

Immune response following vaccination against *Salmonella* Enteritidis using 2 commercial bacterins in laying hens

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Abstract

The humoral and cell-mediated immune (CMI) response to 2 commercial killed *Salmonella* Enteritidis (SE) vaccines (Layermune and MBL SE4C) was evaluated in laying hens. Layers were distributed in 2 experimental groups. The first received a single immunization at 16 wk of age, while the second experimental group was immunized at 12 wk of age and again at 18 wk of age. Serum immunoglobulin (Ig)G antibodies were measured using a commercial SE ELISA kit and showed persistent levels from 3 to 32 and 34 wk post-vaccination. The vaccination protocol using 2 immunizations showed a higher seroconversion level than the single vaccination. However, our results for bacterial intracellular survival indicated that IgG titers were not linked with bacterial killing. Local IgA production was measured in the intestines and oviducts with an in-house SE whole cell antigen ELISA. Only the MBL SE4C vaccine elicited IgA antibody production when tested on intestine and oviduct mucosal secretions, 3-weeks post-vaccination in both immunization protocol groups. To evaluate the CMI response, the splenic T-cells and B-cells populations were analyzed using flow cytometry. The CD3/B-cell ratio decreased 3 wk after the second immunization in the twice vaccinated Layermune group due to an increase in B-cells.

Résumé

La réponse immunitaire humorale et à médiation cellulaire suite à l'administration de vaccins tués contre *Salmonella* Enteritidis (SE) (Layermune ou MBL SE4C) a été évaluée chez des poules pondeuses. Les poules ont été séparées en deux groupes, soit le premier recevant une seule immunisation à l'âge de 16 semaines, et le second groupe recevant deux immunisations à l'âge de 12 et 18 semaines. Les anticorps sériques (Ig)G mesurés à l'aide d'une trousse ELISA commerciale pour SE ont démontré des niveaux persistant de la 3^e aux 32^e et 34^e semaines post-vaccination. Le protocole vaccinal avec 2 immunisations a démontré la séroconversion la plus élevée comparativement au protocole avec une seule immunisation. Cependant, nos résultats concernant la survie intracellulaire bactérienne indiquent que ces titres IgG ne sont pas associés à la mort bactérienne. La production locale d'IgA a été mesurée dans les sécrétions mucosales de l'intestin et de l'oviducte avec un test ELISA maison. Seul le vaccin MBL SE4C a stimulé la production d'IgA locaux, 3 semaines post-vaccination et ce, avec les deux protocoles vaccinaux. Afin d'évaluer la réponse à médiation cellulaire, les populations de cellules spléniques T et B ont été analysées à l'aide de la cytométrie en flux. Le ratio CD3/cellules B a diminué 3 semaines après la seconde immunisation dans le groupe vaccinés deux fois avec le vaccin Layermune et ce, suite à une augmentation du nombre de cellules B.

(Traduit par les auteurs)

Introduction

During the past 2 decades, the number of human cases of *Salmonella* Enteritidis (SE) infections in industrialized countries has increased, with most of these SE foodborne infections associated with egg consumption (1). Various strategies, including vaccination, have been used in the industry to control SE infection and contamination in layers. Both live and killed *Salmonella* vaccines are commercially available. Since vaccination with live SE products is prohibited in many countries, many must rely on bacterins. Most of the studies pertaining to these bacterins have investigated post-vaccine SE shedding over time (2–4). Only a few have looked at the immune response following vaccination, and these studies were limited to young chickens (< 4 wk of age) (5–8).

Bacterins have been used to control host non-specific *Salmonella* infections in poultry, but variation in protection has been observed (3,9–12). For example, autologous vaccines have been used with little information available on their efficacy in a field challenge (13). Killed *Salmonella* vaccines can confer partial protection against intestinal colonization, fecal shedding, systemic spread, and egg contamination (2,8,11,14). However, clinical observations reported that flocks vaccinated with a bacterin showed similar organ and egg contamination levels compared to unvaccinated flocks (2). In another study, the post-vaccination cell-mediated immune response to SE with a bacterin or a subunit vaccine was transient and mainly limited to the SE flagella (7). Several studies have suggested that live attenuated vaccines are more effective than bacterins in protecting birds from SE infection (5,9,15–17). Because of the potential risks associated with

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the use of live attenuated vaccines, such as reversion to virulence, this option is not acceptable for many countries, hence the need to further study the efficacy of killed vaccines.

Therefore, this project was conducted to evaluate the effect of 2 available commercial bacterins on the immune response of laying hens. In our study, SE free pullets from commercial laying farms were vaccinated using 2 killed vaccines (Layermune and MBL SE4C) and subjected to 2 different vaccination regimens. Humoral responses were evaluated using an enzyme-linked immunosorbent assay (ELISA) and cellular responses were assessed using flow cytometry. Results of this study may provide useful information to optimize protection of laying hens against SE and for managing future SE control programs.

Materials and methods

The experimental procedures and protocols were undertaken in accordance with the standards relating to the animal care and management guidelines of the University of Montreal and McGill University with authorization from their respective Ethics Committee of Animal Experimentation.

Laying hens and housing

Four hundred 11 week-old Lohmann White LSL hens were purchased from a commercial source, previously tested and approved SE-free by the Quebec Egg Board and transported to the McGill University's Donald McQueen Shaver Poultry complex. Pullets were randomly distributed in cages (3 pullets per cage), wing tagged according to their treatment group, fed according to the company program, and received water ad libitum. Room temperature was set at 20°C degrees and the lighting program was similar to that of commercial pullets of similar age. The SE free status of the birds was verified in the week following birds' placement by cloacal swabbing.

Vaccines and vaccination

Two killed vaccines (bacterins) were evaluated. Both bacterins contain inactivated strains of SE in a water-in-oil emulsion (Layermune SE; Biomune Company, Kansas, USA and MBL SE4C; Maine Biological Laboratories/Lohmann Animal Health International, Maine, USA). Birds were injected with the Layermune vaccine (0.5 mL/hen) and with the MBL SE4C vaccine (0.25 mL/hen) subcutaneously in the neck according to field practice for both vaccination protocols. Control hens were injected with sterile saline (0.9% NaCl). For each experiment, 200 hens were assigned to 1 of 3 groups: control (no vaccine, sterile saline), vaccination with Layermune, or MBL SE4C.

