Studies on Salmonella Enterica spp Isolated From Egg Farm Environment

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

A Thesis submitted for the fulfilment of the requirements of
the Doctor of Philosophy

THE UNIVERSITY
of ADELAIDE

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The University of Adelaide
Adelaide, South Australia, Australia
August 2016
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Thesis Abstract

Infection with *Salmonella* spp is among the most common causes of foodborne outbreaks of human gastrointestinal disease. Humans acquire *Salmonella* through the consumption of contaminated food items, including eggs and undercooked egg products. There are over 2500 *Salmonella enterica* serovars but globally, *Salmonella* Enteritidis and *Salmonella* Typhimurium are responsible for causing the majority of human disease. *S*. Enteritidis is, however, not endemic in Australia; strains of *S*. Typhimurium have filled this niche and are frequently isolated during cases of egg related foodborne illness. Over the last decade, cases of salmonellosis have increased, representing a significant public health issue. The experiments presented in this thesis were designed to investigate aspects of antimicrobial resistance, layer hen infection, biofilm forming ability of *Salmonella* spp on eggshell and the efficacy of organic acids on controlling *Salmonella* biofilms.

Antimicrobial resistance was characterised for multiple *Salmonella* isolated from the layer hen environment. The majority of *Salmonella* isolates (91.72%; 133/145) were susceptible to all antimicrobials tested. This finding indicates that there is minimal public health risk associated with emergence of antibiotic resistant *Salmonella* within the Australian layer industry.

An infection trial investigated the ability of *S*. Typhimurium to colonise reproductive organs and contaminate developing eggs. Intermittent but persistent faecal shedding of *Salmonella* after oral infection was observed for 15 weeks post infection. Further, *S*. Typhimurium caused eggshell contamination and colonised the reproductive tissue however, *S*. Typhimurium was not isolated from the internal egg contents. These findings indicate that horizontal transmission through contaminated faeces is the main route of egg contamination with *S*. Typhimurium in laying hens.
Biofilm formation was dependent on both temperature and serovar. At ambient temperature, *Salmonella* isolates attached and formed biofilm on eggshells however, differences between strains of *Salmonella* serovars were evident in eggshell biofilm formation.

The anti-bacterial and anti-biofilm activity of commercial organic acid products against *S. enterica* isolates was further investigated. The result of this experiment showed no significant differences between isolates of representative *Salmonella* serovars in respect of inhibitory and bactericidal dilutions of each product. Two out of three commercial organic acid products tested in this study significantly reduced viable cells from 3 and 5 day old biofilms in a dose and time dependent manner. None of the tested products completely eliminated biofilm cells under any of the tested conditions.

In conclusion, the results presented here improve our understanding of the risks of egg associated *S. enterica* spp. Knowledge gained from these experiments could contribute to the development of improved guidelines for food safety and public health in Australia.
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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Vivek Vinayakrao Pande
Acknowledgements

“I may not have gone where I intended to go, but I think I have ended up where I intended to be” —Douglas Adams

First and foremost, I would like to express my sincere gratitude to my supervisor Dr Kapil Chousalkar, for giving me an opportunity to pursue my PhD. Kapil has been a brilliant mentor and without his continuous guidance, this work would not have been possible. Kapil, you believed in me and my ability to complete this daunting task. I shall remain in your debt throughout my life for making my dream of PhD come true.

I would like to express my profound indebtedness to my co-supervisor Dr Andrea McWhorter, for her encouragement, critical evaluation, valuable suggestions, and excellent co-operation throughout my thesis.

My special thanks to Dr Gordon Howarth, Dr Milton McAllister, Dr Darren Trott, Dr Ryan O'Handley, Dr Bec Forder, Dr Farhid Hemmatzadeh, Dr Manouchehr Khazandi, and Dr Charles Caraguel for the guidance during my research work.

I would like to acknowledge The University of Adelaide for providing the Postgraduate Research Scholarship to pursue my higher degree research.

I am thankful to Angela Mills, Marie Kozulic, Jeremy Winchester, and all other staff of Library at Roseworthy campus, The University of Adelaide for their help with Adobe and EndNote.

Words are few to owe my special and heartiest thanks to my friends for their moral support and refreshing company; Vaibhav Gole, Pardeep Sharma, Talia Moyle, Sasikumar, Vinod Kadam, Lesley Menzel, Rebecca Dunbar, David Purdie, Saad Gilani, Rebecca Devon, Janet Pandi, Michael Dom, Jane McNicholl, Noor Haliza Hasan, Sugiyono Saputra, Saad Gilani, Rebecca Abraham, Sam Abraham, Amanda Kidsley and Elizabeth Hickey.
I do not have words to express my sincere thanks to Leena, Mahesh, Jaya, and Vaibhav for the wonderful family support. All of you have made my journey of PhD very joyful and a memorable one.

My special thanks to Hasmukh and Jignya Patel for their wonderful family support during critical periods of thesis writing.

I would also like to thank Dr Jaswinder Kaur and Dr Emad Ehsan from Gawler medical clinic for providing the best health care service to my family during PhD.

A special thanks to all my mentors and my professional colleagues in India for their tremendous motivation; Dr Kurkure, Dr Kalorey, Dr Vishal Pavitrakar, Dr Jaiprakash Bhelonde, Dr Pankaj Shelar, Dr Swapnil Chilgunde, Anand Bokare, Dr Amol Fiske, and Dr Jitendra Ramgaokar. My heartfelt thanks to Dr Umesh Wankhade, Dr Pradyumna Baviskar and Dr Nitin Kamble for continued encouragement during my research work.

A big thank you to my family members who have always been supportive and proud of what I am today. My special thanks to my wife Poonam, my beautiful daughters Unnati and Aastha, my mother, father, brother and his wife for their great understanding, sacrifice, unconditional love and belief in my abilities. My all-family members have been my real pillars of emotional strength while pursuing this degree. My special thanks to my in-laws, Nilesh Tayde, Rucha Kangte and Deepak Kangte for great support and love during my stay in Australia.

Finally, thanks to God for the joy and opportunity to worship him through learning more about this small part of his creation.
List of Publications


Conference Oral Presentations

**Vivek V. Pande**, Andrea R. McWhorter, Kapil K. Chousalkar. *Salmonella enterica* spp isolated from egg farm environments are able to form biofilm on the eggshell. The Australian Society for Microbiology annual scientific meeting, 3-6 July 2016, Perth, Australia.

1.1 Introduction to thesis

_Salmonella enterica subsp. enterica_ is a common cause of foodborne human gastroenteritis, a disease characterised by gut inflammation and diarrhoea (Winter et al., 2010). Consumption of contaminated food products such as pork, meat, egg and egg related products are among the most common sources of _Salmonella_ infection (Hur et al., 2012). It is estimated that worldwide, 93.9 million cases of gastroenteritis and 155,000 deaths are caused annually by _Salmonella_ spp (Majowicz et al., 2010). In Australia, consumption of undercooked egg and egg products are frequently implicated in outbreaks of human salmonellosis (OzFoodNet Working Group, 2012a, 2015b). Over the past decade, human cases of _Salmonella_ infection have increased in Australia. A total of 11,992 _Salmonella_ cases were reported in 2010, representing 53.7 cases per 100,000 people. This is higher in comparison to the previous 5 years, with an average infection rate of 41.8 cases per 100,000 people (OzFoodNet Working Group, 2012a).

Non-typhoidal _Salmonella_ infections are asymptomatic in adult birds (Mead & Barrow, 1990; Revolledo et al., 2006). In humans, infection with non-typhoidal _Salmonella_ is self-limiting and cause mild gastroenteritis however, severe infection is common in elderly and immunocompromised individuals (Parry & Threlfall, 2008). Generally, treatment for self-limiting gastroenteritis is not required however, in case of severe and systemic human salmonellosis, antimicrobials such as fluoroquinolones and extended-spectrum cephalosporins are commonly used (Parry & Threlfall, 2008). The use of antimicrobial agents in the prevention and treatment of many infectious diseases and as a growth promoter is a common practice both in veterinary and human medicine (Hur et al., 2012). Indiscriminate use of antibiotics in both animal and human populations has led to an emergence of multidrug resistant _Salmonella_ strains (Anjum et al., 2011; Hur et al., 2012). The emergence and dissemination of antibiotic resistance to _Salmonella_ is of significant global concern for both animal and public health (Hur et al., 2012).
Globally, *Salmonella* Enteritidis is the dominant serovar in commercial poultry industries, is often isolated from eggs, and is frequently associated with egg related food poisoning in humans (Foley et al., 2011). *S.* Enteritidis, however, is not endemic in commercial Australian poultry flocks (OzFoodNet Working Group, 2009). This niche has been filled by *S.* Typhimurium, which is most frequently linked with egg related foodborne outbreaks (OzFoodNet Working Group, 2009). Foodborne outbreaks associated with raw eggs or egg related products is a major public health issue and highlights the importance of understanding egg contamination in laying hens. One of the mechanisms *Salmonella* spp utilizes for survival in harsh physical and chemical environments is by forming biofilms (Costerton et al., 1999).

*Salmonella* spp are able to attach and form biofilm on a large number of abiotic surfaces including stainless steel, plastics, cement, rubber, and glass (Steenackers et al., 2012). Biofilm is a community of interacting bacterial cells attached to biotic or abiotic surfaces, embedded in self-produced extracellular polymeric matrix (Costerton et al., 1999). Bacteria in a biofilm remain viable and may lead to cross contamination of a variety of food products. *Salmonella* within a biofilm display higher resistance to environmental stressors, antibiotics and disinfectants, compared to planktonic counterparts, thus making the eradication of this bacteria extremely difficult from surfaces commonly used in food and poultry industry (Joseph et al., 2001; Steenackers et al., 2012). The ability of *Salmonella* to form a biofilm represents a significant public health risk for many industries including those involved in food production and processing (Shi & Zhu, 2009). Food safety and public health impacts associated with biofilm forming foodborne pathogens emphasises the importance of understanding eggshell biofilm formation by *Salmonella* spp.

Several intervention strategies have been developed to reduce the risk of *Salmonella* at farm level. These intervention strategies include biosecurity, vaccination, cleaning, disinfection, prebiotics, probiotics, feed and water acidification with organic acids, and egg washing (Chousalkar et al., 2015; Cox & Pavic, 2010; Ricke, 2014). Although the application of these
strategies has reduced the *Salmonella* contamination level, complete elimination of this pathogen is yet to be achieved.

In the poultry industry, several commercial organic acid products are commonly used in feed and water as preventive measures against *Salmonella* contamination. Organic acids are generally regarded as safe (GRAS) and have been used extensively for many years to ensure water hygiene and reduce feed contamination in poultry (Ricke, 2003). Due to increased antimicrobial resistance and a ban on antibiotic growth promoters in many countries, organic acids are a suitable alternative for pathogen control, under a wide variety of food processing conditions (Wales et al., 2010). Organic acids are relatively stable and naturally present in living organisms, especially in the gut environment. Organic acids are either metabolised by food animals or may be excreted unabsorbed, and thus do not present an issue of residues in food (Wales et al., 2010). In addition to anti-microbial action, organic acids have ability to reduce biofilm development of foodborne pathogens on different surfaces (Akbas & Cag, 2016; Borges et al., 2012).

The following section of review summarises the literature relevant to the experiments conducted as a part of this thesis.
1.1.1 *Salmonella* taxonomy and epidemiology

The genus *Salmonella* is a member of *Enterobacteriaceae* family and is a non-spore forming, motile, Gram-negative facultative anaerobe. *Salmonella* is oxidase negative, capable of reducing nitrates to nitrites, and ferments glucose (Ricke et al., 2015). This genus is comprised of two species, *S. enterica*, and *S. bongori* (V). *S. enterica* is further divided into 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) and *S. enterica subsp. Indica* (VI). There are more than 2500 serovars in *Salmonella* genus and more than 1500 serovars belong to species *S. enterica* (Dunkley et al., 2009; Grimont & Weill, 2007; Guibourdenche et al., 2010).

*S. Enteritidis* and *S. Typhimurium* have dominated the epidemiology of *Salmonella* and are the most common causes of human salmonellosis (Hendriksen et al., 2011). Globally, *S. Enteritidis* is a dominant serovar isolated from eggshell and egg contents and is frequently involved in egg and egg product associated foodborne outbreaks (Wales & Davies, 2011). However, in Australia, human disease caused by this serovar occur because of overseas travel. *S. Typhimurium*, in contrast, is the predominant cause of Australian foodborne outbreaks and is often linked with the consumption of contaminated egg and egg related products (OzFoodNetWorking Group, 2009; Wales 2011; OzFoodNetWorking Group, 2012a). The incidence of human cases of *Salmonella* infection have increased over the past few years in Australia. A total of 11,992 *Salmonella* cases were reported in 2010, representing 53.7 cases per 100,000 people. This is higher in comparison to the previous 5 years, with an average infection rate of 41.8 cases per 100,000 people (OzFoodNet Working Group, 2012a). Between 2001 and 2011, *S. Typhimurium* was the predominant causative agent for 150 (90%) of the 166 egg associated outbreaks in Australia (Moffatt et al. 2016).
1.1.2 *Salmonella* pathogenesis

Once ingested, *Salmonella* attaches to oropharyngeal and intestinal epithelial cells via type 1 fimbriae or pili (Ricke et al., 2013b). The pathogenesis of *Salmonella* is mediated through type III secretion system encoded by *Salmonella* pathogenicity islands 1 (SPI-1) and other SPI’s which play an important role in systemic *Salmonella* infection.

Efficient colonisation and invasion of the gastrointestinal tract is attributed to *Salmonella* Pathogenicity Islands (SPI’s) that encode multiple virulence factors present in the *Salmonella* genome (Foley et al., 2013; Foley et al., 2008). For different serovars, several pathogenicity islands have been documented however, SPI-1 to SPI-5 are common in many serovars (Foley et al., 2008; Marcus et al., 2000). In general, SPI-1 is involved in host cell invasion and macrophage apoptosis by encoding type 3-secretion system (T3SS). SPI-2 is associated with systemic infection and intracellular survival. SPI-3 encoding genes are required for intracellular proliferation and *Salmonella* growth in low magnesium environments. SPI-4 harbours genes for toxin secretion and apoptosis or death of host cell. SPI-5 has been found to encode numbers of different T3SS effector proteins (Amavisit et al., 2003; Foley et al., 2008; van Asten & van Dijk, 2005).

1.2 Antimicrobial resistance in *Salmonella*

In humans, *Salmonella* infection occurs 12-72 hours after ingestion of contaminated food and clinical symptoms include fever, abdominal pain, vomiting, nausea, diarrhoea, and headache. In healthy adults symptoms of Salmonellosis are self-limiting and usually resolve in a week however, in infants or immunocompromised patients, symptoms are severe and could lead to life threatening septicaemia (Grant et al., 2016; Li et al., 2013; Olsen et al., 2001; Parry & Threlfall, 2008; Ricke et al., 2013b). Severe, systemic salmonellosis may require treatment with effective antimicrobials such as fluoroquinolones and extended-spectrum cephalosporins (Parry & Threlfall, 2008). However, the increased use of antibiotics in human and veterinary medicine
has led to an emergence of multidrug resistant *Salmonella* strains (Davies & Davies, 2010; Marshall & Levy, 2011). Moreover, the transfer of multidrug resistant *Salmonella* spp to humans through food producing animals can further compromise the treatment options (Hur et al., 2012).

Compared with many other countries, Australia has a cautious approach to antibiotic usage in commercial egg layer flocks. Antimicrobials, such as fluoroquinolones, are prohibited and ceftiofur, a 3rd generation cephalosporin is not approved for mass administration in food producing animals (Cheng et al., 2012; Obeng et al., 2012). To date, there is little information available on the characterization of antimicrobial resistance (AMR) in *Salmonella* isolated from commercial Australian egg layer flocks. The emergence and dissemination of antibiotic resistance to the foodborne pathogen is a serious animal and public health concern for developed and developing countries (Bounar-Kechih et al., 2012; Parsons et al., 2013). Therefore, understanding and monitoring antimicrobial resistance in *Salmonella* spp would help to understand the spread and control of *Salmonella* infection both in animal and human population.

### 1.2.1 Mode of action of antimicrobial agents

Since the middle of the 21st century, antimicrobials have been successfully used to treat various diseases and extensively used in human and veterinary medicine. The antimicrobial agents are classified according to their mechanism of action. The four important mechanisms of action of antimicrobial agents are; (1) inhibition of cell wall synthesis, (2) protein synthesis inhibition, (3) interference with nucleic acid synthesis and (4) metabolic pathway inhibition (Frye & Jackson, 2013; Tenover, 2006). Antimicrobial agents along with their mode of action are illustrated in Table 1.
Table 1. Mode of action of various antimicrobial agents

<table>
<thead>
<tr>
<th>Mode of action</th>
<th>Antimicrobial and or its class</th>
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<tr>
<td><strong>I. Interference of cell wall synthesis</strong></td>
<td>β-lactams</td>
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<tr>
<td></td>
<td>Penicillins</td>
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<td></td>
<td>- Cephalosporins</td>
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<td></td>
<td>- Carbapenem</td>
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<td></td>
<td>- Monobactams</td>
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<td>Glycopeptides</td>
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<td></td>
<td>- Vancomycin</td>
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<td></td>
<td>- Teicoplanin</td>
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<td><strong>II. Protein synthesis inhibition</strong></td>
<td></td>
</tr>
<tr>
<td>- Binds to 30S subunit of ribosome</td>
<td>Aminoglycosides</td>
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<td></td>
<td>Tetracyclines</td>
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<tr>
<td>- Binds to 50S subunit of ribosome</td>
<td>Chloramphenicol</td>
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<td></td>
<td>Macrolides</td>
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<td><strong>III. Interference of nucleic acid synthesis</strong></td>
<td></td>
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<tr>
<td>- DNA synthesis inhibition</td>
<td>Fluoroquinolones</td>
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<tr>
<td></td>
<td>- Ciprofloxacin</td>
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<tr>
<td></td>
<td>- Levofloxacin</td>
</tr>
<tr>
<td>- RNA synthesis inhibition</td>
<td>Rifampin</td>
</tr>
<tr>
<td><strong>IV. Metabolic pathway inhibition</strong></td>
<td>Sulfonamides and Trimethoprim</td>
</tr>
<tr>
<td>- Folic acid synthesis pathway</td>
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</tbody>
</table>
1.2.2 Mechanism of antibiotic resistance

Bacteria may become resistant to various antimicrobial agents through several mechanisms. Major mechanisms of antibiotic resistance in bacteria includes: (1) modification in target site so the antibiotic cannot recognise the target; (2) enzyme production that will inactivate or modify the drug before its effect; (3) expelling or extruding the antibiotic outside the cell by one or more efflux pumps so the drug is unable to reach the target site to exert its antibacterial action; and (4) alterations in the cell membrane permeability that inhibits the access of drug into the cell (Périchon & Courvalin, 2009; Verraes et al., 2013).

Antimicrobial resistance could be intrinsic or acquired. Intrinsic resistance is an inherent capacity of organisms making them insensitive to all antibiotics, whereas acquired resistance is a result of chromosomal mutation in bacterial genome or acquisition of resistance conferring genetic material from a different source (Périchon & Courvalin, 2009; Sefton, 2002; Tenover, 2006). Some bacteria are intrinsically or naturally resistant to many antimicrobials. This type of antimicrobial resistance in bacteria is often due to the absence of target site for the action of antibiotic or the inability of the drug to pass through the cell wall or bacterial membrane for its action. Bacteria, which are sensitive to antimicrobials, may become resistant by mutation in genetic material or acquire new genetic material from resistant bacteria by horizontal gene transfer (HGT) (Verraes et al., 2013). There are three mechanisms of HGT among bacteria: conjugation, transformation, and transduction (Davies, 1994; McManus, 1997; Verraes et al., 2013).

1.2.3 Mechanism of antimicrobial resistance in Salmonella to selected classes of antimicrobials

The development of antimicrobial resistance in Salmonella is determined by one of multiple mechanisms such as: enzyme production to deactivate antimicrobial agent through degradation or structural modification, decrease in cell permeability to antimicrobial agents, efflux pumps activation by antibiotics and modifications of the cellular target site of drug action (Sefton,
Resistance to cephalosporins and penicillin by *Salmonella* is related to production of β-lactamase enzymes produced by different *Salmonella* serovars. The chemical structures of limited antimicrobial agents are degraded by β-lactamase enzymes, whereas a wide array of antimicrobial agents are degraded by broad spectrum β-lactamases (Finch et al., 2003). The most common and important mechanism for β-lactam antibiotic resistance is attributed to β-lactamases production by *Salmonella* spp (Finch et al., 2003; Revathi et al., 1998). AmpC enzyme is one of the β-lactamase enzymes, which are encoded by *bla*CMY and has been shown to mediate resistance to most β-lactam antibiotics including ampicillin, ceftiofur and ceftriaxone (Aarestrup et al., 2004).

In *Salmonella*, resistance to tetracycline is encoded by the *tet* genes. Most of the *tet* genes code for efflux pumps and several others code for ribosomal protection proteins. The majority of *tet* genes in bacteria are present on the mobile genetic elements such as plasmids, transposons, conjugative transposons and integrons (Chopra & Roberts, 2001). Antimicrobial resistance in *Salmonella* to chloramphenicol is encoded by *flor* or *cml* (Butaye et al., 2003; Chopra & Roberts, 2001). Resistance to tetracycline and chloramphenicol is accomplished by the reduction in intracellular levels of antimicrobial agent by expelling the antibiotic from the cell at a rate equal or higher than its uptake. The resistance by efflux mechanism is the most common and well-studied mechanism for tetracycline (Foley & Lynne, 2008; Speer et al., 1992). In addition, chloramphenicol resistance in *Salmonella* is associated with the production of chloramphenicol acetyltransferases encoded by *cat* genes (Murray & Shaw, 1997). The *cat* genes are further divided into *cat* A and *cat* B groups, which differ from each other (Nogrady et al., 2005).

Resistance to aminoglycoside compounds in *Salmonella* is mediated by three classes of enzymes; phosphotransferases, acetyltransferases and adenyltransferases. This chemical modification by enzymes interferes with dry transport or binding of the drug at the site of action (Poole, 2005; Smith & Baker, 2002). Aminoglycoside acetyltransferase encoded by *aaac* can
cause gentamicin resistance, whereas aminoglycoside phosphotransferase encoded by \textit{aphA} leads to kanamycin resistance. Similarly, aminoglycoside adenyltransferases encoded by \textit{aadA} and \textit{aadB} are involved in streptomycin and gentamycin resistance respectively (Randall et al., 2004; Welch et al., 2007).

Resistance to quinolone and fluoroquinolone in bacteria is associated with mutations in bacterial DNA gyrase (\textit{gyrA} and \textit{gyrB}) and topoisomerase IV (\textit{parC} and \textit{parE}) genes. These mutations prevent binding of antimicrobial agents at the target site to carry out the antimicrobial action (Bebear et al., 2003; Dutta et al., 2005; Hooper, 1999). Both, trimethoprim and sulphonamide drugs affect the function of enzymes involved in folic acid synthesis pathway of bacterium (Eliopoulos & Huovinen, 2001).

Resistance to sulphonamides is mediated by drug resistant plasmid encoded by dihydrofolate reductase or dihydropteroate synthetase, which is encoded by \textit{sulI} or \textit{sulII} genes. Both, \textit{sulI} and \textit{sulII} genes are identified in Gram-negative enteric bacteria resistant to sulphonamides with almost equal frequency (Eliopoulos & Huovinen, 2001; Huovinen et al., 1995). Resistance to trimethoprim is mediated by expression of \textit{dhfr} genes that inhibit binding of trimethoprim (Huovinen et al., 1995). The dihydrofolate reductase genes \textit{dhfr1}, \textit{dhfr12}, and \textit{dhfr13} were identified in trimethoprim resistant \textit{Salmonella} isolates (Chen et al., 2004; Doublet et al., 2003).

1.2.4 Antibiotic resistance in \textit{Salmonella} from eggs or egg farm

In order to prevent antimicrobial residues in egg and egg related products, the use of antimicrobials in layer flocks is restricted. As a result, in developed countries the occurrence of antimicrobial resistance in layers is low compared to broilers (Gyles, 2008). In the USA \textit{Salmonella} isolates (n=45) from eight different serovars, recovered from commercial layer farm, were characterised for antibiotic resistance profile. Thirty-five percent (16 of 45) of the \textit{Salmonella} isolates were resistant to at least one antibiotic. A high percentage of the \textit{Salmonella} isolates were resistant to tetracycline, ampicillin, streptomycin, and ceftiofur, whereas no
resistance was detected to gentamycin, kanamycin, and nalidixic acid. Among the eight identified serotypes, S. Kentucky, untypeable, Typhimurium (var. 5-), and Montevideo were antibiotic resistant, whereas S. Heidelberg and Senftenberg, 8, (20): Nonmotile and 8, (20):-z6 were susceptible to all of the antibiotics tested (Li et al., 2007). Another study in the USA characterised antimicrobial resistance in *Salmonella* recovered from shell eggs collected from three commercial egg-processing plants. The results of this study showed that 60.1% *Salmonella* isolates were resistant to more than 11 antimicrobial compounds (Musgrove et al., 2006). Antibiotic resistance was predominantly found in *S.* Typhimurium isolates for tetracycline (63.4%), nalidixic acid (63.4%), and streptomycin (61%), whereas *S.* Kentucky isolates exhibited susceptibility to all antimicrobials (Musgrove et al., 2006). In the UK, between 2004 and 2005, the analysis of antimicrobial susceptibility pattern of 177 *Salmonella* isolates from commercial layer flocks holdings showed 77.4% of *Salmonella* isolates were susceptible to all 16 antimicrobials tested (Snow et al., 2007). The most frequent resistance was observed to ampicillin (15.3%), tetracycline (13.6%), sulphamethoxazole/trimethoprim (11.3%), sulphonamide compounds (10.7%), chloramphenicol (6.8%), and nalidixic acid (5.1%). *Salmonella* isolated in this study did not show resistance to amikacin, amoxicillin/clavulonic acid, apramycin, ceftazidine, ciprofloxacin, cefotaxime, gentamycin, and neomycin. Most antimicrobial resistance was observed amongst *S.* Enteritidis and *S.* Typhimurium isolates (Snow et al., 2007). In Japan, a total of 28 *Salmonella* isolates were recovered from faeces of layer hens from 2000 to 2003 (Asai et al., 2006). The antimicrobial resistance rates of layer chicken isolates to two or more antimicrobials (10.7%, 3/28) were the lowest as compared to *Salmonella* isolates from cattle, pig and broilers (Asai et al., 2006). In the Morocco regions, *Salmonella* strains (n=64) were isolated from three different egg laying farms and tested for antimicrobial susceptibility. Result of antimicrobial susceptibility testing showed that 65.6% (42/64) of *Salmonella* were resistant to at least 1 antibiotic and 29.70% (19/64) of *Salmonella* strains were resistant to two or more antimicrobial agents (Ziyate et al., 2016). *Salmonella* strains showed the highest percentage of
resistance to nalidixic acid (61%), ciprofloxacin (25%), amoxicillin (21%), tetracycline (25%), cephalothin (25%), streptomycin (18%), sulphonamides (14%), and gentamycin (8%). The level of resistance to antimicrobial agents was highest in _S_. Kentucky isolates (25%) followed by _S_. Typhimurium (4.6%) (Ziyate et al., 2016). Altogether, this data suggests that resistance to antimicrobials is serovar dependent and shell eggs could pose a risk of resistant bacteria in the environment and food chain.

### 1.2.5 Antibiotic use in Australian poultry industry

Use of antibiotics in food animals as a growth promoter and for prevention and treatment of infectious diseases is well known. After a recommendation of the Swann report, Australia has adopted a very cautious approach to registration of antibiotics for use in commercial intensive production industry (Barton et al., 2003; Ndi & Barton, 2011; Swann et al., 1969). The antibiotics important for both animals and humans are not used as growth promotants. The intention is to minimise the amount of antibiotic residues in animal products and to minimise the transfer of antibiotic resistance from animals to humans through the food chain.

