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Executive Summary

This sub-project aimed to examine the efficacy of the Vaxsafe ST attenuated live vaccine, administered as two oral doses followed by an intramuscular dose, in reducing contamination of the environment in layer sheds and in reducing egg contamination with *Salmonella*e.

Vaxsafe® ST (Bioproperties Pty Ltd, Australia) is the only live attenuated *Salmonella* vaccine registered for use in poultry in Australia. Vaxsafe ST was developed for oral administration to short lived birds (such as broilers) and the duration of immunity it induces is relatively short due to the level of attenuation of the vaccine strain. Small scale experimental studies have demonstrated that the administration of three live oral doses and a fourth intramuscular dose at ~10 weeks of age provided some level of protection against experimental challenge with S. typhimurium and S.infantis at ~15 wk intervals up to 65wks of age, the long term efficacy of the vaccine in pullets introduced to commercial flocks that are actively shedding *S.* Typhimurium remains unclear. Uptake of vaccination as a supplement to other control measures in the Australian egg laying industry remains low due to the lack of scientific evaluation of the efficacy of the vaccine under a range of commercial conditions. Therefore these studies were initiated to obtain information to guide the use of this vaccine in layer flocks.

Two separate field trials were conducted, in South Australia and in Victoria. Both trials examined the efficacy of a novel dosage regimen for Vaxsafe® ST – three oral doses and a single intramuscular injection. The South Australian trial examined effects of the vaccine in two multi-age caged flocks, while the Victorian study examined effects in 10 single age caged and free range flocks.

Vaccinated birds were found to shed the vaccine into their environment and could transmit the vaccine to unvaccinated birds nearby. Antibody responses against *Salmonella* Typhimurium were only detectable by a commercial ELISA in birds after the final intramuscular injection.

Shedding of *Salmonella*e in infected flocks was found to be much lower than expected and was not evenly distributed around the flock. As a result of this heterogeneous distribution of contamination, infection of a flock could be missed unless a systematic randomised approach to sampling is used.

Contamination of eggs with *Salmonella*e was highest around the onset of lay, with contamination rates dropping by the peak of lay and continuing to be low until flocks were 40 weeks old, when the trial was stopped.

Because the rate of shedding of *Salmonella*e was so low, the studies were not large enough to establish whether vaccination with Vaxsafe® ST reduced the level of environmental and egg contamination.

Because the prevalence of *Salmonella* Typhimurium is relatively low in Australian layer flocks, larger field trials will be needed to determine how efficacious Vaxsafe® ST is in reducing the level of *Salmonella* contamination of eggs in the field. This low prevalence also indicates that quite specific sampling methods are needed to reliably detect *Salmonella*e on layer farms. Egg contamination with *Salmonella*e appears to be particularly high at the onset of lay, suggesting that control may need to be focussed on eggs collected during this period.

Vaxsafe® ST appears to be shed at a higher rate by vaccinated birds than previously thought and there appears to be some transmission of the vaccine between birds.

Contents

Chapter 1

Introduction

Salmonella enterica subsp *enterica (S. enterica)* is a rod-shaped Gram-negative member of the family Enterobacteriaceae and causes foodborne disease worldwide, with an estimated 153 million cases per year (1). This highly ubiquitous species contains more than 2600 different serovars that are differentiated into typhoidal or non-typhoidal *Salmonella* (NTS) serovars (2). Among the NTS serovars, *Salmonella enterica subsp enterica serovar* Enteritidis (*S.* Enteritidis) and *Salmonella enterica subsp enterica serovar* Typhimurium (*S.* Typhimurium) are the two most common serovars that infect poultry and cause food borne outbreaks of enteritis associated with consumption of eggs and egg by-products (3). Although *S.* Enteritidis is the main serovar responsible for outbreaks in Europe and the United States (4-6), in Australia, *S.* Typhimurium is the predominant pathogen responsible for outbreaks associated with consumption of eggs and egg related products (7, 8). *Salmonella* contamination of eggs and egg products, in particular with *S.* Typhimurium, is considered a significant cause of foodborne illness in Australia. Despite a number of control strategies in the form of pre-harvest techniques (flock and feed management, biosecurity, use of prebiotics) and post-harvest techniques (egg storage, egg washing, egg decontamination) focussed on reducing the risk of human salmonellosis (9, 10) there has been no clear cut decline in number of reported cases of salmonellosis. As a result of increases in the number of human salmonellosis cases between 2010 and 2015, there has been pressure from health authorities for improvements in the safety of eggs supplied to the consumer. Vaccination was recently mandated by health authorities as a method to reduce *Salmonella* contamination of eggs in a Victorian egg laying flock associated with a large foodborne outbreak. AECL sponsored workshops (2015) on *Salmonella* in eggs indicated that there was considerable interest, from both health authorities and industry members, in scientific evidence of the efficacy of vaccination as a method for *Salmonella* control under Australian conditions. All parties indicated they were not confident of the benefits of its use without further scientific evidence. *Salmonella* spp can be hosted by multiple animal species, and may survive and multiply in the environment. In addition flocks may be concurrently infected with multiple *Salmonella* serovars. *Salmonella* spp infect the gastrointestinal tract of chickens and may persist in the caeca for several months, with no observable clinical signs. Its presence may have no demonstrable effect on either production or productivity of an infected flock and detection relies on frequent environmental surveillance. Shedding of *Salmonella* spp. by persistently infected carriers may be prolonged or intermittent. Environmental stressors, temperature, water, transportation and the onset of sexual maturity can trigger *Salmonella* shedding in carrier birds. Contamination of shell eggs may occur as a result of either vertical or horizontal transmission, but it is generally accepted that horizontal transmission is the most likely route of foodborne contamination in Australia.

The capacity of *S.* Typhimurium to infect layer flocks and contaminate eggs continues to present a significant public health and food safety challenge. Salmonella control on farms is complex and requires a multi-faceted approach that targets all possible sources of Salmonella exposure. Vaccination to reduce the transmission of Salmonella to eggs has been accepted worldwide. Vaccination of pullets, to either prevent infection or reduce the duration of shedding of Salmonella in exposed flocks, is one measure for achieving better control.

Pavic et al (11) used an inactivated / killed autologous *Salmonella* vaccine (Intervet - MSD, NSW Australia) and had success in reducing prevalence of *Salmonella* serovars in meat chicken breeder flocks. However, inactivated / killed vaccines have multiple disadvantages, including induction of a limited antibody response and a failure to induce a cell-mediated immune response (CMI), which thought to be needed for *Salmonella* control and for long term protection of laying hens (12-15).

Live *Salmonella* vaccines are more effective than killed vaccines against both intestinal and systemic infections (16). Live vaccines generate both humoral and cellular immune responses (17-20) and a stronger Th1 response, which is required for clearance.

Vaxsafe® ST (Bioproperties Pty Ltd, Australia) is the only live attenuated *Salmonella* vaccine registered for use in poultry in Australia. Vaxsafe ST is an *aroA* deletion *S.* Typhimurium mutant and was developed for oral administration to short lived birds (such as broilers). However the duration of immunity it induces is relatively short due to the level of attenuation of the vaccine strain. This vaccine has not been widely used in commercial broiler breeder flocks for this purpose. Small scale experimental studies in commercial layers have demonstrated that the administration of three live oral doses and a fourth intramuscular dose at ~10 weeks of age provided some level of protection against challenge with both homologous and heterologous *Salmonella* serovars (21) for up to 55 weeks post vaccination (65 wks of age). The studies were insufficiently powerful to account for between-bird variation in *Salmonella* shedding. The long term efficacy of the vaccine in pullets introduced to commercial flocks that are actively shedding *S.* Typhimurium remains unclear. Uptake of vaccination as a supplement to other control measures in the Australian egg laying industry remains low due to the lack of scientific evaluation of the efficacy of the vaccine under a range of commercial conditions. This proposed sub-project aimed to investigate the efficacy of the Vaxsafe® ST live vaccine in commercial pullets introduced to single- or multi-age egg laying flocks that had been naturally infected with *S.* Typhimurium. The novel application of two oral doses followed by an intramuscular injection prior to the onset of egg production had not been evaluated in field trials.

The studies were performed by investigators at the University of Adelaide and the University of Melbourne over the period from day old to 40 weeks (i.e. 28 weeks post vaccination) to cover periods of stress and exposure to natural infection by both homologous and heterologous *Salmonella* serovars. The studies included both caged and free range flocks. The University of Adelaide studies described in Chapters 2 and 3 were performed on a commercial pullet rearing farm, and two multi-age, caged layer farms receiving birds from this farm, in South Australia, while the University of Melbourne studies were performed on three production sites in Victoria on 3 single age caged and 7 single age free range flocks.

The objectives of the project were

- To investigate the effect of introducing a live *Salmonella* vaccination programme involving oral and parenteral administration of Vaxsafe® ST into pullet rearing flocks prior to transfer onto naturally-infected multi-age and single aged commercial layer flocks during early lay.
- To measure the level of *S.* Typhimurium shedding in faeces and on egg shell in vaccinated verses unvaccinated flocks under field conditions.

- To study the effects of stressors (such as transport and onset of sexual maturity) on the level of *Salmonella* shedding in vaccinated and unvaccinated flocks.

Chapter 2

Shedding of *Salmonella* Typhimurium Vaxsafe® ST (STM-1 strain) during pullet rearing - a field study

(This work was conducted by Mr. Pardeep Sharma as part of his PhD at the University of Adelaide. Pardeep received International Post Graduate Research Scholarship at the University of Adelaide)

Aims

1. This field study was conducted to understand the level of STM1 shedding in the rearing phase
2. To compare *Salmonella* detection by various detection methods such as culture and PCR.

Materials and methods

Animal ethics

All the experimental procedures were performed in accordance to the Australian Code for the Care and Use of Animals for Scientific Purposes and was approved by the University of Adelaide Animal Ethics Committee (approval number: S-2015-227).

Salmonella vaccines

A commercially available live attenuated *aroA* deletion mutant *S.* Typhimurium vaccine (Vaxsafe® ST; Strain STM-1: ≥ 10^7 cfu/dose, batch no. STM 142921B, Bioproperties Pty Ltd, Australia) was used in this study.

Selection of pullet rearing farm

A commercial pullet rearing farm was selected for this study based on the willingness of the pullet grower to participate in this study. The farm had three sheds (A, B and C). Shed C had capacity to house 15,000 birds and sheds A and B could accommodate 500 birds each. Dust (n=8) and litter samples (n=8) were collected from each shed while the previous batch was reared. This sampling was performed to detect any possible wild type of *Salmonella* spp on the rearing farm. The sheds were cleaned, sanitised and left empty (resting period) for four weeks prior to placement of day-old chicks supplied directly from the hatchery. Dust (n=8) and clean wood shavings (n=8 from) were again collected from each shed after the clean-up and two days prior to the arrival of chicks.

Experimental design

Meconium samples were collected from day old chicks (n=100) at the hatchery. Day old chicks were randomly divided into two groups. Group 1 (n = 10,000) received Vaxsafe® ST by coarse spray. Day old chicks in-group 2 (n = 15,000) were left unvaccinated. Vaccinated chicks were dubbed at one day old for identification. The vaccinated and unvaccinated chicks were placed in different boxes and transported to the commercial pullet rearing farm. Vaccinated chicks (group 1) were placed in shed A and Shed B while unvaccinated chicks were placed in shed C (Figure 2.1). At 6 weeks birds in shed A and B received Vaxsafe® ST vaccine in the drinking water and

at 12 weeks, these flocks were inoculated with a third dose of Vaxsafe® ST by intramuscular injection. The vaccine was reconstituted using a commercial Marek's vaccine diluent as per the manufacturer's instructions and administered as a 0.5 mL dose at the same time as a commercial multi-valent Egg drop syndrome (EDS) / Newcastle disease (ND) killed vaccine (Nobilis® EDS+ND, MSD Animal Health). All birds were reared in a deep-litter, floor-based shed. Antibiotic-free feed was provided by a commercial feed mill and a standard lighting protocol provided by Specialised Breeders Australia was adopted during rearing.

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Sample collection after placement of chicks

After the placement of the chicks, the rearing sheds were sampled at 4 weeks (after the 1st vaccination), 8 (2 weeks after the second vaccination) and 13 weeks (1 week after the third vaccination). At each time point, 31 composite litter samples and dust swabs were collected from both groups (shed A =8, shed B = 8 and shed C = 15) in sterile Whirl-Pak plastic bags (150 x 230 mm, Thermo Fisher Scientific, Australia) from the floor and were processed for *Salmonella* isolation. For the collection of dust swabs, Whirl-Pak speci-sponge bags (115 x 239 mm, Thermo Fisher Scientific, Australia) were pre-moistened using 20 mL of buffered peptone water (BPW; Oxoid, Australia). Separate disposable gloves were used to avoid cross-contamination. After completing the sampling from each shed, shoe covers were removed and placed in a Whirl-Pak sterile plastic bag (Thermo Fisher Scientific, Australia). Thirty one blood samples (shed A =8, shed B = 8 and shed C = 15) were also collected in lithium heparin tubes (BD Vacutainer® Plus plastic plasma tube**,** UK) at each sampling time**.** Plasma samples were stored in aliquots and frozen at -20°C for further analysis.

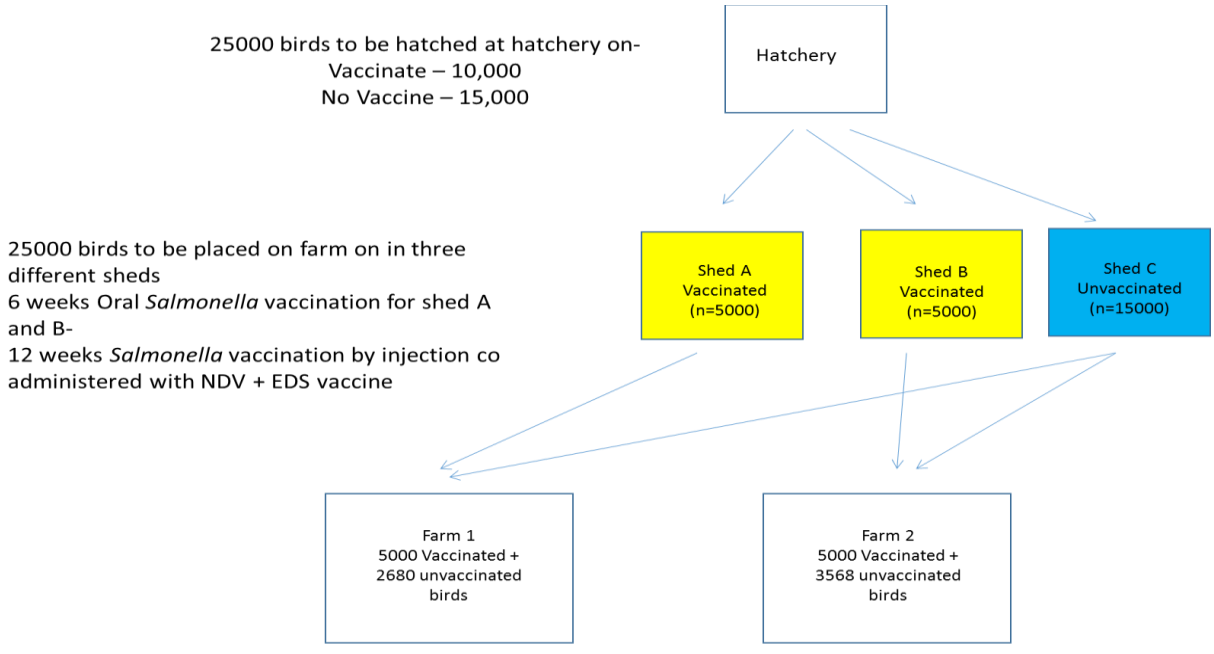

Figure 2.1. Experimental design of the *Salmonella* Typhimurium vaccine field trial.

Isolation of Salmonella and Salmonella Typhimurium vaccine strain from different samples

Litter, dust and shoe cover samples were processed for enumeration of *Salmonella* as previously described (22). Briefly, for isolation of *Salmonella* spp. and the *Salmonella* Typhimurium vaccine strain (STM1), 2 g of litter or faecal sample was inoculated into 10 mL of BPW (1:5). The inoculated samples were incubated for 24-48 hrs at 37 °C and 100 µL of this sample was transferred into 10 mL of Rappaport Vassiliadis Soya peptone broth (RVS, Oxoid, Australia), which was then incubated at 42 °C for 24 h. A loopful of the incubated RVS broth was streaked onto Brilliance *Salmonella* agar (BSA, Oxoid Australia) and xylose lysine deoxycholate agar (XLD, Oxoid, Australia) plates and the plates incubated at 37 °C overnight for confirmation of isolation of *Salmonella* spp. and the vaccine strain. Purple colonies on BSA were presumed to be *Salmonella* spp. The vaccine strain did not produce H_2S on XLD agar, allowing presumptive identification as STM-1. The STM-1 strain did not grow in BSA. Presumptive wild type *Salmonella* and STM-1 colonies were stored in 80% glycerol at -80°C for further analysis by multiplex (for detection and or typing of wild type *Salmonella* spp) or standard PCR (for the detection of STM-1).
Dust samples were moistened (to avoid drying of swabs) with 20 mL BPW and processed for *Salmonella* isolation as described above, with 10 mL of the mixture stored at -20°C for further analysis. The inoculated peptone water was also stored at -20°C for conventional PCR testing.

