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Executive Summary

The current study involved longitudinal and point in time surveys of *Salmonella* carriage and environmental contamination on two commercial multi-age cage layer farms (flock A with age = 32 weeks and flock B with age = 34 weeks) known to be positive for *Salmonella* Typhimurium. Faecal samples were collected from 80 cages from each flock. From flock A, 25.9% (Confidence Interval CI: 16.8-36.8) of the tested cages were positive for *Salmonella* spp. Similarly, for flock B, 39.2% (CI: 28.4-50.9) cages were reported positive for *Salmonella* spp. Based on these results, five *Salmonella* positive and two *Salmonella* negative cages from both farms were selected for longitudinal study and followed up with monthly samplings for up to 40 weeks. In longitudinal samplings, out of all samples tested from flock A and B, 14.42% (130/901) were *Salmonella* positive. Serotyping results confirmed that *S.* Oranienburg was the most frequently (76.92%) reported serovar followed by *S.* Typhimurium PT 9 (11.54%), *S.* Worthington (8.46%), *S.* Agona (3.08%), *Salmonella* subsp.1. ser. 4, 5, 12:-:- (1.54%) and *Salmonella* subsp.1 ser rough: g,s,t:- (0.77%). The results of logistic regression indicated that the *Salmonella* positive status of faeces, egg belt and dust were significant predictors ($p<0.001$) of eggshell contamination by *Salmonella*. When faecal, egg belt and dust samples, were *Salmonella* positive, the odds of eggshell positive *Salmonella* was 91.7, 61.5 and 18.2 times higher, respectively. In the final model (conditional association), only faecal and dust were kept as a predictor with the results indicating that *Salmonella* positive faecal ($p<0.001$, odds ratio=58.87) and dust ($p=0.007$, odds ratio=9.22) samples were the only significant predictors of eggshell contamination of *Salmonella.* Kappa statistics results suggested that there was an almost a perfect agreement between a traditional culture method and a real-time polymerase chains (qPCR) assay to identify *Salmonella* positive eggshell samples (Agreement = 99.19%,) as well as egg belt samples (Agreement = 95%). However, there was a moderate agreement between culture method and qPCR to identify *Salmonella* positive faecal sample (Agreement = 87.14%) and dust samples (Agreement = 80.61%). On the basis of the qPCR results, one log increase in the load of *Salmonella* in faecal samples resulted in 34% increase (odds ratio=1.34) in *Salmonella* positive eggshells. Similarly, one log increase in the load of *Salmonella* on egg belt and dust samples resulted in 43% (odds ratio=1.43) and 45% (odds ratio=1.45) increase in *Salmonella* positive eggshells, respectively.

The shedding of *Salmonella* in a single age commercial egg layer flock was investigated at the onset of lay (18 weeks) followed by two longitudinal samplings at 24 and 30 weeks. The prevalence of *Salmonella* in faeces collected from the low tier cages was significantly higher ($p=0.009$) as compared samples from the high tier cages. In all types of samples, *S.* Mbandaka was the most frequently (54.40%) isolated serovar followed by *S.* Worthington (37.60%), *S.* Anatum (0.8%) and *S.* Infantis (0.8%). The observed agreement between the culture method and the qPCR in detecting *Salmonella* positive dust and egg belt samples was 100%. There was almost perfect agreement (observed agreement = 99.21%) for detection of *Salmonella* positive eggshells. Observed agreement between culture method and qPCR for detecting *Salmonella* positive shoe cover and faecal samples was, however, moderate (80%) and low (54.27%) respectively. The qPCR results showed that there was a significant increase in the load of *Salmonella* on egg belt, dust and shoe cover samples at the 24 and 30 weeks of lay as compared to the 18 weeks of lay. While the qPCR provided a more rapid detection of *Salmonella* on all dry sample types, the traditional culture method proved much more reliable when trying to detect *Salmonella* in faecal samples. *Salmonella* isolates (91.72%; 133/145) were susceptible to the majority of the antimicrobial agents tested. Low levels of resistance were observed to amoxicillin and ampicillin (5.51%), tetracycline (4.13%), cephalothin (2.06%) and trimethoprim (0.68%). Isolates were not resistant to cefotaxime, ceftiofur, ciprofloxacin, chloramphenicol, gentamycin, neomycin and streptomycin. Of these except for Chlortetracycline and Neomycin, other antimicrobials are not used in the layers during lay. The incubation temperature, 22 or 37°C, had a strong influence on the colony morphology of *Salmonella* isolates on Congo red agar plates. The

crystal violet assay revealed that biofilm formation was significantly higher ($P \leq 0.05$) at 22°C compared to that at 37°C.

Contents

Executive Summary iii
Contents iv
Chapter 1: Introduction 1
Objectives 2
Chapter 2: Methodology 3
Chapter 3: A longitudinal study of *Salmonella* contamination in multiage caged layer flocks
Introduction 7
Materials and Methods 7
Results 10
Discussion 20
Chapter 4: A longitudinal study of *Salmonella* contamination in single age caged layer flocks 23
Introduction 23
Materials and Methods 23
Results 27
Discussion 32
Chapter 5: Antimicrobial resistance and biofilm forming ability of *Salmonella* spp. isolated from commercial egg layer flocks of Australia 34
Introduction 34
Materials and Methods 34
Results 35
Discussion 43
General Discussion 45
Implications 46
Recommendations 47
Acknowledgements 47
References 48
Publications arising from project 54
Research Students associated with project 52
Plain English Compendium Summary 53

Chapter 1: Introduction

It is widely recognised that *Salmonella* are a potential threat to the chicken meat and egg industry. Although egg producers are diligent in fulfilling standards for the production of safe food, the egg industry in Australia is often implicated in outbreaks of food poisoning. The industry therefore needs to be vigilant in monitoring the presence of food borne pathogens such as *Salmonella* on or in eggs to enable informed management decisions to be made. Although there is the potential for vertical transmission of *Salmonella* from bird to egg (as has been shown overseas to occur with *Salmonella* Enteritidis), it is generally accepted that horizontal transmission is the most likely source of contamination of shell eggs by most bacteria. Small defects in the egg shell may provide the means for the predominant bacterial species on the egg shell to penetrate and move into the egg contents and the internal properties of eggs favour survival and growth of contaminating organisms which are Gram-negative (such as *Escherichia coli* and *Salmonella*). Gram-negative bacteria have a relatively simple nutritional requirement and have the ability to reproduce, albeit slowly, at low temperatures. Contact between faecal material and the egg shell is often unavoidable. There is a possibility that cracking of egg shells during washing or collection can enhance the entry of microorganisms into the egg. To reduce faecal contamination, egg washing is a common practice in the layer industry, however, there is still a debate as to the benefits or otherwise of washing eggs and current CRC projects are addressing this aspect.

In Europe and the USA, *Salmonella* Enteritidis PT4 is of greatest concern because of the occurrence of trans-ovarian transmission into the egg with this serovar. *Salmonella* Enteritidis PT4 has not been detected in the Australia egg industry, however, other *Salmonella* serovars are still of interest. For example, there is no doubt that serovars isolated from layer farms such as *Salmonella* Typhimurium and *Salmonella* Infantis pose a significant threat to public health compared to other less pathogenic serovars. In Australia, very few studies have characterised the prevalence of different *Salmonella* serovars within the commercial laying industry, especially during active clean-up and control activities. Some of the main challenges for Australia are the costs associated with intensive sampling and *Salmonella* characterisation and the fact that there are no readily available, cost-effective and rapid tools for the detection and differentiation of *Salmonella* serovars for the egg industry. To the egg industry, a good quality egg means the provision of an egg acceptable to the consumer. Production of visually clean eggs, free from dirt and faecal contamination, is the primary concern in the supply of table eggs, although, clean eggs do not necessarily guarantee food safety. On commercial laying farms, environmental *Salmonella* contamination is considered to be the predominant problem which has been very well investigated for the *Salmonella* Enteritidis in other countries. However the dynamics of *Salmonella* serovars which are relevant to Australian Poultry industry such as *Salmonella* Typhimurium and *Salmonella* Infantis have not been investigated. The current project will conduct longitudinal and point-in time surveys of *Salmonella* carriage and environmental contamination on a commercial layer farm in South Australia. This will provide critical information for the egg industry about dynamics of *Salmonella* shedding and the possible link between environment/bird/egg transmission of *Salmonella* serovars of public health significance on environmentally controlled commercial layer farm.

Objectives

The objectives of this Sub-Project, stated in the original application, were to:

- To determine the link between environment/bird/egg transmission of *Salmonella* spp in environmentally controlled commercial layer farm.
- To study the effect of stage of lay on *Salmonella* shedding
- To study the possible relationship between level of egg contamination and *Salmonella* load in the poultry shed environment.
- Using the existing rapid quantitative diagnostic tool to monitor *Salmonella* serovars isolated from layer farms.
- To study the Antimicrobial susceptibility of *Salmonella* isolates of various serovars (isolates obtained in the present project)
- To screen the capacity to produce biofilm by *Salmonella* isolates of various serovars (isolates obtained in the present project)
- *In vitro* work testing bacteriostatic activities of various organic acids against *Salmonella* isolates of various serovars (isolates obtained in the present project)

Chapter 2: Methodology

Stage 1: Cross sectional survey to select cages for longitudinal study

Two commercial layer cage farms, A (flock age = 32 weeks, flock size = 6600 birds) and B (flock age = 34 weeks, flock size = 6500 birds), were selected for this study. In both farms, multi-aged flocks were housed in the same shed. Systematic random sampling was used, to ensure, faecal samples were collected from all representative areas of the flock. In flock A, out of 1320 cages (5 birds per cage), faecal samples were collected from 81 cages (at 95% confidence level, C.I. = 39.4 - 60.6). Similar for flock B, faecal samples were collected from 79 cages (at 95% confidence level, C.I. = 39.2 - 60.7). Accounting for field constraints, two adjacent cages were selected at equal interval along the three lowest tiers (tier 1, 2, and 3 respectively) out of the five tiers.

For isolation of *Salmonella* spp., the faecal samples (1:4) or swabs (1 swab soaked in 25 mls) were inoculated into buffered peptone water (BPW, Oxoid, Australia)(1:4). The inoculated samples were incubated at 37°C overnight and 100 µl of this sample was transferred into Rappaport Vasidalis (RV) broth (Oxoid, Australia) which was then incubated at 42°C for 24 h. A loopful of incubated RV broth was streaked onto Brilliance *Salmonella* agar (BSA, Oxoid Australia) and Xylose Lysine Deoxycholate agar (XLD, Oxoid, Australia) plates. Two to three presumptive *Salmonella* colonies from BSA and XLD agar were selected and used to stab inoculate triple sugar iron agar slopes (TSI; Oxoid, Australia). After incubation at 37°C, the inoculated TSI slopes were examined at intervals of 24 h up to 72 h, for typical *Salmonella* reactions. The presumptive *Salmonella* colonies were also tested for ortho-nitrophenyl-β-D-galactopyranoside (Oxoid, Australia), lysine decarboxylase (LDC) and urease (Oxoid, Australia) activity. Depending upon the results of biochemical reactions, the presumptive *Salmonella* isolates were sent for serotyping to *Salmonella* Reference Laboratory, Adelaide, Australia. Based on *Salmonella* typing results, for longitudinal study, five *Salmonella* positive cages from farm A (*S*. Typhimurium phage type 9 = 3 cages, *S*. Infantis and *S.* Orion = 1 cage each) and farm B (*S*. Typhimurium PT 9 = 2, *S*. Infantis, *S*. Agona and *S*. Oranienburg = 1 cage each) as well as two cages per farm which were negative for *Salmonella* spp. were selected.

Stage 2: Longitudinal study to investigate the association between eggs and environmental contamination

Based on *Salmonella* typing results, five *Salmonella* positive cages each from farm A (*S*. Typhimurium phage type 9 = 3 cages, *S*. Infantis and *S.* Orion = 1 cage each) and farm B (*S*. Typhimurium PT 9 = 2 cages, *S*. Infantis, *S*. Agona and *S*. Oranienburg = 1 cage each), as well as two *Salmonella* negative cages per farm were selected for the longitudinal study. The reason for selecting cages positive with different *Salmonella* serovars was to investigate the dynamics of *Salmonella* shedding of various serovars over a prolonged period of time during longitudinal samplings. The selected cages were sampled at four week intervals. Both farms were sampled with a gap of one week. For each flock, 10 longitudinal samplings were performed over the period of 40 weeks (i.e. 4 week intervals). Figure 3.1 and 3.2 shows the layout of layer shed along with sample collection areas.

Faeces

Faecal samples were collected in sterile Whirl-pak plastic bags (150 X 230 mm, Thermo Fisher Scientific Australia) from underneath the seven selected cages. The full length of the manure belt under each cage was covered while collecting faecal samples. To avoid cross-contamination, gloves (nitrile medium powder free, Pacific Laboratory Products Australia) were changed after collecting every faecal sample.

Egg belt

Egg belt samples were collected from the cage front. Whirl-Pak speci-sponge bags (115 x 239 mm Thermo Fisher Scientific Australia) were used for sample collection. The swabs

were pre-moistened using 25 ml of BPW and dragged to cover whole area in front of individual cage front.

Dust

In each sampling, five dust samples were collected from the different part of poultry shed. Dust was collected in gamma sterile containers (Pacific Laboratory Products, Australia).

Eggs

All the eggs at the front of the seven selected cage were collected. Each egg was collected in separate sterile Whirl-pak plastic bag to avoid cross-contamination. In flock A, moulting was performed in 67th week of lay, therefore, no eggs were obtained in the following (9th) sampling.

Sample processing for *Salmonella* isolation

For faeces and dust, 2 gm of samples were added to 8 ml of BPW while the egg belt swabs were placed in25 ml of BPW. These BPW were then processed as as mentioned above to isolate *Salmonella* spp. Eggshell and egg internal content samples were individually processed. Individual eggs were placed in 10 ml of BPW in Whirl-Pak bags and rinsed by massaging for 2 min. Before rinsing, peptone water was pre-warmed to 37°C to facilitate bacterial recovery. After a rinse sample was obtained, each egg was removed and transferred to a different sterile bag. The BPW samples were incubated at 37°C overnight and 100 µl of this sample was inoculated into RV broth (Oxoid, Australia) which was then incubated at 42°C for 24 h. The samples were further processed for *Salmonella* isolation as mentioned above. 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 samples were further processed for *Salmonella* isolation as mentioned above.

Multi-locus Variable Tandem Repeat Analysis (MLVA) of *S.* Typhimurium isolates

All *S.* Typhimurium strains isolated from farm A and farm B were sent for MLVA to the *Salmonella* Reference Laboratory, Adelaide, Australia.

Quantitative Polymerase Chain Reaction (qPCR)

All samples, after pre-enrichment in BPW at 37°C for overnight, were frozen, freeze dried and used for nucleic acid extraction using the South Australian Research and Development Institute (SARDI) propriety method (16). All qPCR assays were run in a 384 well format with master mix and template being dispensed using a Biomek 3000 Laboratory automation Workstation (Beckman Coulter, USA). The *Taq*Man *Salmonella enterica* detection kit system (Applied Biosystems, Australia) was used to perform qPCR. All reactions were run on a 7900HT Sequence Detection System (Applied Biosystems, Australia) with the following conditions: 95°C for 10 min followed by 45 cycles of 95°C for 15 sec and 60°C for 60 sec. All data were analysed using the 7900HTv2.3 SDS software (Applied Biosystems, Australia). Raw data were analysed for target specific *Salmonella enterica* and internal positive control (IPC) using a threshold cycle (Ct) of 0.8 and baseline of 3-10. *Salmonella* copies were calculated using a standard curve prepared by serial 10 fold dilution of a cultured *Salmonella enterica* serovar Infantis. A cut off Ct of 34, which corresponded to 200 colony forming units (CFU) of *Salmonella,* was used to exclude detection of false positives. A Ct of 34 corresponded to 200 CFU.

Multiplex PCR to identify *S.* Typhimurium and *S.* Infantis positive samples.

Two multiplex PCRs, one for the detection of *S.* Typhimurium and the other for *S.* Infantis, were standardized using the primers published by Akiba et al. (17). The details of the primers and expected size of amplified product are described in Table 2.1. The standardization was performed using various *Salmonella* serovars isolated from the Australian layer industry. For the *S.* Typhimurium multiplex PCR, each reaction mixture

contained 1× reaction buffer (Fisher Scientific, Australia), 2.5 mM $MgCl_2$, 1.6 mM dNTPs, 0.5 µM of invAF, invAR, TMP2F, TMP2R primers, 0.3 µM of TMP1F, TMP1R, TMP3F, TMP3R primers, 0.13 U *Taq* polymerase, and 5 ng DNA template made up to 20 µl with nuclease free water. For the *S.* Infantis multiplex PCR, each reaction mixture contained 1× reaction buffer (Fisher Scientific, Australia), 2.5 mM $MgCl_2$, 1.6 mM dNTPs, 0.5 µM of invAF, invAR,IMP3F, IMP3R primers, 0.3 µM of IMP1F, IMP1R, IMP2F, IMP2R primers, 0.13 U *Taq* polymerase, and 5 ng DNA template made up to 20 µl with nuclease free water. Samples were amplified using a Bio-Rad Thermal Cycler with an initial denaturation step at 95°C for 2 min followed by 35 cycles of amplification (denaturation at 95°C for 10 sec, annealing temperature 60°C for 30 sec and extension at 72°C for 30 sec), with a final extension step at 72°C for 10 min, followed by a holding temperature of 10°C.PCR products were separated by 2% agarose gel electrophoresis in Tris-Acetate-EDTA (TAE) buffer. Gel red was used to visualize bands under ultra-violet light. The size of the PCR products was determined by comparison with a 100 bp DNA ladder (Qiagen, Australia).

