ASSESSMENT OF KILLED SALMONELLA VACCINE EFFICACY IN BROILER BREEDERS AND THEIR PROGENY

by

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(Under the Direction of CHARLES HOFACRE)

ABSTRACT

This study compares efficacy of various *Salmonella* bacterins following priming with two doses of live *Salmonella* Typhimurium vaccine in broiler breeders. Vaccination is one of the most practical measures to reduce *Salmonella* contamination of poultry and to prevent foodborne disease in humans. Commercial live and killed *Salmonella* vaccines have proven to be effective in commercial poultry production. This is supported by several field studies that have demonstrated vaccination increases *Salmonella*-specific antibody titers in vaccinated breeders and significantly reduces breeder colonization by challenge strains. In this study protection from commercially available live and inactivated *Salmonella* vaccine combinations were compared following challenge with three different serotypes of *Salmonella*. Broiler breeders were housed in a controlled setting of six pens; five pens were vaccinated with two doses of live *Salmonella* Typhimurium vaccine and four pens were also vaccinated with inactivated *Salmonella* vaccine containing whole cells of *Salmonella* Enteritidis, *Salmonella* Typhimurium and *Salmonella* Heidelberg. One pen was occupied with unvaccinated breeders that served as controls. At 10, 14, 20, 30, and 45 weeks of age twenty breeders from each treatment were challenged by oral gavage with *Salmonella* Enteritidis. In addition, protection passed to the progeny from the broiler breeders was assessed by collecting eggs from vaccinated and unvaccinated pens. Hatched chicks were placed into eighteen pens, three repetitions for each treatment group. Four progeny studies were done by challenging chicks by oral gavage with *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Salmonella* Kentucky and *Salmonella* Heidelberg. Protection provided by vaccination of broiler breeders was assessed by bacterial culture of liver/spleen pool and ceca for *Salmonella* prevalence, most probable numbers (MPN) of ceca, and enzyme-linked immunosorbent assay (ELISA) using a combined SE/ST Biochek kit on serum and an IgA assay on crop and intestinal lavages. *Salmonella* prevalences and loads in broiler progeny were assessed by bacterial culture and MPN of ceca.

INDEX WORDS: Vaccination, Protection, Salmonella

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DEDICATION

This thesis is dedicated to God, my husband, my parents, and my sister. You have encouraged me and stood by me through every journey I decided to partake on. You will never know how grateful I am for your support and encouragement you have given me throughout the years.

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CHAPTER 1

INTRODUCTION

The Center for Disease Control and Prevention estimates that each year *Salmonella* is the cause of one million illnesses in the United States with 19,000 hospitalizations and 380 deaths (Centers for Disease Control and Prevention [CDC], 2015). Due to the rising number of human *Salmonella* infections each year, the USDA Food Safety and Inspection Service monitors and regulates procedures taken to reduce *Salmonella* incidence. Poultry has been implicated as the largest source of *Salmonella* illnesses and for this reason the poultry industry is trying to reduce the incidence of these organisms in poultry products (Young et al. 2007).

Salmonella can be reduced during all stages of poultry production. A major source of *Salmonella* in broilers is by vertical transmission from the broiler breeder, in turn causing horizontal transmission in the broiler houses (Heyndrick et al. 2002). *Salmonella* species found in the broiler processing plant have been traced to the parent broiler breeder flock and to the hatchery (Bailey et al. 2002). Since these findings, the poultry industry has focused on reducing *Salmonella* incidence in live production in order to also reduce the *Salmonella* numbers and overall load of organisms entering the processing plant (Young et al. 2007).

Vaccination is one of the most practical measures to reduce *Salmonella* contamination of poultry and to prevent disease in humans. Vaccination of chickens with killed bacterins has been shown to decrease intestinal colonization. *Salmonella* Enteritidis bacterins are effective at reducing colonization of internal organs as well as the intestine, however, it has only been shown to reduce shedding not eliminate *Salmonella* all together (Revolledo et al. 2012). Also, attenuated live vaccination has been shown to be effective in protecting against fecal shedding of *Salmonella* more than killed vaccine alone (Ghosh 1989, Timms et al. 1990, Gast et al. 1992, Gast et al. 1993, Smith 1956).

Commercial live and killed *Salmonella* vaccines have been proven to be effective in commercial poultry production. Past studies have shown maternal antibodies for *Salmonella* from vaccinated breeders can be transmitted to the progeny providing some protection to colonization. Transmission of the maternal antibodies not only reduces the number of positive colonization in chicks from vaccinated broiler breeders, but it has also been shown to reduce the colonization numbers, but this maybe dependent on the serotype of *Salmonella* used in the vaccine (Young et al. 2007). In broiler breeders the objective of vaccination is to reduce the chances of vertical transmission of *Salmonella* from the broiler breeders in or on the egg, and provide maternal antibodies to the progeny potentially reducing the prevalence and load of *Salmonella* in the broilers at processing (Berghaus et al. 2011).

Both live attenuated and killed *Salmonella* vaccines are used in broiler breeder flocks with some companies using both vaccine types in the same flocks. Due to cross-protection against different serogroups being limited, a common approach in commercial poultry is to vaccinate broiler breeders with a commercial water-in-oil-emulsion vaccine containing two or more *Salmonella* serotypes that are common in a company's poultry farms (Bailey et al. 2007).

Although field studies on commercial farms have been performed to evaluate the effectiveness of SE vaccination, few studies have been conducted where commercial and autogenous vaccines are compared for serological response as well as protection to challenge in parent hens and their offspring. The objective of this study was to compare efficacy of various

Salmonella bacterins following priming with two doses of live *Salmonella* Typhimurium vaccine in broiler breeders in a controlled setting (Table 1).

The broiler breeders were housed in a controlled setting of six pens. Five pens were vaccinated with two live *Salmonella* Typhimurium, and four of those five pens received inactivated *Salmonella* vaccine containing *Salmonella* Enteritidis, *Salmonella* Typhimurium and *Salmonella* Heidelberg. One pen was occupied with unvaccinated breeders that served as controls. At 10, 14, 20, 30 and 45 weeks of age twenty breeders from each treatment were challenged orally with *Salmonella* Enteritidis. Protection passed to progeny from the broiler breeders was assessed by collecting eggs from vaccinated and unvaccinated pens. Hatched chicks were placed into eighteen pens, three repetitions from each treatment group. Four progeny studies were done by orally gavaging chicks with *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Salmonella* Kentucky and *Salmonella* Heidelberg at day of age. Protection provided by vaccination of broiler breeders was assessed by bacterial culture of liver/spleen pool and ceca, MPN of ceca, ELISA serology on blood serum using Biochek SE/ST ELISA, and IgA ELISA on crop and intestinal lavage using an IgA ELISA assay. Salmonella prevalences and loads in broiler progeny were assessed by bacterial culture and MPN of ceca.

CHAPTER 2

MATERIALS AND METHODS

Broiler Breeders

In April 2012, 1500 day old broiler breeder females (Ross 708) and 50 males (Ross 344) were delivered to the Poultry Diagnostic and Research Center from Aviagen Inc., Huntsville, AL. These chicks were immunized with Marek's HVT-SB-1 vaccine and sprayed with Cocccivac D^{TM} (Merck Animal Health, Inc.), at day of age at primary breeder hatchery. Live *Salmonella* typhimurium vaccine containing a live avirulent strain of *Salmonella* Typhimurium manufactured by Ceva (serial number 382-59) was applied to all broiler breeders, except negative controls, at 3 days and 5 weeks of age via coarse spray. Prior to use, the live vaccine (10,000 doses) was reconstituted using 10 ml of sterile distilled water for each vial of lyophilized vaccine. The 3 vials of reconstituted vaccine was then diluted using 2.1 liters of sterile distilled water for a target dose of $8x10^7$, and titered to determine that the actual dose given was $2.1x10^7$. Killed *Salmonella* vaccine was administered via intramuscular injection in the breast at 10 and 16 weeks of age. These broiler breeders were fed daily. Feed was weighed and fed to the broiler breeders according to Aviagen recommendation based on body weight. Body weight was taken weekly from approximately 25% of the birds and the average body weight was used to calculate feed conversion. The day length was increased at 18 weeks of age to bring the pullets into production.

All broiler breeders were housed in one large concrete floor pen with soft wood shavings, except negative controls, until 10 weeks of age. The negative controls were housed in a large concrete floor pen in a separate building on softwood shavings to prevent spread of the live vaccine. All birds were housed and managed under identical conditions. At ten weeks the broiler breeders were moved into 6 individual concrete floor pens, one for each treatment group, with soft wood shavings. Five pens were vaccinated with two live *Salmonella* Typhimurium and killed *Salmonella* vaccine containing *Salmonella* Enteritidis, *Salmonella* Typhimurium and *Salmonella* Heidelberg, and one pen was occupied with unvaccinated pullets that served as negative controls. Treatment 1 received two doses live Ceva Salmune *Salmonella* Typhimurium vaccines at 3 days and 5 weeks of age and Ceva Layermune *Salmonella* Enteritidis vaccine at 10 weeks, treatment 2 received two doses of live Ceva Salmune *Salmonella* Typhimurium vaccines at 3 days and 5 weeks of age and Ceva Layermune *Salmonella* Enteritidis vaccine at 10 weeks and 16 weeks, treatment 3 received two doses of live Ceva Salmune *Salmonella* Typhimurium vaccines at 3 days and 5 weeks of age, Ceva Layermune *Salmonella* Enteritidis vaccine at 10 weeks and a Ceva Autogenous containing *Salmonella* Enteritidis, *Salmonella* Typhimurium, and *Salmonella* Heidelberg at 16 weeks, treatment 4 received two doses live Ceva Salmune *Salmonella* Typhimurium vaccines at 3 days and 5 weeks of age and Lohmann Animal Health Avipro 109 SE4 *Salmonella* Enteritidis vaccine at 10 weeks and 16 weeks, treatment 5 received two doses of live Ceva Salmune *Salmonella* Typhimurium vaccines at 3 days and 5 weeks of age, and treatment 6 were unvaccinated controls that were not vaccinated. Treatment groups are listed in Table 1.

Table 1. Broiler breeder vaccination groups

Treatment	Vaccination	SE Challenge
1	2 Live Salmune ST+ Layermune SE (0.25 ml) 10 wks	10, 14, 20, 30 and 45 WOA
2	2 Live Salmune ST+ Layermune SE (0.25 ml) 10 wks+16 wks	10, 14, 20, 30 and 45 WOA
3	2 Live Salmune ST+ Layermune SE (0.25 ml) 10 wks+ Ceva Autogenous (SE, ST, SH) 16 wks	10, 14, 20, 30 and 45 WOA
4	2 Live Salmune ST+ Avipro109 SE4 (0.25 ml) 10 wks+16 wks	10, 14, 20, 30 and 45 WOA
5	2 Live Salmune ST	10, 14, 20, 30 and 45 WOA
6	Non-vaccinated	10, 14, 20, 30 and 45 WOA

Environmental sampling was done using Tubigrip bootsocks every 4 weeks to ensure the birds remained *Salmonella* negative. Tubigrip bootsocks were submerged into 100ml buffered peptone water (BPW) to moisten, and two bootsocks were taken in each pen. Bootsocks were placed on top of plastic boots and walked over all areas of each pen. After all samples were collected, each bootsock was placed back into whrilpak bag containing 100 ml of BPW, 10 ml of 10X tetrathionate brilliant green broth and 2 ml of iodine was added to each bootsock which was then incubated for 24 hours at 42^{o} C. Following incubation, each sample was plated to xylose lysine tergitol-4 (XLT-4) agar plates, and then plates were incubated for 24 hours at 37^{o} C. Any *Salmonella* suspect colonies were subbed to blood agar and then incubated overnight at 37^{o}C for *Salmonella* identification and serogrouping.

