Research Note

Response to a *Salmonella* Enteritidis challenge in old laying hens with different vaccination histories

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ABSTRACT Extending the laying period of laying hens is beneficial for economic and sustainability purposes. Because vaccines were designed with a shorter laying period envisaged, it is unclear whether current *Salmonella* vaccines can provide sufficient levels of protection against infection at an older age. The purpose of this experiment was to determine the efficacy of early rearing vaccination schemes against *Salmonella* challenge late in the laying period. There were four treatment groups: birds that had not been vaccinated (Group 1), birds vaccinated with live *Salmonella* Enteritidis (SE) (Group 2), with live and inactivated SE (Group 3), or with live SE and live *Salmonella* Typhimurium (Group 4). At the end of the laying period, the birds were transported from the laying farm to the research facility where they were orally challenged with 2.06×10^9 colony-forming units SE at around 82 wk of age. Hens were euthanized and bacteriology was performed on cecum, liver, spleen, and follicular fluid samples to determine SE colonization 7 and 14 d after challenge. Clinical and bacteriological findings of hens vaccinated with different vaccination schemes and the non-vaccinated control group were compared. No significant differences in SE colonization were found for vaccinated groups compared to the non-vaccinated control group. This may be a result of waning immunity due to the long time between vaccination and challenge. Also, as vaccination took place in the rearing period in the field, initial levels of immunity may not have been optimal due to shortcomings in the vaccination technique. Furthermore, the results of this study may have been affected by differences in age, breed, and origin between the groups. Therefore, controlled studies from early age onwards are necessary for more accurate comparisons between vaccines.

Key words: *Salmonella*, vaccination, Enteritidis, poultry, chicken

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INTRODUCTION

Around 100,000 cases of human salmonellosis are reported in the European Union every year (EFSA and ECDC, 2015). A major source of human food infections is *Salmonella* Enteritidis (**SE**) and *Salmonella* Typhimurium (**ST**) in eggs (Schoeni et al., 1995). Contamination of egg contents with SE mostly occurs through colonization of ovaries, oviducts, and vaginal tissue (De Buck et al., 2004). Also, fecal contamination of the shell can result in penetration of the egg contents with SE and ST and *Salmonella* Heidelberg (Humphrey, 1994; Schoeni et al., 1995; Messens et al., 2005). In addition, cross-contamination of egg shells can occur during processing and storage (EFSA and ESDC, 2014).

Live or inactivated *Salmonella* vaccines are used to vaccinate pullets during the rearing period to reduce fecal shedding, ovarian transmission, and within-flock prevalence (EFSA Panel on BIOHAZ, 2004), thereby reducing *Salmonella* contamination of table eggs and the environment (EFSA and ESDC, 2014; Trampel et al., 2014). As Atterbury et al. (2009, 2010) have shown, different live and attenuated vaccines reduced the percentage of birds positive for SE based on liver swabs, egg content, and cecal carriage but could not completely prevent colonization. Although vaccination will not completely prevent fecal excretion and spread through the flock (Davies and Breslin, 2003, 2004; Arnold et al., 2014), the decrease of internal-egg contamination levels due to vaccination does contribute to reduced public health risk of *Salmonella* infections (Davies and Breslin, 2003, 2004; Arnold et al., 2014).

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Longitudinal studies have shown that *Salmonella* is most likely introduced in a flock early in the laying period (Schulz et al., 2011) and can show long-term persistence in laying hen houses (Carrique-Mas et al., 2008; Schulz et al., 2011; Trampel et al., 2014). Reactivation of shedding, i.e., the renewed excretion of SE by latently infected chickens, or re-infections from the environment make it likely that once SE is introduced into a flock, it remains present throughout a whole production period (Davies and Breslin, 2004). Therefore, vaccines have to protect against *Salmonella* contamination of eggs from start until the end of the production period.

A standard laying hen production cycle is ended at around 70 to 80 wk of age, after which the exhausted hens are slaughtered and replaced by new pullets. There is a trend toward extending the laying period of laying hens to 90 to 100 wk. This may reduce environmental impact and financial costs per egg produced, for example because less breeding hens are needed and the rearing period is shorter in relation to the laying period (Bain et al., 2016; Abín et al., 2018). As current vaccines were developed and validated to provide protection throughout the duration of the laying period, it is unclear whether sufficient levels of protection can be maintained until the end of an extended laying period.