In Australia, antibiotics prohibited for use in all classes of poultry include ampicillin-clavulanic acid, fluoroquinolones, gentamycin, chloramphenicol, and nitrofurans (Australian Veterinary Association, 2014; Barton et al., 2003; Barton & Wilkins, 2001). Only, first, third and fourth generation cephalosporin (Ceftiofur) products are specifically registered for use in cattle to treat respiratory infections (Barton et al., 2003). Antibiotics such as ampicillin, tetracycline, neomycin, spectinomycin, erythromycin lincomycin and tylosin are used as therapeutic and prophylactic agents in meat chicken industry. Bacitracin zinc, virginiamycin, avoparcin, and flavophospholipol are growth-promoting antibacterial used in the chicken meat industry for the control of chronic enteric infections (Barton et al., 2003; Barton & Wilkins, 2001). In layers, antibacterial agents such as bacitracin zinc, chlortetracycline, flavophospholipol, neomycin tylocin phosphate, tylocin tartarate, amoxicillin trihydrate, and sulfadiazine/trimethoprim are used under permit or prescribed by only registered veterinarians (Wilson, 2012). Ionophore
compounds such as narasin, lasalocid and salinomycin are used as coccidiostats in poultry 
(Barton et al., 2003; Barton & Wilkins, 2001; Wilson, 2012).

1.2.6 Antibiotic resistance in \textit{Salmonella} from Australian poultry industry

Egg and chicken meat are the two major sectors of the Australian poultry industry. Poultry 
products are a major source of \textit{Salmonella} infection in humans. In Australia, currently there is 
less information describing antimicrobial resistance patterns in \textit{Salmonella} isolated from 
commercial egg farms or eggs. The results from the Australian Reference Centre have shown 
differences in resistance pattern in \textit{Salmonella} isolates recovered from egg and meat producing 
chickens (Australian \textit{Salmonella} Reference Centre, 2009). Of the 1475 meat chicken isolates, 
31, 10, 7 and 6 \% were resistant to streptomycin, tetracycline, sulphonamides and ampicillin 
respectively. Whereas, of the 265 isolates from egg farms, 2, 4, 2 and 5\% were resistant to 
streptomycin, tetracycline, sulphonamides and ampicillin respectively (Australian \textit{Salmonella} 
Reference Centre, 2009). Resistance to fluoroquinolones and ceftiofur was not detected in any 
of the isolates tested. Multidrug resistance (resistance to 4 or more antibiotics) was observed in 
3\% and 0.4\% of chicken meat and egg farm isolates respectively (Australian \textit{Salmonella} 
Reference Centre, 2009; Ndi & Barton, 2011). In Australia, antimicrobial resistance in 
\textit{Salmonella} spp isolated from poultry is low compared to other parts of the world due to the 
restricted use of antibiotics however, the contamination of egg and egg products remains a 
significant concern for the industry (Chousalkar et al., 2015).

1.3 Egg contamination and \textit{S. Typhimurium} infection in laying 
hens

During 2014-15, total commercial egg production in Australia was 421.3 million dozen eggs 
with per capita consumption of 221 eggs per year (Australian Egg Corporation Limited, 2015). 
Egg contamination by \textit{Salmonella} spp remains a significant challenge to the Australian poultry 
industry. In 2011, egg associated outbreaks comprised 48\% (29/61) of all foodborne \textit{Salmonella}
outbreaks, affecting 498 people in Australia (OzFoodNet Working Group, 2015a). Egg contamination can occur when eggs come in contact with different contaminated sources such as faeces, blood, soil, water, cage, egg belts, insects, and hands (Board & Tranter, 1995; Davies & Breslin, 2003b). Egg and egg related products are the major vehicles of Salmonella foodborne outbreaks in Australia. Thus, improvement in egg safety standards is a major challenge to the Australian poultry industry, public health professionals, and Government agencies.

1.3.1 Mechanism of egg contamination

Previous experimental studies have shown that both S. Enteritidis and S. Typhimurium are able to colonise reproductive organs (ovary and oviduct) and contaminate egg and contents. In most parts of the world, S. Enteritidis is a common cause of foodborne illness linked to contaminated eggs however, in Australia, the majority of the egg and egg related outbreaks are caused by S. Typhimurium (Moffatt et al., 2016; Wales & Davies, 2011).

External and internal egg contamination by Salmonella during poultry production is influenced by many variables such as; cleaning and disinfection routines, flock size, flock age, stress, feed, vaccination, egg production process, storage, and handling of eggs (Whiley & Ross, 2015). As a result, implementation of appropriate control measures is extremely difficult. Egg contamination can occur by two routes, vertical or horizontal. Vertical transmission is a result of reproductive organ colonization (ovary and oviduct) before shell formation, whereas horizontal transmission occurs when Salmonella penetrates the eggshell membrane and contaminates egg contents after the egg is laid (De Reu et al., 2006).

Previous studies have demonstrated a relationship between increased Salmonella shedding in faeces and subsequent eggshell contamination (Gole et al., 2014b). There are several environmental stressors, which could contribute to Salmonella shedding and egg contamination in laying hens. Throughout the production cycle, birds encounter many stress events, which have negative effects on both cellular and humoral immunity of birds (El-Lethey et al., 2003).
Decreased immunity as a result of stress could make birds more susceptible to *Salmonella* infection, which could lead to increased shedding of *Salmonella* in faeces (Ricke et al., 2013a). Onset of lay or sexual maturity is stressful for hens and may induce *Salmonella* shedding in faeces and egg (Gole et al., 2014a; Okamura et al., 2010). Previous longitudinal survey determined shedding of *Salmonella* in caged commercial layer flocks at the onset of lay (18 weeks), at 24 and 30 weeks (Gole et al., 2014a). The highest shedding of *Salmonella* coincided with the onset of sexual maturity. The work indicated that the load of *Salmonella* in faeces was highest (82.14%) at 18 weeks of age and significantly reduced to 38.88% and 12.95% at the age of 24 and 30 weeks respectively (Gole et al., 2014a). The influence of onset of sexual maturity on egg contamination after oral infection of pullets with *S. Typhimurium* DT104 was examined in an earlier study and results showed that egg contamination risk increased in hens when infected at the onset of lay (Okamura et al., 2010).

### 1.3.2 *S. Typhimurium* infection in laying hens

The ability of *S. Enteritidis* to colonise the reproductive organs of laying hens and contaminate forming egg is well known (Gantois et al., 2009). Previous epidemiological investigations have also demonstrated links between *S. Enteritidis* and egg contamination (Doorduyn et al., 2006; Gillespie et al., 2005; Marcus et al., 2007; Molbak & Neimann, 2002). Unlike US and parts of Europe, *S. Enteritidis* is not endemic in commercial Australian poultry flocks, instead, *S. Typhimurium* is frequently implicated in egg and egg product related outbreaks (Dyda et al., 2009; Jardine et al., 2011; Moffatt & Musto, 2013; Reynolds et al., 2010; Stephens et al., 2007). A recent Australian study analysed outbreak data between 2001 and 2010 and found a significant increase in the proportion of foodborne *Salmonella* outbreaks linked to egg and egg products (Moffatt et al., 2016).

In Australia, given the absence of *S. Enteritidis* in commercial flocks, soiling of eggshell with faeces and contamination of layer farm environment could be the likely sources of egg contamination. In addition, other factors such as, cross contamination of food products,
improper handling of eggs in the food processing area, and undercooking may likely contribute to multiple egg related outbreaks (Moffatt et al., 2016). Horizontal route of egg contamination cannot solely explain the outbreak situation in Australia, therefore transfer of S. Typhimurium by the vertical route has been hypothesised as a possible mechanism of egg contamination (Wales & Davies, 2011). In order to improve the understanding of horizontal and vertical egg contamination by S. Typhimurium, infection studies are required in laying hens.

Experimental Salmonella infection of commercial laying hens helps to determine the course of reproductive organ invasion and subsequent contamination of egg and egg contents (Cox et al., 2002). Previous experimental infection studies have examined reproductive organ colonisation and egg contamination by S. Typhimurium in laying hens and in comparison with other Salmonella serovars. Results from these experiments are inconsistent due to variation in experimental design, age of birds, route of inoculation, inoculum dose as well as the strain of S. Typhimurium selected (Baker et al., 1980; Williams et al., 1998; Leach et al., 1999; Okamura et al., 2001a; Okamura et al., 2001b; Okamura et al., 2010). The majority of earlier infection studies examined the capability of S. Typhimurium to colonise reproductive organs or egg contamination frequency up to 3 weeks post-infection (p.i.), which could fail to unveil the ability of S. Typhimurium to cause egg contamination over a prolonged period (Wales, 2011). Altogether, there is a lack of published data arising from long-term experiments aimed at faecal shedding, reproductive organ colonisation, and egg contamination by S. Typhimurium in laying hens. The next section of this review describes the experimental S. Typhimurium infection studies in laying hens.

1.3.2.1 S. Typhimurium infection studies in laying hens with intravenous route

A number of previous studies have examined reproductive organ colonisation and egg contamination by S. Typhimurium after intravenous infection of laying hens. The intravenous injection of 4.65x10^6 CFU of S. Typhimurium strain in laying hens did not result in faecal shedding during the experiment and bacteria was not isolated from shell or egg contents of laid
eggs (Baker et al., 1980). In another experimental study by Okamura et al., (2001a), mature laying hens were intravenously inoculated with 5×10^6 CFU of S. Enteritidis, S. Typhimurium, S. Heidelberg, S. Hadar, S. Infantis and S. Montevideo. On necropsy, S. Enteritidis was isolated more frequently and in higher numbers from cloaca, vagina, liver, spleen, caecal contents, ovaries, and reproductive tract organs than other serovars. Further, internal egg contamination was only observed in hens inoculated with S. Enteritidis (Okamura et al., 2001a). These findings suggest that S. Enteritidis has the ability to cause colonisation of reproductive organs, contamination of eggshell and egg contents amongst six serovars used in this study. In contrast, intravenous infection of 22 week old laying hens at higher doses (1×10^8CFU) of S. Enteritidis, S. Typhimurium, S. Heidelberg, S. Virchow, and S. Hadar, showed no significant differences in colonisation of spleen and reproductive organs (oviduct and ovaries) between S. Enteritidis and S. Typhimurium, 4, 7 and 14 days p.i. (Gantois et al., 2008). Both S. Enteritidis and S. Typhimurium showed higher frequency (40 to 70%) of internal egg contamination than other serovars used in this study (Gantois et al., 2008). The results of these studies suggest that colonisation of reproductive organs and egg contamination is not serovar specific.

1.3.2.2 S. Typhimurium infection studies in laying hens with oral and crop route

The oral route is the most realistic and natural way of Salmonella infection, in comparison to intravenous administration. Oral infection of mature hens with 10^6 CFU of S. Typhimurium, S. Senftenberg and S. Thompson for 10 consecutive days was not associated with contamination of egg contents and visceral organs. However, shedding of all Salmonella strains in faeces was recorded in almost all birds and frequency of eggshell contamination ranged from 6.3 to 9.5 % for all three serovars (Cox et al., 1973). In a short term experiment (4 days), the ability of various strains of S. Enteritidis and S. Typhimurium by oral inoculation with 1x10^8 CFU was compared to colonise internal organs, oviduct and forming eggs of young and mature laying hens (Keller et al., 1997). The results of this study revealed both S. Enteritidis and S. Typhimurium were capable of colonising internal organs, oviduct, and forming eggs however,
only *S. Enteritidis* was recovered from internal egg contents of laid eggs (Keller et al., 1997). In this study, significant differences in tissue invasiveness was observed between *S. Typhimurium* and two of the three *S. Enteritidis* strains (Keller et al., 1997). Studies were also performed using oral infection of point in lay pullets with $10^7$ CFU/ml of eight wild type strains of *S. Typhimurium* DT104 (Williams et al., 1998). The results showed both colonisation and internal egg contamination by the strains of *S. Typhimurium* however, variations in the frequency of internal egg contamination were evident among the strains. Faecal contamination was significantly different between strains and decreased over the two-week period. Faecal carriage of *Salmonella* was not correlated with contamination of egg contents in this study (Williams et al., 1998). Based on the studies described above, substantial variations in degree of systemic and reproductive organ colonisation by *S. Typhimurium* and or *S. Enteritidis* were evident after oral infection. However, these variations were not associated with the probability of colonisation of eggs forming in the oviduct (Keller et al., 1997) or with the contamination of freshly laid eggs (Keller et al., 1997; Williams et al., 1998).

The relationship between oral dose and egg contamination is still unclear and lack of a positive correlation has been observed in previous studies. Oral infection with *S. Typhimurium* at the point of lay and in older hens with $10^{10}$ CFU resulted in substantial mortality and morbidity, along with decreased egg production (Brown & Brand, 1978). Although invasion of tissues including ovaries was frequent, contamination of eggshell was not detected up to three-week p.i. (Brown & Brand, 1978). In another study, oral inoculation of mature laying hens with $2.3 \times 10^8$ CFU of *S. Typhimurium* did not result in contamination of eggshell and egg contents for up to 17 days p.i., even though *Salmonella* was excreted in faeces (Baker et al., 1980). A recent study investigated the potential of 10 different Japanese isolates of *S. Typhimurium* DT104 to contaminate egg after oral infection of old (319 or 329 day old) and point in lay (139 day old) hens at high doses (Okamura et al., 2010). Oral infection of $1 \times 10^8$ to $1 \times 10^{10}$ CFU caused a significant drop in egg production however, no internal egg contamination was
observed in old hens. In contrast, oral infection of pullets at the onset of lay resulted in internal egg contamination of 1.7% eggs (11 of 634) in two weeks and inoculated strains were recovered from the liver, spleen, cecal contents, ovary, and follicles (Okamura et al., 2010).

**1.3.2.3 S. Typhimurium infection studies in laying hens with other routes**

In addition to oral and intravenous administration, contamination of egg by S. Typhimurium has also been examined with aerosol and intravaginal routes. Aerosol delivery of S. Typhimurium DT104 with 2x10\(^2\) to 2x10\(^4\) CFU in point of lay pullets caused systemic infection and shedding in faeces for up to 14 days p.i. (Leach et al., 1999). The frequency of internally contaminated eggs and muscle was significantly higher following aerosol challenges compared to oral challenge with S. Typhimurium strain (Leach et al., 1999). These findings suggest greater virulence of S. Typhimurium by the aerosol route than oral infection. Similarly, aerosol infection of S. Enteritidis phage type 4 in pullets was associated with systemic colonisation, including reproductive organs (ovary and oviduct) however, S. Enteritidis was not isolated from eggs examined up to 28 day p.i. (Baskerville et al., 1992).

Mature laying hens were inoculated intravaginally with 5x10\(^6\) CFU of S. Enteritidis, S. Typhimurium, S. Infantis, S. Hadar, S. Heidelberg, and S. Montevideo to compare their abilities to invade reproductive organs and to contaminate eggs (Okamura et al., 2001b). All Salmonella strains were recovered from the outer surface of eggshells however, the contamination rate was significantly higher in hens inoculated with S. Enteritidis in comparison to S. Typhimurium or any of the other four strains. Egg contents were contaminated with S. Enteritidis (7.5%) and S. Typhimurium (3.1%). The rate of cloaca and vaginal contamination was higher for S. Enteritidis compared with other serovars up to one week. S. Enteritidis was recovered from ovaries and all portions of oviduct however, S. Typhimurium was not recovered from any portion of the oviduct. In the same study, *in vitro* adherence to the vaginal epithelium by all six serovars was investigated and the results indicated the attachment of S. Enteritidis to the vaginal epithelium was significantly higher than all other serovars examined. The results of this study suggested
that *S. Enteritidis* had high affinity to attach to vaginal epithelium of reproductive tissues and may have a significant role in production of *S. Enteritidis* contaminated eggs (Okamura et al., 2001b).

It has been hypothesized that inoculation of semen contaminated with *Salmonella* could infect ovaries and reproductive organs of hens and result in egg contamination. Experiments were conducted to determine the effect of insemination of hens with semen contaminated with $1 \times 10^3$ CFU of *S. Enteritidis* or *S. Typhimurium* (Reiber et al., 1995). The result of this study showed that eggshell contamination was much higher in hens challenged with *S. Enteritidis* than hens challenged with *S. Typhimurium* (Reiber et al., 1995). Both, *S. Enteritidis* and *S. Typhimurium* were recovered from ovaries and different parts of the oviduct. Both, *S. Enteritidis* and *S. Typhimurium* were found in faecal samples taken 24 hours after insemination however, only *S. Enteritidis* was recovered from faeces 7 days after insemination. There was no correlation between faecal shedding, oviduct infection and production of contaminated eggs. This data suggests that semen could serve as a vehicle to infect ovaries and reproductive organs of laying hens and result in production of contaminated eggs (Reiber et al., 1995).

The above literature suggests that *S. Typhimurium* was able to colonise reproductive organs and contaminate eggs either at a similar or lower frequency compared with *S. Enteritidis*. However, most of the experimental infection studies in laying hens with *S. Typhimurium* were conducted for a very short duration and used relatively high doses. These conditions may fail or mask the real potential of *S. Typhimurium* in terms of its infectivity over the whole production cycle (Wales & Davies, 2011).

### 1.3.3 *S. Typhimurium* outbreaks related to egg and egg products across Australia

In 2002, OzFoodNet was established to enhance the surveillance, management and prevention of foodborne diseases across Australia (Hundy et al., 2002). The number of *Salmonella*
outbreaks linked to egg and egg products increased significantly in Australia between 2001 and 2011. These outbreaks had a significant public health impact, affected more than 3200 people, and resulted in 20% of those affected being hospitalised (Moffatt et al., 2016). Several phage types of *S. Typhimurium* were responsible for the egg associated *S. Typhimurium* outbreaks in Australia (Moffatt et al., 2016).

Data obtained from January 2010 to January 2014 by OzFoodNet suggested, a total of 121 outbreaks of *S. Typhimurium* were related to the consumption of raw egg and egg related products in different food preparation settings across Australia (OzFoodNet Working Group Quaterly Reports, from 2010 to 2015). These outbreaks resulted in 2343 cases with 347 hospitalisations. *S. Typhimurium* phage type 170/108 was most frequently (n=37/121, 30.57%) identified followed by phage type 9 (n=26/121, 21.48%) and phage type 135/135a (20/121, 16.52%). Together, these three phage types were responsible for 69% of 121 egg related *S. Typhimurium* outbreaks (Table 2). The rate of outbreak occurrence was variable across Australian states and territories. Highest outbreak rate was reported in Victoria (49/121, 40.49%) followed by New South Wales (38/121, 31.40%). The three most populous states of Australia, New South Wales, Queensland, and Victoria accounted for 80% of 121 outbreaks. Commercial food providers including restaurant, commercial caterers, takeaways, and bakeries accounted for 69.42% (78/121) of outbreaks. Twenty six percent of outbreaks occurred in private residences but total number of affected individuals were lower than commercial settings. In all outbreak events, raw eggs, or partially cooked egg products containing food items such as aioli, hollandaise and desserts (tiramisu, fried ice cream, and mousse) were the most commonly identified food vehicles (Table 2). This data highlights the importance of eggs as a major source of *S. Typhimurium* outbreaks in Australia.
### Table 2. Outbreaks of S. Typhimurium associated with egg or egg products reported by OzFoodNet Australia from January 2010 to January 2014


<table>
<thead>
<tr>
<th>State/Territory</th>
<th>Outbreak period</th>
<th>Setting</th>
<th>Causative agent</th>
<th>Numbers affected</th>
<th>Hospitalised</th>
<th>Food vehicles</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT</td>
<td>March 2010</td>
<td>Private residence</td>
<td>S. Typhimurium PT 170</td>
<td>4</td>
<td>0</td>
<td>Suspected chocolate mousse containing raw egg</td>
</tr>
<tr>
<td>NSW</td>
<td>January 2010</td>
<td>Private residence</td>
<td>S. Typhimurium PT 170</td>
<td>5</td>
<td>4</td>
<td>Suspected mayonnaise prepared with raw eggs</td>
</tr>
<tr>
<td>NSW</td>
<td>January 2010</td>
<td>National franchised fast</td>
<td>S. Typhimurium PT 9</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Aioli prepared with raw egg</td>
</tr>
<tr>
<td>NSW</td>
<td>March 2010</td>
<td>Private residence</td>
<td>S. Typhimurium</td>
<td>9</td>
<td>1</td>
<td>Suspected tiramisu prepared with raw eggs</td>
</tr>
<tr>
<td>VIC</td>
<td>January 2010</td>
<td>Private residence</td>
<td>S. Typhimurium PT 170</td>
<td>12</td>
<td>1</td>
<td>Suspected eggs</td>
</tr>
<tr>
<td>VIC</td>
<td>January 2010</td>
<td>Restaurant</td>
<td>S. Typhimurium PT 9</td>
<td>13</td>
<td>1</td>
<td>Suspected eggs</td>
</tr>
<tr>
<td>VIC</td>
<td>February 2010</td>
<td>Restaurant</td>
<td>S. Typhimurium PT 9</td>
<td>8</td>
<td>1</td>
<td>Suspected eggs</td>
</tr>
<tr>
<td>VIC</td>
<td>March 2010</td>
<td>Private residence</td>
<td>S. Typhimurium PT 135a</td>
<td>5</td>
<td>0</td>
<td>Suspected chicken or eggs</td>
</tr>
<tr>
<td>WA</td>
<td>December 2010</td>
<td>Restaurant</td>
<td>S. Typhimurium PT 170</td>
<td>7</td>
<td>1</td>
<td>Scrambled eggs</td>
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<tr>
<td>WA</td>
<td>January 2010</td>
<td>Restaurant</td>
<td>S. Typhimurium PT 170</td>
<td>25</td>
<td>5</td>
<td>Aioli and Caesar salad</td>
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<tr>
<td>NSW</td>
<td>March 2010</td>
<td>Restaurant</td>
<td>S. Typhimurium 170</td>
<td>6</td>
<td>3</td>
<td>Tartare sauce, prepared with raw egg</td>
</tr>
<tr>
<td>State</td>
<td>Date</td>
<td>Location</td>
<td>Strain</td>
<td>Cases</td>
<td>Source Description</td>
<td></td>
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<td>--------</td>
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<td>-------------------</td>
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<td>-------</td>
<td>-------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>NSW</td>
<td>April 2010</td>
<td>Takeaway</td>
<td>S. Typhimurium 170</td>
<td>9</td>
<td>Mayonnaise made with raw egg</td>
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<tr>
<td>NSW</td>
<td>June 2010</td>
<td>Takeaway</td>
<td>S. Typhimurium 170</td>
<td>45</td>
<td>Chicken, hummus, tabouli</td>
<td></td>
</tr>
<tr>
<td>QLD</td>
<td>June 2010</td>
<td>Restaurant</td>
<td>S. Typhimurium</td>
<td>34</td>
<td>Citrus aioli</td>
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<tr>
<td>NSW</td>
<td>July 2010</td>
<td>Private residence</td>
<td>S. Typhimurium (MLVA: 3-9-7-13-523)</td>
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<td>Unknown, possibly mousse cake with raw eggs</td>
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<tr>
<td>VIC</td>
<td>August 2010</td>
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<td>S. Typhimurium PT 170</td>
<td>4</td>
<td>Eggs</td>
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<td>NSW</td>
<td>October 2010</td>
<td>Takeaway</td>
<td>S. Typhimurium 170</td>
<td>15</td>
<td>Suspected Vietnamese pork rolls- commonly knows to contain egg</td>
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<tr>
<td>NSW</td>
<td>November 2010</td>
<td>Restaurant</td>
<td>S. Typhimurium</td>
<td>2</td>
<td>Suspected salmon patties made with egg</td>
<td></td>
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<tr>
<td>NSW</td>
<td>December 2010</td>
<td>Takeaway</td>
<td>Suspected S. Typhimurium</td>
<td>8</td>
<td>Suspected Vietnamese pork rolls</td>
<td></td>
</tr>
<tr>
<td>QLD</td>
<td>December 2010</td>
<td>Private residence</td>
<td>S. Typhimurium</td>
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<td>Banana milkshake containing raw egg</td>
<td></td>
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<tr>
<td>TAS</td>
<td>December 2010</td>
<td>Restaurant</td>
<td>S. Typhimurium PT 170</td>
<td>38</td>
<td>Homemade ice cream containing raw egg</td>
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<td>QLD</td>
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<td>Location</td>
<td>Salmonella Type</td>
<td>MLVA Profile</td>
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<td>Month</td>
</tr>
<tr>
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<tr>
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<td>October 2013</td>
<td>Restaurant</td>
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<td><em>S. Typhimurium</em> PT 9</td>
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<td>Bakery</td>
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<td>Restaurant</td>
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<td>Other</td>
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<td>State</td>
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<td>Location</td>
<td>Pathogen</td>
<td>Cases</td>
<td>outbreaks</td>
<td>Description</td>
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<tr>
<td>VIC</td>
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<td>Restaurant</td>
<td>S. Typhimurium PT 9</td>
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<td>S. Typhimurium PT 9</td>
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<td>Feb 2014</td>
<td>Restaurant</td>
<td>S. Typhimurium PT 9</td>
<td>13</td>
<td>1</td>
<td>Suspect raw egg aioli</td>
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<td>Restaurant</td>
<td>S. Typhimurium PT 9</td>
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<td>5</td>
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<td>S. Typhimurium PT 44</td>
<td>6</td>
<td>3</td>
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<tr>
<td>VIC</td>
<td>Mar 2014</td>
<td>Private residence</td>
<td>S. Typhimurium PT 9</td>
<td>3</td>
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<tr>
<td>VIC</td>
<td>Mar 2014</td>
<td>Restaurant</td>
<td>S. Typhimurium PT 9</td>
<td>3</td>
<td>2</td>
<td>Raw egg aioli</td>
</tr>
<tr>
<td>VIC</td>
<td>Mar 2014</td>
<td>Restaurant</td>
<td>S. Typhimurium PT 9</td>
<td>14</td>
<td>5</td>
<td>Undercooked eggs in hollandaise sauce</td>
</tr>
</tbody>
</table>

The month of outbreak represents the month of onset of outbreak
* No foodborne outbreaks were reported by the Northern Territory
† Suspected/confirmed egg associated outbreaks
MLVA- Multi-locus variable number of tandem repeat analysis
PFGE- Pulsed-field gel electrophoresis
PT- Phage type
ACT- Australian Capital Territory
NSW- New South Wales
NT- Northern Territory
QLD- Queensland
SA- South Australia
TAS- Tasmania
VIC- Victoria
Several intervention strategies are described in the literature to reduce the risk of *Salmonella* contamination both at pre-harvest and post-harvest level. Although, application of single or a combination of intervention strategies has reduced levels of *Salmonella* contamination, complete elimination of this pathogen has yet to be achieved (Galiş et al., 2013; Ricke et al., 2015). Biofilm formation could be one of the mechanisms that *Salmonella* spp utilizes for overcoming some interventions and surviving harsh physical and chemical environments. A biofilm is a community of bacterial cells attached to surfaces, and encased in self-produced extracellular polymeric matrix (Costerton et al., 1999). Previous studies have shown that *Salmonella* spp are able to form biofilm on food processing surfaces and remain viable for prolonged periods. It is well known that the cells in biofilm are resistant to commonly used disinfectants and as a result, their elimination is difficult. Biofilm formation is a serious food safety concern as cells in biofilm could act as a continuous source of infection and cross contaminate other food products in the food production environment (Steenackers et al., 2012).

In Australia, there is limited information available in the literature on the biofilm formation ability of *Salmonella enterica* spp isolated from layer farm environments.