In order to determine the limit of detection of the multiplex PCR, faecal samples were spiked with various concentrations (10^8 CFU/ml to 10^2 CFU/ml) of *Salmonella*. Genomic DNA was extracted from *Salmonella* spiked faecal samples and the multiplex PCR was performed as mentioned above. The limit of detection was determined by running the PCR products on 2% agarose gel.

Biofilm formation assay

Phenotypic characterisation by Congo red morphology

The colony morphology of all *Salmonella* isolates was determined on Congo red agar plates for curli fimbriae and cellulose production as described previously (12) with some modifications. Briefly, stock *Salmonella* cultures were grown on nutrient agar plates (Oxoid Australia) at 37°C overnight. A single colony of *Salmonella* was grown in 5 mls of Luria-Bertani (LB) broth in a shaking incubator for 6 hours. Each *Salmonella* isolate was spotted (3 µl) onto LB agar containing no salt supplemented with Congo red (40 µg/ml, Sigma Aldrich) and Coomassie brilliant blue (20 µg/ml, Sigma Aldrich). The inoculated plates were incubated at 22°C and 37°C for 96 h and colonies were visualised macroscopically. *Salmonella* ATCC14028 was used a positive control strain for this study.

Quantitation of biofilm formation by crystal violet staining assay

Salmonella isolates were grown on LB agar plates with overnight incubation at 37°C. A single colony was inoculated into 10 ml of LB broth without sodium chloride and incubated at 37°C overnight. A 20 µl aliquot of overnight grown bacterial culture was mixed into 180 µl of LB broth without salt in a round bottom 96 well plate (Sarstedt, Australia). Negative control wells contained 200 µl of LB broth only. The inoculated plates were incubated statically at 22 and 37°C for 96 hrs. After incubation, the plate contents were poured off and the wells washed gently three times with sterile distilled water to remove loosely bound bacteria. The plates were air dried and stained with 200 µl of 0.1% crystal violet for 30 min at room temperature. Following staining, the wells were gently washed three times with sterile distilled water and air dried. Bound crystal violet stain in the wells was resuspended with ethanol-acetone (70:30) for 10 min at room temperature. The absorbance of each well was measured at 590 nm (A_{590}) using a microplate spectrophotometer system (Benchmark plus, Biorad). The mean absorbance of negative controls was subtracted from absorbance of all the test wells. All experiments were conducted in duplicate and repeated once for a total of four replicates for each isolate. For all experiments, mean and standard error were calculated and the difference in degree of biofilm formation between *Salmonella* isolates were analysed by ANOVA using SPSS for windows (version 21, IBM Chicago, IL, USA). P value < 0.05 was considered statistically significant.

Statistical analysis

This is explained in individual chapters.

Table 2.1 Primers used in multiplex PCR

Region	primer	Sequence	Amplicon size (bp)
InvA	invAF	5′-AAACCTAAAACCAGCAAAGG	605
	invAR	5′-TGTACCGTGGCATGTCTGAG	
TSR1	TMP1F	5′-ATGCGGGTATGACAAACCCT	94
	TMP1R	5′-TTAGCCCCATTTGGACCTTT	
TSR2	TMP2F	5′-CAGACCAGGTAAGTTTCTGG	196
	TMP2R	5′-CGCATATTTGGTGCAGAAAT	
TSR3	TMP3F	5′-TTTACCTCAATGGCGGAACC	303
	TMP3R	5′-CCCAAAAGCTGGGTTAGCAA	
ISR1	IMP1F	5′-GGTCATTGTCGGAAACCTGC	95
	IMP1R	5′-ACATTCCCCCTTCCACTGCC	
ISR2	IMP2F	5′-CGCGAAGAAGTGCATAAACC	198
	IMP2R	5′-CGCCACTTTCGTTATCTGAG	
ISR3	IMP3F	5′-ACCTACTACTATCCCTGATG	304
	IMP3R	5′-GCGAATTTTGCTACTTGAAG	

TSR: Typhimurium-specific (genomic) region; ISR: Infantis-specific (genomic) region
InvA: *Salmonella* Invasion Gene A

Chapter 3: A longitudinal study of *Salmonella* on multi-age commercial layer farms

Introduction

Eggs and derived products are often linked to cases of *Salmonella* food poisoning. *Salmonella* outbreaks have been associated with uncooked products like mayonnaise, ice-cream, and cold desert which contain raw egg (1). A very low dose of *Salmonella*, 10 to 20 colony forming unit (CFU), can cause human salmonellosis (2, 3). In Australia, the incidence risk of *Salmonella* infection was 53.7 cases per 100,000 people in 2010, almost 30% higher compared to the five previous years average risk of 41.8 cases per 100,000 people (1).

Salmonella Enteritidis is a major concern for most egg industries around the world. Although *S.* Enteritidis is associated with the majority of egg related outbreaks of human salmonellosis occurring in the European Union (77.2%) (4), it is not endemic to Australian layer flocks (5). Instead, *Salmonella* Typhimurium was the most frequently reported serovar in the 21 egg related food poisoning outbreaks in Australia in 2010 (1). In Australia, a study showed that *Salmonella* Infantis was the most frequently reported serovar from egg shell wash of eggs collected from 31 flocks (6). Furthermore, in Australia, *S*. Infantis has had the largest percentage increase in reported human infections, with 2.2 times more notifications nationally in 2010 than the previous year (1).

Residual contamination of the environment with *Salmonella* is a major problem in commercial layer farms (7, 8, 9). Davies and Breslin (10) concluded that, in cage systems, environmental samples such as egg belt, dust near cages, and pooled accumulated faecal samples should be tested while screening flocks for *Salmonella*. There is little information available in the literature about the risks of *Salmonella* contamination of eggs from infected birds and contaminated shed environment. Chemaly et al. (11) investigated the prevalence of *Salmonella* on eggshells in infected layer flocks, whereas Wales et al. (12) correlated the environmental contamination with faecal contamination by *Salmonella*. However, the rate at which an infected flock can produce *Salmonella* contaminated eggs is unclear. The possible transmission of *Salmonella* from the environment to the egg could be explained with the help of longitudinal studies (12). However, cooperation from egg producers over a period of months or years and the requirement of resources are limiting factor to such studies (12). There are a few reports in which the levels of *Salmonella* contamination in laying houses and hens were examined over time during lay (12, 13, 14). However, these studies did not investigate the degree of internal or external egg contamination. Furthermore, the focus of these studies was mainly on *S*. Enteritidis. Although *S*. Typhimurium has an established ability to be transmitted to humans via contaminated shell eggs, there is little published data on field studies, natural infections and long term experiments (15).

In the present study, longitudinal and point in time surveys were conducted on two known *S*. Typhimurium contaminated commercial layer farms both with multi-aged flocks housed in the same shed. The primary objectives of this study were: 1) To evaluate the association between *Salmonella* load in the shed environment and the probability of eggshells being contaminated with *Salmonella*; 2) To investigate the dynamics of *Salmonella* shedding of various serovars over prolonged period of time during longitudinal samplings; 3) To detect *S*. Typhimurium and *S*. Infantis positive samples using multiplex PCR; 4) To investigate relatedness of various *S*. Typhimurium strains using Multi-locus Variable Tandem Repeat Analysis (MLVA).

Materials and methods

This study was conducted in two stages. In stage 1, with the help of a cross-sectional study, cages infected with various serovars of *Salmonella* spp. were identified. Based on the results of the cross sectional study, in stage 2, *Salmonella* positive and negative cases were selected for longitudinal study and the association between eggs and environmental *Salmonella* contamination was investigated.

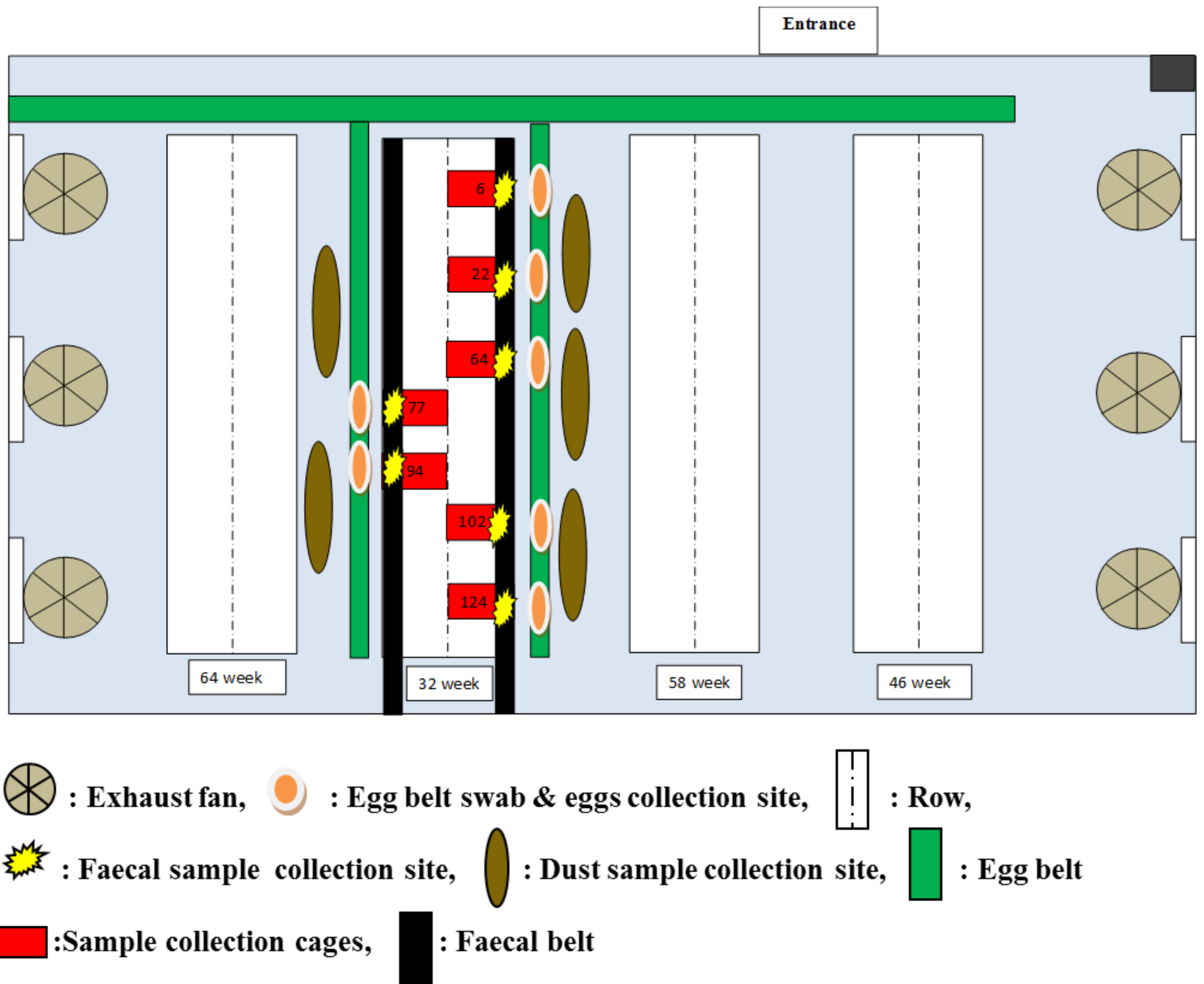

Fig.3.1: The layout of shed with flock A showing the areas of sample collection

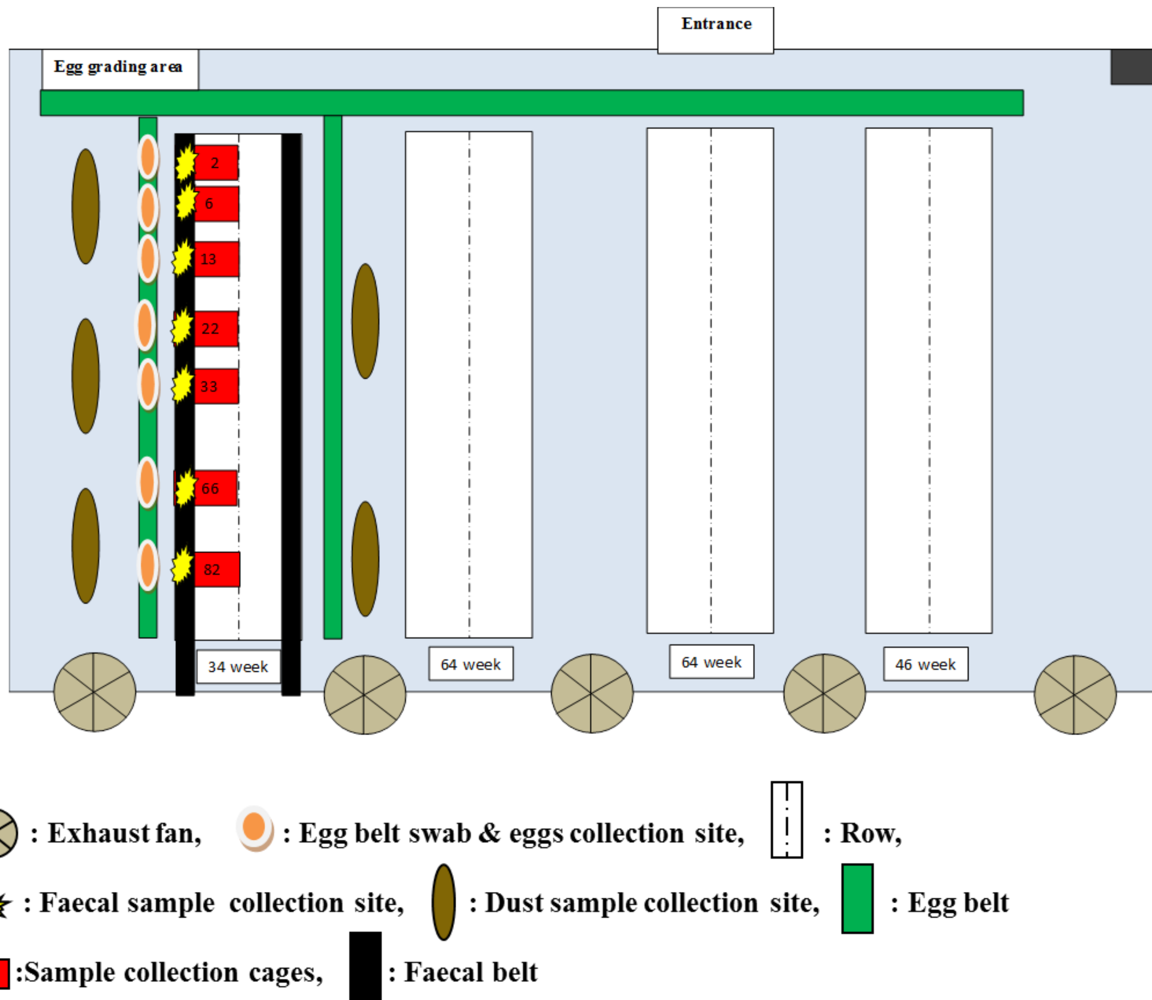

Fig.3.2: The layout of shed with flock B showing the areas of sample collection.

All the eggs at the front of the seven selected cage were collected. Each egg was collected in a separate sterile Whirl-pak plastic bag to avoid cross-contamination. Flock A moulted at 67th week of lay; therefore, no eggs were obtained in the ninth week of sampling.

Multi-locus Variable Tandem Repeat Analysis (MLVA) of *S.* Typhimurium isolates

After serotyping, all *Salmonella* strains which were identified as *S.* Typhimurium were further analysed by MLVA, as described by Ross et al. (16), at *Salmonella* Reference Laboratory, Adelaide, Australia.

Quantitative Polymerase Chain Reaction (qPCR) detection of *Salmonella enterica*

As described in Chapter 2

Multiplex PCR to identify *S.* Typhimurium and *S.* Infantis positive samples

The procedure is outlined in Chapter 2.