Most vaccine efficacy studies have used young birds in the rearing period and assessed protection against bacteriological colonization of organs shortly after vaccination (De Buck et al., 2004, 2005; Gantois et al., 2006; Atterbury et al., 2009, 2010; Deguchi et al., 2009; Filho et al., 2009). In these studies, different types of vaccines and delivery methods were used, and it was shown that vaccination reduced the colonization of *Salmonella* in organs, reduced the number of *Salmonella*-positive eggs and fecal shedding of *Salmonella*. Only a few studies describe protection against SE and ST infections in older hens vaccinated in the rearing period (Woodward et al., 2002; Springer et al., 2011). The number of SE-positive tissues and fecal samples after challenge was significantly reduced in laying hens up to 59 wk of age, vaccinated with a killed SE vaccine in the rearing period (Woodward et al., 2002). Springer et al. (2011) tested the response in laying hens vaccinated with either a live vaccine or inactivated SE vaccine in the rearing period. Vaccinated hens as old as 73 wk of age had a significant reduction in SE colonization in the liver and ceca compared to the unvaccinated control (Springer et al., 2011). However, there are no published reports of the response of laying hens to a challenge above 80 wk old.

In this study, we evaluated the remaining levels of protection against a late SE challenge in chickens subjected to 3 different Salmonella vaccination schemes in the rearing period. We compared clinical findings and colonization of cecum, liver, spleen, and follicle fluid in vaccinated hens compared to a non-vaccinated control group after challenge with SE at around 82 wk of age.

MATERIALS AND METHODS

Birds

A total of 208 birds between 72 to 81wk of age (4 groups of 52 birds each) were transported to the research facility at the end of the laying period. During the rearing period, the birds had not been vaccinated (**CON**), or had been vaccinated with live *Salmonella* Enteritidis (**EL**) (AviPro *Salmonella* Vac E, Lohmann Animal Health GmbH, Cuxhaven, Germany), with live and inactivated *Salmonella* Enteritidis (**EL+ED**) (AviPro *Salmonella* Vac E, Lohmann Animal Health GmbH, Cuxhaven, Germany and Nobilis Salenvac E, Intervet, de Bilt, the Netherlands) or with live *Salmonella* Enteritidis and live *Salmonella* Typhimurium (**EL+TL**) (AviPro *Salmonella* Vac E and AviPro *Salmonella* Vac T, both Lohmann Animal Health GmbH, Cuxhaven, Germany). EL and TL were administered through drinking water and ED through intramuscular injection. Because hens subjected to different vaccination schemes were needed, each group was obtained from a different commercial laying farm, and consequently these were different with regard to age, breed, and country of origin. Hens for groups 1, 2, and 3 had been housed in aviaries and for group 4 in an aviary with covered veranda before transport to the research facility. Each group had been subjected to local standard monitoring programs to verify *Salmonella*-free status. Prior to transport to the research facility, blood samples were collected from at least 20 randomly selected birds of each flock at the laying farm, to test for the presence of *Salmonella* antibodies with a *Salmonella* B&D ELISA on flagellar antigen. The birds arrived at the research facilities at least 12 d before start of the experiment for acclimatization.

Management

At the research facility, each group was housed in a separate research unit. The hens were housed on wood shavings at a density of 4.5 birds/m^2 with 7 to 8 nest boxes and a light regime of 15 light h/d. The birds were fed with a commercial feed for layers, without antibiotics. Feed and drinking water were available ad libitum. Birds were observed daily, and the presence of clinical signs, abnormal behavior, mortality, and egg production per group was recorded. All animal procedures in this experiment were approved by the Animal Ethical Committee of Utrecht University (Utrecht, the Netherlands) under registration number 2014.II.03.016, in full compliance with all relevant legislation.

Experimental design

Twelve days before the start of the experiment (**D-12**), absence of a current *Salmonella* infection was confirmed in all birds with blood samples to test for

Salmonella antibodies (as described previously), and cloacal swabs, as described below. A total of 40 chickens from each group of 52 birds that had been transported to the research facility were selected for the experiment at day 0 (**D0**). The selected chickens were clinically healthy looking birds that were still in production and were 85 (CON), 82 (EL and EL+ED), and 83 wk (EL+TL) of age. After selection, individual cloacal swabs were taken and all hens were orally challenged with SE. Necropsy was performed on 20 randomly selected birds of each group at D7 and on the remaining 20 birds at D14. After euthanization by cervical dislocation and exsanguination, samples for bacteriology were taken aseptically from liver, spleen, ceca, and follicles from each bird. On D14, the same procedures were performed for the remaining 20 birds of each group.

