Food Borne Pathogens and Commercial Eggs in Australia

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(B.V.Sc. & A.H., M.V.Sc.)

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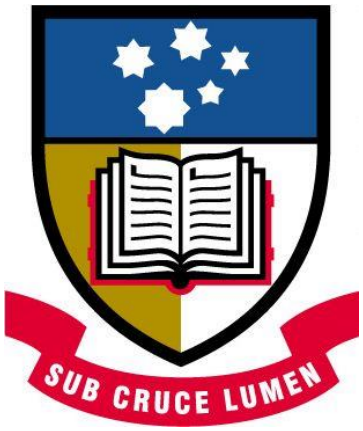

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Abstract

A low level of bacterial contamination on an eggshell is important from both food safety and storage perspectives. A high number of bacteria present on the eggshell surface increases the chances of eggshell penetration and contamination of internal contents. Overall, food quality and sanitary processing conditions can be judged by *Enterobacteriaceae* populations. In chapter 2, no significant differences were detected in the *Enterobacteriaceae* counts of the eggshell surface or in the eggshell pores, of visibly clean eggs collected from Australian layer flocks at various stages of lay. Out of all eggs tested, 4.51% (14/310) eggshell samples were reported *Salmonella* positive.

The rate at which *Salmonella* infected flocks produce *Salmonella* contaminated eggs is unclear. In chapter 3, the possible transmission of *Salmonella* from the environment to the egg was investigated with the help of longitudinal studies on commercial egg farms. Results indicated that the odds of an eggshell being positive for *Salmonella* were 91.76, 61.5 and 18.2 times higher when faecal, egg belt and dust samples, respectively, tested positive for *Salmonella*. On other hand, a one log increase in the load of *Salmonella* detected in faecal, egg belt and in the floor dust samples, respectively, resulted in 35%, 43% and 45% increase ($p<0.001$) in the odds of an eggshell testing positive for *Salmonella*.

In chapter 4, the shedding of *Salmonella* in a single age commercial egg layer flock was also investigated at the onset of lay (18 weeks) followed by two longitudinal samplings at 24 and 30 weeks. At the age of 18 weeks, when the first sampling was performed, the prevalence of *Salmonella* in faeces was 82.14%. However, in later samplings, at the age of 24 and 30 weeks, the prevalence of *Salmonella* in faeces was significantly reduced ($p<0.001$) to 38.88% and 12.95% respectively. 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.

There are various methods to decontaminate the eggshell surface; egg washing is one of them. Egg washing can reduce the level of bacteria on the eggshell surface and horizontal transmission across the eggshell. However, egg washing can damage the cuticle which is the outmost layer on the eggshell surface. The effect of egg washing on *Salmonella* Typhimurium (*S.* Typhimurium) penetration was investigated using agar and whole egg penetration techniques. The results in chapter 5 indicated that eggshell penetration was higher in washed eggs as compared to unwashed eggs. Hence, appropriate attention is essential to make sure eggs are kept at appropriate storage and drying conditions after washing. Statistical analysis also indicated that eggshell penetration by *S.* Typhimurium was related to the incidence of various eggshell ultrastructural features such as cap quality, alignment, erosion, confluence, Type B bodies and cuticle cover. All the *S.* Typhimurium strains used in this study were able to survive on the eggshell surface and in egg internal contents 21 days after infection. Other egg industry associated *Salmonella* serovars such as *S.* Singapore, *S.* Adelaide, *S.* Worthington and *S.* Livingstone had the capacity to penetrate the eggshell. However, these serovars had little or no capacity to survive in the egg internal contents 21 days after inoculation (Chapter 6).

Eggshell quality and safety are important for the consumer's impression of the product. A good quality eggshell protects the egg internal contents from bacterial penetration. A cracked or damaged egg encourages bacteria to move across the eggshell. *Mycoplasma synoviae* (*M. synoviae*) have been found to be associated with poor egg shell quality. The association between egg shell quality parameters and the seroprevalence of *M. synoviae* in eggs collected from Australian commercial layer flocks was investigated in chapter 7. Seroprevalence of *M. synoviae* was found to be

high at 69% (95% confidence interval (CI) = 41.3–89.0). Statistical analysis showed an association between serological status for *M. synoviae* and the incidence of egg quality parameters such as translucency, shell breaking strength, % shell reflectivity and shell deformation. Thus, *M. Synoviae* infection could compromise eggshell quality and as a consequence the eggs from *M. Synoviae* positive flocks may be at risk of becoming contaminated by potentially pathogenic bacteria.

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List of Abbreviations

%	Percent
°C	Degree Celsius
<	Less than
µL	microlitre
µm	Micrometre
AECL	Australian Egg Corporation Limited
ANOVA	Analysis of variance
BHI	Brain heart infusion broth
bp	Base pair
BPW	Buffered peptone water
BSA	Brilliance Salmonella agar
CFU	Colony forming unit
CI	Confidence interval
cm	Centimetre
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
dNTP	Deoxy-nucleotide triphosphate
DT	Definitive type
EAA	Egg apex abnormalities
EDTA	Ethylene diamine tetra acetic acid
EFSA	European food safety authority
ELISA	Enzyme-linked immune-sorbent assay
FSANZ	Food Standards Australia New Zealand
gm	Gram
h	Hour
INVA	Invasion Gene A
IPC	Internal positive control
ISR	Infantis-specific (genomic) region
KW	Kauff-White-Le Minor
LDC	Lysine decarboxylase
min	Minute
mL	Mililitre

MLST	Multi-locus sequence typing
MLVA	Multi-locus variation number tandem repeat analysis
mm	Milimetre
N	Newtons
NA	Not applicable
ND	Not detected
nm	Nanometre
NSWFA	New South Wales Food Authority
ONPG	Ortho-nitrophenyl-β-D-galactopyranoside
p.i.	Post inoculation
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PFGE	Pulse field gel electrophoresis
PT	Phage type
Q-PCR	Quantitative Polymerase Chain Reaction
RH	Relative humidity
RT-PCR	Real time polymerase chain reaction
RV	Rappaport Vassiliadis
Se	Sensitivity
sec	Second
SEM	Scanning electron microscopy
Sp	Specificity
SPI	*Salmonella* Pathogenicity Island
spp	Species
STTR	*Salmonella* Typhimurium tandem repeat
TAE	Tris-borate-EDTA
TBGB	Tetrathionate brilliant green broth
TG-ROC	Two graph receiver operating characteristics
TSI	Triple sugar iron
TSR	Typhimurium-specific (genomic) region
VNTR	Variable number tandem repeat
XLD	Xylose lysine deoxycholate

List of Publications

Journal articles

Gole, V. C., Chousalkar, K. K., & Roberts, J. R. (2012). Prevalence of antibodies to *Mycoplasma synoviae* in laying hens and possible effects on egg shell quality. *Preventive Veterinary Medicine*, *106*, 75-78.

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Gole, V. C., Chousalkar, K. K., Roberts, J. R., Sexton, M., May, D., Tan, J., & Kiermeier, A. (2014). Effect of Egg Washing and Correlation between Eggshell Characteristics and Egg Penetration by Various *Salmonella* Typhimurium Strains. *PloS one*, *9*(3), e90987.

Gole, V. C., Roberts, J. R., Sexton, M., May, D., Kiermeier, A., & Chousalkar, K. K. (2014). Effect of egg washing and correlation between cuticle and egg penetration by various *Salmonella* strains. *International Journal of Food Microbiology*, *182*, 18-25.

Gole, V. C., Torok, V., Sexton, M., Caraguel, C. G. B., Fowler, C., & Chousalkar, K. K. (2014). Association between the indoor environmental contamination of *Salmonella* with egg contamination on layer farms. *Journal of Clinical Microbiology*, *52*, 3250-3258.

Gole, V. C., Caraguel, C. G. B., Sexton, M., Fowler, C., & Chousalkar, K. K. (2014). Shedding of *Salmonella* in a single age caged commercial layer flock at an early stage of lay. *International Journal of Food Microbiology*, *189*, 61-66.

Peer reviewed symposium papers

Gole, V. C., Chousalkar, K. K., Lievaart, J., & Roberts, J. R. (2012) Prevalence of Mycoplasma Synoviae in eggs from laying hens using ELISA. *Australian Poultry Science Symposium*, 23: 205-208.

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Chapter 1 Literature Review

1.1 Introduction

In Australia, there has been a continuous increase in egg production over the last decade. The egg industry produced 397 million eggs in 2012 and per capita consumption of the eggs increased to 214 eggs (AECL, Annual Report 2013).

Eggs free from bacterial contamination are essential to improve their shelf life and to avoid food poisoning outbreaks. However, bacterial contamination of eggshells can occur due to the various surfaces with which eggs come in contact (Board and Tranter, 1995). Uterine tissue, blood, faeces, soil, water, the egg belt, caging material, broken eggs, nesting material, hands and insects are the most important sources of eggshell contamination (Board and Tranter, 1995; Ricke *et al*., 2001; Davies and Breslin, 2003a).

In recent decades, food borne illness has emerged as a serious problem through out the world. In 2010, around Australia approximately 30,035 food poisoning cases were reported. *Salmonella* (11,992 cases) was one of the most important pathogens responsible for these food borne infections (The OzFoodnet Working group, 2012). Many outbreaks were traced back to raw egg products. Hence, the Australian egg industry is under continuous pressure to address public health issues associated with eggs.

There are three potential routes of egg contamination with *Salmonella* spp. (Duguid and North, 1991): 1) the transovarian route (infection occurs when the yolk is connected to the ovary), 2) the oviducal route (contamination of vitelline membrane and/or albumen occurs when the egg passes through the oviduct) and 3) the trans-shell route (bacteria present on the eggshell surface penetrate across the eggshell to contaminate the egg internal contents). There are reports which suggest

that transovarian and oviducal contamination are important for some of the bacterial serovars (Barnhart *et al*., 1991; Gast *et al*., 1992; Baumler *et al*., 2000; Ricke *et al*., 2001) such as *Salmonella* Enteritidis (*S*. Enteritidis). However, this serovar is not endemic in the Australian egg industry. According to Humphrey (1994), trans-shell transmission is the most common route for *Salmonellae* other than *S.* Enteritidis.

For the economics of the poultry farm, eggshell quality is very important and egg quality could play a vital role in trans-shell penetration of *Salmonella* spp. The eggshell plays an important role in protecting the embryo from physical damage and also regulates embryo metabolism. A good quality eggshell significantly protects the internal contents from bacterial penetration. A cracked or damaged egg encourages bacteria to move across the eggshell which may result in food poisoning.

1.2 Formation of the egg

Solomon (1991) and Johnson (2000) describe the whole process of formation of the egg in a laying hen. Even though two oviducts and two ovaries exist at the time of early embryonic development, the right ovary and right oviduct undergo atrophy. Hence, the left ovary produces yolk along with the ovum and releases both into the left oviduct. The infundibulum, which is the funnel shaped upper portion of the oviduct, captures the yolk. Within 15 minutes, the formation of the chalazae and perivitelline membrane occurs in the infundibulum. Also, it is the part of the oviduct where fertilization occurs in breeder birds.

From the infundibulum, the developing egg enters into the magnum which is the albumen secreting portion of the oviduct. The developing egg stays there for approximately 3 hours. Next, the developing egg enters the isthmus where it remains for around 75 minutes. The isthmus is responsible for the formation of inner and outer shell membranes. These membranes play a key role in developing the final shape of the egg.

The egg then undergoes the process of 'plumping' (water and electrolytes enter the albumen) in the tubular shell gland and this is followed by the formation of the mammillary core over a period of 5 hours. The shell gland pouch is the part of the oviduct where the developing egg spends the longest time (18-20 hours), where plumping is completed and the eggshell is formed. From the shell gland pouch, the egg enters into the vagina which is the final section of the oviduct. The vagina has no role in egg formation. The vagina only expels the egg once it leaves the shell gland pouch.

1.3 Eggshell structure and measurement of eggshell quality

The eggshell consists of different layers including the mammillary layer, palisade layer, surface crystal layer (all of which are made up of calcite crystals: the most stable polymorph of calcium carbonate) and the cuticle (Roberts, 2004). The cuticle is a non-calcified layer and is composed of glycoprotein, polysaccharides, lipid and hydroxyapatite crystals (Johnson 2000; Fernandez *et al*., 2001).

There are different techniques for measuring eggshell quality. They are mainly divided into direct and indirect methods. These methods also represent the mechanical and physical properties of eggs respectively (Hammerle, 1969). Examples of indirect methods are specific gravity and non-destructive deformation (Hamilton, 1982). There is another indirect method to measure eggshell quality (density, thickness and eggshell structure) known as 'beta backscatter' which was used by Fox *et al*. (1975) in wildlife studies and by Voisey and Hunt (1976) for evaluating domestic hen eggshell quality. The major advantages of these methods are: they are rapid and inexpensive, there is no need to break the eggs, and multiple measurements can be measured on the same egg (Hamilton, 1982). De Ketelaere *et al.* (2002) developed a new technique to measure dynamic stiffness of eggshells. Direct methods to determine eggshell quality include the quasi-static compression

test and the impact fracture test. Using these tests, the exact force required to break the eggshell can be estimated and also these forces mimic to important dangers that the egg faces under the field conditions (Hamilton *et al*., 1979). In these methods, it is essential to break the egg so only one measurement can be taken on the one egg and it is not possible to use the egg for further purposes. Hunt *et al.* (1977) describe another direct method to measure eggshell quality named 'the puncture test'.

Besides the direct and indirect methods, eggshell quality can be judged by candling, shell colour, amount of shell and shell thickness (Roberts, 2004). On the commercial layer farms, cracks in the eggshell are detected by candling or an electronic crack detector (Roberts, 2004). Eggshell colour can also be judged by shell reflectivity which is the amount of reflection of the light from the eggshell surface. As the egg becomes paler, reflectivity increases and consumers do not prefer such eggs. Eggshell weight is used to determine the percent shell of the egg (shell weight as a percentage of egg weight). In order to measure shell thickness, it is recommended that three pieces of eggshell should be taken from around the equator and using a suitable gauge such as a Mitutoyo model 2109-10 dial comparator gauge, shell thickness is determined (Roberts, 2004). A number of studies indicate that construction of the eggshell is also important in determining shell quality. Using a scanning electron microscope, quality of construction of the mammillary layer of the eggshell can be examined (Solomon, 1991; Roberts and Brackpool, 1994). When the mechanical properties of eggshell such as shell thickness and percentage shell are good but shell breaking stength is poor, the examination of ultrastructure of the eggshell can be useful to explain this discrepancy. Similarly, scanning electron microscopy can be used to evaluate the quality of the eggshell cuticle. The cuticle acts as a first physical barrier to bacterial penetration and protects the egg by covering the eggshell and pore openings.

1.4 Bacteria isolated from table eggs

Bacteria from different genera found on the eggshell surface include *Micrococcus, Streptococcus, Sarcina, Proteus, Serratia, Aeromonas, Staphylococcus, Aerobacter, Arthrobacter, Escherichia, Bacillus, Cytophaga, Pseudomonas, Flavobacterium, Acinetobacter* and *Alcaligens* (Board and Tranter, 1995). Besides these bacteria, Moats (1980) reported the presence of *Kurthia, Morexella, Propionibacterium* and *Yeast* on eggs.

Gram negative bacteria and occasionally a few gram positive microorganisms are usually present in rotten table eggs. Common bacteria found in spoiled eggs are *Serratia, Pseudomonas Flurorescens, Pseudomonas Putida, Alcaligens, Proteus, Escherichia, Xanthomonas, Maltophia, Aeromonas, Hafnia, Citrobacter, Pseudomonas aeroginosa, Acinetobacter, Cytophaga, Bacillus, Micrococcus, Streptococcus* and *Arthrobacter.* Thus, the internal content environment of the egg favors the growth of gram negative bacteria (Board and Tranter, 1995).

1.4.1 Methods for the recovery of bacterial population from eggs

Various methods have been used in order to recover microorganisms from the eggshell surface. Many shell rinsing methods have been based on Gentry's method (1972) in which an egg was placed in bag containing 10 ml of sterile PBS, massaged for 1 min and kept for 5 min. The egg was again massaged for 1 min and used for further processing. Another method involves cracking the eggs aseptically, and putting the eggshells in a bag containing diluent (Berrang *et al*., 1991). Microorganisms were obtained by blending the eggshells and membranes (Brant and Starr, 1962; March, 1969) and also by egg swabbing and blending (Penniston and Hedrick, 1947).

One study found that blending was more effective as compared to surface rinsing in order to recover microorganisms (Gunaratne and Spencer, 1973).

However, other studies found that both methods were equally effective (Penniston and Hedrick, 1947). Moats (1980, 1981) stated that the presence of microorganisms on the eggshell surface, in eggshell pores or in the shell membrane is a deciding factor for the recovery of microorganisms by either surface rinsing/swabbing or by shell blending. In order to recover the microbial populations from eggshell surface and in eggshell pores, the shell rinsing and the shell crush method was used by Cox *et al*. (2002) respectively. In this method, after shell rinsing with Tetrathionate Brilliant Green Broth (TBGB), eggs were dipped in 2% iodine (to kill any leftover bacteria on eggshell surface) for 1 min followed by air drying. The eggshells were cracked and placed in a sterile bag containing TBGB and used for further processing. With Cox's method, it was possible to determine the number of bacteria on eggshell surface and in eggshell pores separately. Hence, this method could be helpful to determine the actual counts of bacteria on eggshell surface and in eggshell pores.

1.4.2 Isolation and characterization of *Enterobacteriacaeae* recovered from washed and unwashed eggs

The family *Enterobacteriaceae* includes bacteria which are gram negative, facultative anaerobic rods, oxidase negative, catalase positive, can ferment sugars to lactic acid as well as reduce nitrate to nitrite. There are more than 28 genera and 80 species (spp.) in this family. They can be roughly divided into three different groups 1) Major pathogens: e.g. *E. coli, Salmonella* serovars, *Yersinia* spp. 2) Opportunistic pathogens: e. g. *Proteus* spp., *Klebiesella pneumoniae, Enterobacter, Edwardsiella* 3) Non pathogens such as *Hafnia and Erwinia.* (Quinn *et al*., 2010).

Musgrove *et al*. (2004) characterized *Enterobacteriaceae* present on the eggshell surface of unwashed and washed eggs using biochemical tests. They reported the presence of *Escherichia coli, Enterobacter cloacae, Enterobacter sakazakii, Enterobacter* spp., *Citrobacter youngae, Klebsiella pneumoniae,*

Klebsiella spp., *Serratia odorifera, Serratia* spp., *Kluyvera* spp., *Providencia rettgeri, Providencia* spp., *Pantoea* spp., *Rahnella aquatilis, Salmonella* spp., *Yersinia* spp., *Flavimonas oryzihabitans, and Xanthomonas maltophilia* on unwashed eggs. From the washed egg, they were able to isolate only *Enterobacter amnigenus* and *Salmonella arizonae.* They found that most of the isolates from the unwashed eggs belonged to genera *Escherichia coli* (*E. coli)* and *Enterobacter*. However, in washed eggs, they were able to recover only a few isolates and concluded that the egg washing removed many of the *Enterobacteriaceae* spp. from the eggshell surface. De Reu *et al*. (2008) investigated the average *Enterobacteriaceae* eggshell contamination, for furnished and non-cage systems and found no significant difference. In a furnished system, 88% of the eggshells and in non-cage system, 94% of the eggshells had a load of < 10 CFU *Enterobacteriaceae*/eggshell. In another study, Jones *et al*. (2004) reported a low level of *Enterobacteriaceae* on unwashed eggshell collected from the accumulator and 0.6 log CFU/mL was the highest concentration detected.

1.5 Genus *Salmonella* and relationship of *S*. Typhimurium with outbreaks related to eggs and egg products

The genus *Salmonella* is mainly divided into two species; *S. enterica* and *S. bongori*. The first one is further classified into six subspecies. The *S. enterica* subspecies *enterica* contains most of the *Salmonella* (EFSA, 2010) and the subspecies is subdivided into numerous serovars (Heyndrickx *et al.,* 2005). Every serovar has its own different host range. Species which are related to only one particular host are called host restricted *Salmonella* serovars. Although, host adapted serovars are specific to one host, they can also cause disease in other animals. On the other hand, host unrestricted serovars can cause gastroenteritis in a broad host range (Uzzau *et al.,* 2000). Host unrestricted serovars such as *S.* Enteritidis, *S.*

Typhimurium and *S.* Infantis are a concern for the egg layer industry and broader poultry industry all over the world.

Many different *Salmonella* serovars (such as Enteritidis, Typhimurium, Agona, Derby, Bredney, Virchow, Infantis, Livingstone, Mbandaka, Newport) have been isolated from eggs (Martelli and Davies, 2012). However, *S.* Enteritidis and *S.* Typhimurium are considered the most important serovars in terms of public health significance. Throughout the world, many egg related food poisoning outbreaks have occurred due to *S.* Enteritidis. However, in Australia, *S.* Enteritidis is not endemic in layer flocks and *S.* Typhimurium has been responsible for most of the egg related *Salmonella* outbreaks (The OzFoodnet Working Group, 2012). Hence, it is essential to investigate the evidence that links *S.* Typhimurium to *Salmonella* outbreaks in eggs and egg related products in other countries.

1.5.1 Evidence of *S.* Typhimurium outbreaks associated with eggs and egg products in the United States of America (USA)

Even though, *S.* Enteritidis has been the most commonly linked serovar to egg related outbreaks, *S.* Typhimurium was also responsible for many shell egg related outbreaks (Louis *et al*., 1988). Homemade ice cream was observed as an important vehicle for the transmission of salmonellosis. Homemade ice creams were responsible for 22 salmonellosis outbreaks during the period 1966 to 1976 (Gunn *et al*., 1978). The most commonly isolated serovar was *S.* Typhimurium which was accounted for 10 out of 22 outbreaks. Most of the outbreaks were associated with ungraded farm eggs and, in all instances, the ice cream custard had not been cooked before freezing. Tyler *et al*. (1984) investigated an outbreak caused by *S.* Typhimurium where all the people (3 adults and 5 children) exposed contracted severe illness and a healthy 13 year old boy died. Investigation indicated that homemade ice cream was responsible for the illness. Furthermore, it was observed

that eggs which were used in the ice cream were the source of *S.* Typhimurium. Important aspects of this outbreak were the death of a healthy child and a very high level of contamination of ice cream (10^6 Salmonellae/g).

During the period, 1973 to 1984, forty four egg containing food vehicles were identified in salmonellosis of known cause and reported to the Centres for Disease Control. *S.* Typhimurium and Enteritidis were responsible for 18 (40%) and 12 (27%) outbreaks, respectively (Louis *et al.*, 1988). Hedberg *et al*. (1993) carried out a case control study to estimate the relationship of egg consumption with sporadic cases of *S.* Typhimurium and *S.* Enteritidis. They observed the consumption of undercooked eggs or egg-containing food, 72 hours before the start of illness, was an important factor for *Salmonella* infection. During the period 1966-1984, egg related salmonellosis outbreaks were dominated by *S.* Typhimurium. However, from 1984 onwards, majority of cases of egg related salmonellosis were due to the serovar Enteritidis. In 2003, a *S.* Typhimurium outbreak related to commercially processed salad was reported by Oregon Health Services and CDC in Oregon which affected 18 people. This was the first reported *S.* Typhimurium outbreak associated with a widely distributed, commercially processed and hard boiled egg product.

1.5.2 The United Kingdom (UK)

Before 1984, food poisoning cases related to *S.* Typhimurium PT 141 were rare in the UK. However, in 1984, this serovar caused 68 human infections and detailed laboratory and epidemiological investigation suggested that hen eggs were the most likely source of *Salmonella* (Chapman *et al*., 1988). Mitchell *et al.* (1989) carried out an investigation to find the source of infection of a food poisoning outbreak that occurred in London. Investigation identified that the probable vehicle of infection was mayonnaise contaminated by *S.* Typhimurium DT 49. Also five out of eight samples examined (from chicken houses of the main egg supplier) were

positive for same definitive type. Results of a case control study indicated that lightly cooked eggs, raw egg products and precooked cooked hot chicken were the sources of human *S*. Enteritidis PT 4 infections in the UK (Cowden *et al*., 1989).

Twelve (12) *S.* Typhimurium egg related outbreaks were reported in Great Britain between the periods of 1984 and 1995 (Martelli and Davies, 2012). *S.* Typhimurium DT 104 was isolated from eggshells in two different surveys conducted in the UK in the 1990s (CVL Weybridge Unpublished data and ACMSF, 2001). In the 1990s, an outbreak of *S.* Typhimurium DT 104 took place across the world and now it is present in the poultry industry of many countries (Martelli and Davies, 2012). The peak of human salmonellosis in UK occurred during 1996 and the population of *S*. Typhimurium DT104 has declined since then (Helms *et al.*, 2005). In 2008, a large national outbreak was reported to be associated with the consumption of pre-pared egg sandwiches where large numbers of cases of *S.* Typhimurium PT U320 were observed (Boxall *et al*., 2011). In Northern Ireland, England and Eire, the outbreaks of human salmonellosis were also reported due to contamination of duck eggs with *S*. Typhimurium DT 8 (HPA, 2010).

1.5.3 France

In a molecular epidemiological investigation of *S.* Typhimurium strains isolated from outbreaks, eggs were reported as a major source of infection (Carraminana *et al*., 1997). In 2009, a food borne outbreak was reported due to the consumption a home-made tiramisu prepared with raw eggs. Investigation indicated that this outbreak was linked to a major layer farm in North-western France and was caused by a non-motile variant of *S.* Typhimurium (Le Hello *et al.*, 2012).

1.5.4 Australia

In Australia, since the 1950s, there has been a gradual increase in *Salmonella* notifications (Communicable Diseases Network Australia and New Zealand, 1997).

However, after the 1980s, cases of *Salmonella* infection increased remarkably (Crerar *et al*., 1995). The serovar *S.* Typhimurium (28 to 38%) was the most predominant *Salmonella* serovar reported to The National Enteric Pathogen Surveillance Scheme (NEPSS) during the period 1990-1995 (Communicable Diseases Network Australia and New Zealand, 1997). From 1980 to 1995, in at least two *Salmonella* outbreaks, eggs were implicated as a source of infection (Communicable Diseases Network Australia and New Zealand, 1997).

In 2001, the overall rate of *Salmonella* infection was 34.1 cases per 100,000 population which were higher than in the USA (15.1 cases per 100,000 population). In 2001, eggs were suspected as a vehicle of infection in three outbreaks (caused *S.* Heidelberg PT 1, Typhimurium PT 9 and PT 135) which infected 29 people, of whom 11 were hospitalized (The OzFoodnet Working Group, 2002).

According to The OzFoodnet working group, during 2001-2005, twenty egg-related *Salmonella* food poisoning outbreaks were reported. These outbreaks affected 280 people and around 18% (50/280) were hospitalized. The highest number of outbreaks was reported in Queensland (8) followed by Victoria (6), Tasmania (2), South Australia (2), New South Wales (2). *S.* Typhimurium was responsible for 75% (15/20) of these outbreaks. In 2002, two salmonellosis outbreaks were responsible for two deaths. Cream cakes and a raw egg dish were implicated in these outbreaks (The OzFoodnet Working Group, 2003). In 2004, eggs were implicated in three *Salmonella* outbreaks and another three were due to cakes and deserts. A source of *S.* Typhimurium PT 126 infections was traced back to a brand of organic eggs in Victoria. However, for other outbreaks, researchers were not successful in linking the implicated eggs to a single farm (The OzFoodnet working group, 2005). In 2005, six egg-related *Salmonella* food poisoning outbreaks were reported.

After 2005, the number of *Salmonella* outbreaks, in which eggs were implicated as a source of infection increased dramatically. From 2006 to 2010, eggs were implicated in 92 *Salmonella* food poisoning outbreaks which resulted in 1740 cases and the rate of hospitalization was 23% (400/ 1740). New South Wales (37) and Victoria (22) recorded the highest number of outbreaks in this period. Outbreaks were more frequent in warmer months (October to March) of the year. Out of 92 outbreaks, most (91%) were due to the various phage types of *S.* Typhimurium. *S.* Typhimurium PT 170 (31.5%) and *S.* Typhimurium PT 193 (19.6%) were the most frequently recorded PTs in egg implicated outbreaks followed by *S.* Typhimurium PT 9 (14%) and PT 135a (8.7%). Other *Salmonella* serovars such as Singapore (3.2%), Anatum (1.08%), Postdam (1.08%), Saintpaul (1.08%), Virchow (1.08%) and Montevideo (1.08%) were rarely reported in the egg related outbreaks (The OzFoodnet Working Group, 2006, 2007, 2008, 2009, 2010, 2012). During this period, *S.* Infantis was not directly observed responsible for egg related food poisoning outbreaks. However, in 2010, there was 2.2 times increase in human cases of this serovar as compared to 2009 (The OzFoodnet Working Group, 2012).

Even though egg were implicated in many outbreaks, historically it was difficult to trace back the eggs to their origin or to find the source of contamination. In Tasmania, during June and December 2005, a series of five *S.* Typhimurium PT 135 outbreaks was reported which involved 125 laboratory confirmed cases. These outbreaks were investigated by personal interviews, cohort studies, microbiological testing, environmental health investigation of food business, trace back, inspection and drag swabbing of egg farm. Investigation revealed that the outbreaks were due to food containing raw eggs or cross contamination of food items due to improper handling and storage. Eggs and packing containers contaminated with faeces was considered as a source of contamination of raw ingredients at food businesses

(Stephens *et al*., 2007). The same egg farm was implicated in two additional egg associated *S.* Typhimurium PT 135 outbreaks (March 2007 and January 2008) which affected 66 people (Stephans *et al*., 2008). In all outbreaks, the investigation was limited to phage typing which cannot differentiate isolates of the same phage type. Hence, it is difficult to definitely prove that the *S.* Typhimurium PT 135 isolated from the egg farm was the same strain circulating in humans.

Rapid identification and differentiation of *Salmonella* isolates is very important in tracing the source of infection. With the use of advanced technology such as MLVA, (multi-locus variable number tandem repeat analysis) which can discriminate the different strains of the same *S.* Typhimurium PT, it is now possible to trace the source/origin of infection. In recent times, there are some outbreaks which were traced back to eggs using MLVA.

In 2007, a series of outbreaks took place in Queensland across various restaurants. The investigation was carried out using MLVA and results indicated that all of the outbreaks were due to multiple genotypes of *S.* Typhimurium 197 which originated from the same egg farm (Slinko *et al*., 2009). In NSW, a homemade raw egg mayonnaise was identified as the source of a gastroenteritis outbreak which affected 68 people of whom 14 were hospitalized. The MLVA results indicated that stool samples of patients and mayonnaise containing raw eggs were positive for *S.* Typhimurium PT 170 with the same MLVA (3-9-8-12-523) pattern (The OzFoodnet Working Group, 2010). In another NSW outbreak, which was due to the consumption of hollandaise sauce prepared with raw egg, *S.* Typhimurium 170 with the same MLVA pattern was isolated from the stool of patients (The OzFoodnet Working Group, 2010).

Even since 2010, the number of egg related *Salmonella* outbreaks have been increasing continuously. In 2011, eggs were implicated in 28 *S.* Typhimurium

outbreaks which affected 514 people, of whom 98 were hospitalized. Victoria (12) and NSW (9) recorded the highest number of outbreaks (OzFoodnet quarterly reports January – December 2011). In 2012, the role of eggs in *Salmonella* outbreaks is highlighted by one of the largest outbreak in Canberra, which affected 140 peoples with 15 hospitalized. Mayonnaise prepared with raw eggs was suspected as a source of infection in this outbreak.

The current data suggest that there is an urgent need for improving farm control of *S.* Typhimurium to minimise food poisoning related outbreaks in Australia and pressure for these improvements will only incease with the application of MLVA technology. The overall data suggest that, unlike USA or Europe, where *S.* Enteritidis has been most frequently associated with egg related outbreaks, in Australia, *S.* Typhimurium is the serovar of concern.

1.6 Epidemiology of *Salmonella* on commercial layer farms and eggs

1.6.1 *Salmonella* Typhimurium

S. Typhimurium was isolated from 1.8% of the UK farms (574) tested during the period 2004 and 2005. Typhimurium was the second most prevalent serovar after Enteritidis (5.8%) (Snow *et al*., 2007). However, Arnold *et al*. (2010) claimed that the true prevalence of Enteritidis and Typhimurium is 14% in UK egg laying holdings. Even though layer flocks were observed positive for *S.* Typhimurium, this serovar was rarely isolated from eggs in the surveys conducted after 1990s. In France, between September 2004 and October 2005, epidemiological investigation was carried out to study the prevalence of *Salmonella* in layer flocks. Out of 519 flocks tested in this survey, 93 (17.7%) were found positive for *Salmonella*. Out of 93 *Salmonella* positive flocks, 23.7% were *S.* Typhimurium positive where as 21.5% reported *S.* Enteritidis positive (Huneau-Salaün *et al*., 2009).

Certain *S.* Typhimurium definitive types like DT 2 and DT 99 are specific to wild birds (Rabsch *et al.,* 2002) and infection of these phage types in laying flocks is short term. Wild bird related *S*. Typhimurium strains may be introduced in free range flocks as a result of feed contamination by wild birds' droppings (EFSA, 2010). In the UK, *S.* Typhimurium is the principal serovar in ducks. Price *et al*. (1962) and Simko (1988) found that *Salmonella* stains isolated from a population of young ducks were predominantly *S.* Typhimurium. This may be due to better ability of *S.* Typhimurium to transmit vertically in ducks (Martelli and Davies, 2012). However, there is little information available regarding the epidemiology of *S*. Typhimurium in egg layer flocks investigated through longitudinal studies of natural infection.

1.6.2 *Salmonella* Infantis

S. Infantis has been reported as a common serovar in the poultry industry worldwide. In Finland, in 1975, a large epidemic of *S.* Infantis in broiler chickens caused human salmonellosis (Raevuori *et al.*, 1978). Poppe *et al.* (1991a) reported that *S.* Heidelberg was the most important serovar isolated from the egg layer flocks of Canada followed by *S.* Infantis. Similarly, *S.* Infantis has also proved to be the second most important serovar after *S*. Enteritidis in Germany (Hinz *et al.*, 1996). In Australia, *S.* Infantis was widely isolated between 1987-1992, particularly from pigs and with a high frequency on eggs (Murray, 1994). Barnhart *et al*. (1991) reported the presence of *S*. Infantis in the ovaries of commercial layer chickens at time of slaughter which raised public health concerns.

The importance of *S.* Infantis to the Australian poultry industry is based on past survey work and recent emerging research. A survey of layer farm was conducted in two different years in the greater Brisbane region in February 1992 and March 1993 to September 1994, respectively. During 1992, out of all samples (litter, feed and faeces) which were *Salmonella* positive, 43% were found to be positive

with serovar Infantis. In the following year, a 6 month longitudinal survey was conducted from September 1993 to March 1994 on a single layer farm with three flocks each of approximately 30,000 birds. The study tested 266 samples, of which 35% were *Salmonella* positive with only 1% prevalence of *S.* Infantis. *S.* Singapore was the most prevalent serovar that persisted till the end of survey, and was also observed in flocks as well as in feed components, especially in meat meal (Cox *et al*., 2002). It was speculated that the prevalence of *S.* Singapore may have prevented the multiplication of other antigenically closely associated serovars such as *S.* Infantis (Barrow *et al.,* 1987).

1.6.3 Other serovars of *Salmonella*

A number of other *Salmonella* serovars such as *S.* Senftenberg, *S.* Livingstone have also been isolated in the worldwide surveys conducted on the prevalence of *Salmonella* in poultry industry. These serovars were primarily isolated from eggshells and also from egg contents. Ebel *et al.* (1992) conducted a study on egg pulp for 52 weeks in the United States. These authors reported seasonal changes affecting the prevalence of *Salmonella* with high temperature during summer increasing the frequency of detection of a wide variety of *Salmonella* serovars from egg pulp and may form a cost effective monitoring system.

1.6.4. Vertical transmission ability of *Salmonella*

Experiments have shown that *S.* Enteritidis PT 4 and *S.* Typhimurium are equally important in infecting the hen's reproductive tract (Gantois *et al*., 2008). *S.* Enteritidis was isolated from eggs when hens were intravenously inoculated, whereas *S.* Typhimurium was isolated from eggs after intra-vaginal inoculation (Okamura *et al.,* 2001a, b). Gantois *et al.* (2008) reported that *S.* Typhimurium has the ability to survive in the albumen during egg laying and to cope with lysozyme better than *S.* Enteritidis.

Under experimental conditions, *S.* Typhimurium DT 104 has been also reported to infect the egg internal contents (Williams *et al.,* 1998) although Okamura *et al.* (2010) observed that *S.* Typhimurium DT104 has a low ability to infect eggs. However, the chances of egg contamination were higher when hens were infected at onset of lay (Okamura *et al.,* 2010).

The potential egg invasiveness for *S.* Enteritidis and other serovars has been studied (Okamura *et al.,* 2001a; Okamura *et al.,* 2001b; Gantois *et al.,* 2008). After intravenous infection of *S.* Hadar and *S.* Enteritidis at a dose rate of 5×10^6 CFU, 10% and 15.8% of hens, respectively, laid contaminated eggs (Okamura *et al.* 2001a). However, in case of other serovars (*S.* Typhimurium, *S.* Heidelberg, *S.* Infantis and *S.* Montevideo), eggs from all hens were *Salmonella* negative after intravenous infection. When the hens were artificially inoculated intra-vaginally at a dose rate of 5×10^6 CFU with the same strains of *Salmonella*, 70% of hens infected with *S.* Enteritidis produced contaminated eggs followed by *S.* Infantis (30%) and *S.* Typhimurium, *S.* Hadar, *S.* Heidelberg, *S.* Montevideo (20% each) (Okamura *et al.* 2001b). This suggests that the route of infection plays an important role in egg contamination. However, under field conditions, a low number of *Salmonellae* are available in the shed environment for the ascending infection of the birds.

Foley and Lynne (2008) reported that, in the United States, there has been an increase in the prevalence of, as well as egg related salmonellosis outbreaks due to, *S.* Heidelberg. In an experiment, laying hens were orally inoculated with *S.* Heidelberg to observe its ability to infect the reproductive tract and to contaminate the eggs internally. *S.* Heidelberg was successful in invading the reproductive tract as well as in contaminating egg internal contents (Gast *et al.,* 2007b). In another study, intravenous inoculation was conducted and *S.* Heidelberg showed the ability to survive in egg albumen during egg formation, while *S.* Hadar and *S.* Virchow were

killed quickly (Gantois *et al.*, 2008). In another experiment, the presence of different *Salmonella* serovars was observed in the ovaries of spent hens in the United States. *S.* Heidelberg (56%) was most frequently isolated and the percentages of other serovars such as *S.* Agona, *S.* Oranienburg, *S.* Mbandaka , *S.* Kentucky, *S.* Montevideo, *S.* London and *S.* Enteritidis was 13, 6.1, 5.2, 3.5, 3.5, 2.6 and 2.4, respectively (Barnhart *et al.*, 1991). When egg contents were experimentally infected with *S.* Virchow, the bacteria were able to multiply in eggs kept at room temperature. At same time, *S.* Virchow remained viable for 6 weeks, when the eggs were stored at 6°C (Lublin & Sela, 2008).

1.6.5 Egg based *Salmonella* prevalence surveys

It is difficult to carry out egg based *Salmonella* prevalence surveys because, even when the flock is infected with *Salmonella*, the frequency of egg contamination is very low (Braden, 2006). Ebel and Schlosser (1993) in the United States reported that the frequency of egg contamination with *Salmonella* is 1 in 20000 eggs. In the United States, some surveys were carried out to study the *Salmonella* contamination of table eggs.

De Louvois *et al*. (1993) conducted a survey to determine *Salmonella* prevalence in eggs produced from the UK hens and also in eggs which were imported to the UK from other European countries. It was observed that the actual prevalence of *Salmonella* in UK eggs (without making a distinction between eggshell and egg internal contents) was 0.9% (65/7045) whereas, in imported eggs, it was 1.6% (138/8630). In UK eggs, out of all *Salmonella* positive eggs (65), the major serovars isolated (72%) were *S.* Enteritidis followed by *S.* Livingstone (12%) and *S.* Typhimurium (9%). In imported eggs, *S.* Infantis (40%), *S.* Livingstone (31%), *S.* Enteritidis (14%) and *S.* Typhimurium (6 %) were predominantly isolated.

Schutze *et al*. (1996) examined one hundred dozen eggs for the prevalence of *Salmonella* on eggshell surface and in internal contents by pooling 12 eggs together. Only one sample was positive for *Salmonella* and the serovar reported was Heidelberg. Jones *et al*. (1995) investigated the level of *Salmonella* contamination in the laying house environment and also on eggs at various stages of the egg processing operation. Seventy two percent of the samples from the laying house were positive for *Salmonella*. Even though, *S.* Typhimurium was isolated from egg belt (5), egg collectors (8), ventilation system (3) samples, none of the egg samples was positive for *S.* Typhimurium. Out of 180 egg samples, eight eggshell wash samples were positive for *Salmonella* Heidelberg and Montevideo serovars. Jones and Musgrove (2007) studied the prevalence of *Salmonella* on restricted eggs (dirty eggs which do not meet quality criteria for retail eggs) and reported that two eggshells (1.1%) were positive for *Salmonella*. Both the samples belonged to Heidelberg serovar.

