A study of non-typhoidal *Salmonella* virulence

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A thesis submitted in fulfilment of the requirements of the degree of

Master of Philosophy

School of Animal and Veterinary Sciences

The University of Adelaide

March 2020

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Abstract

Salmonella is a major cause of foodborne gastroenteritis world-wide. *Salmonella* serovars are broadly classified as typhoidal or nontyphoidal. Nontyphoidal *Salmonella* serovars include *Salmonella* Typhimurium (*S.* Typhimurium) and *Salmonella* Enteritidis (*S.* Enteritidis). In Australia, *S*. Typhimurium is the most commonly identified serovar during human outbreaks relating to eggs and egg products. Recently, during egg related outbreaks, *Salmonella* Hessarek (*S.* Hessarek) has increasingly been identified in South Australia. The aims of this thesis were to determine whether *S*. Typhimurium and *S.* Hessarek have an enhanced ability to penetrate the egg shell, to determine the effects of storage temperature on growth of *S*. Typhimurium and *S*. Hessarek within the egg, to examine the effect of storage temperature of eggs infected with *S.* Typhimurium when infected eggs were consumed and finally to study the effects of storage time and storage temperature on aioli infected with *S.* Typhimurium when infected aioli was consumed.

Egg shells were infected with *S*. Typhimurium and *S.* Hessarek by dipping them in a bacterial suspension either immediately after lay or after being cooled to room (shed) temperature, then stored at either 5°C or 25°C. Egg contents were also infected with *S*. Typhimurium and *S.* Hessarek and stored at either 5°C or 25°C to examine bacterial growth in the egg contents. When eggs were infected immediately after lay, before the eggs cooled to room temperature, *S*. Typhimurium and *S*. Hessarek were able to penetrate into the shell pores, but not into the contents. Egg contents that were infected and stored at 5°C were negative for bacterial growth after direct plating, whereas eggs stored at 25°C were positive for bacterial growth after direct plating. Infected eggs were then stored at either 5°C or 25°C prior to being fed to mice to examine the effect of storage temperature of *S.* Typhimurium infected eggs when consumed. No mice fed infected egg components stored at 5°C exhibited signs of Salmonellosis. Mice fed infected yolk stored at 25°C quickly developed disease symptoms. It

took longer for the animals fed infected egg albumen and shell wash stored at 25°C to begin showing symptoms of disease. Aioli, a raw egg product, was then infected and stored at either 5°C or 25°C for different periods of time (12, 24, 36, 48 or 72 hours). It was then fed to mice, to examine the effect of storage temperature and storage time on *S.* Typhimurium infected aioli when consumed. No infection occurred in mice fed infected aioli stored at 25°C, whereas two mice from the infected aioli stored for 24 hours 5°C and one mouse from the infected aioli stored for 12 hours at 5°C did succumb to salmonellosis. The bacteria enumerated from the organs of these mice was comparable to the number of bacteria enumerated from the control mice fed infected aioli, that were culled. Results of these experiments indicated the effect of storage temperature on bacterial growth within the egg and growth/survival in eggs and egg products prior to infection and the subsequent occurrence of salmonellosis.

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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I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Signed

Talia Sheryli Moyle

Date: ……

Acknowledgements

There are many people I need to thank for their support to enable me to complete my study. The assistance and support of my supervisors Kapil Chousalkar and Andrea McWhorter has been invaluable. I've immensely appreciated their presence and encouragement over the years. I've worked with some wonderful people in our research group during the past few years. Special thanks to Samiullah Khan, Nitish Joat, Sarah Sims, Geoffrey Holds, Nicky-Lee Wilson and Sonali Deshmukh for all their support, technical assistance and conversations about this and that while working in the lab. Thanks also to Angus Missan and Phil Downey for assistance with collecting eggs during some of the trials.

There are so many of my fellow HDR students I need to thank, but in particular I would like to thank my office mates Chelsea Graham, Wei Yee Chan, Ale Arbe and Petra Edwards. Thank you for our good chats and support when it was needed. I need to give an extra special thanks to Rebecca George for not only being a wonderful office mate, but for her support when this degree became a little too much. Her encouragement to keep going is a big part of what has gotten me to this point.

My family, Mum, Dad, Shannyn and Luke have been my constant support throughout my entire time at university. I hope I've made you proud and I can't express how much I appreciate everything you have ever done for me. You mean the world to me, thank you.

Finally, to my wonderful husband Joel. Your support and understanding have been unwavering. Through every triumph and every tear, you have been there, holding down the fort and suppling me with endless cups of tea. I love you more than you will ever know. Thank you for being you.

1.0 Literature review

1.1 Introduction

Salmonella spp. are gram negative, facultative anaerobes from the Enterobacteriaceae family (Keerthirathne et al., 2016; Food standards Australia New Zealand, 2013). Members of the genus, *Salmonella*, are a major cause of foodborne gastroenteritis world-wide (Chaudhuri et al., 2013; Ford et al., 2016; Majowicz et al., 2010). *Salmonella* is comprised of two species, *Salmonella enterica* (*S. enterica*) and *Salmonella bongori (Eng et al., 2015)*. *S. enterica* is further divided into six subspecies that include over 2500 different serovars (Ford et al., 2016; Jones et al., 2008) and can be spread through the consumption of contaminated food or water, animals, people and the environment. *Salmonella* serovars are broadly classified into typhoidal and nontyphoidal *Salmonella*. Nontyphoidal *Salmonella* serovars include *Salmonella* Typhimurium (*S.* Typhimurium) and *Salmonella* Enteritidis (*S.* Enteritidis), which are pathogens with a wide-range of host specificity (Keerthirathne et al., 2016). Serovars within the *S. enterica* subspecies have adapted to avian and mammalian hosts and cause more than 99% of *Salmonella* infections in humans (Suez et al., 2013). Serovars such as Typhi, Sendai, and Paratyphi A, B, and C are highly adapted to humans as a host and cause enteric fever (Keerthirathne et al., 2016). Even though it is thought surveillance data for salmonellosis is underrepresented, figures suggest the rate of infection, over the last decade in particular, has been increasing (Ford et al., 2016).