Prior to vaccination, birds were given a 7-day acclimation period to their new environment. The 2 vaccination regimens consisted of either vaccinating the birds once at 16 wk of age, or twice at 12 and 18 wk of age.

Sample collection and measurements

Local vaccine reaction — The local inflammatory reaction following vaccination was evaluated at the injection site by palpation using the same evaluator 5 d post-vaccination. The level of local reaction was based on the following score: score 0: no palpable lesion; score 1: a

palpable lesion less than 25 mm in diameter; score 2: a lesion with a diameter more than 25 mm.

Blood sample collection — Birds were randomly bled within their treatment group ($n = 25$ per group per sampling period) from the brachial vein before and 4 wk after the first immunization; 2, 16, 30, and 40 wk after the booster dose for groups receiving 2 vaccinations; and 2, 4, 20, 32, and 42 wk post-vaccination for groups receiving 1 vaccination.

Intestinal and oviduct secretions — Birds were euthanized with an intravenous injection of phenobarbital. A 10-cm portion of ileum and oviduct (magnum region) was removed aseptically at necropsy from each hen at weeks 1 and 4 after the primary vaccination (PV) for groups receiving 1 vaccination, 3 wk after the primary vaccination (PPV), and 2 wk after the secondary vaccination (PSV) for groups receiving 2 vaccinations. Serosal connective tissues and fat were removed aseptically and placed in phosphate buffered saline (PBS, pH 7.4) (Invitrogen, Burlington, Ontario). Ileum and magnum were cut longitudinally with scissors to expose their respective lumen. Mucosal secretions (ileum/magnum length = 1 cm \times 10 cm) were aseptically scraped with a sterile microscope slide and suspended in 5 mL of PBS (pH 7.4) (Invitrogen). This preparation was vortexed for 10 s and centrifuged (3000 \times g, 20 min at 4°C). The supernatant was then filtered through a 0.45 μ m filter and conserved at -20°C.

Detection of sera antibodies (IgG)

Sera were separated using centrifugation at 5000 \times g, for 10 min. The immunoglobulin (Ig) G antibodies in sera were measured using the *Salmonella* Enteritidis antibody test kit (Flockchek SE; Idexx laboratories, Westbrook, Maine, USA). The SE assay was done in a microtiter well coated with purified SE antigen (g,m flagella). The quantity of antibody to SE is inversely proportional to the absorbance at 650 nm.

Detection of mucosal antibodies (IgA)

Antigen preparation for ELISA assays — Whole cells of SE strain STF-04-4689 supplied by LEAQ (Laboratoire d'épidémiologie-surveillance animale du Québec) were used as the antigen for the ELISA. Briefly, a colony isolated on blood agar was incubated in 100 mL LB Broth Miller (EMD Chemicals, Gibbstown, New Jersey, USA) overnight at 37°C with shaking at 150 rpm. The suspension was centrifuged at 3500 rpm (2500 g) for 20 min at room temperature and the pellet was suspended in 5 mL of PBS (pH 7.4). The suspension of bacterial cells was sonicated 15 \times 10 s on ice. Five microliters (750 U) of DNase (Invitrogen) was added. This bacterial suspension was shaken at 150 rpm at 37°C. Cells were lysed by passing them through a French press cell (SLM Aminco, Spectronic Instruments, Rochester, New York, USA) at 38 000 psi., the suspension was centrifuged [12 500 rpm (18 677 g); 10 min; 4°C], and the supernatant was stored at -20°C.

ELISA procedure — The ELISA was assessed as described previously by Letellier et al (18) with some modifications. Briefly, flat-bottomed 96 polysorp well ELISA plates (Nunc-immuno plate; Thermo Fisher Scientific, Rochester, New York, USA) were coated with 100 μ L of diluted *Salmonella* whole antigen 1:10 (100 μ g/mL) overnight at 4°C. Non-specific binding was blocked by incubating the plate with 2% milk powder for 1 h at 37°C. Plates were washed 3 times for 5 min in chloride sodium (0.1M) containing (0.05%) (v/v) Tween 20 (Sigma Aldrich, St. Louis, Missouri, USA).

Intestinal and oviduct mucosal secretion samples were diluted 1:2 in PBS-Tween (0.05%) (v/v) and were tested in duplicate (100 µL/well) for 1 h at room temperature on each plate. The plates were washed again (3 × 5 min) and conjugate (goat anti-chicken IgA coupled with Horseradish peroxidase) (USBiological, Massachusetts, USA) (diluted 1: 5000 in PBS containing 0.05% Tween 20) was added to each well (100 µL/well).

The plates were incubated for 1 h at room temperature, washed, and the bound horseradish peroxidase activities revealed by adding 100 µL substrate solution [2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)] (ABTS; Sigma Aldrich) to each well. The plates were incubated for 30 min at room temperature and the absorbance was read at 5, 15, and 30 min (405 nm). Data were collected and the samples were considered positive if their ELISA absorbance values exceeded the mean absorbance value for the negative control samples by more than 2 standard deviations.

Oxidative burst assay for heterophils

The reactive oxygen species (ROS) production during heterophil oxidative burst was measured using 2', 7'-dichlorofluorescein diacetate (DCFH-DA) as a fluorescent probe. The DCFH-DA is oxidized by ROS to fluorescent DCF, which has been widely used for measurement of oxidative response in heterophils by flow cytometric assay (19).

Blood sampling for heterophil collection — Blood samples were taken from the brachial vein into heparin lithium coated vials at 0, 2, 4, 20, and 36 wk post-vaccination in the single immunization groups, while birds from the 2 vaccination protocol were bled at 0 and 4 wk after the primary vaccination and 2, 20, and 36 wk post secondary vaccination.