Statistical analysis

Binomial exact confidence intervals were computed for the prevalence of *Salmonella* positive cage estimate in each flock. Multilevel logistic regression was used to estimate the association between an eggshell being *Salmonella* positive and *Salmonella* positive faeces from the cage where the egg came from, *Salmonella* positive egg belt at the front of this cage, and *Salmonella* positive floor dust at the front of this cage. Random effects for '*flock*' and for '*cage within flock*' were added to the model to account for the fact that eggs were clustered within cage and within flock. Multilevel logistic regression was also used to evaluate the association among the *Salmonella* test outcomes of the corresponding cage faeces, egg belts, and floor dust (only included '*flock*' as random effect). Kappa statistics was computed to assess the agreement between culture isolation and qPCR. The association between the *Salmonella* burden (using log transformed Ct values from qPCR) in faeces, egg belt, and floor dust with the odds of an eggshell testing positive for *Salmonella* was investigated using the same structure multilevel logistic regressions. All models' parameters (odds ratio) were interpreted at 5% significance level. Models assumptions were assessed using standard diagnostic plots. Statistical analyses were performed using the statistical package STATA v12.1 (20).

Results

Selection of cages for longitudinal study

Culture isolation results indicated that in flock A, 21 cages (26.9%; CI: 17.5-38.2) were positive for *Salmonella* spp. at 32 weeks. Flock B had a higher prevalence of *Salmonella* positive cages with 31 cages (39.7%; CI: 28.8-51.5) reported positive at 34 weeks. Based on *Salmonella* typing results, five *Salmonella* positive cages each from farm A (*S.* Typhimurium phage type 9 = 3 cages, *S.* Infantis and *S.* Orion = 1 cage each) and farm B (*S.* Typhimurium PT 9 = 2 cages, *S.* Infantis, *S.* Agona and *S.* Oranienburg = 1 cage each), as well as two *Salmonella* negative cages per farm were selected for the longitudinal study.

Salmonella prevalence in flock A and B in longitudinal study

The details of number of samples which were reported *Salmonella* positive, along with type of serovars identified over the period of 40 weeks from flock A and B, are described in Tables 3.1 and 3.2 respectively. Figure 3.3 shows the prevalence of *Salmonella* in different type of samples. In both flocks, the *Salmonella* prevalence was higher in dust samples compared to egg belt, faeces and eggshells. In flock B, from 6th sampling (58 week onwards), there was an increase in prevalence of *Salmonella* in all types of samples but was highest in dust samples. It was observed that there was a higher fluctuation in *Salmonella* contamination of faeces compared to the dust and egg belt samples. Out of all eggs tested,

in flock B, 7.17% (19/265) eggshells were *Salmonella* positive; however, in flock A, only one eggshell 0.39% (1/256) was reported *Salmonella* positive. All of the egg internal contents from flock A and B were *Salmonella* negative.

Serotyping results confirmed that *S*. Oranienburg was the most frequently (76.92%) reported serovar followed by *S*. Typhimurium PT 9 (11.54%), *S*. Worthington (8.46%), *S*. Agona (3.08%), *Salmonella* subsp.1 ser. 4, 5, 12:-:- (1.54%) and *Salmonella* subsp.1 ser rough: g,s,t:- (0.77%). Table 3.3 provides the percentage of various *Salmonella* serovars isolated from different type of samples. The results of MLVA indicated that the *S*. Typhimurium strains isolated from flock A and flock B were genetically distinct. In flock B, all the *S*. Typhimurium isolates possessed same MLVA pattern (03 15 07 11 550). On other hand, *S*. Typhimurium strains isolated from flock A, exhibited three different MLVA patterns (03 24 11 10 523; 03 24 11 11 523; 03 24 11 12 523).

Relationship between the environmental contamination of *Salmonella* with *Salmonella* positive eggshells

Salmonella positive faecal samples, egg belt, and dust were all unconditionally (analysis did not account for other factors) associated with eggshells testing positive for *Salmonella*. The odds of an eggshell testing positive for *Salmonella* were 91.8 times higher when the faecal sample from this cage tested positive for *Salmonella* (odds ratio= 91.8, p<0.001, CI= 11.2-749.7). The odds of an eggshell testing positive for *Salmonella* were 61.5 times higher when the corresponding section of the egg belt was tested positive to *Salmonella* (odds ratio= 61.5, p<0.001, CI= 7.65-494.8). The odds of an eggshell testing positive for *Salmonella* were 18.2 (odds ratio= 18.2, p<0.001, CI=3.93-84.2) times higher when the corresponding floor dust tested positive for *Salmonella*. In the final multifactorial model (designed to study the possible environment/bird/egg transmission of *Salmonella*) faecal and dust sample results were conditionally (analysis account for other factors) associated with an eggshell testing positive for *Salmonella*. The odds of an eggshell testing positive for *Salmonella* were 58.9 times higher when the faecal sample from the cage tested positive for *Salmonella* (odds ratio= 58.9, p<0.001, CI= 6.9-501.0), and 9.2 times higher when the corresponding floor dust tested positive for *Salmonella* (odds ratio= 9.2, p=0.007, CI=1.8-45.8).

Quantification of *Salmonella* load in environmental samples using qPCR and its relationship with *Salmonella* eggshell contamination

The *Taq*Man *Salmonella enterica* detection system does not provide quantification of positive samples. Therefore, to determine the limit of detection of the assay, a standard curve prepared from a known concentration of *S*. Infantis (2×10^6 to 2×10^0 CFU *Salmonella* per qPCR reaction) was used. The standard curve produced a slope of -3.2, a y intercept of 41 and R^2 of 0.99. Despite the good PCR assay efficiency (105%), confident detection was not possible at less than 200 CFU per qPCR reaction or 25 CFU/µl extracted nucleic acid template. When a cut-off Ct of 34 was used (CFU greater than 200 per qPCR reaction), the qPCR identified 87 *Salmonella* positive samples of which 7 were not detected by the culture based method. The qPCR failed to detect *Salmonella* in 38 samples from which *Salmonella* was cultured. The latter analysis resulted in 68% (80/118) of samples identified as containing *Salmonella* by microbiological culturing also testing positive by qPCR.

Table 3.4 provides the details of the *Salmonella* positive and negative samples detected by culture based analysis and qPCR. Agreement between culture based methodology and qPCR in detecting *Salmonella* was almost perfect for eggshell (observed agreement=99.19%, Kappa coefficient=0.94), and egg belt samples (observed agreement= 95%, Kappa coefficient=0.88), and substantial for faecal (observed agreement=87.14%, Kappa coefficient=0.47) and floor dust samples (observed agreement=80.61%, Kappa coefficient=0.58). The overall (in all samples) agreement between culture based and qPCR detections of *Salmonella* was good (observed agreement=91.02%; Kappa coefficient=0.73).

Using the qPCR standard curve, the load of *Salmonella* in faecal, egg belt, eggshells and dust was determined. Figure 3.4 shows the load of *Salmonella* (average log colony

forming unit (CFU) per PCR reaction) in faeces, egg belt, dust and eggshells. Results indicated that the levels of *Salmonella* detected in faeces, egg belt, and floor dust were unconditionally associated with an eggshell testing positive for *Salmonella*. One log increase in the load of *Salmonella* detected in faecal samples resulted in 35% increase (odds ratio=1.35, $p<0.001$) in the odds of an eggshell testing positive for *Salmonella*. Similarly, one log increase in the load of *Salmonella* detected on egg belt and in the floor dust resulted in 43% (odds ratio=1.43, $p<0.001$) and 45% (odds ratio=1.45, $p<0.001$) increase in the odds of an eggshell testing positive for *Salmonella*, respectively. When averaging *Salmonella* environmental burden across the faeces, egg belt, and dust samples, one log increase in environmental *Salmonella* burden resulted in 51% (odds ratio=1.51, $p<0.001$) increase in the odds of an eggshell testing positive for *Salmonella*. In the final multifactorial model (not considering combined environment burden), only the *Salmonella* burden detected in the egg belt appeared to be conditionally associated with an eggshell testing positive for *Salmonella* (odds ratio=1.43, $p<0.001$).

TABLE 3.1 Prevalence of *Salmonella* in flock A during longitudinal sampling

Collection	Week of lay	Eggshells	Faeces	Egg belt	Dust	*Salmonella* Serovar
1	36	0/33	0/7	0/7	1/5	Dust: *S*. Typhimurium PT 9
2	40	0/33	0/7	1/7	1/5	Egg belt & Dust: *S*. Worthington
3	44	0/30	1/7	0/7	3/5	Faeces & Dust: *S*. Typhimurium PT 9
4	48	0/30	0/7	0/7	0/5	-
5	52	0/30	0/7	0/7	1/5	Dust: *S*. Typhimurium PT 9
6	56	0/30	0/7	0/7	1/5	Dust: *S*. Typhimurium PT 9
7	60	0/32	1/7	1/7	1/5	Faeces: *S*. Worthington. Egg belt: *S*. subsp.1 ser. 4,5,12. Dust: *S*. Typhimurium PT 9
8	64	0/28	1/7	1/7	0/5	Faeces: S. Worthington. Egg belt: *S*. subsp.1 ser. 4,5,12:-:-
9	68	-	2/7	1/7	2/5	Faeces & Egg belt: *S*. Worthington. Dust: *S*. Worthington (1), *S*. Typhimurium PT 9 (1)
10	72	1/10	0/7	1/7	3/5	Egg: *S*. Worthington. Egg belt: *S*. Typhimurium PT 9. Dust: *S*. Typhimurium PT 9 (2), *S*. Worthington

Count of positive isolation/ total number of samples for each sample type (eggshells, faeces, egg belt, and dust). *S*.: *Salmonella*; PT: phage type.

TABLE 3.2 Prevalence of *Salmonella* in flock B during longitudinal sampling

Collection	Week of lay	Egg-shells	Faeces	Egg belt	Dust	*Salmonella* Serovar
1	38	0/31	1/7	0/7	1/5	Faeces & Dust: *S*. Oranienburg
2	42	0/36	0/7	0/7	1/5	Dust: *S*. Oranienburg
3	46	0/39	0/7	0/7	1/5	Dust : *S*. Agona
4	50	0/27	0/7	2/7	2/5	Egg belt: *S*. Oranienburg (1), *S*. Agona (1) Dust: *S*. Oranienburg (1), *S*. Agona (1)
5	54	0/27	2/7	3/7	3/5	Faeces, Dust, Egg belt: *S*. Oranienburg
6	58	1/30	5/7	5/7	1/5	Faeces, Dust, Egg belt, Eggshells: *S*. Oranienburg
7	62	10/34	5/7	6/7	5/5	Faeces, Dust, Egg belt: *S*. Oranienburg Eggshells: *S*. Oranienburg (10), *S*. Typhimurium PT 9 (1)
8	66	8/33	6/7	7/7	5/5	Eggshells, Faeces, Egg belt: *S*. Oranienburg Dust: *S*. Oranienburg (5), *S*. Agona (1)
9	70	0/1	2/7	6/7	5/5	Faeces, Dust, Egg belt: *S*. Oranienburg
10	74	0/4	2/7	6/7	5/5	Faeces: *S*. Oranienburg (1), *S*. Typhimurium PT 9 (1).Egg belt: *S*. Oranienburg (5), *S*. Typhimurium PT 9 (2). Dust: *S*. Oranienburg (5), *S*. subsp.1 ser rough: g,s,t:-

Count of positive isolation/ total number of samples for each sample type (eggshells, faeces, egg belt, and dust). *S*.: *Salmonella*; PT: phage type.

TABLE 3.3 Serovars detected in various *Salmonella* positive sample types from layer farms

Salmonella serovar	Faeces	Egg belt	Dust	Eggshells	Total
S. Typhimurium PT 9	7.14% (2/28)	7.50% (3/40)	21.43% (9/42)	5% (1/20)	11.54% (15/130)
S. Oranienburg	78.57% (22/28)	82.5% (33/40)	64.28% (27/42)	90% (18/20)	76.92% (100/130)
S. Worthington	14.28% (4/28)	5% (2/40)	9.52% (4/42)	5% (1/20)	8.46% (11/130)
S. Agona	0% (0/28)	2.5% (1/40)	7.14% (3/42)	0% (0/20)	3.08% (4/130)
S. subsp.1. 4,5,12:-:-	0% (0/28)	5% (2/40)	0% (0/42)	0% (0/20)	1.54% (2/130)
S. subsp.1 ser rough: g,s,t:-	0% (0/28)	0% (0/40)	2.38 (1/42)	0% (0/20)	0.77% (1/130)

S.: *Salmonella*; PT: phage type.

TABLE 3.4 Agreement between culture method and real-time PCR to detect *Salmonella* positive and negative samples

Sample type	Samples identified by culture method	Samples identified by QPCR			Observed agreement (%)	Kappa coefficient
		Positive	Negative	Total		
Eggshells	Positive	8	1	9	99.14%	0.94
	Negative	0	114	114		
	Total	8	115	123		
Faeces	Positive	10	18	28	87.14	0.47
	Negative	0	112	112		
	Total	10	130	140		
Egg belt	Positive	37	3	40	95	0.87
	Negative	4	96	100		
	Total	41	99	140		
Dust	Positive	25	16	41	80.61	0.58
	Negative	3	54	57		
	Total	28	70	98		
All sample types	Positive	80	38	118	91.02	0.73
	Negative	7	376	383		
	Total	87	414	501		

Multiplex PCR to detect *S.* Typhimurium and S. Infantis positive samples

The multiplex PCR, specific for *S*. Typhimurium and *S*. Infantis was used to test various *Salmonella* serovars isolated from Australian layer farms (Table 3.5). All tested *Salmonella* serovars were amplified by the InvA primers which identifies *Salmonella* spp. All three primer pairs specific to either *S*. Typhimurium or *S*. Infantis were able to detect the respective serovar correctly. The primers specific for *S*. Typhimurium or *S*. Infantis did not produce specific PCR amplification for the other *Salmonella* serovars. However, there was an exception of *Salmonella* subsp.1 ser. 4,5,12:-:- which produced PCR amplification patterns similar to *S*. Typhimurium. In addition to the *Salmonella* specific InvA amplicon, the following serovars also produced an addition single amplicon with one of the TSR or ISR primer pairs: *S*. Agona *S*. Adelaide, *S*. Havana, *S*. Kiambu, *S*. Livingstone, *S*. Mbandaka, and *S*. Ohio (Table 3.5). To determine the limit of detection of multiplex PCR, faecal samples were spiked with the known concentration of *Salmonella*. Results indicated that the limit of detection by multiplex PCR was either 2,000 CFU/PCR reaction or 400 CFU/µl extracted nucleic acid template.

The samples which were *Salmonella* positive by qPCR (n=87) were all analysed by *S*. Typhimurium and *S*. Infantis multiplex PCR. Multiplex PCR identified six potential *S*. Typhimurium and no *S.* Infantis positive samples. The latter is in agreement with the serotyping results. Of the six samples identified as potentially *S*. Typhimurium positive by multiplex PCR, only one sample had *S*. Typhimurium (in addition to *S*. Oranienburg) isolated by microbial culturing. The other five identified *S*. Typhimurium multiplex PCR positive samples had either *S*. Worthington (n=1) or *S*. Oranienburg (n=3) isolated by culturing. These multiplex positive samples all contained *Salmonella* at levels greater than 10,000 CFU/qPCR or 1,250 CFU/µl nucleic acid template. None of the four qPCR positive samples which had *S*. Typhimurium isolated by culturing tested positive by the *S*. Typhimurium multiplex PCR assay.

Results of MLVA indicated that *S*. Typhimurium strains isolated from flock A and flock B, were genetically distinct. In flock B, all the *S*. Typhimurium isolates possessed same MLVA pattern (03 15 07 11 550). On other hand, *S*. Typhimurium strains isolated from flock A, exhibited three different MLVA patterns (03 24 11 10 523; 03 24 11 11 523; 03 24 11 12 523).