Challenge

All birds were challenged at D0 by oral gavage with 1 mL, containing 2.06×10^9 cfu SE strain K285/93 Nal^{res}. The strain was provided by Lohmann Animal Health GmbH, Cuxhaven, Germany. From a fresh overnight (**O/N**) culture on blood agar (Oxoid, the Netherlands), a single colony of the strain was used to inoculate brain heart infusion broth (Oxoid, the Netherlands) and was incubated O/N at 37°C aerobically while shaking. The O/N culture (5×10^8 cfu/mL) was concentrated by centrifugation and resuspended to a final concentration of 2×10^9 cfu/mL. We corrected for 15% loss during centrifugation as determined in a pilot study (data not shown). The concentration of the inoculum was determined by counts of plated serial dilutions.

BACTERIOLOGY

Screening for the presence of Salmonella before inoculation

Before start of the experiment (D-12), and at D0 prior to inoculation, cloacal swabs were taken from all birds. Swabs of 5 birds from the same unit were pooled. The pooled swabs were pre-enriched in buffered peptone water (Oxoid, the Netherlands) at 37°C and afterwards enriched in tetrathionate broth (Oxoid, the Netherlands) at 37°C, both overnight. Then, a loopful of the tetrathionate broth was plated on modified Brilliant Green Agar (**mBGA**; Oxoid, the Netherlands). The plates were incubated O/N at 37°C and screened for suspect colonies.

Processing of organ samples for bacteriology

Ceca, spleen, liver, and (at least) 1 mL follicular fluid of at least two different follicles were collected aseptically during necropsy. The samples were immediately transported to the laboratory where they were weighed, diluted 10 times in buffered peptone water and homogenized (referred to as original sample 1:10).

Qualitative bacteriology of spleen and follicular fluid samples

All original samples 1:10 were incubated O/N at 37°C followed by a second O/N incubation at 37°C in enriched in tetrathionate broth. A loopful of the tetrathionate broth was then plated on mBGA with nalidixic acid (mBGA+; Oxoid, the Netherlands), an antibiotic to select for the challenge strain. The selective plates were incubated O/N at 37°C and screened for suspect colonies. Suspect colonies were subcultured on blood agar for identification by matrix-assisted laser desorption ionization time-of-flight mass spectrometry and latex agglutination to confirm *Salmonella* sp. group D.

Quantitative bacteriology of liver and ceca samples

From liver and cecal content samples, the number of cfu of *Salmonella* bacteria per gram was determined by direct plating of 10-fold dilutions of the original sample 1:10 on mBGA+. The detection limit of this procedure was 500 cfu/g liver or cecal content. On D14, 100 μL of the 1:10 dilutions of liver and cecal content samples was spread out on an mBGA+ plate with a spatula, which lowered the detection limit to 100 cfu/g liver or cecal content. Plates were incubated O/N at 37°C. If colonies were present on the plates, cfu per gram was determined. If no colonies were detected on the plates, the enrichment procedure described above was applied.

STATISTICAL ANALYSIS

Descriptive analyses were performed on the $\log_{10}$-transformed cfu data from the direct plating procedure for cecum and liver for D7 and D14. The large amount of censored data, consisting of positive samples according to enrichment procedures but below detection limit of the direct plating, did not allow for reliable quantitative analyses.

Cecum, liver, spleen, or follicular fluid samples were considered positive if SE colonies were detected after direct plating or after the enrichment procedure, and was negative when both tests were negative. Because almost all cecum and liver samples were positive on D7, and almost all follicular fluid samples were negative at D7 and D14, no statistical analyses were performed on these data. The remaining binary data were analyzed for each sample type with a logistic regression, to compare vaccinated groups with the control group. Akaike's information criterion (**AIC**) was used for model reduction (Burnham and Anderson 2002). A group effect was found if the difference in AIC between

Table 1. Number and percentages of organ samples positive for the *Salmonella* Enteritidis challenge strain based on quantitative and qualitative bacteriology tests for the different groups at D7 and D14 after challenge.