Isolation of peripheral blood heterophils — Four millilitres of the heparin lithium-anticoagulated blood was layered over a discontinuous histopaque (Sigma-Aldrich) gradient (specific gravity 1.077 over specific gravity 1.119). The gradient was then centrifuged at 700 × g for 30 min. After centrifugation, the 1.077/1.119 interface and 1.119 band containing the heterophils were collected following the manufacturer's instructions and resuspended in D-PBS-G (1g/L D-glucose, GIBCO; Invitrogen). The suspension was then centrifuged (200 × g, 10 min) and, after 2 consecutive washes in D-PBS-G, the supernatant was collected. Heterophils were counted under a light microscope using a hemacytometer. The cell population was adjusted to a concentration of 1.25 × 10⁶ heterophils/mL in D-PBS-G to obtain 1 × 10⁶ cells in a 0.8 mL/sample and kept on ice until used.

Cells were loaded with 1 µL DCFH-DA (2', 7'-Dichlorofluorescein diacetate; Molecular Probes, Eugene, Oregon, USA) 5 mM in ethanol 100%. The loaded cells were incubated at 39°C in 5% CO₂ for 15 min. One hundred microliters of PMA (Phorbol 12-myristate 13-acetate; Sigma-Aldrich) and 100 µL D-PBS-G were added in each tube.

Flow cytometric oxidative burst assay — After the addition of PMA to the cells, kinetics was recorded using flow cytometry (FACS Calibur; Becton Dickinson, Germany). Oxidative burst was measured using the geomean in the cytometric assay (The geomean final = the geomean after 60 min of incubation with PMA agonist — the geomean before incubation with PMA agonist). Caution was taken to adequately gate the cell population to block out lymphocytes or monocytes.

Intracellular survival assays

The SE PT4 cells were grown overnight until the end of the exponential growth phase in LB Broth Miller (EMD) and standardized to an optical density of 0.5 at 600 nm.

Briefly, 10 µL of SE culture was added to each falcon tube (BD falcon polystyrene; BD Biosciences, Mississauga, Ontario) containing 100 µL of blood. Adhesion and subsequent entrance of bacteria into cells was allowed to proceed for 60 min at 39°C on a rotator. The samples were then washed twice using Dulbecco's modified PBS 1X (D-PBS; Invitrogen). At this point, colistin was added at a concentration of 600 µg/mL to kill extracellular bacteria, viability of intracellular bacteria was not affected.

To determine the number of intracellular SE, the sample was incubated at 39°C for 18 h. Subsequently, the cells were lysed by adding appropriate dilutions in sterile water to each sample and incubating the lysate for 5 min at room temperature to release intracellular *Salmonella*. Appropriate dilutions in sterile water were then plated onto LB agar to determine the number of viable intracellular *Salmonella* (CFU) by colony counting.

Lymphocytes subpopulation measurements

Isolation of splenic mononuclear cells — The spleen was removed at necropsy. The splenic mononuclear cells (MNC) were isolated from the spleen as previously described (9). Briefly, spleens were washed and minced in Hank's balanced salt solution (HBSS, GIBCO; Invitrogen) and residual tissues were removed using a cell strainer 40 µm nylon (BD Falcon; BD Biosciences). Single cell suspensions from the spleens were overlaid onto the histopaque (density 1.077 g/mL) (Sigma-Aldrich) and centrifuged at 400 × g for 30 min at room temperature. The MNC, obtained from the interface, were washed twice with HBSS. The cell concentrations were adjusted to 1 × 10⁷ cells/mL with staining buffer (DPBS + 2% heat inactivated fetal calf serum [FCS; Sigma-Aldrich] + 0.09% [w/v] sodium azide, pH 7.4).

Labelling antibodies and flow cytometric analysis — Chicken monoclonal antibodies against chicken CD3, CD4, CD8 T lymphocytes (CD = Cluster of differentiation) and IgM expressed on B-cells were used for flow cytometry. The MNC were incubated with monoclonal antibodies in a dilution of 1:125 with staining buffer. Duplicate samples of MNC were stained with R-phycoerythrin-conjugated anti-chicken CD3 and FITC-conjugated anti-chicken IgM (Southern Biotech, Birmingham, Alabama, USA). Duplicate samples of MNC were stained with R-PE-anti-chicken CD8, and FITC- anti-chicken CD4 (Southern Biotech). The cells were washed twice using PBS at 4°C. Samples were analyzed using a flow cytometer (FACS Calibur; Becton Dickinson). Data on 3 × 10⁴ viable cells were collected using computer software (Cell Quest program 3.3; BD BioSciences Canada, Mississauga, Ontario). For gating purposes, the viable MNC population was determined by forward light scatter versus side light scatter analysis.

Statistical analysis

Results for antibodies responses, heterophil oxidative burst, splenocytes population ratios, and intracellular survival were analyzed using a linear model with immunization protocol as a factor.