Salmonella challenge was performed by oral gavage with Nalidixic Acid resistant (25 µg/ml) *Salmonella* Enteritidis at 10, 14, 20, 30 and 45 weeks of age. *Salmonella* Enteritidis challenge was prepared by streaking isolate (frozen in trypticase soy broth and glycerol 1:1) to blood agar and incubating overnight at 37^{o}C. Using one colony of fresh *Salmonella* Enteritidis culture 3 ml of brain heart infusion (BHI) broth was inoculated and incubated overnight at 37^{0}C, then 0.1 ml of overnight culture was transferred into 20 ml BHI broth and incubated for approximately 2 hours. After 2 hours the optical density (OD) at 600nm wavelength was checked every 30 minutes until an OD_{600} of 0.5 was reached. The target dose of the culture at 0.5 OD_{600} was $1x10^8$. Once the culture reached the target OD_{600} it was then diluted 1:100, using sterile phosphate buffered saline (PBS), making the inoculum a target of $1x10^6$ cfu/ml. Using sterile PBS the inoculum was titered to determine the actual dose given to the broiler breeders. Each bird was given 1 ml of $1x10^6$ cfu/ml inoculum containing *Salmonella* Enteritidis. Necropsy was performed 1 week post challenge with collection of liver/spleen pool, ceca, and ovaries. Liver/spleen pool and ovaries and ceca were cultured for prevalence, and MPN (most probable numbers) was also performed on ceca to analyze the load of *Salmonella* Enteritidis in each bird. Samples were collected in sterile Whirl Pack bags. Upon arrival ceca were weighed in order to calculate the total *Salmonella* cfu/g on MPNs. After ceca weights were collected tetrathionate brilliant green broth with iodine was added to all samples making an approximate 1:10 and stomached for 30 seconds. A 1 ml aliquot was removed for MPN analysis. Samples were incubated overnight at 42°C. After overnight incubation 10ul of incubated sample was struck onto XLT-4 plates containing 25µg/ml of Nalidixic acid and incubated overnight at 37°C. Up to 3 black *Salmonella* suspect colonies were selected and confirmed as *Salmonella* Enteritidis using Poly-O and Group D *Salmonella* specific antiserum.

Salmonella prevalences were compared between treatments and broiler breeder ages using a logistic regression model with age and treatment as fixed effects. Interactions between age and treatment were not evaluated because cell prevalences of 0% or 100% precluded the estimation of interaction terms. Log_{10} transformed *Salmonella* MPNs were compared between treatments and broiler breeder ages using two-way analysis of variance. For the comparison of

Salmonella MPNs, samples with a negative culture result by the MPN method but a positive culture result by enrichment were arbitrarily assigned an MPN value equal to one half the minimum detection limit of the MPN assay. Pairwise comparisons were performed using the Bonferroni procedure to limit the type I error probability to 5% over all comparisons. All tests assumed a two-sided alternative hypothesis and $P < 0.05$ was considered statistically significant. Analyses were performed using commercially available statistical software (Stata version 14, StataCorp LP, College Station, TX).

The enumeration and micro-MPN method used on ceca samples allows for calculation of cfu/g of ceca. A 1 ml sample of stomached ceca was transferred to 3 adjacent wells, triplicate of each sample, in the first row of a 96 well 2 ml deep block. A 0.1ml aliquot of sample was transferred to 0.9ml of tetrathionate brilliant green broth containing iodine in the second row. This process was repeated for the remaining rows (producing 5 ten-fold dilutions). Blocks were incubated overnight at 42^{o}C. A 1 µl sample from each well of incubated blocks was transferred onto XLT-4 agar using a pin-tool replicator and plates were incubated overnight at 37^{o}C. Incubated plates produce black colonies and the final dilution of each sample was recorded and entered into the MPN calculator to calculate the cfu/g of *Salmonella* in each ceca sample. Figure 1 shows the MPN block layout.

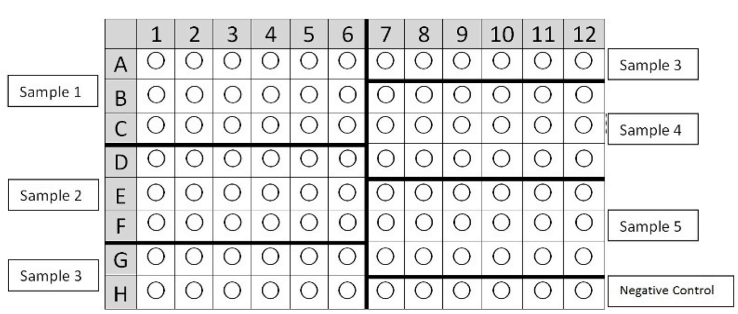

Figure 1. MPN block layout

Vaccine induced immune response evaluation was assessed by serology using SE/ST Biochek ELISA. Blood was collected from the broiler breeders at 10, 11, 14, 15, 18, 21, 45 and 46 weeks of age into EDTA tubes. All plasma was stored at -20^{o}C and all ELISA's were run at one time to minimize variability. In addition, only one lot number of ELISA kit was used. ELISA's were performed by following the protocol provided in the Biochek ELISA kit.

Crop and intestinal lavage was performed on 10 pullets post challenge at 1 day, 11, 15, 21, and 46 weeks of age for IgA ELISAs. The lavage was done using a Tris-Glycine+Tween buffer. For the crop lavage, five milliliters of buffer was injected into the crop lumen via a 10 ml syringe and a 14-gauge needle, the solution was aspirated and injected several times to flush the secretions from the crop wall, and the contents were collected back into the syringe and dispelled into a tube. For the intestinal lavage, small intestines from the jejunum to the duodenum were pulled from birds. Five ml of buffer was injected into one end of the intestine and contents were caught into a tube at the other end of the intestine. All lavage samples were stored at -20^{o}C until analysis. For IgA ELISA assay the crop and intestinal samples were thawed and serial two-fold dilutions using phosphate-buffered saline containing 0.05% Tween 20 were made of the neat samples. The dilutions were added to 96 well enzyme-linked immunosorbent assay trays to which 10ug/ml *Salmonella* Enteritidis lipopolysaccharide had been adsorbed. Following room temperature incubation for 60 minutes the plates were washed three times and then assayed for IgA anti-*Salmonella* Enteritidis antibodies (Holt et al. 2002).

Progeny

Eggs were collected and hatched from broiler breeders 30 weeks of age and older to analyze *Salmonella* colonization protection provided by maternal antibodies passed from the parents to the progeny. Eggs were hatched at PDRC hatchery. Chicks from broiler breeders were challenged by oral gavage with $1x10^5$ cfu/ml per bird of *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Salmonella* Heidelberg and *Salmonella* Kentucky at day of age. All *Salmonella* strains used were resistant to 25 μg/ml of nalidixic acid. At each challenge there were three repetitions of each treatment group with 20 birds per repetition. Chick papers and drag swabs were taken at day of challenge for environmental samples to ensure no *Salmonella* was present in the hatches. Drag swabs were also taken at 7 days post challenge and the week of necropsy. At 42 days of age 12 birds per pen were necropsied for *Salmonella* isolation and 5 of the 12 for enumeration by MPN on cecas. Progeny ceca samples were processed and analyzed using the same methods as used for the broiler breeders.

Salmonella prevalences were compared between treatment groups for each hen age and *Salmonella* challenge serotype using a generalized estimating equations (GEE) logistic model to account for the correlation of responses between birds from the same pen. Log-transformed *Salmonella* MPNs were compared between treatment groups using linear mixed models with pen as a random effect. For the comparison of *Salmonella* MPNs, samples with a negative culture result by the MPN method but a positive culture result by enrichment were arbitrarily assigned an MPN value equal to one half the minimum detection limit of the MPN assay. Pairwise comparisons were performed using the Bonferroni procedure to limit the type I error probability to 5% over all comparisons. All tests assumed a two-sided alternative hypothesis and $P < 0.05$

was considered statistically significant. Analyses were performed using commercially available statistical software (Stata version 14, StataCorp LP, College Station, TX).

CHAPTER 3

RESULTS

Broiler Breeders

Salmonella Enteritidis Prevalences

Ceca. *Salmonella* Enteritidis prevalences in ceca are summarized in Table 2. Overall, there were significant differences between ages ($P < 0.001$) and between treatments ($P < 0.001$). The marginal prevalences at weeks 11 and 21 were similar to each other but lower than any other ages; prevalences at weeks 15 and 31 were similar to each other but higher than any other ages; and prevalences at 46 weeks were significantly different from all other ages. With respect to the treatments, the marginal prevalence of the Salmune ST + Avipro SE 10+16 group was similar to that of the Salmune ST + Layermune + Ceva Autogenous SE, ST, SH 10+16 group but was significantly lower than all other treatments. The marginal prevalence of the non-vaccinated group was significantly higher than that of the Salmune ST + Avipro SE 10+16 and Salmune ST + Layermune + Ceva Autogenous SE, ST, SH 10+16 treatments. The non-vaccinated group had a total of 83 out of 98, or 84.7% positive samples, whereas, the Salmune ST + Avipro SE 10+16 group had a total of 58 out of 99, or 58.6% positive samples, and the Salmune ST + Layermune + Ceva Autogenous SE, ST, SH 10+16 group had 69 out of 99, or 69.7% positive samples.

At 11, 15, 21, 31, and 46 weeks of age twenty broiler breeders from each treatment group were challenged by oral gavage with $1x10^6$ cfu/ml of *Salmonella* Enteritidis. At 11 weeks of age, prevalence of *Salmonella* Enteritidis in the ceca was:

- 2 Live Salmune ST + Layermune SE 10: 10 out of 20, or 50% positive samples

- 2 Live Salmune ST + Layermune SE 10+16: 9 out of 20, or 45% positive samples
- 2 Live Salmune ST + Layermune SE + Ceva Autogenous SE/ST/SH 10+16: 8 out of 20, or 40% positive samples
- 2 Live Salmune ST + Avipro SE 10+16: 4 out of 20, or 20% positive samples
- 2 Live Salmune ST: 10 out of 20, or 50% positive samples
- Novaccinated: 10 out of 20, or 50% positive samples.

At 15 weeks of age prevalence of *Salmonella* Enteritidis in the ceca was:

- 2 Live Salmune ST + Layermune SE 10: 19 out of 19, or 100% positive samples
- 2 Live Salmune ST + Layermune SE 10+16: 19 out of 20, or 95% positive samples
- 2 Live Salmune ST + Layermune SE + Ceva Autogenous SE/ST/SH 10+16: 18 out of 19, or 94.7% positive samples
- 2 Live Salmune ST + Avipro SE 10+16 group had 19 out of 19, or 100% positive samples
- 2 Live Salmune ST group had 18 out of 18, or 100% positive samples
- Novaccinated :18 out of 18, or 100% positive samples.

At 21 weeks of age prevalence of *Salmonella* Enteritidis in the ceca was:

- 2 Live Salmune ST + Layermune SE 16: 14 out of 20, or 70% positive samples
- 2 Live Salmune ST + Layermune SE 10+16: 14 out of 20, or 70% positive samples
- 2 Live Salmune ST + Layermune SE + Ceva Autogenous SE/ST/SH 10+16: 12 out of 20, or 60% positive samples
- 2 Live Salmune ST + Avipro SE 10+16: 2 out of 20, or 10% positive samples;
- 2 Live Salmune ST : 12 out of 20, or 60% positive samples

- Novaccinated: 16 out of 20, or 80% positive samples.

At 31 weeks of age prevalence of *Salmonella* Enteritidis in the ceca was:

- 2 Live Salmune ST + Layermune SE 10 group had 20 out of 20, or 100% positive samples
- 2 Live Salmune ST + Layermune SE 10+16 group had 18 out of 20, or 90% positive samples
- 2 Live Salmune ST + Layermune SE + Ceva Autogenous SE/ST/SH 10+16 group had 18 out of 20, or 90% positive samples
- 2 Live Salmune ST + Avipro SE 10+16: 19 out of 20, or 95% positive samples
- 2 Live Salmune ST group had 19 out of 20, or 95% positive samples
- Novaccinated: 20 out of 20, or 100% positive samples.

At 46 weeks of age prevalence of *Salmonella* Enteritidis in the ceca was:

- 2 Live Salmune ST + Layermune SE 10: 12 out of 20, or 60% positive samples
- 2 Live Salmune ST + Layermune SE 10+16: 15 out of 20, or 75% positive samples
- 2 Live Salmune ST + Layermune SE + Ceva Autogenous SE/ST/SH 10+16: 13 out of 20, or 65% positive samples
- 2 Live Salmune ST + Avipro SE 10+16:14 out of 20, or 70% positive samples
- 2 Live Salmune ST: 17 out of 20, or 85% positive samples
- Novaccinated group had 19 out of 20, or 95% positive samples

Table 2. *Salmonella* Enteritidis prevalences (%) in ceca of broiler breeders by age and treatment group.