### 1.4 *Salmonella* biofilms

*Salmonella* spp are able to form biofilm on many surfaces, such as stainless steel (Hood & Zottola, 1997; Joseph et al., 2001; Nguyen et al., 2014; O'Leary et al., 2015), plastics, cement (Joseph et al., 2001), rubber (Arnold & Yates, 2009) glass, (De Oliveira et al., 2014; Prouty & Gunn, 2003; Solano et al., 2002), epithelial cells (Bodicker et al., 2002) and gall stones (Prouty & Gunn, 2003). However, there is limited information available in the literature on the ability of *Salmonella* spp to form a biofilm on eggshell surface. Biofilm formation is a serious concern for water, shipping, food industry and human and animal health (Simm et al., 2014). Biofilms formed on food preparation surfaces and equipment in domestic and industrial settings could act as a vehicle for contaminating food products, reducing their shelf life, or further spreading the pathogenic bacteria to other hosts risking public health (Shi & Zhu, 2009). Additionally, it
has been demonstrated that cells in biofilms are more resistant to commonly used disinfectants, antibiotics and environmental stressors compared to their planktonic counterparts (Corcoran et al., 2014; Olson et al., 2002; Steenackers et al., 2012; Wong et al., 2010a). Understanding the mechanism of Salmonella biofilm formation, composition and its susceptibility to various antimicrobials, will aid in the management of this pathogen.

1.4.1 Control measures against biofilm

There are several conventional methods such as physical and or mechanical removal, chemical removal and the use of antimicrobials, disinfectants, or sanitizers to control and remove biofilms in food industry. However, none of these procedures is fully effective against biofilms due to the emergence of resistant phenotypes (Sadekuzzaman et al., 2015). Recent advances in the area of biofilm research has led to the development of novel strategies for prevention and control of biofilms. Several new methods such as quorum sensing antagonistic molecules, bacteriophages, enzyme based detergents, pulsed light /laser decontamination devices, ultrasonication, nanotechnology, photodynamic therapy, biosurfactants, bacteriocin, and natural compounds (plant extracts, essential oils and organic acids) have been successfully shown to be an effective alternative against biofilm formation (Sadekuzzaman et al., 2015; Simões et al., 2010). Moreover, combination of these innovative anti-biofilm approaches coupled with conventional techniques (chemical and physical) could mitigate the losses associated with biofilm formation in the near future (Sadekuzzaman et al., 2015).

In recent years, most of the research focus has shifted towards developing active biofilm targeted treatments using natural compounds. These compounds are easily degradable in the environment, efficiently penetrate biofilm structure, and have a high level of activity against pathogens (Bridier et al., 2015). Moreover, with the changing regulatory requirements, antimicrobial agents considered as standard today could be banned in coming years (Reach EU directive on biocides, 98/8EC). Therefore, it would be appropriate to find suitable alternatives, which are environmentally friendly and residue free, for efficient eradication of surface biofilms.
In recent years, use of antimicrobials derived from natural sources against biofilms has gained much attention due to their accepted safe status. Organic acids are generally recognised as safe and are approved by Food and Drug Administration (Mani-López et al., 2012). Antimicrobial properties of organic acids are well known however, their activity against biofilms is less studied.

1.4.2 *Salmonella* biofilm formation and composition

Biofilm formation is a complex process divided into several stages: 1) initial reversible attachment of bacterial cells, driven by weak interactions (van der Walls forces) with surface; 2) irreversible attachment to the surface occurs via hydrophilic or hydrophobic interaction with the help of several attachment structures such as flagella, fimbriae, lipopolysaccharides or adhesive proteins; 3) early development of biofilm architecture (micro colony formation) resulting from proliferation and production of extracellular matrix; 4) formation of mature biofilm containing open water channels through which nutrients and signalling molecules are effectively distributed within the biofilm; 5) detachment of biofilm cells individually or in clumps due to increased fluid shear, endogenous enzymatic degradation, release of exopolysaccharides or surface binding proteins; and 6) dispersion of cells is the last step in biofilm formation cycle that allows colonisation of new niches (Sadekuzzaman et al., 2015; Srey et al., 2013). The formation of biofilm is influenced by several factors such as, *Salmonella* strain (Castelijn et al., 2012; Lianou & Koutsoumanis, 2012), serovar (Schonewille et al., 2012; Vestby et al., 2009), geographic location of isolates (Seixas et al., 2014), type of surface material (De Oliveira et al., 2014; Nguyen et al., 2014; O'Leary et al., 2013), temperature (De Oliveira et al., 2014; Nguyen et al., 2014) and nutrients available (Castelijn et al., 2012).

Biofilm matrix is mainly composed of extracellular material containing polymeric substances. The composition of biofilm may differ between bacterial species and environmental conditions. The rdar (red, dry and rough) morphotype defining the composition of extracellular matrix components is the most studied morphotype of *Salmonella* multicellular behaviour. *Salmonella*
biofilms are structurally composed of proteinaceous compounds and exopolysaccharides. The proteinaceous fraction consists of adhesive curli fimbriae (Tafi or aggregative fimbriae (agf) in *Salmonella*), and secreted large surface protein BapA. The exopolysaccharide fraction is mainly composed of cellulose, the O-antigen capsule, capsular polysaccharides, and lipopolysaccharides (Steenackers et al., 2012).

Curli fimbriae and cellulose are the predominant components of biofilm matrix and their co-expression results in formation of a highly hydrophobic network with tightly packed cells embedded in rigid matrix (Solano et al., 2002; Zogaj et al., 2001). Previous studies have shown that curli fimbriae and cellulose enhances resistance of *Salmonella* to desiccation and is linked to long-term survival of *Salmonella* in the harsh environment (White et al., 2006).

The expression of curli and cellulose is regulated by a complex regulatory network (Figure 1). *csgD* is a transcriptional regulator of the LuxR superfamily that positively regulates the expression of *Salmonella* biofilm associated extracellular matrix components, including curli fimbriae and cellulose (Gerstel & Römling, 2003; Grantcharova et al., 2010; White et al., 2008). *csgD* stimulates the curli production through transcriptional activation of the *csgBAC* operon, which encodes the major curli subunit CsgA, as well as nucleator protein CsgB. *csgD* also indirectly regulates the cellulose synthesis by activating transcription of *adrA*. Being a member of the GGDEF protein family, AdrA synthesizes c-di-GMP as diguanylate cyclase and its transcription is regulated by *csgD* (Liu et al., 2014). BapA, a large surface protein involved in biofilm formation and the expression of *bapA*, is coordinated with that of genes encoding curli fimbriae and cellulose, through the action of *csgD* (Latasa et al., 2005; Wang et al., 2016). Deletion of *bapA* resulted in the loss of capacity to form a biofilm, whereas the overexpression of *bapA* was associated with increased biofilm biomass formation (Latasa et al., 2005).
**Figure 1.** A diagrammatic illustration of the role of CsgD in the curli fimbriae and cellulose production during *Salmonella* biofilm formation. The curli subunits CsgA and CsgB are encoded by csgBAC operon which is positively regulated by the master regulator CsgD. Cellulose synthesis is regulated by CsgD at post-transcriptional level by controlling the activity of several cellulose synthases including BcsA through the synthesis of c-di-GMP by AdrA. C-di-GMP is synthesized as diguanylate cyclase by AdrA and its transcription is regulated by CsgD (Liu., 2014). BapA is a large surface protein required for biofilm formation. The expression of bapA are activated through the genes that encodes curli and cellulose via the action of CsgD (Jonas et al., 2007).

### 1.4.3 Identification of biofilm formation based on colony morphology

A number of previous studies have examined biofilm development in *Salmonella* isolates by colony morphotypes on Congo red agar. This method involves growing *Salmonella* on Luria Bertani agar plates supplemented with Congo red, coomassie brilliant blue and calcofluor dyes. These indicator dyes aid in detection of curli fimbriae and cellulose, which are the major components of *Salmonella* biofilm (O'Leary et al., 2013; Romling et al., 1998; Zogaj et al., 2001). Multicellular behaviour by *Salmonella* spp is often categorised according to the colony
morphology into rdar (red, dry and rough) expressing curli fimbriae and cellulose, bdar (brown, dry and rough) expressing only curli fimbriae, pdar (pink, dry and rough) expressing only cellulose and saw (smooth and white) expressing neither curli fimbriae nor cellulose (Giaouris & Nesse, 2015). In addition to Congo red assays, there are several other methods such as, light microscopy, electron microscopy, atomic force microscopy, confocal microscopy, liquid-air pellicles and microtiter plate assay that are routinely used to demonstrate the role of key components of *Salmonella* biofilm (Castelijn et al., 2012; Giaouris et al., 2015; Jonas et al., 2007; Simm et al., 2014). Biofilm formation is one of the major challenges in controlling *Salmonella* contamination outside the host environment. Control of biofilm is complicated due to their inherent nature and resistance to commonly used antimicrobials.

1.4.4 *Salmonella* biofilm resistance to antimicrobials

Both in the host and non-host environments, biofilm formation confers resistance in *Salmonella* to numerous stress factors such as antimicrobial agents, desiccation, stress, heat, low pH and ionizing radiation (Steenackers et al., 2012). Several studies have investigated and compared the efficiency of different antimicrobial agents against *Salmonella* biofilms and planktonic cells. It has been concluded that cells in biofilm are more resistant to commonly used disinfectants compared to their planktonic counterparts (Joseph et al., 2001; Olson et al., 2002; Wong et al., 2010a). Age of biofilms is one of the key factors that influences the susceptibility of biofilms to various antimicrobial compounds. There is a general consensus that age or maturity of biofilms is associated with increased resistance to antimicrobial substances however, conflicting results were observed between studies. Previous studies have documented increased resistance to antimicrobials with increasing age of biofilm (Anwar & Costerton, 1990; Corcoran et al., 2014; Fraud et al., 2005; Nguyen & Yuk, 2013; Shen et al., 2011). In contrast, the age of biofilm was not associated with increased resistance to antimicrobials (Gilbert et al., 2001; Kim et al., 2007; Wilson et al., 1996; Wong et al., 2010b).
Structure and physiological attributes of biofilm cells confer an inherent resistance to antimicrobial agents such as antibiotics, disinfectants and germicides (Donlan & Costerton, 2002). Several factors such as, reduced diffusion of antimicrobial agent through exopolysaccharide matrix, alterations in the growth rate of biofilm cells and other physiological changes such as specific gene expression in response to direct environmental conditions, development of stress response, expression of multi drug efflux pumps and presence of persister cells, have been suggested to contribute to antimicrobial resistance in biofilm cells (Bridier et al., 2015; Donlan & Costerton, 2002; Mah & O'Toole, 2001). Although commonly known mechanisms of biofilm resistance have been suggested above, exact mechanisms of resistance of biofilms remain unclear or not fully understood. Given that, biofilm development is a multicellular process, it could be possible that multiple defence mechanisms are involved in antimicrobial resistance in biofilms.

1.4.5 Intervention strategies for *Salmonella* control on farm

The environmental contamination of layer flocks by *Salmonella* is a worldwide issue. There are several pre-harvest and post-harvest methods that are widely used to reduce *Salmonella* contamination of layer flocks. Pre-harvest methods include; 1) genetic lines of laying hens resistant to *Salmonella*, 2) flock management such as biosecurity, pest control, cleaning and disinfection, 3) feed management practices, 4) vaccination and 5) natural antimicrobial products such as; bacteriophage, competitive exclusion flora, probiotics, prebiotics and organic acids. Post-harvest methods involve; 1) egg storage at ambient temperature, 2) chemical methods such as; egg washing with sanitisers, electrolysed water, ozone and hydrogen peroxide, 3) physical methods such as irradiation, UV, microwave, pulsed light, gas plasma technology and 4) biological methods such as plant extracts (Galiş et al., 2013).

A common and basic control method is routine cleaning and disinfection of sheds in layer flocks, although its efficacy is highly variable. Previous studies have demonstrated reduction in *Salmonella* contamination in laying houses after cleaning and disinfection, but complete
elimination of *Salmonella* from layer farms was not achieved (Davies & Breslin, 2003a; Wales et al., 2007). Wildlife species have also been identified as an important vector of *Salmonella* contamination of layer farms (Davies & Breslin, 2003b; Wales et al., 2007). There is evidence of several wildlife vectors such as mice, rats, flies, litter beetles and foxes involved in maintaining *Salmonella* infection on farms (Davies & Breslin, 2003c; Liebana et al., 2003). Therefore, control of these different vectors would reduce the introduction of *Salmonella* into layer flocks.

Another important method of *Salmonella* prevention in poultry is vaccination. There are currently two types of *Salmonella* vaccines, live, and killed (Howard et al., 2012). Live vaccines are produced by spontaneous mutations or attenuation by chemical or ultraviolet mutagenesis (De Buck et al., 2004). In Australia, there is no recognised vaccination control program in layer flocks against *Salmonella* serovars that are relevant to human health. There are variations between individual producers and across the states in vaccination activities (Moffatt et al., 2016) because long-term efficacy of the currently available *S.* Typhimurium vaccine in field conditions is uncertain. For post-harvest control, egg washing is extensively used in Australia, Canada, USA, and Japan to reduce eggshell contamination (Galiş et al., 2013; Hutchison et al., 2004). However, the advantages of egg washing have been debated due to risk of cuticle damage and *Salmonella* penetration from the shell surface into egg contents. Previous work has indicated that egg washing may increase the likelihood of penetration of *S.* Typhimurium through the eggshell, particularly during storage and drying conditions, if egg-washing procedures are below standard (Gole et al., 2014c).

### 1.5 Organic acids

Organic acids and their salts are widely used as food additives and preservatives, but also have applications in pre and post-harvest food production and processing (Ricke, 2003). Organic acids have several advantages as antimicrobials because they are a generally recognised as safe
(GRAS), have no acceptable daily intake, are low cost and easy to manipulate (Mani-López et al., 2012). Organic acids are a group of compounds with a –COOH bond and pH dependent proton dissociation property. There are different types of organic acids such as, simple short chain, medium chain, long chain fatty acids, side chained simple, complex acids and aromatic acids (Van Immerseel & Atterbury, 2013). Organic acids, such as acetate, propionate, and butyrate are produced in smaller quantities in the gut environment of food animals and humans, with higher concentration in regions where anaerobic microflora are abundant (Ricke 2003). Organic acids are relatively stable and are usually metabolised by food animals or are excreted unabsorbed, therefore do not create an issue of residues in the food (Wales et al., 2010).

1.5.1 Mechanism of antimicrobial action of organic acids

The anti-microbial mechanism of organic acids is derived from their ability to cross bacterial cell membranes. Once internalised in the cell cytoplasm, organic acids dissociate into anions and protons and interfere with the pH homeostasis of the cell. In addition to alterations in cellular pH gradient, other toxicity effects of organic anions that attribute membrane structure, osmolality and macromolecule synthesis have also been investigated (Cherrington et al., 1990; Ricke, 2003; Russell, 1992; Van Immerseel et al., 2006; Wales et al., 2010). The antibacterial activity of organic acids differs between molecules and is dependent on chemical structure. There are large variations in minimum inhibitory concentrations (MIC) for certain acids when different bacterial species are exposed. For example, the MIC of acetic acid is 250 times lower for Bacillus subtilis than for lactobacilli (Hsiao & Siebert, 1999). The lactobacilli were much more resistant to acetic, benzoic, butyric and lactic acids while E. Coli was most resistant to citric, malic and tartaric acid.

1.5.2 Effects of organic acid on survival and virulence of Salmonella

Medium chain fatty acids (MCFA; caproic, caprylic, carpic and lauric acid-C6 to C12) are much more effective against Salmonella than short chain fatty acids (SCFA) such as formic, acetic,
propionic and butyric acid). As little as 25 mM concentration of C6-C10 acids were bacteriostatic to S. Enteritidis, but the same strain tolerated 100 mM of SCFA (Van Immerseel et al., 2003; Van Immerseel et al., 2004b). Similarly, S. Enteritidis and S. Typhimurium were incubated with low concentrations of monocaprin (5 nM) that had been combined with an emulsifier and the bacteria did not survive (Thormar et al., 2006). This data suggests MCFA are more effective against Salmonella however, large-scale studies are lacking to support this claim.

Organic acid products are generally used in poultry with the aim to reduce Salmonella shedding, internal organ and gut colonisation. Penetration of intestinal epithelial cells is the initial step in Salmonella pathogenesis. Thus, invasion of intestinal epithelial cells must be controlled because this virulence attribute is directly associated with gut colonisation in poultry (Bohez et al., 2008). Invasion genes are located on Salmonella Pathogenicity Islands 1 (SPI-1) and promotes the activity of invasion however, several other SPI's are also required for advancement of pathogenesis. hilA, the environmentally regulated gene is the most important regulatory gene of SPI-1 (Van Immerseel et al., 2006). Organic acids such as propionic acid and butyric acid have been shown to differentially regulate hilA expression. S. Enteritidis and S. Typhimurium when pre-incubated with propionic and butyric acids, were less invasive in human and porcine intestinal cell lines and chicken caecal epithelial cells in vitro however, the invasion potential was more when pre-incubated with acetic acid supplemented medium. The invasion was found to be associated with effects of hilA expression (Durant et al., 1999; Lawhon et al., 2002; Van Immerseel et al., 2004a; Van Immerseel et al., 2004b). The effect of organic acids in feed and water to reduce Salmonella colonisation has been studied extensively in broilers however, such studies in layers are lacking.

1.5.3 Studies on efficacy of organic acids in poultry

Commercial organic acid formulations are widely used in the poultry industry as feed and water additives to combat specific pathogens, including Salmonella. Every commercial organic acid
product is not tested for their inhibitory and bactericidal properties against *Salmonella* serovars isolated from layer farm environment. However, some data on efficacy of organic acids that are potentially used as feed and water additives to control *Salmonella* contamination is available in poultry.

Organic acids are used in feed, drinking water and other matrices to prevent *Salmonella* colonisation in animal tissue and further transmission through the food chain (Van Immerseel et al., 2006). Contaminated poultry feed is considered to be a major source of *Salmonella* in layer farm environments (Williams, 1981). It is believed that incorporating organic acids in feed would decontaminate feed and reduce the uptake of *Salmonella* by chickens. A commercial brand of formic acid significantly reduced the number of *Salmonella* positive breeder feed samples from 4.1% to 1.1% after the treatment of feed with 0.5% formic acid (Humphrey & Lanning, 1988).

In a large-scale study (three years), incidence of *Salmonella* infection in newly hatched chicks was reduced when breeder hens were fed with formic acid treated feed (Humphrey & Lanning, 1988). Breeder hens that received feed treated with formic acid had fewer numbers of *Salmonella* in the litter (4.3% vs 1.4%), hatchery waste (15.3% vs 1.2%) and insert papers (4.6% vs 1.4%). These decreases were evident from the moment breeder hens received formic acid treated feed and illustrate the effects of formic acid on vertical transmission of *Salmonella* (Humphrey & Lanning, 1988).

Researchers have developed new range of products in which SCFA are encapsulated in mineral carriers resulting in slow release during the transport of these acid products through the intestinal tract. Previous study has examined the efficacy of acetic acid (0.24%), formic acid (0.22%) or propionic acid (0.27%) as film coated microcapsules on colonisation of *S.* Enteritidis in the caeca, liver, and spleen. The results showed significant increase in caecal colonisation of *S.* Enteritidis when acetic acid was added, but a significant decrease in caecal colonisation was
observed when butyric acid was added to the feed. Internal organ colonisation was increased when acetic or formic acid was added to the feed (Van Immerseel et al., 2004c). The effect of powder and coated butyric acid were compared for their ability to control *Salmonella* colonisation of caeca and internal organs after infection of young chickens with *S. Enteritidis*. Coated butyric acid was superior to uncoated butyric acid in reducing *S. Enteritidis* colonisation of the caeca and internal organs of specific pathogen free layer chickens (Van Immerseel et al., 2005).

Drinking water is one source of *Salmonella* contamination, thus maintaining water free of *Salmonella* is critical. Water acidification is also one of the methods for controlling *Salmonella* infections and or contamination on farm. SCFA are commonly used in water as sanitizers (Van Immerseel et al., 2006). Commercial organic acids with blends of acids or salts are commonly marketed in the poultry industry. These products differ in their composition, ability to reduce pH and appear to have highly variable antibacterial activity from product to product in water and feed (Wales et al., 2010). A recent study examined the effects of 13 commercial organic acid products (water and feed products) on *S. Enteritidis* and *S. Typhimurium*. All products tested against both *Salmonella* strains revealed little variations between strains with respect to inhibitory and bactericidal activity of each product. However, significant variations were observed between products. The water source had a strong influence on the organic acid activity in this study. The ability of organic acid products to reduce *Salmonella* contamination was higher in tap water in comparison with mineral or river water (Wales et al., 2013). The antibacterial activity of organic acids against *Salmonella* is studied extensively in different matrices however, the efficacy of commercial formulations against *Salmonella* biofilms is not widely studied.

### 1.5.4 Use of organic acids against biofilms

Although several studies have demonstrated the effect of organic acid against *Salmonella* contamination in poultry, there are few studies that examined the effect of organic acid against
*Salmonella* biofilms. Phenolic products or polyphenols are one of the most ubiquitous groups of phytochemicals. Ferulic and gallic acids are examples of phenolic acids of plant origin and their activity against biofilms formed by *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Listeria monocytogenes* was evaluated in a recent study (Borges et al., 2012). Experiments conducted using microtiter plate assay revealed that both ferulic acid and gallic acid (0.1%) had preventive action on biofilm formation and showed higher potential to reduce biofilm mass formed by Gram-negative bacteria (Borges et al., 2012). The efficiency of organic acid treatment can be influenced by cell contact surface. It has been shown that 2% lactic and acetic acid significantly reduced *E. coli* biofilms on polystyrene surface however, the same acids did not reduce *E. coli* biofilms formed on stainless steel surfaces (Park & Chen, 2015). The effects of citric, malic and gallic acids on prevention and removal of *Bacillus subtilis* biofilms on microtiter plates and stainless steel surfaces were evaluated (Akbas & Cag, 2016). The results of this study showed that citric acid was the most effective in preventing and removing biofilms from both surfaces. The anti-biofilm effect of malic acid was higher than gallic acid but less than citric acid. The prevention and control of biofilms by these acids was significantly higher on microtiter plates compared to stainless steel, and this effect was dependent on concentration and exposure time of acids on both surfaces (Akbas & Cag, 2016). The effects of malic acid, ozone alone or combination of both were examined for inhibition of biofilm formation by *S. Typhimurium* on different food surfaces (Singla et al., 2014). The results showed that malic acid alone was not able to inhibit biofilm formation however, malic acid and ozone combination significantly reduced biofilm formation on the food surfaces. These findings suggest that malic acid and ozone could be effective disinfectants against biofilms formed on food contact surfaces (Singla et al., 2014). Cantaloupe rind samples were electrostatically spread with malic and lactic acid solutions alone or in combination to examine reduction in *S. Typhimurium* attachment (Almasoud et al., 2015). The results of this study showed that a combined treatment of 2% malic and lactic acid had 3.6-log reduction of *S. Typhimurium* SD10. This study also revealed that lactic acid was more effective than malic
acid in reducing attachment of S. Typhimurium (Almasoud et al., 2015). The studies described above suggest that organic acids are able to reduce biofilm formation however, complete elimination of biofilms is not possible. In addition, organic acids could be used as a potent anti-biofilm agent in the food industry to avoid toxic hazards of commonly used chemical disinfectants (Akbas & Cag, 2016). However, their prudent use is important to avoid development of acid resistant Salmonella strains (Mani-López et al., 2012).

1.5.5 Salmonella acid tolerance response (ATR)

The antibacterial effects of organic acid depends on concentration, pH and dissociation constant of each acid (Mani-López et al., 2012). Exposure of Salmonella to sub optimal/ sub lethal organic acid concentrations can induce development of adaptive response in the organism. Several studies have reported acid tolerance response (ATR) in S. Typhimurium (Alvarez-Ordonez et al., 2009; Foster & Hall, 1990; Greenacre et al., 2003). This feature of S. Typhimurium represents a serious food safety concern for the meat and poultry industry since during meat product processing, bacteria experience number of mildly acidic conditions, which may induce ATR in Salmonella. The development of ATR could increase the ability of Salmonella to survive in other lethal stresses including low pH in gut and intracellular environment (Mani-López et al., 2012). In order to reduce development of ATR, it is important to inactivate bacteria with an appropriate decontamination method. Appropriate doses, acid type, temperature, and hygienic practices are vital to reduce the risk of the development of ATR in Salmonella (Mani-López et al., 2012).
1.6 Objectives of thesis

Based on research gaps identified within the literature, the objectives of this thesis were:

1. To examine antimicrobial resistance of *S. enterica* isolates from egg farm environment.

2. To study faecal shedding, reproductive organ colonisation and egg contamination after oral infection of laying hens with *S. Typhimurium*.

3. To study the ability of *S. enterica* isolates to form biofilm on eggshell surfaces.

4. To evaluate anti-bacterial and anti-biofilm activity of commercial organic acid products against *S. enterica* isolates recovered from egg farm environment.
1.7 References


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CHAPTER 2 ANTIMICROBIAL RESISTANCE OF NON-TYPHOIDAL *SALMONELLA* ISOLATES FROM EGG LAYER FLOCKS AND EGG SHELLS
**Statement of Authorship**

**Title of Paper**
Antimicrobial resistance of non-typhoidal *Salmonella* isolates from egg layer flocks and egg shells.

**Publication Status**
- ✔ Published
- Submitted for Publication
- Unpublished and Unsubmitted work written in manuscript style

**Publication Details**

**Principal Author**

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<th>Vivek V. Pande</th>
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<tr>
<td>Contribution to the Paper</td>
<td>Designed and performed the experiment. Data compilation, analysis and interpretation of data. Wrote manuscript. Responded to comments and suggestions by co-authors and journal editor</td>
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<td>Overall percentage (%)</td>
<td>80%</td>
</tr>
<tr>
<td>Certification:</td>
<td>This paper reports 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.</td>
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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 to 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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Antimicrobial resistance of non-typhoidal Salmonella isolates from egg layer flocks and egg shells

Vivek V. Pande, Vaibhav C. Gole, Andrea R. McWhorter, Sam Abraham, Kapil K. Chousalkar *
School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy, SA 5371, Australia

ABSTRACT
This study was conducted to examine the antimicrobial resistance (AMR) of Salmonella spp. isolated from commercial caged layer flocks in New South Wales and South Australia. All Salmonella isolates (n = 145) were subjected to phenotypic and genotypic characterisation of AMR and carriage of integrons. The majority of Salmonella isolates (91.72%) were susceptible to all antimicrobials tested in this study. Limited resistance was observed to amoxicillin and ampicillin (5.51%), tetracycline (4.13%), cephalothin (2.06%) and trimethoprim (0.68%). None of the isolates were resistant to cepotaxime, cefotiofur, ciprofloxacin, chloramphenicol, gentamycin, neomycin or streptomycin. A low frequency of Salmonella isolates (4.83%) harboured antimicrobial resistance genes and a class 1 integron. The most commonly detected AMR genes among the Salmonella isolates were blaclav (2.07%), tet A (1.38%) and qnrS (0.69%). Overall, Salmonella enterica isolates exhibited a low frequency of AMR and represent a minimal public health risk associated with the emergence of multidrug resistant Salmonella spp. from the Australian layer industry.

1. Introduction
Salmonella spp. is a major cause of foodborne illness worldwide. Consumption of contaminated food products such as pork, meat, egg and egg related products are among the most common sources of Salmonella infection (Hur et al., 2012). In Australia, outbreaks of human salmonellosis are associated with the consumption of contaminated food products containing chicken meat or egg products (OzFoodNet Working Group, 2012). In Australia, a total of 11,992 Salmonella cases were reported in 2010, representing 53.7 cases per 100,000 people which is higher compared to the previous 5 years with an average infection rate of 41.8 cases per 100,000 people (OzFoodNet Working Group, 2012).