DNA extraction from culture (presumptive colonies), inoculated buffered peptone water and litter

The Chelex® method was used to extract DNA from stored culture samples or inoculated buffered peptone water (23). The Isolate Faecal DNA kit (Bioline, Australia) was used to extract DNA from litter samples as per the manufacturer's instructions. The concentration of isolated DNA in a sample was determined using a spectrophotometer (Nano drop ND 1000, Biolab, Australia). For qPCR, dilution was performed using nuclease free water to achieve a final DNA concentration of 5 ng/µL. Finally, these diluted DNA samples were used in real-time PCR.

Conventional and Quantitative PCR (qPCR)
For the detection and/or confirmation of wild type *Salmonella* spp, presumptive colonies or inoculated peptone water were screened by multiplex PCR as described previously (24). For the detection of STM-1, primers (Forward 5'-3'GTTTTAAGTGTAATTCGGGG; Reverse 5'-3' TATGATCAAATGGTTTCGCC resulting amplicon of 164 bp) were designed within a 360 bp region of sequence centred on the transposon / *aroA* insertion point junction. In order to optimise the standard PCR reaction for the detection of STM-1, a gradient PCR was performed using MyTaq™ DNA Polymerase (Bioline, Australia) according to the manufacturer's directions, in a total reaction volume of 20 µL containing 2 µL of DNA template, 0.5 µM each forward and reverse primer, 1.5 mM $MgCl_2$, 2.5 µM of each dNTP and 2.5 U of *Taq* polymerase. The STM-1 vaccine specific TMP3 and *invA* gene amplifications involved an initial heating step of 94°C for 2 minute followed by denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 68°C for 30 seconds for 30 cycles and a final extension at 72°C for 5 minutes in a T100 thermal cycler (Bio-Rad, Australia). Annealing temperatures of 60°C and higher yielded no additional

bands in the gradient PCR, so this temperature was selected for conventional and qPCR. For quality control and detection of possible cross contamination, the newly designed STM-1 PCR primers were tested against 28 different *Salmonella* serovars isolated from layer farm environments (Table 2.1). DNA extracted from tenfold serial dilutions of cultured STM-1 vaccine in faeces was tested by standard PCR to determine the detection limit of the standard PCR. Briefly, STM-1 strain was inoculated in peptone water and incubated overnight at 37^0C. Tenfold dilutions were prepared and added to the faecal samples. The Isolate Faecal DNA kit (Bioline, Australia) was used to extract DNA from spiked faecal samples, as per the manufacturer's instructions.

Standard curve and qPCR for Salmonella Typhimurium vaccine in litter samples

The qPCR was performed in a total reaction volume of 10 µL composed of the following: 2 µL sample (5 ng/µL), 5 µL of 2 x Quantifast SYBER Green Master Mix and 1 µM of reverse and forward primers. Quantifast® SYBER® Green qPCR kit (Qiagen, Australia) was used for the qPCR. Thermocycling was initiated by incubation at 95°C for 10 minutes, followed by 40 cycles of denaturing at 95°C for 15 seconds and annealing/elongation at 60°C for 60 seconds on a 7900HT sequence detection system (Applied Biosystems). Data were analysed assuming a cycle threshold (C_T) of 0.8 and baseline of 3 to 10. A standard curve was prepared for calculation of the limit of detection and quantification of bacterial cells using a 10-fold serial dilution of cultured STM-1 vaccine. An STM-1 colony was inoculated in peptone water and incubated overnight at 37^0C. Briefly, 0.2-gram samples of faeces were inoculated with a 10-fold dilution series of cultured STM-1. The Bioline kit was used for DNA extraction from these spiked faecal samples and qPCR was performed using diluted DNA samples (5 ng/µL). Negative and positive controls were used in each PCR reaction cycle. A cut off C_T of 34 was used to exclude the detection of false positives. A C_T of 34 corresponded to ~1,000 bacterial cells of *Salmonella* (Figure 2.2).

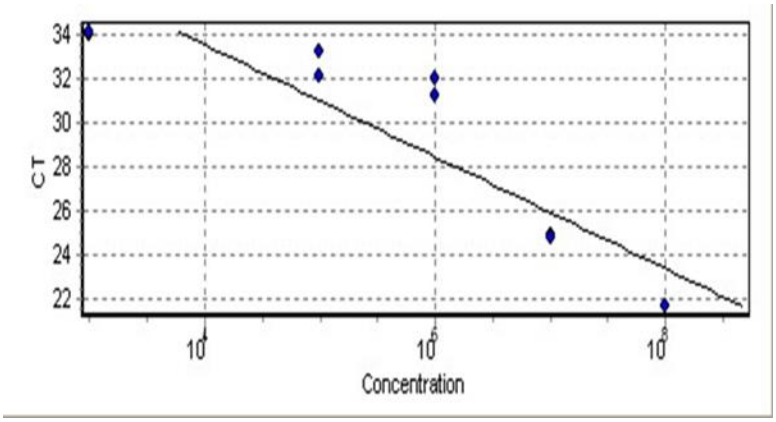

Figure 2.2. qPCR standard curve for the detection of STM-1.

Detection of anti-LPS antibodies by ELISA

ELISAs were performed using 96 well flat-bottomed microtitre plates coated with inactivated group B LPS antigen (BioChek, Holland) to detect antibodies in the plasma and antibody titres were calculated according to the manufacturer's recommendations.

Statistical analysis

Data were analysed using IBM®SPSS Statistics® version 24.0 and GraphPad Prism version 6. The prevalences of the *Salmonella* Typhimurium vaccine strain in litter, dust swabs and shoe cover samples from vaccinated and unvaccinated birds were compared using Fisher's exact test. The vaccine and bacterial loads, average log CFU and plasma antibody titres were analysed using a two way analysis of variance (ANOVA) followed by a Tukey's multiple comparison test of the mean. The significance of differences between antibody titres in the vaccinated and unvaccinated birds was assessed with Student's t-test. P values <0.05 were considered significant.

Table 2.1: *Salmonella* serovars tested against newly designed STM-1 primer set

Typhimurium DT 9
Typhimurium DT 170 (= 108)
Typhimurium DT193
Typhimurium DT135
Typhimurium DT 44
Adelaide
Infantis
Orion
Agona
Mbandaka
Johannesburg
Livingstone
subsp I ser 4,12:d:-
Singapore
Orion var 15+,34+
Chester
Zanzibar
Kiambu
Virchow
Cerro
Lille
Ohio
Anatum
Bredeney
Havana
Senftenberg
Oranienburg
Worthington
Montevideo
Isangi
DNA extracted from chicken faeces

Results

All sheds (A, B and C), including both the samples from the previous flock and from the shed two days prior to placement of the vaccinated flock) were *Salmonella* negative prior to arrival of the chicks. Meconium samples that were randomly collected from chicks prior to administration of Vaxsafe® ST were *Salmonella* negative. During rearing 6 litter samples (2 at each time point) from the vaccinated group were PCR positive for wild type *Salmonella* spp. These samples were culture negative. Birds from the unvaccinated group were negative for wild type *Salmonella* spp. For the detection of STM-1 strain from field samples, the standard PCR assay was more sensitive than culture or qPCR methods. The overall agreement between culture and standard PCR was 26.3 %, with a kappa coefficient 0.263, suggesting minor agreement.

Table 2.2: Comparison of culture and the standard PCR (St PCR) method for detection of STM-1 during rearing.

Sampling	Week of age	Culture	St PCR	Agreement
1	4	4/16	7/16	60%
2	8	2/16	8/16	25%
3	13	3/16	14/16	6.4%
Total		9/48	29/48	26.3%

The limit of detection of the standard PCR was 1 $\log_{10}$ CFU/gm of faeces and the limit of detection for the qPCR assay was 3 $\log_{10}$ CFU/gm of faeces. During this study, the standard PCR was more sensitive. Hence, to avoid false negative results, all litter samples were screened using the standard PCR in the first instance and positive samples were further tested by qPCR.

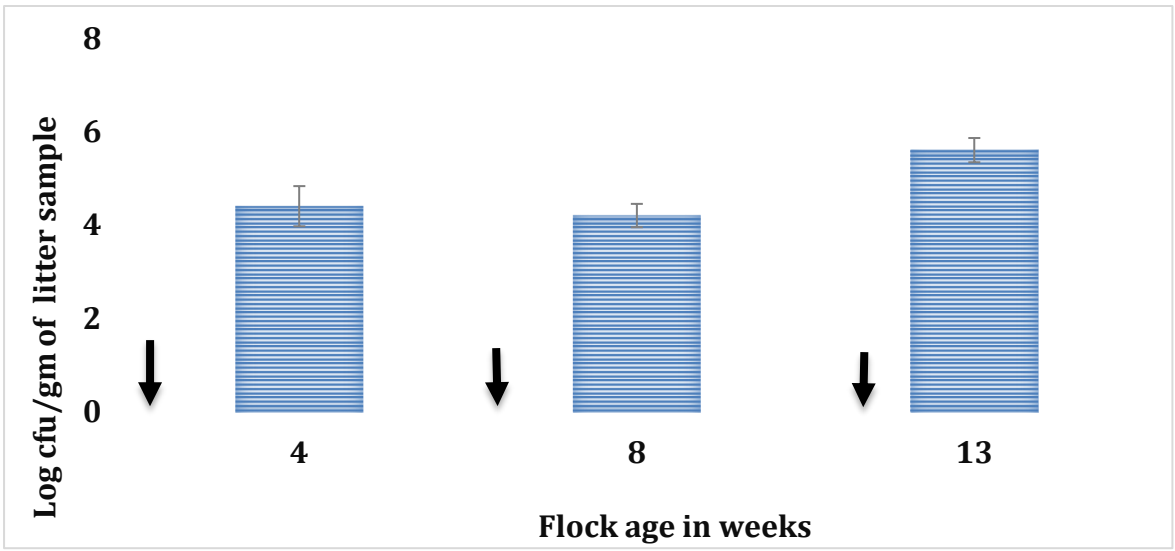

Figure 2.3 Shedding pattern of STM-1 during rearing. Arrows indicate vaccination at day 1, week 6 and week 13.

During rearing, 6 faecal samples (2 at each time point) from the vaccinated group were PCR positive for wild type *Salmonella* spp. These samples were culture negative. Multiplex PCR results indicated that these wild type *Salmonella* were predominantly *S*. Mbandaka. There were no detectable clinical signs after administration of the vaccine by the intramuscular route. The qPCR data suggested that, compared to

weeks 4 and 8, the shedding of STM-1 at week 13 was significantly higher (P = 0.025). The serological results indicated that the birds in both vaccinated and unvaccinated groups had titres below the cut off value for the assay prior to administration of the intramuscular dose. After intramuscular injection, the titres in the vaccinated group were above the cut off value and were significantly higher than those of the unvaccinated pullets.

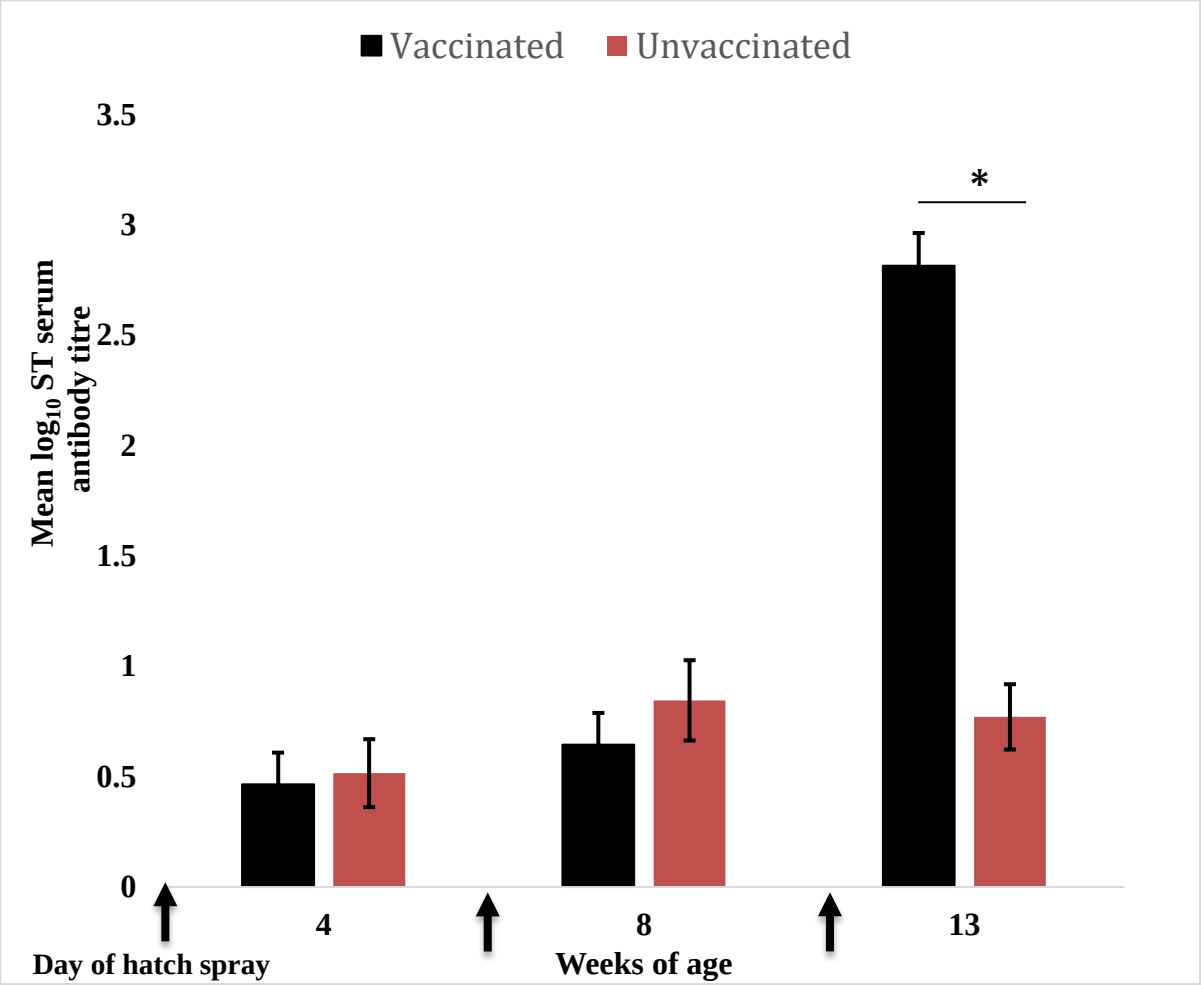

Figure 2.4. Antibody titres of vaccinated and unvaccinated pullets during rearing. Dotted line indicates the cut off value for ELISA kit.

Discussion

The goals of this study were to assess the shedding of and immune responses following the administration of a live attenuated *Salmonella* Typhimuirum STM-1 vaccine in rearing under field conditions and compare various STM-1 detection methods. In this study the sensitivity of the standard PCR assay was greater than that of the qPCR assay. Although qPCR is considered to be more sensitive in general, this

wasn't the case in this study. The newly designed primers for STM-1 did not amplify a product from wild type *Salmonella* spp or DNA extracted from faeces. This suggested that the primers were specific, but in future a probe-based qPCR could be designed to the improve sensitivity and detection of STM-1 in clinical samples. Furthermore there was only a minor level of agreement between culture and the standard PCR assay for the detection of STM-1. The standard PCR assay was more sensitive than culture for the detection of STM-1. However it is important to note that PCR can detect the DNA of non-viable STM-1 in clinical samples. There is also a possibility that during the limit of detection experiment for standard and qPCR, the bacteria cultured in pre-enrichment broth had passed stationary phase and non-viable bacterial cells were detected during the PCR assay. The other limitation of this study was that the detection limit of the PCR assays was determined using faecal samples and litter samples were tested. The load of STM-1 DNA in the litter samples increased gradually and was significantly higher at week 13. This could be because of the gradual build-up of viable or non-viable bacteria (STM-1 in this case) in the litter samples. It could also be hypothesised that the stress induced by the handling of birds during intramuscular injection resulted in increased shedding of bacteria. However further controlled experiments are necessary to confirm this hypothesis. Viable STM-1 was detected for up to 26 days (25) and 35 days (26) post vaccination in meat chickens during a controlled experiment. In this study the viable STM-1 was detected at 4 weeks after administration of first dose of vaccine. Intramuscular administration of STM-1 vaccine is presently an off label application method in Australia (27), however, Bioproperties have submitted an application to the Australian Pesticides and Veterinary Medicines authority (APVMA) to extend the label claim to include administration by IM injection. In this study, intramuscular administration of STM-1 did not result in any clinical signs in pullets, while, a previous report suggested that there was an adverse reaction to parental administration of this vaccine (28).