	D7			D14		
Group[1]	Positive	% Positive	OR (95% PCI)[2]	Positive	% Positive	OR (95% PCI)[2]
Cecum samples						
CON	20	100		6	30	
EL	19	95	–	16	80	9.33 (2.35 to 44.91)*
EL+ED	20	100	–	8	40	1.56 (0.42 to 5.98)
EL+TL	19	95	–	17	85	13.22 (3.10 to 74.22)*
Total	78	98		47	59	
Liver samples						
CON	20	100		13	65	
EL	19	95	–	18	90	4.85 (0.98 to 36.25)
EL+ED	20	100	–	13	65	1.00 (0.27 to 3.72)
EL+TL	18	90	–	14	70	1.26 (0.33 to 4.87)
Total	77	96		58	73	
Spleen samples						
CON	19	95		17	85	
EL	12	60	0.08 (0.00 to 0.51)*	17	85	1.00 (0.16 to 6.08)
EL+ED	15	75	0.16 (0.01 to 1.12)	14	70	0.41 (0.08 to 1.86)
EL+TL	14	70	0.12 (0.01 to 0.83)*	13	65	0.33 (0.06 to 1.43)
Total	60	75		61	76	
Follicular fluid samples						
CON	0	0		0	0	
EL	0	0	–	0	0	–
EL+ED	0	0	–	1	5	–
EL+TL	1	5	–	2	10	–
Total	1	1		3	4	

$n = 20$ per group.

[1]CON = non-vaccinated control group, EL = birds vaccinated with live *Salmonella* Enteritidis, EL+ED = birds vaccinated with live *Salmonella* Enteritidis and inactivated *Salmonella* Enteritidis, EL+TL = birds vaccinated with live *Salmonella* Enteritidis and live *Salmonella* Typhimurium (logistic regression analysis).

[2]OR = odds ratio. 95% PCI = 95% profile (log-) likelihood confidence intervals. OR are based on logistic regression analysis with CON as reference group.

Effect sizes with the control group on the same day are marked with an asterisk.

the models was more than 2. For spleen samples, the starting model contained group, time (D7 and D14), and their interaction as independent variables. Cecum and liver samples at D14 were analyzed with group as independent variable. Results of the logistic regression model are reported as odds ratio (**OR**) and 95% profile (log-) likelihood confidence intervals (**95% PCI**).

Statistical analyses were carried out using R (R Core Team, 2015).

RESULTS

The absence of a current *Salmonella* infection for all groups was verified with negative outcomes of bacteriology of the cloacal swabs taken before the start of the experiment. Also, all blood samples of the non-vaccinated control group were negative. In the vaccinated groups, both negative and positive titers were found. The positive titers were suggestive for the absence of a recent *Salmonella* infection (BioChek, 2012), and positive titers were deemed related to the vaccinations in the rearing period.

During the experiment, no clinical findings were observed that could indicate a *Salmonella* infection, e.g., diarrhea or a drop in egg production, or other disease. After the challenge, no mortality was seen in any of the groups.

The numbers and percentages of organ samples positive for the SE challenge strain for the different groups at D7 and D14 after challenge are summarized in Table 1. The total number of positive cecum, liver, and spleen samples for all groups combined shows that the majority of birds in the experiment tested positive for SE on D7 and on D14. In contrast, follicular fluid samples were only positive in 1 and 3 birds at D7 and D14, respectively.

The results from qualitative testing for the different groups at D7 and D14 are summarized in Table 2. The number of positive samples after direct plating ranged between 8 and 15 per 20 samples for cecum at D7 and were much lower, ranging between 0 and 6 per 20 samples for liver (D7 and D14) and cecum (D14). This indicates that a large proportion of samples remained below the detection limit of the direct plating procedure and therefore lack a (log)cfu/g value. As quantitative data were unavailable for the majority of positive birds, mean and standard deviation for the logcfu/g could not be calculated.

There were no group effect in the level of protection against SE colonization between vaccinated groups and

Table 2. Results *Salmonella* Enteritidis bacteriology tests for 20 cecum and 20 liver samples per different group at D7 and D14 after challenge.[1]

		Cecum samples				Liver samples			
Day	Group[1]	*n* positive after direct plating[2]	*n* enrichment positive	Enrichment only/total positive[3]	*n* negative	*n* positive after direct plating[2]	*n* enrichment positive	Enrichment only/total positive[3]	*n* negative
D7	CON	15	5	0.25	0	4	16	0.80	0
	EL	8	11	0.58	1	6	13	0.68	1
	EL+ED	14	6	0.30	0	1	19	0.95	0
	EL+TL	15	4	0.21	1	6	12	0.67	2
D14	CON	3	3	0.50	14	0	13	1	7
	EL	3	13	0.81	4	2	16	0.89	2
	EL+ED	2	6	0.75	12	0	13	1	7
	EL+TL	2	15	0.88	3	0	14	1	6

$n = 20$ per group.
[1]Groups are explained under Table 1.
[2]The detection limit of direct plating on D7 and D14 was 500 cfu/g and 100 cfu/g, respectively.
[3]The detection limit of the enrichment procedure on D7 was $0 <$ cfu/g < 500 and on D14 was $0 <$ cfu/g < 100.

