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Full-Length Article

Efficacy of a killed *Salmonella Enterica* serovar Typhimurium bacterin vaccine administration in layer birds challenged with heterologous *Salmonella Enterica* serovar Enteritidis

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ABSTRACT

In this study, we evaluated the efficacy of administering a killed *Salmonella enterica* ser. Typhimurium bacterin (ST) vaccine with an adjuvant intramuscularly on humoral immunity, cellular immunity, and SE load reduction in layers. The ST vaccine was prepared with 97% *S*. Typhimurium and an adjuvant of 3% Immune Plus® with preservatives. Eighty 14-week-old *Salmonella*-free Hy-Line W-36 pullets were randomly allocated into two groups: unvaccinated control and ST vaccinated, with 40 birds per group. Birds were immunized intramuscularly with 500 μL (Endovac) vaccine at week 17 and a booster dose at week 19. At 27 weeks of age, both groups were challenged with 5×10^8 CFU/mL of nalidixic acid-resistant *Salmonella enterica* ser. Enteritidis. At 22, 23, and 24 weeks of age, ST-vaccinated birds showed higher serum anti-*Salmonella* IgY levels than the control group by 186%, 202% ($P < 0.05$), and 2700% ($P > 0.05$), respectively. At 28 weeks of age, vaccinated birds had 8.3% lower levels ($P > 0.05$) of anti-*Salmonella* IgA in bile and 240% greater levels ($P < 0.05$) of anti-*Salmonella* IgY in serum compared to control group. At 28 weeks of age, splenocytes from the ST-vaccinated birds had increased antigen-specific T-lymphocyte proliferation ($P > 0.05$). There were no significant differences in CD4+/CD8+-T-cell ratios, IL-10, IL-4, IL-1β, IFNγ mRNA levels in the spleen and cecal tonsil between vaccinated birds compared to control. However, the vaccine did not reduce the *Salmonella* Enteritidis load in ceca, spleen, and liver. It can be concluded that the intramuscular administration of the killed ST vaccine with the adjuvant Immune Plus can increase serum antibody titers and induce a humoral immune response specific to *Salmonella*. However, the increase in serum antibody titers were not successful in reducing the *Salmonella* load in ceca, spleen, and liver.

Introduction

Salmonella is a significant zoonotic pathogen present in poultry causing an estimated 93.8 million infection cases, of which 80.3 million were foodborne, and 155,000 deaths annually (Majowicz et al., 2010). According to the CDC, approximately 1.35 million cases of *Salmonella* infection is reported annually in the United States, resulting in a significant economic burden (Scallan et al., 2011). The most common reservoir of *Salmonella* infection is poultry, with more than 70% of human salmonellosis cases in the US associated with the consumption of contaminated chicken meat and eggs (Andino et al., 2015).

Salmonella enterica ser. Enteritidis (SE) and *Salmonella enterica* ser. Typhimurium (ST) are the most important poultry-linked non-typhoidal *Salmonella* serotypes in the United States, followed by Heidelberg, Newport, and Infantis (Andino and Hanning, 2015). *Salmonella* serovars are known to be very well adapted to invade the intestinal epithelial and lymphoid cells due to their ability to survive the acidic pH of the GI tract. In contrast to humans, chickens can host high loads of *Salmonella* (up to 5 log $_{10}$ CFU/g) (Stern et al., 2001) and remain asymptomatic. The resulting subclinical infection in poultry is associated with the incidence of human illnesses (Shah et al., 2017).

With the advent of multi-drug-resistant *Salmonella*, the use of vaccination has increased in the poultry industry to combat *Salmonella*. Vaccination programs are developed to combat the public health hazard

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of *Salmonella* contamination of poultry meat and eggs. Live-attenuated vaccines comprise most of the commercially available vaccines. Other available vaccines include killed and subunit vaccines (Saenz et al., 2022). The main drawback of the *Salmonella* live vaccine is the ability of the strain to revert to a virulent form that the killed vaccine doesn't possess (Lauring et al., 2010). The currently available killed vaccines against *Salmonella* are shown to give partial protection against different serovars and are not successful in the complete elimination of *Salmonella* loads in layers (Jia et al., 2020). Hence, a killed vaccine administered intramuscularly that can confer cross-protection against a broad range of serovars is essential.

Further to improve the immune response, the *Salmonella* strain in vaccine is modified. Briefly, *Salmonella* is a gram-negative bacterium, and the cell wall is made up of lipopolysaccharide (LPS). LPS has three regions: the outer O-antigen, the middle core oligosaccharide, and an inner lipid A moiety (Kong et al., 2012). The *Salmonella* Typhimurium strain in the present vaccine lacks the capsular O side-chain carbohydrate, and it exposes the inner lipid A component of the bacterial cell wall. Lipid A activates nuclear factor κB (NF-κB) and upregulates co-stimulatory molecules and proinflammatory cytokines by inducing a potent innate immune response through the Toll-like receptor 4 (TLR4)-MD2-CD14 pathway (Kawasaki et al., 2012). Because the lipid A inner core antigens are common to all *Salmonella* (Luderitz et al., 1966), cross-protective immunity is expected. The objective of this study is to evaluate the efficacy of a killed *Salmonella* vaccine, based on the adjuvant Immune Plus, against *Salmonella* in layer birds. The study hypothesizes that the vaccine will elicit a potent cell-mediated and humoral immune response when delivered intramuscularly to layers.

Materials and methods

Ethics statement

All animal protocols followed the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Georgia. The Animal Care and Use ethical approval number is A2022 10-015-A1. The experiment was conducted at the Poultry Research Center at the University of Georgia, Athens, Georgia.