Typically, infection with Salmonella is self-limiting producing mild gastroenteritis, however, severe infection occurs common in elderly and immunocompromised individuals (Parry and Threlfall, 2008). Severe, systemic salmonellosis may require treatment with antimicrobials such as fluoroquinolones and extended-spectrum cephalosporins (Parry and Threlfall, 2008). The use of antimicrobial agents in the prevention and treatment of many infectious diseases and as a growth promoter is well known both in veterinary and human medicine (Hur et al., 2012). Indiscriminate use of antibiotics in both animal and human populations has, however, led to an emergence of multidrug resistant Salmonella strains (Anjum et al., 2011). The emergence and dissemination of antibiotic resistance to Salmonella is of significant global concern for both animal and public health. Moreover, the transfer of multidrug resistant Salmonella spp. to humans through food producing animals can compromise the treatment options (Hur et al., 2012). Compared with many other countries, Australia currently has a very conservative approach for antibiotic usage in commercial egg layer flocks. Antimicrobials, such as fluoroquinolones, are prohibited and cefotiofur is not approved for mass administration in food producing animals (Cheng et al., 2012; Obeng et al., 2012). To date, there is little information available characterising antimicrobial resistance (AMR) in Salmonella isolated from commercial Australian egg layer flocks. In this study, the phenotypic and genotypic AMR was characterised for multiple Salmonella isolates recovered from layer flocks and egg shells.

2. Materials and methods
2.1. Bacterial strains and serotyping
A total 145 Salmonella isolates were used in this study. Samples were isolated from 33 commercial caged layer flocks sourced from a total of 13 farms from New South Wales (10 farms) and South Australia (3 farms). All Salmonella isolates used in this study were previously isolated in our laboratory during epidemiological studies (Chousalkar and Roberts, 2012; Gole et al., 2014a, 2014b). Details of Salmonella isolates, sources and their distribution are presented in Table 1. All isolates were serotyped by the Salmonella Reference Laboratory, Institute of Medical and Veterinary Science, SA Pathology (Adelaide, South Australia).
2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of each Salmonella isolate to 12 antibiotics (Table 2) was determined using the broth microdilution method and the results were interpreted according to the established Clinical and Laboratory Standards Institute (CLSI, guidelines (CLSI, 2013)). In cases where CLSI breakpoints were absent, results were evaluated according to the National Antimicrobial Resistance Monitoring guidelines (National Antimicrobial Resistance Monitoring System, 2012) or Swedish Veterinary Antimicrobial Resistance Monitoring guidelines (Swedish Veterinary Antimicrobial Resistance Monitoring, 2012). All tests were performed using Escherichia coli ATCC 25922 as control strain. Salmonella isolates showing resistance to more than three classes of antimicrobial agents were classified as multi-drug resistant (MDR).

2.3. DNA extraction

DNA extraction from all Salmonella isolates was performed using 6% Chelex® (Bio-Rad, Sydney, NSW, Australia) prepared in TE (10 mM Tris 1.5 mM MgCl2, 2.5 mM each primer pool and 1 U of Hotstart Taq polymerase (Qiagen, Australia)). Uniplex PCR reactions were also performed in a total reaction volume of 25 μL containing 2 μL DNA template. Each uniplex PCR reaction mixture consisted of final concentration of 1.5 mM MgCl2, 2.5 μM each of dNTP (Bioline, Australia), 25 pM of each primer, and 1 U of Taq polymerase (Bioline, Australia). DNA amplification was carried out in T100 thermal cycler (Bio-Rad, Australia) using the following conditions: 15 min initial denaturation at 95 °C, following 30 cycles of denaturation at 94 °C for 30 s, annealing at various temperatures for 30 s (Table S1) and extension at 72 °C for 60 s and final amplification cycle at 72 °C for 10 min. PCR products were electrophoresed at 80 V for 2.5 h on 2% agarose gel. The size of PCR products was determined by comparing with standard 100 bp ladders (Thermo Fisher, Australia). The details of antimicrobial resistance genes, integrons and primer sequences are described in Supplementary Table S1.

3. Results

3.1. Antimicrobial susceptibility screening of Salmonella isolates

The Salmonella isolates selected for this study displayed a low but wide spectrum of antibiotic resistance (Table 2). A total of 91.72% (133/145) of the Salmonella isolates were susceptible to all tested antimicrobials. Overall, resistance was observed to amoxicillin and ampicillin (5.51%), tetracycline (4.13%), cephalothin (2.06%) and trimethoprim (6.85%) (Table 2). Resistance to cefotaxime, ceftiofur, ciprofloxacin, chloramphenicol, gentamicin, neomycin, or streptomycin was not observed for any isolate. S. Mbandaka, S. Typhimurium and S. Worthington showed resistance to amoxicillin, ampicillin and tetracycline whereas S. Agona, S. Anatum, S. Infantis and S. Oranienburg were susceptible to all tested antimicrobials.

No multidrug resistant phenotypes were identified from any Salmonella isolate included in this study. Results of resistance patterns of all Salmonella isolates are described in Table 3.

3.2. Detection of resistance genes and integrons

All 145 Salmonella isolates belonging to seven different serovars recovered from layer flocks were investigated for antimicrobial resistance genes and integrons by multiplex and uniplex PCR. A total of 4.83% (7/145) Salmonella isolates harboured antimicrobial resistance genes and class 1 integron (Table 4). The resistance genes most commonly detected were blaTEM (2.07%), tet A (1.38%), dhfrT and class 1 integron (0.69%). One isolate, S. Mbandaka was positive for class 1 integron and contained the dhfrT gene conferring resistance to

### Table 1

<table>
<thead>
<tr>
<th>Salmonella (S.) serovar</th>
<th>Source</th>
<th>Dust</th>
<th>Egg belt</th>
<th>Fences</th>
<th>Shell wash</th>
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<tr>
<td>S. Agona</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S. Anatum</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>S. Infantis</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>S. Mbandaka</td>
<td>7</td>
<td>11</td>
<td>12</td>
<td>0</td>
<td>30</td>
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<td>S. Oranienburg</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>6</td>
<td>30</td>
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<tr>
<td>S. Typhimurium</td>
<td>14</td>
<td>5</td>
<td>6</td>
<td>1</td>
<td>26</td>
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<tr>
<td>S. Worthington</td>
<td>7</td>
<td>8</td>
<td>14</td>
<td>2</td>
<td>31</td>
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<tr>
<td><strong>Total</strong></td>
<td>42</td>
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<td>45</td>
<td>20</td>
<td>145</td>
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### Table 2

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<th>Antimicrobials</th>
<th>No. of isolates tested</th>
<th>AMC</th>
<th>AMP</th>
<th>CTX</th>
<th>CEF</th>
<th>CPL</th>
<th>CIP</th>
<th>CHL</th>
<th>GEN</th>
<th>NEO</th>
<th>STR</th>
<th>TET</th>
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<td>S. Agona</td>
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<td>0.0</td>
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<td>0.0</td>
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<tr>
<td>S. Anatum</td>
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<td>0.0</td>
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<tr>
<td>S. Infantis</td>
<td>16</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
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<tr>
<td>S. Mbandaka</td>
<td>30</td>
<td>6.66 (2)</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>3.33 (1)</td>
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<td>S. Oranienburg</td>
<td>30</td>
<td>0.0</td>
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<tr>
<td>S. Typhimurium</td>
<td>26</td>
<td>3.84 (1)</td>
<td>0.0</td>
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<td>0.0</td>
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<tr>
<td>S. Worthington</td>
<td>31</td>
<td>16.12 (5)</td>
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<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
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<td><strong>Total</strong></td>
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<td>5.51 (8/145)</td>
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<td>0.0</td>
<td>206 (3/145)</td>
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<td>413 (6/145)</td>
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Abbreviations: AMC — Amoxycillin; AMP — Ampicillin; CTX — Cefotaxime; CEF — Ceftiofur; CPL — Cephalothin; CIP — Ciprofloxacin; CHL — Chloramphenicol; GEN — Gentamicin; NEO — Neomycin; STR — Streptomycin; TET — Tetracycline; TRM — Trimethoprim.
trimethoprim. Several antimicrobial resistance genes and integrons were not detected in some Salmonella isolates including, 
\( \text{carT1, floR, aadA, bla}_{\text{OXA}}, \text{cmA}, \text{bla}_{\text{CMY-2}}, \text{dfrH1, aphA1, bla}_{\text{SHV}}, \text{bla}_{\text{MER-1}, \text{bla}_{\text{TEM-1}}, \text{aac(3)-Ia}, \text{bla}_{\text{TEM-1}}, \text{tetB, tetC, tetD, tetG, integron class 2 and integron class 3.} \)

The association of phenotypic resistance and the presence of antimicrobial resistance genes were variable among Salmonella serovars. For example, phenotypic resistance to one or more antimicrobials was observed for both S. Mbandaka (n = 5) and S. Worthington (n = 5), however, the corresponding resistance genes were not detected by PCR. One S. Typhimurium isolate tested positive for the presence of the \( \text{bla}_{\text{TEM}} \) gene but did not show resistance by broth microdilution method.

### 4. Discussion

The widespread use of antibiotics both in both human and veterinary medicine is well known, but their imprudent use may give rise to multidrug resistant strains of Salmonella (Anjum et al., 2011). This is the first Australian-based descriptive study characterising the occurrence of phenotypic and genotypic antimicrobial resistance of Salmonella isolates recovered from commercial layer flocks. It is noteworthy that the majority of Salmonella isolates (91.72%) in this study were susceptible to all antimicrobials tested and no resistance was observed to fluoroquinolones or extended spectrum cephalosporins which are commonly used for the treatment of human salmonellosis (Cheng et al., 2012). Though the degree and prevalence of antimicrobial resistance in Salmonella isolates was low, the most common resistance was to amoxicillin, ampicillin, cephalothin, tetracycline and trimethoprim. A large scale study in the UK reported that 76% Salmonella isolates from laying flocks were susceptible to antimicrobials tested, with the frequency of resistance being highest to ampicillin (15.3%), tetracycline (13.6%), chloramphenicol (6.8%) and streptomycin (10.7%) (Snow et al., 2007). The findings presented here are in contrast with previous reports from the USA and China showing high rates of resistance to antimicrobials in Salmonella isolates from retail chicken meat samples, food animals or food products (Musgrove et al., 2006; Chen et al., 2004; Louden et al., 2012). Globally, resistance to fluoroquinolones or extended spectrum cephalosporins among Salmonella spp. isolated from food animals is prevalent (Hur et al., 2012). The results obtained in this study demonstrating the absence antimicrobial resistance in Salmonella spp. isolated from Australian caged layer flocks is an important finding for public health. The lack of fluoroquinolone resistance in this study could be attributed to a ban on the use of fluoroquinolones in Australian food animals. As a result, resistance to fluoroquinolones has not been detected in many bacterial strains including Salmonella (Cheng et al., 2012). Australia has strict regulations and controlled usage for antibiotic in food producing animals in contrast with other countries where fewer regulations are imposed on usage of critical antimicrobials (Obeng et al., 2012). Antibiotics used in human medicine are generally not used to treat commercial egg layers. This strategy has helped minimise the amount of antibiotic residues in eggs or egg products and transfer of antibiotic resistance and resistance genes from animals to humans through the food chain (Barton et al., 2003). As a result of these restrictions, a recent Australian study reported very low levels of antimicrobial resistance in Salmonella isolates from confirmed cases of salmonellosis in livestock (Abraham et al., 2014). The current study has extended these earlier studies and has demonstrated a lower prevalence of antimicrobial resistance in Salmonella serovars recovered from Australian layer flocks.

In this study, antimicrobial resistance was associated with specific Salmonella serovars, S. Mbandaka, S. Typhimurium and S. Worthington. Serovar specific differences for resistance have been described in previous studies (Musgrove et al., 2006; Aslam et al., 2012). The serovar specific differences in resistance could be the result of selective transfer of mobile genetic elements or Salmonella serovars may possess the genetic determinants of antimicrobial resistance (Aslam et al., 2012). Further studies designed to characterise the genetic mechanisms of serovar specific resistance are essential.

The discordance between phenotypic resistance and the presence of antimicrobial resistance genes among S. Mbandaka and S. Worthington isolates was consistent with (Abraham et al., 2014) but differed from (Anjum et al., 2011). In the present study, one S. Typhimurium isolate found to be PCR positive for the \( \text{bla}_{\text{TEM}} \) gene was susceptible to all antimicrobials (Anjum et al., 2011). Such a phenomenon could occur due to the presence of silent, non-expressing genes or existence of non-integrated gene cassettes as described for \( \text{qnrA}, \text{bla}_{\text{CMY}}, \text{tetB} \) and \( \text{strA-strB} \) genes in Salmonella isolates (Anjum et al., 2011).

Consistent with previous work, \( \text{bla}_{\text{TEM}} \) was the most commonly detected \( \beta \)-lactamase gene in Salmonella isolates which were resistant to ampicillin and amoxicillin (Aslam et al., 2012). Tetracycline resistance in Salmonella is encoded most frequently by class \( \text{A, B, C, D, and G} \) genes and these genes were screened in all Salmonella isolates in this study. Two of our isolates (1.38%) contained only \( \text{tetA} \) gene and none of the other \( \text{tet} \) genes were observed in Salmonella isolates. Resistance to trimethoprim is primarily mediated by expression of \( \text{dfrG} \) genes encoding drug-resistant dihydrofolate reductase (DHFR) (Hoppenin et al., 1995). The \( \text{dfrG} \) gene was identified in a trimethoprim resistant S. Mbandaka isolate and is consistent with other reports (Abraham et al., 2014). A class 1 integron was also detected in a single S. Mbandaka isolate which carried \( \text{dfrG} \) gene conferring resistance to trimethoprim, a finding which has also been reported earlier in Salmonella spp. isolated from livestock (Abraham et al., 2014).

The work described here highlights the low rates of antimicrobial resistance in Salmonella isolated from Australian layer flocks. Regular surveillance over a larger geographical area and comprehensive nationwide sampling is, however, needed to identify any changes in antimicrobial resistance patterns in Salmonella isolates in the egg industry. Collective efforts to ensure the appropriate use of antimicrobials in
food animals remain a high priority to minimise the risk of spread and development of antimicrobial resistance.

**Competing interests**

All authors declare no competing interest.

**Acknowledgements**

This research was conducted within the Poultry CRC (Grant no. 3.3.2), established and supported under the Australian Government’s Cooperative Research Centres Program. Mr. Vivek Pande is an International Postgraduate Research Scholarship recipient at University of Adelaide. We would like to acknowledge Nikita Nzewi for technical help during this research work. We would also like to thank all egg producers who participated in this study.

**Appendix A. Supplementary data**

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2015.02.025.

**References**


Table S1 Details of primers used in PCR for antimicrobial resistance genes.

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<th>PCR pool set/ annealing temperature</th>
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<th>Amplicon size (bp)</th>
<th>Reference</th>
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CHAPTER 3 STUDY OF *SALMONELLA* TYPHIMURIUM INFECTION IN LAYING HENS
# Statement of Authorship

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<th>Study of <em>Salmonella Typhimurium</em> Infection in Laying Hens</th>
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## Principal Author

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<td>Overall percentage (%)</td>
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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 to 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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<th>Dr Kapil K. Chousalkar</th>
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Study of Salmonella Typhimurium Infection in Laying Hens

Vivek V. Pande, Rebecca L. Devon, Pardeep Sharma, Andrea R. McWhorter and Kapil K. Chousalkar*

School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy, SA, Australia

Members of Salmonella enterica are frequently involved in egg and egg product related human food poisoning outbreaks worldwide. In Australia, Salmonella Typhimurium is frequently involved in egg and egg product related foodborne illness and Salmonella Mbandaka has also been found to be a contaminant of the layer farm environment. The ability possessed by Salmonella Enteritidis to colonize reproductive organs and contaminate developing eggs has been well-described. However, there are few studies investigating this ability for Salmonella Typhimurium. The hypothesis of this study was that the Salmonella Typhimurium can colonize the gut for a prolonged period of time and that horizontal infection through feces is the main route of egg contamination. At 14 weeks of age hens were orally infected with either S. Typhimurium PT 9 or S. Typhimurium PT 9 and Salmonella Mbandaka. Salmonella shedding in feces and eggs was monitored for 15 weeks post-infection. Egg shell surface and internal contents of eggs laid by infected hens were cultured independently for detection of Salmonella spp. The mean Salmonella load in feces ranged from 1.54 to 63.35 and 0.31 to 98.38 most probable number/g (MPN/g) in the S. Typhimurium and S. Typhimurium + S. Mbandaka group, respectively. No correlation was found between mean fecal Salmonella load and frequency of egg shell contamination. Egg shell contamination was higher in S. Typhimurium + S. Mbandaka infected group (7.2% S. Typhimurium, 14.1% S. Mbandaka) compared to birds infected with S. Typhimurium (5.66%) however, co-infection had no significant impact on egg contamination by S. Typhimurium. Throughout the study Salmonella was not recovered from internal contents of eggs laid by hens. Salmonella was isolated from different segments of oviduct of hens from both the groups, however pathology was not observed on microscopic examination. This study investigated Salmonella shedding for up to 15 weeks p.i which is a longer period of time compared to previously published studies. The findings of current study demonstrated intermittent but persistent fecal shedding of Salmonella after oral infection for up to 15 weeks p.i. Further, egg shell contamination, with lack of internal egg content contamination and the low frequency of reproductive organ infection suggested that horizontal infection through contaminated feces is the main route of egg contamination with S. Typhimurium in laying hens.

Keywords: Salmonella Typhimurium, laying hens, oviduct, egg contamination
INTRODUCTION

Foodborne gastric infections due to *Salmonella enterica* are of major concern worldwide. Typically contaminated eggs and egg related products are primary vehicles for human salmonellosis. Globally, *S. Enteritidis* represents a dominant serotype in commercial poultry isolated from eggs and is frequently involved in egg related food poisoning in humans (Foley et al., 2011). *S. Enteritidis*, however, is not endemic in Australian poultry flocks (OzFoodNet Working Group, 2009). This niche has been filled by *S. Typhimurium*, which is a leading cause of foodborne outbreaks linked to contaminated egg and egg related products (OzFoodNet Working Group, 2009). In 2010, *S. Typhimurium* was the most commonly notified *Salmonella* serotype accounting for 5241 (44%) cases of all *Salmonella* notified infections in Australia (OzFoodNet Working Group, 2012).

The external and internal egg contamination by *Salmonella* during poultry production is a complex issue, influenced by many variables. As a result, implementation of appropriate control measures is extremely difficult (Whiley and Ross, 2015). Egg contamination can occur by two routes, vertical or horizontal. Vertical transmission is a result of reproductive organ colonization (ovary and oviduct) before shell formation, whereas horizontal transmission occurs due to external egg shell contamination (De Reu et al., 2006).

Oral challenge of both *S. Enteritidis* and *S. Typhimurium* has the potential to invade the reproductive organs. However, only *S. Enteritidis* has been recovered from egg contents (Keller et al., 1997; Okamura et al., 2001a; Gast et al., 2004, 2007, 2013; Gantois et al., 2008). The intrinsic properties and resistance to antibacterial compounds enabling *S. Enteritidis* to colonize the oviduct and contaminate egg internal contents are well-known (Gantois et al., 2009). There is, however, limited information on the long term shedding, colonization of reproductive organs and egg contamination by *S. Typhimurium*.

Previous studies have examined reproductive organ colonization and egg contamination by *S. Typhimurium* in laying hens. Results from these experiments, however, are inconsistent due to variation in experimental design, route of inoculation, inoculum dose as well as the strain of *S. Typhimurium* selected (Baker et al., 1980; Williams et al., 1998; Leach et al., 1999; Okamura et al., 2001a,b, 2010). Moreover, the majority of these previous studies examined the capability of *S. Typhimurium* to colonize reproductive organs and/or egg contamination frequency up to 3 weeks post-infection, which could fail to unveil the ability of *S. Typhimurium* to cause egg contamination over a prolonged period (Wales and Davies, 2011). Altogether, there is a lack of published data arising from long term experiments aimed at fecal shedding, reproductive organ colonization and egg contamination by *S. Typhimurium* in laying hens.

On commercial layer farms environmental contamination with multiple *Salmonella* serovars is common and represents a serious concern for poultry industries worldwide (Gole et al., 2014c; Im et al., 2015). A recent epidemiological survey examining the prevalence of *Salmonella* spp. on layer farms demonstrated that *S. Mbandaka* (54.40%, 68/125) was the most frequently recovered serovar along with *S. Typhimurium* (11.54%, 15/130) (Gole et al., 2014a,c). *S. Mbandaka* has also been isolated from egg shell, animals, feed, and sporadic cases of human salmonellosis (Hoszowsky and Wasyl, 2001; Little et al., 2007; Im et al., 2015). Given the diversity of poultry associated *Salmonella* serovars, there are few reports on how the presence of commonly isolated serovars from layer farm environments (such as *S. Mbandaka*) might influence the shedding patterns of *S. Typhimurium*. In addition, how two *Salmonella* serovars have an effect upon organ invasion and egg contamination *in vivo* is still unclear.

Given the potential public health threat by *S. Typhimurium* associated with consumption of contaminated egg and egg products, this study sought to investigate the dynamics of egg contamination over an extended time course. In this study the duration of fecal shedding, its relation to frequency of egg contamination and reproductive organ colonization after oral infection with *S. Typhimurium* alone and in combination with *S. Mbandaka* was investigated in commercial layer hens. To our knowledge, this is a first report of a *Salmonella* oral challenge model conducted in controlled environment employing strict biosecurity measures for up to 30 weeks of age.

MATERIALS AND METHODS

Experimental Animals

Fertile eggs were obtained from a commercial layer parent flock. Eggs were fumigated using formaldehyde as previously described (Samberg and Meroz, 1995) and incubated for 21 days at 37.7°C. Relative humidity was maintained at 45–55% until day 18 and increased to 55–65% up to hatching. A total of 32 birds were hatched, raised in pens until week 10 and then shifted in cages contained within positive pressure rooms at Roseworthy Campus of *The University of Adelaide*, until the end of experiment (week 30).

Sample size for this study was calculated using Openepi-Tool (Dean et al., 2011). This tool along with the sample size determines the power of the experimental trial. For sample size calculation, assumed percent with outcome in *S. Typhimurium* and *S. Typhimurium + S. Mbandaka* infected group was 20% and 70% respectively with the confidence interval of 95%. This gave an 80% chance of detecting differences between treatment groups with normal approximation.

Prior to experiments all animal rooms and equipment were fumigated with formaldehyde and cleaned with commercial disinfectants (Chemet, Australia). Throughout the experiment, feed was sterilized by fumigation (Samberg and Meroz, 1995) and water purification tablets (Aquatabs, Ireland) were added to drinking water. Feed and water was provided *ad libitum*. The recommended lighting program specified in the commercial management guide of Hy-Line Australia Pty Ltd was followed in this study. Feces, feed, and water samples were tested at fortnightly intervals for detection of *Salmonella* spp. by the culture method as described previously (Gole et al., 2014a). All experiments were conducted according to the protocol approved by the institutional animal ethics committee of *The University of Adelaide* (Protocol No. S-2014-008) and in compliance with regulations
the Australian code for the care and use of animals for scientific purposes.

**Bacterial Strains, Culture, and Inoculum Preparation**

*Salmonella* isolates used for oral infection in this study were recovered previously from layer hen fecal samples (Gole et al., 2014a,c). *S. Typhimurium* PT 9 has been frequently implicated in egg product related human Salmonellosis in Australia (OzFoodNet Working Group, 2009, 2012). Hence, this strain was selected. The antimicrobial resistance profile of *Salmonella* isolates was characterized earlier (Pande et al., 2015). *S. Typhimurium* PT 9 isolate used in this study was resistant to amoxicillin, ampicillin, and tetracycline. This isolate was susceptible to trimethoprim, cefotaxime, cephalothin, chloramphenicol, gentamycin, neomycin, and streptomycin. On other hand, S. Mbandaka isolate used in this study was resistant to amoxicillin, ampicillin, and trimethoprim and susceptible to cefotaxime, cephalothin, chloramphenicol, gentamycin, neomycin, streptomycin, and tetracycline (Pande et al., 2015).

For oral inoculation, stocks of bacterial strains were cultured overnight at 37°C on nutrient agar. Twenty-four hours prior to infection, a single colony of each *Salmonella* strain was added to a separate tube containing 5 ml of Luria Bertani (LB) broth (Oxoid, Australia) and incubated 6 h with shaking (110 rpm). From this LB culture, 10 μl was transferred to 5 ml of LB and grown overnight at 37°C with shaking. Bacterial suspensions were diluted to 10^8 bacteria per ml for oral inoculation. Bacterial cell counts (CFU) were determined by plating 10-fold serial dilutions of the inoculum on nutrient agar to confirm dose.

**Experimental Design**

At week 10 after hatch, birds were divided in three treatment groups and housed in separate rooms in individual cages. At the age of 14 weeks, birds were orally challenged with either 10^6 CFU of *S. Typhimurium* PT 9 (T group, n = 14) or 10^9 CFU of *S. Typhimurium* PT 9 and S. Mbandaka (TM group, n = 14). Control birds (C group, n = 4) received only sterile LB broth. Following infection, all experimental birds were monitored twice a day for clinical signs of infection. All hens were humanely euthanized at the age of week 30. Ovary and segments of the oviduct (infundibulum, magnum, isthmus, uterus (shell gland) and vagina) were collected in sterile Whirl-Pack plastic bags (Thermo Scientific, Australia) followed by the addition of 90 ml Buffered peptone water (BPW, Oxoid, Australia) (1:10); bags were then homogenized for 1 min. From this bag 10 ml of homogenate was placed into three different sterile tubes (10^6 dilution). Then 1 ml of homogenate sample was transferred to three different tubes containing 9 ml of BPW, and then serially diluted in triplicate tubes of BPW. The tubes were incubated overnight at 37°C. After incubation, 10 μl of BPW from each MPN tube was plated on modified semisolid Rappaport–Vassiliadis (MSRV, Oxoid, Australia) agar plates and incubated at 42°C for 24 h. A loopful of media from the leading edge of white zones from MRSV plate was streaked onto XLD and or *Salmonella* Brilliance agar plates (Oxoid, Australia) for confirmation of *Salmonella*.

**Bacteriological Analysis of Egg Shell and Internal Contents**

Eggs from both control and *Salmonella* infected hens were collected aseptically in individual Whirl-Pack plastic bags. Each egg was processed for the presence of *Salmonella* on the egg shell and in the internal contents. Briefly, an individual egg was immersed in 10 ml of BPW in Whirl-Pack plastic bag, massaged for 2 min and then removed. The egg shell rinse was then processed for *Salmonella* isolation as previously described (Gole et al., 2014a). Each egg was dipped in 70% ethanol for 2 min to prevent internal content contamination from the egg shell surface. Each egg was then broken aseptically and contents emptied into a Whirl-Pack plastic bag. The egg contents were homogenized thoroughly. Five ml of internal egg contents were mixed with 45 ml of BPW (1:10) and incubated at 37°C overnight. *Salmonella* enrichment and isolation from egg shell and internal content samples was carried out as described previously (Gole et al., 2014a). *Salmonella* positive egg shell wash enriched in Rappaport-Vassiliadis (RVS) broth was stored in 20% glycerol at −80°C for further PCR testing.

**Bacteriological Analysis of Reproductive Organs**

Samples (0.1–0.2 g) of the ovary, infundibulum, magnum, isthmus, uterus (shell gland), and vagina were collected in sterile tubes. The tissue samples were homogenized using a Bullet Blender® (Next Advance Inc. USA) at full speed for 2 min and serial 10-fold dilutions were prepared in phosphate buffer saline (PBS). From each dilution 100 μl was spread directly onto XLD agar plates (Oxoid, Australia) and incubated overnight at 37°C. After 24 h the number of colonies was enumerated and concentration of *Salmonella* in tissues was expressed as mean log_{10} CFU/g of tissue.

