Council for the Control of Animal Experimentation (CONCEA/MCTI) also recognize the ethical nuances of using embryos. According to Informative Note No. 01/2024, projects involving embryos beyond 50% of their developmental period must undergo ethical review by Institutional Animal Care and Use Committees (CEUAs) (CONCEA/MCTI, 2024). Our research adheres not only to regulatory frameworks but also to a broader ethical commitment: respecting the developmental stage of the embryo and applying humane practices throughout the study.

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CHAPTER 2

NON-TARGETED PROTEOMICS CAN SHED LIGHT ON POTENTIAL BIOMARKERS OF INFLAMMATION AND INFECTION IN CHICKEN EMBRYOS AS AN EXPERIMENTAL MODEL

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ABSTRACT

In previous study, we inoculated *Salmonella* Pullorum (SP) as a model for inflammation in late-stage chicken embryo (CE) and it has proven to be an interesting model for studying infection and inflammation. In the present study, to refine its use ethically, we evaluated CE inoculated at 9.5 days of embryonic development (DED) with SP as an infection experimental model via the allantoic cavity in low dose, and assessed after 24 or 48 hours as an alternative model for acute inflammation monitoring clinical outcomes of mortality and weight alongside proteomics. Serum proteomics was performed by LC-MS/MS, followed by enrichment analysis using PANTHER and KEGG. Statistical analyses included t-tests, Tobit mixed-effects models (proteomics), and PERMANOVA (multivariate). Embryo mortality significantly increased at 48h post-inoculation, reaching 45%. Among the surviving embryos, there was a tendency for a reduction in weight ($p < 0.0506$). Proteomic analysis revealed modest but consistent increases in protein abundance and functional activity in *Salmonella*-infected chicken embryos, despite high zero inflation. Notably, alpha-2-macroglobulin-like protein 1 was significantly upregulated at 48 hours post-infection, suggesting an endogenous immune response and positioning this protein as a potential biomarker in the CE model. When we studied the metabolic pathways, biological processes, and molecular functions, we found a p-value <0.05 for plasminogen activating cascade, mitophagy (animal), carbon metabolism,

pyruvate metabolism, blood coagulation, and PPAR signaling pathway. However, after adjusting for multiple comparisons, the significance was no longer supported. Although most differences were not statistically significant after p-correction, Bayesian modeling highlighted subtle but credible effects of infection on host proteome dynamics, supporting the utility of the CE model before 12 DED for studying early immune responses.

INTRODUCTION

Understanding systemic responses to infection and inflammation, particularly in the context of identifying early molecular events triggered by pathogenic exposure, is one of the key goals in biomedical research. Traditionally, mammalian models have been used to investigate host responses (Domínguez-Oliva et al., 2023). However, out of respect for animal welfare and in line with ethical research practices, scientists are increasingly turning to alternative models that follow the principles of Replacement, Reduction, and Refinement (the 3Rs). In this context, the chicken embryo (CE) presents a promising experimental platform (Zosen et al., 2021).

The CE offers several practical advantages, including low cost, high accessibility, and faster results (Garcia et al., 2021). Importantly, embryos younger than 12 days of development (DED) are widely considered incapable of perceiving pain due to incomplete maturation of nociceptive circuits (Kollmansperger et al., 2023). As such, the CE before 12 DED represents a biologically relevant and ethically acceptable model for studying systemic effects of infection and inflammation. Despite its increasing use in virology (Banfield et al., 1998) and toxicology (Ribeiro et al., 2022), its application as an inflammation model by bacterial infection, particularly with a focus on systemic proteomic changes, remains limited.

Proteomic profiling enables the detection of subtle shifts in protein expression and functional activity, offering valuable insights into the molecular mechanisms underlying host responses (Horvatić et al., 2019). However, studying the proteome in embryonic tissues introduces analytical challenges, such as high biological variability, low protein abundance, and zero-inflated datasets. Additionally, limited functional annotation for *Gallus gallus* in current databases further complicates interpretation.

Here, we investigated the early systemic non-target proteomic response of CE to *Salmonella* Pullorum (SP) as an infection inflammatory model at 24 and 48 hours post-

inoculation, using a combination of Bayesian statistical modeling, multivariable analysis and functional annotation across multiple biological levels.

MATERIAL AND METHODS

The experiments were conducted at the Poultry Incubation Laboratory (LIAVE), Laboratory of Infectious and Contagious Diseases (LADOC) and Nanobiotechnology Laboratory Prof. Luiz Ricardo Goulart Filho (NANOS) at the Federal University of Uberlândia (UFU). The project was evaluated and approved by the Animal Ethics Committee (CEUA) of UFU, under protocol numbers 008/21 and 23117.010881/2024-41. All procedures followed the standards and regulations established by the National Council for the Control of Animal Experimentation (CONCEA).

Chicken embryos assays

We used SP as a model for infection and inflammation, based on our previous experience with this bacterium in CE at later developmental stages (de Souza et al., 2024). Since we already knew it could cause complete embryo mortality at earlier stages, we used a much lower concentration of the pathogen. A total of 50 embryonated eggs from the Hy-Line strain of *Gallus gallus* were used and maintained in an automatic incubator (Premium Ecológica) at 37°C and 55% humidity. On day 9.5 DED, the CE were divided into two groups: 30 CE formed the positive control (PC), inoculated with 100µL of 2 log CFU/mL of SP and 20 CE formed the negative control (NC), inoculated with 100 µL of 0.85% saline solution. The embryonaded eggs were weighed on the day of inoculation and monitored daily for mortality assessment using candling. At 10.5 DED and 11.5 DED (24 and 48 hours post-inoculation, respectively), the mortality and gross damage were analyzed, the survived embryos were weighed, blood samples were collected from each group (PC and NC). After clot formation, blood was centrifuged at 5,000 rpm for 5 minutes to obtain the serum, which was transferred to sterile tubes and stored at -80°C until further analysis.

Proteomics Analysis

A total of 10 samples of each group from embryos collected 24 and 48 hours post-inoculation was used for proteomics analysis. For in-solution protein digestion, 50 µL of the blood serum was treated in an ultrasonic bath for 5 min with 1% RapiGest SF (w/v) (Waters, Milford, MA) and 0.5 M dithiothreitol (DTT). Samples were then incubated at 60 °C for 45 min under constant agitation, followed by alkylation with 0.5 M iodoacetamide (IAA) and incubation at 37 °C for 60 min in the dark. Proteins were digested with trypsin (20 ng/µL) at 37 °C overnight with continuous agitation. Digestion was stopped by acidification with 0.5% trifluoroacetic acid (TFA), followed by incubation at room temperature for 60 min. Precipitates were removed by centrifugation at 14,500 g for 10 min. The resulting supernatants were desalted and concentrated using ZipTips (Sigma-Aldrich) containing a C18 stationary phase, preconditioned and washed with 0.1% TFA. Peptides were eluted using a 50:50 acetonitrile:water solution containing 0.1% TFA for subsequent proteomic analysis.

Mass spectrometry analysis was performed using a high-resolution quadrupole time-of-flight (Q-TOF) mass spectrometer (Agilent 6520B) coupled to a liquid chromatography system (Agilent Infinity 1260) equipped with an electrospray ionization (ESI) source. Peptide separation was achieved on an AdvanceBio Peptide Mapping column (Agilent Technologies) using a multistep gradient of acetonitrile over 400 minutes at a constant flow rate of 0.4 mL/min. Ionization settings included a drying gas flow of 8 L/min at 325 °C, nebulizer pressure set to 45 psi, and a capillary voltage of 4 kV. Proteins were identified based on high-resolution mass measurements, with a mass accuracy threshold of <10 ppm and tandem MS/MS spectra. Data interpretation was conducted using the Spectrum Mill MS Proteomics Workbench (Agilent Technologies), with database searches restricted to *Gallus gallus* sequences from UniProt.

Functional Enrichment Analysis

Functional enrichment analysis was conducted to explore the biological roles of all proteins identified in the proteomic dataset. The Protein ANalysis THrough Evolutionary Relationships (PANTHER) classification system (http://pantherdb.org) was used to annotate biological processes, molecular functions, and pathways (Mi et al., 2021), while the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was applied exclusively for pathway analysis (Sollis et al., 2023).

Statistical analysis

For weight and mortality assessments, comparisons between groups were performed using the chi-square test, followed by pairwise comparisons of proportions and calculation of odds ratios to quantify effect sizes considering $p<0.05$ using the Graph PadPrism program 10.02.

For proteomics analysis, at both time points, the distribution of protein concentration values exhibited strong zero inflation, indicating potential left-censoring at zero. Therefore, we applied Bayesian Tobit mixed-effects models using the brms package in R. Protein identity ("Compound") was included as a random intercept to account for protein-specific variability, while sample category ("positive" or "negative") was modeled as a fixed effect. All models assumed a Gaussian likelihood with censoring from below at zero. Models were estimated using four MCMC chains of 4,000 iterations each (1,000 warm-up), with adapt_delta = 0.95 and max_treedepth = 15. Convergence was assessed using the Rhat statistic and effective sample sizes.

Multivariate composition analysis was conducted for the 24-hour protein dataset. PCA and PCoA were used to explore variance structure and group separation, while PERMANOVA assessed statistical differences in composition. Differential abundance was assessed using limma for each feature type, except proteins at 48 hours, which were modeled separately using a Tobit Bayesian approach.

For both time points, functional annotation was evaluated across three gene ontology levels: Pathway, Biological Process, and Molecular Function. To assess differences between the sample categories, Bayesian hierarchical linear models were employed using the *brms* package, incorporating sample category as a fixed effect and functional annotations (e.g., pathway ID or molecular function) as random intercepts. These models assumed a Gaussian distribution and followed the same priors and sampling settings described previously.

In parallel, compositional and ordination analyses were performed to explore overall patterns in functional profiles. Bray–Curtis dissimilarity metrics were computed on sample-by-function matrices—log-transformed and scaled when appropriate—and used as input for Principal Coordinates Analysis (PCoA) and Principal Component

Analysis (PCA). Group-level differences in overall functional composition were then statistically tested using PERMANOVA with 999 permutations (adonis2 function).

Finally, differential abundance testing was conducted using the *limma* package. Linear modeling was followed by empirical Bayes moderation (via the *eBayes()* function), and the most differentially abundant features were extracted using *topTable()*. To account for multiple testing, p-values were adjusted using the False Discovery Rate (FDR) correction.

RESULTS

Chicken embryo weight and mortality

There was no mortality in embryos after 24 hours post inoculation. After collection of 10 embryos of each group, the remaned embryos continued to incubated and after 48 hours, before new collection, the mortality was access by candeling again and there was 6 embryos died of a total of 20 remained embryos (Table 1).

Table 1. Embryo mortality following bacterial inoculation.

Development day on inoculation	**Treatment Group**	**No. of Embryos**	**Mortality at 24 h**	**Mortality at 48 h**
9.5	Inoculated	30	0/30 (0%)	6/20* (30%)
9.5	Negative Control	20	0/20 (0%)	0/10* (0%)

*Since that ten of the embryos were euthanized with 24 hours

Surviving embryos inoculated at 9.5 DED and euthanized 48 hours later, were weighed using an analytical balance (Bel LW303iH - 0,001g x 310g). Although no statistically significant difference was found, the p-value (0.0506) is very low suggesting a trend toward significance (Figure 1).

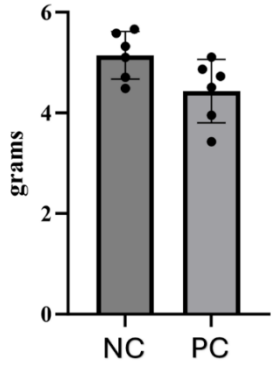

Figure 1. Weights of surviving embryos inoculated with saline (NC) or *Salmonella* Pullorum (PC) at 9.5 (B) embryonic days, evaluated 48 hours post-inoculation. Although no statistically significant difference was found, the p-value (0.0506) is very low suggesting a trend toward significance.

Protein-level analysis

A total of 1,164 proteins were identified in samples collected 24 hours post-inoculation, and 1,463 proteins were identified at 48 hours. Approximately 90% of the observations at both time points were zero. This high proportion of zero values, which is consistent with patterns reported in untargeted proteomic datasets, is often attributed to signals falling below the detection limit (Koopmans et al., 2014).

Only 5.76% and 56.6% of the proteins identified at 24- and 48-hours post-inoculation, respectively, could be functionally classified using PANTHER and KEGG databases. This limitation is primarily due to the lack of experimental annotation available for *Gallus gallus* in current reference databases. Consequently, our functional analysis was conducted based solely on the subset of proteins with available annotations.

Results are presented separately for each time point to highlight temporal dynamics in the host response.