	Age (weeks)					
Treatment	11	15	21	31	46	Total
2 Live Salmune ST +Layermune SE 10	10/20 (50.0)	19/19 (100)	14/20 (70.0)	20/20 (100)	12/20 (60.0)	75/99 (75.8)[b,c]
2 Live Salmune ST +Layermune SE 10+16	9/20 (45.0)	19/20 (95.0)	14/20 (70.0)	18/20 (90.0)	15/20 (75.0)	75/100 (75.0)[b,c]
2 Live Salmune ST +Layermune SE+Ceva Autogenous SE/ST/SH 10+16	8/20 (40.0)	18/19 (94.7)	12/20 (60.0)	18/20 (90.0)	13/20 (65.0)	69/99 (69.7)[a,b]
2 Live Salmune ST +Avipro SE 10+16	4/20 (20.0)	19/19 (100)	2/20 (10.0)	19/20 (95.0)	14/20 (70.0)	58/99 (58.6)[a]
2 Live Salmune ST	10/20 (50.0)	18/18 (100)	12/20 (60.0)	19/20 (95.0)	17/20 (85.0)	76/98 (77.6)[b,c]
Nonvacc	10/20 (50.0)	18/18 (100)	16/20 (80.0)	20/20 (100)	19/20 (95.0)	83/98 (84.7)[c]
Total	51/120 (42.5)[a]	111/113 (98.2)[c]	70/120 (58.3)[a]	114/120 (95.0)[c]	90/120 (75.0)[b]	436/593 (73.5)

Marginal percentages with a superscript in common do not differ with a level of significance of 5% over all comparisons.

Liver/Spleen. *Salmonella* Enteritidis prevalences in liver/spleen samples are summarized in Table 3. There were significant differences between ages ($P < 0.001$) and between treatments ($P < 0.001$). The marginal prevalences at weeks 11, 21, and 46 were similar to each other but were significantly lower than the prevalences at 15 and 31 weeks. With respect to treatments, the marginal prevalences of the 2 Live Salmune ST +Layermune SE 10, 2 Live Salmune ST

+Layermune SE 10+16, 2 Live Salmune ST +Layermune SE+Ceva Autogenous SE/ST/SH 10+16, and 2 Live Salmune ST +Avipro SE 10+16 groups were similar to each other but were significantly lower than the prevalences of the 2 Live Salmune ST and non-vaccinated groups.

At 11 weeks of age, prevalence of *Salmonella* Enteritidis in the liver/spleen was:

- 2 Live Salmune ST + Layermune SE 10: 2 out of 20, or 10% positive samples
- 2 Live Salmune ST + Layermune SE 10+16: 0 out of 20, or 0% positive samples
- 2 Live Salmune ST + Layermune SE + Ceva Autogenous SE/ST/SH 10+16: 3 out of 20, or 15% positive sample
- 2 Live Salmune ST + Avipro SE 10+16: 2 out of 20, or 10% positive samples
- 2 Live Salmune ST: 2 out of 20, or 10% positive samples
- Novaccinated: 6 out of 20, or 30% positive samples.

At 15 weeks of age, prevalence of *Salmonella* Enteritidis in the liver/spleen was:

- 2 Live Salmune ST + Layermune SE 10: 8 out of 19, or 42.1% positive samples
- 2 Live Salmune ST + Layermune SE 10+16: 6 out of 20, or 30% positive samples
- 2 Live Salmune ST + Layermune SE + Ceva Autogenous SE/ST/SH 10+16: 6 out of 19, or 31.6% positive samples
- 2 Live Salmune ST + Avipro SE 10+16: 2 out of 19, or 10.5% positive samples
- 2 Live Salmune ST: 12 out of 18, or 66.7% positive samples
- Non-vaccinated: 17 out of 18, or 94.4% positive samples

At 21 weeks of age, prevalence of *Salmonella* Enteritidis in the liver/spleen was:

- 2 Live Salmune ST + Layermune SE 16: 0 out of 20, or 0% positive samples
- 2 Live Salmune ST + Layermune SE 10+16: 0 out of 20, or 0% positive samples

- 2 Live Salmune ST + Layermune SE + Ceva Autogenous SE/ST/SH 10+16: 1 out of 20, or 5% positive samples
- 2 Live Salmune ST + Avipro SE 10+16: 0 out of 20, or 0% positive samples
- 2 Live Salmune ST: 1 out of 20, or 5% positive samples
- Non-vaccinated: 5 out of 20, or 25% positive samples

At 31 weeks of age, prevalence of *Salmonella* Enteritidis in the liver/spleen was:

- 2 Live Salmune ST + Layermune SE 10: 11 out of 20, or 55% positive samples
- 2 Live Salmune ST + Layermune SE 10+16: 10 out of 20, or 50% positive samples
- 2 Live Salmune ST + Layermune SE + Ceva Autogenous SE/ST/SH 10+16: 6 out of 20, or 30% positive samples
- 2 Live Salmune ST + Avipro SE 10+16: 4 out of 20, or 20% positive samples
- 2 Live Salmune ST: 18 out of 20, or 90% positive samples
- Non-vaccinated: 19 out of 20, or 95% positive samples.

At 46 weeks of age, prevalence of *Salmonella* Enteritidis in the liver/spleen was:

- 2 Live Salmune ST + Layermune SE 10: 1 out of 20, or 5% positive samples
- 2 Live Salmune ST + Layermune SE 10+16: 0 out of 20, or 0% positive samples
- 2 Live Salmune ST + Layermune SE + Ceva Autogenous SE/ST/SH 10+16: 1 out of 20, or 5% positive samples
- 2 Live Salmune ST + Avipro SE 10+16: 1 out of 20, or 5% positive samples
- 2 Live Salmune ST: 8 out of 20, or 40% positive samples
- Non-vaccinated group had 8 out of 20, or 40% positive samples (Table 3).

Table 3. *Salmonella* Enteritidis prevalences (%) in combined liver and spleen samples of broiler breeders by age and treatment group.

	Age (weeks)					
Treatment	11	15	21	31	46	Total
2 Live Salmune ST +Layermune SE 10	2/20 (10.0)	8/19 (42.1)	0/20 (0.0)	11/20 (55.0)	1/20 (5.0)	22/99 (22.2)[a]
2 Live Salmune ST +Layermune SE 10+16	0/20 (0.0)	6/20 (30.0)	0/20 (0.0)	10/20 (50.0)	0/20 (0.0)	16/100 (16.0)[a]
2 Live Salmune ST +Layermune SE+Ceva Autogenous SE/ST/SH 10+16	3/20 (15.0)	6/19 (31.6)	1/20 (5.0)	6/20 (30.0)	1/20 (5.0)	17/99 (17.2)[a]
2 Live Salmune ST +Avipro SE 10+16	2/20 (10.0)	2/19 (10.5)	0/20 (0.0)	4/20 (20.0)	1/20 (0.05)	9/99 (9.1)[a]
2 Live Salmune ST	2/20 (10.0)	12/18 (66.7)	1/20 (5.0)	18/20 (90.0)	8/20 (40.0)	41/98 (41.8)[b]
Nonvacc	6/20 (30.0)	17/18 (94.4)	5/20 (25.0)	19/20 (95.0)	8/20 (40.0)	55/98 (56.1)[b]
Total	15/120 (12.5)[a]	51/113 (45.1)[b]	7/120 (5.8)[a]	68/120 (56.7)[b]	19/120 (15.8)[a]	160/593 (27.0)

Marginal percentages with a superscript in common do not differ with a level of significance of 5% over all comparisons.

Ovaries. *Salmonella* Enteritidis prevalences in ovaries are summarized in Table 4. There was no significant difference between ages ($P = 0.205$) or treatments ($P = 0.744$). Ovaries were only collected at 31 and 46 weeks of age. At 31 weeks of age, prevalence of *Salmonella* Enteritidis in the ovaries was:

- 2 Live Salmune ST + Layermune SE 10: 1 out of 20, or 5% positive samples
- 2 Live Salmune ST + Layermune SE 10+16: 3 out of 20, or 15% positive samples
- 2 Live Salmune ST + Layermune SE + Ceva Autogenous SE/ST/SH 10+16: 0 out of 20, or 0% positive samples
- 2 Live Salmune ST + Avipro SE 10+16: 0 out of 20, or 0% positive samples

- 2 Live Salmune ST: 2 out of 20, or 10% positive samples
- Nom-vaccinated: 1 out of 20, or 5% positive samples

At 46 weeks of age, prevalence of *Salmonella* Enteritidis in the ovaries was:

- 2 Live Salmune ST + Layermune SE 10: 0 out of 20, or 0% positive samples
- 2 Live Salmune ST + Layermune SE 10+16: 0 out of 20, or 0% positive samples
- 2 Live Salmune ST + Layermune SE + Ceva Autogenous SE/ST/SH 10+16: 0 out of 20, or 0% positive samples
- 2 Live Salmune ST + Avipro SE 10+16: 1 out of 20, or 5% positive samples
- 2 Live Salmune ST: 0 out of 20, or 0% positive samples
- Non-vaccinated: 2 out of 20, or 10% positive samples

There were no significant differences in the overall prevalences of *Salmonella* Enteritidis in the ovaries between treatment groups.

Table 4. *Salmonella* Enteritidis prevalences (%) in ovaries of broiler breeders by age and treatment group.

	Age (weeks)					
Treatment	11	15	21	31	46	Total
2 Live Salmune ST +Layermune SE 10	NC	NC	NC	1/20 (5.0)	0/20 (0.0)	1/40 (2.5)[a]
2 Live Salmune ST +Layermune SE 10+16	NC	NC	NC	3/20 (15.0)	0/20 (0.0)	3/40 (7.5)[a]
2 Live Salmune ST +Layermune SE+Ceva Autogenous SE/ST/SH 10+16	NC	NC	NC	0/20 (0.0)	0/20 (0.0)	0/40 (0.0)[a]
2 Live Salmune ST +Avipro SE 10+16	NC	NC	NC	0/20 (0.0)	1/20 (5.0)	1/40 (2.5)[a]
2 Live Salmune ST	NC	NC	NC	2/20 (10.0)	0/20 (0.0)	2/40 (5.0)[a]
Nonvacc	NC	NC	NC	1/20 (5.0)	2/20 (10.0)	3/40 (7.5)[a]
Total	NC	NC	NC	7/120 (5.8)[a]	3/120 (2.5)[a]	10/240 (4.2)

NC – Not collected. Marginal percentages with a superscript in common do not differ with a level of significance of 5% over all comparisons.

Salmonella Enteritidis *MPNs*

Ceca. $\log_{10}$ *Salmonella* Enteritidis MPNs are summarized in Table 5, and the distribution of MPNs is illustrated in Figure 2. There were significant differences between ages ($P < 0.001$) and between treatments ($P < 0.001$). The marginal mean $\log_{10}$ MPN at 21 weeks was significantly lower than all other ages, and the mean $\log_{10}$ MPN at 31 weeks was significantly higher than all other ages. The marginal means at 11, 15, and 46 weeks did not differ from one another. With respect to the treatments, the marginal mean of the 2 Live Salmune ST +Avipro SE 10+16 group was significantly lower than that of the 2 Live Salmune ST and non-vaccinated groups. The mean of the non-vaccinated group was significantly higher than that of all the other treatments.

Table 5. Summary of $\log_{10}$ *Salmonella* Enteritidis MPN/ceca in broiler breeders by age and treatment group. Numbers are the mean, standard deviation, and sample size, respectively.