Experimental design and diets

Hy-Line W-36 birds were obtained from the poultry research center (Athens, GA) and reared on the floor until the pullets reached 17 weeks of age. At week 17 of age, a total of 80 *Salmonella*-free Hy-Line W-36 pullets were randomly allocated into two treatment groups of unvaccinated and ST-vaccinated birds. All birds were screened for *Salmonella* by cloacal swab examination at 17 weeks of age. The birds were then placed into individual cages equipped with a nipple drinker and trough feeder. The birds had access to *ad libitum* feed and water throughout the study. The birds were given layer stock feed to meet the nutrient requirement as per the Hy-Line International, 2020 guide. During the experimental period (17- 28 weeks of age) birds were provided with a 16:8-hour light: dark period. For this experiment, the experimental unit was the bird. The study was terminated on week 28. There was no mortality throughout the study.

Vaccine

The vaccine used for the study was Endovac *Salmonella* killed bacterin vaccine (Endovac Animal Health, Columbia, MO, USA). The vaccine composition was 97% *Salmonella* Typhimurium ((R/17 strain) and 3% Immune Plus® with preservatives. The vaccine was derived from a genetically mutated *Salmonella* Typhimurium that lacks the capsular O side-chain carbohydrate. At 17 and 19 weeks of age, birds in ST vaccine treatment group were inoculated with 500 μL of the ST vaccine. The vaccines were administered intramuscularly into the right pectoralis muscle. The control birds were mock vaccinated with PBS.

Treatment group and heterologous challenge

At 20, 21, 22, 23 and 24 weeks of age, blood was collected from all treatment groups. Samples were collected from randomly selected 6 birds/treatment (n = 6) at each time point. Birds were examined daily for physical distress and mortality. Egg production was recorded daily from 20 to 27 weeks of age. A weekly hen day egg production was calculated by dividing the total number of eggs produced by the total hen day in that week.

For the challenge study, a nalidixic acid-resistant *Salmonella enterica* ser. Enteritidis (SE) isolate was selected. The challenge *Salmonella* inoculum was prepared. At week 27 of age, birds were orally inoculated with 1ml of 5×10^8 CFU/mL of nalidixic acid-resistant SE using an oral gavage needle. All birds in control and vaccinated groups were challenged. The birds were euthanized by cervical dislocation and samples were collected one-week post-challenge, at 28 weeks of age.

Effect of ST vaccine on heterologous serum IgY and bile IgA anti-Salmonella antibodies of vaccinated birds

Blood samples (1 mL) were collected from birds every week from week 20 until week 24 and on week 28 after necropsy, from the wing vein using a 22G needle. Blood samples were collected in 1.5 mL Eppendorf tubes and were centrifuged at 10,000 rpm for 10 minutes for the separation of serum. The serum was collected in an autoclaved microcentrifuge tube. Samples were stored frozen at -20°C. An indirect enzyme-linked immunosorbent assay (ELISA) was performed to quantify *Salmonella*-specific IgY antibody titers in the chicken serum both pre- and post-*Salmonella* challenge infections.

An insulin syringe was used to collect bile on week 28 after necropsy. The aliquots of bile samples were stored at -80°C until use. Further, *Salmonella*-specific IgA antibody titers in the bile post-*Salmonella* challenge was determined as previously described (Renu et al., 2020).

A total of 12 samples, 6 from each treatment, from each week was used. Samples were analyzed in duplicates. Checkerboard titrations were carried out with different dilutions of serum and bile, coating antigens, and secondary antibodies for finding the concentrations of primary and secondary antibody. Flat-bottom 96- well plates (ThermoFisher Scientific, Waltham, MA, USA) were coated with R-17 bacterin stock ((Endovac Animal Health, Columbia, MO)-solid phase antigen (10 μg/mL) diluted in 0.1 mmol/L carbonate coating buffer (pH 9.6) and incubated overnight at 4°C. The plates were washed three times with PBS Tween-20 (0.05% Tween 20, pH 7.4) (PBST) to remove the unbound antigen. The wells were blocked with 200 μL of SuperBlock™ Blocking Buffer in PBS (ThermoFisher Scientific, Waltham, MA, USA) for 1 hour at 37°C for IgY analysis. Plates were washed three times with PBST. Hundred microliters of serum (1: 2000 dilution) and bile (1: 1600 dilution) in SuperBlock™ Blocking Buffer (ThermoFisher Scientific, Waltham, MA, USA) was added in duplicates to the plates and incubated for 1 hour at 37°C. Plates were washed three times with PBST and 100 μL/ well of 1: 100,000 dilutions of HRP (horse radish peroxidase) conjugated goat anti-chicken IgY secondary antibody (Cat. No. A16054, Invitrogen, Waltham, MA, USA) or HRP-conjugated goat anti-chicken IgA (Novus Biologicals, Littleton, CO, USA) in SuperBlock™ Blocking Buffer was added in duplicates and incubated for 30 minutes at room temperature.

The plates were washed three times with PBS-Tween 20, and 100 μL/ well of TMB peroxidase substrate (3,3,5,5-tetramethylbenzidine) solution (eBioscience, San Diego, CA, USA) was added. The reaction was stopped after 10 min by adding 100 μL/ well of 1 mol/L hydrochloric acid. An ELISA plate reader (BioTek, VT, USA) was used for measuring the OD values at 450 nm. IgY and IgA antibody levels were reported as the mean optical density (OD 450).

Table 1
The sequence and annealing temperatures of the real- time PCR primers for cytokine gene expression analysis.