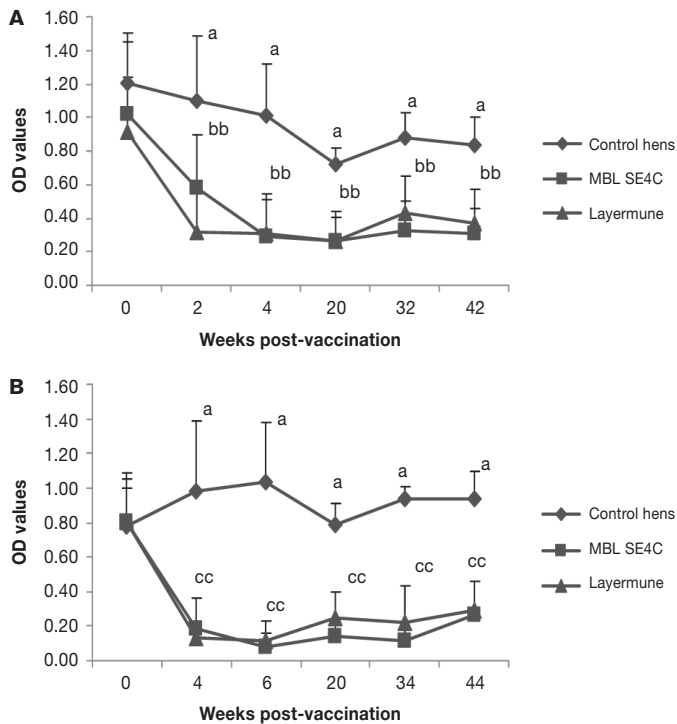


Figure 1. A — Detection of antibodies (IgG) in sera of laying hens vaccinated once analyzed using *Salmonella* Enteritidis (SE) antibody test kit. **B** — Detection of antibodies (IgG) in sera of laying hens vaccinated twice. Antibodies (IgG) analyzed using SE antibody test kit. Vertical bars indicated the standard errors. Significant differences in mean titers were determined by using the Bonferroni test at $P < 0.01$. The mean titers of MBL SE4C vaccinated group (■) and Layermune vaccinated group (▲) were compared with those of control group (◆). The titer of SE-antibodies is inversely proportional to the optical density (OD) values at 650 nm. Data represent mean \pm standard deviation ($n = 25$ hens/group/sampling period). ^{a,b,c} $P < 0.0001$.

Contrasts between protocol means at different time periods were done using the sequential Bonferroni procedure. Intracellular survival data were log₁₀ transformed before analysis to normalize the data. Data for local inflammatory reaction were analyzed with the exact chi-squared test. The level of statistical significance was set at $P < 0.05$ throughout. Statistical analyses were carried out using computer software (SAS version 9.1; SAS, Cary, North Carolina, USA).

Results

Sera antibodies (IgG) responses

The SE-specific serum IgG titers were compared using ELISA for all groups. The mean optical density (OD) values of sera are shown in Figure 1A for the groups vaccinated once and Figure 1B for the groups vaccinated twice. The OD values of vaccinated hens were significantly lower than those of control hens at each time point after vaccination ($P < 0.0001$), indicating a higher IgG titer. The IgG levels remained elevated during the laying period in both vaccination protocols. There was no statistical difference in antibody levels between the 2 vaccinated groups at any sampling time or with either vaccination protocol. But both groups vaccinated twice showed statistically higher sera titers than groups with a single immunization, until the 32 to 34 wk post-vaccination sampling ($P < 0.02$).

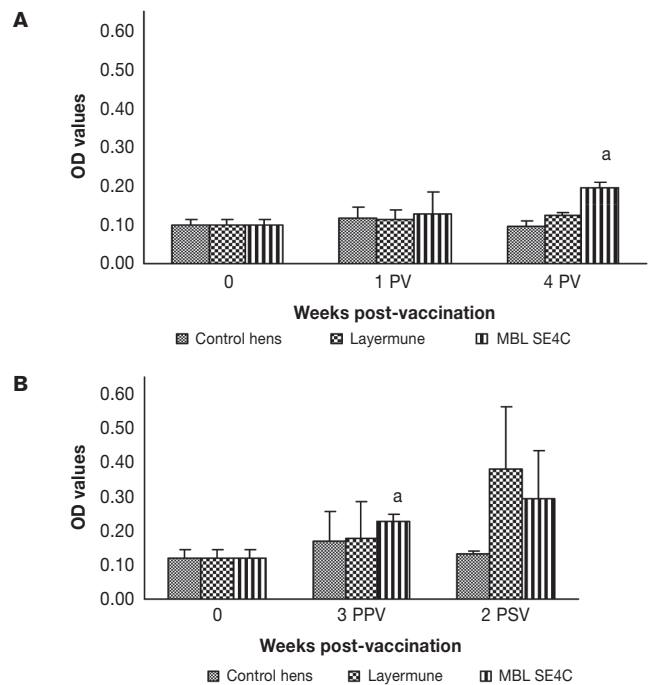


Figure 2. A — Immunoglobulin (IgA) levels in the intestinal mucus of laying hens vaccinated once with subcutaneous *Salmonella* Enteritidis (SE) vaccine. **B** — IgA levels in the intestinal mucus of laying hens subcutaneous SE vaccinated twice. *Salmonella* Enteritidis whole cell-specific antibodies (IgA) were detected using ELISA. Data represent mean \pm standard deviation ($n = 3$ hens/group/sampling period). OD — optical density; PV — post-vaccination; PPV — post primary vaccination; PSV — post second vaccination. ^a $P < 0.01$.

Mucosal antibodies (IgA) response

Although the intestinal IgA antibody levels (Figure 2A,B) were low throughout the experiment, there were differences between the 2 vaccines. The MBL SE4C bacterin group vaccinated once showed a statistically significant increase in intestinal IgA antibody values at week 4 post-vaccination compared with those in the control group ($P = 0.0007$) and Layermune bacterin ($P = 0.001$). Similar results were observed with the 2 vaccination protocols ($P = 0.004$) at 3 wk after the primary vaccination.

The IgA antibody levels in the oviduct (Figure 3A, B) were higher than those measured in the intestines. Similarly to the local intestinal immune response, oviduct IgA titers were significantly higher 3 to 4 wk post-vaccination in the groups vaccinated with MBL SE4C than in the control groups ($P = 0.0001$ with single immunization and $P < 0.04$ with 2 immunizations).

There was no statistical difference in IgA response between the controls and the groups vaccinated with Layermune for either the intestinal or the oviduct local immune response.