In Australia, *S*. Typhimurium is the most commonly identified serovar during human outbreaks of salmonellosis (Ford et al., 2016). *S.* Typhimurium can survive in a very broad range of hosts and environments, making it difficult for researchers to determine the exact factors which enable its survival in such diverse conditions (Chaudhuri et al., 2013; Foley et al., 2013). Animals such as pigs, cattle and chickens as well as their associated products are major reservoirs for infection with *Salmonella* (Chaudhuri et al., 2013; Stevens et al., 2009). Dairy products, fresh produce, nuts (Ford et al., 2016), egg and egg products (Hawkey et al., 2013;

OzFoodNet, 2012) have also been found to be associated with *Salmonella* outbreaks. Furthermore, it has been shown that 90% of identified outbreaks relating to eggs in Australia are caused by *S.* Typhimurium (Moffatt et al., 2016). Specific serovar identification is vital to facilitate traceback of the outbreak to the source. Multi Locus Variable Number Tandem Repeat Analysis (MLVA) has been adopted as the preferred method for *S.* Typhimurium identification during outbreaks in Australia (Lindstedt et al., 2003; Octavia et al., 2015; Sintchenko et al., 2012). Identification then aids in improving food safety for the public. There are obvious public health benefits and economic gains to be made by minimising the occurrence of *Salmonella* infections (Humphrey, 2004).

This review will cover several aspects of *S.* Typhimurium relating to its pathogenicity, virulence and food safety in relation to eggs and raw egg products. Particular points of interest focussed on are the effects of storage temperature on bacterial pathogenicity and examples of *in vitro* and *in vivo* studies previously conducted examining *Salmonella* and its pathogenicity.

1.2 *Salmonella* pathogenicity and virulence genes

S. Typhimurium possesses a number of genes associated with virulence, including survival, invasion and cellular adhesion (Foley et al., 2013; McWhorter et al., 2015). Gene clusters known as *Salmonella* pathogenicity islands (SPIs) encode virulence factors. These SPIs are distributed in the *Salmonella* genome and give *Salmonella* the ability to efficiently colonise the host (Foley et al., 2013). There are several main pathogenicity islands reported for different *Salmonella* serovars, with some being specific to serovars (Hensel, 2004). However, SPI-1 to SPI-5 are commonly present in most *Salmonella* serovars (Amavisit et al., 2003; Foley et al., 2013; Foley and Lynne, 2008). A summary of the function of these SPIs is as follows. SPI-1 genes are required for the bacteria to invade host cells and induce macrophage apoptosis. Genes in SPI-2 enable systemic infection and replication within the macrophages. SPI-3 genes

allow the bacteria to survive in the macrophages and grow in less than ideal conditions, such as high magnesium environments. SPI-4 genes facilitate intramacrophage survival and also contains genes for toxin secretion and apoptosis. SPI-5 groups genes that encode several T3SS effector proteins, for the type III secretion systems (T3SS) (Foley et al., 2013; Foley and Lynne, 2008; Hensel, 2004; Rychlik et al., 2009). It has been demonstrated that *Salmonella* persistence is influenced by SPI-1 and SPI-2 (Foley et al., 2013) as they encode T3SS which enable the transport of effector proteins into other cells (Ibarra et al., 2010; Rychlik et al., 2009). Fibronectin binding proteins, such as MisL, enable *Salmonella* colonisation which is host specific and are encoded by SPI-3. SPI-4 has also been found to be host specific and important for invasion and colonisation of intestinal cells (Morgan et al., 2004). The presence of SPIs and their encoded virulence genes allow for bacterial pathogens to be distinguished from other closely related non-pathogenic strains (Suez et al., 2013). Virulence genes have been characterised by PCR by several research groups in order to determine *Salmonella* virulence (Shah et al., 2011; Ziemer and Steadham, 2003). Environmental growth conditions, such as oxygen availability and growth phase of the bacteria, are known to affect the virulence capabilities of *Salmonella* and therefore the result of *Salmonella*-host cell interaction (Ibarra et al., 2010). If virulence capabilities are increased, which can occur when *Salmonella* grows in optimal conditions or when co-expression of flagella and SPI-1 genes occurs, invasion levels have been found to increase (Ibarra et al., 2010).

It has been demonstrated that different *Salmonella* serovars possess varying pathogenicity, usually due to genomic differences (Suez et al., 2013), however it is common for *Salmonella* serovars to possess the same virulence genes. McWhorter *et al.* 2015 tested several different *Salmonella* serovars for 23 genes (including avrA, invA, sipB, sptP, sitC, misL, pipD, pefA, spvC and fliC) commonly associated with invasion, adhesion and survival of *Salmonella* spp. They showed that the majority of the serovars tested were positive for tested genes, but not all of the virulence genes, with the exception of *S.* Typhimurium phage types which were positive

for all virulence genes tested. Horizontal gene transfer is primarily responsible for genomic variability between bacterial strains (Suez et al., 2013). This variability is most likely the cause for varying pathogenicity between *Salmonella* strains (McWhorter et al., 2015). Acquisition of foreign genes with virulence functionality can differentiate these pathogens from closely related non-pathogenic strains (Suez et al., 2013). An appropriate combination of genes must be acquired to facilitate specific functional changes (Peterson, 1996).