In our study, there was no increase in antibody titres after spray vaccination when one day old or after oral immunization at 6 weeks. The plasma IgG concentrations against *S.* Typhimurium specific antigens were significantly higher in the vaccinated group at 13 weeks of age, after intramuscular vaccination at 12 weeks of age. Alderton et al (29) reported an increased antibody response after oral administration of an *aroA* deletion mutant *S.* Typhimurium, but the dose rate was higher (10^{10} CFU/ml) than in this study (10^{7} CFU/ml). The culture, standard PCR and qPCR results from the current study suggest that STM-1 successfully colonized the chicken gut but did not induce a systemic antibody response until after parental administration.

Finding vaccines that work effectively in inhibiting colonization of newly hatched chicks by pathogens is important as they are highly susceptible to colonization by bacteria at this stage of their lives (30). During this study, six wild type *Salmonella* isolates were obtained from litter and dust samples, most of which were *S*. Mbandaka. *S*. Mandaka has been isolated frequently from layer farms during epidemiological investigations (22, 31). These data do not allow any conclusion about induction of cross protection against heterologous *Salmonella* serovars by administration of *S*TM-1. *Salmonella* survives in dust, feed, equipment and other environmental samples and its removal from the environment and from flocks with conventional disinfection is difficult, so there is always a challenge to improve vaccines and the effectiveness of vaccination programmes. This short term study for 13 weeks post vaccination showed that STM-1 was detectable in the faeces and in environmental samples by culture, conventional and qPCR of pre-enrichment samples.

In summary, oral administration of the *Salmonella* Typhimurium STM -1 strain present in the commercially available live vaccine (Vaxsafe® ST) colonizes the chicken gut (pullets in this case) and is shed after vaccination in faeces into the litter and the environment. This information is of value for the industry and the vaccine manufacturer.

Chapter 3

Shedding of *Salmonella* Typhimurium in vaccinated and unvaccinated hens during early lay: a field study

(This work was conducted by Mr. Pardeep Sharma as part of his PhD at the University of Adelaide. Pardeep received International Post Graduate Research Scholarship at the University of Adelaide)

Introduction

Vaccination in poultry is one practical measure to reduce shedding of *Salmonella* (15, 32). Vaccination of laying hens has been shown to confer protection against *Salmonella* infection and to decrease the level of on-farm contamination (5, 12, 14, 33). Previous studies have indicated that vaccination can help in reducing the incidence of egg contamination by reduction of *Salmonella* colonization in the reproductive organs and intestinal tracts of laying hens (34, 35). Studies have been conducted to test the efficacy of various vaccines in chickens (36-40) under experimental conditions, but there is limited information on the efficiency of vaccination in hens challenged naturally under field conditions in early lay. Various ST DTs, such as DT 108/170, 44, 9, 135 and 135a, are mainly responsible for egg associated outbreaks of disease in humans in Australia (8, 41). Therefore, it is important to design a vaccination programme in laying hens that can provide protection against different DTs. This study was focused on studying the efficacy of Vaxsafe® ST (STM-1) vaccination at rear in caged layer flocks challenged with wild type ST under field conditions.

Materials and Methods

Selection of farms

Two commercial cage farms (A and B) were selected for this study. Both farms had multi-age flocks within the sheds. The farms were included based on their history of detection of *S.* Typhimurium and willingness of the farmers to be included in this study. Farm A had a cross flow ventilation, while farm B had tunnel type ventilation (Figure 3.1).

Vaccination and placement of pullets

All pullets were vaccinated during rearing as described in Chapter 2. Pullets were transported to the layer farm at the age of 16 weeks. Vaccinated and unvaccinated pullets were housed in the same shed to allow assessment of any horizontal transfer of vaccine from vaccinated to unvaccinated birds. Each cage contained six birds.

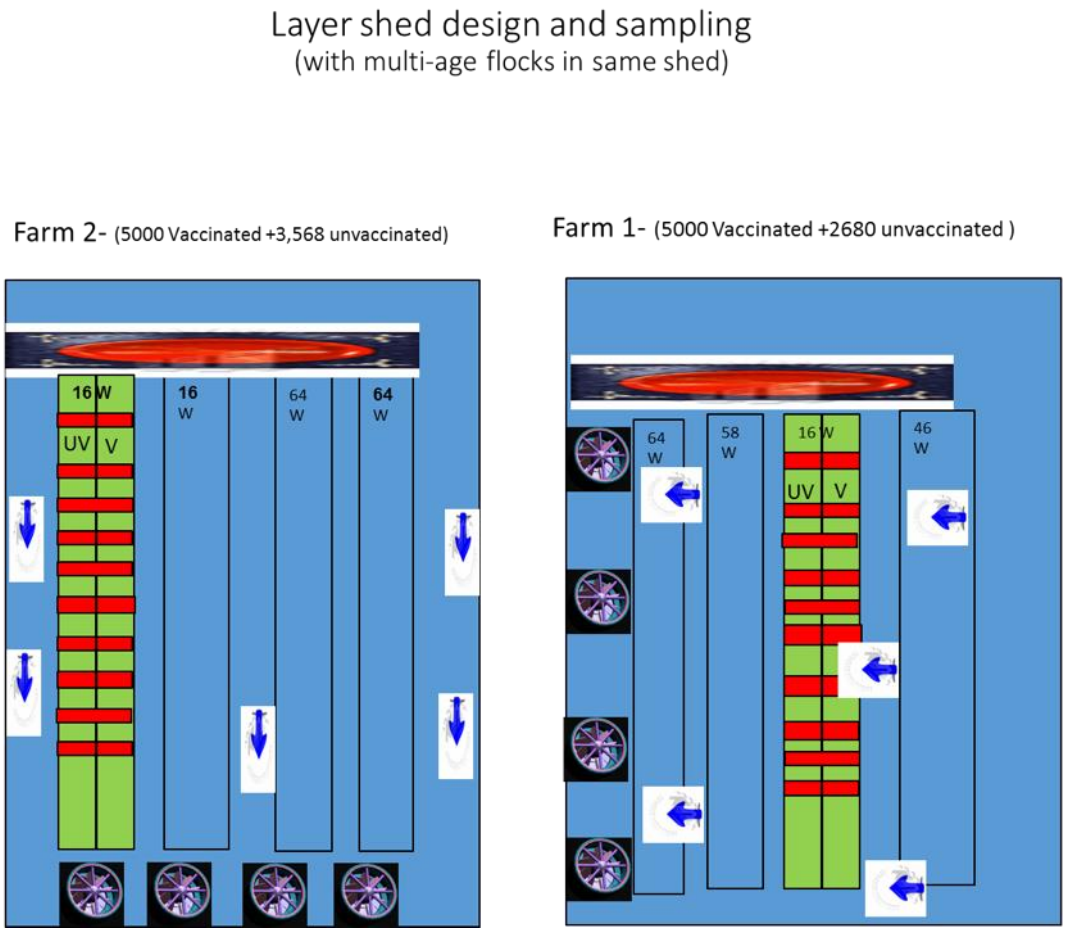

Figure 3.1. Layer shed design and sampling locations (highlighted in red). Blue arrows indicate the direction of airflow. UV, unvaccinated birds; V, vaccinated birds.

Serology

Blood samples (n=10) were collected from each treatment group. Plasma was separated and stored at -20°C for further testing. Antibody titres were determined using using the Biochek ELISA kit (BioChek, NL) as described in Chapter 2.

Collection and processing of environmental samples

Dust and cage swabs (n=8 each) were collected prior to the placement of pullets on farms A and B. This sampling was conducted to confirm the presence of viable wild type *S.* Typhimurium on both farms. After placement of pullets at farm A and B, at 17 weeks, thirty cages from each treatment group were selected. Fresh faecal samples were collected from the manure belt underneath the cages. Based on the results from thirty cages, ten *Salmonella* positive cages from each treatment group were selected for further longitudinal sampling. Faecal samples, egg belt swabs, eggs and dust swabs (1 m^2 area) were collected at each sampling time point. All samples were processed for *Salmonella* isolation by culture as described in Chapter 2. Suspected colonies of STM-1 or wild type *Salmonella* spp were stored and tested using the standard PCR as described in Chapter 2. To study the possible flow of vaccine between vaccinated and unvaccinated birds housed in the same shed, DNA extracted

from faecal samples from both treatment groups (n=200) were tested using the standard PCR for detection of STM-1.

Processing of eggs

Eggs were collected from each cage (n=120) at each sampling time point. Six eggs were pooled together for processing. Egg shell wash and internal contents were processed separately. Individual eggs were placed in 10 ml of sterile BPW in Whirl-Pak bags (Thermoscientific, Australia). To recover bacteria from the eggshell surface, the egg was massaged in the Whirl-pak bag for 2 min. Before rinsing, BPW was warmed to 37°C to facilitate bacterial recovery. After a rinse sample was obtained, each egg was removed and transferred to a new sterile bag. The BPW samples were incubated at 37°C overnight and 100 µl of this sample was inoculated into Rappaport-Vassiliadis Soya Peptone Broth (RVS) broth (Oxoid, Australia) which was then incubated at 42°C for 24 h. The incubated RVS broths were further processed for *Salmonella* isolation as described above. After eggshell surface processing, each egg was dipped into 70% alcohol for 60 sec to eliminate any bacteria present on the outside of the shell and was allowed to air dry in a biosafety cabinet. After drying, the eggs were cracked open into a sterile container. The egg internal contents, collected in sterile containers, were thoroughly mixed and 2 ml of egg internal content was inoculated into 8 ml of BPW. The inoculated BPW was further processed for *Salmonella* isolation as described above.

Results

Serology suggested that the antibody titres in vaccinated hens were significantly higher than in the unvaccinated group. Mean antibody titres in the vaccinated group were above the cut off value, but the mean titres in the unvaccinated group were below the positive threshold (mean $\log_{10}$ antibody titre = 2.8) (Figure 3.2).

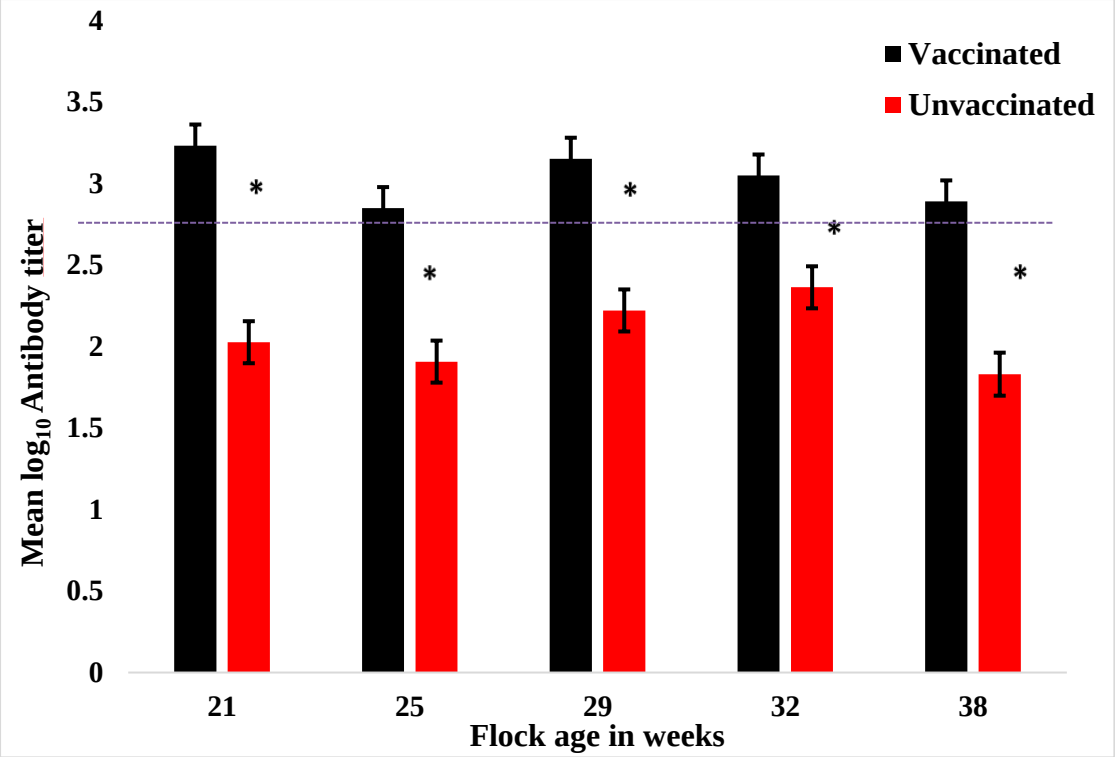

Figure 3.2. Antibody titres in vaccinated and unvaccinated hens during early lay. There was no significant difference in the prevalence of *Salmonella* spp. in faeces in vaccinated and unvaccinated groups at early lay. Similarly, multiplex PCR results indicated that there was no significant difference in the prevalence of *S.* Typhimurium in the vaccinated and unvaccinated groups at early lay. The *Salmonella* prevalence was significantly higher (P=0.04) at week 17 than at weeks 25, 29 and 32 (Figure 3.3).

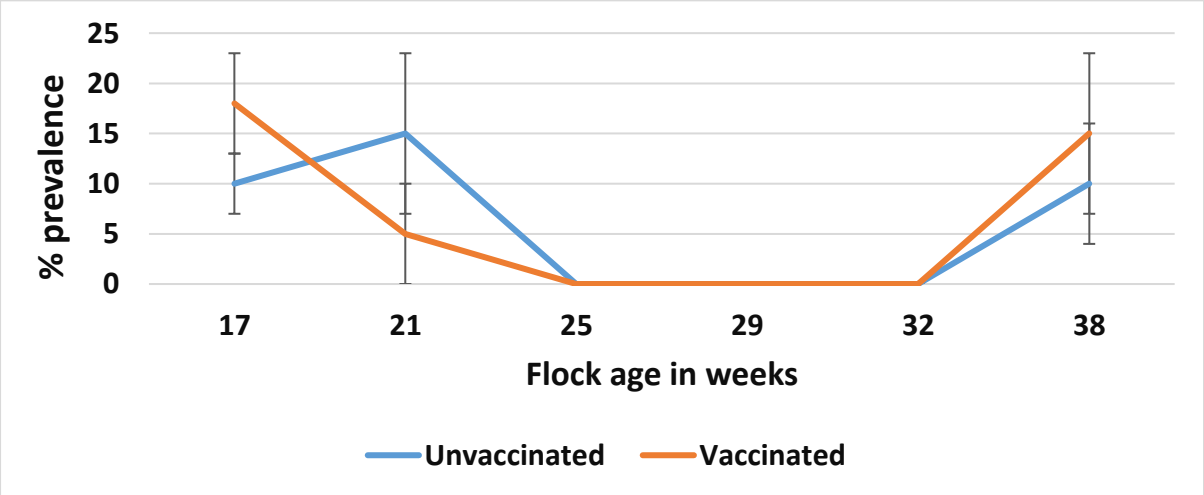

Figure 3.3. Prevalence of *Salmonella* spp. and *S.* Typhimurium in faeces

There was no significant difference in the prevalence of *Salmonella* in the vaccinated and unvaccinated groups on the egg belt in early lay (Figure 3.4) and no significant difference in the prevalence of *S.* Typhimurium in the vaccinated and unvaccinated groups in early lay (Figure 3.5).

