Study of Salmonella typhimurium infection and vaccination in laying hens

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

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Thesis Abstract

Salmonella Typhimurium is responsible for the majority of egg-associated foodborne outbreaks in Australia. It colonises sub clinically in commercial laying hens, and these hens can shed bacteria in the poultry environment resulting in egg contamination; hence, it is of significant public health concern. Therefore, understanding the biology of *S.* Typhimurium shedding, host-pathogen interaction, and its management in laying hens is essential. In our study we conducted a 16-week infection trial, using layer hens reared free from exogenous *Salmonella*. The results highlighted that *S.* Typhimurium continued to persist in the internal organs for 16 weeks post challenge and infection was not eliminated despite measurable antibody response from the asymptomatic *S.* Typhimurium carrier adult birds. The stress of onset of lay led to the recrudescence of *S.* Typhimurium, further leading to its proliferation, intermittent shedding and causing eggshell contamination.

Live attenuated *Salmonella* vaccine is commonly used for the vaccination of chickens to control the contamination of poultry products. Vaxsafe® ST; (Strain STM-1, $\geq 10^{7.0}$cfu/dose, Bioproperties Pty Ltd) is the only commercially available live attenuated *aroA* deletion mutant *S. enterica* serovar Typhimurium vaccine in Australia and was used in this study. The antibody response to vaccine and efficacy of Vaxsafe® ST during pullet rearing and early production were investigated. The pullets vaccinated after intramuscular injection at 12 weeks produced significantly higher antibody response ($p < 0.001$) to *S.* Typhimurium vaccine strain. The vaccine strain STM-1 successfully colonised the chicken gut but did not induce a systemic antibody response until after parenteral administration. The load of STM-1 in litter samples increased gradually and was significantly higher at week 13, highlighting that Vaxsafe® ST has a potential as an antigen delivery system.

During laying, Vaxsafe® ST was tested in naturally infected *S.* Typhimurium laying hen flocks. At the onset of lay there was no significant difference in prevalence of *Salmonella* spp. in faeces in vaccinated and unvaccinated groups, although antibody titre was significantly higher in vaccinated than unvaccinated group at all sampling points during this study. The prevalence of wild-type *S.* Typhimurium did not vary significantly in subsequent samplings. *S.* Typhimurium was consistently found in dust and shoe cover samples, throughout the study. Given that *S.* Typhimurium and other serovars can survive/persist in the shed environment (such as in dust), regular cleaning, disinfection and or removal of dust from shed is necessary. *Salmonella* spp. can form biofilms on various surfaces hence cleaning of the shed could be challenging.

Three commercial disinfectants (Product A, B and C: containing a chlorinated compound, quaternary ammonium compounds (QAC), and twin-chain QAC; respectively) tested in this experiment significantly reduced viable biofilm cells; however, none of the product eliminated the biofilm cells. The results of this study showed that biofilm age was associated with the increased resistance to disinfectant treatments. These findings may have future implications for the use of disinfectants such as required concentration and exposure time in the poultry industry to control biofilm.

From this work, it can be concluded that *S.* Typhimurium persists in the internal organs of hens for a prolonged period and these hens act as a latent carrier with a continuous source of egg and environmental contamination. Vaccination of poultry, which would decrease *S.* Typhimurium contamination of flocks, is considered as an effective measure to reduce human cases of salmonellosis. However, use of the Vaxsafe® ST vaccine in laying hens is "not an ultimate intervention" for reduction of *S.* Typhimurium, hence, implementation of more than one or several interventions strategies is essential.

Thesis Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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List of Publications

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Manuscripts submitted for Publications

Sharma, P., Moyle, T. S., Sexton, M., McWhorter A. R., Holden, K., Underwood, G. and Chousalkar, K. K. (2017). Shedding of a live attenuated *Salmonella* Typhimurium vaccine Vaxsafe® ST (strain STM-1) during pullet rearing - a field study. *Avian Pathology* (Submitted).

Sharma, P., Pande, V. V., Moyle, T. S., Caraguel, C., Sexton, M., McWhorter A.R. and Chousalkar, K. K. (2017). Shedding of *Salmonella* Typhimurium in vaccinated and unvaccinated hens during early lay: a randomised controlled trial. *Vaccine* (Submitted).

Sharma, P., Pande, V. V. and Chousalkar, K. K. (2017). Efficacy of commercial disinfectants against biofilms formed by *Salmonella enterica* serovars Typhimurium isolates. *Animal* (Submitted).

Conference Oral and Poster Presentations

Sharma, P., Pande, V. V., Devon, R. L., McWhorter, A. R. & Chousalkar, K. K. (2015), Dynamics of *Salmonella* Typhimurium shedding from early to peak lay in laying hens (Oral). <http://www.saysoft.net/meat15/proceedings/papers/100075.pdf>. "XVI European Symposium on the Quality of Eggs & Eggs Products & XXI European Symposium on the Quality of Poultry Meat, 10-14 May, 2015 at Nantes, France".

Pande, V. V., Devon, R. L., **Sharma, P.,** McWhorter, A. R. & Chousalkar, K. K. (2016). *Salmonella* Typhimurium shedding and egg contamination in experimentally infected laying hens (Oral). The 43rd Annual General Meeting of the Australian Society for Microbiology, 12-15 July 2015, Canberra, Australia.

Sharma, P., Pande, V. V., Devon, R. L., McWhorter, A. R. & Chousalkar, K. K. (2016). Serological responses to *Salmonella* Typhimurium infection in laying hens. (Poster) <http://www.asap.asn.au/wp-content/uploads/abstract-2015/272/attach_brief.pdf>. Animal Production 2016 joint conference of the Australian Society of Animal Production and the New Zealand Society of Animal Production held on July 4 -7, Adelaide, Australia.

Chapter 1 Literature Review

1. Introduction to thesis

World-wide, members of the bacterial genus, *Salmonella,* are among the most common causes of foodborne gastrointestinal disease (Majowicz et al. 2010; Ford et al. 2016). *Salmonella* is highly diverse and is comprised of two species, *Salmonella bongori* and *Salmonella enterica* (*S. enterica*) each containing subspecies. Within *S. enterica*, over 2600 serovars have been described and can be further differentiated into typhoidal or non-typhoidal *Salmonella* (NTS) serovars (Guibourdenche et al. 2010). Among NTS serovars, *S.* Enteritidis and *S.* Typhimurium are responsible for the majority of food-borne salmonellosis in humans, although the pattern of servovar dominance varies geographically and over time (Voetsch et al. 2004).

NTS primarily cause a self-limiting gastroenteritis in humans. Annually, an estimated 94 million infections and 155,000 deaths worldwide are documented with 80.3 million of these identified as foodborne related cases of salmonellosis (Majowicz et al. 2010). Animal or animal products including poultry, eggs, milk and milk products can become contaminated with NTS at different points in the food production chain and serve as a source of human infection (Foley & Lynne 2008). Each year, 4.1 million cases of food poisoning are reported in Australia, costing approximately $1.2 billion per year. Of these incidents, 39,600 cases have been attributed to infection with *Salmonella* (Gillian et al. 2005; Kirk, 2014). During Australian outbreaks of salmonellosis, eggs are frequently identified as the primary source for *Salmonella* (Food Standards Australia New Zealand, 2011).

Globally, *S.* Enteritidis is commonly associated with human disease, but to date, it is not endemic within the Australian commercial poultry industry. *S.* Typhimurium is principally regarded as the most significant egg contaminant (Ford 2016; Moffatt et al. 2016). Thus, there are clear benefits to both the economy and public health to improve management of *Salmonella* within the food supply chain.

In the farm environment, chicks, pullets and hens are repeatedly exposed to *Salmonella*. Once infected, the intestinal tract is colonised by bacteria that ultimately establish a persistent infection within the bird (Gast & Holt 1998). Peak *Salmonella* shedding in faeces is correlated with the onset of lay and decreases as a commercial egg laying flock ages (Li et al. 2007). Stress experienced by hens during the onset of lay has been shown to increase susceptibility to *Salmonella* colonisation and increased rates of faecal shedding (Skov et al. 2002) which can result in a higher proportion of eggs becoming contaminated (Pande et al. 2016a). The persistent nature of *Salmonella* in laying hens additionally increases risk of transmission to other flocks and continuous production of contaminated eggs (Gast et al. 2009). The risk of egg-borne *S.* Typhimurium outbreaks is therefore a food safety issue and highlights the importance of understanding host-pathogen interaction, colonisation and persistence in laying hens so that mitigation programmes for *S.* Typhimurium infection in egg laying flocks can be designed and implemented.

The following review summarises the literature relevant to the experiments conducted as a part of this thesis.

1.1.*Salmonella* Taxonomy and Epidemiology

Salmonella are rod-shaped Gram-negative, facultative-anaerobic bacteria that are members of the *Enterobacteriaceae* family. They are generally motile with peritrichous flagella, ferment glucose, and reduce nitrate to nitrite. The genus, *Salmonella* (*S.*), contains two species, *S. bongori* and *S. enterica*. The species *S. enterica* is further divided in to six subspecies: *S. enterica* subsp, *enterica* (I), *S. enterica* subsp, *salamae* (II), *S. enterica* subsp, *arizonae* (IIIa), *S. enterica* subsp, *diarizonae* (IIIb), *S. enterica* subsp, *houtenae* (IV), *S. enterica* subsp, *indica* (VI) (Ryan et al. 2017). Members of the group *S. enterica* subspecies *enterica* (I) commonly infect warm-blooded animals and are responsible for causing the

majority of human cases of salmonellosis (Ryan et al. 2017). Within the *S. enterica* subspecies *enterica* (*S. enterica)*, approximately 2600 serovars have been identified using serological characterisation of both O (somatic) and H (flagellar) antigens (Nataro et al. 2011).

Members of *S. enterica* have a wide range of host specificity. Certain serovars are only able to cause disease in a single host species (host-restricted); the most common example being *S.* Typhi, the causative agent of human typhoid fever. Other serovars can either be host-adapted (associated with one species but able to cause disease in other hosts) or have a broad host range (causes disease in numerous vertebrate species). The last two groups are commonly referred to as non-typhoidal serotypes (NTS) and are largely responsible for causing diarrheal disease in humans as well as many other vertebrate species.

Many *S. enterica* serovars have the ability to colonise commercial layer flocks and may be transmitted vertically or horizontally on-farm and persist throughout processing stages during production (Liljebjelke et al. 2005; Chaudhuri et al. 2013). Globally, *S.* Enteriditis and *S.* Typhimurium are commonly isolated from egg farms.

S. Typhimurium strains can be subdivided into definitive phage types (PTs), according to their susceptibility to a series of bacteriophages (Anderson et al. 1977; Rabsch et al. 2002). Many *Salmonella* Typhimurium PTs, such as PT104 and PT49 have been found to be associated with layer hens and egg contamination overseas (Williams et al. 1998; Okamura et al. 2010). *S.* Typhimurium PT108/170, PT44, PT9, PT193, PT135 and PT135a, however, are the primary definitive types identified during egg-associated outbreaks in Australia (Sintchenko et al. 2012; The OzFoodNet Working group 2015b). During the period between 2006 and 2010, a total of 92 *Salmonella* food poisoning outbreaks were reported. These outbreaks of salmonellosis involved 1,750 individuals, 400 of which required hospitalisation (The OzFoodNet Working Group 2015a; Chousalkar & McWhorter 2017). Of these cases,

the majority (91%) were due to different PTs 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 DT 9 (14%) and DT 135a (8.7%) (Stephens et al. 2008). Since 2010, the number of egg-related *Salmonella* outbreaks have continued to increase in Australia.

1.2. Pathogenesis of *Salmonella* Typhimurium in laying hens

Salmonella infects its host primarily thorough the oral-faecal route and once ingested, bacteria interact with enterocytes to establish intestinal colonisation. Certain serovars, such as *S.* Typhimurium, are able to infect and multiply within macrophages which facilitates bacterial dissemination from the gastrointestinal tract to internal organs resulting in systemic disease and ultimately establishing persistence (Hensel, 2000). The ability of *Salmonella* strains to persist intracellularly is crucial for pathogenesis; strains lacking this ability are considered non-virulent (Bakowski et al. 2008). Variation in virulence and pathogenicity of different *Salmonella* serovars is likely due to genomic variability that arises primarily as a consequence of horizontal gene transfer (Suez et al. 2013).

The overall pathogenesis of *Salmonella* is controlled by a number of multiple virulence factors encoded in the genome and are, in general, located within *Salmonella* pathogenicity islands (SPI). In *S. enterica*, 23 SPIs have been identified. Five SPIs (SPI-1 - SPI-5) have clear roles in contributing to *Salmonella* virulence and pathogenesis (Groisman & Ochman 1996; Marcus et al. 2000; Sabbagh et al. 2010). *Salmonella* utilizes two specialized type III secretion system (T3SS) that are encoded within *Salmonella* pathogenicity islands, SPI-1 and SPI-2. SPI-1 T3SS is mainly responsible for bacterial invasion of the host epithelium and invoking the inflammatory response (Zhou & Galan 2001; Chakravortty et al. 2005). The SPI-2 T3SS is responsible for bacterial survival and multiplication within eukaryotic

cells, including macrophages and maintenance of the *Salmonella*-containing vacuoles (SCV) (Hensel, 2000; Hensel, 2004). The maintenance of long-term infection in chickens has been contributed to the genes carried on SPI-2 (Monack, 2012). It has been shown that *S*. Typhimurium requires both SPI-1 and SPI-2 for gastrointestinal colonization and systemic spread in chickens (Jones et al. 2007). The studies have also found that SPI-1 is essential for caecal colonization (Bohez et al. 2006) and SPI-2 is necessary for avian reproductive tract colonisation by *S*. Enteritidis (Bohez et al. 2008).

The other *Salmonella* pathogenicity islands, SPI-3, SPI-4 and SPI-5 also contribute to bacterial pathogenesis. SPI-3 contains ten open reading frames that encode *Salmonella* virulence determinants with highly diverse functions (Fàbrega & Vila 2013). SPI-4 is a 27 kilobase region within the *Salmonella* genome and during infection it acts in consort with SPI-1 to initiate invasion in to host epithelial cells (Gerlach et al. 2008). SPI-5 encodes six genes that play a role in the enteropathogenesis of *Salmonella* spp. (Wood et al. 1998).

Salmonella pathogenesis depends on multiple factors, such as overall bacterial virulence, infective dose, route of infection, genetic makeup, as well as immune status of the host (Mastroeni, 2006). Depending on age, infection with NTS can have varied outcomes in poultry birds. In young chicks, NTS infection can lead to significant morbidity and mortality however, mature birds are more resistant and frequently do not exhibit any clinical signs. The immune system of a chick is not fully developed and hence chicks are considered immunologically incompetent rendering them more susceptible to *Salmonella* infection (Bar-Shira & Friedman 2006). The extensive bacteraemia caused by *S*. Typhimurium in chicks has been found to cause occasional high mortality (Gast, 2008). The oral dose delivered is directly correlated with the incidence of both death and gastrointestinal colonisation in chicks (Sadler et al. 1969; Fagerberg et al. 1976). Adult hens, in contrast, typically exhibit very limited signs of morbidity and low rates of mortality upon primary

infection *Salmonella* (Bumstead & Barrow 1993; Hogue et al. 1997; Prevost et al. 2006). This resistance has often been correlated to the acquisition of protective gut microflora that produce antagonistic factors that inhibit *Salmonella* growth (Stavric et al. 1987).

Intestinal colonisation and systemic dissemination are the two most consistently observed features of NTS infections in mature hens. *S.* Enteritidis strains are able to persist in the intestinal tract of laying chickens for several months after oral inoculation and have also been found to persist for extended periods of time in the liver (22 weeks) and spleen (up to 40 weeks) of orally infected hens (Gast & Beard 1990). *S.* Enteritidis has also been isolated from ovary, oviduct, heart blood, and peritoneum (Gast & Beard 1990; Shivaprasad et al. 1990; Gast, 2008; Maciel et al. 2017). The dynamics of persistent *Salmonella* infection in hens has been predominantly studied using *S.* Enteritidis, far less is known, however, about long-term infection with *S.* Typhimurium.

S. Typhimurium is endemic in Australian layer flocks and intermittent shedding is a continuous source for the environmental contamination (The OzFoodNet Working group 2012, 2015b; Moffatt & Musto 2013; Gole et al. 2014; Ford et al. 2016; Moffatt et al. 2016). Recently, hens orally infected with *S.* Typhimurium have exhibited intermittent bacterial shedding over a 16-week period (Pande et al. 2016a). More long-term experiments focussed on understanding the mechanisms controlling *S.* Typhimurium persistence and the effect of host immune response to infection with this bacterium are needed.

1.3. Immune responses to *Salmonella* Typhimurium infection

The avian immune system lacks lymph nodes and is composed of unique lymphoid tissues and organs (Kaiser, 2010). The thymus, bursa of Fabricus, and bone marrow are the primary avian lymphoid organs while spleen, mucosal associated lymphoid tissues, diffuse lymphoid

tissues and germinal centres are considered as secondary lymphoid organs (Pope, 1997). The avian immune system is comprised of two types of immunity, innate and adaptive.

Innate immunity is less specific and considered the first line of defence during early infection (Erf, 2004). This form of immunity depends on pattern recognition receptors (PRRs) and toll-like receptors (TLRs) for the detection of pathogen-associated molecular patterns (PAMPs) which initiate signalling cascades and control the development of the innate immune response (Chausse et al. 2011). Chicken TLR has many unique properties at the level of ligand specificity, the formation of TLR receptor complexes, and activated TLR signalling pathways. Genome scanning of the chicken TLR repertoire revealed that the presence two TLR2 isoforms (TLR2 types 1 and 2), two TLR1/6/10 orthologs, and a single TLR3, TLR4, TLR5, and TLR7. In addition, chickens have two TLRs that appear absent in the mammalian species, namely TLR15 and TLR21. Chickens also lack an ortholog of mammalian TLR9 (Keestra et al. 2013). TLRs contribute to host resistance to microbial pathogens and stimulate the adaptive immune response through the control of dendritic cell maturation (Iwasaki & Medzhitov, 2004). TLR2, TLR4 and TLR5 are mainly responsible for the identification of *Salmonella* (Uematsu et al. 2006). Immune cells and cytoplasm of intestinal epithelial cells express TLR2 which recognise bacterial lipoproteins (Kawai & Akira 2005), TLR4 recognises lipopolysaccharide (LPS) (Crhanova et al. 2011) while TLR5 plays a fundamental role in *S*. Typhimurium infection in chicken (Iqbal et al. 2005).

During early infection, the host cytokine response plays an important role in controlling bacteria. Contact with bacterial lipopolysaccharide stimulates dendritic cells (DCs) and macrophages which release pro-inflammatory cytokines causing inflammation in infected tissues. Macrophages, heterophils, B lymphocytes, T lymphocytes, mast cells etc. contribute to the protective effects against intestinal infection (Methner et al. 2010) through the production of cytokines (IL-1β, IL-12, IL-18) and chemokines (IL-8). In immune cells, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor and synaptotagmins

have been found to regulate processes of cytokine trafficking to cell migration and phagocytosis (Arango Duque & Descoteaux 2014). During the initial phase of *Salmonella* infection interleukin (IL)-12 induces interferon-gamma (IFN-γ) production which activates macrophages restricting intracellular bacterial replication (Ismail et al. 2002). Infected macrophages can also enhance killing mechanisms by producing high intracellular concentrations of nitric oxide by iNOS (Braukmann et al. 2015) which contributes to the bactericidal activity against *Salmonella* (Chakravortty & Hensel 2003).

Although the macrophage is considered a "powerful professional phagocyte", *Salmonella* being an intracellular organism with the help of salmonellosis Pathogenicity Island 2 (SPI-2) type III secretion system overcomes this phagocytic process by surviving in the phagolysosome (Hensel 2000; Erf 2004; Chappell et al. 2009; Wigley 2014). *Salmonella*-infected macrophages move through the reticuloendothelial system dispersing the bacteria to the spleen and liver resulting in a systemic infection (Wigley et al. 2014). This ability of *Salmonella* ultimately results in a decreased capacity of the host to clear infection and facilitates the establishment of a persistent infection.

Adaptive immunity consists of both the humoral and cell-mediated immune (CMI) responses. DCs and macrophages serve as antigen-presenting cells (APCs) that act as an important channel between innate and adaptive immune responses by presenting processed antigens to B and T lymphocytes. B lymphocytes, upon activation, proliferate and differentiate into plasma cells responsible for the production of antibodies. The avian immune system consists of three classes of immunoglobulins: IgM, IgY (IgG) and IgA.

Salmonella-specific serum IgG produced by B cells on an encounter with antigen is essential for protection against virulent *Salmonella.* The role of antibody in immunity to *Salmonella* infection, however, remains poorly defined. B cells are not required for the resolution of attenuated *Salmonella* infection although antibody does accelerate clearance of secondary

infection when studied in BALB/c mice infected with attenuated serovar Typhimurium (McSorley & Jenkins 2000). Generally, in murine model immune serum appears to increase phagocytosis and superoxide production primarily mediated by Fcγ receptor 1 and in addition to production of specific antibodies that can be used to confer protection, B cells support the production of IFN-γ by CD4+ and CD8+ T cells, probably by virtue of their capacity to present antigen (Beal & Smith 2007). The strong T cell response is associated with the *Salmonella* clearance in aged chickens, while the antibody response does not always correlate with increased resistance to disease, strong B-cell responses are induced, including production of IgA, which could interact directly with *Salmonella* in the gut lumen. Beal et al. (2006) also showed that antibody response is not essential for gut clearance of *S.* Typhimurium.

IgA responses and polymorphonuclear leukocytes (heterophils) appear to be more critical for intestinal clearance of *Salmonella* (Berthelot-Herault et al. 2003; Beal et al. 2004b; Beal & Smith 2007). The IgA response works by the three mechanisms (i) interference with epithelial receptor binding; (ii) immune exclusion; and (iii) direct impact on the activation or expression of virulence determinants (*e.g.*, motility and T3SS) and aids in *Salmonella* clearance (Mantis & Forbes 2010). Heterophils enhance phagocytosis, by opsonisation of *Salmonella* and induce upregulation of various cytokine mRNA levels (Kogut et al. 2003). Beal et al. (2004a) also suggested that the adaptive immunity is more crucial for the clearance of *S.* Typhimurium infection and clearance of secondary challenge is faster than seen with the primary challenge.

Cell-mediated immunity (CMI) is mainly regulated by T lymphocytes, the antigen-specific cells with the ability to identify various pathogens. CMI responses work mainly against intracellular antigens and are regulated by helper T cells. Adaptive immunity through antibodies and T-cells such as $CD4^+$ (T helper cells) and $CD8^+$ (T cytotoxic cells) recognise

specific molecular features of an antigen (Erf, 2004). In response to flagellin, DCs initiate the differentiation of naïve B cells into IgA plasma cells independent of the gut-associated lymphoid tissue (Uematsu & Akira 2009). Sufficient numbers of memory cells are necessary to attain protection, and they must remain as a long-lived functional population (Chabalgoity et al. 2007). Following the establishment of systemic infection chickens may clear or control the replication of *S.* Typhimurium through adaptive immune response. If replication is not controlled bacteria replicates in internal organs leading to septicaemia and death commonly in young chicks.

CMI is also characterised by a Th1 lymphokine profile which is associated with activation of macrophages and cytotoxic lymphocytes and appears to be a critical part of effective anti-*Salmonella* immunity. IFN-γ protects chickens against *Salmonella* invasion into organs and its levels correlate with the clearance of *Salmonella* from organism (Beal et al. 2004a). The CMI is reduced at the onset of lay (Wigley et al. 2005) which has been proposed as one of the reason for increased *S.* Typhimurium shedding and egg contamination at the start of lay (Holt, 1999). In chickens, cell-mediated immunity is more important than humoral responses for tissue clearance of virulent *Salmonella* strain (Beal et al. 2004a; Beal & Smith 2007). B cells are not involved in the removal of primary *S.* Typhimurium infection (Beal et al. 2006); moreover *S.* Typhimurium clearance from the older chicken gut is associated with strong T cell response (Beal & Smith 2007). It has been hypothesised that *S.* Typhimurium generates more effective and stronger immune response than *S.* Enteritidis which limit the disease progression and quickly clears the *S.* Typhimurium infection from birds (Wales & Davies 2011).

1.4.*Salmonella* Typhimurium infection and egg contamination in laying hens

Eggs are an important component of Australian agricultural commodity. In 2013–14, the Australian commercial egg industry produced 397.4 million dozen eggs from 16.55 million commercial laying hens (AECL, Annual Report, 2005). Poultry, however, are often persistently infected with non-typhoidal serovars of *Salmonella enterica* which can lead to the downstream contamination of eggs and products containing raw or undercooked eggs (Moffatt et al. 2016). The primary route of *Salmonella* infection for poultry is horizontally via contact with contaminated faeces, litter, feed, water, insects or farm equipment (FAO/WHO 2002). Vertical transmission has also been described and arises when an ovarian follicle or developing egg becomes infected (Poppe, 2000). The capacity of *S.* Typhimurium to infect laying flocks and contaminate eggs has become a significant problem as new egg invasive strains of *S.* Typhimurium such as PT104, PT99, PT170/108, PT9 and PT135/135a have emerged (The OzFoodNet Working group 2010).

Salmonella colonise the avian intestinal tract with the crop and ceca being the primary sites of colonisation (Impey & Mead 1989). *Salmonella* infection in poultry produces systemic disease that is cleared through cellular immunity within two to three weeks (Beal et al. 2004b). *Salmonella* can survive within and replicate in the macrophages, and if not cleared by the host immune system can lead to the colonisation and spread to internal organs, such as liver and spleen, where bacteria can be found in large numbers (Barrow, 1999). Adult healthy birds infected with *S.* Typhimurium often become symptomless carriers with continuous shedding (Carter et al. 2009).

Egg contamination can occur through either vertical or horizontal mechanisms. Vertical transmission occurs when *Salmonella* colonise the oviduct of an infected hen and contaminate egg membranes and internal contents during development (De Reu et al. 2006b). *S*. Enteritidis and *S*. Typhimurium serovars when administered orally has shown to invade reproductive organs of the laying hens. However, *S*. Enteritidis possesses the capacity

of vertical transmission and has been isolated from the egg internal contents (Okamura et al. 2001a; Gantois et al. 2008; Gast et al. 2013;); intrinsic factors such as the presence of cuticle, shell characteristics (shell quality, porosity, shell defects) and shell membrane allowing a specific interaction with the reproductive organs and resistance to antibacterial compounds which enable *S*. Enteritidis to colonize the oviduct and contaminate egg internal contents of laying hens (Messens et al. 2007; Gantois et al. 2009; Wales & Davies 2011).

Horizontal transmission of *Salmonella* occurs when an egg is laid in a contaminated environment and the bacteria penetrate the egg shell membrane infecting the egg internal contents (De Reu et al. 2006b). Any subsequent bacterial replication on the eggshell increases the chances of *Salmonella* penetrating the eggshell as well as the internal egg contents (De Reu et al. 2006a). Although vertical transmission of *Salmonella* from bird to egg has been demonstrated (as has been shown for *S*. Enteritidis), a recent study has shown that horizontal transmission is the most likely source of *S*. Typhimurium contamination of shell eggs (Pande et al. 2016a). Previous experimental infection trials have examined egg contamination and internal organ colonization of layer hens. These studies, however, have infected birds at different ages, using a variety of inoculation methods (Williams et al. 1998; Leach et al. 1999; Okamura et al. 2001b; Okamura et al. 2010) and have collected samples over varied time periods limiting the degree to which the data can be directly compared. The productive lifetime of a layer hen, can extend beyond 50 weeks of age and few studies have investigated extended bacterial shedding dynamics, egg contamination and host responses to infection.

During their productive lifespan, layer hens may also experience many physiological and environmental stressors, such as overcrowding, extreme temperature variation and the onset of lay that may lead to increased faecal shedding of *Salmonella* (Holt, 1993; Nakamura et al. 1994; Quinteiro-Filho et al. 2012; Borsoi et al. 2015). Stress has also been linked with

impaired immunity (Mashaly et al. 2004; Shini et al. 2008; Quinteiro-Filho et al. 2012; Borsoi et al. 2015) that may increase intestinal colonisation by enteric pathogens such as *Salmonella* (Borsoi et al. 2015). The effect environmental stressors have on layer hens and how they contribute to *Salmonella* shedding patterns have been studied in both controlled and field trials (Nakamura et al. 1994; Seo et al. 2000). Both studies reported the short-term increase in shedding of *S*. Enteritids with short-term exposure to stress.