the non-vaccinated control groups for cecum, liver at D7, and follicular fluid at D7 or D14 according to AIC. Based on logistic regression analysis, the odds of SE colonization was significantly lower for the EL group and the EL+TL group in spleen samples at D7 (OR = 0.08; 95% PCI 0.00 to 0.51; $P = 0.02$ and OR = 0.12; 95% PCI 0.00 to 0.83) compared to the non-vaccinated control group. At D14, significant differences between groups were only found for cecum samples. Here, the odds of a positive result were increased for the EL group (OR = 9.33; 95% PCI 2.35 to 44.91; $P < 0.01$) and EL+TL (OR = 13.22; 95% PCI 3.10 to 74.22; $P < 0.01$) group compared to the non-vaccinated control group.

DISCUSSION

In light of a trend toward extending egg-producing cycles of laying hens for sustainability purposes, we tested whether SE vaccines administered in the rearing period could provide protection against SE challenge in laying hens of around 82 wk of age. Bacteriology of liver, cecum, spleen, and follicular fluid samples for 3 different vaccination schemes was compared to a non-vaccinated control group. In all groups, the majority of cecum, liver, and spleen samples were positive for the challenge strain after challenge, which indicates that the vaccinated birds were not better protected against an SE challenge than non-vaccinated birds.

In this study, 98% of the hens had positive cecum samples at D7 after challenge, and the proportion of positive samples was not lower in the vaccinated groups compared to the non-vaccinated group. This is in line with findings of Atterbury et al. (2009), who found that 22 to 56% of 17-wk-old vaccinated young hens had colonized ceca after challenge with a low dose (1.5×10^2 cfu) and 72 to 89% after challenge with a high dose (1.5×10^8 cfu) of SE. In our study, the percentage of positive cecum samples was higher, but this can be explained by the higher challenge dose of 2.06×10^9 cfu. However, in contrast with other studies (Woodward et al., 2002; Burns et al., 2004; Atterbury et al., 2009, 2010; Springer et al., 2011), vaccinated hens did not show lower proportions of positive ceca compared to non-vaccinated controls. This might be due to the long period between vaccination and the challenge.

When birds with SE-colonized ceca would excrete lower numbers of SE, this would still reduce the total fecal shedding of SE on flock level. In our study, numbers of positive ceca were not significantly reduced in the vaccinated groups. In a study by Springer et al. (2011), a statistically significant lower challenge strain burden was found in cecal scrapings for vaccinated compared to non-vaccinated birds up to week 73 of age. In other studies with younger birds, cecal bacterial counts were reduced in vaccinated compared to non-vaccinated birds (Deguchi et al., 2009; Atterbury et al., 2010). A decrease in the counts of *Salmonella*-positive liver and cecum samples was found between 7 and 14 d after challenge, which was also found in other studies (Atterbury et al., 2009; Deguchi et al., 2009; Gast et al., 2011, 2015).

In addition to reducing fecal shedding, another goal of vaccination was reduction of colonization of reproductive tissues and internal egg contamination. Before the reproductive organs can become colonized, the infection has to become systemic. Therefore, we also determined whether the bacteria could be found in liver, spleen, and follicular fluid. The high proportions of positive livers and spleens found in this study indicate that, even after vaccination, the bacteria were able to establish a systemic infection. Only 4 follicular fluid samples of a total of 80 were challenge strain positive. This was expected, as in flocks naturally infected with SE, the incidence of "in ovo" infection is relatively rare (0.06 to 1.0%) (Poppe et al., 1992; Humphrey, 2006). Also, investigation of table eggs samples from EU member states in 2013 showed that only approximately 0.1% of the samples were *Salmonella*-positive (EFSA and ECDC, 2015). In vaccine efficacy studies, generally a high-dosed intravenous challenge is used to facilitate evaluation of the effects of vaccination on colonization of the reproductive tract and internal egg

contamination (De Buck et al., 2005; Gantois et al., 2006; Nandre et al., 2015). After intravenous inoculation, differences between non-vaccinated controls and vaccinated groups could be detected, although the number of positive reproductive organs and eggs was low compared to other organs. Consequently, in our study and in many other vaccine efficacy studies, cecum, liver, and spleen samples, rather than samples of reproductive organs or eggs, are mainly used for the evaluation of vaccine efficacy (Woodward et al., 2002; Burns et al., 2004; De Buck et al., 2005; Gantois et al., 2006; Atterbury et al., 2009; Deguchi et al., 2009; Filho et al., 2009; Atterbury et al., 2010; Filho et al., 2010; Desin et al., 2011; Parker et al., 2011; Springer et al., 2011; Wisner et al., 2011; Cho et al., 2013; Nandre et al., 2014). The birds showed no clinical signs or mortality after the challenge. This is similar to field conditions, where the birds become orally infected with *Salmonella* and often show no clinical signs either. In other studies, with an intravenous challenge, clinical signs have been seen (Gantois et al., 2006; Deguchi et al., 2009; Nandre et al., 2015).

