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# Evaluation of protective immune response against fowl typhoid in chickens vaccinated with the attenuated strain *Salmonella* Gallinarum Δ*cob*SΔ*cbi*A

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ABSTRACT

*Salmonella enterica* serovar Gallinarum biovar Gallinarum (SG) causes fowl typhoid in chickens, a septicemic infection which results in high mortality rates. This disease causes high economic impact to the poultry industry worldwide because of the mortality or elimination of positive flocks to control bacterial dissemination. Live vaccines are used in the fields, however the characterization of immune mechanisms important for protection are being studied to improve the efficacy of vaccination schemes. In this study, we evaluated the immune response in brown layer-hens, vaccinated or not, during the most critical period of infection. Cellular and humoral immunity were extensively evaluated until 7 days post-infection (DPI), by flow cytometry and ELISA, respectively. Furthermore, we evaluated the expression of important pro-inflammatory cytokines after infection of bone marrow derived macrophages (BMDMs) with the live attenuated SG vaccine and with the wild SG strain. The results showed an increasing production of IgG and IgM during the first week post-infection, in vaccinated layer-hens, which was absent in unvaccinated birds. The population of $CD8^+CD44^+$ and $CD4^+CD44^+$ T cells in spleen and cecal tonsils constantly decreased in unvaccinated birds in comparison with vaccinated layers. The expression of IFN-γ and TNF-α in BMDMs was induced by both SG strains (attenuated and wild) at similar levels ($p>0.05$). Vaccination with live SG vaccine reduced systemic infection by challenge strain of SG and prevented the mortality rate of 85% that occurred in unvaccinated layer-hens during 30 dpi. Furthermore, the immunization enhanced the proliferation of effector $CD4^+$ and $CD8^+$ T cells after challenge.



## 1. Introduction

*Salmonella enterica* serovar Gallinarum biovar Gallinarum (SG) is an avian host-specific serovar that causes fowl typhoid (Barrow and Freitas Neto, 2011). Despite biosecurity measures, this disease still affects commercial poultry flocks worldwide (O.I.E., 2015). The outcome of the infection is marked by high morbidity and up to 80% mortality of naturally infected birds (Shivaprasad, 2000). During outbreaks, severe protocols are followed to restrain and eliminate the spread of SG, and in case of infections of breeders, the complete removal of the flock and quarantine are required by strict legislation (Barrow and Freitas Neto, 2011; BRASIL, 2003).

The control of infections by biosecurity measures and vaccination is adopted in many countries to prevent outbreaks in commercial flocks (Feberwee et al., 2001; Meunier et al., 2016; Penha Filho et al., 2009). Currently the live vaccines have shown better efficacy to protect chickens against fowl typhoid in comparison with the killed vaccines available, however the mechanisms involved in the protective immune response against this pathogen are still being clarified (Wigley et al., 2005). The infection occurs by oral route, however SG quickly invades the epithelial barrier, the gut associated lymphoid tissue (GALT) or can be passively captured by M cells, consequently spreading to internal organs, leading to a severe septicemic infection (Shivaprasad, 2000; Tahoun et al., 2012). The bacterial colonization of organs and multiplication in these tissues can cause acute death of the host after a short incubation period of 4 days-post infections, marked by a profound hepatosplenomegaly (Chappell et al., 2009). Immunity to protect against SG requires significant participation from both the innate and acquired branches of the immune system (McSorley, 2014; Santos et

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al., 2001). Due to the capability to survive and replicate in the intracellular micro-environment especially inside macrophages, the cellular mediated immunity (CMI) is important for the control of *Salmonella* infection (Chappell et al., 2009). The relevance of humoral immune response is not completely elucidated, but evidences point out an important involvement of B cells for immunity against this pathogen, independently from the production of antibodies (Nanton et al., 2012). Although brown layer-hens and broilers (meat type chickens) are not capable to control bacterial proliferation and are highly susceptible to mortality caused by SG, which has high capacity to inhibit and evade the innate immune response during infection of these varieties of chickens (Barrow and Freitas Neto, 2011; Shivaprasad, 2000). The vaccination and priming of B and T cells with attenuated live vaccines has shown to be capable to stimulate a protective immune response which can control bacterial dissemination and mortality of brown layer-hens, thus this animal model is frequently used to evaluate SG infection (Wigley et al., 2005).

The first vaccine against fowl typhoid was introduced in the late 1950s with the rough strain of SG (SG9R) obtained by chemical mutagenesis (Smith, 1956). Since then, the efforts of many researchers to improve the safety and obtain novel vaccines resulted in different live vaccines that are being tested experimentally (Jawale and Lee, 2014; Mitra et al., 2013; Shehata et al., 2013). It is essential that vaccines for invasive host-specific *Salmonella* serovars stimulates development of antigen-specific memory and effector $CD4^+$ T cells and $CD8^+$ T cells (Wahid et al., 2015). T cells express CD44 on the membrane after activation in response to invading pathogens and the expression remains at high levels in effector and memory T cells after activation (Baaten et al., 2010).

The strain SGΔ*cob*SΔ*cbi*A is a mutant strain of SG 287/91, with deletion on genes *cob*S and *cbi*A, previously shown to be attenuated and capable to colonize internal organs, in reduced numbers in comparison with wild SG strain (de Paiva et al., 2009). Previous studies have also shown that this strain is immunogenic and one vaccine dose elicited protection against SG and cross protection against *S.* Enteritidis (Penha Filho et al., 2010). A higher local influx of $CD8^+$ T cells in the cecal tonsils of white layer-hens and faster reduction of *S.* Enteritidis intestinal burden were noticed in the group vaccinated with SGΔ*cob*SΔ*cbi*A (Penha Filho et al., 2010). However little is known about the immunological mechanisms involved in the control of mortality and systemic infection by SG in vaccinated chickens. Thus, the present work was designed considering that experimental development of fowl typhoid starts during the first week, and the onset of the disease clinical signs and mortality occurs after 5 days post-infection (DPI). We aimed to study the elements of acquired immune response that are effective to protect susceptible chickens against SG during this critical period of onset of the disease, in comparison with unvaccinated susceptible chickens. For this, an extensive evaluation of the cellular and humoral immune responses was performed, before and after the challenge with wild SG.

## 2. Materials and methods

### 2.1. Experimental birds

Two hundred and forty commercial Brown layer-hens, free of immunizations against *Salmonella*, were purchased at day of hatch of eggs from grandparents breeder flocks with high biosecurity standard. Birds were reared in SPF facilities with controlled ambient conditions with water and food supplied *ad libitum* during the experiments. At arrival, pools of spleen samples from 10 extra birds and feces on the transport boxes were analyzed by bacteriological culture and PCR for *inv*A gene (Dobhal et al., 2014) for confirmation of the *Salmonella sp.* free status of the chicken flock. Animal experimentation was approved by the Brazilian Committee of Animal Welfare and Ethics (permit number 00155813).

### 2.2. Bacterial strains and vaccines

The live vaccine (LV) consisted of the attenuated SGΔ*cob*SΔ*cbi*A (de Paiva et al., 2009; Penha Filho et al., 2010), resistant to kanamycin. An invasive highly pathogenic SG strain (SG 287/91), kindly donated by Prof. Paul Barrow (University of Nottingham, UK) was used to challenge birds. Bacterial cultures were prepared in Luria-Bertani (LB) broth (Invitrogen, USA) at 100 rpm in a shaking incubator at 37ºC/Overnight. The inocula for vaccination and challenge were previously counted by serial dilutions method to obtain the $OD_{600}$ value corresponding to $10^8$CFU/mL.

