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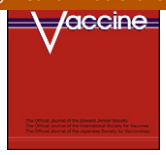

Humoral and cellular immune response generated by different vaccine programs before and after *Salmonella* Enteritidis challenge in chickens

Rafael Antonio Casarin Penha Filho[a,*], Bruna Silva Moura[a], Adriana Maria de Almeida[a], Hélio José Montassier[a], Paul A. Barrow[b], Angelo Berchieri Junior[a]

[a] School of Agricultural and Veterinary Sciences – São Paulo State University, Jaboticabal 14884-900, SP, Brazil
[b] School of Veterinary Medicine and Science – University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, UK

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ABSTRACT

The poultry industry has a high demand for *Salmonella* vaccines in order to generate safer *Salmonella*-free food for consumers around the world. Vaccination against *S.* Enteritidis (SE) is vastly undertaken in many countries, although the criteria for the use of live vaccine (LV) or killed vaccine (KV) should also depend on the immune mechanisms triggered by each. In this study, a commercial bacterin (KV) and an attenuated SG mutant (LV) were used in four different vaccine programs (LV; LV + LV; KV; LV + KV). At 1 day before (dbi) and 1, 6 and 9 days after SE challenge (dpi), humoral (IgM, IgG and secretory IgA) and cellular (CD8$^+$ T cells) immune responses were evaluated along with the production of IL-10, IL-12 and IFN-γ. Although after challenge, all birds from each group had an influx of CD8$^+$ T cells, birds which received KV had lower levels of these cells in organs and significantly higher levels of immunoglobulins. The expression of the cytokines was up-regulated in all groups post-vaccination, although, after challenge, cytokine expression decreased in the vaccinated groups, and increased in the unvaccinated group A. IL-10 levels were significantly higher at 1 day post-infection in the group that received KV, which may be involved in the weak cellular immune response observed within this group. In caecal tonsils, IFN-γ expression at 1 dbi was higher in birds which received two vaccine doses, and after challenge, the population of CD8$^+$ T cells constantly increased in birds that were only vaccinated with the LV. This study demonstrated that the development of a mature immune response by CD8$^+$ T cells, provided by the use of the LV, had better efficacy in comparison to the high antibody levels in the serum stimulated by the KV. However, high secretory IgA levels in the intestinal lumen associated with influx CD8$^+$ T cells may be indicative of protection as noticed in group E (LV + KV).

1. Introduction

Salmonella enterica subsp. *enterica* serovar Enteritidis (SE) is a pandemic pathogen, present in countries with industrial poultry production since the 1990s [1]. Each year, millions of foodborne salmonellosis cases occur worldwide, resulting in an estimated 155,000 deaths [2]. Poultry meat and eggs are largely implicated in SE foodborne infections [3], and the use of vaccine programs has shown great application for SE control in poultry flocks [4,5].

Salmonella vaccines can act by distinct mechanisms. Killed vaccines are vastly adopted in many countries, for vaccination of commercial table-egg layers. Most of these vaccines contain SE antigens and adjuvants, and stimulate an enhanced humoral immune response, with variable levels of protection [6,7]. Otherwise, live vaccines containing attenuated *Salmonella* strains stimulate cell mediated immunity (CMI), not necessarily producing high antibody titers [8]. Due to the low risk of human infection and the host-specificity, attenuated strains of *Salmonella enterica* subsp. *enterica* serovar Gallinarum biovar Gallinarum (SG) have been extensively used as live vaccines against salmonellosis in chickens [9–12].

The immune response against these intracellular bacteria involves many factors, including detection of pathogen associated molecular patterns (PAMPs) by the host pattern recognition receptors (PRRs), e.g. Toll-like receptors (TLRs), and signaling through production of cytokines, which have an important role in modulating the nature of the immune response [13]. Pro-inflammatory cytokines trigger the innate immune response, and its chemoattractant activity recruits phagocytic monocytes, natural killer cells, macrophages and heterophils, important cells for the primary immune response against SE [14–17]. Although the innate immune

* Corresponding author at: Department of Veterinary Pathology, School of Agricultural and Veterinary Sciences, São Paulo State University (FCAV-Unesp), Via de Acesso Prof. Paulo Donato Castellane s/n, Jaboticabal 14884-900, SP, Brazil.
Tel.: +55 16 3209 2662; fax: +55 16 3209 2600.
E-mail address: rafaelpenha12@yahoo.com.br (R.A.C. Penha Filho).

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response has proven to be important in preventing colonization by SE, the acquired immunity can provide a faster and more specific immune response to this pathogen [18].

$CD8^+$ T cells can recognize and destroy infected cells. Antigenic stimulation of naïve $CD8^+$ T cells, by antigen presenting cells (APCs) can lead to the development of two lines; memory $CD8^+$ T lymphocytes and effector $CD8^+$ cytotoxic T lymphocytes (CTLs). The search for live bacterial vaccines that stimulate $CD8^+$ T cell response has been studied previously [9–21]. Differentiation to CTLs is dependent mainly upon the production of IL-12 [22]. Nonetheless, IL-12 induces the production of Interferon-γ (IFN-γ), an essential cytokine for protective immunity against primary infection with *Salmonella* [23]. IL-10 is a regulatory cytokine that causes down-regulation of inflammatory responses and deactivates macrophages [24]. IL-10 has a negative influence on IFN-γ expression by T helper 1 (Th1) cells and promotes proliferation of Th2 cells and antibodies [25,26].