**DNA Extractions from Fecal Samples, Egg Shell Wash, and Reproductive Organs**

DNA was extracted from all fecal samples of control, T and TM groups using QIAamp DNA Stool Mini Kit (Qiagen, Australia) according to manufacturer instructions. DNA extraction from all *Salmonella* isolates recovered from egg shell washes of T and TM hens was performed as previously described (Pande et al., 2015). Briefly, the frozen stock of RVS broth was thawed and 50 μl of

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*Pande et al.*
broth was mixed with 450 μl of LB broth and incubated overnight at 37°C. One hundred microliter of overnight bacterial culture was mixed to 1 ml of sterile water and centrifuged at 14,000 g for 2 min. After decanting the supernatant, the bacterial pellet was re-suspended in 200 μl of 6% Chelex® (Bio-Rad, Sydney, NSW, Australia) prepared in TE (10 mM Tris and 1 mM EDTA). Tubes were incubated at 56°C for 20 min, vortexed and further incubated at 100°C for 8 min. Samples were placed on ice for 5 min and centrifuged at 14,000 g for 10 min. Supernatants were recovered from each sample and used as a DNA template for PCR.

DNA was extracted from reproductive organs using DNeasy Blood & Tissue Kit (Qiagen, Australia) as per manufacturer instructions.

**PCR Detection of S. Typhimurium and S. Mbandaka**

*Salmonella* positive egg shell wash samples from T and TM group, all fecal samples and culture positive reproductive organs from T and TM groups were screened for *Salmonella* specific invA gene and S. Typhimurium serovar specific genomic region TSR3 (Akiba et al., 2011) by multiplex PCR to detect S. Typhimurium PT9. TSR3 gene was not amplified in S. Mbandaka isolates (Akiba et al., 2011). Further, to differentiate S. Mbandaka from S. Typhimurium PT 9 in the TM group, DNA extracted from feces, egg shell wash and reproductive organs were tested for *dhfrV* gene that confers resistance to trimethoprim (Pande et al., 2015). Samples from T infected group were also tested for *dhfrV* gene. S. Typhimurium PT9 used in this study was sensitive to trimethoprim and negative for *dhfrV* gene (Pande et al., 2015). PCR reactions for *invA* and TSR3 gene were performed in a total reaction volume of 20 μl including 2 μl DNA template. PCR reaction mixture consisted of final concentration of 1.5 mM MgCl2, 2.5 μM of each dNTP (Bioline, Australia), 0.5 μM each forward and reverse primer and 2.5 U of Taq polymerase (Bioline, Australia). DNA amplification was carried out in T100 thermal cycler (Bio-Rad, Australia) using the following protocol: 2 min initial denaturation at 94°C, following 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 68°C for 30 s and a final extension at 72°C for 5 min.

PCR reactions for *dhfrV* gene were performed in a total reaction volume of 25 μl including 2 μl DNA templates. Each PCR reaction mixture consisted of final concentration of 1.5 mM MgCl2, 2.5 μM of each dNTP (Bioline, Australia), 0.28 μM of each primer and 2.5U of Taq polymerase (Bioline, Australia) using the following PCR cycle conditions: 2 min initial denaturation at 95°C, following 30 cycles of denaturation at 94°C for 30 s, annealing at 64°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 min.

PCR products were electrophoresed at 60 V for 1.5 h on 1.5% agarose gel in 0.5X Tris borate EDTA buffer and stained with GelRed™ nucleic acid gel stain (Biotium, USA). The size of PCR products was determined by comparing with standard 100 bp ladders (Thermo Fisher, Australia). Negative and positive controls were used in each PCR reaction for all the samples.

In order to investigate the detection limit of S. Typhimurium by multiplex PCR, *Salmonella* negative fecal samples were spiked with S. Typhimurium or S. Typhimurium + S. Mbandaka at doses ranging from 10^1 to 10^9 CFU/ml. Following DNA extractions from spiked samples using QIAamp DNA Stool Mini Kit (Qiagen, Australia), multiplex PCR was performed as described above.

**Histopathology of Reproductive Organs**

Infundibulum, magnnum, isthmus, uterus, and vagina were collected individually to evaluate histomorphological alterations in response to *Salmonella* infection. Tissue samples of reproductive organs were fixed in 10% neutral buffered formalin, embedded in paraffin wax and 5 μm sections were stained with Haematoxylin and Eosin stain (H &E).

**Statistical Analysis**

Significant differences between groups in the isolation rate of *Salmonella* from feces and eggs were determined by Fisher’s exact probability test. MPN data was analyzed by two way analysis of variance. The relationship between recovery of *Salmonella* (MPN/g) from feces and isolation of *Salmonella* from egg shell was determined by Pearson correlation test ($R^2$-value). All data generated in this study was analyzed statistically either using GraphPad Prism version 6 software or IBM® SPSS Statistics® version 21. P < 0.05 were considered statistically significant.

**RESULTS**

**Clinical Symptoms and Mortality**

During the first week p.i., mucoid and blood tinged feces were observed in two birds from each treatment group. No mortality was recorded in any of the treatment groups throughout this study.

**Fecal Shedding of Salmonella at Different p.i. Intervals**

All fecal, water and feed samples collected from experimental birds before oral challenge were negative for *Salmonella* spp. The number of *Salmonella* positive fecal samples for both T (S. Typhimurium) and TM (S. Typhimurium and S. Mbandaka) groups over the course of the experiment is presented in Table 1. No significant difference ($p = 0.848$) was observed in number of fecal samples positive for *Salmonella* between T (152/168, 90.47%) and TM groups (154/168, 91.66%). There were more fecal positive samples until week 5 p.i.

An overall decline after week 5 in the number of birds shedding *Salmonella* in feces was observed in both groups. Overall, persistent *Salmonella* shedding in feces was observed in both groups throughout the experimental period after oral infection. *Salmonella* spp. was not isolated from any bird in the control group (data not shown).

**Enumeration of Salmonella from Feces by MPN Method**

The viable counts of *Salmonella* (MPN/g) in feces over the course of the experiment are presented in Figure 1. Throughout the
### TABLE 1 | Detection of *Salmonella* from fecal samples by culture and PCR.

<table>
<thead>
<tr>
<th>Days p.i.</th>
<th>T group</th>
<th>TM group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Salmonella</em> detection by culture method</td>
<td><em>S. Typhimurium</em> detection by PCR</td>
</tr>
<tr>
<td>Day 1</td>
<td>14/14 (100%)</td>
<td>14/14 (100%)</td>
</tr>
<tr>
<td>Day 3</td>
<td>13/14 (93%)</td>
<td>14/14 (100%)</td>
</tr>
<tr>
<td>Day 6</td>
<td>14/14 (100%)</td>
<td>14/14 (100%)</td>
</tr>
<tr>
<td>Day 9</td>
<td>14/14 (100%)</td>
<td>14/14 (100%)</td>
</tr>
<tr>
<td>Day 12</td>
<td>14/14 (100%)</td>
<td>13/14 (92.85%)</td>
</tr>
<tr>
<td>Week 3</td>
<td>14/14 (100%)</td>
<td>12/14 (85.71%)</td>
</tr>
<tr>
<td>Week 5</td>
<td>11/14 (78.57%)</td>
<td>11/14 (78.57%)</td>
</tr>
<tr>
<td>Week 7</td>
<td>10/14 (71%)</td>
<td>12/14 (85.71%)</td>
</tr>
<tr>
<td>Week 9</td>
<td>9/14 (64.28%)</td>
<td>11/14 (78.57%)</td>
</tr>
<tr>
<td>Week 11</td>
<td>9/11/14 (71%)</td>
<td>10/14 (71%)</td>
</tr>
<tr>
<td>Week 13</td>
<td>11/14 (78.57%)</td>
<td>11/14 (78.57%)</td>
</tr>
<tr>
<td>Week 15</td>
<td>11/14 (100%)</td>
<td>11/14 (100%)</td>
</tr>
<tr>
<td>Total</td>
<td>152/168 (90.47%)</td>
<td>155/168 (92.26%)</td>
</tr>
</tbody>
</table>

*a*Number of fecal samples positive/total numbers of fecal samples tested by culture method.  
*Numbers of fecal samples positive/total numbers of fecal samples tested by PCR.

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### Analysis of Salmonella from Egg Shell

All eggs tested (*n* = 136) from control hens were negative for *Salmonella*. The frequency of egg shell contamination after oral infection ranged from 0 to 16.67 and 21.11% in T and TM group, respectively. Overall the frequency of egg shell contamination was significantly higher (*p* = 0.001) in the TM group (18.69%, 83/444) as compared to the T group (5.66%, 24/424) (Table 2).

In order to determine the effect of co-infection (TM group) on the recovery rate of *S*. Typhimurium on egg shell surface, multiplex PCR that specifically differentiates *S*. Typhimurium from *S*. Mbandaka was carried out. Overall the frequency of recovery of *S*. Typhimurium from egg shells of TM group (7.20%, 32/444) did not differ significantly from T group (5.66%, 24/424) (Table 2). PCR results indicated that overall, 14.1% (63/444) egg shell samples were positive for *S*. Mbandaka (Table 2). Correlation between *Salmonella* shedding in feces (MPN/g) and subsequent egg shell contamination was analyzed using a Pearson correlation test. No correlation was evident between mean fecal *Salmonella* load and observed frequency of contaminated eggs laid by orally infected birds of T and TM group (*p* = 0.624, *R*² = 0.002 T group; *p* = 0.177, *R*² = 0.022 TM group). Fecal Shedding and egg contamination data per bird/egg over time is presented in Supplementary Table 1. In TM group, *Salmonella* shedding in feces and eggs was variable in individual birds across 15 weeks p.i.

### Comparison between Culture and PCR Based Detection of *S*. Typhimurium

The sensitivity of multiplex PCR for invA and TSR3 gene to detect *S*. Typhimurium was determined by spiking fecal samples with various doses of *S*. Typhimurium or *S*. Typhimurium + *S*. Mbandaka. The PCR detection limit for *S*. Typhimurium

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![Graph showing the number of *Salmonella* MPN/g in feces of birds orally infected with 10⁹ CFU of *S*. Typhimurium (T group) or *S*. Typhimurium + *S*. Mbandaka (TM group). Values are Mean ± SEM. Comparison between group marked with an asterisk (*) is significantly different at *p* < 0.05 based on ANOVA.](image-url)
was $10^6$ CFU/reaction whereas it was $10^4$ CFU/reaction when fecal samples were spiked with both S. Typhimurium and S. Mbandaka. The PCR detection limit for $dfrV$ gene to detect S. Mbandaka was $10^4$ CFU/reaction, when fecal samples were spiked with both S. Typhimurium and S. Mbandaka. The details of fecal and egg shell samples positive and negative for $Salmonella$ at different days p.i. by culture and PCR method are described in Tables 1, 2. Fecal samples from T infected group tested negative for $dfrV$ gene. Overall, S. Typhimurium was detected in 133/168 (79.16%) fecal samples and 32/444 (7.20%) egg shell samples in TM group. Similarly, S. Mbandaka was detected in 121/168 (72.02%) fecal samples and 63/444 (14.18%) egg shell samples in TM group.

### Analysis of $Salmonella$ from Internal Egg Contents

Over the course of the experiment, $Salmonella$ was not isolated from the internal content of eggs ($n = 1004$) laid by either control or infected hens.

### Bacteriological and Histopathological Analysis of Reproductive Organs

The recovery rate of $Salmonella$ from reproductive organs is summarized in Table 3. Colonization of $Salmonella$ in reproductive organs of laying hens after oral infection was observed in both groups. In the T group birds $Salmonella$ was recovered from different segments of oviduct: ovary (1/14), infundibulum (2/14), magnum (2/14), isthmus (3/14), uterus (3/14), and vagina (3/14) collected after 15 weeks p.i. However, in the TM group $Salmonella$ was only recovered from infundibulum (1/14), uterus (2/14), and vagina (1/14) (Table 3). Mean concentration of $Salmonella$ (mean $\log_{10}$ CFU/g) was highest in vagina (3.54 ± 0.64) and uterus (3.00 ± 0.45) of the T and TM group birds, respectively (Table 3). Colonization of reproductive organs was not frequent and only 0–3 hens of the 14 hens for each of the groups showed $Salmonella$ in the bacteriological analysis of their reproductive organs, and no histopathological lesions were detected in any case.

### Detection of S. Typhimurium in Reproductive Tissues by PCR

The reproductive organs from the T and TM groups found positive for $Salmonella$ by culture method were analyzed by multiplex PCR to detect S. Typhimurium. Only one reproductive tissue (uterus) from T group was found positive for S. Typhimurium by multiplex PCR assay (data not shown). All other samples from T group tested negative by PCR for $Salmonella$ spp.

### DISCUSSION

The present experiment was designed to study the long term shedding, egg contamination and colonization of oviduct by S. Typhimurium. It is considered that adult birds are more resistant to salmonellae than young chicks due to the developed gut microflora (Gast, 2008). Continued harboring of the organism and intermittent fecal shedding has also been noted for up to 1 year following infection of day old chicks (Gast, 2008) however, in our study older birds (14 wk) were infected with $Salmonella$. Previous studies reported low colonization of S. Typhimurium.
in adult birds (Groves, 2011), however, the results of the current study demonstrate that S. Typhimurium can colonize the gut and shed bacteria up to 15 weeks p.i.

In this study, intermittent but prolonged fecal shedding of bacteria was observed in both infected groups. A significant difference between the T and TM group at day 12 p.i. could be due to the intermittent Salmonella shedding. The magnitude of Salmonella shedding was higher up to 5 week p.i. Thereafter, the level of Salmonella in feces dropped but persisted for 15 weeks p.i. The increased Salmonella shedding in feces observed up to 5 week p.i. in this study could be attributed to the stress associated with the onset of lay. In layer birds, the stress occurring as a result of lay could negatively impact their immunity (El-Lethey et al., 2003; Humphrey, 2006) consequently resulting in higher shedding of Salmonella. Higher rate of fecal Salmonella shedding at the early onset of lay has also been reported previously (Gole et al., 2014a). The decrease in Salmonella load in feces after 5 weeks p.i. in both treatment groups could be the result of recovery from laying stress or development of effective humoral response. In addition, previous studies have reported that gastrointestinal microflora of older birds was responsible for protection against food poisoning Salmonella serovars (Barrow et al., 1988; Gast, 2008).

Fecal Salmonella counts from this study could not be compared with previous reports because the majority of these studies have examined post-infection fecal shedding of Salmonella for a shorter duration. A field survey investigating the prevalence of Salmonella shedding on commercial layer farms found significant variability in Salmonella prevalence at various stages of lay (Gole et al., 2014a). On farm, shedding of S. Typhimurium from the known positive laying hens can be intermittent and remain undetected for several weeks (Gole et al., 2014c). Such results suggest that Salmonella spp. can remain in the caeca for long periods of time and persistently infected hens could transmit the infection to unexposed and susceptible birds thereby maintaining the Salmonella infection cycle in the flock (Lister and Barrow, 2008). Hence, it is essential to frequently monitor the Salmonella free status of the birds used for the infection trials.

No correlation between fecal Salmonella counts and the recovery of bacteria from egg shell surface in experimentally infected birds was observed in this study. A recent longitudinal survey on two commercial layer farms found a significant relationship between Salmonella fecal contamination and egg shells testing positive for Salmonella (odds ratio 9.18; p < 0.001) (Gole et al., 2014c). In contrast, egg shells were found negative for S. Typhimurium in experimental infections although the bacterium was excreted in the feces (Baker et al., 1980; Okamura et al., 2001a, b). In the present study, though the egg shell contamination failed to positively relate with fecal shedding of Salmonella, fecal carriage of Salmonella was observed throughout the experimental period. The egg shell surface contamination observed in this study stresses the importance of proper egg handling and hygienic practices in food preparation and processing premises to avoid cross contamination of other food products.

The multiplex PCR was validated to detect S. Typhimurium positive samples in T and TM groups. In experimentally spiked fecal samples, the multiplex PCR demonstrated a good sensitivity and was able to detect 10^2 CFU/reaction of S. Typhimurium. On the other hand, PCR assay was able to detect 10^4 CFU/reaction of S. Typhimurium and S. Mbandaka in the fecal samples spiked with S. Typhimurium + S. Mbandaka. The poor detection limit observed in the feces experimentally spiked with S. Typhimurium + S. Mbandaka may under-represent the positive samples detected in the TM group using the PCR assay. The poor PCR sensitivity compared with the standard culture method to detect S. Typhimurium in fecal samples is similar to previous studies and could be attributed to the gradual reduction in Salmonella in feces, presence of PCR inhibitors and other abundant microflora DNA interfering with the PCR assays (Wilson, 1997; Gole et al., 2014a, c).

This study has examined egg shell contamination following oral infection with Salmonella for a prolonged period (15 weeks p.i.) compared to previous short term experimental infection studies (up to 3 weeks) and our results demonstrated that egg shell contamination by Salmonella occurred for longer p.i. intervals. Egg shell contamination following oral infection of S. Typhimurium observed in this study has also been reported previously (Cox et al., 1973). In the current study, the overall rate of egg shell contamination by Salmonella was significantly higher in the co-infected group (TM group) compared to the T group. However, the effect of co-infection on egg shell contamination analyzed by PCR demonstrated no significant difference in number of S. Typhimurium positive egg shells between T (S. Typhimurium) and TM groups (S. Typhimurium + S. Mbandaka). There is a little literature indicating the effect of mixed Salmonella infection on egg contamination after oral infection. The high experimental infection doses used in our study does not mimic field situations and had non-significant effects on the recovery rate of S. Typhimurium from the egg shell in the coinfected group. To compare these results with the field scenario further experiments using different routes and doses of multiple Salmonella serotypes are needed.

In the present study internal egg contents laid down by birds infected with S. Typhimurium alone or in combination with S. Mbandaka were negative for Salmonella up to week 15 p.i. The results of this study are also in agreement with the field surveys in Australia (Daughtry et al., 2005; Gole et al., 2013, 2014c) and previous reports in which oral or crop infection with S. Typhimurium was not associated with the contamination of egg contents (Cox et al., 1973; Baker et al., 1980; Keller et al., 1997; Okamura et al., 2010). On the other hand, contamination of egg internal contents with S. Typhimurium has been documented after experimental infection of hens at the onset of lay via oral and aerosol routes (Williams et al., 1998; Leach et al., 1999; Okamura et al., 2010). Altogether, the possibility of egg content contamination with S. Typhimurium seems to be a rare event. However, in those studies where experimental infection has caused internal contamination, sexual maturity, or the onset of lay was found to be an important factor for internal egg contamination.
It is well-known that colonization of reproductive organs with S. Enteritidis results in the deposition of bacteria within the egg contents of developing eggs in experimentally infected laying hens (Thiagarajan et al., 1994; Keller et al., 1995). However, the frequency of S. Typhimurium isolation from reproductive organs and corresponding frequency of internal egg content contamination is unclear. The present study determined that colonization of reproductive organs of S. Typhimurium infected (T group) hens and coininfected (TM group) hens varied after oral infection. The magnitude of S. Typhimurium recovery from each section of oviduct except for uterus was higher in the T group than TM group where Salmonella was localized to certain parts of the oviduct. To assess the effect of mixed infection, reproductive tissues from T and TM groups found positive for Salmonella by culture method were also analyzed by multiplex PCR to detect S. Typhimurium. In spite of positive culture results, S. Typhimurium was recovered from only one reproductive tissue (uterus) by multiplex PCR assay. This finding suggests that culture methods are more sensitive than multiplex PCR in detecting S. Typhimurium. The lack of additional stand-alone S. Mbandaka group and sacrifice of birds at regular intervals are some of the limitations of this study. However, it is interesting to note that despite the low Salmonella colonization in the oviduct of hens from TM group, frequency of egg shell contamination was significantly higher in the TM group (particularly for S. Mbandaka) as compared to the T group.

The results of prolonged Salmonella fecal shedding observed in this study indicated that colonization was present somewhere within the animal after several weeks p.i. However, though the persistence of Salmonella in the reproductive tissues of very few infected birds was evident after a long p.i. interval, the internal egg contents were negative throughout the experimental period in both T and TM groups. Moreover, this study demonstrates that the mere presence of S. Typhimurium in reproductive tissues would not give rise to the production of internally contaminated eggs.

The observations of the present study also support the previous findings which concluded that S. Typhimurium has the potential to colonize both the reproductive organs and developing eggs prior to oviposition but cannot be recovered from internal egg contents after oviposition (Keller et al., 1997; Okamura et al., 2001a; Gantois et al., 2008). Overall, the results of the present and previous studies demonstrate that S. Typhimurium was found to colonize the reproductive organs of laying hens. However, why S. Typhimurium is not associated with contamination of laid eggs is still unclear.

S. Typhimurium is able to penetrate and survive in the egg albumin and the yolk at 20 or 25 °C (Gantois et al., 2008; Gole et al., 2014b). In addition, the S. Typhimurium genome possesses virulence associated genes involved in cellular adhesion, invasion and survival of S. Typhimurium (McWhorter et al., 2015). Therefore, it could be possible that S. Typhimurium is unable to survive and proliferate in egg contents during egg formation at host body temperature (42° C) or there could be down regulation of genes critical to colonization of S. Typhimurium. This could partly explain why S. Typhimurium despite their colonization in reproductive organs was never isolated from egg contents in this study. Salmonella pathogenicity islands (SPIs) are the gene clusters that encode virulence factors present in Salmonella genome (Foley et al., 2013). It has been observed that SPI-1 and SPI-2 contribute to the colonization of caecum, liver, and spleen in chickens (Dieye et al., 2009; Rychlik et al., 2009). A recent study also demonstrated that poultry body temperature may regulate systemic colonization (Troxell et al., 2015). However, the role of these pathogenicity islands in reproductive organ colonization in laying hens is less understood and needs further research. In addition, the possible role of several factors such as immunoglobulins, iron sequestering, and proteins inhibiting bacterial protease and antibacterial enzymes present in the egg yolk and albumin have been identified to inhibit the growth of Salmonella before shell formation is complete and eggs are laid (Keller et al., 1995; Gantois et al., 2009; Bedrani et al., 2013).

In order to determine the course of reproductive organ invasion after oral Salmonella infection, histopathology of reproductive tissues was carried out in this experiment. The regions of reproductive tract which were found positive after cultural analysis did not show lesions suggestive of bacterial infection. As there is lack of published information on histopathological alterations in oviduct tissue after prolonged infection interval, these findings could not be compared to previous studies. In addition, examination of infected birds at periodic intervals was not a part of this study but may have identified a time window for establishment of oviduct lesions as a result of bacterial infection. The possible explanation for the absence of inflammatory lesions after a long p.i. interval in response to oral Salmonella infection in this study could be related to either the low level of tissue colonization or development of strong immune response to clear the infection. Further, research examining the localization of Salmonella at different time intervals, cellular involvement and why Salmonella clearance from reproductive tissues does not take place is warranted.

In summary, intermittent but persistent fecal shedding of Salmonella after oral infection was observed up to 15 weeks p.i. Further, egg shell contamination together with lack of internal egg contents contamination and the low frequency of reproductive organ infection suggested that horizontal infection through contaminated feces is the main route of egg contamination with S. Typhimurium during lay. Previously, it has been hypothesized that effective and more immune response generated by S. Typhimurium compared to S. Enteritidis is likely to limit the disease progression and quickly clears the S. Typhimurium infection from birds (Wales and Davies, 2011). The egg shell contamination observed in this study also stresses the importance of proper egg handling and hygienic practices in food preparation and processing premises to avoid cross contamination of other food products. Considering the productive life span of commercial laying hens (75–80 weeks) further studies are required to study the shedding of S. Typhimurium beyond 15 weeks p.i.

**AUTHOR CONTRIBUTIONS**

Conception and designed the experiments: KC, AM, and VP. Performed the experiments: VP, RD, PS, KC, and AM. Data
acquisition and analysis: VP, KC, and AM. Drafting of article and revisions: VP, AM, and KC.

ACKNOWLEDGMENTS

This research work was supported by Australian Egg Corporation Limited (AECL) Australia. Mr. VP is a recipient of post-graduate research scholarship of The University of Adelaide Australia. We thank Dr Vaibhav Gole and Dr Rebecca Forder for technical help in this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00203

REFERENCES


Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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**Supplementary Table 1.** PCR detection of *S*. Typhimurium and *S*. Mbandaka in fecal and eggshell wash samples of individual bird (TM group) over 30 wk.

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<sup>a</sup> Number of *Salmonella* positive eggs by individual bird, □ = Positive for *S*. Typhimurium, □ = Positive for *S*. Mbandaka
CHAPTER 4 *Salmonella enterica* Isolates from Layer Farm Environments are Able to Form Biofilm on Eggshell Surfaces

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<td>✔ Published □ Accepted for Publication □ Submitted for Publication □ Unpublished and Unsubmitted work written in manuscript style</td>
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## Principal Author

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<td>Contribution to the Paper</td>
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</tr>
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<td>Overall percentage (%)</td>
<td>80%</td>
</tr>
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<td>Certification</td>
<td>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.</td>
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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.

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<th>Dr Kapil K. Chousalkar</th>
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Vivek V. Pande, Andrea R. McWhorter & Kapil K. Chousalkar

To cite this article: Vivek V. Pande, Andrea R. McWhorter & Kapil K. Chousalkar (2016) Salmonella enterica isolates from layer farm environments are able to form biofilm on eggshell surfaces, Biofouling, 32:7, 699-710, DOI: 10.1080/08927014.2016.1191068

To link to this article: http://dx.doi.org/10.1080/08927014.2016.1191068
Salmonella enterica isolates from layer farm environments are able to form biofilm on eggshell surfaces

Vivek V. Pande, Andrea R. McWhorter and Kapil K. Chousalkar
School of Animal and Veterinary Sciences, The University of Adelaide, Roseworthy, Australia

ABSTRACT
This study examined the eggshell biofilm forming ability of Salmonella enterica isolates recovered from egg farms. Multicellular behaviour and biofilm production were examined at 22 and 37°C by Congo red morphology and the crystal violet staining assay. The results indicated that the biofilm forming behaviour of Salmonella isolates was dependent on temperature and associated with serovars. Significantly greater biofilm production was observed at 22°C compared with 37°C. The number of viable biofilm cells attached to eggshells after incubation for 48 h at 22°C was significantly influenced by serovar. Scanning electron microscopic examination revealed firm attachment of bacterial cells to the eggshell surface. The relative expression of csgD and adrA gene was significantly higher in eggshell biofilm cells of S. Mbandaka and S. Oranienburg. These findings demonstrate that Salmonella isolates are capable of forming biofilm on the eggshell surface and that this behaviour is influenced by temperature and serovar.

INTRODUCTION
Members of the bacterial species Salmonella enterica are common causes of human gastroenteritis, a disease characterised by gut inflammation and diarrhoea (Winter et al. 2010). It is estimated that gastroenteritis caused by Salmonella spp. accounts for 93.8 million cases and 155,000 deaths worldwide each year (Majowicz et al. 2010). In Australia, contaminated food products of animal origin, particularly egg and egg products, are frequently associated with outbreaks of human salmonellosis (OzFoodNet Working Group 2015, 2012). The formation of a biofilm is one of the mechanisms Salmonella spp. utilise for survival in harsh physical and chemical environments (Costerton et al. 1999). A biofilm is a community of interacting bacterial cells attached to biotic or abiotic surfaces, embedded in a self-produced extracellular polymeric matrix (Costerton et al. 1999). The extracellular matrix of a biofilm is predominantly comprised of curli, fimbriae and cellulose, which promote linkage and interaction between bacterial cells (Steenackers et al. 2012). Differential multicellular behaviour contributes to the formation of distinct colony morphotypes on Congo red agar plates supplemented with Coomassie brilliant blue (Romling et al. 2003; Seixas et al. 2014). Salmonella within a biofilm displays higher resistance to environmental stressors, antibiotics and disinfectants compared to planktonic counterparts, thus making the eradication of this bacterium extremely difficult from surfaces commonly used in food and the poultry industry (Joseph et al. 2001; Steenackers et al. 2012).