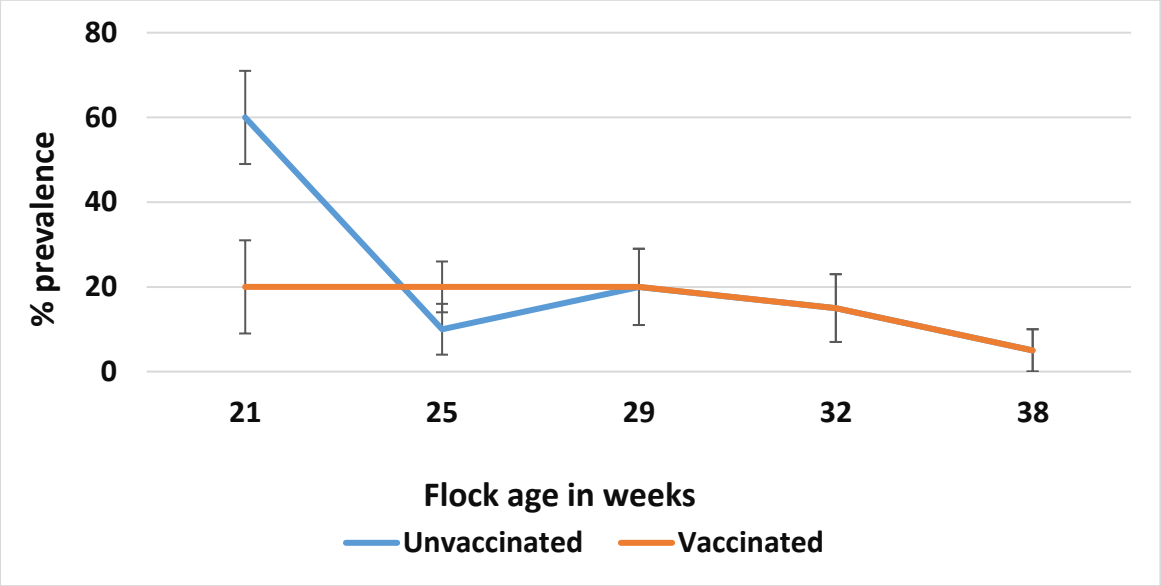

Figure 3.4. Prevalence of *Salmonella* spp. on egg belt

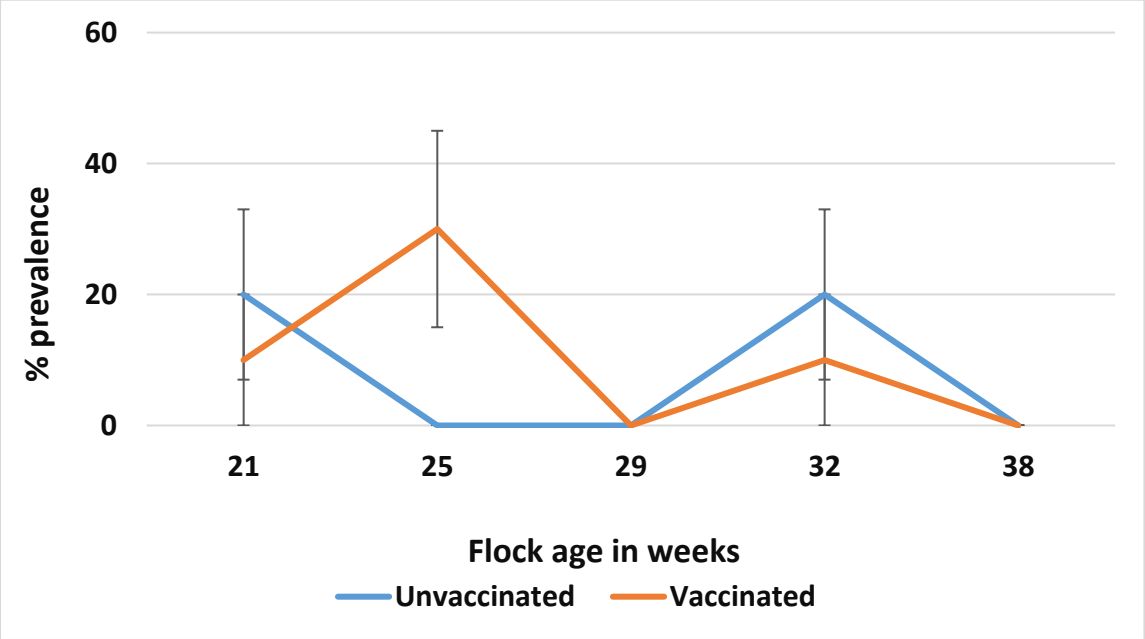

Figure 3.5. Prevalence of *Salmonella* Typhimurium on egg belt

Wild type *Salmonella* spp were consistently found in dust and shoe cover samples (Figure 3.6). *S*. Typhimurium were also consistently found in the dust and was detected throughout the study (Figure 3.7), although its prevalence did not vary significantly over the period of sample collection.

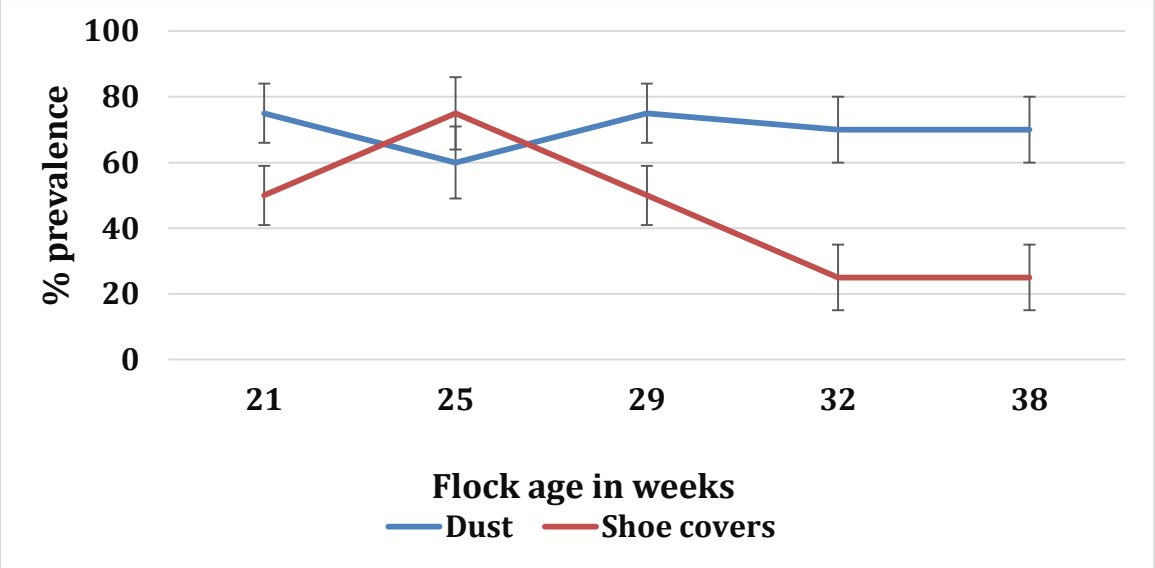

Figure 3.6. Prevalence of *Salmonella* spp. in dust and on shoe covers

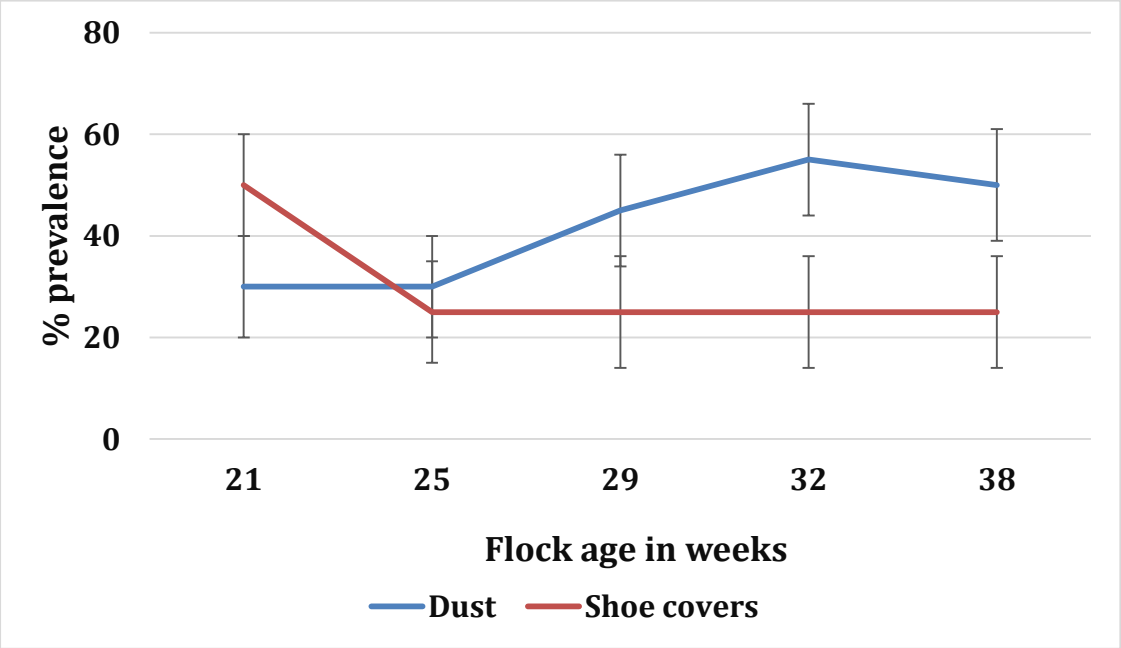

Figure 3.7. Prevalence of *S.* Typhimurium in dust and on shoe covers

Only one egg shell was positive among the samples collected from the vaccinated group. The *Salmonella* spp. in the egg shell wash was *S.* Typhimurium. All egg internal contents were negative for *Salmonella* spp. Four samples (3 egg belts and 1 faecal sample) were positive for STM-1 like bacteria by culture at week 21 and 25. Egg-belt samples were collected in front of cages and the faecal sample was collected from the manure belt directly under cages housing of non-vaccinated hens.
These STM-1 like positive samples were negative for *InvA* gene but positive for STM-1 specific standard PCR (as described in Chapter 2) and their phenotypic appearance (lack of H_2S production and colony appearance) on XLD agar. Both PCR and culture

results suggest that the vaccine may have potential to spread horizontally within the shed.

Discussion

In poultry *Salmonella* spp. readily colonise the gastrointestinal tract without usually causing clinical signs in birds (42). This means that it is more challenging to control the load of these microorganisms in poultry because there are no clinical signs suggestive of higher bacterial loads in the flock. As a result, the failure of on-farm or supply chain interventions to control *Salmonella* contamination are often only identified after outbreaks in humans (42).

The majority of poultry vaccines are developed to prevent disease, but as *S.* Typhimurium infection in adult hens does not cause disease, the rationale underlying vaccination is to reduce shedding. In this study the IgG titres of unvaccinated birds were below the positive threshold, but the titre was above the threshold in vaccinated hens. This finding was in agreement with a previous field study (27), which found that titres in unvaccinated field challenged hens were below the threshold. During an experimental *S.* Typhimurium challenge study the antibody titres of infected hens was well above the threshold (43). These hens were orally inoculated with 10^9 CFU of *S.* Typhimurium. It has been hypothesised that virulent serovars, such as *S.* Typhimurium, are more likely to invade and induce a greater systemic immune response (44). From these previous observations, it could be deduced that during the current field trial, hens were not challenged with a high enough bacterial load to induce a systemic immune response.

In this study, there was no significant difference in the prevalence of *S.* Typhimurium infection in the vaccinated and unvaccinated groups in early lay. Our findings are in agreement with a previous study by Tan et al (25) who reported that an *aroA* deletion mutant *S*. Typhimurium vaccine did not reduce faecal shedding of wild type *S.* Typhimurium under experimental conditions. Barrow et al (26) suggested that reductions in faecal shedding of wild type *S.* Typhimurium in birds vaccinated with an *aroA* deletion mutant *S*. Typhimurium were not long lasting. Although the antibody response contributes to the clearance of extracellular bacteria, facultative intracellular bacteria such as *Salmonella* spp. can persist in the host inside cells, so a cell mediated immune response is essential for clearance of *S.* Typhimurium (45). Cell-mediated immunity (CMI) is characterized by a T-helper 1 (Th1) cytokine profile, which is associated with activation of macrophages and cytotoxic lymphocytes and appears to be a critical part of effective anti-*Salmonella* immunity (46-48). R. K. Beal, et al. (49) showed that an antibody response was not essential for gut clearance of *S.* Typhimurium but suggested that an effective vaccine should activate both cellular and humoral immune response. In this study although there was an increased antibody response in the vaccinated group, *Salmonella* spp. shedding in faeces was not significantly different from the unvaccinated group. Further work on the effects of STM-1 administration on the cell mediated immune response will be helpful to understand the biology of this vaccine.

Our findings about the persistence of *S.* Typhimurium in dust samples is consistent with a previous report (22). In this study, shedding of viable STM-1 was seen for up to 25 weeks (13 weeks post IM vaccination). Previously, shedding of an *aroA* deletion mutant *S.* Typhimurium vaccine was detected in the faeces of vaccinated chickens for up to 26 days after vaccination (25). The standard STM-1

specific PCR indicated that DNA of this strain was present in unvaccinated birds, suggesting that there was transmission of STM-1 from vaccinated to unvaccinated hens housed in the same shed. STM-1 like bacteria were detected by culture (based on colony morphology and colony PCR). However further confirmation using whole genome sequencing and/or serotyping of STM-1 like isolates during production is essential.

In this study, only one egg shell positive sample was detected which was not sufficient to assess whether STM-1 had any effect on the shedding of wild type *Salmonella* spp on eggs. However, given that faecal samples and egg belts are the possible indicators of egg contamination (22), it could be deduced that the STM-1 vaccine may not have any effect on the level of egg contamination, although larger controlled studies are necessary to investigate this further. It is important to note that vaccination against *S.* Typhimurium is one of the intervention strategies to reduce the shedding of bacteria and not the ultimate prevention. Given that wild type *S.* Typhimurium is able to be detected in dust over several weeks, regular removal of the dust from the shed is likely to be important.

Chapter 4

Shedding of *Salmonella* Typhimurium and *Salmonella* Infantis in vaccinated and unvaccinated caged and free-range layer hens throughout production: a longitudinal field study

Aims

The Victorian field study aimed to compare the effect of vaccination with Vaxsafe® ST on contamination of the environment of sheds and of eggs with *Salmonella* spp during production in commercial single aged caged and layer flocks.

Methodology

Study Design

The study aimed to sample selected flocks at least three times during production and, if possible, twice during pullet rearing. The study design aimed to have a power of detection of an effect due to vaccination of 80% at the 95% confidence level. Sample size calculations were conducted assuming a reduction in the proportion of positive samples from 30% to 15% and assuming a conservative intra-class correlation co-efficient to account for clustering at the farm level and the effect of repeated observations at the farm level of time. To achieve this study design at least ten treatment flocks were required.

Egg sampling

At each egg sampling event 300 eggs were collected. This number of eggs was sufficient to detect a 1% prevalence of egg contamination with 95% confidence.

Estimates of the appropriate sample size for environmental sampling were calculated based on sampling of ecological environments (50). To ensure that the sampling strategy was replicable and reflected an unbiased sample of the shed, the sampling method was validated to ensure representative coverage of the shed environment by modelling using R (51).

Selection of flocks

Ten single aged flocks from farms with a history of *Salmonella* Typhimurium were recruited for vaccination, 3 caged flocks and 7 free range flocks, on three production sites. Eight unvaccinated control flocks from the same sites, at various stages of production, were sampled during the study and previous records were obtained to determine historical flock and farm *Salmonella* status. An unvaccinated single aged flock was replaced with a vaccinated flock.

Vaccination

Most pullets were vaccinated by coarse spray when one day old at the source hatchery using a conventional spray cabinet used for the application of live viral vaccines. For those flocks not able to be vaccinated at the hatchery, they were vaccinated orally by drinking water on arrival on farm. Pullets were vaccinated at 4 to 6 weeks of age via drinking water in accordance with the routine on-farm vaccination schedule. The third vaccination was conducted by contract vaccinators at 12 weeks of age. Vaxsafe® ST vaccine was reconstituted with sterile diluent (3 to 5ml) and then added to the NDV/EDS killed vaccine prior to intramuscular administration.

Sampling Methodology
All sheds were sampled (see below) after cleaning, prior to chick arrival or pullet transfer, to determine the status of the shed prior to the onset of the study period. All samples were collected from the environment as per the environmental sampling method, unless otherwise stated.

Pullet Rearing
Chicks were sampled at day old on delivery to pullet rearing facility by collection of chick papers or box liners. Thirty chick box liners were collected if available. If chick papers were not available, flocks were sampled within a week of arrival to determine the level of environmental contamination after arrival.

Production
All flocks were sampled at least three times during production, from immediately after transfer till they were 40 weeks of age. Sampling occasions were post transfer (16-18 weeks), peak lay 24-26 weeks and 40 weeks of lay (peak egg mass).