Subsequently, Chemaly *et al*. (2009) studied the level of *Salmonella* contamination in eggs from 28 known positive farms. The French study indicated that 1.05% (44 out of 4200) of the eggshells was found positive for *Salmonella* and the serotyping of *Salmonella* isolates revealed five different serovars: Typhimurium, Enteritidis, Infantis, Virchow and Montevideo.

1.6.6 *Salmonella* and The Australian egg industry

The *Salmonella* contamination of eggs and egg products is a major public health issue, according to food safety bodies such as New South Wales Food Authority (NSWFA) and Food Standards Australia New Zealand (FSANZ). The Australian Egg Industry is under continuous pressure to improve its approach towards *Salmonella* control. *Salmonellae* are frequently isolated from the environment of poultry farms, sometimes involving the presence of serovars which

are regarded as pathogenic and of importance to human health (especially *S.* Typhimurium).

Cox *et al*. (2002) conducted a major *Salmonella* survey in the egg industry in Queensland and studied the significance of *S*. Infantis for the egg industry. These researchers also isolated other serovars such as Singapore, Virchow, Cerro, Orion var 15+ 34+, subspecies 1 serovar 4, 12:d, Bredeney, Zanzibar, Anatum, Agona, Lille, Orion, Senftenberg, Mbandaka, and Johannesburg (Cox *et al*., 2002), although the extent of pathogenicity of these Australian Egg Industry related *Salmonella* serovars has not yet been investigated.

Sexton *et al*. (2008) conducted a *Salmonella* survey on raw ingredients in South Australian feed mills. The canola meal samples were most frequently positive for *Salmonella* (24.6%) followed by meat meal (20.3%), pollard (7.7%) and soybean meal (1.9%). The most important risk found in this survey was the presence of *S*. Infantis in meat meal which is commonly used in egg layer diets, but meat meals were found to be *S*. Typhimurium negative.

In Australia, two surveys were conducted in 1986 and 1989 which tested 360 and 190 eggs for the presence of *Salmonella*. All the eggs tested during these surveys were *Salmonella* negative (Douglas, 2004). Daughtry *et al*. (2005) carried out a large survey to investigate the prevalence of *Salmonella* in commercial eggs. All eggshell surfaces (10,000) and egg internal content (20000) samples was reported *Salmonella* negative. Chousalkar and Roberts (2012) conducted a small study on isolation of *Salmonella* from egg shell wash, egg shell pores and internal contents. This study was performed using relatively small number of samples compared to Daughtry's study and the eggshell surface samples were reported positive for *S*. Infantis and egg internal contents tested negative. Egg based surveys are important but laborious, and large numbers of eggs are required for *Salmonella* testing.

Moreover, the negative *Salmonella* results from eggs may not clearly reflect *Salmonella* negative status of a flock. The contamination of eggshells with faeces could indicate the level of bacterial eggshell contamination and *Salmonella* shedding in faeces (Gast and Beard, 1990; De Louvois, 1993); however, this could be highly variable (Morris, 1990).

In order to reduce egg related salmonellosis outbreaks, the implementation of egg quality assurance programs (Australian Egg Corporation Limited, 2005) has been considered. As part of this program, accredited egg producers participate in regular *Salmonella* testing. Environmental drag swabs are usually collected as part of the testing. In Australia, *S.* Enteritidis has been isolated from poultry products a number of times (Arzey, 2002). However, egg related salmonellosis outbreaks related to this serovar have been not reported in Australia and *S.* Typhimurium remains the serovar of concern (The OzFoodnet Working group, 2012). Due to the low rate of isolation *S.* Enteritidis from poultry products, as well as the lack of epidemiological evidence to link poultry products with human *S.* Enteritidis infection, the Australian poultry industry is considered free from S. Enteritidis (Sergeant *et al*., 2003). Most *S.* Enteritidis infections in Australia are believed to be acquired overseas.

1.7 Detection and characterization of different *Salmonella* serovars

Bacterial subtypes originating from the same source are likely to be similar compared to subtypes originating from different sources. In epidemiological studies, it is possible to trace pathogens/bacteria to the origin of the outbreak by identifying serovars that are relatively similar or indistinguishable to those involved in the outbreak. Subtyping is also useful to identify new and emerging pathogens. Based on the subtyping, evolutionary studies can be carried out. Traditionally, for the typing of the *Salmonella enterica* serovars, phenotyping methods like serotyping, phage typing

and biochemical profiling are routinely used. The current classification of *Salmonella enterica* subspecies *enterica* serovars is based on serotyping (Anonymous, 1934). Serotyping classifies the bacterial strain based on the reactions of the somatic (O) and flagellar (H) antigens with the specific antisera. Even though the importance of serotyping is widely utilized in surveillance programs, it lacks the capacity to discriminate closely related isolates (Herikstad *et al*., 2002). Pulse field gel electrophoresis (PFGE) rapidly became popular because of its capacity to identify the bacterial strain at the origin of outbreak. PFGE is also considered as the gold standard for the molecular subtyping of *Salmonella* (Wattiau *et al*., 2011). During the last two decades, a number of other molecular subtyping techniques have also been developed, optimized and tested for distinguishing closely related isolates. Even though none of them emerged as a clear ideal method for *Salmonella* typing, these techniques have higher sensitivity for subtyping, need little technical expertise and they utilize common laboratory reagents (Foley *et al*., 2007).

1.7.1 *Salmonella* typing using phenotypic methods

1.7.1.1 Slide agglutination serotyping

The Kauff-White-Le Minor (KW) scheme is used for the serotyping of *Salmonella* spp. The scheme is based on the 1930's original scheme with some modifications (Anonymous, 1934; Grimont *et al*., 2007; Guibourdenche *et al*., 2010). The *Salmonella* serotyping scheme is based on the reactivity of somatic antigen to specific antisera (Grimont *et al*., 2007). In serotyping, sera able to identify the variants of somatic and flagellar antigens are used. The somatic and flagellar antigens are highly variable; 64 somatic and 114 flagellar antigens have been detected so far (Grimont *et al*., 2007; McQuiston *et al*., 2008). Many somatic antigens can be expressed on the surface of *Salmonella* at the same time. Even though most of the *Salmonella* have two copies of genes which code for the flagellar

protein, only one flagellar protein is expressed at one time (McQuiston *et al*., 2008). Based on the flagellar protein, most of the isolates are named as diphasic (Phase I & II or H1 & H2). Even though tri-phasic and quadri-phasic subtypes are rare, monophasic salmonellae are not exceptional (Burnens *et al*., 1996). An antigenic formula is used to indicate the antigen (O, H1, H2 - separated by semicolons) identified on the strain (Wattiau *et al*., 2011).

Most of the *Salmonellae* of veterinary importance belong to subspecies *enterica* (Quinn *et al*., 2010) which has more than 1500 different serovars (Grimont *et al*., 2007). Even though some serovars have the same antigenic formula, they differ in their biochemical properties. Based on the biochemical properties, *Salmonellae* are divided into different subspecies: 1) Subspecies I – *enterica* 2) Subspecies II – *salamae 3*) Subspecies IIIa – *arizonae 4*) Subspecies IIIb – *diarizonae 5*) Subspecies IV – *houtenae 6*) Subspecies VI – *indica* (Wattiau *et al*., 2011). Hence, serotyping is useful to differentiate strains within a subspecies and define serotypes/serovars/subtypes within it; however weak and non-specific agglutination may result in false positive reactions (Schrader *et al*., 2008). Serotyping involves use of over 150 specific anti-sera but still this method is used as a reference method and commonly preferred for initial screening. There are some other limitations to this technique such as it is not possible to type a strain due to auto-agglutination or due to the loss of antigen expression. Secondly, by this method, it is not possible to trace the strain to the point of outbreak nor does it define the phyletic relationships (Wattiau *et al*., 2011).

1.7.1.2 Serotyping by anti-body microarray

Cai *et al*. (2005), using SuperEpoxy microarray slide spotted with antibody, developed a serotyping assay. This technique was successful in typing 20 commonly isolated serovars in Canada. As compared to the traditional serotyping, it requires

less time and is more sensitive. Due to high sensitivity, the phase inversion step can be eliminated (Wattiau *et al*., 2011).

1.7.1.3 Phage typing

In order to differentiate *Salmonella* strains belonging to the same serovar, phage-typing is used. The ability of specific phage to lyse the strain of concern is used to assign the phage type (Anderson and Williams, 1956). Phage typing was initially used for *Salmonella* serovars Typhi, Paratyphi A and Typhimurium. Phage typing was also used for Enteritidis (Ward *et al*., 1987). The main advantage of this method is its simplicity. However, it may give ambiguous lysis reactions and also has limited capacity due to the small number of phages (Wattiau *et al*., 2011).

1.7.2 Molecular typing techniques

1.7.2.1 Pulse field gel electrophoresis

With the application of alternative polarity, PFGE separates twenty times larger DNA fragments as compared to traditional gel electrophoresis. PFGE has been used for *Salmonella* typing since 1990 (Garaizar *et al*., 2000; Murase *et al*., 2004; Olsen *et al*., 1994; Weide-Botjes *et al*., 1998). PFGE is one of the earliest molecular sub-typing techniques capable of differentiating serovars at the level of an outbreak investigation**.** However, it is essential to compare PFGE patterns between laboratories. The PulseNet network developed by the Centre for Disease Control and Prevention (http://www.cdc.gov/pulsenet) provides facilities to compare the different PFGE patterns. However, this tool is becoming unreliable and instinctive because of the huge number of profile clusters characterized by few differences.

1.7.2.2 Multi-locus sequence typing (MLST)

MLST involves the direct sequencing of 450 to 500 base pair (bp) fragments of 5 to 10 housekeeping genes and detection of mutations in their sequences. After finding the mutation of the base pair in a sequence, it is designated as an allele type. The combinations of all mutations in the loci are used to define MLST type of the

particular strain (Hyytiä-Trees *et al*., 2007). A major advantage of MLST is that its data are non-ambiguous, portable and can be easily shared between laboratories by using the MLST website (http://pubmlst.org). Kidgell *et al*. (2002) developed the first MLST scheme, for *Salmonella* serovar Typhi based on 7 housekeeping genes. This method later became popular especially for the non-typhoidal *Salmonella* serovars (Wattiau *et al*., 2011). Even though some studies indicated that MLST schemes can compete with PFGE, the major drawbacks are the cost and the amount of laboratory work involved as well as the requirement for high level expertise to proofread nucleotide sequences (Wattiau *et al*., 2011).

1.7.2.3 Real-time and Multiplex PCR

Malorny *et al*. (2003) standardized PCR for identification of food borne pathogens. However, along with detection, it is important to determine the load of *Salmonella* in an environmental sample. In the case of egg laying hens and other poultry samples, a molecular quantitative method such as real-time PCR could be helpful in determining the environmental loads of *Salmonella*. In real-time PCR, absolute quantification of the target microorganism can be performed using a standard curve prepared from known serial dilutions of standards. The absolute quantification may help to understand the risks of having *Salmonella* contaminated eggshells from a contaminated environment. There is a dilemma of whether or not to perform a pre-enrichment step before real-time PCR. Some researchers have developed direct quantification real-time PCR protocols which do not involve an enrichment step (Wolffs *et al*., 2006; Guy *et al*., 2006). However, DNA sample preparation for real time PCR, which involves the processing of an appropriate amount of sample, extraction and concentrating target DNA in small volumes, is a difficult task. If a sample contains a very low level of *Salmonella*, pre-enrichment of samples is essential before performing real-time PCR (Malorny *et al.*, 2008). The

pre-enrichment step also helps to recover stressed or damaged cells from clinical samples. However, it is unclear whether pre-enrichment of samples can alter chosen threshod (Ct) values or not. Real-time PCR is less laborious, quick and cheap as compared to the traditional quantitative culture method.

Serovar specific PCR has the capacity to differentiate between the serovars of *Salmonella* spp. The genes involved in coding somatic and flagella antigens have been frequently used to develop serovar specific PCR. Desai *et al*. (2005) developed an allele specific PCR based on variation in *rfbS* gene sequence to identify the *S.* Pullorum serovar. Similarly, some researchers developed multiplex PCR assays to selectively identify *S.* Typhimurium serovars (Lim *et al*., 2003; Salehi *et al*., 2007). On the other hand, Woods *et al*. (2008) used a comparative genomics approach to differentiate Paratyphi C and Cholerasuis serovars using multiplex PCR. However, the specificity of these assays was not completely established. Akiba *et al.* (2011) developed a multiplex PCR assay to differentiate seven *Salmonella* serovars. These researchers targeted *Salmonella* specific genomics regions to differentiate Typhimurium, Gallinarum, Cholerasuis, Dublin, Infantis, Enteritidis and Hadar serovars.

The combination of real-time PCR and multiplex PCR assay could be helpful in determining the environmental load of *Salmonella* and presence of different serovars in a layer flock. However, the sensitivity of these assays in naturally contaminated samples is currently unknown.

1.7.2.4 Multi locus variable number tandem repeat analysis **(MLVA)**

In prokaryotic cells, the amount of repetitive DNA is increasing (Van Belkum *et al*., 1998) and these repeats can vary in size and location. The variable number tandem repeat (VNTR) loci are loci in the genome where repeats are organized in tandem and show inter-individual unit number variability (Nakamura *et al*., 1987). In

MLVA analysis, using a simple multiplex PCR followed by capillary electrophoresis, the number of tandem repeats present at different loci can be determined. MLVA is automated and fast compared to MLST; capillary electrophoresis can differentiate the fragments that are different by only a few base pairs.

Lindstedt *et al*. (2003) used tandem repeat analysis for typing of *S.* Typhimurium. Lindstedt *et al*. (2003) used eight different loci for typing. Capillary electrophoresis was used to determine the size of the VNTR loci. By this method, a very high resolution of variants of DT 104 was obtained which provided a tool to trace a DT 104 outbreak. A number of different protocols have been developed for the subtyping of Typhimurium (Chiou *et al*., 2010; Lindstedt *et al*., 2004; Ross *et al*., 2009; Witonski *et al*., 2006).

Comprehensive genome analysis has suggested that five different loci (STTR 3, STTR 5, STTR 6, STTR 9 and STTR 10) provide sufficient allelic variation to differentiate the various strains of *S.* Typhimurium and these loci are used in Australia for MLVA typing (Heuzenroeder *et al*., 2013). Once the fragment size of each locus is determined by multiplex PCR followed by capillary electrophoresis, a profile number for an isolate can be assigned based on actual fragment sizes. However, in Australia, a different MLVA coding system is adopted. In this system, codes indicating the number of tandem repeats in each fragment are used as profile numbers. As flanking regions are conserved, only variable regions of each loci are used to determine fragment length. Briefly, as described by (Heuzenroeder *et al*., 2013), if there is no amplification of PCR product then it represents code '0'. On other hand, if there is PCR product present with the absence of tandem repeat then it indicates code '1'. A fragment containing one tandem repeat is coded as '2'. Hence, profile number for a loci/fragment (p)= n+1 where n is the number of tandem repeats

in a fragments. This coding is applied to all loci except STTR 3 which contains the truncations of three or more base pairs which may not affect number of tandem repeats. Hence, for STTR 3 loci, actual fragment size in base pairs is expressed when assigning profile number to indicate minor differences fragment lengths. Finally, by comparing the profile number of *S.* Typhimurium strains, it is possible to decide the relatedness between these strains. Generally, STTR 3 and STTR 9 loci have been considered as stable. If there is a variation in either this locus then, regardless of variation in other loci, the isolates of interest are considered as distinct or unrelated. However, variations in STTR 5, STTR 6 and STTR 10 loci have been observed more frequently. One or two tandem repeat changes in these loci can occur relatively more rapidly. In such cases, further instinctive interpretation is essential to decide the relatedness of *S.* Typhimurium strains under observation. With the use of MLVA, which can discriminate in the different strains of the same *S.* Typhimurium phage type, it is now possible to quickly trace source/origin of infection. In Australia, in a number of egg related salmonellosis outbreaks, MLVA has been very useful in identifying an origin of infection (Slinko *et al*., 2009; Reynolds *et al*., 2010; Moffatt *et al*., 2012). MLVA is less expensive, easy to perform and data can be easily shared between laboratories using the same scheme for genomic interpretation. In South Australia, MLVA could be easily performed using a standardized protocol developed by *Salmonella* Reference laboratory, Adelaide.

1.8 Measures to control *Salmonella* contamination of eggs

The chances of *Salmonella* egg contamination can be reduced by decreasing the prevalence of *Salmonella* in egg layer flocks. Reducing the environmental load of *Salmonella* in the layer shed, by adopting good management practices (such as regular cleaning of sheds), could also reduce the incidence of egg contamination.

1.8.1 Methods

Various methods have been used to control *Salmonella* in layer flocks. Some of these methods are pre-harvest and others are post-harvest (Galis *et al*., 2013). Pre-harvest methods includes 1) Genetic selection for resistance to *Salmonella* 2) Flock management involving sanitation, flock testing, biosecurity 3) Vaccination 4) Use of natural antimicrobial products such as prebiotics, probiotics, organic acid. Post-harvest methods involve eggshell decontamination using 1) Chemical methods e.g. egg washing by sanitizers, ozone, electrolysed water 2) Physical methods e.g. irradiation, U.V. light, gas plasma technology 3) Biological methods e.g. plant extracts (Galis *et al*., 2013).

1.8.2 Egg washing

Egg washing with sanitizers is one of the most common methods of reducing eggshell contamination. This technique is adopted in many countries such as Australia, Japan and USA (Hutchison *et al*. 2004). However, in European countries, washing of class-A table egg is banned (Nys and Van Immerseel, 2009). Egg washing protocols involve multiple steps 1) pre-washing 2) egg washing with a surfactant 3) egg washing with sanitizer 4) drying (May *et al*., 2013). During egg washing, the maintenance of rinse water temperature is important. If an egg is placed in a cooler environment, egg internal contents contract resulting in pulling of membranes away from the eggshell. This creates a negative pressure and movement of air across the eggshell (Messens, 2011). If there is water on the eggshell surface or in pores, the contamination of egg internal contents can occur due to the presence of contaminants in water or in eggshell pores (Haines and Morgan, 1940; Lorenz and Starr, 1952). Hence, the temperature of wash water should be maintained 6.7°C (20°F) higher than egg internal contents temperature and should be always kept at 32.2°C (90°F) or higher (Galis *et al*., 2013). Hutchison *et al*. (2003) observed that,

under field conditions, the majority of commercial eggs washing plants use either ammonium or chlorine based sanitizers. To perform effective egg washing, in the presence of solid debris e.g. faeces on the eggshell surface, 47.4°C temperature as well as 10.8 pH was required (Leclair *et al*., 1994). It has been observed that *S.* Typhimurium was able to multiply at pH <9.5 at wash water temperatures ranging between 38°C and 42°C. However, *S.* Typhimurium was not able to survive when pH increased above 10 (Holley and Proulx, 1986). Humphrey *et al*. (1993) reported that pH range of 10-11.4 is ideal for controlling *Salmonella*. In a study conducted by Northcutt *et al*. (2005) at a commercial egg washing plant, generally pH of wash water ranged between 10 and 11.4.

The major advantages of egg washing are: 1) it removes faecal debris present on eggshell surface, 2) it reduces the level of bacteria on the eggshell surface which may help to reduce the chances of horizontal transmission across the eggshell and avoid contamination of egg internal contents, 3) due to the reduction of bacteria on eggshell surface, the chance of cross contamination of other food stuffs in a kitchen is reduced (Galis *et al*., 2013). However, some studies suggested that chemicals used in egg washing have the potential to alter the eggshell surface and also can damage the cuticle layer (Wang and Slavik, 1998; EFSA, 2005). This may also reduce egg internal quality due to the loss of moisture. All these factors may favour horizontal transmission of *Salmonella* across the eggshell especially when post egg washing storage conditions are poor. Hence, benefits and losses of egg washing are still controversial. Wang and Slavik (1998) also reported that the degree of damage to the eggshell surface varies among egg washing protocols which suggests that the chances of horizontal transmission may vary with egg washing protocol.

1.8.3 *Salmonella* control strategies in Australia

The control of *Salmonella* shedding on farm still remains a challenge. *Salmonella* control on farm requires a multi-prolonged approach targeting all the possible sources of *Salmonella* exposure (Jackson and Underwood, 2005). Effective *Salmonella* control depends upon the good farm management and husbandry practices (including all aspects of feed, birds, management, cleaning and disinfection, control of rodents, etc). Recently Sharp *et al*. (2012) conducted a study on the protective effects of *Salmonella* vaccines available in Australia. The various routes of vaccine delivery and their effectiveness against homologous and heterologous serovars of *Salmonella* were evaluated. The best overall protection was achieved by the combination of subcutaneous injection of the live vaccine at 6 weeks followed by the intramuscular injection of inactivated vaccine at 12 weeks. However, one of the important findings of this study was the difficulty in challenge and the recovery of *Salmonella* serovars from challenged birds during the experiment (Groves *et al*., 2011). The impact of vaccination on the level of *Salmonella* contamination on eggs was not tested in any of these studies.

1.9 Eggshell penetration studies

1.9.1 Physical and chemical barriers to bacterial penetration

There are three physical barriers which protect the egg from the bacterial penetration. The first physical barrier is the cuticle, a proteinaceous layer which covers the eggshell and pore openings. The next barrier to the cuticle is the crystalline eggshell followed by a final physical barrier, the membranes, which divide the albumen and the eggshell (Haigh and Betts, 1991). There are three distinct layers of membranes: inner membrane, outer membrane and limiting membrane. A meshwork of arbitrarily oriented fibers leads to the formation of the inner and outer membranes whereas the limiting membrane consists of homogenous electron dense

material (Bruce and Drysdale, 1994) and is interconnected with the internal part of the inner membrane (Wong *et al.*, 1997). Even though bacteria penetrate through shell membranes, egg internal contents are proctected by a number of antimicrobial chemical components in albumen. These chemical compontents include Lysozyme, Ovotransferrin, Avadin, Ovomucoid, Ovoinhibitor, Ovomucin. Because of all these components, it is difficult for bacteria to survive and grow in egg albumen.

1.9.2 Methods used to study bacterial penetration of eggs

Different methods have been used to measure the penetration of bacteria across the eggshell. In the most common method, whole eggs were dipped in a *Salmonella* suspension and, after incubation, the membranes and / or egg internal contents are analyzed for the presence of *Salmonella*. The movement of *Salmonella* in the direction of the albumen and yolk can be investigated, in addition to penetration of the eggshell, using this technique (Messens *et al*., 2005a). Board and Board (1967) developed an alternative method referred to as the agar penetration approach where the egg contents were removed and substituted with agar containing an antibiotic as well as tetrazolium. The agar filled eggs were dipped in a suspension of antibiotic resistant *S.* Enteritidis. After incubation, dark red colonies at the places of penetration were identified by candling (Berrang *et al.,* 1998). Another method involved the immersion of whole eggs in a culture containing bioluminescent *Salmonella*. And use of an imaging system for the detection of luminescence at the places of penetration (Messens *et al*., 2005). The major disadvantage of this second method was a low sensitivity of detection of *Salmonella* penetration. To obtain visible fluorescence, 10^4 bacteria per mL of albumen were essential and to obtain luminescence an additional detection step was needed (Chen *et al.*, 1996; Chen and Griffiths, 1996). Nascimento *et al.* (1992) used a small piece of eggshell placed on the surface of the agar in a petri dish to investigate eggshell penetration. A droplet of

suspension containing *S.* Enteritidis was put on the surface of the eggshell (1 cm^2). After incubation (20 min at 23°C), the eggshell was separated from the agar and the plates were incubated. The eggshell penetration was determined based on the colonies on agar plates.

The agar penetration approach is regarded as the most suitable for investigating the eggshell penetration ability of various *Salmonella* serovars. Also, this approach could be more suitable for studying the relationship between *Salmonella* eggshell penetration and various eggshell quality parameters including ultra-structural variations in the mammillary layer. However, the whole egg penetration approach matches better with the real life situation and is most suitable for comparing the survival ability of different *Salmonella* serovars on eggshell surface as well as in the egg internal contents.

1.9.3 External factors affecting *Salmonella* egg penetration

1.9.3.1 Bacterial strain

The ability of *S.* Enteritidis and *Pseudomonas* Fluorescens (*P*. Fluorescens) to penetrate the eggshell was investigated by Anderson *et al*. (1995) who reported that both bacteria penetrated the eggshell with equal capacity. There was a difference in the survival rate of *S.* Enteritidis and *P*. Fluorescens depending upon the part of the eggshell that was exposed to the bacteria. The exterior surface was more favorable for survival of *S.* Enteritidis. On other hand, *P*. Fluorescens had greater capacity to penetrate the eggshell membranes and survive in the egg internal contents. Miyamoto *et al.* (1998) reported that *S.* Enteritidis and *S.* Typhimurium possess equal eggshell penetration ability. Similarly, Schoeni *et al.* (1995) revealed that *S.* Enteritidis, *S.* Typhimurium and *S.* Heidelberg had the same capacity to penetrate through the eggshell in order to contaminate the egg internal contents during storage at 25°C. However, Sauter and Petersen (1974) reported that *S.* Typhimurium had a better

capacity to penetrate eggs than the other serovars. De Reu *et al.* (2006) studied the egg penetration ability of different bacteria which can be isolated from the egg contents and observed that *Pseudomonas* spp, *S.* Enteritidis and *Alcaligenes* spp penetrated the eggshells more regularly. In a whole egg experiment, penetration by *S.* Enteritidis was highest followed by *Corynebacterium* spp and *Serratia* Marcescens. Cogan *et al.* (2004) reported that the expression of flagella as well as curli fimbriae was important for the survival of *Salmonella* serovars in albumen. Using an *in vitro* penetration model, Gast *et al.* (2007a) demonstrated that the penetration frequency and yolk multiplication of *S.* Enteritidis was significantly higher as compared to *S.* Heidelberg.

1.9.3.2 Temperature difference between egg and bacterial suspension

The contraction of egg contents occurs when warm eggs are subjected to cooling and this creates negative pressure which helps the bacteria to penetrate across the eggshell pores (Bruce and Drysdale, 1994). Miyamoto *et al.* (1998) observed a higher penetration rate in eggs which were exposed to a suspension of either *S.* Enteritidis or *S.* Typhimurium at 20°C, as compared to eggs which were cooled down to 4°C, before dipping them in the bacterial suspension.

1.9.3.3 Moisture

Some studies have found that moisture is essential and plays an important role in eggshell penetration studies (Bruce and Drysdale, 1994; Berrang *et al.* 1999a). Although *S.* Typhimurium penetration was increased by the existence of water on the shell surface, penetration can occur even in the absence of the water on the eggshell (Padron, 1990). Eggs may ‘sweat’ and condense moisture on the eggshell surface when transferred from the refrigerated storage to the room temperature (Bruce and Drysdale, 1994). However, Ernst *et al.* (1998) reported that 3 h of sweating did not increase the penetration of *S.* Enteritidis as compared to the control group. The

variation in the results of these studies, however, may be due to the differences in the protocol used to investigate eggshell penetration.

1.9.3.4 The load of bacteria used for egg inoculation

A number of reports have indicated that the rate of contamination of eggs is directly proportional to the number of *Salmonella* in the suspension used for egg immersion (Chen *et al*., 1996; Miyamoto *et al.,* 1998; Braun *et al.,* 1999). Braun *et al.* (1999) reported that, after 20 days of storage at 15°C, eggs exposed to higher bacterial dose were highly contaminated (10.1%) compared to the low dose (1%). Similarly, Schoeni *et al.* (1995) found that, when faeces containing high inoculums (4 or 6 log CFU/g) of bacteria were applied to the surface of the eggshell, the contents as well as membranes were contaminated more frequently.

1.9.3.5 pH of inoculating media

The effect of pH on eggshell penetration by *Salmonellae* was investigated by Sauter *et al.* (1979). Eggs were infected with *S.* Typhimurium, *S.* Saint-paul, or *S.* Derby culture in water (3 log CFU/mL) with the pH varying from 5.0 to 9.5. It was observed that, at pH 5, penetration rates were significantly lower compared to higher pH values.

1.9.3.6 Temperature and relative humidity (RH) during incubation

Braun *et al.* (1999) found that, with an increase in temperature and RH, the rate of *S.* Enteritidis penetration into egg internal contents was increased. However, Wang and Slavik (1998) reported that incubation temperature did not affect eggshell penetration by *S.* Enteritidis. Schoeni *et al.* (1995) reported that 50% of the egg internal contents were contaminated at 25°C after 3 days of incubation while there was no contamination at 4°C. Following a positive temperature differential, Rizk *et al.* (1966) were able to recover *Salmonellae* from egg contents when the infected eggs were incubated at 22-23°C or 10-13°C. On other hand, the recovery of

Salmonellae at 2°C was very low. Gast *et al.* (2006) demonstrated that the prompt refrigeration of eggs at 4°C may help to limit the multiplication of *S.* Enteritidis in the egg yolk of contaminated eggs. Chousalkar *et al.* (2010) reported significantly lower bacterial penetration of the eggshell when eggs were incubated at 4°C as compared to the eggs stored at room temperature, with a 10^7 CFU inoculum of *S.* Infantis.

1.9.4 Egg factors affecting *Salmonella* penetration

1.9.4.1 Cuticle

The cuticle plays an important role in protecting against bacterial penetration. It is the first physical barrier between the egg and external contamination. Some reports have demonstrated that, in the first few minutes following oviposition, the cuticle is generally moist and immature. This immature cuticle has been observed to be less efficient as a barrier, compared to the mature cuticle (Miyamoto *et al*., 1998). The mature cuticle closes the pores on the eggshell and protects the egg from water and bacterial penetration (Berrang *et al*., 1999a). When the cuticle was removed either by EDTA treatment or eggshell abrasion, there were increased incidences of eggshell invasion by bacteria (Board *et al.*, 1979). Board and Halls (1973) found that there was no significant cuticle in 3.5% of the eggs examined whereas, in 8.0% of eggs, the cuticle was absent at the apex or blunt end. Nascimento *et al.* (1992) using a scanning electron microscopy (SEM) revealed that, throughout the laying cycle in most of the eggs, the cuticle was either patchy or absent. Nascimento *et al.* (1992) also questioned the efficiency of cuticle in protecting the eggs from the bacterial penetration, as at all stages of laying cycle, there was a lack of uniformity in cuticle cover over the whole eggshell. The cuticle shrinks with the age of an egg as a result of drying and may lead to exposure of the pores to the environment (Mayes and Takeballi, 1983). De Reu *et al.* (2006) reported that the average cuticle deposition

was inferior in penetrated eggshells as compared to the unpenetrated eggshells. However, Messens *et al.* (2005b) found that *Salmonella* eggshell penetration was not affected by the level of cuticle present on a shell. A natural variation in cuticle could affect bacterial penetration (Bain *et al*., 2013).

1.9.4.2 Eggshell quality and bacterial penetration

Sauter and Petersen (1974) found that eggs with a poor specific gravity and thin shells were more susceptible to *Salmonella* penetration. Orel (1959) reported similar findings with *Pseudomonas.* However, Williams *et al.* (1968) found that penetration ability of *S*. Typhimurium was not affected by shell thickness. Eggs with a visually poor shell quality were more prone to *S.* Enteritidis penetration (Nascimento and Solomon, 1991).

The architecture of the eggshell is also a very important factor in determining the quality of the eggshell and this can be examined by ultra-structural studies (Roberts and Brackpool, 1994). The highest variation in eggshell ultra-structure occurs in the mamillary layer where various abnormalities have been reported (Roberts and Brackpool, 1994). Roberts and Brackpool (1994) reported that various factors like the genetic strain of hen, age, environmental temperature, dietary factors, disease and stress can affect eggshell quality at the ultra-structural level. Using the mamillary layer abnormalities as criteria for judging eggshell quality, Nascimento *et al.* (1992) demonstrated that eggshell quality decreases with increase in age of the hen and was associated with increased incidence of eggshell penetration by the bacteria. However, Berrang *et al.* (1998) reported that there was no correlation between the age of the flock and *S.* Typhimurium penetration of shell and membranes.

Chousalkar *et al.* (2010) studied the ultra-structure of the eggshell using SEM and hypothesized that, during the early phases of eggshell formation, changes in the

mammillary layer of the eggshell may lead to the development of translucent eggs which could then be responsible for bacterial penetration. Bain *et al.* (2006) also reported that microcracks can give rise to translucent areas on otherwise intact eggshells.

1.9.4.3 Porosity

The number of the eggshell pores per egg varies from 7000-17000 (Mayes and Takeballi, 1983). These pores are covered with cuticle (Board, 1980). However, there are often some pores that lack either sufficient cuticle cover or a cuticle plug (Board and Tranter, 1995). It is through these 'patent' pores that bacteria may infect the egg internal contents (Board and Tranter, 1995). Haigh and Betts (1991) reported that the blunt end of an egg had higher porosity as compared to the apex or pointed end and Messens *et al.* (2005b) found that more bacterial invasion occurred through the blunt end as compared to the apex. As the age of hen advances, the pore numbers in the eggshell increases (Nascimento *et al*., 1992). However, there are some reports which indicate that bacterial penetration is independent of the porosity (De Reu *et al.*, 2006; Messens *et al.*, 2005b).

1.9.4.4 Defects in eggshell due to cooling

The rate of cooling after oviposition also affects the eggshell integrity. Fajardo *et al.* (1995) found that there was increase in the bacterial penetration of *S.* Enteritidis through the microscopical cracks produced by the rapid cooling of the eggs, Thompson *et al.* (2000) and well as Chen *et al.* (2002) did not find increased bacterial penetration with rapid cooling.

1.9.4.5 Membranes

Board and Tranter (1995) reported that, as the inner membrane is attached to the limiting membrane, it is more capable of preventing bacterial penetration than the outer membrane. When eggs were dipped in a suspension containing either *S.*

Heidelberg, *S.* Enteritidis or *S.* Typhimurium and stored at 25°C for three days, 50% of the membranes along with the contents were infected (Schoeni *et al.*, 1995). Hartung and Stadelman (1963) reported that the membranes from fresh eggs were less susceptible to the bacterial penetration as compared to the membranes from old eggs. Berrang *et al.* (1999b) speculated that the network of the inner membrane was related with bacterial invasion and the charge of chemical structure of the membrane had some impact on bacterial penetration ability.

1.10 Infectious agents and eggshell quality

Many diseases have been found to be associated with poor eggshell quality. Any disease which has the potential to damage the reproductive tract may affect eggshell quality. Charlton *et al.* (2000) reported that diseases like infectious bronchitis virus, Newcastle disease, avian influenza and avian encephalomyelitis may cause production loss as well as shell deformities. Charlton *et al.* (2000) reported that Mycoplasmas have the potential to alter eggshell quality. Recent research has suggested that the Australian strains of infectious bronchitis virus were not directly responsible for eggshell quality defects (Chousalkar *et al.*, 2009); however there is still uncertainty about the direct effects of other infectious agents such as *Mycoplasma spp* on egg quality and these agents remain unrecognised in the majority of circumstances.

The mycoplasmas are microorganisms belonging to the class Mollicutes. The Class Mollicutes consist of nine different genera. Out of these nine genera, five are of veterinary importance. The genera *Mycoplasma* and *Ureaplasma* contain the species of pathological significance for domestic animals. Several pathogenic mycoplasmas are responsible for Avian mycoplasmosis. Out of these, *Mycoplasma synoviae* (*M. synoviae*) is the most important one (Kleven & Bradbury, 2008). *M. synoviae* is mainly responsible for the infectious synovitis in chickens. It is mainly

transmitted by the aerosol route with vertical transmission being less important. The main clinical features include synovitis, arthritis and respiratory signs (Quinn *et al*., 2010).

In England, a 78.6% prevalence of *M. synoviae* was reported in commercial layer flocks (Hagan *et al*., 2004). Similarly, in Southern California, a high prevalence (86.7%) of *M. synoviae* was observed by Mohammed *et al*. (1986). Multiple age flocks as well as low biosecurity standards at the layer farms were identified as a risk factors for the high prevalence and persistence of *M. synoviae* infections (Stipkovits and Kempf, 1996; Kleven, 2003). *M. synoviae* infected commercial layer flocks therefore pose a significant epidemiological risk for other categories of poultry. Suzuki *et al*. (2009) reported a high seroprevalence (up to 53%) of *M. synoviae* in backyard flocks in Paraquay. However, in Serbia, seroprevalence of *M. synoviae* was reduced to 22.2% from 47.5% during 2000- 2009 (Kapetanov *et al*., 2010).

Economic losses due to *M. synoviae* include increased mortality (1 to 4%) in broilers (Shapiro, 1994); drop in egg production in layers by 5 to 10% and decreased hatchability of breeders by 5 to 7% (Mohammed *et al*., 1987; Stipkovits and Kempf, 1996). In an experimental study, Lott *et al*. (1978) reported that *M. synoviae* infection had no effect on eggshell strength but a Dutch strain of *M. synoviae* was observed to be responsible for the formation of egg apex abnormalities (Feberwee *et al*., 2009a). The incidence of egg apex abnormality decreased significantly with the application of a live *M. synoviae* vaccine (Feberwee *et al*., 2009b). The mechanism by which *M. synoviae* affects the normal eggshell calcification process and eggshell quality is not clear. More controlled studies are required to study the direct effects of *M. synoviae* on the fully functional oviduct of laying hens. Even though *M. synoviae* has been observed responsible for the formation of egg apex abnomalilities, there is a lack of awareness in the Australian egg industry about this infection. To complement

the studies on eggshell structure and bacterial penetration into eggs, some preliminary studies on *M. Synoviae* were undertaken.

1.11 Aim and objectives

A low level of bacterial contamination on an eggshell is important from both food safety and storage perspectives. A high number of bacteria present on the eggshell surface increases the chances of eggshell penetration and contamination of internal contents. *Enterobacteriaceae* populations are used as an indicator for judging hygienic and sanitary conditions of raw food (Mercuri and Cox, 1979). However, in Australia, very little is known about the load of *Enterobacteriaceae* on eggs and the actual prevalence of *Salmonella* on eggshell surface or in eggs collected from different layer flocks across Australia is poorly understood.

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 (Wales *et al*., 2007). 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 (Wales and Davies, 2011). 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 (El-Lethey *et al*., 2003). 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) (Humphrey, 2006). 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 the literature regarding the shedding of *Salmonella* at the initial stages of the laying period.

Egg washing can reduce the microbial load on the eggshell surface (Messens *et al*., 2011) and thus may lower the rate of penetration of *Salmonella* across the eggshell and decrease the incidence of food poisoning. However, there is little known about the effect of egg washing on eggshell penetration by *Salmonella.* Intrinsic factors that may affect egg penetration include shell porosity, shell thickness and the extent of cuticle present on the shell (Messens *et al*., 2005a). However, there is a lack of substantial literature on the relationship between translucency, eggshell ultrastructure and the penetration of bacteria.

Eggshell quality could be also affected by infectious agents such as *M. synoviae.* The possible effects of *M. synoviae* on eggshell quality are poorly understood. The present investigation was a preliminary step to investigate the prevalence of *M. synoviae* in Australian layer flocks. The study was mainly focused the association between the prevalence of *M. synoviae* in Australian layer flocks and its effects on eggshell quality.

Considering the different perspectives and literature gaps outlined in the review, specific objectives of this study were:

1) To estimate the load of *Enterobacteriaceae* on eggs collected from various layer flocks across Australia.

2) To investigate association between the indoor environmental contamination of *Salmonella* with egg contamination on layer farms.

3) To investigate the shedding of *Salmonella* in a single age caged commercial layer flock at an early stage of lay.

4) To study the effect of egg washing and correlation between eggshell characteristics and egg penetration by various *Salmonella* Typhimurium strains.

5) To study the effect of egg washing and correlation between cuticle and egg penetration by various *Salmonella* strains (*S.* Singapore, *S.* Adelaide, *S.* Worthington and *S.* Livingstone) isolated from Australian layer flocks.

6) To determine the prevalence of antibodies to *Mycoplasma synoviae* in laying hens and possible effects on eggshell quality.

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Chapter 2 Survey of *Enterobacteriaceae* contamination of table eggs collected from layer flocks in Australia

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2.1 Abstract

In the present study, eggs from 31 commercial caged layer flocks at different stages of lay in Australia were collected. *Enterobacteriaceae* populations from eggshell surface and eggshell pores were enumerated and these populations characterized using API® Rapid 20E strips. The eggshell surface, eggshell pore and egg internal content samples were also processed for the isolation of *Salmonella* and these isolates were tested for the presence or absence of several virulence genes (*prgH, sopB, spiC, orfL, invA, sifA, sitC, misL*). Results indicated that there was no significant difference in total *Enterobacteriaceae* count on the eggs of the flock from early, mid or late lay flocks. *Enterobacteriaceae* isolates were of 11 different genera which included: *Cedecea, Citrobacter, Enterobacter, Escherichia, Klebsiella, Kluyvera, Leclercia, Pantoea, Salmonella, Serratia* and *Yersinia.* Out of all 153 identified *Enterobacteriaceae* isolates, the *Escherichia* genus was reported most frequently (60.78%). Results also indicated that overall there were 4.51% (14/310) *Salmonella* positive pooled samples. In this study, 14 *Salmonella* strains were isolated, serotyping confirmed that 12 out of them were *Salmonella* Infantis and the 2 others were *Salmonella enterica* subsp. *enterica* serovar 4,12:d: Polymerase chain reaction results indicated that all *Salmonella* Infantis isolates harboured *invA, misL, orfL, prgH, sifA, sitC, sopB* and *spiC* genes which suggest that *Salmonella* Infantis strains isolated from eggshell surface may have the capacity to invade and survive in macrophages.