### 2.3. Experimental design

For each experiment three groups containing 30 birds each were formed. Chickens in vaccinated group (VAC) were immunized with the LV at 25 days of age and challenged at 45 days of age. Chickens in infected group (INF) were not vaccinated and were challenged at 45 days of age with wild SG287/91. Another group of 30 chickens was kept as negative control (unvaccinated and unchallenged) for the normalization of cytokine quantification. Twenty additional chickens in groups VAC and INF were used to record the mortality rates during 30 DPI. The vaccine and challenge inocula were administered orally, into the crop and consisted of $10^8$ CFU of the corresponding bacteria, eluted on 1mL of Phosphate Buffer Saline (PBS) pH 7.4 (Merck, Germany). The same experimental designs were repeated to evaluate vaccine efficacy against SG, analyzing the bacterial numbers, clinical signs and mortality.

### 2.4. Sampling and bacteriology

At day 1 and 15 before infection (DBI) and 1, 3 and 7 DPI, five birds from each group were euthanized for sampling. Spleen and cecal tonsil samples were harvested, snap-frozen in liquid nitrogen and stored at -80ºC for cytokine quantification or used fresh for flow cytometry analysis and bacterial enumeration as described previously (Wigley et al., 2005). Blood was harvested and the sera was used for IgG and IgM quantification. SG numbers were expressed as $Log_{10}$ per gram of tissue. Enriched positive samples ($\leq 10^2$ CFU/g) were expressed as 2 ($Log_{10}$ of CFU/g) for calculations. To confirm the absence of SGΔ*cob*SΔ*cbi*A in internal organs at 1 DBI and 1 DPI, pools of liver, spleen and cecal tonsil samples from group VAC were enriched on Rappaport broth for 24h at 37C and plated in Brilliant Green agar containing kanamycin (30μg/mL), for selective growth of the LV strain. Additionally, a PCR to detect SGΔ*cob*SΔ*cbi*A was carried out with the enriched samples, according to methods previously described (Penha Filho et al., 2010).

### 2.5. Antibody production

Indirect Enzyme-linked immunosorbent assay (ELISA) using SG soluble protein antigen was applied to quantify IgG (IgY) and IgM in the sera of five birds at each moment, as previously described (Penha Filho et al., 2012). Briefly, SG was cultured in LB broth until OD600 reached 1, bacterial pellet was obtained after centrifugation for 10 min at 10000 *g* and washed in PBS pH 7.4, the bacterial pellet was suspended in 10 mL of PBS and submitted to sonication (Branson Sonifier 250, USA), using 8 cycles of 85 watts with 30s intervals. After sonication and centrifugation at 13000 *g* for 30 min, the supernatant containing SG soluble proteins was used as the soluble antigen diluted at 1:10000 for detection of IgG or IgM. Conjugated antibodies anti-chicken IgG and anti-chicken IgM were used at 1:1000 (Bethyl Laboratories, USA). The optical density values (OD) were used to calculate the adjusted *E* values using the following formula:

$$E\ value = (OD\ sample - OD\ negative\ control)/OD\ positive\ control - OD\ negative\ control)$$

### 2.6. Cytokine quantification by real time PCR

Spleen and cecal tonsils were used for RNA extraction and cDNA synthesis, as described previously (Carvajal et al., 2008). The cDNA was stored at -20ºC until use. The real time PCR was carried out in 25μL containing 50ng of cDNA; 0.5μM of primers; 12.5μL of SYBR Green 2X MasterMix (ThermoScientific, USA) and nuclease free water. Primers are detailed in Table 1. The fold change in the mRNA expression of each cytokine was calculated in comparison with unvaccinated and unchallenged birds, using five replicates at each moment. The normative genes 18S and GAPDH were adopted using the $2^{-\Delta\Delta Cp}$ method (Livak and Schmittgen, 2001).

### 2.7. Quantification of effector-memory $CD4^+$ and $CD8^+$ T cells

Phenotypes of T cells were quantified by flow cytometry, analyzing the population of effector $CD3^+CD4^+CD44^+$ and $CD3^+CD8^+CD44^+$ T cells in 5 replicates of spleen and cecal tonsils samples, at each moment. Tissue samples were disrupted in the 70μm cell strainer (Becton Dickinson Labware, NJ, USA). The leukocytes were obtained by gradient centrifugation in Ficoll-Paque (Sigma-Aldrich, USA) equally diluted and $10^6$ cells were incubated for 30 min on ice with anti-chicken CD3-PE, CD4αβ-FITC or CD8αβ-FITC and CD44-APC antibodies (5ng/mL; SouthernBiotech, USA) and subsequently fixed in ice cold formaldehyde 1%. Isotype control for each fluorochrome was used to set the cutoff value. Samples were evaluated in the BD FACSCanto instrument (BD Biosciences, USA), recording 50000 events per sample. The mean percentage values of each cell population were calculated with FlowJo Software v.10 (FlowJo, USA).

### 2.8. Bone marrow derived macrophages (BMDMs) infection with SG

BMDMs were obtained as previously described (Marim et al., 2010) from fresh bone marrow from an uninfected and unchallenged chicken. Briefly, the femur was aseptically harvested and rinsed in 70% ethanol for disinfection. The bone marrow was collected by rinsing with RPMI complete media in the interior of the opened epiphysis. The adherent monocytes were harvested on 6 well plates and cultured with chicken rGM-GSF (5 ng/mL). The BMDMs were infected with the wild or the attenuated SG strain using the multiplicity of infection (MOI) of 25:1 and incubated for 2h at 37ºC and 5% $CO_2$. Concanavalin A (ConA, 10 μg/mL) and LPS (5 μg/mL) were used as controls. After incubation, the cells were harvested for quantification of IFN-γ, TNF-α, IL-1β and IL-4 levels by RT-qPCR, as described in item 2.6 (primers in Table 1). Each sample was analyzed in three replicates and in two repetitions.

### 2.9. Statistical analysis

The Kruskall-Wallis method was used to analyse the incidence of different values between all groups at each sampling day. The Bonferroni test was further applied to compare differences between groups separately. The $\chi^2$ test was used to evaluate the mortality rate results. Values were considered statistically different at $p<0.05$.