Gene	Primer sequence (5′ - 3′)	Annealing temperature	References
Ribosomal protein S-13 (RPS-13)	F- CAAGAAGGCTGTTGCTGTICG R- GGCAGAAGCTGTCGATGATT	55.5°C	(Hutsko et al., 2016)
Interleukin-1β (IL-1β)	F- CTACACCCGCTCACAGTCCT R- TCACTTTCTGGCTGGAGGAG	57.4°C	(Shanmugasundaram et al., 2013)
Interferon- γ (IFN-γ)	F-GTGAAGAAGGTGAAAGATATCATGGA R- GCTTTGCGCTGGATTCICA	57°C	(Shanmugasundaram et al., 2013)
Interleukin-10 (IL-10)	F- CATGCTGCTGGGCCTGAA R- CGTCTCCTTGATCTGCTTGATG	57.5°C	(Shanmugasundaram et al., 2013)
Interleukin-4 (IL-4)	F- AACATGCGTCAGCTCCTGAAT R- TCTGCTAGGAACTTCTCCATTGAA	60°C	(Renu et al., 2018)

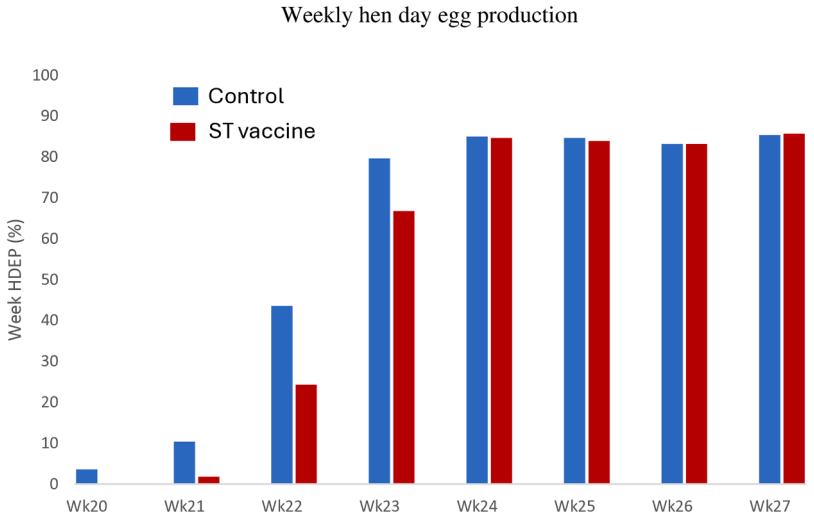

Fig. 1. Weekly hen day egg production of vaccinated and control birds from week 20 to 27. Layer birds were randomly assigned to two groups, namely unvaccinated control and vaccinated groups. Egg production was measured daily, and at 20, 21, 22, 23, 24, 25, 26, and 27wk of age, weekly HDEP was calculated.

Effect of ST vaccine on heterologous Salmonella Enteritidis load in the ceca, liver, spleen and ovary of vaccinated birds

At 28 weeks of age (one-week post-challenge), ceca, spleen, liver, and ovary from 8 chickens (n = 8) per treatment were collected. Briefly, for the quantification and identification of *Salmonella,* ceca, liver, spleen, and ovary samples were aseptically collected into a stomacher bag, weighed and 50 mL of Tetrathionate broth was added and homogenized for 1 minute. The samples were then incubated at 42°C for 24h. To aid in the selection of the antimicrobial-resistant challenge organisms, a loop full of incubated sample was struck to xylose lysine tergitol-4 (XT-4) plates with 25 μg of nalidixic acid/mL. Plates were then incubated for 24 hours at 37°C. Poly-O *Salmonella*-specific antiserum was used for verifying and serogrouping suspected *Salmonella* colonies (Dorea et al., 2010). A three-tube MPN technique was employed for enumerating *Salmonella* in the samples as described earlier (Price et al., 2020). *Salmonella* enumeration data were recorded as MPN/g and were log-transformed for statistical analysis.

Effect of ST vaccine on heterologous ex-vivo recall- response of splenic PBMCs of vaccinated birds

On week 28, spleen samples were aseptically collected from 8 birds per treatment. For the ex vivo lymphocyte proliferation assay, single-cell suspension of PBMCs were obtained as described previously (Renu et al., 2020). In brief, mononuclear cells were obtained by passing the whole spleen over a 40 μm cell strainer (Sigma Aldrich, St. Louis, MO) with 5 mL of RPMI. Density centrifugation was done using Ficoll-pague plus solution (Fisher Scientific, MA, United States) and further separation of the splenic lymphocytes in the interface. The cells were then washed by centrifugation. Cells were resuspended in 100 μL of RPMI-1640 ((Sigma Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum, 1% Penicillin and Streptomycin to a cell density of approximately 5×10^5 mononuclear cells per mL. Cells were plated in 96-well flat bottom plates in triplicates per sample in 100 μL volume. The cells were then stimulated with 10μg/mL of *Salmonella* Typhimurium antigen in 100 μL complete RPMI and the plates were incubated for 72 hrs at 37°C in a 5% CO_2 incubator. As a positive control, 20μg/mL of concanavalin A (Con A) was used. After 72 hrs of incubation, MTT ([3-(4,5-di methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to measure the proliferation of lymphocytes as earlier described (Zhao et al., 2012). The optical density (OD_{570}) was measured using a spectrometer at 570 nm.