2.2 Introduction

Food borne illness costs Australia an estimated $1.2 billion per year (Hall *et al*., 2005). In Australia, *Salmonella* Typhimurium is the principal cause of egg-associated Salmonellosis outbreaks (The OzFoodNet Working Group, 2010). Control

of *Salmonella* shedding on farm still remains a challenge. Cage laying production systems are the major source of whole shell eggs in Australia (Chousalkar and Roberts, 2012). For the shelf life of an egg and from a food safety perspective, it is important to lower the level of bacterial contamination on eggs. Studies on microbial contamination of eggshells have been performed earlier (Musgrove *et al*., 2004; Musgrove *et al*., 2005b). It has been observed that abnormalities in eggshells (thin shells, increased shell pore numbers) can potentiate the entry of food borne pathogens into the eggs (De Reu *et al*., 2008). With an increase in number of bacteria present on the eggshell surface, the chances of eggshell penetration and contamination of internal contents by bacteria increases (Smith *et al*., 2000). There is some evidence suggesting that eggshell translucency increases the incidence of bacterial penetration (Chousalkar *et al*., 2010). At oviposition, 90% of eggs are germ free (Board, 1966). However, the eggshell can be contaminated by any surface with which egg comes in contact (Board and Tranter, 1995). Faeces, water, caging material, nesting material, insects, hands, broken eggs, dust on egg belt, blood, soil are the most common sources of eggshell contamination (Board and Tranter, 1995; Ricke *et al*., 2001). Overall, food quality and sanitary processing conditions can be judged by coliforms, *Enterobacteriaceae* and *E. coli* populations (Kornacki and Johnson, 2001; Ricke *et al*., 2001). The annual report of the OzFoodnet network (2010) reported 9,533 cases of *Salmonella* infection in Australia. It is important to study the prevalence of *Salmonella* on the eggshell surface because of increased risks of bacterial penetration into the internal contants. Also contaminated eggshells may play an important role in cross contamination in the kitchen.

Complex pathogenesis is a characteristic of *Salmonella* infections. The virulence capacity of *Salmonella* is encoded by multiple genes which are clustered together on *Salmonella* Pathogenicity Islands (SPI) (Hensel, 2004). Using the Type

III secretion system, various pathogens deliver effector proteins into the cytosol of host cells (Marcus *et al*., 2000). SPI 1 and SPI 2 encode distinct type III secretion systems. By delivering effector proteins, SPI 1 helps the *Salmonella* to penetrate the intestinal epithelium and also it induces apoptosis in macrophages (Lostroh and Lee, 2000). SPI 2 is important in systemic disease as it contains genes which are essential for survival and replication of *Salmonella* within host macrophages and epithelial cells (Shea *et al*., 1996). SPI 3 encodes the high affinity Mg^{2+} uptake system (Marcus *et al*., 2000) which is important for survival of *Salmonella* in macrophages, whereas SPI 4 is involved in secretion of toxins (Gassama-Sow *et al*., 2006). Hence, it is essential to investigate the presence or absence of virulence genes located on different SPIs. Currently, in Australia, there is limited information on the presence of virulence genes in *Salmonella* strains isolated from eggs. Keeping this perspective in mind, in the present study, visibly clean eggs collected from commercial egg farms from hens at various stages of lay were tested for the presence of *Salmonella* spp. The *Enterobacteriaceae* populations on the eggshell surface and in the eggshell pores were monitored. *Salmonella* isolates from eggs were tested for the presence of a wide range of virulence genes.

2.3 Materials and Methods

2.3.1 Collection of eggs and preparation of *Salmonella* Infantis inoculums in egg samples

Visually clean eggs (n=1860, 60 eggs from 31 flocks), collected from the cage fronts of commercial layer farms, were processed for isolation of *Salmonella* spp. from the eggshell surface, eggshell crush and internal contents. Selection of the flocks in present study was based on the willingness of the producers to participate in the study. 60 eggs were collected from each flock. De Reu *et al*. (2005a) reported that sampling of at least 40 eggs is necessary in order to get statistically reliable

results for determination of bacterial contamination of eggshell. The farms included in this study had either HyLine or Isa Brown laying hens at different stages of lay. Eggs were candled to ensure they were intact eggs without obvious cracks. All the 31 flocks were divided into three different categories based on the age of flock. Early lay (22-40 weeks), Mid lay (41-55 weeks) and Late lay (above 55 weeks) categories, including 10, 8 and 13 flock respectively.

Salmonella Infantis strain (Obtained from *Salmonella* Reference Laboratory, IMVS, Adelaide, Australia. This strain of *Salmonella* was originally isolated from the layer flock). Bacteria stored at -80°C in 50% glycerol were plated on Xylose lysine deoxycholate agar (XLD; Oxoid, Australia) and incubated overnight at 37°C. The single colony was suspended in sterile PBS and serial dilutions were prepared to achieve the concentration from 10^0 cfu/mL to 10^8 cfu/mL

Six eggs were placed in a sterile bag with Sixty mL of Sterile PBS. *Salmonella* strain was grown as described above. The samples were spiked with an appropriate quantity of *Salmonella* Infantis suspension and plated on XLD agar. Altogether 5 spiked samples were constructed in the study. For each trial set, 2 nonseeded samples were analyzed as the negative control.

2.3.2 Eggshell surface wash

Six pooled eggs were placed in 60 mL of sterile phosphate buffer saline (PBS) (Merck, Australia) in Whirl-Pak bags and rinsed by shaking for 2 min. Before rinsing, PBS was 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. Intact eggs were then stored at 4°C overnight for future use. A 100 µL of the PBS rinsate was plated on violet red bile glucose agar (Oxoid, Australia) to enumerate *Enterobacteriaceae* counts. Also, 1 mL of rinsate was transferred to 4 mL of buffered peptone water (Oxoid, Australia) for *Salmonella* isolation. Plates or

buffered peptone water was incubated at 37°C overnight. After incubation, colonies on the plates were counted.

2.3.3 Shell crush methodology

For isolation of bacteria from eggshell pores, eggshells were processed as described by Musgrove *et al*. (2005a, b). Briefly, after eggshell surface processing, each eggshell surface was dipped into 70% alcohol for 30 sec to kill any bacteria present on the outside of the shell and was allowed to air dry in a biosafety cabinet. The eggs were cracked open into a sterile container. The inside of the eggshells was then washed with sterile phosphate buffered saline to remove the adhering egg albumen because of the antimicrobial activity of albumen. Shell and shell membranes of six pooled egg samples were transferred to a sterile bag and crushed gently. To each bag, 60 mL of PBS was added. A 100 µL of PBS was plated on violet red bile glucose agar and also, 1 mL of PBS was transferred to 4 mL of buffered peptone water. Plates and buffered peptone water was incubated at 37°C overnight. After incubation, colonies on the plates were counted.

2.3.4 Egg internal contents

The egg internal contents from pooled eggs were collected in the sterile containers and were thoroughly mixed. 1 mL of egg internal content was inoculated with 4 mL of buffered peptone water for further processing.

2.3.5 Isolation and identification of *Salmonella* spp.

The isolation of *Salmonella* was carried as described earlier by Cox *et al*. (2002). To isolate *Salmonella* spp., inoculated buffered peptone water (from shell surface, shell crush and egg internal contents) was 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 the same sample was streaked on Xylose lysine deoxycholate agar (XLD; Oxoid, Australia) and

Bismuth Sulphite agar (BSA; Oxoid, Australia) plates. Presumptive *Salmonella* colonies from BSA or 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 tested by slide agglutination reaction using Poly O and Poly H antigens (BD, Australia) along with API© Rapid 20E strips (Biomerieux, Australia). Slopes of isolates were sent to the Institute of Medical and Veterinary Sciences (IMVS), Adelaide, Australia for *Salmonella* serotyping.

2.3.6 Characterization of *Enterobacteriaceae*

Inoculated buffered peptone water (eggshell surface and eggshell crush) was plated on violet red bile glucose agar. After overnight incubation at 37°C, an individual colony was selected and characterized using API© Rapid 20E strips (Biomerieux, Australia). Strips were inoculated, handled as per the manufacturer's instructions and reactions were recorded using API webplus software (Biomerieux, Australia).

2.3.7 DNA extraction and Polymerase chain reaction (PCR) for *Salmonella* Infantis typing

Salmonella Infantis isolated from eggshell wash were grown in 5 mL Brain heart infusion broth (BHI, Oxoid, Australia) overnight at 37°C with shaking. The cells were pelleted using a centrifuge at 1,500 g for 10 min. DNA was extracted and purified using Wizard® Plus Minipreps DNA purification system (Promega, Australia) as per manufacturers' instructions. The extracted DNA suspended in nuclease free water was stored at -20°C until further use. *Salmonella* Infantis isolates were tested for eight different virulence genes (Table 2.1). For PCR, each reaction mixture contained 1 X reaction buffer (Fisher Scientific, Australia), 1.8 mM $MgCl_2$,

200μM dNTPs, 1μM of each primer, 1U Taq polymerase, and 50 pg 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 5 min followed by 30 cycles of amplification (denaturation at 95°C for 30 sec, annealing temperature as per primer- for 30 sec and extension at 72°C for 1 min 30 sec), with a final extension step at 72°C for 5 minutes, followed by a holding temperature of 10°C.

The details of primers used in the PCR reactions, annealing temperature and size of amplified product are described in Table 2.1. PCR products were separated by 2% agarose gel electrophoresis in Tris-borate-EDTA (TBE) buffer. Gel red was used to visualize bands under ultra-violet light. Size of the PCR products was determined by 100 bp DNA ladder (Qiagen, Australia).

2.3.8 Statistical analysis

Statistical analysis was performed following log transformation of the bacterial counts. One way analysis of variance (ANOVA) and Kruskal Wallis test were used with Graph Pad Prism 5 software to compare bacterial counts of early, mid and late lay.

2.4 Results and Discussion

A high bacterial load present on the eggshell surface could increase the chance of eggshell penetration and contamination of internal contents (Smith *et al*., 2000). The spiking method indicated that the limit of detection for *Salmonella* Infantis by culture method was approximately 1 log CFU/mL. In the present study, shell rinse and crush methods were used to recover *Enterobacteriaceae* from commercial shell eggs as described earlier by Musgrove *et al*. (2005a, b). Limit of detection was not calculated for *Enterobacteriaceae* isolation/count from eggs. It is possible that the limit of detection of *Enterobacteriaceae* isolates from 11 different genera could be variable and further investigations are necessary to determine the

detection limits of these different genera. We found a relatively low average *Enterobacteriaceae* count on the eggshell (1.46 log CFU/eggshell), which is in agreement with De Reu *et al*. (2009) who reported 1.51 log CFU/eggshell count in eggs from furnished cages. Musgrove *et al*. (2005b) reported an average *Enterobacteriaceae* count of 2.29 log CFU/eggshell from the eggs which were collected at a commercial egg processor. The difference in the count might be due to the variation in sampling, as in the present study, eggs were directly collected from the cage front whereas, in Musgrove's study, eggs were collected from a commercial facility. Jones and Musgrove (2007) reported a higher *Enterobacteriaceae* count (3.40 log CFU/eggshell) from eggshell wash. However, the study of Jones and Musgrove (2007) was conducted on restricted shell eggs which did not meet the quality standard for retail. As bacteria can move from eggshell surface into eggshell pores and further into egg internal contents, it is important to study the bacterial count in eggshell pores. In this current study, the average *Enterobacteriaceae* count in eggshell pores was 0.34 log CFU/eggshell. In the present study, we did not find any significant difference in *Enterobacteriaceae* count (on eggshell and in shell pore) across early, mid and late lay (Table 2.2). There is a dearth of literature regarding *Enterobacteriaceae* counts in eggshell pores or effect of flock age on *Enterobacteriaceae* count on eggs, which precludes comparison of our findings with those of other workers. However, there are number of studies which have investigated the effect of flock age on bacterial contamination of eggshells. De Reu *et al*. (2005) and Protais *et al*. (2003) reported that there was no significant difference in eggshell contamination between beginning and end of the laying period in furnished cages or aviaries. Huneau-Salaun *et al*. (2010) found that eggshell contamination increased significantly with increasing age of flock, but Mallet *et al*. (2003) reported that contamination decreased with age. However, both of these

authors attributed the variation in their results to seasonal or environmental effects rather than flock age.

API Rapid 20E was used to identify the various members of *Enterobacteriaceae* at genus and species level. Identified isolates belonged to 11 different genera which included: *Cedecea, Citrobacter, Enterobacter, Escherichia, Klebsiella, Kluyvera, Leclercia, Pantoea, Salmonella, Serratia* and *Yersinia* (Table 2.3). Out of all isolates identified, isolates from *Escherichia* genus were reported most frequently (60.78%), followed by *Salmonella* (9.15%), *Enterobacter* (8.49%) and *Serratia* (5.22%) (Figure 2.1). All other genera were identified less frequently (< 5%). Isolates reported at least once were: *Cedecea* spp., *Citrobacter freundii, Enterobacter carcinogenous, Enterobacter* spp., *Escherichia coli, Escherchia fergusonii, Klebsiella pneumonia, Kluyvera* spp., *Leclercia adecarboxylata, Pantoea* spp., *Pantoea* spp. 2, *Pantoea* spp. 3, *Salmonella* Infantis, *Salmonella enterica* subsp. *enterica* serovar 4,12:d:, *Serratia odorifera, Serratia plymuthica, Serratia* spp., *Yersinia enterocolitica.* Also, three *Escherichia coli* isolates were identified from eggshell pores. Our findings regarding the presence of various genera of *Enterobacteriaceae* on eggshells are in agreement with Musgrove *et al*. (2004) who also reported *Escherichia, Salmonella, Enterobacter, Serratia, Yersinia, Klebsiella, Pantoea, Kluyvera and Citrobacter* on eggshell surface. However, *Cedecea* spp. and *Leclercia adecarboxylata* were not reported in their study. Out of these, *Leclercia adecarboxylata* is infrequently isolated from eggshells. This microorganism was rarely reported in humans (Hess *et al*., 2008). There are very few reports of *Cedecea* isolation from eggshells and the clinical significance of *Cedecea* is not fully understood (Abate *et al*., 2011). In the present study, out of all *Enterobacteriaceae* genus reported, *Escherichia* was reported most frequently. This

finding is in agreement with earlier reports by Musgrove *et al*. (2004) and Stępień-Pyśniak (2010).

In the present study, from a total of 310 pooled eggshell wash samples, fourteen pooled eggshell wash samples were found positive for *Salmonella.* Serotyping confirmed that twelve samples were *Salmonella* Infantis whereas two were *Salmonella enterica* subsp. *enterica* serovar 4,12:d: isolates. All of the eggshell pore and egg internal content samples were negative for *Salmonella.* Overall there were 4.51% (14/310) *Salmonella* positive pooled samples. Similar findings were reported by Stępień-Pyśniak (2010) who reported 3.2% prevalence of *Salmonella* on eggshells. Cox *et al*. (1973) reported that less than 10% of the eggshells were contaminated in hens individually artificially infected with *Salmonella enterica* serovars Senftenberg, Thompson and Typhimurium, whereas Jones *et al*. (1995) reported that 7.8% of the eggshells from eggs sampled before processing were contaminated. In all these three studies, eggs were processed individually for *Salmonella* isolation. In the present study, *Salmonella* spp. were not detected in any of the egg internal contents. This finding is in agreement with Daughtry *et al*. (2005) who undertook a microbiological survey of commercial eggs in Australia to determine the prevalence of *Salmonella* contamination. During Daughtry's study, *Salmonella* spp. was not isolated from the internal contents of any of the 20,000 eggs sampled.

All of the *Salmonella* Infantis isolates from the present study possessed *invA, misL, orfL, prgH, sifA, sitC, sopB* and *spiC* virulence genes. These genes play an important role in invasion of macrophages and are also essential for survival of *Salmonella* within macrophages. Out of these genes, *prgH, sopB, invA* and *sitC* are located on *Salmonella* pathogenicity island (SPI) 1 whereas *spiC and sifA* are located on SPI-2. The *misL* and *orfL* genes are located on SPI-3 and SPI 4 respectively

(Skyberg *et al*., 2006; Gassama-Sow *et al*., 2006). SPI 1, SPI 2 and SPI 3 play an important role in invasion of macrophages and survival of *Salmonella* within macrophages (Hughes *et al*., 2008) whereas SPI 4 is involved in secretion of toxins (Gassama-Sow *et al*., 2006) and survival of *Salmonella* in macrophages. For *Salmonella* Typhimurium, SPI 1 and SPI 2 are important in causing systemic and gastrointestinal tract infection in young chicks (Jones *et al*., 2007). *Salmonella* Infantis isolates in the present study possessed genes located on SPI 1 (*prgH, sopB, invA, sitC*) and SPI 2 (*spiC, sifA*) which suggest that these isolates may have the capacity to cause systemic and gastrointestinal infection in day old chicks. However, further animal trials are essential to confirm these findings. PCR results also indicated that all the *Salmonella* Infantis isolates possessed *orfL* and *misL* genes which are involved in survival of *Salmonella* in macrophages. However it is essential to note that possession of a single or a few virulence genes does not endow a strain with pathogenic/virulent status unless that strain has acquired the appropriate virulence gene cluster to cause disease in a specific host species (Gilmore and Ferretti, 2003). *Salmonella* Infantis strains isolated from eggshells may have capacity to invade and survive in macrophages. However, further studies such as a macrophage invasion assay are essential to confirm these findings.

Even though, in the present study, the prevalence of *Salmonella* on eggshells was low, proper handlings of eggs in the kitchen is essential as improper handling may cause cross contamination of other food materials leading to food poisoning outbreaks (Slinko *et al*. 2009). It is essential to adopt safe food handling practises in the food service sector, so that cross contamination can be avoided which will, potentially, reduce the risks of *Salmonella* food poisoning cases.

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Table 2.1 Details of the genes used for *Salmonella* typing

Gene	Function	Forward Primer (F) (5' - 3') and Reverse Primer (R) (5' - 3')	Annealing temperature	Product Size	Reference
prgH	Invasion of macrophages	F-GCCCGAGCAGCCTGAGAAGTTAGAAA R-TGAAATGAGCGCCCCTTGAGCCAGTC	55°C	755 bp	Hughes *et al.* (2008)
sopB	Invasion of macrophages	F-GAAGACTACCAGGCGCACTT R-TTGTGGATGTCCACGGTGAG	55°C	804 bp	This study
InvA	Invasion of macrophages	F-CTGGCGGTGGGTTTTGTTGTCTTCTCTATT R-AGTTTCTCCCCCTCTTCATGCGTTACCC	60°C	1062 bp	Hughes *et al.* (2008)
sitC	Invasion of macrophages/ iron acquisition	F-CAGTATATGCTCAACGCGATGTGGGTCTCC R-CGGGGCGAAAATAAAGGCTGTGATGAAC	64°C	740 bp	Hughes *et al.* (2008)
spiC	Survival in macrophages	F-CCTGGATAATGACTATTGAT R-AGTTTATGGTGATTGCGTAT	56°C	300 bp	Hughes *et al.* (2008)
sifA	Survival in macrophages	F-TTTGCCGAACGCGCCCCCACACG R-GTTGCCTTTTCTTGCGCTTTCCACCCATCT	62°C	448 bp	Hughes *et al.* (2008)
misL	Survival in macrophages	F-GTCGGCGAATGCCGCGAATA R-GCGCTGTTAACGCTAATAGT	58°C	540 bp	Hughes *et al.* (2008)
orf L	Survival in macrophages/ colonisation	F-GGAGTATCGATAAAGATGTT R-GCGCGTAACGTCAGAATCAA	56°C	331 bp	Hughes *et al.* (2008)

Table 2.2 Total *Enterobacteriaceae* count (log CFU/eggshell) at different stages of lay

Bacterial count	Early lay	Mid lay	Late lay
Total *Enterobacteriaceae* count (shell surface)	1.78±0.33	1.46±0.22	1.22±0.15
Total *Enterobacteriaceae* count (shell pore)	0.32±0.17	0.44±0.28	0.29±0.10

Mean ± SEM

Table 2.3 *Enterobacteriaceae* isolated from egg

Flock Number	Age (weeks)	*Enterobacteriaceae* Reported (Number isolated)
1	22	*Yersinia enterocolitica* (2), *Klebsiella pneumoniae* (1), E. *coli* 1 (1)
2	24	*Serratia plymuthica* (1), *E.* coli1 (1)
3	24	*E. coli* 1 (8), *Klebsiella pneumoniae* (1)
4	26	*Serratia plymuthica* (1)
5	27	*E. coli* 1 (1), *Klebsiella pneumoniae* (1),
6	27	*E. coli* 1 (10)
7	30	*E. coli* 1 (8), *E. coli* 2 (2), *Salmonella* Infantis (1)
8	30	*E. coli* 1 (1), *E. coli* 2 (1)
9	31	*Enterobacter* (2), *Kluyvera* spp. (1), *Serratia* spp. (1), *E. coli* 1 (2), *Leclercia adecarboxylata* (1)
10	34	*E. coli* (3), *Enterobacter carcinogenous* (1), *Serratia odorifera* (1)
11	41	*E. coli 1* (5), *E. coli* 2 (2), *E. fergusonii* (1), *Klebsiella pneumonia* (1), *Salmonella* Infantis (1)
12	45	*E. coli 1* (2), *Pantoea* spp. 3 (1), *Pantoea* spp. 2 (1)
13	45	*E. coli* 1 (1)
14	46	*E. coli* 1 (8), *E. fergusonii* (1), *Salmonella* Infantis (1)
15	50	-
16	52	*Kluyvera* (1), *Klebsiella pneumonia* (1), *E. coli* 1 (2), *Salmonella* Infantis (2)
17	53	*Serratia plymuthica* (1), *Pantoea* spp. (1)
18	55	*E. coli* 1 (1)
19	56	*E. coli* 1 (5), *Pantoea* spp. (1), *E. coli 1* (2)
20	58	*E. coli* 1 (5), *Escherichia* genus (1), *Serratia odorifera* (1), *Enterobacter cloacae* (2), *Salmonella* Infantis (4)
21	59	*Yersinia entercolitica* (1), *Serratia plymuthica* (1), *Kluyvera* spp. (1)
22	62	*Cedecea* spp. (1), *E. coli 1* (1), *Citrobacter freundii* (1), *Enterobacter* (2)
23	63	*E. coli* 1 (1)
24	64	*Leclercia adecarboxylata* (1), *E. coli* 2 (1), *Enterobacter cloacae* (1)
25	66	*Enterobacter* (1), *E. fergusonii* (1), *E. coli* 1 (3), *E. coli* 2 (1), *Salmonella* Infantis (1)
26	66	*Yersinia entercolitica* (2), *E. coli* 1 (1)
27	66	*E. coli* 1 (1)
28	68	*Enterobacter cloacae* (1), *E. coli* 1 (5), *E. coli* 1 (1)
29	72	*Pantoea* spp. 3 (1), *Pantoea* spp. 1 (1), *Yersinia entercolitica* (1), *Enterobacter cloacae* (1)
30	74	*Leclercia adecarboxylata* (1), *Salmonella* Infantis (2), *Salmonella enterica* subsp. *enterica* serovar 4,12:d: (2)
31	80	*E. coli* 1 (3), *Enterobacter cloacae* (1), *Enterobacter aerogenes* (1), *Serratia odorifera* (1)

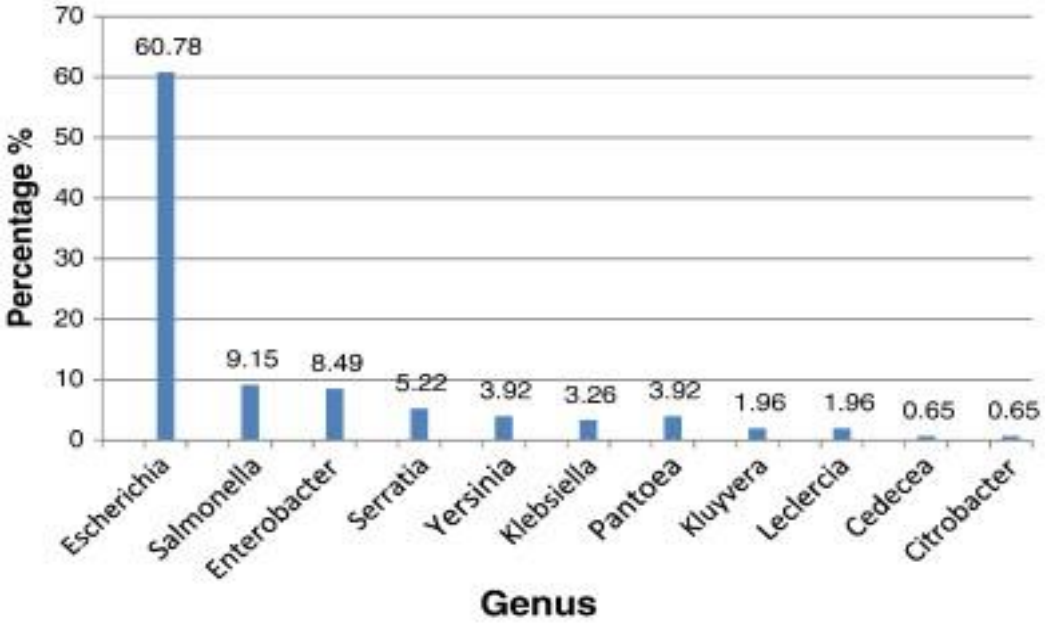

Figure 2.1 Percentage of different genus isolated from egg

Chapter 3 Association between the indoor environmental contamination of *Salmonella* with egg contamination on layer farms

Statement of Authorship

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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Vaibhav C. Gole		
Contribution to the Paper	Performed the experiment, compiled, analyzed and interpreted data, wrote manuscript, responded to editing suggestions by co-authors		
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Author Contributions

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Contribution to the Paper	Performed the experiment, compiled, analyzed and interpreted data, wrote manuscript, responded to editing suggestion by co-authors.		
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Contribution to the Paper	Assisted in analysis and interpreting the complex data, edited the manuscript.		
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3.1 Abstract

The current study involved longitudinal and point in time surveys of *Salmonella* carriage and environmental contamination on two commercial cage layer farms positive for *Salmonella* Typhimurium and associated with tracebacks from human salmonellosis cases (flock A age = 32 weeks; and flock B age = 34 weeks). *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. Agreement between culture based methods and real-time polymerase chain reaction on pre-enriched broths 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). One log increase in the load of *Salmonella* detected in faecal, egg belt and floor dust samples resulted in 35%, 43% and 45% increase ($p<0.001$) in the odds of an eggshell testing positive for *Salmonella* respectively**.** Multi-locus variable number tandem repeat analysis (MLVA) of *S.* Typhimurium strains isolated from flock A were distinct from flock B. *S.* Typhimurium strains detected from human food poisoning cases exhibited similar MLVA pattern to the strains isolated from flock A and B.

3.2 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 (The OzFoodNet Working Group, 2012). A very low dose of *Salmonella*, 10 to 20 colony forming unit

(CFU), can cause human salmonellosis (Kapperud *et al*., 1990; Vought and Tatini, 1998). 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 (The OzFoodNet Working Group, 2012).

Although *S.* Enteritidis is associated with the majority of egg related outbreaks of human salmonellosis occurring in the European Union (77.2%) (EFSA, 2010), it is not endemic to Australian layer flocks (Sergeant, 2003). Instead, *Salmonella* Typhimurium (*S.* Typhimurium) was the most frequently reported serovar in the 21 egg related food poisoning outbreaks in Australia in 2010 (The OzFoodNet Working Group, 2012). In Australia, a study showed that *Salmonella* Infantis (*S.* Infantis) was the most frequently reported serovar from egg shell wash of eggs collected from 31 flocks (Gole *et al*., 2013). 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 (The OzFoodNet Working Group, 2012).

Residual contamination of the environment with *Salmonella* is a major problem in commercial layer farms (Van de Giessen *et al*., 1994; Davies and Breslin, 2003; Gradel *et al*., 2004). Davies and Breslin (2001) 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.* (2009) investigated the prevalence of *Salmonella* on eggshells in infected layer flocks, whereas Wales *et al*. (2007) 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 (Wales *et al*., 2007). However, cooperation from egg producers over a period of months or years and the requirement of resources are limiting factor to such studies (Wales *et al*., 2007). There are a few reports in which the levels of *Salmonella* contamination in laying houses and hens were examined over time during lay (Davison *et al*., 1999; Kinde *et al*., 2005; Wales *et al*., 2007). 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 (Wales and Davies, 2011).

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.

3.3 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 cages were selected for longitudinal study and the association between eggs and environmental *Salmonella* contamination was investigated.

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

Two commercial layer conventional cage sheds were selected from two different farms with a history of *Salmonella* infection. The study sheds, Shed A (from Farm A) and Shed B (from Farm B), included multi-aged flocks with each age-class housed in separate rows and only a single age-class flock was selected for sampling in each shed. In Shed A, the selected flock included 1,320 cages of 32-week old birds (5 birds per cage for an approximate total of 6,600 birds), while the selected flock in Shed B included 1,300 cages of 34-week old birds (5 birds per cage for an approximate total of 6,500 birds). To ensure that at least several cages positive for *Salmonella* would be identified, a representative sample size of 78 cages per flock was targeted. Accounting for field constraints, two adjacent cages were selected at equal intervals along the three lowest tiers (tier 1, 2, and 3 respectively) out of the five tiers. Appendix 2 Figure 1a and 1b shows the layout of layer shed along with sample collection areas.

Composite faecal samples were collected in sterile Whirl-Pak plastic bag (150 × 230 mm, ThermoFisher Scientific, Australia) from manure belts of 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. For isolation of *Salmonella* spp., the faecal samples were inoculated in 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 Soya peptone (RVS) broth (Oxoid, Australia) which was then incubated at 42°C for 24 h. A loopful of the incubated RVS broth was streaked onto Brilliance *Salmonella* agar (BSA, Oxoid Australia) and Xylose lysine deoxycholate agar (XLD, Oxoid, Australia) plates. 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 (ONPG, 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.

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

Based on *Salmonella* typing results, five *Salmonella* positive cages each from farm A (*S*. Typhimurium PT 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).

3.3.3 Environmental and egg sampling

A composite faecal sample (one per study cage, n=7) was collected in a sterile Whirl-pak plastic bag (150 X 230 mm, Thermo Fisher Scientific, Australia) from underneath the individual selected cages in each flock. 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 faecal sample collection. Egg belt samples (one per study cage, n=7) were collected from the front

of the cage. 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 the whole area in front of individual cages. During each sampling period, five dust samples were collected from different parts of the poultry shed and from the floor near to the selected cages for longitudinal study. Dust (n=5) was collected in gamma irradiated sterile containers (Pacific Laboratory Products, Australia).

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.

3.3.4 Sample processing for *Salmonella* isolation

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

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

After serotyping, all *Salmonella* strains which were identified as a Typhimurium were further analysed by MLVA, as described by Ross *et al.* (2009), at *Salmonella* Reference Laboratory, Adelaide, Australia.

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

Total nucleic acid was extracted from samples using a modification (Torok *et al*., 2008) of a South Australian Research and Development Institute (SARDI, Adelaide, Australia) proprietary method (Stirling *et al*., 2004). All samples had been subjected to pre-enrichment in BPW; 2 gms of faeces or dust samples in 8 mL BPW, egg belt swabs in 25 mL BPW, and six pooled individual eggshell washes in 10 mL BPW each. Following incubation at 37°C overnight, the incubated broths were frozen and freeze-dried. Ten mL of extraction buffer (Torok *et al*., 2008) was added to the freeze dried samples and incubated at 70°C for an hour before proceeding with the SARDI proprietary extraction method. The qPCR detection of *Salmonella* was done using the TaqMan *Salmonella enterica* detection kit system (Applied Biosystems, Australia) in a total reaction volume of 20 µL containing 8 µL sample, 10 µL of 2 x Environmental Master Mix and 2 µL of 10 x Target Assay Mix. All real-time PCR 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). 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 Ct (cycle threshold) 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 was used to exclude detection of false positives. A Ct of 34 corresponded to 200 CFU of *Salmonella*.

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

Two multiplex PCR reactions, one for the detection of *S*. Typhimurium and the other for *S.* Infantis, were performed using the primers published by Akiba *et al.* (2011). Primer sequences and the expected amplification product sizes are shown in Appendix 1, Table 1. The multiplex PCR assays were used on various *Salmonella* serovars isolated from the Australian layer industry to confirm the specificity of the assays (Appendix 1, Table 2). For the *S*. Typhimurium multiplex PCR, each reaction mixture contained 1 x PCR buffer II (Applied Biosystems, Australia), 2.5 mM $MgCl_2$, 1.6 mM dNTPs (Invitrogen, Australia), 0.5 μM each of InvAF, InvAR, TMP2F, TMP2R primers, 0.3 μM each of TMP1F, TMP1R, TMP3F, TMP3R primers, 1 U of AmpliTaq DNA polymerase LD (Applied Biosystems, Australia) and 5 μL DNA template in a reaction volume of 20 μl. For the *S*. Infantis multiplex PCR, each reaction mixture contained 1 x PCR buffer II (Applied Biosystems, Australia), 2.5 mM $MgCl_2$, 1.6 mM dNTPs, 0.5 μM each of InvAF, InvAR, IMP3F, IMP3R primers, 0.3 μM each of IMP1F, IMP1R, IMP2F, IMP2R primers, 1 U of AmpliTaq DNA polymerase LD (Applied Biosystems, Australia), and 5 μL DNA template in a reaction volume of 20 μl. Samples were amplified in a MJ Research PTC-225 Peltier thermal cycler (GeneWorks, Adelaide, Australia) with an initial denaturation step at 95°C for 2 min followed by 35 cycles of amplification (denaturation at 95°C for 10 s, annealing temperature 60°C for 30 s and extension at 72°C for 30 s), with a final extension step at 72°C for 10 min. PCR products were separated by 2% agarose gel electrophoresis in Tris-Acetate-EDTA (TAE) buffer,

stained with GelRed (Jomar Diagnostic, Australia) and visualized under ultra-violet light.

In order to determine the limit of detection for each multiplex PCR assay, faecal samples were spiked with various concentrations (10^8 CFU/mL to 10^2 CFU/mL) of the corresponding *Salmonella* serovar (*S.* Typhimurium or *S*. Infantis). DNA was extracted from *Salmonella* spiked faecal samples and the multiplex PCRs were performed as mentioned above. The limit of detection was determined by running the PCR products on 2% agarose gel.

3.3.8 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 real-time PCR. 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.

3.4 Results

3.4.1 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.

3.4.2 *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 Table 3.1 and 3.2, respectively. Figure 3.1 and 3.2 shows the prevalence of *Salmonella* in different type of samples in flock A and B respectively. In both flocks, the *Salmonella* prevalence was higher in dust samples compared to egg belt, faeces and eggshells. In flock A, the prevalence of *Salmonella* was highest in dust samples (26%) followed by faeces (7.14%) and egg belt (7.14%). Similarly, in flock B, the *Salmonella* the prevalence of *Salmonella* was highest in dust (58%) followed by egg belt (50%) and faeces samples (32.86%) respectively. In flock B, from 6th sampling (58 week onwards), there was an increase in prevalence of *Salmonella* in all types of samples but it was highest in the dust samples. It was observed that there was a higher fluctuation in *Salmonella* contamination of faeces compared to the dust and egg belt samples in flock B.

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. 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).

3.4.3 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 multi-factorial 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).

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

The TaqMan *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) 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 or 25 CFU/µL extracted nucleic acid template. When cut-off Ct of 34 was used (CFU greater than 200 per PCR reaction), 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 (Appendix 1, Table 3).

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 3.3 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, $p<0.001$).

3.4.5 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 (Appendix 1, Table 2). 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 in case 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 (Appendix 1, Table 2). 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.

3.5 Discussion

The current study involved cross sectional and longitudinal 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, from combined data from two flocks, 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 (McDermid and Lever, 1996). A low dose of *S*. Typhimurium DT 104 infection (2×10^2 or 2×10^4 CFU per bird) resulted in increased *Salmonella* contamination of eggs (Leach *et al*., 1999). In the present study, qPCR results indicated that the level of *Salmonella* in dust samples peaked up to 5 log 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 (Wales *et al*., 2007). 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 (Carriquemas and Davies, 2008).

The prevalence of *Salmonella*, in both flock A and B, increased during the latter 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, resulting in an increased shedding of *Salmonella*. However, further studies are essential to confirm stress induced *Salmonella* shedding.

Flock A moulted at the 67th week of lay. In the following week (68), it was observed that shedding of *Salmonella* in faeces increased and subsequently contamination of dust was also increased. As a result of moulting, eggs could not be obtained for *Salmonella* isolation in 68th week. However, in the 72nd week, one eggshell was reported *Salmonella* positive. Moulting along with immunosuppression can alter gut microbiota and physiology, and these changes may influence the host-pathogen relationship (Golden *et al*., 2008). Holt (2003) reported that induced moulting resulted in higher shedding of *Salmonella* Enteritidis in faeces and increased colonization of internal organs and a higher number of *S.* Enteritidis positive eggs were produced within the first 5 weeks after moulting (Holt, 2003).

Out of all eggs tested, only 4% (20/521) of the eggshells were reported *Salmonella* positive. The serovars which were observed on the positive eggshells were the same as those detected from farm environmental samples. However, all of the egg internal contents were *Salmonella* negative. These findings are in agreement with our previous survey (Gole *et al*., 2013), where all egg internal contents were *Salmonella* negative. Egg penetration experiment have demonstrated that *S.* Worthington has the capacity to penetrate across the eggshell but lacks the ability to survive in the egg internal contents (Gole *et al*., 2014b), whereas *S*. Typhimurium has the capacity to penetrate and survive in egg internal contents at 20°C (Gole *et al*., 2014a). There is a lack of reliable information regarding the ability of *S.*

Typhimurium to transmit vertically. In the present experiment, even though chickens were positive for *S*. Typhimurium, egg internal contents were *Salmonella* negative.

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. However, in Germany a large chocolate related outbreak of this serovar was reported in 2005 (Werber *et al*., 2005).

The statistical modelling used in this study suggested that *Salmonella* positive faecal, egg belt, and dust samples 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 suggests that faecal contamination of *Salmonella* is the most important factor for the production of *Salmonella* positive eggshells. The qPCR results presented in this study however demonstrated that a one log increase in the load of *Salmonella* detected in faecal, egg belt and dust samples resulted in 35%, 43% and 45% increases in the odds of an eggshell testing positive for *Salmonella*, respectively.

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, 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 (Jensen *et al*., 2013). 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. 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.

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 (Heuzenroeder *et al*., 2013), this variation was not significant to call them as unrelated or distinct *S.* Typhimurium isolates. A quarterly report released from the 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 (Australian *Salmonella* Reference Centre, 2013). 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:-.

Multiplex PCR assays were used to identify *S*. Typhimurium and *S*. Infantis from samples which had been identified as *Salmonella enterica* positive by qPCR. All the samples were negative for *S*. Infantis, which is in agreement with the microbiological culture based results. Of the 87 qPCR positive samples further analysed by *S*. Typhimurium multiplex PCR, six potential *S*. Typhimurium positives were identified. Of these only one sample had a positive *S*. Typhimurium strain isolated by culturing, while the others were positive for *S*. Oranienburg or *S*. Worthington. Four samples known to be positive for *S*. Typhimurium by culturing did not test positive by multiplex PCR. These conflicting 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 the multiplex PCR assay for *S*. Typhimurium was 2,000 CFU/reaction. This may explain why know positive samples identified by culturing did not test positive by multiplex PCR. These findings are in agreement with previous experiment of Soumet *et al*. (1999) who also reported a poor sensitivity (10^7 CFU/mL) of multiplex PCR for the samples (obtained from poultry houses) pre-enriched in BPW. Furthermore, in the present study, when testing pure isolates of a range of *Salmonella*, noted that *Salmonella* subsp.1. ser. 4, 5, 12:-:- was amplified by all three *S*. Typhimurium primer pairs in multiplex PCR and hence were 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*. (2011). 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 (Le Hello *et al*., 2012). The present study is the first report of non-motile variant of *S*. Typhimurium in Australian laying flocks. 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 moulting 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. The culture method was more sensitive than multiplex PCR for the detection of *S.* Typhimurium. 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*. Moulting and the transition into egg production represent significant shifts in physiological and immunological equillibria which are associated with significant increases in *Salmonella* shedding.