1.3 *Salmonella* pathogenesis

In humans and mammals, the most common means of infection with *Salmonella* is via the faecal-oral route (Foley et al., 2013; Foley and Lynne, 2008; Grassl and Finlay, 2008). When *Salmonella* contaminated food is ingested, it must survive the acidic environment of the stomach before progressing further down the digestive tract. *Salmonella* has an adaptive mechanism for such environments called the acid tolerance response (ATR) which requires the implementation of several acid shock proteins (ASP) (Foley et al., 2013; Foley and Lynne, 2008). Up to 50 ASP are induced when the ATR occurs. Eight of those proteins require an alternative sigma factor encoded by *rpoS*, which is an important regulator during shock events. Levels of sigma factor have been shown to increase upon acid shock thereby explaining the induction of specific ASPs (Baik et al., 1996). Other common regulatory genes associated with acid response include, *fur*, *phoP* and *phoQ* (Foley et al., 2013). It has also been suggested that *Salmonella* strains that have been pre-exposed to acidic conditions, such as those found in some foods, may be better equipped to withstand exposure to the acidic conditions of the gut (Humphrey, 2004). After surviving the low pH environment of the stomach, the bacteria proceed to the gastrointestinal tract (GIT) organs. These include the small intestine, colon and caecum. Immune and epithelial cells in the GIT are the first line of defence against bacterial invasion (Foley et al., 2013; Foley and Lynne, 2008). *Salmonella* also

has to compete with microflora present in the system for adherence to and then invasion of enterocytes and M cells to enable colonisation of the GIT. The bacteria adhere to intestinal cells via fimbriae and flagella present on the surface of the bacteria (Foley et al., 2013; Foley and Lynne, 2008). *Salmonella* has been shown to possess both host specific and conserved factors to enable colonisation. Once adhered to intestinal cells, *Salmonella* will express a T3SS protein associated with SPI-1 to enable invasion of the cell. T3SS allows transport of toxins and other effector proteins into the cells and is the vital component for *Salmonella* invasion (Foley et al., 2013). Effector proteins such as invJ, spaO, prgI, sipA, sipB, sipC, sptP, avrA, sopA and sopB are secreted through the T3SS. SopB plays an important role in facilitating inflammation and altering ion balances within cells causing the occurrence of diarrhea. Other proteins, such as sopA, sopD, sopE, and sipA, have been found to play a role in *Salmonella* gastroenteritis (Foley et al., 2013). When *Salmonella* interacts with the epithelial and immune cells, it induces production of proinflammatory cytokines and chemokines in the host, which then trigger other immune cells to respond, including macrophages, T and B cells, neutrophils and dendritic cells (Grassl and Finlay, 2008). *Salmonella* has many adaptive functions, simple nutritional requirements and can survive for a long period of time in foods and other substrates. However, there are many factors that affect *Salmonella* virulence, growth and survival such as; environmental conditions, temperature, pH and water activity (Food standards Australia New Zealand, 2013). Survival in food, such as eggs, is of particular concern, given the threat to public health.

1.4 *Salmonella* and layer hens

Intact eggs can be contaminated with *Salmonella* either through vertical (which is common in the case of *S.* Enteritidis) or horizontal transmission (Fajardo et al., 1995). Horizontal transmission, a more common route of transmission in Australia, occurs when bacteria

penetrates through the egg shell (Miyamoto et al., 1998). As it is laid, the egg passes through the highly contaminated cloacal area. Immediately after the egg is laid, it comes in contact with many sources of contamination in the environment, including faecal material and dirty bedding in the nesting area. Previous research has indicated that eggs laid into a highly contaminated environment are at risk for higher levels of bacterial contamination compared with eggs laid in a clean environment (De Reu, 2006).

Although not common in Australian eggs, vertical transmission occurs when the yolk, the albumen and/or the membranes are directly contaminated, due to bacterial infection of the reproductive organs. Infection can come from the ovaries or oviduct tissue, before the eggs are covered by the shell (Messens et al., 2005; Miyamoto et al., 1998). When infection of the reproductive organs occurs, it is commonly due to *S.* Enteritidis (Messens et al., 2005). Vertical transmission can originate from an infection of the ovaries due to a systemic infection, or from an ascending infection from the contaminated cloaca to the vagina and lower regions of the oviduct (Keller et al., 1995).

1.5 Food safety in eggs

There has been an increase in the occurrence of *Salmonella* infection over the last decade. Between the year 2000 and 2013 notifications per 100,000 increased from approximately 18 to approximately 34 (Ford et al., 2016). The occurrence of *S.* Typhimurium in egg related outbreaks has also increased, therefore it is necessary to control the spread and incidence of this pathogen in the food chain. Eggs can become infected with *Salmonella* at any point in the production chain, from production on farm, through processing, preparation to consumption (Fajardo et al., 1995). Outbreaks of *Salmonella* infections have an obvious effect on public health and subsequently there are often substantial financial cost involved with outbreaks. The economic gains to be made in minimising the rate of infection in eggs is significant

(Humphrey, 2004). There are, however, precautions that can be put in place to minimise its effect from the farm to the table. These include on farm biosecurity, correct storage temperature, discarding any cracked or dirty eggs, correct handling and minimising cross contamination (SA Health, 2012; Humphrey, 2004). The end point of the chain is most often the kitchen, where improper handling, improper storage and cross contamination occurs (Humphrey, 2004).