Effect of ST vaccine on spleen and cecal tonsil CD4+:CD8+ cell ratio of vaccinated birds

The percentage of CD4+and CD8+ cells were analyzed by flow cytometry as described previously (Renu et al., 2018). In brief, cecal tonsil and spleen samples were collected from 8 birds per treatment on week 28. Single-cell suspensions of the cecal tonsil and spleen were prepared and lymphocytes isolated from the respective organs by density centrifugation using Ficoll-pague plus solution (Fisher Scientific, MA, United States). Cells (1×10^6) were then incubated in a 96-well plate at 1: 200 dilution with PE-conjugated mouse anti-chicken CD4, 1: 200 dilution of FITC-conjugated mouse anti-chicken CD8 (Southern Biotech, Birmingham AL, USA), or 1: 500 dilution of unlabeled mouse IgG for 20 minutes. Cells were washed twice after incubation, to remove

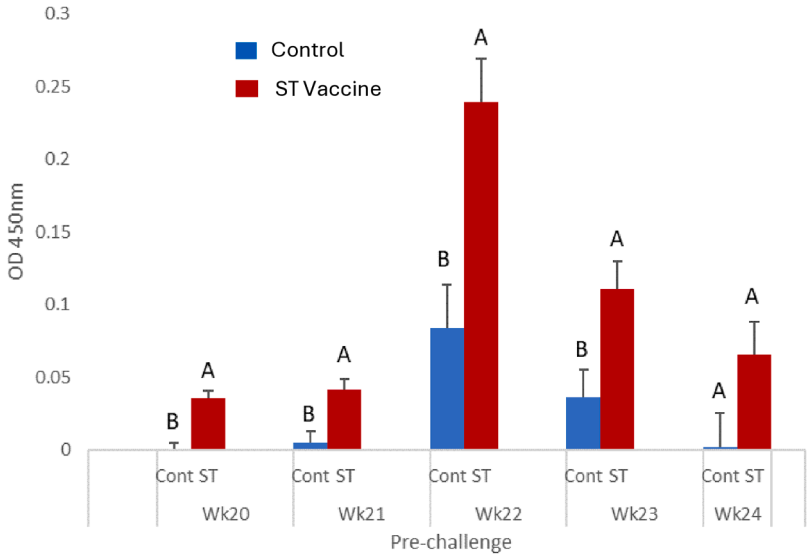

Fig. 2. Effect of ST vaccine on serum IgY anti-*Salmonella* antibodies of vaccinated birds. Layer birds were randomly assigned to two groups, namely unvaccinated control and vaccinated groups. Blood was collected pre-challenge on week 20, 21, 22, 23 and 24 and analyzed for anti-*Salmonella* IgY levels in the serum by ELISA. Results were reported as average optical density (OD) values. Means with no common superscript differ significantly ($P < 0.05$). n = 6. P values: wk20, P=0.001; wk21, P=0.008; wk22, P=0.004; wk23, P=0.017; wk24, P=0.08.

Table 2
Salmonella Enteritidis prevalence in ceca samples in control and vaccinated layer birds one week after challenge with SE at 28 weeks of age.

Treatment	Number of samples	Number of positive (%)	Average MPN value [log10 MPN/g (SE)]
Control	8	6(75)	1.59[a]
ST Vaccine	8	8(100)	2.24[a]
P-value	P>0.05		

Abbreviations: ST, *Salmonella* Typhimurium
At 27 weeks, birds were challenged with 5 × 10^8 CFU/mL of SE. On week 28, ceca, liver, spleen and ovary samples were collected, and *Salmonella* enumeration was done using MPN method. Data was recorded as MPN/g and log-transformed for statistical analysis. Mean values with no common superscript differ significantly.

Table 3
Salmonella Enteritidis prevalence in liver and spleen samples in control and vaccinated layer birds one week after challenge with SE at 28 weeks of age.

Treatment	Number of samples	Number of positive (%)	Average MPN value [log10 MPN/g (SE)]
Control	8	5(62.5)	0.05[a]
ST Vaccine	8	6(75)	0.02[a]
P-value	P>0.05		

Abbreviations: ST, *Salmonella* Typhimurium
At 27 weeks, birds were challenged with 5 × 10^8 CFU/mL of SE. On week 28, ceca, liver, spleen and ovary samples were collected, and *Salmonella* enumeration was done using MPN method. Data was recorded as MPN/g and log-transformed for statistical analysis. Mean values with no common superscript differ significantly.

unbound antibodies, by centrifugation at 400 × g for 5 minutes. CD4+ and CD8+ cell percentages were analyzed based on forward scatter and side scatter plots using CytoSoft software (Guava Easycyte, Millipore, Billerica, MA). The result was reported as gated cell percentage and CD4+/CD8+ cell ratio was expressed.

Effect of ST vaccine on cytokine gene expression in the cecal tonsils of vaccinated birds

At week 28 of age, 8 birds/treatment was selected, euthanized and one cecal tonsil was collected in 5 mL tubes filled with 3 mL of RNA later (Qiagen, Germantown, MD). After seven days of storage at 4°C to allow the samples to be permeated with RNA later, followed by removal of excess RNA later, the samples were kept at -80°C until processing. The total RNA from cecal tonsil samples was extracted using TRIzol reagent (Invitrogen, CA). After dissolving the extracted RNA in Tris- EDTA Buffer (pH 7.5), the purity of RNA was assessed by using NanoDrop™ 2000c Spectrophotometer (Thermo Fisher Scientific). The extracted RNA was reverse transcribed into cDNA as described previously (Shanmugasundaram et al., 2021). The relative expression of IL-10, IL-1β, IL-4, IFNγ mRNA transcripts were analyzed by real-time PCR using iQ™ SYBR® Green Supermix (Bio-Rad, CA, USA), after normalizing for the housekeeping gene β-actin mRNA. Relative mRNA expression was reported as fold change from the reference calculated using $2^{(Ct\ Sample\text{-}Housekeeping)}/2^{(Ct\ Reference\text{-}Housekeeping)}$, where Ct is the threshold cycle (Schmittgen et al., 2008). The primers used for analysis are listed in Table 1.

Statistical analysis

A parametric Student T-test was used to determine the statistical difference among groups. The non-parametric MPN data was Log10 transformed before statistical analysis. The results were considered statistically significant at $P < 0.05$. All statistical analyses were performed using JMP Pro 16 software (JMP Statistical Discovery LLC, Cary, NC).