Stress experienced by a hen during the onset of lay can also trigger increased *S.* Typhimurium shedding and egg contamination. Stress can stimulate the recrudescence of bacteria from internal organs resulting in high bacterial load in faeces (Rostagno et al. 2006). A similar observation was reported (Okamura et al. 2010) with increased egg contamination at the onset of lay in pullets infected with *S*. Typhimurium DT104. A decline in the cell-mediated immune response has been observed in hens at point of lay typically at the age of 17-20 weeks of age (Wigley et al. 2005). This impairment in CMI may be one of the reasons for increased *S*. Typhimurium shedding. Molting-related stress in hens has also been shown to enhance *Salmonella* shedding in eggs and faeces (Holt et al. 2003) and could also cause higher levels of colonisation in internal organs (Holt, 1995).

Due to the public health importance of contaminated eggs, understanding the dynamics of *S.* Typhimurium shedding patterns and associated host responses to infection is of critical importance. Oral *S.* Typhimurium infection can lead to cloacal contamination followed by faecal shedding and horizontal egg contamination during lay (Pande et al. 2016a). Additional long-term layer hen infection trials would improve our understanding of the correlation between faecal shedding and egg contamination and add to our current knowledge of the immunological consequences of *Salmonella* colonisation.

1.5. Interventions limiting *Salmonella* in egg production system

Eggs used for human consumption represent a risk for the spread of foodborne salmonellosis through the consumption of raw or uncooked food items. Between 2005 and 2009, extensive control procedures were implemented throughout the European Union food supply chain which contributed to a 50% decrease in the incidence of salmonellosis highlighting the importance of their use (Keerthirathne et al. 2017). From farm to table, there are many possible points where *Salmonella* can infect the egg supply chain. A wide range of management strategies and intervention measures exist to control *Salmonella* in pre- and post-harvest production. The goal of these strategies is to minimise the introduction, persistence, and transmission of food-borne pathogens such as *Salmonella* in the flock and therefore reducing contamination on eggs.

1.5.1. Pre-harvest Interventions

Pre-harvest interventions are employed to either prevent bacterial infection or reduce the duration of *Salmonella* shedding in pullets and adult layer hens (Kilroy et al. 2015). Vaccination, strong biosecurity programs, as well as the use of feed free of animal by-products are keys to reducing the prevalence of *Salmonella* on farm.

1.5.1.1. *Salmonella* Typhimurium vaccination scenario in chickens

Due to the public health risk, vaccination of poultry against *Salmonella* as a control measure has been extensively researched. Many countries world-wide have implemented vaccination programs that in combination with other on-farm control measures showed reduction of *Salmonella* on farm and subsequently human cases of salmonellosis have been reduced. In

the early 1990s, for example, there was sharp increase in human cases of salmonellosis due to infection with *S*. Enteriditis in the UK. Both government and industry groups implemented flock vaccination programs against *S.* Enteriditis that along with other control measures is credited with marked decrease in human cases of salmonellosis (Desin et al. 2013).

Vaccination is one control measure that is widely employed in the poultry industry world-wide to reduce *Salmonella* colonisation of a flock. For broiler birds, the goal is to achieve sufficient immunity for the 5-8 weeks of life that prevents birds from becoming infected with *Salmonella*. The challenge with layer hens is two-fold; the productive lifespan of the bird is substantially longer (about 80 weeks) so maintaining long term immunity is key. In addition, the vaccine regime needs to be competitive enough to prevent *Salmonella* from establishing a persistent infection. Persistent infection results in the intermittent shedding of the bacteria over the productive lifetime of hen, which can increase during periods of stress (Humphrey, 2006; Gast et al. 2009).

1.5.1.1.1. Killed or inactivated *Salmonella* vaccines

Killed or inactivated vaccines are produced through physical or chemical methods such as heat treatment, formalin fixation, or acetone and alum precipitation while preserving antigenicity (Singh, 2009; Barrow & Methner 2013). One of the major advantages of this class of vaccines is the lack of reversion to virulence, minimizing the potential risks of downstream contamination of the food supply chain. The use of inactivated vaccines has been linked experimentally with reduced *Salmonella* loads in faeces, internal organs and eggs following challenge (Gast, 2017).

Multivalent inactivated vaccines are also commercially available and exhibit effectiveness at reducing multiple serovars in laying hen flocks. A bivalent vaccine containing both *S.* Enteriditis and *S.* Typhimurium significantly reduced faecal shedding of both serovars and

egg contamination after intravenous and intraperitoneal challenge in layer flocks (Okamura et al. 2007). Trivalent inactivated vaccines containing representatives from multiple serogroups provides cross protection for multiple serovars (Deguchi et al. 2009; Pavic, 2010). Maternal transfer of antibodies has also been demonstrated in vaccinated breeder hens (Pavic et al. 2010), potentially providing protection for newly hatched chicks against systemic infection and intestinal colonisation (Young et al. 2007).

Inactivated vaccines, however, have several drawbacks. They elicit an incomplete antibody response and do not stimulate a cell-mediated immune response (CMI) for providing long-term protection in laying hens (Van Immerseel et al. 2005; Barrow, 2007). The inactivated vaccines when used with strong adjuvants could induce secretory immunoglobulins (sIgA) to enhance immune response, thus further protecting mucosal surface from *Salmonella* colonisation (Mastroeni et al. 2001; Barrow, 2007; Beal & Smith 2007; Singh, 2009). Moreover, administration of inactivated vaccine requires handling of birds which is labour intensive, and age of the birds leaves them susceptible to bone breakage during handling (Holt et al. 2003).

1.5.1.1.2. Live-attenuated vaccines

Live attenuated vaccines are produced through the introduction of mutations or deletions in genes required for bacterial metabolism, virulence or survival. These mutations generate avirulent strains that retain the ability to stimulate the host immune response (De Cort et al. 2013; Desin et al. 2013; De Cort et al. 2017). Several live attenuated *Salmonella* vaccines have been produced and are widely used.

Mutations targeted at bacterial metabolic genes that limit *in vivo* growth have been among the most common vaccine strains. Among these are the *aroA* mutants, which lack the ability to synthesize certain aromatic compounds necessary for growth. These mutant strains remain

invasive within the host and are able to induce a strong immune response which is required for early clearance of wild-type challenge strains from the gut (Barrow & Methner 2013).

Several *S.* Typhimurium *aroA* mutant strains have been made and tested experimentally for their vaccine potential. A*roA* mutant *S.* Typhimurium strains have been shown to be protective against challenge from a wild-type strain and reduced faecal shedding (Barrow et al. 1990; Alderton et al. 1991; Abs el-osta et al. 2015). Vaccination with *aroA* mutants produces strong IgG, IgM and IgA responses in both sera and the intestine which is important for the control of *Salmonella* within a bird (Alderton et al. 1991). Vaccine dose and administration, however, have been shown to be important factors in eliciting an effective immune response (Alderton et al. 1991; Groves et al. 2015). Cross-protection has also been demonstrated for *S.* Typhimurium *aroA* vaccine strains. Vaccinated pullets challenged with *S.* Enteriditis exhibited decrease faecal shedding of the challenge strain and lower contamination of the environment (Parker et al. 2001).

Other live attenuated *S.* Typhimurium vaccines include the Δ*cya*-Δ*crp* which is unable to produce functional adenylate cyclase and cyclic adenosine monophosphate receptor proteins. Oral immunization with this vaccine also induces a strong immune response in vaccinated birds (Hassan & Curtiss 1990). The Δ*cya*-Δ*crp* vaccine was effective at protecting challenged birds against intestinal and internal organ colonisation by both homologous and heterologous *Salmonella* strains (Hassan & Curtiss 1994; Hassan & Curtiss 1997). A Δ*phoP rpoS S.* Typhimurium mutant strain also reduced faecal shedding in birds challenged with *S.* Typhimurium (Papezova et al. 2008). Experiments with the *S.* Typhimurium triple mutant, Δ*lon*Δ*cp*xRΔ*asd*, revealed that vaccinated layer hens produced a strong IgG and IgA response against *S.* Typhimurium, *S.* Gallinarum and *S.* Enteritidis infection in layer chickens (Lee, 2015).

Live attenuated *S.* Typhimurium vaccine strains have several advantages over inactivated vaccines and are considered as a useful component of *Salmonella* control plans (Hassan & Curtiss 1994). They stimulate both the cell-mediated and the humoral immune responses (Van Immerseel et al. 2005). Other advantages of this class of vaccines are that they are frequently less costly, easier to store (no cold chain is needed) and can also be used as carriers for delivery of recombinant antigens (Tan et al. 1997; Mastroeni et al. 2001; Dueger et al. 2003; Van Immerseel et al. 2005; Desin et al. 2013). Although live attenuated vaccines are thought to be the most efficient forms of immunoprophylaxis against *Salmonella*, there are drawbacks to their use such as, prolonged survival in the environment which may pose a hazard to humans (Tan et al. 1997), the vaccinated animals may continue to shed the vaccine strain over a period of time, making it difficult to differentiate between vaccinated and infected animals and, the potential of reversion to virulence (Gast et al. 2007).

1.5.1.1.3. Subunit vaccines

A subunit vaccine is comprised of a single antigen or multiple defined antigens such as bacterial outer membrane proteins (OMPs), porins, toxins, or ribosomal fractions proteins that stimulate the immune system (Singh, 2009; Desin et al. 2013). When administered with an appropriate adjuvant, subunit vaccines have been shown to provide effective but short-term immunity (Mutwiri et al. 2011). A number of subunit *S.* Typhimurium vaccines have been tested under experimental conditions and have shown to provide protective effect through the induction of systemic and mucosal immune responses (Prejit et al. 2013; Jawale, & Lee 2014). A *S.* Typhimurium ghost vaccine candidate, for example, elicited a dose dependent immune response and protection against challenge with wild-type *S.* Typhimurium (Jawale, & Lee 2014, 2016).

Recombinant outer membrane protein (OmpC) of *S.* Typhimurium has also been tested as a vaccine candidate (Jha et al. 2015). Prejit et al. (2013) used OmpC against challenge with

virulent *S.* Typhimurium in layer birds and found decreased faecal shedding with minimal detection of *Salmonella* from internal organs and eggs, suggesting that *S. enterica* OMP protein could be an ideal target for a future vaccine. New and advanced vaccine delivery systems such as nano-drug delivery particles, liposomes and immune stimulating complexes (ISCOMs) have been reported effective and are important in generating cell mediate immune responses. The r-OmpC used with these adjuvants can act as an immunomodulatory and induce a stronger immune response and could represent a new vaccine candidate (Jha et al. 2015).

While subunit vaccines have been shown to provide significant protection against *Salmonella* infection in laying hens the complexity of their manufacture combined with the high cost of production are limiting factors. Moreover, subunit vaccine development is in its early stages require further experimentation. These vaccines may represent the next generation of vaccines that enable the safe delivery of antigens from multiple *Salmonella* serovars and provide improved protection against egg contamination in laying hens.

1.5.1.1.4. *Salmonella* vaccines in Australia

Salmonella Typhimurium remains a primary concern for the Australian commercial egg industry. Historically, only two *S.* Typhimurium vaccines registered for use in Australia, a live attenuated strain produced by Bioproperties Pty Ltd (Vaxsafe® STM-1) and an inactivated autogenous multivalent vaccine produced by Intervet (now MSD Animal Health) (Sharpe et al. 2012; Abs el-osta et al. 2015; Groves et al. 2016).

Currently, the Vaxsafe® STM-1 strain is the only vaccine available for use in Australia (Alderton et al. 1991; Groves et al. 2016). A reduction in faecal shedding, colonisation and excretion of *S.* Typhimurium has been reported by Bioproperties following oral administration of Vaxsafe® STM-1 strain in day old chicks followed by a booster at two

weeks of age (Vaxsafe® ST (Strain STM-1). This vaccine is registered to use by spray and drinking water applications and has been shown to reduce *Salmonella* shedding in broilers (Alderton et al. 1991). The long-term efficacy of the STM-1 vaccine strain in commercial layer flocks under farm conditions, however, remains unclear.

1.5.1.2. Competitive Exclusion

Vaccination against *S.* Typhimurium does not confer ultimate prevention. The combination of live and inactivated *Salmonella* vaccines, sanitation throughout the production stages along with competitive exclusion (CE) represent the most important methods to increase the resistance of both young and adult chickens (European Food Safety Authority, 2004). Vaccination in layers is needed for many months, therefore, should be compatible with the use of CE (European Food Safety Authority, 2004). CE is defined as the protective effect of the natural bacterial flora of the intestine, which limits the colonisation of some bacterial pathogens. Commercial preparations of CE products such as probiotics or direct-fed microbial CE cultures are often used in chickens. CE causes obstruction of attachment sites for *Salmonella* by the native flora lining the intestine, competition for essential nutrients by the native flora limits the ability of salmonellae to grow. Such protective flora may produce volatile fatty acids (especially in the ceca) that restrict the growth of salmonellae (Jeffrey, 1999). Administration of the combination of *Salmonella* vaccine and CE culture produced a better protective effect than either single administration. The phenomenon has been widely tested in poultry as a means of controlling colonisation by paratyphoid salmonellas, and potential benefits have been demonstrated under laboratory and field conditions (Lloyd et al. 1977; Revolledo et al. 2006). The combined live *S.* Enteritidis phage type 4 (PT4) vaccines (Salmovac SE, 10^9 CFU; IDT Biologika) was used in day old chicks, and CE culture was also administered on day 2 (Aviguard, Microbial Developments Ltd.) via crop instillation in White Leghorn chickens. On day 3, chickens were infected orally with *S.*

Enteritidis 147R, PT4. The challenge strain was enumerated in caecal contents and liver from infected birds and the combination of live *S.* Enteritidis vaccine with CE culture has completely prevented the caecal colonisation and the systemic invasion of the challenge strain in the liver. The CE culture diminished the intestinal colonisation of *S.* Enteritidis challenge strain. The live vaccine has resulted in the influx of heterophils, monocytes and lymphocytes from blood into the caecal mucosa leading to decreased *S.* Enteritidis challenge organism. In this study, it was deduced that live vaccine had produced an early innate immune response that was sufficient enough to strongly inhibit the systemic invasion and dissemination of the *S.* Enteritidis challenge strain. The combined use of a commercial SE-LV with a CE culture did not induce an additive intestinal colonisation-inhibition effect; however, it did result in an additive protective effect that prevented the systemic dissemination of the *S.* Enteritidis challenge strain completely (Braukmann et al. 2016). To further exploit the efficacy of the combined use of CE and vaccination, live *Salmonella* vaccines are needed that are not only sufficiently attenuated but also still capable of inducing both colonisation- and invasion-inhibition effects by stimulating polymorphonucleated cell migration to the intestinal cell walls, thus preventing initial colonisation by wild type *Salmonella*. These strategies can be applied and used in the preparation of live ST vaccines.

1.5.1.3. Biosecurity

On farm biosecurity measures including the control of wildlife, rodents, insects, unauthorised human visitors, and fomites (e.g., feed truck and farm equipment) from entering the layer farm all contribute toward *Salmonella* control (Gradel & Rattenborg 2003). Worker sanitation and hygiene measures including footbaths, hand washing facilities, and the use of disposable overalls and shoe covers are additional biosecurity measures that can be implemented to further improve on farm control of zoonotic pathogens (Gradel & Rattenborg 2003). Strict biosecurity measures on breeder farms/houses could also contribute

substantially towards bacterial control. Such measures of control include shower-in–shower-out (i.e., shower prior to and after entry to the breeder farm), farm dedicated footwear, coveralls, and hair covering.

Cleaning and disinfection of poultry houses is also a critical control measure. Shed sanitation following litter removal is considered a significant factor for reduction of *Salmonella* in the next placement flock (Marin et al. 2011).

Other pre-harvest interventions include prevention of feed contamination and recontamination through chemical and physical interventions such as treating feed with acids (e.g., organic and propionic acids) and formaldehyde, heat treatments, irradiation (Ricke, 2003). Combinations of acids and heat treatments of poultry feed have also been shown to reduce levels of *Salmonella* (Matlho et al. 1997). Vaccination programs for breeder flocks should also be considered with the intention of reducing vertical transmission of *Salmonella* spp. in poultry production. The hatcheries employ some interventions in the form of disinfection of incubators and hatching cabinet surfaces. The disinfection of the hatching eggs could be done using UV light, ozone, electrostatic charging, and electrostatic spraying to control the transmission of *Salmonella* (Russell, 2003; Perry et al. 2008).

1.5.2. Post-harvest interventions to reduce the risk of egg contamination by *Salmonella*

Post-harvest methods are targeted at reducing total *Salmonella* counts, especially on the eggshell surface to ensure the microbiological quality of the marketed shell eggs (Galis et al. 2013). Maintenance of temperature during storage, egg washing with disinfectants, electrolysed water, ozone, irradiation of egg with ultraviolet light and pulsed light, using microwaves, and gas plasma techniques are additional methods used to further reduce the risk of egg contamination with *Salmonella* (Galis et al. 2013).

1.5.2.1. Eggshell decontamination to control *Salmonella* spp. penetration and prevention of egg contamination

The following post-harvest methods have been studied for eggshell decontamination by *Salmonella*. These methods can be divided in to following classes the chemical, physical and biological. Chemical methods include washing (through sanitizers), hydrogen peroxide, electrolysed water, and Ozone. The physical methods involve irradiation, microwave technology, ultraviolet light technology, pulsed light technology, gas plasma technology and ultrasounds. The third type is biological methods comprises the use of plant extracts; the effectiveness of these plant extracts has not been fully demonstrated until now (Galis et al. 2013).

Many of these post-harvest procedures are not widely accepted and have had very limited implementation. Further research into their efficacy is required.

1.6. Cleaning and disinfection

Cleaning and disinfection are key components of routine biosecurity in poultry farming. The process of decontamination using disinfectants inhibit or kill a broad-spectrum of microorganisms (White & McDermott 2001). Commercial disinfectants contain a variety of active ingredients, and are routinely used in medical and food processing industries, as well as in private house hold conditions (Beier et al. 2008).

The primary aim of disinfection is to kill remaining infectious agents left after cleaning. Disinfectant chemicals, however, lose their effectiveness after contacting organic materials such as manure, blood, dust or dirt (Bessems, 1998; Ward et al. 2005). The presence of large amounts of organic material may protect infectious agents from the action of disinfectants.

Disinfectants can be applied through spray, aerosol, or fumigation methods and should be applied only after the building and equipment have been thoroughly cleaned, ideally right after rinsing. Disinfection is the least reliable step of biosecurity because it depends on many factors including the quality of cleaning, the hardness of water, quality and suitability of disinfectant, as well as use of the correct dilution and application (Sigal, 2017).

Current proposals for a new Animal Health Strategy for the EU and new industry guides for good hygiene practices in broiler and layer production include special mention of on-farm biosecurity including cleansing and disinfection (Anonymous, 2010). The laying hen house environment can act as a continuous source of egg contamination with *Salmonella*. Thus, routine cleaning and disinfection of layer shed is commonly used preventive methods to control *Salmonella.* However, previous studies (Davies & Breslin 2003a; Wales et al. 2007) showed that despite using cleaning and disinfection *Salmonella* prevalence can remain high in laying hen houses suggesting ineffectiveness of commonly available disinfectants against *Salmonella*. Incorrect dilutions and insufficient cleaning are possible reasons for the failure of a disinfectant to eliminate infectious diseases in a farm environment (Ward et al. 2005).

A number of commercial disinfectants are routinely used on layer farms to remove microorganisms on equipment surfaces and eggs to reduce bacterial contamination, thereby preventing contamination of food products with pathogen and spoilage organisms (McDonnell & Russell 1999). There are six major classes of disinfectants, namely quaternary ammonium compounds (QAC), halogens (chlorine, iodophores), peroxygens, aldehydes, phenols and biguanides (Singh, 2016). The low permeability of the cell wall or decreased accessibility to the target due to active efflux mechanisms can reduce the efficacy of the disinfectant (Fraise, 2002). Bacteria may be exposed to disinfectant concentrations lower than that required to deliver a lethal insult (Chapman, 2003).

1.7.*Salmonella* biofilm formation and use of disinfectants

Salmonella on food surfaces represents an important public health risk. Recent work has shown that multiple *Salmonella* isolates are capable of forming biofilm on the eggshell surface (Pande et al. 2016b). Biofilms on food surfaces such as eggshells as well as farm or kitchen equipment represent a potential source for food contamination (Shi & Zhu 2009). A biofilm is any group of microorganisms which adhere to a surface together and are enclosed in self-produced matrix of extracellular polymeric substances (EPS). Cell surface hydrophobicity and the expression of specific appendages, including fimbriae, curli, and outer membrane proteins, can influence bacterial attachment to surface (Goulter et al. 2009). A mature biofilm is highly heterogeneous due to the mixture of flat-cell monolayer biofilm and three-dimensional mushroom-shaped biofilm (Karatan & Watnick 2009; Steenackers et al. 2012). Bacterial attachment is an initial and essential step of biofilm formation by pathogens such as *S. enterica* (Yaron & Römling 2014). The ability of *Salmonella* to form biofilms, contributes to its defensive mechanism and persistence in both host and non-host environments and is especially important in food processing environments in response to stress (Patel et al. 2013; Yaron & Römling 2014). This ability is a significant food safety and public health concern due to increase difficulty to eradicate biofilms and the possibility of to the development of resistance to antimicrobials and common disinfectants (Corcoran et al. 2013). Lower levels of disinfectants can influence the formation of biofilms and antimicrobial resistance (AMR) (Capita et al. 2014).

The chemical properties and mechanism of action of the disinfectants that are commonly used on the livestock farms have been studied (McDonnell & Russell 1999). Glutaraldehyde and formaldehyde are known to alkylate and create cross-links within protein molecules and to bind to cell wall peptidoglycans. Glutaraldehyde is more microbicidal at alkaline pH and works quickly via damaging the cell envelope (Gorman et al. 1980). Halogen-releasing agents expose pathogens to active forms of chlorine or iodine further causing oxidative damage to bacterial membranes and to DNA. Peroxygens are oxidizing agents that uses

peracetic acid to disrupt lipid membranes, proteins and nucleic acids via attack by reactive species such as the hydroxyl radical (McDonnell & Russell 1999). QAC's are the cationic surfactants and are active on the bacterial membranes (Chapman, 2003). Phenols and cresol compounds, causes denaturation of protein at increased concentration and have coagulative effects on cytoplasm (Russell, 2004). The limitations of cleaning and disinfection in *Salmonella*-contaminated layer farms have been documented (Davies & Breslin 2003b; Wales et al. 2006), and some disinfectants with proven performance in the standard tests do not appear to be as efficacious in field situations (McLaren et al. 2011). Biofilms formation with extracellular matrix in areas such as water systems is also associated with enhanced bacterial resistance to disinfectants (McDonnell & Russell 1999). The variations in susceptibility to disinfectants have been observed between *Salmonella* strains (Sander et al. 2002) and even within the Typhimurium serogroup (Thomson et al. 2007). The susceptibility of disinfectants including QAC and substituted phenols to *Enterobacteriaceae* pathogen, may increase or decrease depending on nutrient availability, cell count and bacterial multiplication (Bjergbæk et al. 2008). The chlorocresol-based, phenolic, peroxygen, QAC's and chlorinated compounds are commonly used as the commercially available disinfectants in poultry sheds (Zeidler, 2002; McLaren et al. 2011) to eradicate biofilms, but, there is a limited information whether the bacteria are resistant to the washing chemicals or not. Moreover, information on disinfectant susceptibility, appropriate concentration and exposure time against biofilm formed by *S*. Typhimurium on egg shells is also lacking.

The strategies to control bacteria must be complete, following food products from the farm to the table (Wachsmuth et al. 1997). *Salmonella* in the food chain sequence are controlled by strategies that include the use of biocides as disinfectants (Beier et al. 2017). Hutchison et al. (2004) used two different wash chemicals a chlorine-based sanitizing agent in a concentration of 3 g/L and a quaternary ammonium based sanitizing agent in a concentration of 25 mL/L. Three different steps in the egg washing process: prewash at 44 ºC, wash at 44

ºC and rinse at 48 ºC, followed by air drying at 42 ºC for 2 min were used (Hutchison et al. 2007). Washing of contaminated eggs under optimum conditions resulted in a more than 5-log reduction of *Salmonella* counts from the shell surface. *Salmonella* was not isolated from the internal contents highlighting that when properly controlled, egg washing did not cause *Salmonella* to enter the contents. Both *S.* Enteritidis and *S.* Typhimurium were shown to enter the egg contents when water temperatures were lowered, indicating that strict temperature control must be maintained in order to prevent the ingress of *Salmonella* into egg contents. The concentration of the washing chemical compounds and the length of the washing period do not appear to influence the contamination of the egg contents.

In commercial processing, eggs are most frequently rinsed with chlorine (sodium hypochlorite) solutions, which act as antimicrobial agents. A chlorinated compound is bactericidal due to its oxidative reaction and interferes with cellular protein function (Ramesh et al. 2002). This compound has been tested as a disinfectant against *Salmonella* biofilms with variable effects (Toté et al. 2010; Wong et al. 2010; Ziech et al. 2016). Chlorine is widely used because of its low cost and it provides some measure of bacterial reduction (Hutchison et al. 2007). A high level of chlorine, however, can be detrimental to the quality of eggs due to remaining residues deposited on the eggshell (Bialka et al. 2004; Keener, 2017).

Commercially available QAC's and twin chain QAC's disinfectants are widely used as egg shell sanitizers in poultry sheds (Zeidler, 2002). Twin chain QAC's are fourth generation quaternaries containing didecyl dimethyl ammonium chloride with greater germicidal performance than QACs. These quaternaries have superior germicidal activity, are low foaming and have a high tolerance to protein loads and hard water. They are recommended for disinfection in the food and beverage industry, given their low toxicity (QuimiNet, 2012). QAC's leads to disruption of the lipid bilayer of the cytoplasmic membrane and outer

membrane of gram-negative bacteria leading to leakage of cytoplasmic components and eventually cell lysis (Quinn et al. 2011). Møretrø et al. (2003) tested three QAC commercial disinfectants against biofilms formed by *Salmonella* strains, isolated from feed industry and found that exposure of QAC for 5 min resulted in the complete reduction of all strains when used at 80% of recommended user concentration.

There is, however, a lack of information regarding the effect of chemical concentration and exposure time on biofilm removal. A few studies have suggested that biofilm formed over a prolonged time are more resistant to disinfectants than short age biofilms (Mangalappalli-Illathu et al. 2008; Habimana et al. 2010). There are, however, conflicting reports that also suggest that biofilm age does not increase resistance to disinfectants (Wong et al. 2010). Wong et al. (2010) studied the susceptibility of 3, 5, and 7-day-old *S.* Typhimurium biofilms grown on MBEC^{TM} system to disinfectants with different exposure times of 1 and 5 minutes and found that age of biofilm did not affect the efficiency of disinfectants. However, there is little known about the effect of egg washing on eggshell penetration by *Salmonella* in the Australian context.

At present, the decontaminated eggs are mainly used as a food product for individuals susceptible to *Salmonella* infection. More research is required on the testing of egg decontamination procedure so that safe food with greater public health protection could be prepared and it should also not affect egg properties (Keerthirathne et al. 2017).

1.8. Major gaps and hypothesis of thesis

In Australia, egg-borne *S.* Typhimurium outbreaks caused by consumption of contaminated eggs and egg products is a major public health issue. Between 2001 and 2011, there was a significant increase in egg or egg product related *Salmonella* outbreaks and majority of these incidents were caused by *S.* Typhimurium (Moffatt et al. 2016). Due to their long productive

lifespan, persistently infected layer hens can serve as a continuous source of *Salmonella* shedding and egg contamination (Revolledo et al. 2006). Thus, it is essential to study host-pathogen interaction, colonisation and persistence in internal organ along with effect of stressors in laying hens so that risk reduction programs for *S.* Typhimurium infection in egg laying flocks can be developed. Most studies of host-pathogen interaction have focussed primarily on elucidating the human side of infection utilising mouse models (Mittrücker & Kaufmann 2000). Far less research has been conducted to understand the long-term shedding kinetics of *S.* Typhimurium in faeces and the subsequent risk of egg contamination. Furthermore, the persistence of *S.* Typhimurium in internal organs is not fully understood.