TABLE 3.5 Multiplex PCR assays targeting *Salmonella* serovars Infantis and Typhimurium

Salmonella serovar	Amplification results of each serovar specific genomic region by multiplex PCR						
	Salmonella	Typhimurium			Infantis		
	InvA (605 bp)	TSR1 (94 bp)	TSR2 (196 bp)	TSR3 (303 bp)	ISR1 (95 bp)	ISR2 (198 bp)	ISR3 (304 bp)
Salmonella Infantis	+	-	-	-	+	+	+
Salmonella Anatum[2]	+	-	+	-	-	-	-
Salmonella Typhimurium phage type 9	+	+	+	+	-	-	-
Salmonella Typhimurium phage type 44	+	+	+	+	-	-	-
Salmonella Typhimurium phage type 135	+	+	+	+	-	-	-
Salmonella Typhimurium phage type 170	+	+	+	+	-	-	-
Salmonella Typhimurium phage type 193	+	+	+	+	-	-	-
Salmonella Oranienburg	+	-	-	-	-	-	-
Salmonella Agona[1]	+	-	+	-	-	-	-
Salmonella Orion	+	-	-	-	-	-	-
Salmonella subsp.1 serovar rough g,s,t:-	+	-	-	-	-	-	-
Salmonella Adelaide[1]	+	-	-	-	-	-	+
Salmonella Bredney	+	-	-	-	-	-	-
Salmonella Cerro	+	-	-	-	-	-	-
Salmonella Havana[1]	+	-	-	-	+	-	-
Salmonella Johannesburg	+	-	-	-	-	-	-
Salmonella Kiambu[1]	+	-	-	-	-	-	+
Salmonella Lille	+	-	-	-	-	-	-
Salmonella Mbandaka[1]	+	+	-	-	-	-	-
Salmonella Montevideo	+	-	-	-	-	-	-
Salmonella Ohio[1]	+	-	-	+	-	-	-
Salmonella Virchow	+	-	-	-	-	-	-
Salmonella Livingstone[1]	+	-	-	+	-	-	-
Salmonella Singapore	+	-	-	-	-	-	-
Salmonella Senftenberg	+	-	-	-	-	-	-
Salmonella Zanzibar	+	-	-	-	-	-	-
Salmonella Worthington	+	-	-	-	-	-	-
Salmonella subsp.1 serovar 4,5,12:-:-[3]	+	+	+	+	-	-	-

[1] Similar result were observed by Akiba et al. (19)
[2] Akiba et al. (19) observed no amplification with *S*. Typhimurium or *S*. Infantis primers
[3] Not investigated by Akiba et al. (19)

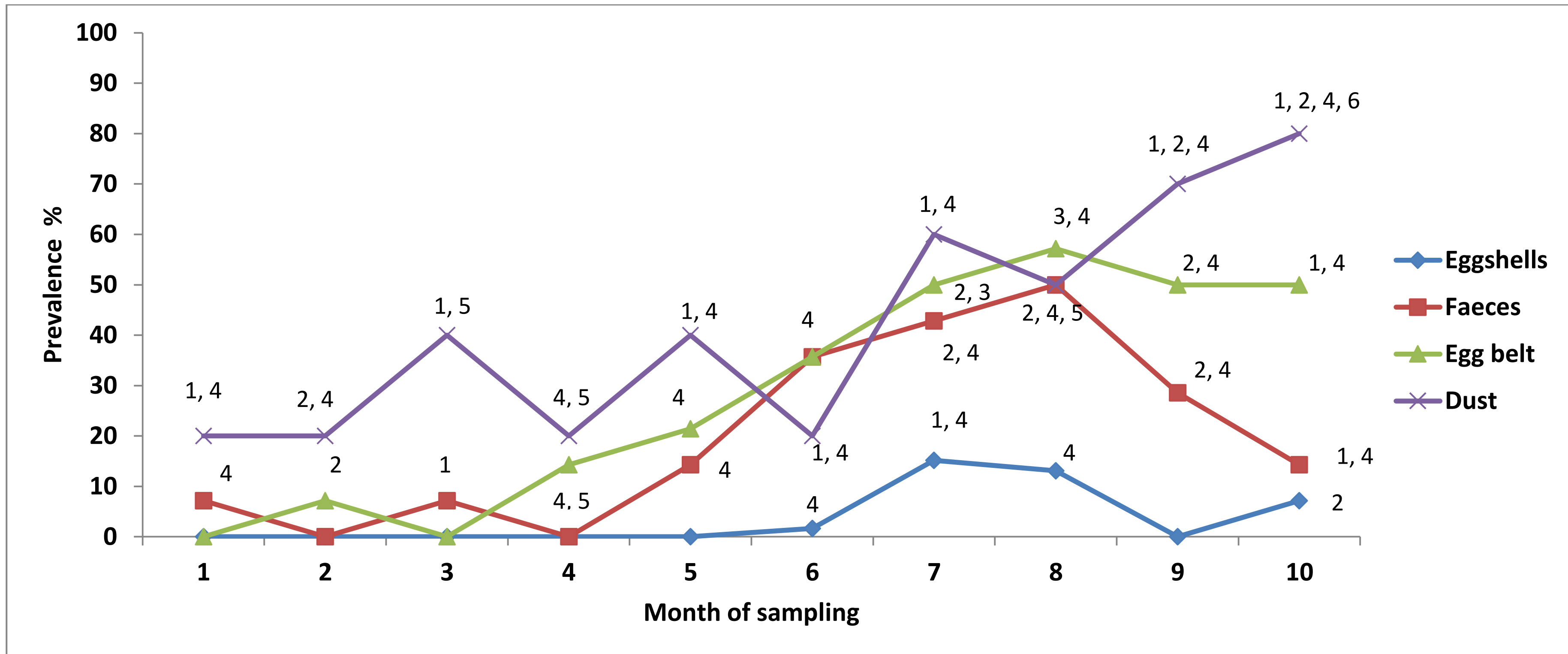

FIG 3.3. Percent prevalence of *Salmonella* in different type of samples over period of 10 month
1: *Salmonella* Typhimurium PT 9
2: *Salmonella* Worthington
3: *Salmonella* subsp.1 ser 4,5,12:-:-
4: *Salmonella* Oranienburg
5: *Salmonella* Agona
6: *Salmonella* subsp.1 ser rough g,s,t:-

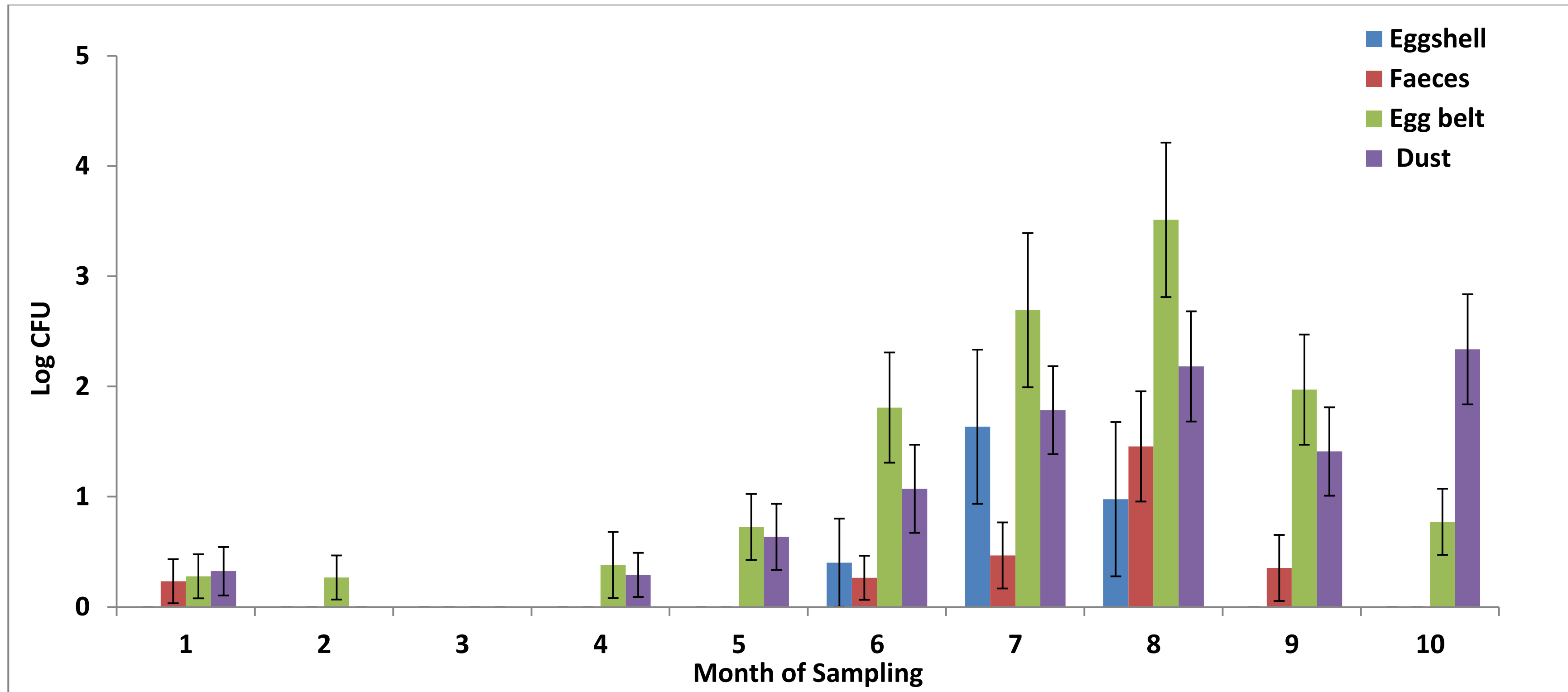

FIG 3.4 The load of *Salmonella* (average log colony forming unit (CFU)/qPCR reaction) in faeces, egg belt, dust and eggshells over period of 10 months.

Discussion

The current study involved longitudinal and point in time surveys of *Salmonella* carriage and environmental contamination on two commercial cage layer farms. Initial prevalence of *Salmonella* (based on faecal sampling n=78) in flock A and flock B was 26.9% and 39.7% respectively.

In the longitudinal study of 40 weeks, the highest prevalence of *Salmonella* was detected in dust samples (42%) followed by egg belt (28%), faecal (20%) and eggshells (4%) samples. The high prevalence of *Salmonella* in the dust may result in an airborne spread of infection in the layer flock within the shed. It has been observed that *S.* Typhimurium is capable of surviving in aerosol for long periods of time (21). A low dose of *S.* Typhimurium DT 104 infection (2 x 10^2 or 2 x 10^4 CFU per bird) resulted in increased *Salmonella* contamination of eggs (22). In the present study, qPCR results indicated that the level of *Salmonella* in dust samples peaked up to 10^5 CFU per qPCR, which may have resulted in the lateral spread of *Salmonella* in the flock. Hence, the presence of *Salmonella* in dust is a risk factor for the spread of infection in layer flock.

Of the 140 faecal samples tested, 20% were reported *Salmonella* positive. It was observed that there was higher variation in *Salmonella* contamination of faeces as compared to the dust samples. This may be due to the increased frequency of removal of faeces from the systems as compared to the dust (12). Faecal samples are believed to be better indicators of the infection status of flocks, whereas dust sample are more likely to indicate previous infection status (23).

The prevalence of *Salmonella*, in both flock A and B, increased during the later stages of lay. There is no clear information available within the literature which indicates the relationship between the stage of lay and *Salmonella* shedding. In flock B, following 58 weeks of age), there was a substantial increase in the prevalence of *Salmonella* in all types of samples. During this period, there was introduction of a new flock into the same shed. This new flock was housed adjacent to the flock which was sampled in the current study. There is a possibility that the introduction of a new batch of birds into the same shed may have stressed the birds under investigation, resulted in increased shedding of *Salmonella*. However, further studies are essential to confirm these observational findings.

Flock A moulted at the 67th week of lay. In the following week, it was observed that shedding of *Salmonella* in faeces increased and subsequently contamination of dust was also increased. As a result of molting, no eggs were obtained for *Salmonella* isolation in 68th week. However, in the 72nd week, one eggshell was reported *Salmonella* positive. Molting along with immunosuppression can alter gut microbiota and physiology, and these changes may influence the host-pathogen relationship (24). Holt (25) reported that induced molting resulted in higher shedding of *S.* Enteritidis in faeces and increased colonization of internal organs. The higher number of *S.* Enteritidis positive eggs were produced within the first 5 weeks after molting (25).

Out of all eggs tested, 4% (20/521) eggshells were reported *Salmonella* positive. The serovars which were observed on the eggshells were the same as those detected from farm environmental samples. However, all the egg internal contents were *Salmonella* negative. These findings are in agreement with our previous survey (6) where all egg internal contents were *Salmonella* negative. Egg penetration experiments have indicated that *S.* Worthington has a capacity to penetrate across the eggshell but lacks the ability to survive in the egg internal contents, whereas *S.* Typhimurium has a capacity to penetrate and survive in egg internal contents at 20°C (26). In the present experiment, even though chickens were positive for *S.* Typhimurium, egg internal contents were *Salmonella* negative. There is a lack of reliable information regarding the ability of *S.* Typhimurium to transmit vertically.

In the current study, of 20 *Salmonella* positive eggshell samples, 18 were positive for *S.* Oranienburg whereas one sample was positive for *S.* Worthington and another positive for *S.* Typhimurium. In Australia, egg associated *S.* Oranienburg outbreaks have not been reported so far. However, in Germany a large chocolate related outbreak of this serovar was reported in 2005 (27).

Salmonella positive faecal samples, egg belt, and dust were all unconditionally associated with eggshells testing positive for *Salmonella*. The odds of an eggshell testing positive for *Salmonella* were 91.8, 61.5 and 18.2 times higher when faecal, egg belt and dust samples tested *Salmonella* positive. This clearly suggests that faecal contamination of *Salmonella* is the most important factor for the production of *Salmonella* positive eggshells. On other hand, qPCR results suggest that not only qualitative score (positive or negative) but also quantitative level of *Salmonella* in environmental samples is very important in order to have *Salmonella* positive eggshells. One log increase in the load of *Salmonella* detected in faecal, egg belt and dust samples resulted in 35%, 43% and 45% increase in the odds of an eggshell testing positive for *Salmonella*, respectively. These findings are very important for developing management strategies for reducing the incidences of *Salmonella* positive eggshells by decreasing the level of *Salmonella* in the environment of layer shed.

The prevalence with which *Salmonella* was detected in samples using qPCR was lower than traditional microbiological culturing, with 68% of known *Salmonella* positives being identified. Furthermore, the sample type also influenced the variation in the agreement between the culture and qPCR based detection. An almost perfect agreement was reported between the two methods in identifying *Salmonella* positive eggshell and egg belt samples, with only a moderate agreement observed when faecal and dust samples were investigated. The microbiological culture based method involved pre-enrichment of samples in BPW followed by selective enrichment in RVS, while qPCR analysis was done on the pre-enriched samples only. This could explain the lower probability of detection by qPCR, especially if samples were contaminated with only low levels of *Salmonella* as has been observed by others (28). Despite the traditional microbiological culture based methods being more sensitive due to selection, the qPCR results did indicate that the probability of eggshell contamination was significantly increased with as little as a 10 fold increase in *Salmonella* levels within the shed environment. Therefore, qPCR does have potential as an initial rapid and high throughput screening tool to identify *Salmonella* in the environment.

In the present study, MLVA was used to investigate the relatedness of the different *S.* Typhimurium strains isolated from the two study flocks. As per the Australian coding system, strains isolated from flock A were distinct and unrelated to the strains isolated from flock B. All the *S.* Typhimurium strains from flock B exhibited no allelic variation. In contrast to this, there was an allelic variation in the strains isolated from flock A. However, as per the Australian MLVA coding system (29), this variation was not significant enough to call them as unrelated or distinct *S.* Typhimurium isolates. A quarterly report released from Institute of Medical and Veterinary Science (IMVS), Adelaide, Australia, indicated that *S.* Typhimurium strains responsible for human food poisoning cases exhibited similar MLVA pattern to the strains isolated from flock A and B (30). Serotyping confirmed that, *S.* Oranienburg was the most frequently reported serovar followed by *S.* Typhimurium PT 9, *S.* Worthington, *S.* Agona, *Salmonella* subsp.1 ser. 4, 5, 12:-:- and *Salmonella* subsp.1 ser rough: g,s,t:-.

These results may be an indication of a mixed *Salmonella* infection within the sample and the microbiological characterization based on a limited number of presumptive *Salmonella* positive colonies. The limit of detection for multiplex PCR was 2,000 CFU/reaction which may account for why know positive samples identified by culturing did not test positive by multiplex PCR. This clearly suggests that culture based methods are more sensitive and specific than multiplex PCR assays in characterising *S.* Typhimurium. These findings are in agreement with previous experiment of Soumet et al. (31). They reported a poor sensitivity (10^7 CFU/ml) of multiplex PCR for the samples (obtained from poultry houses) pre-enriched in BPW and directly tested by multiplex PCR. Furthermore, when testing pure isolates of a range of *Salmonella,* it was noted that *Salmonella* subsp.1. ser. 4, 5, 12:-:- was amplified by all three *S.* Typhimurium primer pairs in multiplex PCR and hence indistinguishable from *S.* Typhimurium. There is no previous information on the expected results for *Salmonella* subsp.1 ser. 4, 5, 12:-:- using the multiplex PCR system described by Akiba et al. (19). In France, genomic analysis has revealed that a non-motile strain of *Salmonella enterica* subsp. *enterica* with the antigenic formula 4, 5, 12:–:– is a non-motile variant of *S.* Typhimurium and responsible for egg related food poisoning outbreak

(32). Within Australia, the present study is the first report of a non-motile variant of *S.* Typhimurium in laying flocks. However, further genomic analysis is essential to reveal similarity of this non-motile variant with *S.* Typhimurium. In the future, such atypical *Salmonella* variants may emerge as a new challenge for the Australian layer industry.