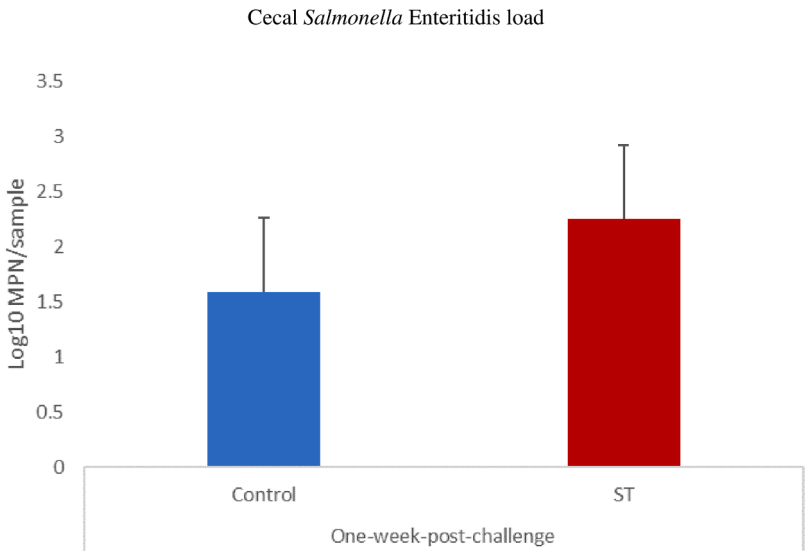

Fig. 3. Effect of ST vaccine on *Salmonella* Enteritidis load in the ceca of vaccinated birds. Layer birds were randomly assigned to two groups, namely unvaccinated control and vaccinated groups. Birds were challenged with 5×10^8 CFU/mL of SE at 27 wk of age. On wk 28, ceca, liver, spleen and ovary samples were collected, and *Salmonella* enumeration was done using Most Probable Number (MPN) method. Data was recorded as MPN/g and were log-transformed for statistical analysis. Means with no common superscript differ significantly ($P < 0.05$). n = 8.

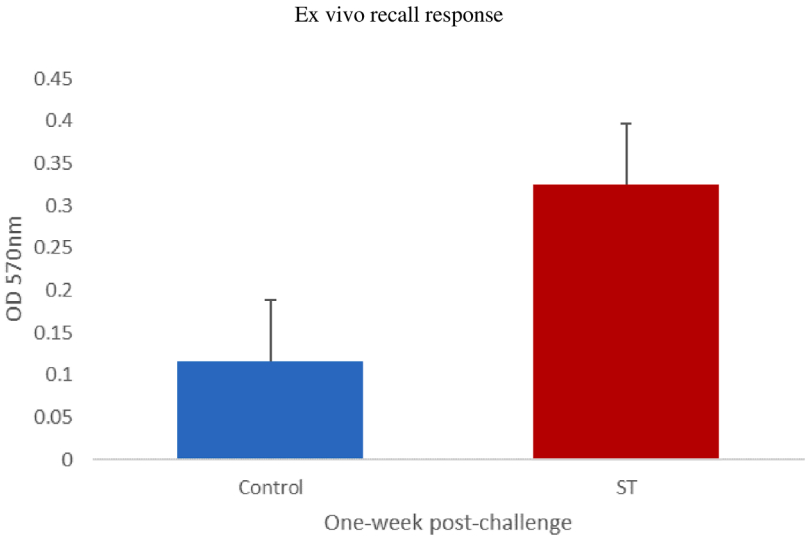

Fig. 4. Effect of ST vaccine on *ex-vivo* recall- response of splenic mononuclear cells of vaccinated birds. Layer birds were randomly assigned to two groups, namely unvaccinated control and vaccinated groups. Birds were challenged with 5×10^8 CFU/mL of SE at 27 wk of age. On wk 28, single cell suspension of spleen was prepared, and cells were stimulated with 10μg/mL of *Salmonella* Typhimurium antigen for 72h. As a negative control splenocytes were stimulated with 0.0 μg/mL of antigen. As a positive control, 20μg/mL of concanavalin A (Con A) was used. MTT assay was used to measure the proliferation of lymphocytes and absorbance was measured at OD570 nm. Results were reported as average optical density (OD_{570}) values. Means with no common superscript differ significantly ($P < 0.05$). n = 8.

Results

Egg production of vaccinated birds

The egg production of birds in the treatment groups from week 20 to 27 is presented in Fig. 1. There was no difference in egg production between the unvaccinated control and vaccinated birds.

Effect of ST vaccine on serum IgY anti-Salmonella antibodies of vaccinated birds

At 21 weeks of age, the birds vaccinated with the ST vaccine had 708% greater levels of anti-*Salmonella* IgY in serum, compared to the control ($P < 0.05$). There was a significant effect ($P < 0.01$) of ST vaccination on serum anti-*Salmonella* IgY antibodies when compared to the control group at 21 weeks of age. At week 22 of age, the vaccinated birds had significantly ($P < 0.01$) increased anti-*Salmonella* IgY serum titers than the control group by 186%. On week 23, birds vaccinated with ST vaccine had 202% greater levels ($P = 0.01$) of serum anti-*Salmonella* IgY, compared to control group. At week 24, both treatment groups had no significant differences in ($P > 0.05$) serum anti-*Salmonella* IgY antibodies (Fig. 2).