Salmonella vaccine research, to date, largely demonstrates that vaccine strains are capable of reducing bacterial shedding in faeces and do not provide full protection against wild-type challenge. On farm, reduced bacterial shedding is believed to correlate with reduced egg contamination, however, the risk remains that *Salmonella* will continue to contaminate the food supply chain. In addition, the majority of *S.* Typhimurium vaccine efficacy studies were focussed on chicks or broilers where a single high-dose challenge was used followed by monitoring and sampling for days to a few weeks to assess vaccine effects. *Salmonella* vaccination studies involving natural challenge in field situations are far less common than small-scale single-dose challenge studies with short-term endpoints. On farm trials would help to determine whether vaccination is effective in limiting *S.* Typhimurium persistence in layer hens and controlling egg contamination.

S. Typhimurium can attach and form a biofilm a wide variety of surfaces. Biofilm formation contributes to the persistence of *Salmonella* in the food processing environment and detachment of biofilm cells from surface could lead to cross contamination of multiple food products (Møretrø et al. 2012). In Australia, different commercial disinfectants are routinely used for egg washing in the layer farms to remove microorganisms, thereby preventing

contamination of food products with pathogen and spoilage organisms (Messens, 2013). Very little, however, is known about the efficacy of commercially available disinfectants against biofilms formed on surfaces, and further research is required to fulfil this gap.

1.9. Objective of thesis

Considering the gaps outlined in this review, the objectives of the thesis were:

1. To correlate faecal shedding and egg contamination patterns with host responses to infection (single and mixed) including faecal corticosterone levels as a marker of the host stress response as well as levels of *S.* Typhimurium specific antibodies in the serum and yolk. A final aim of this study was to characterise persistence of *Salmonella* infection in peripheral organs.

2. i) To understand the level of the vaccine colonisation, shedding and immune response generated after administration in pullets, (ii) To develop vaccine specific PCR in order to detect and differentiate vaccine from wild type *Salmonella* serovars.

3. To evaluate the effectiveness of a live vaccine (Vaxsafe® ST) against egg contamination in commercial egg laying cage flocks, naturally infected with *S.* Typhimurium during early lay.

4. To test the anti-biofilm efficacy of commercially available disinfectants at different concentrations against *S.* Typhimurium isolates from layer farms.

1.10. References

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Chapter 2 Correlating bacterial shedding with fecal corticosterone levels and serological responses from layer hens experimentally infected with *Salmonella* Typhimurium

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VETERINARY RESEARCH

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Correlating bacterial shedding with fecal corticosterone levels and serological responses from layer hens experimentally infected with *Salmonella* Typhimurium

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Abstract

Salmonella Enteriditis and *Salmonella* Typhimurium are commonly isolated during egg-related outbreaks of salmonellosis and represent a significant international public health issue. In Australia, *Salmonella* Typhimurium is the most common serovar identified in egg product related foodborne outbreaks. While a number of studies have investigated *Salmonella* shedding and host responses to infection, they have been conducted over a short time period. The present study sought to characterise bacterial shedding and host responses to infection in hens infected with only *Salmonella* Typhimurium or co-infected with both *Salmonella* Typhimurium and *Salmonella* Mbandaka over a 16 week period. *Salmonella* shedding was quantified using the most probable number and qPCR methods and was highly variable over the course of the experiment. On day 1, fecal corticosterone metabolites in birds infected with *Salmonella* Typhimurium (674.2 ± 109.3 pg/mg) were significantly higher than control (238.0 ± 12.62 pg/mg) or co-infected (175.4 ± 8.58 pg/mg) birds. The onset of lay occurred between weeks 6–8 post-infection (pi) and Fecal corticosterone metabolite (FCM) concentrations increased in both control and co-infected birds. Antibody responses to infection were monitored in both serum and yolk samples. *Salmonella* Typhimurium specific antibody was lower in co-infected animals than monoinfected animals. Bacterial loads in internal organs were characterised to determine persistence. Spleen, liver and caecal tonsils were positive for bacteria in both groups, indicating that *Salmonella* was not cleared from the birds and internal organ colonization could serve as a reservoir for continued bacterial shedding.

Introduction

Commercial poultry are often persistently infected with non-typhoidal serovars of *Salmonella enterica*. Eggs and raw egg based food products are often identified as the source of *Salmonella* during outbreaks of human gastrointestinal disease [1]. Thus, the zoonotic potential of *Salmonella* represents a significant global public health concern. In North America and Europe, the most common serovar isolated during egg-related outbreaks is *Salmonella* Enteritidis followed by *Salmonella* Typhimurium [2]. Strains of *Salmonella* Typhimurium, however, are most frequently identified during Australian outbreaks of egg-related cases of salmonellosis [1].

Over the past several years, the incidence of human cases of salmonellosis in Australia has been increasing. In 2011, the total number of food related disease outbreaks had increased to over 150 and 38.4% were attributed to *Salmonella* [1]. Over the same period, the number of cases linked directly with eggs increased from 20.8 to 44.8% [1]. Despite improvements of on-farm control strategies, *Salmonella* Typhimurium remains a significant problem within the Australian layer industry [3].

Due to the public health importance of contaminated eggs, understanding the dynamics of *Salmonella* Typhimurium shedding patterns and associated host responses to infection is of critical importance. Previous experimental infection trials have examined

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egg contamination and internal organ colonization of layer hens. These studies, however, have infected birds at different ages, using a variety of inoculation methods [4–7] limiting the degree to which the data can be directly compared. Moreover, the data obtained from these investigations was collected for 3–4 weeks pi. The productive lifetime of a layer hen, however, can extend beyond 50 weeks of age and few studies have investigated extended bacterial shedding dynamics, egg contamination and host responses to infection.During productive lifespan, layer hens may also experience many physiological and environmental stressors, such as overcrowding, extreme temperature variation and the onset of lay that may lead to increased fecal shedding of *Salmonella* [8–11]. Stress has also been linked with impaired immunity [8, 9, 12, 13] which may increase intestinal colonization by enteric pathogens such as *Salmonella* [14]. The host immune response to *Salmonella* infection may also contribute to increased corticosterone levels however, relationship between persistent *Salmonella* colonisation and stress in birds is unclear.

In the Australian egg industry, *Salmonella* Typhimurium is frequently isolated from eggshell surfaces but it is not the only serovar isolated from egg farms [15, 16]. The poultry farm environment is often contaminated with multiple serovars [15–17]. Field epidemiological investigations suggested that *Salmonella* Mbandaka was commonly isolated along with *Salmonella* Typhimurium in layer flocks without any clinical signs in chickens [16, 18]. *Salmonella* Mbandaka has not been associated with any egg related outbreaks in Australia [19], although this serovar has been associated with egg product related *Salmonella* outbreaks in the US [20].

Competition between co-infecting strains may affect the dynamics of one or more serovars. Layer hens environmentally infected with *Salmonella* Kentucky, for example, mitigated *Salmonella* Enteritidis colonisation of internal organs [21]. In addition, coinfection of layer hens with *Salmonella* Enteritidis, *Salmonella* Gallinarum and *Salmonella* Isangi has recently been shown to enhance disease in infected birds [22]. To date, there have been limited studies investigating how co-infection affects the dynamics of *Salmonella* Typhimurium shedding as well as host responses to infection.

Our hypothesis was that *Salmonella* Mbandaka can affect the shedding of *Salmonella* Typhimurium and internal organ colonization. We have conducted a 16 week infection trial, using layer hens reared free from exogenous *Salmonella.* Results from a companion study demonstrated that over the 16 week infection period, bacterial shedding was variable and that vertical transmission of *Salmonella* Typhimurium DT9 into egg internal did not occur [23]. The aims of the present study were to correlate fecal shedding and egg contamination patterns with host responses to infection (single and mixed) including fecal corticosterone levels as a marker of the host stress response as well as levels of *Salmonella* Typhimurium specific antibodies in the serum and yolk. A final aim of this study was to characterise persistence of *Salmonella* infection in peripheral organs.

Materials and methods

Birds

Fertile eggs were obtained from a commercial brown layer flock hatchery. Eggs were fumigated using formaldehyde and incubated for 21 days. A total of 32 pullets were hatched and raised in floor pens in positive pressure rooms within an animal housing facility located on the Roseworthy Campus of the University of Adelaide. The rooms within this facility, all animal cages, trays, and feeders had previously been cleaned and decontaminated using FoamCleanS and SaniGuard (Chemtall, Australia). At 10 weeks of age, birds were divided into three treatment groups: control ($n = 4$), *Salmonella* Typhimurium ($n = 14$) and *Salmonella* Typhimurium + *Salmonella* Mbandaka ($n = 14$) and housed individually in cages in separate rooms. Fumigated feed and sanitised water (Aquatabs, Ireland) were provided ad libitum to all birds. Feed, water and fecal samples were screened for *Salmonella* fortnightly by culture method as described previously [16]. This experiment was performed according to the Australian Code for the Care and Use of Animals for Scientific Purposes and was approved by the University of Adelaide Animal Ethics Committee (approval number: S-2014-008).

Bacterial isolates

Single isolates of *Salmonella* Typhimurium definitive type 9 (DT9) and *Salmonella* Mbandaka were used in this study. These *Salmonella* had been previously isolated from samples collected from layer hen farms during a previous epidemiology study [16] and serotyped at the *Salmonella* Reference Laboratory, Institute of Veterinary Medical Science (IMVS), Adelaide, South Australia.

Challenge experiment

At 14 weeks of age, just prior to lay, hens were orally inoculated with 1×10^9 colony forming units (CFU) of either *Salmonella* Typhimurium DT9 or a combination containing equal amounts of both *Salmonella* Typhimurium DT9 and *Salmonella* Mbandaka (5.0×10^8 CFU of each serovar) suspended in Luria–Bertani (LB) broth (Oxoid, Australia). Serial tenfold dilutions of the inoculum were prepared and plated onto nutrient agar to confirm the total number of bacteria. Control birds received a sham inoculum containing only sterile LB broth. Clinical signs

of infection were recorded throughout the experiment. At 30 weeks of age, [16 weeks post-infection (pi)] all birds were euthanized with Lethabarb (Virbac, Australia). Bone marrow, spleen, liver and caecal tonsils were collected from each bird for bacteriological examination.

Enumeration of *Salmonella* in fecal samples

A total of 320 fecal samples from individual hens were collected aseptically using sterile plastic bags on day 1 post-infection (pi) followed by 1, 2, 4, 6, 8, 10, 12, 14 and 16 weeks pi. *Salmonella* enumeration using the three tube most probably number (MPN) method was performed on all faecal samples as described previously [24]. *Salmonella* suspected samples were streaked onto xylose lysine deoxycholate (XLD) agar plates (Oxoid, Australia) and *Salmonella* Brilliance agar plates (Oxoid, Australia) for confirmation of *Salmonella* spp.

Bacterial DNA extractions from fecal samples, egg shell wash and internal organs

DNA was extracted from fecal samples using the Isolate Fecal DNA Kit (Bioline, Australia) following manufacturer instruction. DNA extraction from eggshell washes (enriched in RVS broth) collected from both infection groups was performed using Chelex® (Bio-Rad, Sydney, NSW, Australia) [25] The Wizard genomic DNA purification kit (Promega, Australia) was used to extract DNA from the tissue samples as per manufacturer instructions.

Standard curve and qPCR for fecal samples for *Salmonella* Typhimurium and *Salmonella* Mbandaka

The PCR detection of *Salmonella* was performed using the Quantifast® SYBER® Green qPCR kit (Qiagen, Australia) in a total reaction volume of 10 µL containing 2 µL sample (5 ng/µL), 5 µL of 2× Quantifast SYBER Green Master Mix and 1 µM of reverse and forward primers. *Salmonella* Typhimurium serovar specific primers *TSR*3 were used to detect *Salmonella* Typhimurium DT9. Further, to differentiate *Salmonella* Mbandaka from *Salmonella* Typhimurium DT9 in the co-infection group, primers for class 1 integron were used to specifically detect *Salmonella* Mbandaka [26]. The qPCR conditions were 5 min of denaturation at 95 °C, followed by 40 cycles of denaturation at 95 °C for 10 s and 60 °C for 30 s each. Rotor-gene 1.7.75 (Corbett Research, Qiagen, Australia) software version was used for the data analysis. A standard curve was generated to establish the limit of detection and quantification of positive samples, by determining a serial tenfold dilution of spiked fecal samples with known concentrations of *Salmonella* Typhimurium or *Salmonella* Typhimurium + *Salmonella* Mbandaka.

Fecal corticosterone analysis

Fecal samples collected at day 1 (pi) followed by 1, 2, 4, 6, 8, 10, 12, 14 and 16 weeks pi were thawed, mixed, and dried at 103 °C overnight. After cooling to room temperature, samples were ground to a fine powder. Corticosterone metabolites were extracted using methods recommended by the DetectX Corticosterone EIA kit manufacturer (Arbor Assays, Ann Arbor, USA). The concentration of fecal corticosterone metabolites (FCM) was measured by DetectX Corticosterone EIA kit as per manufacturer instruction.

Survey of egg shell and egg internal contents for *Salmonella* contamination

Eggs laid daily during 6, 8, 10, 12 and 14 weeks pi were collected and processed for *Salmonella* detection from both the eggshell and internal contents (Total eggs: 892; Control = 118, *Salmonella* Typhimurium only = 365, *Salmonella* Typhimurium + *Salmonella* Mbandaka co-infection = 409) using previously described methods [16]. Eggshell wash enriched in Rappaport–*Vassiliadis* broth (RVS; Oxoid, Australia) was stored in 80% glycerol at −80 °C to differentiate between *Salmonella* Typhimurium DT9 and *Salmonella* Mbandaka by standard PCR.

PCR for egg shell wash and internal organ samples for *Salmonella* Typhimurium and *Salmonella* Mbandaka

Salmonella positive eggshell wash and internal organ samples from both infection groups were screened for the amplification of *invA* and *TSR*3 gene for detection of *Salmonella* Typhimurium by multiplex PCR [26]. *TSR*3 gene was not amplified in *Salmonella* Mbandaka isolates [26]. Samples from both groups were also tested for the presence of *Salmonella* Mbandaka.

Bacteriology of internal organs

Bone marrow, spleen, liver and caecal tonsils were collected at week 16 pi and processed for bacteriology. Briefly, 0.1–0.2 grams of tissue sample were homogenised and serial tenfold dilutions were prepared in phosphate buffer saline (PBS). One hundred micro litre of each dilution was spread onto XLD agar plates and incubated overnight at 37 °C. After 24 h, the bacterial colonies were enumerated and the number of *Salmonella* in tissues was expressed as mean $\log_{10}$ CFU/g of tissue.

Serum and egg yolk sample collection and serologic examination by ELISA

On day 0 and at 1, 2, 4, 6, 8, 10, 12, and 14 weeks pi, 2 mL blood samples were collected from each bird and placed into serum clot activator tubes (Vacuette® tube, Greiner Bio-One, Australia). A total of 145 (Control; n = 20,

Salmonella Typhimurium only; $n = 57$, Co-infection group; $n = 68$) egg samples collected at weeks 6, 8, 10, 12 and 14 pi were processed for the antibody extraction from the yolk samples. Egg yolk antibodies were extracted as described previously [27]. Dilutions of chloroform-extract egg yolk antibody were prepared from the pools of known positive and known negative eggs from control birds. Samples were tested in duplicate for the following dilutions; 1:10, 1:50, 1:100, 1:500 and 1:1000. From the curve produced, the linear part was expanded. Readings of known positive and negative samples individually at the selected dilution produced a cut-off value for the test. Threshold value were determined by plotting sensitivity and specificity against the cut off value using two graph receiver operating characteristics (TG-ROC) analysis as described [28]. A dilution factor of 1:100 was selected because it was on the linear part of the standard curve.

Antibody detection from both serum and egg yolk samples was tested using the Chicken *Salmonella* Typhimurium Antibody Kit LPS Group B (BioChek, Holland) and antibody titres were calculated according to manufacturer instruction.

Statistical analysis

The data for average $\log_{10}$ CFU/qPCR, corticosterone level, and serum and egg yolk was analyzed using a two way analysis of variance (ANOVA) followed by a Tukey's multiple comparison of the mean. Significance between bacterial titres in organs was tested using a Mann–Whitney test. The correlation between MPN/g fecal count and *Salmonella* positive eggshell wash, average $\log_{10}$ CFU/qPCR and corticosterone concentration was determined by Pearson correlation test (r^2 value). All data was analysed using either by GraphPad Prism version 6 software or IBM®SPSS Statistics® version 21. p values <0.05 were considered statistically significant. A D'Agostino-Pearson omnibus normality test was conducted for all data. Serum and egg yolk antibody titres were normally distributed. MPN data were not normally distributed. MPN data was analysed by a Kruskal–Wallis with a Dunn's comparison of the means.

Results

Shedding and viable bacterial counts of *Salmonella* in fecal samples

Bacterial shedding varied significantly over time ($p < 0.01$) in both experimental treatment groups (Figure 1). The greatest number of viable bacteria observed in birds infected with only *Salmonella* Typhimurium occurred during week 1 pi, with a mean MPN/g of 48.53 ± 16.55. Samples collected from the *Salmonella* Typhimurium infection group in week 10 exhibited the lowest mean MPN/g, 1.535 ± 1.05. For birds infected with both *Salmonella* Typhimurium and *Salmonella* Mbandaka, the greatest number of viable *Salmonella* was detected on day 1 pi with a mean MPN/g of 44.80 ± 18.30. The lowest mean MPN/g, 0.78 ± 0.27, was observed in the multi-serovar infection group at week 6 pi.

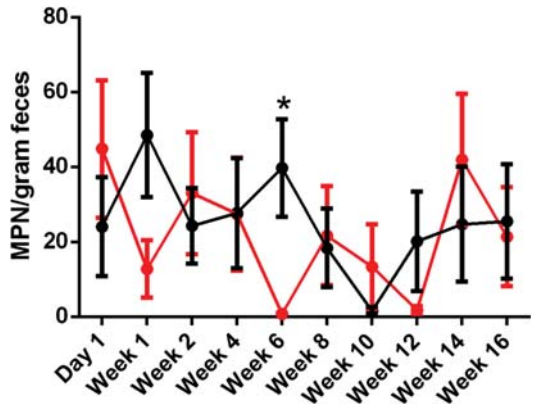

Figure 1 Enumeration of *Salmonella* in feces using most probable number (MPN) method. Fecal samples were collected from birds orally infected with 1×10^9 CFU of *Salmonella* Typhimurium (Black line) or a combination of both *Salmonella* Typhimurium + *Salmonella* Mbandaka (5×10^8 CFU of each serovar) (Red line) on day 1 and weeks 1, 2, 4, 6, 8, 10, 12, 14 and 16 pi. Data is presented as mean MPN/gram feces ± standard error of the mean. Bacterial shedding in both infection groups varied significantly over the course of the experiment ($p < 0.01$). At week 6 pi, bacterial shedding was significantly higher in birds infected with only *Salmonella* Typhimurium group (*$p < 0.05$).

Over the entire experiment, no significant effect of time or treatment was detected between single and multi-serovar treatment groups ($p > 0.05$). At week 6 pi, however, birds infected with only *Salmonella* Typhimurium exhibited a significantly greater mean MPN/g than birds infected with both *Salmonella* Typhimurium and *Salmonella* Mbandaka ($p < 0.05$). This difference correlated with the onset of lay. No *Salmonella* was detected in uninfected birds over the course of the experiment.

Quantification of *Salmonella* in fecal samples using a serovar specific qPCR

A quantitative PCR was developed to detect total *Salmonella* Typhimurium in single infection fecal samples and differentiate between *Salmonella* Typhimurium and *Salmonella* Mbandaka co-infection samples. A standard curve was generated by spiking uninfected, control feces spiked with known quantities of *Salmonella* Typhimurium. A cut-off Ct of 32 was used to exclude the detection of false positives and corresponded to 100 CFU of

Salmonella. For fecal samples spiked with both *Salmonella* Typhimurium and *Salmonella* Mbandaka, a cut-off Ct of 33 was used to exclude the detection of false positives. A Ct of 33 represented 1000 CFU of *Salmonella*. Data are presented as mean $\log_{10}$ CFU/gram feces ± standard error of the mean.

The number of *Salmonella* detected by qPCR varied significantly in both treatment groups over the course of the experiment (Figure 2). The greatest amount of *Salmonella* detected in all groups was observed at week 1 pi (Figure 2) with *Salmonella* Mbandaka in the co-infection group exhibiting the highest mean $\log_{10}$ CFU/gram feces (8.13 ± 0.65). Interestingly, *Salmonella* Mbandaka had the highest mean $\log_{10}$ CFU/gram feces between weeks 1 through 14 pi, though this difference was not significant than *Salmonella* Typhimurium. After week 1, *Salmonella* detection was relatively stable and consistent and did not vary significantly. No significant correlation was observed between MPN counts and qPCR results.

Fecal corticosterone metabolites in dried fecal extracts

Measuring fecal corticosterone metabolites (FCM) is a non-invasive method enabling the measurement of one stress parameter [29, 30]. It has been previously shown that during point of lay, birds experience increased physiological stress and are thought to be immunocompromised [31]. Infection, however, has also been shown to affect plasma corticosterone levels [12]. Therefore, it was hypothesized that corticosterone should increase in all chickens around the onset of lay, and infection may lead to further increase in level of corticosterone.

Fecal samples collected for enumeration of bacteria were also processed for FCM. A significant effect of time ($p < 0.05$) and treatment ($p < 0.001$) were observed between FCM concentrations (Figure 3). At day 1 pi, the FCM in birds infected with *Salmonella* Typhimurium (674.2 ± 109.3 pg/mg) was significantly higher than the FCM observed for control birds (238.0 ± 12.62 pg/mg) or birds infected with a mixed inoculum of both *Salmonella* Typhimurium and *Salmonella* Mbandaka (175.4 ± 8.58 pg/mg) ($p < 0.001$).

At week 6 pi, the mean FCM (625.2 ± 113.2 pg/mg) increased in birds co-infected with both *Salmonella* Typhimurium and *Salmonella* Mbandaka. At this time point, no significant difference between the two infection groups was detected. The mean FCM in control birds (268.7 ± 24.19 pg/mg), however, was significantly less than both treatment groups ($p < 0.01$). At weeks 8, 12, 14 and 16 pi, the mean FCM obtained for all groups varied but did not differ significantly (Figure 3).

No significant correlation was detected between the mean FCM concentration and MPN counts in singly or co-infected birds ($r^2 = -0.036$, $p = 0.699$). A significant but weak positive correlation ($r^2 = 0.26$, $p = 0.02$) was observed between the mean log copy number/gram and FCM concentration in birds infected with *Salmonella* Typhimurium only.

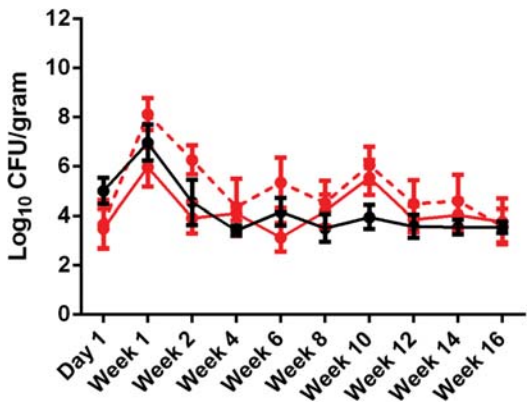

Figure 2 Quantification and differentiation of *Salmonella* by qPCR. Bacterial loads of fecal samples collected from birds infected with *Salmonella* Typhimurium only (black line) or co-infected with *Salmonella* Typhimurium (red line) and *Salmonella* Mbandaka (red hashed line) were quantified using a serovar specific qPCR. *Salmonella* Typhimurium was detected using a primers designed to the *TSR3* gene while *Salmonella* Mbandaka was detected using the *dhrfV* gene. Data are presented as mean $\log_{10}$ CFU/gram feces ± SEM. The amount of bacteria varied significantly over time ($p < 0.01$).

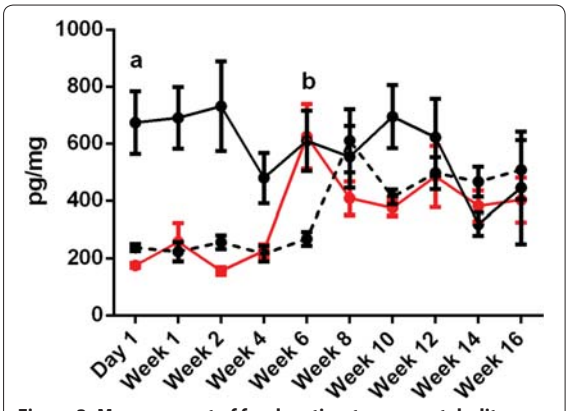

Figure 3 Measurement of fecal corticosterone metabolites (FCM). FCM concentrations were measured at day 1 and weeks 1, 2, 4, 6, 8, 10, 12, 14, and 16 pi. Data is presented as mean pg/mg feces ± SEM. A significant effect of time ($p < 0.05$) and treatment ($p < 0.001$) was detected for FCM concentrations. At day 1 pi, the mean FCM in birds infected with *Salmonella* Typhimurium (black line) was significantly higher than either control (black hashed line) or co-infected birds (red line) (a; $p < 0.001$). At week 6 pi, the mean FCM in co-infected birds increased. At this time point, both infection groups were exhibited significantly higher FCM concentrations than control birds (b; $p < 0.01$).

Detection of *Salmonella* from eggshell wash and internal contents

Eggs were collected at weeks 6, 8, 10, 12, and 14 pi and tested for the presence of *Salmonella* on the shell surface and within the internal contents. *Salmonella* was isolated throughout the experiment from the eggshell wash of experimentally infected hens. In birds infected with only *Salmonella* Typhimurium, the percentage of eggshell contamination ranged from 9.52 to 21.74%. Birds infected with both *Salmonella* Typhimurium and *Salmonella* Mbandaka exhibited a similar level of eggshell contamination, 10.89–33.33% (Table 1). By culture methods, the percentage of eggshell contamination was highest in both the groups at week 6 pi (onset of lay). No significant difference in eggshell contamination frequency was detected between *Salmonella* infection treatment groups. PCR results of egg shell samples indicated that the recovery rate of *Salmonella* Typhimurium (11.74%) was higher than *Salmonella* Mbandaka (6.60%) in co-infection group (Table 1).

No linear correlation was observed between the *Salmonella* MPN count in feces and eggshell contamination of infected birds ($r^2 = 0.001$, $p = 0.99$). *Salmonella* was not detected in egg internal contents of either infection treatment group at any point during this experiment. Eggshells and internal contents from control hens were also negative for *Salmonella*.

Salmonella Typhimurium antibody titres in serum and egg yolk samples

The titres of *Salmonella* Typhimurium specific serum and yolk antibodies were measured over the course of the experiment (Figures 4A and B). The lowest mean antibody titre (antilog) in birds infected with only *Salmonella* Typhimurium was observed at week 1 pi (1286 ± 168.1) and peaked at week 6 pi (2678 ± 179.5). After week 6 pi, antibody titres remained constant during the remainder of the experiment. A similar pattern was observed for *Salmonella* Typhimurium antibodies measured from the co-infection group. In birds infected with both *Salmonella* Typhimurium and *Salmonella* Mbandaka, the mean titre was lowest at week 1 pi (997.7 ± 170.5) and highest at week 6 pi (1949 ± 239.1). Mean antibody titres of birds infected with *Salmonella* Typhimurium only were significantly higher than those obtained for the co-infection group at weeks 6, 8, 10, 12, and 14 pi ($p < 0.01$). Control birds were negative for *Salmonella* Typhimurium antibodies over the course of the experiment.

Eggs collected from both infection groups tested positive for *Salmonella* yolk antibodies (Figure 4B). A significant effect of treatment was detected between the experimental groups ($p \leq 0.01$).