24 Hours Post-Inoculation

The Bayesian Tobit model estimated a small positive effect of the infected category on protein concentration (estimate = 0.20, 95% CI: [0.08, 0.31]; Figure 2), with

a baseline concentration of 1.53 [1.38, 1.68] in the control group. We found 10 proteins with large absolute log2 fold changes (positive or negative) and p-values < 0.05 in the comparison between the negative and positive groups. However, none of these proteins remained statistically significant after multiple testing correction (adjusted p > 0.48; Table 2). Model diagnostics were satisfactory (Rhat = 1.00), and substantial variation across proteins was observed (random intercept SD = 2.08; residual SD = 4.74).

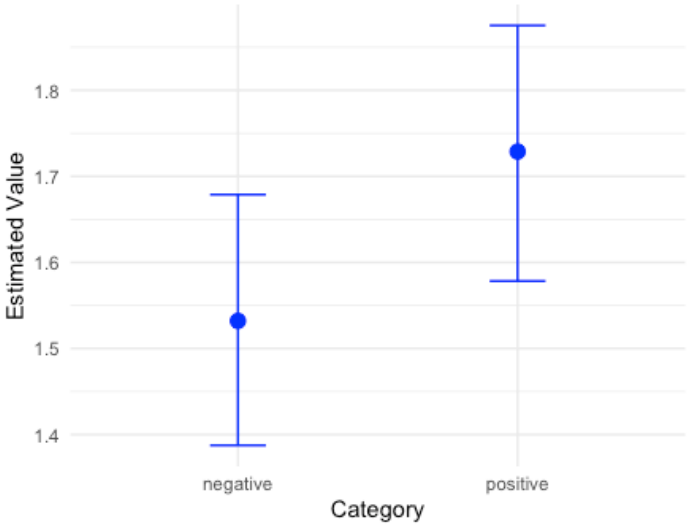

Figure 2. Estimated effect of infection on protein concentration based on the Bayesian Tobit model. Posterior mean estimate and 95% credible interval for the effect of the infected group on overall protein concentration.

Table 2. Top 10 proteins with the largest absolute log fold change and corresponding significance values.

Protein	logFC	p-value	adj.p-value
PocR ligand-binding domain-containing protein	-7,36218	0,007443	0,481581
DNA-directed RNA polymerase subunit beta'	-5,88011	0,010626	0,481581
C40 family peptidase (Fragment)	-7,11622	0,023203	0,481581
Hemoglobin subunit alpha-D	5,981186	0,030754	0,481581
Large ribosomal subunit protein bL27	5,464271	0,030937	0,481581
Winged helix-turn-helix domain-containing protein	-6,161	0,031186	0,481581
Rne/Rng family ribonuclease (Fragment)	-5,09971	0,033392	0,481581

Amidohydrolase family protein (Fragment)	-5,0408	0,033854	0,481581
C4-type zinc ribbon domain-containing protein	-4,99478	0,03431	0,481581
DNA protecting protein DprA	4,929941	0,034848	0,481581

Negative values of logFC indicate lower expression in the infected group compared to controls, while positive values indicate higher expression. logFC: log fold change; p-values: unadjusted p-values; adj.p-value: adjusted p-values (FDR). No proteins reached statistical significance after FDR correction.

For functional pathway analysis (n = 38), the Bayesian linear mixed-effects model estimated a modest effect of infection (estimate = 0.36, 95% CI: [–0.09, 0.79]), although the credible interval included zero (Table 3). Pathway-level variability was high (SD = 6.16), as was residual variance (SD = 5.77). Although several pathways exhibited notable fold changes, none met the criteria for statistical significance after FDR adjustment (Table 3). Global compositional analyses (PERMANOVA: $R^2 = 0.055$, $F = 1.05$, $p = 0.392$; PCA and PCoA) revealed substantial overlap between infected and control samples.

Table 3. Top 10 functional pathways ranked by evidence of differential abundance.

Pathway	logFC	P.Value	adj.p-value
huntington_disease_p00029	-3.400735	0.05434367	0.5358081
vitamin_d_metabolism_and_pathway_p04396	5.001452	0.12413911	0.5358081
egf_receptor_signaling_pathway_p00018	2.782077	0.1437023	0.5358081
fgf_signaling_pathway_p00021	2.782077	0.1437023	0.5358081
parkinson_disease_p00049	2.782077	0.1437023	0.5358081
cadherin_signaling_pathway_p00012	-2.32676	0.19241542	0.5358081
wnt_signaling_pathway_p00057	-2.32676	0.19241542	0.5358081
vitamin_digestion_and_absorption	3.144938	0.2008284	0.5358081
influenza_a	4.051805	0.20700077	0.5358081
neuroactive_ligand_receptor_interaction	4.051805	0.20700077	0.5358081

Negative values of logFC indicate lower expression in the infected group compared to controls, while positive values indicate higher expression. logFC: log fold change; p-values: unadjusted p-values; adj.p-value: adjusted p-values (FDR). No pathways reached statistical significance after FDR correction.

In the analysis of biological processes (n = 9), a slightly stronger effect was detected (estimate = 0.60, 95% CI: [0.17, 1.03]). Among the processes, *developmental process* showed a nominally significant unadjusted p-value ($p < 0.05$); however, this

result did not remain significant after correction for multiple comparisons. Overall, none of the biological processes met the significance threshold following FDR adjustment (Table 4). The intercept for the control group was estimated at 5.92 [1.72, 9.89], with high residual (SD = 6.83) and random effect variability (SD = 6.53). PERMANOVA showed no significant group separation ($R^2 = 0.073$, $F = 1.41$, $p = 0.36$), and ordination analyses confirmed strong group overlap.

Table 4. Differential abundance results for biological processes.

Biological Process	logFC	p-value	adj.p-value
developmental_process	-1.27621554	0.03595366	0.3595366
localization	1.94344828	0.09656887	0.4828444
homeostatic_process	3.14072237	0.17732355	0.5330334
metabolic_process	1.60190989	0.21321337	0.5330334
multicellular_organismal_process	-0.69732012	0.38378537	0.7675707
cellular_process	0.36860625	0.62442156	0.8604248
interspecies_interaction	0.3022397	0.6883398	0.8604248
response_to_stimulus	0.15187429	0.83933781	0.9325976
biological_regulation	0.02177744	0.96911855	0.9691185

Negative values of logFC indicate lower expression in the infected group compared to controls, while positive values indicate higher expression. logFC: log fold change; p-values: unadjusted p-values; adj.p-value: adjusted p-values (FDR). No biological process reached statistical significance after FDR correction.

Analysis of molecular functions (n = 9) showed a similar result (estimate = 0.56, 95% CI: [0.15, 1.00]; control intercept = 4.26 [1.63, 6.82]; residual SD = 6.50; random effect SD = 4.13). As in previous levels, global analysis did not indicate significant differences (PERMANOVA: $R^2 = 0.069$, $F = 1.33$, $p = 0.22$), and none of the functions passed the FDR threshold for statistical significance (Table 5), with ordination analyses confirming strong group overlap.

Table 5. Differential abundance results for molecular functions.

Molecular Function	logFC	p-value	adj.p-value
molecular_adaptor_activity	-1.832773	0.107470	0.639810
transporter_activity	2.404668	0.129623	0.639810
structural_molecule_activity	-1.316140	0.198367	0.639810
binding	0.972101	0.269256	0.639810

catalytic_activity	1.205032	0.319905	0.639810
antioxidant_activity	1.473245	0.473227	0.738612
atp_dependent_activity	1.675976	0.517028	0.738612
transcription_regulator_activity	0.109857	0.900841	0.971577
molecular_function_regulator_activity	0.051439	0.971577	0.971577

Negative values of logFC indicate lower expression in the infected group compared to controls, while positive values indicate higher expression. logFC: log fold change; p-values: unadjusted p-values; adj.p-value: adjusted p-values (FDR). No molecular function reached statistical significance after FDR correction.

48 Hours Post-Inoculation

As observed at 24 hours, protein expression at 48 hours post-inoculation remained highly zero-inflated (~89.5%). The Bayesian Tobit model estimated a modest increase in overall protein concentrations in the infected group compared to controls (estimate = 0.26, 95% CI: [0.12, 0.40]), with a control group intercept of 1.84 [1.70, 1.98]. This global effect suggests a mild but consistent shift in proteomic expression following infection. The overall distribution of posterior estimates is visualized in the credible interval plot (Figure 3). Despite the modest average change, ten proteins presented $p<0.05$ (Table 6) but only one protein (alpha-2-macroglobulin-like protein 1) stood out with a highly significant and biologically meaningful increase. This protein exhibited a $\log_2$ fold change of 13.13 and remained significant after FDR correction (adjusted p = 0.0081), representing an over 8,000-fold elevation in the infected group (Table 6).

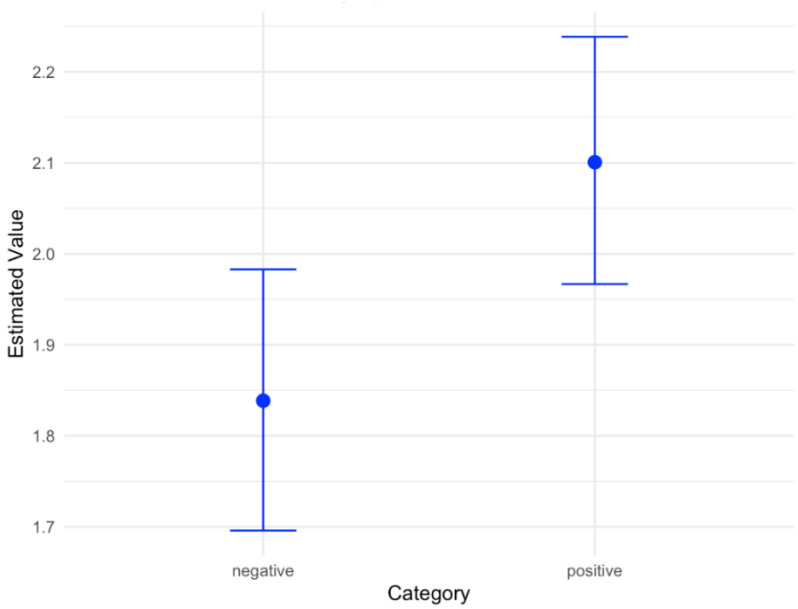

Figure 3. Estimated effect of infection on protein concentration at 48 hours post-infection. Posterior mean estimate and 95% credible interval for the effect of infection on protein concentration at 48 hours, based on the Bayesian Tobit model.

Table 6. Top 10 proteins ranked by evidence of differential abundance at 48 hours post-infection.

Protein	logFC	p-value	adj.p-value
alpha_2_macroglobulin_like_protein_1	13,1272	5,52E-06	0,0080
eh_domain_binding_protein_1_like_protein_1	9,5960	0,00281	0,5453
cadherin_domain_containing_protein	-7,7774	0,00315	0,5453
pit54_protein	8,2560	0,00978	0,5453
Plasminogen	7,1136	0,01245	0,5453
leucine_zipper_protein_1	-7,0448	0,01386	0,5453
fibrinogen_c_domain_containing_1	8,07286	0,01486	0,5453
rous_sarcoma_virus_transcription_enhancer_factor_iii	7,57050	0,01609	0,5453
protein_tyrosine_phosphatase	7,41027	0,01625	0,5453
ciliogenesis_and_planar_polarity_effector_complex_subunit_1	-6,4227	0,01629	0,5453

Negative values of logFC indicate lower expression in the infected group compared to controls, while positive values indicate higher expression. logFC: log fold change; p-values: unadjusted p-values; adj.p-value: adjusted p-values (FDR).

At the pathway level, the Bayesian hierarchical model estimated a significant increase in expression values in the infected group (estimate = 0.31, 95% CI: [0.12, 0.51]), indicating consistently higher pathway-associated concentrations compared to controls. The model accounted for moderate variability across pathways (random effect SD = 3.28; residual SD = 5.23), with a control group intercept of 3.13 [1.54, 4.70]. Despite these differences in magnitude, multivariate analysis did not detect significant compositional shifts (PERMANOVA: $F = 1.14$, $R^2 = 0.066$, $p = 0.284$), and ordination confirmed overlapping group structures. Several pathways exhibited relatively large $\log_2$ fold changes, and six pathways showed nominal significance (unadjusted $p < 0.05$). However, none of these results remained statistically significant after FDR adjustment (Table 7).

Table 7. Top molecular pathways ranked by evidence of differential abundance at 48 hours post-infection.

Pathway	logFC	p-value	adj.p-value
plasminogen_activating_cascade_p00050	5.865	0.008789	0.65879
mitophagy_animal	-6.065	0.019005	0.65879
carbon_metabolism_gga01200	6.843	0.024512	0.65879
pyruvate_metabolism_gga00620	6.843	0.024512	0.65879
blood_coagulation_p00011	3.136	0.030551	0.65879
ppar_signaling_pathway	4.905	0.033103	0.65879
pi3_kinase_pathway_p00048	2.763	0.051977	0.65879
hormone_signaling_gga04081	2.701	0.055584	0.65879
transcription_regulation_by_bzip_transcription_factor_p00055	-3.344	0.059852	0.65879
angiotensin_ii_stimulated_signaling_through_g_proteins_and_beta_arrestin_p05911	2.759	0.066855	0.65879

Negative values of logFC indicate lower expression in the infected group compared to controls, while positive values indicate higher expression. logFC: log fold change; p-values: unadjusted p-values; adj.p-value: adjusted p-values (FDR). No pathways reached statistical significance after FDR correction.