Effect of ST vaccine on salmonella enteritidis load in the ceca, liver, spleen and ovary of vaccinated birds

On week 28 (one-week post-challenge), there was no difference in the prevalence of heterologous *Salmonella* Enteritidis load in ceca, liver,

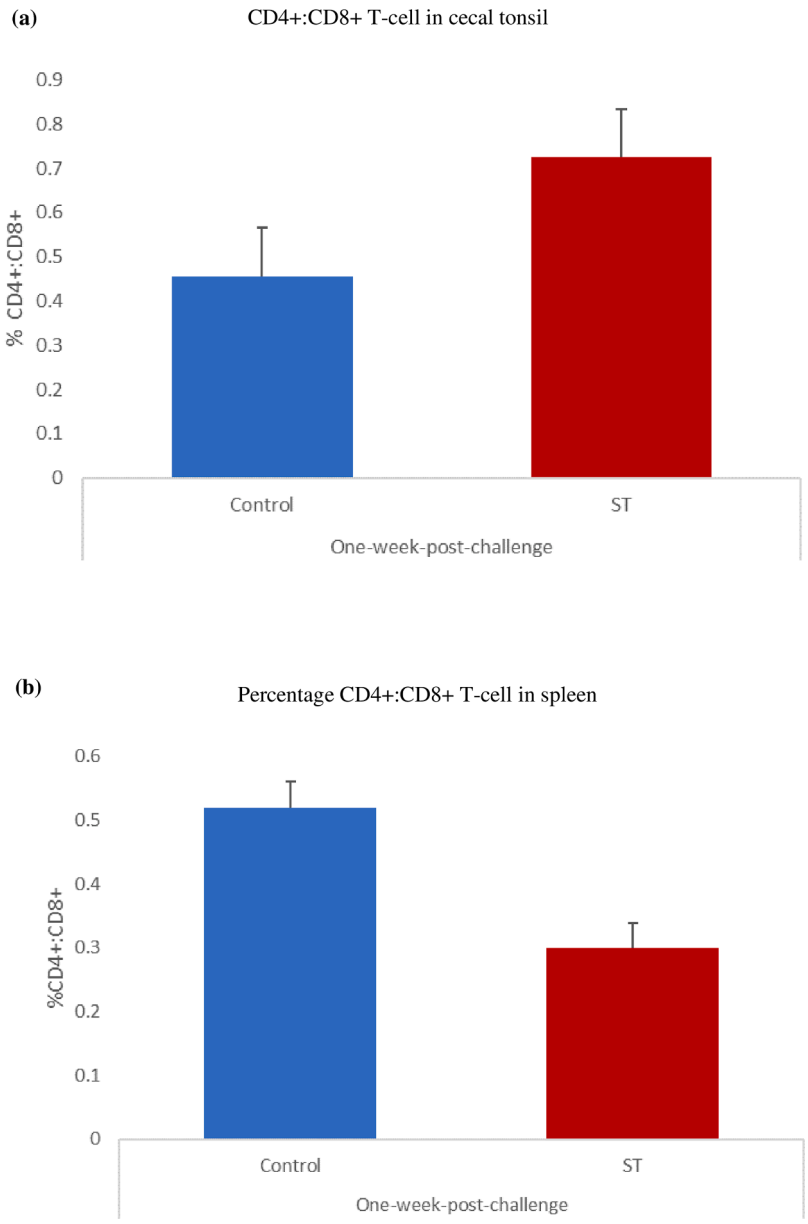

Fig. 5. Effect of ST vaccine on cecal tonsil (5a) and spleen (5b) CD4+:CD8+ T cell ratios of vaccinated birds. Layer birds were randomly assigned to two groups, namely unvaccinated control and vaccinated groups. Birds were challenged with 5×10^8 CFU/mL of SE at 27 wk of age. At 1wk post-challenge on week 28, CD4+:CD8+ T cell percentage in the cecal tonsil and spleen were analyzed by flow cytometry (Guava Eascyte; Millipore). The result was reported as gated cell percentage and CD4+/CD8+ cell ratio was expressed. Means with no common superscript differ significantly ($P < 0.05$). n = 8.

and spleen among the treatment groups ($P > 0.05$). Serogroup D was found to be present in all *Salmonella* isolates recovered from all organs, which was in line with the SE challenge strain. Table 2 and Table 3 show the prevalence of *Salmonella* in ceca and spleen/liver samples, respectively. The mean of SE population in the ceca (Fig. 3) and liver of vaccinated birds was 2.20 and 0.02LogMPN/g, respectively ($P > 0.05$). Statistical analysis of the *Salmonella* population in the ovary was not done as the MPN average was below the detectable limit in all layer birds (Hofacre et al., 2018).

Effect of ST vaccine on ex-vivo recall- response of splenic mononuclear cells of vaccinated birds

At 10μg/mL *Salmonella* antigen stimulation concentration, vaccinated birds had no significant difference in the splenic mononuclear cell proliferation compared to the control birds, on week 28 ($P = 0.06$, Fig. 4).

Effect of ST vaccine on cecal tonsil and spleen percentage CD4+:CD8+ cell ratio of vaccinated birds

There were no significant differences in the CD4+:CD8+ T cells ratio in the cecal tonsil (Fig. 5a) and spleen (Fig. 5b) of vaccinated birds at 28 weeks, compared to the control.

Effect of ST vaccine on serum IgY and bile IgA anti-Salmonella antibodies of vaccinated birds post-Salmonella challenge

There were significant differences between the two treatment groups on serum anti-*Salmonella* IgY ($P = 0.01$) on week 28 (one-week post-challenge) (Fig. 6a). Birds immunized with the ST vaccine had 240% higher levels ($P = 0.01$) of serum anti-*Salmonella* IgY when compared to