Environmental Sampling in Cage Sheds
From each cage shed the sampling design took into account the structure of the facilities to ensure that ~30 samples were collected from each shed. The sampling design for each shed was replicated at each sampling event to ensure repeatability of the results. A total of 29 samples, 10 egg-belt, 10 dust, 5 manure belt and 4 boot swabs, were collected from each cage shed. Samples were systematically collected from each cage frame on both rows of birds where flocks were housed back to back in frames. Dust, egg belt and boot swabs were collected from the length of each row, while manure belts were sampled at the end of each frame (2 rows comprise a single frame). Sampling locations are indicated in Figure 4.1.

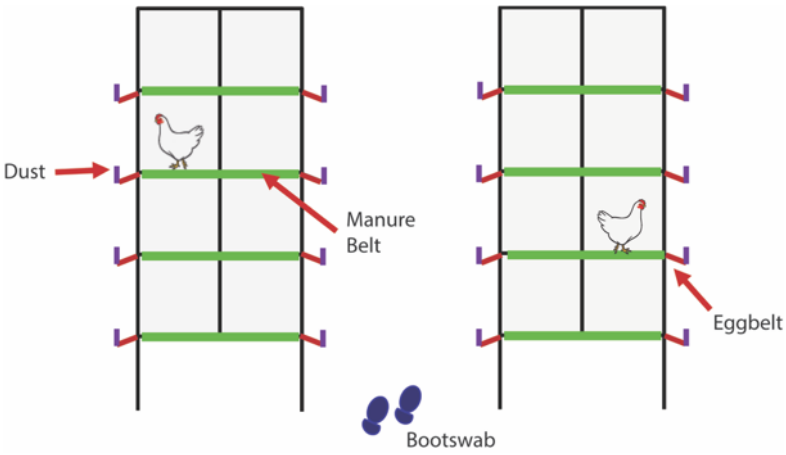

Figure 4.1. Sampling locations within a cage shed

Egg belts were sampled using four 10 x 10 cm cotton gauze swabs (swabs) to wipe the surface of the entire length of the egg belt of each row. Dust samples were collected using two swabs wiped along the uppermost surface of the nest box cover for the length of each row. Four swabs were used to collect manure belt samples. Each swab was wiped over all exposed surfaces, including the manure belt, and any exposed guarding or pipes exposed to manure when the belts were operated.

Clean dry boots, with new plastic boot covers, were worn on entry into the shed. Boot swabs pre-moistened with sterile BPW were worn over the plastic boot covers while walking within the shed to collect the other samples. Two pairs of boot swabs (4 in total) were collected, with each pair representing half the shed.

Sampling in Free Range Sheds

The sampling strategy varied depending on the shed design. In each shed 24 samples were collected, 4 boot swabs, 4 perch swabs, 4 fan or wall swabs, 4 nest box swabs and 4 feeder swabs. Samples were systematically collected from each half of the shed to ensure that samples were collected across the entire bird contact space. Perches were sampled using four swabs to wipe the surface of perching areas for the length of at least two perches in each half of the shed. Two fans on each side of the shed were sampled using four swabs wiped over the surface of the fan guard. Nest box samples were collected by wiping four swabs the length of the nest boxes on each half of the shed. Four feeders or feeder lines in each shed were sampled by wiping four swabs the length of the feeder line, or two swabs for each feeder. Again, samples were collected from each half of the shed.

Each sample type was pooled by row or sample location into a Whirlpak™ bag and identified by shed, sample type and row ID or location ID and shed half. Samples were immediately refrigerated and transported to the laboratory for processing the same day.

Egg Sampling

In caged flocks, 300 eggs were collected randomly from within each shed on each sampling occasion. Thirty eggs were collected from each frame and row, from four egg belts. Samples were identified by frame and row and processed to maintain traceability to the location of sampling. In flocks at the onset of egg production eggs were collected from the front of cages prior to the egg belts being run for the first egg collection, so did not travel over egg belts. Floor eggs from free range flocks were collected for sampling. On each sampling occasion 300 eggs were sampled when available. Opportunistically available eggs were collected from 2 vaccinated and 1 control flock at 5-15% egg production.

Sample Processing

All samples were collected and processed the same day.

Primary Samples

The primary sample types for each location, material used for sample collection and the sample pool sizes for microbiological testing are summarized in Table 4.1. Details of primary sample processing post sample collection are provided below, with microbiological testing conditions outlined.

Table 4.1. Primary sample collection material and pool size for testing

Sample Type	Sample Material	Pool Size*
Dust	*10 x 10 cm Gauze Swab*	*2 or 4*
Manure Belt	*10 x 10 cm Gauze Swab*	*4*
Egg Belt	*10 x 10 cm Gauze Swab*	*4*
Boot Swab	*Boot Swab*	*1*
Egg	*Whole Egg*	*1 or 3*
Egg components	*Outside wash*	*1*

	Shell and membrane	*1*
	Internal content (yolk and white)	*1*
Chick Papers	*Hatchery basket liner*	*10*

*No. sample materials per primary sample

Chick Papers
To each sample of ten chick papers 900 ml of buffered peptone water (BPW) was added and the sample left at room temperature to soak for approximately 30 minutes until the paper was wet through. The sample was then macerated manually by massaging the paper within the bag and incubated.

Boot Swabs
Each pair of boot swabs collected from were processed as individual swabs. BPW (200 ml per boot swab) was added with minimal mixing and incubated.

Eggs
Individual whole eggs (not cracked or broken as determined by candling) were placed in a sterile Whirlpak™ bag containing BPW (25 ml). Each egg was manually macerated and the contents gently mixed for 1 – 2 minutes. For egg component examination, each egg was placed in a sterile Whirlpak™ bag containing BPW (25 ml). The surface of the egg was gently massaged for 1-2 minutes to remove as much surface debris as possible. The Whirlpak™ bag containing the rinse was incubated (outside wash). The egg was aseptically removed from the Whirlpak™ bag and sterilized by full immersion in 100% ethanol at room temperature and air dried on a sterile surface. The dried egg was then aseptically broken into two Whirlpak™ bags, one containing the egg white and yolk only (inside contents) and the other containing the shell and membranes (shell). To each sub-sample 25 ml of BPW was added and each sample was gently massaged for 1-2 minutes to manually macerate and mix the samples. Whole eggs or egg components were incubated at 37°C for 48 hours.

Egg Pools
Pools of 3 eggs were placed in a sterile Whirlpak™ bag containing BPW (75 ml). Eggs were macerated manually and the contents gently mixed for 1 to 2 minutes. Egg pools were incubated at 37°C for 48 hours.

Microbiological Testing
Microbiological testing was conducted as per the Australian Standard[1] 5013.10-2009 (Food Microbiological Method 10: Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp. (ISO 6579:2002, MOD)) (52). To each primary sample, unless indicated above, 9 volumes of BPW was added with little mixing and each suspension was statically incubated at 37°C for 18-24 hours. After incubation, three 33 µl aliquots (total 0.1 ml) were taken from each primary sample and inoculated onto Modified Semi–solid Rappaport Vassiliadis (MSRV) plates.

MSRV plates were aerobically incubated at 41.5°C and visually examined at 12, 24 and 48 hours after inoculation. Plates with evidence of swarming growth have a

[1] Equivalent to the European Standard ISO 6579:2002 - differs only in the *Salmonella* positive control isolate.

grey-white turbid zone extending from the inoculation point with a clearly defined edge. Positive plates were sub-cultured by streaking in duplicate onto xylose–lysine–desoxycholate (XLD) agar. XLD agar plates were incubated for 24 hours at 37°C. When *Salmonella* was identified after the first 24 hr on XLD agar, no further plating from MSRV was conducted.

Suspect *Salmonella* spp positive colonies on XLD agar were confirmed biochemically in triplicate using Triple Sugar Iron agar (TSI) and Lysine Iron agar (LIA), incubated for 24 hours at 37°C. At least two suspect *Salmonella* positive colonies from each sample were confirmed positive by PCR using a *Salmonella* specific multiplex PCR developed in at the Asia-Pacific Centre for Animal Health. If more than one morphologically distinct colony type was observed in the primary culture, then additional colonies were tested. All *Salmonella* positive samples were screened by PCR to identify *Salmonella* Typhimurium or *Salmonella* Infantis positive isolates. All *Salmonella* Typhimurium or *Salmonella* Infantis positive samples were sub-cultured into *Salmonella* maintenance media for long-term storage.

Salmonella Multiplex PCR conditions

A multiplex screening PCR was developed to screen all isolates for the presence of *Salmonella* Subspecies enterica, *Salmonella* enterica subspecies enterica serovar Typhimurium and *Salmonella* enterica subspecies enterica serovar Infantis.

Primers were sourced from the literature; *Salmonella* Infantis (53), *Salmonella enterica (54)* and *Salmonella* Typhimurium (55) and are presented in **Table 4.2**.

PCR requirements per sample: Add 1ul template to 4ul dNTPs (1.25mM), 1ul of each primer set (10uM), 2ul of $MgCl_2$ (25mM), 4ul of 5x Gotac buffer, 1.8ul water, 0.2ul of GoTaq (5U/ul). Total volume 20ul.

PCR run conditions were as follows: step 1, 95°C 5min, 35 cycles of step 2 95°C for 1min, step 3 62°C for 30s, step 4, 72°C for 1min and final step 5, 72°C for 10min.

Table 4.2. Primers for Multiplex PCR for screening *Salmonella spp.*, *Salmonella* Typhimurium and *Salmonella* Infantis

Primer ID	Gene	PRIMER (5'-3')	Product Size	Reference
STM4497F STM4497R	STM	GGAATCAATGCCCGCCAATG CGTGCTTGAATACCGCCTGTC	523bp	(55)
878F 1275R	FliB	TTGCTTCAGCAGATGCTAAG CCACCTGCGCCAACGCT	413bp	(53)
139-141F 139-141R	InvA	ACAGTGCTCGTTTACGACCTGAAT AGACGATGGTACTGATCGATAAT	244bp	(54)

Enumeration

Enumeration was conducted using the most probable number (MPN) technique by serial dilution in accordance with the Australian standard 5013.14.1-2010 (56), and modified using MSRV as described by the SARDI laboratory (pers. Comm.). A 10 ml sub-sample was collected from each primary sample prior to incubation. Three tube,

10 ml, 1:10 and 1:2 dilution series were inoculated with a starting aliquot of 1 ml (1:9 ml) or 5 ml (5:5 ml) of the subsample respectively, in each dilution series. From each dilution in the series three 33 µl (total 0.1 ml) aliquots were inoculated onto MSRV plates. MSRV plates were aerobically incubated at 41.5°C and visually examined 12, 24 and 48 hours after inoculation. Positive plates were sub-cultured by streaking onto XLD agar. XLD agar plates were incubated for 24 hours at 37°C. When *Salmonella* were identified after the first 24 hr on XLD agar, no further plating from MSRV was conducted. This process was replicated in triplicate for each sub-sample tested.

Statistical Methods

All sample collection data and testing results were stored in a Filemaker Pro relational database (57) or Excel spreadsheet (58). Data mining, variable screening, and identification of outliers, missing values or incorrectly coded variables were conducted using Excel and Tableau (59). Univariate, multivariate and advanced statistical analyses were conducted using R (51). Measures of association were conducted using the epiR package v0.9-69 in R (60). Where odds were unable to be calculated due to a zero in the numerator, tests were calculated using an alternative method (61). Evaluation of egg and environmental prevalence was conducted using logistic or linear regression using a mixed effects model to take into account of variation between multiple flocks and locations using the lme4 and nlme packages in R.

Prevalence Calculations

Pooled prevalence was estimated for a fixed pool size with known sensitivity and specificity (62, 63). Apparent prevalence was calculated by estimating prevalence from the results of a screening test assuming an imperfect test (64, 65). Estimates for test sensitivity and specificity were 0.88 and 0.99 respectively. Sensitivity and specificity estimates for the culture method were obtained from EU inter-laboratory comparison studies (66-68).

Most Probable Number Calculations

Most probable numbers of *Salmonella* spp. per unit of sample material tested were calculated using the method described in the USFDA bacteriological analytical manual (69).

Results

Ten single aged treatment flocks from farms with a history of *S.* Typhimurium carriage were recruited for the study, 3 caged flocks and 7 free range flocks. Eight unvaccinated (control) flocks from the same farms, at various stages of production, were sampled during the study.

In each shed an unvaccinated single aged flock was replaced with a vaccinated flock. Unfortunately, four free range flocks withdrew from the trial prior to the onset of production, leaving six vaccinated flocks in the study. Vaccination and sampling was conducted until the point of transfer (6 or 16 weeks), when the flocks were lost to follow-up. Their loss occurred too late in the trial to enable replacement flocks to be identified.

Environmental Sampling

Sample size calculations

Two sampling strategies were evaluated, 5 randomly selected samples (collected similarly to the EU regulatory sampling strategy (70)) or 30 samples collected using a systematic random sampling strategy. Each strategy was replicated 500 times and the sampling location plotted on a two-dimensional, georeferenced representation of a cage environment as shown in **Error! Reference source not found.**. Each potential sampling location is represented as a point and two cage frames are illustrated within the plot for ease of understanding. Wall, fan and floor sampling locations are illustrated between the sampling rows.

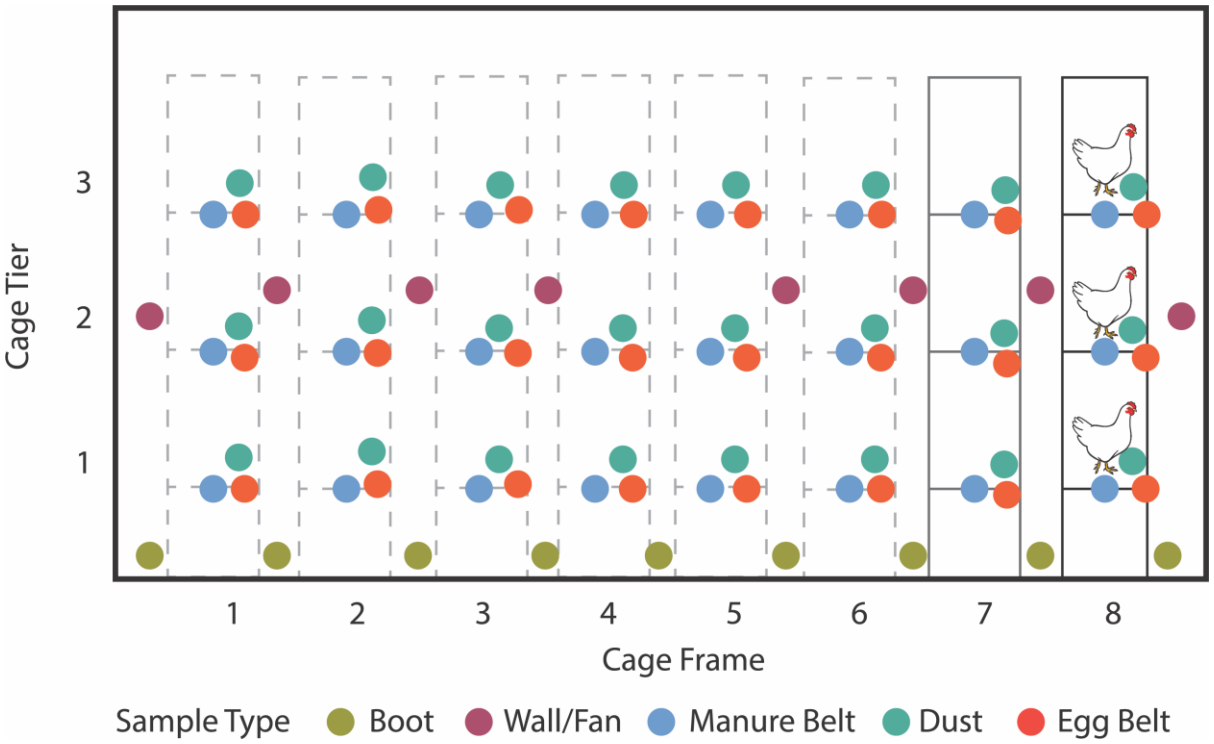

Figure 4.2. Two-dimensional, georeferenced spatial representation of a three dimensional cage environment. Layer cage environments are three dimensional spaces, to enable modelling of the sampling environment, each sampling location is represented as a point within a two dimensional XY plane. Each point identified in the plot represents a potential sampling location, with each sample type illustrated by a colour. Cage frames are represented within the plot for illustrative purposes with birds within a tier to enable the reader to understand the plot. Floor, fan and wall sampling locations are indicated as points between the cage frames and bird contact area sampling locations - egg belt, manure belt and dust within the cage frame.