We did not perform ANOVA analysis or Student's t test to compare the log-transformed cfu per gram bacteriology data between groups as described by others (De Buck et al., 2005; Okamura et al., 2007; Filho et al., 2009, 2010; Parker et al., 2011). Commonly, samples negative with direct plating and only positive after enrichment are assigned a certain log-transformed value, while samples that are negative in both tests are assigned a log-transformed value of 0. The data of this study showed that the majority of positive samples were only positive after enrichment, and therefore many samples would require a fictive logcfu value of 1 (Table 2). This would have a large impact on the mean logcfu, resulting in a large bias in the outcome of the statistical analysis. Also, such data are not normally distributed and differences between groups are likely to be underestimated as demonstrated by Lorimer and Kiermeier (2007). An alternative method would be to use an ANOVA with left censoring of the enrichment-positive samples that remain below the detection limit of the direct plating. Because too much data were censored, we could not analyze the logcfu data of the positive samples using this censored regression approach (Klein and Moeschberger, 2003; Lorimer and Kiermeier, 2007) (Table 2).

The groups differed more from each other than only with regard to vaccination history. Each group of birds in this study came originally from different farms and countries, were of different breeds and ages, and were not placed at the research facility at the same time. Hens of different breeds can have a different immune response to a *Salmonella* spp. infection (Li et al., 2013). It remains unclear whether these varying circumstances between groups influenced the results of this study.

In conclusion, in this study we did not detect any effect of any of the vaccination schemes in the rearing period on protection against SE challenge at around 82 wk of age. This may be a result of reduced immunity due to the long duration between initial vaccination and the challenge. However, as the birds were vaccinated under field conditions at the rearing farm and vaccine efficacy could not be assessed directly after vaccination, it is not clear whether the vaccination was sufficient to start with. Controlled studies from early age onward are necessary for more accurate comparisons between vaccines.

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REFERENCES

Abín, R., A. Laca, A. Laca, and M. Díaz. 2018. Environmental assesment of intensive egg production: a Spanish case study. J. Cleaner Prod. 179:160–168.

Arnold, M. E., R. J. Gosling, R. M. La Ragione, R. H. Davies, and F Martelli. 2014. Estimation of the impact of vaccination on faecal shedding and organ and egg contamination for *Salmonella* Enteritidis, *Salmonella* Typhiumurium and monophasic *Salmonella* Typhimurium. Avian Pathol. 43:155–163.

Atterbury, R. J., J. J. Carrique-Mas, R. H. Davies, and V. M. Allen. 2009. *Salmonella* colonisation of laying hens following vaccination with killed and live attenuated commercial *Salmonella* vaccines. Vet. Rec. 165:493–496.

Atterbury, R. J., R. H. Davies, J. J. Carrique-Mas, V. Morris, D. Harrison, V. Tucker, and V. M. Allen. 2010. Effect of delivery method on the efficacy of *Salmonella* vaccination in chickens. Vet. Rec. 167:161–164.

Bain, M. M., Y. Nys, and I. C. Dunn. 2016. Increasing persistency in lay and stabilising egg quality in longer laying cycles. What are the challenges? Br. Poult. Sci. 57:330–338.

Biochek. 2012. Interpretation and Application of Results Manual. Accessed Oct. 2015. http://www.biochek.com/media/BIOCHEK INTERPRETATION Manual 2012 V3.pdf.

Burnham, K. P., and D. R Anderson. 2002. Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach. 2nd ed. Springer, New York.

Burns, K., S. Kelly-Aehle, and J. Lawrence. 2004. Megan® Egg–protection for commercial layers against *Salmonella* Enteritidis infection for consumer protection. Accessed Feb. 2016. http://www.fda.gov/ohrms/dockets/dockets/00n0504/00N-0504_emc-001650-02.pdf.