Persistence of *Salmonella* in internal organs

At 30 weeks of age (week 16 pi), the experiment was terminated and birds were euthanized. Spleen, liver, bone marrow and caecal tonsils from all hens were collected and processed for *Salmonella* to characterise the persistence of the bacteria in these organs. All samples collected from control hens were negative for *Salmonella*. Bacteria were detected in all tissues except for the bone marrow samples. The total number of positive samples was greatest in the spleen, followed by the liver and caecal tonsils (Figure 5). The mean splenic bacterial load observed in birds infected with only *Salmonella* Typhimurium (757.4 ± 301.1 CFU/g tissue) was significantly greater than the mean titre observed for birds inoculated with both *Salmonella* Typhimurium and *Salmonella* Mbandaka (236.0 ± 54.51 CFU/g tissue) ($p < 0.01$).

Birds infected with both *Salmonella* Typhimurium and *Salmonella* Mbandaka exhibited the highest number of individuals positive for *Salmonella* in the liver

Table 1 Percentage of isolation of *Salmonella* and *Salmonella* Typhimurium by culture and PCR method respectively from eggshell samples of orally infected birds at different weeks of pi

Weeks pi	*Salmonella* Typhimurium only group		Co-infection group		
	Salmonella detection by culture method	*Salmonella* Typhimurium detection by PCR	*Salmonella* detection by culture method	*Salmonella* Typhimurium detection by PCR	*Salmonella* Mbandaka detection by PCR
Week 6	21.74[c] (5/23)[a]	17.39 (4/23)	33.33 (8/24)	8.33 (2/24)[b]	8.33 (2/24)[b]
Week 8	9.52 (8/84)	8.33 (7/84)	10.89 (11/101)	8.91 (9/101)	0.00 (0/101)
Week 10	15.85 (13/82)	15.85 (13/82)	15.22 (14/92)	10.87 (10/92)	5.43 (5/92)
Week 12	13.48 (12/89)	12.36 (11/89)	22.92 (22/96)	14.58 (14/96)	13.54 (13/96)
Week 14	11.49 (10/87)	10.34 (9/87)	21.88 (21/96)	13.54 (13/96)	7.29 (7/96)
Total	13.15 (48/365)	12.05 (44/365)	13.20 (54/409)	11.74 (48/409)	6.60 (27/409)

[a] Number of positive eggs/total number of eggs tested.

[b] Results confirmed by PCR.

[c] Values in %.

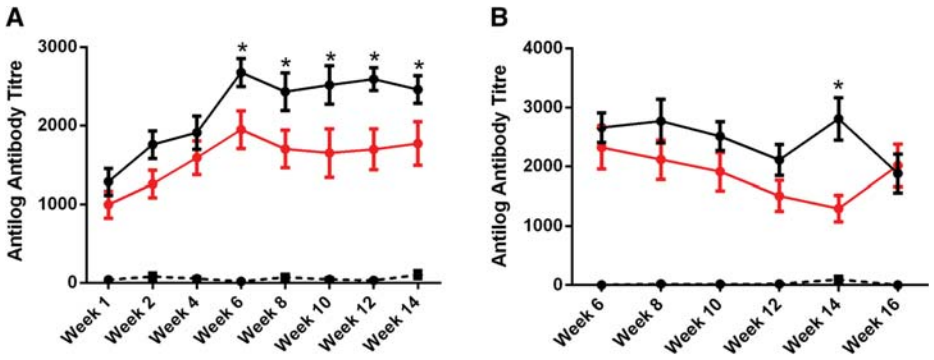

Figure 4 Quantification of *Salmonella* Typhimurium specific antibodies in serum and yolk. *Salmonella* Typhimurium specific antibody titers (antilog antibody titres ± SEM) were characterised over the course of the experiment in both serum (**A**) and yolk (**B**) in control birds (black hashed line) as well as hens infected with only *Salmonella* Typhimurium (black line) or a combination of both *Salmonella* Typhimurium and *Salmonella* Mbandaka (red line). In the serum, the amount of *Salmonella* Typhimurium antibody was significantly higher in singly infected birds compare with co-infected birds from week 6 pi till the end of the experiment (*$p < 0.01$). Mean antibody titres detected in yolk samples collected from *Salmonella* Typhimurium infected birds were only significantly different from co-infected birds at week 14 pi (*$p < 0.01$).

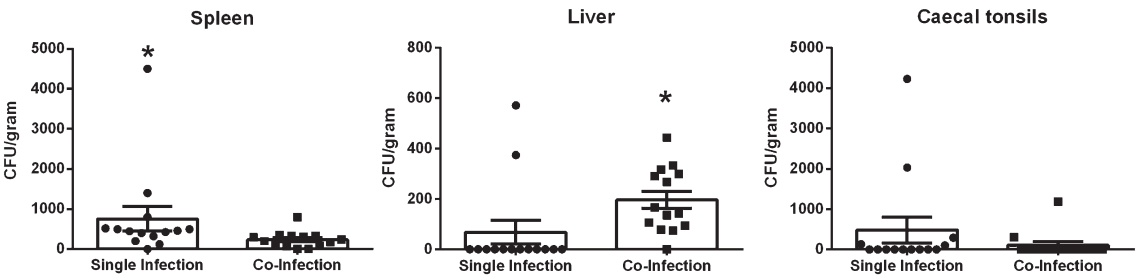

Figure 5 Bacterial persistence in spleen, liver and caecal tonsils. The total amount of viable bacteria was quantified from internal organs at week 16 pi. Data is presented as mean CFU/gram tissue ± standard error of the mean. Individual data points have also been included to highlight the variation within each group. In splenic samples, bacterial loads in birds infected with only *Salmonella* Typhimurium were significantly higher than the titre observed in the co-infection group (*$p < 0.01$). Only 2/14 liver samples from the *Salmonella* Typhimurium treatment group were positive for bacteria, while 13/14 positive samples were detected for co-infected birds. The mean for the liver was significantly higher in the co-infection group (*$p < 0.01$). No significant difference was detected in bacterial loads from the caecal tonsils.

(92.9%) (Figure 5). The mean bacterial titre for the co-infection group was 197.1 ± 34.17 CFU/g tissue and was significantly higher than the mean titre observed for birds infected with *Salmonella* Typhimurium, 68.46 ± 46.97 CFU/g tissue (Mann–Whitney, $p < 0.01$). The lowest overall level of *Salmonella* colonisation was observed in the caecal tonsils with 35.7% positive individuals in the single infection group and 14.3% positive birds in the multi-serovar group (Figure 5). Bacterial titres in caecal tonsils collected from birds infected with only *Salmonella* Typhimurium ranged from 0 to 4.2×10^3 CFU/g tissue with a mean titre of 485.7 ± 321.9 CFU/g tissue. Birds infected with both *Salmonella* Typhimurium and *Salmonella* Mbandaka ranged from 0 to 1.2×10^3 CFU/g tissue with a mean of 107.1 ± 85.79 CFU/g tissue. No significant difference was detected between the two infection treatment groups.

The culture positive internal organs were further tested by PCR to differentiate *Salmonella* Typhimurium and *Salmonella* Mbandaka in the co-infection group (Table 2). In splenic samples, 1 of 14 was positive for *Salmonella* Typhimurium while 4/14 samples were positive for *Salmonella* Mbandaka. Three of 14 liver samples from the co-infection group were positive for *Salmonella* Mbandaka, however, no *Salmonella* Typhimurium was detected. In the caecal tonsils, 1/14 samples were positive for *Salmonella* Typhimurium and 1/14 tested positive for *Salmonella* Mbandaka.

Table 2 Recovery and enumeration of *Salmonella* from internal organs

Organ	*Salmonella* Typhimurium only group			Co-infection group			
	***Salmonella* detection by culture method**	**Mean $\log_{10}$ CFU/g and SEM**	***Salmonella* Typhimurium detection by PCR**	***Salmonella* detection by culture method**	**Mean $\log_{10}$ CFU/g and SEM**	***Salmonella* Typhimurium detection by PCR**	***Salmonella* Mbandaka detection by PCR**
Spleen	13/14 (92.85%)	2.52 ± 0.22 ($n = 13$)	3/14 (21.43%)	12/14 (85.71%)	2.01 ± 0.24 ($n = 12$)	1/14 (7.14%)	4/14 (28.57%)
Liver	2/14 (14.29%)	0.38 ± 0.26 ($n = 2$)	2/14 (14.29%)	13/14 (92.85%)	2.10 ± 0.17 ($n = 13$)	0/14 (0.00%)	3/14 (21.43%)
Caecal tonsils	5/14 (35.71%)	0.97 ± 0.38 ($n = 5$)	2/14 (14.29%)	2/14 (14.29%)	0.40 ± 0.27 ($n = 2$)	1/14 (7.14%)	1/14 (7.14%)

Discussion

This study indicated that layers infected with *Salmonella* Typhimurium DT9 became persistently infected causing intermittent bacterial shedding in faeces. At week 6 pi, the MPN count in *Salmonella* Typhimurium infected group was significantly higher than multi-serovar infection group. Week 6 corresponded to the onset of lay in experimental birds and it was postulated that this increase could be related to physiological stress induced by onset of lay [16].

FCM levels in the *Salmonella* Typhimurium group were higher than either the control or multi-serovar treatment groups from day 1 until week 6 pi. Bacterial lipopolysaccharide (LPS) can induce inflammation within a host and has been associated with increased serum and corticosterone [12]. The LPS of *Salmonella enterica* is variable, serovar dependent and contributes to different degrees of virulence. This may account for lower mean FCM concentrations in the multi-serovar group. Increase in FCM in all treatment groups between 6-8 weeks could be attributed to the onset of lay, and infection may lead to further increase in level of FCM. However, it important to note that no positive correlation was observed between bacterial shedding and FCM levels in this study.

During this study, higher rates of eggshell contamination at the onset of lay could be attributed to increased *Salmonella* shedding in feces at that point [5, 16]. There was no linear correlation between *Salmonella* shedding in feces and egg shell contamination of infected birds and this is in agreement with earlier reports [32]. Of note, in this study *Salmonella* was not detected from egg internal contents.

The *Salmonella* Typhimurium IgG antibody titres increased after week 1 pi and peaked at week 6 pi. Birds were seropositive till the end of the trial at week 14 pi but the immune response did not result in complete clearance of *Salmonella spp*. It is also important to note that the antibody response contributes to the clearance of extracellular bacteria, intracellular bacteria can persist in the host thus cell mediated immune response is essential for clearance of *Salmonella* Typhimurium (reviewed in [33]). Overall decreased IgG antibody response in multi-serovar infection group could perhaps be due to the competitive and immunoprotective mechanism between both *Salmonella* strains. However, the absence of an infection treatment with only *Salmonella* Mbandaka in this study limits this conclusion.

In the multi-serovar group, a low MPN was obtained at week 6 yet qPCR results revealed similar loads of both *Salmonella* Typhimurium and *Salmonella* Mbandaka. The discrepancy may be due to the detection of both live and dead bacteria using PCR method. However it is unclear why that has happened specifically at week 6 pi. Laying hens mounted immune response to invasive strain of *Salmonella* Typhimurium DT9 without inducing clinical signs. Variation in qPCR and MPN results could be attributed to the sensitivity of the tests used. Several factors such as heterogeneous distribution of the pathogen in sample, number of stressed cells, sample matrix, enrichment time and enrichment media can influence the accuracy of quantitation [34].

In *Salmonella* Typhimurium infected group there were increased levels of FCM concentrations, antibody titres and bacterial shedding (as detected by MPN method) at week 6 pi (onset of lay) which supports the theory that the presence of stress hormones can stimulate *Salmonella* growth and enhance bacterial colonisation in the intestine [35]. However present data suggests that this theory may not apply when host is infected with multiple *Salmonella* serovars. Concentration of corticosterone levels in sera can increase or decrease the antibody response [36]. In this study the high corticosterone levels did not suppress the humoral immune response against *Salmonella* Typhimurium.

Stress can stimulate the recrudescence of bacteria from internal organs resulting in high bacterial load in feces [37]. Our findings indicated that *Salmonella* Typhimurium persisted in internal organs despite high levels of circulating specific IgG antibody. Previous studies reported *Salmonella* Typhimurium clearance from liver and other internal organs due to Th-1 dominated responses and high levels of interferon-γ expression at around 14–28 days pi [38]. Some studies reported low frequency of *Salmonella* Enteritidis in liver and other internal organs for up to 22 weeks pi [39]. It has also been suggested that age at exposure did not affect recovery of *Salmonella* Typhimurium from liver [40]. Our observations could not be compared with previous reports because such studies were performed using broilers for short period of time. It could be hypothesised that persistence of *Salmonella* Typhimurium in internal organs including liver could be due to the timing of challenge (prior to lay in this case). Sexual maturity can induce immunosuppression by altering cellular and humoral immune response [33]. This could ultimately cause bacteria to avoid clearance and dominate host leading to a recrudescence of infection. However further studies are required to confirm this hypothesis. It is interesting to note that in mixed infection group, only *Salmonella* Mbandaka was detected from liver.

Previous literature stated that intestinal persistence of *Salmonella* Typhimurium in chickens was longer when birds were challenged at day old compared to day 7 and that older birds are considerably more resistant to salmonellae than are young chicks [41, 42]. Our study indicated that infection of adult birds (14 weeks old in this study)

can also result in continued harbouring of the *Salmonella* Typhimurium and intermittent faecal shedding. This shedding can be associated with the stress event such as onset of lay. However interplay between stress, immune response and *Salmonella* Typhimurium shedding in single or mixed infection group at the onset of lay is more complex to understand.

To conclude, *Salmonella* Typhimurium DT9 persistently infected hens causing intermittent bacterial shedding in faeces. At the onset of lay shedding of *Salmonella* Typhimurium was affected in mixed infection group. Increased immune response did not result in clearance of *Salmonella spp* (except for *Salmonella* Typhimurium at week 6 pi). There was no correlation between FCM and *Salmonella* shedding. This long term *Salmonella* Typhimurium infection model provided useful insights on the continued persistence and or recrudescence of *Salmonella* Typhimurium, although further investigation is necessary to understand the immunobiology of long term and systemic *Salmonella* Typhimurium infection.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
PS, VP, AM and KC designed the experiments and were involved with preparation of this manuscript. PS, VP, AM and KC conducted the layer hen trial. PS performed all sampling and microbiological processing. TM performed corticosterone extractions from fecal samples. PS and TM conducted FCM experiments. TM and KC performed qPCR. All authors read and approved the final manuscript.

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Chapter 3 Shedding of a live attenuated *Salmonella* Typhimurium vaccine Vaxsafe® ST (strain STM-1) during pullet rearing - a field study

Statement of Authorship

Title of Paper	Shedding of a live attenuated *Salmonella* Typhimurium vaccine Vaxsafe® ST (strain STM-1) during pullet rearing - a field study.
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Principal Author

Name of Principal Author	Pardeep Sharma
Contribution to the Paper	Performed the experiment. Sampling, processing, data compilation, analysis and interpretation of data. Wrote manuscript, responded to comments and suggestions by co-authors.
Overall percentage (%)	70%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 31.05.2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dr Kapil K. Chousalkar
Contribution to the Paper	Supervised the development of experiment. Liaised with farm owners for obtaining required samples, assisted in sample collection and performing experiment, edited the manuscript. Acted as a corresponding author.
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Name of Co-Author	Dr Andrea R. McWhorter
Contribution to the Paper	Designed primers for ST1 detection
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Name of Co-Author	Dr Karen Holden
Contribution to the Paper	Provided input in isolation of ST1, primer design and writing manuscript
Signature	Date: 30/05/2017

Name of Co-Author	Dr Gregory Underwood
Contribution to the Paper	Provided input in obtaining funding for this work. Facilitated discussions between industry and research personel. Prvided critical comments for manuscript.
Signature	Date: 30/5/2017

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Contribution to the Paper	Assisted in PCR analysis.
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Name of Principal Author	Pardeep Sharma
Contribution to the Paper	Performed the experiment. Sampling, processing, data compilation, analysis and interpretation of data. Wrote manuscript, responded to comments and suggestions by co-authors.
Overall percentage (%)	70%
	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dr Margaret Sexton		
Contribution to the Paper	Liaised with farm owners for obtaining required samples, assisted in sample collection.		
Signature		Date	1/6/2017

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

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Abstract

Vaxsafe® ST (STM-1, Bioproperties Pty Ltd, Australia) *Salmonella* Typhimurium vaccine is the only live attenuated *Salmonella* vaccine registered for use in poultry in Australia. The use of Vaxsafe® ST in commercial layer pullet flocks has increased significantly in Australia over the past few years. Little is known, however, about the colonisation and shedding characteristics of the vaccine strain under commercial conditions. The present study was designed to monitor the levels of STM-1 in the environment after a routine vaccination programme was applied to a commercial pullet flock. The multi-dose vaccination regimen included a total of 3 separate applications of Vaxsafe® ST including coarse spray in the hatchery, drinking water at six weeks (wks), and intramuscular (IM) injection at 12 wks. Serological results indicated that birds in both the vaccinated and unvaccinated groups had titres below the cut-off value for the assay prior to administration of the IM dose. A significant increase in serum antibody levels was observed following IM inoculation at 12 wks. STM-1 was detected in environmental samples using both culture and PCR methods at each of the sample intervals up to 13 wks of age, with comparable sensitivity between culture and PCR. A qPCR assay was developed to detect STM-1 from environmental samples. This qPCR was specific for STM-1 and did not detect wild-type *Salmonella*. The STM-1 specific qPCR had a detection limit of 10^3 bacterial cells/g of faeces, compared to 10^1 cells/g for the conventional PCR. The qPCR data suggested that environmental levels of STM-1 were highest at 13 wks of age, 7 wks after the last oral vaccination and 1 wk after the IM vaccination. These findings suggest that STM-1 can be recovered from the environment in vaccinated flocks up to 13 wks of age, and suggests that Vaxsafe® ST, when delivered as a multi-dose regimen to a commercial pullet flock, leads to intestinal colonisation and shedding of STM -1 in faeces and thus the environment.

Keywords: *Salomonella* Typhimurium, Pullets, Salmonellosis, Vaccination, ELISA, PCR.

1. Introduction

Salmonella enterica is a rod-shaped Gram-negative member of the family *Enterobacteriaceae* and causes foodborne disease worldwide, with an estimated 153 million cases per year (Kirk *et al.*, 2015). Although *S.* Enteritidis is the leading serovar responsible for outbreaks in Europe and the United States (Elaine *et al.*, 2011; O'Brien, 2013; Wales & Davies, 2011), in Australia, *S.* Typhimurium is the predominant serovar responsible for outbreaks associated with consumption of eggs and egg-related products (Moffatt & Musto, 2013; The OzFoodNet Working group, 2015). Despite a number of control strategies in the form of pre-harvest techniques (such as flock and feed management, biosecurity and use of prebiotics) and post-harvest techniques (such as egg storage, egg washing and egg decontamination) focussed on reducing the risk of human salmonellosis (Galis *et al.*, 2013; Gast, 2007) there has been no clear-cut decline in the number of reported cases of salmonellosis (Moffatt *et al.*, 2016).

Reduction in faecal shedding, colonisation and generation of IgG immune response against *S.* Typhimurium has been observed after oral administration of Vaxsafe STM-1 strain in day old chicks followed by an oral booster at two weeks of age (Alderton *et al.*, 1991). Vaxsafe STM-1 is an *aroA* deletion mutant vaccine and was generated by transferring an Aro transposon insertion Aro A -554: Tn*10*, to a wild-type *S.* Typhimurium (Alderton *et al.*, 1991). For a *Salmonella* vaccine to be effective, it must retain its colonising and invasion capacity to ensure that selected antigen remains accessible to the host defence mechanism for an extended period of time. Experimental laboratory studies have already been conducted with the STM-1 vaccine strain to study protection against *S.* Typhimurium infection in meat chickens (Alderton *et al.*, 1991) and in layers (Groves *et al.*, 2016). These prior studies were mainly focused on examining the efficacy of the vaccine against colonisation, invasion and shedding of wild-type *S.* Typhimurium under experimental conditions. Little is known about the colonisation and shedding of the STM-1 vaccine in faeces and environment under natural

field conditions during rearing where *Salmonella* challenge is relatively low. To fulfil these gaps, this study was conducted with the following objectives (i) To understand the level of the vaccine colonisation, shedding and humoral immune response generated after administration in pullets, (ii) To develop vaccine specific PCR in order to detect and differentiate vaccine from wild type *Salmonella* serovars.

2. Materials and methods

2.1. Animal ethics

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

2.2. *Salmonella* vaccines

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

2.3. Selection of pullet rearing farm

A commercial pullet rearing farm was selected for this study based on the willingness of the pullet grower to participate. The farm had a history of *Salmonella* Livingstone infection. The rearing facility had three sheds (A, B and C). Shed C had the capacity to house 15,000 birds and sheds A and B could accommodate 5,000 birds each. Dust (n=8) and litter samples from one square meter area (n=8) were collected from all three sheds where the previous batch was reared. This sampling was performed to detect any possible wild type *Salmonella* spp. on the rearing farm. The sheds were cleaned, sanitised and left empty (resting period) for four weeks prior to placement of day-old chicks supplied directly from the hatchery. Surface swabs (n=8) and clean wood shavings (n=8) from one square meter area were again collected from each shed after the clean-up and two days prior to the arrival of chicks. Litter samples

were collected from front, middle and rear end of the shed. Dust swabs were collected from extraction fans and sidewalls. Sample numbers (dust and litter) were determined based on previous findings (Mahe *et al.*, 2008) which suggested that 2 dusts and 4 faecal samples (total of 6 samples from overall shed) were sufficient to ensure detection of *Salmonella* spp in alternate systems. Previous study also suggested that more samples (such as dust) increased sensitivity of detection (Mahe, et al., 2008), hence 16 dust and faecal samples from shed A and B (vaccinated group) and 15 dust and faecal samples from shed C were collected from each shed in this study.

2.4. Experimental design

Meconium samples (pooled sample from 90 chicks) were collected from day old chicks (n=100) at the hatchery. Day old chicks were randomly divided into two groups. Group 1 (n = 10,000) received Vaxsafe® ST by coarse spray. Day old chicks in group 2 (n = 15,000) were left unvaccinated. Vaccinated chicks were dubbed at one day old for identification. The vaccinated and unvaccinated chicks were placed in different boxes and transported to the commercial pullet rearing farm. Vaccinated chicks (group 1) were placed in shed A and shed B while unvaccinated chicks were placed in shed C. At 6 wks birds in sheds A and B received Vaxsafe® ST vaccine in the drinking water, and at 12 wks, these flocks were inoculated with a third dose of Vaxsafe® ST by intramuscular injection. The vaccine was reconstituted using a commercial Marek's disease vaccine diluent under veterinary supervision and administered as a 0.5 mL dose as a separate injection at the same time as a commercial multi-valent Egg drop syndrome (EDS) / Newcastle disease (ND) killed vaccine (Nobilis® EDS+ND, MSD Animal Health). All birds were reared in a deep-litter, floor-based shed. The antibiotic-free feed was provided by a commercial feed mill and a standard lighting protocol provided by Layer Company was adopted during rearing. Birds did not receive organic acid or probiotics during this study.

2.5. Sample collection after placement of chicks

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

2.6. Isolation of *Salmonella* and Vaxsafe® ST vaccine strain from different samples

Litter, dust and shoe cover samples were processed for enumeration of *Salmonella* as previously described (Gole *et al.*, 2014). Briefly, for isolation of *Salmonella* spp. and the *Salmonella* Typhimurium vaccine strain (STM-1), 2 g of litter sample was inoculated into 10 mL of BPW (1:5). The inoculated samples were incubated for 24-48 hrs at 37 °C and 100 µL of this sample was transferred into 10 mL of Rappaport Vassiliadis Soya peptone broth (RVS, Oxoid, Australia), which was then incubated at 42 °C for 24 h. Ten microliter of the incubated RVS broth was streaked onto Brilliance *Salmonella* agar (BSA, Oxoid Australia) and xylose lysine deoxycholate agar (XLD, Oxoid, Australia) plates and the plates incubated at 37°C overnight for confirmation of isolation of *Salmonella* spp. and the vaccine strain. Purple colonies on BSA were presumed to be *Salmonella* spp. The vaccine strain did

not produce H_2S on XLD agar, allowing presumptive identification as STM-1. The STM-1 strain did not grow on BSA. Likely STM-1 colonies from XLD and *Salmonella* positive colonies from BSA were mixed with 0.5 ml of Brain Heart Infusion broth (BHI, Oxoid, Australia) and incubated at 37°C overnight. Next day 0.5 ml of 80% glycerol was added, mixed with the incubated cultures and were stored at -80°C for further analysis by conventional PCR. Dust samples were moistened (to avoid drying of swabs) with 20 mL BPW and processed for *Salmonella* isolation as described above, with 10 mL of the mixture stored at -20°C for further analysis. All BPW samples were tested by multiplex PCR using previously described methods (Gole *et al.,* 2014).

2.7. DNA extraction from culture (presumptive colonies) and litter

The Chelex® method was used to extract DNA from stored culture samples (Malorny *et al.*, 2009). The Isolate Faecal DNA kit (Bioline, Australia) was used to extract DNA from faecal and dust samples as per manufacturer instruction. The concentration of isolated DNA in a sample was determined using a spectrophotometer (Nano drop ND 1000, Biolab, Australia). For qPCR and conventional PCR, dilution was performed using nuclease free water to achieve a final DNA concentration of 5 ng/µL. Finally, these diluted DNA samples were used in real-time qPCR and conventional PCR.

2.8. Conventional and Quantitative PCR (qPCR)

Salmonella positive (both wild type and vaccine) samples were screened for the amplification of *invA* and *TSR*3 gene for detection of *Salmonella* Typhimurium by multiplex PCR as described previously (Gole *et al.*, 2014). For the detection of STM-1, primers (Forward 5'-GTTTTAAGTGTAATTCGGGG-3'; Reverse 5'-TATGATCAAATGGTTTCGCC-3' resulting amplicon of 164 bp) were designed within a 360 bp region of sequence centred on the transposon / *aroA* insertion point junction. In order to optimise the conventional PCR reaction for the detection of STM-1, a gradient PCR was

performed using MyTaq™ DNA Polymerase (Bioline, Australia) according to the manufacturer's directions, in a total reaction volume of 20 µL containing 2 µL of DNA template, 0.5 µM each forward and reverse primer, 1.5 mM $MgCl_2$, 2.5 µM of each dNTP and 2.5 U of *Taq* polymerase. The STM-1 vaccine specific gene amplifications involved an initial heating step of 94°C for 2 minutes followed by denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 68°C for 30 seconds for 30 cycles and a final extension at 72°C for 5 minutes in a T100 thermal cycler (Bio-Rad, Australia). Annealing temperatures of 60°C and higher yielded no additional bands in the gradient PCR, so this temperature was selected for conventional and qPCR. To assess assay specificity for quality control and detection of possible cross contamination, the newly designed STM-1 PCR primers were tested against 28 different *Salmonella* serovars isolated from layer farm environments (Table 1) and *Escherichia fergusonii*. Genome analysis of *Escherichia fergusonii* indicated that this strain has a transposon similar to Tn21 (Forgetta *et al.*, 2012). Tn*21* is carried by a plasmid and transposon Tn*10* is one of component of the same plasmid (Liebert *et al.*, 1999). DNA extracted from ten-fold serial dilutions of the cultured STM-1 vaccine in faeces was tested by conventional PCR to determine the detection limit of the conventional PCR.

2.9.Standard curve and qPCR for *Salmonella* Typhimurium vaccine in faecal samples

The qPCR was performed in a total reaction volume of 10 µL composed of the following: 2 µL sample (5 ng/µL), 5 µL of 2 x Quantifast SYBR Green Master Mix and 1 µM of reverse and forward primers. Quantifast® SYBR® Green qPCR kit (Qiagen, Australia) was used for the qPCR. Thermocycling was initiated by incubation at 95°C for 10 minutes, followed by 40 cycles of denaturing at 95°C for 15 seconds and annealing/elongation at 60°C for 60 seconds on a 7900HT sequence detection system (Applied Biosystems). Data were analysed assuming a threshold of 0.3 and baseline of 3 to 10. A standard curve was prepared for calculation of the limit of detection and quantification of bacterial cells using a 10-fold serial

dilution of cultured STM-1 vaccine. Briefly, 0.2 g samples of faeces were inoculated with a 10-fold dilution series of cultured STM-1. The Bioline kit was used for DNA extraction from these spiked faecal samples and qPCR was performed using diluted DNA samples (5 ng/µL). Template control and positive controls (DNA extracted from known *Salmonella* Typhimurium culture) were used in each PCR reaction cycle. The qPCR assay was able to detect ≥1,000 of bacterial cells of *Salmonella* (STM-1)/ g of faeces. Before qPCR assay, all samples were tested for presence of the *InvA* gene (Akiba *et al.*, 2011; Gole *et al.*, 2014). *InvA* positive samples were then subjected to qPCR assay.