Heat-mapping was used to indicate which locations were sampled: heat maps of the simple random sampling strategy and the systematic random sampling strategy are illustrated in **Figure 4.3**. The more frequently a location was sampled the more frequently that location was sampled. The frequency with which a sample was selected is indicated by colour, as the frequency increases the “hotter” (indicated by yellow) the sampling location was coloured. Where no sampling location is selected, the colour is cool (indicated by blue). Effective spatial representation of the

environment was achieved when all locations were evenly coloured indicating that each location was as likely to be sampled. The difference between the two sampling strategies is illustrated in the figure and clearly demonstrates that collection of 5 samples (indicated by the uneven distribution of “hot spots” in the rows and tiers Figure 4.3 A) would be insufficient to effectively cover the full environment evenly, no matter how many times sampling is repeated. Conversely collection of 30 systematically random samples (indicated by yellow bands across each row and cage tier Figure 4.3 B) was sufficient to ensure even coverage across the full cage environment and allowed a heterogeneous distribution of *Salmonella*e to be detectable.

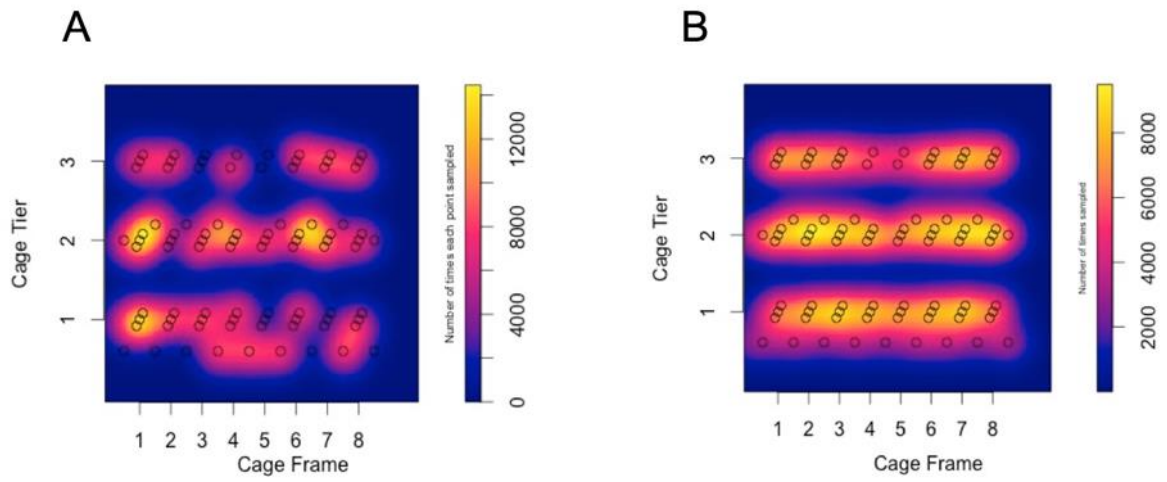

Figure 4.3. Heat map of the frequency of sample location selection based on two sampling strategies replicated 500 times. A. Five randomly selected sampling locations. B. Thirty systematically random sampling locations.

Pullet rearing

Two of four pullet rearing sites were able to be sampled. Pullet rearing sites comprised both cage and floor (litter) rearing facilities. Six of the seven free range flocks were reared in cages until 6 weeks of age, then transferred to floor rearing facilities prior to transfer to production facilities. Floor rearing facilities were only able to be sampled after cleaning prior to flock placement. One free range pullet flock was unable to be sampled during rearing, but routine flock test records indicated that no *Salmonella* spp were detected during rear. The details of the *Salmonella* testing methodology at pullet rearing for this flock was unable to be obtained. No control flocks were able to sampled during rearing, so comparisons of the effectiveness of vaccination during this period, which were outside the original intended scope of the study, were unable to be conducted.

The two caged rearing sites were sampled on ten occasions; each site was sampled prior to flock placement and each placed flock was sampled at least three times. The total number of samples taken, the environmental sample prevalence by sample type and the prevalence of *Salmonella* Infantis are indicated in Table 4..

Table 4.3. Pullet site environmental sampling results summarized by sample type for all sampling occasions.

Sample Type	*Positive Samples*	*Total Samples*	*Apparent Prevalence (95% CI)*	
			Salmonella spp.	*S. Infantis*
Boot Swabs	*26*	*64*	*0.41 (0.29-0.53)*	*0.031 (0.009-0.11)*
Dust	*35*	*224*	*0.16 (0.12-0.22)*	*0.013 (0.005-0.039)*
Manure Belt	*18*	*50*	*0.36 (0.24-0.50)*	*0.006 (0.021-0.162)*
Chick papers	*0*	*84*	*0.00*	*0.00*
All	*71*	*422*	*0.17 (0.14-0.21)*	*0.19 (0.01-0.037)*

Salmonella spp. were detected at each sampling event and the prevalence of *Salmonella* spp. positive environmental samples increased as the pullet flocks aged despite vaccination (**Error! Reference source not found.**).

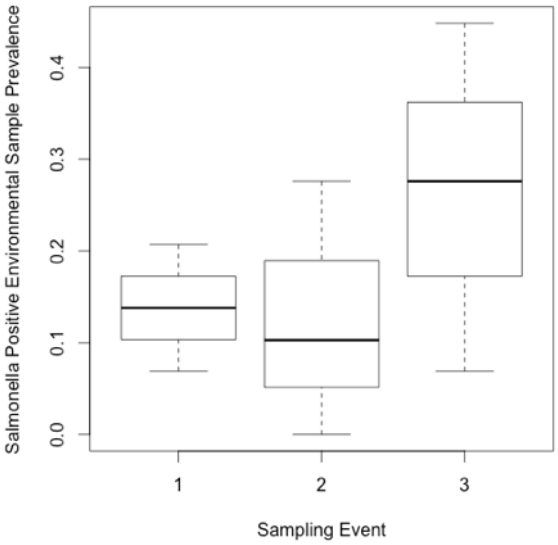

Figure 4.4. Cage rearing site *Salmonella* spp. positive environmental sample prevalence for each sampling date aggregated for all pullet flocks

The distribution of *Salmonella* spp. within the cage rearing facilities varied by sample event and was clearly not homogenous. The very low environmental sample prevalence and distribution over time is illustrated in **Error! Reference source not found.**.

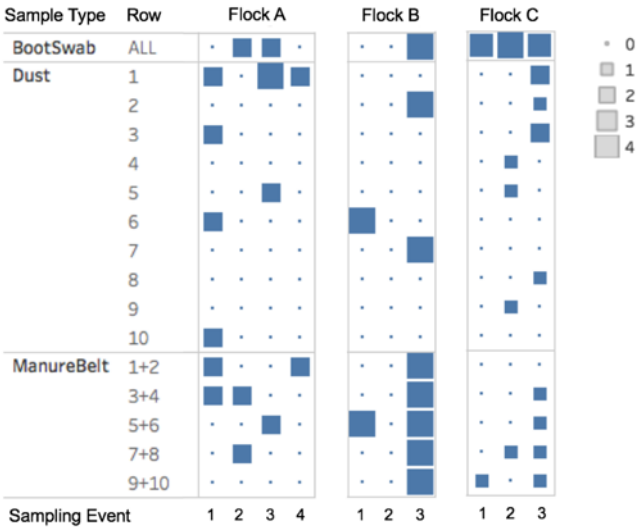

Figure 4.5. Heterogeneous environmental distribution of detection of *Salmonella* spp over time in three pullet flocks. Positive environmental locations are indicated as a blue square and negative locations are represented by a point. Time point 1 represents post cleaning sampling, time 2 indicates post 6-week vaccination and time 3 sampling after the 12-week vaccination.

The distribution of positive environmental samples varied for each flock. Only boot swabs collected in flock C were positive on every sampling occasion and only in flock B and flock C did the environmental sample prevalence and distribution within the shed increase at the final sampling date. Simple random sampling within the shed would have been unlikely to have detected *Salmonella*e on any of the sampling occasions, with possible exception of in flocks B and C on the third sampling occasion.

Aggregated environmental sampling results for all flocks sampled on the caged rearing sites are presented in Table 4.. The mean environmental sample prevalence increased as the flocks aged, but at the second sampling event, post the second vaccination, some flocks were negative for *Salmonella* spp.

Table 4.4. Pullet rearing environmental sampling results summarized by sample event for all caged rearing sites

Sampling Event	*Flock Age*	*Sample Timing*	*Apparent Prevalence*	
			Salmonella spp. Mean +/- S.D.	*Salmonella Infantis Mean (95% CI)*
1	*Not applicable*	*Post cleaning*	*0.14 +/- 0.07*	*0.034 (0.01-0.18)*
2	*6 weeks*	*Post 2nd vaccination*	*0.13 +/- 0.14*	*0.069 (0.02-0.22)*

3	*14- 16 weeks*	*Post 3rd vaccination*	*0.26 +/- 0.19*	*0.034 (0.01-0.18)*

Multiple *Salmonella enterica enterica* serovars were detected during sampling, but *Salmonella* Typhimurium was not detected on the caged rearing site on any sampling occasion. *Salmonella* Infantis was detected in all pullet flocks during rearing at a very low sample prevalence (Table 4.). The timing of detection of *Salmonella* Infantis varied by flock and location and it was not detected in the same location on consecutive sampling dates. The heterogeneous distribution of *Salmonella* Infantis contamination within the shed environment is shown in **Error!**

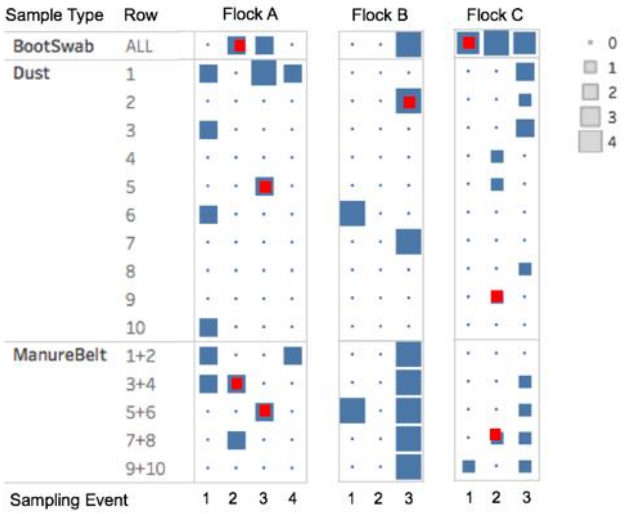

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Figure 4.6. Heterogeneous distribution of detection of *Salmonella* Infantis during pullet rearing in three flocks. *Salmonella* positive locations are indicated in blue and *Salmonella* Infantis positive locations are indicated in red. Sites where no samples were detected positive are indicated by a blue point.

To evaluate vaccine efficacy the proportion of contaminated environmental samples prevented by the vaccine in vaccinated flocks was estimated using the attributable fraction (Table 4.) (71). Post cleaning environmental sample results were used as the unvaccinated control point. The effect of vaccination on the environmental sample prevalence of *Salmonella* spp. and *S*. Infantis was estimated for all periods post vaccination or by sampling point – after the second and third vaccinations. The odds of environmental contamination with *Salmonella* varied over the time, with the odds of *Salmonella* spp. contamination higher post cleaning than after the second vaccination and the odds of *Salmonella* Infantis contamination higher in the environment post cleaning than after the third vaccination. Overall, at the end of pullet rearing, vaccination did not prevent contamination of the environment in the vaccinated group when using the post cleaning sample results as the baseline for comparison.

Table 4.5. Efficacy of vaccination in reducing the prevalence of *Salmonella* spp. and *Salmonella* Infantis in environmental samples

Time Period	*Salmonella spp.*		*S. Infantis*	
	OR (95% CI)	*AF (95% CI)*	*OR (95%)*	*AF (95% CI)*
Post 2nd vaccination	*1.07 (0.48-2.39)*	*0.06 (-0.93-0.54)*	*0.22 (0.03-1.88)*	*-3.33 (-34.31-0.47)*
Post 3rd Vaccination	*0.52 (0.24-1.11)*	*-0.72 (-2.28-0.09)*	*1 (0.06-16.24)*	*0 (-14.74-0.94)*
All time points	*0.74 (0.37-1.48)*	*-0.3 (-1.38-0.29)*	*0.33 (0.04-2.75)*	*-1.93 (-22.49-0.63)*

OR, Odds ratio; AF, Attributable fraction

Production Period

Four vaccinated flocks have been sampled to the study design end point (40 weeks), and three flocks remain in the study with two sampling occasions in each flock still remaining to be conducted. Eight control flocks of varying ages were sampled during the study period and three recruited control flocks remain to be sampled. A total of 2901 samples were processed for this part of the study; 656 environmental samples and 2245 egg samples.

***Salmonella* spp. environmental sample prevalence**

A total of 656 environmental samples were collected from all sampled production flocks. The overall *Salmonella* spp. prevalence was high, with 76% of samples testing positive for multiple *Salmonella enterica enterica* serovars. *Salmonella* Typhimurium prevalence was lower than expected, with only 0.5% (range 0.2-0.8%) of the environmental samples positive for *Salmonella* Typhimurium. Dust swabs had the highest *Salmonella* Typhimurium prevalence of all the sample types. The prevalence of *Salmonella* Infantis positive samples was substantially higher, at 19% (range 15-24%) of the samples tested. Manure belt samples had the highest sample prevalence of all sample types, for both *Salmonella* Infantis and all *Salmonella* spp. Environmental sample prevalence results aggregated for all flocks for all sample types are summarized in Table 4..

Table 4.6. Production site environmental sample results summarized by sample type and *Salmonella* serovar for all sampling occasions.

Sample Type	*Positive Samples*	*Total Samples*	*Apparent Prevalence (95% CI)*		
			S. Typhimurium	*S. Infantis*	*Salmonella spp.*
Boot swab	*71*	*100*	*0.04 (0.02-0.09)*	*0.19 (0.13-0.28)*	*0.71 (0.62-0.79)*
Dust	*171*	*276*	*0.08 (0.06-0.12)*	*0.15 (0.11-0.20)*	*0.62 (0.56-0.68)*
Egg Belt	*157*	*180*	*0.02 (0.01-0.06)*	*0.20 (0.15-0.27)*	*0.87 (0.81-0.91)*
Manure Belt	*94*	*100*	*0.02 (0.01-0.07)*	*0.24 (0.17-0.33)*	*0.94 (0.88-0.97)*

All	*498*	*656*	*0.05 (0.04-0.07)*	*0.19 (0.16-0.21)*	*0.76 (0.72-0.79)*

The environmental sample prevalence by stage of production for all flocks is illustrated in **Error! Reference source not found.**. The environmental sample prevalence for all *Salmonella* spp. varied considerably by age of flock, from 0 to 1. As the flocks aged the mean prevalence increased compared to the baseline (empty shed) sample prevalence and then declined after peak egg production (26-30 weeks of age) to peak egg mass (38-44 weeks of age).

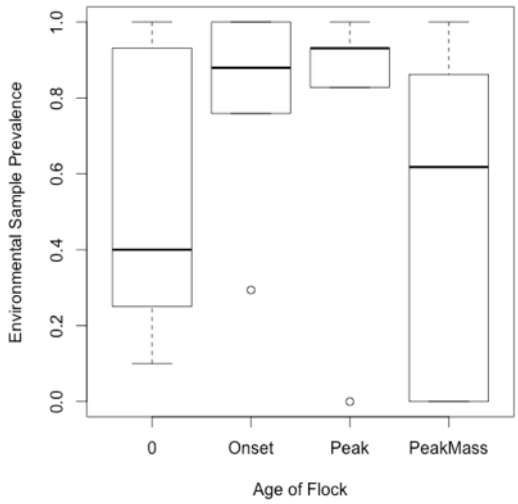


Figure 4.7. Environmental sample *Salmonella* spp. prevalence by stage of production for all flocks. Age of Flock 0: shed sampling results prior to flock placement. Onset, onset of lay to 5% production (16 to 22 weeks of age); Peak, peak lay (26-31 weeks of age); PeakMass, peak egg mass (38 to 42 weeks of age).

There was a significant difference between flock type and the effect of vaccination, if this factor was considered on its own. As can be seen in Figure 4.8, there was a substantial difference in the environmental prevalence between the different production systems. The results are confounded by flock type due to the large difference in prevalence between the two production systems in environmental sample prevalence. After accounting for the large difference in production type using a mixed effects logistic regression model to remove the excessive variation between the two production systems, there was no significant difference between the flocks in the prevalence of *Salmonella* spp or *Salmonella* Typhimurium as a result of vaccination. *Salmonella* Infantis prevalence was increased in vaccinated flocks (OR 3.2, P<0.001).

To examine the effect of flock age on the prevalence of infection in vaccinated and unvaccinated flocks, the sampling period was investigated by age and stage of production. Sampling dates were aggregated into four sampling periods corresponding to age and production level, as follows: prior to flock placement, onset of egg production to 5% lay (16-22 weeks), peak egg production (26 to 31 weeks), and peak egg mass (38-42 weeks). The apparent prevalence of *Salmonella* spp. in the environmental samples was calculated for each time period and is presented for all groups in

Table 4., with a comparison of each production stage for vaccinates and controls for each *Salmonella* serovar provided in Figure 4.9.