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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 flock A and B

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 from flock A and B

Sample type	Samples identified by culture method	Samples identified by Real-time PCR			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		

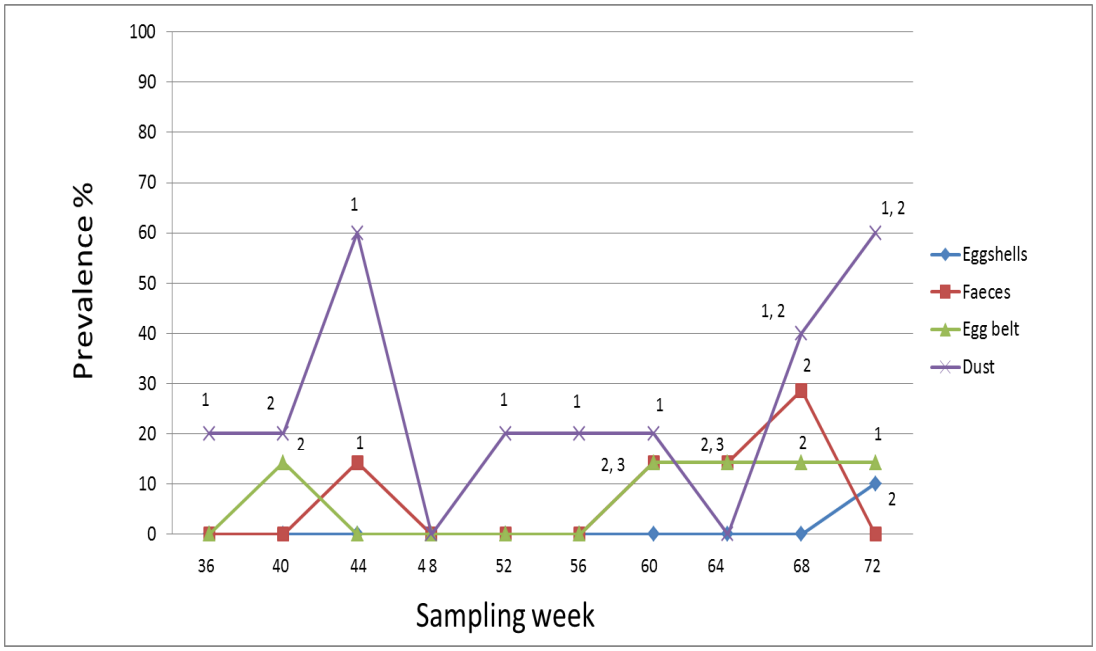

Figure 3.1 Percent prevalence of *Salmonella* in different type of samples over period of 10 samplings in flock A
1: *Salmonella* Typhimurium PT 9
2: *Salmonella* Worthington
3: *Salmonella* subsp.1 ser 4,5,12:-:-

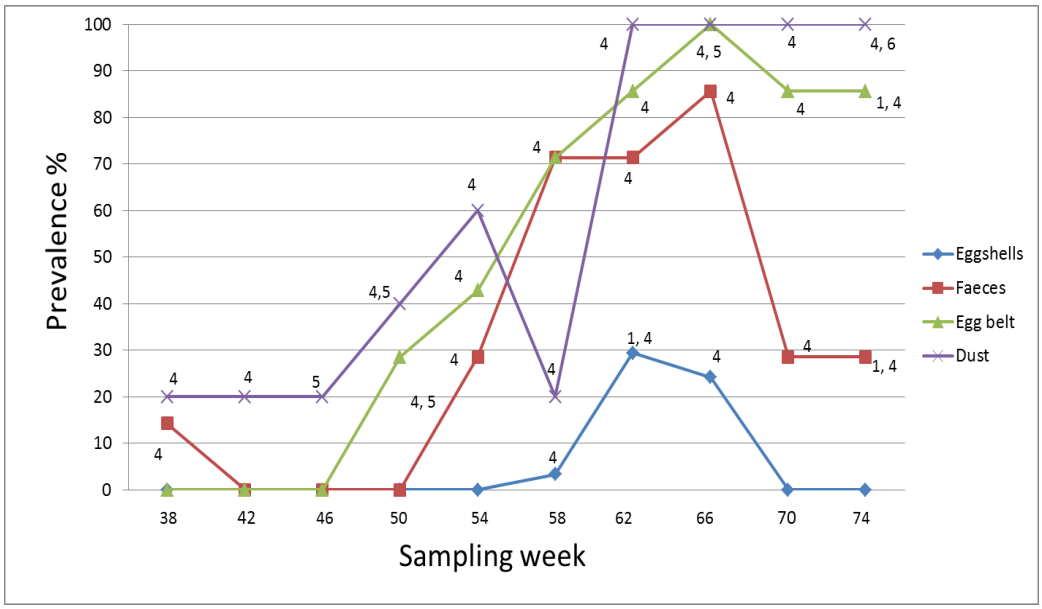

Figure 3.2 Percent prevalence of *Salmonella* in different type of samples over period of 10 samplings in flock B
1: *Salmonella* Typhimurium PT 9
4: *Salmonella* Oranienburg
5: *Salmonella* Agona
6: *Salmonella* subsp.1 ser rough g,s,t:-

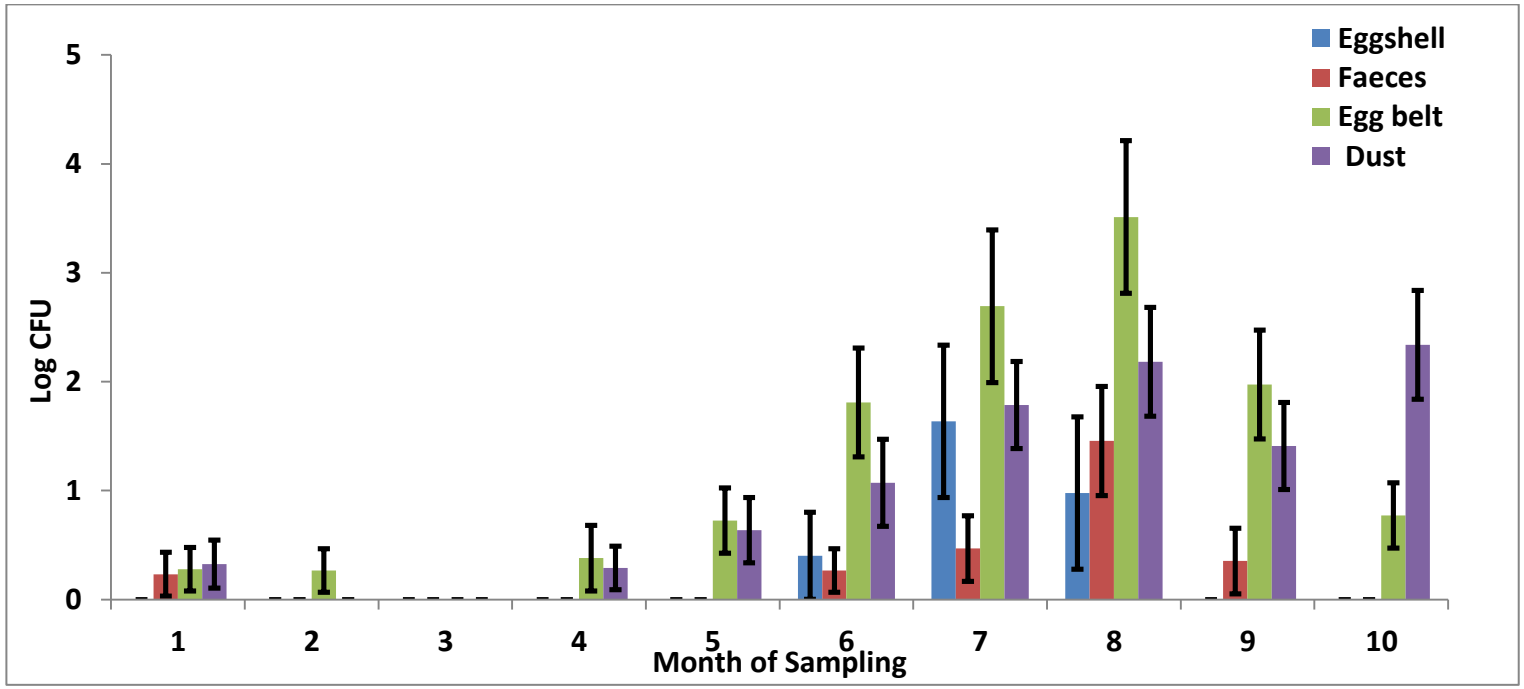

Figure 3.3 The load of *Salmonella* (average log colony forming unit (CFU)/qPCR reaction) in faeces, egg belt, dust and eggshells over period of 10 months

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

Statement of Authorship

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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

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Contribution to the Paper	Performed the experiment, compiled, analyzed and interpreted data, wrote manuscript, responded to editing suggestions by co-authors.		
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Contribution to the Paper	Supervised the development of experiment, Liaised with egg producers for obtaining required samples, assisted in sample collection & performing experiment, edited the manuscript.		
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Contribution to the Paper	Assisted in analysis and interpreting the complex data, edited the manuscript.		
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Contribution to the Paper	Performed experiment, compiled, analyzed and interpreted data, wrote manuscript, responded to editing suggestions by co-authors		
Signature		Date	6/6/14

Name of Co-Author	Chelsea Fowler		
Contribution to the Paper	Assisted in sample processing & preparing farm layout, edited the manuscript.		
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Signature		Date	

4.1 Abstract

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. At the age of 18 weeks, when the first sampling was performed, the prevalence of *Salmonella* in faeces was 82.14%. However, in later samplings, at the age of 24 and 30 weeks, the prevalence of *Salmonella* in faeces was significantly reduced ($p<0.001$) to 38.88% and 12.95% respectively. 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%), *S.* Infantis (0.8%). One of the objective of this study was to compare efficacy of the two methods ie culture method and real-time PCR in detecting *Salmonella* positive samples. The observed agreement between culture method and real-time PCR 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 real-time PCR for detecting *Salmonella* positive shoe cover and faecal samples was, however, moderate (80%) and low (54.27%) respectively. Real-time PCR 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.

4.2 Introduction

The genus *Salmonella* is a member of *Enterobacteriaceae* family. *Salmonella* is a rod shaped Gram negative, non-spore forming bacteria. In Australia, the rate of *Salmonella* infection has been reported to be 53.7 cases per 100,000 people in 2010 and eggs are often implicated as a major source of *Salmonella* infection (The

OzFoodNet Working Group, 2012). At oviposition, 90% of eggs are free of bacteria (Board, 1966). The eggshell surface can, however, become contaminated during contact with any surface. Furthermore, the contamination of eggs with *Salmonella* on layer farm could be variable. The risk assessment analysis performed by Ebel and Schlosser (2000) indicated that the rate of egg contamination by *Salmonella* Enteritidis was 1 in 20,000 eggs. Even though, the incidence of *Salmonella* egg contamination is very low, the numbers of eggs produced/consumed per year contributes to the large number of *Salmonella* contaminated eggs entering the consumer market. This sporadic rate of egg contamination could affect the trace back of egg related *Salmonella* food poisoning outbreaks. The presence of *Salmonella* in layer flock can be identified with the help of environmental sampling (Holt *et al*., 2011). The present infection status of the flock can be better indicated by faecal samples whereas dust samples may indicate prior *Salmonella* infection (Carrique-Mas and Davies, 2008).

During the laying production cycle, birds could experience various stressful events. It was observed that stress can impair humoral and cell mediated immune response of the birds (El-Lethey *et al*., 2003). 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 increase in the shedding of *Salmonella* in faeces could increase the chances of eggshell contamination (Gole et al., 2014 Unpublished). 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) (Humphrey, 2006). 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 no

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 (Edwards, 2011). 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*. (1997) 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. 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 (Uyttendaele *et al*., 2003). The use of a real-time polymerase chain reaction (RT-PCR) 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 real-time PCR to detect *Salmonella* positive samples.

4.3 Materials and methods

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 conventional 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.

4.3.1 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) as these cages were closer to exhaust fans. 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), based on the results of culture method, 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). Rows selected for egg sampling belonged to low and high traffic areas to investigate the difference the *Salmonella* prevalence in egg in these areas. Also, processing egg samples for *Salmonella* isolation is very time consuming as well as laborious. Hence, we have to restrict egg sampling to tiers belonging to two rows (one from low and high traffic areas each) and not all three. 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.

4.3.2 Isolation of *Salmonella* from different samples using culture method

For isolation of *Salmonella* spp., the 2 gm of faecal, feed and dust samples were inoculated in BPW (1:4) (Cox *et al*., 2002). The inoculated samples were incubated at 37°C overnight and 100 µL of this sample was transferred into Rappaport Vasidalis Soya peptone broth (RVS, Oxoid, Australia) which was then incubated at 42°C for 24 h. A loopful of the incubated RVS broth was streaked onto

Brilliance *Salmonella* agar (BSA, Oxoid Australia) and xylose lysine deoxycholate agar (XLD, Oxoid, Australia) plates. 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.

In the laboratory, egg belt samples were again (to avoid drying of swabs) moistened with BPW and processed for *Salmonella* isolation as mentioned above. Eggshell and egg internal content samples were individually processed. Individual sample (a pool of six eggs) was placed in 60 mL of sterile BPW in Whirl-Pak bags and rinsed by massaging for 4 min. Before rinsing, BPW was pre-warmed to 37°C to facilitate bacterial recovery. After a rinse sample was obtained, each pool was removed and transferred to a new sterile bag. The egg internal contents, collected in sterile containers, were thoroughly mixed and 2 mL of egg internal content per pool was inoculated into 8 mL of BPW. The inoculated BPW were further processed for *Salmonella* isolation as mentioned above.

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

4.3.3.1 DNA extraction from pre-enriched samples

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. Cells were resuspended in 600 µL nuclei lysis solution followed by incubation at 80°C for 5 min. Samples were allowed to cool down at room temperature and 3 µL of RNase solution was added to the cell lysate. Samples were vortexed and incubated at 37°C for 30 min. To the RNase treated cell lysate, a 200 µL protein precipitation solution was added, followed by centrifugation at 14000 g for 3 min. The supernatant containing was transferred to a clean 1.5 mL microcentrifuge tube with 600 µL of isopropanol and centrifuged at 14000 g for 2 min. The DNA pellet was further washed with 600 µL of 70% ethanol. A 100 µL of DNA rehydration solution was finally added to microcentrifuge tube to rehydrate DNA by incubating tubes at 4°C overnight. The concentration of DNA in sample was determined using a spectrophotometer (Nano drop ND 1000, Biolab, Australia). Further dilution was performed using nuclease free water to achieve final 5 ng/µL DNA concentration. Finally, these diluted DNA samples were used in real-time PCR.

4.3.3.2 The DNA extraction from raw faecal samples

DNA was also extracted directly from faecal samples using QIAamp DNA Stool Mini Kit (Qiagen, Australia) as per manufacturer instructions. A 0.2 gm faecal sample, was weighed and dispensed into microcentrifuge tubes containing 2 mL ASL buffer. The samples were vortexed and heated in a 70°C water bath for five minutes. The samples were centrifuged at 4800 x g for 10 min and 120 µL of the superannuant was transferred to another clean microcentrifuge tube containing an Inhibitex tablet (Qiagen, Australia). The samples were vortexed and stored at room temperature for 1 min. The samples were then centrifuged at 4800 x g for 10 min and 200 µL of resulting supernatant was treated with 15 µL of proteinase K and 200 µL of AL buffer. The mixture was reheated at 70°C and transferred to a spin column. Washing and elution was performed according to the manufacturer's instructions. The elution volume was 100 µL. Extracted DNA was quantified using Nanodrop and stored at -

20°C until used for real-time PCR. 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.

4.3.3.3 The real-time PCR reaction

The PCR detection of *Salmonella* was carried out using the TaqMan *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 TaqMan *Salmonella enterica* detection kit does not provide quantification of positive samples. To determine the limit of detection and and to allow the 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 real-time PCR was performed on the diluted DNA samples (5 ng/µL). In each PCR 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*.

4.3.4 Statistical analysis

The prevalence of *Salmonella* positive cages was estimated at each sampling point with 95% binomial exact confidence intervals. The data from flock A and B was kept separate only for computing the flock prevalence of *Salmonella* (using

exact binomial confidence interval model). In all other cases, data was pooled from both flocks. The data was pooled, in order to reduce the sources of heterogeneity in two flocks. Because of larger sample sizes, pooled analyses also offer the opportunity to minimize variation in individual studies. Hence, aggregated data may provide greater statistical power and more certainty. 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 real-time PCR) 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.

4.4 Results

4.4.1 Prevalence of *Salmonella* in faecal samples

Sample numbers and the test outcomes for each type of specimen collected at the 3 sampling points are presented in table 4.1. 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.

4.4.2 Prevalence of *Salmonella* in other type of samples

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 55 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.

4.4.3 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%), *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).

4.4.4 Comparison between Real-time PCR and culture

The limit of detection for real-time PCR 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 69 positive samples, 12 were negative by the culture method. On other hand, RT-PCR failed to detect 70 samples which were positive by culture method (Table 4.3).

Table 4.3 provides the details of agreement between culture method and real-time PCR 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 real-time PCR, samples were also pre-enriched in BPW. Agreement between raw and BPW enriched faecal samples tested with real-time PCR was moderate (86.6%) with more samples testing positive with the raw vs pre-enriched BPW faecal samples (19 and 11 positives, respectively).

4.4.5 *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 ($p<0.05$) 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 ($p<0.05$).

4.5 Discussion

In the present study, the shedding of *Salmonella* in single age layer flock was investigated at the onset of lay. Results of 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 (Van de Giessen *et al*., 1994; Gradel *et al*., 2004). 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 (El-Lethey *et al*., 2003; Humphrey, 2006) 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. Another possibility could be that pullets were *Salmonella* carriers, and the transport stress may have induced *Salmonella* shedding in faeces at 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 (1996) 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 no information in literature to compare these findings. Experiments are necessary involving the estimating the level of corticosterone (as stress indicting parameter) in faeces in high and low traffic areas, and further, investigating the relationship of corticosterone with *Salmonella* shedding.

The agreement between culture method and real-time PCR 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 culture method and real-time PCR. The real-time PCR 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, real-time PCR was also performed using pre-enriched BPW from faecal samples. However, with this protocol, real-time PCR was able to detect only 14.86% (11/74) of culture positive samples (data not presented). This clearly suggested that BPW pre-enrichment or single enrichment did not improve the detection by PCR of *Salmonella* from faecal samples. 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*. (2013) who also reported a low relative sensitivity of real-time PCR (20%) as compared to a culture method. Complex polysaccharides in faeces can acts as PCR inhibitors

(Monteiro *et al*., 1997). Low sensitivity of real-time PCR compared to culture method could be attributed to the 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 (Van Hoorebeke *et al*., 2011). The bird holding capacity of the flock sampled in the present study was 36750. Larger flock size increases the risk of introduction of *Salmonella* infection (Van Hoorebeke *et al*., 2011). However, it has been observed that the persistence of *Salmonella* was not significantly related to flock size (Carrique-Mas *et al*., 2009a; 2009b). 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 (Carrique-Mas *et al*., 2008). The commercial egg layer farm sampled during this study had three different sheds connected with a common egg conveyer belt. Each shed housed single aged flock (early lay < 40 week, mid lay 40 to 65 week and late lay > 65 week). Another important factor for the continuous presence of *Salmonella* in cage layer farms is difficulty in cleaning and disinfection of interior of cages, egg belt and feeders (Davies and Breslin, 2003; Carrique-Mas *et al*., 2009b).

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 of being moved 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. 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 Australia, food poisoning outbreak associated with *S.* Mbandaka has been reported in Australia in 2007. However, the source of infection was unclear. 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 (Cox *et al*., 2002). *Salmonella* Mbandaka has been isolated from egg shell surface (Little *et al*., 2007). 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 in faeces 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 real-time PCR was lower as compared to culture method in detecting *Salmonella* positive faecal samples. The sensitivity of real-time PCR was also not improved with use of a pre-enrichment step. This might be due to the presence of PCR inhibitory factors in faeces, a low number of target microorganism as well as large number of competing bacteria in faeces. However, real-time PCR offer's a rapid method of monitoring other types of contamination such as egg belt, shoe cover, dust and eggs. As per real-time PCR 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.

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Table 4.1 Prevalence of *Salmonella* during three longitudinal sampling using culture method

Sampling	Week of lay	Faeces	Egg belt	Dust	Shoe cover	Eggshells	*Salmonella* serovars*
1	18 (t0)	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 (t+6)	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 (t+12)	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).
S.: *Salmonella* **Salmonella* serovar isolated (number of positive samples).
t0: first sampling at week 18; t+6: second sampling at week 24; t+12: third sampling at week 30 of lay.

Table 4.2 Serovars detected in various *Salmonella* positive sample types between 18 and 30 weeks

Salmonella serovar	Faeces	Egg belt	Dust	Shoe cover	Eggshells	Total
S. Worthington	60.27 % (44/73)	6.66% (2/30)	0% (0/11)	11.11% (1/9)	0% (0/2)	37.60% (47/125)
S. Mbandaka	38.36% (28/73)	73.33% (22/30)	81.81% (9/11)	77.78% (7/9)	100% (2/2)	54.40% (68/125)
S. Infantis	1.37% (1/73)	0% (0/30)	0% (0/11)	0% (0/9)	0% (0/2)	0.8% (1/125)
S. Anatum	0% (0/73)	0% (0/30)	0.09% (1/11)	0% (0/9)	0% (0/2)	10.8% (1/125)
S. Worthington + *S.* Mbandaka	0% (0/73)	10% (3/30)	0.09% (1/11)	11.11% (1/9)	0% (0/2)	4% (5/125)
S. Worthington + *S.* Mbandaka + *S.* Infantis	0% (0/73)	3.33% (1/30)	0% (0/11)	0% (0/9)	0% (0/2)	0.8% (1/125)
S. Mbandaka + *S.* Anatum	0% (0/73)	6.66% (2/30)	0% (0/11)	0% (0/9)	0% (0/2)	1.6% (2/125)

Table 4.3 Agreement between culture method and real-time PCR to detect *Salmonella* positive negative samples between 18 and 30 weeks

		Positive and negative samples identified by Real-time PCR			
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	8	4	12	100
	Negative	0	0	0	
	Total	8	4	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 between 18 and 30 weeks

Sample type	Week of lay (sampling)			P value			
	18 (t0)	24 (t+6)	30 (t+12)	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

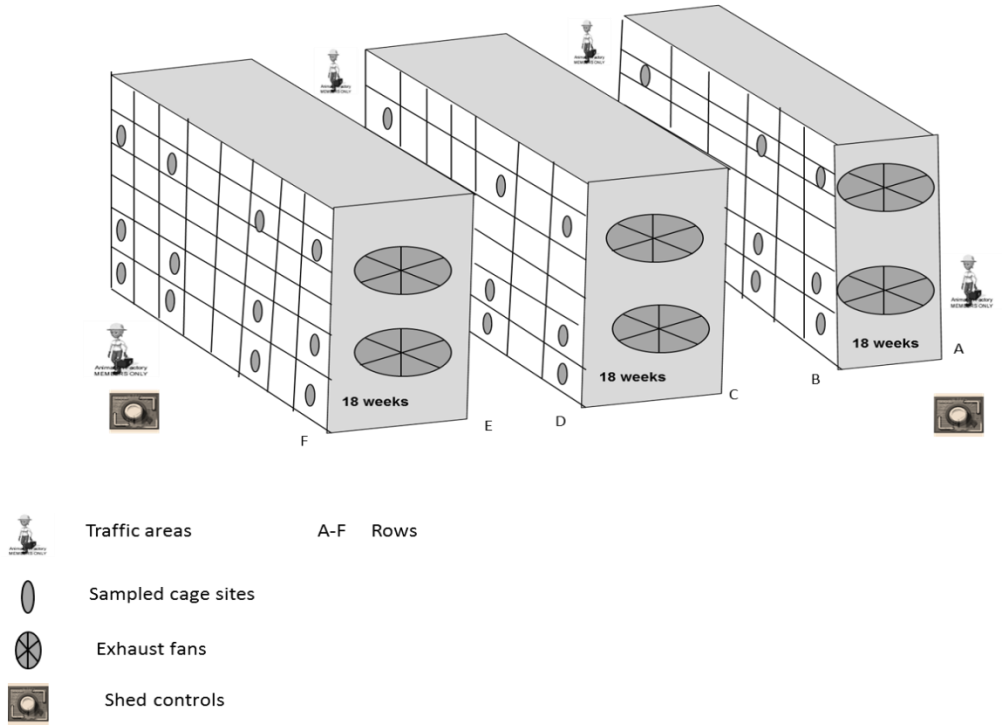

Figure 4.1 The layout of shed showing the areas of sample collection at t0 sampling

Chapter 5 Effect of egg washing and correlation between eggshell characteristics and egg penetration by various *Salmonella* Typhimurium strains

Statement of Authorship

Title of Paper	Effect of Egg Washing and Correlation between Eggshell Characteristics and Egg Penetration by Various Salmonella Typhimurium Strains
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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Vaibhav C. Gole		
Contribution to the Paper	Performed the experiment, compiled, analyzed and interpreted data, wrote manuscript, responded to editing suggestions by co-authors		
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Contribution to the Paper	Supervised the development of experiment, Liaised with egg producers for obtaining required egg samples, assisted in performing experiment, edited the manuscript.		
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Contribution to the Paper	Supervised development of experiment, assisted in developing eggshell ultrastructure scoring system, edited the manuscript.		
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Contribution to the Paper	Designed egg washing machine,standardized egg washing protocol edited the manuscript.		
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Name of Principal Author (Candidate)	Vaibhav C. Gole		
Contribution to the Paper	Performed experiment, compiled, analyzed and interpreted data, Wrote manuscript, responded to editing suggestion by co-authors.		
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Contribution to the Paper	Assisted in the interpretation of complex data, edited the manuscript.		
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Name of Co-Author	Andreas Kiermeier		
Contribution to the Paper	Designed egg washing machine, standardized egg washing protocol, edited the manuscript.		
Signature		Date	4/6/14

5.1 Abstract

Salmonella is an important foodborne pathogen, causing an estimated 11,992 cases of infection in Australia per year. Egg or egg product related salmonellosis is a major concern for the egg industry. Worldwide, *S.* Typhimurium is one of the most common serovars identified in *Salmonella* food poisoning cases. The current study investigated the ability of five *S.* Typhimurium strains to penetrate washed and unwashed eggs using whole egg and agar egg penetration methods. All *S.* Typhimurium strains were able to penetrate eggshells and survive in egg albumen (at 20°C) according to whole egg penetration results. Polymerase Chain Reaction results demonstrated that *S.* Typhimurium strain 2 (10^3 and 10^5 CFU/mL), and strain 5 (10^3 and 10^5 CFU/mL) egg penetration was significantly higher ($p<0.05$) in washed eggs when compared to unwashed eggs. Statistical analysis of the agar penetration experiment indicated that *S.* Typhimurium was able to penetrate washed eggs at a significantly higher rate when compared to unwashed eggs ($p<0.05$). When compared to unwashed eggs, washed eggs also had significantly damaged cuticles. Statistical analysis also indicated that eggshell penetration by *S.* Typhimurium was related to various eggshell ultrastructural features such as cap quality, alignment, erosion, confluence, Type B bodies and cuticle cover.

5.2 Introduction

Salmonella spp. have been one of the most important food poisoning pathogens throughout the last century and remain a challenge for microbiology and public health (Hardy, 2004). It is estimated that 1.3 billion incidences of non-typhoidal salmonellosis occur throughout the world annually (Coburn *et al*., 2007). The annual report of the OzFoodnet network (2012) reported 11,992 cases of *Salmonella* infection in Australia in 2010, with an estimated annual cost due to all food borne illness of $1.2 billion (Hall *et al*., 2005). Eggs are often implicated in the

cases of food poisoning due to salmonellosis (The OzFoodnet Working Group, 2012), which can be acquired by the ingestion of raw or undercooked eggs. Intact eggs can be contaminated by *Salmonella* using two possible routes: vertical transmission and horizontal transmission. Vertical transmission occurs as a result of *Salmonella* infection of the reproductive organs i.e. ovaries or oviduct hence also called the transovarian route. In the transovarian route, the egg yolk membranes or surrounding albumen are directly contaminated (Messens *et al*., 2005b). Horizontal transmission is also called the trans-shell route in which *Salmonella* penetrates through the eggshell during or following oviposition (Miyamoto *et al*., 1998). A major cause of egg related *Salmonella* food poisoning cases and the most prevalent serovar in the egg layer industry across the world is *S*. Enteritidis; however, it is not endemic in Australian layer flocks (Sergeant *et al*., 2003). In Australia and other parts of the world, *S.* Typhimurium, is one of the most common serovars identified in egg borne salmonellosis cases (The OzFoodnet Working Group, 2012; Wales and Davies, 2011). Horizontal transmission is believed to be the most common route by which salmonellae other than *S.* Enteritidis contaminate egg internal contents (Martelli and Davies, 2012).

Important extrinsic factors such as the bacterial strain, temperature differential, moisture on the eggshell, the number of microorganisms in the inoculum and the storage conditions may affect eggshell penetration by *Salmonella* spp (Messens *et al*., 2005b). Intrinsic factors that may affect egg penetration include shell porosity, shell thickness and the extent of cuticle present on the shell (Messens *et al*., 2005b). There is also some evidence to suggest that eggshell translucency is associated with greater microbial penetration (Chousalkar *et al*., 2010). However, there is a lack of substantial literature on the relationship between translucency, eggshell ultrastructure and the penetration of bacteria.

Faeces, water, caging material, nesting material, insects, hands, broken eggs, blood, soil or dust on the egg belt are the most common sources of microbial contamination of the eggshell (Board and Tranter, 1995; Ricke *et al*., 2001). Egg washing can reduce the microbial load on the eggshell surface (Messens *et al*., 2011) and thus may lower the rate of penetration of *Salmonella* across the eggshell and decrease the incidence of food poisoning. Egg washing is therefore used to reduce eggshell contamination in many countries such as the United States, Australia and Japan (Hutchison *et al*., 2004). However, some researchers claim that egg washing chemicals can damage the cuticle layer of the eggshell (Wang and Slavik, 1998) which may result in moisture loss and deterioration of the internal quality of the egg. It is also possible that egg washing may favour the transmission of *Salmonella* across the eggshell particularly when the post-washing storage and drying conditions are substandard. As a result, there is currently a global debate over the benefits of egg washing. Damage to the cuticle or alteration of the eggshell surface may change with different egg washing protocols (Wang and Slavik, 1998) and may result in variation in the penetration of bacteria across the eggshell. There is a lack of information in this area in the Australian context.

In Australia, *S.* Typhimurium has been identified as the most prevalent serovar involved in cases of salmonellosis food poisoning (The OzFoodnet Working Group, 2012). Additionally, STm PTs (STm PT 9, STm PT 44, STm PT 135, STm PT 170 and STm PT 193) were frequently isolated or detected from egg products related to food poisoning cases in Australia (The OzFoodnet Working Group, 2010; 2012). However, there is a lack of information available regarding how well these *S.* Typhimurium strains survive on the eggshell surface and penetrate the shell to contaminate the internal contents of the egg. A preliminary study suggested that

eggshell ultrastructure may have an impact on eggshell penetration by *Salmonella* (Samiullah *et al*., 2013), but there is a lack of direct evidence.

The objectives of this study were to examine the effect of egg washing on the survival of various *S.* Typhimurium strains on the eggshell surface, to investigate the penetration ability of different *S.* Typhimurium strains, and to study the effect of egg washing on the bacterial penetration of the eggshell. The effect of egg washing on cuticle ultrastructure and the relationship between eggshell quality parameters and bacterial penetration were also able to be systematically investigated. Finally, the relationship between translucency and eggshell ultrastructure parameters and ease of bacterial penetration was studied.

5.3 Materials and methods

Fresh and visibly clean table eggs were collected from hens 40 weeks old from a commercial Hyline layer farm located in South Australia near Adelaide. This study did not involve endangered or protected species. All table eggs used in this study were collected from cage front which did not involve handling of birds. Birds were not sacrificed for this study and animal ethics approval was therefore not required. The farm was selected based on the willingness of the producers to participate in the study and specific permission was not required for egg collection. In the present experiment, five different *S.* Typhimurium strains were used, which had been initially characterised by reference laboratory from Australian layer farms, were obtained from the Australian *Salmonella* Reference Centre, Institute of Veterinary Medical Science (IMVS) in Adelaide, Australia. Each of these five strains was belonged to the different *S.* Typhimurium phage types (STm PT): Stain 1/group 1: STm PT 9; Strain 2/group 2: STm PT 44; Strain 3/group 3: STm PT 135; Strain 4/group 4: STm PT 170 and Strain 5/group 5: STm PT 193.

5.3.1 Egg washing

The egg washing process used in this study involved the stages of pre-washing, washing with the aid of a surfactant, sanitizing and drying. A laboratory based washer which could hold 15 eggs in three rows of five rotating rollers was used for the physical mechanics of the egg washing. Washing was performed using a hydroxide and hypochlorite based solution at the concentration of 0.45% (v/v) which equates to a pH of ~12 and ~200 ppm hypochlorite in the working solution at 40°C. Washing was followed by a compatible sanitizer (at a concentration of 0.16% (v/v)) which equated to ~200 ppm hypochlorite in the working solution at 32°C. Eggs were washed and sanitized for 46 and 22 seconds, respectively. The pressure of the spray was 3 psi without brushes. Eggs were left on the bench for 15 minutes to dry and used for further experiments.

5.3.2 Inoculum preparation

Strains of *S.* Typhimurium stored at -80°C in 80% glycerol were plated on xylose lysine deoxycholate (XLD) agar (Oxoid, Australia) and incubated overnight at 37°C. Colonies were selected from XLD agar and resuspended in phosphate buffered saline (PBS) to match the turbidity equivalent with a 0.5 McFarland standard (BioMerieux, Australia). Enumeration of viable bacteria was performed by serial dilution and spread plating on XLD agar and incubation overnight at 37°C. Following enumeration, a 200 mL inoculum containing 10^3 and 10^5 colony forming units (CFU) per mL was prepared for each serovar. Agar filled eggs and whole eggs were immersed for 90 sec in one of three dilutions: PBS (control), ~10^3, and ~10^5 CFU/ mL of *S.* Typhimurium.

5.3.3 Whole egg penetration experiment to investigate the survival of *S.* Typhimurium on eggshell surface and in egg internal contents

The effects of egg washing on *S.* Typhimurium survival on the eggshell surface and penetration across the eggshell, as well as the survival of *S.* Typhimurium in the internal contents of the egg, were investigated using a 'whole egg penetration' approach. Ninety eggs were collected from HyLine Brown hens in early lay and were divided into two groups: washed (n=30) and unwashed (n=60). Washed eggs were divided into one control (PBS) and two treatment groups (10^3 and 10^5 CFU/mL) with 10 eggs each. All the washed eggs were incubated at 20°C after exposure to *S.* Typhimurium or the sham PBS treatment. Unwashed eggs were divided into two groups of 30 eggs. Group 1 was further divided into one control and two treatment groups (10^3 and 10^5 CFU/mL) of 10 eggs each. Eggs from group 1 were incubated at 20°C after exposure to *S.* Typhimurium or the sham PBS treatment. Group 2 was also divided into one control and two treatment groups (10^3 and 10^5 CFU/mL) of 10 eggs each. These unwashed eggs were incubated at 37°C. The reason that only unwashed eggs were incubated at 37°C is that washed eggs are not used for hatching purposes. Each egg was dipped into 70% ethanol for 30 sec to sterilize the outer shell and allowed to air dry in a biosafety cabinet for 10-15 min. Eggs were then immersed for 90 sec in 10^3 CFU/mL or 10^5 CFU/mL of *S.* Typhimurium. After inoculation, eggs were incubated at 20°C or 37°C for 21 days.

5.3.3.1 Isolation of S. Typhimurium from eggshell surface and egg internal contents from whole egg penetration experiment

Eggshell surface samples and egg internal contents samples were processed separately by pooling two eggs together. After incubation, each egg was placed in a Whirl-Pak bag (Nasco, USA) containing 10 mL of buffered peptone water (BPW; Oxoid, Australia) and each egg was massaged for 1 min. A 100 µL aliquot of the

mixture was spread plated onto XLD plates and incubated overnight at 37°C, and subsequently quantified.

To investigate the penetration and survival of *S.* Typhimurium in the internal contents of the egg, after the eggshell wash, eggs were dipped in 70% ethanol for 30 sec. Eggs were then aseptically opened, emptied into the Whirl-Pak bags and mixed. A 2 mL aliquot of the internal contents was transferred to 8 mL of BPW and 100 µL of this mixture was plated on XLD agar and incubated overnight at 37°C. Plates were then observed for *Salmonella* growth. Slopes of suspected *Salmonella* isolates were sent to the Institute of Medical and Veterinary Sciences (IMVS), Adelaide, Australia for confirmation.

5.3.3.2 Polymerase Chain Reaction amplification for detection of S. Typhimurium

Detection of *S.* Typhimurium on the eggshell surface and in internal contents was achieved using a polymerase chain reaction (PCR) assay that targeted the *Salmonella*-specific *InvA* gene (Rahn *et al*., 1992). The pooled BPW (from two eggs), from the whole egg penetration experiment, for the eggshell surface and egg internal contents samples, was incubated overnight at 37°C. These overnight incubated pooled BPW samples were used for DNA extraction using the Wizard PlusMinipreps DNA purification system (Promega, Australia) as per manufacturer's instructions. Extracted DNA was suspended in nuclease free water, and stored at -20°C until further use. Each PCR reaction mixture contained 10×reaction buffer (Fisher Scientific, Australia), 1 mM $MgCl_2$, 0.25 mM dNTPs, 5 µM forward *InvA* primer (5'- CTGGCGGTGGGTTTTGTTGTCTTCTCTATT-3'), 5 µM reverse *InvA* primer (5'-GTTTCTCCCCCTCTTCATGCGTTACCC-3'), 1.65 U *Taq* polymerase, and 10 ng DNA template, made up to 20 µL with nuclease free water. Samples were amplified using a Kyratech automated thermal cycler (Adelaide, Australia) with an initial denaturation step at 95°C for 5 min, followed by 30 cycles of amplification

(denaturation at 95°C for 30 sec, annealing temperature 60°C for 30 sec and extension at 72°C for 1 min 30 sec), with a final extension step at 72°C for 5 min, followed by a holding temperature of 8°C. The separation of PCR products was done by 1.5% agarose gel electrophoresis in a Trisborate-EDTA (TBE) buffer. GelRed was used to visualize bands under ultra-violet light. The size of the PCR products was estimated using a 1 kb DNA ladder (Qiagen, Australia), with bands identified at 1062 bp. The sensitivity of PCR was determined using serial dilutions. Cultures of *S.* Typhimurium stored at -80°C in 80% glycerol were plated on xylose lysine deoxycholate (XLD) agar (Oxoid, Australia) and incubated overnight at 37°C. Colonies were selected from the XLD agar and resuspended in BPW to match the turbidity of a 0.5 McFarland standard (BioMerieux, Australia). Enumeration of viable bacteria was performed by serial dilution and spread plating on XLD agar. DNA extraction and *Salmonella* specific PCR (as described above) was performed using serial dilutions of BPW. DNA extraction and *Salmonella* specific PCR was also performed from serially diluted BPW incubated overnight at 37°C.

5.3.4 Agar method for assessment of the eggshell penetration with respect to washing, translucency and eggshell ultrastructural parameters

The effects of washing, eggshell quality and translucency on the bacterial penetration of the eggshell were assessed by the 'agar egg' method as described previously (De Reu *et al*., 2006). Fresh eggs were obtained from the cage front of layers. All eggs were candled, scored for translucency, and allocated to two translucency groups based on candling score, until each group contained 32 eggs; where 1= low translucency, and 2= high translucency. For scoring translucency, a quantitative approach was used where a 1 cm^2 area of eggshell was marked and the numbers of lighter coloured spots on the eggshell (as viewed over a light source) were counted. Eggshells with less than 10 spots/cm^2 were considered to have low

translucency. Eggs from each group were then allocated to washed and unwashed groups (n=16 each) and subsequently allocated to inoculated (n=10) and control groups (n=6). Each egg was dipped into 70% ethanol for 30 sec for sterilization of the egg shell surface and aseptically air dried for 10-15 min.

The internal contents of each egg were removed using an 18 g needle (BD, Australia) at the blunt end of the egg. Eggs were also washed internally with sterile PBS (pH 7.2) to remove residual albumen. Eggs were then filled with XLD agar and sealed after the agar solidified. Agar-filled eggs from each treatment group (washed and unwashed) were immersed for 90 sec in 200 mL of approximately 10^5 CFU/mL solution of *S.* Typhimurium. Eggs from the control groups (washed and unwashed) were immersed in sterile PBS for 90 sec. After inoculation, agar-filled eggs were incubated at 20°C for 21 days. After incubation, the eggs were aseptically opened and the penetration of *Salmonella* spp. was assessed by the blackening of the interior eggshell.