	Age (weeks)					
Treatment	11	15	21	31	46	Total
2 Live Salmune ST +Layermune SE 10	2.18	2.98	1.68	4.31	2.54	$2.91^{a,b}$
	1.23	1.24	1.47	1.53	1.37	1.65
	10	19	14	20	12	75
2 Live Salmune ST +Layermune SE 10+16	2.34	3.24	1.50	4.13	2.17	$2.81^{a,b}$
	1.51	1.15	1.58	1.63	1.06	1.65
	9	19	14	18	15	75
2 Live Salmune ST +Layermune SE+Ceva Autogenous SE/ST/SH 10+16	3.33	2.30	1.48	3.86	3.44	$2.90^{a,b}$
	2.06	1.22	1.66	1.31	1.60	1.71
	8	18	12	18	13	69
2 Live Salmune ST +Avipro SE 10+16	3.08	2.37	1.25	3.49	2.14	2.69^{a}
	1.99	1.38	0.00	1.90	1.17	1.63
	4	19	2	19	14	58
2 Live Salmune ST	2.69	2.64	1.51	5.58	3.21	3.33^{b}
	1.78	1.00	0.83	1.22	1.53	1.89
	10	18	12	19	17	76
Nonvacc	3.52	4.83	3.04	5.69	4.46	4.45^{c}
	1.58	1.60	1.56	1.03	1.56	1.71
	10	18	16	20	19	83
Total	2.82^{b}	3.06^{b}	1.88^{a}	4.53^{c}	3.08^{b}	3.23
	1.66	1.51	1.54	1.66	1.62	1.81
	51	111	70	114	90	436

Marginal means with a superscript in common do not differ with a level of significance of 5% over all comparisons.

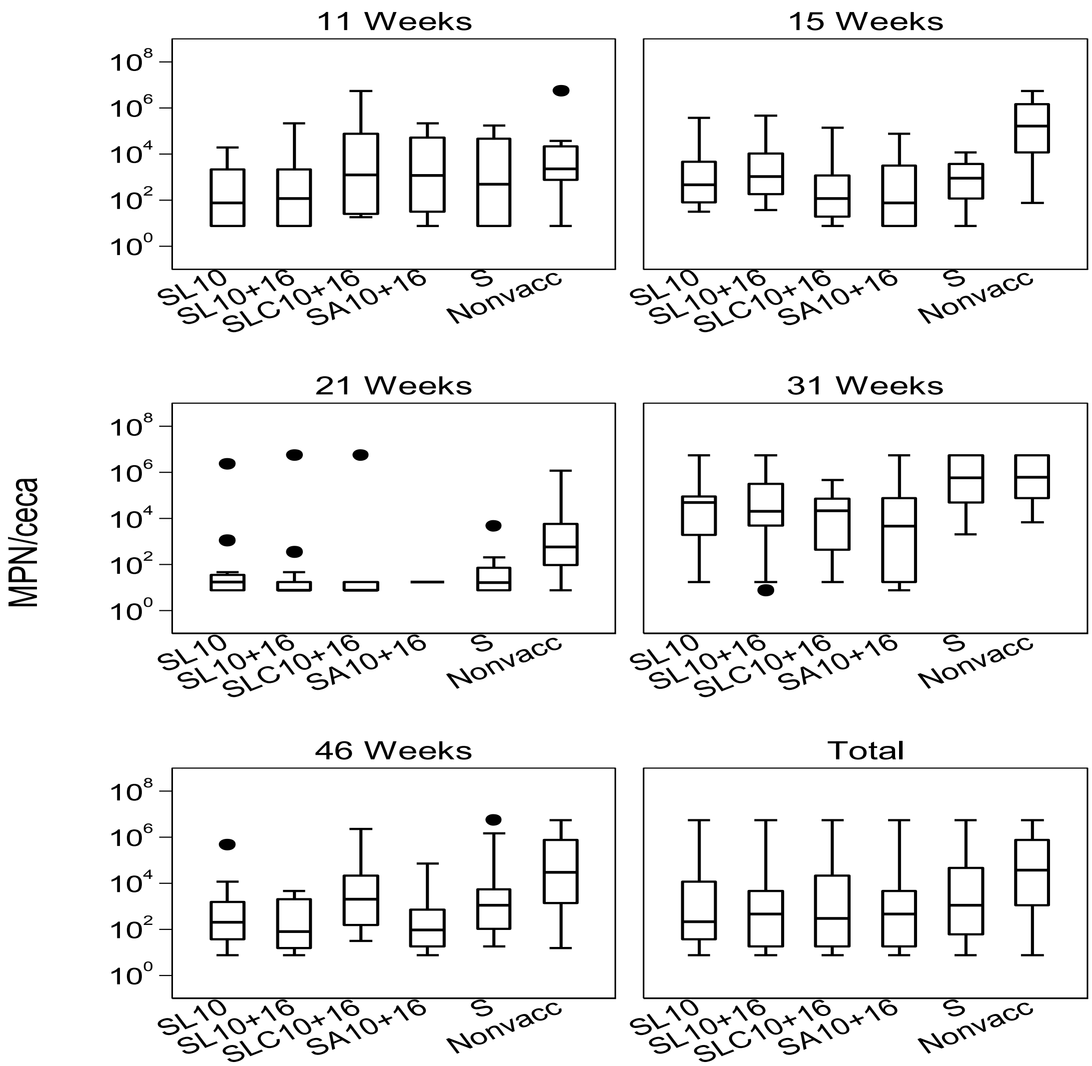

Figure 2. Boxplots of *Salmonella* Enteritidis MPN/ceca for breeder hens by age and treatment group. See Table 5 for sample sizes. Treatments are defined as follows: SL10 (2 Live Salmune + Layermune SE 10 weeks); SL 10+16 (2 Live Salmune + Layermune SE 10 weeks and 16 weeks); SLC10+16 (2 Live Salmune + Layermune SE 10 weeks + Ceva Autogenous SE/ST/SH 16 weeks); SA10+16 (2 Live Salmune + Avipro 109 SE4 10 weeks and 16 weeks); S (2 Live Salmune); Nonvacc (Non-vaccinated).

Serology

Salmonella Biochek SE/ST ELISA *titers*. Geometric mean *Salmonella* ELISA titers are summarized in Table 6, and the distributions of *Salmonella* ELISA titers are illustrated in Figures 3 and 4. Titers differed significantly between treatments at all time points ($P < 0.001$). Results of pairwise comparisons between treatments are summarized in Table 6.

Salmonella titers in the 2 Live Salmune ST and non-vaccinated groups remained lower than the other vaccinated groups throughout the study. There was an increase in titers at 12 weeks of age in the 2 Live Salmune ST + Layermune SE 10+16, 2 Live Salmune ST + Layermune SE + Ceva Autogenous SE/ST/SH 10+16, and 2 Live Salmune ST + Avipro SE 10+16 groups and a smaller peak in the 2 Live Salmune ST + Layermune SE 10 group. Titers also increased at 15 and 18 weeks of age and continued to increase at 21 weeks for the 2 Live Salmune ST + Layermune SE + Ceva Autogenous SE/ST/SH 10+16, and 2 Live Salmune ST + Avipro SE 10+16 groups. After 21 weeks of age ELISA titers in all groups leveled out and remained fairly consistent through the end of the study.

Table 6. Geometric mean *Salmonella* Biochek SE/ST ELISA titers (95% CI) by treatment group and week of age for 20 birds in each group.

	Treatment						
Age (weeks)	SL10	SL10+16	SLC10+16	SA10+16	S	Nonvacc	†P-value
10	244[b] (172; 317)	204[a,b] (155; 270)	284[b] (209; 386)	186[a,b] (127; 275)	274[b] (207; 362)	106[a] (77; 147)	< 0.001
11	274[a,b] (190; 396)	221[a] (152; 322)	227[a] (168; 306)	595[b,c] (489; 723)	736[c] (548; 988)	55[a] (20; 149)	< 0.001
12	841[b,c] (270; 2620)	2606[c] (2064; 3290)	1760[c] (740; 4185)	2617[c] (1078; 6353)	368[a,b] (278; 488)	53[a] (26; 110)	< 0.001
15	3317[b] (2861; 3847)	3489[b] (3098; 3930)	2288[b] (972; 5386)	3096[b] (1272; 7535)	384[a] (184; 802)	234[a] (96; 570)	< 0.001
18	5228[b] (4386; 6230)	3807[b] (2248; 6446)	2288[b] (662; 7912)	1150[b] (247; 5352)	238[a] (136; 415)	224[a] (101; 496)	< 0.001
21	3953[b] (2977; 5250)	1965[b] (583; 6629)	4550[b] (4191; 4941)	5272[b] (4961; 5603)	166[a] (89; 309)	425[a] (322; 562)	< 0.001
28	2249[b] (1688; 2997)	2887[b] (2375; 3510)	2841[b] (2398; 3366)	4917[c] (3641; 6642)	639[a] (415; 982)	280[a] (201; 390)	< 0.001
31	3788[b] (3014; 4760)	4898[b] (4191; 5725)	4184[b] (3495; 5010)	5985[b] (4687; 7642)	436[a] (205; 925)	603[a] (442; 824)	< 0.001
43	2822[b,c] (2036; 3914)	4764[c,d] (3541; 6411)	4793[c,d] (3488; 6585)	7502[d] (5608; 10036)	334[a] (166; 674)	782[a,b] (553; 1106)	< 0.001
46	2457[b] (1875; 3219)	3187[b,c] (2514; 4040)	3011[b,c] (2206; 4110)	5234[c] (3444; 7954)	441[a] (303; 640)	603[a] (476; 764)	< 0.001

†Kruskal-Wallis test of treatment effects.

Within rows, treatments with a superscript in common do not differ with a level of significance of 5% over all comparisons. Treatments are defined as follows: SL10 (2 Live Salmune + Layermune SE 10 weeks); SL 10+16 (2 Live Salmune + Layermune SE 10 weeks and 16 weeks); SLC10+16 (2 Live Salmune + Layermune SE 10 weeks + Ceva Autogenous SE/ST/SH 16 weeks); SA10+16 (2 Live Salmune + Avipro 109 SE4 10 weeks and 16 weeks); S (2 Live Salmune); Nonvacc (Non-vaccinated).

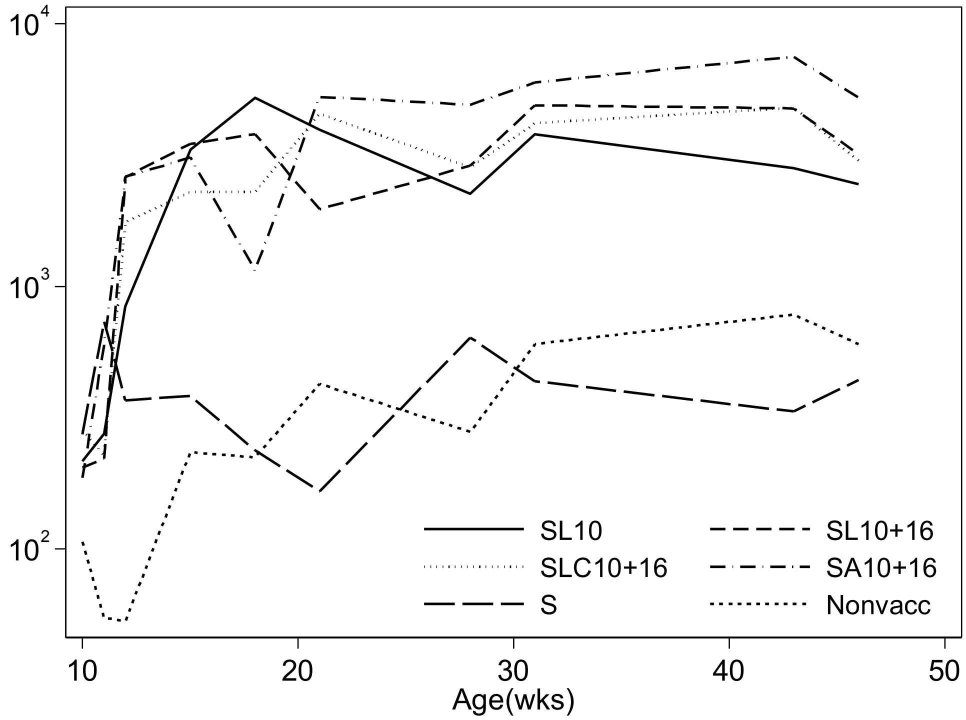

Figure 3. Geometric mean *Salmonella* Biochek SE/ST ELISA titers by treatment group and week of age for n = 20 birds per treatment. Treatments are defined as follows: SL10 (2 Live Salmune + Layermune SE 10 weeks); SL 10+16 (2 Live Salmune + Layermune SE 10 weeks and 16 weeks); SLC10+16 (2 Live Salmune + Layermune SE 10 weeks + Ceva Autogenous SE/ST/SH 16 weeks); SA10+16 (2 Live Salmune + Avipro 109 SE4 10 weeks and 16 weeks); S (2 Live Salmune); Nonvacc (Non-vaccinated).