## 3. Results

### 3.1. Protection against SG infection, symptoms and mortality rate

In vaccinated chickens (group VAC), no chicken died or developed apparent clinical symptoms characteristic of fowl typhoid. As shown in Fig. 1, mortality of chickens in group INF started after 6 DPI and reached a total of 85% (17/20) of chickens in this group at 30 DPI. However, birds that died at 6 DPI did not present clinical signs and mortality was acute. Classical disease symptoms were visible after 7 DPI in group INF, with greenish diarrhea, weakness, feeding interruption and ruffled feathers. At necropsy, affected chickens demonstrated enlarged liver and spleen, however, necrotic lesions were not present in chickens dead before 10 DPI. Surviving animals from INF group, showed large necrotic lesions in the liver. In vaccinated chickens (Group VAC), internal organs were normal, however 3 chickens presented small lesions suggestive of necrotic foci, in the spleen at 3 DPI. The bacterial numbers are presented in Table 2. At 15 DBI the attenuated LV strain was still isolated in cecal tonsil samples (4.7 CFU/g). At 1 DBI and 1 DPI the LV strain was not found in spleen, liver or cecal tonsils examined. The enumeration of the challenge strain showed higher numbers in samples of liver, spleen and cecal tonsils of unvaccinated chickens (group INF) throughout the evaluated moments, reaching a peak at 7 DPI, with values above 5.1 CFU/g in all analyzed organs. As shown in Table 2, the bacterial numbers were significantly lower in vaccinated chickens ($p<0.05$), at all moments, not exceeding 2.5 CFU/g at 7 DPI.

### 3.2. Serum antibody titers against SG

The serum levels of IgG and IgM against SG in vaccinated and unvaccinated chickens are shown in Fig. 2. The levels of IgM and IgG were significantly higher ($p<0.05$) in vaccinated chickens at all moments evaluated, in comparison with the same moments in unvaccinated chickens (group INF). In group INF, IgM was detected at low levels, only at 3 and 7 DPI, however IgG was not present in this group, during the evaluated period. In group VAC, IgG and IgM induced by vaccination were detected at 1 and 15 DBI. Challenge induced increase in both classes of antibodies, thus, at 1, 3 and 7 DPI, levels were higher in group VAC than at 1 DBI.

**Table 1**
List of cytokines and primers used for the RT-qPCR.

| Target gene | Oligonucletodes | Annealing | Size (bp) | GenBank ID |
|---|---|---|---|---|
| GAPDH | F5′-TCAAATGGGCAGATGCAGGT-3′<br>R5′-AGCTGAGGGAGCTGAGATGA-3′ | 60ºC | 125 | NM_204305.1 |
| 18S | F5′-CATGGCCGTTCTTAGTTGGT-3′<br>R5′-GGCGTAGGGTAGACACAAGC-3′ | 58ºC | 232 | FM165414 |
| IFN-γ | F5′-AGCTGACGGTGGACCTATTAT-3′<br>R5′-GGCTTTGCGCTGGATTC-3′ | 56ºC | 259 | HQ739082.1 |
| TNF-α | F5′-ATCCTCACCCCTACCCTGTC-3′<br>R5′-AACTCATCTGAACTGGGCGG-3′ | 60ºC | 171 | NM_204267.1 |
| IL-1β | F5′-ACCCGCTTCATCTTCTACCG-3′<br>R5′-TAGGTGGCGATGTTGACCTG-3′ | 60ºC | 155 | DQ393267.1 |
| IL-4 | F5′-TGGAGAGCATCCGGATAGTGA-3′<br>R5′-ATTCAGGAGCTGACGCATGT-3′ | 60ºC | 197 | AJ621249.1 |
| IL-12 | F5′-ACCAGCCGACTGAGATGTTC-3′<br>R5′-GTGCTCCAGGTCTTGGGATA-3′ | 59ºC | 163 | FJ788636.1 |

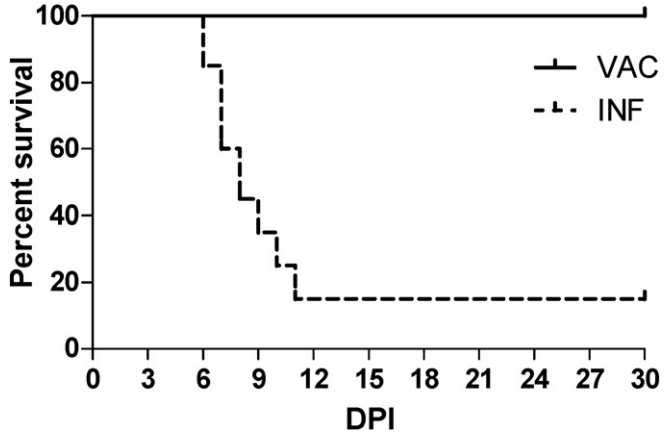


**Fig. 1.** Mortality of brown layer-hens after challenge with wild SG287/91 in group VAC and group INF, recorded during 30 DPI.

### 3.3. Flow cytometry

The different T cell populations were measured in spleen and cecal tonsils by flow cytometry. The results of the cellular immune response by effector T helper cells ($CD3^+CD4^+CD44^+$) and effector cytotoxic T cells ($CD3^+CD8^+CD44^+$) are demonstrated in Fig. 3A and supplementary Fig. 3B. The mean percentages of $CD4^+CD44^+$ and $CD8^+CD44^+$ cells were obtained after selecting $CD3^+$ T cells population, and the results are shown in Fig. 3A and represented in Fig. 3B. As shown in Fig. 3A, the $CD4^+CD44^+$ population was significantly higher ($p<0.05$) in group VAC at 15 DBI (21.9%), 1 DBI (24.7%), 3 DPI (24.9%) and 7 DPI (37.2%) in spleen samples in comparison with unvaccinated chickens in group INF, which showed 6.8%, 14.5%, 13.8% and 20.5%, respectively. In cecal tonsils, this population was significantly augmented in group VAC at 15 DBI (42.2%), 1 DBI (34.3%), 1 DPI (48.9%) and 7 DPI (35.4%), in comparison with the control group INF which presented reduced populations at 15 DBI (18.1%), 1 DBI (22.5%), 1 DPI (24.5%) and 7 DPI (17.4%).

The population of effector cytotoxic $CD8^+CD44^+$ T cells in spleen was significantly higher at all moments evaluated in group VAC (45.3%, 59.7%, 53.4%, 50.8% and 58.6%) in comparison with chickens that were only challenged in group INF which showed respectively, 7.6%, 12.9%, 20.1%, 22.7% and 20.5% of $CD8^+CD44^+$ T cells, in spleen. The population of $CD8^+CD44^+$ T cells in cecal tonsils of chickens in group VAC was significantly higher at 15 DBI (37.7%), 1 DBI (30.9%), 1 DPI (50.2%), 3 DPI (37.5%) and 7 DPI (34.2%) in comparison with chickens in group INF which showed 12.2%, 11.8%, 20.9%, 21.1% and 13.7%, respectively.

**Table 2**
Bacterial numbers in $Log_{10}$ of SGΔ*cob*SΔ*cbi*A before challenge and strain SG287/91 after challenge in different tissues.

| Moments | Organs | | | | | |
|---|---|---|---|---|---|---|
| | Spleen | | Liver | | Cecal Tonsils | |
| | VAC | INF | VAC | INF | VAC | INF |
| 15 DBI | 0 | 0 | 0 | 0 | 4.70* | 0 |
| 1 DBI | 0 | 0 | 0 | 0 | 0 | 0 |
| 1 DPI | 0.66[a] | 0.66[a] | 0 | 0 | 1.33[a] | 2.26[b] |
| 3 DPI | 1.33[a] | 2.01[a] | 2.55[a] | 3.07[a] | 0.76[a] | 1.7[b] |
| 7 DPI | 2.54[a] | 5.16[b] | 2.83[a] | 5.47[b] | 2.02[a] | 5.17[b] |

At 1, 3 and 7 DPI only the challenge strain (strain SG287/91) was enumerated, the absence of the LV strain was confirmed by PCR at 1 DBI and 1 DPI. *At 15 and 1 DBI bacterial numbers refer to the LV strain. Data represent the mean values. Different letters in the same line means statistical difference ($p<0.05$).