Heterophil oxidative burst

The heterophil oxidative burst response of laying hens to 2 bacterins and immunization protocols was compared. The oxidative burst response was significantly increased 2 wk post-vaccination in groups with a single immunization ($P < 0.001$) and 4 wk post-vaccination in groups with 2 immunizations ($P < 0.001$). An increased age effect

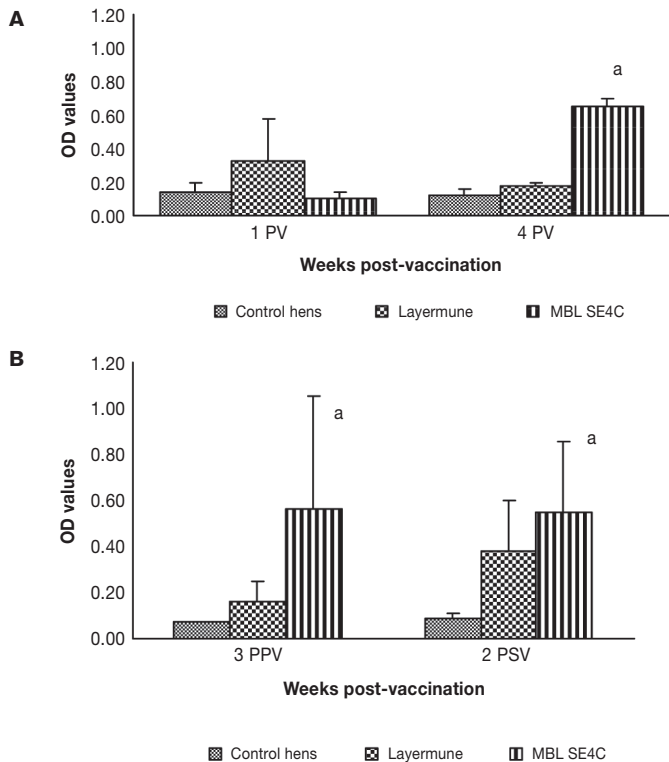


Figure 3. A — Immunoglobulin (Ig)A levels in the oviductal mucus of laying hens vaccinated once with subcutaneous *Salmonella* Enteritidis (SE) vaccine. **B** — IgA levels in the oviductal mucus of laying hens vaccinated twice with subcutaneous SE vaccine. *Salmonella* Enteritidis whole cell-specific antibodies (IgA) were detected using ELISA. Data represent mean \pm standard deviation ($n = 3$ hens/group/sampling period). OD — optical density; PV — post-vaccination; PPV — post primary vaccination; PSV — post second vaccination. ^a $P < 0.01$.

with the oxidative burst response was observed in all treatment groups and controls at 20 and 30 wk post-vaccination.

Effects of vaccination on splenocytes population ratios

Results on the evaluation of cellular immune response within the splenocytes population are presented in Figure 4A and Figure 4B for the CD3/B-cells ratio. There was an increased CD3/B-cells ratio the 1st wk post-vaccination when compared to pre-vaccination ($P < 0.05$) in the MBL SE4C group vaccinated once, but this ratio returned to pre-vaccination values at 3 wk post-vaccination (Figure 4A). There was a decreased CD3/B-cells ratio in the Layermune group 2 wk after administering the booster ($P < 0.05$) (Figure 4B). No significant change in the CD3/B-cells ratio was observed in the control groups pre-vaccination versus post-vaccination.

Vaccination of 16-week-old hens using a single immunization resulted in increased splenic CD4/CD8 ratio at 3 wk post-vaccination in the MBL SE4C vaccinated group ($P < 0.05$). In the groups receiving 2 immunizations, the CD4/CD8 ratio was significantly higher at 3 wk after the 1st vaccination in the Layermune group when compared to pre-vaccination ($P < 0.05$), but returned to its initial level 2 wk after the booster ($P > 0.05$).

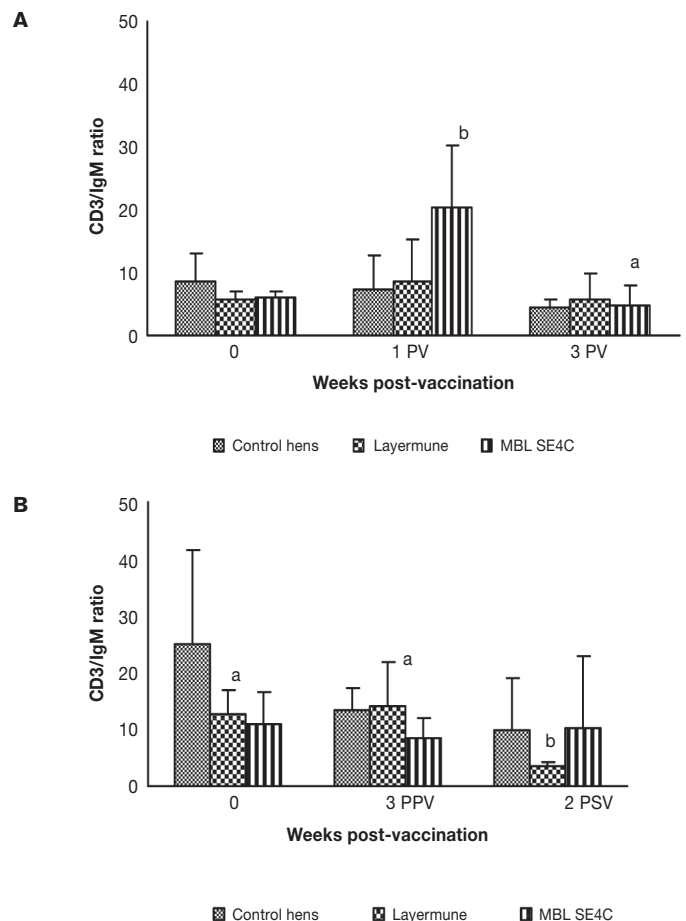


Figure 4. The CD3/IgM cells ratio after immunization with single vaccination (A) and 2 immunizations (B). Data represent mean \pm standard deviation ($n = 3$ hens/group/sampling period). PV — post-vaccination; PPV — post primary vaccination; PSV — post second vaccination. ^{a,b} $P < 0.05$.

Intracellular survival assays

The number of *Salmonella* surviving in the leucocytes at all sampling times in all groups (before vaccination, 4 mo post-vaccination in and with either vaccination protocols) was not different (Table I).

Four months post-vaccination, the number of surviving intracellular bacteria tended to be higher compared with the number of bacteria that survived prior to vaccination. This increase was significant in the single immunization groups; Layermune ($P = 0.002$), MBL SE4C ($P = 0.035$), and controls ($P = 0.042$). The double immunization protocol induced similar results with higher numbers of intracellular surviving SE 4 mo post-vaccination for the Layermune ($P = 0.004$), MBL SE4C ($P = 0.005$), and control ($P = 0.0047$) groups.