Consuming undercooked eggs and raw egg products is often the cause of *Salmonella* outbreaks (Denehy et al., 2011; Humphrey, 2004; Stephens et al., 2008). A variety of egg products can be categorised as moderate to high risk to the consumer due their method of preparation and the potential for *Salmonella* contamination. These products are often partially cooked or even raw. These include desserts such as tiramisu and mousse, drinks such as protein drinks containing raw eggs and eggnog and sauces and dressings such as mayonnaise and aioli (SA Health, 2012). Yolk has been shown to be a high-risk food if contaminated, due to the rate of *Salmonella* growth when infected (Bradshaw et al., 1990; Gast and Holt, 2000a). Given it is the main ingredient of aioli, aioli is of particular interest as being a high-risk food (Denehy et al., 2011). If *Salmonella* contaminated eggs are used to make aioli, the bacteria will then also contaminate the end aioli product. This contamination could originate from the farm from whence the eggs were produced. Poor food safety and handling is also of concern in regard to aioli production, as common issues identified in restaurants and kitchens include poor hygiene by not washing hands after handling eggs, inadequate cleaning of the mixer used to make aioli and failure to monitor the temperature it is kept at (Stephens et al., 2008). With safety recommendations often not being followed when handling aioli (e.g. not storing it below 5°C, not preparing a new batch daily, not discarding at the end of the day and storing it for more than 24 hours) (SA Health, 2012), it is necessary to examine what effects these mishandlings may have on the food safety of aioli when consumed.

Little research has been conducted using an *in vivo* model to study the effect of storage temperature on bacterial growth/survival in raw egg products such as aioli, and the subsequent effects when consumed. Estimates of the infectious dose of *Salmonella* in food products has been variable, but it is typically thought that a dose of 10^6-10^8 colony forming units (CFU) (Acheson, 2003; Humphrey, 2004) would be effective. This could be affected by the bacteria's pre-infection environment (Humphrey, 2004) and lower doses may be effective depending on the host (Acheson, 2003).

In addition to *S.* Typhimurium and *S.* Enteritidis, other *Salmonella* serovars have also been implicated in human food borne outbreaks. Recently, there have been reported cases of *Salmonella* Hessarek (*S*. Hessarek) being identified to cause illness during egg related outbreaks in South Australia (SA Health, 2017). It is unknown whether it has the same potential and ability to be as infectious as *S.* Typhimurium, therefore this needs to be explored. ***S.*** Hessarek was originally isolated from a common raven (*Corvus corax*) in Iran in 1953 and has been detected in outbreaks of septicaemic salmonellosis in wild birds and in European mammals (Kenny et al., 2019). It is an uncommon serotype in Australia, however from January 2012 to the end of December 2016, there were 96 notifications of ***S.*** Hessarek nationally. Of the 96 notifications, 54% were from South Australian residents. The rate of ***S.*** Hessarek notifications in South Australians was seven times higher than the rate in other states. Between March 2017 and 3 July 2018, 25 human cases were reported. Of the reported human cases, 96% reported eating eggs (Kenny et al., 2019). Given the limited research on *S.* Hessarek and its association with egg related outbreaks, further study needs to be conducted to determine its capabilities in regard to survivability and virulence.

1.6 Effect of storage temperature of eggs infected with *Salmonella*

Growth and survival of *Salmonella* is influenced by many factors, one of which being temperature. The temperature range for growth of *Salmonella* is 5.2–46.2°C, but the optimal temperature range for replication is 35–43°C (Food standards Australia New Zealand, 2013). In Australia, the recommended temperature for egg storage on farm is below 15 °C (+ /- 3°C) (Australian Egg Corporation Limited, 2010), but once the eggs leave the farm, there are no prescriptive regulatory requirements for the storage of eggs in retail shops. Consequently, it is not uncommon to find eggs kept both in the refrigerator and on the shelf. It is thought the storage temperature of eggs may play an important role in minimising bacterial growth within the egg and therefore lessening the risk of salmonellosis (Fajardo et al., 1995; Schoeni et al., 1995). Storage temperatures greater than 7°C are thought to facilitate bacterial growth within the egg if it is contaminated (Food standards Australia New Zealand, 2009) and it has been shown there is an increase in the number of *Salmonella* Enteritidis (*S*. Enteritidis) in infected eggs with the increase in the storage temperature (Kim et al., 1989). However, in respect to *S*. Typhimurium and *S.* Hessarek, not enough research has been undertaken in examining the effect of storage temperature on egg penetration of these serovars. Similarly, limited research has been conducted looking at the effect of storage temperature on growth of both these types of *Salmonella* within eggs. To examine the effects of *S.* Typhimurium and other pathogens, *in vitro* and *in vivo* models are utilised to study their capabilities in realistic scenarios.

1.7 In vitro and in vivo *Salmonella* models

There are many *in vitro* and *in vivo* models that have been previously utilised in *Salmonella* research. Pathogenicity can be investigated using *in vitro* testing involving cell invasion. It is important *in vitro* cell models accurately represent the *in vivo* situation to be tested (Cencič

and Langerholc, 2010). As *Salmonella* infection most often affects the gastrointestinal system, which is a complex system, with many different cell types and microorganisms, cell models need to take these factors into consideration (Cencič and Langerholc, 2010). When testing *Salmonella* invasion capabilities, it is common for cell invasion to be studied in human colon tumorigenic cell lines Caco-2(Cencič and Langerholc, 2010; McWhorter et al., 2015), T84 and HT-29 (Cencič and Langerholc, 2010), however, Caco-2 cells are the most widely used due to their ability to express characteristic differentiation and functionality (Cencič and Langerholc, 2010; Shah et al., 2011). HeLa cells have also been used in *Salmonella* invasion studies (Ibarra et al., 2010). A study tested *S.* Enteritidis invasion in Caco-2 cells and found significant differences in invasiveness between isolate strains, indicating invasiveness isn't uniform across all *Salmonella* strains (Shah et al., 2011). This has also been demonstrated in a study examining the virulence potential of *S.* Typhimurium of differing MLVA types in Caco-2 cells. The mean percent recovery of the isolates ranged from 7.1 to 33.3% (McWhorter et al., 2017). As previously mentioned, environmental growth conditions have been found to affect the ability of *Salmonella* to express virulence genes (Ibarra et al., 2010). The effect of growth conditions on invasive ability of different *Salmonella* serovars has been shown by testing bacteria cultured in Luria Bertani (LB) broth, grown until stationary phase. Observations consistent with other studies showed an increase in invasion capabilities of the bacteria grown in LB broth (McWhorter et al., 2015). Chicken macrophage cells, HD11 (Shah et al., 2011) and MQ-NSCU (Dieye et al., 2009) have also been used in *in vitro* testing to ascertain the ability of *Salmonella* isolates to invade and survive within chicken cells.