At the biological process level (n = 18), the Bayesian hierarchical model estimated a small but statistically credible increase in the infected group (estimate = 0.21, 95% CI: [0.08, 0.33]), indicating slightly higher biological process–associated concentrations compared to controls. The model accounted for moderate variability across processes (random effect SD = 3.28). Despite this shift in average abundance, global compositional

differences were not significant (PERMANOVA: $F = 0.24$, $R^2 = 0.015$, $p = 0.89$), and ordination confirmed strong overlap between groups. Differential abundance analysis revealed moderate $\log_2$ fold changes in some biological processes; however, none reached statistical significance, either before or after adjustment for multiple comparisons (Table 8).

Table 8. Top biological processes ranked by evidence of differential abundance at 48 hours post-infection.

Biological Process	logFC	p-value	adj.p-value
rhythmic_process_go_0048511	1.5904	0.1816	0.9068
cellular_process_go_0009987	1.9694	0.2364	0.9068
multicellular_organismal_process_go_0032501	0.6508	0.2927	0.9068
locomotion_go_0040011	-0.9851	0.3234	0.9068
developmental_process	0.3288	0.5572	0.9068
biological_process_involved_in_interspecies_interaction_between_organisms_go_0044419	0.5457	0.5831	0.9068
metabolic_process_go_0008152	0.2566	0.6115	0.9068
homeostatic_process_go_0042592	0.3278	0.6793	0.9068
localization_go_0051179	0.1862	0.7008	0.9068
response_to_stimulus_go_0050896	0.1768	0.7541	0.9068

Negative values of logFC indicate lower expression in the infected group compared to controls, while positive values indicate higher expression. logFC: log fold change; p-values: unadjusted p-values; adj.p-value: adjusted p-values (FDR). No biological process reached statistical significance after FDR correction.

At the molecular function level ($n = 14$), the Bayesian hierarchical model estimated a small but statistically credible increase in the infected group (estimate = 0.25, 95% CI: [0.08, 0.42]), suggesting slightly higher molecular function–related values compared to controls. The model accounted for moderate variability across functions (random effect SD = 1.04). Despite this shift in average concentration, multivariate analysis did not indicate significant global compositional differences between groups (PERMANOVA: $F = 0.99$, $R^2 = 0.059$, $p = 0.42$), and ordination confirmed substantial overlap. Differential abundance testing highlighted some functions with relatively high $\log_2$ fold change, but none of these were statistically significant, either before or after FDR correction (Table 9).

Table 9. Top molecular functions ranked by evidence of differential abundance at 48 hours post-infection.

Molecular Function	logFC	p-value	adj.p-value
antioxidant_activity_go_0016209	3.61626	0.13907	0.68963
transcription_regulator_activity_go_0140110	0.64910	0.21559	0.68963
structural_molecule_activity_go_0005198	0.83609	0.21573	0.68963
translation_regulator_activity_go_0045182	2.05640	0.26655	0.68963
molecular_function_regulator_activity_go_0098772	0.43516	0.29336	0.68963
cargo_receptor_activity_go_0038024	1.90976	0.35872	0.68963
atp_dependent_activity_go_0140657	-0.66552	0.39407	0.68963
catalytic_activity_go_0003824	0.34276	0.51208	0.79658
molecular_transducer_activity_go_0060089	-0.37933	0.56914	0.79680

Negative values of logFC indicate lower expression in the infected group compared to controls, while positive values indicate higher expression. logFC: log fold change; p-values: unadjusted p-values; adj.p-value: adjusted p-values (FDR). No molecular function reached statistical significance after FDR correction.

DISCUSSION

Salmonella Pullorum (SP) is a highly virulent embryonic pathogen that causes high mortality in chicken embryos (CE). In previous studies, we observed that the older the embryos, the more resistant they become to infection, depending on the bacterial dose (de Souza et al., 2024). Therefore, in this study, we inoculated a very low dose (1 log CFU/CE) in an attempt to evaluate the surviving embryos. In this model, we observed an embryo mortality rate of 30% after 48 hours, which was similar to findings in older embryos exposed to higher doses and evaluated over the same period. This high mortality confirms that the bacteria were active and suggests that some embryos may have mounted an immune response to limit bacterial proliferation (Li et al., 2019).

This hypothesis is supported by our weight data: embryos in the SP-challenged group had lower body weights compared to controls, with a p-value close to significance ($p = 0.0506$), indicating a trend. Similarly, embryos inoculated with SP at 13 days of embryonic development (DED) and evaluated at 17 DED also exhibited weight loss (de Souza et al., 2024). These findings support our hypothesis that, despite their young age, some embryos initiate an immune response to counteract the pathogenicity of SP. To

further explore the molecular mechanisms involved in this response, we performed a non-targeted proteomic analysis.

Non-targeted proteomics is a powerful approach to explore the biological responses and molecular pathways activated during infection. While we acknowledge the limitations posed by the incomplete protein database for our target species (*Gallus gallus*), with only 5.76% and 56.6% of the proteins successfully identified at 24 and 48 hours respectively, we emphasize that the results obtained are highly reliable.

Our findings are supported by robust statistical analyses, which remain valid despite the inherent challenges of zero-inflated data. Therefore, although we recognize that some pathways, biological processes, and molecular functions may not have been detected or discussed due to database limitations, we are confident in the accuracy and biological relevance of the proteins and pathways that were identified.

Many of the values obtained for proteins, metabolic pathways, biological processes, and molecular mechanisms in our comparison between two groups (CP and CN) showed statistical significance at the unadjusted level ($p < 0.05$). However, these differences did not remain significant after correction for multiple testing. Despite this, we chose to discuss these findings as biological trends, in line with the approach supported by (O'Connell et al., 2018), who emphasize that in proteomics analyses with small sample sizes and missing values, strict p-value adjustment can mask biologically meaningful signals and increase false negatives. Additionally, Rubin (2024) argues that in simple comparisons involving only two groups, adjusting p-values may not be necessary and can unnecessarily reduce statistical power. These perspectives suggest that, particularly in exploratory studies, consistent trends supported by biological plausibility and fold-change directionality may provide valuable insights, even in the absence of adjusted statistical significance (Diz et al., 2011).

Although only one protein reached statistical significance after multiple testing correction, our results consistently revealed small but credible increases in protein abundance and functional activity in infected samples. These effects were detected through Bayesian modeling under zero-inflated conditions, a common challenge in proteomic datasets (Koopmans et al., 2014).

At 24 hours post-inoculation, the Bayesian Tobit model indicated a modest increase in overall protein concentration in the infected group. Although no individual proteins passed the significance threshold after FDR adjustment, the general upward trend

may reflect the embryo's early systemic response to infection. For example, hemoglobin subunit alpha-D, known to function as a mitochondrial antioxidant in non-erythroid cells (Reed et al., 2025), was upregulated in positive samples and could suggest early oxidative stress responses in infected embryos.

The protein annotated as "Winged helix-turn-helix domain-containing protein" showed a tendency toward reduced abundance in infected embryos. Although this difference did not remain statistically significant after p-value correction, it was in line with the only biological process with a negative fold change and significant unadjusted p-value: "developmental process." This alignment suggests that infection may interfere with key regulatory mechanisms active during early embryogenesis. Biological processes were classified using the PANTHER system, which incorporates Gene Ontology data (Carbon et al., 2021), and describes how living systems evolve over time. At early developmental stages, proper genome activation and transcription factor activity are crucial. Supporting this, Liao et al. (2022) reported dynamic expression of key transcription factor families during chicken embryogenesis, including Fox, belonging to the winged helix-turn-helix class, reinforcing the relevance of this protein's downregulation within the broader context of developmental regulation.

The minimal changes observed in inflammatory pathways, evidenced by only one inflammation-related protein showing an unadjusted p-value < 0.05, combined with the downregulation of development-associated proteins, align with the outright absence of mortality at 24 hours post-infection. Considering that even highly virulent *Salmonella* strains typically cause significant embryo mortality only after ~48 hours (Li et al., 2019).

By 48 hours post-inoculation, the expression pattern remained highly zero-inflated, yet the overall shift in protein abundance became more pronounced. The Bayesian Tobit model estimated a credible increase in protein levels in the infected group (estimate = 0.26, 95% CI: [0.12, 0.40]), reflecting a consistent global effect of infection. While most individual proteins did not reach statistical significance after multiple testing correction, alpha-2-macroglobulin-like protein 1 was significantly upregulated, indicating a targeted and biologically relevant response. This suggests that, alongside a subtle and diffuse modulation across many proteins, infection can also induce marked changes in selected components, which may reflect early activation of immune or stress pathways in the developing embryo (Vandooren & Itoh, 2021).

Although α2-macroglobulin (A2M) is known to be a ubiquitous plasma protein across vertebrates (Bergwik et al., 2021; Sottrup-Jensen, 1989) its immunomodulatory role has been thoroughly documented only in mammalian species (Lagrange et al., 2022; Rehman et al., 2013; Vandooren & Itoh, 2021). In contrast, avian studies have focused almost exclusively on A2M (referred to as ovostatin in birds) in egg white, where it is synthesized by the oviduct and acts as an antibacterial, protease-inhibiting defense factor for the embryo (Lim et al., 2011; Pathirana et al., 2016; Wang et al., 2019).

Importantly, no studies to date have examined A2M in chicken blood or liver in response to infection, and its presence in circulating avian tissues remains largely unexplored. Intriguingly, however, multiple proteomic analyses of embryonic chicken liver, originally aimed at metabolic profiling (Peng et al., 2018; Shen et al., 2023; Yang et al., 2021), consistently report the presence of A2M isoforms in supplementary data. This strongly suggests that A2M is endogenously synthesized by the embryo rather than merely maternally deposited. Therefore, our findings of differential A2M abundance in infected embryos indicate not a residual from egg white, but rather a true embryonic immune response, with production and regulation by the embryo itself. The significant upregulation of α2-macroglobulin-like protein 1 at 48 hours post-infection (coinciding with the onset of embryo mortality) raises the possibility that this protein may act as a biomarker of inflammation and disease progression in the embryonic context.

In mammals, A2M is a well-established acute-phase protein induced primarily by innate immune signals such as IL-6 and TNF-α, and functions as a broad-spectrum protease inhibitor, cytokine carrier, and modulator of immune cell activity, particularly macrophages and neutrophils. Besides, its immunomodulatory properties extend to adaptive immune processes, making it a key player in both arms of immunity (Vandooren & Itoh, 2021). Although adaptive immunity is not fully functional at this embryonic stage, the observed increase in A2M may reflect innate immune activation in response to systemic infection. Its emergence only at 48 hours, and not earlier, suggests a role in later-stage responses, potentially associated with attempts to limit tissue damage or regulate inflammation. Given the temporal association with increased mortality, it is plausible that α2-macroglobulin contributes to survival pathways under infectious stress, a hypothesis that remains unexplored in avian models and warrants further investigation. The present study not only identified a potential biomarker for inflammation in chickens but also highlighted a critical knowledge gap: whether A2M truly does not play a role in the

adaptive immune response in chickens, as it does in mammals, or whether its function in this species remains largely unexplored.

At the functional level, pathways, biological processes, and molecular functions showed slightly higher values in the infected group. Although these differences did not reach statistical significance after correction for multiple testing, the credible intervals of the model estimates excluded zero in several comparisons, suggesting weak but consistent biological effects. As observed in the 24-hour dataset, PERMANOVA and ordination analyses revealed no statistically significant global differences between groups, which supports the idea of subtle and distributed proteomic modulation.

Despite the absence of statistically robust findings, some pathways exhibited relatively large fold changes ($p<0.05$ before the adjusted p-value) that may indicate biologically meaningful trends. One notable example is the Plasminogen activating cascade, which promotes the conversion of plasminogen into active plasmin. This pathway plays a central role not only in fibrinolysis and extracellular matrix remodeling, but also in inflammation and tissue repair (Syrovets et al., 2012), and may reflect early host responses to infection-induced tissue stress.

Similarly, the blood coagulation pathway was upregulated in infected embryos, likely reflecting the activation of systemic inflammatory responses. Previous studies have shown that bacterial infection can trigger inflammation-driven coagulation cascades emphasizing the strong link between coagulation and immune activation in chickens (Li et al., 2023). In this context, increased coagulation activity may serve as an early and sensitive indicator of infection-induced inflammation.