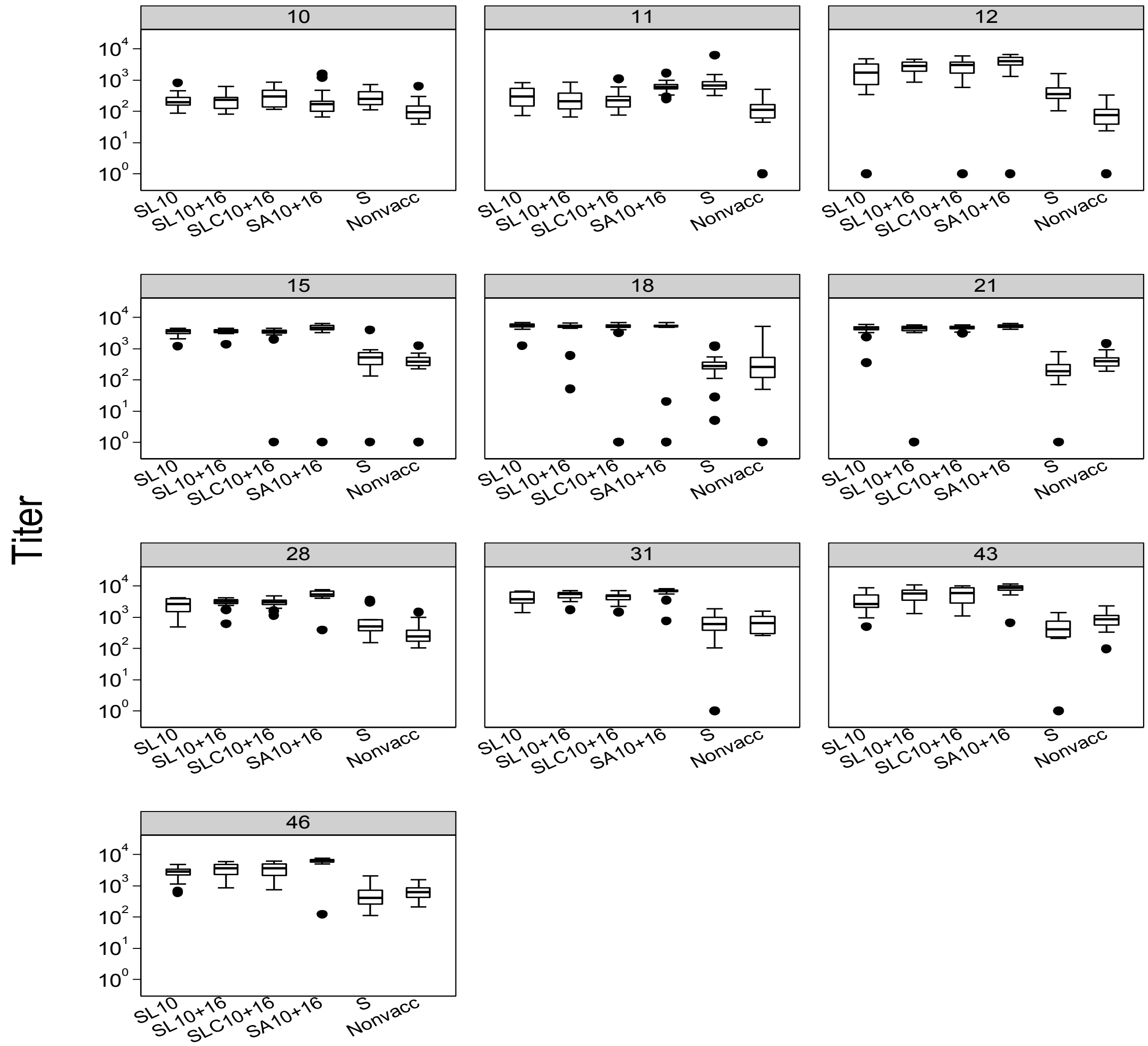

Figure 4. Boxplots of *Salmonella* Biochek SE/ST ELISA titers by treatment group and week of age for n = 20 birds per treatment. Treatments are defined as follows: SL10 (2 Live Salmune + Layermune SE 10 weeks); SL 10+16 (2 Live Salmune + Layermune SE 10 weeks and 16 weeks); SLC10+16 (2 Live Salmune + Layermune SE 10 weeks + Ceva Autogenous SE/ST/SH 16 weeks); SA10+16 (2 Live Salmune + Avipro 109 SE4 10 weeks and 16 weeks); S (2 Live Salmune); Nonvacc (Non-vaccinated).

Crop IgA ELISA. Optical densities for the crop IgA ELISA are summarized in Table 7, and the distributions of optical densities for the crop IgA ELISA are illustrated in Figure 5. The optical densities differed between treatments at 0, 11, and 46 weeks of age. Results of pairwise comparisons between treatments are summarized in Table 7. There were no comparable differences between treatments on the crop IgA ELISA.

Table 7. Summary of crop IgA optical densities. Numbers reported in the table are the median, (minimum, maximum), and sample size, respectively.

	Week					
Treatment	0	11	15	21	31	46
2 Live Salmune ST +Layermune SE 10	0.094[a,b] (0.087; 0.098) 5	0.109[a,b] (0.088; 0.203) 10	0.094[a] (0.088; 0.207) 10	0.104[a] (0.090; 0.168) 10	0.101[a] (0.091; 0.143) 10	0.136[a,b] (0.091; 0.243) 10
2 Live Salmune ST +Layermune SE 10+16	0.093[a,b] (0.091; 0.097) 5	0.101[a,b] (0.089; 0.247) 10	0.140[a] (0.089; 0.252) 10	0.096[a] (0.089; 0.146) 10	0.101[a] (0.093; 0.134) 10	0.200[b] (0.092; 0.403) 10
2 Live Salmune ST +Layermune SE+Ceva Autogenous SE/ST/SH 10+16	0.086[a] (0.077; 0.091) 5	0.095[a] (0.090; 0.100) 10	0.097[a] (0.088; 0.172) 10	0.107[a] (0.095; 0.156) 10	0.099[a] (0.095; 0.139) 10	0.122[a,b] (0.094; 0.194) 10
2 Live Salmune ST +Avipro SE 10+16	0.138[b] (0.115; 0.170) 5	0.093[a] (0.089; 0.102) 10	0.094[a] (0.086; 0.204) 10	0.137[a] (0.086; 0.264) 10	0.120[a] (0.101; 0.228) 10	0.126[a,b] (0.087; 0.293) 10
2 Live Salmune ST	0.136[b] (0.100; 0.141) 5	0.097[a,b] (0.090; 0.214) 10	0.094[a] (0.082; 0.106) 10	0.098[a] (0.089; 0.159) 10	0.107[a] (0.098; 0.162) 10	0.112[a] (0.092; 0.141) 10
Nonvacc	0.132[b] (0.126; 0.201) 5	0.185[b] (0.096; 0.233) 10	0.092[a] (0.082; 0.136) 10	0.111[a] (0.090; 0.377) 10	0.101[a] (0.091; 0.176) 10	0.157[a,b] (0.093; 0.247) 10
†*P*-value	< 0.001	0.001	0.097	0.195	0.055	0.011

†Kruskal-Wallis test of treatment effects. Within columns, treatments with a superscript in common do not differ with a level of significance of 5% over all comparisons.

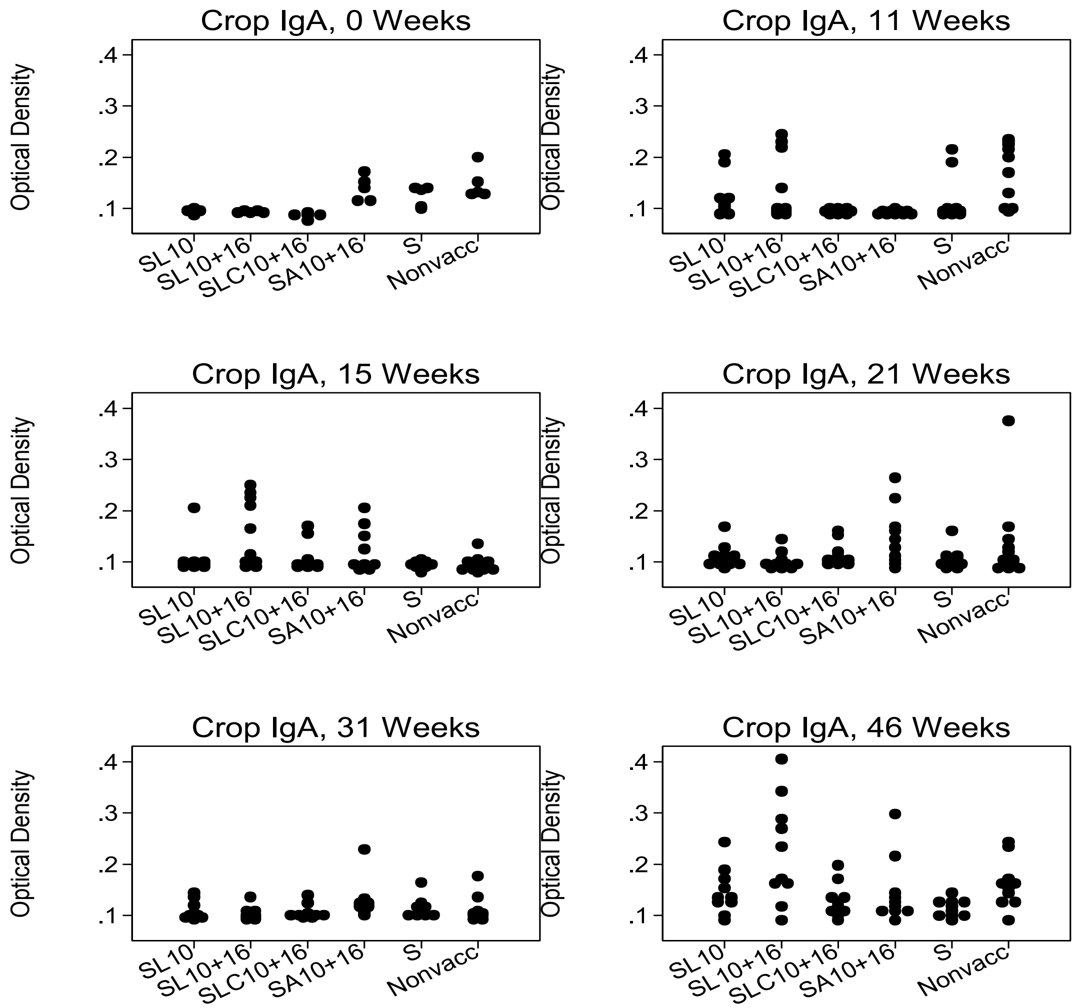

Figure 5. Dotplots of individual crop IgA ELISA optical densities by treatment and age. Treatments are defined as follows: SL10 (2 Live Salmune + Layermune SE 10 weeks); SL 10+16 (2 Live Salmune + Layermune SE 10 weeks and 16 weeks); SLC10+16 (2 Live Salmune + Layermune SE 10 weeks + Ceva Autogenous SE/ST/SH 16 weeks); SA10+16 (2 Live Salmune + Avipro 109 SE4 10 weeks and 16 weeks); S (2 Live Salmune); Nonvacc (Non-vaccinated).

Intestine IgA ELISA. Optical densities for the intestine IgA ELISA are summarized in Table 8, and the distributions of optical densities for the intestine IgA ELISA are illustrated in Figure 6. The optical densities differed between treatments at 0, 21, 31, and 46 weeks of age. Results of pairwise comparisons between treatments are summarized in Table 8.

Table 8. Summary of intestine IgA optical densities. Numbers reported in the table are the median, (min, max) and sample size.