2.10. Detection of anti-LPS antibodies by ELISA

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

2.11. Statistical analysis

Data were analysed using IBM®SPSS Statistics® version 24.0 and GraphPad Prism version 6. The prevalence of the Vaxsafe® ST vaccine strain in the litter, dust swabs and shoe cover samples from vaccinated and unvaccinated birds were compared using Fisher's exact test. The vaccine and bacterial loads, average Log_{10} CFU and serum antibody titres were analysed using a two-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison test of the mean. The significance of differences between antibody titres in the vaccinated and unvaccinated birds was assessed with Student's t-test. P values < 0.05 were considered significant.

3. Results

All three sheds (A, B and C) were *Salmonella* negative prior to the arrival of the chicks (including both the samples from the previous flock as well as from the shed two days before placement of the vaccinated flock). Meconium samples collected randomly from chicks

before administration of Vaxsafe® ST were also negative for *Salmonella*. There were no detectable clinical signs after administration of the vaccine by the intramuscular route. During rearing, six litter samples from the vaccinated group (2 at each time point from shed A) were PCR positive for wild type *Salmonella* spp. following enrichment in BPW. Multiplex PCR results from enriched BPW indicated that these wild type Salmonellae were predominantly *S*. Mbandaka. These samples were, however, culture negative. Birds from the unvaccinated group were negative for wild type *Salmonella* spp.

For the detection of STM-1 strain from field samples, the conventional PCR assay was more sensitive than culture or qPCR methods. During the first sampling at four wks of age, 25% of the floor litter samples was STM-1 positive by culture method followed by 12.25% at eight wks of age, and there was a further increase in STM-1 shedding to 18.75% at 13 wks of age. STM-1 vaccine testing by standard PCR revealed 43.75%, 50% and 87.50% positive results at four, eight and thirteen weeks of age, respectively. The overall agreement between culture and conventional PCR was 26.3 %, with a kappa coefficient 0.263, suggesting minor agreement (Table 2). All dust and shoe cover samples collected from both treatment groups were tested negative by culture and qPCR test. During the experiment, overall, six dust samples (2 at each time point from shed A) were PCR positive for STM-1 following enrichment in BPW. Two shoe covers (one each from shed A and B) were PCR positive.

The limit of detection of the conventional PCR for STM-1 detection was 10 CFU/g of faeces, and the limit of detection for the qPCR assay was 1000 CFU/g of faeces. During this study, the conventional PCR was more sensitive so to avoid false negative results, all litter samples were screened using the conventional PCR in the first instance, and positive samples were further tested by qPCR for quantification of STM-1 The primers designed for STM-1 did not amplify a product from thirty wild type *Salmonella* spp, however STM-1 primers designed during this study also detected 164 bp amplicon from *Escherichia fergusonii*.

The load of STM-1 (average log colony forming unit (CFU) / RT-PCR reaction) in litter samples across the three different sampling points were also calculated. At week 4, the average load of STM-1 in the litter samples was 4.4 log CFU ± 0.43, followed by 4.2 log CFU ± 0.25 at 8 weeks and significantly increased ($p = 0.025$) at 13 weeks of age (5.60 log CFU ± 0.26) after booster at 12 weeks. The qPCR data suggested that, compared to weeks 4 and 8, the shedding of STM-1 at week 13 was significantly higher ($P = 0.025$) (Figure 1).

The serological results indicated that the birds in both vaccinated and unvaccinated groups had titres below the cut-off value for the assay prior to administration of the intramuscular dose. After intramuscular injection, the titres in the vaccinated group were above the cut-off value and were significantly higher ($p = < 0.0001$) than those of the unvaccinated pullets (Figure 2).

4. Discussion

The primary aims of this study were to assess the bacterial shedding and host immune response following the administration of a live attenuated *S.* Typhimurium STM-1 vaccine during pullet rearing under field conditions and compare multiple STM-1 detection methods. In this study, the sensitivity of the conventional PCR assay was greater than that of the qPCR assay. This finding contradicts previous investigation suggesting that qPCR is more sensitive than conventional PCR for the detection of pathogens (Law *et al.*, 2015), however, a recent commentary suggested that qPCR is not necessarily more sensitive than conventional PCR (Bastien *et al.*, 2008). The difference in sensitivity between conventional PCR and qPCR could be attributed to the total reaction volume used in the assay because higher reaction volumes can dilute the inhibitory factors present in template DNA (Bastien *et al.*, 2008). The primers designed for STM-1 did not amplify a product from thirty wild type *Salmonella* spp but did cross amplify with *Escherichia fergusonii*. *Escherichia fergusonii* has a transposon similar to Tn*21* (Forgetta *et al.*, 2012). Tn*21* is carried by a plasmid and transposon Tn*10* is one of component of the same plasmid (Liebert *et al.*, 1999). The mutation in STM-1 used

in this study was induced by transferring an Aro transposon insertion Aro A -554: Tn*10*, which is the likely reason for cross amplification with *Escherichia fergusonii*. Hence, for PCR assay, it is essential to also test suspected positive STM-1 samples for the *Salmonella* specific *InvA* gene. Furthermore, there was only a minor level of agreement between culture and the conventional PCR assay for the detection of STM-1. The conventional PCR assay was more sensitive than culture for the detection of STM-1. However, it is important to note that PCR could detect the DNA of non-viable STM-1 in litter samples. The load of STM-1 DNA in the litter samples increased gradually and was significantly higher at week 13. This could be because of the gradual build-up of viable or non-viable bacteria (STM-1 in this case) in the litter samples. It could also be hypothesised that the stress induced by the handling of birds during intramuscular injection resulted in increased shedding of bacteria. However further controlled experiments are necessary to confirm this hypothesis. Other live *S.* Typhimurium vaccines have been recovered from vaccinated birds or their environment for up to 26 days (Tan *et al.*, 1997) and 35 days (Barrow *et al.*, 1990) post vaccination in meat chickens during a controlled experiment. In this study, viable STM-1 was detected at least four weeks after administration of the first dose of Vaxsafe® ST vaccine to commercial layer pullets being reared on the floor.

Intramuscular administration of Vaxsafe® ST vaccine is presently an off-label application method in Australia (Groves *et al.*, 2016). Bioproperties has, however, submitted an application to the Australian Pesticides and Veterinary Medicines Authority (APVMA) to extend the label claim to include administration by IM injection. In this study, intramuscular administration of Vaxsafe® ST did not result in any clinical signs of disease in pullets, while, a previous study reported adverse reaction to parenteral administration of this vaccine (Groves *et al.*, 2015).

In our study, there was no increase in antibody titres after spray vaccination when one day old or after oral immunisation at six weeks. The serum IgG concentrations against *S.*

Typhimurium specific antigens were significantly higher in the vaccinated group at 13 weeks of age, one week after IM injection at 12 weeks of age. Alderton *et al.,* 1991 reported an increased antibody response after oral administration of a pre-Masterseed strain of STM-1 used in pilot laboratory studies prior to registration; however, the dose rate administered was 5,000-fold higher (10^{10} CFU/mL) compared to this study (2 x 10^{7} CFU/mL).

In order for a *Salmonella* vaccine strain to be effective, it must retain its colonising and invasion capacity to ensure that selected antigens remain accessible to the host defence mechanism for prolong periods (Alderton *et al.*, 1991). The results of isolation by culture, conventional PCR and qPCR from the current study suggest that STM-1 successfully colonised the chicken gut but did not induce a systemic antibody response until after parenteral administration. It should be noted, however, that intestinal samples were not tested in this study. This suggests that Vaxsafe® ST has the potential as an antigen delivery system and vaccination with STM-1 could help in reduction of wild type ST infection.

The first line of defence against enteric pathogens in the gastrointestinal tract is the normal gut microflora. Nurmi *et al.* (1992) suggested that live vaccines increased the competitive advantage of the vaccine strain against the invading pathogens, whereas Berchieri and Barrow (1990) suggested that live vaccines provide protection by competitive exclusion effect and also helps in the development of classical immunity. Finding vaccines that work efficiently in inhibiting colonization of newly hatched chicks by pathogens is important as they are highly susceptible to colonisation by bacterial pathogens at this stage of their lives (Gast & Benson, 1996). During this study, six wild-type *Salmonella* isolates were obtained from litter and dust samples, most of which were *S.* Mbandaka. *S.* Mbandaka has frequently been isolated from layer farms during epidemiological investigations (Chousalkar *et al.*, 2016; Gole *et al.,* 2014). These data do not allow any conclusion about induction of cross-protection against heterologous *Salmonella* serovars by administration of STM-1.

Salmonella survives in dust, feed and other environmental samples (Gole *et al.,* 2014; Wales *et al.,* 2007) and its removal from the environment and flocks with conventional disinfection is difficult, so there is always a challenge to improve vaccines and the efficacy of vaccination programmes. This short-term study demonstrates that STM-1 was detectable in the litter and environmental samples by culture, conventional and qPCR assay.

In summary, oral administration of the *Salmonella* Typhimurium STM -1 strain present in the commercially available live vaccine (Vaxsafe® ST) colonises the chicken gut (pullets in this case) and is shed after vaccination in faeces into the litter and the environment. This information is of value for the industry and the vaccine manufacturer. Conventional PCR methods enabled the detection of more STM-1 positive samples than the culture method used in this study. The successful application of the molecular techniques would be beneficial to poultry industries as well as vaccine producers as this method can be used to identify the reliability and execution time make it possible for PCR to be used in conjunction with conventional microbiological methods to detect vaccine strain in the environment.

For this study, the recruitment of farms was based on the willingness of the farmers to participate. The shedding and persistence of *Salmonella* vaccine in the environment could be explained with the help of longitudinal studies and by recruiting several farms with different management practises. Recruitment of large number of pullet rearing farms would have been ideal. Cooperation from pullet growers over a period of several months and the demand on their resources are limiting factors to such studies. Further pen trials/controlled vaccine challenge experiments will be useful to determine the persistence of vaccine strain in internal organs and or lymphoid tissues.

Conflict of Interest

Dr. Karen Holden and Dr. Greg Underwood are employed by Bioproperties Pty Ltd.

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Table 1. *Salmonella* serovars and other samples tested against newly designed STM-1 primer set

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

*, Same phage type referred as 170/108

Table 2. Comparison of culture and conventional PCR method for detection of STM-1 from litter samples during rearing phase of vaccinated group

Sampling	**Week of age**	**Culture**	**CPCR**	**Agreement**	***S.* Mbandaka**
		% (no. positive/total no.)			
1	4	25 (4/16)	43.75 (7/16)	60%	2
2	8	12.25 (2/16)	50 (8/16)	25%	2
3	13	18.75 (3/16)	87.50 (14/16)	6.4%	2
Total		18.75 (9/48)	60.42 (29/48)	26.3%	6

CPCR: conventional PCR

Figure captions

Figure 1

The load of STM-1 (average $\log_{10}$ cfu/qPCR) in litter samples of vaccinated group over a period of 13 weeks during rearing. Error bars indicate standard errors. Black arrows indicate vaccination at 1 day, 6 and 12 weeks. Asterisks (*) indicate statistically significant differences ($p < 0.05$) in the load of STM-1 (average $\log_{10}$ cfu/qPCR) at 13 weeks in comparison with 4 and 8 weeks.

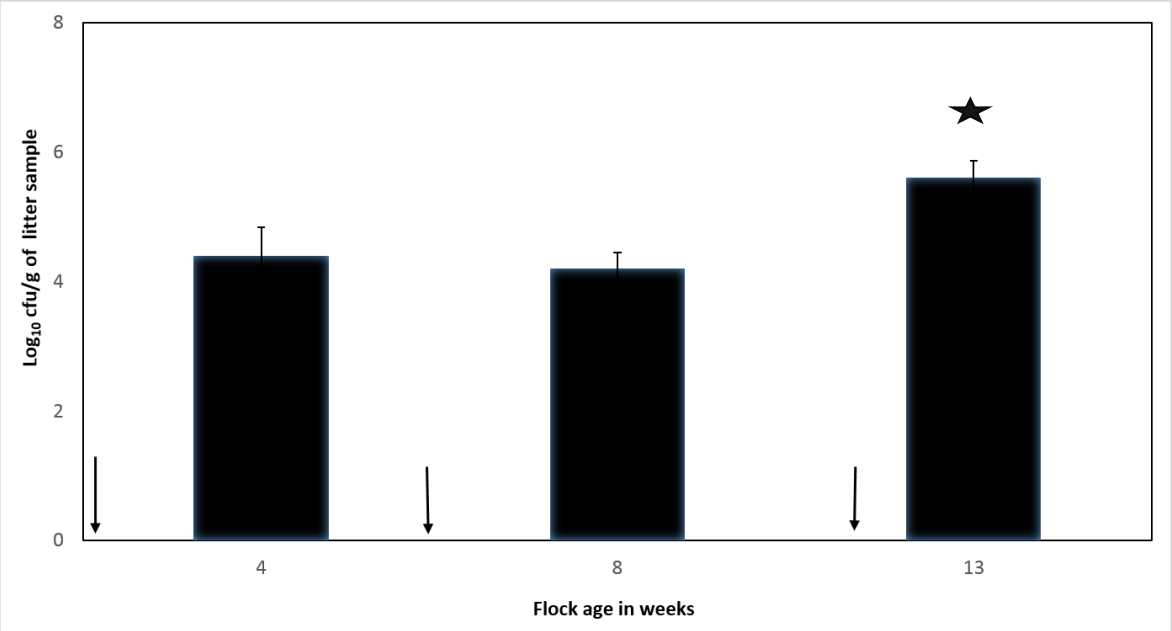
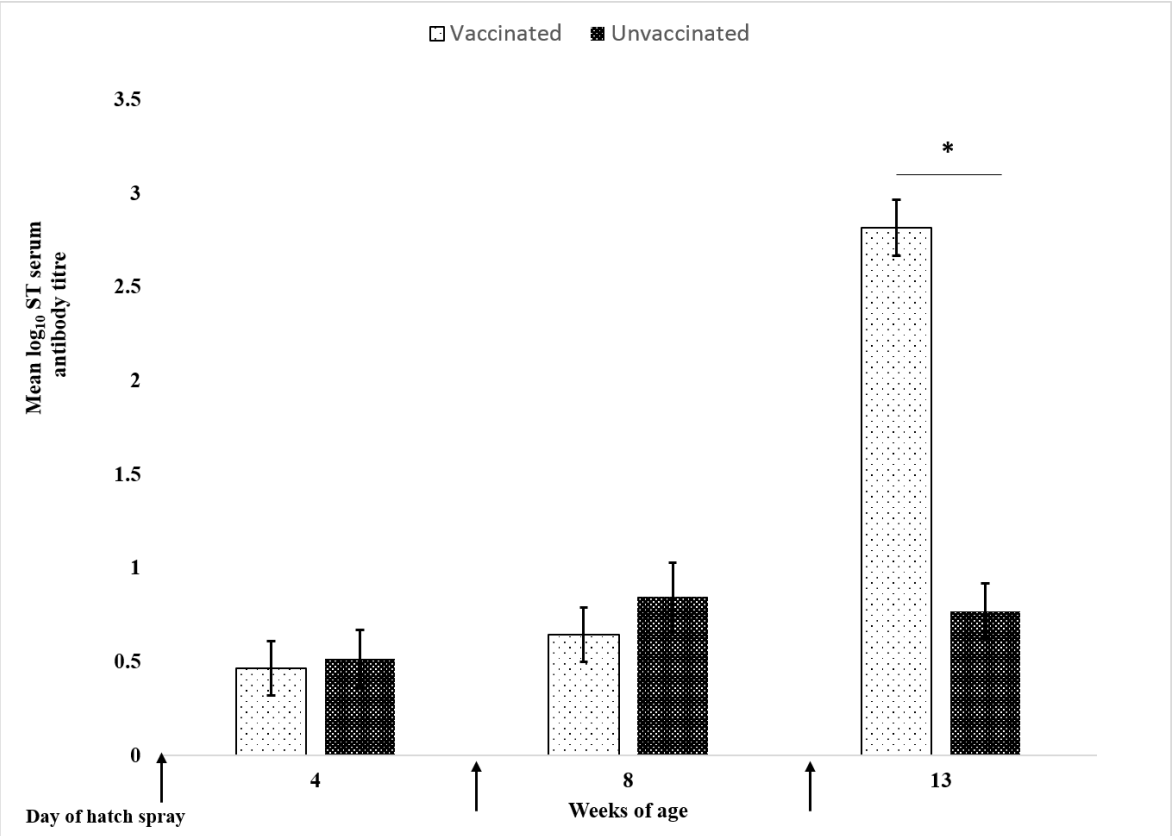


Figure 2

Antibody titres (IgG) of vaccinated and unvaccinated pullets during rearing. Black arrows indicate the vaccination at 1 day, 6 and 12 weeks. Asterisks (*) indicate statistically significant differences ($p < 0.05$) between vaccinated and unvaccinated groups.



Chapter 4 Shedding of *Salmonella* Typhimurium in vaccinated and unvaccinated hens during early lay: a randomised controlled trial

Statement of Authorship

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Name of Principal Author (Candidate)	Pardeep Sharma
Contribution to the Paper	Performed the experiment. Sampling, processing, data compilation, analysis and interpretation of data. Wrote manuscript, responded to comments and suggestions by co-authors.
Overall percentage (%)	75%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 31.05.2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
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iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Shedding of *Salmonella* Typhimurium in vaccinated and unvaccinated hens during early lay: a randomised controlled trial

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Abstract

This project aimed to examine the efficacy of the Vaxsafe® ST (Strain STM-1) attenuated live vaccine, administered as two oral doses followed by an intramuscular dose, in reducing contamination by *Salmonellae* of both eggs and the environment in the layer sheds. A randomised controlled trial was conducted up to 26 weeks post last vaccine on two different multi-age caged egg farms. The antibody titres were significantly higher in the vaccinated group at all sampling points during this trial. There was no significant difference in the prevalence of *Salmonella* (detected by culture and PCR method) between the vaccinated and unvaccinated groups on the egg belt and faeces in early lay. Wild-type *Salmonella* spp. were consistently found in dust and shoe cover samples. Only one eggshell positive sample was detected which was not sufficient to assess whether STM-1 had any effect on the shedding of wild type *Salmonella* spp. on eggs. Quantitative PCR (QPCR) assay was able to differentiate between the live vaccine strain and wild type *Salmonella*. The load of wild-type *Salmonella* in shed environment was relatively low (1.3 $\log_{10} \pm 0.48$ CFU/m^2 of surface area). It is important to note that vaccination against *S.* Typhimurium is one of the possible intervention strategies to reduce the shedding of *Salmonella* and not the ultimate prevention. Given that wild type, *S.* Typhimurium was able to be detected in dust over several weeks, regular removal of the dust from the shed is likely to be important.

1. Introduction

In Australia, contamination of eggs and egg products with *Salmonella enterica* serovars, in particular, *Salmonella* Typhimurium, represents a significant public health risk. During the period between 2001 and 2011, a marked increase in *Salmonella* outbreaks linked to eggs was observed, and 90% of these incidents were caused by *Salmonella* Typhimurium [1].

Salmonella vaccination of poultry is one practical measure farmers can use to reduce shedding of *Salmonella* in their flocks [2, 3]. Vaccination has been shown to confer immunological protection against infection to layer hens as well as decreasing the level of on-farm contamination [4-6]. Both live and killed *Salmonella* vaccines have been used with variable success against *Salmonella* serovars in laying hens [7]. Gantois et al. [8], tested a live metabolic drift mutant vaccine TAD *Salmonella* vac® E and TAD *Salmonella* vac® T against *Salmonella* Enteritidis (SE) challenge in laying hens and found that vaccination reduced the colonisation of SE in the reproductive organs and intestinal tracts, which helped reduce egg contamination levels. However, there is a paucity of information regarding the efficacy of live ST vaccines in laying hens, which is particularly important given ST is a major serovar in the Australian egg industry, and Vaxsafe® ST (Bioproperties Pty Ltd, Australia) is the only live attenuated vaccine registered for the control of ST infection in poultry in Australia. Vaxsafe® ST was developed for short-lived birds (such as broilers) and registered for oral and coarse-spray application routes. The mutant was engineered from a virulent wild-type *S.* Typhimurium by knocking out the *aroA* gene using a transposon (aroA-554: Tn 10) insertion method [9]. While studies have been conducted to test the efficacy of a range of different *Salmonella* vaccines in chickens under experimental conditions [10-14], there is limited information on the efficiency of the use of STM-1 in hens challenged naturally under field conditions in early lay. The primary aim of this randomized controlled trial was to test the efficacy of the Vaxsafe® ST live vaccine in commercial egg laying flocks, naturally infected with S. Typhimurium during early lay. Furthermore, two live vaccinations

(oral) followed by parenteral administration (injection) prior to the onset of egg production, has not been evaluated in randomized controlled trials. Environmental stressors, temperature, water, transportation and the onset of sexual maturity can trigger *Salmonella* shedding in carrier birds [15]. This trial was carried out over the period from day-old to 38 weeks (i.e. 26 weeks post last vaccination) to cover periods of stress and exposure to natural infection with *Salmonella* homologous or heterologous to the live vaccine strain. As caged production systems supply the majority (> 60%) of eggs for human consumption in Australia, the experimental trials evaluated the effectiveness of vaccination in a multi-age, high-challenge system.

2. Materials and Methods

2.1. Vaccination and placement of pullets

Commercial Hy-Line Brown chicks were obtained from a commercial hatchery in Victoria and randomly divided into two parallel groups. Group 1 (n = 10,000) received Vaxsafe® ST by coarse spray in the hatchery while Group 2 (n = 15,000) chicks were left unvaccinated. The Group 1 vaccinated chicks were debeaked in the hatchery for identification. The vaccinated and unvaccinated chicks were then placed into different boxes and transported to the commercial pullet rearing farm in the same vehicle and placed into separate rearing sheds. At 6 weeks of age, Group 1 vaccinated chicks received a second dose of Vaxsafe® ST vaccine in the drinking water, and at 12 weeks of age, the Group 1 vaccinated birds were inoculated with a third dose of Vaxsafe® ST by intramuscular (I/M) injection. For the I/M dose, the vaccine was reconstituted using a commercial Marek's vaccine diluent as per the manufacturer's instructions and administered as a 0.5 mL dose at the same time as a commercial multivalent Egg drop syndrome (EDS) / Newcastle disease (ND) killed vaccine (Nobilis® EDS+ND, MSD Animal Health). All birds were reared in a deep-litter, floor-based shed situated in South Australia. The antibiotic-free feed was provided by a commercial feed mill and a standard lighting protocol provided by layer company was adopted during rearing.

2.2. Selection of commercial egg farms

Two commercial caged egg farms were selected for this randomized controlled trial based on a) geographical location being situated in South Australia, b) a history of *S*. Typhimurium infection, and c) the willingness of the producers to participate in the trial. Shed A (from Farm A) and Shed B (from Farm B) were multi-aged flocks situated on two different farms with each age-class housed in separate rows. Other flocks housed in these sheds on the day of placement of the vaccinated and unvaccinated (control) flocks were as follows: Shed A had flocks aged 46, 58 and 64 weeks and Shed B had a single 64-week old flock. The sheds and cages housing test flocks were not cleaned out or disinfected prior to placing the new flock, and thus the new flocks would have been exposed to high challenge levels when introduced to their respective sheds. Each flock was sampled five times at four-week intervals from June to October (early to mid-lay). In shed A, the selected flock included 1280 cages of 17 weeks old birds (6 birds per cage, for an approximate 7680 birds (5000 vaccinated + 2680 unvaccinated) and shed B had 1428 cages of 17 weeks old birds with 6 birds per cage, for an approximate 8568 birds (5000 vaccinated + 3568 unvaccinated). To ensure that at least several cages positive for wild-type *Salmonella* would be identified, a representative sample size of thirty vaccinated and thirty unvaccinated cages per flock from each side was targeted from both the farms. Accounting for field constraints and based on previous findings [16] the cages were selected from the three lowest tiers (tiers 1, 2, and 3) out of the five tiers.

To check the *Salmonella* infection status of the farms, dust and cage swabs (n=8 each) from both trial sheds were collected one week prior to the arrival of point of lay pullets (16 weeks of age). Vaccinated and unvaccinated pullets were transported from the rearing farm to the production farm on the same vehicle by a contractor specialising in live bird transport. The vaccinated and unvaccinated parallel group birds were housed in the same shed on each layer farm to provide a consistent challenge for both treatment groups, and to allow direct

assessment of *Salmonella* sp. shedding, including the potential horizontal transfer of STM-1 vaccine to unvaccinated birds (Figure 1a, b). To enhance the sensitivity of detecting *Salmonella* sp. shedding, a representative sample size of 30 cages per flock were randomly selected throughout the shed for sampling at each interval. The vaccinated and control flocks were sampled at approximately monthly intervals after transfer to the production farm from 17 weeks (17, 21, 25, 29, 32 and 38wks). Based on our previous findings, *Salmonella* shedding was found to be most prevalent in the lower three cage tiers [14]; thus, cages were selected at equal intervals along the three lowest tiers of the five tiers in each trial shed. Fresh faecal samples (n=30 each from a vaccinated and unvaccinated parallel groups) were also collected from the manure belt underneath the cages. Based on the initial faecal sample test results (17 weeks of age), ten *Salmonella* positive cages were selected for further longitudinal sampling. Faecal samples (n=10), egg belt swabs (n=10), eggs (n=60) and dust swabs (1 m^2 area) (n=5) were collected during each sampling.

2.3. Collection and processing of environmental samples

All samples were processed for *Salmonella* and vaccine strain isolation by culture as described previously [15, 16]. Purple colonies on Brilliance *Salmonella* agar (BSA, Oxoid Australia) were presumed to be *Salmonella* spp. STM-1 vaccine strain is easily demarcated from wild type *Salmonella* as it did not produce H_2S on xylose lysine deoxycholate agar (XLD, Oxoid, Australia), allowing presumptive identification as STM-1 and this STM-1 strain does not grow on BSA [17]. Likely STM-1 colonies and *Salmonella* positive were mixed with 0.5 mL of Brain Heart Infusion broth (BHI, Oxoid, Australia) and incubated overnight at 37°C. Colonies suspected as being either STM-1 or wild type *Salmonella* spp. were stored in 0.5 mL of 80% glycerol and tested using standard PCR (see Section 2.8). Egg belt swabs, dust samples and shoe covers were moistened with 20 mL buffered peptone water (BPW) and processed for *Salmonella* isolation as described above. The prevalence of *Salmonella*-positive cages (detected by culture and PCR method) between the vaccinated

and unvaccinated groups on the egg belt and faeces was estimated at each sampling point with 95% binomial exact confidence intervals. To study the possible flow of vaccine between vaccinated and unvaccinated birds housed in the same shed, DNA extracted from faecal samples from both treatment groups were tested using the standard PCR for detection of STM-1 (see Section 2.8).

2.4. Processing of eggs

Eggs were collected directly from the egg belt in front of each sampling cage into a sterile plastic bag at each sampling time point. The egg belt in front of each sample cage was also swabbed after collection of eggs. Six eggs were pooled together for processing. Eggshell wash and internal contents were processed separately. Pooled eggs were placed in 10 mL/egg of sterile BPW in Whirl-Pak bags (Thermoscientific, Australia). The processing of eggs was performed as described previously [16].

2.5. Serology

At each sampling, ten blood samples from each treatment group were collected in lithium heparin tubes (BD Vacutainer® Plus plastic plasma tube**,** UK**)**. Plasma samples were stored in aliquots and frozen at -20°C for analysis. ELISAs were performed using 96 well flat-bottomed microtiter plates coated with inactivated group B LPS antigen (BioChek, Holland) to detect IgG antibodies in the plasma and IgG antibody titres were calculated according to the manufacturer's recommendations.