In contrast, *in vivo* studies look at the actual effect on an organism and allow for tissue and other samples to be collected for investigation. Mouse models are often used for *in vivo* testing and often in conjunction with *in vitro* testing to see the similarities/differences between invasion in both models (McWhorter et al., 2015; Shah et al., 2011; Suez et al., 2013).

S. Typhimurium causes systemic infection in mice that resembles typhoid fever caused by *S. enterica* serovar Typhi in humans (Chaudhuri et al., 2018). Systemic disease models are commonly performed in inbred mouse strains such as C57BL/6 and BALB/c mice (Bearson and Bearson, 2009). These strains are utilised because they have a mutation in the *Slc11a1* gene, which results in a non-functioning Nramp1 protein. Nramp1 is an ion transporter responsible for the transport of cations out of phagosomes, which limits the availability of iron and other ions for ingested microorganisms, which impairs their growth in phagocytes (Nilsson et al., 2019). The most common technique to deliver an infective dose in mouse studies is via oral galvage. It allows for precise delivery of inoculum directly into the stomach. After oral infection of susceptible mice, *Salmonella* does not replicate efficiently in the intestine. It penetrates the epithelial barrier by invasion of M cells or via dendritic cells. After penetration it colonises Peyer's patches and mesenteric lymph nodes and spreads to the liver and spleen. The mice then usually succumb to systemic infection (Barthel et al., 2003; Muotiala and Makela, 1993). It has been shown when mice are infected with *Salmonella* serovars with a high invasion capability, there is a higher mortality rate than those infected with low or medium invasion capabilities, where no clinical signs of infection or mortality were observed (McWhorter et al., 2015). Mice can also shed *Salmonella* in their faeces if they are infected. This can continue persistently, which is common with many varieties of *Salmonella.* This persistence is what enables the faecal-oral route of transmission (Swearingen et al., 2012).

There are many advantages to using a mouse model. These include lesser space requirements for housing, easier handling and decreased expenses. Larger animals such as chickens, pigs and cattle are more natural hosts for *Salmonella,* however there is more genetic variability between animals therefore variation in disease susceptibility and bacterial colonisation occurs. The other significant difference is *Salmonella* causes a systemic infection in mice, whereas in their more natural hosts, infection is usually localised to the gastrointestinal tract (Bearson and Bearson, 2009), which could be seen as a disadvantage of the mouse model.

However, it has been shown that if the intestinal microbiota of susceptible mouse strains is disrupted by antibiotic treatment, then the mice will develop intestinal inflammation similar to the occurrence of salmonellosis in humans (Barthel et al., 2003).

1.8 Aims and hypothesis

As highlighted, limited research has been undertaken examining the effect of storage temperature on egg penetration of *S*. Typhimurium and looking at the effect of storage temperature on *S*. Typhimurium growth within eggs. Overall there is a lack of research on *S.* Hessarek, therefore studying its capabilities in egg infection is required. It would also be beneficial to compare egg penetration capabilities of *S.* Hessarek to *S.* Typhimurium given its importance to the egg industry. Limited research has been conducted using an *in vivo* model to study the effect of storage temperature on bacterial growth/survival in eggs and raw egg products such as aioli, and the subsequent effects when these are consumed.

Given the gaps in knowledge and the importance of *S.* Typhimurium to the Australian egg industry, the aims of this thesis are as follows;

- To determine whether *S*. Typhimurium and *S.* Hessarek have an enhanced ability to penetrate the eggshell.
- To determine the effects of storage temperature on growth of *S*. Typhimurium and *S*. Hessarek within the egg.
- To examine the effect of storage temperature of eggs infected with *S.* Typhimurium when consumed.
- To study the effects of storage time and storage temperature on aioli infected with *S.* Typhimurium when consumed.

It is hypothesised that an ambient storage temperature (25°C) of *Salmonella* infected eggs would give rise to favourable conditions for bacterial growth within the egg and that in an *in*

vivo trial it will result in clinical symptoms and illness in the mice that have consumed infected eggs, in comparison to eggs infected and stored at a refrigerated temperature (5°C). Similarly, an ambient storage temperature (25°C) of infected aioli will give rise to favourable conditions for bacterial growth/survival, resulting in clinical symptoms and illness in the mice, in comparison to infected aioli stored at a refrigerated temperature (5°C).

By combining the use of *in vitro* and *in vivo* models to study the effects of storage temperature on *Salmonella* infected eggs, this will hopefully lead to a clear indication of the importance of storage temperature regulations, to improve the food safety of Australian eggs and egg products.