	Week					
Treatment	0	11	15	21	31	46
2 Live Salmune ST +Layermune SE 10	$0.146^{b,c}$ (0.137; 0.168) 5	0.215^{a} (0.088; 0.544) 10	0.175^{a} (0.093; 0.420) 10	$0.266^{a,b,c}$ (0.108; 0.867) 10	$0.173^{a,b}$ (0.092; 0.850) 10	$1.024^{a,b}$ (0.117; 3.02) 10
2 Live Salmune ST +Layermune SE 10+16	0.177^{c} (0.135; 0.287) 5	0.249^{a} (0.097; 0.791) 10	0.231^{a} (0.096; 0.493) 10	$0.144^{a,b}$ (0.103; 0.732) 10	0.110^{a} (0.103; 0.402) 10	1.251^{b} (0.291; 3.00) 10
2 Live Salmune ST +Layermune SE+Ceva Autogenous SE/ST/SH 10+16	$0.135^{a,b,c}$ (0.106; 0.161) 5	0.185^{a} (0.097; 0.840) 10	0.155^{a} (0.087; 0.317) 10	$0.169^{a,b,c}$ (0.132; 0.358) 10	0.387^{b} (0.142; 0.777) 10	0.234^{a} (0.127; 0.330) 10
2 Live Salmune ST +Avipro SE 10+16	$0.095^{a,b,c}$ (0.089; 0.143) 5	0.145^{a} (0.098; 0.321) 10	0.133^{a} (0.094; 0.308) 10	$0.416^{b,c}$ (0.118; 1.88) 10	$0.203^{a,b}$ (0.101; 0.392) 10	1.137^{b} (0.209; 3.39) 10
2 Live Salmune ST	0.085^{a} (0.082; 0.094) 5	0.127^{a} (0.089; 0.499) 10	0.135^{a} (0.104; 0.619) 10	0.669^{c} (0.135; 1.25) 10	$0.248^{a,b}$ (0.102; 0.521) 10	$0.775^{a,b}$ (0.135; 2.83) 10
Nonvacc	$0.087^{a,b}$ (0.082; 0.104) 5	0.308^{a} (0.088; 1.24) 10	0.132^{a} (0.088; 0.264) 10	0.121^{a} (0.100; 0.494) 10	$0.208^{a,b}$ (0.124; 1.02) 10	$0.338^{a,b}$ (0.128; 2.43) 10
†*P*-value	< 0.001	0.336	0.450	< 0.001	0.036	0.009

†Kruskal-Wallis test of treatment effects. Within columns, treatments with a superscript in common do not differ with a level of significance of 5% over all comparisons.

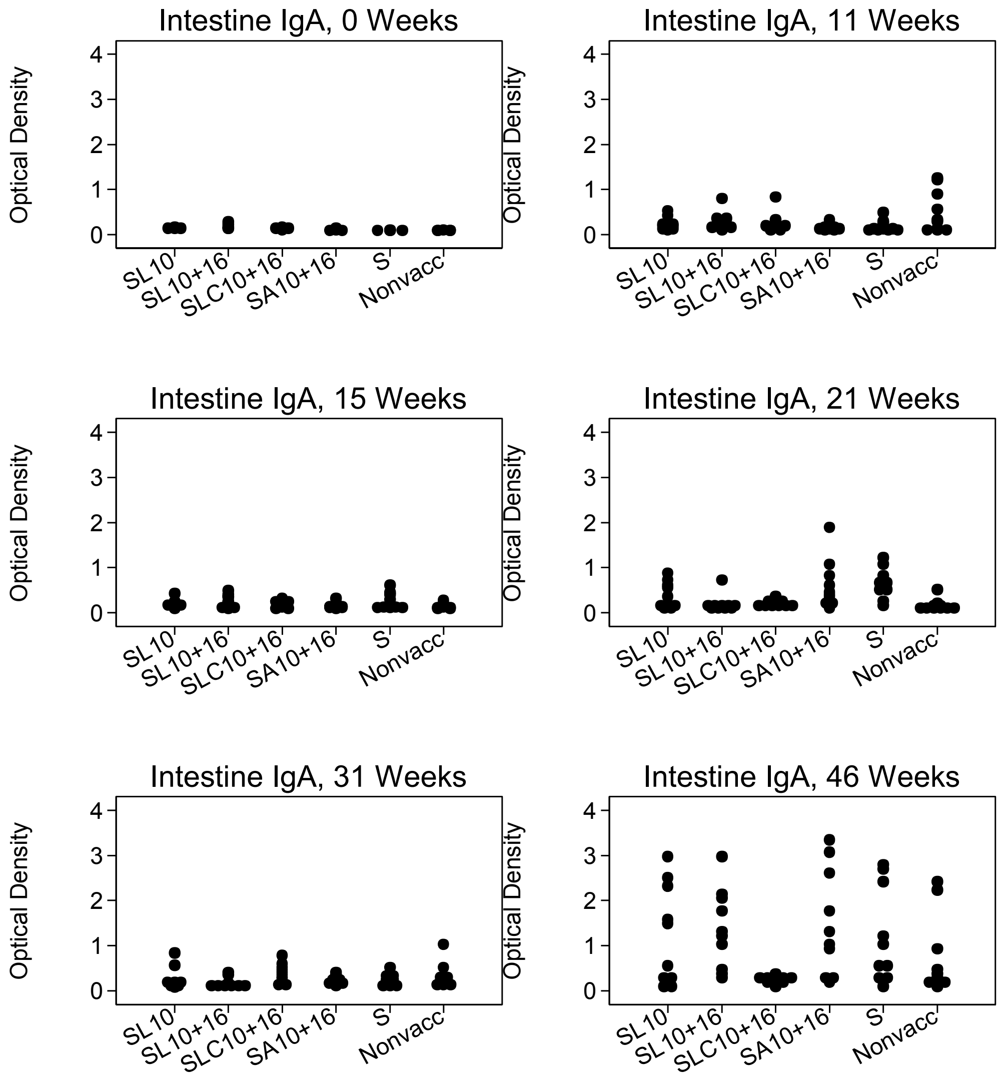

Figure 6. Dotplots of individual intestinal IgA ELISA optical densities by treatment and age. Treatments are defined as follows: SL10 (2 Live Salmune + Layermune SE 10 weeks); SL 10+16 (2 Live Salmune + Layermune SE 10 weeks and 16 weeks); SLC10+16 (2 Live Salmune + Layermune SE 10 weeks + Ceva Autogenous SE/ST/SH 16 weeks); SA10+16 (2 Live Salmune + Avipro 109 SE4 10 weeks and 16 weeks); S (2 Live Salmune); Nonvacc (Non-vaccinated).

Progeny

Salmonella prevalences

Salmonella prevalences are summarized by treatment group, breeder age, and challenge serotype in Table 9, and the results by each of the three pen-level distribution of prevalences is illustrated in Figure 7. There was no significant difference in *Salmonella* prevalence between treatments for progeny of 34 week-old hens challenged with either *S*. Enteritidis ($P = 0.195$) or *S*. Typhimurium ($P = 0.074$). Likewise, there was no significant difference between treatments for progeny of 45 week-old hens challenged with either *S*. Heidelberg ($P = 1.00$) or *S*. Kentucky ($P = 1.00$). There was a significant difference between treatments for progeny of 53 week-old hens challenged with *S*. Enteritidis ($P < 0.001$), with birds from the 2 Live Salmune ST+ Avipro SE 10+16 and 2 Live Salmune ST groups having significantly lower prevalences than birds from the 2 Live Salmune + Layermune SE 10+16 and non-vaccinated groups. There was also a significant overall test of the difference between treatments for progeny of 53 week-old hens challenged with *S*. Heidelberg ($P = 0.022$), but none of the post-hoc pairwise comparisons were statistically significant.

Table 9. *Salmonella* prevalences (%) in progeny of vaccinated breeders by treatment group, breeder age, and challenge serotype. Pen-level replicates for each treatment had 12 birds per pen.

		Salmonella Challenge Serotype				
Age(wk)	Treatment	None	Enteritidis	Heidelberg	Kentucky	Typhimurium
34	2 Live Salmune ST+ Layermune SE 10		30/36 (83.3)[a]			25/36 (69.4)[a]
	2 Live Salmune ST+ Layermune SE 10+16		34/36 (94.4)[a]			24/36 (66.7)[a]
	2 Live Salmune ST+ Layermune SE+Ceva Autogenous SE/ST/SH 10+16		35/36 (97.2)[a]			20/36 (55.6)[a]
	2 Live Salmune ST+ Avipro SE 10+16		35/36 (97.2)[a]			27/36 (75.0)[a]
	2 Live Salmune ST		32/36 (88.9)[a]			29/36 (80.6)[a]
	Nonvacc	0/36 (0.0)	36/36 (100)[a]			30/36 (83.3)[a]
45	2 Live Salmune ST+ Layermune SE 10			36/36 (100)[a]	36/36 (100)[a]	
	2 Live Salmune ST+ Layermune SE 10+16			36/36 (100)[a]	36/36 (100)[a]	
	2 Live Salmune ST+ Layermune SE+Ceva Autogenous SE/ST/SH 10+16			36/36 (100)[a]	36/36 (100)[a]	
	2 Live Salmune ST+ Avipro SE 10+16			36/36 (100)[a]	36/36 (100)[a]	
	2 Live Salmune ST			24/24 (100)[a]	36/36 (100)[a]	
	Nonvacc	40/47 (85.1)		36/36 (100)[a]	36/36 (100)[a]	
53	2 Live Salmune ST+ Layermune SE 10		29/36 (80.6)[a,b]	28/36 (77.8)[a]		
	2 Live Salmune ST+ Layermune SE 10+16		34/36 (94.4)[b]	31/36 (86.1)[a]		
	2 Live Salmune ST+ Layermune SE+Ceva Autogenous SE/ST/SH 10+16		27/36 (75.0)[a,b]	35/36 (97.2)[a]		
	2 Live Salmune ST+ Avipro SE 10+16		27/36 (75.0)[a]	32/36 (88.9)[a]		
	2 Live Salmune ST		28/36 (77.8)[a]	36/36 (100)[a]		
	Nonvacc	65/72 (90.3)	34/36 (94.4)[b]	36/36 (100)[a]		

For each age and *Salmonella* serotype, treatment percentages with a superscript in common did not differ with a level of significance of 5% over all comparisons.

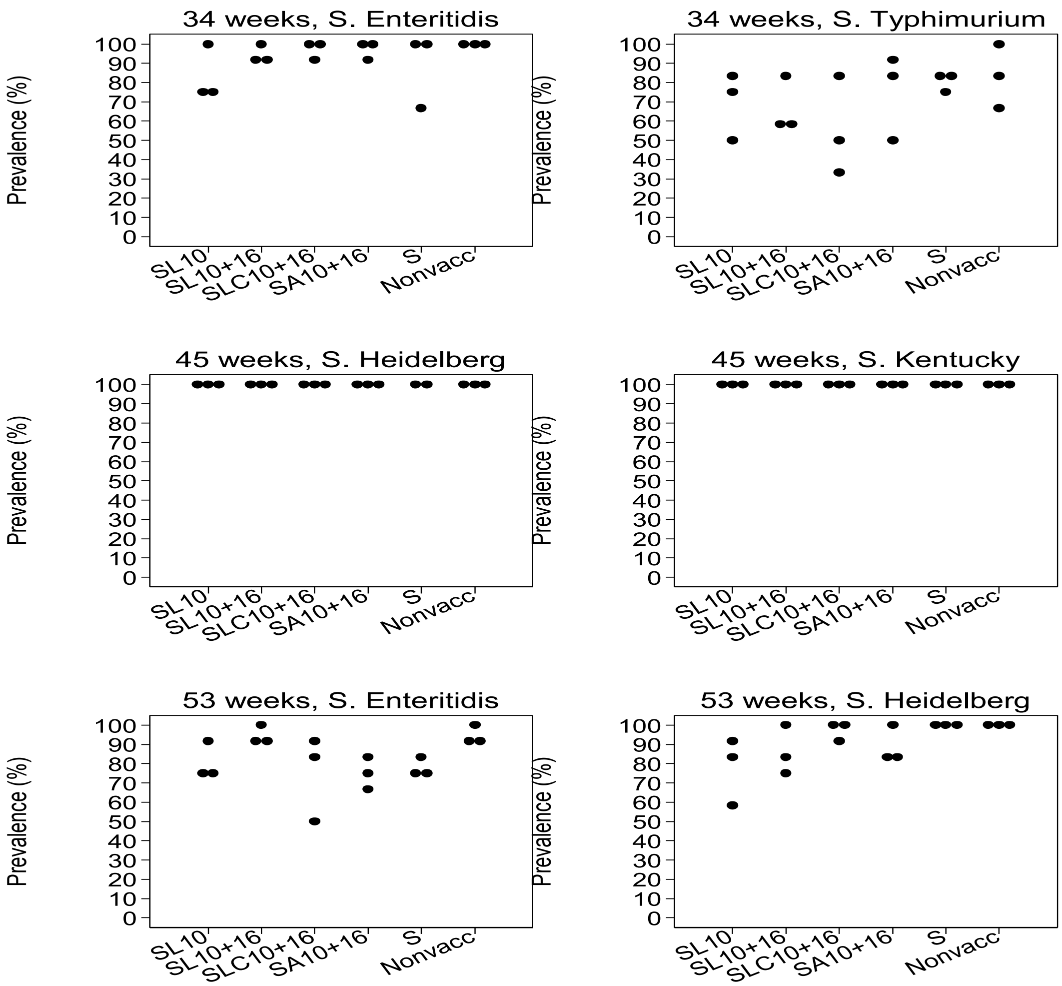

Figure 7. Dot plots of pen-level *Salmonella* prevalence for each treatment by breeder age and challenge serotype. Treatment groups were defined as follows: SL10 (2 live Salmune + Layermune SE 10 weeks); SL10+16 (2 Live Salmune + Layermune SE 10 weeks and 16 weeks); SLC10+16 (2 Live Salmune + Layermune SE 10 weeks + Ceva Autogenous SE/ST/SH 16 weeks); SA10+16 (2 Live Salmune + Avipro 109 SE4 10 weeks and 16 weeks); *S* (2 Live Salmune); Nonvacc (Non-vaccinated).