2.6. DNA extraction from faecal, egg belt and dust samples

DNA was extracted from faecal, egg belt and dust samples using the Isolate Faecal DNA Kit (Bioline, Australia). DNA was extracted according to manufacturer's instructions. The DNA yield (ng) and purity (absorbance ratio at 260/280) of extracted genomic DNA were determined spectrophotometrically using the NanoDrop® ND-1000 (Biolab, Australia).

Dilutions were prepared using nuclease free water to achieve a working concentration of 5 ng/ µL DNA for use in real-time PCR.

2.7. Standard PCR for the detection of STM-1

All DNA samples extracted from faeces were screened for the amplification of *invA* and *TSR3* genes for the detection of *S.* Typhimurium by multiplex PCR as described previously [16]. To differentiate between wild-type and vaccine strains, primers (Forward 5'-3'GTTTTAAGTGTAATTCGGGG; Reverse 5'-3' TATGATCAAATGGTTTCGCC) were designed using a sequence that included the transposon / *aroA* gene junction that is unique to the STM-1 strain. This generated an amplicon of 164 base pairs. If a sample was positive for all three PCR products, it was considered STM-1 positive. For the detection and differentiation of wild-type *Salmonella* serovars from vaccine strain, forward: 5'-TCTTTTTTCATCCCCACG-3' and reverse primer: 5'-CGGTTTTACCACAAGCTAA-3'. These primers were designed from the region that included the *aroA* gene junction which is conserved for the *Salmonella* strains. The specificity of these designed primers was tested against 22 different *Salmonella* serovars. All *Salmonella* serovars except for *S*. Mbandaka generated the amplicon of 182 bp from this primer set (Table 1). These primers did not generate any amplicon from vaccine strain STM-1. This primer set was also used for qPCR.

2.8. Standard curve and qPCR for wild type *Salmonella* from faecal samples

All reactions were run in a total volume of 10 µL using the TaqMan *S. enterica* detection kit system (Applied Biosystems, Australia). The reaction components and final concentrations used for the qPCR were 2 µL of 5 ng/µL diluted DNA sample, 5 µL of 2 x Quantifast SYBER Green Master Mix and 1µM of both forward and reverse primers. The quantitative PCR was performed with the Quantifast® SYBER® Green qPCR kit (Qiagen, Australia). DNA amplification was performed using a thermocycler under the following cycling conditions: initial denaturation at 95°C for 10 min.; followed by 40 cycles of denaturation at 95°C for

15 sec. with subsequent annealing and extension at 60°C for 60 sec. on a 7900HT sequence detection system (Applied Biosystems). Data were analysed assuming a threshold of 0.3 and baseline of 3 to 10. Serial ten-fold dilutions of ST spiked faecal samples were used to generate a standard curve to determine the limit of detection and quantification of ST cells. Briefly, 0.2 g of faecal sample was mixed with a ten-fold dilution of ST culture. Bioline faecal DNA purification kit was used to purify DNA from spiked faecal samples, and 5 ng/µL of DNA sample was used for doing the qPCR. Negative (unspiked) and positive (ST spiked faecal samples) controls were used in every PCR reaction. The limit of detection for the qPCR assay was ≥100 of *Salmonella* cells/g of faeces. The primers designed for detection of wild type *Salmonella* spp. did not cross amplify with the vaccine strain (STM-1).

3. Results

Mean antibody titres in the vaccinated group were above the cut-off value, but the mean titres in the unvaccinated group were below the positive threshold (mean $\log_{10}$ antibody titre = 2.8) (Figure 2). Antibody titres in vaccinated hens were significantly higher at all time points i.e. 21, 25, 29, 32 and 38 weeks of age than in the unvaccinated group.

There was no significant difference in the prevalence of *Salmonella* spp. in faeces, as detected by culture, in vaccinated and unvaccinated groups at early lay. Similarly, multiplex PCR results indicated that there was no significant difference in the prevalence of *S.* Typhimurium in the faeces of the vaccinated and unvaccinated groups at early lay. The *S.* Typhimurium prevalence was significantly higher by culture method (p=0.04) at week 17 than at weeks 25, 29 and 32 (Figure 3a).

There was no significant difference in the prevalence of *Salmonella* (Figure 3b) or *S.* Typhimurium (Figure 4a), as detected by culture, between the vaccinated and unvaccinated groups on the egg belt in early lay. Wild-type *Salmonella* spp. were consistently found, in dust and shoe cover samples by culture method (Figure 4b). *S.* Typhimurium was

consistently isolated from the dust throughout the trial (Figure 5), although its prevalence did not vary significantly over the period of sample collection. Only one eggshell was culture positive among the samples collected from the vaccinated group. The *Salmonella* spp. in the eggshell wash was *S.* Typhimurium. All egg internal contents were negative by culture for *Salmonella* spp. Four faecal samples were positive for STM-1 by PCR method, although STM-1 was not detected by culture.

There was no significant difference in the level of wild type *Salmonella* spp. on egg belt, as detected by culture method, between vaccinated and unvaccinated groups (Figure 6a). The level of *Salmonella* spp. in dust samples did not vary significantly over the period of sample collection (Figure 6b). The load of wild type *Salmonella* spp. in dust samples at 25 weeks was 1.3 $\log_{10} \pm 0.48$ CFU/m^2 of surface area.

4. Discussion

In poultry nontyphoidal salmonellae readily colonise the gastrointestinal tract without usually causing clinical symptoms of disease in birds [18]. This means that it is more challenging to control these microorganisms in poultry because birds do not exhibit clinical signs suggestive of higher bacterial loads. As a result, the failure of on-farm or supply chain interventions to control *Salmonella* contamination are often only identified after outbreaks in humans [18].

Vaccination is an intervention strategy that has been widely used to reduce *Salmonella* infections. Killed vaccines have been used with success against Salmonellosis [19], however; they have some disadvantages; they elicit an incomplete antibody response, and they are unable to elicit a cell-mediated immune response (CMIR) that is needed for *Salmonella* control and for long-term protection of laying hens [2].

Live vaccines generate both humoral and cellular immune responses and are considered better than inactivated vaccines because they can stimulate mucosal immunity and thus can provide effectiveness against intestinal and systemic infection [20].

Most poultry vaccines are developed to prevent disease, but as *S.* Typhimurium infection in adult hens does not cause disease, the rationale underlying vaccination is to reduce shedding. Two live vaccinations (oral) followed by parenteral administration (injection) in combination with inactivated oil-emulsion vaccine (EDS + ND) prior to the onset of egg production provides a convenient vehicle for the administration of the Vaxsafe® ST through a multi-dose method and saves on preparation and administration costs. In this trial, *S.* Typhimurium IgG serum antibody titres in unvaccinated birds were below the positive threshold, but the titre was above the threshold in vaccinated hens. This finding was in agreement with a previous field trial [21], which found that titres in unvaccinated field challenged hens were below the threshold. During an experimental *S.* Typhimurium challenge trial, involving oral inoculation with 10^9 CFU of *S.* Typhimurium, the antibody titres of infected hens was well above the threshold [22]; however, another trial [21] found that oral administration of Vaxsafe® ST was unable to induce humoral immune response with transitory reduction in *Salmonella* caecal colonisation. It has been hypothesised that virulent serovars, such as *S.* Typhimurium, are more likely to invade and induce a greater systemic immune response [23]. Based on these previous observations, it could be deduced that during the current randomized controlled trial, the hens were not challenged with a high enough bacterial load to induce a systemic immune response.

In this trial, there was no significant difference in the prevalence of *S.* Typhimurium infection, as detected by culture method using faecal sampling, in the vaccinated and unvaccinated groups in early lay. Our findings are in agreement with a previous trial [24] that reported that an *aroA* deletion mutant *S.* Typhimurium vaccine did not significantly reduce the frequency of faecal shedding of wild-type *S.* Typhimurium under experimental

conditions. A study has found a reduction in faecal shedding of wild-type *S.* Typhimurium in chicks vaccinated with an oral and intramuscular administration of *aroA* mutant *S.* Typhimurium at four days old, although this mutant initially reduced the faecal excretion for 14 days of the challenge strain this effect did not persist [25]. Although the antibody response contributes to the clearance of extracellular bacteria, facultative intracellular bacteria such as *Salmonella* spp. can persist intracellularly, so a CMIR is essential for clearance of *S.* Typhimurium [26]. Beal, Powers [27] showed that an antibody response was not essential for gut clearance of *S.* Typhimurium but suggested that an effective vaccine should activate both cellular and humoral immune responses. In the present trial, there was an increased antibody response in the vaccinated group after parenteral administration although cell-mediated immunity was not evaluated. In this trial vaccinated birds were dubbed for differentiating vaccinated and unvaccinated flocks. Dubbing can reduce heat transfer from adult bird making these birds more heat stressed [28]. Stress could potentially induce *S.* Typhimurium shedding in faeces which could further increase the chances of egg contamination [29]; however, it is important to note that in this trial, birds were housed in environmentally controlled sheds. Further work on the effects of Vaxsafe® ST administration on the CMIR and measurement of stressors such as faecal corticosterone metabolites will be helpful to understand the biology of this vaccine.

Our findings about the persistence of *S.* Typhimurium in dust samples is consistent with a previous report [16]. In this trial, the environmental samples such as dust swab, egg belt and shoe covers were positive for *S.* Typhimurium at all sample intervals. Only four (2.5%) faecal samples and one (0.5%) eggshell was found positive for *S.* Typhimurium. In a poultry shed, the external surface of eggs can either be contaminated by dust, contact with the egg belt or contact with faeces. One eggshell positive sample was detected which was not sufficient to assess whether STM-1 had any effect on the shedding of wild type *Salmonella* spp. on eggs. However, given that faecal samples, dust and egg belts are the possible

indicators of egg contamination [16], it could be deduced that the STM-1 vaccine may not have any effect on the level of egg contamination, although larger controlled studies are necessary to investigate this further. Eggs were collected directly from the egg belt which was already contaminated with *Salmonella*. Contact with the egg belt, should, therefore have been the primary source of eggshell contamination; however, it was interesting to note that every egg (except for one) collected from contaminated egg belt samples was *Salmonella* negative. Live vaccines may not be very effective in multi-age sheds because older *Salmonella* infected birds in the shed may act as a continuous source of infection to the newly arrived pullets (vaccinated pullets in this trial).

QPCR data suggested that the level of *Salmonella* load in the commercial farms that were sampled was relatively low, hence it could be concluded that these birds received low challenge during the trial. Most of the experimental vaccine challenge studies used relatively higher challenge dose (up to 10^9 CFU/mL). During our previous epidemiological investigations, we were able to detect more than 4 $\log_{10}$ CFU in dust samples [16] so this suggests that the level of *Salmonella* spp. could be variable between different flocks housed in the same shed.

For this trial, the recruitment of farms was largely based on the willingness of farmers to participate. Recruitment of a larger number of egg farms would have been ideal; however, cooperation from egg producers over a period of months and the requirement of resources are limiting factor to such studies [30].

Given that *S.* Typhimurium and other serovars can survive/persist in the shed environment (such as in dust), regular cleaning and or removal of dust from shed is also important. Use of the Vaxsafe® ST vaccine in multi-age flocks is "not an ultimate intervention" for reduction of *S.* Typhimurium because of the complexities involved in achieving control, such as the efficacy of cleaning of sheds, the lack of resting periods between batches and the possible carry over of contamination from existing flocks. It is important to note that the goal of

administering a live *Salmonella* vaccine is to reduce the shedding of *S*. Typhimurium onto poultry products destined for human consumption. Administration of live vaccine is one of the intervention strategies, and if combined with an effective biosecurity programme, such as good sanitation, appropriate egg handling full shed depopulation, and management practices, it could assist in reducing the risk of product contamination.

Ethics statement

All experimental procedures involving animals were in compliance with the recommended protocol approved by the institutional animal ethics committee of The University of Adelaide (Protocol No. S-2014-008) and in compliance with the Australian code for the care and use of animals for scientific purposes.

Competing interests

The authors declare that they have no competing interests.

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Table 1

Salmonella enterica serovar strains used and other samples tested for newly designed wild type ST PCR results.

***Salmonella* isolate details**	**Result**	***Salmonella* isolate details**	**Result**
Typhimurium DT 170/108*	+	Zanzibar	+
Typhimurium DT193	+	Kiambu	+
Typhimurium DT135	+	Virchow	+
Typhimurium DT 5	+	Cerro	+
Adelaide	+	Lille	+
Orion	+	Ohio	+
Agona	+	Bredeney	+
Johannesburg	+	Havana	+
Livingstone	+	Senftenberg	+
subsp I ser 4,12: d: -	+	Oranienburg	+
Chester	+	Montevideo	+
Mbandaka	-		

*- Same phage type referred as 170/108.
+- Positive result
-- Negative result

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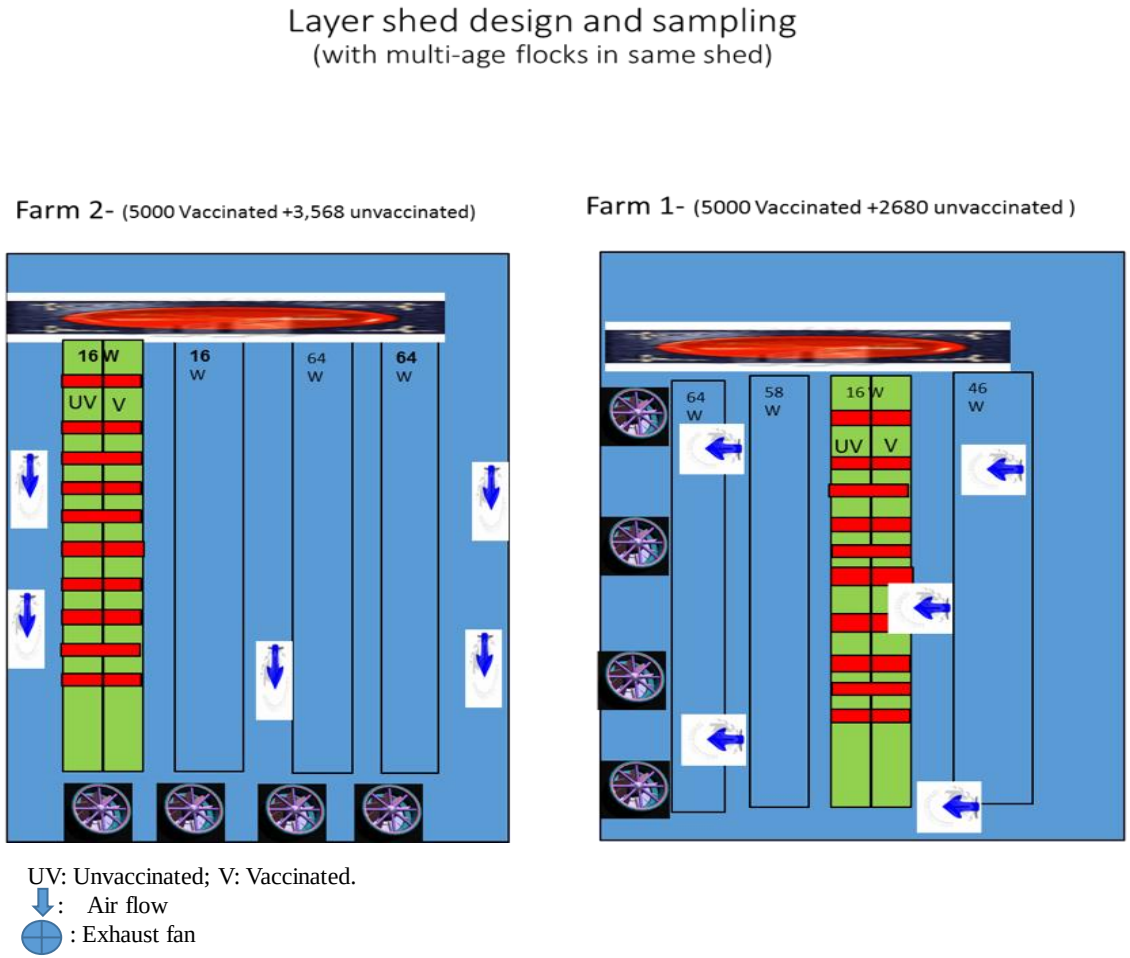
Figure 1. Layouts of the shed A as Farm 1 and shed B as Farm 2 showing the areas of sample collection from vaccinated and unvaccinated hens.



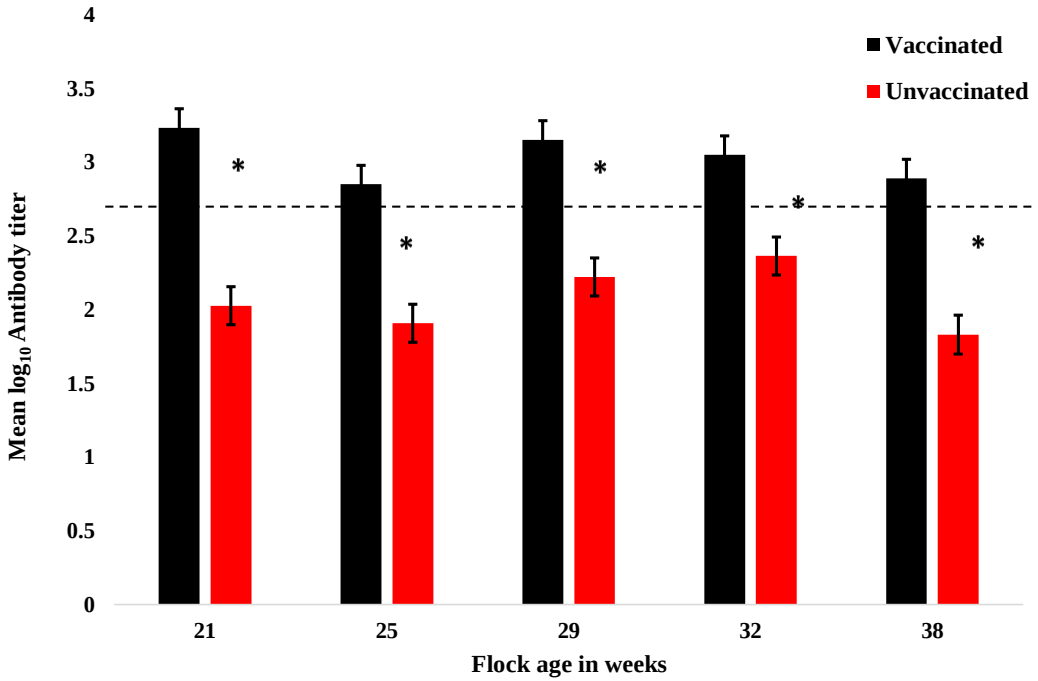


Figure 2. Mean $\log_{10}$ *S.* Typhimurium IgG antibody titres in vaccinated (n=10) and unvaccinated (n=10) hens over time during early lay. Asterisks (*) indicate statistically significant differences ($p < 0.05$) in both the groups for the corresponding time.

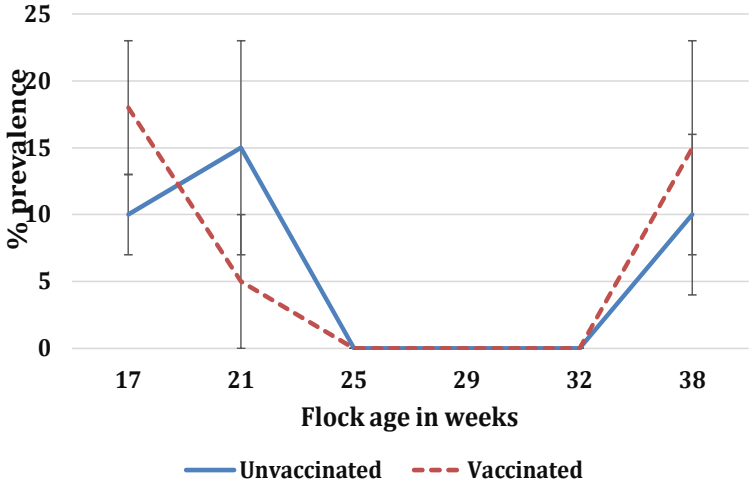


Figure 3a. Culture results for prevalence of *S. Typhimurium* in faeces of vaccinated (n=10) and unvaccinated (n=10) hens over the time. Bars indicates standard error.

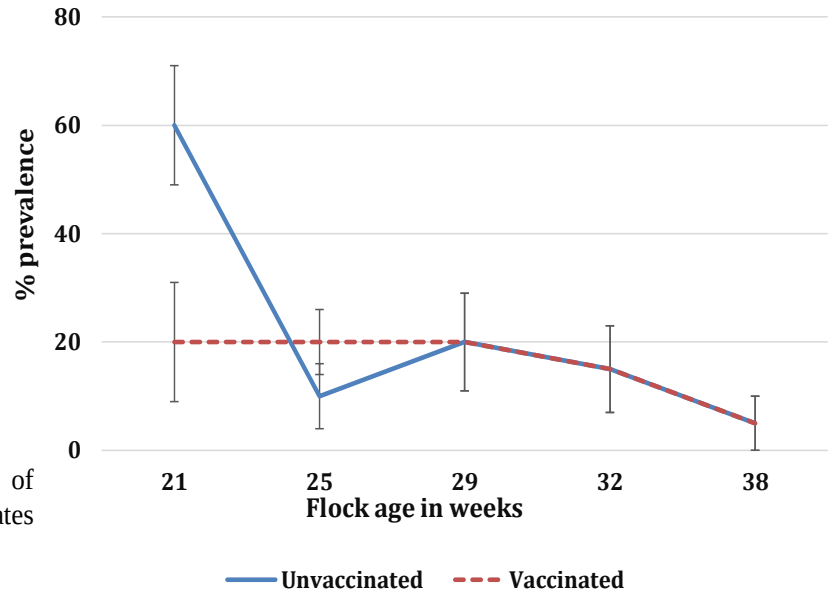


Figure 3b. Culture results for prevalence of *Salmonella* spp. on egg belt of vaccinated (n=10) and unvaccinated (n=10) hens over the time. Bars indicates standard error.

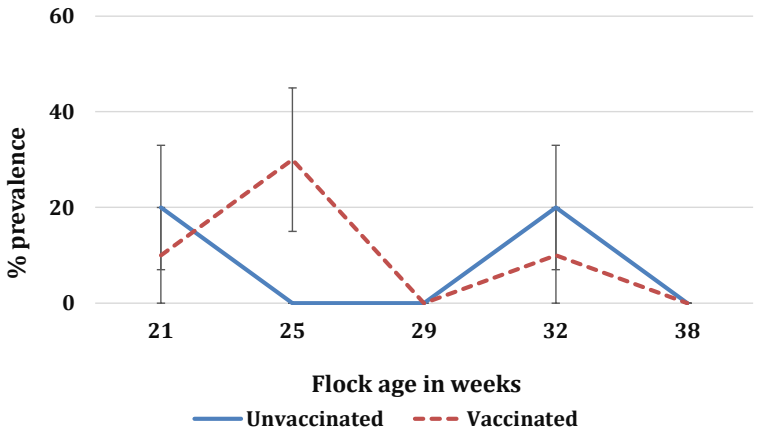


Figure 4a. Culture results for prevalence of *Salmonella* Typhimurium on egg belt of vaccinated (n=10) and unvaccinated (n=10) hens over the time. Bars indicates standard error.

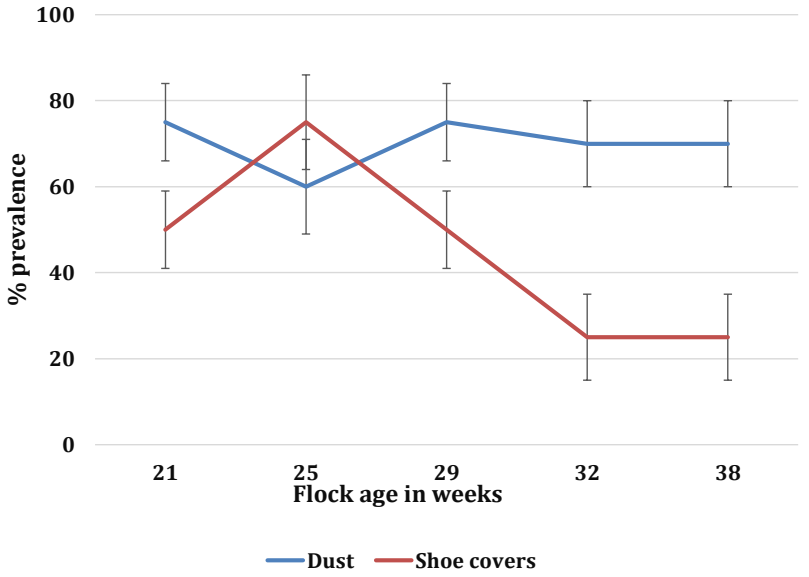


Figure 4b. Culture results for prevalence of *Salmonella* spp. in dust and on shoe covers over the time. Bars indicates standard error.

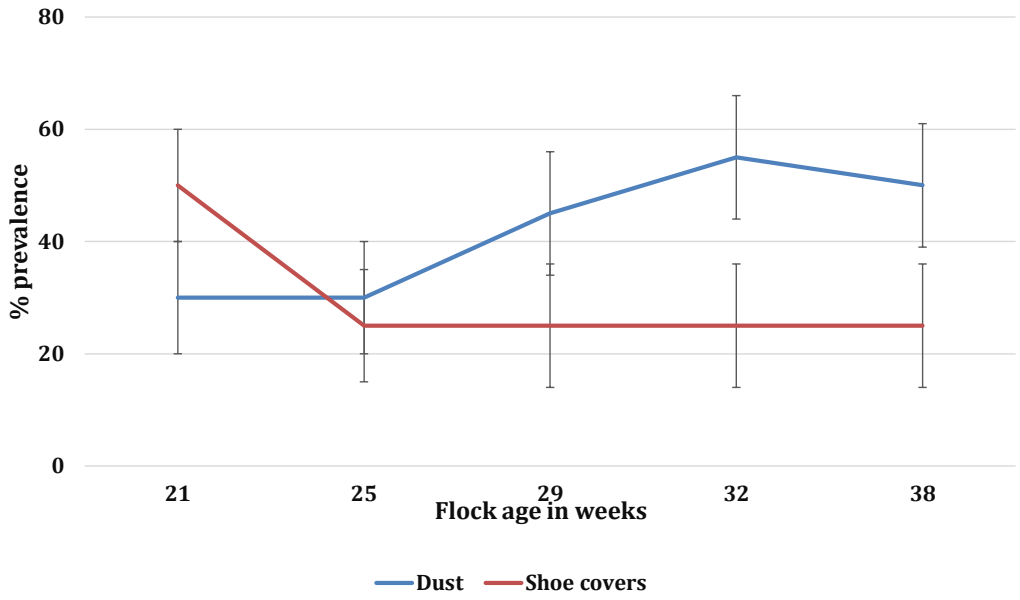


Figure 5. Culture results for prevalence of *S.* Typhimurium in dust and on shoe covers over the time. Bars indicates the standard error.

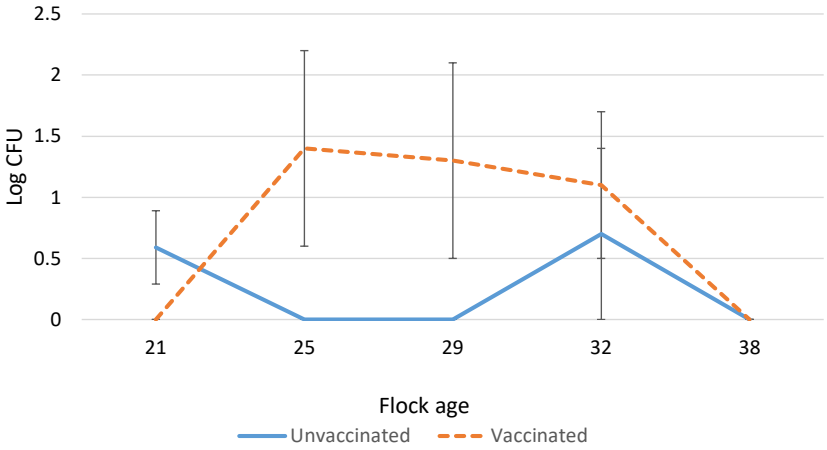


Figure 6a. Culture results for the level of wild type *Salmonella* on egg belt samples from vaccinated (n=10) and unvaccinated (n=10) hens over the period of this experiment. Bars indicates standard error.