2.0 General materials and methods

2.1 Bacterial inoculum preparation

2.1.1 Bacterial suspension for egg infection

Bacterial suspensions were prepared by reviving bacterial culture (*S.* Typhimurium isolated from an egg belt in a chicken layer farm or *S.* Hessarek obtained from the *Salmonella* reference laboratory, IMVS, SA Health) on nutrient agar (Oxoid, Australia). Then a 0.5 McFarland standard (BioMerieux, Australia) was used to prepare a $1x10^8$ CFU bacterial suspension in saline, which was then further diluted in saline to reach the desired infection dose ($1x10^3$ CFU/mL - $1x10^6$ CFU/mL depending on the treatment) (Gole, et al., 2014b).

2.1.2 Bacterial suspension aioli infection

A sample of stored *Salmonella* Typhimurium bacteria (isolated from an egg belt in a chicken layer farm) was cultured on sheep blood agar (SBA) (Oxoid, Australia) and incubated overnight at 37°C. One colony was then placed in five millilitres of Luria Bertani (LB) broth (Oxoid, Australia) and incubated in a shaker incubator for at least 6 hours at 37°C. 100µl of this culture was then added to 100mL of LB broth and incubated in the shaker incubator overnight at 110 rpm (McWhorter and Chousalkar, 2015).

2.1.3 Positive controls

Positive controls were prepared by reviving a *Salmonella* Typhimurium bacterial culture (isolated from an egg belt in a chicken layer farm) on nutrient agar (Oxoid Australia) and incubating overnight at 37°C. One colony was then placed in five millilitres of LB broth (Oxoid Australia) and incubated in a shaker incubator for at least 6 hours at 37°C. 10 µl of this culture was then added to 10 mL of LB broth and incubated in the shaker incubator overnight at 110rpm (McWhorter and Chousalkar, 2015). This stationary phase of bacterial culture was then used to infect the positive controls.

2.2 Egg shell inoculation

Eggs were first sanitised by immersion in 75% ethanol for 90 seconds and then left to air dry in a biosafety cabinet. Eggs were then immersed in bacterial suspension ($1x10^3$ CFU/mL or $1x10^6$ CFU/mL) (at room temperature) for 90 seconds. The eggs were then air dried in a biosafety cabinet (Gole, et al., 2014b). After inoculation, infected eggs were stored at either 5°C or 25°C for four days.

2.3 Egg contents inoculation

Eggs were sanitised by immersion in 75% ethanol for 90 seconds and then left to air dry in a biosafety cabinet. Bacteria were inoculated directly into the albumen or yolk by passing a sterile 23-gauge needle into the egg contents through the shell (Cogan et al., 2001). The holes were sealed after inoculation with nail polish. Infected eggs were then stored at either 5°C or 25°C for 4 days. Uninfected control groups were stored under the same conditions.

2.4 *Salmonella* isolation

Egg shell crush, yolk and albumen samples were processed for *Salmonella* isolation. Egg shell was placed in 10 mL of buffered peptone water (BWP) (Oxoid, Australia) and crushed thoroughly by hand (Samiullah et al., 2014). Albumen and yolk were processed separately (1mL egg component in 9 mL BWP (Oxoid, Australia)) (Samiullah et al., 2014). Bacteria present in the samples were counted by direct plating on xylose lysine deoxycholate (XLD) agar (Oxoid, Australia) (Gole, et al., 2014b). In the event of no bacterial growth on a plate after direct plating, BPW mixed samples were incubated overnight at 37°C and further enriched in Rappaport-Vassiliadis soya peptone (RVS) broth (Oxoid, Australia) then streak

plated on XLD agar and results were recorded as positive or negative for the presence of *Salmonella*.

2.5 Mouse infection

Animal ethics approval (S-2017-079) was granted by the University of Adelaide Animal Ethics Committee for the use of mice in these studies. Specific pathogen free, 6-8 week old, female BALB/c mice were sourced from Laboratory Animal Services (University of Adelaide). They were raised in cages and fed an irradiated commercial diet. Food and water were provided ad libitum during the experiment. Each group contained seven animals. Mice received 100μl of designated treatment (see table 4.1 and 5.1 for specific treatment groups), via oral gavage. Control groups were infected with LB broth containing the *Salmonella* Typhimurium strain as a positive control and negative control mice were inoculated with LB broth. The mice were observed, four times a day during the first week post infection and then twice daily for the remainder of the trial. During each visit, mice were observed for clinical symptoms of disease (e.g. lethargy, hunching, ruffled fur etc.) and mortality (with clinical records kept) for up to 21 days post infection (McWhorter and Chousalkar, 2015). At the end of the experiment, all surviving mice were humanely culled by carbon dioxide asphyxiation. Each clinical symptom observed received a score of one and mice with a clinical score of five or above (five or more clinical symptoms) during the trial were also humanely culled. Faecal samples were collected at day 3, 6, 9, 12, 15 and 18 post infection. The cage and bedding were changed at every faecal sampling to minimise the carryover of contamination/ shedding. Faecal and tissue samples collected from mice were processed for bacteriology.

2.6 Sample processing

2.6.1 Faecal samples

From the collected faecal samples, 100 mg of sample was placed in a 1.5 mL Eppendorf tube with 1 mL of buffered peptone water (BPW) (Oxoid, Australia) and incubated overnight at 37°C. One hundred microlitres was then added to 10 mL of Rappaport-Vassiliadis soya peptone (RVS) broth (Oxoid, Australia) and incubated overnight at 42°C. The RVS was streaked on Brilliance *Salmonella* agar (Oxoid, Australia) and incubated overnight at 37°C (Gole, et al., 2014a). The plates were then examined to determine if the samples were positive or negative for *Salmonella* presence.