5.3.4.1 Scanning electron microscopy (SEM)

A scanning electron microscope (SEM) (JCM-5000 NeoScope, JEOL, Japan) was used to score the ultrastructural features of the mammillary layer of the eggshell. A Dremel high speed rotary, model tool, 300 series was used to cut piece of eggshell (approximately 1 cm^2) from around the equator of all 64 eggs. Eggshell pieces were soaked overnight in tap water. Shell membranes were then removed and eggshell pieces were air dried. Plasma etching of dried eggshell pieces was then performed using a BioRAD RF Plasma Barrel Etcher PT 7150 for 4 hours. Next, an air duster was used to remove ash particles. Each eggshell piece was mounted on a 9 mm diameter aluminium stub using I005Aqueous conductive silver liquid SEM adhesive (ProSciTech, Australia). The specimens were sputter coated in a Neocoater for 5 min, and viewed under the SEM (JCM-5000 NeoScope, JEOL, Japan) at various

magnifications. Eggshell ultrastructural features of the mammillary layer were scored as per the following criteria: Cap size: similar = 1, variable = 2; Confluence: low = 1, high = 2; Cap quality: good = 1, poor = 2 ; Alignment: low = 1, high = 2, Type A bodies: low = 1, high = 2; Type B bodies: low = 1 , high = 2; Argonite: Absent = 1, present = 2; Erosions: absent = 1, present = 2; Depression: absent = 1, present = 2.

Assessment of the cuticle was carried out using all the eggs in the control and treatment groups. Shell pieces (64 eggs) of approximately 1 cm^2 were cut out from around the equator of the eggshell using a Dremel tool, mounted on a 9 mm diameter aluminium stub, sputter coated and viewed under the SEM, as described above. Scoring of the cuticle was done according to Samiullah *et al*. (2013) with following criteria: Cuticle score 1 = 90 to 100 % cuticle cover, score 2 = 60 to 90 % cuticle cover, score 3 = 20 to 60 % cuticle cover, score 4 = 0 to 10 % cuticle cover.

From all eggs, three pieces of shell with intact shell membranes were taken from around the equator of the egg to measure shell thickness. A custom-built gauge (based on a Mitutoyo Dial Comparator Gauge model 2019-10, Japan) was used to measure the shell thickness in micrometers.

5.3.5 Statistical analysis of whole egg and agar penetration experiment

All statistical analyses were performed with the statistical software R version 2.15.0. Statistical analysis, for the whole egg penetration experiment, was conducted using the t-test (for eggshell surface samples- direct agar culture method) and Fisher's exact test (for internal contents samples- direct agar culture method). On the other hand, all the results from PCR were analysed using Fisher's exact test.

In the agar egg experiment, a logistic regression was used to assess the effects of washing, eggshell translucency and their interaction on eggshell penetration of inoculated eggs. Logistic regression was also used to explore the relationship between the overall egg shell structure and *S.* Typhimurium penetration. The overall

eggshell structure was defined by the ultrastructural parameters of cap size, confluence, caps, alignment, the number of Type A and B bodies, the level of argonite, depression, erosion and shell thickness. An ordered logistic regression was used to model the effects of washing, translucency and treatment on the cuticle score of all 64 eggs. In addition, the relationship between translucency score and egg ultrastructure parameters was investigated using logistic regression. Models were assessed using Analysis of Variance (ANOVA) and based on a significance level of $p<0.05$, non-significant interactions were removed step-wise until only significant terms remained in the model. Model fit was assessed using standard diagnostic plots.

5.4 Results

5.4.1 Whole egg penetration experiment to study the survival of *S*. Typhimurium on eggshell surface and the contamination of egg internal contents

5.4.1.1 Survival of S. Typhimurium on the eggshell surface after 21 days of incubation

As the penetration of bacteria across the eggshell is dependent on the survival of bacteria on the eggshell, we compared the survival of *S.* Typhimurium strains on the eggshell surface of washed and unwashed eggs. Results indicated that there was no significant difference in the survival rate of *S.* Typhimurium strains on the eggshell surface of washed and unwashed eggs (Table 5.1). There was variation in the survival ability of different strains on the eggshell surface of washed and unwashed eggs. The effect of temperature (Table 5.2) on the survival of *S.* Typhimurium on the eggshell surface of unwashed eggs was studied using two different temperatures (20°C and 37°C). The survival rate of *S.* Typhimurium strain 2 ($p=0.02$) and strain 5 ($p=0.0001$) was significantly higher at 20°C (Table 5.2). For all *S.* Typhimurium strains, the overall trend indicated that a temperature of 20°C was more favourable for *S.* Typhimurium survival (Table 5.2). Using two different doses of inoculation (10^3 and 10^5 CFU/mL), the effect of dose on survival was studied and,

as expected, results indicated that survival rate was higher in eggs inoculated with a 10^5 CFU/mL dose. However, a significant difference was observed only in the case of *S.* Typhimurium strain 4 (p=0.02) (Table 5.3).

5.4.1.2 Penetration of eggs and contamination of internal contents by S. Typhimurium using the direct agar culture method

Statistical analysis indicated that, at a dose of 10^5 CFU/mL, the penetration of *S.* Typhimurium strain 2 into washed eggs was significantly higher (p=0.04) compared to unwashed eggs (Table 5.4a, 5.4b). In contrast, *S.* Typhimurium strain 3 penetration at 10^5 CFU/mL was higher (p=0.04) in unwashed eggs. For the other *S.* Typhimurium strains (1, 4 and 5), there was no significant difference in the *S.* Typhimurium penetration of washed and unwashed eggs. The effect of temperature on *S.* Typhimurium egg penetration was studied at 20°C and 37°C. *S.* Typhimurium strain 3 penetration (inoculated with 10^5 CFU/mL) was significantly higher (p=0.04) at 20°C (Appendix 1, Table 4). Temperature had no significant effect on the egg penetration of other *S.* Typhimurium strains. The effect of dose on egg penetration was also investigated using two different doses (10^3 and 10^5 CFU/mL). At 10^5 CFU/mL, the penetration of strain 3 (at 20°C) was significantly higher (p=0.04) compared to 10^3 CFU/mL. However, for the remaining *S.* Typhimurium strains, there was no significant effect of dose on egg penetration.

5.4.1.3 PCR amplification for detection of S. Typhimurium

Results of PCR indicated that, in the case of all *S.* Typhimurium strains, there was no significant difference in the number of *Salmonella* positive eggshells from washed and unwashed eggs. When the effect of incubation temperature on the detection of *Salmonella* on the eggshell surface was studied, it was observed that *S.* Typhimurium strain 5 (10^5 CFU/mL) detection was significantly higher (p=0.04) at 20°C as compared to 37°C.

Results of PCR demonstrated that, *S.* Typhimurium strain 2 (inoculated with 10^5 CFU/mL) and strain 5 (inoculated with 10^3 and 10^5 CFU/mL) penetrations were significantly higher ($p<0.05$) in washed eggs than unwashed eggs (Table 5.4a, 5.4b). The effect of temperature on the *S.* Typhimurium egg penetration was studied and results suggested that strain 3 (inoculated with 10^5 CFU/mL) penetration was significantly higher ($p=0.047$) at 20°C compared to 37°C. When the effect of dose on egg penetration was studied, results showed that *S.* Typhimurium strain 3 (20°C) egg internal content contamination was significantly higher ($p=0.04$) for eggs treated with 10^5 CFU/mL as compared to 10^3 CFU/mL. In other cases (except *S.* Typhimurium strain 1 at 20°C), egg penetration tended to be higher at an inoculation dose of 10^5 CFU/mL as compared to 10^3 CFU/mL but the difference was not significant.

5.4.1.4 Comparison of direct agar culture method and non-selective enrichment PCR

The limit of detection for the direct agar culture method and the combination of BPW enrichment without overnight incubation/PCR was 50 CFU/mL. The limit of detection of the combination of BPW enrichment with overnight incubation/PCR was 0.5 CFU. After 21 days of incubation, using the direct agar culture method, 51% of the eggshells were reported positive for *Salmonella* whereas, using PCR, a significantly higher ($p=0.0001$) number of eggshells (82%) were observed to be *Salmonella* positive.

Using the direct agar culture method, it was observed that 16% of eggs were penetrated by *S.* Typhimurium but PCR results indicated that 49.33% egg internal contents were *Salmonella* positive. PCR detected a significantly higher ($p=0.0001$) number of positive egg internal contents as compared to the direct agar culture method.

At 20°C and as per the direct agar culture method, 18% of washed eggs and 16% of unwashed eggs were penetrated by *S.* Typhimurium. However, PCR results indicated a very different scenario where a significantly higher (p=0.0003) number of washed eggs (74%) were penetrated by *S.* Typhimurium as compared to unwashed eggs (36%), though it is not possible to determine whether these detections related to viable organisms.

5.4.2 Agar egg penetration experiment for investigating the eggshell penetration with respected to washing, translucency and eggshell ultrastructural parameters

5.4.2.1 Relationship of washing and translucency with egg penetration by S. Typhimurium

A summary of the results for the number of washed and unwashed eggs which were penetrated for each *S.* Typhimurium strain and translucency score is given in Table 5.5. An analysis of only inoculated eggs indicated that *S.* Typhimurium penetrations were significantly higher for washed eggs than for unwashed eggs. All eggs –irrespective of their washing status or translucency score – were penetrated when inoculated with *S.* Typhimurium strain 2. For all *S.* Typhimurium strains, 80-100% of washed eggs were penetrated while only 40-70% of unwashed eggs were penetrated. In most cases, there was no significant difference in the number of penetrated eggs with low and high translucency scores, but translucency did have a significant relationship with the penetration of *S.* Typhimurium strain 5 (p=0.02).

5.4.2.2 Relationship of washing and translucency with egg cuticle score

The results of the ordered logistic regression identified egg washing as having a significant effect on the cuticle score for all groups (Table 5.6). Figure 5.1 and Figure 5.2 show the good quality cuticle of an unwashed egg and the damaged cuticle/eggshell surface of a washed egg respectively. The interaction between washing and translucency was also significant for *S.* Typhimurium strain 3

(p=0.002). Depending on the *S.* Typhimurium strain, 60-100% of washed eggs have a cuticle score of 3 or 4. This is compared to the cuticle scores of unwashed eggs being more evenly distributed between the four categories and 30-75% of unwashed eggs had cuticle scores of 3 or 4.

5.4.2.3 Relationship between eggshell structure and eggshell penetration

To study the relationship between the eggshell structure and the susceptibility of the eggshell to penetration, we considered only the inoculated eggs and identified relationships between penetration and certain ultrastructure parameters, for each phage type. In Table 5.7 and 8, the details of statistical analysis are explained. Excluding variables that did not vary amongst inoculated eggs, the level of Type B bodies (Figure 5.3) was a significant predictor of *S.* Typhimurium group 1 (strain 1) penetration (p=0.01) (Table 5.8). In the case of *S.* Typhimurium group 3 (strain 3), cuticle (p=0.001), confluence (p=0.03) (Figure 5.4) and cap quality (p=0.0004) were significant predictors of eggshell penetration (Table 5.7). Results indicated that the levels of confluence (p=0.01), alignment (p=0.04) and erosion (p=0.007) (Figure 5.5) were significantly related to *S.* Typhimurium group 4 (strain 4) eggshell penetration (Table 5.7, 5.8). It was also observed that *S.* Typhimurium group 5 (strain 5) penetration was significantly related with eggshell ultrastructure parameters such as the level of confluence (p=0.01), cap quality (p=0.02), alignment (p=0.03) (Figure 5.6) and erosions (p=0.009) (Table 5.7, 5.8). Statistical analysis also showed that, in most cases (except strain 3; p=0.02), shell thickness was not related to eggshell penetration.

5.4.2.4 Relationship between eggshell ultrastructure parameters and translucency score

To investigate the relationship between translucency and eggshell ultrastructure parameters, the analysis identified different results for each *S.* Typhimurium strain. For *S.* Typhimurium group 1 (strain 1), none of the

ultrastructure parameters were significantly related with translucency score and for group 4 (strain 4), there was a significant relationship only between cap size and translucency (p=0.04). All eggs with high translucency (score 2) had a variable cap size (score 2) and approximately 9% of eggs with low translucency (score 1) had a similar cap size. For *S.* Typhimurium group 2 and 3 (strain 2 and strain 3), there were significant differences in erosion counts between eggs of differing translucency scores (p=0.04 and 0.004 respectively). The analysis of shell thickness indicated that eggs with low translucency have significantly thicker shells on average compared to eggs with high translucency for *S.* Typhimurium group 2 (strain 2) (p=0.02) and group 5 (strain 5) (p=0.04). In contrast, eggs with high translucency and inoculated with *S.* Typhimurium group 3 (strain 3) have significantly thicker shells on average than eggs with low translucency (p=0.02).

5.5 Discussion

The penetration of bacteria across the eggshell is dependent on the survival of bacteria on the eggshell surface and egg storage conditions. Results from this study indicated that there was no significant difference in the survival rate of *S.* Typhimurium on the eggshell surface of washed and unwashed eggs.

For detecting *Salmonella*, PCR is a rapid, reliable and sensitive technique (Rahn *et al*., 1992). Hence, PCR was used in the present study to detect *Salmonella* on the eggshells as well as in egg internal contents in the whole egg penetration experiment. In the present experiment, the combination of Non-selective enrichment with overnight incubation/PCR was observed to be more sensitive as it was able to detect more positives. This result was not unexpected because PCR was performed on DNA extracted from bacterial cells in exponential phase. PCR results also indicated that, in the case of all *S.* Typhimurium strains, there was no significant difference in the number of *Salmonella* positive eggshells of washed and unwashed

eggs. There is no information available in the literature to compare these findings. Direct agar method provided the actual counts of *Salmonella* whereas the results of PCR were qualitative. When the effect of temperature (20°C and 37°C) on the survival of *S.* Typhimurium on the eggshell surface was studied, the overall trend indicated that a temperature of 20°C is more favourable for *S.* Typhimurium survival on the eggshell surface at day 21 post inoculation (p.i.). These findings are in agreement with previous experiments which also reported better survival of *Salmonella* on the eggshell surface at lower temperatures (Baker, 1990; Radkowski, 2002; Botey-Salo *et al*., 2012), however, there were differences in the incubation temperatures and type of *Salmonella* serovar used in the previous study (*S.* Enteritidis).

For all *S.* Typhimurium strains, after 21 days incubation, 51% of the eggshells were positive for *S.* Typhimurium. These findings are in agreement with De Reu *et al.* (2006) who found a high survival rate of *S.* Enteritidis on the eggshell surfaces after 21 days of incubation.

Results from whole egg penetration study also indicated that all *S.* Typhimurium strains used in the present study (at 20°C) were capable of penetrating the eggshells and surviving in the egg albumen which is considered to be a hostile environment for the survival of bacteria. Using the whole egg penetration approach, out of all the eggs tested, 16% of internal contents were observed to be positive for *S.* Typhimurium. This could be due to the antimicrobial properties of albumen. It was found that *S.* Typhimurium strain 2 penetration was significantly higher in washed eggs than unwashed eggs. For other *S.* Typhimurium strains (1, 4 and 5), there was no significant difference in the *S.* Typhimurium penetration of washed and unwashed eggs. Even though the results of the direct agar culture method showed that *S.* Typhimurium strain 3 penetration (at inoculation of 10^5 CFU/mL) was higher in

unwashed eggs, PCR results indicated that there was no significant difference. PCR results also indicated that *S.* Typhimurium strain 2 (10^5 CFU/mL) and strain 5 (10^3 and 10^5 CFU/mL) egg penetration was significantly higher in washed eggs than unwashed eggs, which could be due to damage to the cuticle by egg washing chemicals.

The effect of temperature on *S.* Typhimurium penetration was studied at 20°C and 37°C. *S.* Typhimurium strain 3 penetrations (at inoculation of 10^5 CFU/mL) was significantly higher at 20°C. For other *S.* Typhimurium strains, there was no significant effect of incubation temperature on egg penetration. Similar results were observed when samples were tested by PCR. Previous studies (Schoeni *et al*., 1995; Braun *et al*., 1999) reported that, with the increase in temperature, the egg penetration of *Salmonella* was also increased. It is difficult to compare findings of these previous studies with the results of present experiment as in all these previous studies (Schoeni *et al*., 1995; Wang and Slavik, 1998; Braun *et al*., 1999) eggs were incubated approximately at 4°C and 20°C whereas in the present experiment 20°C and 37°C temperatures were used to incubate eggs. The lower penetration at 37°C may be due to the reduced survival of *Salmonella* on the eggshell surface during incubation at this temperature.

The effect of dose on egg penetration was also investigated using two different doses (10^3 and 10^5 CFU/mL). At 10^5 CFU/mL, the penetration of *S.* Typhimurium strain 3 (at 20°C) was significantly higher ($p=0.047$) as compared to 10^3 CFU/mL. PCR results also confirmed that the egg penetration by *S.* Typhimurium strain 3 was dependent on the dose of inoculation. These findings are consistent with a number of previous studies which indicated that the rate of contamination of eggs is directly proportional to the number of *Salmonella* in the culture used for infecting the eggs (Schoeni *et al*., 1995; Miyamoto *et al*., 1998;

Braun *et al*., 1999). In agar penetration experiment, statistical analysis showed that *S.* Typhimurium strains penetration of washed eggs was significantly higher ($p<0.005$) than unwashed eggs. This may be due to the damage of cuticle by egg washing chemicals. To evaluate this further, the effects of washing on cuticle deposition was investigated using SEM. Results from the ordered logistic regression indicated that washed eggs had a significantly higher cuticle score (poor cuticle quality) as compared to unwashed eggs. These findings were not in agreement with a previous experiment where it was observed that egg washing had no significant effect on the quality of the cuticle (Leleu *et al*., 2011). The difference in the findings may be due to the variation in the age of laying hens and the difference in the protocol and chemicals of egg washing. In the present study, eggs were collected from younger hens (< 45 weeks) in contrast to the previous experiment where eggs were collected from old laying hens (> 54 weeks) (Leleu *et al*., 2011). It was previously observed that increasing age of laying hens has a negative impact on cuticle thickness (Sparks *et al*., 1984; European Food Safety Authority, 2005). The variation in results of different experiments might result from the difference in the egg washing protocol (Wang and Slavik, 1998). In the present study, in case of *S.* Typhimurium strain 3, cuticle quality was observed as a significant predictor of *Salmonella* eggshell penetration. The mature cuticle closes the pores on the eggshell and protects the egg from the water and bacterial invasion (Berrang *et al*., 1999) and the removal of cuticle or lower cuticle can result in increase in bacterial penetration (Alls *et al*., 1964; De Reu *et al*., 2006). In the present study, using the agar approach, the relationship of translucency with the *S.* Typhimurium eggshell penetration was studied. Results indicated that, in most cases (except for strain 5), there was no significant relationship between translucency and eggshell penetration. However, Chousalkar *et al*. (2010) reported a significant correlation between eggshell

translucency and eggshell penetration by *S.* Infantis and *E. coli*. It is also essential to note that, in these two experiments, different bacterial strains were used to study eggshell penetration.

The SEM results were also analysed to study the relationship of eggshell quality parameters with the eggshell penetration. A higher incidences of alignment, erosions, poor cap quality, Type A mammillary bodies, Type B mammillary bodies may result in the weakening of the eggshell (Solomon, 1992a). Small spherical bodies (Figure 5.3) in the mammillary layer which may or may not have contact with membrane layer are known as Type B Bodies (Roberts and Brackpool, 1994). On other hand, the condition in which mammillary caps attach to each other is known confluence (Roberts and Brackpool, 1994) (Figure 5.4). Confluence is required for a stronger eggshell region (Roberts and Brackpool, 1994). Our results indicated that, for *S.* Typhimurium group 3 (strain 3) ($p=0.03$), group 4 (strain 4) ($p=0.01$) and group 5 (strain 5) ($p=0.01$), eggshell penetration was negatively related to the level of confluence. The abrasion in mammillary layers are known as erosions (Figure 5.5) which is believed to create the areas of weakness in eggshells (Roberts and Brackpool, 1994). Alignment in the eggshells is a situation where mammillae appeared to "line up" which may help to propagate a crack in eggshell (Roberts and Brackpool, 1994) (Figure 5.6). Results also indicated that, for *S.* Typhimurium strain 4 and strain 5, eggshell penetration was positively related to a higher incidence of alignment and erosion. In case of *S.* Typhimurium group 3 and 5 (strain 3 and 5), eggshell penetration was negatively related to good cap quality. All these results are in agreement with the previous findings of Solomon (1992b) who reported that good mammillary caps and confluence can resist bacterial penetration whereas alignment, erosion and Type B bodies assist bacterial penetration. However, in the present study it was not clear as to why Type B bodies were negatively related to the incidence of

S. Typhimurium group 1 (strain 1) eggshell penetration. Statistical analysis also showed that, in most cases, shell thickness was not related to the eggshell penetration. Similarly, a number of studies have observed that the shell thickness did not affect whole egg and agar egg penetration (Messens *et al*., 2005a; De Reu *et al*., 2006; Samiullah *et al*., 2013).

Our finding underlines the importance of proper storage and careful handling of eggs in the food industry and the domestic environment. Egg washing can reduce the level of *Enterobacteriaceae* (up to 4 $\log_{10}$) on the eggshell surface very efficiently (May *et al*., 2013) but, at the same time, results from agar penetration experiment indicated that the trans-shell penetration was higher in washed eggs than unwashed eggs. Hence, appropriate attention is essential to make sure eggs are kept at appropriate storage and drying conditions so that they will not come in contact with *Salmonella* after washing. In one study, swabs taken from multiple premises of grading machinery were reported positive for *Enterobacteriaceae* (May *et al*., 2013); such situation could pose a higher risk of contamination of washed eggs. Hence, regular cleaning of the egg washing machine and grading equipment is essential to avoid recontamination of eggs once they are washed. Only in case of *S.* Typhimurium strain 2, 100% eggshell penetration was observed in washed and unwashed eggs. This suggests that *S.* Typhimurium strain 2 may have more capacity for trans-shell penetration compared to other *S.* Typhimurium strains used in this experiment. Luo *et al*. (2012), using comparative genome analysis, showed that even highly similar *S.* Typhimurium strains could vary in their genome. In the current experiment, each *S.* Typhimurium strain belonged to different *S.* Typhimurium phage type. It is possible that strain variation was linked to phage type. However, further investigation using multiple isolates of same *S.* Typhimurium phage type is essential to confirm the variation in penetration ability of different phage types.

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Table 5.1 Survival of *Salmonella* Typhimurium strains on eggshell surface after 21 days of incubation: Comparison between washed and unwashed eggs at 20°C

***Salmonella* Typhimurium strain**	**Dose of infection (CFU/mL)**	**Washing status**	**Eggshell contamination after incubation (Log CFU/ eggshell) Mean ± SE**	**p-value**
S. Typhimurium strain 1	10^3	Washed	3.09 ± 0.82	0.68
		Unwashed	2.51 ± 1.08	
	10^5	Washed	4.09 ± 0.27	0.34
		Unwashed	4.41 ± 0.16	
S. Typhimurium strain 2	10^3	Washed	2.69 ± 1.09	0.54
		Unwashed	3.58 ± 0.89	
	10^5	Washed	4.49 ± 0.01	0.37
		Unwashed	3.58 ± 0.89	
S. Typhimurium strain 3	10^3	Washed	2.23 ± 0.92	0.33
		Unwashed	0.89 ± 0.89	
	10^5	Washed	3.42 ± 0.92	0.34
		Unwashed	1.90 ± 1.17	
S. Typhimurium strain 4	10^3	Washed	0.89 ± 0.89	0.62
		Unwashed	1.56 ± 0.96	
	10^5	Washed	4.34 ± 0.21	0.27
		Unwashed	3.22 ± 0.85	
S. Typhimurium strain 5	10^3	Washed	1.67 ± 1.04	0.59
		Unwashed	2.51 ± 1.05	
	10^5	Washed	2.12 ± 0.88	0.06
		Unwashed	4.42 ± 0.20	

Table 5.2 Survival of *Salmonella* Typhimurium strains on eggshell surface of unwashed eggs after 21 days of incubation: Comparison between 20°C and 37°C

***Salmonella* Typhimurium strain**	**Dose of infection (CFU/mL)**	**Temperature (°C)**	**Eggshell contamination after incubation (Log CFU/ eggshell) Mean ± SE**	**p-value**
S. Typhimurium strain 1	10^3	20	2.51 ± 1.08	0.76
		37	3.04 ± 1.24	
	10^5	20	4.41 ± 0.16	0.29
		37	4.69 ± 0.18	
S. Typhimurium strain 2	10^3	20	3.58 ± 0.89	0.02
		37	ND	
	10^5	20	3.58 ± 0.89	0.02
		37	ND	
S. Typhimurium strain 3	10^3	20	0.89 ± 0.89	0.37
		37	ND	
	10^5	20	1.90 ± 1.17	0.18
		37	ND	
S. Typhimurium strain 4	10^3	20	1.56 ± 0.96	0.18
		37	ND	
	10^5	20	3.22 ± 0.85	0.05
		37	0.69 ± 0.69	
S. Typhimurium strain 5	10^3	20	2.51 ± 1.05	0.07
		37	ND	
	10^5	20	4.42 ± 0.20	0.0001
		37	ND	

ND: not detected

Table 5.3 Survival of *Salmonella* Typhimurium strains on the eggshell surface after 21 days of incubation: Comparison between different doses (10^3 and 10^5 CFU/mL) of infection

***Salmonella* Typhimurium strain**	**Temperature (˚C)**	**Washing status**	**Dose of infection (CFU/mL)**	**Eggshell contamination after incubation (Log CFU/ eggshell) Mean ± SE**	**p-value**
S. Typhimurium strain 1	20	Washed	10^3	3.09 ± 0.82	0.30
			10^5	4.09 ± 0.27	
		Unwashed	10^3	2.51 ± 1.08	0.15
			10^5	4.41 ± 0.16	
	37	Unwashed	10^3	3.04 ± 1.24	0.26
			10^5	4.69 ± 0.18	
S. Typhimurium strain 2	20	Washed	10^3	2.69 ± 1.09	0.17
			10^5	4.49 ± .013	
		Unwashed	10^3	3.58 ± 0.89	1.00
			10^5	3.58 ± 0.89	
	37	Unwashed	10^3	ND	NA
			10^5	ND	
S. Typhimurium strain 3	20	Washed	10^3	2.23 ± 0.92	0.39
			10^5	3.42 ± 0.92	
		Unwashed	10^3	0.89 ± 0.89	0.51
			10^5	1.90 ± 1.17	
	37	Unwashed	10^3	ND	NA
			10^5	ND	
S. Typhimurium strain 4	20	Washed	10^3	0.89 ± 0.89	0.02
			10^5	4.37 ± 0.21	
		Unwashed	10^3	1.56 ± 0.96	0.23
			10^5	3.22 ± 0.85	
	37	Unwashed	10^3	ND	0.37
			10^5	0.69 ± 0.69	
S. Typhimurium strain 5	20	Washed	10^3	1.67 ± 1.04	0.75
			10^5	2.12 ± 0.88	
		Unwashed	10^3	2.59 ± 1.05	0.11
			10^5	4.47 ± 0.20	
	37	Unwashed	10^3	ND	NA
			10^5	ND	

NA: Not applicable, ND: Not detected

Table 5.4a Whole egg penetration by different *Salmonella* Typhimurium strains: Comparison between washed and unwashed eggs at 20°C

***Salmonella* Typhimurium strain**	**Dose of inoculation (CFU/mL)**	**Method of analysis**	**Group**	**Number of penetrated pools (of 2 eggs)**	**Number of non-penetrated pools (of 2 eggs)**	**p-value**
S. Typhimurium strain 1	10^3	Direct agar culture	Washed	1	4	1.00
			Unwashed	0	5	
		PCR	Washed	4	1	1.00
			Unwashed	5	0	
	10^5	Direct agar culture	Washed	1	4	1.00
			Unwashed	1	4	
		PCR	Washed	3	2	1.00
			Unwashed	4	1	
S. Typhimurium strain 2	10^3	Direct agar culture	Washed	2	3	1.00
			Unwashed	1	4	
		PCR	Washed	4	1	0.21
			Unwashed	1	4	
	10^5	Direct agar culture	Washed	4	1	0.04
			Unwashed	0	5	
		PCR	Washed	5	0	0.04
			Unwashed	1	4	
S. Typhimurium strain 3	10^3	Direct agar culture	Washed	0	5	1.00
			Unwashed	0	5	
		PCR	Washed	3	2	0.17
			Unwashed	0	5	

Table 5.4b Whole egg penetration by different *Salmonella* Typhimurium strains: Comparison between washed and unwashed eggs at 20°C

***Salmonella* Typhimurium strain**	**Dose of inoculation (CFU/mL)**	**Method of analysis**	**Group**	**Number of penetrated pools (of 2 eggs)**	**Number of non-penetrated pools (of 2 eggs)**	**p-value**
S. Typhimurium strain 3	10^5	Direct agar culture	Washed	0	5	0.04
			Unwashed	4	1	
		PCR	Washed	4	1	1.00
			Unwashed	4	1	
S. Typhimurium strain 4	10^3	Direct agar culture	Washed	0	5	1.00
			Unwashed	0	5	
		PCR	Washed	1	4	1.00
			Unwashed	0	5	
	10^5	Direct agar culture	Washed	0	5	1.00
			Unwashed	1	4	
		PCR	Washed	4	1	0.52
			Unwashed	2	3	
S. Typhimurium strain 5	10^3	Direct agar culture	Washed	1	4	1.00
			Unwashed	0	5	
		PCR	Washed	4	1	0.04
			Unwashed	0	5	
	10^5	Direct agar culture	Washed	0	5	1.00
			Unwashed	1	4	
		PCR	Washed	5	0	0.04
			Unwashed	1	4	

Table 5.5 Relationship of washing and translucency with agar egg penetration by *Salmonella* Typhimurium

	Number of penetrated (non -penetrated) eggs					
	Low Translucency		**High Translucency**			
***Salmonella* Typhimurium strain**	**Unwashed**	**Washed**	**Unwashed**	**Washed**	**p-value (Relationship of washing with agar egg penetration)**	**p-value (Relationship of translucency with agar egg penetration)**
S. Typhimurium strain 1	5 (5)	9 (1)	5 (5)	8 (2)	0.02	0.73
S. Typhimurium strain 2	10 (0)	10 (0)	10 (0)	10 (0)	1.00	1.00
S. Typhimurium strain 3	4 (6)	10 (0)	7 (3)	9 (1)	0.002	0.41
S. Typhimurium strain 4	4 (6)	9 (1)	4 (6)	9 (1)	0.0005	1.00
S. Typhimurium strain 5	8 (2)	10 (0)	4 (6)	8 (2)	0.02	0.02

Table 5.6 Effect of egg washing on cuticle score

***Salmonella* Typhimurium strain**	**Washing status**	**Number of eggs**	**Average cuticle score (Mean ± SE)**	**p-value**
S. Typhimurium strain 1	Washed	32	3.60 ± 0.09	0.0001
	Unwashed	32	2.75 ± 0.15	
S. Typhimurium strain 2	Washed	32	3.25 ± 0.15	0.05
	Unwashed	32	2.72 ± 0.19	
S. Typhimurium strain 3	Washed	32	3.50 ± 0.13	0.0001
	Unwashed	32	2.59 ± 0.14	
S. Typhimurium strain 4	Washed	32	3.19 ± 0.16	0.01
	Unwashed	32	2.59 ± 0.16	
S. Typhimurium strain 5	Washed	32	2.81 ± 0.16	0.01
	Unwashed	32	2.28 ± 0.15	

Table 5.7 Relationship between eggshell ultrastructural parameters and penetration by *Salmonella* Typhimurium

Salmonella Typhimurium strain	Number of penetrated (non-penetrated) eggs												
	Alignment			Cuticle score					Confluence			Cap quality	
	Low	High	p-value	1	2	3	4	p-value	Low	High	p-value	Good	Poor	p-value
S. Typhimurium strain 1	4 (4)	23 (9)	0.44	1 (0)	4 (3)	6 (7)	16 (3)	0.09	21 (8)	6 (5)	0.51	9 (6)	18 (7)	0.97
S. Typhimurium strain 2	11 (0)	29 (0)	1.00	4 (0)	8 (0)	12 (0)	16 (0)	1.00	20 (0)	20 (0)	1.00	20 (0)	20 (0)	1.00
S. Typhimurium strain 3	11 (3)	19 (7)	0.83	0 (0)	5 (8)	9 (1)	16 (1)	<0.01	22 (6)	7 (4)	0.03	5 (3)	25 (7)	<0.01
S. Typhimurium strain 4	4 (7)	22 (7)	0.04	1 (1)	4 (7)	11 (4)	10 (2)	0.09	23 (7)	3 (7)	0.01	6 (11)	20 (3)	0.35
S. Typhimurium strain 5	9 (4)	21 (6)	0.03	6 (1)	5 (4)	13 (4)	6 (1)	0.46	29 (7)	1 (3)	0.01	2 (7)	28 (3)	0.002

Table 5.8 Relationship between eggshell ultrastructural parameters and penetration by *Salmonella* Typhimurium

***Salmonella* Typhimurium strain**	**Number of penetrated (non-penetrated) eggs**													
	Type A bodies			**Type B bodies**			**Erosion**			**Argonite**			**Depression**		
	Low	High	p-value	Low	High	p-value	Absent	Present	p-value	Absent	Present	p-value	Absent	Present	p-value
S. Typhimurium strain 1	25 (12)	2 (1)	0.39	13 (4)	14 (9)	0.007	15 (6)	12 (7)	0.07	27 (13)	0 (0)	NA	26 (13)	1 (0)	0.35
S. Typhimurium strain 2	37 (0)	3 (0)	1.00	24 (0)	16 (0)	1.00	25 (0)	15 (0)	1.00	31 (0)	9 (0)	1.00	39 (0)	1 (0)	1.00
S. Typhimurium strain 3	30 (10)	0 (0)	NA	3 (2)	27 (8)	0.64	13 (8)	17 (2)	0.09	30 (10)	0 (0)	NA	30 (10)	0 (0)	NA
S. Typhimurium strain 4	23 (14)	3 (0)	0.55	11 (7)	15 (7)	0.77	8 (6)	18 (8)	0.01	22 (14)	4 (0)	0.07	26 (14)	0 (0)	NA
S. Typhimurium strain 5	24 (10)	6 (0)	0.38	10 (5)	20 (5)	0.30	6 (2)	24 (8)	0.01	30 (10)	0 (0)	NA	30 (10)	0 (0)	NA

NA: not applicable

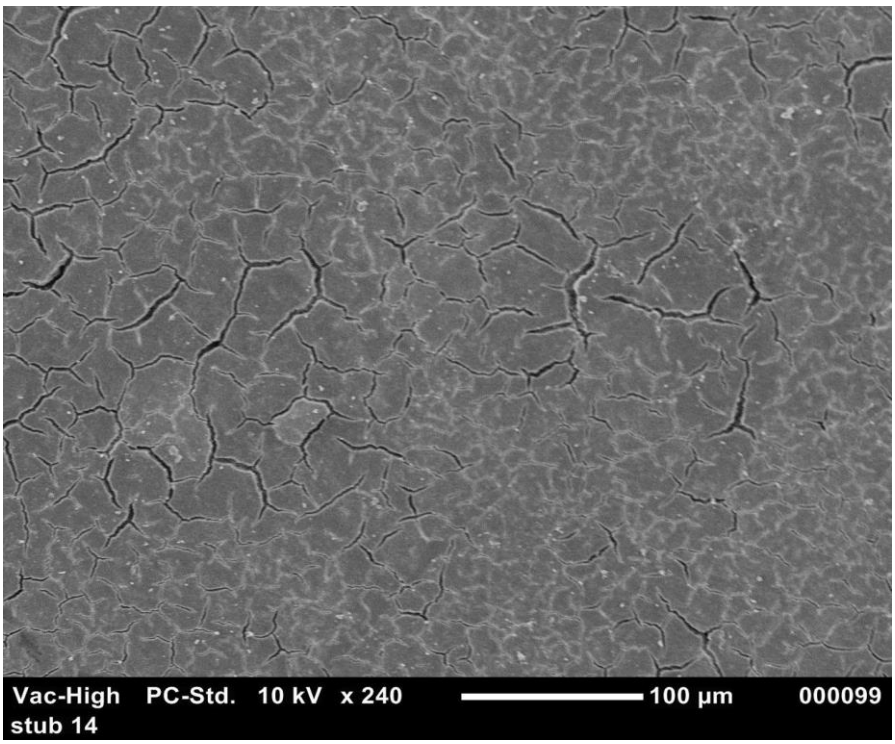

Figure 5.1 SEM image of good quality cuticle in unwashed eggs with no eggshell pores exposed

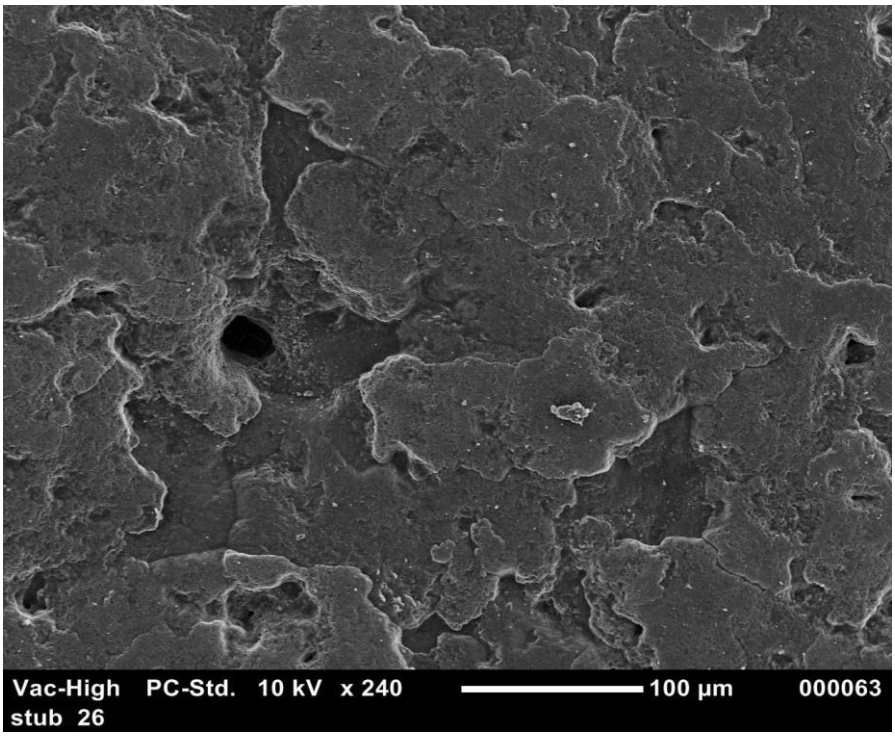

Figure 5.2 SEM image of damaged eggshell surface and exposed eggshell pore in washed eggs