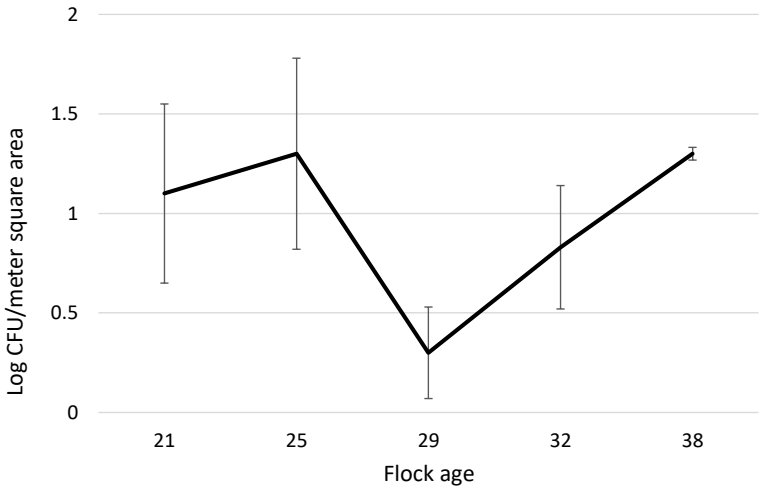


Figure 6b. Culture results for the **l**evel of wild type *Salmonella* spp in the dust collected from shed the period of this experiment. Bars indicates standard error.

Chapter 5 Efficacy of commercial disinfectants against biofilms formed by *Salmonella enterica* serovars Typhimurium isolates

Statement of Authorship

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Principal Author

Name of Principal Author	Pardeep Sharma
Contribution to the Paper	Designed and conducted the experiment. Acquired, analysed and interpreted experimental data. Drafting and revision of paper.
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 31/05/2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature	Date 31.5.17

Name of Co-Author	Dr Vivek V. Pande
Contribution to the Paper	Designed, assisted in performing experiment. Helped in data interpretation and manuscript editing.
Signature	Date 31/05/2017

Efficacy of commercial disinfectants against biofilms formed by *Salmonella enterica* serovars Typhimurium isolates

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Abstract

Salmonella enterica serovar Typhimurium (*S*. Typhimurium) is one of the major pathogens associated with egg-borne outbreaks of Salmonellosis in Australia. *S*. Typhimurium is able to attach to and form biofilm on various surfaces. Biofilm formation is a major cause of cross contamination of food products and a significant public health issue. In this study the susceptibility of *S*. Typhimurium biofilms to three different commercial disinfectants (Product A, B and C) was investigated. Biofilms were formed for 24 and 48 hour (h) using the MBEC^{TM} system at 22 ºC and treated with commercial disinfectants at 0.4, 0.5, 0.8 or 1.0% concentrations for 30 s (s). All the three disinfectants showed a significant reduction in viable biofilm cells at both times (24 and 48 h) in a dose dependent manner. The age of biofilm was associated with the resistance towards all disinfectants at both concentration. In conclusion, the commercial disinfectants used in this study were able to reduce the viable cells in biofilms however; complete eradication of biofilm cells was not possible. These findings also highlight the need to consider the optimisation of disinfectants and novel strategies to control biofilms formed by *S*. Typhimurium.

Keywords: biofilm; disinfection; *Salmonella* Typhimurium; layer farm; egg industry

Implications

The study was conducted to explore the antibiofilm efficacy of routinely used commercially available disinfectants against *Salmonella* Typhimurium isolates from the layer farms. Biofilms from the layer farm isolates were formed and treated with disinfectants at single and double dose concentrations. Disinfectants showed a significant reduction in viable biofilm cells, however; complete eradication of biofilm cells was not possible. Age of biofilm was associated with the resistance towards disinfectants at both concentrations. This study has expanded our understanding of the susceptibility of *S.* Typhimurium biofilms to commercially available disinfectants and findings have implications for usage, development, and optimisation of disinfectants.

Introduction

Non-typhoidal Salmonellae (NTS) infections are a leading cause of foodborne gastric illness in people in developed and developing countries (Moore *et al.*, 2011). It is estimated that almost 94 million cases of gastroenteritis are caused by non-typhoidal *Salmonella* spp. worldwide (Majowicz *et al.*, 2010). Globally, *Salmonella* Enteritidis have dominated the epidemiology of foodborne outbreaks associated with consumption of raw egg and egg products however, this serovar is not endemic in commercial poultry flocks in Australia (Wales and Davies, 2011). Contamination of eggshell by *S.* Typhimurium is a major public health and food safety issue to the Australian poultry industry. In Australia, the majority of the egg and egg related foodborne outbreaks are caused by *Salmonella* Typhimurium (The OzFoodNet Working group, 2015). *S.* Typhimurium was responsible for 90% (150/166) egg associated outbreaks in Australia from 2001 to 2010 (Moffatt *et al.*, 2016).

There are several intervention strategies to control *Salmonella* at pre- and post-harvest level such as flock management, biosecurity, vaccination, and egg washing (Galis *et al.*, 2013). In Australia, Canada, USA, and Japan egg washing with commercial disinfectants is

extensively used to reduce eggshell contamination (Hutchison *et al.*, 2004, Galis *et al.*, 2013). Previous studies demonstrated that egg washing with commercial disinfectant was able to reduce *Salmonella* load on the eggshell surface (Messens *et al.*, 2011) however there is less information on the activity of commercial disinfectants against *Salmonella* biofilms formed on equipment.

Biofilm formation is one of the survival mechanisms utilised by *Salmonella* spp. against physical and chemical stress factors in the environment (Steenackers *et al.*, 2012). Biofilm is a community of cells attached to biotic and abiotic surfaces in a self-produced extracellular matrix component (Donlan and Costerton, 2002). Cells in biofilm exhibit a greater resistance to antimicrobials and environmental stressors than their planktonic counterparts (Steenackers *et al.*, 2012). Hence, their eradication from domestic and industrial settings is difficult. In addition, previous studies have demonstrated that susceptibility of biofilm cells to commonly used disinfectants was also associated with the biofilm age (Habimana *et al.*, 2010, Shen *et al.*, 2011).

Biofilm formation contributes to the persistence of *Salmonella* in the food processing environment and detachment of biofilm cells from surface could lead to cross contamination of other food products compromising the food quality (Møretrø *et al.*, 2012). Biofilm formation is a significant public health and food safety issue and its control is critical. The objectives of this study were to test the susceptibility of 24 and 48 h old biofilms formed on plastic surface to different concentrations of commercial disinfectants following 30 s exposure. All commercial disinfectants tested in this study are currently being used in the Australian poultry industry. In this study each product was tested at the user recommended concentration or twice recommended concentration against *S.* Typhimurium biofilms.

Materials and methods

Salmonella isolates, growth conditions

Twelve isolates of *S.* Typhimurium isolated during epidemiological studies were used for biofilm formation (Gole *et al.*, 2014). All isolates were serotyped at the Australian *Salmonella* Reference Centre, Microbiology and Infectious Diseases, SA Pathology, Adelaide, South Australia.

Stock cultures of *Salmonella* isolates were streaked onto xylose lysine deoxycholate (XLD) agar plates (Thermo Fisher Scientific, Australia) and stored in Luria-Bertani (LB) broth (Oxoid, Australia) at -80°C in 20% glycerol solution.

Inoculum preparation for biofilm study

The stock culture of *S.* Typhimurium was streaked onto XLD agar plates and incubated at 37 °C for 24 h for the preparation of biofilm inoculum. Briefly, a single colony of each *S.* Typhimurium isolate was selected from XLD agar plate and resuspended in 10 ml tube containing LB broth. The inoculated broth was incubated at 37°C overnight. Next day, bacterial culture was diluted (1:100) in LB without NaCl to achieve 10^7 CFU ml^{-1} for biofilm formation. Viable cells were enumerated by plating 10-fold serial dilutions of bacterial inoculum on LB agar plates to confirm dose.

Biofilm formation

The MBEC™ Assay system (Innovotech Inc. Edmonton, Canada) was used to form *S.* Typhimurium biofilms. Biofilms were formed by adding 200 µl of *S.* Typhimurium inoculum in each well of MBEC plate and plates were incubated at 22 ± 2°C for 24 or 48 h on a rocking platform shaker (ROCKit, Select Bio-Products, NJ, USA).

Biofilm disinfectant challenge

Three commercial disinfectants (Table 1) commonly used in the Australian poultry industry were tested in this study against *S.* Typhimurium biofilms according to the manufacturer

procedural manual version 1.1 (Innovotech Inc. Edmonton, Canada). The details of commercially available disinfectants with chemical composition and intended use are listed in Table 1. Products A & C were tested at 0.5 or 1.0%, and product B was tested at 0.4 or 0.8%. Freshly prepared working solutions of each commercial disinfectant were prepared from stock solution and used immediately on the challenge day. Sterile water was used as a negative control and *S.* Typhimurium formed biofilm without any addition of disinfectant was used as a positive control in this experiment.

After biofilm establishment, peg lids were aseptically removed at 24 or 48 h and rinsed with 0.9% normal saline solution (NSS) for 1 min in 96 well flat bottom microtiter plates (Nunc™, Thermo Scientific, Australia). For each disinfectant studied, the peg lids were placed in disinfectant plate and exposed for 30 s Sterile NSS was used as a negative control in the disinfectant challenge plate. After 30 s exposure, the peg lids were rinsed two times (one min each rinse) in NSS, immersed in the neutralising broth (Difco™ Neutralizing Broth) for one min. The peg lids were then aseptically transferred to 96 well plate containing LB broth. The plates were sonicated at high speed for 5 min (Model 160TD, Soniclean Pty Ltd, Australia) and number of viable cells were enumerated by plate count method. In this study, two biological replicates of each *S.* Typhimurium isolate were used to test the activity of each disinfectant against biofilms.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6 Software, Inc. CA, USA. Mean recovery of viable cells from control and disinfectant treated group at different concentrations and days of biofilm formation were analysed by two-way ANOVA followed by Tukey's multiple comparisons test. Data were expressed as mean log CFU peg^{-1} ± standard error of mean (SEM). P values < 0.05 were considered statistically significant.

Results

Effect of product A challenge against biofilms

In comparison to positive control (7.05 log CFU peg^{-1}) product A significantly reduced 4.38 and 4.43 log CFU peg^{-1} viable cells of 24 h biofilm at 0.5 and 1 % concentration, respectively (Figure 1). Similarly, in comparison with positive control (7.02 ± 0.09 log CFU peg^{-1}) product A, significantly reduced 2.72 and 3.38 log CFU peg^{-1} from 48 h biofilms at 0.5 and 1% concentration, respectively (Figure 1). However, no significant difference was observed between both concentrations in the recovery of viable biofilm cells after 24 and 48 h of biofilm formation.

In this study, increasing age of biofilm was associated with the resistance towards treatment with product A. The sensitivity of 48 h biofilm cells to product A at 0.5 % was significantly reduced (P=0.0002) and more cells were recovered from 48 h biofilms (4.29±0.19 log CFU peg^{-1}) than the 24 h biofilm (2.67±0.34 log CFU peg^{-1}). There was a trend (P=0.053) towards decrease in sensitivity of product A between 24 (2.63±0.29 log CFU peg^{-1}) and 48 h (3.65±0.26 log CFU peg^{-1}) biofilm at 1% concentration.

Effect of product B challenge against biofilm

Compared to positive control (7.05 log CFU peg^{-1}) product B reduced viable cells for up to 4.35 and 4.57 log CFU peg^{-1} from 24 h biofilm at 0.4% and 0.8% concentration, respectively (Figure 2). However, no significant difference in the recovery of viable biofilms cells after 24 h was observed between 0.4 and 0.8% concentration. In comparison with positive control (7.02 ± 0.09 log CFU peg^{-1}) product B, significantly reduced 1.86 and 2.79 log CFU peg^{-1} from 48 h biofilms at 0.4 and 0.8% concentration, respectively (Figure 2). However, no significant difference was observed between both concentrations in the recovery of viable biofilm cells after 48 h of biofilm formation.

The resistance of biofilm cells towards product B at all concentrations was associated with the increasing age of biofilm. The sensitivity of 48 h biofilm cells to product B at 0.4 % was significantly reduced (P=0.0001) and more cells were recovered from 48 h biofilms (5.16±0.22 log CFU peg^{-1}) than the 24 h biofilm (2.70±0.41 log CFU peg^{-1}). Similarly, more cells were recovered from 48 h biofilms (4.23±0.22 log CFU peg^{-1}) than the 24 h biofilm (2.48±0.38 log CFU peg^{-1}) when treated with 0.8% concentration of product B.

Effect of product C challenge against biofilm

In comparison with positive control (7.05 log CFU peg^{-1}) product C at 0.5% and 1% concentration significantly reduced 4.95 and 4.92 log CFU peg^{-1} of 24 h biofilm, respectively (Figure 3). However, no significant difference in the recovery of viable biofilms cells after 24 h was observed between 0.5 and 1% concentration.

In comparison with positive control (7.02 log CFU peg^{-1}) product C, significantly reduced 1.91 and 2.24 log CFU peg^{-1} from 48 h biofilms at 0.5 and 1% concentration, respectively (Figure 3). However, no significant difference was observed between both concentrations in the recovery of viable biofilm cells after 48 h of biofilm formation.

There was significant difference in the resistance to product C at all concentrations between biofilm ages. The sensitivity of 48 h biofilm cells to product C at 0.5 % was significantly reduced (P=0.0001) and more cells were recovered from 48 h biofilms (5.11±0.11 log CFU peg^{-1}) than the 24 h biofilm (2.10±0.40 log CFU peg^{-1}). Similarly, more cells were recovered from 48 h biofilms (4.78±0.09 log CFU peg^{-1}) than the 24 h biofilm (2.13±0.34 log CFU peg^{-1}) when treated with 1% concentration of product C.

Discussion

Globally, non-typhoidal *Salmonella enterica* serovars (NTS) are mainly responsible for the foodborne diseases (Crump and Mintz, 2010, Ford *et al.*, 2016). The consumption of contaminated egg and egg related products are mainly responsible for the majority of human

salmonellosis outbreaks (Moffatt *et al.*, 2016). *S.* Typhimurium have the ability to persist in the environment for a longer duration by forming biofilms (Vestby *et al.*, 2009, Yahya *et al.*, 2017). Biofilm forming ability of *Salmonella* is a defensive mechanism and can protect the bacterium against several stressors including chemical treatment. As a result, removal of *Salmonella* biofilm by chemical disinfectants is challenging (Marin *et al.*, 2009). This study investigated the activity of commercial disinfectants against *S.* Typhimurium biofilm of different ages.

The three commercial disinfectants (Product A, B and C) tested in this experiment significantly reduced viable biofilm cells, however, none of the product have completely eliminated the biofilm cells. The effects of disinfectants are highly concentration dependent (Russell and McDonnell, 2000), and it is shown that high concentrations of disinfectants were able to reduce more viable cells from biofilms, or even demonstrated 100% reduction in viable cells (Møretrø *et al.*, 2009, Steenackers *et al.*, 2012). In contrast, in this study, even at twice the recommended user concentration, complete elimination of biofilm cells was not achieved by any of the products.

The type of disinfectants can also influence the susceptibility of *Salmonella* biofilms (Møretrø *et al.*, 2009). Product A, used was a chlorinated compound, and has already been tested with variable effects as a disinfectant against biofilms (Toté *et al.*, 2010, Wong *et al.*, 2010, Ziech *et al.*, 2016). The chlorinated compound is bactericidal due to its oxidative reaction with cellular proteins and it interferes with cellular function (Ramesh *et al.*, 2002). Product A was effective in reducing viable biofilm cells at both 0.5 and 1% concentrations, however was not able in eliminate all viable cells. However, previous studies have shown that sodium hypochlorite (chlorinated compound) when used at a concentration of 52.5 mg/ml for 1 and 5 min was able to completely remove viable cells of 5 or 7 day (d) old *Salmonella* biofilms (Toté *et al.*, 2010, Wong *et al.*, 2010). The differences in concentration, exposure time (30 s) and age of biofilm (24 and 48 h) could have contributed to the

discrepancies in the results. Similarly, a limited effect of chlorine against *Salmonella* biofilms has also been documented in the previous study (Møretrø *et al.*, 2009). Another study, by Ziech et al. found that chlorinated alkaline reagent (Sanifoam) was more effective than acetic acid in removing biofilms formed by *Salmonella* on poultry processing surfaces (Ziech *et al.*, 2016), and a combination of chlorine, lactic acid and ethanol disinfectants was more effective to reduce *Salmonella* biofilms formed on the stainless-steel surface (Zhang *et al.*, 2017). These findings suggest that blends of acids and/or chlorinated reagents have an additive effect to reduce *Salmonella* biofilms. It remains poorly understood why Product A was not able to completely remove viable biofilm cells in the current study.

Product B contained quaternary ammonium compounds (QAC), and product C is a twin-chain QAC. QAC have powerful disinfectant activity and are more effective against gram-positive than gram-negative bacteria (Hegstad *et al.*, 2010). QAC leads to disruption of the lipid bilayer of the cytoplasmic membrane and outer membrane of gram-negative bacteria leading to leakage of cytoplasmic components and eventually cell lysis (Quinn *et al.*, 2011). In our study, both products B and C, were able to reduce the number of viable biofilm cells of two different ages at both the concentrations. The decrease in viable cell numbers was greater in Product C which is a fourth-generation twin chain quaternary containing didecyl dimethyl ammonium chloride with greater germicidal performance than QACs (QuimiNet, 2012). Møretrø *et al.* (2003) tested three QAC commercial disinfectants against biofilms formed by *Salmonella* strains, isolated from feed industry and found that exposure of QAC for 5 min resulted in the complete reduction of all strains when used at 80% of recommended user concentration. These quaternaries have superior germicidal activity, are low foaming and have a high tolerance to protein loads and hard water. They are recommended for disinfection in the food and beverage industry, given their low toxicity (QuimiNet, 2012).

A study (Ramesh *et al.*, 2002) tested thirteen different disinfectants containing QACs, sodium chlorite (1%) and hypochlorite (0.05%) for two min exposure against the 3 and 4 d

old formed biofilm by the mixture of *S.* Typhimurium, *S.* Thompson, *S.* Berta, *S.* Hadar, and *S.* Johannesburg strains isolated from poultry transport containers and found that sodium chlorite containing disinfectants were more effective than QACs and hypochlorite. It has been reported that increase in the concentration and contact time of disinfectants can increase their effectiveness against *Salmonella* gram-negative bacteria (Wong *et al.*, 2010). In contrast, in our experiment all three products reduced the number of viable biofilm cells, however increasing their concentration did not alter the results significantly. The difference in concentrations, biofilm (24 and 48 h age) formed for short time and short disinfectant exposure time (30 s) may be one of the reasons for this limited effect and future studies by increasing these parameters could be conducted

The results of this study showed that biofilm age was associated with increased resistance to disinfectant treatments. The findings of the present study are in agreement with previous reports that age of biofilm was significantly related to increased resistance to commonly used disinfectants or antimicrobials (Corcoran *et al.*, 2014, Nguyen *et al.*, 2014). In contrast, other studies showed that age of *S.* Typhimurium biofilms was not associated with the increased resistance towards commonly used disinfectants (Wong *et al.*, 2010). The discrepancies in results could be attributed to the diverse experimental conditions, such as product, growth, strains of bacteria, temperature, and device used. There is a paucity of literature on the efficacy of commercial disinfectants against biofilms formed by *S.* Typhimurium isolates recovered from the layer farm environment therefore the result of the present study could not be compared with earlier work.

None of the products in this study were able to remove biofilm cells completely (100%), even at the double recommended user concentration. These findings could be compared with earlier study (Wong *et al.*, 2010) which tested six different compounds (sodium hypochlorite, citric acid, BC, a QAC based disinfectant, chlorhexidine gluconate and ethanol) against 3 d-old biofilm of *S.* Typhimurium strain and found that higher doses of

sodium hypochlorite, chlorhexidine gluconate and ethanol failed to eliminate this bacterium. Cells in biofilm are embedded in self-produced extra cellular matrix components which may act as chemical and mechanical protection against environmental stressors including chemical disinfectants (Scher *et al.*, 2005). As a result, the complete biofilm elimination was not achieved in this study.

In our study, only *S.* Typhimurium isolates were used during biofilm experiments, however, in commercial settings contamination of the farm environment with different serovars of *S. enterica* is common and is of serious concern worldwide (Im *et al.*, 2015). Thus, further studies examining the activity of commercial disinfectant against biofilms formed by multiple *Salmonella* serovars are needed.

Conclusions

In conclusion, our data demonstrated that the commercial disinfectants tested in this study were effective in reducing viable biofilm cells, however none of the product succeeded in eliminating 24 or 48 h old biofilms. In addition, the age of the biofilm was associated with increased resistance to disinfectants tested in this study. These findings may have future implications for use of disinfectants such as required concentration and exposure time in the poultry industry to control biofilm.

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Table 1 *Details of commercial disinfectants used in the study*

Product Name	Composition	Intended use and dose
A	Chlorinated liquid (10% potassium hydroxide + 10% sodium hypochlorite)	Egg shell sanitizer; 1:10, 1:100
B	QAC (100g/l Quaternary Ammonium compound)	Egg shell sanitizer, used as sanitizer in poultry sheds, animal pens, farm equipment; 1:50, 1:100
C	Twin-chain QAC (>= 10 - < 30 % didecyldimethylammonium chloride + < 10% ethanol + < 10% w/w alcohols, C12-14, ethoxylated)	Sanitizer in food processing plants; 1:10

Figure captions

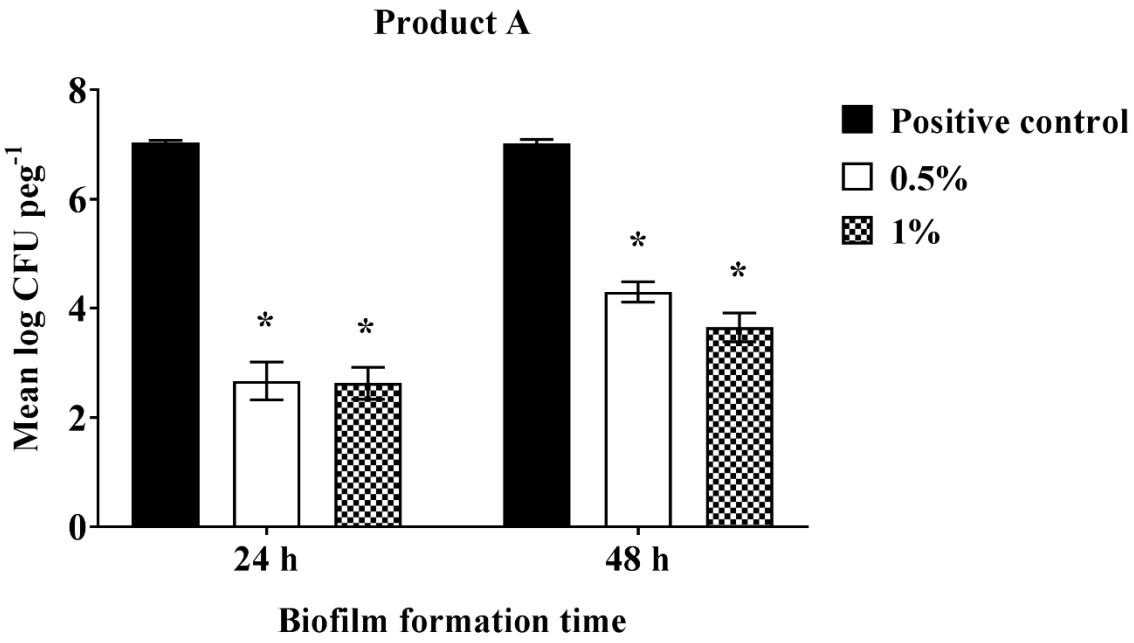

Figure 1 Effect of disinfectant (Product A) on 24 and 48 h old *S.* Typhimurium biofilms. The data are presented as mean log CFU peg^{-1} ± SEM with two replicates for each *S.* Typhimurium isolate (n=12). Asterisks (*) indicate statistically significant differences ($p < 0.05$) in comparison with positive control biofilms for the corresponding time.

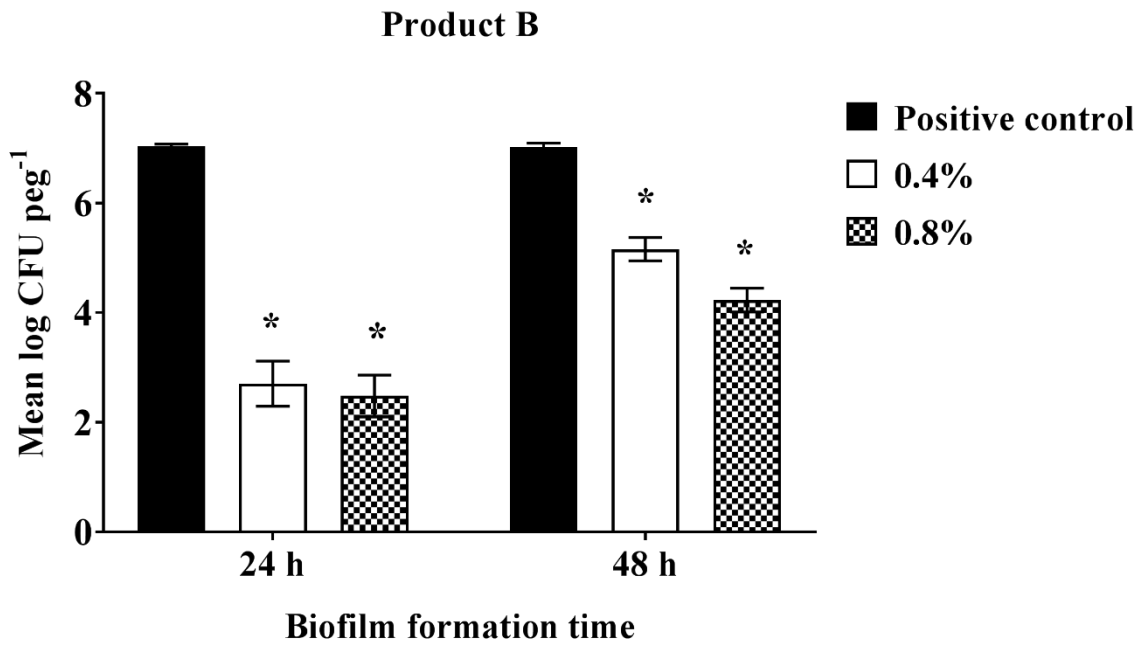

Figure 2 Effect of disinfectant (Product B) on 24 and 48 h old *S.* Typhimurium biofilms.

The data are presented as mean log CFU peg^{-1} ± SEM with two replicates for each *S.* Typhimurium isolate (n=12). Asterisks (*) indicate statistically significant differences ($p < 0.05$) in comparison with positive control biofilms for the corresponding time.

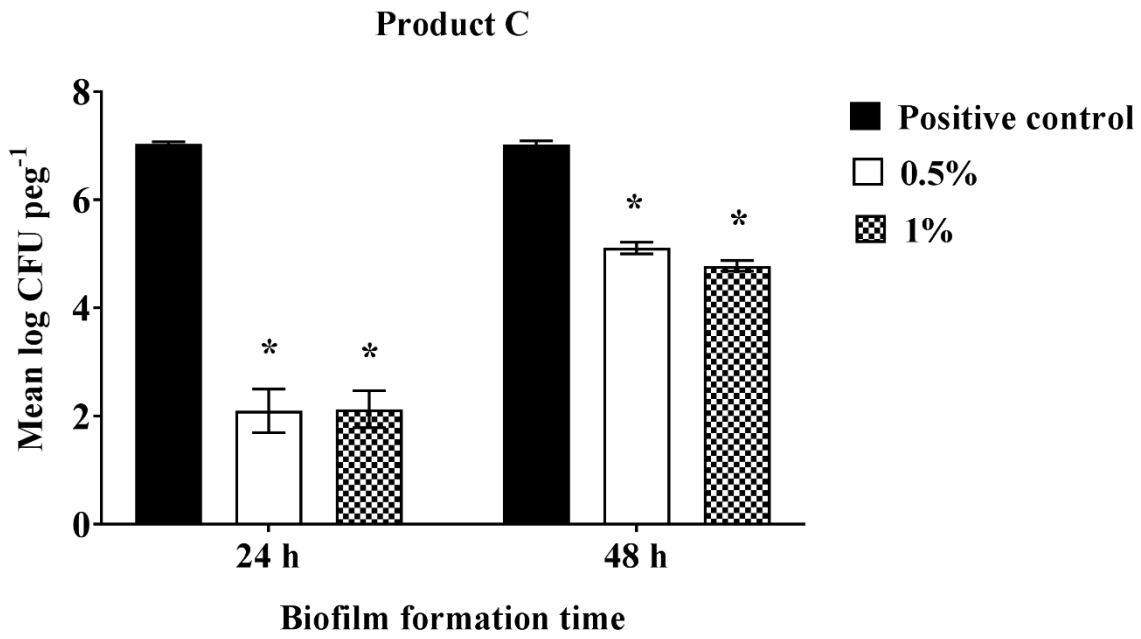

Figure 3 Effect of disinfectant (Product C) on 24 and 48 h old *S.* Typhimurium biofilms.