In parallel, activation of the PPAR signaling pathway in infected embryos suggests an attempt to regulate inflammation and maintain metabolic balance. PPARs are nuclear receptors involved in lipid metabolism and immune modulation, and their activation can promote anti-inflammatory responses or facilitate pathogen persistence, depending on the context (Li et al., 2025). In this study, PPAR signaling may reflect a compensatory mechanism aimed at modulating the host's inflammatory state. These immunometabolic adaptations are further supported by the upregulation of pathways related to carbon and pyruvate metabolism, which may indicate increased energy demands in response to infection-induced stress. Such metabolic shifts are consistent with reprogramming events described in broiler chickens, where PPAR activation was associated with attenuation of

inflammation and modulation of energy metabolism under challenging conditions (Li et al., 2025)

Finally, the observed downregulation of mitophagy in infected embryos may compromise mitochondrial quality control during a critical period of embryonic development. Mitophagy is essential for eliminating damaged mitochondria and ensuring proper cell differentiation (Chen et al., 2021). Its suppression could therefore contribute to the developmental disturbances already observed in our dataset. Similar findings have been reported in embryos exposed to environmental toxins, where impaired mitophagy led to oxidative damage and embryotoxicity (Liu et al., 2023), supporting a broader role for mitochondrial stress in mediating infection-related developmental impairment.

The use of the CE as a model system, particularly before 12 DED, introduces several analytical challenges. Low protein levels, high biological variability, limited detection, and the lack of functional information for *Gallus gallus* make it difficult for conventional analyses to detect clear differences between groups. These limitations were addressed using Bayesian hierarchical models, including Tobit regression to handle censoring and mixed-effects models to account for variability across proteins and functional terms. These models proved effective in capturing nuanced, low-intensity signals associated with infection, providing a more interpretable representation of the host response under these constrained experimental conditions.

To our knowledge, this is the first study to characterize proteomic changes in the blood of chicken embryos infected prior to 12 DED. Moreover, this is the first report to associate A2M with an endogenous immune response in the embryonic or even adult bloodstream of *Gallus gallus*. While previous studies have documented the presence of this protein in the egg white, deposited during oogenesis, no prior work has demonstrated its differential expression in embryonic liver or circulation because of infection.

This finding highlights not only the sensitivity of the model but also expands our understanding of innate immune responses in early avian ontogeny. Further validation is needed to confirm the role of A2M in the avian immune system. However, its consistent upregulation in response to infection suggests that A2M may play a similar immunomodulatory role in birds as described in mammals, extending its relevance to early embryonic development in avian species. In this context, A2M could represent a promising target for future studies aiming to identify new biomarkers of systemic

inflammation in chickens. Altogether, these findings support the utility of the CE before 12 DED as a powerful and tractable model for infection and inflammation.

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CHAPTER 3

CHICKEN EMBRYOS BEFORE 12 DAYS OF DEVELOPMENT AS AN ALTERNATIVE MODEL FOR INFECTION AND INFLAMMATION STUDIES

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ABSTRACT

The chicken embryo (CE) model offers a practical and physiologically relevant system for studying infection and inflammation, while supporting animal welfare through reduced use of post-hatch animals and adherence to the 3Rs. In a previous study, we used *Salmonella* Pullorum (SP) to model inflammation in late-stage CE. To ethically refine this approach, we inoculated embryos at 7 or 9.5 days of development via the allantoic cavity and assessed them 48 hours later as a model of acute inflammation. Embryos were monitored for mortality, serum and allantoic fluid were collected to quantify acute phase proteins (Serum Amyloid A, Alpha-1-Acid Glycoprotein, Ovotransferrin), cytokines (IL-4, IL-10), and corticosterone by ELISA, and kidneys were collected for histopathological analysis. In a separate experiment, CE were inoculated with SP at 9.5 days and blood, liver and spleen samples were collected at 11.5 and 17 days for flow cytometry and cytokines gene expression analysis by RT-PCR to evaluate the immunological response throughout development. Embryo mortality significantly increased at 48h post-inoculation about 50%. SP infection triggered early inflammation, marked by increased SAA in allantoic fluid and decreased IL-10 in serum. There was a decrease of corticosterone in CE challenge with SP. Flow cytometry revealed age-related changes in lymphocyte and antigen-presenting cell populations. A decline in $CD8^+$ T cells at 11.5 DED and an increase in $TCR\gamma\delta^+$ cells at 17 DED were observed in infected embryos, while $CD4^+$ T cell frequencies remained stable. T lymphocytes were more prevalent than B lymphocytes, likely due to earlier thymic development and their central role in early immune responses. Gene expression analysis showed decreased TGF and increased IL-8 levels with advancing age, suggesting developmental regulation of immune signaling. Overall, CE infected with SP before 12 days of development exhibit an early inflammatory response, supporting their applicability as a model for early-stage inflammation.

INTRODUCTION

Compared to traditional models like rodents, the chicken embryo (CE) model stands out for being easier to handle, faster and cheaper to use, and suitable for testing a wide variety of compounds and therapies. It also offers high reproducibility and reliability, supported by its well-understood biological and physiological features (Garcia et al., 2021).

First systematically studied by Aristotle, CE has long been recognized as a valuable model for embryological research. Its developmental progression is conventionally described according to incubation time, maintaining its historical significance in comparative and developmental biology (Hamburger & Hamilton, 1992). Beyond its classical embryological relevance, CE also serve as a bridge between *in vitro* and *in vivo* studies, providing an essential early step in evaluating toxicity and drug effects that require whole-organism responses not possible in cell cultures (Campitelli et al., 2025).

Ethically, the use of CE offers additional advantages. In most countries, CE are not classified as live animals until 17 days of embryonic development (DED), which exempts their experimental use from requiring prior ethics committee approval (Ribatti & Annese, 2023). The European Directive 2010/63/EU states that ethical clearance is unnecessary for research involving CE. Similarly, both the Institutional Animal Care and Use Committee (IACUC) and the USA National Institutes of Health consider embryos under 14 DED incapable of experiencing pain, allowing their use in studies without ethical restrictions or protocol submission (IACUC, 2022).

However, in Brazil, recent regulations have introduced specific ethical considerations for the use of CE. The Brazilian National Council for the Control of Animal Experimentation (CONCEA/MCTI), through Informative Note CONCEA No. 01/2024, states that research projects and teaching protocols involving embryos beyond 50% of their developmental stage should be submitted for ethical review and approval by Institutional Animal Care and Use Committees (CEUAs), to ensure animal welfare and uphold ethical standards in research (CONCEA/MCTI, 2024).

Although the CE's nervous system starts forming around 1 DED, the anatomical and functional components necessary for pain perception are not fully developed until approximately 13 DED (Sarnella et al., 2024). Therefore, the use of CE in biomedical research offers an ethical and effective alternative to more sentient animal models, especially when embryos are used before day 15 of development, which is considered the

threshold for the onset of pain perception in chickens (Süß et al., 2023), while still being sufficiently developed to exhibit essential physiological responses.

One such physiological response is the acute phase response (APR), which in chickens is defined by the manifestations of fever, anorexia, inflammation, and production of acute phase proteins (APPs) predominantly in the liver. Thus, the presence of increased APPs in serum serves as a useful physiological biomarker to characterize a disease challenge (Riva et al., 2024).

In CE most APPs are synthesized by hepatocytes around 7 DED, reaching its peak by 14 DED, which coincides with increased capacity for APP production (Hanafi Sulong et al., 2021). In parallel, stress-related biomarkers such as corticosterone are also relevant. The adrenal glands of CE become functionally active by 8 DED, with corticosterone secretion showing slight fluctuations between 10 and 14 DED, followed by a marked increase approaching 16 DED (Hanafi Sulong et al., 2021).

To establish a reliable animal model for studying inflammation, it is essential to evaluate both cellular and molecular components of the immune response(Garcia et al., 2021). Monitoring the distribution and dynamics of immune cell populations (such as T and B lymphocytes and macrophages) provides insight into the progression and regulation of inflammation. In parallel, the analysis of cytokine expression offers a molecular perspective on the balance between pro- and anti-inflammatory signals(Alkie et al., 2019). Together, these parameters contribute to a comprehensive understanding of immunomodulation and are fundamental for characterizing the inflammatory potential and responsiveness of the model.

The thymus and bursa of Fabricius, the primary lymphoid organs responsible for T and B cell development, begin to form within the first days of embryogenesis and reach significant structural and functional maturation by mid-embryonic development (Fellah et al., 2013; Ribatti et al., 2019). In parallel, several cytokines involved in innate and adaptive immune responses (such as IL-1β, IL-8, IL-12, IL-18, IL-4, IL-10, and IFN-γ) are already detectable between embryonic days 3 and 12 DED (Abdul-Careem et al., 2007; Anastasiadou & Michailidis, 2016).

Despite the broad use of CE as a research model, its application in inflammation studies still lacks standardization and validation at earlier stages of development. In a previous study, we successfully used CE at 17 days of embryonic development (DED) as an inflammation model by inoculating SP at 11 DED (de Souza et al., 2024). Although late-stage embryos offer advantages over post-hatch models, including reduced stress,

investigating earlier stages (before 12 DED) is essential to strengthen the ethical, sustentability and scientific value of CE as a model for infection and inflammation. Therefore, this study aimed to assess the suitability of early-stage embryos as a model for inflammation by inoculating SP as experimental model and analyzing the progression of lymphocyte and monocyte populations, as well as cytokine expression, up to 17 DED.

MATERIAL AND METHODS

The experiments were conducted at the Poultry Incubation Laboratory (LIAVE), the Laboratory of Infectious and Contagious Diseases (LADOC) and the Nanobiotechnology Laboratory Prof. Luiz Ricardo Goulart Filho (NANOS) at the Federal University of Uberlândia (UFU). Embryonated eggs from the Hy-Line strain of *Gallus gallus* were used and maintained in an automatic incubator (Premium Ecológica) at 37°C and 55% humidity. The project was evaluated and approved by the Animal Ethics Committee (CEUA) of UFU, under protocol numbers 008/21 and 23117.010881/2024-41. All procedures followed the standards and regulations established by the National Council for the Control of Animal Experimentation (CONCEA).

Pilot test of inflammation

We used SP as a model for infection and inflammation, based on our previous experience with this bacterium in CE at later developmental stages (de Souza et al., 2024a). Since we already knew it could cause complete embryo mortality at earlier stages, we used a lower concentration of the pathogen in a pilot test to evaluate whether SP could be a feasible model for early-stage infection in CE. A total of 20 embryonated eggs from the Hy-Line strain of *Gallus gallus* were used and maintained in an automatic incubator (Premium Ecológica) at 37°C and 55% humidity. On day 7 DED, the CE were divided into two groups: 10 CE formed the positive control (PC), inoculated with 100µL of 2 log CFU/mL of SP and 10 CE formed the negative control (NC), inoculated with 100 µL of 0.85% saline solution. The embryonaded eggs were monitored daily for mortality assessment using candling. At 24 hours post-inoculation, no mortality was observed. Two embryos were euthanized and blood collection was proceeded on day 8 DED, but the volume of blood was less than 100 µL, which means a volume too small for the necessary

analyses. A sharp increase in mortality was observed starting at 48 hours post-inoculation, reaching a 90% death rate at 72 hours.

Chicken embryos assay for ELISA and histopathological analysis

Based on the results of the pilot test, two independent assays were conducted using different inoculation times. In the first assay, 20 embryonated eggs were inoculated with 100µL of 2 log CFU/mL of SP, and another ten received 100 µL of 0.85% saline solution (NC), both via the allantoic fluid at 7 DED. Eggs were monitored daily for mortality using candling. At 9 DED (48 hours post-inoculation), allantoic fluid and blood serum were collected for the analysis of inflammatory proteins using ELISA kits.

In the second assay, 25 embryonated eggs were used. On day 9.5 of embryonic development (DED), 15 eggs were inoculated with the same concentration of SP, and the remaining 10 received 100 µL of 0.85% saline solution (NC), both via the allantoic cavity. Eggs were monitored daily for viability. At 11.5 DED (48 hours post-inoculation), mortality was assessed, and samples were collected from the surviving embryos in each group (PC and NC). The increased embryo size at this stage enabled the collection of larger volumes of blood and additional tissue samples, supporting a broader range of analyses. Allantoic fluid and blood serum were collected for the analysis of inflammatory proteins, corticosterone, and anti-inflammatory interleukins using ELISA kits, while kidneys were harvested for histopathological evaluation.

Inflammation assay for flow cytometry and RT-PCR analysis

Considering the relatively large volume of whole blood required for flow cytometry, and the limited blood availability in early-stage CE, we designed an independent experiment and included older embryos samples to evaluate the maturation of the immune response in relation to age. A total of 138 embryonated chicken eggs were used and allocated into two experimental assays. In the first assay, embryos were inoculated at 9.5 days of embryonic development (DED), and blood samples were collected at 11.5 DED. In the second assay, blood collection occurred at 17 DED. Due to the early developmental stage and limited blood volume in the first assay, flow cytometry was performed in three separate experiments, each corresponding to one antibody panel.