The investigation of antibodies for protection against *Salmonella* has presented conflicting results. In different studies, high titers of serum IgG could not be associated with reduction of intestinal SE burden after an experimental challenge [27,28]. Otherwise, in field experiments, lower *Salmonella* prevalence in vaccinated flocks was associated with high antibody titers [5,29]. IgA has an important role in local role in local immunity. This isotype is secreted in mucosal surfaces and helps to prevent is secreted in mucosal superficies, helping to prevent bacterial colonization in the intestinal lumen [30]. Additionally, IgA can be transferred to the offspring by passive immunity, protecting newly hatched chicks [31].

Immunity to salmonellosis has been studied and summarized [18,32], however it is important to study the acquired immunity generated by vaccine programs, applicable in the fields. In the present work, a commercial bacterin and a novel vaccine candidate (attenuated SG) were used in four different combinations to investigate the efficacy to control SE challenge and the effector mechanisms triggered, such the influx of $CD8^+$ T cells, antibodies and the expression of regulatory cytokines.

2. Materials and methods

2.1. Experimental birds

One hundred and twenty white layer-hens, susceptible to SE infection [9], were obtained at day of hatch and submitted to bacteriological and serological tests in order to check the *Salmonella*-free status. All birds used tested negative for *Salmonella* infection.

Animal experimentation was approved by the Brazilian Committee of Animal Welfare and Ethics (permit number 6236-09). Birds were reared in controlled ambient conditions.

2.2. Bacterial strains and vaccines

A bacterin in oil emulsion containing SE phagotype (PT) 4, PT8 and PT13a antigens, was administered subcutaneously in the nape (0.3 mL/bird) as the killed vaccine (KV). An attenuated mutant SG strain, with deletion on genes *cob*S and *cbi*A, unable to synthesize ciano-cobalamin and immunogenic against SE, was used as the live vaccine strain (LV) [10]. An invasive SE PT4 strain [31] was used to challenge birds. Bacterial cultures were prepared in Luria-Bertani (LB) broth (Invitrogen, USA) at 100 rpm at 37 °C/24 h. The LV and SE challenge inocula consisted of 10^8 CFU in Phosphate Buffer Saline (PBS) pH 7.4 (Merck, Germany), administered orally, into the crop.

2.3. Experimental design

Five groups containing 20 birds each were allocated and vaccination was carried out at 5 and/or 25 days of age, as described in Table 1. At 45 days of age, all birds were challenged. Unvaccinated and unchallenged birds were used as a negative control for cytokine quantification.

Table 1
Vaccine schemes tested against *S.* Enteritdis.

Groups	Number of birds	1st dose (5 days)	2nd dose (25 days)
A	20	Unvaccinated	
B	20	LV	–
C	20	LV	LV
D	20	–	KV
E	20	LV	KV

LV, live vaccine (0.5 mL via oral); KV, killed vaccine (0.3 mL subcutaneous). At 45 days of age, all birds in all groups were challenged with *S.* Enteritidis.

2.4. Sampling and bacteriology

At 1 day before infection (dbi) and 1, 6 and 9 days post-infection (dpi), blood was harvested from five birds in each group, which were then euthanized by cervical dislocation for sampling. After necropsy, the intestinal lumen was washed with 2 mL phenylmethyl sulfonyl fluoride (PMSF) buffer [33] and centrifuged at 2000 rpm, at 4 °C for 30 min, supernatants were then stored at −20 °C. Spleen, liver and caecal tonsil samples were aseptically harvested, snap-frozen in liquid nitrogen and stored at −80 °C for immunohistochemistry or quantitative PCR.

Spleen and caecal contents were used in bacteriology as described previously [34]. SE counts were expressed as $\log_{10}$ per gram of sample. Positive samples after enrichment ($\leq 10^2$ CFU/g), are expressed as 2 ($\log_{10}$ of CFU/g) in calculations.

2.5. Antibodies production

Indirect enzyme-linked immunosorbent assay (ELISA) using SE antigen was applied to quantify IgG (also known as IgY) and IgM in the sera, and secretory IgA in the intestinal lumen (lavage), as described before [35]. The optical density values (OD) were used to calculate the adjusted *E* values using the following formula:

$$E \text{ value} = \frac{\text{OD sample} - \text{OD negative control}}{\text{OD positive control} - \text{OD negative control}}$$

2.6. Immunohistochemistry

Immunohistochemistry was used to determine the influx of $CD8^+$ T cells as described previously [36]. Briefly, frozen tissue sections (8 μm) of liver and caecal tonsil samples were fixed in ice-cold acetone. Sections were incubated overnight at 4 °C with anti-chicken $CD8\alpha^+$ antibody (5 ng/mL, SouthernBiotech, USA). Reaction was developed with Envision-HRP Kit and 3,3′-diaminobenzidine (DAB, Dako, USA). Tissue sections were randomly photographed in light microscope (Eclipse Moticam, Nikon, Japan). The percentage of positively stained areas was analyzed using Image Pro Plus Software (MediaCybernetics, USA).

2.7. RNA isolation and reverse transcription (RT-PCR)

Spleen and caecal tonsil were used for RNA extraction and cDNA synthesis was performed as described previously [36]. The RNA quality was determined using the NanoDrop 1000 (ThermoScientific, USA) and by agarose gel electrophoresis. The cDNA was stored at −20 °C until use.

Table 2
Primer sequences used in the qPCR in real time for cytokine quantification.