Local inflammatory reaction

All pullets vaccinated with a bacterin showed a local inflammatory reaction when compared to controls ($P < 0.0001$). The score of the inflammatory reaction at vaccination site in the Layermune vaccine group (score 1: 46.59%, score 2: 7.95%) was greater than for those receiving the MBL SE4C vaccine (score 1: 26.14%, score 2: 1.14%) at 16 wk after a single immunization ($P < 0.0001$). There

Table I. Intracellular survival of *Salmonella* Enteritidis in leucocytes following variant immunization protocols in hens, expressed in number of bacteria ($\times 10^3$ CFU/mL)

Treatments	Single vaccination		Double vaccination	
	Pre-vaccination	4 mo post-vaccination	Pre-vaccination	4 mo post-vaccination
Saline solution ^a	81.33 \pm 103.58	1136.67 \pm 1251.57 ^b	35.20 \pm 46.80	2896.66 \pm 957.09 ^b
Layermune ^a	14.00 \pm 7.54	1770.00 \pm 873.09 ^b	42.80 \pm 27.58	3206.66 \pm 1374.09 ^b
MBL SE4C ^a	66.00 \pm 54.44	1066.67 \pm 547.93 ^b	36.60 \pm 39.81	2490.00 \pm 2867.28 ^b

Data are presented as mean \pm standard deviation ($n = 3$ hens/group/sampling period).

^a Values expressed as mean \pm standard error.

^b $P < 0.05$.

was an increase in severity of the inflammatory lesion after administration of the 2nd vaccine at 18 wk of age (score 1: 58.44%, score 2: 11.69%) compared with the score of the lesions observed after the 1st immunization at 12 wk of age (score 1: 28.21%, score 2: 2.56%) in Layermune vaccinated groups ($P < 0.0001$).

Discussion

Our main objective was to better characterize the humoral and cell-mediated immune responses of laying hens vaccinated with 2 commercially available *Salmonella* Enteritidis bacterins using 2 vaccination protocols. We were particularly interested in assessing the immune response following a single immunization since this is a protocol producers prefer due to the cost of the vaccine and the stress of handling.

Both vaccination protocols showed an increased seroconversion level with antibody titers being higher in the protocol using 2 immunizations until 32 wk post-vaccination. Immunization with both bacterins induced serum IgG responses, which persisted to the end of our sampling period at 42 to 44 wk post-vaccination.

With regard to systemic humoral response, there are different opinions on the protective effect of vaccination against SE. The serum antibody titers are of uncertain value as indicators of the extent of protection against *Salmonella* because, even if a killed vaccine can induce a high SE-specific antibody level, it does not necessarily protect the chickens against SE infection (5,20).

Since mucosal surfaces are the major sites for SE invasion, the local humoral response might play an important role against *Salmonella* colonization. Initial colonization of the intestines is often followed by invasion through mucosal epithelial cells and dissemination to specific internal organs like spleen, liver, ovaries, and oviduct (21). Experimental SE infection has shown that IgA antibodies predominate in the intestinal secretions, peaking 1 wk after SE infection and persisting during intestinal colonization (22) for up to 9 wk post-infection (23). In our study, mucosal immunity was induced 3 wk post-vaccination following MBL SE4C injection, as shown by the increased intestinal IgA levels. Such response could limit the replication of pathogens within the mucosal lumen, as well as their adhesion (24). It would be interesting to see how long these vaccine generated IgA titers remain and whether they are able to protect birds against an infection for the whole laying period (up to 72 wk of age).

The main goal in vaccinating laying hens against SE is to prevent food contamination by reducing or preventing egg contamination.

Bacterial infection in the reproductive organs of laying hens may cause contamination of eggs either via the yolk or the albumen. The immune response, therefore, plays an essential role in preventing infection by *Salmonella* organisms in the reproductive organs (25). After vaccination, the oviduct IgA titers in our MBL SE4C vaccinated group increased 3 wk post-vaccination, with higher oviduct IgA levels than those measured in the intestines. A study by Withanage et al (26) suggested a relationship between the SE-specific antibodies measured in the oviduct and the bacterial clearance. Although a *Salmonella*-specific humoral response in infected laying hens cannot eliminate the bacteria from the reproductive tract, the presence of Ig in the oviduct could be sufficient to reduce reproductive tract colonization and the subsequent egg contamination (27).

In chickens, phagocytosis is one of the mechanisms used to avoid host infection by *Salmonella* (28). From our results, the number of surviving intracellular SE cells was similar in vaccinated hens to unvaccinated hens, indicating that vaccination did not enhance phagocytosis 4 mo post-vaccination. We observed that vaccinated laying hens maintained high blood antibodies (IgG) levels during the whole laying period. Antibodies are effective opsonins that can mediate phagocytosis through antibody receptors, which are specific for phagocytic cells (29). Our findings suggest that IgG titers are not a reliable indicator of bacterial killing because these antibodies appear to be non-opsonizing antibodies for phagocytosis. Furthermore, increased intracellular survival, as observed 4 mo after vaccination, not only in vaccinated groups but also in control groups, suggested an age-dependent effect in the laying hens in the experiment rather than a vaccination effect. Other authors have reported that phagocytic capacity of heterophils increased over the 1st wk of life (30). However, to our knowledge, there have been no reports regarding the increase in *Salmonella* intracellular survival over time in either chickens or other species.