Biofilm formation is a complex process and regulated by several sets of genes involved in extracellular matrix production and adhesion. csgD, a transcriptional regulator of the LuxR superfamily, positively regulates the expression of Salmonella biofilm associated extracellular matrix components, including curli, fimbriae and cellulose (Gerstel & Römling 2003; White et al. 2008; Grantcharova et al. 2010). csgD stimulates curli production through transcriptional activation of the csgBAC operon, which encodes the major curli subunit CsgA as well as the nucleator protein CsgB. csgD also indirectly regulates cellulose synthesis by activating transcription of adrA. Being a member of the GGDEF protein family, AdrA synthesises c-di-GMP as diguanylate cyclase, and its transcription is regulated by csgD (Liu et al. 2014). BapA, a large surface protein involved in biofilm formation and the expression of bapA, is coordinated with that of genes encoding curli, fimbriae and cellulose, through the action of csgD (Latasa et al. 2005; Wang et al. 2016).
Biofilm formation is influenced by several factors including environmental conditions, e.g., the type of culture medium and the surface material, as well as strain origin and serovar (Vestby et al. 2009; Castelijn et al. 2012; Lianou & Koutsoumanis 2012; Schonewille et al. 2012; De Oliveira et al. 2014). The ability of Salmonella to attach and form a biofilm represents a significant public health risk for many industries, including those involved in food production and processing. Biofilm on food preparation surfaces and equipment could serve as a persistent source of cross contamination, compromising the safety of food products and human health (Shi & Zhu 2009).

Salmonella spp. are able to form biofilm on a large number of abiotic surfaces including stainless steel, plastics, cement, rubber and glass (Steenackers et al. 2012). Biofilm formation on the surfaces of eggshells, however, has to date not been documented for any bacterial species. Worldwide egg and eggshell contamination by Salmonella is a major concern for poultry industries. For its survival and growth on the outer surface of an egg Salmonella must overcome low nutrient availability and temperature stress (Gantois et al. 2009). Over the past decade, the number of foodborne salmonellosis cases traced to egg or egg products has substantially increased (OzFoodNet Working Group 2012). This suggests that Salmonella spp. are able to attach and or form biofilm on the eggshell surface, and remain viable, resulting in the cross contamination of a variety of food products. Food safety and public health impacts associated with biofilm forming foodborne pathogens emphasises the importance of understanding eggshell biofilm formation by Salmonella spp. The main objective of the present study was to determine the biofilm forming ability of egg farm related Salmonella serovars on the eggshell surface. In addition, the impact of temperature and serovar variation on biofilm formation was also investigated.

Materials and methods

Bacterial strains and serotyping

One hundred and forty-five Salmonella isolates of seven different serovars: S. Agona (n=6), S. Anatum (n=6), S. Infantis (n=16), S. Mbandaka (n=30), S. Oranienburg (n=30), S. Typhimurium (n=26), and S. Worthington (n=31) were used to study biofilm formation. All Salmonella isolates used in this study were previously isolated in the authors’ laboratory from 33 caged layer flocks across 13 different egg farms during epidemiological studies (Chousalkar & Roberts 2012; Gole et al. 2014a, 2014b) and were serotyped at the Australian Salmonella Reference Centre, Microbiology and Infectious Diseases, SA Pathology, Adelaide, South Australia.

Biofilm formation assay

Phenotypic characterisation by Congo red morphology

The colony morphology of Salmonella isolates (n=145) was determined on Congo red agar plates for curli, fimbriae and cellulose production, as described previously (Castelijn et al. 2012) with some modifications. Briefly, stock Salmonella cultures were grown on nutrient agar plates at 37°C overnight. Single colonies of Salmonella were grown in 5 ml of Luria-Bertani (LB) broth (10 g bacto tryptone) (Oxoid, Adelaide, Australia), 5 g yeast extract (Oxoid), 10 g sodium chloride (Fischer Chemicals, Melbourne, Australia) with shaking (110 rpm) at 37°C for 6 h. Each Salmonella isolate was plated (3 μl) onto LB agar without sodium chloride, supplemented with Congo red (40 μg ml⁻¹, Sigma Aldrich, St Louis, MO, USA) and Coomassie brilliant blue (20 μg ml⁻¹, Sigma Aldrich). The inoculated plates were incubated at 22 and 37°C for 96 h and colonies were visualised macroscopically. Salmonella enterica subsp. enterica serovar Typhimurium ATCC 14028 was used as a positive control strain during this study.

Quantitation of biofilm formation by crystal violet staining assay

Salmonella isolates (n=145) were grown on LB agar plates overnight at 37°C. Single bacterial colonies were inoculated in 10 ml of LB broth without sodium chloride and incubated at 37°C overnight. Twenty μl of overnight grown bacterial culture were mixed with 180 μl of LB broth without sodium chloride in polystyrene round bottom 96 well plates (Sarstedt, Adelaide, Australia). Negative control wells contained 200 μl of LB broth only. Inoculated plates were incubated statically at either 22 or 37°C for 96 h. After incubation, the plate contents were decanted and wells were washed gently three times with sterile distilled water to remove loosely bound bacteria. Plates were air dried and stained with 200 μl of 0.1% (w/v) crystal violet for 30 min at room temperature. Following staining, wells were gently washed three times with sterile distilled water and air dried. Bound crystal violet stain in the wells was resuspended for 6 h. Each plate was washed three times with sterile distilled water and air dried. Bound crystal violet stain in the wells was resuspended for 6 h. Each plate was washed three times with sterile distilled water and air dried.

Studies on biofilm formation on eggshell

Salmonella isolates and egg source

Four Salmonella isolates from each Salmonella serovar: S. Agona, S. Anatum, S. Infantis, S. Mbandaka,
S. Oranienburg, S. Typhimurium and S. Worthington, isolated from layer farms, were used to investigate their biofilm forming ability on the eggshell. All isolates were examined for their potential to survive and form biofilm on eggshell surfaces at 22°C for 48 h. Based on the results of biofilm formation at 22°C, ambient temperature was selected to study eggshell biofilm formation. In this study, fresh unwashed eggs were obtained from the cage front of a 39-week-old caged layer flock housed at Roseworthy campus, The University of Adelaide.

**Biofilm formation on eggshell**

To ensure that a defined surface area was covered by bacterial culture, an individual egg was marked with a pencil (1.2 cm² area) and disinfected with formaldehyde fumigation (Samberg & Meroz 1995). Eggshell surfaces were further sanitised by immersing the eggs in 70% ethanol for 2 min, allowing them to dry in a class II biosafety cabinet, and then immediately used for experimentation. Biofilm formation on the eggshell was assessed at 22°C on individual eggs in triplicate for each isolate of representative serovar. All experiments were performed in duplicate.

Stock *Salmonella* cultures were grown on nutrient agar plates at 37°C overnight. Single colonies of *Salmonella* were grown in 20 ml of LB broth with overnight incubation at 3°C. Eggs were placed horizontally in Whirlpack bags (Fischer Scientific, Melbourne, Australia) containing 5 ml of overnight grown bacterial culture and 45 ml of LB broth without sodium chloride (10 g bacto tryptone and 5 g yeast extract, Oxoid) (1:10), ensuring the marked surface area was covered with bacterial suspension. The bags were then incubated statically at 22°C for 48 h. Negative control bags contained LB broth only. The cell concentration (CFU) in working bacterial suspensions was calculated by plating 10-fold serial dilutions on nutrient agar.

The number of bacterial cells contained within the eggshell biofilm was enumerated by the plate count method, as described earlier (Castelijn et al. 2012) with some modification. Following incubation at 22°C for 48 h, eggs were aseptically removed from the Whirlpack bags using sterile forceps and kept in Petri plates. The eggshell (1.2 cm² area) was rinsed thrice with sterile 0.85% normal saline solution to remove unbound cells. Attached cells were collected with a sterile cotton swab to remove biofilm-associated bacteria. The cotton swab was placed into a microcentrifuge tube containing 0.85% normal saline and vortexed with acid washed glass beads (710–1180 μm, Sigma-Aldrich) at full speed for 1 min. Serial dilutions were prepared in 0.85% normal saline and plated on xylose lysine deoxycholate (XLD) agar plates (Thermo Scientific, Adelaide, Australia). Plates were incubated at 37°C overnight and colonies were counted to determine the number of colony forming units (CFU). The results were expressed as log₁₀ CFU cm⁻². In this study, three biological replicates of each *Salmonella* isolate were used to ensure reproducibility of the results and the experiments were conducted in duplicate.

**RNA extraction and quantitative real time PCR (RT-PCR)**

The biofilm cells on the eggshell surface were removed as described above, cotton swabs were suspended in 1 ml of RNAlater® Stabilisation Solution (Ambion™, Austin, TX, USA) and cells were harvested by centrifugation at 5,000 g for 10 min. RNA was extracted from triplicate independent cultures of each *Salmonella* isolate from each serovar using a RNeasy mini kit (Qiagen, Melbourne, Australia) according to the manufacturer’s guidelines. The concentration of RNA was analysed using a NanoDrop™ 1000 spectrophotometer (Thermo Fischer Scientific, Wilmington, DE, USA) and samples were stored at −80°C.

Quantitative PCR was performed using Rotor gene 600 real time thermal cycler (Corbett Research, Qiagen) with Quantifast SYBR® Green RT-PCR Kit (Qiagen) as per the manufacturer’s recommended protocol. PCRs were performed in a total reaction volume of 20 μl and contained 10 μl of 2x Quantifast SYBR Green RT-PCR master mix, 2 μl (1 μM) each of the forward and reverse primers, 0.2 μl of Quantifast RT Mix, 2.8 μl of RNase-free water and 5 ng of template RNA. Real time PCR was performed using the following cycling conditions: 10 min reverse transcription at 50°C, 5 min initial PCR activation step at 95°C followed by 40 cycles of denaturation at 95°C for 10 s and combined annealing and extension at 60°C for 30 s. Each RNA sample was analysed in duplicate and each PCR reaction included a non-template control. Primer sets synthesised by Geneworks (Adelaide, Australia) were used to amplify *adrA bapA, csgB* and *csgD* genes (Supplementary material Table S1). To generate a standard curve, the serially diluted RNA standard (1,000 ng to 0.001 ng) was quantified in each quantitative RT-PCR run. The slopes of the standard curves were used to measure the amplification efficiency for each of these primers. Quantitative RT-PCR results were analysed by comparative threshold cycle (Ct) method and an internal calibrator was used to normalise the transcription levels (Livak & Schmittgen 2001). The relative gene expression was calculated by the 2⁻ΔΔCt formula, where ΔΔCt is equivalent to the Ct of the internal calibrator subtracted from the Ct of the target gene.

**Scanning electron microscopy (SEM)**

SEM was performed to examine the attachment of bacterial cells and cell density on the eggshell during biofilm formation. For SEM, following incubation at 22°C for 48 h, eggs were aseptically removed from Whirlpack bags and the eggshell (1.2 cm² area) was rinsed three times with
Table 1. Congo red agar morphotypes from various Salmonella serovars after incubation for 96 h at 22 and 37°C.

<table>
<thead>
<tr>
<th>Morphotypes (Temperature)</th>
<th>S. Agona (n=6)</th>
<th>S. Anatum (n=6)</th>
<th>S. Infantis (n=16)</th>
<th>S. Mbandaka (n=30)</th>
<th>S. Oranienburg (n=30)</th>
<th>S. Typhimurium (n=26)</th>
<th>S. Worthington (n=31)</th>
<th>Total (n=145)</th>
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<tr>
<td>rdar (22°C)</td>
<td>6 (100%)</td>
<td>6 (100%)</td>
<td>16 (100%)</td>
<td>30 (100%)</td>
<td>29 (96.66%)</td>
<td>26 (100%)</td>
<td>19 (61.29%)</td>
<td>132 (91.03%)</td>
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<tr>
<td>saw (22°C)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (3.33%)</td>
<td>0</td>
<td>12 (38.71%)</td>
<td>13 (8.96%)</td>
</tr>
<tr>
<td>rdar (37°C)</td>
<td>6 (100%)</td>
<td>6 (100%)</td>
<td>16 (100%)</td>
<td>30 (100%)</td>
<td>29 (96.66%)</td>
<td>26 (100%)</td>
<td>29 (96.66%)</td>
<td>145 (100%)</td>
</tr>
<tr>
<td>saw (37°C)</td>
<td>6 (100%)</td>
<td>6 (100%)</td>
<td>16 (100%)</td>
<td>30 (100%)</td>
<td>30 (100%)</td>
<td>26 (100%)</td>
<td>31 (100%)</td>
<td>145 (100%)</td>
</tr>
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</table>

Abbreviations: rdar – red, dry and rough; saw – smooth and white.

sterile 0.85% normal saline solution to remove unbound cells. Eggshell samples were removed carefully and fixed in a solution containing 1.25% glutaraldehyde, 4% paraformaldehyde, and 4% sucrose in phosphate buffered saline (PBS) (pH 7.2). Samples were rinsed once in wash buffer containing 4% sucrose in PBS, post fixed in 2% osmium tetroxide (OsO₄) for 30 min and dehydrated in ascending grades of ethanol. Samples were immersed in a 1:1 mixture of 100% ethanol and hexamethyldisilazane, (ProSciTech, Townsville City, QLD, Australia), then in two changes of 100% hexamethyldisilazane before drying in a fume hood. Samples were mounted on aluminium stubs and coated with platinum for observation under a scanning electron microscope (Philips XL30 FEGSEM) at Adelaide Microscopy, the University of Adelaide.

Statistical analysis
For all experiments, the data are presented as mean ± the standard errors of the mean. To determine the significant mean differences between Salmonella serovar, data of biofilm formation and attachment of viable biofilm cells to the eggshell was analysed by one-way analysis of variance (ANOVA) followed by Turkey’s post hoc multiple comparison test.

Significant differences in the expression levels of genes between Salmonella serovars were analysed statistically using the Kruskal–Wallis test. All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, Inc. La Jolla, CA, USA). In all cases, a p-value of < 0.05 was considered statistically significant.

Results
Colony morphology on Congo red agar plates
In order to determine curli, fimbriae and cellulose production, the multicellular behaviour of all Salmonella isolates was examined on Congo red agar plates after incubation for 96 h at 22 and 37°C. Two major colony morphotypes were observed in this study: (1) red, dry and rough (rdar), indicative of curli, fimbriae and cellulose production, and (2) smooth and white (saw), indicating a lack of both curli, fimbriae and cellulose production (Figure 1). At 22°C, rdar and saw morphotypes were displayed by 91.03% (132/145) and 8.96% (13/145) Salmonella isolates respectively (Table 1). In contrast, 100% of the Salmonella isolates (145/145) displayed saw morphology on Congo red agar plates at 37°C (Table 1).

Crystal violet staining assay
To quantify biofilm production formed by Salmonella isolates at 22 and 37°C after incubation for 96 h, the crystal violet staining assay was used. Overall, the amount of biofilm formation was significantly influenced by temperature (Figure 2A) and representative isolates of Salmonella serovar (Figure 2B and C). Biofilm formation was significantly higher (p < 0.05) at 22°C (OD₅₉₀=2.24 ± 0.02) compared with 37°C (OD₅₉₀=0.21 ± 0.01). Among the seven serovars, isolates of S. Anatum (OD₅₉₀=2.71 ± 0.05) produced significantly more (p < 0.05) biofilm at 22°C compared to S. Agona (OD₅₉₀=2.27 ± 0.04); S. Infantis (OD₅₉₀=2.03 ± 0.05); S. Mbandaka (OD₅₉₀=1.98 ± 0.03); S. Oranienburg (OD₅₉₀=2.21 ± 0.03); S. Typhimurium (OD₅₉₀=2.41 ± 0.06) and S. Worthington isolates (OD₅₉₀=2.43 ± 0.04) (Figure 2B). At 37°C, biofilm formation was weak and increased variability was observed between serovars when compared with 22°C (Figure 2C). At 37°C, only S. Oranienburg (OD₅₉₀=0.72 ± 0.05) and S. Typhimurium (OD₅₉₀=0.21 ± 0.02) formed significantly more (p < 0.05) biofilm compared with the isolates of other serovars: S. Agona (OD₅₉₀=-0.05 ± 0.01); S. Anatum (OD₅₉₀=-0.01 ± 0.01); S. Infantis (OD₅₉₀=0.02 ± 0.01); S. Mbandaka (OD₅₉₀=0.06 ± 0.01) and S. Worthington (OD₅₉₀=0.04 ± 0.01) (Figure 2C).

Enumeration of Salmonella biofilm cells on eggshell
To determine whether Salmonella isolates are able to attach and form biofilm on eggshell, the bacterial cells were removed by swabbing and enumerated by plate count and the results are shown in Figure 3. Control eggs cultured for Salmonella were negative. The numbers of viable biofilm cells attached to the eggshell varied significantly between representative isolates of Salmonella serovars. The number of viable cells recovered from eggshells inoculated with S. Anatum...
Scanning electron microscopy

To obtain detailed information on the architecture of biofilm formation following incubation for 48 h at 22°C by representative isolates of *Salmonella* serovars, the eggshells were visualised by SEM. SEM revealed that the bacterial cells were firmly adhered to the eggshell surface and had formed a dense multilayer. A characteristic extracellular matrix was observed after incubation for 48 h, indicating (6.65 ± 0.12 log_{10} CFU cm⁻²) were significantly (*p* < 0.05) greater than *S. Agona* (5.95 ± 0.15 log_{10} CFU cm⁻²), *S. Infantis* (5.58 ± 0.09 log_{10} CFU cm⁻²), *S. Mbandaka* (5.69 ± 0.11 log_{10} CFU cm⁻²), *S. Oranienburg* (5.88 ± 0.08 log_{10} CFU cm⁻²) and *S. Typhimurium* isolates (5.72 ± 0.21 log_{10} CFU cm⁻²). Eggs inoculated with *S. Worthington* (6.27 ± 0.13 log_{10} CFU cm⁻²) had significantly more (*p* < 0.05) viable biofilm cells than eggs treated with *S. Infantis* isolates (5.58 ± 0.09 log_{10} CFU cm⁻²).

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**Figure 1.** Colony morphology on Congo red agar plates. Three μl of overnight grown culture in LB broth, without sodium chloride, were plated on Congo red agar plates, incubated at either 22 or 37°C and visualised after 96 h. Differences in colony morphotypes were evident between the two temperatures. The red, dry and rough (rdar) morphotype was observed at 22°C for both experimental isolates (A) and the control strain *S. Typhimurium* ATCC 14028 (B). Smooth and white (saw) colonies on Congo red agar plates at 37°C were observed for all experimental isolates (C) as well as *S. Typhimurium* ATCC 14028 (D). Images in this figure are representative samples of the rdar and saw morphotypes observed at 22 or 37°C.

(6.65 ± 0.12 log_{10} CFU cm⁻²) were significantly (*p* < 0.05) greater than *S. Agona* (5.95 ± 0.15 log_{10} CFU cm⁻²), *S. Infantis* (5.58 ± 0.09 log_{10} CFU cm⁻²), *S. Mbandaka* (5.69 ± 0.11 log_{10} CFU cm⁻²), *S. Oranienburg* (5.88 ± 0.08 log_{10} CFU cm⁻²) and *S. Typhimurium* isolates (5.72 ± 0.21 log_{10} CFU cm⁻²). Eggs inoculated with *S. Worthington* (6.27 ± 0.13 log_{10} CFU cm⁻²) had significantly more (*p* < 0.05) viable biofilm cells than eggs treated with *S. Infantis* isolates (5.58 ± 0.09 log_{10} CFU cm⁻²).
study produced a dense layer of cells encapsulated by an abundant extracellular matrix over the observed eggshell surface area.

**Expression of biofilm forming genes by quantitative RT-PCR**

Genes associated with curli, fimbriae and cellulose production can be used as a measurement of biofilm formation. Quantitative RT-PCR was used to measure the relative transcription levels of csgD, csgB, *adrA* and *bapA* genes in eggshell biofilm cells of *S. Anatum*, *S. Agona*, *S. Infantis*, *S. Mbandaka*, *S. Oranienburg*, *S. Typhimurium* and *S. Worthington* isolates after incubation for 48 h at 22°C.

The relative gene expression levels of *csgD* in eggshell biofilm cells of *S. Mbandaka* (1.517 ± 0.387) were significantly higher than the expression observed for *S. Typhimurium* isolates (0.287 ± 0.142, *P* = 0.028, Figure 5A). *S. Oranienburg* (0.303 ± 0.100) exhibited higher expression levels of *adrA* compared with *S. Agona* isolates (0.082 ± 0.031, *p* = 0.010, Figure 5C). No significant differences in the relative gene expression levels of either *csgB* or *bapA* genes in eggshell biofilm cells were observed between representative isolates of different *Salmonella* serovars (Figure 5B and D).

**Discussion**

This study investigated the ability of egg farm associated *Salmonella* serovars to form biofilm and the results demonstrate that biofilm formation was significantly influenced by both temperature and serovar. All the isolates tested during this study were found to possess the ability to form biofilm on the eggshell surface at 22°C. The ability to form a biofilm represents a significant risk not only to contamination of food items prepared with eggs but also in the kitchen environment.

At 22°C, the majority (91.03%) of isolates exhibited the rdar (red, dry and rough) morphotype that has been linked with curli, fimbriae and cellulose production. In contrast, all *Salmonella* isolates (100%) displayed the saw (smooth and white) morphotype at 37°C indicating the absence of curli, fimbriae and cellulose production. Similar morphotypes have been reported previously for different isolates of *S. Typhimurium* and *S. Enteritidis* (Romling et al. 1998, 2003; Castelijn et al. 2012; O’Leary et al. 2013). It has been proposed that bacteria expressing the rdar morphotype form tight attachments on abiotic surfaces and as a consequence are able to persist long-term in the environment (Romling et al. 1998; Vestby et al. 2009).

Findings of the crystal violet assay indicated that although there were significant differences between
Salmonella serovars in biofilm formation, the Salmonella isolates exhibited dense biofilm production at 22°C compared with 37°C. The results presented here are consistent with the findings of a previous study that examined the biofilm formation capacity of 78 different isolates of eight Salmonella serovars and showed that biofilm formation is strongly dependent on serovar and that the greatest biofilm formation was observed at 20°C (Schonenville et al. 2012).

In Salmonellae, csgD (previously agfD) promoter is the key target for expression of multicellular behaviour (the rdar morphotype) and is regulated by environmental conditions. Expression of csgD positively regulates the production of polymers, thin aggregative fimbriae and cellulose that form the extracellular matrix of biofilm (Romling et al. 2000; Gerstel & Romling 2001; Liu et al. 2014). Previously it has been observed that the rdar morphotype and expression of thin aggregative fimbriae were restricted to low temperatures (below 37°C) (Römling et al. 1998; Grantcharova et al. 2010; Castelijn et al. 2013). Moreover, in the stationary phase of bacterial cell growth, nitrogen and phosphate depletion were found to induce csgD promoters that enhanced the rdar morphotype (Gerstel & Romling 2001). This could partially explain the higher biofilm formation at 22°C in this study. Faster depletion of nutrient availability and changes in oxygen tension and pH are among some of the factors responsible for causing weak biofilm production at 37°C (Gerstel & Romling 2001; Stepanović et al. 2003). Altogether, the data obtained from Congo red agar morphology and the crystal violet staining assay suggest that biofilm composition and regulation is dependent on temperature and vary between Salmonella serovars.

Figure 3. Enumeration of Salmonella biofilm cells from the eggshell after incubation for 48 h at 22°C. Data are expressed as mean log_{10} CFU cm^{-2} ± SEM from two independent experiments. Bars marked with different lowercase letters indicate significant differences (p < 0.05, ANOVA).

In Australia, Salmonella foodborne outbreaks are often associated with eggshell contamination and not due to bacterial colonisation of the egg internal contents (McAuley et al. 2015). There are no published data reporting the ability of Salmonella spp. to produce biofilm on an eggshell surface. Based on the data obtained from colony morphology and the crystal violet staining assay, further experiments were conducted to examine biofilm formation by Salmonella isolates on eggshell at 22°C. The attachment of Salmonella to the eggshell is the primary step in egg contamination and the biofilm formation process. The findings of this study demonstrated that all Salmonella serovars were able to attach to the eggshell surface but significant differences between representative isolates of various Salmonella serovars were observed. The survival of S. Typhimurium PT 135, an egg product related outbreak strain, was assessed on eggshells and the results indicated the persistence of this strain on the egg shell up to four weeks (McAuley et al. 2015). Previous findings from the authors’ laboratory showed that S. Typhimurium strains were able to survive on an eggshell for up to 21 days (Gole et al. 2014c). While the formation of biofilm was not assessed in either study, this could be one of the likely mechanisms that bacteria use to persist on eggshell. Previous studies on Salmonella Typhimurium isolates from poultry and pork production chains examined their ability to attach to stainless steel surfaces at 20 or 25°C for up to seven or 14 days. The results of these studies revealed long term survival of viable cells attached to the surface of stainless steel coupons. However, survival was influenced by different serovars (Castelijn et al. 2013), the condition of the medium and the incubation period (O’Leary et al. 2013; Wang et al. 2016).

Bacterial attachment to a surface is a complex process and influenced by several factors including temperature. The temperature of 22°C used in the present study to examine bacterial attachment makes it difficult to define the precise temperature range suitable for the attachment of Salmonella to the eggshell. Therefore, further studies at refrigeration temperature analysing the mechanism of survival and/or attachment of Salmonella to the eggshell are needed. The results of biofilm formation on the eggshell suggest that cleaning and disinfection in the food processing environment is necessary, as the persistence of biofilm cells could cross-contaminate other food products and increase the risk of food-borne outbreaks associated with eggs and egg related products.

The architecture of Salmonella attachment and biofilm development by representative isolates of different Salmonella serovars was visualised by SEM and the images showed a dense layer of closely attached cells encapsulated within an extracellular matrix. Such encapsulation of eggshell biofilm cells suggest that curli, fimbriae and cellulose
Figure 4. SEM images showing biofilm formation by *Salmonella* serovars on eggshells after incubation for 48 h at 22°C. A dense layer of *Salmonella* cells surrounded by extracellular matrix components was observed on inoculated eggshells. Representative images of biofilm formation on the eggshell surface by the different *Salmonella* serovars used in this study. (A) Control; (B) *S*. Agona; (C) *S*. Anatum; (D) *S*. Infantis; (E) *S*. Oranienburg; (F) *S*. Mbandaka; (G) *S*. Typhimurium; (H) *S*. Worthington.
have a role, as most of the biofilm formation by representative isolates of Salmonella serovar exhibited the rdar morphotype on Congo red agar plates at 22°C. Similar microscopic observation has been described previously for Salmonella biofilm formed on stainless steel surfaces (Castelijn et al. 2012). The microscopic appearance of Salmonella biofilm formed on the food processing surfaces changes over time. Morphologically more adherent and dense biofilm was observed by SEM at 168 h compared with 48 h on surfaces in food processing environments (Corcoran et al. 2014). The SEM images in the present study provided evidence that isolates of Salmonella serovars recovered from layer farm environments are capable of attaching and forming biofilms on the eggshell surface.

Subsequent, quantitative RT-PCR analysis showed that the relative expression of selected genes (csgD, csgB, adrA and bapA) involved in the production of curli, fimbriae and cellulose was induced in the eggshell biofilm cells at 22°C. However, significant differences in the expression levels of only csgD and adrA genes were observed between different isolates of Salmonella serovars. These data suggest the role of curli, fimbriae and cellulose in eggshell biofilm formation in a low nutrient medium at an ambient temperature of 22°C.

csgD, a transcriptional regulator of the LuxR superfamily, positively regulates the expression of Salmonella biofilm associated extracellular matrix components, including curli and cellulose (Gerstel & Römling 2003; Fabrega et al. 2014). csgD also indirectly regulates cellulose synthesis by activating transcription of adrA (Romling et al. 2000; Gerstel & Römling 2003; Castelijn et al. 2012).