Target gene	Oligonucleotides	Annealing	Size (bp)	GenBank ID
18S	F 5′-CATGGCCGTTCTTAGTTGGT-3′ R 5′-GGCGTAGGGTAGACACAAGC-3′	50 °C	232	FM165414
IL-10	F 5′-CGGGAGCTGAGGGTGAA-3′ R 5′-GTGAAGAAGCGGTGACAGC-3′	60 °C	272	AJ621614
IL-12 p40	F 5′-ACCAGCCGACTGAGATGTTC-3′ R 5′-GTGCTCCAGGTCTTGGGATA-3′	54 °C	163	FJ788636.1
IFN-γ [49]	F 5-AGCTGACGGTGGACCTATTATT-3′ R 5′-GGCTTTGCGCTGGATTC-3′	56 °C	259	HQ739082.1

F, forward primer; R, reverse primer.

2.8. Quantitative real time PCR

The real time PCR was carried out in 25 μL reactions using 50 ng of cDNA; 0.5 μM of forward and reverse primers; 12.5 μL of Maxima SYBR Green 2X (ThermoScientific, USA); 0.2 μL Platinum Taq DNA polymerase (Invitrogen, USA) and nuclease free water. Primer sequences and annealing temperatures are detailed in Table 2. The fold change in the mRNA expression of each cytokine encoding gene was calculated in comparison the normative gene 18S and unvaccinated and unchallenged birds, using the $2^{-\Delta\Delta Cp}$ method [37].

2.9. Statistical analysis

The Kruskall–Wallis method was used to analyze the incidence of different values between all groups at each sampling day. The Bonferroni test was further applied to compare differences between groups separately. Values were considered statistically different at $p < 0.05$.

3. Results

3.1. Recovery of SE

The efficacy of each vaccination scheme was first evaluated by bacterial counting of the SE challenge strain in spleen and caecal content (Fig. 1). At 1 dpi, the challenge strain was detected in the spleen samples only after enrichment in groups A, B and E with no differences between groups ($p > 0.05$). At 6 dpi, SE was recovered in spleen from all groups. In group E, the bacterial burden was significantly decreased in comparison with the unvaccinated group A. At 9 dpi, SE numbers in spleen samples were low and not statistically different between groups ($p > 0.05$). After challenge, SE was recovered in high numbers in the caecal contents of the unvaccinated group A. At 1 dpi, all vaccinated groups had lower amounts of the challenge strain in the caecal contents compared to group A ($p < 0.05$). At 6 and 9 dpi the bacterial burden was significantly lower in vaccinated groups B, C and E ($p < 0.05$), whilst in group D, which received only one dose of the KV, SE numbers were not different from the unvaccinated group A.

3.2. Antibody levels in serum

IgM and IgG levels were significantly higher in groups D and E ($p < 0.05$; Fig. 2). In groups A, B and C, IgM and IgG levels were relatively low throughout sampling. Although IgM slightly increased at 9 dpi in groups A and C. IgG levels in groups A (unvaccinated), B and C increased at 6 dpi.

3.3. Secretory IgA in the intestinal lumen

The levels of IgA (Fig. 2) were similar in all groups at 1 dbi. After challenge, groups D and E had increasing levels of IgA until 6 dpi ($p < 0.05$). At 9 dpi, it was still significantly higher in group E than the other groups ($p < 0.05$). Groups B and C demonstrated increasing levels of secretory IgA until 9 dpi, although it did not reach the same levels of groups D and E, whilst in group A levels were low.

3.4. IL-12, IFN-γ and IL-10 expression

The transcript level of IL-12 in spleen and caecal tonsil (Fig. 3) was higher in all vaccinated groups before challenge, when compared to the unvaccinated group ($p < 0.05$). After challenge, the expression of IL-12 was reduced and in caecal tonsils it did not differ between vaccinated groups and unvaccinated group A ($p > 0.05$). IFN-γ levels were significantly augmented in vaccinated groups in comparison to unvaccinated birds, in spleen and caecal tonsils (Fig. 3) before challenge. IFN-γ expression in caecal tonsils was significantly elevated in groups C and E at 1 dbi, and at 6 dpi in group E, in comparison with the other groups ($p < 0.05$). IL-10 was highly expressed in spleen samples of all vaccinated groups in comparison with group A at 1 dbi ($p < 0.05$). At 1 dpi, the expression of this cytokine in spleen decreased in all groups, except in group D. In caecal tonsils, IL-10 levels were higher in groups C and E before challenge, and a peak was seen at 6 dpi in group E (Fig. 3).