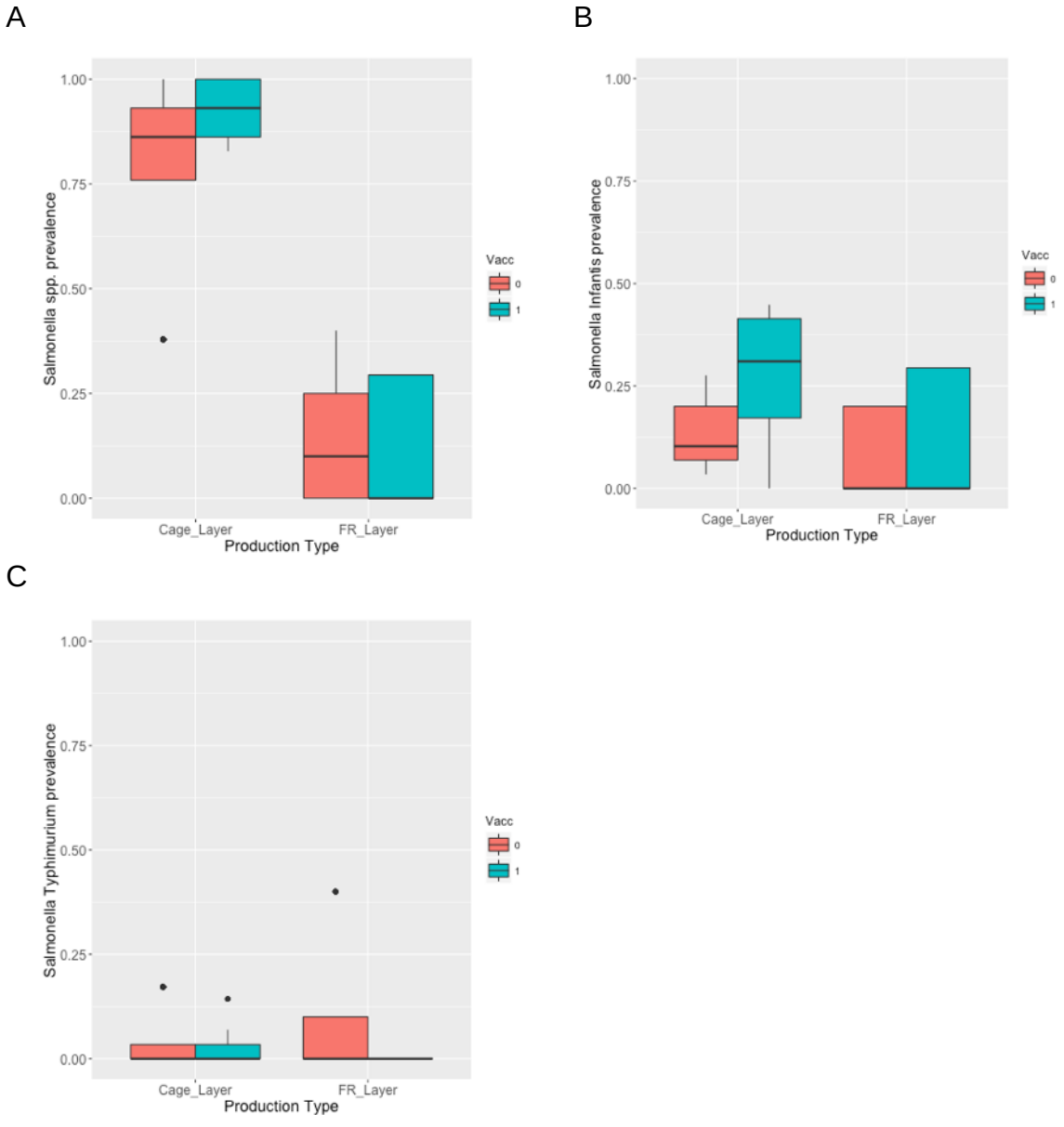


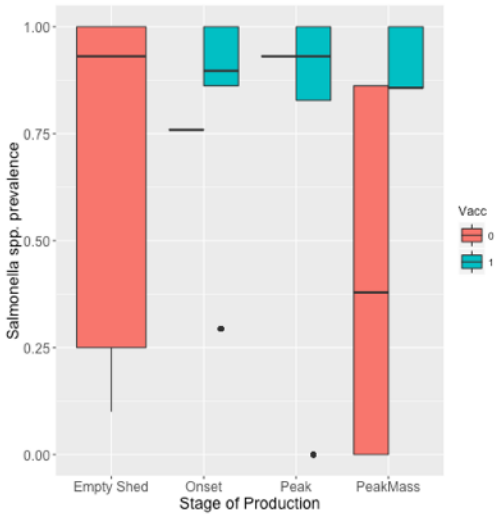
Figure 4.8. Environmental sample *Salmonella* prevalence in vaccinates and controls for both production types, Cage Layer and Free Range. A, *Salmonella* spp. sample

prevalence; B, *Salmonella* Infantis sample prevalence; C, *Salmonella* Typhimurium sample prevalence.

Table 4.7. Apparent *Salmonella* spp. prevalence for production site environmental samples summarized by production age to peak egg mass and *Salmonella* serovar for all sampling occasions.

Sampling Age	*Positive Samples*	*Total Samples*	*Apparent Prevalence (95% CI)*		
			S. Typhimurium	*S. Infantis*	*Salmonella spp.*
Empty Shed	*62*	*94*	*0.11 (0.06-0.19)*	*0.16 (0.099-0.247)*	*0.66 (0.56-0.75)*
Onset	*136*	*162*	*0.01 (0.001-0.034)*	*0.19 (0.138-0.259)*	*0.78 (0.71-0.84)*
Peak Egg	*107*	*136*	*0.02 (0.004-0.052)*	*0.26 (0.198-0.345)*	*0.78 (0.71-0.85)*
Peak Egg Mass	*47*	*94*	*0.02 (0.006-0.074)*	*0.05 (0.023-0.119)*	*0.51 (0.41-0.61)*
All	*352*	*486*	*0.03 (0.02-0.05)*	*0.18 (0.15-0.21)*	*0.71 (0.66-0.75)*

A



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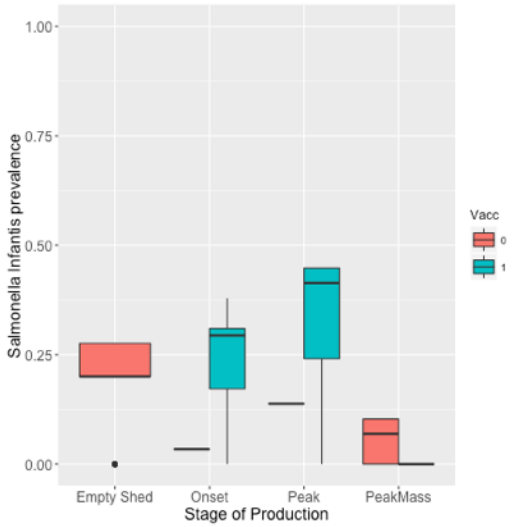

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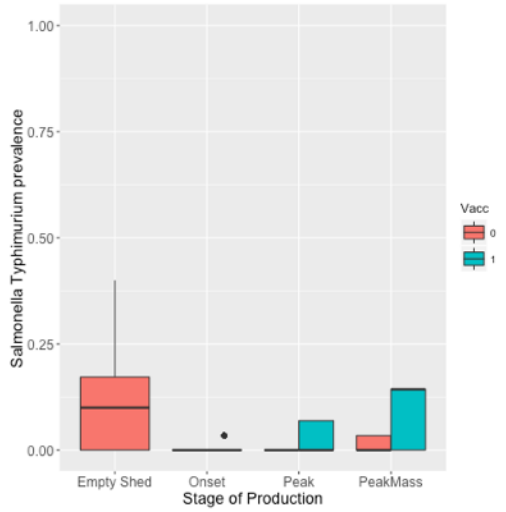

Figure 4.9. Environmental sample *Salmonella* prevalence in vaccinated and controls flocks as the flock ages to 40 weeks of production. Null, empty shed sample prevalence; Onset, egg production onset of lay to 5% production (16 to 22 weeks); Peak, peak egg production (26 to 31 weeks); and PeakMass, peak egg mass (38-42 weeks). A. *Salmonella* spp. sample prevalence, B. *Salmonella* Infantis sample prevalence. C. *Salmonella* Typhimurium sample prevalence.

The sampling date prior to flock placement was used as the baseline for shed contamination of the environment and likely represents the level of environmental challenge each control or vaccinated flock was subjected to at placement. A logistic regression mixed effects model was evaluated to examine the effect of production on the vaccinated groups, taking into account repeated measures (multiple sampling dates in the same flock), the flock effect and the stage of production. There was no significant effect of vaccination on the prevalence of *Salmonella* spp. or *Salmonella* Typhimurium. However, there was a significant effect on *Salmonella* Infantis environmental sample prevalence. The full model was unable to converge, but there were significant effects associated with a truncated model of vaccination and flock type only (Table 4.). The influence of vaccination is dependent on the flock type and, although significant, there is large variation between flock type, and we only have a small sample size for each group to date. Results are likely to change as more time points and flocks are sampled. The full model suggests that there is an important association with stage of production, as indicated in the boxplots, with the prevalence of infection varying by stage of production, the most significant difference being at peak egg mass, when the prevalence of *Salmonella* Infantis declines.

Table 4.8. Logistic regression mixed effects model results for *Salmonella* Infantis environmental sample prevalence taking into account flock type and vaccination status.

Variable	*Odds Ratio (95%CI)*	*Significance*
Vaccination	*3.26 (1.59-6.51)*	*P < 0.001*
Free Range	*0.22 (0.02 - 0.68)*	*P < 0.1*

Quantification of environmental contamination with *Salmonella* spp.
Due to the high sample prevalence for *Salmonella* spp., enumeration of *Salmonella* spp. by most probably number (MPN) in environmental samples was undertaken to determine if a change in the number of *Salmonella* in a sample type could be used to detect differences in shedding, as there are relatively few reports on quantification of *Salmonella* spp. in environmental samples. A single flock was followed until 20 weeks of age, with samples collected at the end of production from the previous flock housed in the shed, post cleaning and then fortnightly. The number of *Salmonella* spp. detected per gram of manure belt sample tested are summarized in Table 4. and **Error! Reference source not found.**.

Table 4.9. Most probable numbers of *Salmonella* spp. per gram of manure belt samples collected from the same shed until 20 weeks of age

Sampling Time	*Most Probable Number (CFU/g)*	
	Mean +/- S.D.	*Range*
Previous Flock	*317.20 +/- 430.50*	*12.70 - 621.60*
Post Cleaning	*18.10 +/- 8.70*	*8.05 - 23.12*
16 Weeks	*7.02 +/- 4.71*	*1.57- 9.73*
18 Weeks	*23.12 +/- 0*	*0*
20 Weeks	*0.48 +/- 0.78*	*0- 0.64*

The manure belt samples collected at the end of production from the previous flock were significantly higher than samples collected post cleaning and at all other time points (R^2=0.478, P = 0.03). There was no significant difference between the samples collected after cleaning.

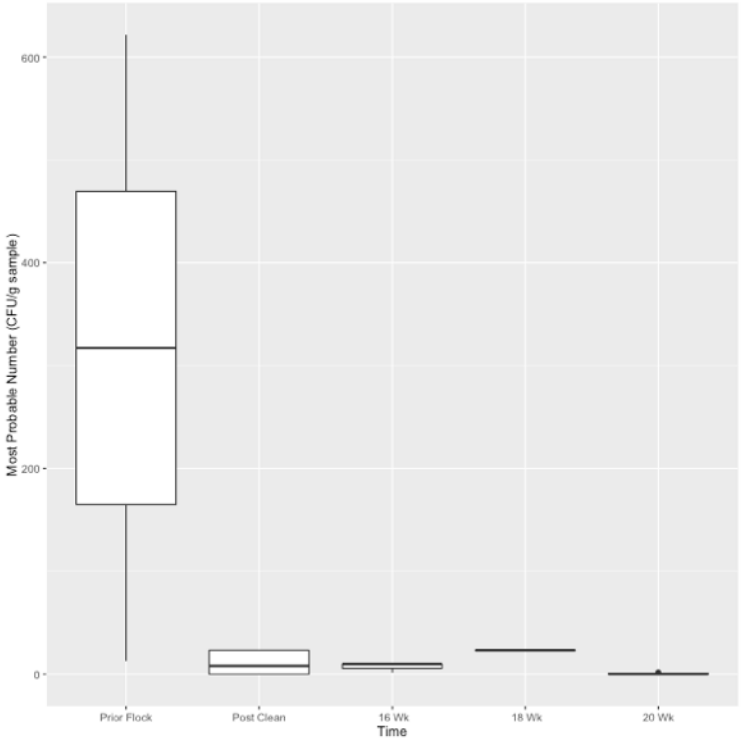

Figure 4.10. Most probable numbers of *Salmonella* spp. per gram of manure belt samples from the same shed in the previous flock, post cleaning, and at time points up to 20 weeks of age in a vaccinated flock.

Dust samples contained less *Salmonella* spp. (mean = 2.73 +/- 3.13 CFU/g) than manure belt samples. Due to the very low numbers of *Salmonella* in each sample, despite the high egg prevalence in samples collected at the same time, no further quantification of samples was undertaken.

Egg Sampling

Eggs were collected from all flocks in production on at least three occasions, at onset of lay (16 to 22 weeks, 5% production), at peak production (26-31 weeks), and at peak egg mass (38-42 weeks). These time frames were selected during the study design to reflect periods of maximum bird stress and presumed shedding of *Salmonella* into both the environment and onto eggs. A total of 7,975 eggs were tested. Three hundred eggs were obtained on each sampling occasion, where possible, and eggs were tested in pools of one, two or three depending on the number of eggs obtained on each sampling occasion.

The apparent prevalence of eggs positive for *Salmonella* spp. for each sampling event was calculated assuming an imperfect test for pooled samples, as described in the methods. Results for egg *Salmonella* spp. prevalence are presented in Table 4., Table 4. and Table 4.. The overall prevalence of *Salmonella* detected in all egg samples tested was 0.05 (S.D. +/-0.05). Multiple *Salmonella* serovars were found in egg pools from the same shed on each sampling occasion. The egg prevalence varied the most at the onset of egg production (range: 0.003-0.017) and rapidly declined at peak and peak egg mass. *Salmonella* Typhimurium was only detected in eggs in vaccinated caged flocks at the onset of lay. *Salmonella* Infantis was detected in both vaccinated and control flocks at the onset of lay and at peak lay. The prevalence of egg contamination with *Salmonella* spp. was highest in vaccinated free range eggs (but these have the smallest number of sampling events and flocks to date). More control flocks and time points for early production are required to ensure this effect is not confounded by a lack of sample points.

Table 4.10. Apparent prevalence of egg contamination with *Salmonella* serovars by flock type for all flocks.

Flock Type	*Apparent Prevalence (Mean + S.D.)*		
	Salmonella Typhimurium	*Salmonella Infantis*	*Salmonella spp.*
Free Range	*0.00*	*0.015 +/- 0.02*	*0.09 +/- 0.11*
Caged Layer	*0.003 +/- 0.01*	*0.007 +/- 0.01*	*0.04 +/- 0.04*
All	*0.003 +/- 0.01*	*0.008 +/- 0.01*	*0.05 +/- 0.05*

Table 4.11. Apparent prevalence of egg contamination with *Salmonella* serovars by stage of production to peak egg mass for all flocks.

Production Stage	*Apparent Prevalence (Mean +/- S.D.)*		
	Salmonella Typhimurium	*Salmonella Infantis*	*Salmonella spp.*

Onset (16-22 weeks)	*0.006 +/- 0.02*	*0.014 +/- 0.02*	*0.07 +/- 0.01*
Peak lay (26-31 weeks)	*0.00*	*0.003 +/- 0.01*	*0.01 +/- 0.01*
Peak mass (38-42 weeks)	*0.00*	*0.00*	*0.02 +/- 0.01*

The prevalence of egg contamination for vaccinates and controls varied by stage of production and was highest in vaccinated flocks at both the onset of production and at peak mass, with a reduction at peak lay (Table 4.).

Table 4.12. Apparent prevalence of egg contamination with all *Salmonella* spp. by stage of production to peak egg mass for vaccinates and controls.