In our study, the decrease of CD3/B-cell ratio in the Layermune vaccine treated group (2 immunizations) likely suggests that this bacterin induced B-cells maturation, since CD3+ cells were not significantly different over time. For the detection of B-cells, an anti-chicken IgM antibody was used. Since the majority of circulating B-cells expresses IgM on their surface, the membrane bound IgM molecule appears to be an excellent antigen for B-cells detection (31). This result is in accordance with the study of Sasai et al (32) in which elevated percentage of IgM + lymphocytes were reported. Since mature B-cells produce 3 types of Ig: IgG, IgM, and IgA (25), an increase in B-cells likely correlates with an increased post-vaccination serum antibody IgG titer. However, the same trend was not observed

with the single immunization protocol. This might suggest: a) a possible age effect since this group was first vaccinated at 16 rather than 12 wk of age; b) an intermediate response 1 wk after a single vaccination, which could not be detected in the 2 immunizations protocol due to our sampling design; or c) changes at 3 wk post single vaccination that were too subtle to be detected. Our results demonstrate that there was an increased CD4/CD8 ratio in the MBL SE4C group after the 1st vaccination, but a 2nd immunization had no effect on this ratio. It is not possible with our data to explain whether there was an age or a vaccination protocol effect for the variations in the CD4/CD8 ratio. *Salmonella* infection generally induces the production of specific CD4+ and CD8+ T-cells, and both T-cell populations appear to be important for protection during primary and secondary responses (33,34).

Poultry heterophils are the first cell type to engage in phagocytosis and killing of pathogens (35). The oxidative burst, which generates reactive radical oxygen species, is one of the main bactericidal mechanisms of heterophils. The results of this study demonstrated that administration of the bacterin induced an increased oxidative burst of heterophils 2 and 4 wk post-vaccination. This reaction would appear to be important as a first line of defence against SE. For example, heterophil-depleted chickens showed a severe morbidity and mortality when a normally sublethal dose of SE was inoculated orally (36). Furthermore, this function appears to be age-dependent since the capability of heterophils to phagocytise increased from day 1 to day 7 in newly hatched chicks (30).

Vaccination with the 2 commercial SE bacterins induced palpable changes at the injection site when compared with the unvaccinated group with larger lesions being associated with the Layermune vaccine. Both bacterins contained inactivated strains of *Salmonella* Enteritidis with oil-based adjuvants. In a recent study, a Layermune bacterin injected group showed histological changes at injection site consisting in an epithelioid granuloma surrounding oily cysts and necrosis with peripheral fibroplasias (37). These lesions are likely a result of the adjuvants, which activate the complement via the alternate pathway and induce an inflammatory reaction at the injection site (38). The potential severity of the vaccine reaction at the injection site should, therefore, be considered when choosing the age, vaccination site, and vaccine.

In summary, our results demonstrated that vaccination with double immunizations was more effective than a single immunization to stimulate the humoral systemic response. Given the limited effect observed on the CMI, it was not possible to assess the role of splenic T-cells and B-cells on the immune response induced by these 2 SE bacterins. In contrast to this lack of significant effect on the CMI, the bacterin induced SE-flagella specific antibodies and demonstrated a higher IgG titer after vaccination. It is not clear if this increased antibody response would be associated with the protection of birds against SE infection. The ability of MBL SE4C to induce a local immune response in the intestine and oviduct could be more effective against SE infection than the systemic response. Our results suggest that both bacterins were able to generate an immune response, but it is not known if this response generated sufficient titers, opsonic antibody production, or both after vaccination to protect birds against a SE challenge.

We are currently studying the SE clearance and immune responses in older laying hens following vaccination and experimental infection. These studies will provide further information regarding the killed vaccines' efficacy to protect the laying hens and their eggs from SE challenge.

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References

1. Methner U DR, Reiche R, Böhlend K. Occurrence of *Salmonellae* in laying hens in different housing systems and inferences for control. *Berl Munch Tierarztl Wochenschr* 2006;119:467-473.
2. Davison S, Benson CE, Henzler DJ, Eckroade RJ. Field Observations with *Salmonella* Enteritidis Bacterins. *Avian Dis* 1999;43:664-669.
3. Woodward MJ, Gettinby G, Breslin MF, Corkish JD, Houghton S. The efficacy of Salenvac, a *Salmonella enterica* subsp. *enterica* serotype Enteritidis iron-restricted bacterin vaccine, in laying chickens. *Avian Pathol* 2002;31:383-392.
4. 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;125:111-119.
5. Babu U DR, Okamura M, Lillehoj HS, et al. *Salmonella* Enteritidis clearance and immune responses in chickens following *Salmonella* vaccination and challenge. *Vet Immunol and Immunopathol* 2004;101:251-257.
6. Okamura M, Lillehoj HS, Raybourne RB, Babu US, Heckert RA. Cell-mediated immune responses to a killed *Salmonella* Enteritidis vaccine: Lymphocyte proliferation, T-cell changes and interleukin-6 (IL-6), IL-1, IL-2, and IFN-[gamma] production. *Comp Immunol Microbiol Infect Dis* 2004;27:255-272.
7. Okamura M, Lillehoj HS, Raybourne RB, Babu U, Heckert R. Antigen-specific lymphocyte proliferation and interleukin production in chickens immunized with killed *Salmonella* Enteritidis vaccine or experimental subunit vaccines. *Avian Dis* 2003;47:1331-1338.
8. Liu W, Yang Y, Chung N, Kwang J. Induction of humoral immune response and protective immunity in chickens against *Salmonella* Enteritidis after a single dose of killed bacterium-loaded microspheres. *Avian Dis* 2001;45:797-806.
9. Babu USM, Myers MJ, Okamura M, et al. Effects of live attenuated and killed *Salmonella* vaccine on T-lymphocyte mediated