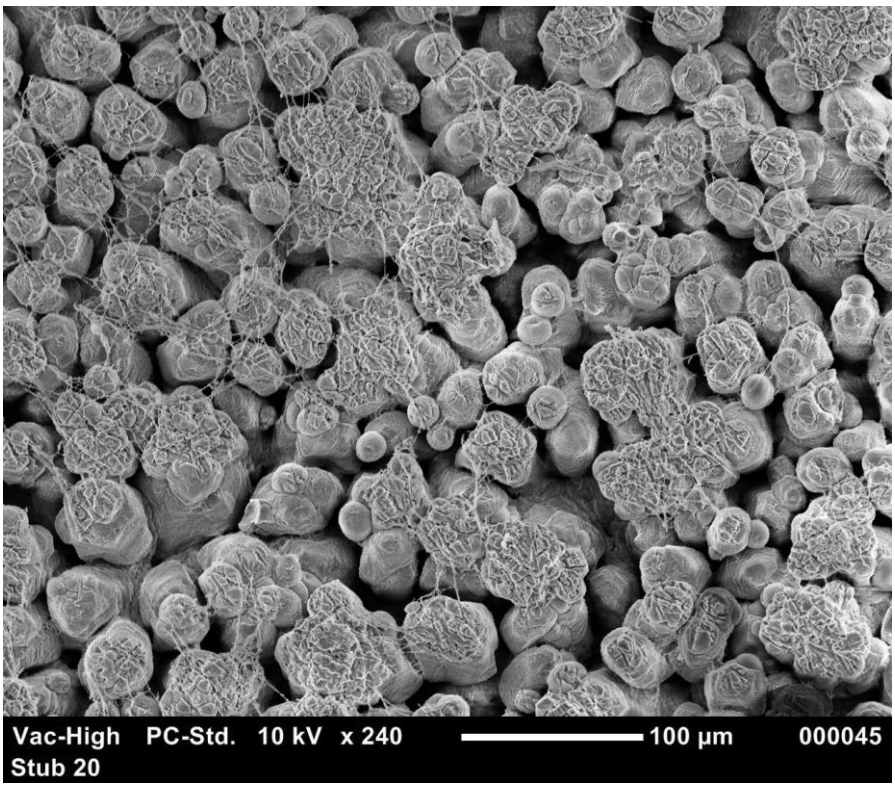

Figure 5.3 SEM image showing large number of Type B bodies in eggshell

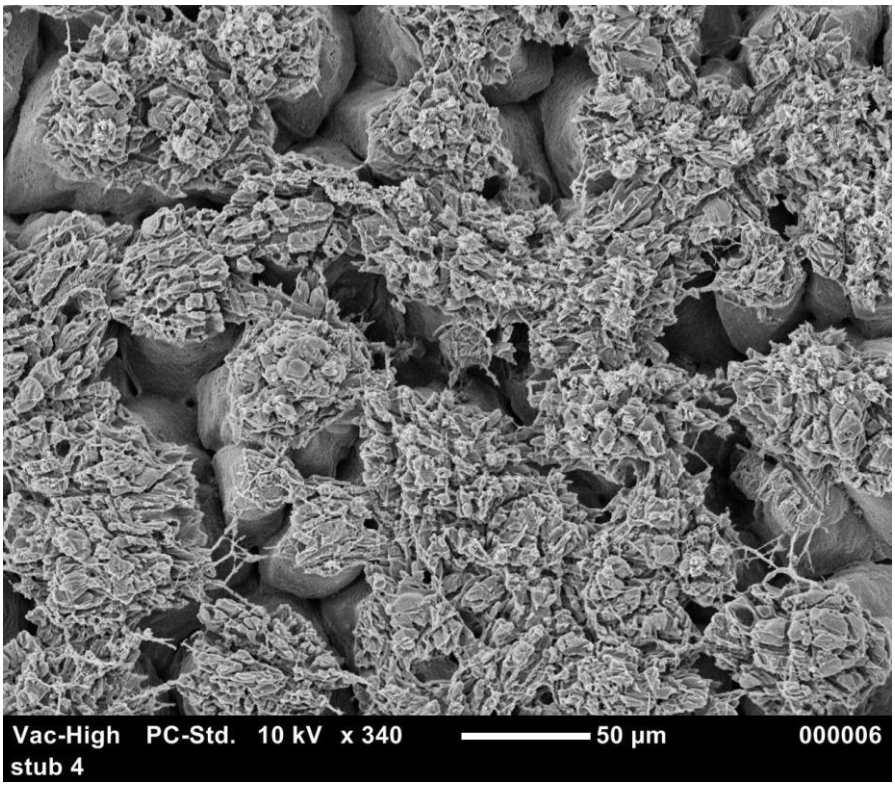

Figure 5.4 SEM image of good quality mammillary caps with confluence

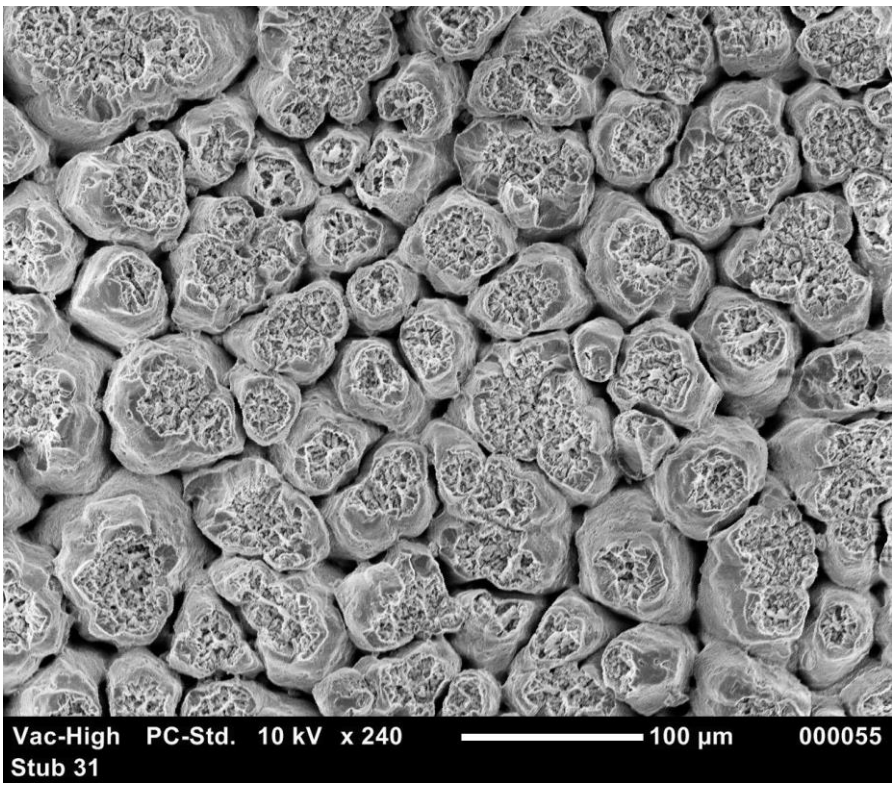

Figure 5.5 SEM image of extensive erosions throughout the eggshell

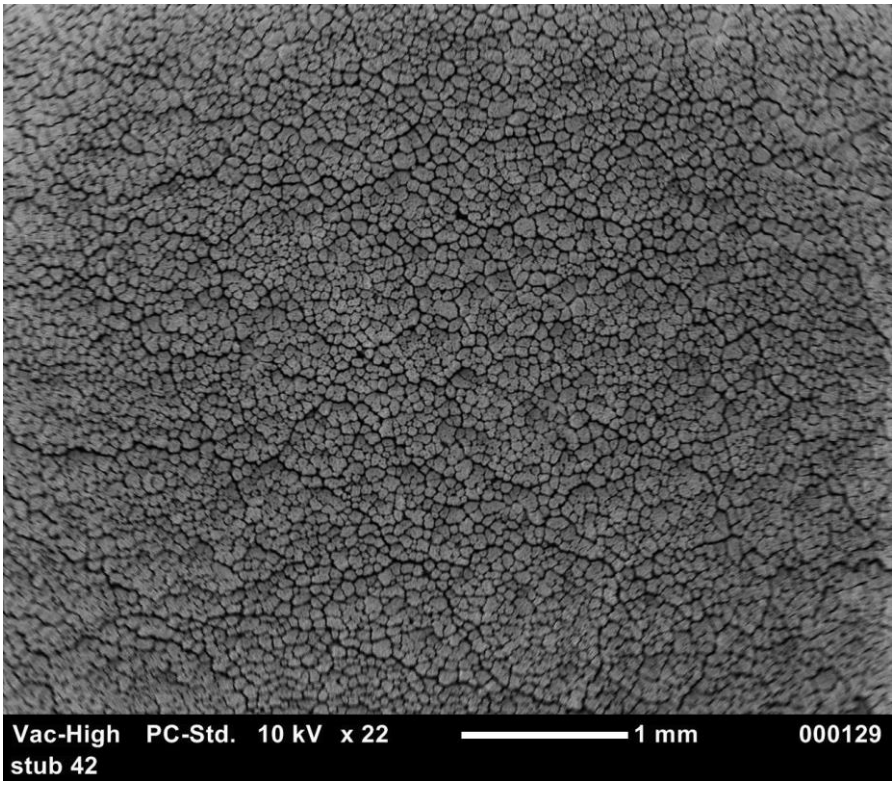

Figure 5.6 SEM image showing alignments in the mammillary layer

Chapter 6 Effect of egg washing and correlation between cuticle and egg penetration by various *Salmonella* strains

Statement of Authorship

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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Vaibhav C. Gole
Contribution to the Paper	Performed the experiment, compiled, analyzed and interpreted data, wrote manuscript, responded to editing suggestions by co-authors.
Signature	Date 6/6/14

Name of Co-Author	Kapil K. Chousalkar
Contribution to the Paper	Supervised the development of experiment, Liaised with egg producers for obtaining required egg samples, assisted in performing experiment, edited the manuscript.
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Contribution to the Paper	Supervised the development of experiment, assisted in developing eggshell ultrastructure scoring system, edited the manuscript.
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Contribution to the Paper	Performed experiment, compiled, analyzed and interpreted data, Wrote manuscript, responded to editing suggestion by co-authors		
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6.1 Abstract

In Australia, Europe and the United States, eggs and egg products are frequently associated with *Salmonella* food poisoning outbreaks. Many of the egg-associated *Salmonella* outbreaks have been due to the products such as mayonnaise, ice-cream and cold desserts which are eaten without cooking following the addition of raw egg. The ability of four *Salmonella* isolates (one each of *S.* Singapore, *S.* Adelaide, *S.* Worthington and *S.* Livingstone) to penetrate washed and unwashed eggs using whole egg and agar egg penetration methods was investigated in the current study. The results of the agar penetration experiment indicated that all the isolates used in the present study have the capacity to penetrate the eggshell. Eggshell penetration by the *S.* Worthington isolate was higher but not significant ($p=0.06$) in washed eggs compared to unwashed eggs. However, for all other isolates (*S.* Singapore, *S.* Adelaide and *S.* Livingstone), there was no significant difference in penetration of washed and unwashed eggs. Statistical analysis indicated that cuticle score was a significant linear predictor of *Salmonella* eggshell penetration. Whole egg penetration results showed that all of the *Salmonella* isolates used in the present study were capable of surviving on the eggshell surface after 21 days of incubation (at 20°C) following a high dose of inoculation (10^5 CFU/mL). The combined data of all isolates demonstrated that, the survival rate of *Salmonella* on eggshells (inoculated with 10^5 CFU/mL) was significantly higher ($p=0.002$) at 20°C as compared to 37°C. *S.* Singapore, *S.* Worthington, and *S.* Livingstone were not detected from egg internal contents whereas *S.* Adelaide was detected in one egg's internal contents.

6.2 Introduction

Salmonellosis, one of the most important foodborne diseases worldwide, is caused by the Gram negative bacteria of genus *Salmonella*. Infection is characterized by acute onset of fever, abdominal pain, diarrhoea, nausea and sometimes vomiting with incubation period of 6 -72 hours (World Health Organisation, 2013). In Australia, Europe and the United States, eggs and egg products are frequently associated with *Salmonella* food poisoning outbreaks (The OzFoodnet working group, 2012; Braden, 2006; European Food Safety Authority, 2012). Many of the egg-associated *Salmonella* outbreaks have been due to the consumption of contaminated products such as mayonnaise, ice-cream and cold desserts which are prepared or consumed without cooking after addition of raw egg (The OzFoodnet working group, 2012). Vertical transmission and horizontal transmission are possible routes by which *Salmonella* can contaminate intact eggs (Messens *et al*., 2005a). Egg contamination by horizontal transmission occurs when *Salmonella* penetrates the eggshell during or following oviposition, contaminating the internal contents (Miyamoto *et al*., 1998). For salmonellae other than *S.* Enteritidis, horizontal transmission is believed to be the most common route for the contamination of egg internal contents (Humphrey, 1994).

Contact between the eggshell and faeces is difficult to avoid completely. The extent of faecal contamination of the eggshell and the level of *Salmonella* shedding in faeces determines the level of eggshell contamination (Gast and Beard, 1990; De Louvois, 1993). Externally contaminated eggshells pose the risk of egg internal content contamination through horizontal transmission as well as the cross contamination of other food items in the kitchen. Epidemiological investigation from *Salmonella* food poisoning outbreaks in Queensland revealed that the use of dirty and cracked eggs in the restaurants was the major source of bacteria in these

outbreaks (Slinko *et al*., 2009). One way to control such outbreaks is egg washing which reduces the microbial load on the eggshell surface, limiting the chances of contamination of egg internal contents as well as cross contamination of other food items. Egg washing is common practise in the United States, Australia and Japan (Hutchison *et al*., 2004). There are, however, some detrimental outcomes associated with egg washing such as damage to the physical barriers of the egg, especially the cuticle (European Food Safety Authority, 2005) which is the first line of defence against bacterial penetration (Board and Halls, 1973). Other factors such as shell porosity and thickness can also affect egg penetration by bacteria (Messens *et al*., 2005a). However, these findings were not confirmed by following experiments as the eggshell penetration by *Salmonella* was independent of shell porosity and thickness (Messens *et al*., 2005b; De Reu *et al*., 2006). Increased eggshell translucency could be associated with greater microbial penetration (Chousalkar *et al*., 2010) but additional research investigating the relationship between translucency and bacterial penetration is required across contaminants.

Although *S.* Typhimurium is the most frequently isolated *Salmonella* serovar from egg products related to food poisoning cases in Australia (The OzFoodnet working group, 2012), other *Salmonella* serovars such as *S.* Singapore, *S.* Adelaide, *S.* Worthington and *S.* Livingstone have been frequently isolated from Australian layer farms (Cox *et al*., 2002; NSW Food Authority, 2012; Gole *et al*., 2013a). Egg related food poisoning outbreaks with these serovars have not been reported but in India, *S.* Worthington has been reported as the most frequently isolated non-typhoidal *Salmonella* serovar from human cases (Kumar *et al*., 2009). In 2004, an outbreak of *S.* Singapore associated with sushi consuption was reported in Queensland, Australia, but, the source of contamination was not clear (Barralet *et al*., 2004). In Norway and Sweden, during 2001, an outbreak of *S.* Livingstone, due to

contaminated processed fish products, resulted in three deaths and 22 hospitalizations (Guerin *et al*., 2004). Around the world, the majority of egg penetration studies have investigated the penetration ability of either *S.* Enteritidis or *S.* Typhimurium. To date, little attention has been given to other *Salmonella* isolates such as *S.* Singapore, *S.* Adelaide, *S.* Worthington and *S.* Livingstone.

The objectives of this study were to examine the effect of egg washing on the survival of these *Salmonella* isolates on the eggshell surface and to investigate the eggshell penetration ability along with the survival of different *Salmonella* isolates in egg internal conten. The study also included an investigation of egg washing and translucency on the penetration of eggshell by *Salmonella* isolates described. The effect of egg washing on cuticle ultrastructure and the relationship between cuticle quality and bacterial penetration were also investigated.

6.3 Materials and methods

All the *Salmonella* strains (*S.* Singapore - 709750, *S.* Adelaide - 709043, *S.* Worthington 703775 and *S.* Livingstone 709041) used in this study were isolated from the Australian layer flocks. The strain *S.* Worthington was isolated from egg shell wash by our laboratory where as the other isolates were obtained from the Australian *Salmonella* Reference Centre (Adelaide, Australia).

6.3.1 Egg washing

Fresh, visibly clean eggs were collected from hens (40 weeks old) a HyLine layer farm in South Australia. Before egg washing, all eggs were candled to identify cracks in eggshells. Egg washing processes used in this study involved washing with the aid of a surfactant followed by sanitisation and drying. A laboratory based washer with the capacity of 15 eggs at a time (three rows of five rotating rollers) was used for the physical mechanics of the egg washing. Washing was performed using a hydroxide and hypochlorite based solution at the concentration of 0.45% (v/v)

equivalent to a pH of ~12 and ~200 ppm hypochlorite in the working solution at 40°C. Washing was followed by a compatible sanitizer (at a concentration of 0.16% (v/v)) which equated to ~200 ppm hypochlorite in the working solution at 32°C. Eggs were washed and sanitized for 46 and 22 seconds, respectively. The pressure of the spray was 3 psi without brushes. Eggs were left on the bench for 15 minutes to dry and used for further experiments.

6.3.2 Inoculum preparation

The *Salmonella* isolates used in these experiments (*S.* Singapore, *S.* Adelaide, *S.* Worthington and *S.* Livingstone) were stored at -80°C in 80% glycerol. Bacteria were recovered from freezing by plating on xylose lysine deoxycholate (XLD) agar (Oxoid, Australia) and incubated overnight at 37°C. Colonies were selected from XLD agar and resuspended in phosphate buffered saline (PBS) to match the turbidity equivalent with a 0.5 McFarland standard (BioMerieux, Australia). Enumeration of viable bacteria was performed by serial dilution and spread plating on XLD agar and incubation overnight at 37°C. Following enumeration, a 200 mL inoculum containing 10^3 and 10^5 colony forming units (CFU) per mL was prepared for each isolate. Agar filled eggs and whole eggs were immersed for 90 sec in one of three dilutions: PBS (control), ~10^3, and ~10^5 CFU/ mL of *Salmonella*.

6.3.3 Whole egg penetration experiment

The effects of egg washing on the survival of *Salmonella* isolates on the eggshell surface and penetration across the eggshell, as well as the survival of *Salmonella* isolates in the internal contents of the egg, were investigated using a 'whole egg penetration' approach. Ninety eggs were collected from HyLine Brown hens in early lay and were divided into two groups: washed (n=30) and unwashed (n=60). Washed eggs were divided into one control (PBS) and two treatment groups (10^3 and 10^5 CFU/mL) with 10 eggs each. All the washed eggs were incubated at

20°C after exposure to *Salmonella* or the control PBS treatment. Unwashed eggs were divided into two groups of 30 eggs. Group 1 was further divided into one control and two treatment groups (10^3 and 10^5 CFU/mL) of 10 eggs each. Eggs from group 1 were incubated at 20°C after exposure to *Salmonella* or the control PBS treatment. Group 2 was also divided into one control and two treatment groups (10^3 and 10^5 CFU/mL) of 10 eggs each. These unwashed eggs were incubated at 37°C. The reason that only unwashed eggs were incubated at 37°C is that washed eggs are not used for hatching purposes in Australia. Each egg was dipped into 70% ethanol for 30 sec to sterilize the outer shell and allowed to air dry in a biosafety cabinet for 10-15 min. Eggs were then immersed for 90 sec in 10^3 CFU/mL or 10^5 CFU/mL of *Salmonella*. After inoculation, eggs were incubated at 20°C or 37°C for 21 days.

6.6.3.1. Isolation of Salmonella from eggshell surface and egg internal contents from whole egg penetration experiment

Eggshell surface samples and egg internal contents samples were processed separately by pooling two eggs together. After incubation at 20°C or 37°C for 21 days, each pair of eggs was placed in a Whirl-Pak bag (Nasco, USA) containing 20 mL of buffered peptone water (BPW; Oxoid, Australia) and each egg was massaged for 1 min. A 100 µL aliquot of the mixture was plated onto XLD plates, incubated overnight at 37°C and subsequently quantified. The limit of detection for isolation of *Salmonella* from eggshell surface and egg internal contents was 2 CFU/100 µL.

To investigate the penetration and survival of *Salmonella* in the contents of the egg, after the eggshell wash, eggs were dipped in 70% ethanol for 30 sec. Eggs were then aseptically opened, emptied into the Whirl-Pak bags and mixed. A 2 mL aliquot of the contents was transferred to 8 mL of BPW and 100 µL of this mixture was plated on XLD agar and incubated overnight at 37°C. Plates were then observed

for *Salmonella* growth. Suspected *Salmonella* isolates were sent to the Institute of Medical and Veterinary Sciences (IMVS), Adelaide, Australia for confirmation.

6.3.4 Agar method for assessment of the eggshell penetration

The effects of washing, translucency, and eggshell quality on the bacterial penetration of the eggshell were assessed by the 'agar egg' method as described previously by De Reu *et al.* (2006). Fresh eggs were obtained from the cage front of layers. All eggs were candled, scored for translucency, and allocated to two translucency groups based on candling score, until each group contained 32 eggs; where 1= low translucency, and 2= high translucency. For scoring translucency, a quantitative approach was used where a 1 cm^2 area of eggshell was marked and the numbers of lighter coloured spots on the eggshell (as viewed over a light source) were counted. Eggshells with fewer than 10 spots/cm^2 were considered to have low translucency. Eggs from each group were then allocated to washed and unwashed groups (n=16 each) and subsequently allocated to inoculated (n=10) and control groups (n=6). Each egg was dipped into 70% ethanol for 30 sec for sterilization of the eggshell surface and aseptically air dried for 10-15 min. The internal contents of each egg were removed using an 18 g needle (BD, Australia) at the blunt end of the egg. Eggs were also washed internally with sterile PBS (pH 7.2) to remove residual albumen. Eggs were then filled with XLD agar and sealed with cellophane tape after the agar solidified. Agar-filled eggs from each treatment group (washed and unwashed) were immersed for 90 sec in 200 mL of approximately 10^5 CFU/mL solution of *Salmonella*. Eggs from the control groups (washed and unwashed) were immersed in sterile PBS for 90 sec. After inoculation, agar-filled eggs were incubated at 20°C for 21 days. After incubation, the eggs were aseptically opened and the penetration of *Salmonella* spp. was assessed by the blackening of the interior eggshell.

6.3.4.1 Scanning electron microscopy (SEM)

A scanning electron microscope (SEM) (Philips XL 30 FEGSEM) was used to score the cuticle of the eggshell. Assessment of the cuticle was carried out using all the eggs in the control and treatment groups. A Dremel high speed rotary, model tool, 300 series was used to cut pieces of eggshell (approximately 1 cm^2) from around the equator of all eggs. Each eggshell piece was mounted on a 12.6 mm diameter pin type stub using I005Aqueous conductive silver liquid SEM adhesive (ProSciTech, Australia). The specimens were sputter coated in a Neocoater for 5 min, and viewed under the SEM (Philips XL 30 FEGSEM) at various magnifications. Scoring of the cuticle was conducted according to Samiullah *et al*. (2013).

6.3.5 Statistical analysis of whole egg and agar penetration experiment

The statistical software IBM SPSS version 20 (2011) was used to perform all statistical analysis. Data analysis for the whole egg penetration experiment was performed using a Student's t-test. The samples that did not produce any colonies on XLD plates were considered negative for *Salmonella* and assigned one CFU score per eggshell for allowing log transformations. In the agar egg experiment, the effect of washing, eggshell translucency and their interaction on eggshell penetration of inoculated eggs was investigated using a binary logistic regression model. Logistic regression was also used to explore the relationship between the cuticle score and *Salmonella* penetration. The effects of washing, translucency and treatment on the cuticle score were analysed using an ordered logistic regression model. Models were assessed using the Wald statistics based on a significance level of $p \leq 0.05$. Non-significant interactions were excluded from models one at a time.

6.4 Results

6.4.1 Whole egg penetration experiment

6.4.1.1 Survival of Salmonella isolates on the eggshell surface after 21 days of incubation

The survival of bacteria on the eggshell surface plays an important role in the horizontal transmission of bacteria across the eggshell to contaminate the egg internal contents. Hence, we compared the survival of various *Salmonella* isolates on the eggshell surface of washed and unwashed eggs. *Salmonella* was not detected from any eggshell surfaces of control group eggs, which was to be expected as they were not inoculated with any isolates. However, a significant difference was not observed between the levels of *Salmonella* on the surface of washed and unwashed eggs (Table 6.1).

Using two different temperatures (20°C and 37°C), the effect of temperature on the survival of various *Salmonella* isolates on the eggshell surface of unwashed eggs was investigated. The survival rate of *S.* Worthington was significantly higher ($p< 0.0005$) at 20°C compared to 37°C. For all isolates, the overall trend indicated that a temperature of 20°C was more favourable for *Salmonella* survival on the eggshell surface. Interestingly, *S.* Singapore, *S.* Worthington and *S.* Livingstone isolates used in this study were not detected on the eggshell surface of unwashed eggs (inoculated with 10^3 CFU/mL) after 21 days of incubation at both temperatures (20°C and 37°C) (Table 6.2). At 37°C, all the eggshells inoculated with 10^3 CFU/mL and 10^5 CFU/mL of *S.* Singapore, *S.* Adelaide and *S.* Worthington were negative after the 21 day post inoculation. The combined data of all isolates demonstrated that the survival rate of *Salmonella* on eggshell surface (inoculated with 10^5 CFU/mL) was significantly higher ($p=0.002$) at 20^0C as compared to 37°C.

The effect of dose on the survival of *Salmonella* was studied using two different doses of inoculation (10^3 and 10^5 CFU/mL) and, as expected, in most cases

(except *S.* Singapore, washed group at 20°C) results indicated that survival rate was higher in eggs inoculated with a 10^5 CFU/mL dose (Table 6.3). However, a significant difference ($p<0.05$) was observed only for *S.* Worthington and *S.* Livingstone. Similar findings were reported with the combined data of all isolates, where there was significantly higher *Salmonella* survival on eggs inoculated with a 10^5 CFU/mL dose as compared to a 10^3 CFU/mL dose.

6.4.1.2 Penetration of eggs and contamination of internal contents by Salmonella isolates

All the internal contents of control group eggs were negative for *Salmonella*. Results of the direct agar culture method demonstrated that, for *S.* Singapore, *S.* Worthington and *S.* Livingstone, *Salmonella* was not detected in any of the egg internal content samples. For *S.* Adelaide, only one egg's internal content was positive from 40 eggs tested.

6.4.2 Agar egg penetration experiment

6.4.2.1 Relationship of washing and translucency with egg penetration by various Salmonella isolates

Table 6.4 summarises the results for the number of washed and unwashed eggs which were penetrated for each *Salmonella* isolate and translucency score. Agar penetration experiments indicated that *S.* Singapore, *S.* Worthington, *S.* Adelaide and *S.* Livingstone were capable of penetrating the eggshells. In the case of *S.* Worthington, eggshell penetration was higher but not significant ($p=0.06$) in washed eggs as compared to unwashed eggs. However, for *S.* Adelaide and *S.* Livingstone, there was no significant difference in the eggshell penetration of washed and unwashed eggs, and for *S.* Singapore, eggshell penetration was identical for washed and unwashed eggs (Table 6.4). Statistical analysis also demonstrated that, for all *Salmonella* isolates, there was no significant difference in the number of penetrated eggs with low and high translucency scores (Table 6.4). Overall, there was no

significant effect of egg washing and translucency on eggshell penetration (Table 6.4).

6.4.2.2 Relationship of washing and translucency with egg cuticle score

The results of the ordered logistic regression identified egg washing as having a significant effect on the cuticle score of eggs belonging to *S.* Singapore, *S.* Adelaide and *S.* Worthington groups (Table 6.5). However, for *S.* Livingstone, there was no significant difference in the cuticle score of washed and unwashed eggs. The good quality cuticle of an unwashed egg and the damaged cuticle/eggshell surface of a washed egg is shown in Figure 6.1 and Figure 6.2 respectively. The interaction between washing and translucency was not significant for any of the *Salmonella* isolates. Table 6.6 provides detailed results about the relationship between translucency and cuticle score. Results indicated that there was no significant relationship between translucency and egg cuticle score.

6.4.2.3 Relationship between cuticle score and penetration by Salmonella isolates

Results indicated that, for *S.* Worthington, cuticle score was a significant predictor (p=0.05) of eggshell penetration (Table 6.7). With the increase in cuticle score, there was an increase in eggshell penetration. However, for all other isolates, no significant relationship was observed between cuticle score and eggshell penetration. The relationship between the cuticle score and eggshell penetration was also investigated by combining data from all *Salmonella* isolates. Results demonstrated that cuticle score was a significant predictor (p=0.04) of eggshell penetration (Table 6.7).

6.5 Discussion

The present study was designed to examine the survival and egg penetration ability of *Salmonella* strains apart from Typhimurium which have been isolated from Australian egg layer farms. It is important to study the survival ability of different

Salmonella isolates on the eggshell surface, as it has been observed that the cross contamination of food items in the kitchen environment may lead to *Salmonella* outbreaks. Our results indicated that all of the *Salmonella* isolates (*S.* Singapore, *S.* Adelaide, *S.* Worthington and *S.* Livingstone) used in the present study were capable of surviving on the eggshell surface after 21 days of incubation (at 20°C) following contamination.

The outermost layer on the eggshell is the cuticle. The thickness of the cuticle varies from 0.5 to 12.8 μm and it is made up of lipids, hydroxyapatite crystals, glycoproteins and polysaccharides (Fernandez *et al*., 2001; Whittow *et al*., 2000). The protein extract derived from the cuticle has antibacterial activity against both Gram-positive and Gram-negative bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus subtilis* (Wellman-Labadie *et al*., 2008). However, some researchers claim that egg washing chemicals can damage the cuticle layer of the eggshell (Wang and Slavik, 1998) which could result in a better survival of bacteria on the eggshell surface of washed eggs as compared to unwashed eggs. To check this hypothesis, we compared the survival of *Salmonella* on the eggshell surface of washed and unwashed eggs. There was no significant difference in the survival rate of *Salmonella* isolates (individual isolate as well as combined data of all isolates) on the eggshell surface of washed and unwashed eggs. However, the overall trend indicated that the survival rate was better on the eggshell surface of washed eggs which may be due to the damage of cuticle by egg washing chemicals. At this stage, it is unclear to what extent the cuticle can inhibit the bacterial contamination of eggshell under natural conditions.

The survival of various *Salmonella* isolates on the eggshell surface was studied using two different incubation temperatures (20°C and 37°C). The Results demonstrated that a temperature of 20°C is more favourable for *S.* Worthington

survival on the eggshell surface. In the case of other *Salmonella* serovars; there was no significant difference in the survival of *Salmonella* on the eggshell surface at 20°C and 37°C. However, combined data of all isolates suggested significantly higher *Salmonella* survival on eggshell surface at 20°C as compared to 37°C. A lower temperature, along with a dry eggshell surface, may have reduced the rate of *Salmonella* metabolism (Radkowski, 2002). Similar findings were reported by previous experiments (Radkowski 2002; Botey-Salo *et al*., 2012).

At 37°C, all the eggshells inoculated with 10^3 CFU/mL and 10^5 CFU/mL *S.* Singapore, *S.* Adelaide and *S.* Worthington were observed negative after the incubation period of 21 days. In contrast to this, the results of *S.* Typhimurium egg penetration study indicated that *S.* Typhimurium has the better capacity to survive on the eggshell at 37°C after 21 days of incubation (Gole *et al*., 2014). Interestingly, *S.* Singapore, *S.* Worthington and *S.* Livingstone were not detected on the eggshell surface of unwashed eggs with a low dose of inoculation (10^3 CFU/mL) after 21 days of incubation at 20°C. Eggshells infected with various strains of *S.* Typhimurium (10^3 CFU/mL), however, have been reported *Salmonella* positive (Gole *et al*., 2014). This clearly indicates that *S.* Typhimurium has better a capacity to survive on the eggshell surface as compared to the other *Salmonella* isolates used in this study.

Using the 'whole egg penetration' approach, the egg penetration ability of *Salmonella* isolates without altering egg structure was studied. All the egg contents for *S.* Singapore, *S.* Worthington and *S.* Livingstone were found to be *Salmonella* negative. It is possible that either egg internal contents may have a very low level of *Salmonella* below the limit of detection (2 CFU/100 µl) or these isolate may lack the ability to survive in egg internal contents. To confirm these findings further experiments is essential using the selective enrichment using Rappaport Vassidalis soya peptone (RVS) broth. *S.* Adelaide was detected in the egg contents in only one

egg internal content sample. In contrast to these findings, Gole *et al*. (2014) reported that 16% of egg internal contents were positive when eggs were inoculated with the different strains of *S.* Typhimurium. Egg albumen contains many antimicrobial components such as lysozyme and ovotransferrin which can inhibit the growth of bacteria. In order for the bacteria multiply in the egg, it is essential for *Salmonella* to move from the egg albumen to egg yolk which is a rich source of nutrients for bacterial multiplication (Gantois *et al*., 2008). *Salmonella* isolates used in present study (*S.* Singapore, *S.* Worthington, *S.* Livingstone and S. Adelaide) either lack the ability to survive in the hostile environment of egg albumen or they do not have a capacity to move from egg albumen to egg yolk which resulted in the killing of all the *Salmonella* that penetrated the eggs. Additionally, further studies are essential to confirm these finding, using more sensitive detection methods such as selective enrichment in RVS broth and/or Real-Time PCR (qPCR).

In order to clean dirty eggs and to reduce the microbial load on the eggshell surface, egg washing is commonly used in Australia (Anonymous, 2009). On the other hand, egg washing is banned in the European Union for first grade eggs and is a subject of rigorous debate (Nys and Van Immerseel, 2009). Hence, to investigate the effect of egg washing on eggshell penetration by *Salmonella* and to study eggshell penetration ability of different *Salmonella* isolates, the 'Agar egg penetration' was adopted. Results indicated that *S.* Singapore, *S.* Worthington, *S.* Adelaide and *S.* Livingstone were capable of penetrating the eggshells. The combined data of all isolates suggested that, even though the eggshell penetration was higher in washed eggs as compared to unwashed eggs, the difference was not significant. However, in the case of *S.* Worthington, eggshell penetration was marginally higher ($p=0.06$) in washed eggs as compared to unwashed eggs. The previous results of Gole *et al*. (2014) also indicated that eggshell penetration of

various strains of *S.* Typhimurium was higher in washed eggs as compared to unwashed eggs. The higher penetration in washed eggs may be due to damage to the cuticle by egg washing chemicals.

Using SEM, the effect of egg washing on cuticle integrity was also evaluated and results of the ordered logistic regression indicated that the cuticle score was significantly higher (less cuticle) in washed eggs as compared with unwashed eggs. In contrast to these findings, Leleu *et al*. (2011) reported that cuticle was not affected by egg washing. This variation may be due to difference in the age of laying hens from which eggs were collected. Previous studies indicated that cuticle thickness is negatively affected by increase in the age of birds (Spark and Board, 1984; Messens *et al*., 2005a). In the present study, eggs were collected from birds at 40 weeks of age, where previously Leleu *et al*. (2011) used eggs from relatively old laying hens (> 54 weeks). Another possible explanation for the variation in the results is differences in the chemicals used for egg washing. The degree of cuticle damage can vary with different type of chemicals used in the egg washing protocol (Wang and Slavik, 1998). In the present experiment, cuticle score was found to be a significant predictor of *S.* Worthington eggshell penetration. A similar result was obtained for the data combined from all *Salmonella* isolates. These findings are in agreement with De Reu *et al*. (2006) who reported that, in penetrated eggs, the mean cuticle deposition was lower as compared to non-penetrated eggs. However, Messens *et al*. (2005b) observed no correlation between the cuticle score and eggshell penetration by *S.* Enteritidis. Similarly, in the present study, for other individual *Salmonella* isolates (*S.* Adelaide, *S.* Singapore and *S.* Livingstone), there was no significant effect of egg washing on eggshell penetration. Also, for these isolates, there was no significant relationship between cuticle score and eggshell penetration. In the present study, SEM was used to score the cuticle where as Messens *et al*. (2005b) scored

cuticle using straing techniques. Hence, the variation in the results between these studies may be attributed to different methods used to quantify cuticle score. Also, the eggshell penetration is not dependent only on cuticle quality.

The relationship between eggshell translucency and egg penetration was also studied by using the agar egg penetration approach. Results indicated that there was no significant relationship between penetration and translucency. However, the results of present experiment are in contrast with the findings of Chousalkar *et al*. (2010) who reported a significant correlation between eggshell penetration by *S.* Infantis and *E. coli* and eggshell translucency. The variation in the results may be due to the difference in the scoring of eggshell translucency. In the present experiment, eggs were allocated to different translucency groups based on a pre-determined cut off. It is essential to note that there was also a difference in the bacterial strains used to study eggshell penetration in these two studies.

In conclusion, all of the *Salmonella* isolates used in the present study were capable of surviving on the eggshell surface after 21 days of incubation (at 20°C) following a high dose of inoculation. Previously studies indicated that these *Salmonella* serovars (*S*. Singapore, *S*. Worthington, *S*. Livingstone and *S*. Adelaide) have a capacity to cause food poisoning outbreaks in humans. However, it is essential to note that these historical outbreaks were not associated with eggs.

In Australia, *Salmonella* serovars such as *S*. Infantis and *S*. subsp. 1 serovar 4,12:d: have been isolated from the eggshell surface (Gole *et al*., 2013b). Hence, careful and hygienic handling of eggs in the supply chain and in kitchen is essential to reduce egg related food poisoning outbreaks. There was a trend towards better survival of *Salmonella* on eggshell surface of washed eggs as compared to unwashed eggs. However, the difference was not significant. The agar egg penetration experiment showed that all the *Salmonella* isolates used in the present study have a

capacity to penetrate across eggshells of washed and unwashed eggs. This also shows that eggshell penetration is not a unique characteristic of *S.* Enteritidis or *S.* Typhimurium. Bacteria other than *Salmonella* such as *Staphylococcus*, *Acinetobacter*, *Alcaligenes*, *Serratia* and *Pseudomonas* also possess eggshell penetration ability (De Reu *et al*. 2006).

In the present study, for washed eggs, *S.* Worthington eggshell penetrations were reported higher as compared to unwashed eggs. Hence, special attention is essential to avoid re-contamination of eggs with *Salmonella* after washing. As per the results of the whole egg penetration experiment, in case of *S.* Singapore, *S.* Worthington and *S.* Livingstone, *Salmonella* was not detected in egg internal contents samples. These serovar may have limited capacity to survive in the egg internal contents. This may be the reason why these serovars were not reported in egg related food poisoning outbreaks to date in Australia. However, further studies are essential using more sensitive techniques such as qPCR to confirm these findings. On the other hand, clearly a better survival ability of *S.* Typhimurium on the eggshell surface and in internal egg contents seems likely to be responsible for the clear association of *S.* Typhimurium with egg related food poisoning outbreaks in Australia.