Carrique-Mas, J. J., M. Breslin, A. R. Sayers, I. McLaren, M. Arnold, and R. Davies. 2008. Comparison of environmental sampling methods for detecting *Salmonella* in commercial laying flocks in the UK. Lett. Appl. Microbiol. 47:514–519.

Cho, Y., Z. Kang, K. Kang, S. Jeong, H. Yoon, S. Suh, and T. Hahn. 2013. Efficacy and clinical trials of Salenvac-T, bivalent killed vaccine containing *Salmonella* Enteritidis and *Salmonella* Typhimurium. Korean J. Vet. Res. 53:43–48.

Davies, R., and M. Breslin. 2003. Effects of vaccination and other preventive methods for *Salmonella* Enteritidis on commercial laying chicken farms. Vet. Rec. 153:673–677.

Davies, R., and M. Breslin. 2004. Observations on *Salmonella* contamination of eggs from infected commercial laying flocks where vaccination for *Salmonella* enterica serovar Enteritidis had been used. Avian Pathol. 33:133–144.

De Buck, J., F. Van Immerseel, F. Haesebrouck, and R. Ducatelle. 2004. Colonization of the chicken reproductive tract and egg contamination by *Salmonella*. J. Appl. Microbiol. 97: 233–245.

De Buck, J., F. Van Immerseel, F. Haesebrouck, and R. Ducatelle. 2005. Protection of laying hens against *Salmonella* Enteritidis by immunization with type 1 fimbriae. Vet. Microbiol. 105:93–101.

Deguchi, K., E. Yokoyama, T. Honda, and K. Mizuno. 2009. Efficacy of a novel trivalent inactivated vaccine against the shedding of *Salmonella* in a chicken challenge model. Avian Dis. 53:281–286.

Desin, S., A. Wisner, P.-K. Lam, E. Berberov, C. Mickael, A. Potter, and W. Köster. 2011. Evaluation of *Salmonella* enterica serovar Enteritidis pathogenicity island-1 proteins as vaccine candidates against *S. Enteritidis* challenge in chickens. Vet. Microbiol. 148:298–307.

EFSA and ECDC (European Centre for Disease Prevention and Control). 2014. The European Union summary report on trends and sources of zoonoses, zoonotic agents and Food-borne outbreaks in 2012. EFSA J. 12:3547.

EFSA Panel on Biological Hazards (BIOHAZ). 2004. Opinion of the Scientific Panel on Biological Hazards on a request from the Commission related to the use of vaccines for the control of *Salmonella* in poultry. EFSA J. 114:1–74.

EFSA and ECDC (European Centre for Disease Prevention and Control). 2015. The European union summary report on trends and sources of zoonoses, zoonotic agents and Food-Borne outbreaks in 2013. EFSA J. 13:3991.

Filho, R. A. C. P., J. B. de Paiva, Y. M. S. Argüello, M. D. da Silva, Y. Gardin, F. Resende, A. Berchieri Junior, and L. Sesti. 2009. Efficacy of several vaccination programmes in commercial layer and broiler breeder hens against experimental challenge with *Salmonella* enterica serovar Enteritidis. Avian Pathol. 38:367–375.

Filho, R. A. C. P., J. B. de Paiva, M. D. da Silva, A. M. de Almeida, A., and Berchieri Junior. 2010. Control of *Salmonella* Enteritidis and *Salmonella* Gallinarum in birds by using live vaccine candidate containing attenuated *Salmonella* Gallinarum mutant strain. Vaccine. 28:2853–2859.

Gantois, I., R. Ducatelle, L. Timbermont, F. Boyen, L. Bohez, F. Haesebrouck, F. Pasmans, and F. van Immerseel. 2006. Oral immunisation of laying hens with the live vaccine strains of TAD *Salmonella* vac® E and TAD *Salmonella* vac® T reduces internal egg contamination with *Salmonella* Enteritidis. Vaccine. 24:6250–6255.

Gast, R. K., R. Guraya, and P. S. Holt. 2011. Frequency and persistence of fecal shedding following exposure of laying hens to different oral doses of *salmonella* enteritidis. Int. J. Poult. Sci. 10:750–756.

Gast, R. K., R. Guraya, D. R. Jones, and K. E. Anderson. 2015. Persistence of fecal shedding of *Salmonella* Enteritidis by experimentally infected laying hens housed in conventional or enriched cages. Poult. Sci. 94:1650–1656.

Humphrey, T. J. 1994. Contamination of egg shell and contents with *Salmonella* Enteritidis: a review. Int. J. Food Microbiol. 21:31–40.

Humphrey, T. J. 2006. Are happy chickens safer chickens? Poultry welfare and disease susceptibility. Br. Poult. Sci. 47:379–391.