In contrast, the second assay comprised a single experiment encompassing all three panels simultaneously.

For the first assay, 32 eggs were used per panel. The positive control (PC) group consisted of 20 embryos inoculated via the allantoic cavity with 100 µL of SP suspension at a concentration of 2 log CFU/mL. The negative control (NC) group comprised 12 embryos that received 100 µL of 0.85% sterile saline solution. Embryo viability was assessed daily through candling. At 11.5 DED (48 hours post-inoculation), blood was collected from viable embryos into whole blood collection tubes containing EDTA and immediately processed for flow cytometric analysis. Additionally, pooled samples of liver, spleen, and intestines were collected and stored at –80 °C for RT-PCR analysis of interleukin gene expression.

In the second assay, 42 eggs were used. On day 9.5 DED, 30 embryos were inoculated with the same concentration of SP, while the remaining 12 received saline solution, both via the allantoic cavity. Embryos were monitored daily for viability. At 17 DED (7 days post-inoculation), mortality was recorded, and blood from surviving embryos in both PC and NC groups was collected into whole blood collection tubes containing EDTA and immediately processed for flow cytometric analysis. Additionally, pooled samples of liver, spleen, and intestines were collected and stored at –80 °C for RT-PCR analysis of interleukin gene expression.

Quantification of inflammatory proteins, corticosterone, and anti-inflammatory cytokines by ELISA kits

The quantification of acute-phase proteins—Serum Amyloid A (SAA), Alpha-1-Acid Glycoprotein, and Ovotransferrin—was performed using ELISA kits (MBS8807507, MBS053462, and MBS944289, respectively; MyBioSource®), following the manufacturers' instructions. Serum and allantoic fluid samples were collected from 9- and 11.5-day-old chicken embryos (CE) for this analysis.

At 17 days of embryonic development, it is possible to assess heart rate as an indicator of stress (Horr et al., 2023). However, since this assessment was not feasible at 12 days, we chose to measure corticosterone levels instead, in order to evaluate the stress response at this earlier stage. Corticosterone quantification was conducted using an ELISA kit (EIACORT; Invitrogen®), according to the manufacturer's instructions, using only serum samples from 11.5-day-old embryos. In a previous experiment, we observed

elevated IL-10 levels at 17 days of development in embryos infected with SP. In the present study, we aimed to determine whether this increase starts earlier, to ensure that the 48-hour post-infection time point does not yet correspond to a resolution or recovery phase of the infection. Anti-inflammatory cytokines IL-4 and IL-10 levels were quantified using ELISA kits (MBS282456 from MyBioSource® and ECH3RB from Invitrogen®, respectively) exclusively in serum from 11.5-day-old embryos. All analyses followed the manufacturers' protocols.

Flow Cytometry Analysis

The whole blood samples were centrifuged at 200 × g for 5 minutes at room temperature to separate the blood components. Following centrifugation, the leukocyte-rich buffy coat layer was carefully collected and transferred to a new tube. To remove residual erythrocytes, the cell suspension was incubated with erythrocyte lysis buffer for 15 minutes at room temperature. The samples were then centrifuged under the same conditions to pellet the leukocytes. After discarding the supernatant, the cells were resuspended in PBS supplemented with 5% BSA and incubated for 10 minutes at room temperature to reduce nonspecific binding prior to flow cytometry analysis.

Cells were then incubated for 30 minutes at 4°C in the dark with fluorochrome-conjugated antibodies according to the following panels: Panel 1 (Mouse Anti-Chicken TCRγδ-FITC, CD4-PE, CD3-PECγ5, and CD8-APC); Panel 2 (Mouse Anti-Chicken Monocyte/Macrophage-FITC, MHC Class II-PE, CD45-PE-Cγ5, and CD4-APC); and Panel 3 (Mouse Anti-Chicken Bu-1-PE, CD3-PE-Cγ5, and CD4-APC)

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Isotype controls and unstained negative controls were included to assess nonspecific antibody binding and cellular autofluorescence, respectively. The positivity threshold for marker expression was set at a relative fluorescence intensity value of 0.1, based on the fluorescence levels of these controls. Mean fluorescence intensity (MFI) values were recorded to quantify marker expression levels.

After staining, samples were washed three times and resuspended in PBS. Flow cytometric acquisition was performed using a Attune-Thermo flow cytometer, and a minimum of 100,000 events were collected per sample. Data were analyzed using software FlowJo.

Gene expression analysis by RT-qPCR

Each sample was analyzed in duplicate. Total cellular RNA was isolated from blood samples using Quick-zol reagent (Trizol) from Ludwig Biotec, following the protocol by (Fonseca et al., 2016) with modifications. Briefly, 250 µL of blood was mixed with 750 µL of Trizol reagent, then 200 µL of chloroform was added. The mixture was centrifuged at 12,000 × g for 15 minutes at 4ºC. The aqueous phase was carefully transferred to a new tube containing 500 µL of isopropanol, homogenized, and incubated at 4ºC for 10 minutes, followed by centrifugation at 12,000 × g for 10 minutes at 4ºC. The resulting pellet was washed with 75% ethanol and centrifuged at 7,500 × g for 5 minutes at 4ºC. After air drying, the RNA pellet was resuspended in nuclease-free water and heated at 56ºC for 10 minutes using a thermoblock.

Quantitative real-time reverse transcription PCR (RT-qPCR) was conducted using primers designed based on the NCBI reference sequences NM_205498.2 for *Gallus gallus* interleukin-8-like 2 (IL8L2) and X00182.1 for *Gallus gallus* cytoplasmic beta-actin (Table 1). The reactions were prepared with the GoTaq® Probe 1-Step RT-qPCR System (Promega). Each 20 µL reaction mixture included 10 µL of 2X GoTaq® Probe qPCR Master Mix with dUTP, 0.4 µL of GoScript™ RT Mix for 1-Step RT-qPCR, 1 µL of primers (10 pmol), 0.75 µL of probe, 4.85 µL of nuclease-free water, and 2 µL of RNA template. The annealing temperature was set at 60ºC for all primer pairs. To validate the RT-qPCR assay for interleukin gene expression, two types of positive controls were used. First, 10-day-old chicken embryos were infected with Newcastle Disease Virus (NDV), and tissues were collected for total RNA extraction. Second, liver and spleen explants from 10-day-old embryos were cultured in Dulbecco's Modified Eagle Medium (DMEM) and stimulated with phorbol 12-myristate 13-acetate (PMA) to induce cytokine expression. Negative controls consisted of elution buffer provided in the kit.

Table1. Primer sequences and characteristics for cytokine genes and reference gene used in RT-qPCR.

Genes	Primers	Sequence	No. Bases
IL-8	Foward	5'-CTC GTC TTT GCC AAG GTA GGA-3'	21
IL-8	Reverse	5'-AGA ATT GAG GTC AGG CTT GGC-3'	21

IL-8	Probe	5’-6-FAM-GTG GTA AAG ATG GGG AAT GAG CTG CG-Tamra-3’	26
IL-6	foward	5’-CTG TTC GCC TTT CAG ACC TAC C-3’	22
IL-6	Reverse	5’-CTT CAT GGC GAT TTA TAC CCA TC-3’	23
IL-6	Probe	5’-6-FAM-GGC GTC GTT TGT GCT GTA GCA-Tamra-3’	24
TGFβ-3	Foward	5’- TGA GTG GCT TCT GCA CAG AGA G -3’	22
TGFβ-3	Reverse	5’-AGT CTT GTT GCT TCT TAG GGC G-3’	22
TGFβ-3	Probe	5’-6-FAM-CAA CCT CGG CCT GGA AAT CAG CAT AC-Tamra-3’	26
BetaActina	Forward	5’-AAT GGC TCC GGT ATG TGC AA-3’	20
BetaActina	Reverse	5’-TCA TCA CCA ACT GGG TAG CTC TTC-3’	24
BetaActina	Probe	5’-6-FAM-CCA TAC CAA CCA TCA CAC CGT GAT GTC TG-Tamra-3’	29

Amplifications were performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) under the following thermal cycling conditions: reverse transcription at 45ºC for 15 minutes; initial enzyme activation and reverse transcriptase inactivation at 95ºC for 2 minutes; followed by 40 cycles of denaturation at 95ºC for 15 seconds and combined annealing/extension at 60ºC for 1 minute. Fluorescence data were collected at the end of each cycle and analyzed using Bio-Rad CFX Maestro 2.3 software.

To quantify gene expression, the cycle threshold (Ct) values were normalized against those of the housekeeping gene beta-actin (ΔCt). Relative expression changes were determined by comparing ΔCt values of treated samples to controls (ΔΔCt) according to a modified (Livak & Schmittgen, 2001) method. Fold change was calculated using the formula:

$$2\text{^}(-|\Delta\Delta Ct|)$$

Histopathological analysis

Kidney fragments were fixed in buffered 10% formalin and processed using routine histological techniques, with slight timing adjustments. The tissues were stained with hematoxylin and eosin (HE), following the protocol described by (Behmer & Tolosa, 2003). The slides were examined under light microscopy. Lesions were initially identified and scored blindly. After this first evaluation, the negative control groups were revealed, allowing for score adjustments based on baseline histological patterns. When necessary, some slides were reanalyzed to improve scoring accuracy.

Histological analysis focused on three main aspects: hydropic degeneration, inflammation, and vascular changes (congestion and hemorrhage). Cytoplasmic edema was assessed semi-quantitatively, with a scoring system ranging from 0 (normal) to 3 (marked), defined relative to the control group, as proposed by (de Souza et al., 2024). Inflammatory response was classified according to the number and intensity of cellular infiltrates: 0 (absent), 1 (mild, with up to two subtle foci), 2 (moderate, with three to four), and 3 (severe, with five or more foci or one intense focus).

For vascular alterations, only internal congestion and hemorrhage were considered as peripheral bleeding could result from tissue handling. These findings were also graded from 0 to 3, using the same severity criteria applied in the other evaluations.

Statistical analysis

For mortality assessments, comparisons between groups were performed using the chi-square test, followed by pairwise comparisons of proportions and calculation of odds ratios to quantify effect sizes. After the removal of outliers and the normality test, a t-test was performed to evaluate the effect of treatment within each age group. To analyze the acute inflammation proteins, corticosterone, and cytokines by ELISA, we constructed a standard curve considering $R > 95\%$. For the analysis of acute inflammation proteins, we performed ANOVA followed by the Tukey test. For cytokines and corticosterone analysis by ELISA, we used a t-test. A two-way ANOVA was conducted to assess the interaction between treatment and age. To cytokine levels we performed the statistical analysis of dCt and we calculates the fold change We considered $p<0.05$ using the Graph PadPrism program 10.02. For the histopathological kidney evaluation, the scores were compared between groups using the Wilcoxon test. Statistical analysis was performed based on the difference in median scores relative to the negative control group. Differences were considered significant when $p < 0.05$.

RESULTS

Chicken embryo mortality

In the pilot test, when we inoculated 1 log UFC/CE at 7 DED, a progressive pattern of mortality was observed among embryos inoculated with SP. Within the first 48 hours post-inoculation, one embryo died, followed by a sharp increase in mortality, with eight additional embryos succumbing by 72 hours. Only one embryo survived until the final evaluation at 120 hours (5 days post-inoculation), resulting in a total mortality rate of 90%.

There was no statistically significant difference in embryo mortality 48 hours post-inoculation between embryos inoculated at 7 DED and those inoculated at 9.5 DED ($p > 0.05$) (Table 2).

Table 2. Embryo mortality following bacterial inoculation in pilot test and two experimental trials

Experiment	**Embryonic day on inoculation**	**Treatment Group**	**No. of Embryos**	**Mortality at 24 hpi**	**Mortality at 48 hpi**	**Mortality at 72 hpi**	**Mortality at 5 dpi**
Pilot test	7	Inoculated	10	0/10 (0%)	8/9* (88,88%)	1/1 (100%)	-
Pilot test	7	Negative Control	10	0/10 (0%)	0/10 (0%)	0/10 (0%)	-
First trial	7	Inoculated	20	1/20 (5%)	9/19 (47,3%)	-	-
First trial	7	Negative Control	10	0/10 (0%)	0/10 (0%)	-	-
Second trial	9.5	Inoculated	15	0/15 (0%)	8/15 (53.3%)	-	-
Second trial	9.5	Negative Control	10	0/10 (0%)	0/10 (0%)	-	-
First trial Citometry	9.5	Inoculated	20	0/20 (0%)	8/20 (40%)	-	-
First trial Citometry	9.5	Negative Control	12	0/12 (0%)	0/12 (0%)	-	-
Second trial Citometry	9.5	Inoculated	30	0/30 (0%)	12/30 (40%)	10/18 (61.1%)	1/8 (12.5%)
Second trial Citometry	9.5	Negative Control	12	0/12 (0%)	0/12 (0%)	0/12 (0%)	0/12 (0%)

*Since that one of the embryos were euthanized with 24 hours; hpi: hours post-inoculation; dpi: days post-inoculation. Chi-square; $p < 0.05$.