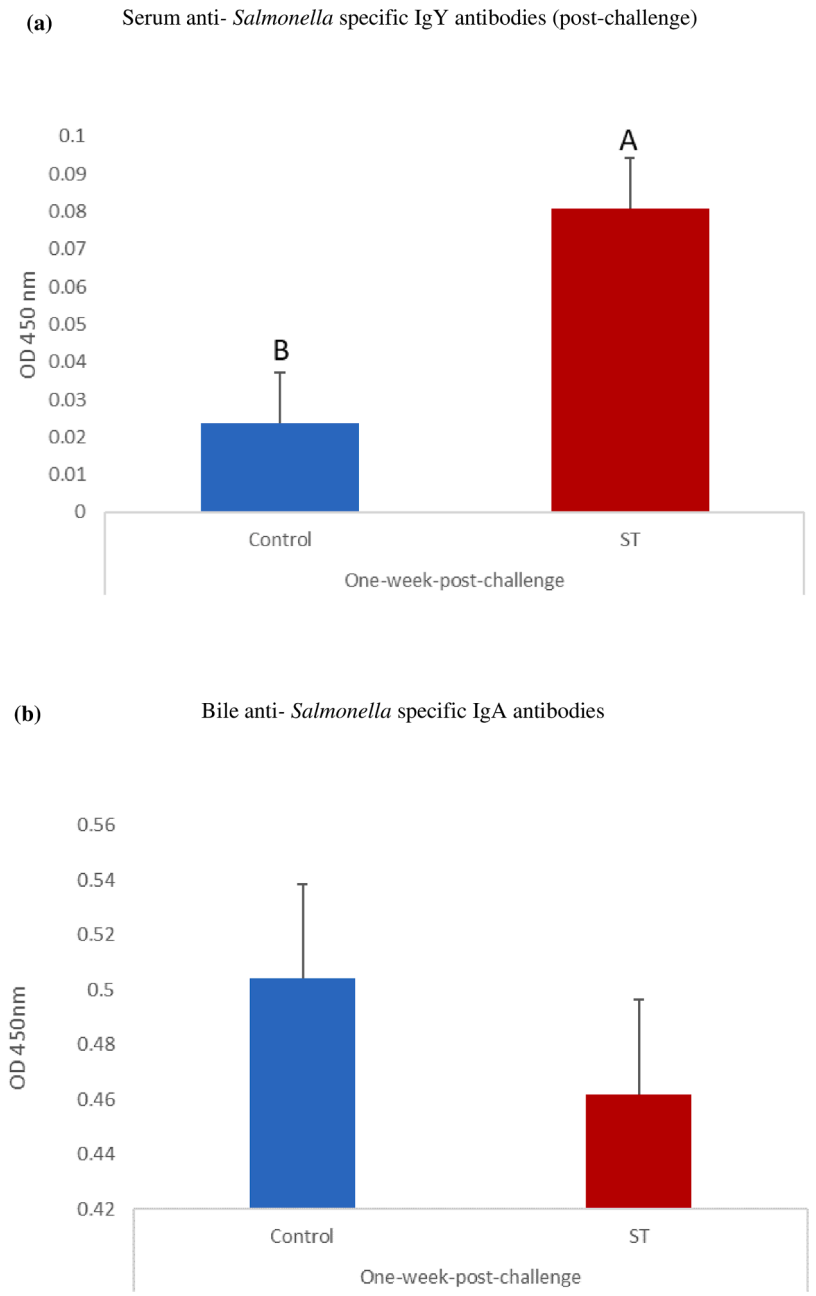

Fig. 6. Serum (6a) and bile (6b) anti- *Salmonella* IgY/IgA of layer birds vaccinated with ST vaccine. Layer birds were randomly assigned to two groups, namely unvaccinated control and vaccinated groups. Birds were challenged with 5×10^8 CFU/mL of SE at 27 wk of age. Blood and bile samples were collected 1wk post challenge and analyzed for anti-*Salmonella* antigen-specific IgY and IgA levels by ELISA. Results were reported as average optical density (OD) values. A- serum anti-*Salmonella* IgY, B- bile anti-*Salmonella* IgA. Means with no common superscript differ significantly ($P < 0.05$). n = 8.

control on week 28. There was no significant difference ($P > 0.05$) in bile anti-*Salmonella*-IgA antibody titers at one-week post-challenge, on week 28 ((Fig. 6b). However, the vaccinated birds have 8.3% lower levels of bile anti-*Salmonella* IgA ($P > 0.05$).

Effect of ST vaccine on cytokine gene expression in the cecal tonsils of vaccinated birds

On week 28, the relative gene expression of cytokines such as IL-10, IL-4, IL-1β and IFNγ in the vaccinated treatment groups was not significantly different from that in the control unvaccinated group ($P > 0.05$, Fig. 7).

Discussion

In this study, we investigated the efficacy of a killed *Salmonella* Typhimurium bacterin vaccine (ST) in layer birds. All birds were vaccinated with the ST vaccine by intramuscular route at 17 weeks of age and were given a booster vaccination at 19 weeks by the same route. This vaccine interval was executed to consider the start of the laying period of birds and to lessen the incidence of horizontal and vertical transmission (Jackson et al., 2006). The vaccine in the study was safe, as we observed no mortality or morbidity in the vaccinated birds and the absence of local reactions at the vaccination site. In this study, we observed no adverse effects of the vaccine on egg production, as the weekly HDEP was similar in both treatment groups from week 23 to week 27 (Crouch et al., 2020). The absence of negative effects on egg