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Table 6.1 Survival of *Salmonella* isolates on eggshell surface after 21 days of incubation at 20 °C: comparison between washed and unwashed eggs

Salmonella isolate	Dose of inoculation (CFU/mL)	Washing status	Contamination after incubation (Log CFU/ egg pool) Mean ± SE (number of *Salmonella* negative egg pool)	p-value
Salmonella Singapore	10^3	Washed	0.89 ± 0.55 (3)	0.18
		Unwashed	ND (5)	
	10^5	Washed	0.40 ± 0.40 (4)	0.38
		Unwashed	1.20 ± 0.76 (3)	
Salmonella Adelaide	10^3	Washed	2.30 ± 0.97 (2)	0.24
		Unwashed	0.76 ± 0.76 (4)	
	10^5	Washed	3.58 ± 0.91 (1)	0.74
		Unwashed	3.04 ± 1.25 (2)	
Salmonella Worthington	10^3	Washed	0.98 ± 0.98 (4)	0.37
		Unwashed	ND (5)	
	10^5	Washed	4.59 ± 0.24 (0)	0.18
		Unwashed	4.11 ± 0.22 (0)	
Salmonella Livingstone	10^3	Washed	ND (5)	NA
		Unwashed	ND (5)	
	10^5	Washed	4.72 ± 0.12 (0)	0.30
		Unwashed	4.49 ± 0.17 (0)	
All *Salmonella* isolates	10^3	Washed	1.04 ± 0.39 (14)	0.06
		Unwashed	0.19 ± 0.19 (19)	
	10^5	Washed	3.32 ± 0.46 (5)	0.86
		Unwashed	3.21 ± 0.45 (5)	

ND: not detected, NA: not applicable

Table 6.2 Survival of *Salmonella* isolates on eggshell surface of unwashed eggs after 21 days of incubation: comparison between 20°C and 37°C

Salmonella isolate	Dose of inoculation (CFU/mL)	Temperature (°C)	Contamination after incubation (Log CFU/ egg pool) Mean ± SE (number of *Salmonella* negative egg pool)	p-value
Salmonella Singapore	10^3	20	ND (5)	NA
		37	ND (5)	
	10^5	20	1.20 ± 0.76 (3)	0.19
		37	ND (5)	
Salmonella Adelaide	10^3	20	0.76 ±0.76 (4)	0.37
		37	ND (5)	
	10^5	20	3.04 ± 1.25 (3)	0.07
		37	ND (5)	
Salmonella Worthington	10^3	20	ND (5)	NA
		37	ND (5)	
	10^5	20	4.11 ± 0.22 (0)	0.00
		37	ND (5)	
Salmonella Livingstone	10^3	20	ND (5)	NA
		37	ND (5)	
	10^5	20	4.49 ± 0.17 (0)	0.94
		37	4.46 ± 0.37 (0)	
All *Salmonella* isolates	10^3	20	0.19 ± 0.19 (19)	0.33
		37	ND (20)	
	10^5	20	3.21 ± 0.45 (6)	0.002
		37	1.11 ± 0.45 (15)	

ND: not detected, NA: not applicable

Table 6.3 Survival of *Salmonella* isolates on the eggshell surface after 21 days of incubation: comparison between different doses (10^3 and 10^5 CFU/mL) of inoculation

Salmonella isolate	Temperature (°C)	Washing status	Dose of inoculation (CFU/mL)	Contamination after incubation (Log CFU/ egg pool) Mean ± SE (number of *Salmonella* negative egg pool)	P-value
Salmonella Singapore	20	Washed	10^3	0.89 ± 0.55 (3)	0.49
			10^5	0.40 ± 0.40 (4)	
		Unwashed	10^3	ND (5)	0.19
			10^5	1.20 ± 0.76 (3)	
	37	Unwashed	10^3	ND (5)	NA
			10^5	ND (5)	
Salmonella Adelaide	20	Washed	10^3	2.30 ± 0.97 (2)	0.36
			10^5	3.58 ± 0.91 (1)	
		Unwashed	10^3	0.76 ± 0.76 (4)	0.16
			10^5	3.04 ± 1.25 (2)	
	37	Unwashed	10^3	ND (5)	NA
			10^5	ND (5)	
Salmonella Worthington	20	Washed	10^3	0.98 ± 0.98 (4)	0.02
			10^5	4.59 ± 0.24 (0)	
		Unwashed	10^3	ND (5)	0.00
			10^5	4.11 ± 0.22 (0)	
	37	Unwashed	10^3	ND (5)	NA
			10^5	ND (5)	
Salmonella Livingstone	20	Washed	10^3	ND (5)	0.00
			10^5	4.72 ± 0.12 (0)	
		Unwashed	10^3	ND (5)	0.00
			10^5	4.49 ± 0.17 (0)	
	37	Unwashed	10^3	ND (5)	0.00
			10^5	4.46 ± 0.37 (0)	
All *Salmonella* isolates	20	Washed	10^3	1.04 ± 0.39 (14)	0.001
			10^5	3.32 ± 0.46 (5)	
		Unwashed	10^3	0.19 ± 0.19 (19)	0.00
			10^5	3.21 ± 0.45 (5)	
	37	Unwashed	10^3	ND (20)	0.02
			10^5	1.11 ± 0.45 (15)	

ND: not detected, NA: not applicable

Table 6.4 Relationship of washing and translucency with agar egg penetration by various *Salmonella* isolates

		Low Translucency (Score 1)		High Translucency (Score 2)			
Salmonella isolate	Number of penetrated/ Not-penetrated eggs	Unwashed	Washed	Unwashed	Washed	p-value (Relationship of washing with agar egg penetration)	p-value (Relationship of translucency with agar egg penetration)
Salmonella Singapore	Not Penetrated	2	3	2	1	1.00	0.43
	Penetrated	8	7	8	9		
Salmonella Adelaide	Not Penetrated	1	1	2	0	0.31	1.00
	Penetrated	9	9	8	10		
Salmonella Worthington	Not Penetrated	1	1	5	0	0.06	0.20
	Penetrated	9	9	5	10		
Salmonella Livingstone	Not Penetrated	2	6	3	4	0.11	0.74
	Penetrated	8	4	7	6		
All *Salmonella* isolates	Not Penetrated	6	11	12	5	0.18	0.11
	Penetrated	34	29	28	35		

Table 6.5 Effect of egg washing on cuticle score

Salmonella isolate	Washing status	Number of eggs	Average cuticle score (Mean ± SE)	p-value
Salmonella Singapore	Washed	32	3.22 ± 0.13	0.002
	Unwashed	32	2.59 ± 0.12	
Salmonella Adelaide	Washed	32	3.37 ± 0.11	0.00
	Unwashed	32	2.69 ± 0.13	
Salmonella Worthington	Washed	32	3.12 ± 0.12	0.00
	Unwashed	32	2.37 ± 0.13	
Salmonella Livingstone	Washed	32	2.72 ± 0.14	0.81
	Unwashed	32	2.69 ± 0.10	

Table 6.6 Relationship between translucency and cuticle score

Salmonella isolate	Translucency	Number of eggs	Average cuticle score (Mean ± SE)	p-value
Salmonella Singapore	Low	32	2.91 ± 0.14	0.45
	High	32	2.91 ± 0.14	
Salmonella Adelaide	Low	32	2.93 ± 0.14	0.47
	High	32	3.12 ± 0.12	
Salmonella Worthington	Low	32	2.84 ± 0.14	0.40
	High	32	2.66 ± 0.15	
Salmonella Livingstone	Low	32	2.69 ± 0.08	0.73
	High	32	2.72 ± 0.15	

Table 6.7 Relationship between cuticle score and eggshell penetration by *Salmonella* isolates

		Cuticle score				
Salmonella isolate	Number of penetrated/non-penetrated eggs	1	2	3	4	p-value
Salmonella Singapore	Penetrated	1	6	18	7	0.13
	Non-penetrated	0	5	2	1	
Salmonella Adelaide	Penetrated	1	6	20	9	0.48
	Non-penetrated	0	2	1	1	
Salmonella Worthington	Penetrated	0	9	18	6	0.05
	Non-penetrated	1	3	3	0	
Salmonella Livingstone	Penetrated	3	11	8	3	0.47
	Non-penetrated	0	11	4	0	
All *Salmonella* isolates	Penetrated	5	32	64	25	0.04
	Non-penetrated	1	21	10	2	

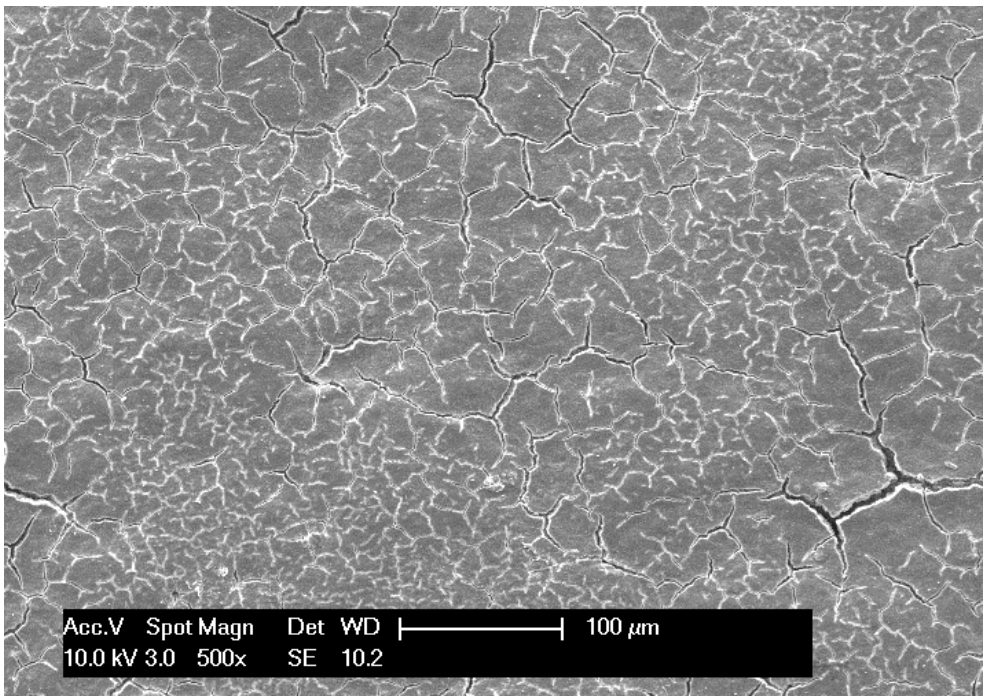

Figure 6.1 Scanning electron microscope image of good quality cuticle on the eggshell surface of unwashed egg

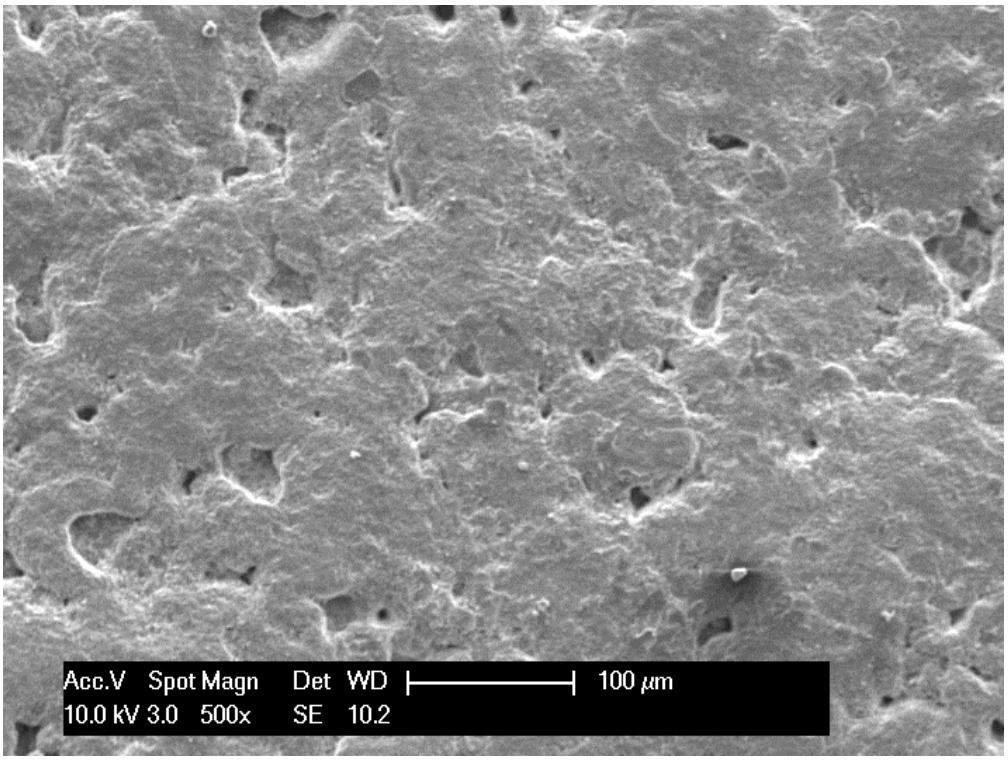

Figure 6.2 Scanning electron microscope image of poor quality cuticle on the egg shell surface of washed egg.

Chapter 7 Prevalence of antibodies to *Mycoplasma synoviae* in laying hens and possible effects on egg shell quality

Statement of Authorship

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By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

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7.1 Abstract

Mycoplasma synoviae (*M. synoviae*) can cause respiratory disease, and synovitis, or result in a silent infection in chickens and turkeys. The importance of *M. synoviae* is well established in broilers, but only a few studies have been conducted in egg layers and these studies suggest that infection may influence eggshell quality. In the present study, the prevalence of *M. synoviae* in commercial layer flocks was estimated using ELISA. For this study, 19 commercial layer flocks were selected randomly from New South Wales and the Queensland regions of Australia from producers who were willing to participate in the survey. Sixty eggs per flocks were randomly collected, out of these 30 eggs were used for ELISA and the remaining 30 eggs were used to estimate various egg shell quality parameters. Subsequently, association between the serological status of eggs for *M. synoviae* and egg shell quality was studied. In the flocks under study, seroprevalence of *M. synoviae* was found to be high at 69% (95 % confidence interval (CI) = 41.3 to 89.0). Statistical analysis showed an association between serological status for *M. synoviae* and egg quality parameters such as translucency, shell breaking strength, % shell reflectivity and shell deformation. On the other hand, there was no significant association between serological status for *M. synoviae* and other egg quality parameters such egg weight, egg shell weight, % egg shell or shell thickness.

7.2 Introduction

Mycoplasma species are well-known pathogens of domestic poultry, causing significant economic losses (Lierz *et al*., 2007). *Mycoplasma synoviae* (*M. synoviae*) is one of the most pathogenic species of this genus and can cause respiratory disease, synovitis or sometimes result in a silent infection in poultry. The importance of *M. synoviae* is well established in broilers but only a few studies have been conducted in

layers (Hagan *et al*., 2004). *M. synoviae* is known to be transmitted vertically through eggs (Jordan, 1975) and the prevalence of egg *M. synoviae* antibodies in egg yolk is reported to be an appropriate way of assessing the flock prevalence of *M. synoviae* infection in laying hens (Hagan *et al*., 2004) which is correlated with serum antibodies (Mohammed *et al*., 1986a). Vertical transmission of a respiratory isolate of *M. synoviae* was confirmed in broiler breeders (Macowan *et al*., 1984) and the Dutch strain of *M. synoviae* was found to be one of the factors causing egg shell translucency (Feberwee *et al.*, 2009a, b). For the economics of the poultry farm, eggshell quality is very important and egg quality could play a vital role in trans-shell penetration of *Salmonella* spp. A good quality eggshell significantly protects the internal contents from bacterial penetration. However, there is little information available regarding the effects of Australian strains of *M. synoviae* on egg shell quality. In the present study, the prevalence of *M. synoviae* in commercial layer flocks was studied using ELISA. The association between egg shell quality parameters and the seroprevalence of *M. synoviae* in eggs from the same flocks was investigated.

7.3 Materials and Methods

Eggs were collected randomly from 19 different commercial layer flocks (aged between 22 to 74 weeks) located in New South Wales and Queensland, Australia from producers who were willing to participate in the survey. Of these 19 flocks, three flocks were vaccinated (Bioproperties, Vaxsafe® MS). *M. synoviae* was expected to spread quickly within a flock so, from each flock 60 eggs were collected**.** Thirty eggs were collected in order to extract antibodies from the egg yolk which were further used in an ELISA. Thirty eggs from each flock were analyzed for egg quality parameters: translucency score (0= No translucency, 1= mild translucency, 2= moderate translucency, 3= Severe translucency), shell reflectivity (%), egg weight

(g), shell breaking strength (Newtons), shell deformation to breaking point (μm), shell weight (g), percentage shell (%) and shell thickness (μm). All the equipment used for egg quality analyses, except egg shell thickness, was supplied by Technical Services and Supply, UK. Egg shell thickness was measured by taking shell pieces from three equidistant points on the equator with shell membrane intact and measuring their thickness using a gauge constructed from a Mitutoyo Dial Comparator gauge Model 2109-10.

7.3.1Antibody extraction from egg yolk and ELISA

The yolk extraction method was adapted from Mohammed *et al.* (1986a). For a saline extraction, 3 mL of egg yolk was collected from each of the 570 eggs (n=30 from 19 flocks) and mixed with 3 mL saline, vortexed and left for 48 h at 4°C. For the chloroform extraction, 0.5 mL saline extraction and 1 mL chloroform were vortexed to a thick paste. This was allowed to stand for 30 min at room temperature before being centrifuged at 850 g for 20 min. The upper aqueous layer was removed and used in the ELISA. These extracted yolk antibodies were stored at -20°C. Each extracted antibody sample was diluted 1:50 ratio in PBS and was then used in the ELISA. The BioChek Mycoplasma synoviae antibody kits (BioChek, catalog code CK 115) were used in this study, according to the manufacturer's instructions, in order to study the prevalence of *M. synoviae* in the sampled commercial layer flocks. Absorbance of controls and test samples was measured at 405 nm (Multiskan Ascent pathtech). Dilutions of chloroform-extract egg yolk antibody were prepared from the pools of known positive (*M. synoviae* vaccinated) and known negative eggs and tested for the following titres; 1:10, 1:50, 1:100, 1:500 and 1:1000. From the curve produced, the linear part was expanded. Reading the known positive and negative samples individually at the selected dilution produced a cut-off point for the test. Test sensitivity, specificity and threshold (cut off) values were determined by

plotting sensitivity and specificity against the cut off value using two graph receiver operating characteristics (TG-ROC) analysis as described by Greiner *et al.* (1995). Based on ELISA results, the flocks were divided into two groups, infected and uninfected. The flocks with 10% or more positive reactions were considered positive serologically, using the criterion of Kleven and Bradbury (2008) and a third group was established based on vaccination history (Bioproperties, Vaxsafe® MS). Using the ELISA results, sero-prevalence of *M. synoviae* was estimated with 95% exact binomial confidence interval model on normal approximation and the one way analysis of variance (ANOVA) of the S-PLUS statistical software was used to compare egg shell quality parameters of infected, uninfected and vaccinated groups.

7.4 Results and Discussion

Egg yolk antibodies were used for studying the prevalence of *M. synoviae* amongst the layer flock. Using the chloroform-extracted egg yolks, a dilution factor of 1:50 was chosen as it was on the linear part of the standard curve produced. The Se and Sp for each threshold value were calculated as the proportion of positive results in the positive reference population and negative results in the negative reference population, respectively (Greiner *et al*., 1995). It was observed that the optimized cut-off point for egg yolk was 0.390 with 90% Se and Sp. Of the 19 flocks screened under this study, numbers of serologically positive (infected) and negative (uninfected) flocks were found to be 11 and 5, respectively, and the remaining 3 flocks were vaccinated. Thus, the prevalence *of M. synoviae* serologically positive flocks in commercial layers was high {11/16 (69%), 95 % CI = 41.3 to 89.0}. Table 7.1 shows the individual flock-wise sero-prevalence of *M. synoviae.* Laying hens are efficient producers of antibodies. Following immunization against a specific pathogen, higher levels of antibodies are usually found in egg yolk than serum of hens (Malik *et al*., 2006). Using egg yolk samples for routine screening is beneficial

as it avoids the expense and stress of blood sampling. The present study was conducted in order to estimate the sero-prevalence of *M. synoviae* infection in the commercial layer flocks by ELISA and a high sero-prevalence of *M. synoviae* in commercial layer stock was found. The prevalence study of Hagan *et al*. (2004), which was also based on the detection of *M. synoviae* antibodies in eggs, reported a prevalence of 78.6% in commercial layer flocks in East England. In another study (Mohammed *et al*., 1986b), prevalence of *M. synoviae* was 87% in commercial layer flocks in Southern California. Our findings are in accordance with other researchers mentioned above. It has been found that multiple age flocks and low biosecurity standards amongst the layer farms are responsible for the high prevalence and persistence of *M. synoviae* infections (Stipkovits & Kempf, 1996; Kleven, 2003). *M. synoviae* infected commercial layer stocks therefore pose a significant epidemiological risk for other categories of poultry. Suzuki *et al.* (2009) reported a high percentage of *M. synoviae* sero-prevalence (up to 53%) in backyard flocks in Paraquay, measured by ELISA test. Kapetanov *et al.* (2010) reported that, in Serbia, the overall seroprevalence of *M. synoviae* in the flocks decreased from 47.5% in 2000 to 22.2% in 2009.

Table 7.2 shows association between *M. synoviae* serological status and different egg shell quality parameters including translucency, egg shell reflectivity, egg weight, shell breaking strength, shell deformation, egg shell weight, % egg shell and shell thickness. Statistical analysis showed that the vaccinated group (3.1±0.1) had the highest translucency score as compared to the infected (2.4±0.1) and uninfected (2.3±0.1) groups. Eggshell colour can be judged by shell reflectivity which is the amount of reflection of the light from the eggshell surface. As the egg becomes paler, reflectivity increases and consumers do not prefer such eggs. The infected group (31.5±0.2 %) had significantly higher % shell reflectivity, followed

by the vaccinated group (29.7±0.5%) and the uninfected group (28.0±0.3 %), respectively.

Shell breaking strength (38.9±0.5N) was found to be significantly lower in the infected group, as compared to the uninfected and vaccinated groups. In the uninfected group, shell deformation (343.5±6.0 μm) was significantly higher as compared to the other two groups. Shell deformation reflects elasticity of the eggshell. The egg with higher shell deformation requires greater force to cause breakage. Hence, with low elasticity, there are more chances of egg breakage which may cause economical losses to egg producers. However, there was no significant difference for other egg quality parameters such as egg weight, egg shell weight, % egg shell and shell thickness among these three groups. Shell breaking strength may have reduced by mechanisms other than shell thickness.

For the economic viability of the egg industry, it is critical to produce eggs with good egg shell quality. Egg quality problems are responsible for significant economic losses within the Australian egg industry so it is important to study the factors which are responsible for decreased egg internal quality and egg shell quality. Many factors have been identified as having direct or indirect effects on egg shell quality including bird strain, nutrition, stress and disease (Roberts, 2004). Age of a bird could also affect the egg production and egg quality (Roberts, 2004), however in this study, eggs were collected from flocks representing various age groups. The mechanism by which *M. synoviae* affects the normal eggshell calcification process and egg shell quality is not clear. *M. synoviae* affect the ciliary motility in the oviduct, which could lead to changes in the uterine fluid content affecting the deposition of calcium carbonate crystals (Dominquez-Vera *et al*., 2000). Feberwee *et al*. (2009a, b) reported that a Dutch strain of *M. synoviae* was associated with

formation of egg apex abnormalities (EAA) and also reported a synergism between *M. synoviae* and infectious bronchitis virus. However, there is little information available regarding synergism between uterotropic strains of Australian infectious bronchitis virus and *M. synoviae*. In the present study, it was found that shell breaking strength was significantly lower in the infected group as compared to the uninfected and vaccinated groups. Also, the vaccinated group had the highest translucency score as compared to the infected and uninfected groups, whereas the infected group had lighter shells followed by the vaccinated and negative groups, respectively. On the other hand, there was no significant difference between serological status of *M. synoviae* and other egg shell quality parameters such as egg weight, egg shell weight, % egg shell and shell thickness. The findings of the study are in contrast to earlier findings by Lott *et al.* (1978) who found that *M. synoviae* infection had no effect on egg shell strength in a laboratory study. The present study was a field investigation and not performed under experimental/controlled conditions, hence there could be other confounding factors affecting egg quality. Feberwee *et al*. (2009b) reported that vaccination with a live *M. synoviae* vaccine reduces the occurrence of *M. synoviae*-induced egg apex abnormality significantly, but the current study reported a high translucency score in the vaccinated group. A poor quality eggshell is unble to protect the internal contents from bacterial penetration and may result in food poisoning outbreak.

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Table 7.1 Individual flock-wise sero-prevalence of *M. synoviae* with 95% exact binomial confidence interval model

Flock age (weeks)	*M. synoviae* serologically positive samples/sample size	Flock-wise (%) sero-prevalence (95% CI)
22	20/30	66.7 (47.2–82.8)
24	0/30	0.0 (0.0–11.6)
26	1/30	3.3 (0.1–17.2)
27	10/30	33.3 (17.3–52.8)
31	1/30	3.3 (0.1–17.2)
41	0/30	0.0 (0–11.6)
45	11/30	36.7 (19.9–56.1)
53	12/30	40.0 (22.7–59.4)
55	4/30	13.3 (3.8–30.7)
58	29/30	96.7 (82.8–99.9)
59	8/30	26.7 (12.3–45.9)
62	15/30	50.0 (31.3–68.7)
64	11/30	36.7 (19.9–56.1)
66	0/30	0.0 (0.0–11.6)
66	3/30	10.0 (2.1–26.5)
72	12/19	63.2 (38.4–83.7)
30[a]	15/30	50.0 (31.3–68.7)
52[a]	14/30	46.7 (28.3–65.7)
74[a]	20/30	66.7 (47.2–82.7)

[a] Vaccinated flocks

Table 7.2 Association between *M. synoviae* serological status and different egg shell quality parameters

Variables	Infected	Uninfected	Vaccinated
Number of flocks	11	5	3
Translucency	$2.4^{a} \pm 0.1$	$2.3^{a} \pm 0.1$	$3.1^{b} \pm 0.1$
Shell reflectivity (%)	$31.5^{a} \pm 0.2$	$28.0^{b} \pm 0.3$	$29.7^{c} \pm 0.5$
Egg weight (gm)	$61.5^{a} \pm 0.4$	$60.2^{a} \pm 0.9$	$59.9^{a} \pm 0.5$
Shell breaking strength (N)	$38.9^{a} \pm 0.5$	$44.7^{b} \pm 0.7$	$46.1^{b} \pm 1.3$
Shell deformation (μm)	$295.8^{a} \pm 3.4$	$343.5^{b} \pm 6.0$	$310.8^{a} \pm 8.8$
Egg shell weight (gm)	$5.8^{a} \pm 0.1$	$5.69^{a} \pm 0.1$	$5.6^{a} \pm 0.1$
% Egg shell	$9.4^{a} \pm 0.1$	$9.5^{a} \pm 0.1$	$9.3^{a} \pm 0.1$
Shell thickness (μm)	$390.9^{a} \pm 2.5$	$385.6^{a} \pm 3.1$	$383.9^{a} \pm 3.4$

Means ± SE with the different superscript in the same row are statistically significantly different ($p \leq 0.05$) from each other.

Chapter 8

General discussion

A low level of bacterial contamination on an eggshell is important from both food safety and storage perspectives. A high number of bacteria present on the eggshell surface increases the chances of eggshell penetration and contamination of internal contents.

In general, food quality and sanitary processing conditions can be judged by *Enterobacteriaceae* populations. In chapter 2, the average *Enterobacteriaceae* count on the eggshell surfaces was log 1.46 CFU/eggshell assessed across 31 flocks. However, these low levels of bacterial contamination have the potential to contaminate egg processing and packing plants. Similarly, they can also act as a source of contamination of egg washing equipment. Recontamination, which frequently occurs due to unhygienic equipment and processing facilities, as well as cross contamination, has been responsible for 25% of outbreaks (Musgrove, 2011). In the future, the level of *Enterobacteriaceae* contamination of egg processing and grading floors could be monitored at regular intervals. However, in Australia, there is a shortage of large scale surveys investigating the level of *Enterobacteriaceae* contamination of egg processing and grading floor. Such surveys may aid in better understanding of reservoirs of bacterial contamination along the egg supply chain. Also, a survey of bacterial contamination, in supermarket eggs as well as at different points of supply chain, could be conducted using quantitative methods. This could provide an insight into bacterial loads on eggs before they reach the consumer. In addition, the variation in the bacterial load on eggs at the farm gate and supermarket could be determined. Findings of such experiments would be helpful in identifying risk factors for bacterial egg contamination along the egg supply chain and establish bench mark standards for bacterial load on eggs at different pont of supply chain.

These bacterial processes have important implications for embryo survival/hatchability in fertile eggs as well as food safety in shell eggs.

In Australia, conventional cage eggs have a 68% market share (AECL, 2013). The benefits of conventional cages are lower operational cost, high hygiene and better biosecurity standards. However, due to welfare implications, there is a pressure to ban cage eggs (Walker *et al*., 2001). Hence, free range production system is becoming a major source of egg production in Australia and in other parts of the world. Ranging in extreme weather conditions could be stressful for the birds and may increase shedding of bacteria. All these factors may result in the increased bacterial contamination of eggs. However, there is a little attention has been given towards estimating the level of bacterial contamination as well as on determining *Salmonella* prevalence on eggs collected from free range production system. Higher bacterial load on eggs may lead to increase in food poisoning outbreaks.

Enterobacteriaceae isolated from eggs belonged to 11 different genera. Of all *Enterobacteriaceae* isolates, 60.78% were related to the *Escherichia* genus followed by *Salmonella* (9.15%) and *Enterobacter* (8.49%). The genus *Salmonella* is responsible for most of the egg related food poisoning outbreaks. In the present study, the prevalence of *Salmonella* on the eggshell surface was 4.51%.

Residual contamination of the environment with *Salmonella* is a major problem in commercial layer farms (Van de Giessen *et al*., 1994; Davies and Breslin, 2003; Gradel *et al*., 2004). However, 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 chapter 3, 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 with the help of formaldehyde. Carrique-Mas *et al*. (2009) 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. However, formalin has health and safety implications for farm workers (Carrique-Mas *et al*. 2009). 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 and free range systems in the same shed may hinder the cleaning procedure.

In 2006, the European Union passed legislation with the aim to reduce prevalence *S.* Enteritidis and *S.* Typhimurium in layer flocks upto 2% or below (Anonymous, 2006). This legislation prepared guidelines for the farmer and sampling requirements which involved the collection of faecal samples or boot swabs at 15 week interval starting from 22 and 26 of the age of the flock since hatch (Gosling *et al*., 2014). As a part of this legislation, from January 2008, the member states which had layer flocks with *Salmonella* prevalence above 10%, were required to use vaccination against *Salmonella* (Anonymous, 2006). As a result, in Great Britain, the prevalence of *S.* Enteritidis/Typhimurium in laying hens was reduced to 0.07% (Anonymous, 2011). This reduction in *Salmonella* prevalence was drastically low compared to 7.95% which was observed during EU baseline survey conducted between 2004 and 2005 (Snow *et al*., 2010).

In chapter 3, even though birds were infected with *S.* Typhimurium, all egg internal content samples were *Salmonella* negative. This supports the arguments that *Salmonella* serovars isolated in the present investigation may lack vertical transmission ability to contaminate egg internal contents. 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 particularly considering that infected eggs had a low prevalence. 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 and it would be necessary to develop models of metabolic sterss to accompany the challenge studies.

S. Typhimurium strains isolated from layer flocks during this study possessed MLVA patterns similar to those of the strains isolated from human food poisioning cases. Certain MLVA types (such as 3 24 11 10 523) are more frequently reported in human food poisoning than others (Australian *Salmonella* Reference Centre, Quarterly Report, 2014). It is unclear whether change in MLVA pattern of *S*. Typhimurium is linked to a variation in virulence and thus capacity to cause illness. This hypothesis could be tested using the human intestinal epithelial cell line, Caco2, as an *in vitro* model for *Salmonella* invasion. Genomic variation within virulence genes may also alter the pathogenic potential and thus the invasive ability of different *S*. Typhimurium MLVA types. Hence, whole genome sequence analysis may provide better insight into the relation between the genomic variations and pathogenic potential of different MLVA types of *Salmonella* Typhimurium.

During the laying production cycle, birds can experience various stressful events. El-Lethey *et al*. (2003) 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 (Humphrey, 2006). 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. This could be tested by comparing corticosterone concentrations in faeces collected from birds before and after transportation (transport stress) and further by investigating the relationship of corticosterone level with *Salmonella* shedding.

In chapter 4, 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.

There are several intervention strategies for controlling *Salmonella spp* on the layer farm and also during the egg supply chain. Egg washing is one of the commonly used methods in Australia to reduce the level of bacteria on the eggshell surface. However, Wang and Slavik (1998) reported that egg washing chemicals have the potential to alter the eggshell surface. This may lead to increased horizontal transmission of bacteria across the eggshell.

In chapter 5, all *S*. Typhimurium phage types were able to survive on eggshell surface as well as in egg contents after 21 days of *Salmonella* infection. This finding underlines the importance of proper handling of eggs in the food industry as well as in the kitchen environment to avoid cross contamination of other food items. Agar egg penetration results suggested that eggshell penetration was higher in washed eggs as compared to unwashed eggs. Hence, it is essential to make sure that eggs are stored properly to avoid contamination with *Salmonella* after egg washing. Results also suggested a trend that *S*. Typhimurium egg penetration was influenced by the

change in eggshell ultrastructure parameters such as cuticle, cap quality, Type B bodies, alignment, confluence and erosion. Further, genetic selection for shell ultrastructure may reduce risks of bacterial penetration. Wellman-Labadie *et al*. (2008) reported that cuticle derived protein extract had an antibacterial effect on gram negative and gram positive bacteria. However, under field conditions, the effectiveness of the cuticle in preventing bacterial contamination of eggshells is unknown; further studies are essential to draw concrete conclusions comparing experimental bench marks to commercial eggs.

In the Australian egg industry, other *Salmonella* serovars such as *S.* Livingstone, *S.* Singapore and *S.* Adelaide have been frequently isolated from egg farms (Cox *et al*., 2002; NSW Food Authority, 2012). However, the survival ability or eggshell penetration capacity of these serovars was unknown. Hence, in chapter 6, the whole egg and agar egg approaches were used to study the egg penetration ability of these serovars. The results of the agar penetration experiment indicated that all the isolates (one each *S.* Singapore, *S.* Adelaide, *S.* Worthington and *S.* Livingstone) had the capacity to penetrate the eggshell. This clearly suggested that eggshell penetration is not only characteristic of *S.* Typhimurium or *S.* Enteritidis. Eggshell penetration by *S.* Worthington showed a trend of higher ($p = 0.06$) penetration in washed eggs as compared to unwashed eggs. This may be due to damage of cuticle by egg washing chemicals as the cuticle score was higher in washed eggs as compared to unwashed eggs. Statistical analysis also indicated that the cuticle score was a significant linear predictor of *Salmonella* eggshell penetration. Whole egg penetration results showed that all of the *Salmonella* serovars used in the present study were capable of surviving on the eggshell surface after 21 days of incubation (at 20°C) ,following a high dose of inoculation (10^5 CFU/mL). All the egg contents tested negative for *S.* Singapore, *S.* Worthington and *S.* Livingstone although *S.*

Adelaide was detected in internal contents of one egg. *Salmonella* isolates used in the present study (*S.* Singapore, *S.* Worthington, *S.* Livingstone and S. Adelaide) either lacked the ability to survive in the hostile environment of egg albumen or they did not have a capacity to move from egg albumen to egg yolk which resulted in the senescence of all the *Salmonella* that penetrated the eggs. Further studies are essential to confirm these findings, using more sensitive detection methods such as selective enrichment or Real-Time quantitative PCR (RT-qPCR). Overnight incubation in BPW followed by selective enrichment in rappaport vassiliadis broth may help to revive stressed *Salmonella* cells in egg albumen. Increased survival ability of *S.* Typhimurium on the eggshell surface and in internal egg contents may be responsible for the clear association of *S.* Typhimurium with egg related food poisoning outbreaks in Australia.

Egg penetration studies conducted during this study suggest that *Salmonella* penetration across the eggshell was influenced by eggshell quality. Eggshell quality can be affected by any disease that can potentially lead to damage of the reproductive tract of the hen. A Dutch strain of *Mycoplasma synoviae* (*M. synoviae*) has been reported to be responsible for formation of egg apex abnormalities (Feberwee *et al*., 2009). In chapter 7, the sero-prevalence of *M. synoviae* in the Australian layer flocks was investigated. The possible association between eggshell quality and the sero-prevalence of *M. synoviae* was investigated using commercial flocks.

The sero-prevalence of *M. synoviae* positive commercial layer flocks was high 11/16 (69%). Multiple age flocks and low biosecurity standards on the layer farms could be responsible for the high prevalence and persistence of *M. synoviae* infections (Stipkovits and Kempf, 1996 and Kleven, 2003). The sero-prevalence of *M. synoviae* was associated various egg quality parameters such as shell deformation, translucency, % shell reflectivity and shell breaking strength. In the infected group,

shell breaking strength was significantly lower as compared to the uninfected group. This may lead to the increased breakage of saleable eggs and economic losses to producers. Similarly, in the infected group, eggs had lighter coloured shells (due to higher shell reflectivity) as compared to uninfected group. Consumers have an eversion to eggs with lighter eggshells. As the present study was a field investigation, further controlled animal infection trials are essential to confirm the association between Australian strains of *M. synoviae* and egg quality parameters.

8.1 Conclusion

The overall prevalence of *Salmonella* on eggs was 4.51%. Environmental *Salmonella* contamination in layer sheds has been observed as a risk factor for the production of *Salmonella* contaminated eggs. As egg production from free range system is increasing in Australia, it is essential to conduct surveys determining the load of bacteria on eggs from free range production system. Also, as shown in the egg penetration experiment, *S.* Typhimurium was able to survive on the eggshell surface as well as in egg internal contents 21 days after infection. These contaminated eggs can act as a source for human salmonellosis infection and also can cause cross-contamination of other food items in the kitchen. According to Bell and Kyriades (2002), salmonellosis can be caused by many factors such as the improper storage of food, inadequate cooking, prolonged storage of food, improper handling of food and consumption of raw food items. Further extension work is essential to educate the general public regarding the careful handling and storage of food items in the supply chain as well as in kitchen. In the present study, epidemiological investigations were conducted on only three caged layer farms in South Australia. There is a need for a nation-wide survey to study the prevalence of *Salmonella* serovars on layer farms (caged, free range and barn production systems) in Australia. Such studies would provide a better understanding of the predominant *Salmonella*

serovars on layer farms and assist with development of control measures to reduce subsequent egg related outbreaks.

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Appendix 1

Table 1 Primers used in multiplex PCR (Akiba *et al*., 2011)

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

Table 2 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*. (2011)

[2] Akiba et al. (19) observed no amplification with *S*. Typhimurium or *S*. Infantis primers

[3] Not investigated by Akiba *et al*. (2011)

Table 3 *Salmonella* serovars isolated and characterised by microbiological culturing and the proportion testing positive by qPCR

Salmonella serovar(s) cultured from n samples	Percentage detected by qPCR
S. Typhimurium PT 9 (n=13)	23%
S. Worthington (n=9)	44%
S. Oranienburg (n=87)	77%
S. subsp. 1 ser. 4,5,12:-:- (n=3)	66%
S. Agona (n=3)	33%
S. Oranienburg and *S.* subsp.1 ser. rough g,s,t:- (n=1)	100%
S .Typhimurium PT 9 and *S.* Oranienburg (n=2)	100%
Total *Salmonella* (n=118)	68%

S: *Salmonella*; PT: phage type.

Table 4 Whole egg penetration by different *Salmonella* Typhimurium strains: effect of temperature on penetration in unwashed eggs

Salmonella Typhimurium strain	Dose of infection (CFU/mL)	Temp-erature (°C)	Number of penetrated pools (of 2 eggs)	Number of Non-penetrated pools (of 2 eggs)	p-value
S. Typhimurium strain 1	10^3	20	0	5	1.00
		37	1	4	
	10^5	20	1	4	1.00
		37	0	5	
S. Typhimurium strain 2	10^3	20	1	4	1.00
		37	0	5	
	10^5	20	0	5	1.00
		37	0	5	
S. Typhimurium strain 3	10^3	20	0	5	1.00
		37	0	5	
	10^5	20	4	1	0.04
		37	0	5	
S. Typhimurium strain 4	10^3	20	0	5	0.17
		37	3	2	
	10^5	20	1	4	1.00
		37	2	3	
S. Typhimurium strain 5	10^3	20	0	5	1.00
		37	1	4	
	10^5	20	1	4	1.00
		37	0	5	

Appendix 2

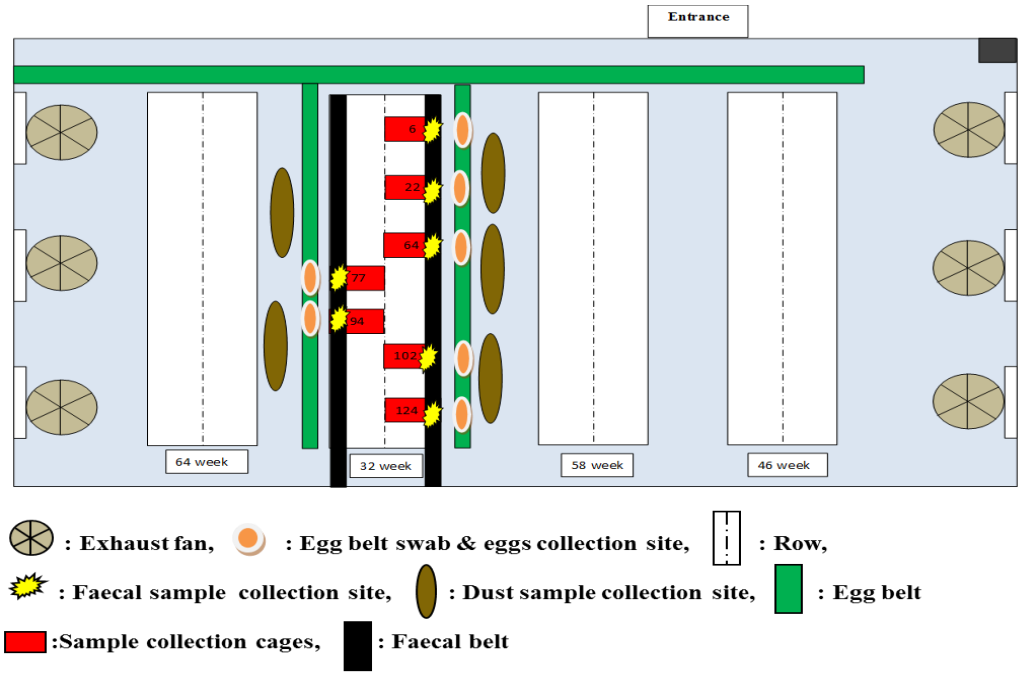

Figure 1a The layout of shed with flock A showing the areas of sample collection

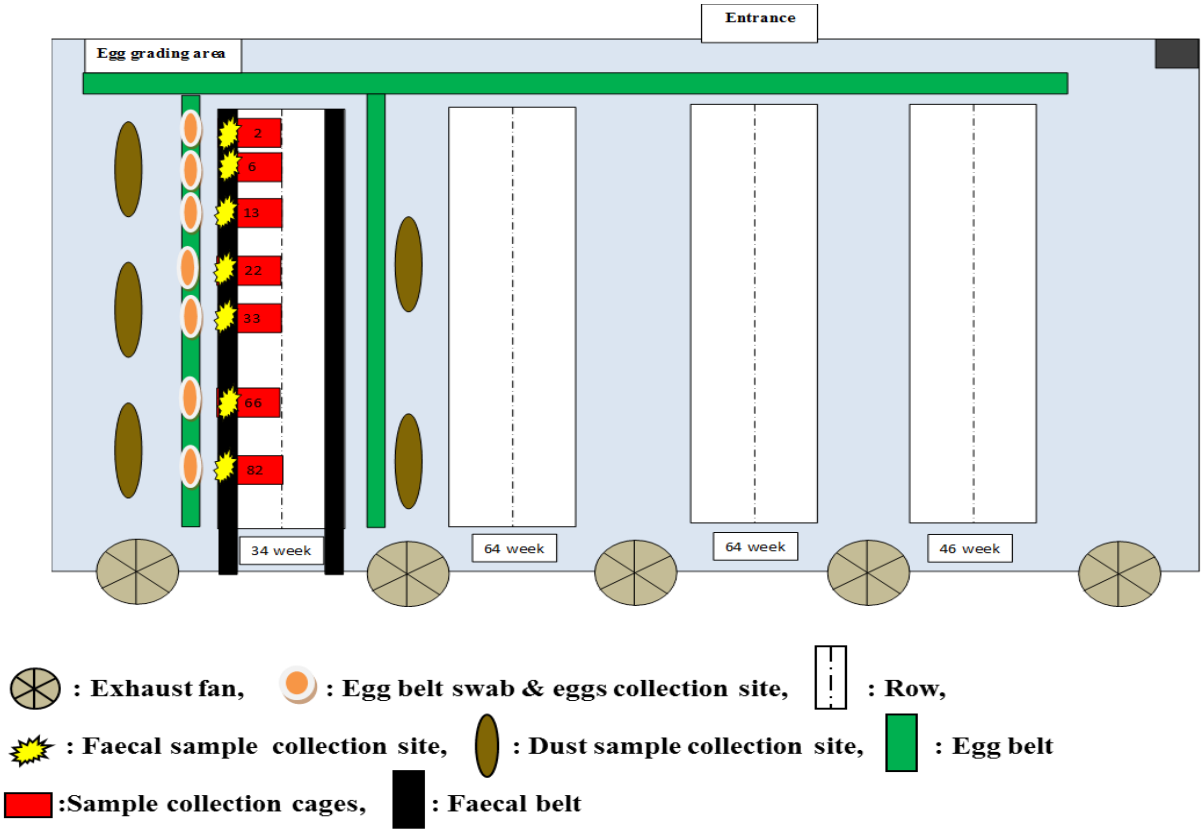

Figure 1b The layout of shed with flock B showing the areas of sample collection.