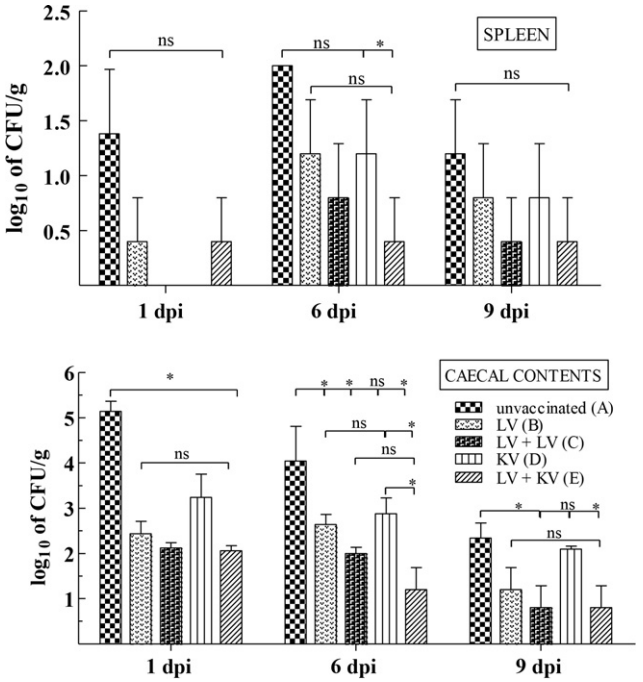

Fig. 1. *S.* Enteritidis count in spleen and caecal tonsil samples after challenge in unvaccinated (group A) and vaccinated chickens (groups B, C, D and E). Numbers of the challenge strain are expressed in $\log_{10}$ of CFU per gram of sample. For samples that were positive after enrichment, the value 2 was used in the calculations. Data represent the means ± SD. *, $p < 0.05$; ns, not significative.

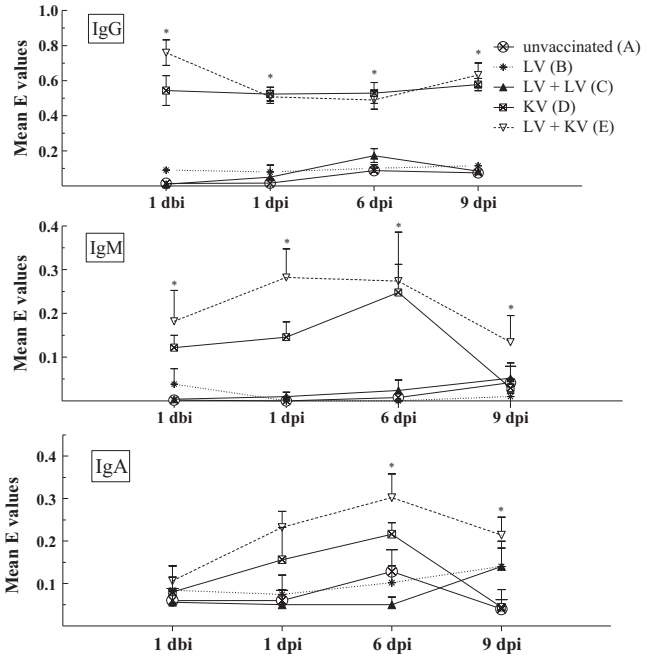

Fig. 2. Levels of IgG, IgM (serum) and IgA (intestinal lumen) in unvaccinated (group A) and vaccinated chickens (groups B, C, D and E), before (1 dbi) and after challenge (1, 6 and 9 dpi). Data represent the means ± SD. *, $p < 0.05$; ns, not significative.

3.5. Influx of CD8$^+$ T cells

The recruitment of CD8$^+$ T cells in liver and caecal tonsils, evaluated by immunohistochemistry, is displayed in Fig. 4. Before the challenge, at 1 dbi, all groups had low levels of CD8$^+$ T cells in caecal tonsil. At 1 dpi, the influx of CD8$^+$ T cells started to increase in all groups, including the unvaccinated group A. At 6 dpi, cell influx was significantly higher in groups A and C, and at 9 dpi, groups B and C showed the highest levels of CD8$^+$ T cells ($p < 0.05$), in caecal tonsil samples however, groups D and E exhibited significantly lower levels of CD8$^+$ T cells, similar to the unvaccinated group A. In liver samples, CD8$^+$ T cells were present at 1 dbi, although, only groups B, C and E were significantly different from the control group A. After challenge, the cell influx in the liver was clearly increased in all groups, and the highest levels were seen in group A; values in group D were constant and had no significant increase during this period. At 6 dpi, the amount of CD8$^+$ T cells was not different between vaccinated groups ($p > 0.05$). However, at 9 dpi, groups B and C showed higher numbers of CD8$^+$ T cells than groups D and E in liver.

4. Discussion

Studies regarding the influence of live and killed vaccines on the immune responses of commercial chickens are important to clarify the specific mechanisms involved. Discussions about the use of *Salmonella* vaccines are always controversial; live vaccines are often questioned about reversion to virulence, whilst killed vaccines are described as weak stimulators of the CMI [18,38]. The present study, and others, demonstrates that bacterins stimulate the humoral response which is ineffective on its own, to control *Salmonella* infection [39]. However, KV can reduce *Salmonella* burden in poultry flocks when used with a biosecurity program [5,40].

Immune responses generated by invasive live vaccines should trigger similar processes as the pathogenic strains. The mutant SG invaded the host organism from the gut and colonized internal organs similarly to the wild strain [10]. Additionally vaccine strains with known genetic deletions (GMO) have reduced risks of reversion to virulence, in comparison with rough strains [41]. After challenge, the LV stimulated the control of SE (Fig. 1). At 9 dpi,