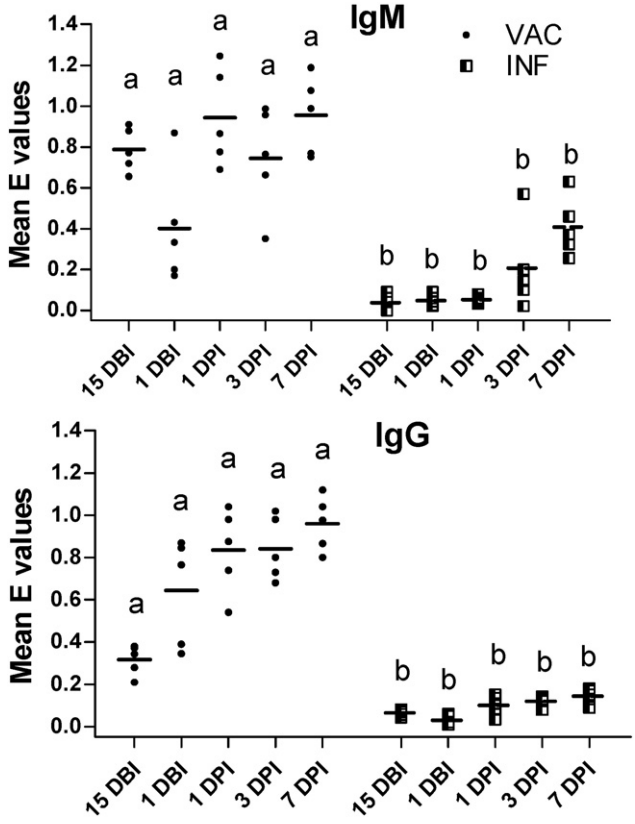


**Fig. 2.** Levels of IgG and IgM in vaccinated (group VAC) and unvaccinated brown layer-hens (group INF), at 15 and 1 DBI and 1, 3 and 7 DPI. Data represent the mean E values from 5 birds at each moments. Different letters indicate statistical difference in comparison with the same moment between groups VAC and INF ($p<0.05$).

### 3.4. Cytokine production in tissues

As shown in Fig. 4, a significantly increased expression of INF-γ in spleen was present in group VAC at 15 DBI, 1 DBI, 1 DPI and 3 DPI in comparison with unvaccinated group INF, furthermore, the levels of INF-γ reached the highest levels in group VAC at 1 DPI ($p<0.05$) in spleen and cecal tonsils. At 1 and 3 DPI the levels of TNF-α and IL-1β increased in both groups, in comparison with previous moments evaluated. However, at 3 and 7 DPI levels of these cytokines were still significantly higher ($p<0.05$) in group INF than in chickens in group VAC, in cecal tonsil and spleen (Fig. 4). The expression of IL-12 and IL-4 were augmented at 15 DBI and 3 DPI in group VAC in spleen samples. Chickens in group INF, showed an increased expression of IL-12 at 1 DPI in cecal tonsils and spleen and also IL-4 in cecal tonsil compared to group VAC.

### 3.5. Cytokine production profile of infected BMDMs

The expression of all evaluated cytokines was upregulated in both groups of infected BMDMs. As shown in Fig. 4, the infection of BMDMs with wild (SG287/91) or attenuated SG strains stimulated the expression of IFN-γ and TNF-α at similar levels without statistical difference between groups ($p>0.05$). The expression levels of IL-1β by BMDMs was significantly increased after infection with wild SG (fold change 40.7) in comparison with attenuated SG (fold change 21.3). The levels of IL-4 were also higher in BMDMs infected with wild SG (fold change 4.3), whilst fold change reached 2.8 in cells infected with attenuated SGΔ*cob*SΔ*cbi*A.

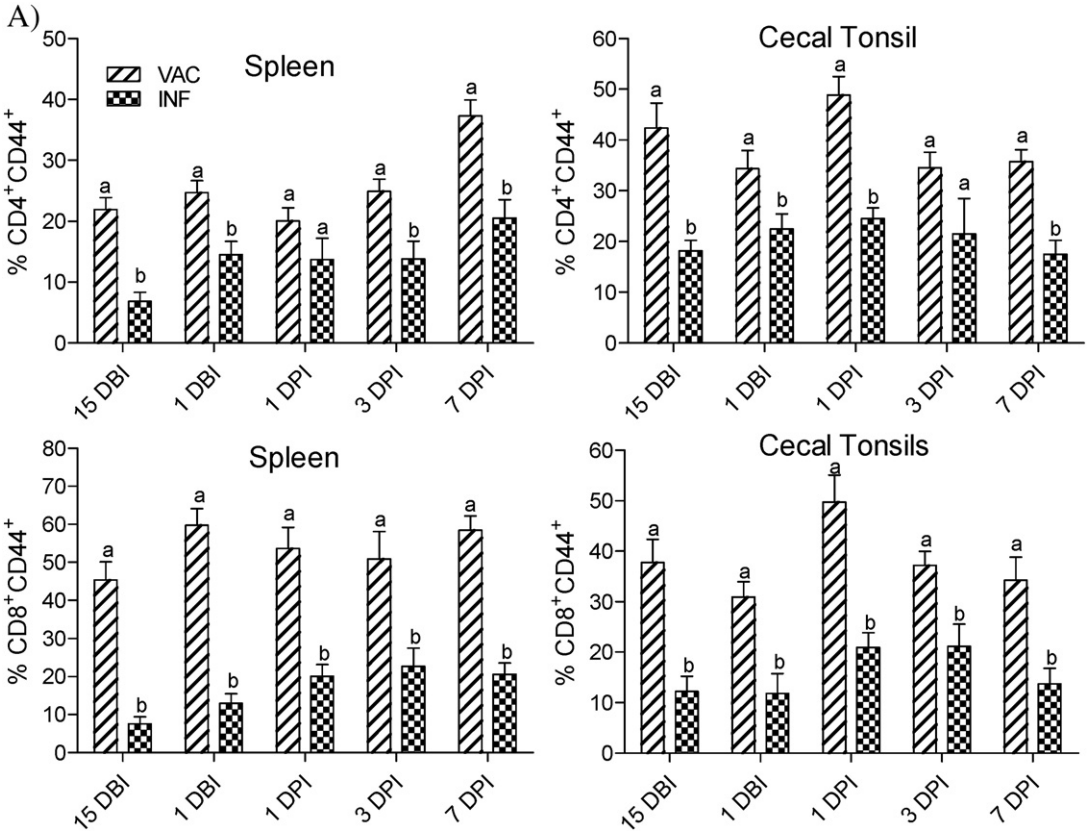


**Fig. 3A.** Flow cytometry analysis of effector T helper cells $CD4^+CD44^+$ and effector cytotoxic T cells $CD8^+CD44^+$ in spleen and cecal tonsils of vaccinated (group VAC) and unvaccinated brown layer-hens (group INF) before and after challenge, at 15 DBI and 1 DBI and 1 DPI, 3 DPI and 7 DPI. Columns represents the mean percent value ± SD (bars) of 5 replicates at each moment. Different letters indicate statistical difference in comparison with the same moment between groups VAC and INF ($p<0.05$).