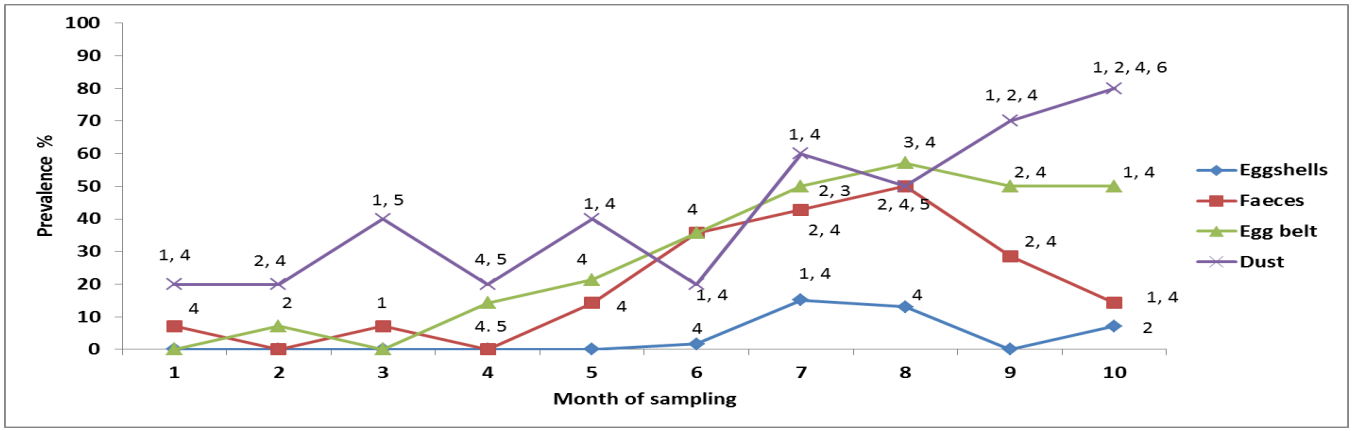

Figure 2 Percent prevalence of *Salmonella* in different type of samples over period of 10 month

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:-

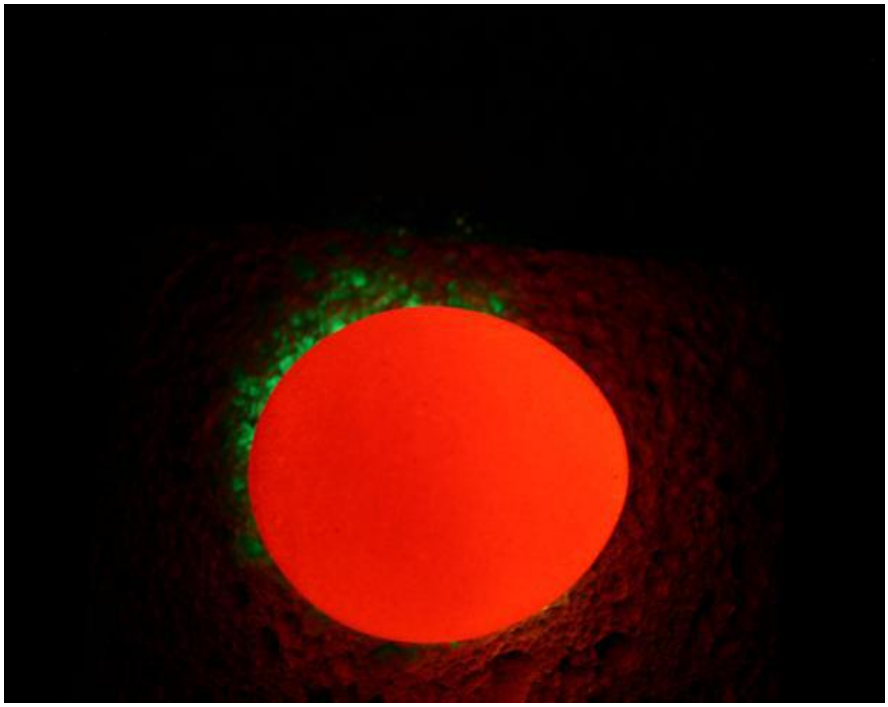

Figure 3 Image of non-translucent egg (score=1)

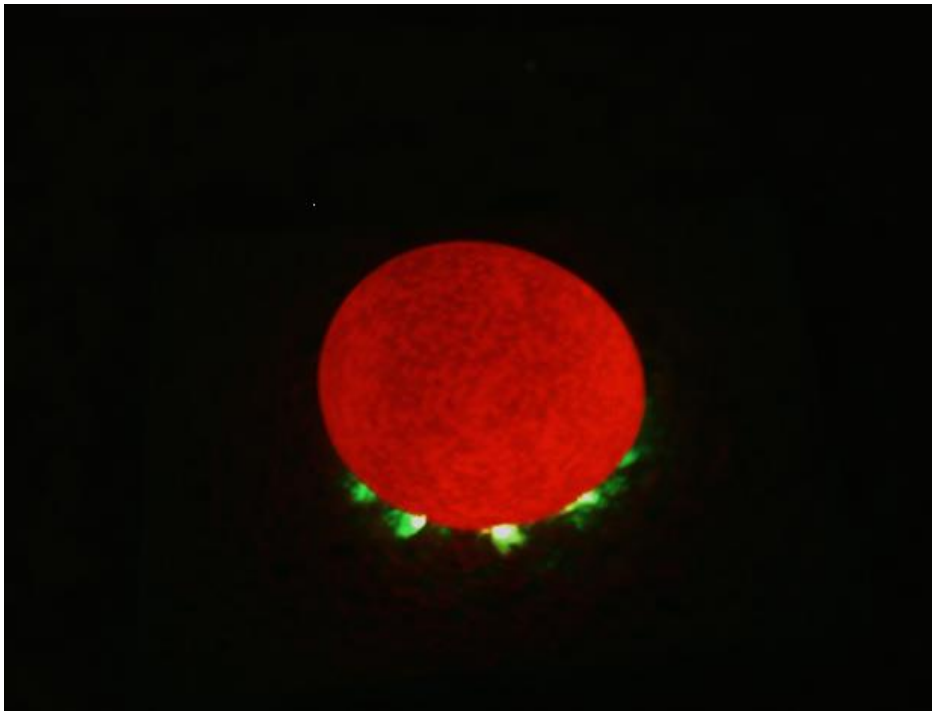

Figure 4 Image of translucent egg (score=2)

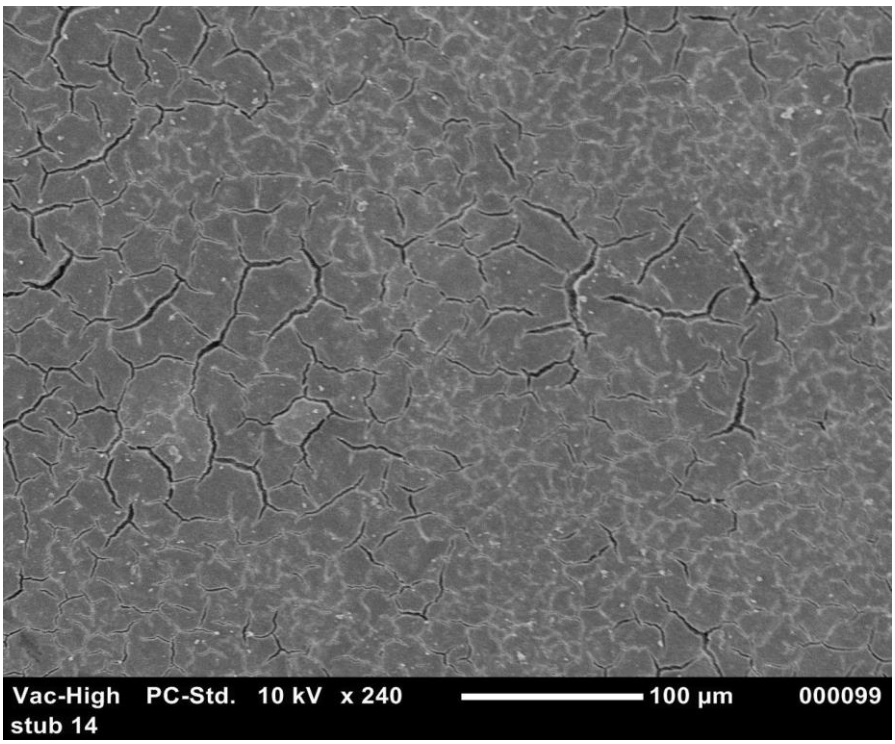

Figure 5 SEM image of good quality cuticle (score =1)

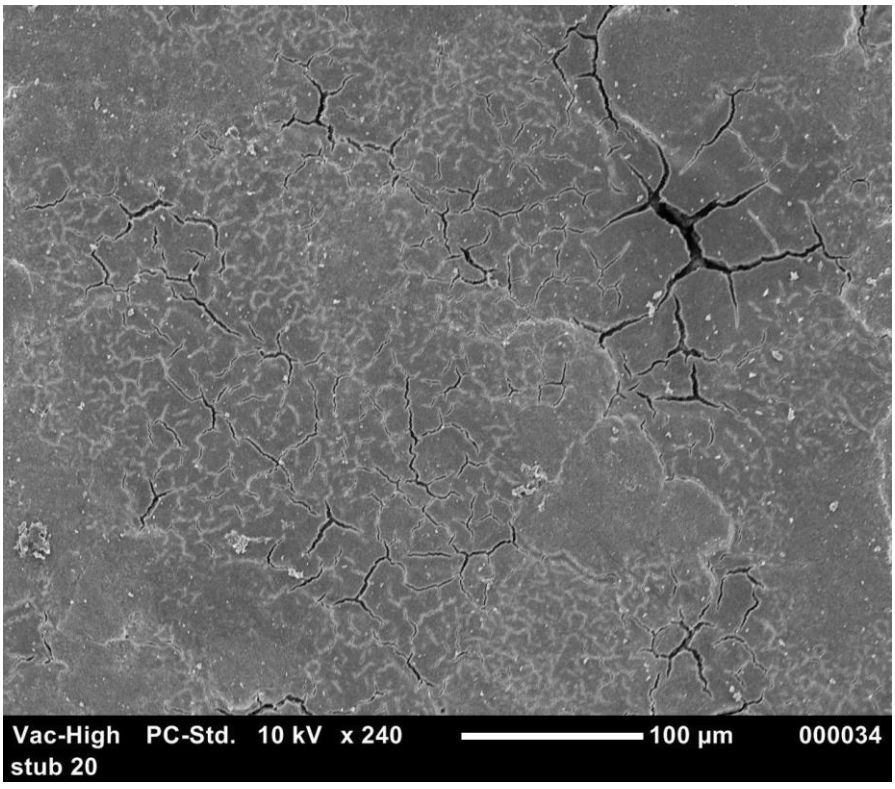

Figure 6 SEM image of cuticle (score =2)

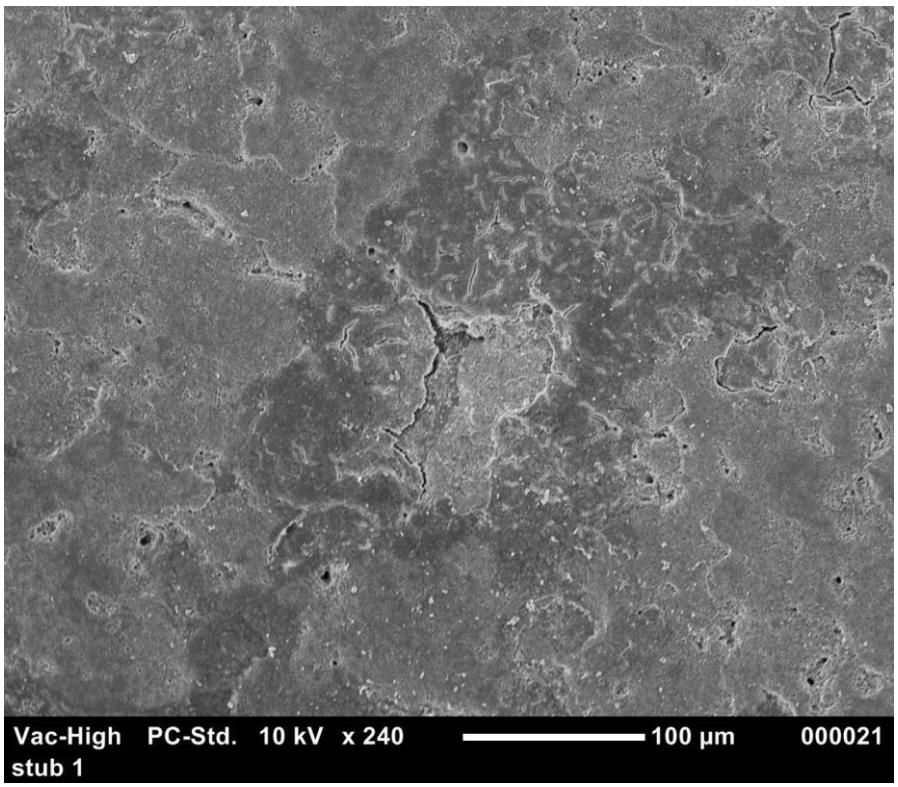

Figure 7 SEM image of cuticle (score = 3)

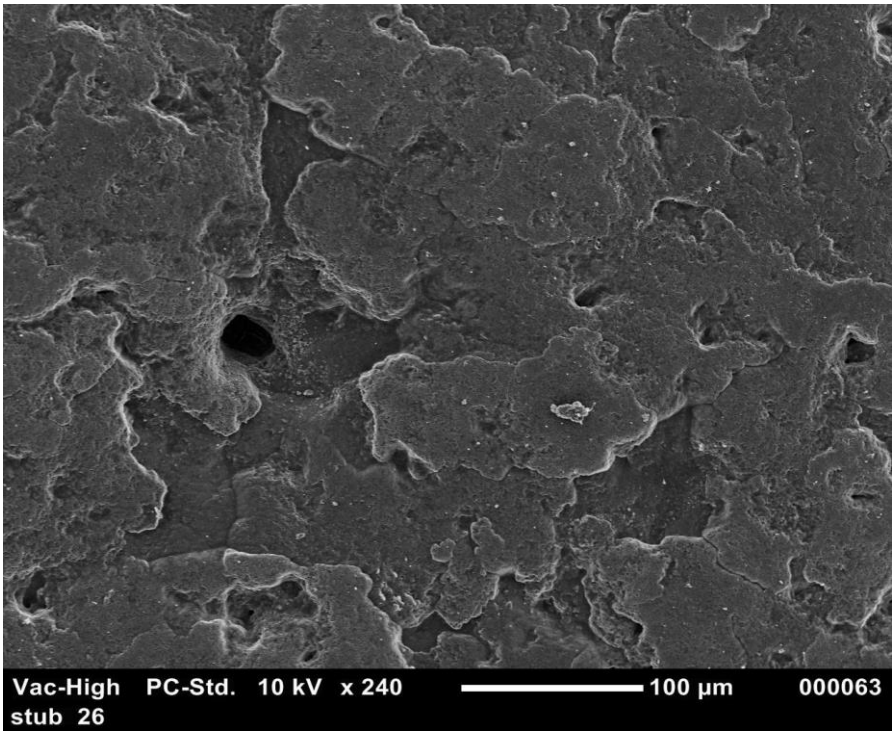

Figure 8 SEM image of damaged eggshell surface and exposed eggshell pore with no cuticle (score = 4)

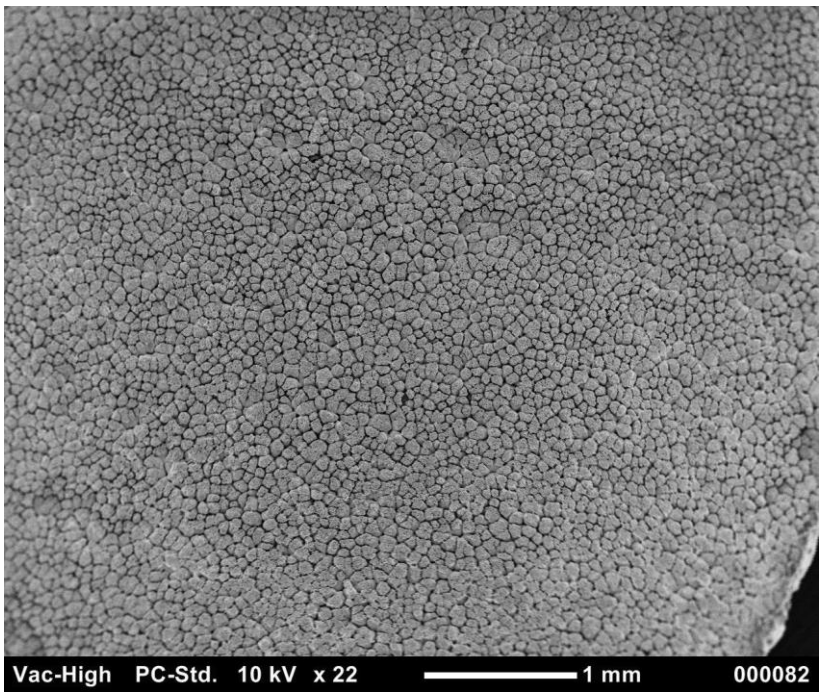

Figure 9 SEM image showing isolated alignments (score=1) in the mammillary layer

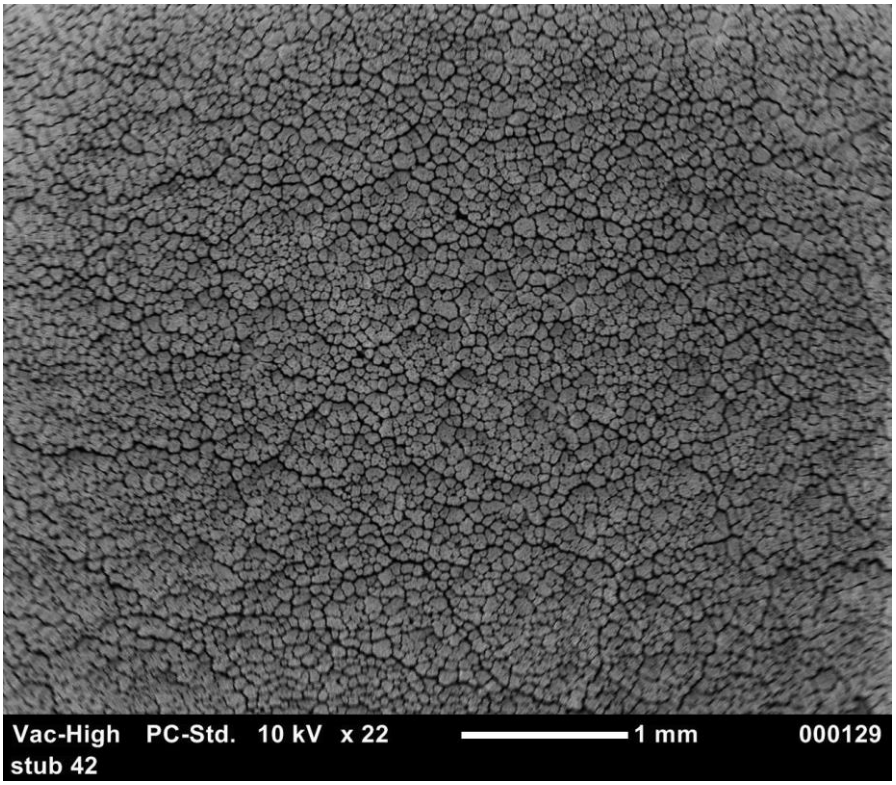

Figure 10 SEM image showing extensive alignments (score =2) in the mammillary layer

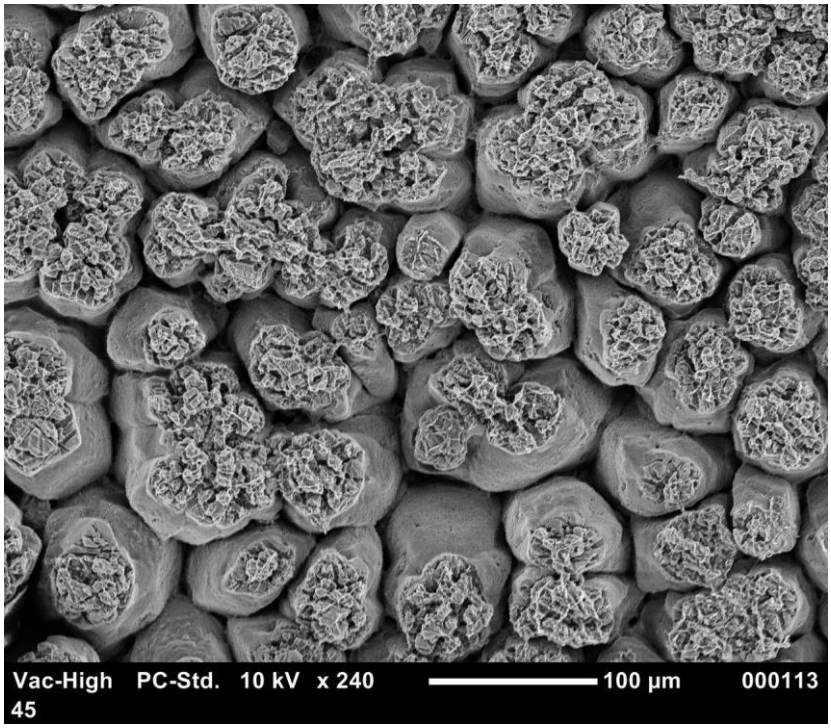

Figure 11 SEM image showing a low number of Type A bodies (score=1) in eggshell

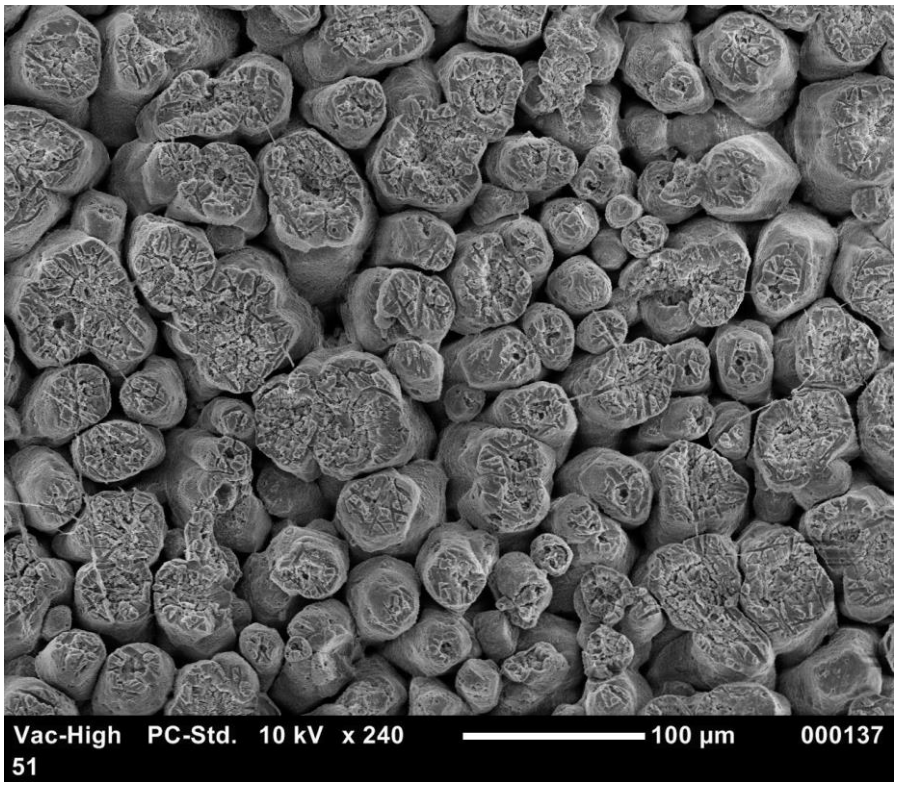

Figure 12 SEM image showing large number of Type A bodies (score=2) in eggshell

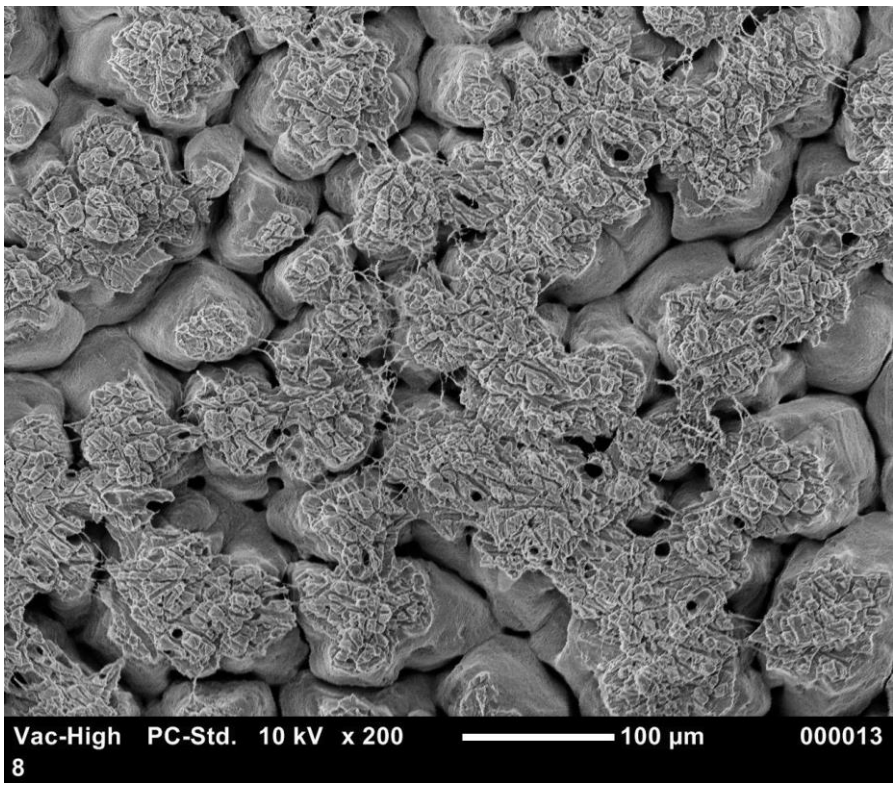

Figure 13 SEM image showinga low number of Type B bodies (score=1) in eggshell

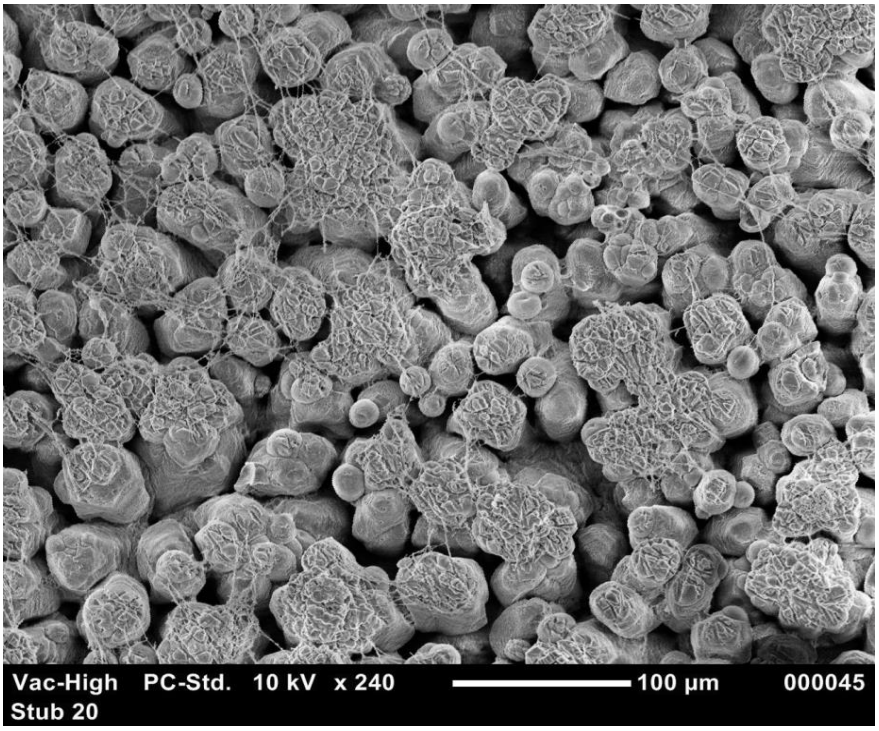

Figure 14 SEM image showing large number of Type B bodies (score=2) in eggshell

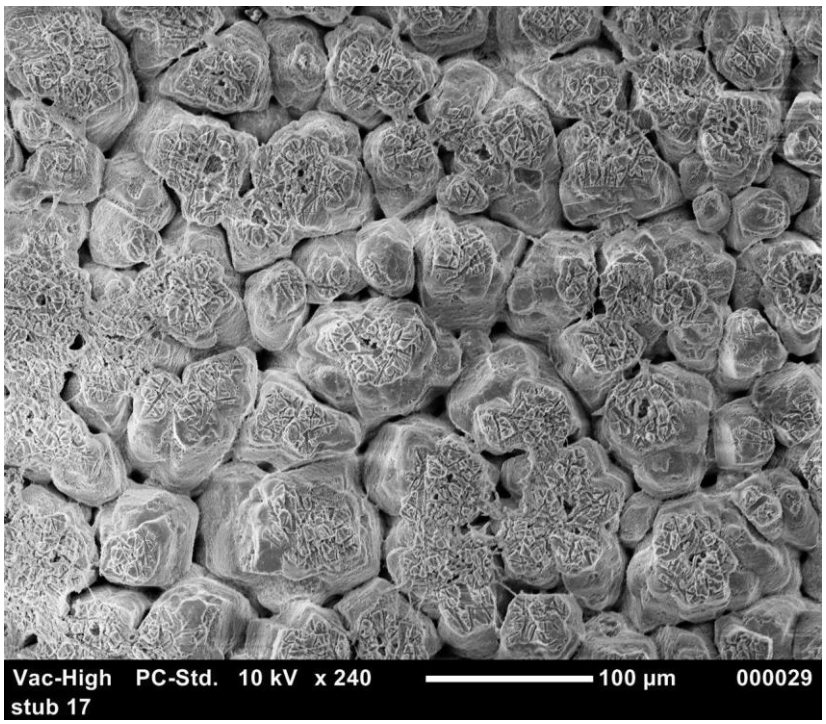

Figure 15 SEM image of poor quality mammillary caps (score=2) with low confluence (score=1)

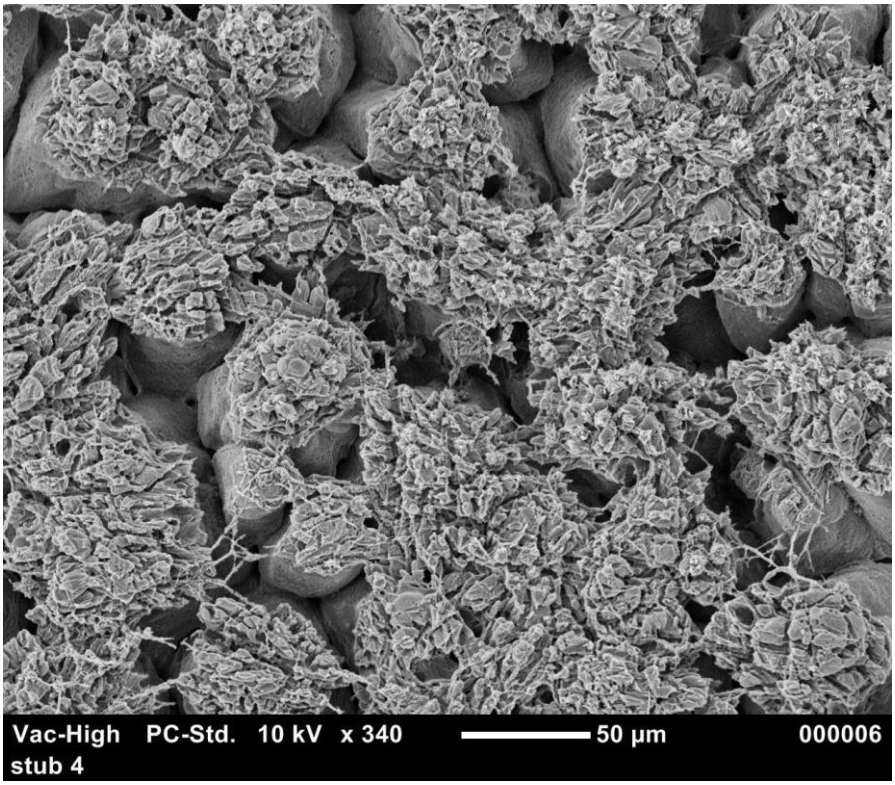

Figure 16 SEM image of good quality mammillary caps (score=1) with high confluence (score=2)

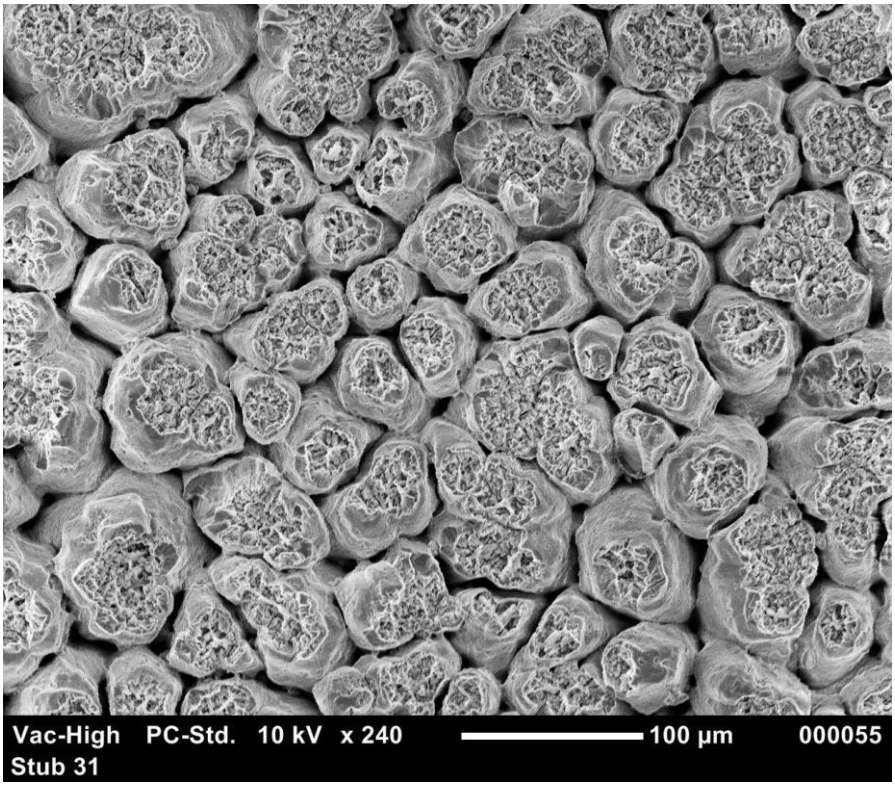

Figure 17 SEM image of erosions (score=2) throughout the eggshell

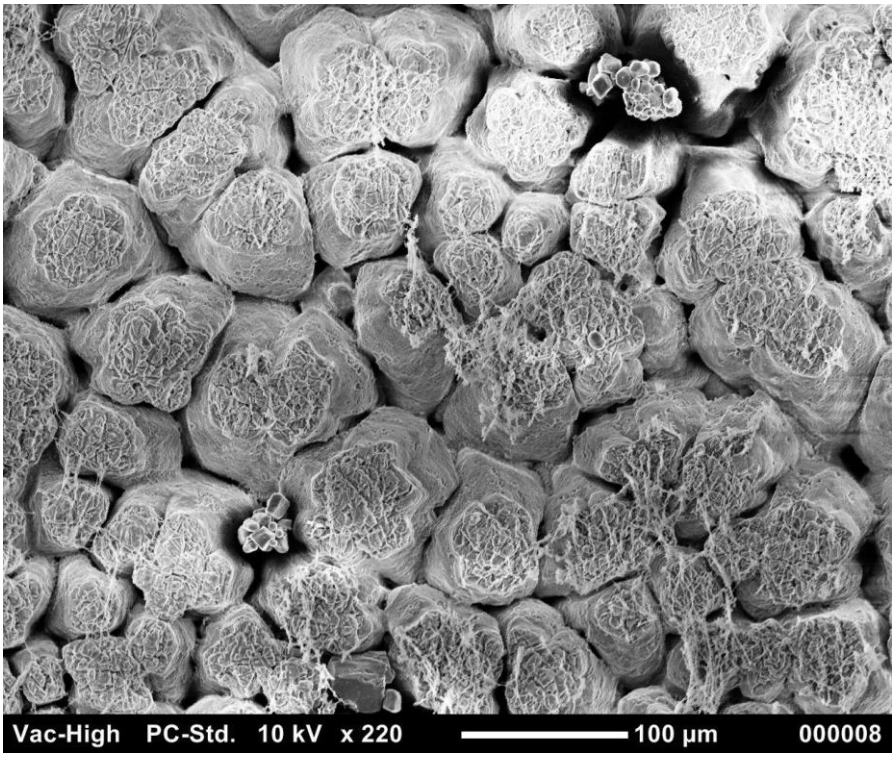

Figure 18 Presence of Aragonite in the mammillary layer of eggshell

Appendix 3

Biochemical test for *Salmonella* confirmation

1 ONPG Test

1) Put one ONPG disc into microcentrifuge tube.
2) Add 0.1 mL of sterile 0.88% sodium chloride.
3) Pick the colony under test with a sterile loop and emulsify it in the tube containing the disc and physiological saline.
4) Incubate at 35°C.
5) Examine tubes (for colour change) at hourly intervals for up to 6 h to detect active lactose fermenters.
6) Organisms that are negative after 6 h should be incubated for up to 24 h to detect the late lactose fermenters.
7) Result: Colourless: ONPG negative.

   Yellow: ONPG positive.

2 Lysine decarboxylase test

1) To prepare decarboxylase broth, add one LDC tablet to 5 mL of distilled water in an appropriate bottle.
2) Sterilize the bottle by autoclaving at 121°C for 15 minutes.
3) Allow to cool down the bottle containing the broth.
4) Pick up the 1 or 2 colonies under test with a sterile loop and mix well them with broth.
5) Cover the surface of the broth with mineral oil (approximately 1 mL).
6) Tighten the caps of bottles and incubate at 35°C.
7) Examine the tubes after 24 h for colour change

8) Results: Purple colour: Positive reaction.

Yellow colour: Negative reaction.

3 Urease test

1) Pick up the 1 or 2 colonies under test with a sterile loop.
2) Inoculate surface of the urease slope with heavy inoculum and also stab the slant making sure bacteria goes all the way through.
3) Incubate the slopes with loosened caps at 35°C for 18- 24 h.
4) After incubation, examine the slopes for colour change.
5) Results: Bright/pale pink: Positive reaction.

Yellow/orange: negative reaction.

Biochemical tests of *Enterobacteriaceae* identification

(Source: http://www.microbelibrary.org/library/laboratory-test/2878-bacterial-identification-by-the-analytical-profile-indexsystem-analytical-profile-index-e20-for-enterobacteriaceae)

1) ONPG: test for b-galactosidase enzyme by hydrolysis of the substrate o-nitrophenyl-b-D-galactopyranoside.
2) Arginine dihydrolase (ADH) test
3) Lysine decarboxylase (LDC) test
4) Ornithine decarboxylase (ODC) test
5) Citrate test (CIT)
6) Hydrogen sulfide production (H2S) test
7) URE: test for the enzyme urease
8) Tryptophan deaminase (TDA) test
9) Indole test (IND)
10) Voges-Proskauer (VP) test
11) Gelatinase (GEL) test
12) Glucose (GLU) test

13) Fermentation of mannose (MAN) test

14) Fermentation of inositol (INO)

15) Fermentation of sorbitol (SOR)

16) Fermentation of rhamnose (RHA)

17) Fermentation of sucrose (SAC)

18) Fermentation of melibiose (MEL)

19) Fermentation of amygdalin (AMY)

20) Fermentation of arabinose (ARA)

21) The OX test: a test for cytochrome oxidase