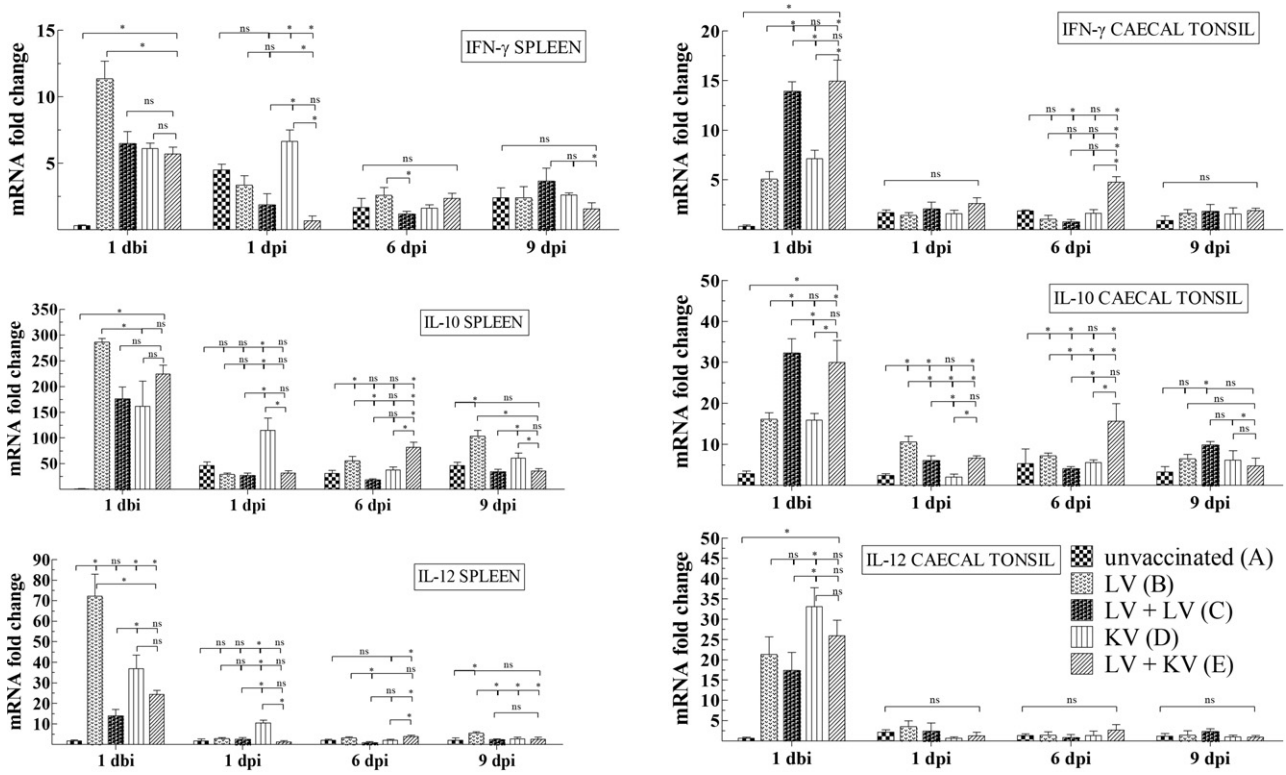

Fig. 3. Relative quantification of IFN-γ, IL-10 and IL-12 expression in spleen and caecal tonsils of unvaccinated (group A) and vaccinated chickens (groups B, C, D and E), before (1 dbi) and after challenge (1, 6 and 9 dpi). Data represent the means ± SD. *, $p < 0.05$; ns, not significative.

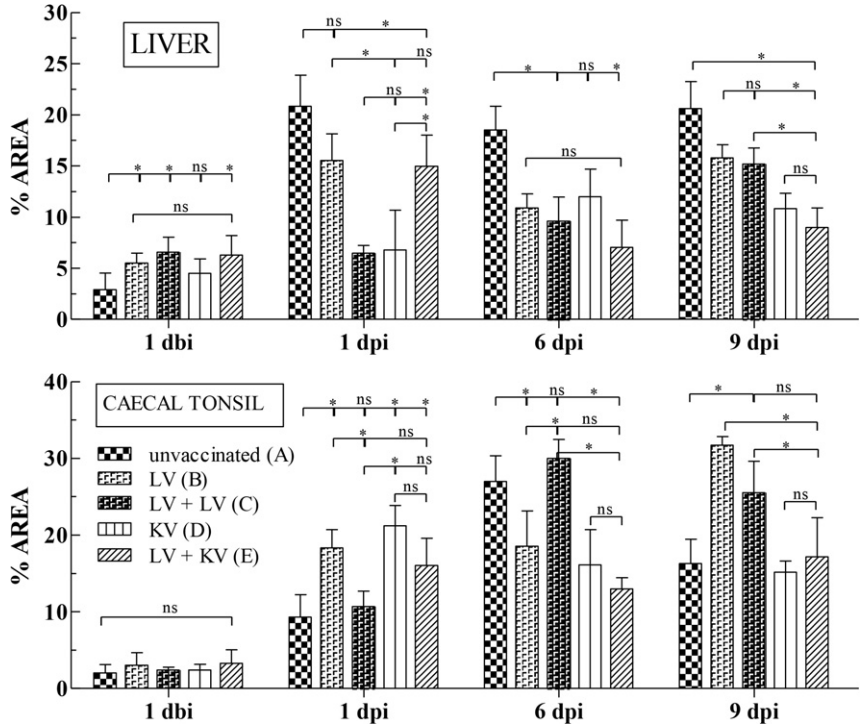

Fig. 4. Immunohistochemistry staining of CD8⁺ T cells in sections of liver and caecal tonsil of unvaccinated (group A) and vaccinated chickens (groups B, C, D and E), before (1 dbi) and after challenge (1, 6 and 9 dpi). Columns represent the mean percentage of stained areas ± SD. *, $p < 0.05$; ns, not significative.

groups B and C, both vaccinated only with LV showed the highest levels of CD8⁺ T cells in caecal tonsils (Fig. 4).