## 4. Discussion

Vaccination is the main control measure against fowl typhoid, however due to biosecurity lapses, reintroduction and persistence SG in the environment, novel outbreaks may occur and are frequently reported worldwide (O.I.E., 2015). The vaccines against SG available for use in the fields are prepared with live attenuated strains, however the immune response elements that are effective against fowl typhoid are still under investigation to improve efficacy of vaccine schemes (Chappell et al., 2009; Matsuda et al., 2011; Setta et al., 2012; Wigley et al., 2005). While the Th2 immune response profile and antibodies triggered by vaccination have shown to play a minor role in the control of septicemic infection, increasing number of studies have demonstrated the effective participation of the Th1 profile, characterized by the cellular immune response, especially by effector and memory T lymphocytes and macrophages is crucial for the reduction of the bacteria proliferation and mortality caused by typhoidal *Salmonella* infections (Wahid et al., 2015). Susceptible chickens are incapable to control the mortality caused by SG infection based on the innate immunity (Chappell et al., 2009). Thus, these varieties of chickens that include brown layer-hens, broiler chickens (meat type) and turkeys need vaccination to induce the acquired immunity and memory T cells against this host-specific pathogen. Although, protection against typhoid salmonellosis is deeply studied in humans and murine models (Lee et al., 2012), the knowledge about the immune response against fowl typhoid in poultry still needs elucidation. Our previous study showed that one dose of the LV was capable to protect chickens against mortality with similar efficacy (Penha Filho et al., 2010). However, the present study was formulated to evaluate the immune response events that support the protection in vaccinated chickens, susceptible to fowl typhoid.

Considering that the onset of experimentally induced fowl typhoid occurs during the first week post-infection susceptible lines of chickens (Chappell et al., 2009; Shivaprasad, 2000), we prioritized to evaluate differences in the immune response of vaccinated and unvaccinated chickens within this critical period. In our study, brown layer hens were highly susceptible to the challenge and the mortality rate reached was 85% in INF group. Although SG is invasive and colonizes the internal organs, it is important to notice that SG also invaded and persisted in the cecal tonsils lymphoid tissue in high numbers in unvaccinated chickens until 7 DPI (Table 2), suggesting that the local immune response, in the gut associated lymphoid tissue (GALT) may also be important to control invasion through the intestinal epithelia, as previously suggested (Berndt et al., 2007). Considering the efficacy of other vaccines used for SG control, the strain SGΔ*cob*SΔ*cbi*A showed similar protection results, demonstrating its potential for field application, for reduction of mortality caused by pathogenic SG strains, which ranges from 60% to 100% in experimentally infected chickens (Matsuda et al., 2011; Mitra et al., 2013; Rosu et al., 2007).

The live vaccine strain protected all chickens in group VAC from mortality and high bacterial dissemination of the challenge strain. The protection against fowl typhoid mortality using one dose of the LV has been previously demonstrated (Penha Filho et al., 2010; Rosu et al., 2007), however the results presented here demonstrate the kinetics of the systemic bacterial dissemination in comparison with the cellular and humoral immunity development. Vaccinated chickens were infected with challenge strain, however lower bacterial numbers were present in comparison with unvaccinated chickens. The highest numbers of SG287/91 in group VAC were 2.83 CFU/g in liver at 7DPI. Challenged layer-hens in group INF showed constantly increasing bacterial numbers in all organs evaluated with peaks at 7 DPI reaching 5.16 CFU/g in spleen and 5.47 CFU/g in liver (Table 2), which corresponded to the moment that mortality started in this group.

The evaluation of humoral immune response showed that IgM and IgG levels were present before infection in group VAC, stimulated by

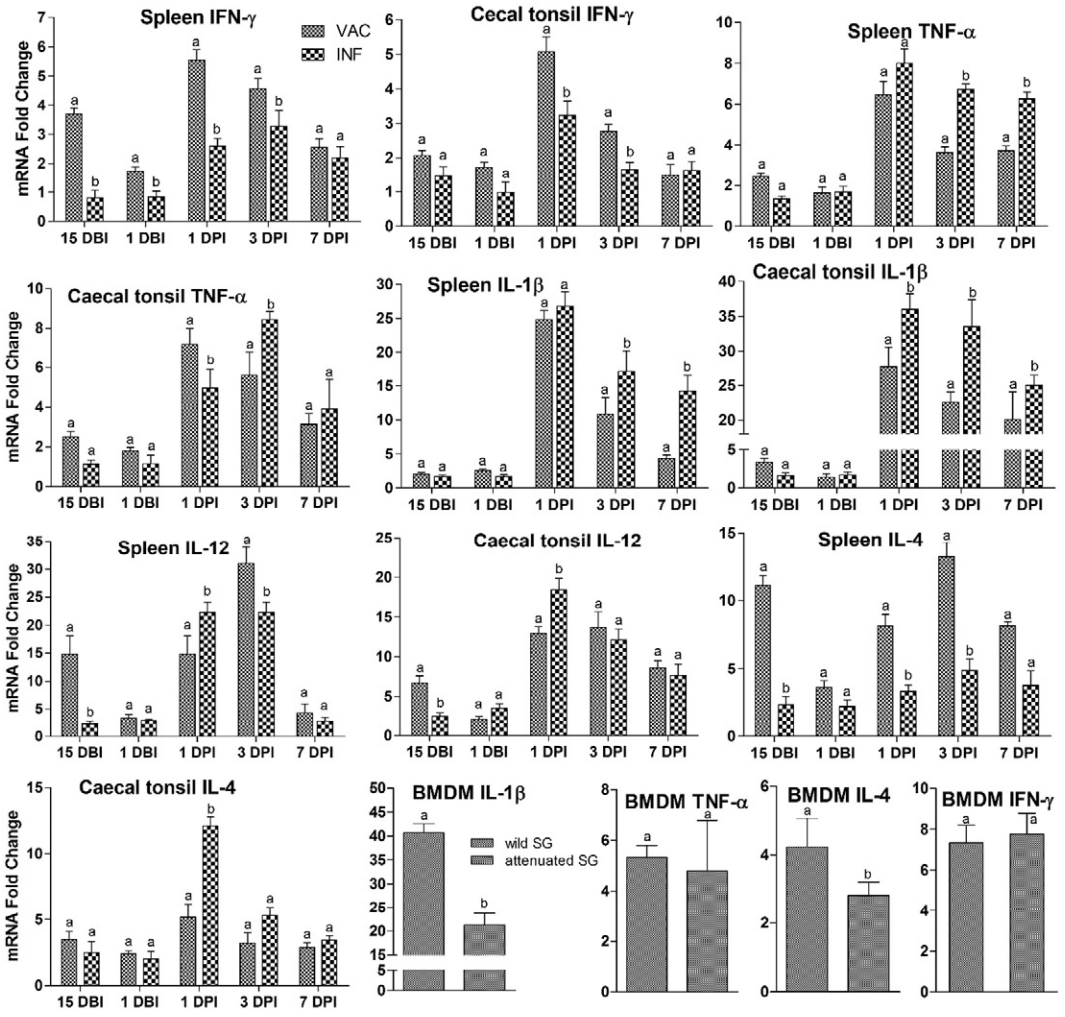


**Fig. 4.** Expression levels of cytokines in spleen and cecal tonsils of vaccinated (group VAC) and unvaccinated brown layer-hens (group INF), at 15 and 1 DBI and 1, 3 and 7 DPI and levels of IFN-γ, TNF-α, IL-1β and IL-4 in BMDMs infected with wild or attenuated SG after 2h of infection. Data on each column represent the mean values obtained by the evaluation of 5 replicates ± SD. Different letters indicate statistical difference in comparison with the same moment between groups VAC and INF ($p<0.05$).