- immunity in laying hens. *Vet Immunol Immunopathol* 2003;91:39–44.
10. Nakamura M, Nagamine N, Takahashi T, Suzuki S, Sato S. Evaluation of the efficacy of a bacterin against *Salmonella* Enteritidis infection and the effect of stress after vaccination. *Avian Dis* 1994;38:717–724.
 11. Nakamura M, Nagata T, Okamura S, Takehara K, Holt PS. The effect of killed *Salmonella* Enteritidis vaccine prior to induced molting on the shedding of *S. Enteritidis* in laying hens. *Avian Dis* 2004;48:183–188.
 12. Uchiya K, Nikai T, Sugihara H. Difference in the protection against infection with different challenge strains of *Salmonella* Enteritidis by killed vaccine. *Kansenshogaku Zasshi* 1991;65:1411–1418.
 13. Van immerseel FMU, Rychlik I, Nagy B, et al. Vaccination and early protection against non-host-specific *Salmonella* serotypes in poultry: Exploitation of innate immunity and microbial activity. *Epidemiol Infect* 2005;133:959–978.
 14. Timms LM, Marshall RN, Breslin MF. Laboratory and field trial assessment of protection given by a *Salmonella* Enteritidis PT4 inactivated, adjuvant vaccine. *Br Vet J* 1994;150:93–102.
 15. Curtiss R, Hassan JO. Nonrecombinant and recombinant avirulent *Salmonella* vaccines for poultry. *Vet Immunol Immunopathol* 1996;54:365–372.
 16. Adriaensen C DGH, Tian JQ, De Craeye S, et al. A live *Salmonella enterica* serovar Enteritidis vaccine allows serological differentiation between vaccinated and infected animals. *Infect Immun* 2007;75:2461–2468.
 17. Betancor L, Schelotto F, Fernandez M, Pereira M, Rial A, Chabalgoity JA. An attenuated *Salmonella* Enteritidis strain derivative of the main genotype circulating in Uruguay is an effective vaccine for chickens. *Vet Microbiol* 2005;107:81–89.
 18. Letellier A MS, Lessard L, Chénier S, Quessy S. Host response to various treatments to reduce *Salmonella* infections in swine. *Can J Vet Res* 2001;65:168–172.
 19. Brousseau P PY, Tryphonas H, Blakley B, Boermans H, Flipo D, Fournier M. *Manual of Immunological Methods*. CRC Press ed, 1999.
 20. Nagaraja KV, Rajashekara G. Vaccination against *Salmonella enterica* serovar Enteritidis infection: Dilemma and realities. *Salmonella enterica* serovar Enteritidis in humans and animals: Epidemiology, Pathogenesis, and Control. 1999:397–404.
 21. Gast RK, Holt PS. Experimental horizontal transmission of *Salmonella* Enteritidis strains (Phage Types 4, 8, and 13A) in chicks. *Avian Dis* 1999;43:774–778.
 22. Berthelot-Hérault F, Mompert F, Zygmunt MS, Dubray G, Duchet-Suchaux M. Antibody responses in the serum and gut of chicken lines differing in cecal carriage of *Salmonella* Enteritidis. *Vet Immunol Immunopathol* 2003;96:43–52.
 23. Zhang-Barber L, Turner AK, Barrow PA. Vaccination for control of *Salmonella* in poultry. *Vaccine* 1999;17:2538–2545.
 24. Fukutome K, Watarai S, Mukamoto M, Kodama H. Intestinal mucosal immune response in chickens following intraocular immunization with liposome-associated *Salmonella enterica* serovar Enteritidis antigen. *Dev Comp Immunol* 2001;25:475–484.
 25. Yoshimura Y. Significance of local immunity in hen reproductive organs. *Anim Sci J* 2004;75:183–191.
 26. Withanage GSK, Sasai K, Fukata T, Miyamoto T, Baba E. Secretion of *Salmonella*-specific antibodies in the oviducts of hens experimentally infected with *Salmonella* Enteritidis. *Vet Immunol Immunopathol* 1999;67:185–193.
 27. De Buck J, Van Immerseel F, Ducatelle HR. Colonization of the chicken reproductive tract and egg contamination by *Salmonella*. *J Appl Microbiol* 2004;97:233–245.
 28. Kramer J, Visscher AH, Wagenaar JA, Jeurissen SHM. Entry and survival of *Salmonella enterica* serotype Enteritidis PT4 in chicken macrophage and lymphocyte cell lines. *Vet Microbiol* 2003;91:147–155.
 29. Tizard IR. *Veterinary Immunology, An Introduction*. 7th ed. Philadelphia: Saunders, 2004:27–31.
 30. Wells LL, Lowry VK, Deloach JR, Kogut MH. Age-dependent phagocytosis and bactericidal activities of the chicken heterophil. *Dev Comp Immunol* 1998;22:103–109.
 31. Berndt A, Methner U. B cell and macrophage response in chicks after oral administration of *Salmonella* Typhimurium strains. *Comp Immunol Microbiol Infect Dis* 2004;27:235–246.
 32. Sasai K YK, Lillehoj HS, Withanage GSK, Fukata T, Baba E, Arakawa A. Analysis of splenic and thymic lymphocyte subpopulations in chickens infected with *Salmonella* Enteritidis. *Vet Immunol Immunopathol* 1997;59:359–367.
 33. Mittrucker HW, Kaufmann SH. Immune response to infection with *Salmonella* Typhimurium in mice. *J Leukoc Biol* 2000;67:457–463.
 34. Berndt A, Methner U. Gamma/delta T cell response of chickens after oral administration of attenuated and non-attenuated *Salmonella* Typhimurium strains. *Vet Immunol Immunopathol* 2001;78:143–161.
 35. He H, Genovese KJ, Swaggerty CL, Nisbet DJ, Kogut MH. In vivo priming heterophil innate immune functions and increasing resistance to *Salmonella* Enteritidis infection in neonatal chickens by immune stimulatory CpG oligodeoxynucleotides. *Vet Immunol Immunopathol* 2007;117:275–283.
 36. Kogut MH TG, Hargis BM, Corrier DE, DeLoach JR. The effect of 5-fluorouracil treatment of chicks: A cell depletion model for the study of avian polymorphonuclear leukocytes and natural host defenses. *Poult Sci* 1993;72:1873–1880.
 37. Toyota-Hanatani Y, Inoue M, Ekawa T, Ohta H, Igimi S, Baba E. Importance of the major Fli C antigenic site of *Salmonella* Enteritidis as a subunit vaccine antigen. *Vaccine* 2008;26:4135–4137.
 38. Vermout SDM, Losson B, Mignon B. Choix d'un adjuvant lors d'essais de vaccination. *Ann Méd Vét* 2003;147:393–401.