Before challenge, low levels of CD8⁺ T cells were detected (Fig. 4), suggesting that levels of CD8⁺ cells returned to basal levels during the interval between vaccination and challenge, as seen before [36,42]. After challenge, the population of CD8⁺ T cells constantly increased in groups B and C. This may suggest a controlled clonal expansion of memory CD8⁺ cells in these vaccinated birds. Furthermore, high numbers of CD8⁺ T cells persisted for longer periods, in birds that were vaccinated only with the LV (groups B and C). Otherwise, the combination of LV and KV (group E), generated lower levels of CD8⁺ T cells, similarly to the KV (group D), whereas unvaccinated birds had rapid influx of cytotoxic T cells in the liver, possibly attracted by invasive bacteria in this organ.

Birds which received one dose of LV (group B) showed the highest levels of IFN-γ in spleen before challenge. This cytokine is important for macrophage activation [42,43], however after challenge of vaccinated birds, the levels of this cytokine decreased. This may be related to the development of acquired immunity mechanisms, obviously different from the innate immune response that is triggered in unvaccinated birds after primary infection (Fig. 3). Paratyphoid salmonellosis is frequently limited to the gastrointestinal tract; thus the control of bacterial invasion must occur primarily at the intestinal mucosae and gut associated lymphoid tissue (GALT), specifically the caecal tonsils. Considering this, the highest production of IFN-γ in the caecal tonsils was seen in groups C and E (Fig. 4). At 6 dpi, the expression of IFN-γ was significantly higher in group E, which could be associated with the ability of birds in this group to control the first phase of SE infection; colonization and invasion. As shown in Fig. 1, control of SE in caecal contents was clearly faster in groups C and E than in the control groups A and D. The association of IFN-γ production and clearance of primary *Salmonella* infection was suggested previously [35,42,44]. However, in this study, IFN-γ levels decreased after challenge (1 dpi) of vaccinated birds, reaching similar levels to the unvaccinated group A, suggesting that the development of acquired immunity in vaccinated birds is not solely dependant on IFN-γ.

IL-12 has an important role in stimulating the production of IFN-γ, recruiting naïve CD8⁺ T cells and CTLs and developing the CD8⁺ memory cells [45,46]. The present study detected high expression of this cytokine in vaccinated birds before challenge (Fig. 3). At 1 dbi IL-12 levels in caecal tonsils were elevated in all vaccinated groups in comparison with unvaccinated birds (group A). The presence of CD8⁺ T cells combined with low expression of IL-12 in group A (unvaccinated), may lead to a weak response by Ag-specific CD8⁺ T cells and birds may become more susceptible to infection.

LV seems to stimulate the development of the CMI in a controlled manner. The influx of CD8⁺ cells in groups B and C was constantly increasing as SE numbers decreased. Therefore, at 6 and 9 dpi, the bacterial burden was lower in all groups which received at least one dose of the LV, whilst the high immunoglobulin levels could not decrease SE burden in group D. The high levels of IL-10 in spleen samples are indicative of the important role developed in vaccinated animals [25]. After challenge, IL-10 levels decreased in all vaccinated groups which may be an important shift to increase antigen presentation and the pro-inflammatory response.

Considering the effective control of the challenge strain, the bacterial burden was significantly decreased in groups C and E. The combination of LV and KV provides a comprehensive immune response. Even though the SG based LV is more efficacious to stimulate the CMI, the KV contains highly immunogenic proteins, like flagellin, and stimulates high antibody titers. The CMI combined with the higher titer of secretory IgA (Fig. 2) could be associated with the good efficacy of the vaccine program used in group E. B cells and related immunoglobulins can be important for the effective control of *Salmonella* infection [47], as they can present *Salmonella* antigens and generate an effective immune response by CTLs [48].

In summary, this study elucidates aspects of the humoral and cellular immune responses triggered by different vaccine programs using LV and KV, and correlates the control of infection with the

efficacy of each vaccine program. It was shown that using KV, only, does not appear to control high bacterial numbers, despite the high immunoglobulin levels generated. The bacterin showed an impaired ability to elicit $CD8^+$ T cells responses, compared to the LV. However, the combination of LV and KV on the same vaccine program showed greater efficacy, together with the use of two doses of LV, both vaccine programs stimulated a protective immunity against this pathogen. Overall, this study reinforced the importance of vaccination for the effective control of SE infections for poultry production and showed novel alternatives for vaccination that may be useful in the fields.