In conclusion, the *Salmonella* positive samples of faeces, egg belt and dust were significant predictors of eggshell contamination. A single log CFU increase in the level of *Salmonella* within the layer shed environment significantly increased the incidence of eggshell contamination. Flocks sampled during this study showed a variation in *Salmonella* shedding over time. Stress induced by molting or introduction of a new batch of birds within the shed may have resulted in higher shedding of *Salmonella* in the environment, however, further controlled studies are required to prove these observational findings. Results of this study could be helpful to determine risks of having *Salmonella* contaminated eggshells and also for developing strategies for risk management programs to control *Salmonella*.

Chapter 4:
Shedding of *Salmonella* in a single age caged commercial layer flock at an early stage of lay

Introduction

During the laying production cycle, birds can experience various stressful events. It was observed that stress can impair humoral and cell mediated immune response of the birds (33). Thus, due to the impaired immune response, birds might become more susceptible to *Salmonella* infection which in turn may lead to increased *Salmonella* shedding in faeces. The results of our previous study indicated that an increase in the shedding of *Salmonella* in faeces could increase the chances of eggshell contamination (Chapter 3). One of the most important stressful events in laying hens is the onset of sexual maturity and or lay which generally also coincides with the transfer of birds from one production system (rearing shed) to another (layer shed) (34). It could, therefore, be hypothesised that when birds reach sexual maturity (with addition of transport stress), they may be more susceptible to *Salmonella* infection. However, there is little information available in literature regarding the shedding of *Salmonella* at the initial stages of the laying period.

Laying birds may also be stressed with the level of traffic (of workers) or noisy cleaning methods in layer shed (35). In a large egg layer farm (having capacity ~ 30000 bird/shed), outer lanes (high traffic area) could be more frequently used by workers as compared to inner lanes (low traffic area) to access shed controls. Even in the presence of biosecurity measures, Davies et al. (36) reported that the footware of workers working on processing plants was identified as a risk factor for *Salmonella* infection of the premises. Similarly, the birds in low tiers may experience greater disturbance with the movement of the workers as compared with birds in higher tiers. It could be hypothesised that all these factors may contribute to an increased susceptibility of birds to *Salmonella* infection. However, there is little known about the relationship between these stress factors and *Salmonella* shedding.

The culture method protocol, for identification of *Salmonella* positive samples, involves multiple steps and generally takes four to six days (37). The use of a qPCR method could be helpful to reduce the time involved in detecting *Salmonella* positive samples and enable he quantification of bacteria in samples. The data generated would be beneficial for the development of *Salmonella* monitoring and control programs.

The objectives of present study were: 1) to investigate the *Salmonella* shedding in early stages of lay 2) to study the effect of traffic (low and high) and the various level of tiers on the shedding of *Salmonella* 3) to compare the efficacy of culture method and qPCR to detect *Salmonella* positive samples.

Materials and methods

The study farm was an egg layer farm that has three different sheds connected with a common egg conveyer belt. Each shed housed bird cohorts of the same age (early lay < 40 week, mid lay 40 to 65 week and late lay > 65 week). The early lay shed was selected for conducting a prospective cohort study. The birds were transferred to the shed two weeks prior to the commencement of the study. The shed included six rows containing five tiers of 49 cages each and in each cage twenty birds were housed. The total size of the flock in the study shed was approximately 36,750 birds. The study shed was first sampled at 18 weeks of bird age (t0) followed by two longitudinal samplings at 24 and 30 weeks (t+6 and t+12, respectively). In each time point, faecal, egg belt, dust, feed, and shoe cover samples were collected. Eggs were collected in only last two sampling from the cages of low and high tiers as the eggs from 18 weeks of age were directly sent for egg pulping.

Specimen Collection

During the first sampling, a larger cross-sectional sampling was conducted to map the initial infection distribution within the flock. In total, 56 composite cage faecal samples, 12 egg belt swabs, 6 dust samples, 4 shoe cover samples and 4 feed samples were collected. The cages were systematically sampled at an approximate interval of 16 cages (cage order: 1, 16, 33, 49) from tier 1, 2 and 5. Additional faecal samples from tier 5 were collected from cages near to fan end (cage number 49). Composite faecal samples were collected in sterile Whirl-pak plastic bag (150 X 230 mm, ThermoFisher Scientific, Australia) from underneath the selected cages. The full length of the manure belt under each cage was covered while collecting faecal samples. To avoid cross-contamination, disposable gloves were changed between each cage.

Egg belt swabs were collected from the front of the respective tiers (one sample per tier) using Whirl-Pak speci-sponge bags (115 x 239 mm Thermo Fisher Scientific, Australia). The swabs were pre-moistened using 25 ml of BPW and dragged to cover the whole length of the egg belt. Dust (one per corridor) and feed (n=4) samples were collected in sterile containers (Pacific Laboratory Products, Australia). During the sample collection of each row, disposable shoe covers were worn to sample the floor dust. At the end of the sampled row, shoe covers were removed and placed in a 250 ml sterile plastic container (Pacific Laboratory Products, Australia).

Following the first sampling (t0), sampling at t+6 and t+12 were restricted to 3 rows, two in high (row A and F) and one in low traffic area (row D) (Figure 4.1). Side rows, which have been most frequently used by farm workers, were considered high traffic areas, and middle rows, which were less frequently used, were considered low traffic areas. From each selected row, six cages were systematically sampled (cage number: 1, 8, 16, 33, 40, 49) from the tier 1, 2 and 5. Altogether, composite faecal samples were collected from 54 cages (6 cages × 3 tiers × 3 rows) and processed for *Salmonella* isolation. Similarly, 9 egg belt (3 tiers × 3 rows), 3 dust, 3 shoe cover and 4 feed samples were collected. During t+6 and t+12 sampling, all the laid eggs at the front of the sampled cages were collected from the low and high tiers of two rows (row A and F). Eggs were placed in a sterile Whirl-pak plastic bag (150 X 230 mm, Thermo Fisher Scientific, Australia). A pool of six eggs was considered as a one sample.

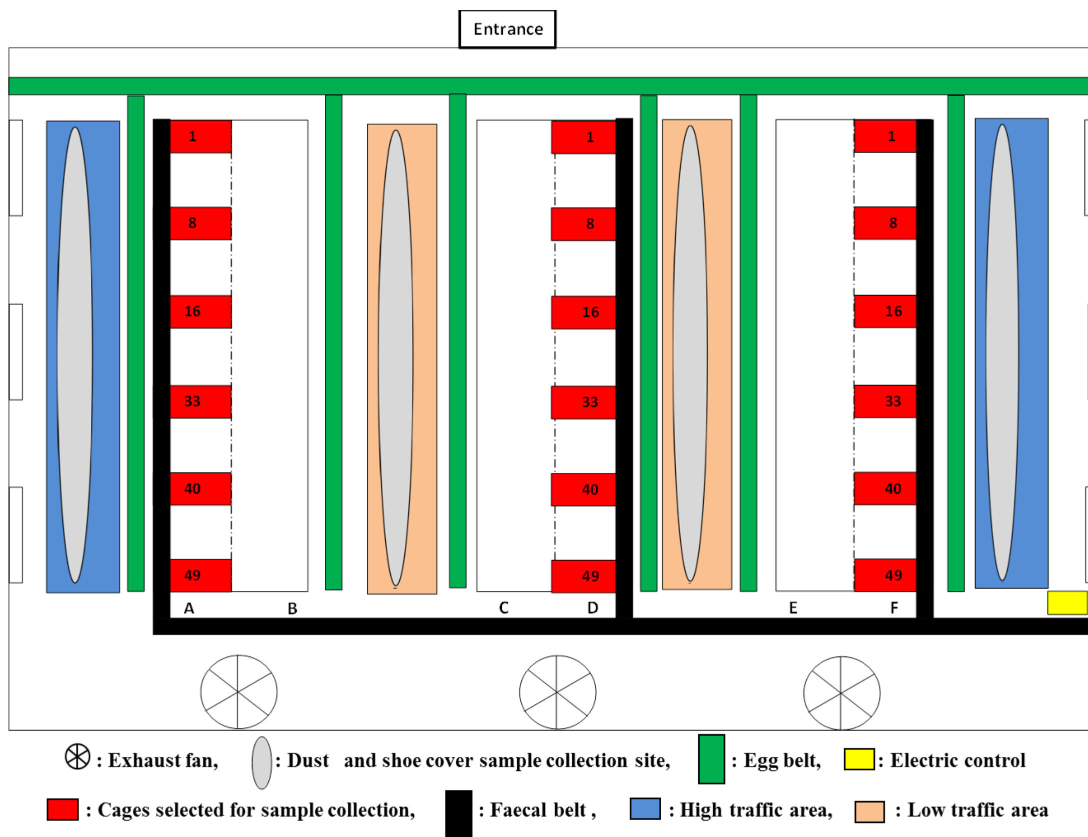

Figure 4.1: The schematic layout of shed showing the sample collection areas

Isolation of *Salmonella* from different samples
The isolation of *Salmonella* spp from different samples is described in Chapter 2.

Real-time Polymerase Chain Reaction detection of *Salmonella enterica*

The Wizard genomic DNA purification kit (Promega, Australia) was used to extract DNA from the pre-enriched BPW samples (faecal, egg belt, dust, feed, shoe cover and eggshell) as per manufacturer's instructions with slight modifications. Briefly, 5 ml of pre-enriched samples were centrifuged at 14000 g for 2 min to pellet bacterial cells. The bacterial pellet was then treated as per manufacturer's instructions.

In order to compare the efficiency of raw faecal samples compared with incubated BPW to detect *Salmonella*, DNA was also extracted from raw faecal samples using QIAamp DNA Stool Mini Kit (Qiagen, Australia) as per manufacturer instructions. Extracted DNA was quantified using Nanodrop and stored at -20°C until used for qPCR. Further dilution was performed using nuclease free water to achieve the final 5 ng/µl DNA concentration. Finally, these diluted DNA samples were used in real-time PCR.

The PCR detection of *Salmonella* was done using the *Taq*Man *Salmonella enterica* detection kit system (Applied Biosystems, Australia) in a total reaction volume of 15 µl containing 6 µl sample (5 ng/µl), 7.5 µl of 2 x Environmental Master Mix and 1.5 µl of 10 x Target Assay Mix. All reactions were run on a Corbett Research (Adelab Scientific, Australia) with the following PCR conditions: 95°C for 10 min followed by 45 cycles of 95°C for 15 sec and 60°C for 60 sec. All data were analysed using the software version Rotor-gene 1.7.75. The *Taq*Man *Salmonella enterica* detection kit does not provide quantification of positive samples. To determine the limit of detection and quantification of positive samples, a standard curve was prepared by generating a serial 10-fold dilution of faecal samples spiked with various concentrations of *Salmonella*. Briefly, 1 gm of faecal samples was spiked with different 10 fold dilutions (3×10^4 CFU to 3 CFU/gm) of *Salmonella*. As described above, DNA extraction from spiked faecal samples was performed using QIAamp DNA Stool Mini Kit (Quigen, Australia). The qPCR was performed on the diluted DNA samples (5 ng/µl). In each qPCR reaction cycle, positive and negative controls were used to confirm the success of reaction. A cut-off Ct of 32 was used to exclude detection of false positives. A Ct of 32 corresponded to 30 CFU of *Salmonella*.

Statistical analysis
The prevalence of *Salmonella* positive cages was estimated at each sampling point with 95% binomial exact confidence intervals. Multilevel logistic regression was used to compare prevalence estimates to account for the fact that cages were sampled repeatedly and the fact that cages were clustered within tier and tiers within row. The default mixed model included random effects for 'row', 'tier within row' and for 'cage within tier'. This model was then used to investigate the fixed effect of the following factors on *Salmonella* positive isolation: sampling points (t0, t+6, t+12), high and low traffic areas, tier level, and cage location within a tier.

Agreement between detection methods (culture and qPCR) was estimated by simply using the proportion of samples for which the test result (either positive or negative) agreed (i.e. observe agreement). Kappa statistics was intentionally not used because of the recognised limitations of this index including its instability with samples with extreme prevalences (<20% or >80%).

Kruskal-Wallis test was used to determine the variation in the load of *Salmonella* (log transformed CFU) in different types of samples over period of three sampling. p-values were interpreted at 5% significance level. Models assumptions were assessed using standard diagnostic plots. Statistical analyses were performed using the statistical package STATA v12.1 (20).

Results

Prevalence of *Salmonella* in faecal samples

During the first sampling t0 (flock was 18 weeks of age), 82.14% (95% CI: 69.6%-91.1%) of the cage faecal samples were *Salmonella* positive. When compared to t0, the prevalence of *Salmonella* positive cages significantly decreased to 38.88% at t+6 (95% CI: 25.9%-54.1%) and to 12.96% (95% CI: 5.4%-24.9%) at t+12 (p<0.001).The prevalence of *Salmonella* in low tier cages (prevalence= 64.38%, CI: 0.53-0.74) was significantly higher (p=0.009) as compared with high tier cages (prevalence=24.39%, CI: 0.15-0.35). There was no significant difference (p>0.05) in the prevalence of *Salmonella* positive cage across cage location, between specific rows, and between high and low traffic areas in the shed.

Prevalence of *Salmonella* in other type of samples

Table 4.1 summarizes the numbers of samples and the test outcomes for each type of specimen collected at the 3 sampling points. For all three sampling points, all egg belt and dust samples tested positive *Salmonella* isolation. Out of the 10 dust samples collected with shoe covers, nine (90%) were *Salmonella* positive. At t+6, out of the 54 eggshells tested, all were *Salmonella* negative, and, at t+12, two of the 72 eggshells were *Salmonella* positive (2.7%). All feed samples and egg internal contents were *Salmonella* negative.

Serotyping of *Salmonella* isolates

Serotyping results confirmed that, in all types of samples, *S.* Mbandaka was the most frequently (54.40%) isolated serovar followed by *S.* Worthington (37.60%), *S.* Anatum (0.8%) and *S.* Infantis (0.8%). In faecal samples, *S.* Worthington was the most prevalent serovar whereas *S.* Mbandaka was predominantly isolated from the egg belt, dust, shoe cover and eggshell samples (Table 4.2).

Comparison between qPCR and culture

The limit of detection for qPCR was 30 CFU/gm of sample (Ct value =32) with reaction efficiency above 100%. The real-time PCR identified 69 positive samples out of 343 tested samples. Out of the 69 positive samples, 12 were negative by the culture method. On other hand, qPCR failed to detect 70 samples which were positive by culture method (Table 4.3).

Table 3 provides the details of agreement between culture method and qPCR to detect *Salmonella* overall and in the different types of specimens. Overall, the two methods agreed on the detection outcome of 76.1% of the tested samples. This observed agreement was perfect (100%) for the dust (n=12) and egg belt (n=30) samples, and almost perfect for eggshells samples (99.2%, n=127). On other hand, this agreement was moderate for shoe cover samples (80%, n=10) and low for faecal samples (54.3%, n=164). For better detection of *Salmonella* in faecal samples with qPCR, samples were also pre-enriched in BPW. Agreement between raw and BPW enriched faecal samples tested with qPCR was moderate (86.6%) with more samples testing positive with the raw vs pre-enriched BPW faecal samples (19 and 11 positives, respectively).

Salmonella quantification

Table 4.4 shows the load of *Salmonella* (average log colony forming unit (CFU) per PCR reaction) in faeces, egg belt, dust, shoe cover and eggshells across the three different sampling points. At t0, the average load of *Salmonella* on the egg belt was 3.02 log CFU±0.26 and increased significantly at t+6 (4.59 log CFU±0.13) and t+12 (5.26 log CFU±0.24). In shoe cover samples, the load of detected *Salmonella* also built-up significantly between t0 (1.48 log CFU±0.59), t+6 (3.98 log CFU±0.46), and t+12 (4.21 log CFU±0.46) (p=0.03). *Salmonella* loads in shoe covers were not significantly different between t+6 and t+12 (p=0.51). Similar results were observed for dust samples where *Salmonella* loads increased between t0 (0.83 log CFU±0.58), t+6 (3.32 log CFU±0.38), and

t+12 (4.13 log CFU±0.44) ($p<0.05$). In faecal samples, the *Salmonella* load was significantly lower at t+12 as compared to t0 and t+6 ($p<0.05$).