immunization. At 1 and 15 DBI, IgG anti-SG was already present in significant higher levels ($p<0.05$) in vaccinated chickens and increased after challenge. Immunoglobulins were not detected in significant levels after challenge of unvaccinated chickens, before the occurrence of mortality (Fig. 2). Despite the known importance of CMI for the clearance of *Salmonella* after primary challenge, the participation of the humoral immune response may also contribute for the opsonization and clearance of infection in vaccinated animals, especially invasive serovars of *Salmonella* that can reach the bloodstream causing sepsis, such as SG. Different conclusions have been drawn about the role of secreted immunoglobulins to control *Salmonella*, however it is known that memory B lymphocytes are essential to stimulate CMI and IFN-γ secretion by $CD4^+$ T cells, and contribute to a protective immunity against this pathogen, independently from the role of antibody production (Nanton et al., 2012). The absence of antibodies against SG in group INF, also shows the failure to develop elements of a protective immune response, in time to control the infection in these susceptible line of layer-hens, without the use of vaccination.

The role of T cells in response to *Salmonella* is known to be crucial, participating in different events such as production of pro-inflammatory cytokines, regulation of local and systemic immune response, activation of macrophages and clearance of intracellular bacteria (Delgoffe and Powell, 2015; Dougan et al., 2011). Stimulating immunological memory in T cells through vaccination is important for the fast turnover and proliferation of effector/memory T cells ($CD44^+$) in case of challenge by pathogenic strains (Holmkvist et al., 2015). A depletion of $CD8^+CD44^+$ T cells in the spleen and cecal tonsils of unvaccinated layer-hens was noticed at 1, 3 and 7 DPI corresponding to less than 40.5% of total lymphocyte population ($CD3^+$). Vaccinated birds, showed constantly higher numbers of $CD8^+CD44^+$, in comparison with unvaccinated group ($p<0.05$). As demonstrated by other authors (Becattini et al., 2015; Ciabattini et al., 2013), the priming of both T cell population ($CD4^+CD44^+$ and $CD8^+CD44^+$) with the live vaccine, demonstrated to be an efficacious toll to prevent the uncontrolled infection and dissemination of the pathogen after challenge. As shown in Fig. 3A and 3B, the number of effector T cells activated through vaccination was significantly higher in group VAC before challenge. In unvaccinated brown layers, a depletion of effector T cells, especially the $CD8^+CD44^+$ was noticed after challenge. As previously shown cytotoxic $CD8^+$ T cells are important to control systemic *Salmonella enterica* infections (Lopez-Medina et al., 2014; Penha Filho et al., 2012). Thus, it could be noticed that vaccination with the LV contributed to the rapid turnover of memory lymphocytes into effector T cells. However, at 7 dpi the T cell population (both $CD4^+$ and $CD8^+$) were still significantly reduced, and the turnover of naive T cells to effector T cells did not occurred efficiently in order to control the SG infection.

Control of primary infections in non-immunized animals, depends mostly on bacterial clearance by NK cells and macrophages, after activation by cytokines produced by $CD4^+$ T cells or bacterial particles, such as lipopolysaccharide (LPS) (Wigley, 2013). In this study, the CMI by both

$CD4^+CD44^+$ and $CD8^+CD44^+$ effector T cells, was significantly reduced in unvaccinated chickens after infection, in comparison with vaccinated chickens. These results suggest that the immunization with live attenuated SG vaccine is capable to stimulate memory T cells, which promptly proliferated after re-stimulation by the challenge strain, accelerating the time of bacterial clearance from the host organism (Fig. 3). Considering the role of $\alpha\beta CD8^+$ cytotoxic T cells (CTLs) in the clearance of intracellular bacterial infection, a significant population of CTLs was only noticed in vaccinated chickens (Fig. 3B). Otherwise in group INF, the chickens demonstrated a failure to mount immune response on time to control bacterial dissemination. The reduced population of T cells in spleen and cecal tonsils at 7 DPI, suggests that the development of the disease in unvaccinated chickens caused lesions in these organs, as previously shown (Chappell et al., 2009), resulting in necrosis and mortality. IL-12 is a key cytokine for the coordination of the innate and acquired immune response(Mescher et al., 2006). This cytokine is known to stimulate a strong effector immune response by $CD8^+$ T cells, including during primary infections (Pearce and Shen, 2007). As shown in Fig. 4, both the vaccine and challenge strains stimulated IL-12 production, however the presence of this cytokine before challenge was only present at significant levels in vaccinated chickens (15 DBI). After challenge, at 1 DPI, the levels were higher ($p<0.05$) on both organs evaluated in unvaccinated and infected chickens from group INF, however this group was not capable to overcome the SG infection. In vaccinated chickens, the higher levels of this cytokine at 3 DPI may have contributed to the development of the strong effective T cell response (Figs. 3A and 3B), capable to control SG challenge and mortality. In contact with the vaccine antigens, of naïve $CD8^+$ T cells are programmed by IL-12, promoting the formation of memory populations which may undergo strong clonal expansion upon challenge with pathogen (Xiao et al., 2009).

Although $CD4^+$ T cells are an important source of IFN-γ, to develop CMI, these are not the only source of this cytokine of crucial role during *Salmonella* clearance (Weintraub et al., 1997). Other authors have shown that NK cells and macrophages may be an important source of IFN-γ during *Salmonella* infection (Lapaque et al., 2009). As shown, the infection of BMDMs infected with wild or attenuated SG strains, expressed IFN-γ in comparison with uninfected control cells used for normative control, however no significant differences were noticed between both infected groups ($p>0.05$; Fig. 4). An important characteristic of the LV strain, is that it was capable to stimulate the expression of this important cytokine, involved in the development of CMI. The detection of *Salmonella* by extracellular receptors is capable to trigger the production of IFN-γ and pro-inflammatory cytokines. As shown in Fig. 4 the *in vivo* experiments also demonstrated an augmented expression of IFN-γ in spleen and cecal tonsils of chickens in group VAC which were vaccinated with LV. Although the higher expression of the cytokine in this group may be due to the higher number of primed T cells in this group, which proliferated at 1 DPI, after contact with the challenge SG strain.