Salmonella MPNs

Log_{10} *Salmonella* MPNs are summarized by treatment group, breeder age, and challenge serotype in Table 10, and the distribution of MPNs is illustrated in Figure 8. There was no significant difference in the level of *Salmonella* (MPN/gram) in the ceca between treatments for progeny of 34 week-old hens challenged with either *S*. Enteritidis ($P = 0.427$) or *S*. Typhimurium ($P = 0.173$). There was a significant difference in the MPN/gram between treatments for progeny of 45 week-old hens challenged with *S*. Heidelberg ($P = 0.042$), with the 2 Live Salmune ST + Layermune SE 10+16 group having a significantly lower mean log_{10} MPN than the 2 Live Salmune ST + Layermune SE + Ceva Autogenous SE/ST/SH 10+16 group. There was also a significant difference between treatments for progeny of 45 week-old hens challenged with *S*. Kentucky ($P = 0.020$), with the 2 Live Salmune ST group having a significantly lower mean log_{10} MPN than either the 2 Live Salmune + Layermune + Ceva Autogenous 10+16 group or the 2 Live Salmune ST + Avipro SE 10+16 group. There was no significant difference between treatments for progeny of 53 week-old hens challenged with either *S*. Enteritidis ($P = 0.268$) or *S*. Heidelberg ($P = 0.477$).

Table 10. Mean $\log_{10}$ *Salmonella* MPN (SD) in progeny of vaccinated breeders by treatment group, breeder age, and challenge serotype. Pen-level replicates for each treatment had 12 birds per pen. Treatments were compared using linear mixed models with pen as a random effect.

		Salmonella Challenge Serotype				
Age (weeks)	Treatment	None	Enteritidis	Heidelberg	Kentucky	Typhimurium
34	2 Live Salmune ST+ Layermune SE 10		2.55^{a} (1.24) n = 12			2.36^{a} (1.35) n = 9
	2 Live Salmune ST+ Layermune SE 10+16		2.68^{a} (1.30) n = 14			2.36^{a} (1.54) n = 10
	2 Live Salmune ST+ Layermune SE+Ceva Autogenous SE/ST/SH 10+16		2.01^{a} (1.10) n = 14			2.17^{a} (1.08) n = 8
	2 Live Salmune ST+ Avipro SE 10+16		3.56^{a} (1.63) n = 14			1.38^{a} (0.94) n = 10
	2 Live Salmune ST		2.81^{a} (2.01) n = 14			1.42^{a} (0.81) n = 10
	Nonvacc		3.68^{a} (2.38) n = 15			2.11^{a} (1.75) n = 13
45	2 Live Salmune ST+ Layermune SE 10			$2.66^{a,b}$ (0.97) n = 15	$3.24^{a,b}$ (0.82) n = 15	
	2 Live Salmune ST+ Layermune SE 10+16			2.00^{a} (0.80) n = 15	$2.94^{a,b}$ (1.48) n = 15	
	2 Live Salmune ST+ Layermune SE+Ceva Autogenous SE/ST/SH 10+16			3.66^{b} (1.05) n = 15	3.68^{b} (0.90) n = 15	
	2 Live Salmune ST+ Avipro SE 10+16			$2.92^{a,b}$ (0.95) n = 15	3.92^{b} (1.20) n = 15	
	2 Live Salmune ST			$3.35^{a,b}$ (1.18) n = 10	2.23^{a} (0.90) n = 15	
	Nonvacc	3.59 (1.94) n = 15		$2.81^{a,b}$ (1.34) n = 15	$2.76^{a,b}$ (1.38) n = 15	
53	2 Live Salmune ST+ Layermune SE 10		2.29^{a} (1.97) n = 13	1.60^{a} (0.67) n = 10		
	2 Live Salmune ST+ Layermune SE 10+16		2.40^{a} (1.36) n = 13	2.33^{a} (1.60) n = 14		
	2 Live Salmune ST+ Layermune SE+Ceva Autogenous SE/ST/SH 10+16		1.68^{a} (1.77) n = 12	2.72^{a} (1.49) n = 15		
	2 Live Salmune ST+ Avipro SE 10+16		1.85^{a} (1.49) n = 11	2.94^{a} (1.75) n = 11		
	2 Live Salmune ST		1.70^{a} (1.12) n = 8	2.51^{a} (1.32) n = 15		
	Nonvacc	3.41 (1.70) n = 29	3.23^{a} (2.05) n = 14	2.48^{a} (1.81) n = 15		

For each age and *Salmonella* serotype, mean $\log_{10}$ MPNs with a superscript in common did not differ with a level of significance of 5% over all comparisons

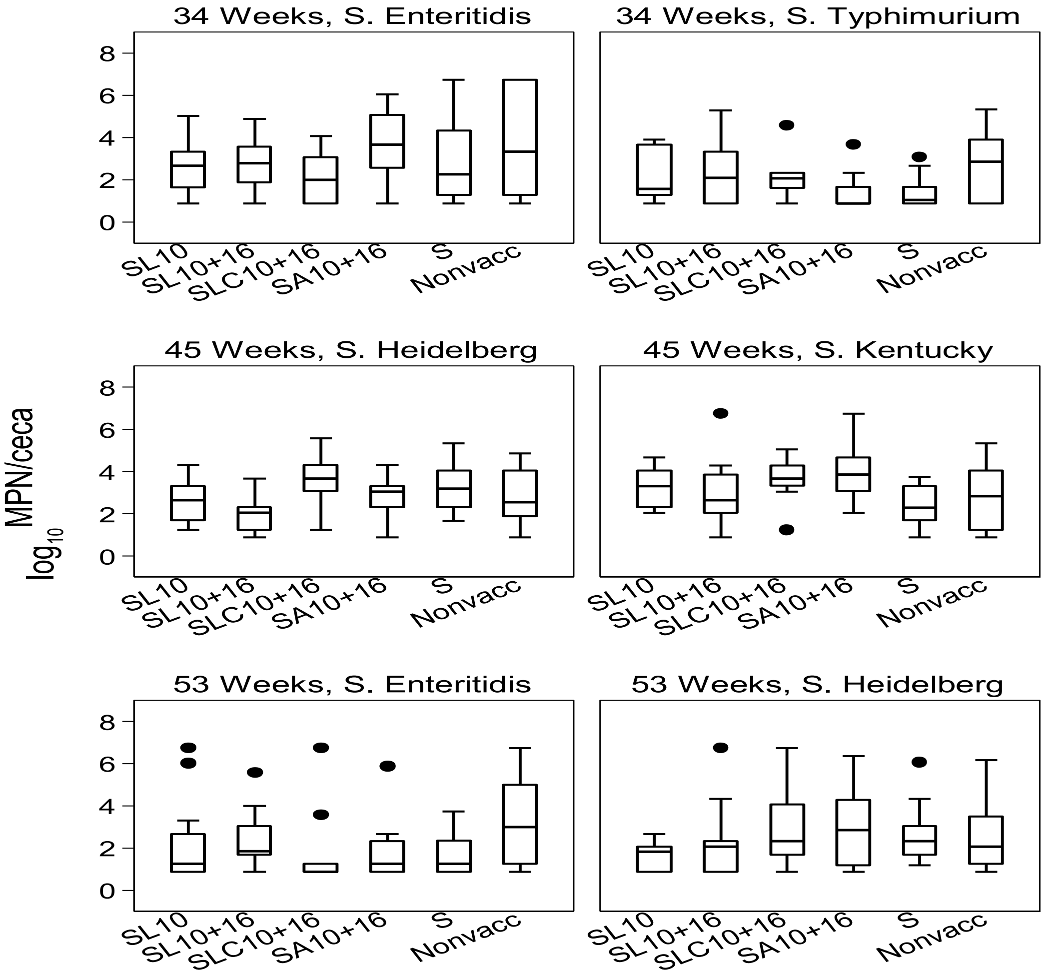

Figure 8. Box plots of bird-level $\log_{10}$ *Salmonella* MPNs for each treatment by breeder age and challenge serotype. Treatment groups were defined as follows: SL10 (2 live Salmune + Layermune SE 10 weeks); SL10+16 (2 Live Salmune + Layermune SE 10 weeks and 16 weeks); SLC10+16 (2 Live Salmune + Layermune SE 10 weeks + Ceva Autogenous SE/ST/SH 16 weeks); SA10+16 (2 Live Salmune + Avipro 109 SE4 10 weeks and 16 weeks); *S* (2 Live Salmune); Nonvacc (Non-vaccinated).

CHAPTER 4

DISCUSSION

Vaccination programs in broiler breeders are used to reduce the problem of *Salmonella* in their progeny, however, there is limited controlled challenge studies in the literature that documents the effectiveness of this strategy other than in a field setting. (Dorea et al. 2010) This study reported on the effectiveness of different *Salmonella* vaccine combinations and their ability to reduce the *Salmonella* prevalence and load in broiler breeders and progeny from a commercial broiler integrator.

Broiler Breeders

At each challenge study 20 birds from each treatment group were removed from the pens, tagged for treatment identification, and moved to one big concrete floor pen or colony house with soft wood shavings. At each challenge there were no symptoms of systemic infection displayed by the birds, and there was no mortality as well. In an attempt to cause positive internal organs (liver, spleen and ovaries) each bird was challenged using 1ml of $1x10^6$ cfu/ml *Salmonella* Enteritidis. This challenge dose was large and may not reflect the load in most field challenges, which could explain the highly positive ceca samples. *Salmonella* Enteritidis was used as the challenge strain because according to the CDC, SE is the number one cause of foodborne illness in people who are infected with *Salmonella* (CDC 2015). Therefore, most commercially available killed bacterins have *Salmonella* Enteritidis as the antigen. In this study we wanted to show which treatment groups would provide the most protection and cross protection to *Salmonella* Enteritidis and to other *Salmonella* serovars of risk to humans.

A concern for broilers is to reduce *Salmonella* colonization of pullets so there is less or no *Salmonella* for either egg transmission by transovarial or by the feces. In this study broiler breeders were challenged with *Salmonella* Enteritidis to determine the level of protection in the ceca and internal organs. At 11 weeks of age the only protection was primarily from the live vaccine given at 3 days and 5 weeks of age. Therefore, it is not unexpected that there would be no differences between groups. The reason there was no difference from the non-vaccinates compared to the vaccinated groups maybe the 6 week time period from the last live vaccination in the vaccinated groups. Bailey found that the duration of immunity to live *Salmonella* vaccination is approximately 28 days (Bailey et al. 2007). This would explain why at 10 and 11 weeks of age the *Salmonella* specific ELISA titers (Table 6) of the vaccinated groups aren't significantly higher than the non-vaccinated group.