TABLE 4.1 Prevalence of *Salmonella* during three longitudinal sampling

Sampling	Week of lay	Faeces	Egg belt	Dust	Shoe cover	Eggshells	*Salmonella* serovars isolates*
1	18	82.14% (46/56)	100% (12/12)	100% (6/6)	100% (4/4)	-	Faeces: *S*. Infantis (1), *S*. Worthington (35), *S*. Mbandaka (10). Egg belt: *S*. Worthington + *S*. Mbandaka (3), *S*. Mbandaka (6), *S*. Worthington + *S*. Mbandaka + *S*. Infantis (1), *S*. Mbandaka + *S*. Anatum (2). Dust: S. Mbandaka (3), *S*. Mbandaka + *S*. Anatum (1), *S*. Worthington + *S*. Mbandaka (1), *S*. Anatum (1). Shoe cover: *S*. Mbandaka (3), *S*. Worthington + *S*. Mbandaka (1).
2	24	38.88% (21/54)	100% (9/9)	100% (3/3)	100% (3/3)	0% (0/55)	Faeces: *S*. Agona (1), *S*. Worthington (4), *S*. Mbandaka (16). Egg belt: *S*. Worthington (2), *S*. Mbandaka (7). Dust: *S*. Mbandaka (3). Shoe cover: *S*. Mbandaka (2), *S*. Worthington (1).
3	30	12.96% (7/54)	100% (9/9)	100% (3/3)	66.33% (2/3)	2.77% (2/72)	Faeces: *S*. Worthington (5), *S*. Mbandaka (2). Egg belt: *S*. Mbandaka (9). Dust: *S*. Mbandaka (3). Shoe cover: *S*. Mbandaka (2). Eggshell: *S*. Mbandaka (2).

Count of positive isolation/ total number of samples for each sample type (faeces, egg belt, dust, Shoe cover and eggshells).
*The number of *Salmonella* positive samples is providd in brackets.

TABLE 4.2 Serovars detected (in %) in various *Salmonella*-positive sample types during this study

Salmonella serovar	Faeces (n = 73)	Egg belt (n = 30)	Dust (n =11)	Shoe cover (n = 9)	Eggshells (n = 2)	Total (n = 125)
S. Worthington	60.27	6.66	0	11.11	0	37.60
S. Mbandaka	38.36	73.33	81.81	77.78	100	54.40
S. Infantis	1.37	0	0	0	0	0.8
S. Anatum	0	0	0.09	0	0	10.8
S. Worthington + *S.* Mbandaka	0	10	0.09	11.11	0	4
S. Worthington + *S.* Mbandaka + *S.* Infantis	0	3.33	0	0	0	0.8
S. Mbandaka + *S.* Anatum	0	6.66	0	0	0	1.6

TABLE 4.3 Agreement between culture method and real-time PCR to detect *Salmonella*-positive negative samples

		Positive and negative samples identified by qPCR			
Sample type	Positive and negative samples identified by culture method	Positive	Negative	Total	Observed agreement (%)
Eggshells	Positive	2	0	2	99.21
	Negative	1	124	125	
	Total	3	124	127	
Faeces	Positive	9	65	74	54.27
	Negative	10	80	90	
	Total	19	145	164	
Egg belt	Positive	30	0	30	100
	Negative	0	0	0	
	Total	30	0	30	
Dust	Positive	12	0	12	100
	Negative	0	0	0	
	Total	12	0	12	
Shoe cover	Positive	8	1	9	80
	Negative	1	0	1	
	Total	9	1	10	
All sample types	Positive	57	70	127	76.09
	Negative	12	204	216	
	Total	69	274	343	

TABLE 4.4 *Salmonella* load (average log colony forming unit (CFU)/real-time PCR reaction) in faeces, egg belt, shoe cover, dust and eggshells over period of three samplings.

	Week of lay (sampling number)			P value			
Sample type	18 (1)	24 (2)	30 (3)	Kruskal Wallis test	18 vs 24 weeks	24 vs 30 weeks	18 vs 30 weeks
Faeces	0.25±0.09[a]	0.49±0.14[a]	ND[b]	0.003	0.29	0.001	0.004
Egg belt	3.02±0.26[a]	4.59±0.13[b]	5.26±0.24[c]	<0.001	0.001	0.04	<0.001
Shoe cover	1.48±0.59[a]	3.98±0.46[b]	4.21±0.46[b]	0.03	0.03	0.51	0.03
Dust	0.83±0.58[a]	3.32±0.38[b]	4.13±0.44[b]	0.03	0.03	0.27	0.03
Eggshells	ND[a]	ND[a]	0.15±0.09[a]	0.13	NA	NA	NA

The different superscripts in the same sample type are statistically significantly different ($p < 0.05$) from each other.
Log CFU ± standard error
ND: Not detected
NA; not applicable

Discussion

In the present study, the shedding of *Salmonella* in single age layer flock was investigated at the onset of lay. The results of the culture method indicated that at t0 (the age of 18 weeks), the prevalence of *Salmonella* in faeces was highest (82.14%). However, in latter samplings, at t+6 and t+12, the prevalence of *Salmonella* in faeces was reduced significantly (p<0.001) to 38.88% and 12.95% respectively. There is a dearth in the literature to compare these findings as layer flocks are rarely sampled at the very early stage of lay. Residual *Salmonella* contamination on the layer farm is responsible for re-introduction of *Salmonella* in a flock (9, 38). It is possible that, in the present study, newly arrived pullets on the farm were suffering from transport, handling and relocation stress along with the stress related to onset of lay. In laying hens, stress can negatively influence immune response (33, 34) which may have increased the susceptibility of young pullets to acquire *Salmonella* infection from the shed environment. This in turn may have resulted in the higher shedding of *Salmonella* at the age of 18 weeks of age. However, further controlled experiments are essential to establish association between stress and *Salmonella* shedding. Once the birds settled in cages (week 24 and 30), the shedding of *Salmonella* was reduced.

The prevalence of *Salmonella* in low tier cages was significantly higher (p=0.009) as compared to high tier cages. The higher prevalence of *Salmonella* in low tier cages could be explained by several factors. First, the birds in lower tier cages were more exposed to the dust on the floor. McDerrnid and Lever (39) demonstrated that *Salmonella* can survive in aerosols, maintained using a rotating drum, for a considerable period of time. In the present study, dust samples, in all three sampling, were consistently positive for *Salmonella* which may have resulted in the higher lateral spread of infection in lower tiers as compared to higher tiers. Secondly, birds housed in lower tiers are more exposed to the movement of workers and cleaning equipment as compared to the birds in higher tiers which may have resulted in stress and ultimately higher *Salmonella* shedding in cages belonging to lower tiers. However, there was no significant difference observed in the shedding of *Salmonella* in the cages belonging to high and low traffic areas. There is little or no information in literature to compare these finding. Further experiments are necessary involving the estimation of stress indicting parameters in high and low traffic areas which may provide better information regarding *Salmonella* shedding.

For qPCR, the limit of detection was 30 CFU/gm of sample. The PCR was able to identify 54% (69/127) of samples which were *Salmonella* positive by the culture method. The agreement between the culture method and qPCR varied based on sample type. The observed agreement between two methods was almost 100% for dust, egg belt and eggshell samples. However, in case of faecal samples, there was a low agreement (54.27%) between the culture method and qPCR. The qPCR was able to detect 25.67% (19/74) of faecal samples which were also culture positive. With the objective to improve the detection of *Salmonella* positive faecal samples, qPCR was also performed using pre-enriched BPW from faecal samples. However, with this protocol, qPCR was able to detect only 14.86% (11/74) of culture positive samples. This clearly suggested that BPW pre-enrichment or single enrichment did not improve the detection by PCR of *Salmonella* from faecal samples. When samples were processed for *Salmonella* detection by qPCR method, the limit of detection was 30 CFU. The comparative results between culture method and real-time PCR assay indicated that, culture method was able to detect less than 30 CFU of *Salmonella* spp. These findings are in agreement with Jensen et al. (28) who also reported a low relative sensitivity of real-time PCR (20%) as compared to a culture method. The low sensitivity of qPCR compared to the culture method could be attributed to the presence of PCR inhibitors in environmental samples.

In present study, most of the egg belt, dust and shoe cover samples were tested positive for *Salmonella* throughout sampling period. *Salmonella* prevalence in a layer farm can be affected by various factors such as farm and flock size (38). The bird holding capacity of the flock sampled in the present study was 36,750. Larger flock size increases the risk of introduction of *Salmonella* infection (38). However, it has been observed that the

persistence of *Salmonella* was not significantly related to flock size (40, 41). The presence of multiple flocks on the same farm enhances the risk of cross contamination between sheds especially when they are connected by common egg conveyor belt (42). In the present study, sheds on farm were connected with common egg conveyer belt. Another important factor for the continuous presence of *Salmonella* in battery cage layer farms is difficulty in cleaning and disinfection of interior of cages, egg belt and feeders (8, 41).

At age of 30 weeks (t+12), in faecal samples, the level of *Salmonella* dropped significantly. This could have been attributed to the recovery of the birds from the stress and acclimatization to the shed environment. However, there was significant increase in the load of *Salmonella* on egg belt, dust and shoe cover samples at t+6 and t+12 as compared to t0. Cleaning of shed and removal of dust (similar to faecal samples) at regular interval may help to reduce the level of environmental contamination in layer shed. In the present study, real-time PCR results indicated that, at t+12 (week 30), three eggshell samples were *Salmonella* positive. The only serovar isolated from all eggshells samples was *S.* Mbandaka. The same serovar was most frequently reported on egg belt, dust and shoe cover samples indicating the source of eggshell contamination. In the present study, even though, birds were infected with *Salmonella*, egg internal contents were *Salmonella* negative. The vertical transmission ability of most prevalent *Salmonella* serovars isolated from this study needs further investigation. Previously, it has been reported that *Salmonella* Infantis was not isolated from egg internal contents of known positive birds (43). *Salmonella* Mbandaka has been isolated from egg shell surface (44), however the controlled studies are essential to study the vertical transmission ability of predominant *Salmonella* serovars isolated during the present investigation. The serovars isolated in the present study may lack the ability to transmit vertically (vertical transmission) or may have little capacity to survive in egg internal contents (horizontal transmission). However, to confirm this, further studies are essential.

In conclusion, during this experiment, at the start of lay (18 weeks), within first week of housing, the shedding of *Salmonella* in faecal samples was at a peak compared with later sampling times. However, over the time, *Salmonella* infection subsided in subsequent samplings. The prevalence of *Salmonella* in birds housed in the lower tiers was higher as compared to birds in higher tiers. The sensitivity of qPCR was lower as compared to the culture method in detecting *Salmonella* positive faecal samples. The sensitivity of qPCR was also not improved with use of a pre-enrichment step. This might be due to the presence PCR inhibitory factors in faeces, a low number of target microorganism, and a large number of competing bacteria in faeces. On the basis of the qPCR results, load of *Salmonella* on egg belt, shoe cover and dust increased with the age of the flock. Hence, regular monitoring and intervention strategies are required to reduce the environmental load of *Salmonella* in layer shed which could be helpful to reduce the chances of eggshell contamination.

Chapter 5: Study of Antimicrobial resistance, biofilm forming ability and organic acid susceptibility of *Salmonella* spp.

Introduction

Although most *Salmonella* infections are self-limiting producing mild gastroenteritis, severe infections are common in elderly and immunocompromised patients (45). Systemic and severe infections caused by *Salmonella* require treatment with effective antimicrobials such as fluoroquinolones, and extended-spectrum cephalosporins (46). The use of antimicrobial agents in the prevention and treatment of many infectious diseases and as a growth promoter is well known both in veterinary and human medicine (47). However, indiscriminate use of antibiotics in animal and human population has led to an emergence of multidrug resistant *Salmonella* (48,49). The emergence and dissemination of antibiotic resistance in *Salmonella* is a serious animal and public health concern for both developed and developing countries (50). Moreover, the transfer of multidrug resistant *Salmonella* spp to humans through food producing animals can compromise the treatment options.

In comparison to other countries, Australia has a very cautious approach to the use of antibiotics in commercial egg layer flocks. Antimicrobials such as fluroquinolones are prohibited and ceftiofur is not approved for mass administration in food producing animals (51, 52). In addition, antibiotics used in human medicine are generally not used to treat commercial egg layers. This strategy has helped to minimise the amount of antibiotic residues in eggs or egg products and transfer of antibiotic resistance and resistance genes from animal to human through the food chain (53). As a result of these restrictions, a recent Australian study reported very low levels of antimicrobial resistance in *Salmonella* isolates from confirmed cases of salmonellosis in livestock (54). To date, there is little or no information available on characterisation of antimicrobial resistance in *Salmonella* isolated from commercial egg layer flocks in Australia. Therefore, monitoring of antimicrobial resistance in *Salmonella* species isolated from layer flocks is of particular interest.

In addition to antimicrobial resistance, *Salmonella* at various levels of food chain are able to persist in food processing environment by forming a biofilm. Biofilm is defined as community of interacting bacterial cells exposed to biotic or abiotic surface, embedded in self-produced extracellular polymeric matrix (55). Earlier studies have demonstrated the biofilm forming ability of *Salmonella* on many surfaces such as stainless steel, plastics, cement, rubber, glass and gallstones (56). *Salmonella* in biofilms are well protected against environmental stresses, antibiotics (57) and disinfectants making the eradication of bacteria extremely difficult from the industrial surfaces and equipment (58). In the past, biofilm formation by *Salmonella* on various abiotic surfaces has been studied extensively (56) however biofilm formation by *Salmonella* isolated from egg layer flocks has yet not been fully explored. In this study, the antimicrobial resistance profile and biofilm formation capability of various *Salmonella* isolates recovered from layer flocks of Australia was investigated.

Materials and Methods

Bacterial strains and serotyping

A total 145 *Salmonella* isolates were used in this study. Samples were isolated from 33 caged layer flocks across 13 farms from New South Wales (10 egg commercial egg farms) and South Australia (3 commercial egg farms). All *Salmonella* isolates used in this study were previously isolated in our laboratory during epidemiological studies (59). Isolates obtained during epdemiological investigation as explained in Chapter 3 & 4). Details of *Salmonella* isolates, sources and their distribution are presented in Table 5.1. All isolates were serotyped at the Institute for Medical and Veterinary Science, Adelaide, Australia.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of each *Salmonella* isolate to 12 antibiotics was determined by a broth microdilution method as per Clinical and Laboratory Standards Institute (CLSI) methods and the results were interpreted according to the established CLSI guidelines (60). In cases where CLSI breakpoints were absent, the results were evaluated according to the National antimicrobial resistance monitoring system (NARMS) (61) or Swedish Veterinary Antimicrobial Resistance Monitoring 2011 (SVARM) (62). All tests were performed using *Escherichia coli* ATCC 25922 as a quality control strain. *Salmonella* isolates showing resistance to more than three classes of antimicrobial agents were classified as multi-drug resistant (MDR).

Biofilm formation assay

The detailed methods for studying biofilm formation by *Salmonella* spp is outlined in Chapter 2.

Determination of maximum inhibitory dilutions (MID) of organic acid (OA) products against *Salmonella* serovars

Salmonella enterica spp *enterica* (n=4 of each serovar) previously isolated investigation as mentioned above were used in this study : *S.* Agona; *S.* Anatum; *S.* Infantis; *S.* Mbandaka; *S.* Oranienburg; *S.* Typhimurium and *S.* Worthington. All isolates were serotyped at Institute for Medical and Veterinary Science, Adelaide, Australia. The details of commercially available water soluble OA products were selected to examine their efficacy against *Salmonella* isolates are mentioned in Table 5.2.

Each *Salmonella* isolate was screened to determine the MID of OA products. Briefly, each OA product (2%) was prepared in nutrient broth (Oxoid, Australia) and diluted serially in 96 well plates. All the isolates were tested in duplicate and one negative control well in each row contained nutrient broth only whereas OA was omitted from positive control well of each row. Each *Salmonella* isolate was grown overnight at 37°C on a nutrient agar plate. A suspension of each *Salmonella* isolate was prepared by suspending bacteria in saline to 0.5 McFarland standard and further diluted (1:20) in normal saline. A 10 µl aliquot of the suspension was added to each challenge well as well as positive control well. Plates were incubated for 18 hours at 37°C and observed for visible growth. The highest concentration of OA products without visible growth was recorded as MID.

Results

Antimicrobial susceptibility screening of *Salmonella* isolates

The *Salmonella* isolates selected for this study displayed a low but wide spectrum of antibiotic resistance (Table 5.3). A total of 91.7 % (133/145) of the *Salmonella* isolates were susceptible to all tested antimicrobials. Overall, resistance was observed to amoxicillin and ampicillin (5.5%), tetracycline (4.1%), cephalothin (2 %) and trimethoprim (0.6%). Resistance to cefotaxime, ceftiofur, ciprofloxacin, chloramphenicol, gentamycin, neomycin, or streptomycin was not observed for any isolate. The *S.* Mbandaka, *S.* Typhimurium and *S.* Worthington isolates showed resistance whereas the *S.* Agona, *S.* Anatum, *S.* Infantis and *S.* Oranienburg isolates were susceptible to all tested antimicrobials.

No specific multidrug resistant phenotypes were associated with *Salmonella* serovars in this study and the results of multidrug resistance patterns of all *Salmonella* isolates are described in Table 5.4.