In this study, the relative gene expression of csgD was significantly higher in eggshell biofilm cells of S. Mbandaka isolates when compared with S. Typhimurium isolates. Interestingly, the higher expression of csgD and adrA genes observed in S. Mbandaka and S. Oranienburg isolates, respectively, was not associated with their enhanced eggshell biofilm formation. It is interesting to note that a
parallel difference in terms of csbD gene expression and eggshell biofilm formation between S. Mbandaka and S. Typhimurium isolates was not observed. Similarly, the expression of adrA was significantly higher in S. Oranienburg compared with S. Agona isolates. adrA is an important gene required for cellulose production and its expression in S. Typhimurium biofilm cells has been found to be influenced by the type of growth medium (Wang et al. 2016). The increased expression levels of csbD and adrA genes observed in S. Mbandaka and S. Oranienburg isolates, respectively, could be the result of early development of mature biofilm in these serovars, whereas the low expression levels of these genes in S. Typhimurium and S. Agona isolates could be the result of a developing phase in biofilm formation. Although differences in the gene expression pattern between representative isolates of serovars were observed, interestingly only S. Typhimurium has frequently been reported from egg product related human food poisoning outbreaks in Australia (OzFoodNet Working Group 2015). It is also important to note that other serovars such as S. Oranienburg, S. Mbandaka and S. Agona were detected in eggs or egg products but they were not reported frequently in human outbreaks in Australia (Taylor et al. 1998; Food Standards Australia New Zealand 2009).

The understanding of differential gene regulation during Salmonella biofilm formation is well defined and previous studies have examined this feature on various abiotic surfaces. However, to the authors’ knowledge this is the first study which examined the expression of the key genes involved during Salmonella biofilm formation on the eggshell. Thus, the results of this study cannot be compared with the earlier investigations. Previously, significant variations between time and the expression levels of biofilm-associated genes were observed in S. Typhimurium biofilm formed on stainless steel coupons using trypticase soy broth (Wang et al. 2016); and Castelijn et al. (2012) showed that the expression levels of csbD and adrA were significantly increased in Salmonella biofilm cells in a low nutrient medium at 25°C compared with 37°C.

Although biofilm formation on eggshells at 22°C appears to be serovar related in this study, comparison between serovars using four representative isolates may not be adequate to define the serovar specific differences in biofilm formation on eggshell. Hence, further studies screening a larger number of Salmonella isolates of various serovars are required. Moreover, the expression levels of biofilm genes were analysed at a single temperature and time point. Future studies at different temperatures and time intervals investigating the kinetics of gene expression and biofilm formation on eggshell by different Salmonella serovars are required.

Complete elimination of Salmonella from the poultry and food industry environment is challenging. Overall food safety improvement is possible through adequate hygienic measures, along with antimicrobial intervention strategies at all levels of the food production chain. To reduce eggshell contamination, egg washing with sanitisers is a common method worldwide, including in Australia (Chousalkar et al. 2015). Given the variation in egg washing protocols, it is unclear whether the current sanitisers, their concentration, and exposure time are able to prevent or completely remove biofilm cells on the eggshell. Further research examining the anti-biofilm effects of egg washing agents is required. The ability of Salmonella serovars to form eggshell biofilm observed in this study is of particular interest to the poultry and food industry, as survival and biofilm formation on eggshells at ambient temperature may increase the risk of food contamination and human Salmonella infections. The findings of this study have improved knowledge of eggshell biofilm formation by Salmonella serovars and the information should be useful in implementing anti-biofilm strategies against foodborne infections associated with Salmonella contamination.

In conclusion, this study has demonstrated that Salmonella isolates recovered from a layer farm environment are able to form biofilm on eggshells. The behaviour of biofilm formation was affected by temperature and representative isolates of various Salmonella serovars. In addition, it has been demonstrated that at ambient temperature, Salmonella isolates are able to attach and form biofilm on eggshells and genes associated with curli, fimbriae and cellulose production contribute to biofilm formation. However, differences between representative isolates of Salmonella serovars were evident in eggshell biofilm formation. Further, biofilm formation on the eggshell observed in this study could be a food safety and public health concern, as the persistence of such biofilm producing Salmonella strains would make their eradication more difficult, and enable them to develop resistance to antimicrobials and common disinfectants.

Acknowledgements

The authors thank Nikita Nevrekar for technical help during this research work. They would also like to thank Lyn Waterhouse for technical help during the scanning electron microscope work at Adelaide microscopy of the University of Adelaide.

Disclosure statement

All authors declare no conflict of interest.
References


### Table S1. List of primers used in this study.

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<td>csgB</td>
<td>Nucleator and minor subunit of curli fibres</td>
<td>TTTGCGATATACTGGCATCGTT</td>
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<td>csgD</td>
<td>Master regulator of biofilm</td>
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<td>adrA</td>
<td>Post transcriptional activator of cellulose biosynthesis</td>
<td>CGCCATATCCGCAGACTTTAGC</td>
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<td>bapA</td>
<td>Large surface adhesins</td>
<td>CGTCAGCGCGCGCGTAGTA</td>
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CHAPTER 5 ANTI-BACTERIAL AND ANTI-BIOFILM ACTIVITY OF COMMERCIAL ORGANIC ACID PRODUCTS AGAINST S. ENTERICA ISOLATES RECOVERED FROM LAYER FARM ENVIRONMENT
## Statement of Authorship

<table>
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<th>Anti-bacterial and anti-biofilm activity of commercial organic acid products against <em>S. enteritis</em> isolates recovered from layer farm environment</th>
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<td>Overall percentage (%)</td>
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<tr>
<td>Certification</td>
<td>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.</td>
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| Signature | Date | 18/07/2016 |

### 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 to 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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Anti-bacterial and anti-biofilm activity of commercial organic acid products against *Salmonella enterica* isolates recovered from layer farm environment

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**Running headline:** Organic acid *Salmonella* biofilms

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5.1. Abstract

**Aims:** This study aimed to evaluate the antibacterial activity of commercial organic acid products against *S. enterica* isolates. The second aim of this study was to examine the susceptibility of 3 and 5 day old *Salmonella* Typhimurium biofilms to organic acid products.

**Methods and Results:** Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of organic acid products were evaluated for *Salmonella* isolates (n=4) of representative serovars. Biofilms were formed at 22 ± 2°C using MBEC™ assay system and exposed for 30 and 90 minutes at 0.2 and 0.4% of commercial organic acid products.

No significant differences were observed between isolates of representative *Salmonella* serovars in respect of inhibitory and bactericidal concentrations of each product. However, between the products wide variations in these tests were evident. Two out of three commercial organic acid products tested in this study significantly reduced viable cells from all ages of biofilms in a dose and time dependent manner. Increased age of biofilm did not enhance resistance towards organic acid treatments. None of the tested products completely eliminated biofilm cells at any concentrations and exposure times.

**Conclusions:** Product composition, exposure time, and concentration of organic acid products were important factors in reducing viable biofilm cells. Incomplete elimination of biofilm from surfaces commonly used in food supply chain may cause further contamination and lead to recurrent infections.

**Significance and Impact of the Study:** This study has expanded our understanding about the susceptibility of *Salmonella* biofilms to commercial organic acid products. These findings have implications in the usage, development, and optimization of organic acid products and their ability to eliminate/reduce biofilms.
5.2. Introduction

Non-typhoidal *Salmonella* (NTS) infections are one of the main causes of foodborne illness worldwide (Kirk et al. 2015). Globally, an estimated 93.8 million cases of gastroenteritis and 155,000 deaths are caused by *Salmonella* spp annually (Majowicz et al. 2010). In Australia, consumption of contaminated food products of animal origin, particularly egg and egg products, are frequently associated with outbreaks of human salmonellosis (OzFoodNet Working Group 2012; 2015).

There are more than 2,500 serovars of *Salmonella enterica* spp however, *Salmonella* Enteritidis and *Salmonella* Typhimurium are the most common causes of human salmonellosis in most parts of the world (Hendriksen et al. 2011). Globally, *S. Enteritidis* is most commonly isolated from eggshell and egg contents and is frequently involved in egg and egg product associated foodborne outbreaks (Wales 2011). *S. Enteritidis*, however, is not endemic in Australian layer flocks and infection in humans with this serovar occurs primarily as a result of overseas travel. In Australia, *S. Typhimurium* has filled this niche and is frequently implicated in foodborne outbreaks linked to the consumption of contaminated egg and egg related products (OzFoodNet Working Group 2009; 2012; 2015). Between 2001 and 2010, *S. Typhimurium* was identified as the causative agent for 150 (90%) of the 166 egg associated outbreaks in Australia (Moffatt et al. 2016).

In order to reduce the risk of *Salmonella* contamination, several intervention strategies have been developed at multiple points both on farm and in the commercial kitchen. In poultry industry cleaning, disinfection, biosecurity, vaccination, prebiotics, probiotics, acidification of feed and water (organic acids) as well as egg washing are the most common practises used at farm level (Cox and Pavic 2010; Ricke 2014; Chousalkar et al. 2015). In the commercial kitchen environment, routine cleaning and disinfection are efficient and cost effective methods to reduce the risk of *Salmonella* contamination. The common classes of chemical disinfectants in food industry include alcohols, aldehydes, halogens, phenicols, oxidising agents, biguanides,
and quaternary ammonium compounds (McDonnell and Russell 1999; Møretrø et al. 2012). Although the application of these strategies has reduced the *Salmonella* contamination level, complete elimination of this pathogen is yet to be achieved.

Organic acids are commonly used to prevent microbial contamination and dissemination of foodborne pathogens in pre-harvest and post-harvest food production and processing (Ricke 2003). Organic acids are generally referred to as volatile fatty acids, fatty acids, weak, or carboxylic acids (Cherrington et al 1991, Ricke 2003). Organic acids, such as acetate, propionate, and butyrate are produced in smaller quantities in the gut environment of food animals and humans (Ricke 2003). Organic acids are relatively stable and usually metabolised by food animals or are excreted unabsorbed therefore, chemical residues are not a food safety risk (Wales et al. 2010). Commercial organic acid products are widely used in the poultry industry. Acid blends and buffered blends including acid salts are favoured for maximum antimicrobial effect (Wales et al. 2010). These products are commonly used in feed and water to reduce shedding of specific foodborne pathogens including *Salmonella* (Van Immerseel et al. 2006; Wales et al. 2013). Previous *in vivo* poultry studies have shown that the addition of organic acids in feed (Hinton and Linton 1988; Iba and Berchieri 1995; Thompson and Hinton 1997) and water (Parker et al. 2007; Menconi et al. 2013) significantly reduced the shedding and caecal colonisation of *Salmonella*. It has also been demonstrated *in vitro* that organic acid products are able to reduce total *Salmonella* counts in different feed types (Koyuncu et al. 2013) and water sources (Wales et al. 2013).

Contaminated drinking water is one source of *Salmonella* infection and re-infection in poultry flocks. *Salmonella* can survive in drinking water for prolonged periods of time, therefore it is important to keep drinking water free of *Salmonella* (Van Immerseel et al. 2006). Despite acidification of drinking water, complete elimination of *Salmonella* is difficult. Bacterial biofilms that form in water tanks and lines are protected, making the eradication of *Salmonella* challenging (Watkins 2006).
A biofilm is a community of bacteria irreversibly attached to biotic or abiotic surfaces and enclosed in self-produced extracellular matrix material (Donlan and Costerton 2002). *Salmonella* is able to attach and form biofilm on different surfaces such as stainless steel, glass, plastic, cement, and rubber, which are commonly used in industry and domestic environments (Steenackers et al. 2012). The extracellular matrix in which biofilm cells are embedded acts as both chemical and mechanical barriers against antimicrobial agents and environmental stressors (Suci et al. 1994; Chen and Stewart 1996; Steenackers et al. 2012).

Minimum inhibitory concentrations and minimum bactericidal concentration testing are traditionally used to evaluate the efficacy of antimicrobial agents. These testings are performed using planktonic phenotypes to measure required concentration of antimicrobial agent for its bacteriostatic or bactericidal activity (Prescott and Baggot 1985). However, the concentration of antimicrobial agent to kill bacteria in biofilm may be thousand times greater than that required to kill planktonic bacteria of exactly same strain (Nickel et al. 1985; Costerton et al. 1995). It has also been demonstrated that cells in a biofilm are more resistant to commonly used disinfectants compared to their planktonic counterparts (Joseph et al. 2001; Wong et al. 2010a). Thus, biofilm formation contributes to the persistence of *Salmonella* in environments and detachment of bacteria from biofilm could be a source of recurrent bacterial contamination in food processing chains, reducing food quality (Møretrø et al. 2012). Previous studies have documented increased resistance to antimicrobials with increasing age of biofilm (Anwar and Costerton 1990; Fraud et al. 2005; Shen et al. 2011; Nguyen and Yuk 2013; Corcoran et al. 2014). In contrast, age of biofilm was not associated with increased resistance to antimicrobials (Wilson et al. 1996; Gilbert et al. 2001; Kim et al. 2007; Wong et al. 2010b). Previous research in our laboratory demonstrated that *Salmonella enterica* isolates from egg farm environment are able to form biofilm on the eggshell surface (Pande et al. 2016).

Although much is known about anti-*Salmonella* efficacy of organic acids in feed and water, studies examining the efficacy of commercial organic acid products against the S. Typhimurium
biofilm are lacking. Biofilm formation is a serious food safety concern, therefore prevention of biofilm in food chains is critical.

In Australia, organic acid products are commonly used by egg producers in feed and water as additives to combat foodborne pathogen including *Salmonella*. Persistent exposure to organic acids could increase their inhibitory and bactericidal concentration. Therefore, the susceptibility of *Salmonella* isolates to organic acid product may differ. In line with this assumption, large variations were observed in the minimum inhibitory concentrations of the different bacterial species (Aarestrup and Hasman 2004) and *Salmonella* serotypes (Long et al. 2016) to the commonly used disinfectants. However, there is limited data available examining the inhibitory and bactericidal concentration of commercial organic acid products against isolates of *Salmonella* serotypes recovered from layer farm environment.

In this study, we investigated the differences between isolates of representative *Salmonella* serovars in minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC) of three commercial organic acid products. The efficacy of commercial organic acid products against *S.* Typhimurium biofilms was also evaluated for different aged biofilms, and exposure time and organic acid product concentration. From amongst isolates of representative *Salmonella* serovars, *S.* Typhimurium was selected in this study because of its frequent involvement in human disease outbreaks in Australia (OzFoodNet Working Group 2012).

### 5.3. Materials and Methods

**Bacterial strains**

Four isolates of each *Salmonella enterica* spp. *enterica*: *S.* Agona; *S.* Anatum; *S.* Infantis; *S.* Mbandaka; *S.* Oranienburg; *S.* Typhimurium (phage type 9) and *S.* Worthington previously isolated during epidemiological studies were used in this study (Chousalkar and Roberts 2012; Gole et al. 2014). All isolates were serotyped at the Australian *Salmonella* Reference Centre, Microbiology and Infectious Diseases, SA Pathology, Adelaide, South Australia.
Organic acid products

Commercial organic acid products commonly used in the Australian poultry industry were selected to examine their efficacy against *Salmonella* isolates. The details of commercial organic acid products tested are mentioned in Table 1.

**Determination of MIC and MBC of organic acid products against *Salmonella* serovars**

**MIC**

Each *Salmonella* isolate from seven representative serovars was screened to determine the MIC of organic acid products as described earlier with some modifications (Wales et al. 2013). Briefly, each organic acid product (2%) was prepared in nutrient broth (Oxoid, Australia) and diluted serially in 96 well plates (Sarstedt, Australia). The range of concentration used to determine the MIC of each organic acid product was 0.003 - 2%. All isolates were tested in duplicate with one negative control well in each row containing nutrient broth only. Organic acid was omitted from positive control well of each row. Each test strain of *Salmonella* was grown overnight at 37°C on nutrient agar. Bacterial suspension of each *Salmonella* isolate was prepared by suspending bacteria in saline to 0.5 McFarland standard and diluted (1:20) in normal saline. For each challenge and positive control well, 10 µL of bacterial suspension was added. Plates were incubated for 18 h at 37 °C and observed for visible growth. The highest concentration of organic acid products without visible growth was recorded as MIC.

**MBC**

To determine minimum bactericidal concentration (MBC), 10-µl aliquot from each well of a 96 well plate showing no visible growth was spotted onto nutrient agar plates. Following overnight incubation of plates at 37 °C, the dilution showing no growth was documented as MBC. The MIC and MBC were determined for all *Salmonella* isolates in duplicate and the experiments were performed twice on different days.


**Salmonella isolates, culture conditions, and inoculum preparation for biofilm study**

Four isolates of *S. Typhimurium* (phage type 9) were used to study biofilm formation. All isolates were cultured onto xylose lysine deoxycholate (XLD) agar plates (Thermo Fisher Scientific, Australia) and maintained in Luria Bertani (LB) broth (Oxoid, Australia) with 20% glycerol at -80 °C. The biofilm inoculum was prepared by culturing the stocks of each bacterial isolate overnight at 37 °C onto XLD agar (Thermo Fisher Scientific, Australia). Briefly, a single colony of each *Salmonella* isolate was added to a separate tube containing 10 ml of LB broth and incubated overnight with shaking (110 rpm). The overnight grown culture was diluted 1:100 in LB with no salt to reach a final concentration of $10^7$ bacteria per ml for biofilm formation. Colony forming unit (CFU) were determined by plating 10 fold serial dilutions of the inoculum on LB agar to confirm dose.

**Biofilm formation**

*S. Typhimurium* biofilm formation was carried out using MBEC™ Assay system (Innovotech Inc. Edmonton, Canada). The top plate of MBEC™ biofilm inoculator consists of 96 pegs and a corresponding base containing 96 individual wells. The design of this plate allows the growth of multiple isolates and testing of multiple biocides at different concentrations. Biofilm formation was established by adding 200 µl of bacterial inoculum in each well of MBEC plate and plates were incubated at $22 \pm 2$ °C for 3 or 5 days on a rocking platform shaker (ROCKit, Select Bio-Products, NJ, USA).

**Activity of organic acid products against *S. Typhimurium* biofilms**

The activity of three commercial organic products listed in Table 1 were examined against *S. Typhimurium* biofilm as previously described with some modifications (Wong et al. 2010a). Due to confidential reasons, the details of the organic acid products and company cannot be revealed in the public domain. Before each experiment, fresh dilutions of organic acids were made from stock solution and used immediately. Two concentrations of each organic acid
product (0.2 and 0.4%) were used to examine their activity against biofilm for 30 or 90 min. Following biofilm establishment, peg lids were aseptically removed at day 3 or 5 of biofilm growth and rinsed with 0.9% normal saline solution for 1 min in individual wells of 96 well flat bottom microtiter plates (Nunc™, Thermo Scientific, Australia). For each organic acid studied, the peg lids were placed in organic acid plate and exposed for 30 or 90 min. Tap water was filtered using Nalgene™ rapid-flow™ sterile filter with PES membrane (Thermo Fischer Scientific, Australia) and used as a negative control in the organic acid challenge plate. After specified exposure time, the peg lids were rinsed twice (1 min each rinse) in normal saline solution, immersed in the neutralising broth (Difco™ Neutralizing Broth) for one min then aseptically transferred to 96 well plate containing 200 µl of LB broth. The plates were sonicated at high speed for 5 min (Model 160TD, Soniclean Pty Ltd, Australia), serially diluted in 0.9% normal saline solution, and plated onto LB agar plates (Thermo Scientific, Australia). Following overnight incubation at 37 °C, the colonies were counted to determine colony forming units (CFU). The testing of each organic acid product was performed in triplicate and repeated twice. The detailed instructions for this assay are provided by the manufacturer in the procedural manual version 1.1 (Innovotech Inc. Edmonton, Canada).

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism version 6 for windows (GraphPad Software, Inc. CA, USA). One-way analysis of variance (ANOVA) was used to detect differences in the MIC and MBC between Salmonella isolates of each serotype for individual organic acid products. The mean cell count recovered from control and organic acid treated group at different days, exposure times and concentrations were analysed by two way ANOVA followed by Tukey’s multiple comparisons test and data were expressed as Mean log_{10} CFU ± SEM. P values <0.05 were considered statistically significant.
5.4. Results

MIC and MBC for *Salmonella* isolates

The mean MIC and MBC values of the three different organic acid products against isolates of *Salmonella* serovars are presented in Table 2. MIC and MBC values for each product did not differ significantly between *Salmonella* isolates of representative serovars. Two products (A and C) were most potent for both MIC and MBC. However, one product (B) was less effective in inhibiting and abolishing the growth of *Salmonella* in both tests.

Efficacy of organic acid product A against *S. Typhimurium* biofilms

In Australia, *S. Typhimurium* is frequently implicated in egg and egg related food poisoning cases in humans, hence *S. Typhimurium* isolates were selected for biofilm formation and their susceptibility to organic acid treatment was evaluated in this study. The mean \( \log_{10} \) CFU reduction in viable biofilm cells after organic acid exposure was calculated by subtracting the mean \( \log_{10} \) CFU of cells from the control group. In comparison with control, significant differences (\( P < 0.05 \)) in the recovery rate of viable cells from 3 and 5 day old biofilms were observed after treatment with organic acid product A and this effect was exposure and/ or concentration dependent (Fig. 1).

Product A, at 0.2 % for 30 min exposure reduced 1 and 1.22 \( \log_{10} \) CFU cells from day 3 and 5 biofilms respectively. At the same concentration, exposure time of 90 min reduced 2.62 and 1.89 \( \log_{10} \) CFU cells at day 3 and 5 of biofilm formation. At 0.4% concentration for 30 min exposure there was 2.52 and 3.26 \( \log_{10} \) CFU reduction in viable cells of day 3 and 5 biofilms respectively. After 90 min exposure at 0.4%, reduction of 4.90 and 2.52 \( \log_{10} \) CFU cells was observed from day 3 and day 5 biofilms. None of the exposure times and concentrations of organic acid product A were able to eliminate day 3 and day 5 viable biofilm cells.
Efficacy of organic acid product B against *S. Typhimurium* biofilms

*S. Typhimurium* biofilms were grown for 3 and 5 days and then exposed for 30 or 90 min at 0.2% or 0.4% concentration of product B. In comparison with control, treatment with organic acid product B for 30 or 90 min at both 0.2% and 0.4% concentration had no significant effect (P > 0.05) on the recovery rate of viable cells from any biofilms (Fig. 2). There was less than 1 log\(_{10}\) CFU reduction in viable cells from 3 and 5 day old biofilms exposed for 30 or 90 min at both concentrations of organic acids (Fig. 2).

Efficacy of organic acid product C against *S. Typhimurium* biofilms

*S. Typhimurium* biofilms were grown for 3 and 5 days and then exposed for 30 or 90 min at different concentrations of organic acid product C. Significant reduction (P<0.05) in viable cells was observed in comparison to control from all ages of biofilm and this effect was related to exposure time and/or concentration of organic acid treatment (Fig. 3).

Product C, at 0.2 % with 30 min exposure reduced only 0.55 and 1.02 log\(_{10}\) CFU cells from day 3 and 5 biofilms respectively. However, 90 min exposure resulted in higher reduction of the number of viable cells with, 1.37 and 2.78 log\(_{10}\) CFU cells from day 3 and 5 biofilms respectively. At 0.4% concentration for 30 min exposure there was 1.11 and 2.19 log\(_{10}\) CFU reduction in viable cells of day 3 and 5 biofilms, respectively. After 90 min exposure at 0.4%, reduction of 2.98 and 3.76 log\(_{10}\) CFU cells was observed from day 3 and day 5 biofilms.

5.5. Discussion

*Salmonella enterica* subsp. *enterica* are the major cause of food-borne illnesses and a serious public health concern (Hendriksen et al. 2011). The majority of human disease outbreaks are associated with consumption of contaminated food particularly egg and egg related products (Moffatt et al. 2016). Reducing *Salmonella* contamination at farm level is a logical step to reduce human infection and ensuring public health and safety. In the poultry industry,
commercial organic acid products are extensively used in feed and water as one of the control measures to reduce the risk of *Salmonella* contamination (Cox and Pavic 2010; Wales et al. 2010). It is now recognised that part of the ecological success of *Salmonella enterica* spp enabling it to survive extreme environmental conditions is due to its ability to form biofilms which is a major food safety issue worldwide (Steenackers et al. 2012). This study has examined inhibitory and bactericidal activity of commercial organic acid formulations against isolates of *Salmonella* serovars. Our results demonstrated that inhibitory and bactericidal concentrations of organic acids did not differ between isolates of representative *Salmonella* serovars and some organic acid products were able to reduce biofilm formation at all ages however, none of the products was able to completely eliminate *S. Typhimurium* biofilms.

Screening of 28 *Salmonella* isolates from seven representative serovars for inhibition and bactericidal activity by three commercial organic acid products showed no significant variation between isolates. However, variations in these tests were observed amongst products. Similarly, significant differences amongst the four commercial organic acid products for MIC and MBC values against *Salmonella* spp have been documented in an earlier study (Wales et al. 2013). These findings suggests that the exposure time could increase the inhibitory and bactericidal activity to the biocide therefore, bacterial isolates may develop different susceptibility patterns to a particular product.

In this study, the efficacy of three organic acid products against *Salmonella* biofilms was evaluated using MBEC assay system. The MBEC system or Calgary biofilm device is a high throughput-screening assay designed to evaluate the efficiency of antimicrobials against biofilms of a variety of pathogens. Previously the MBEC system has been used to determine the efficacy of commonly used disinfectants and antibiotics against biofilms of pathogenic bacteria including *Salmonella* (Ceri et al. 1999; Wong et al. 2010a; Wong et al. 2010b).
The results of this study showed that biofilm age was not associated with increased resistance to organic acid treatments. Exposure time, concentration, and organic acid type were responsible for the reduction of viable biofilms cells. There is a paucity of literature on the efficacy of commercial organic acid products against *S. Typhimurium* biofilms, therefore the result of present study could not be compared with previous work. Nevertheless, the findings of present study could be partially compared with previous reports that showed 3 and 5 day old *S. Typhimurium* biofilms were not associated with the increased resistance towards commonly used disinfectants (Wong et al. 2010b). In contrast, other studies reported that age of biofilm was significantly related to increased resistance to commonly used disinfectants or antimicrobials (Shen et al. 2011; Nguyen and Yuk 2013; Corcoran et al. 2014). The discrepancies in results could be attributed to the diverse experimental conditions, such as product, growth, strains of bacteria, temperature, and device used.

The extracellular matrix (ECM) is an important constituent of biofilm that provides protection to biofilm cells against the harsh effects of many biocides and other environmental factors by acting either as a barrier or by reducing the diffusion of active products (Suci et al. 1994; Chen and Stewart 1996; Steenackers et al. 2012). ECM development by *S. Typhimurium* on day 3 and 5 day old biofilms may have provided protection against action of organic acids. As a result, complete biofilm elimination was not achieved in this study.

This study indicated that the efficacy of three commercial organic acid products varied greatly against *Salmonella* biofilm under the conditions of this study using MBEC assay system. Of the organic acid products tested, two products (A and B) were able to reduce viable cells in the biofilm of all ages and this effect was associated with exposure time and concentration. No significant difference (P< 0.05) was observed in the effectiveness of product B when used on biofilms cells of all ages at different exposure time and concentration. Even at twice the recommended user concentration, complete elimination of biofilm cells was not achieved by any of the products tested under the conditions of this study. These findings could be compared
with earlier study, which reported that gallic, and ferulic acids were not able to completely remove biofilms formed by *E. coli, Listeria monocytogenes, Pseudomonas aeruginosa, and Staphylococcus aureus* (Borges et al. 2012). The anti-microbial mechanism of organic acids is derived from their ability to cross bacterial cell membrane and once internalised into the cell cytoplasm, organic acid dissociate into anions and protons and interfere with the pH homeostasis of the cell. In addition to alterations in cellular pH gradient, other toxicity effects of organic anions that attribute to the membrane structure, osmolality and macromolecule synthesis have also been proposed (Cherrington et al. 1990; Russell 1992; Ricke 2003; Van Immerseel et al. 2006; Wales et al. 2010). The differences in the activity of organic acid products against biofilms may relate to product composition, concentration of active ingredients, mechanism of action and pH of the solutions. Therefore, due to these differences and proprietary issues the comparison of three organic acid products A, B, and C is not suitable.