At 15 weeks of age the broiler breeders in four of the six treatments had received one inactivated vaccine 5 weeks previously by injection. The challenge dose was high in order to enable internal organ colonization, and this may have been too great of a challenge for the level of immunity developed in the ceca to prevent colonization. This trend continued throughout the other challenges, therefore, the total positive ceca were statistically analyzed. In the total ceca prevalence the inactivated product that was 100% antigens for SE were the Layermune SE and the Avipro SE. In this study with an SE challenge we saw no significant difference between these vaccines and the vaccine that had two other *Salmonella* serovars in addition to the *Salmonella* Enteritidis. However, the Avipro SE did have a significantly lower overall ceca prevalence (58.6%) than the other vaccine groups. Since this is a different manufacturer there maybe a difference in adjuvant or in volume of antigen. The non-vaccinated broiler breeders had significantly higher prevalences of *Salmonella* Enteritidis in ceca than the 2 Live Salmune ST +

Layermune SE + Ceva Autogenous SE/ST/SH 10+16 and Salmune ST + Avipro SE 10+16 groups. In addition, the 2 Live Salmune ST + Layermune SE 10 and 2 Live Salmune ST + Layermune SE 10+16 groups had a greater *Salmonella* Enteritidis prevalence reduction than that of the non-vaccinated group.

As mentioned before, a concern for broilers is to reduce *Salmonella* colonization of pullets and hens so there is less or no *Salmonella* transmitted to them. The goal of vaccination is not only to reduce the prevalence of *Salmonella,* but to also reduce the load of *Salmonella* in the birds. The load of *Salmonella* in the ceca was measured by MPNs in this study. The mean $\log_{10}$ MPN at 21 weeks was significantly lower than at all other ages. This could be the age when the immunity provided by the vaccine was the highest, or the challenge may not have colonized as well as at other ages throughout the study. The fact that the mean $\log_{10}$ MPN of the non-vaccinated group at this age was higher (3.04) than all vaccinated groups supports that this age provided the highest immunity from the vaccine. The 2 Live Salmune ST + Avipro SE 10+16 had a significantly lower marginal mean than all other treatment groups, and the non-vaccinated group had a significantly higher marginal mean than all other treatment groups.

With the high challenge given to all of the birds in each treatment, it was expected to see translocation of *Salmonella* into the internal organs. The prevalence of the liver/spleen was highest in the vaccinated groups at 31 weeks of age. This could be a result of the length of time since the last vaccination. The overall prevalences of *Salmonella* Enteritidis in the liver/spleen samples were much higher in the 2 Live Salmune ST group, which had a total of 41 out of 98, or 41.8% positive samples, and the non-vaccinated group, which had a total of 55 out of 98, or 56.1% positive samples. In comparison, the 2 Live Salmune ST + Layermune SE 10 group had a total of 23 out of 99, or 22.2% positive samples; the 2 Live Salmune ST + Layermune SE 10+16 group had a total of 16 out of 100, or 16% positive samples; the 2 Live Salmune ST + Layermune SE + Ceva Autogenous SE/ST/SH 10+16 group had a total of 17 out of 99, or 17.2% positive samples, and the 2 Live Salmune ST + Avipro SE 10+16 group had a total of 9 out of 99, or 9.1% positive samples. As in the ceca, the 2 live Salmune ST + Avipro SE 10+16 had a significantly lower overall liver/spleen prevalence (9.1%) than other vaccine groups. These results show that the live vaccine alone did not provide sufficient immunity to prevent translocation of the *Salmonella* Enteritidis into internal organs. As shown in Figure 3, the antibody titers on the Biochek SE/ST ELISA provide proof that treatment groups with killed vaccine provide a higher level of antibodies to vaccinated broiler breeders than those vaccinated with the live vaccine only. All vaccinated groups showed significantly lower overall liver/spleen prevalence as well, when compared with the 2 Live Salmune ST and non-vaccinated groups.

One of the main concerns in broiler production is to reduce the load of *Salmonella* in broiler breeder flocks to, in turn, reduce the possibility of egg transmission to their progeny. Contaminated eggs, in some instances, have been traced back to flocks of laying hens that were culturally or serologically positive for *Salmonella* Enteritidis (Gast et al. 1990). In this study ovaries were collected at 31 and 46 weeks of age. Even with the large challenge dose, there was very little recovery of *Salmonella* Enteritidis from the ovaries. The prevalence was highest in the non-vaccinated group as expected, but also in the 2 Live Salmune ST + Layermune SE 10+16, however, there was still only a total prevalence of 7.5%. The 2 Live Salmune ST + Layermune SE + Ceva Autogenous SE/ST/SH was the only group with 0% overall prevalence. Since there was not a large number of positive ovaries in these challenges it is difficult to tell if there was any difference in the vaccines in preventing egg transmission.

Several studies have demonstrated that vaccination increases the *Salmonella*-specific titers of vaccinated breeders (Bailey et al. 2007). Breeders vaccinated with live and killed vaccines had significantly higher titers than the 2 Live Salmune ST and non-vaccinated groups. Prior to killed vaccination at 10 weeks all groups had low *Salmonella*-specific serum antibody titers. Titers did not start to increase until 15 weeks of age, 5 weeks post the first killed vaccination. The ELISA data demonstrates an antibody response to the killed vaccine, not to live *Salmonella* vaccination. However, the two doses of live *Salmonella* vaccination does provide some protection to the pullets prior to killed vaccination although immunity from a live *Salmonella* Typhimurium vaccine is known to be short-lived, and most of the immunity is cell-mediated, not humoral (Young et al. 2007). The 2 Live Salmune ST + Avipro SE 10+16 group had higher *Salmonella*-specific antibody titers throughout the study.

Maternal IgA is deposited in the amniotic fluid, which is swallowed by the embryo prior to hatching; therefore, it is important for broiler breeders to secrete IgA to provide protection to the progeny (Rose et al. 1974). In this study, we looked at the amount of IgA antibodies found in the mucosal lining of the broiler breeders' crop and intestine at 0 days of age and 7 days post inoculation with *Salmonella* Enteritidis at 11, 15, 21, 31, and 46 weeks of age. We were unable to clearly demonstrate comparable IgA levels found in the crop and intestine of the vaccinated breeders due to the low optical densities given by the IgA ELISA assay and some variability between plates. However, there were more significant differences found in the intestine than in the crop. At 46 weeks of age, all vaccinated groups except the 2 Live Salmune ST + Layermune SE + Ceva Autogenous 10+16 had higher intestine IgA optical densities than the non-vaccinated group. The intestine IgA optical densities rose from 0 days of age to 21 weeks of age which demonstrates vaccination increases IgA antibodies.

In conclusion, vaccination of broiler breeders with killed *Salmonella* vaccine provided a reduction in *Salmonella* prevalence and loads, and significantly increased *Salmonella*–specific antibody titers in vaccinated broiler breeder hens.

Progeny

Increased maternal antibody titers in the yolks of eggs from vaccinated breeders and in the hatched broiler chicks is important, with some studies finding that broiler chicks from vaccinated hens were less susceptible to *Salmonella* colonization than were chicks from unvaccinated hens (Berghaus et al. 2011).

There were no significant differences between treatments for progeny of 34 weeks old broiler breeders challenged with *Salmonella* Enteritidis or *Salmonella* Typhimurium for ceca prevalence. Although there were no differences for the progeny challenged with SE, there were differences between treatments for the progeny challenged with ST; all treatment groups had a lower percentage of ceca prevalences than the 2 Live Salmune ST and non-vaccinated groups. There was also no significant difference between treatments for progeny of 45 week old broiler breeders challenged with either *Salmonella* Heidelberg or *Salmonella* Kentucky. Only one vaccine, 2 Live Salmune + Layermune SE + Ceva Autogenous SE/ST/SH 10+16, contained SH, and none of the vaccines contained SK. These isolates were used to explore the possibility of cross protection between serovars. This is a possible explanation for the high percentage of positive samples in all groups for the *Salmonella* Kentucky challenge. The *Salmonella* Kentucky results are consistent with what is known about short-lived cross-immunity, and the progeny exposed with *Salmonella* Kentucky would not have been expected to have much maternal protection (Young et al. 2007). There was a significant difference between treatments for progeny of 53 week old broiler breeders challenged with *Salmonella* Enteritidis. Birds from the 2

Live Salmune ST + Avipro 10+16 and 2 Live Salmune ST groups had significantly lower prevalences than birds from 2 Live Salmune ST + Layermune SE 10+16 and non-vaccinated groups. The breeders from the 2 Live Salmue ST group may have still had some cross-protective antibodies to the *Salmonella* Enteritidis that were passed to the progeny providing them with protection to the challenge with *Salmonella* Enteritidis. The reason for the high prevalence in the 2 Live Salmune + Layermune SE 10+16 results could have been due to the age of the broiler breeders at the time of this egg collection. The immunity provided by the vaccine could have been lower than the immunity provided at previous ages.

There was no significant difference in ceca $\log_{10}$ MPN levels between treatments for progeny of 34 week old broiler breeders challenged with either *Salmonella* Enteritidis or *Salmonella* Typhimurium. Although there were no differences for the progeny challenged with SE, there were differences between treatments for the progeny challenged with ST; 2 Live Salmune ST + Avipro 10+16 and 2 Live Salmune ST groups had lower $\log_{10}$ MPN means and standard deviations that all other treatment groups. This immunity to the ST could be left over from the live vaccine, although it is known to be short-lived, or it could have been cross protection provided by the killed SE vaccine with the live ST vaccine. There was a significant difference between treatments for progeny of 45 week old broiler breeders challenged with *Salmonella* Heidelberg with the 2 Live Salmune ST +Layermune SE 10+16 group having a significantly lower mean $\log_{10}$ MPN than the 2 Live Salmune ST + Layermune SE + Ceva Autogenous SE/ST/SH 10+16 group. Although we would have expected to see lower mean $\log_{10}$ MPN levels in the 2 Live Salmune ST +Layermune SE + Ceva Autogenous SE/ST/SH 10+16 group, the protection provided to the 2 Live Salmune ST + Layermune SE 10+16, in this instance, could have been provided by cross-protective antibodies from the Live ST or killed SE vaccine. There was also a significant difference between treatments for progeny of 45 week old broiler breeders challenged with *Salmonella* Kentucky, with the 2 Live Salmune ST group having a significantly lower mean $\log_{10}$ MPN than either the 2 Live Salmune + Layermune + Ceva Autogenous 10+16 group or the 2 Live Salmune ST + Avipro SE 10+16 group. There was no significant difference between treatments for progeny of 53 week old broiler breeders challenged with either *Salmonella* Enteritidis or *Salmonella* Heidelberg. Although there were no significant differences, progeny challenged with SE from the 2 Live Salmune ST + Layermune SE + Ceva Autogenous SE/ST/SH 10+16 , 2 Live Salmune ST + Avipro 10+16, and 2 Live Salmune ST groups had lower $\log_{10}$ MPN means and standard deviations than the other treatment groups. When looking at the dot plots in Figure 7, it can be seen that the 2 Live Salmune ST + Layermune SE + Ceva Autogenous SE/ST/SH group had individual pens trending lower in prevalence and MPN levels of *Salmonella* Enteritidis. This may indicate an advantage of combining two different killed vaccines. Progeny challenged with SH from the 2 Live Salmune ST + Layermune 10 group had lower $\log_{10}$ MPN means and standard deviations than all other treatment groups, this reduction could have been provided by cross-protective antibodies in the live and killed vaccine passed through maternal antibodies.

The challenge dose in this study was 1×10^6 cfu/ml per bird in broiler breeders and 1×10^5 per bird in the progeny was a larger dose and every bird was exposed to that dose, which is greater than what is seen in most field challenges. Such a large dose may not reflect the natural exposure load that is normally seen in field challenges. Even with the large challenge dose the differences seen in this study show that vaccination with a combination of live and killed vaccine can lower *Salmonella* prevalence and load in ceca and prevent *Salmonella* from invading internal organs significantly more than not vaccinating or vaccinating with live vaccine alone. Even small decrease of *Salmonella* in the field could potentially decrease the amount of *Salmonella* in the processing plant and in turn decrease the number of *Salmonella* related food borne illnesses (Young et al. 2007).

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