Among embryos inoculated at 7 DED, the odds of death at 48 hours post-inoculation were 20 times higher than at 24 hours (Odds Ratio (OD) = 20.0; p = 0.0014). Similarly, for embryos inoculated at 9.5 DED, the odds of death at 48 hours post-inoculation were 16 times higher compared to 24 hours (OR = 16.0; p = 0.0142).

For the experiment designed for flow cytometry sample collection, embryos in the first trial had 28 times higher odds of death at 48 hours post-inoculation compared to 24 hours (OR = 27.88; p = 0.0033). In the second trial, the odds of death were 41 times higher at 48 hours (OR = 41.2; p = 0.00012) and 75 times higher at 72 hours (OR = 75.4; p = 0.0000067), both relative to 24 hours.

Inflammatory proteins, and corticosterone

Differences in serum concentrations of the acute-phase proteins SSA, alpha-1-acid glycoprotein, and ovotransferrin were not statistically significant (p > 0.05) between embryos inoculated with SP at 7 and 9.5 DED and collected 48 hours post-inoculation, and those inoculated with saline (NC). In the allantoic fluid, however, only serum amyloid A showed increased concentration in embryos inoculated with SP (1,054µg/mL) at 9.5 days compared to the NC group (0,1193 µg/mL) (Figure 1).

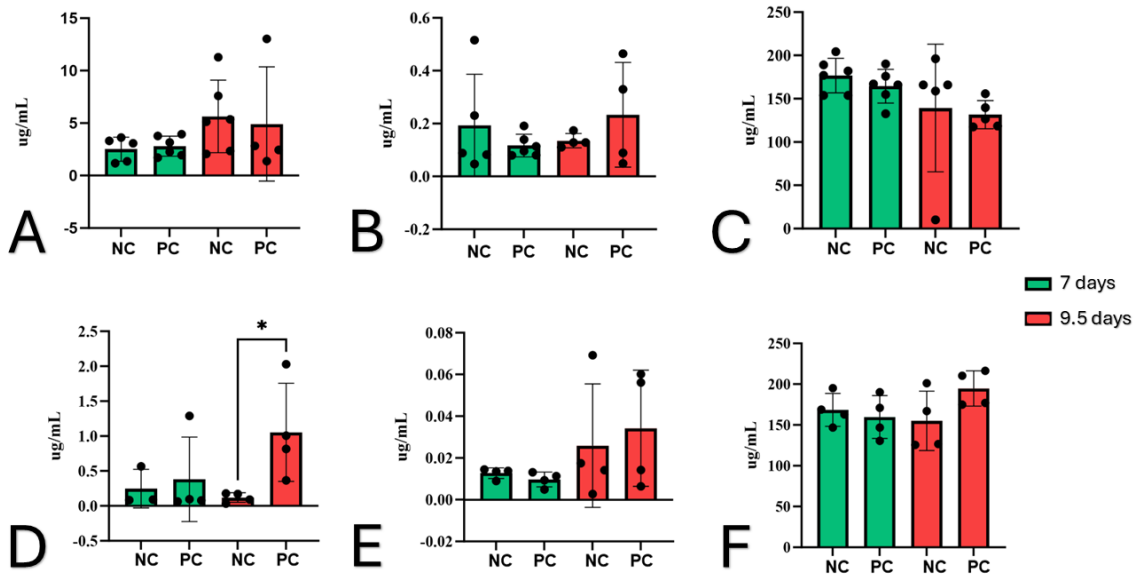

Figure 1. Serum concentrations of serum amyloid A (A), alpha-1-acid glycoprotein (B), and ovotransferrin (C), and allantoic fluid concentrations of serum amyloid A (D), alpha-1-acid glycoprotein (E), and ovotransferrin (F) in embryos inoculated with *Salmonella*

Pullorum (PC) or saline (NC) at 7 (7D) and 9.5 (9.5D) days of embryonic development and sampled 48 hours post-inoculation. Student's *t*-test was performed within each age group. $p < 0.05$. Significant difference is indicated by *.

When evaluating serum corticosterone levels, some values from the positive control group exceeded the maximum detectable concentration, and only three samples could be reliably quantified. Corticosterone levels were lower in embryos inoculated with SP compared to those in the NC group (Figure 2).

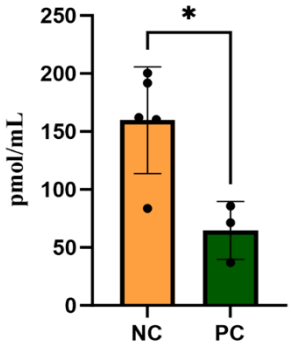

Figure 2. Serum corticosterone concentration in embryos inoculated with *Salmonella* Pullorum (PC) or saline solution (NC) at 9.5 days of embryonic development and collected 48 hours post-inoculation. Two samples exceeded the assay detection range and were therefore excluded from the analysis. Student's *t*-test was performed. $p < 0.05$. Significant difference is indicated by *.

In the ELISA results for the interleukins tested, no significant difference was observed in the serum concentration of IL-4 between CE inoculated at 9.5 DED and sampled at 11.5 DED (48 hours post-inoculation) (Figure 3A). For IL-10, it was not possible to determine the concentration, as the absorbance values in the saline-treated group (negative control) exceeded the upper limit of the ELISA kit's standard curve. Therefore, a relative analysis based on absorbance values was performed, revealing that

IL-10 levels were lower in the SP-treated group compared to the NC group (Figure 3B).

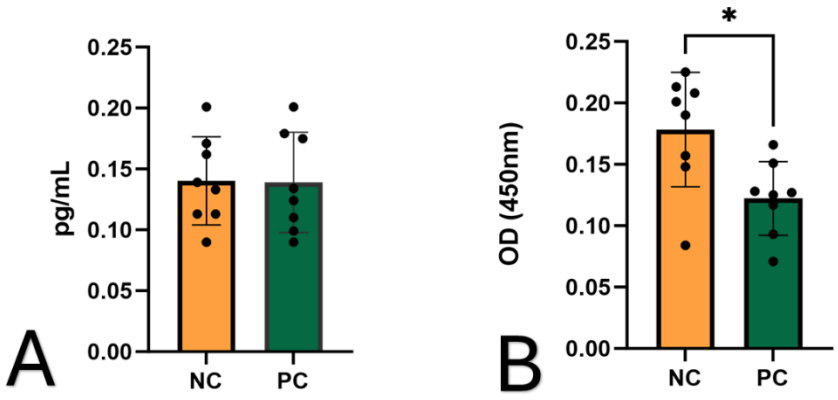

Figure 3. Concentrations of IL-4 (A), and absorbance values of IL-10 (B) in the serum of embryos inoculated with SP at 9.5 DED and sampled at 11.5 DED, as determined by ELISA. Student's *t*-test was performed. $p < 0.05$. Significant difference is indicated by *.

Flow Cytometry Analysis

The frequency of $CD3^+$ cells within the total cell population progressively decreased with age in the positive group, reaching a maximum of approximately 0.2% in the infected group (Figure 4A). The percentage of TCRγδ cells within the $CD3^+$ population increased with age, with a significant rise also observed at 17 days of age in the PC compared to the NC group (Figure 4B). The frequency of $CD4^+$ cells within the $CD3^+$ population remained stable across all age groups and treatments (Figure 4C). In contrast, the frequency of $CD8^+$ cells within the $CD3^+$ population showed a marked decline in the PC group at 11.5 days of age (Figure 4D). As a result, the $CD8^+/CD4^+$ ratio decreased progressively with age (Figure 4E).

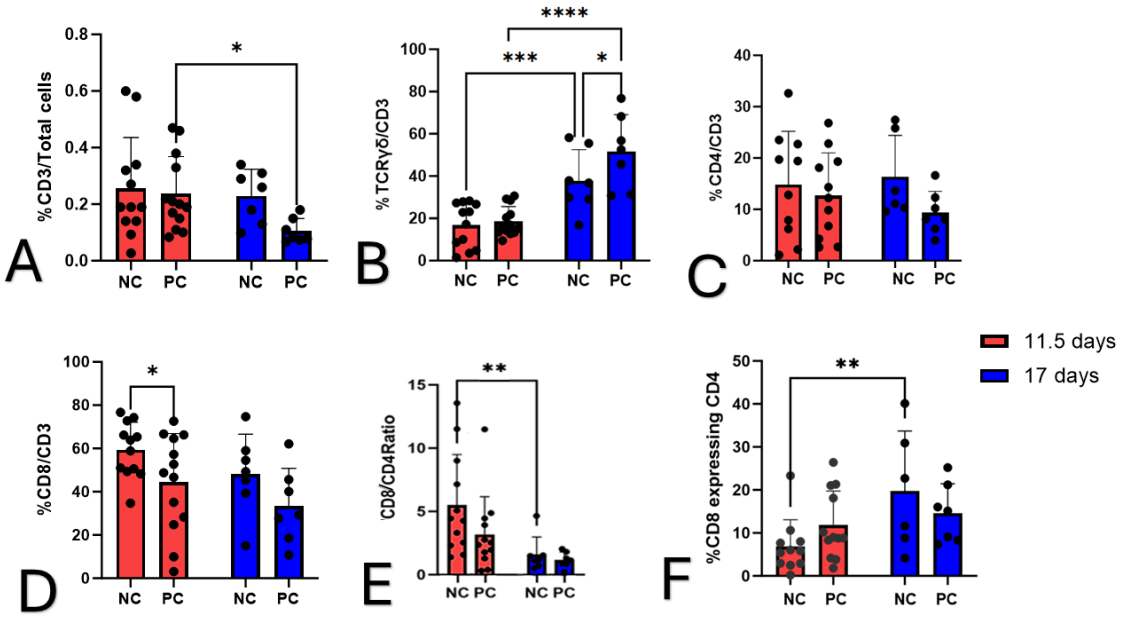

Figure 4. Age-dependent modulation of T cell subpopulations in the blood following *Salmonella* Pullorum infection. A: percentage of CD3+ cells of the total cells; B: percentage of TCRγδ + cells of the CD3+ cells; C: percentage of CD4+ cells of the CD3+ cells; D: percentage of CD8+ cells of the CD3+ cells; E: CD4/CD8 ratio, F: percentage of CD8+ expressing CD4+ cells. Asterisks indicate statistical significance based on two-way ANOVA: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

The frequency of CD45$^+$ cells, representing total leukocytes, progressively decreased with embryonic age (Figure 5A). The frequency of MHCII$^+$ cells within the CD45$^+$ population increased with age in both the control (NC) and infected (PC) groups (Figure 5B). In contrast, the frequency of MRC1$^+$ cells within the CD45$^+$ population decreased with age in the NC group but increased in the PC group at 17 days of embryonic development (Figure 5C).

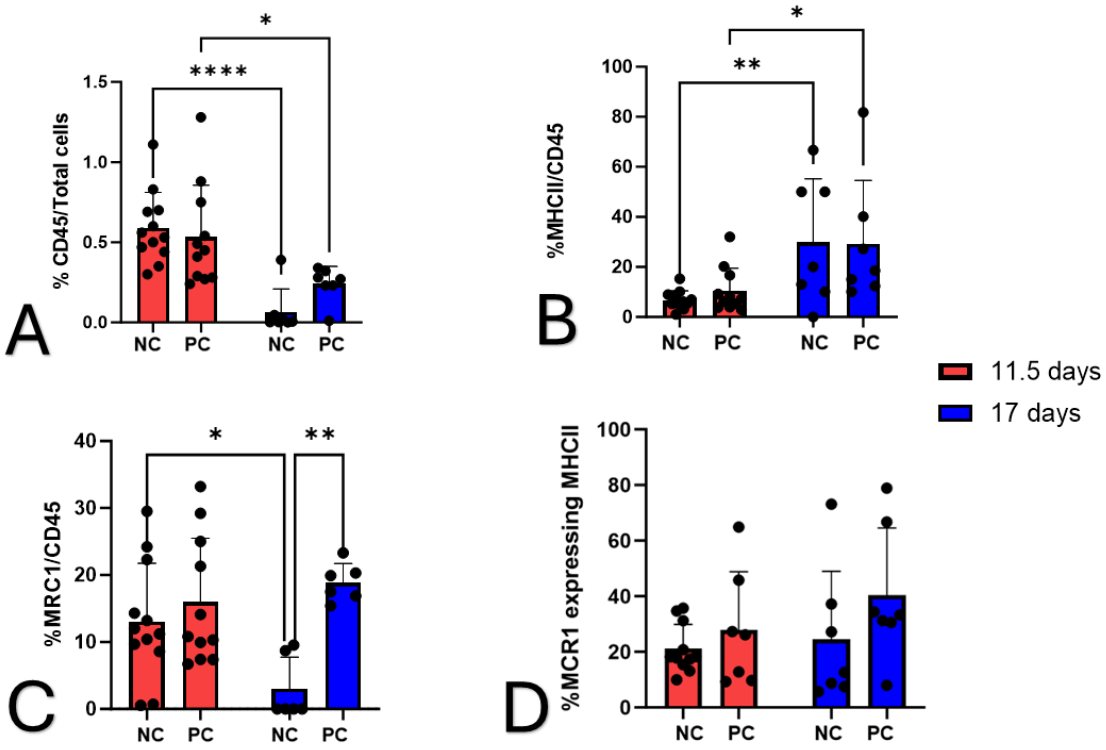

Figure 5. Age-dependent changes in leukocyte frequency and antigen-presenting cell markers in blood following Salmonella Pullorum infection. A: percentage of CD45+ cells of the total cells; B: percentage of MHCII+ cells of the CD45+ cells; C: percentage of MRC1+ cells of the CD45+ cells. Asterisks indicate statistical significance based on two-way ANOVA: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.0001$ (****).