Production Stage	*Apparent Prevalence (Mean +/- S.D.)*	
	Controls	*Vaccinates*
Onset (16-22 weeks)	*0.035 +/- 0.00*	*0.08 +/-0.06*
Peak lay (26-31 weeks)	*0.02 +/- 0.01*	*0.01 +/- 0.01*
Peak mass (38-42 weeks)	*0.02 +/-0.01*	*0.03 +/- 0.00*
All	*0.02 +/-0.01*	*0.06 +/- 0.06*

The difference in the prevalence of egg contamination between vaccinated and unvaccinated flocks is shown in Figure 4.11 for production type, stage of production and *Salmonella* serovar. The range in prevalence egg contamination was significantly higher at the onset of lay and substantial differences in egg prevalence (range: 0.003-0.17) were observed between eggs collected at the immediate onset of lay (16-18 weeks) and at 5% lay (18-22 weeks). All eggs tested at this age (range: 0.15-0.17) were collected from vaccinated flocks, so it is not possible to establish whether this increased level of egg contamination is influenced by vaccination.

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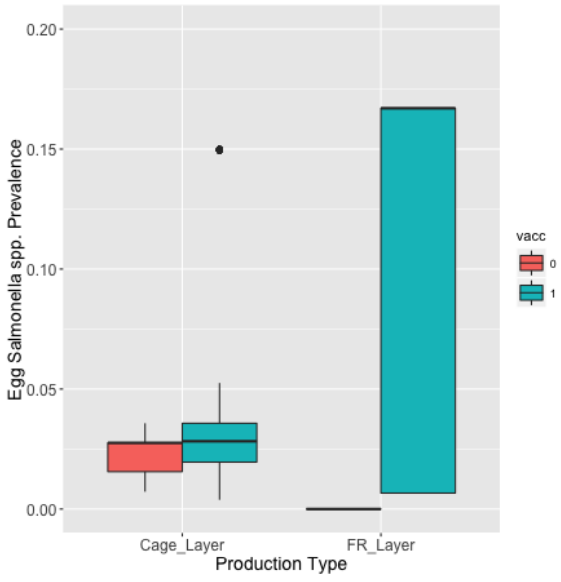

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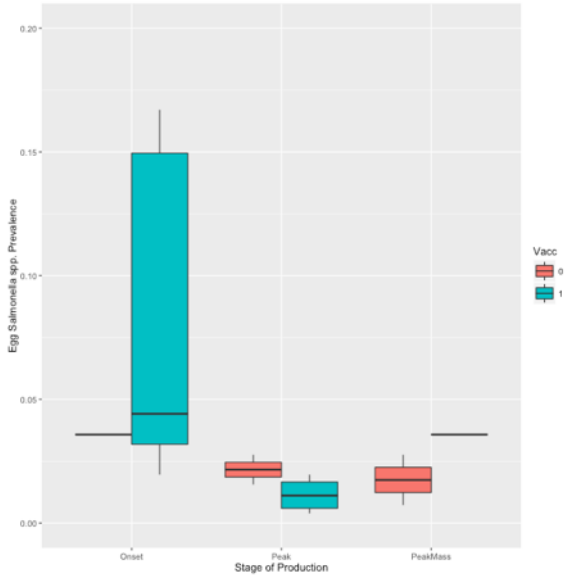

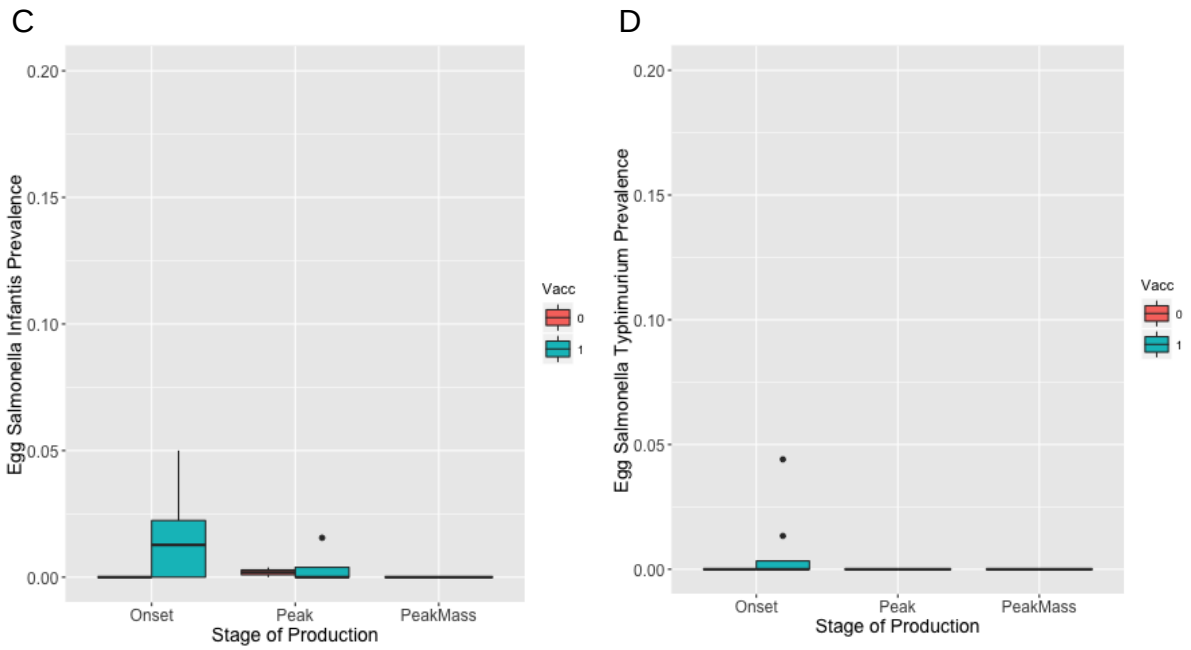

Figure 4.11. Apparent prevalence of egg contamination. A, prevalence for all *Salmonella* spp. by flock type for vaccinated and unvaccinated flocks; B, prevalence for all *Salmonella* spp. by vaccination status and stage of production; onset (16-22 weeks), peak (26-31 weeks), and peak egg mass (38-42 weeks); C, prevalence of *Salmonella* Infantis by stage of production; and D, prevalence of *Salmonella* Typhimurium by stage of production.

Egg contamination

Examination of egg components for *Salmonella* spp. was conducted on randomly selected sub-samples of 30 eggs on four occasions. A randomly selected sample of eggs was chosen from the primary sample and processed as described in the methods. In two sub-samples, internal egg contamination (yolk and white) was detected. *Salmonella* Infantis was detected in one sample and another *Salmonella* spp. (not typed) was detected in the other. The results of this testing are summarized in Table 4.. A single component from each positive egg was positive for *Salmonella* spp., indicating that in these samples egg contamination occurred either internally, eternally or in the shell and membranes, but not in any of the locations on each egg simultaneously. An attempt was made to enumerate the level of contamination in egg samples but the level of contamination was estimated to be <1 CFU per g of sample tested.

Table 4.13. *Salmonella* spp. prevalence in egg components from a randomly selected sub-sample of primary egg pools.

Sub Sample	Pooled Egg Primary Sample Prevalence	Egg Prevalence		
		Outside	*Shell and Membranes*	*White and Yolk*
1	*0.02*	*0.05*	*0.10*	*0.00*
2	*0.15*	*0.00*	*0.00*	*0.03*
3	*0.29*	*0.00*	*0.03**	*0.03**

4	*0.10*	*0.00*	*0.00*	*0.00*

**Salmonella* Infantis

Mixed Effects Model Results

Estimates of the prevalence of contamination of eggs are confounded by production type, as with the environmental results. To remove this confounding effect the egg prevalence results were analyzed separately by flock type for cage eggs only, as there are currently insufficient results for the free range flocks. There was no significant effect of vaccination on the prevalence of *Salmonella* spp., *Salmonella* Typhimurium, or *Salmonella* Infantis in cage eggs.

Discussion

The principals of environmental sampling for the detection of *Salmonella* spp. have been well established and validated, as appropriate methods for both the detection of infection and the level of environmental contamination (sample prevalence) correlates well with flock prevalence in studies conducted overseas (70, 72, 73). It is known that faecal sampling underestimates the true prevalence of *Salmonella* infection in infected hens (74) and it is widely accepted that environmental sampling for *Salmonella* spp. is more sensitive for the detection of infected flocks than bird sampling.

This study design demonstrated that, despite a high *Salmonella* spp. sample prevalence, the proportion of *Salmonella* Typhimurium positive samples in flocks with known infection was very low. The environmental sample prevalence of *Salmonella* Typhimurium was at the limit of detection and well below the study design prevalence that would have enabled us to demonstrate an effect from the novel application of Vaxsafe® ST in this study.

The low apparent prevalence of *Salmonella* Typhimurium at both the pullet rearing site (0%) and during production (0.5%) is an important finding with regards to *Salmonella* sampling and detection in the field. The number of *Salmonella* spp. positive samples detected was high, but not unexpected, but the comparatively low numbers of *Salmonella* Typhimurium in an environment with a known history was surprising. Additionally, the lower prevalence in manure belt samples compared to dust samples suggests that it is possible that flock infection with *Salmonella* Typhimurium may go undetected if the sampling strategy does not include dust and is not sufficiently robust to ensure adequate coverage of the contaminated environment.

Multiple *Salmonella enterica enterica* serovars were detected in all flocks sampled during the study period. All sheds had multiple serovars detected at any one sampling event and the proportion of *Salmonella* serovars detected on each sampling occasion varied, despite the same number of samples being taken at each sampling event, indicating that infection of flocks is dynamic and environmental contamination and or survival does not remain static over time.

Pullet Rearing

Vaccination of pullet flocks did not prevent environmental contamination with *Salmonella* Infantis. Infection at a very low prevalence was able to be detected at all

pullet sites sampled during rearing. No *Salmonella* Typhimurium was detected in the environment during the rearing period in the flocks that were able to be sampled.

Production

This study validated the appropriateness of our methodology in a layer environment for the detection of *Salmonella* serovars, despite the unexpectedly low prevalence of *Salmonella* Typhimurium in the flocks sampled. Egg sampling and testing was found to be much more useful to demonstrate changes in prevalence in response to vaccination. Collection of fewer samples would have led to incorrect assumptions about the prevalence of *Salmonella* infection and the effect of the age of the flock on egg contamination. Although the study was not sufficiently powerful to demonstrate a clear effect of vaccination, because the prevalence of infection was much lower than anticipated, important findings were made about the risk periods for infection and egg contamination that will enable development of future studies to understand the epidemiology of infection and the timing of intervention.

Despite the high sample prevalence of all *Salmonella* spp., the amount of environmental contamination was low. The number of organisms detected in the environment in this study were much lower than those expected based on reports of the quantities of *Salmonella* shed in faeces by birds infected with *Salmonella* spp., including *Salmonella* Typhimurium (10^5 -10^6 CFU/g (75, 76)) and *Salmonella* Infantis (10^3 -10^8 CFU/g, (77)). These results further support the importance of appropriate sampling methodology in the detection of *Salmonella*.

The egg prevalence findings are consistent with those reported in the international literature (38) for all *Salmonella* spp. and *Salmonella* Typhimurium, and were low, with the exception of at the onset of lay. Internal egg contamination rates have never been reported for *Salmonella* Infantis in Australia. No *Salmonella* Typhimurium was detected in the egg component study. In addition, the number of *Salmonella* spp. in these samples was very low - below the limit of quantification per gram of sample tested - consistent with the levels reported in the literature for *Salmonella* Enteritidis (78).

Implications

Egg contamination with *Salmonella* spp., *Salmonella* Infantis and *Salmonella* Typhimurium were generally low with the exception being at the onset of egg production in vaccinated flocks. The observation that the prevalence of *Salmonella* contaminated egg pools appears to peak at around 5% egg production is important and needs to be further investigated to determine what effect this may have on consumer risk. These eggs are typically small and below market size, but there is a rapid increase in both production and egg size at this age (which varies by flock and flock type) and eggs are being collected for market and may pose an enhanced risk to the marketplace. Understanding of the level of contamination of eggs from this phase of production to peak is critically important for effective decision making for producers and determining food safety outcomes for consumers.

Salmonella contamination of both egg content and shell and membrane components of the egg, in addition to surface contamination, has not been reported in Australia previously and requires further investigation.

The low prevalence of *S.* Typhimurium detected in these flocks, and the relative efficacy of the different sample sites, will facilitate design of future studies to establish the efficacy of differing approaches to *Salmonella* control on Australian egg farms.

Recommendations

This initial study was limited to understanding the protective effect of Vaxsafe® ST up to 40 weeks of age due to the short timeframe required for the project. A number of vaccinated flocks remain in production. The opportunity remains to continue to collect information from the flocks remaining in production to evaluate whether there is any benefit of vaccination at mid and late lay to the end of production. The prevalence of *Salmonella* Typhimurium in the recruited flocks was well below that expected during the design of study and precluded detection of an effect of vaccination. The recruitment of more flocks is necessary to fully evaluate the effect of vaccination under current commercial conditions, and the assessment of vaccination control in those flocks should be targeted at the early onset of lay period when contamination levels seem to be greatest. Given that *Salmonella* Typhimurium and other serovars are able to survive/persist in the shed environment (such as in dust), regular cleaning and or removal of dust from shed is also important. Use of the Vaxsafe® ST vaccine in multi-age flocks is “not an ultimate intervention” for reduction of *Salmonella* Typhimurium because of the complexities involved in achieving control, such as the efficacy of cleaning of sheds, the lack of resting periods between batches and the possible carry over of contamination from existing flocks. Hence implementation of more than one or several interventions strategies is essential.

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Research Students associated with the project

Mr Pardeep Sharma, PhD student, The University of Adelaide (Current)

Plain English Compendium Summary

Sub-Project Title:	**Field trials to study the efficacy of a *Salmonella* Typhimurium live vaccine in egg layers**
Poultry CRC Sub-Project No.:	3.2.7
Researcher:	Kapil Chousalkar, Helen Crabb and Glenn F. Browning
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Sub-Project Overview	This sub-project aimed to examine the efficacy of the Vaxsafe ST attenuated live vaccine, administered as three oral doses followed by an intramuscular dose, in reducing contamination of the environment in layer sheds and in reducing egg contamination with *Salmonella*e.
Background	Vaxsafe® ST (Bioproperties Pty Ltd, Australia) is the only live attenuated *Salmonella* vaccine registered for use in poultry in Australia. Vaxsafe ST was developed for oral administration to short lived birds (such as broilers) and the duration of immunity it induces is relatively short due to the level of attenuation of the vaccine strain. Small scale experimental studies have demonstrated that the administration of three live oral doses and a fourth intramuscular dose at ~10 weeks of age provided some level of protection against experimental challenge with *Salmonella* over a short study period, but the long term efficacy of the vaccine in commercial flocks that are actively shedding *S.* Typhimurium remains unclear. Uptake of vaccination as a supplement to other control measures in the Australian egg laying industry remains low due to the lack of scientific evaluation of the efficacy of the vaccine under a range of commercial conditions. Therefore these studies were initiated to obtain information to guide the use of this vaccine in layer flocks.
Research	Two separate field trials were conducted, in South Australia and In Victoria. Both trials examined the efficacy of a novel dosage regimen for Vaxsafe® ST – three oral doses and a single intramuscular injection. The South Australian trial examined effects of the vaccine in two multi-age caged flocks, while the Victorian study examined effects in 10 single age caged and free range flocks. Vaccinated birds were found to shed the vaccine into their environment and could possibly transmit the vaccine to unvaccinated birds nearby. Antibody responses against *Salmonella* were only seen in birds after the final intramuscular injection. Shedding of *Salmonella*e in infected flocks was found to be much lower than expected and was not evenly distributed around the flock. As a result, infection of a flock could be missed unless the approach to sampling considered this uneven distribution of shedding was taken into account. Contamination of eggs with *Salmonella*e was highest around the onset of lay, with contamination rates dropping by the peak of lay and continuing to be low until flocks were 40 weeks old, when the trial was stopped. Because the rate of shedding of *Salmonella*e was so low, it was not possible to establish whether vaccination with Vaxsafe® ST reduced the level of environmental and egg contamination.
Implications	Because the prevalence of *Salmonella* Typhimurium is relatively low in Australian layer flocks, larger field trials will be needed to determine how efficacious Vaxsafe® ST is in reducing the level of *Salmonella* contamination of eggs in the field. This low prevalence also indicates that quite specific sampling methods are needed to reliably detect *Salmonella* on layer farms. Egg contamination with *Salmonella*e appears to be

	particularly high at the onset of lay, suggesting that control may need to be focussed on eggs collected during this period. Vaxsafe® ST appears to be shed at a higher rate by vaccinated birds than previously thought and there appears to be some transmission of the vaccine between birds.
Publications	None as yet.