Colony morphology on Congo red agar plates

All *Salmonella* isolates were examined morphologically for their ability to form biofilms on Congo red agar plates at 22 and 37˚C after 96 hrs. In this study, three major morphotypes rough, dry and red (RDAR), pink, dry and rough (PDAR) and smooth and white (SAW) were

observed and the results of the colony morphotypes are presented in Table 5.5. The incubation temperature 22 and 37˚C had a strong influence on colony morphology on Congo red agar plates by *Salmonella* isolates. Amongst all *Salmonella* isolates, the most common morphotypes at 22˚C observed were RDAR and PDAR, produced by 91% and 8.2% *Salmonella* isolates respectively. At 22˚C only one *Salmonella* isolate, *S*. Oranienburg, displayed the SAW morphotype. In contrast, all *Salmonella* isolates revealed SAW morphology on Congo red agar plates at 37˚C which is indicative of no production of either curli fimbriae or cellulose. Overall, the results of this study indicated that *Salmonella* isolates have the potential to form extracellular matrix components of biofilm such as curli fimbriae and cellulose when grown at 22˚C.

Crystal violet biofilm assay

Biofilm production by *Salmonella* isolates at 22 and 37°C after 96 hrs of incubation was quantified using crystal violet staining assay. Overall, the amount of biofilm formation was significantly influenced by temperature and *Salmonella* serovars (Fig. 1). The biofilm formation was significantly higher ($P \leq 0.05$) at 22°C (OD_{590}= 2.24±0.01) compared to biofilm formation at 37°C (OD_{590}= 0.21±0.01).

Among the seven serovars, the *S*. Anatum (OD_{590}= 2.71±0.05) isolates produced significantly more ($P \leq 0.05$) biofilm at 22°C than the rest of the serovars: *S*. Agona (OD_{590}= 2.27±0.03); *S*. Infantis (OD_{590}= 2.03±0.05); *S*. Mbandaka (OD_{590}= 1.98±0.02); *S*. Oranienburg (OD_{590}= 2.21±0.03); *S*. Typhimurium (OD_{590}= 2.41±0.06); *S*. Worthington (OD_{590}= 2.43±0.03).

At 37°C, biofilm formation by *Salmonella* isolates was weak and greater variability was observed compared to 22°C (Fig 5.2). At 37°C, only the *S*. Oranienburg (OD_{590}= 0.72±0.04) and *S*. Typhimurium (OD_{590}= 0.21±0.02) isolates formed significantly more ($P \leq 0.05$) biofilm compared to other serovars: *S*. Agona (OD_{590}= -0.05±0.00); *S*. Anatum (OD_{590}= -0.01±0.00); *S*. Infantis (OD_{590}= 0.02±0.02); *S*. Mbandaka (OD_{590}= 0.06±0.01) and *S*. Worthington (OD_{590}= 0.04±0.01) (Fig 5.3).

Maximum inhibitory dilutions (MID) of organic acid (OA) products

The mean MID values of five different OA products against seven *Salmonella* serovars are presented in Figure 5.4. There were no variations seen amongst the five commercial OA products for MID values. One OA product (C) was comparatively less potent for MID against all seven *Salmonella* serovars. Similarly, OA product (B) was comparatively less potent for MID against *S*. Anatum. However, all the *Salmonella* isolates tested were susceptible at the recommended dose (except for product A where the recommended dose could not be found).

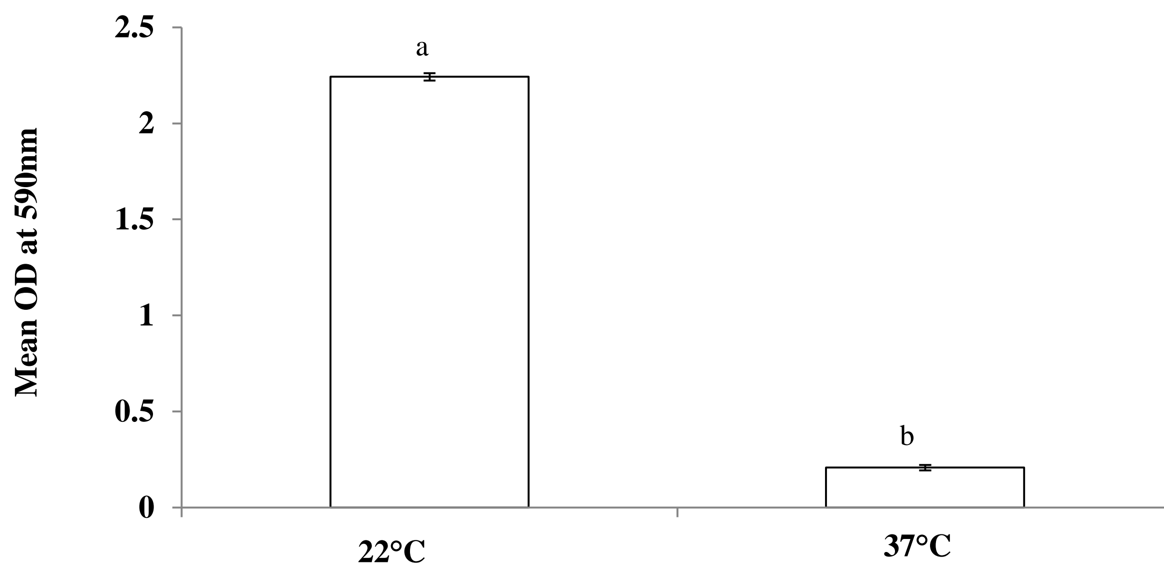

Figure 5.1: ***Salmonella*** **Biofilm formation at two different temperatures**

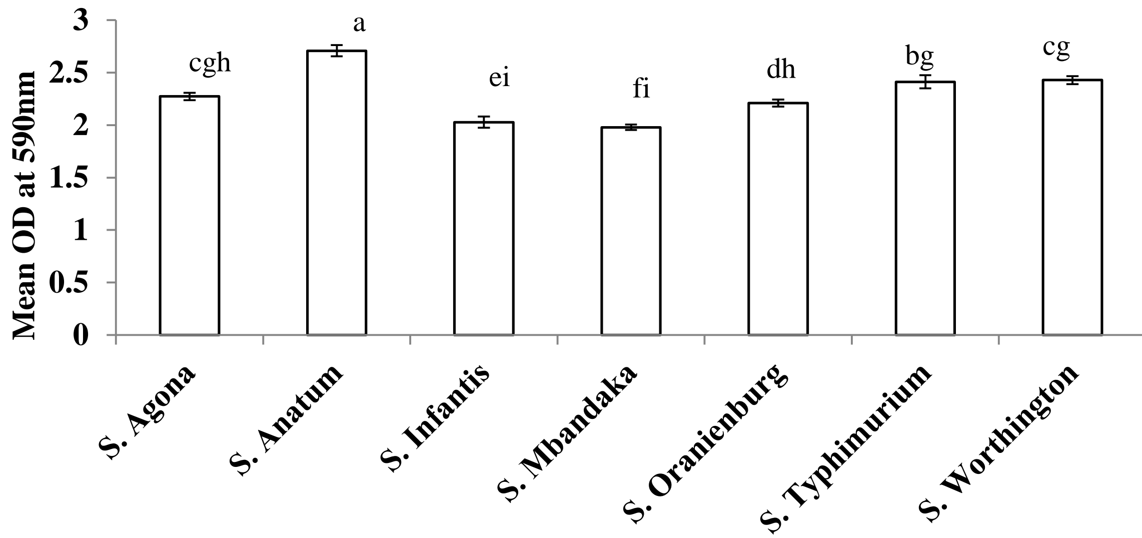

Figure 5.2. ***Salmonella*** **Biofilm formation at 22 ^{0}C**

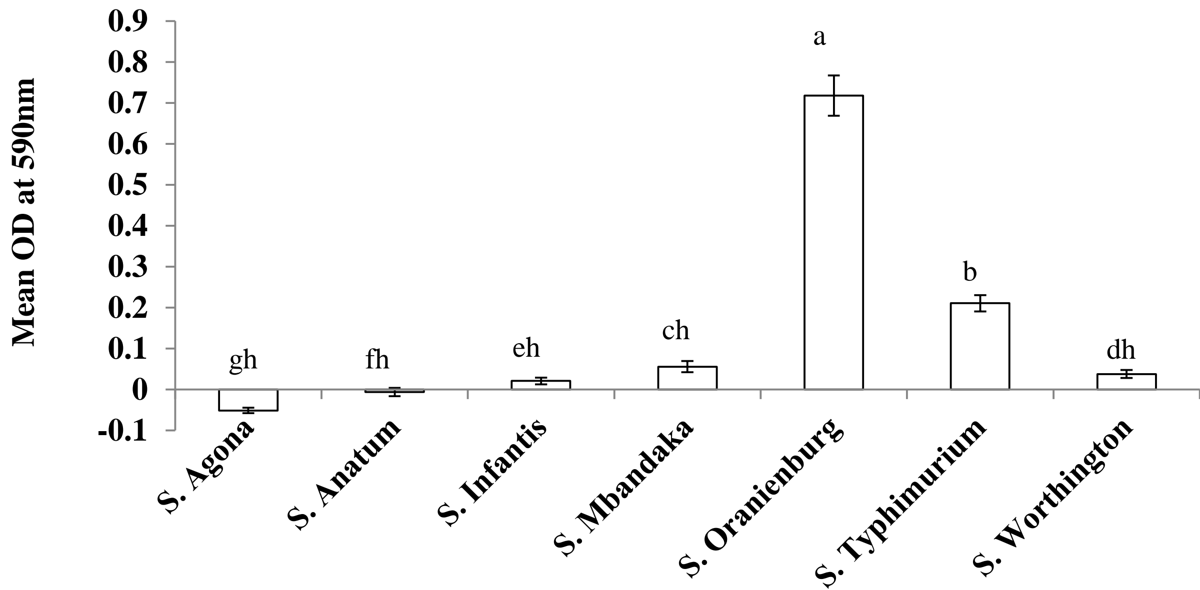

Figure 5.3: ***Salmonella*** **Biofilm formation at 37 ^{0}C**

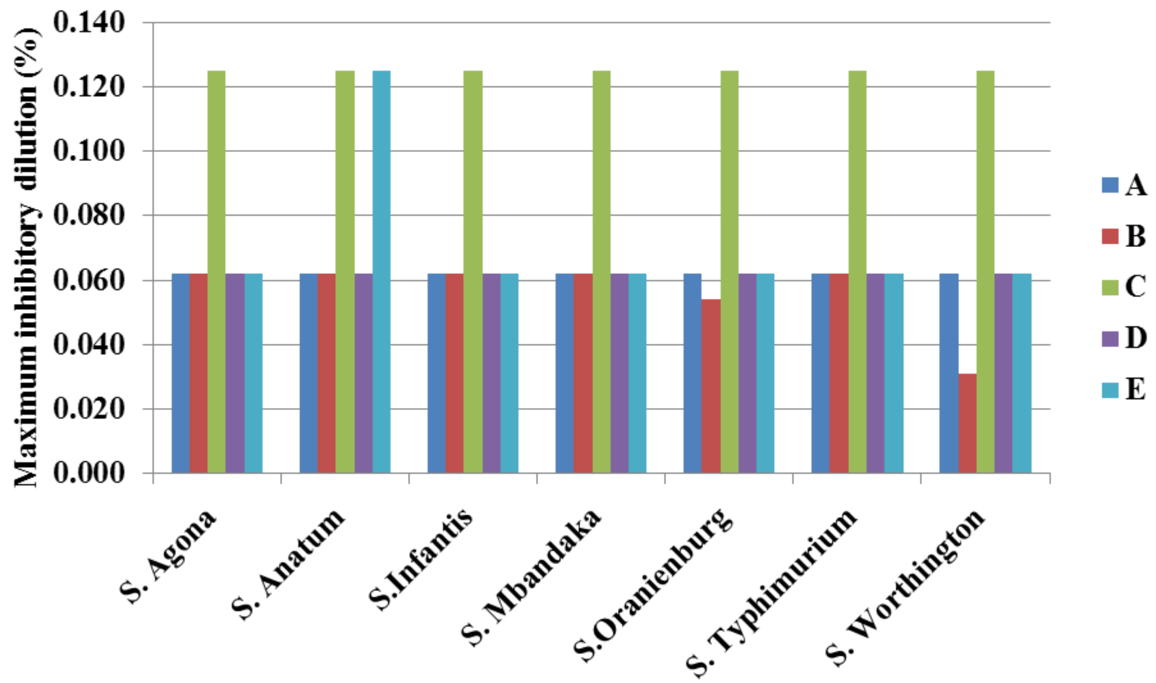

Figure 5.4: Maximum inhibitory dilutions of five commercial OA products against four test strains of seven different *Salmonella* serovars.

Table 5.1 Sources of *Salmonella* serovars.

Serovar	Source				Total
	Dust	Egg belt	Faeces	Shell wash	
S. Agona	4	2	0	0	6
S. Anatum	2	4	0	0	6
S. Infantis	0	2	3	11	16
S. Mbandaka	7	11	12	0	30
S. Oranienburg	8	8	8	6	30
S. Typhimurium	14	5	6	1	26
S. Worthington	7	8	14	2	31
Total	42	40	43	20	**145**

Table 5.2 Details of commercial OA products used in the study.

Product Name	Composition	Intended use and dose
A	Liquid, synergistic combination of acidity-regulating salts	Drinking water, Dose not mentioned
B	Mixture of formic acid and sodium formate	Feed/ 3-12 kg/t feed, 2- 3% in Drinking water
C	Propionic acid formic acid, ammonium propionate and ammonium formate	For ensiling corn, corn cob mix, crushed grain and forage. 2- 3% in Drinking water
D	Liquid, synergistic combination of pathogen-inhibiting organic acids and their salts.	Feed, Label starts- can be mixed with water in any proportion
E	Synergistic blend of free and buffered organic acids	2 % in Drinking water

Table 5.3 Percentage (%) of antimicrobial resistance among different serovars of *Salmonella*.

		Antimicrobials				
Serovar	No. of isolates tested	AMC	AMP	CPL	TET	TRM
S. Infantis	16	0.0	0.0	0.0	0.0	0.0
S. Mbandaka	30	6.66(2)	6.66(2)	3.33(1)	16.66(5)	3.33(1)
S. Typhimurium	26	3.84 (1)	3.84(1)	0.0	3.84(1)	0.0
S. Worthington	31	16.12 (5)	16.12(5)	6.45(2)	0.0	0.0
Total	145	5.51 (8/145)	5.51 (8/145)	2.06 (3/145)	4.13 (6/145)	0.68 (1/145)

Abbreviations: AMC-Amoxycillin; AMP-Ampicillin; CTX- Cefotaxime; CEF- Ceftiofur ; CPL- Cephalothin; CIP- Ciprofloxacin CHL-Chloramphenicol; GEN- Gentamycin; NEO-Neomycin; STR- Streptomycin; TET- Tetracyline; TRM-Trimethorpim
S. Agona (n=6), S. Anatum (n=6), S. Infantis (n=16) and S. Oranienburg (n=30) isolates showed no resistance to all tested antibiotics.
All Salmonella isolates showed no resistance to Cefotaxime, Ceftiofur, Ciprofloxacin, Chloramphenicol, Gentamycin, Neomycin and Streptomycin

Table 5.4 Multidrug resistance pattern among various *Salmonella* serovars recovered from layer flocks.

Serovar (no. of isolates)	Resistance to number of antibiotics	Resistance pattern	Number of isolates
Mbandaka (30)	1	Tet	4
Mbandaka (30)	3	AMP+AMC+TRM	1
Mbandaka (30)	4	AMP+AMC+CPL+TET	1
Typhimurium (26)	3	AMC+AMP+TET	1
Worthington (31)	2	AMC+AMP	3
Worthington (31)	3	AMP+AMC+CPL	2

Abbreviations: AMC-Amoxycillin; AMP-Ampicillin; CPL- Cephalothin; TET- Tetracyline; TRM-Trimethorpim
S. Agona (n=6), S. Anatum (n=6), S. Infantis (n=16) and S. Oranienburg (n=30) isolates showed no resistance to all tested antibiotics.
All Salmonella isolates showed no resistance to Cefotaxime, Ceftiofur, Ciprofloxacin, Chloramphenicol, Gentamycin, Neomycin and Streptomycin

Table 5.5 Congo red agar morphotypes by various *Salmonella* serovars after 96 h of incubation at 22 and 37°C.