The frequency of $CD3^+$ cells, representing T lymphocytes, progressively decreased with age in the blood of the infected group, reaching a maximum of approximately 0.2% of the total cell population (Figure 6A). Similarly, Bu^+ cells, representing B lymphocytes, also declined with age in both the control (NC) and infected (PC) groups (Figure 6B).

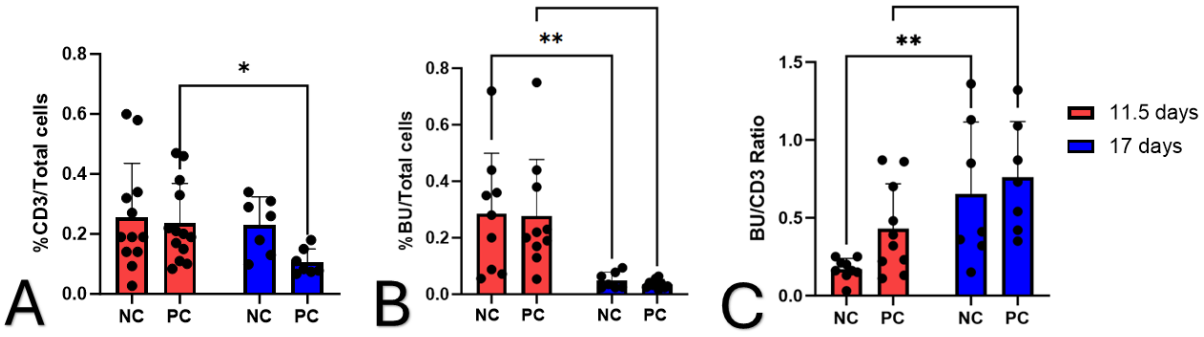

Figure 6. Age-dependent frequency of T lymphocytes ($CD3^+$) and B lymphocytes (Bu^+) in blood following *Salmonella* Pullorum infection; A: percentage of CD3+ cells of the total cells; B: percentage of Bu+ cells of the total cells; C: CD3+/BU+ ratio. Asterisks indicate statistical significance based on two-way ANOVA: $p < 0.05$ (*), and $p < 0.01$ (**).

Gene expression analysis by RT-qPCR

In the RT-PCR analysis, gene expression was evaluated based on the ΔCT values, calculated as the difference between the CT of the target gene and that of the constitutive gene. Therefore, higher ΔCT values indicate lower gene expression. TGF expression was higher in 11.5 DED embryos compared to 17 DED embryos, suggesting a decrease in TGF levels with advancing embryonic age (Figure 7A). IL-6 expression did not differ significantly between groups or developmental stages, indicating that it was not influenced by either the infection or the embryo's age (Figure 7B). In contrast, IL-8 expression increased with embryonic age in both groups. A slight increase at 11.5 DED in the infected group (PC) may reflect a transient inflammatory response (Figure 7C). Regarding the fold change analysis, which compares gene expression between the infected (PC) and control (NC) groups, IL-8 showed an increase, suggesting a possible infection-related upregulation despite the non-significant variation in ΔCT values (Figure 7D).

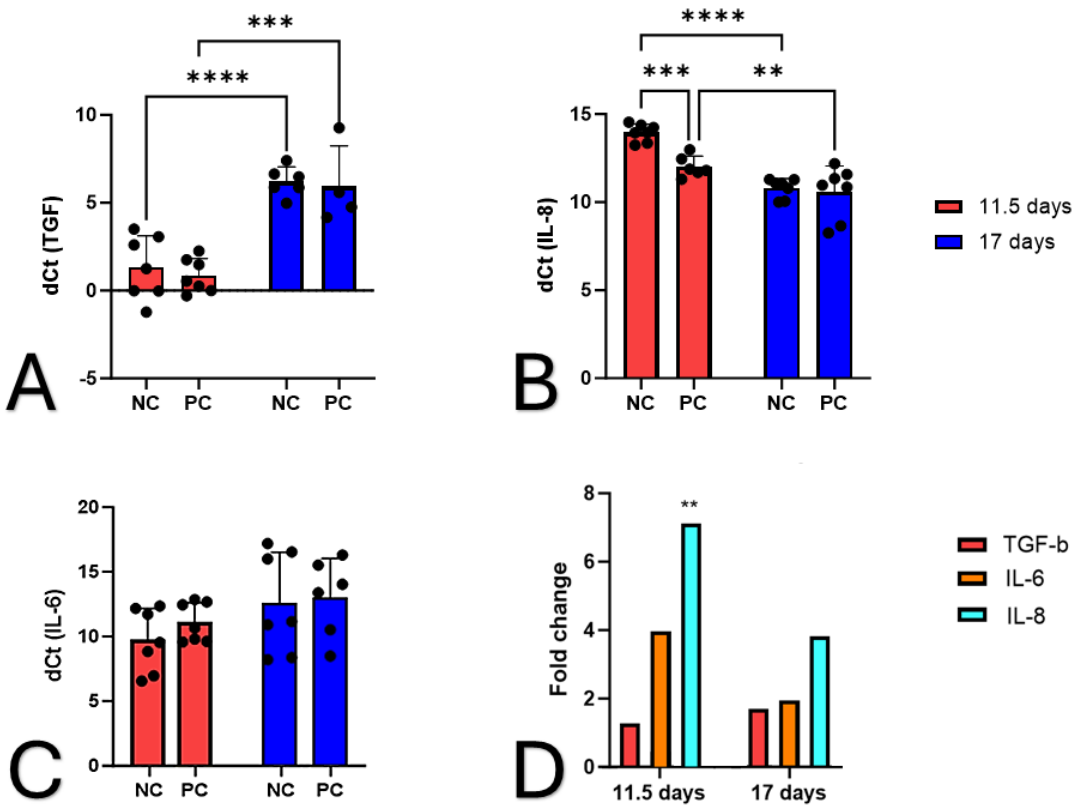

Figure 7. Relative gene expression of TGF, IL-6, and IL-8 in chicken embryos at different developmental stages in control (NC) and *Salmonella*-infected (PC) groups; A: ΔCT values of TGF; B: ΔCT values of IL-8; C: ΔCT values of IL-6; D: Fold change of TGF, IL-8 and IL-6.

Histopathological analysis

Histopathological evaluation of the kidneys revealed that only hydropic degeneration was significantly increased in the infected embryos compared to the control group (Figure 8; Figure 9A). No significant differences were observed between groups for the other evaluated parameters, including inflammation, vascular congestion, and hemorrhage (Figure 9B, 9C and 9D).

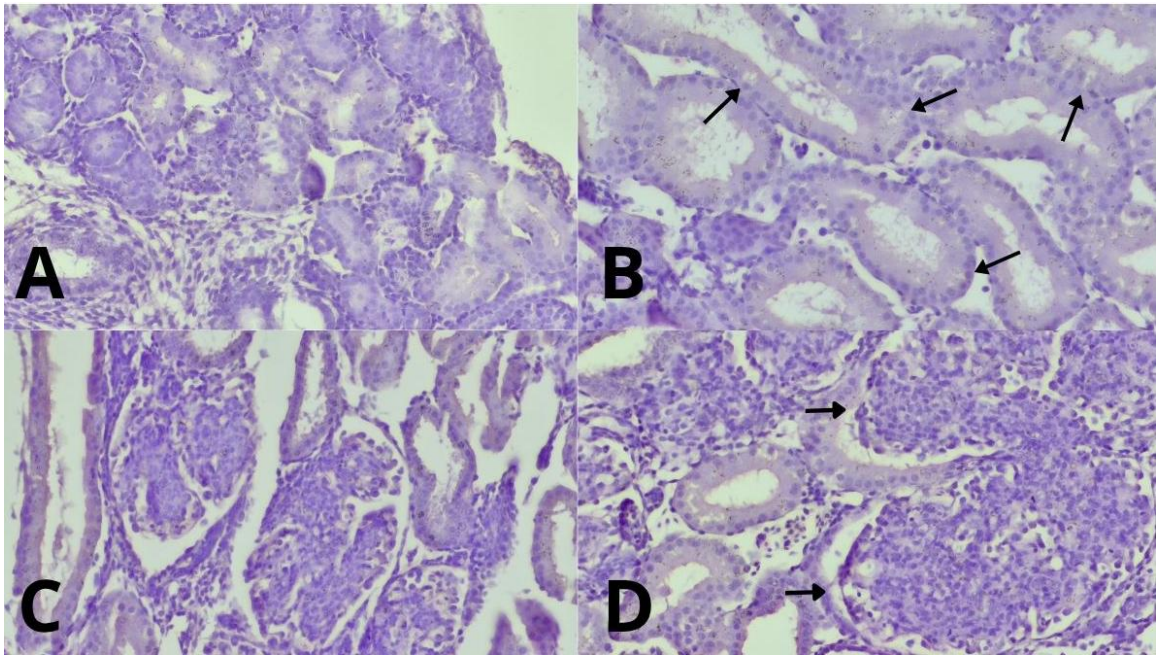

Figure 8. Histological sections of renal tubules in chicken embryos. (A) Negative control group showing normal tubular morphology; (B) Positive control group (infected with *Salmonella* Pullorum) showing epithelial cells with increased volume and an expanded, rounded profile. The cytoplasm is pale eosinophilic or slightly vacuolated, with poorly defined clear cytoplasmic vacuoles (arrow); (C) Negative control group showing normal glomerulus morphology; (D) Positive control group showing glomerular enlargement with features of proliferative nephropathy (arrow). Hematoxylin and eosin stain, 400×.

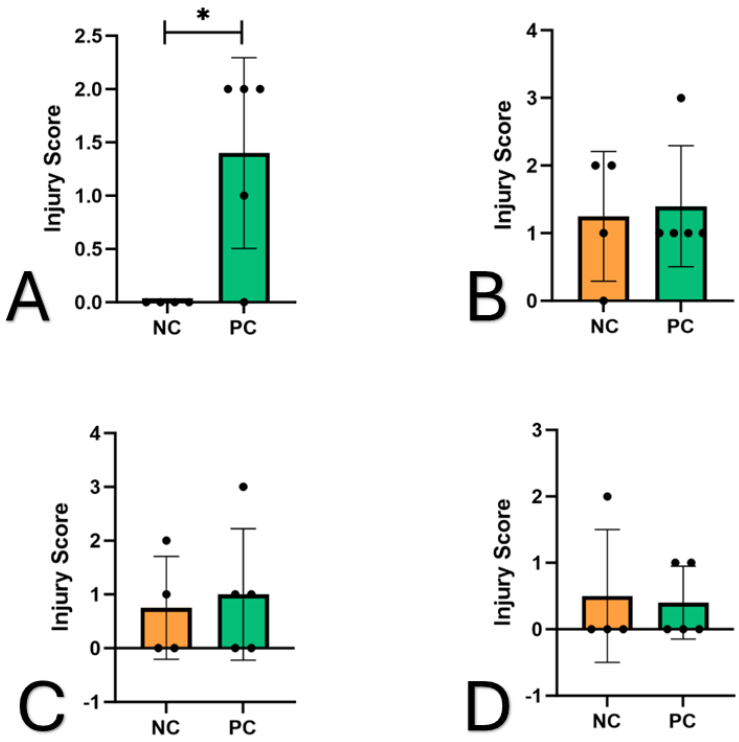

Figure 9. Histopathological Kidney Scores in Chicken Embryos; A. Hydropic degeneration; B. Hemorrhage; C. Congestion; D. Inflammation. Median scores were compared to the negative control group using the Wilcoxon test. $p < 0.05$ was considered significant (*).

DISCUSSION

Although several studies have explored the use of CE as an experimental model for infections caused by viruses (Pogorzelska et al., 2024; Schilling et al., 2018; Xie et al., 2018), bacteria (Gripenland et al., 2014; Li et al., 2019), and fungi (Verdi et al., 2018), there are still important gaps to be addressed, particularly regarding their use as an alternative model studying inflammatory responses. This highlights the need to better understand and standardize the use of the CE as an alternative inflammation model.