2.6.2 Organ samples

Liver, spleen, caecum and small intestine samples were collected from all mice. Organ samples were placed in safe lock tubes containing 500µl of sterile saline and stainless-steel homogenisation beads ranging in size from 0.5 mm to 2.0 mm. Samples were homogenised at full speed for 5 minutes and serial 10-fold dilutions were prepared. One hundred microlitres of all dilutions was spread plated onto XLD agar (Oxoid, Australia) and incubated overnight at 37°C. Colonies were then counted to determine bacterial load. Pre-emptively, in the event of no bacterial growth on the plate initially, samples were enriched in BPW, then in RVS broth, before plating on XLD agar and results were recorded as positive or negative for the presence of *Salmonella*.

3.0 Investigating the ability of *Salmonella* Typhimurium and *Salmonella* Hessarek to penetrate egg shell and their growth in eggs stored at different temperatures.

3.1 Introduction

Various *Salmonella* species are commonly associated with occurrences of foodborne gastroenteritis globally (Chaudhuri et al., 2013; Ford et al., 2016; Majowicz et al., 2010). Surveillance data for Salmonellosis suggests the rate of infection, over the last decade has been increasing (Ford et al., 2016). Of over 2500 known serovars (Ford et al., 2016; Jones et al., 2008), *Salmonella* Typhimurium (*S*. Typhimurium) definitive type 9 (DT-9) is the most commonly identified serovar during outbreaks of salmonellosis amongst humans, in Australia. It has also been shown to be the most frequently identified serovar during egg related outbreaks (Moffatt et al., 2016). A common route of transmission of *Salmonella* in eggs is via horizontal transmission. After oviposition, eggs can come into contact with a contaminated environment, giving *Salmonella* the opportunity to pass through the egg shell into the egg contents. This is the most common route of transmission in Australia. Contamination of eggs can occur at any point during the production chain, from farm through to table (Fajardo et al., 1995). Recently, there have been reported cases of *Salmonella* Hessarek (*S*. Hessarek) being identified during egg related outbreaks in South Australia (SA Health, 2017). It is not known if *S.* Hessarek is capable of vertical transmission, but egg contents can be contaminated through the horizontal route. According to the code of practice for shell egg production, grading, packing and distribution, the recommended temperature for egg storage is below 15 °C (+ /- 3°C) (Australian Egg Corporation Limited, 2010), but once they leave the farm, there are no regulatory requirements to store eggs in refrigerated conditions, hence it is not uncommon to find eggs kept on the shelf at ambient temperature in some Australian retail shops. It is important to note however, a previously conducted risk assessment concluded, eggs that are cracked or visibly dirty (soiled) lead to an increased potential for microorganisms such as *Salmonella* to gain access to the egg content. It also determined that storage temperature was not a contributing factor in *Salmonella* gaining

access to egg contents, but it can become a contributing factor once the egg is contaminated and stored at greater than 7℃ (Food standards Australia New Zealand, 2009). So far, limited research has been undertaken examining the effect of storage temperature on egg penetration of *S*. Typhimurium DT9 and *S*. Hessarek. Similarly, limited research has been conducted looking at the effect of storage temperature on *S*. Typhimurium and *S.* Hessarek within eggs. The temperature of a freshly laid egg is comparable to hen's body temperature (40-42℃) (Wilson, 1948), accordingly, our hypothesis was that negative pressure induced by rapid cooling of freshly laid eggs to the shed temperature (21°C on commercial farms), enhances bacterial penetration across the egg shell. Accordingly, the aims of this study were to determine whether *S*. Hessarek and *S*. Typhimurium have the ability to penetrate the egg shell, to determine the effects of storage temperature on growth of *S*. Hessarek and *S*. Typhimurium within the egg and to examine any differences in growth between *S.* Typhimurium and *S.* Hessarek in eggs stored at different temperatures.

3.2 Materials and methods

3.2.1 Cooling rate of eggs post lay

To determine the cooling rate of eggs, post lay, thirty-two Hyline Brown hens that were thirty-two weeks old, were individually housed in cages at The University of Adelaide, Roseworthy campus, South Australia (animal ethics approval (S-2018-009) granted by the University of Adelaide Animal Ethics Committee). Cage size was a per the industry standard, 550cm^2 per hen (Primary Industries Standing Committee, 2002). Lighting conditions for the hens were sixteen hours of light and eight hours of dark per day (Hy-Line International, 2019). Freshly laid eggs were collected from the cage front immediately after lay and the temperature of the egg contents was recorded. Eggs were then collected at 10 minute intervals and the temperature recorded to determine the length of time for an egg to reach

room temperature. Five eggs were tested at each time point. This study was conducted twice. The shed temperature ranged from 20-23°C.

3.2.2 Egg shell penetration

Thirty-two freshly laid eggs were collected from cage front of above mentioned flock and transported immediately to the lab for infection (as per 2.2). Eggs, with temperature of 34-35°C (immediately after lay) and 22-23°C (cooled for 60 minutes), were immersed in bacterial suspension (at room temperature) that ranged between $1x10^3$-$1x10^4$ CFU/mL of *S.* Hessarek (obtained from *Salmonella* reference laboratory, IMVS, SA Health) or *S.* Typhimurium DT9 (obtained from layer hen environment sample from previous work within our research group (Gole, et al., 2014a)). Bacterial suspensions were prepared as per 2.1.1. After immersion in the bacterial suspension, eggs were dried in a biosafety cabinet before eggs were stored at either 5°C or 25°C for 24 hours and 72 hours. There were five eggs in each infection group and 3 eggs in each control of which there were control groups for both egg temperature and storage temperatures. Control eggs were immersed in saline. Egg shell crush, yolk and albumen samples from all eggs were processed for *Salmonella* isolation (Moyle et al., 2016), with samples that were negative after direct plating (on XLD agar) being further enriched in Rappaport-Vassiliadis Soya Peptone (RVS) broth (Oxoid, Australia).