Although attenuated due to metabolic alterations, the LV strain has unaltered membrane components, such as LPS, and the interaction with BMDMs or the host lymphoid tissues induced detectable levels of pro-inflammatory cytokine IL-1β. In fact, the expression of all cytokines was increased after infection of BMDMs with attenuated and wild SG strains in comparison with unvaccinated and uninfected cells or chickens, used as normative controls for gene expression calculations. Previous study showed variable invasion capability after the infection of chicken abdominal macrophages with attenuated and wild type strains of SG (Matsuda et al., 2010). The higher expression of IL-1β by BMDMs infected with wild SG in comparison with the attenuated SG, may have occurred due to higher intracellular proliferation capacity of this strain, as noticed in the bacterial numbers in the evaluated tissues. IL-1β was also significantly increased ($p<0.05$) after challenge in group INF, in spleen and cecal tonsils samples. The stimulation of pro-inflammatory cytokines is considered a relevant issue for the development of vaccine immune response, such that inflammatory molecules are frequently used as adjuvants (Pasetti et al., 2011). The vaccine strain of SG was capable to induce lower, however significant levels of IL-1β and IFN-γ and TNF-α, which may be associated with the CMI present *in vivo*. The live SG vaccine also stimulated the humoral immune response as demonstrated in Fig. 2, complementing the CMI noticed. It was possible to notice in chickens from group VAC, specific IgG response to the soluble fraction of SG antigens, which corresponded to intracellular cytoplasmic proteins, demonstrating that during antigen processing of the LV strain, not only Th1 specific response is produced, but also Th2 antigens are processed. Despite the levels of IL-4 expression by the BMDMs stimulated *in vitro* with attenuated SG (LV) were not significantly elevated, the processing of vaccine antigens *in vivo* is much more complex, and involves different cell populations controlling the immune response polarization though cytokines. Other cell populations are capable to produce of Th2 cytokines, such as activated $CD4^+$ T cells and NK cells, which may interfere on the macrophage phenotypes (Chavez-Galan et al., 2015; Mosser, 2003).

Overall, the importance of immunization to protect susceptible lines of chickens has been demonstrated, once these birds are unable to control infection depending on the innate immunity mechanisms. It has been shown that vaccinated brown layer-hens are capable to mount a proliferative response by effector $CD4^+$ and $CD8^+$ T cells, whilst unvaccinated birds quickly succumb to a systemic infection, loss of lymphoid tissues, often resulting in death. The live vaccine was capable to infect chicken BMDMs and stimulate the production of pro-inflammatory cytokines. Thus, as shown by previous studies and this, live vaccines against SG have a consolidated importance since its first report (Smith, 1956), as an effective tool for prevention and control of fowl typhoid in the fields.

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## Conflicts of interest

The authors declare no conflict of interest.

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## References

Baaten, B.J.G., Li, C.-R., Deiro, M.F., Lin, M.M., Linton, P.J., Bradley, L.M., 2010. CD44 Regulates Survival and Memory Development in Th1 Cells. Immunity 32, 104–115.

Barrow, P.A., Freitas Neto, O.C., 2011. Pullorum disease and fowl typhoid–new thoughts on old diseases: a review. Avian Pathol. 40, 1–13.

Becattini, S., Latorre, D., Mele, F., Foglierini, M., De Gregorio, C., Cassotta, A., Fernandez, B., Kelderman, S., Schumacher, T.N., Corti, D., Lanzavecchia, A., Sallusto, F., 2015. T cell immunity. Functional heterogeneity of human memory CD4(+) T cell clones primed by pathogens or vaccines. Science 347, 400–406.

Berndt, A., Wilhelm, A., Jugert, C., Pieper, J., Sachse, K., Methner, U., 2007. Chicken cecum immune response to *Salmonella enterica* serovars of different levels of invasiveness. Infect. Immun. 75, 5993–6007.

BRASIL, 2003. Ministério da Agricultura, Pecuária e Abastecimento. Programa Nacional de Sanidade Avícola. Instrução Normativa 78. Diário Oficial da União, República Federativa do Brasil, Brasília - DF.

Carvajal, B.G., Methner, U., Pieper, J., Berndt, A., 2008. Effects of *Salmonella enterica* serovar Enteritidis on cellular recruitment and cytokine gene expression in caecum of vaccinated chickens. Vaccine 26, 5423–5433.

Chappell, L., Kaiser, P., Barrow, P., Jones, M.a., Johnston, C., Wigley, P., 2009. The immunobiology of avian systemic salmonellosis. Vet. Immunol. Immunopathol. 128, 53–59.