The data are presented as mean log CFU peg^{-1} ± SEM with two replicates for each *S.* Typhimurium isolate (n=12). Asterisks (*) indicate statistically significant differences ($p < 0.05$) in comparison with positive control biofilms for the corresponding time.

Chapter 6 General Discussion and Conclusions

General Discussion and Conclusions

Gastrointestinal disease caused by nontyphoidal *Salmonella* (NTS) serovars is one of the most commonly identified foodborne illnesses with an estimated 80.3 million cases every year (Majowicz et al. 2010). Chicken meat, egg, and egg based products can become contaminated with NTS and serve as a mechanism of transmission for the bacteria (Gould et al. 2004; Foley & Lynne 2008). In Australia, from 2009 to 2015, the incidence of human salmonellosis has increased with a total of 16,952 notifications (NNDSS, 2016; The OzFoodNet Working group, 2012). Of the 2600 *S. enterica* serovars, *Salmonella* Typhimurium (*S.* Typhimurium) is the serovar most commonly isolated during egg associated foodborne outbreaks in Australia (The OzFoodNet Working group, 2012). Despite on-farm control strategies, *S.* Typhimurium remains a significant food safety concern within the Australian egg industry (Moffatt et al. 2016). Therefore, understanding the shedding of *S.* Typhimurium and egg contamination in laying hens is of significant importance to the industry.

S. Typhimurium infected adult hens do not typically exhibit symptoms of disease and can act as carriers with intermittent and prolonged shedding in faeces, potentially resulting in egg contamination (Van Immerseel et al. 2005; Coburn et al. 2007; Grassl & Finlay 2008; Filho et al. 2009; Dougan et al. 2011). Such carrier hens are natural reservoirs and can introduce pathogens into the food chain. To date, the majority of work on *S.* Typhimurium pathogenesis in poultry has been performed using broilers or breeders for a relatively short period (Bailey et al. 2007a; Berghaus et al. 2011). There is a gap in current knowledge related to long-term faecal shedding of *S.* Typhimurium and its relation to egg contamination in single and mixed infection group specially at the onset of lay. Previous study suggested that the combination of live attenuated *S.* Typhimurium and an autogenous inactivated multivalent vaccine when used in experimental laboratory conditions has provided protection against *S.* Typhimurium challenge in layer hens (Groves et al. 2016).

However, there is a lack of research investigating the effectiveness of such farm interventions in reducing egg contamination in Australian field conditions. Therefore, the experiments described in this thesis were conducted with the following objectives: (i) to correlate faecal shedding and egg contamination with host immune responses to infection (*S.* Typhimurium (single) and *S.* Typhimurium and *S.* Mbandaka (mixed)) at the onset of lay as well as to characterise persistence of *Salmonella* infection in peripheral organs; (ii)to understand the level of vaccine colonisation (Vaxsafe® ST1), shedding and immune response generated after administration in pullets and to evaluate the effectiveness of this vaccine during early lay against egg contamination in naturally infected egg laying cage flocks. The final aim was to test anti-biofilm efficacy of commercially available disinfectants against *S.* Typhimurium isolates from layer farms at different concentrations.

To achieve these aims the first experiment was designed to further our understanding of the dynamics of *S.* Typhimurium (single) and *S.* Typhimurium and *S.* Mbandaka (mixed) shedding in hens. Coinfection of layer hens with different *Salmonella* serovars has been shown to aggravate disease in infected birds (Pulido-Landinez et al. 2014). In this study, increased most probable number (MPN/g) count was observed in *S.* Typhimurium-infected group at the week 6 pi. (onset of lay) compared to co-infected group and it was postulated that this increase could be related to physiological stress induced by the onset of lay. Similarly, Gole et al. (2014a) during a lonitudinal study investigated *Salmonella* shedding in a single age commercial egg layer flock and concluded that at the onset of lay (18 weeks of age) the shedding of *Salmonella* was peaked when compared with later 24 and 30 weeks sampling. However, it remained unclear why MPN/g count was lower in the co-infected group. A recent study in laying hens has suggested that coinfection with *S.* Kentucky (a non-pathogenic *Salmonella* serotype) has reduced the recovery and colonisation of *S.* Enteritidis (pathogenic serotype) in internal organs highlighting that various serovars effect the ecology of pathogenic *Salmonella* on layer farm (Guard et al. 2015).

Physiological stressors, such as the onset of lay may lead to an increase in faecal shedding of *Salmonella* (Nakamura et al. 1994). Results of previous field (Gole et al. 2014a) and pen trials (Okamura et al. 2010) indicate that the onset of lay is linked with increased *Salmonella* shedding and egg contamination. It was hypothesised that stress at point-of-lay could induce *S.* Typhimurium shedding. In this study, no significant correlation ($r^2 = -0.036$, $p = 0.699$) was observed between the mean FCM concentration and *Salmonella* shedding (MPN counts) in single or co-infected birds. A weak positive correlation ($r^2 = 0.26$, $p = 0.02$), however, was observed in birds infected with only *S.* Typhimurium. These observations are in agreement with a recent field study (Gole et al. 2017). In the present study, no linear correlation was observed between the *Salmonella* MPN count in faeces and eggshell contamination in both single and co-infected birds which is in agreement with a related study (Pande et al. 2016). Eggshell contamination in both single and co-infected groups was similar. This finding is in contrast to results obtained from a laboratory trial which found eggshells were negative for *S.* Typhimurium (Okamura et al. 2001). This difference in eggshell contamination may be due to differences in experimental conditions such as age, dose and route of infection. Okamura et al. (2001) intravenously infected 34 weeks old laying hens with 10^6 CFU units of *S.* Typhimurium; however, in our study 14 weeks old hens were orally infected with either of *S.* Typhimurium DT9 or a combination containing equal amounts of both *S.* Typhimurium DT9 and *S.* Mbandaka. The percentage of eggshell contamination with *S.* Typhimurium was highest in both groups at week 6 pi. which could be attributed to the onset of lay. This shedding could be associated with the stress induced by the onset of lay that could negatively impact immunity (El-Lethey et al. 2003; Humphrey, 2006), consequently resulting in higher egg contamination.

Salmonella was not detected in internal egg contents of either infection treatment group at any point during this experiment, and these results are consistent with the study where egg

contents laid by *S.* Typhimurium infected hens were negative for bacteria (Okamura et al. 2010).

In the *S.* Typhimurium-infected treatment group, increased levels of FCM concentration, antibody titres and bacterial shedding (as detected by MPN method) at week 6 pi. (onset of lay) supports the theory that onset of lay can induce *Salmonella* shedding (Verbrugghe et al. 2016). It has been speculated that *S.* Typhimurium being a virulent serovar, is more effective in invading and inducing a higher systemic immune response thus further aggravating the stress of the onset of lay in birds (Barrow et al. 1988). The stress of onset of lay could lead to immune cell dysfunction and thus increases the bacterial shedding (Humphrey, 2006).

The antibody response between *S.* Typhimurium and *S.* Typhimurium and *S.* Mbanadka coinfected hens was also investigated. A significant effect of treatment was detected between the experimental groups ($p \leq 0.01$) and birds were found seropositive until the end of the trial, but the humoral immune response did not result in complete clearance of *Salmonella* spp. It is also important to note that the antibody response contributes to the removal of extracellular bacteria, intracellular bacteria can persist in the host thus cell-mediated immune response is essential for clearance of *S.* Typhimurium (Chappell et al. 2009). T cells play an essential role in developing immunity against *Salmonella* infection. T cells not only provide clearance of primary infection but also ensure resistance to successive infection (Pham & McSorley 2015). On interaction with host cells, the antibody produced from B cells generate protective immunity against ongoing *Salmonella* infection. For instance, activation and differentiation of $CD4^+$ T cells occur in response to cytokines expressed by antigen presenting cells. Further cytokines released by $CD4^+$ T cells causes recruitment and activation of phagocytes and stimulate B cells (Cummings et al. 2009). B cells require additional secondary signals for antigen presentation after interaction and proliferation of *Salmonella*-specific T cells with the dendritic cells of the lymphoid organ. This is further suggestive of a significant role of $CD4^+$ T cells and contributory role of $CD8^+$ T cells and B

cells in acquired immunity to *Salmonella* infection and its clearance (Pham & McSorley 2015). Cell-mediated immune response which is triggered through induction of protective cytokines is important for protection against *Salmonella* infections in chickens. Future studies on induction of protective cytokines through quantitative analysis of cytokine mRNA by real-time PCR and flow cytometric analysis of CD 4 & CD 8 T-cells to measure cell-mediated immune response during *S.* Typhimurium infection is a prerequisite for the better understanding of adaptive immunity in the chicken model.

At 16-weeks post-infection, spleen, liver, and caecal tonsils from all euthanised hens were found positive for the *Salmonella*. These results demonstrated that infection of adult birds could result in continued harbouring of *S.* Typhimurium and intermittent faecal shedding with persistence in internal organs despite high levels of humoral immune response. However, it remains unclear that despite higher antibody response than control group *S.* Typhimurium persisted in the hens, and further studies on host-pathogen interactions and *S.* Typhimurium dissemination throughout internal organs in the laying hens during *S.* Typhimurium infections are needed. This would allow surveillance of the specific cell types such as of macrophages, heterophils, B and T-lymphocytes where *S.* Typhimurium are residing within the chickens.

Many intervention strategies have been investigated to control *Salmonella* contamination on commercial layer farms. On-farm preventive approaches include flock testing, sanitation, biosecurity and vaccination (Galis et al. 2013). However, these strategies do not provide complete protection, as they are unable to prevent *S.* Typhimurium shedding completely. Vaccination has been widely used to reduce the *Salmonella* shedding in poultry and subsequently the transmission to humans. However, vaccination of hens has had a varying degree of success against *Salmonella* infection, depending on the vaccine and the *Salmonella* serotype. Berghaus et al. (2011) found that multivalent vaccine with inactivated *S.* Typhimurium, *S.* Enteritidis and *S.* Kentucky increased the immunity of breeder hens and

reduced *Salmonella* prevalence in their broiler progeny, but did not reduce *Salmonella* in the layer farm environment. In another study, vaccination did not affect the shedding of *S.* Enteritidis and *S.* Typhimurium in hens; however, there was a decrease in the proportion of *S.* Enteritidis and *S.* Typhimurium positive eggshells (Arnold et al. 2014). Much of the work with *Salmonella* vaccine against non-typhoidal *Salmonella* infections and egg contamination in laying hens have been conducted for *S.* Enteritidis serovar (De Buck et al. 2005; Gantois et al. 2006; Kilroy et al. 2016). There is lack of data demonstrating the efficacy of *S.* Typhimurium vaccine on reducing faecal shedding and ultimately bacterial egg contamination under farm conditions. Hence, a field experiment was conducted to test the effectiveness of commercially available Vaxsafe® ST vaccine in laying hens against *S.* Typhimurium infection in field conditions.

For a *Salmonella* vaccine to be effective, it must retain its colonising and invasion ability to ensure that selected antigens remain accessible to the host defence mechanism for prolonged periods (Alderton et al. 1991). Isolation by culture, conventional PCR and qPCR from the current study suggested that STM-1 successfully colonised the chicken gut indicating that Vaxsafe® ST has a potential as an antigen delivery system and vaccination with STM-1 could help in reduction of wild type *S.* Typhimurium infection; however, this vaccine did not induce a systemic antibody response until after parenteral administration at 12 weeks.

For the detection of STM-1 strain from field samples, the conventional PCR assay was more sensitive than culture or qPCR methods. The difference in sensitivity between conventional PCR and qPCR could be attributed to the total reaction volume used in the assay because higher reaction volumes can dilute the inhibitory factors present in template DNA (Bastien et al. 2008).

Conventional PCR was found more sensitive than culture method for the detection of STM-1, and there was a minor agreement (kappa coefficient 0.263) between culture and conventional PCR. Given that PCR assay can detect the DNA of nonviable bacteria, higher

sensitivity of this assay in comparison to culture is not surprising. At week 13, the load of STM-1 in litter sample was higher than week 4 and 8, and this change took place because of the gradual build-up of viable or non-viable STM-1 in the litter. Viable wild strains of *Salmonella* were not detected in this study hence the protective effect of the vaccine against wild-type *Salmonella* could not be assessed during rearing. Future studies on shedding and persistence of STM-1 vaccine strain in field environment could be performed by recruiting several farms which would be useful to determine the antigenic potential and efficacy of STM-1vaccine strain.

In 2006, the European Union (EU) approved legislation with the aim to reduce the prevalence of *S.* Enteritidis and *S.* Typhimurium in layer flocks. As a part of this legislation, the layer flocks with *Salmonella* prevalence above 10%, were required to vaccinate flocks 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 lower compared to 7.95% which was observed during EU baseline survey conducted between 2004 and 2005 (Snow et al. 2010). Field and experimental studies have shown the efficacy of vaccination programmes for the reduction of *S.* Enteritidis contamination of poultry products in the European Union and UK (DEFRA, 2008; European Food Safety Authority, 2010; Arnold et al. 2014). Egg borne salmonellosis caused by *S.* Typhimurium is a significant public health issue in Australia. Previous study demonstrated that the live attenuated *S.* Typhimurium vaccination provided protection against *S.* Typhimurium challenge in the layer flocks (Groves et al. 2016). However, little is known about the efficacy of *S.* Typhimurium vaccine in reducing faecal shedding and egg contamination in naturally infected flocks in Australia. To fulfil this gap the next experiment has been performed to test *S.* Typhimurium live vaccine in Australian layer flocks. A field trial was designed and performed with an objective to evaluate the effectiveness of a live vaccine (Vaxsafe® ST) during early lay in production flocks that were

naturally infected with *S.* Typhimurium. Live *Salmonella* vaccines are better than inactivated vaccines because of their effectiveness against both intestinal and systemic infection. Live vaccines induce humoral, mucosal and cell-mediated immune responses (Zhang-Barber et al. 1999). Live *S.* Typhimurium vaccines could have better and longer protective effect that helps in the development of classical immunity, provide protection against invading pathogens by competitive exclusion effect (Berchieri et al. 1990).

Most poultry vaccines are developed to prevent disease, but as *S.* Typhimurium infection in adult hens does not cause clinical symptoms, the rationale underlying vaccination is to reduce shedding. Bailey et al. (2007b) showed that vaccination increases the *Salmonella*-specific titre of vaccinated breeders. Similarly, this study demonstrated that antibody titre in the vaccinated group was significantly higher than the unvaccinated group at all sampling points. *S.* Typhimurium wild-type being a pathogenic serotype, is more invasive and likely to generate an intense immune reaction (Barrow et al. 1988).

Beal et al. (2006) showed that humoral mediated immune response was not essential for clearance of *S.* Typhimurium from the gut and further suggested that an effective vaccine should activate both cellular and humoral immune response. In the present trial, there was an increased antibody response in the vaccinated group after parenteral administration which demonstrates a secondary response with a strong IgG immune response occurred. This indicates that live vaccine has an effect on the humoral response, although cell-mediated immunity was not evaluated.

A non-significant difference in the prevalence of *S.* Typhimurium between vaccinated and unvaccinated groups suggested that Vaxsafe® ST is not effective in reducing the *S.* Typhimurium faecal shedding which is in accordance to Tan et al. (1997) and they found that *aroA* deletion mutant *S.* Typhimurium vaccine strain failed to reduce frequency of faecal shedding of wild-type *S.* Typhimurium when inoculated with 10^8 CFU under laboratory conditions.

In this trial, the environmental samples such as dust swab, egg belt and shoe covers were positive for *S.* Typhimurium at all sampling points. It was not possible to conclude the effect of the STM-1 vaccine on the level of egg contamination because only 0.5% eggshell was *S.* Typhimurium culture positive among the samples collected from the vaccinated group. Given that *S.* Typhimurium was detected in dust over several weeks, regular cleaning of the shed is essential because a small reduction of *Salmonella* in the environment could potentially reduce the amount of *Salmonella* in poultry products (Young et al. 2007). All internal egg contents laid were found negative for *S.* Typhimurium detected by culture method. This highlights that vertical transmission through *S.* Typhimurium is not the primary route for egg contamination and is accordance with Pande et al. (2016).

Eggs were collected directly from the egg belt which was contaminated with wild-type *Salmonella* spp. Contact with the egg belt, therefore, could be the source of eggshell contamination; however, it was interesting to note that every egg (except for one) collected from contaminated egg belt was *Salmonella* negative.

Live vaccines may not be very useful in multi-age sheds because older *Salmonella*-infected birds in the shed may act as a continuous source of infection to the newly arrived pullets (vaccinated pullets in this trial); however, future field studies in natural farm conditions are required to investigate effect of Vaxsafe® ST vaccine from day-old hens to eighty weeks till end of their life in *Salmonella*-infected laying flocks. However, such findings are valuable for the understanding efficacy of live vaccine on *Salmonella* shedding and egg contamination, the successes of such studies also depend on the willingness of producers to participate.

QPCR data suggested that the level of wild-type *Salmonella* load in the commercial farms that were sampled was relatively low; hence it could be concluded that these birds received low challenge during the trial. Future studies with a high dose of *Salmonella* are needed to know the efficacy of the STM-1 vaccine in field conditions.

Usage of Vaxsafe® ST vaccine as a method for treatment and control of *S.* Typhimurium infection alone is not ideal for egg laying birds, but a combination of different practices such as good sanitation, biosecurity, flock and farm management practices is needed. This vaccine trial was performed only on two commercial cage layer farms, recruitment of multiple farms would provide a better understanding of the efficacy of Vaxsafe® ST against *S.* Typhimurium infection.

Many post-harvest techniques have been employed to reduce the risk of *Salmonella* contamination of eggs and egg-borne human salmonellosis. Cleaning and disinfection of farm and egg grading equipment are routinely preformed. Some *Salmonella* serovars such as *S.* Typhimurium can remain on surfaces and equipment used for handling washing, transport and storage of eggs; hence, routine cleaning and sanitation may fail to eradicate *S.* Typhimurium from such surfaces (McKee et al. 2008). *Salmonella* readily forms biofilms and persists on food contact surfaces as part of their adaptation to stress (Steenackers et al. 2012) and extracellular adhesion capabilities (Rodrigues et al. 2011). Thus, biofilm formation is likely to be responsible for the long-term persistence of *Salmonella* on surfaces which could act as a further source of egg contamination. Several studies have reported biofilm formation and the survival of bacteria on both biotic and abiotic surfaces, and their susceptibility to disinfectants (Larsen et al. 2014; Park & Chen 2015). However, there is limited information on the effect of disinfectants against *S.* Typhimurium biofilms. To fulfil this gap in current knowledge, this experiment was designed to test the anti-biofilm efficacy of commercially available disinfectants at different concentrations against *Salmonella* Typhimurium isolates from layer farms.

Three commercial disinfectants (Product A, B and C: containing a chlorinated compound, quaternary ammonium compounds (QAC), and twin-chain QAC; respectively) were not able to eliminate the biofilm cells. The effects of disinfectants are highly concentration dependent (Russell & McDonnell 2000), and it has been shown that high concentrations of disinfectants

are able to reduce more viable cells (Møretrø et al. 2009; Steenackers et al. 2012). In contrast, in this study, even with a high concentration of disinfectants (twice the recommended dose), complete elimination of biofilm cells was not achieved. Concentration is one of the critical factors in regulating the disinfectant activity and means of resistance of biocidal-type agents. Inappropriate dilutions of prepared products could increase the chances of cross-resistance among less sensitive bacteria. It is essential to use an accurate concentration of disinfectants for removal of biofilms; also, it is important to note that disinfectant concentration is only one of the many factors which should be critically examined in determining the actual antibiofilm activity of formulated products.

The type of disinfectants can also influence the susceptibility of *Salmonella* biofilms in suspension or on the surface, and it has been shown that disinfectants are more effective against *Salmonella* in the suspension than surface test (Møretrø et al. 2009). Product A used was a chlorinated compound, studies have shown that sodium hypochlorite, when used at a concentration of 50 mg/ml for one and five min. was able to completely remove five or seven days old *Salmonella* suspension biofilms (Toté et al. 2010; Wong et al. 2010). It remains unclear as to why Product A was not able to completely remove viable suspension biofilm cells in this study.

Product B and C tested contained quaternary ammonium compounds (QAC), and a twin-chain QAC, respectively. QAC has strong disinfectant activity and are more effective against gram-positive than gram-negative bacteria (Hegstad et al. 2010). In our study, the decrease in viable cell numbers was greater with Product C which is a fourth-generation twin chain quaternaries containing didecyl dimethyl ammonium chloride with greater germicidal performance than QACs (QuimiNet, 2012). It has been reported that increase in the concentration and contact time of disinfectants can increase their effectiveness against *Salmonella* (Wong et al. 2010). In contrast, in our experiment all three products reduced the number of viable biofilm cells but, increasing their concentration did not alter the results

significantly. The difference in concentrations, biofilm (24 and 48 h age) formed for short time and short disinfectant exposure time (30 s) may be one of the reasons for this effect.

This study showed that 48 h old biofilms were more resistant to commercial disinfectants as compared 24 h biofilms suggesting that biofilm age was associated with increased resistance to disinfectants. Cells in a biofilm are embedded in self-produced extracellular matrix components which may protect against environmental stressors including chemical disinfectants (Scher et al. 2005). As a result, the complete biofilm elimination was not achieved in this study. The findings of the present study are in agreement with previous reports that age of biofilm was significantly related to increased resistance to commonly used disinfectants or antimicrobials (Corcoran et al. 2014; Nguyen et al. 2014).

In our study, only *S.* Typhimurium isolates were used during biofilm experiments, however, in commercial settings contamination of the farm environment with different serovars of *S. enterica* is common (Im et al. 2015). Hence, further studies on the antibiofilm activity of disinfectants against *Salmonella* serovars other than Typhimurium are necessary. The incomplete removal of biofilms could potentially favour the bacterial persistence in a food chain. During this experiment, the short exposure of chlorinated, quaternary ammonium compounds and twin-chain quaternary ammonium compounds at 0.4, 0.5, 0.8, and 1% concentrations in experimental conditions were unable to remove *S.* Typhimurium biofilms. However, further experiments are necessary to investigate the extended exposure time of disinfectants in laboratory and field settings.

In summary, this study has demonstrated that adult layer hens infected with *S.* Typhimurium become persistently infected and intermittently shed bacteria over a prolonged period. Intermittent faecal shedding of *S.* Typhimurium is a risk factor linked with the contamination of eggs. Vaxsafe® ST vaccine was not effective in reducing the *S.* Typhimurium faecal shedding and egg contamination in production flocks. This study reinforced that vaccination along with other biosecurity measures in the form of flock and farm management should be

used for the effective control of *S.* Typhimurium infections in laying hens. The disinfectants tested were not able to eliminate the *S.* Typhimurium biofilm cells; however, further regular research and scientific data relating the basis of *S.* Typhimurium biofilm formation, and persistence within the layer farm to help in the management of *S.* Typhimurium is needed. Overall, the results of these studies could be helpful for developing strategies for risk management programs to control *S.* Typhimurium infections in laying hens.

Future Research

A long-term *S.* Typhimurium challenge studies are needed to investigate host cellular immune response and *S.* Typhimurium dissemination throughout internal organs in the laying hens. Our findings have demonstrated that *Salmonella* was not cleared from infected birds over a 16-week period and internal organ colonization could serve as a reservoir for continued bacterial shedding and egg contamination. Surveillance of the specific cell types using flow cytometry for cell counting or cell sorting of macrophages, heterophils, B-lymphocyte, CD4, CD8 cells and γδ T-lymphocytes where *S.* Typhimurium are residing would be useful to study the host immune response. Further, after isolation of infected phagocytic cells from challenged layers, the changes in the specific proinflammatory cytokines and chemotactic chemokines such as interleukin (IL) 1β, IL6 or IL8, Th1 cytokines such as IFN-γ and Th17 cytokines such as IL17 and IL22 after *S.* Typhimurium infection in laying hens would be helpful to understand the role of T cells and cell-mediated immunity in infected hens. Since T lymphocytes represent the essential component of adaptive immunity, future work must continue to use the available techniques to screen functional properties of these cells.

This study was conducted to monitor IgG antibody response in the *S.* Typhimurium infected hens. Since *S.* Typhimurium is primarily restricted to the intestinal mucosa and IgA antibodies are most likely to protect mucosal surfaces from the invading pathogens, further

investigation to measure *S.* Typhimurium IgA ELISA titres in *S.* Typhimurium infected hen is required.

Vaccination of poultry is one of the useful control measures to control *S.* Typhimurium shedding and egg contamination in laying hens. In this study, the efficacy of Vaxsafe® ST was studied on naturally infected caged layer farms; birds in field conditions could be exposed to a low dose *S.* Typhimurium; hence there is a need to recruit a larger number of egg farms in future research to allow valid statistical analysis. *S.* Typhimurium infected hens harbour bacteria in the visceral organs, intestinal tract and act as a subclinical carrier. When stressed, birds start shedding these bacteria. Further work is required to generate an efficient vaccine strain that will reduce shedding of *Salmonella* in stressed hens and ultimately reduce egg contamination. Previous studies formulating safe vaccine candidates such as *S.* Enteritidis ghost vaccines has reduced the bacterial counts in internal organs of the chickens (Jawale & Lee 2014). However, *Salmonella* is a commensal organism in poultry and can survive well in the environment. Hence, vaccination is "not an ultimate intervention" for reduction of *S.* Typhimurium because of the complexities involved in achieving control. Therefore, implementation of several interventions strategies in the form of biosecurity along with farm and flock management is essential.

Salmonella homologous and heterologous challenge is very low in field conditions, and it moves slowly in the flocks (Lister & Barrow 2008), as multiple *Salmonella* serovars with different antigenic structure are prevalent in field conditions, vaccine that provides cross-protection against different serovars is needed. Few rough mutants of *S. enterica* have shown to be immunogenic and relatively defensive against heterologous *Salmonella* serovar challenge in a murine model (Nagy et al., 2008). Thus, strains with partial or conditional suppression of O-antigen might achieve both increased cross-reactivity as the core region of surface lipopolysaccharide is mainly preserved in *S. enterica* and, if used as a live vaccine, a suitable degree of attenuation could be achieved. This research on immune responses to *S.*

Typhimurium infection routinely used in murine models can be used in laying hens so that one can understand the mode of action of immunity and it would be helpful in the development of safe and effective *S.* Typhimurium layer vaccine. In the present study, antibiofilm efficacy for *S*. Typhimurium isolates was tested only on 24, and 48 h old formed biofilms against three commercially available disinfectants with an exposure time of half a minute. Only twelve isolates of *S.* Typhimurium from layer farms were tested during this study, further studies with the multiple isolates to compare susceptibility between *S.* Typhimurium biofilms can be designed. Contact exposure time longer than 30 seconds may eliminate the *S.* Typhimurium biofilm for these disinfectants, but this was not examined in this study and further studies with an increased exposure time of 1 and 5 minutes could be conducted. Future studies on the susceptibility of *S.* Typhimurium biofilms at different ages 3, 5 and 7 days to different concentrations of disinfectants could also be investigated. Such studies would provide a better understanding of antibiofilm efficacy against commercially available disinfectants and would be helpful in the development of control measures to reduce subsequent egg-related outbreaks. Moreover, further research to understand the basis of *S.* Typhimurium biofilm formation in shed environment and in supply chain will help in the management of this organism.

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