Klein, J. P., and M. L. Moeschberger. 2003. Survival Analysis: Techniques for Censored and Truncated Data. 2nd ed. Springer-Verlag, New York.

Li, L., Y. Shi, M. J. Cheserek, G. Su, and G. Le 2013. Antibacterial activity and dual mechanisms of peptide analog derived from cell-penetrating peptide against *Salmonella* typhimurium and *Streptococcus pyogenes*. Appl. Microbiol. Biotechnol. 97:1711–1723.

Lorimer, M. F.,, and A. Kiermeier. 2007. Analysing microbiological data: Tobit or not Tobit?. Int. J. Food Microbiol. 116:313–318.

Messens, W., K. Grijspeerdt,, and L. Herman. 2005. Eggshell penetration by *Salmonella*: a review. World Poult. Sci. J. 61:71–86.

Nandre, R. M., S. K. Eo, S. Y. Park, and J. H. Lee. 2015. Comparison of a live attenuated *Salmonella* Enteritidis vaccine candidate secreting Escherichia coli heat-labile enterotoxin B subunit with a commercial vaccine for efficacy of protection against internal egg contamination by *Salmonella* in hens. Can. J. Vet. Res. 79:235–240.

Nandre, R. M., K. Matsuda,, and J. H. Lee. 2014. Efficacy for a new live attenuated *Salmonella* Enteritidis vaccine candidate to reduce internal egg contamination. Zoonoses Public Health. 61:55–63.

Okamura, M., H. Tachizaki, T. Kubo, S. Kikuchi, A. Suzuki, K. Takehara, and M. Nakamura. 2007. Comparative evaluation of a bivalent killed *Salmonella* vaccine to prevent egg contamination with *Salmonella* enterica serovars Enteritidis, Typhimurium, and Gallinarum biovar Pullorum, using 4 different challenge models. Vaccine. 25:4837–4844.

Parker, W. D., B. Lungu, R. D. Berghaus, H. S. Sellers, I. R. Alvarado, and C. L. Hofacre. 2011. Comparison of real-time PCR with conventional PCR and culture to assess the efficacy of a live attenuated *Salmonella* enterica Serovar Typhimurium vaccine against *Salmonella* enterica Serovar Enteritidis in commercial leghorn chicks vaccinated under field and laboratory conditions. Avian Dis. 55:248–254.

Poppe, C., R. P. Johnson, C. M. Forsberg, and R. J. Irwin. 1992. *Salmonella* Enteritidis and other *Salmonella* in laying hens and eggs from flocks with *Salmonella* in their environment. Can. J. Vet. Res. 56:226–232.

R Core Team. 2015. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Schoeni, J. L., K. A. Glass, J. L. McDermott, and A. C. L. Wong. 1995. Growth and penetration of *Salmonella* Enteritidis, *Salmonella* Heidelberg and *Salmonella* Typhimurium in eggs. Int. J. Food Microbiol. 24:385–396.

Schulz, J., S. van Hoorebeke, B. Hald, J. Hartung, F. van Immerseel, I. Radtke, S. Kabell, and J. Dewulf. 2011. The dynamics of *Salmonella* occurrence in commercial laying hen flocks throughout a laying period. Avian Pathol. 40:243–248.

Springer, S., T. Lindner, M. Ahrens, G. Woitow, F. Prandini, and H.-J. Selbitz. 2011. Duration of immunity induced in chickens by an attenuated live *Salmonella* Enteritidis vaccine and an inactivated *Salmonella* Enteritidis/Typhimurium vaccine. Berl. Munch. Tierarztl. 124:89–93.

Trampel, D. W., T. G. Holder, and R. K. Gast. 2014. Integrated farm management to prevent *Salmonella* Enteritidis contamination of eggs. J. Appl. Poult. Res. 23:353–365.

Wisner, A. L. S., T. S. Desin, P.-K. S. Lam, E. Berberov, C. S. Mickael, H. G. Townsend, A. A. Potter, and W. Köster. 2011. Immunization of chickens with *Salmonella* enterica subspecies enterica serovar Enteritidis pathogenicity island-2 proteins. Vet. Microbiol. 153:274–284.

Woodward, M. J., G. Gettinby, M. F. Breslin, J. D. Corkish, and S. Houghton. 2002. The efficacy of Salenvac, a *Salmonella* enterica subsp. Enterica serotype Enteritidis iron-restricted bacterin vaccine, in laying chickens. Avian Pathol. 31:383–392.