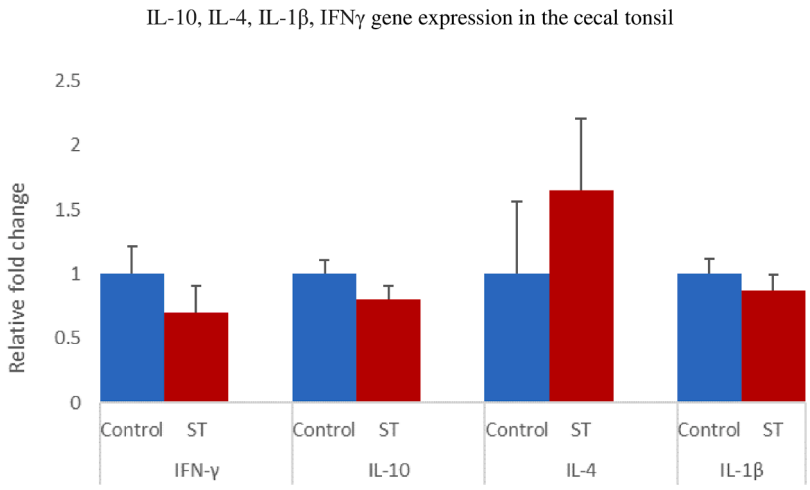

Fig. 7. IL-10, IL-4, IL-1β, IFNγ gene expression in the cecal tonsil of vaccinated birds. Layer birds were randomly assigned to two groups, namely unvaccinated control and vaccinated groups. Birds were challenged with 5×10^8 CFU/mL of SE at 27 wk of age. Cecal tonsils were collected at 1wk post-challenge and anlyzed for IL-10, IL-4, IL-1β, IFNγ mRNA after normalizing for β-actin mRNA by RT-PCR. Results were expressed as fold change from the control. Means with no common superscript differ significantly ($P < 0.05$). n = 8.

production and mortality throughout the study period indicate that the intramuscular administration of the ST vaccine was safe.

In this study, we quantified the *Salmonella*-specific IgY antibody levels in serum samples from vaccinated birds before the experimental challenge. The two doses of the vaccine induced antigen-specific humoral response against *Salmonella*. Results agree with other studies that show that inactivated vaccines elevated antigen-specific serum IgY (Okamura et al., 2007). We observed significantly higher serum antibody responses to the vaccine in vaccinated birds from week 20 (one--week post-booster dose) to week 23. This level was not significantly higher than that of unvaccinated control birds at week 24. The decrease in antibody response with two doses of vaccine was consistent with previous studies that showed a third IM inoculation of the vaccine enhanced the immune response (McWhorter et al., 2018).

Salmonella can invade and colonize poultry digestive tracts (Shaji et al., 2023). After the heterologous challenge of birds with SE on week 27, we did not observe any signs of systemic infection or mortality in the birds. The findings in this study observed no significant differences in the SE load in ceca, liver, or spleen. The ovary samples did not exhibit any SE colonization. These findings could be due to the short duration (one-week) of the study (Price et al., 2020). Our findings indicate that the heterologous SE challenge strain crossed the intestine and invaded the spleen and liver. The highly positive ceca samples may be explained by the fact that this challenge dose was high and might not accurately represent the load in typical field challenges (Baxer, 2015). The killed vaccine used in this study was anticipated to confer protection against other *Salmonella* serovars.

In this study, we looked at the level of antigen-specific IgA in bile samples collected one week after challenging the birds. The bile IgA levels in the vaccinated birds were not significantly higher than in the control birds. Our findings showed 8.3% higher levels of bile anti-*Salmonella* IgA in unvaccinated control birds one-week post-*Salmonella* inoculation. This is probably because, at one-week post-SE infection, the vaccinated groups still had SE infection, whereas the unvaccinated group had started recovering from the infection by then (Luoma et al., 2017). This is consistent with the significant increase in cecal *Salmonella* colonization in the vaccinated group obtained in this study. The study identified that the ST vaccine elevated serum anti-*Salmonella* IgY levels one week after the SE challenge. This was consistent with previous studies that showed an increase in the humoral immune response in layer birds (Deguchi et al., 2009).

We measured the cell-mediated immune response of vaccinated birds against *Salmonella* by quantifying the antigen-recall response using an antigen-specific lymphocyte proliferation assay (Giambrone et al., 1980). The development of protective immunity brought on by vaccination also depends on cell-mediated immune (CMI) responses and, consequently, immunological memory for the antigen (Janeway et al., 2001). As per previous studies, killed vaccines induce a weak cell-mediated immune response (Valero-Pacheco et al., 2020). However, our results showed that ST-vaccinated birds had an increased, but not statistically significant ($P = 0.06$) antigen-specific proliferation after being re-exposed to *Salmonella* antigen (Acevedo-Villanueva et al., 2022). Hence, ST vaccination can trigger a strong cellular immunological response, as evidenced by its ability to trigger a notable proliferation of antigen-specific lymphocytes against ST antigens.

In our current study, we did not find any statistically significant differences in the CD4+:CD8+ T cell ratio in ST-vaccinated birds compared to the control ($P > 0.05$). We investigated the impact of the ST vaccination on important immune-related cytokines that are biologically relevant to infections caused by *Salmonella* in layers. We investigated the induction of both pro- and anti-inflammatory cytokine responses. The vaccine in the study was expected to elicit a stronger Th1 immune response and induce significant levels of pro-inflammatory cytokines (IFNγ). There were no significant differences for cytokine mRNA expression. ST vaccination had no significant effects on the anti-inflammatory cytokine IL-10 mRNA expression levels in the cecal tonsils of birds. However, in contrast to the ST-vaccinated birds, the cecal tonsils of the control group showed an increase in both IFNγ and IL-10 mRNA expression (Penaloza et al., 2018). The higher levels of IL-10 may act to balance the proinflammatory cytokine produced and thereby lessen the inflammation and host tissue damage brought on by *Salmonella* infections (Shanmugasundaram et al., 2015). Overall, it can be concluded that the vaccination has no negative consequences on the bird's production characteristics.

Conclusion

We conclude from this study that vaccination of layer birds with the killed *Salmonella enterica* ser. Typhimurium bacterin (ST) vaccine significantly increased *Salmonella*–specific antibody titers. However, the vaccine used in this study did not offer resistance to the cecal colonization by the heterologous SE challenge strain. However, modifying the vaccine dose or composition might confer protection against heterologous serovars, which warrants further research.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Ramesh K Selvaraj reports financial support was provided by US Department of Agriculture. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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