Chavez-Galan, L., Olleros, M.L., Vesin, D., Garcia, I., 2015. Much More than M1 and M2 Macrophages, There are also CD169$^+$ and TCR$^+$ Macrophages. Front. Immunol. 6, 263.
Ciabattini, A., Pettini, E., Medaglini, D., 2013. CD4$^+$ T Cell Priming as Biomarker to Study Immune Response to Preventive Vaccines. Front. Immunol. 4, 421.
de Paiva, J.B., Penha Filho, R.A., Arguello, Y.M., Berchieri Junior, A., Lemos, M.V., Barrow, P.A., 2009. A defective mutant of *Salmonella enterica* Serovar Gallinarum in cobalamin biosynthesis is avirulent in chickens. Braz. J. Microbiol. 40, 495–504.
Delgoffe, G.M., Powell, J.D., 2015. Feeding an army: The metabolism of T cells in activation, anergy, and exhaustion. Mol. Immunol.
Dobhal, S., Zhang, G., Rohla, C., Smith, M.W., Ma, L.M., 2014. A simple, rapid, cost-effective and sensitive method for detection of *Salmonella* in environmental and pecan samples. J. Appl. Microbiol. 117, 1181–1190.
Dougan, G., John, V., Palmer, S., Mastroeni, P., 2011. Immunity to salmonellosis. Immunol. Rev. 240, 196–210.
Feberwee, A., de Vries, T.S., Hartman, E.G., de Wit, J.J., Elbers, A.R., de Jong, W.A., 2001. Vaccination against *Salmonella* Enteritidis in Dutch commercial layer flocks with a vaccine based on a live *Salmonella* Gallinarum 9R strain: evaluation of efficacy, safety, and performance of serologic *Salmonella* tests. Avian Dis. 45, 83–91.
Holmkvist, P., Roepstorff, K., Uronen-Hansson, H., Sanden, C., Gudjonsson, S., Patschan, O., Grip, O., Marsal, J., Schmidtchen, A., Hornum, L., Erjefalt, J.S., Hakansson, K., Agace, W.W., 2015. A major population of mucosal memory CD4$^+$ T cells, coexpressing IL-18Ralpha and DR3, display innate lymphocyte functionality. Mucosal Immunol. 8, 545–558.
Jawale, C.V., Lee, J.H., 2014. A novel approach for the generation of *Salmonella* Gallinarum ghosts and evaluation of their vaccine potential using a prime-booster immunization strategy. Vaccine 32, 6776–6782.
Lapaque, N., Walzer, T., Meresse, S., Vivier, E., Trowsdale, J., 2009. Interactions between human NK cells and macrophages in response to *Salmonella* infection. J. Immunol. 182, 4339–4348.
Lee, S.J., Liang, L., Juarez, S., Nanton, M.R., Gondwe, E.N., Msefula, C.L., Kayala, M.A., Necchi, F., Heath, J.N., Hart, P., Tsolis, R.M., Heyderman, R.S., MacLennan, C.A., Felgner, P.L., Davies, D.H., McSorley, S.J., 2012. Identification of a common immune signature in murine and human systemic Salmonellosis. Proc. Natl. Acad. Sci. U. S. A. 109, 4998–5003.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif.) 25, 402–408.
Lopez-Medina, M., Perez-Lopez, A., Alpuche-Aranda, C., Ortiz-Navarrete, V., 2014. *Salmonella* modulates B cell biology to evade CD8$^+$ T cell-mediated immune responses. Front. Immunol. 5, 586.
Marim, F.M., Silveira, T.N., Lima Jr., D.S., Zamboni, D.S., 2010. A method for generation of bone marrow-derived macrophages from cryopreserved mouse bone marrow cells. PLoS One 5, e15263.
Matsuda, K., Chaudhari, A.A., Kim, S.W., Lee, K.M., Lee, J.H., 2010. Physiology, pathogenicity and immunogenicity of lon and/or cpxR deleted mutants of *Salmonella* Gallinarum as vaccine candidates for fowl typhoid. Vet. Res. 41, 59.
Matsuda, K., Chaudhari, A.A., Lee, J.H., 2011. Comparison of the Safety and Efficacy of a New Live *Salmonella* Gallinarum Vaccine Candidate, JOL916, with the SG9R Vaccine in Chickens. Avian Dis. 55, 407–412.
McSorley, S.J., 2014. Immunity to intestinal pathogens: lessons learned from *Salmonella*. Immunol. Rev. 260, 168–182.
Mescher, M.F., Curtsinger, J.M., Agarwal, P., Casey, K.a., Gerner, M., Hammerbeck, C.D., Popescu, F., Xiao, Z., 2006. Signals required for programming effector and memory development by CD8$^+$ T cells. Immunol. Rev. 211, 81–92.
Meunier, M., Guyard-Nicodeme, M., Dory, D., Chemaly, M., 2016. Control strategies against Campylobacter at the poultry production level: biosecurity measures, feed additives and vaccination. J. Appl. Microbiol. 120, 1139–1173.
Mitra, A., Loh, A., Gonzales, A., Laniewski, P., Willingham, C., Curtiss Iii, R., Roland, K.L., 2013. Safety and protective efficacy of live attenuated *Salmonella* Gallinarum mutants in Rhode Island Red chickens. Vaccine 31, 1094–1099.
Mosser, D.M., 2003. The many faces of macrophage activation. J. Leukoc. Biol. 73, 209–212.
Nanton, M.R., Way, S.S., Shlomchik, M.J., McSorley, S.J., 2012. Cutting edge: B cells are essential for protective immunity against *Salmonella* independent of antibody secretion. J. Immunol. 189, 5503–5507.
O.I.E., 2015. World Animal Health Information System. Available from: http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Diseasetimelines.
Pasetti, M.F., Simon, J.K., Sztein, M.B., Levine, M.M., 2011. Immunology of gut mucosal vaccines. Immunol. Rev. 239, 125–148.
Pearce, E.L., Shen, H., 2007. Generation of CD8 T cell memory is regulated by IL-12. J. Immunol. 179, 2074–2081.
Penha Filho, R.A., de Paiva, J.B., Arguello, Y.M., da Silva, M.D., Gardin, Y., Resende, F., Berchieri Junior, A.B., Sesti, L., 2009. Efficacy of several vaccination programmes in commercial layer and broiler breeder hens against experimental challenge with *Salmonella enterica* serovar Enteritidis. Avian Pathol. 38, 367–375.
Penha Filho, R.A., de Paiva, J.B., da Silva, M.D., de Almeida, A.M., Berchieri Jr., A., 2010. Control of *Salmonella* Enteritidis and *Salmonella* Gallinarum in birds by using live vaccine candidate containing attenuated *Salmonella* Gallinarum mutant strain. Vaccine 28, 2853–2859.
Penha Filho, R.A., Moura, B.S., de Almeida, A.M., Montassier, H.J., Barrow, P.A., Berchieri Junior, A., 2012. Humoral and cellular immune response generated by different vaccine programs before and after *Salmonella* Enteritidis challenge in chickens. Vaccine 30, 7637–7643.
Rosu, V., Chadfield, M.S., Santona, A., Christensen, J.P., Thomsen, L.E., Rubino, S., Olsen, J.E., 2007. Effects of *crp* deletion in *Salmonella enterica* serotype Gallinarum. Acta Vet. Scand. 49, 14.
Santos, R.L., Zhang, S., Tsolis, R.M., Kingsley, R.A., Adams, L.G., Baumler, A.J., 2001. Animal models of *Salmonella* infections: enteritis versus typhoid fever. Microbes Infect. 3, 1335–1344.
Setta, A.M., Barrow, P.A., Kaiser, P., Jones, M.A., 2012. Early immune dynamics following infection with *Salmonella enterica* serovars Enteritidis, Infantis, Pullorum and Gallinarum: Cytokine and chemokine gene expression profile and cellular changes of chicken cecal tonsils. Comp. Immunol. Microbiol. Infect. Dis.
Shehata, A.A., Sultan, H., Hafez, H.M., Kruger, M., 2013. Safety and efficacy of a metabolic drift live attenuated *Salmonella* Gallinarum vaccine against fowl typhoid. Avian Dis. 57, 29–35.
Shivaprasad, H.L., 2000. Fowl typhoid and pullorum disease. Rev. Sci. Tech. 19, 405–424.
Smith, H.W., 1956. The use of live vaccines in experimental *Salmonella* Gallinarum infection in chickens with observations on their interference effect. J. Hyg. 54, 419–432.
Tahoun, A., Mahajan, S., Paxton, E., Malterer, G., Donaldson, D.S., Wang, D., Tan, A., Gillespie, T.L., O'Shea, M., Roe, A.J., Shaw, D.J., Gally, D.L., Lengeling, A., Mabbott, N.A., Haas, J., Mahajan, A., 2012. *Salmonella* transforms follicle-associated epithelial cells into M cells to promote intestinal invasion. Cell Host Microbe 12, 645–656.
Wahid, R., Fresnay, S., Levine, M.M., Sztein, M.B., 2015. Immunization with Ty21a live oral typhoid vaccine elicits crossreactive multifunctional CD8$^+$ T-cell responses against *Salmonella enterica* serovar Typhi, *S.* Paratyphi A, and *S.* Paratyphi B in humans. Mucosal Immunol.
Weintraub, B.C., Eckmann, L., Okamoto, S., Hense, M., Hedrick, S.M., Fierer, J., 1997. Role of alphabeta and gammadelta T cells in the host response to *Salmonella* infection as demonstrated in T-cell-receptor-deficient mice of defined Ity genotypes. Infect. Immun. 65, 2306–2312.
Wigley, P., 2013. Immunity to bacterial infection in the chicken. Dev. Comp. Immunol. 41, 413–417.
Wigley, P., Hulme, S., Powers, C., Beal, R., Smith, A., Barrow, P., 2005. Oral infection with the *Salmonella enterica* serovar Gallinarum 9R attenuated live vaccine as a model to characterise immunity to fowl typhoid in the chicken. BMC Vet. Res. 1, 2.
Xiao, Z.G., Casey, K.A., Jameson, S.C., Curtsinger, J.M., Mescher, M.F., 2009. Programming for CD8 T Cell Memory Development Requires IL-12 or Type I IFN. J. Immunol. 182, 2786–2794.