In previous studies, we proposed the use of SP as an inflammatory agent in this model, inoculating embryos at 13 DED and collecting samples four days post-infection (17 DED). The results were satisfactory, showing reduced weight in infected embryos compared to the negative control group inoculated with 0.85% saline solution, along with macroscopic lesions suggestive of inflammation, such as hyperemia and an increase in IL-10 levels indicated that this cytokine may be involved in the recovery phase (de Souza et al., 2024). While our previous results indicate that CE from 13 to 17 DED can serve as a suitable model for studying inflammation, offering several advantages over post-hatch animals, such as non-invasive inoculation and the absence of the need for physical restraint, it is necessary to further refine our study within an ethical framework.

Although many countries do not require ethics committee approval for studies involving CE, or only after later developmental stages, research shows that brain activity related to sensory processing begins around 13 DED (Kollmansperger et al., 2023), and behavioral responses to pain, such as beak movements, appear by 15 DED (Süß et al., 2023). Thus, using embryos before these stages helps avoid potential suffering and aligns with the 3Rs (Replacement, Reduction, and Refinement), as early-stage embryos serve as a partial replacement for more developed animal models, reducing ethical concerns (Sarnella et al., 2024).

In the pilot test, two embryos at 8 DED were euthanized for blood collection; however, the volume obtained was less than 100 µL, making further analyses unfeasible.

Furthermore, despite the low inoculum dose (1 log CFU/embryo), a mortality rate of 90% was recorded within 96 hours post-inoculation. These findings indicate that the bacterial inoculation had a severe and time-dependent impact on embryo viability, with most deaths occurring after three days. This outcome highlights the pathogenic potential of SP in the early stages of embryonic development and emphasizes the need for precise timing in subsequent inflammatory experimental designs. Based on these findings, we selected 9 DED as the minimum age for sampling in the experimental design.

There were no differences in serum concentration of acute phase proteins tested with ELISA kits in both ages. However, in allantoid fluid of embryos inoculated at 9.5 DED and collected 48 hours post-inoculation, there was an increase in SAA of the embryos inoculated with SP. SAA is a highly sensitive acute phase protein produced mainly by hepatocytes during early infection. Its levels can rise over 1000-fold, playing a key role in host defense by opsonizing gram-negative bacteria like *Salmonella* and activating immune cells such as macrophages and neutrophils (Pal Singh et al., 2020). In addition to serving as a reservoir for embryonic excreta, the allantoic fluid also receives amino acids, peptides, and proteins resulting from embryonic metabolism (Ahmed et al., 2022) including acute-phase proteins produced by the liver. Additionally, the direct transfer of gut-derived proteins to this compartment is likely, particularly considering the extrahepatic production of acute-phase proteins in the intestine (Hincke et al., 2019).

Since the blood volume collected from embryos inoculated at 7 DED was very limited (approximately 300 μL) and no changes in acute phase protein levels were observed, subsequent analyses were performed using only the serum from embryos inoculated at 9.5 DED. To evaluate whether, 48 hours post-inoculation, CE were in the recovery phase, which is characterized by the increase of anti-inflammatory interleukins as a mechanism of disease resolution (Kogut & Arsenault, 2017; Tang et al., 2018), we assessed IL-10 and IL-4.

The absence of differences in IL-4 may indicate that the Th2 response had not yet been fully activated at 24–48 hours post-infection, or that its modulation occurs in specific tissues and is not reflected in the serum at this stage. IL-10 was reduced in the group inoculated with SP, and this indicates that the response to the infection may temporarily suppress IL-10 production confirming that the CE were still in the inflammatory phase (B. Yin et al., 2025). It is generally recognized that decreased IL-10 levels can enhance resistance to primary infection by allowing a stronger inflammatory response (Milby-Blackledge et al., 2024; Rothwell et al., 2004). Consistently, some poultry studies have

reported reduced IL-10 gene expression following *Salmonella* Typhimurium or *Salmonella* Enteritidis challenge (Fasina et al., 2008; X. Li et al., 2016; Redmond et al., 2009)

To assess whether the embryos experienced stress or pain 48 hours after inoculation, we measured corticosterone levels. Contrary to our expectations, ELISA results showed significantly lower concentrations in infected embryos compared to controls. Since the Hypothalamic–Pituitary–Adrenal (HPA) axis (responsible for corticosterone release during stress) is still developing between days 4 and 11 of embryonic life (De Groef et al., 2008), it is possible that the infection interfered with its maturation or activation. Although our initial goal was to detect stress as a marker of discomfort, the absence of increased corticosterone may reflect the immaturity of this system at this stage. Nonetheless, we proceeded with the model, as the literature supports that embryos in this developmental window are not yet capable of perceiving pain (Kollmansperger et al., 2023; Süß et al., 2023; Weiss et al., 2023).

Although the overall frequency of $CD3^+$ cells among total leukocytes decreased with age in the infected group, this finding may reflect the natural increase in total cellularity during embryonic development rather than a true reduction in T lymphocyte numbers. In support of this, the proportion of specific T cell subsets, such as $TCR\gamma\delta^+$ cells, increased significantly with age, particularly following SP infection at 17 DED. These findings suggest that SP infection may promote an enrichment of γδ T cells during later stages of embryonic development. Notably, γδ T cells are among the first T cell subsets to develop and mature in the embryonic thymus (Lee et al., 2024). Moreover, an expansion of γδ T cells in response to *Salmonella* challenge has been previously reported in the spleens of post-hatch chicks, supporting their role in early immune responses to bacterial infection (Berndt & Methner, 2001).

The $CD4^+$ cells within the population of CD3 remained stable across all age groups and infection, indicating a preserved proportion of helper T cells among the total T lymphocyte population. In contrast, the $CD8^+$ cells within the population of $CD3^+$ cells showed a marked decline in the PC group at 11.5 DED, suggesting that SP infection may lead to a reduction in cytotoxic T cell representation during this developmental stage. Although some studies have reported an increase in $CD8^+$ T cell populations following *Salmonella* infection in chickens (Berndt & Methner, 2001), other findings suggest that this response may be more complex. In chickens orally or intratracheally infected with *Salmonella* Typhimurium, a transient suppression of lymphocytes, and atrophy of

lymphoid organs such as the spleen and thymus were observed as early as two days post-infection (Hassan & Curtiss, 1994). In late stages, *Salmonella* can negatively regulate $CD8^+$ T cell cytotoxic response in order to remain within the host for a long period of time (López-Medina et al., 2015).

The higher $CD8^+/CD4^+$ ratio observed in 11.5 DED may reflect a relative predominance of $CD8^+$ cells at an early stage of thymic development (Fellah et al., 2013). At 17 DED, the increased proportion of $CD8^+$ cells co-expressing $CD4^+$ suggests an expansion of double-positive T cells ($CD4^+CD8^+$), consistent with the expected profile of TCRαβ T cell development in the thymus, as described in the literature (Fellah et al., 2013).

The progressive decline in $CD45^+$ cell frequency with embryonic age likely reflects a relative dilution of circulating leukocytes in the growing embryo, as total blood volume and non-hematopoietic cell populations expand during development. Despite this overall decrease in leukocyte frequency, the proportion of $MHCII^+$ cells within the $CD45^+$ population increased with age in both control and infected groups, suggesting maturation and expansion of antigen-presenting cell subsets such as dendritic cells, B cells, or activated monocytes (Fellah et al., 2013). The apparent decline in $MRC1^+$ cell frequency with age observed in the control group may follow a similar pattern seen with $CD45^+$ cells, not representing a true reduction, but rather a relative decrease due to the expansion of other leukocyte populations. This suggests that $MRC1^+$ cells may be proportionally diluted as the immune system develops and diversifies, rather than actively reduced. Notably, the increase in $MRC1^+$ cells in the infected group at 17 DED suggests that *Salmonella* infection triggers an immune response capable of promoting recruitment or phenotypic activation of these mononuclear phagocytes(Huang et al., 2020).

In line with the trends observed for $CD45^+$ and $MRC1^+$ populations, the apparent decline in $CD3^+$ and BU^+ cell frequencies with embryonic age is likely due to a relative dilution effect, as the total cellularity of the embryo increases during development. The increase in the $BU^+/CD3^+$ ratio at 17 DED may reflect the natural rise in B cell maturation and expansion toward the end of embryonic development, as the bursa of Fabricius becomes functionally active around day 16. In contrast, T cell ($CD3^+$) populations may remain stable or decline under infectious challenge, resulting in a relative predominance of B cells at this stage (Garcia et al., 2021).

TGF expression was higher in 11.5 DED compared to 17 DED, suggesting a decrease in TGF levels with advancing embryonic age. Although TGF-β is widely

recognized for its roles in adult physiology (including the regulation of inflammation, hematopoiesis, steroidogenesis, and tissue repair) it also plays a crucial role during embryonic development by controlling differentiation, morphogenesis, and tissue remodeling.Jakowlew et al. (1994) demonstrated that the expression of TGF-β3 mRNA is detectable as early as 1.5 days of incubation and increases gradually until approximately day 12, after which it declines. Therefore, our results may reflect a natural downregulation of TGF-β3 with the age.

In contrast, IL-8 expression increased with embryonic age in both groups. Its presence has been detected early during embryonic development (Anastasiadou & Michailidis, 2016) and remains active in the perinatal period, with higher intestinal expression observed at hatching compared to earlier embryonic stages (Sharma et al., 2025; Terada et al., 2018). IL-8 is a chemokine produced by cells such as macrophages and epithelial cells in response to pro-inflammatory stimuli, and plays a key role in the innate immune response by recruiting heterophils to sites of infection or tissue injury. A slight increase at 11.5 DED in the infected group (PC) may reflect a transient inflammatory response. Fold change analysis, which compares gene expression between the infected (PC) and control (NC) groups, also revealed an increase in IL-8 expression, indicating activation of inflammatory signaling pathways in response to SP infection.

In the histopathological analysis, hydropic degeneration was significantly increased in the kidneys of infected embryos, suggesting that renal integrity may have been compromised, potentially allowing the leakage of low molecular weight proteins such as SAA into the allantoic compartment. The detection of SAA in the allantoic fluid (but not in the serum) supports the hypothesis of renal protein loss associated with tissue damage. Okafor et al. (2025) reported proteinuria and abnormal protein excretion following *Salmonella* infection in the context of renal degeneration. While further analyses are required to confirm this mechanism, our findings suggest a potential link between early kidney damage and altered protein distribution during infection. The proliferative glomerulonephritis observed may be related to immune complex deposition triggered by the systemic immune response to *Salmonella* infection.

No significant differences were observed between groups for the other evaluated parameters, including inflammation, vascular congestion, and hemorrhage. However, our findings are consistent with those of Shchebentovska et al., 2021), who reported inflammatory and vascular lesions in several organs, while observing only degenerative changes in the renal epithelium.

We evaluated CE 48 hours after SP inoculation, during which approximately 40–50% mortality was observed. Following this peak, the surviving embryos remained viable up to 17 DED. The use of SP, a highly pathogenic bacterium for chickens, was intended to establish a comprehensive model of infection and inflammation. The observed outcomes indicate that even at this early stage, the embryonic immune system can mount an active response against a virulent pathogen at the tested dose.

Taken together, the findings suggest that the embryonic immune response to SP infection evolves dynamically over time. At 12 DED, the increase in $CD8^+$ T cells, despite the absence of a corresponding rise in $CD4^+$ cells, may reflect an early cytotoxic response during a phase of high mortality, in which helper T cell expansion had not yet occurred. Similarly, cytokine analysis revealed reduced IL-10 and unchanged IL-4 levels at this stage, indicating that the anti-inflammatory response was not yet established. The increased frequency of $MRC1^+$ macrophages together with elevated IL-8 expression suggests macrophage activation contributing to the inflammatory response. The concurrent rise in γδ T cells further indicates early adaptive immune activation. Histopathological evidence of renal lesions alongside increased SAA in the allantoic fluid suggests inflammation-induced kidney damage contributing to protein leakage.

These results demonstrate that chicken embryos younger than 12 days of development exhibit measurable and dynamic immune responses to SP infection, including early activation of cytotoxic T cells, macrophages, and γδ T cells, alongside modulations in cytokine profiles and acute phase proteins. The presence of histopathological lesions further supports the establishment of an inflammatory process in this early developmental window. Therefore, embryos below 12 DED represent a promising and reproducible experimental model to study early innate and adaptive inflammatory responses, enabling controlled investigations of host-pathogen interactions and immunomodulatory interventions during embryogenesis.

These results demonstrate that chicken embryos younger than 12 DED exhibit measurable and dynamic innate and adaptive immune responses to SP infection. The evidence of early cellular activation and inflammation supports the suitability of embryos below 12 DED infected with SP as a reproducible experimental model to investigate early inflammatory processes and host-pathogen interactions during embryogenesis, enabling controlled studies of immunomodulatory interventions.

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