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References

[1] Rodrigue DC, Tauxe RV, Rowe B. International increase in *Salmonella* enteritidis: a new pandemic? Epidemiol Infect 1990;105(1):21–7.
[2] Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'Brien SJ, et al. The global burden of nontyphoidal *Salmonella* gastroenteritis. Clin Infect Dis 2010;50(6):882–9.
[3] Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, et al. Food-related illness and death in the United States. Emerg Infect Dis 1999;5(5):607–25.
[4] Van Immerseel F, Methner U, Rychlik I, Nagy B, Velge P, Martin G, et al. Vaccination and early protection against non-host-specific *Salmonella* serotypes in poultry: exploitation of innate immunity and microbial activity. Epidemiol Infect 2005;133(6):959–78.
[5] Berghaus RD, Thayer SG, Maurer JJ, Hofacre CL. Effect of vaccinating breeder chickens with a killed *Salmonella* vaccine on *Salmonella* prevalences and loads in breeder and broiler chicken flocks. J Food Prot 2011;74(5):727–34.
[6] Piao Z, Toyota-Hanatani Y, Ohta H, Sasai K, Tani H, Baba E. Effects of *Salmonella enterica* subsp. *enterica* serovar Enteritidis vaccination in layer hens subjected to *S.* Enteritidis challenge and various feed withdrawal regimens. Vet Microbiol 2007;125(1–2):111–9.
[7] de Freitas Neto OC, Mesquita AL, de Paiva JB, Zotesso F, Berchieri Júnior A. Control of *Salmonella enterica* serovar Enteritidis in laying hens by inactivated *Salmonella* Enteritidis vaccines. Braz J Microbiol 2008;39(2):390–6.
[8] Babu U, Dalloul RA, Okamura M, Lillehoj HS, Xie H, Raybourne RB, et al. *Salmonella* enteritidis clearance and immune responses in chickens following *Salmonella* vaccination and challenge. Vet Immunol Immunopathol 2004;101(3–4):251–7.
[9] Penha Filho RA, de Paiva JB, Arguello YM, da Silva MD, Gardin Y, Resende F, et al. Efficacy of several vaccination programmes in commercial layer and broiler breeder hens against experimental challenge with *Salmonella enterica* serovar Enteritidis. Avian Pathol 2009;38(5):367–75.
[10] Penha Filho RA, de Paiva JB, da Silva MD, de Almeida AM, Berchieri A. Control of *Salmonella* Enteritidis and *Salmonella* Gallinarum in birds by using live vaccine candidate containing attenuated *Salmonella* Gallinarum mutant strain. Vaccine 2010;28(16):2853–9.
[11] Matsuda K, Chaudhari AA, Lee JH. Evaluation of safety and protection efficacy on cpxR and lon deleted mutant of *Salmonella* Gallinarum as a live vaccine candidate for fowl typhoid. Vaccine 2011;29(4):668–74.
[12] Matsuda K, Chaudhari AA, Lee JH. Comparison of the safety and efficacy of a new live *Salmonella* Gallinarum vaccine candidate JOL916, with the SG9R vaccine in chickens. Avian Dis 2011;55(3):407–12.
[13] Ferro PJ, Swaggerty CL, Kaiser P, Pevzner IY, Kogut MH. Heterophils isolated from chickens resistant to extra-intestinal *Salmonella* enteritidis infection express higher levels of pro-inflammatory cytokine mRNA following infection than heterophils from susceptible chickens. Epidemiol Infect 2004;132(6):1029–37.
[14] Kogut MH, Rothwell L, Kaiser P. IFN-gamma priming of chicken heterophils upregulates the expression of proinflammatory and Th1 cytokine mRNA following receptor-mediated phagocytosis of *Salmonella enterica* serovar enteritidis. J interferon Cytokine Res 2005;25(2):73–81.
[15] Johansson C, Ingman M, Jo Wick M. Elevated neutrophil, macrophage and dendritic cell numbers characterize immune cell populations in mice chronically infected with *Salmonella*. Microb Pathog 2006;41(2–3):49–58.
[16] Dougan G, John V, Palmer S, Mastroeni P. Immunity to salmonellosis. Immunol Rev 2011;240(1):196–210.
[17] Setta AM, Barrow PA, Kaiser P, Jones MA. 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 2012;35(5):397–410.
[18] Barrow PA. *Salmonella* infections: immune and non-immune protection with vaccines. Avian Pathol 2007;36(1):1–13.
[19] Domenech VE, Panthel K, Meinel KM, Russmann H. Rapid clearance of a recombinant *Salmonella* vaccine carrier prevents enhanced antigen-specific CD8 T-cell responses after oral boost immunizations. Microbes Infect 2005;7(5–6):860–6.
[20] Jones-Carson J, McCollister BD, Clambey ET, Vazquez-Torres A. Systemic CD8 T-cell memory response to a *Salmonella* pathogenicity island 2 effector is restricted to *Salmonella enterica* encountered in the gastrointestinal mucosa. Infect Immun 2007;75(6):2708–16.
[21] Sevil Domenech VE, Panthel K, Winter SE, Russmann H. Heterologous prime-boost immunizations with different *Salmonella* serovars for enhanced antigen-specific CD8 T-cell induction. Vaccine 2008;26(15):1879–86.
[22] Mescher MF, Curtsinger JM, Agarwal P, Casey KA, Gerner M, Hammerbeck CD, et al. Signals required for programming effector and memory development by CD8+ T cells. Immunol Rev 2006;211:81–92.
[23] Jouanguy E, Doffinger R, Dupuis S, Pallier A, Altare F, Casanova JL. IL-12 and IFN-gamma in host defense against mycobacteria and Salmonella in mice and men. Curr Opin Immunol 1999;11(3):346–51.