Organic acid products (A and C) contain buffering components and blends of organic acids. These two products resulted in a significant reduction of viable bacterial cells within a biofilm. Product B contains multiple organic acids but no buffering components and was not associated with significant reduction in biofilm cells under test conditions. This suggests that rather than only blends of acids, buffering capacity of the product may maintain and stabilise the pH required to limit the growth of pathogenic bacteria. Significant reduction in biofilm formation with decreasing pH values has been observed in earlier studies (Nguyen et al. 2014; Wang et al. 2016). The product A and C were associated with higher reductions in viable biofilms cells and had lowest MIC and MBC values than product B. In contrast, product B found to be least effective in reducing viable cells in biofilms and showed higher MIC and MBC values than products A and C. Therefore, the variations in biofilm activity by these products are not surprising.

All of the products tested in this study are marketed in poultry industry for use in drinking water. Previous study reported that the activity of commercial organic acid products against
Salmonella was enhanced in tap water in comparison with mineral or river water (Wales et al. 2013). In this study, organic acid products were tested in tap water hence further studies examining anti-biofilm effects of organic acid products in different water sources are needed. Such studies would have relevance to field conditions where organic and inorganic matter in water from different sources could influence the effect of organic acid products against pathogenic bacteria.

In this study only S. Typhimurium was used during biofilm experiments however, in commercial settings contamination of farm environment with multiple serovars is common and a serious concern worldwide (Gole et al. 2014; Im et al. 2015). Biofilm formation by mixed species is the most likely phenomenon under the conditions of harsh environment in food processing industry. Microbial communities can be formed by several species of microorganisms, such mixed species biofilm may have different susceptibility and resistance pattern towards organic acid treatment. Thus, further studies examining the activity of organic acid product against biofilm formed by consortium of microorganism are needed.

An important risk associated with incomplete removal of biofilm cells after organic acid treatment is the development of enhanced acid tolerance response in foodborne pathogens such as Salmonella. It has been shown that acid adapted S. Typhimurium were more resistant to extremely acidic conditions as compared to non-adopted populations (Berk et al. 2005; Alvarez-Ordonez et al. 2009; Álvarez-Ordóñez et al. 2012). Development of acid tolerance in the surviving biofilm cells in the food production environment may favour their survival, growth and may also serve as a source of cross contamination or even resist subsequently applied decontamination treatments (Álvarez-Ordóñez et al. 2012). Persistence of such biofilm cells in food processing facility may compromise food safety and is a serious public health concern. In the literature there are no findings documented with regard to acid tolerance response in Salmonella isolates that has been exposed to lethal or sub lethal concentrations of organic acids.
on the poultry farm. Further comparative research, monitoring organic acid resistance level in *Salmonella enterica* strains with or without organic acid exposure is essential.

In conclusion, our data demonstrated that there are no significant differences between isolates of *Salmonella* serovars in respect of inhibitory (MIC) and bactericidal (MBC) concentrations of each commercial organic acid products. Age of biofilm was not associated with increased resistance to organic acids tested in this study. Although organic acid treatment was effective in reducing viable biofilm cells, none of the organic acid product eliminated 3 or 5 day old biofilms. However, the product type, concentration, and exposure time were important factors for reduction in the number of viable biofilm cells. These findings may have future implications for use of organic acids against biofilm control in poultry industry.

**Acknowledgements**

Mr. Vivek Pande is a recipient of postgraduate research scholarship of The University of Adelaide Australia.
5.6. References


**Table 1.** Details of commercial organic acid products used in the study.

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Composition (As per the information available with the product leaflet)</th>
<th>Intended use and dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Synergistic combination of acidity-regulating salts</td>
<td>Drinking water; 0.2%</td>
</tr>
<tr>
<td>B</td>
<td>Propionic acid, formic acid, ammonium propionate and ammonium formate</td>
<td>Drinking water; 0.2%</td>
</tr>
<tr>
<td>C</td>
<td>Synergistic blend of free and buffered organic acids</td>
<td>Drinking water; 0.2%</td>
</tr>
</tbody>
</table>
Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of three commercial organic acid products against *Salmonella* isolates (n=4) of representative serovars.

<table>
<thead>
<tr>
<th><em>Salmonella</em> serovars</th>
<th>MIC</th>
<th>MBC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A product</td>
<td>B product</td>
</tr>
<tr>
<td></td>
<td>A product</td>
<td>B product</td>
</tr>
<tr>
<td>S. Agona</td>
<td>0.062±0.00</td>
<td>0.0125±0.00</td>
</tr>
<tr>
<td>S. Anatum</td>
<td>0.062±0.00</td>
<td>0.0125±0.00</td>
</tr>
<tr>
<td>S. Infantis</td>
<td>0.062±0.00</td>
<td>0.0125±0.00</td>
</tr>
<tr>
<td>S. Mbandaka</td>
<td>0.062±0.00</td>
<td>0.0125±0.00</td>
</tr>
<tr>
<td>S. Oranienburg</td>
<td>0.062±0.00</td>
<td>0.0125±0.00</td>
</tr>
<tr>
<td>S. Typhimurium</td>
<td>0.062±0.00</td>
<td>0.0125±0.00</td>
</tr>
<tr>
<td>S. Worthington</td>
<td>0.062±0.00</td>
<td>0.0125±0.00</td>
</tr>
</tbody>
</table>

* MIC or MBC values for each organic acid product in the respective column do not differ significantly between the isolates of *Salmonella* serovars. Values of MIC and MBC are the concentrations of organic acid product associated with inhibition or elimination of *Salmonella* growth and are expressed as Mean±SEM (n=4 *Salmonella* isolates of each serovar).
**Figure 1.** Recovery of viable biofilm cells at day 3 and day 5 after exposure to organic acid product A. 3 and 5 day old *S.* Typhimurium biofilms exposed for 30 or 90 min to 0.2 or 0.4% concentration of organic acid and cells were enumerated. A significant reduction in viable biofilm cells from 5 day old biofilms was observed after 30 min exposure to product A at 0.2% concentration whereas 90 minute exposure has significantly reduced the viable biofilm cells from 3 and 5 day old biofilms. Product A at 0.4% concentration significantly reduced viable cells from 3 and 5 day biofilms at all exposure times. Data are presented as Mean \(\log_{10} \text{CFU} \, \text{peg}^{-1} \pm \text{SEM} \) from two independent experiments with three replicates for each *Salmonella* isolate (n=4). Asterisks (*) indicate statistically significant differences (P<0.05, ANOVA) in comparison with control biofilms of corresponding day.

**Figure 2.** Recovery of viable biofilm cells at day 3 and day 5 after exposure to organic acid product B. 3 and 5 day old *S.* Typhimurium biofilms exposed for 30 or 90 min at 0.2 or 0.4% concentration of organic acid and cells were enumerated. No significant differences in the recovery of viable biofilm cells were observed from 3 and 5 day old biofilms at all exposure times and concentrations of product B. Data are presented as Mean \(\log_{10} \text{CFU} \, \text{peg}^{-1} \pm \text{SEM} \) from two independent experiments with three replicates for each *Salmonella* isolate (n=4).

**Figure 3.** Recovery of viable biofilm cells at day 3 and day 5 after exposure to organic acid product C. 3 and 5 day old *S.* Typhimurium biofilms exposed for 30 or 90 min to 0.2 or 0.4% concentration of organic acid and cells were enumerated. No significant reduction in viable biofilm cells was observed from 3 and 5 day old biofilms after 30 min exposure at 0.2% concentration of product C whereas 90 minute exposure has significantly reduced the viable biofilm cells from 5 day old biofilm only. Product C at 0.4% concentration significantly reduced viable cells from 5 day biofilm only at 30 min exposure however, 90 min exposure has significantly reduced viable cells from 3 and 5 day old biofilms. Data are presented as Mean \(\log_{10} \text{CFU} \, \text{peg}^{-1} \pm \text{SEM} \) from two independent experiments with three replicates for each
Salmonella isolate (n=4). Asterisks (*) indicate statistically significant differences (P<0.05, ANOVA) in comparison with control biofilms of corresponding day.
Figure 1.

Product A, 0.2% concentration

Product A, 0.4% concentration
Figure 2.

**Product B, 0.2% concentration**

- Control Day 3
- Test Day 3
- Control Day 5
- Test Day 5

**Product B, 0.4% concentration**

- Control Day 3
- Test Day 3
- Control Day 5
- Test Day 5
Figure 3.
Foodborne disease can have a variety of etiological agents, including bacteria, viruses, parasites, prions, as well as chemicals or natural toxins. Amongst bacteria, *Bacillus*, *Campylobacter*, *Clostridium*, *Escherichia*, *Salmonella*, and *Staphylococcus* are some of the common bacterial foodborne pathogens and are responsible for a significant proportion of gastrointestinal infections in humans. In Australia, *Salmonella enterica* infections are the second most prevalent cause of foodborne illness after *Campylobacter*. It is estimated that gastric illness caused by non-typhoidal *Salmonella* spp accounts for 93.8 million cases and 155,000 deaths worldwide each year (Majowicz et al., 2010). Infection with *Salmonella* is a public health and financial burden for both industrialised and non-industrialised countries.

Humans acquire non-typhoidal *Salmonella* through consumption of contaminated food items such as pork, meat, milk, fruits, vegetables, as well as egg, and egg products (Hur et al., 2012). In Australia, the majority of human disease outbreaks of non-typhoidal salmonellosis are associated with consumption of contaminated raw or undercooked egg or egg-based products. *Salmonella* Typhimurium (*S. Typhimurium*) is the most frequently isolated organism during outbreak investigations (OzFoodNet Working Group, 2012, 2015) and has been identified as the causative agent for 150 (90%) of the 166 egg associated outbreaks reported between 2001 and 2011 (Moffatt et al., 2016).

On farm intervention strategies including biosecurity, vaccination, organic acids, egg washing, as well as cleaning and disinfection, have been implemented worldwide to reduce the risk of *Salmonella* within the food supply chain. The emergence of antimicrobial resistant *Salmonella* strains and the decreased susceptibility of *Salmonella* biofilms to antimicrobials remain major obstacles in controlling these bacteria. In this thesis, the antibacterial sensitivity of *S. enterica* spp recovered from Australian egg farm environments, the reproductive organ colonisation and egg contamination by *Salmonella* after oral infection in layers, the role of biofilms in transmission of *Salmonella* infection and *in-vitro* susceptibility of biofilms to commercial organic acid products were investigated.
Rate of antimicrobial resistance (AMR) in Australian layer farm isolates

Antimicrobial resistance in *Salmonella* and its adverse effects on public health is a major focus of scientific research at both national and international levels. Indiscriminate use of antimicrobial agents in human and veterinary medicine favours the emergence of antimicrobial resistant strains of *Salmonella*. In Australia, antimicrobial resistance surveillance data in *Salmonella* isolates from broilers is available (Page, 2009), however, such data from layer farm related isolates is very limited.

One of the first Australian studies characterising phenotypic and genotypic AMR of *S. enterica* isolates recovered from commercial cage-egg farm environments is described in Chapter 2. Results of this study showed low prevalence of AMR and the majority of *Salmonella* isolates (91.72%) were susceptible to antimicrobials. The low prevalence of AMR in *Salmonella* isolates is an encouraging finding from the perspective of the egg industry and public health. These data are consistent with previous work, which demonstrated low levels of AMR among clinical *Salmonella* isolates isolated from Australian food animals (Abraham et al., 2014). A finding of significant importance is the absence of isolates resistant to fluoroquinolones and extended-spectrum cephalosporins, which are commonly used drugs for severe and systemic human salmonellosis. The low rate of antimicrobial resistance observed in this study is an outcome of a conservative approach to registration of new antimicrobial agents, high-quality poultry production standards and prudent selection of use of antimicrobial agents in the industry (Page, 2009). In comparison to other countries, Australia has strict regulations on antibiotic usage in food animals. The use of fluoroquinolones is not permitted in layer birds or other food-producing animals. As a result, no resistance to fluoroquinolones has been previously detected in *Salmonella* isolates from Australian food animals (Abraham et al., 2014; Barton, 2010). Other factors such as Australia’s geographical location, quarantine regulations and the minimum level of human contact or exposure to food animals, significantly contributes to restricting the emergence of antimicrobial resistance in *Salmonella* strains.
Antibiotic resistance genes are ubiquitous in nature and confer resistance to antibiotics. Bacteria can acquire antibiotic resistance genes through either spontaneous mutations or exchange of genetic material between bacteria. In this study, 4.83% (7/145) *S. enterica* isolates harboured antimicrobial resistance genes and class 1 integron. The presence of resistant genes with integron indicates that some genes are carried on transferable mobile gene cassettes placed either on chromosomal DNA or on plasmids (Carattoli, 2003; Carattoli et al., 2005; Levings et al., 2005). Although the distribution of antimicrobial resistance genes and integron 1 amongst *Salmonella* isolates was low, this could be a serious concern. These mobile genetic elements have the potential to transfer or acquire resistance determinant into the genome of other bacterial populations, if antibiotic selection pressure is further intensifed.

This study was limited in part due to the inclusion of *Salmonella* isolates from egg farms located within two Australian states. In order to address the issue of antimicrobial resistance, a national screening of *Salmonella* isolates from multiple egg farms is required. Continuous AMR surveillance monitoring is also essential for tracking the distribution of antimicrobial resistance in the Australian egg industry. Although antimicrobial resistance in *Salmonella* spp isolated from egg farms is low, public health authorities, the egg industry, and the general community often express concerns about the risk of egg contamination and egg related disease outbreaks.

**Reproductive organ colonisation and egg contamination by *S. Typhimurium* after oral infection in laying hens**

The ability of *S. Typhimurium* to colonise reproductive organs and contaminate eggs have been examined previously, however, the results from these studies are inconsistent. In addition, there is limited data on the long term faecal shedding of *S. Typhimurium*. Given the increase in a number of egg-associated outbreaks in Australia (OzFoodNet Working Group, 2012; Moffatt et al., 2016), there is no conclusive information on the precise mode of egg contamination by *S. Typhimurium*. In this study, *S. Typhimurium* phage type 9 (PT9) with multi locus variable tandem repeat analysis (MLVA) profile 03-24-11-11-523 was chosen based on its frequent
involvement in human food poisoning outbreaks. In South Australia during 2011, *S. Typhimurium* PT9 with MLVA profile 03-24-11-11-523 was most frequently been associated with food poisoning cases associated with egg or egg related products.

Experimental *Salmonella* infection of commercial laying hens in a controlled environment is an effective method to study the reproductive organ invasion and subsequent egg contamination. Such studies are however, expensive, and labour intensive. Furthermore, it is difficult to maintain *Salmonella* free birds over prolonged period of time. In experiment 2 (outlined in chapter 3), layer hens were orally challenged with *S. Typhimurium* PT9 to investigate long-term bacterial shedding in the faeces, oviduct colonisation, and egg contamination. Shedding in faeces was high up to 5 weeks post infection (p.i.), but persisted up to 15 weeks p.i. The increased *Salmonella* shedding in faeces observed up to 5-week p.i. was attributed to stress associated with the onset of lay. The results of previous field (Gole et al., 2014a) and experimental infection studies (Okamura et al., 2010) indicated that sexual maturity or onset of lay was associated with increased *Salmonella* shedding and egg contamination.

The present study examined *Salmonella* shedding and egg contamination for 15-week p.i. Commercial laying hens, however, have an extended productive cycle (up to 70-80 weeks) and may experience many physical or environmental conditions that could induce stress such as, onset of lay/ sexual maturity, transportation, farm management, induced moulting, extreme environmental variations, or concurrent infections (Gole et al., 2014b; Humphrey, 2004). Stress caused by these conditions could increase colonisation of *Salmonella* in the gut, and favour the translocation of *Salmonella* to the reproductive tract, where contamination of eggs could occur during formation (Burkholder et al., 2008). Therefore, extending the research beyond 15 weeks would give more information about *S. Typhimurium* shedding and egg contamination in response to various stress events in laying hens during their productive lifespan. It is also important to note that such long-term studies are expensive and laborious. Previous studies have shown that stress caused by induced molt or feed restriction markedly increases the level
of corticosteroids hormones (Freeman et al., 1981; Harvey & Klandorf, 1983) and decreases the cell-mediated immunity, resulting in decrease in the number of CD4 T lymphocytes (Holt, 1992a, 1992b). The mechanism of stress was not investigated during this study, hence further research should be directed to study the level of stress hormones, immune response, its influence on *Salmonella* shedding and subsequent egg contamination in laying hens.

*S. Typhimurium* PT9 colonised the reproductive organs and caused eggshell contamination over the course of the experiment, however, no bacteria were isolated from the egg internal contents (albumen and yolk). These findings imply that horizontal transmission/ external soiling of shell eggs with faeces, or contamination from the layer farm environment, is the likely route of egg contamination for the *S. Typhimurium* PT 9 isolate used in this study. Other factors such as poor egg handling and hygienic measures, undercooking and cross- contamination of other food products during food preparation could increase the likelihood of foodborne outbreaks in humans. The eggshell contamination observed in this study indicates that intensive efforts to minimise *Salmonella* contamination pressure at the primary production level are required to mitigate the high number of egg-associated disease outbreaks. The results of this study will help educate regulators and commercial food providers to recognise the risk of eggshell contamination and strictly adhere to the food safety standard associated with egg handling in a food-processing environment.

The colonisation of reproductive organs can lead to internal egg contamination prior to oviposition (Gantois et al., 2009). In this study, although *S. Typhimurium* was able to persist in the reproductive organs, it remains unclear why it was unable to contaminate the internal egg contents. The egg itself possesses several antibacterial properties. Antibacterial compounds within egg albumen such as lysozyme, ovotransferin, ovomucoid, ovoinhibitors, cystatin, ovostain, β-defensins, and immunoglobulins have shown to inhibit the growth of *Salmonella* spp (Gantois et al., 2009). The results of an *in vitro* egg penetration study revealed that *S. Typhimurium* at 20°C was able to penetrate the eggshell and survive in egg internal contents.
after 21 days of infection (Gole et al., 2014c). The successful survival of *S*. Typhimurium in egg albumen in that study may be due to deterioration of egg albumen's antimicrobial activity during storage period of 21 days. Hence, it could be concluded that the *S*. Typhimurium PT9 isolate used in this study was not able to survive in the fresh albumen *in vivo*.

Another possible explanation for the failure of *S*. Typhimurium to contaminate egg internal content (even after colonisation of oviduct) could be lack of the Regions of difference (ROD) in *S*. Typhimurium genome. Previously it has been demonstrated that ROD 9, 21, and 40 plays an important role in chicken reproductive tract colonization (Raspoet et al., 2014). These ROD's are present in *S*. Enteritidis but not in *S*. Typhimurium (Thomson et al., 2008). It could be hypothesised that the inability of *S*. Typhimurium to survive in fresh egg albumen resulted from the lack of ROD’s. However, further, microarray based experiments are essential to confirm this hypothesis.

There are several genes that are important for persistence of *Salmonella* in the chicken reproductive tract. Previous studies have identified the genes using *in vivo* expression technology (Gantois et al., 2008) and genome wide microarray based transposon library (Raspoet et al., 2014). The results of these studies demonstrated virulence genes within *Salmonella* Pathogenicity Islands (SPI)-1, 2, and 3, involved in amino acid synthesis, nucleic acid metabolism, motility, cell wall integrity, virulence plasmid and stress responses were induced during oviduct colonisation and egg contamination with *S*. Enteritidis (Gantois et al., 2008; Raspoet et al., 2014). Further mechanistic research is required to unveil the role of these genes during oviduct colonisation and egg contamination by *S*. Typhimurium. Such information could improve our understanding of the underlying molecular mechanism behind *S*. Typhimurium colonisation in the oviduct of laying hens.
Role of *Salmonella* biofilms in food safety

Despite pre- and post-production control measures, including biosecurity, sanitation, vaccination, organic acids, and egg washing, residual *Salmonella* contamination of the layer farm environment remains a major challenge for the poultry industry. The formation of biofilm is a mechanism utilised by the *Salmonella* spp for survival in harsh physical and chemical environments. Biofilm formation is influenced by several factors such as *Salmonella* serovar, bacterial strain, as well as environmental conditions. Bacterial cells in a biofilm are resistant to commonly used antimicrobials, hence their eradication from food preparation surfaces from domestic and industrial settings is difficult. Eggshell biofilm formation by *Salmonella* poses a significant risk of cross-contamination of other food items prepared in a kitchen environment and biofilm cells may become a source of human infections.

In Chapter 4, *Salmonella* isolates (n=145) from seven representative serovars, *S*. Agona, *S*. Anatum, *S*. Infantis, *S*. Mbandaka, *S*. Oranienburg, *S*. Typhimurium and *S*. Worthington were examined for their ability to form biofilms at two different temperatures (22 and 37°C), using crystal violet and Congo red assay. These serovars were isolated from a commercial layer farm environment and some of them have been associated with egg related outbreaks of food poisoning (Glass et al., 2016; Gole et al., 2014a; Gole et al., 2014b). This study showed that biofilm-forming behaviour was significantly influenced by temperature and *Salmonella* strains. Biofilm formation was higher at 22°C compared to 37°C. Furthermore, curli fimbriae and cellulose, major components of biofilm matrix, contribute to biofilm formation at ambient temperature (22°C). The ability of *Salmonella* serovars to form biofilm at ambient temperature (22°C) is of particular interest to the poultry industry because commercial layer sheds are usually maintained at similar temperature. Survival and biofilm formation by *Salmonella* at ambient temperature may increase the risk of food contamination and human *Salmonella* infections.
It was further hypothesized that *Salmonella* spp possesses the ability to attach, remain viable, and form biofilms on eggshell surface. Representative isolates of *Salmonella* serovars (*S.* Agona, *S.* Anatum, *S.* Infantis, *S.* Mbundaka, *S.* Oranienburg, *S.* Typhimurium, and *S.* Worthington) were examined for their ability to attach and form biofilms on eggshell at ambient temperature (22°C). The results demonstrated that *Salmonella* spp are able to attach and form biofilm, however, this behaviour was significantly influenced by *Salmonella* strains. The molecular mechanism underlying biofilm formation revealed that the key genes (*adrA*, *bapA*, *csgA* and *csgD*) involved in the production of curli fimbriae and cellulose were induced during eggshell biofilm formation. Scanning electron microscopy examination of the eggshell revealed a dense layer of biofilm cells encapsulated in an extracellular matrix. These results suggest that biofilm is one of the likely mechanisms *Salmonella* utilises to persist on eggshell. In this study, limited isolates of *Salmonella* serovars were examined for their ability to form biofilms on eggshell, making it difficult to ascertain serovar-specific differences. Future investigation including large number of isolates of representative serovars is required. Although the results of the current study indicate that *S. enterica* isolates are able to form biofilms at 22 °C, this study did not examine such behaviour at refrigeration temperature, which is commonly used to store eggs in supermarkets and consumer households. To our knowledge, this is the first report describing biofilm formation on eggshells.

**Anti-bacterial and anti-biofilm activity of organic acids against *S. enterica* spp**

Complete eradication of *Salmonella* on farm and through the food supply chain is unlikely. Improved food safety practises, hygiene, and antimicrobial strategies will, however, help to reduce the risk of *Salmonella* contamination. Several intervention strategies have been employed to reduce *Salmonella* contamination at farm level and during food supply chain. In Australia, organic acids are commonly used in feed and water to prevent *Salmonella* contamination. Given the differences in composition and the active ingredients of commercial
organic products, their inhibitory and bactericidal activity may differ between products. Moreover, the activity of commercial organic acid products against *Salmonella* biofilm is unclear.

The experiments described in Chapter 5 sought to identify inhibitory and bactericidal concentrations of three commercial organic acid products against representative isolates of *Salmonella* serovars. In addition, susceptibility of *S. Typhimurium* biofilms to organic acid products was characterised using high throughput MBEC™ assay. The results of this experiment showed no significant differences between isolates of representative *Salmonella* serovars with respect to inhibitory and bactericidal dilutions of each product. However, wide variations in inhibitory and bactericidal effects between products were evident. Of the organic acid products evaluated in this study, two products were able to reduce viable cells in the biofilm of all ages in a dose and time-dependent manner. In general, higher concentration and increased contact time with organic acid were critical to prevent the growth of biofilm-associated cells. There is a general consensus that older biofilms are more resistant to antimicrobials than younger biofilms. In contrast to earlier research, this study demonstrated that older biofilms (Day 5) were not more resistant than younger biofilms (Day 3) to organic acid treatments. The product type, concentration, and exposure time were identified as important parameters in the successful reduction of viable biofilm cells. Organic acid products, however, even at twice the recommended user concentration were unable to completely eradicate biofilm cells. The incomplete removal of biofilms may favour the bacterial persistence and as a result, their eradication becomes more difficult from both domestic and commercial settings.

The repeated exposure of *Salmonella* biofilms cells to lethal or sub-lethal concentrations of organic acid during routine sanitation process may give rise to increased organic acid tolerance or development of acid tolerance response. The emergence and dissemination of acid resistant *Salmonella* strains could compromise the control of *Salmonella* and pose a significant public health risk. The extracellular matrix is an important constituent of biofilm, as it acts as a
chemical and mechanical barrier and prevents the diffusion of active products. Although this study did not elucidate the mechanism of increased resistance of S. Typhimurium biofilm to organic acid treatments, the scanning electron microscopic examination of eggshell biofilm support the hypothesis that extracellular matrix of the biofilm could protect the cells against the action of organic acids.

Organic acid products marketed in the poultry industry for use in drinking water were evaluated in this study. However, the activity of organic acid products known to differ considerably depending on the type of water source. The previous study has shown substantial anti-Salmonella activity of commercial organic acid products in tap water in comparison with river or mineral water (Wales 2013). In this study, organic acid products were diluted in tap water and screened against the biofilm activity however, further studies on the anti-biofilm activity of organic acid products in different water sources are needed. Such studies will be relevant to field conditions where organic and inorganic matter in the water from different sources could vary and influence the effect of organic acids against pathogenic bacteria.

In both domestic and commercial settings, multiple species of zoonotic bacteria can attach, survive, and form biofilms on abiotic surfaces. However, it has not yet been determined whether interactions between members of bacterial community in the biofilm influence the virulence, susceptibility, and resistance towards antimicrobial agents. Further studies on the activities of organic acid against biofilm formed by the consortium of microorganism are needed.

**Conclusions**

The major conclusions of this thesis are that S. enterica isolates recovered from layer farm environments exhibited low frequency of antimicrobial resistance and presents a low public health risk associated with the emergence of multidrug resistant Salmonella spp isolated from Australian egg industry. Although S. Typhimurium PT9 was able to colonise the reproductive organs and contaminate eggshell, throughout the study, S. Typhimurium was not isolated from
the egg internal contents. This data indicate that horizontal transmission is the main route of egg contamination with *S. Typhimurium* PT9 in laying hens. *S. enterica* isolates were able to attach and form biofilm on eggshell surface at 22°C however, biofilm-forming behaviour was significantly influenced by temperature and *Salmonella* strains. The inhibitory and bactericidal dilutions of each commercial organic acid products did not differ between representative isolates of *Salmonella* serovars. Organic acid treatment was effective in reducing viable biofilm cells, but none of the product eradicated the viable cells from 3 or 5 day old biofilms. The age of biofilms was not associated with resistance towards organic acid treatments. However, organic acid products, exposure time, and concentration were critical factors for the reduction in the number of viable biofilm cells. The results of these studies may assist in highlighting the risks of *Salmonella* contamination and developing policies to mitigate the risk. Intervention strategies to control *Salmonella* is not limited to farm level, but the collaborative efforts from public, food industry and regulatory authorities are essential for the control of *Salmonella* throughout the food supply chain.
References