Morphotypes (Temperature)	*Salmonella* isolates							Total (n=145)
	S. Agona (n=6)	S. Anatum (n=6)	S. Infantis (n=16)	S. Mbandaka (n=30)	S. Oranienburg (n=30)	S. Typhimurium (n=26)	S. Worthington (n=31)	
RDAR (22°C)	6 (100%)	6 (100%)	16 (100%)	30 (100%)	29 (96.66%)	26 (100%)	19 (61.29%)	132 (91.03%)
SAW (22°C)	0	0	0	0	1 (3.33%)	0	0	1 (0.68%)
PDAR (22°C)	0	0	0	0	0	0	12 (38.71%)	12 (8.27%)
RDAR (37°C)	0	0	0	0	0	0	0	0
SAW (37°C)	6 (100%)	6 (100%)	16 (100%)	30 (100%)	30 (100%)	26 (100%)	31 (100%)	145 (100%)

PDAR- pink, dry and rough; RDAR- red, dry and rough; SAW- smooth and white

Salmonella isolates did not show PDAR morphology at 37°C

Discussion

The widespread use of antibiotics both in both human and veterinary medicine is well known however their imprudent use is giving rise to multidrug resistant strains of *Salmonella*, a major zoonotic food borne pathogen (63). Analyses of antimicrobial resistant and biofilm producing *Salmonella* in layer production system have been often overlooked in Australia. This is the first descriptive Australian-based study and has characterised the occurrence of phenotypic and genotypic antimicrobial resistance and biofilm formation ability of 145 *Salmonella* isolates recovered from commercial layer flocks in two states of Australia.

It is noteworthy that majority of the *Salmonella* isolates (91.72%) in this study remained susceptible to the all antimicrobials tested and no resistance was observed to fluroquinolnes and extended spectrum cephalosporins which are drug of choice for treatment of human salmonellosis (51). In the present study, although the overall antimicrobial resistance in *Salmonella* isolates was low, the most common resistance seen was to amoxicillin, ampicillin, cephalothin, tetracycline and trimethoprim. The findings of the present study contrast with a previous study where 77.4% *Salmonella* isolates from laying flocks of UK were susceptible to antimicrobials tested, with the frequency of resistance being highest to ampicillin (15.3%), tetracycline (13.6%), chloramphenicol (6.8%) and streptomycin (10.7%) (63). Similarly, antibiotic resistance to *Salmonella* isolates collected from commercial eggplants in the USA showed higher resistance (≥ 60%) to the majority of antimicrobials tested (64). In general, our findings also contrast with previous studies showing greater degree of resistance to antimicrobials in *Salmonella* isolates from retails meat samples, food animals or food products in USA and China (63, 64, 65).

The absence of resistance to these critical antimicrobials in this study is a welcome feature from the public health perspective. Globally, the prevalence of resistance to fluroquinolones or extended spectrum cephalosporins in *Salmonella* spp isolated from food animals is concerning (47, 63). The absence of fluroquinolone resistance in this study could be attributed to the fact that, in Australia usage of fluoroquinolone in food animals is banned and as a result resistance to fluoroquinolone is absent in many bacterial strains including *Salmonella* (51). In comparison with other countries where fewer regulations are imposed on usage of critical antimicrobials, Australia has strict regulations and controlled usage for antibiotic in food producing animals (52). The current study has extended these earlier studies and has demonstrated a lower prevalence of antimicrobial resistance in *Salmonella* isolates recovered from layer flocks of Australia.

In this study, antimicrobial resistance was associated with *Salmonella* isolates; *S.* Mbandaka, *S.* Typhimurium and *S.* Worthington. Serovars specific differences for resistance have already been observed in previous studies (64, 66). The serovar specific differences in resistance could be the result of selective transfer of mobile genetic elements or *Salmonella* serovars may possess the genetic determinants of antimicrobial resistance (66). However, further studies aimed to determine the genetic mechanism of serovar specific resistance are essential.

The cells in biofilm are potential source of cross contamination which is a serious food safety concern and *Salmonella* in biofilms can exhibit increased antimicrobial resistance (56). In recent years, biofilm formation by zoonotic foodborne pathogens including *Salmonella* is becoming a major focus of research. This study has evaluated biofilm formation by *Salmonella* isolates by colony morphology and crystal violet assay under different conditions. The most important components of biofilm formation are curli fimbriae and cellulose. Presence or absence of either one component or both produces characteristic morphology on Congo red agar plates (67). In this study, temperature conditions had a strong influence on biofilm formation. The Congo red agar plate analysis of major biofilm components such as curli fimbriae and cellulose showed that these components favoured the biofilm formation at low temperature (22°C) compared to 37°C. Such differences in biofilm formation by *Salmonella* isolates has also been reported earlier at 25 and 37°C using low and rich nutrient conditions[31]. In the present study, the morphotype RDAR suggestive of curli fimbriae and cellulose production was most prevalent in *Salmonella* isolates at low temperature,

consistent with previous findings (68). At the higher temperature (37°C), all *Salmonella* isolates displayed SAW morphotypes, indicative of failure to produce extracellular components of biofilm which is also in agreement with an earlier report (67). Over the decades, various methods have been reported to study *in vitro* biofilm development and formation. Crystal violet is a basic dye binds to negatively charged molecules and extracellular matrix components (69). Crystal violet staining is the most common and convenient technique (69) used to evaluate biofilm formation and was employed in this study to evaluate this feature in *Salmonella* isolates incubated at 22 and 37°C. Our findings suggest that biofilm formation was observed at 22°C. At 37°C, the majority of the *Salmonella* isolates were unable to form biofilm and this data also corresponded with Congo red agar plate morphotypes, where all isolate exhibited SAW morphology. The findings of a previous study has also demonstrated a temperature and strain dependent variation in biofilm formation of *Salmonella,* with biofilm formation higher at 22°C compared to 30 or 37°C (70). Altogether, the data obtained from Congo red agar morphology and crystal violet assay suggest that 22°C is the optimum temperature for *Salmonella* to form biofilm, a temperature that mimics the typical shed environment/temperature condition of the layer farm. However, future studies examining the biofilm forming potential of *Salmonella* isolates onto stainless still, egg conveyer belts, egg shell or any farm equipment in the shed is essential. Although the level of antimicrobial resistance amongst the *Salmonella* isolates tested in the present study was low, the majority of the isolates were able to form biofilm at 22°C.

The efficacy of commercially available water soluble OA products was studied *in vitro*. Although the results indicated that the Salmonella serovars were susceptible for the recommended dose, further studies are required to test the potency of these OA in different water sources (such as rain, dam or bore well water).

Chapter 6: General Discussion

Residual contamination of the environment with *Salmonella* is a major problem in commercial layer farms (7, 8, 9). However, in Australia there is little information in the literature regarding the risks of *Salmonella* contamination of eggs from infected birds and or a contaminated shed environment. Furthermore, the rate at which an infected flock can produce *Salmonella* contaminated eggs is unclear. In the present study, the possible transmission of *Salmonella* from the environment to the egg was investigated with the help of longitudinal studies on commercial egg farms. Results of the culture method demonstrated that the likelihood of an eggshell testing positive for *Salmonella* was 91.8, 61.5 and 18.2 times higher when faecal, egg belt and dust samples, respectively, were also *Salmonella* positive. Also as determined by qPCR, a log increase in the load of *Salmonella* detected in faecal, egg belt and floor dust samples resulted in 35%, 43% and 45% increases ($p<0.001$), respectively, in the frequency of obtaining *Salmonella* positive eggshells.

Results of this study could be helpful in determining risks of *Salmonella* contaminated eggshells and also for developing control strategies such as vaccination, strict biosecurity, cleaning and disinfection of layer sheds which could reduce the shedding and environmental level of *Salmonella* in the layer shed. *Salmonella* spp persisted in the shed environment on both farms sampled over a ten month period. Regular cleaning and disinfection of layer sheds could lower egg contamination. After depopulation, a thorough cleaning of layer shed equipment (cages, egg belt, egg belt brushes, feeders) and areas such as ventilation fans and cage tops is essential. Similarly, disinfection of shed can be carried out appropriately. Carrique-Mas *et al.* (40) reported that 10% formalin resulted in a significant reduction in the prevalence of *Salmonella* in samples collected from cage laying houses as compared to other disinfectants. The effectiveness of cleaning and disinfection procedures can vary based on type of chemical and disinfectant used on layer farms. Investigating the efficiency of cleaning and disinfection methods to reduce *Salmonella* contamination on layer farms could be helpful in designing or developing standard operating procedures across Australia. However, the presence of multi-age flocks in the same shed may hinder the cleaning procedure. In the present study, even though birds were infected with *S.* Typhimurium, all egg internal content samples were *Salmonella* negative. This suggests that *Salmonella* serovars isolated in the present investigation may have lacked the ability to tranmsit vertically. However the infected birds or cages were sampled at four weekly intervals so there is a possibility that any internally contaminated eggs (laid during that period) remained undetected. Hence, further experimental studies with more frequent egg sampling are essential to confirm this finding. Another way to confirm these findings would be to perform *in vivo* infection controlled trials to study the vertical transmission ability of predominant *S.* Typhimurium phage types isolated from egg farms. Raising *Salmonella* free commercial flocks from day old to point of lay, however is challenging and costly.

During the laying production cycle, birds can experience various stressful events. El-Lethey *et al.* (33) reported that cell mediated and humoral immune responses could be impaired as a result of stress. One of the most stressful events for laying hens is the onset of sexual maturity and/or lay which generally also coincides with the transfer of birds from one production system to another (34). In Chapter 4, it was hypothesised that birds reaching the stage of sexual maturity (with the addition of transport stress) are more susceptible to *Salmonella* infection due to an impaired immune response as a result of stress. Hence, the shedding of *Salmonella* in a single aged commercial layer flock was investigated by performing three longitudinal samplings after transport of hens at an early stage of lay. At the start of lay (18 weeks), within the first week after transport, the shedding of *Salmonella* in faecal samples was at a peak. However, over time, the *Salmonella* infection subsided in subsequent samplings. This could be due to the acclimatization of birds to the shed environment during later samplings. To confirm these findings, further experiments investigating the direct effect of stress on *Salmonella* shedding are essential.

The prevalence of *Salmonella* in birds housed in the lower tiers of cage sheds was found to be higher as compared to birds in upper tiers. Increased exposure of lower tiers to dust as well as movements of shed workers may be responsible for greater *Salmonella* prevalence in the lower tiers. With increasing age of the flock, there was a significant increase in the load of *Salmonella* in dust, egg belt and shoe cover samples. This underlines the importance of regular cleaning of sheds even during the laying period. The stress caused by the onset of sexual maturity or transport could have led to the higher susceptibility of birds to *Salmonella* infection and or to the onset of shedding.
The current work has also shown a low level of antimicrobial resistance in *Salmonella* isolated from layer flocks in Australia. However, regular surveillance covering a greater geographical area and comprehensive nationwide sampling is needed to determine any shift in antimicrobial resistance pattern in *Salmonella* isolates in the egg industry. Collective efforts to ensure the appropriate use of antimicrobials in food animals remain a high priority to minimise the risk of spread and development of antimicrobial resistance. This study has also demonstrated the ability of all the examined *Salmonella* isolates to form biofilms, though the production varied with different environmental conditions.

Implications

The current project conducted longitudinal and point-in time surveys of *Salmonella* carriage and environmental contamination on multi-age and a single aged commercial layer farms in South Australia. The data provides critical information for the egg industry about dynamics of *Salmonella* shedding and the possible link between environment/bird/egg transmission of *Salmonella* serovars of public health significance on environmentally controlled commercial layer farm. *Salmonella* positive samples of faeces, egg belt and dust were significant predictors of eggshell contamination. A single log CFU increase in the level of *Salmonella* within the layer shed environment significantly increased the incidence of eggshell contamination. During this study, the internal content of eggs laid by *S.* Typhimurium positive hens was negative for *Salmonella*. *Salmonella* Typhimurium strains responsible for human food poisoning cases exhibited similar MLVA pattern to the strains isolated from flock A and B. The prevalence of *Salmonella* in single aged flock housed in the lower tiers was higher as compared to birds in higher tiers. The sensitivity of qPCR was lower as compared to culture method in detecting *Salmonella* positive fecal samples. The sensitivity of qPCR was also not improved with use of a pre-enrichment step. The culture method was more sensitive than the qPCR and multiplex PCR assays. The antibiotic resistance of *Salmonella* serovars isolated during this study was low. The *Salmonella* serovars were able to form biofilm at 22^0C. Biofilm formation may be a serious food safety and public health concern. Thus, along with prudent usage of antibiotics in food producing animals at the farm level, strict hygienic measures and sanitation programs in food processing facilities and the food chain in general are required.

Recommendations

- The investigators recommend that the Australian Egg Industry continues to support research, development and extension in the area of food safety, with *Salmonella* serovars and phage types being the main target.
- Extension activities arising from the outcomes of this sub-project could include seminars and workshops for the egg producers and health department officials.
- The egg industry should continue to promote the prudent and responsible use of antibiotics
- Similar studies to those described here (on caged layer operations) should be undertaken on free range flocks in future.
- After depopulation, a thorough cleaning of layer shed equipment (cages, egg belt, egg belt brushes, feeders) and areas such as ventilation fans and cage tops is essential.
- Stressful events in the flock could influence the *Salmonella* shedding hence efforts should be directed to minimise the stress level in flock.
- It is also essential to study the shedding of *Salmonella* in pullets during rearing as current study investigated shedding during lay.
- A nationwide epidemiological survey of *Salmonella* could be conducted in future. The current study was conducted on layer flocks in SA.
- Studies could be conducted to investigate the formation of biofilms on shed equipment and on egg shell surface.

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Publications Arising from Project

Journal Articles

1 Gole VC, Torok, V., Sexton M., Caragual, C., Fowler C., Chousalkar, K (2014). Shedding of *Salmonella* in a single age caged commercial layer flock at an early stage of lay. ***International Journal of Food Microbiology*** *(Accepted in Press)*

2 Gole VC, Torok, V., Sexton M., Caragual, C., Chousalkar, K (2014). Association between the indoor environmental contamination of *Salmonella* with egg contamination on egg layer farm. ***Journal of Clinical Microbiology*** (Accepted, in Press, JCM.00816-14. [Epub ahead of print])

3 Pande VV, Gole, VC, Mcwhorter M, Abhram S. Chousalkar KK. (2014). Antibiotic resistance and biofilm forming ability of *Salmonella* serovars isolated from egg farms. ***Journal of Global Antimicrobial Resistance*** (Submitted)

Non-refereed Conference Papers

1 Gole, V.C., Sexton, M., Chousalkar, K.K., 2013 Epidemiology of *Salmonella* on egg farms. Proceedings of the XXI European Symposium on the Quality of Poultry Meat and the XV European Symposium on the Quality of Eggs and Egg Products, Bergamo, Italy September 15-19 (Oral Presentation).

2 Gole, V.C., McWhorter, A. Sexton, M., Chousalkar, K.K., 2014. Which *Salmonella* spp are of concern on egg farms. Proceedings of Poultry Information Exchange. Gold coast. Australia.

Research Students associated with the project

Vaibhav Gole, PhD student, The University of Adelaide (completed)
Vivek Pande, PhD student, The University of Adelaide (Current)

Plain English Compendium Summary

Sub-Project Title:	Eggshell quality and the risks of food borne pathogens
Poultry CRC Sub-Project No.:	3.2.2
Researcher:	Kapil Chousalkar
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Sub-Project Overview	The current project investigated shedding dynamics of *Salmonella* srovars on *Salmonella* positive multi-age and single aged commercial layer farms. Several *Salmonella* serovars were isolated from farms. One log increase in the load of *Salmonella* in faecal samples resulted in 34% increase (odds ratio=1.34) in *Salmonella* positive eggshells. Similarly, one log increase in the load of *Salmonella* on egg belt and dust samples resulted in 43% (odds ratio=1.43) and 45% (odds ratio=1.45) increase in *Salmonella* positive eggshells, respectively. In caged environment, birds housed in housed in lower tiers shed high level of *Salmonella* compared to birds in top tiers. The antimicrobial resistance in *Salmonella* isolates obtained during this study was low. *Salmonella* isolates were able to form biofilm and 22^{0}C.
Background	It is widely recognised that *Salmonella* spp are a potential threat to the chicken meat and egg industry. Although egg producers are diligent in fulfilling standards for the production of safe food, the egg industry in Australia is often implicated in some outbreaks of food poisoning. The industry therefore needs to be vigilant in monitoring the presence of food borne pathogens such as *Salmonella* on farm to enable informed management decisions to be made.
Research	The dynamics of *Salmonella* shedding single age or multi-age flocks was monitored to establish possible link between environment/bird/egg transmission of *Salmonella* serovars of public health significance. The load of Salmonella was quantified using qPCR. The culture method of Salmonella isolation was compared with molecular methods such as qPCR and multiplex PCR. Salmonella strains isolated from this study were also tested for antimicrobial susceptibility and biofilm forming ability at various temperatures. In-vitro susceptibility of Salmonella isolates to commercially available water soluble organic acid products was investigated.
Implications	The data provides important information for the egg industry about dynamics of *Salmonella* shedding and the possible link between environment/bird/egg transmission of *Salmonella* serovars of public health significance on environmentally controlled commercial layer farm. *Salmonella* positive samples of faeces, egg belt and dust were significant predictors of eggshell contamination. The incidence of *Salmonella* spp needs to be monitored regularly in the Australian egg industry.
Publications	Journal Articles – 1 published, 1 in press, 1 submitted Non-refereed Conference Papers – 2 published