[24] O'Farrell AM, Liu Y, Moore KW, Mui AL. IL-10 inhibits macrophage activation and proliferation by distinct signaling mechanisms: evidence for Stat3-dependent and -independent pathways. EMBO J 1998;17(4):1006–18.
[25] Rothwell L, Young JR, Zoorob R, Whittaker CA, Hesketh P, Archer A, et al. Cloning and characterization of chicken IL-10 and its role in the immune response to Eimeria maxima. J Immunol 2004;173(4):2675–82.
[26] Xu Q, Katakura Y, Yamashita M, Fang S, Tamura T, Matsumoto SE, et al. IL-10 augments antibody production in in vitro immunized lymphocytes by inducing a Th2-type response and B cell maturation. Biosci Biotechnol Biochem 2004;68(11):2279–84.
[27] Gast RK, Stone HD, Holt PS. Evaluation of the efficacy of oil-emulsion bacterins for reducing fecal shedding of *Salmonella* enteritidis by laying hens. Avian Dis 1993;37(4):1085–91.
[28] Tran TQL, Quessy S, Letellier A, Desrosiers A, Boulianne M. Immune response following vaccination against *Salmonella* Enteritidis using 2 commercial bacterins in laying hens. Can J Vet Res 2010;74(3):185–92.
[29] Feberwee A, de Vries TS, Elbers AR, de Jong WA. Results of a *Salmonella* enteritidis vaccination field trial in broiler-breeder flocks in the Netherlands. Avian Dis 2000;44(2):249–55.
[30] Pasetti MF, Simon JK, Sztein MB, Levine MM. Immunology of gut mucosal vaccines. Immunol Rev 2011;239(1):125–48.
[31] Inoue AY, Berchieri A, Bernardino A, Paiva JB, Sterzo EV. Passive immunity of progeny from broiler breeders vaccinated with oil-emulsion bacterin against Salmonella enteritidis. Avian Dis 2008;52(4):567–71.
[32] Mastroeni P. Development of acquired immunity to *Salmonella*. J Med Microbiol 2003;52(6):453–9.
[33] Okamoto A, Andreatti Filho R, Lima E, Pereira R, Menconi A, Marietto-Gonçalves G. Immunological evaluation of the intestinal mucosa of broiler chicks treated with *Lactobacillus* spp. and challenged with *Salmonella* Enteritidis. Braz J Poult Sci 2007;9(4):259–62.
[34] Methner U, Barrow PA, Berndt A. Induction of a homologous and heterologous invasion-inhibition effect after administration of *Salmonella* strains to newly hatched chicks. Vaccine 2010;28(43):6958–63.
[35] Withanage GSK, Wigley P, Kaiser P, Mastroeni P, Brooks H, Powers C, et al. Cytokine and chemokine responses associated with clearance of a primary *Salmonella enterica* serovar typhimurium infection in the chicken and in protective immunity to rechallenge. Society 2005;73(8):5173–82.
[36] Carvajal BG, Methner U, Pieper J, Berndt A. Effects of *Salmonella enterica* serovar Enteritidis on cellular recruitment and cytokine gene expression in caecum of vaccinated chickens. Vaccine 2008;26(42):5423–33.
[37] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−delta delta C(T)) method. Methods 2001;25(4):402–8.
[38] Babu U, Scott M, Myers MJ, Okamura M, Gaines D, Yancy HF, et al. Effects of live attenuated and killed *Salmonella* vaccine on T-lymphocyte mediated immunity in laying hens. Vet Immunol Immunopathol 2003;91(1):39–44.
[39] Beal RK, Powers C, Davison TF, Barrow PA, Smith AL. Clearance of enteric *Salmonella enterica* serovar typhimurium in chickens is independent of B-cell function. Infect Immun 2006;74(2):1442–4.
[40] Davison S, Benson CE, Henzler DJ, Eckroade RJ. Field observations with *Salmonella* enteritidis bacterins. Avian Dis 1999;43(4):664–9.
[41] Frey J. Biological safety concepts of genetically modified live bacterial vaccines. Vaccine 2007;25(30):5598–605.
[42] Bao S, Beagley KW, France MP, Shen J, Husband AJ. Interferon-gamma plays a critical role in intestinal immunity against *Salmonella* typhimurium infection. Immunology 2000;99(3):464–72.
[43] He H, Genovese KJ, Kogut MH. Modulation of chicken macrophage effector function by T(H)1/T(H)2 cytokines. Cytokine 2011;53(3):363–9.
[44] Beal RK, Powers C, Wigley P, Barrow PA, Smith AL. Temporal dynamics of the cellular, humoral and cytokine responses in chickens during primary and secondary infection with *Salmonella enterica* serovar typhimurium. Avian Pathol 2004;33(1):25–33.

[45] Xiao Z, Casey KA, Jameson SC, Curtsinger JM, Mescher MF. Programming for CD8 T cell memory development requires IL-12 or type I IFN. J Immunol 2009;182(5):2786–94.
[46] Degen WG, van Daal N, van Zuilekom, Burnside J, Schijns VE. Identification and molecular cloning of functional chicken IL-12. J Immunol 2004;172(7):4371–80.
[47] Mittrücker HW, Raupach B, Köhler A, Kaufmann SH. Cutting edge: role of B lymphocytes in protective immunity against *Salmonella* typhimurium infection. J Immunol 2000;164(4):1648–52.
[48] de Wit J, Souwer Y, Jorritsma T, Klaasse Bos H, ten Brinke A, Neefjes J, et al. Antigen-specific B cells reactivate an effective cytotoxic T cell response against phagocytosed *Salmonella* through cross-presentation. PLoS ONE 2010;5(9):e13016.
[49] Hong YH, Lillehoj HS, Lee SH, Dalloul RA, Lillehoj EP. Analysis of chicken cytokine and chemokine gene expression following Eimeria acervulina and Eimeria tenella infections. Vet Immunol Immunopathol 2006;114(3–4):209–23.