3.2.3 Inoculation of egg contents

One hundred and fifty freshly laid eggs from a caged flock (aged between 35-40 weeks) were obtained from a commercial flock and inoculated with bacterial suspension that (as mention in 3.2.2) ranged between $1x10^3$-$1x10^4$ CFU/mL of *S.* Hessarek or *S.* Typhimurium. After inoculation, infected eggs (15 per treatment group) were stored at either 5°C or 25°C (Table 3.1). Three eggs from each treatment group were opened at regular intervals; days 4, 7, 14,

21 and 28 days post infection (P.I.). Albumen and yolk were processed separately (1mL egg component in 9 mL Buffered Peptone Water (BWP) (Oxoid, Australia)) for *Salmonella* isolation and enumeration from each egg. Bacteria present in the yolk and albumen were counted by direct plating. In the event of no bacterial growth on a plate after direct plating, samples were further enriched in RVS broth, and results were recorded as positive or negative for the presence of *Salmonella*. *Salmonella* Typhimurium ATCC strain (obtained from the *Salmonella* reference laboratory, IMVS, SA Health) was used as a positive control. The trial was run three times with each strain of *Salmonella*.

Table 3. 1 Treatment groups for egg inoculation trials.

ST= *Salmonella* Typhimurium, SH= *Salmonella* Hessarek and DT= Definitive type.

Treatment	**Storage temperature**	
Control	5°C	25°C
Yolk infected with ST DT9 or SH	5°C	25°C
Yolk infected with ST ATCC	5°C	25°C
Albumen infected with ST DT9 or SH	5°C	25°C
Albumen infected with ST ATCC	5°C	25°C

3.2.4 Statistical analysis

Inoculation data was analysed, and graphs prepared using GraphPad Prism version 7.0c. Statistical analysis undertaken included 2way ANOVA and Tukey's multiple comparisons test.

3.3 Results

3.3.1 Cooling rate of eggs post lay

Periodical temperature measurements of eggs post lay showed it took approximately 50 minutes for the eggs to cool completely to shed temperature (figure 3.1). During egg cooling, the shed temperature ranged from 20-23°C.

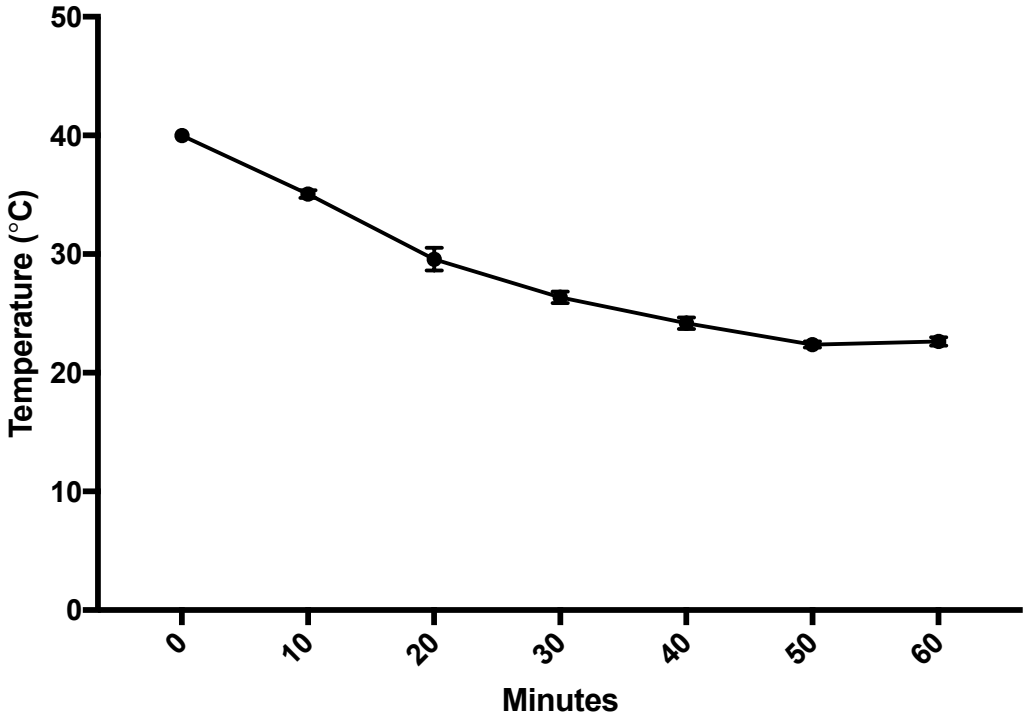

Figure 3. 1 Cooling rate of eggs post lay. Measurements taken at 10 minute intervals.

3.3.2 Egg shell penetration

All yolk and albumen samples were negative for all treatment groups for both *S.* Typhimurium and *S*. Hessarek infection indicating the bacteria did not penetrate through the egg shell pores into the egg contents. Figure 3.2 shows the results of egg shell crush (pores). Group 1 contained eggs infected immediately after lay and group 2 contained eggs completely cooled before infection. Eggs from group 2 also had no bacterial growth in the egg shell pores. After enrichment, all of the egg shell pore samples from the group 1, 25°C storage treatment group (Figure 3.2), were positive for *Salmonella* for both *S.* Typhimurium and *S*. Hessarek infection. These results were the same after 24 hour and 72 hours of storage for both *S.* Typhimurium and *S*. Hessarek infection, except for the amount for bacteria enumerated after direct plating from the group 1, 25°C storage treatment group. In eggs infected with *S*. Hessarek, the average log CFU/egg shell (pores) after 24 hours of storage was 2.5 ±0.14 and the average log CFU/egg shell (pores) after 72 hours of storage was 3.3±0.41. There was an increase in *S*.

Hessarek count in egg shell pore, but this increase was not statistically significant. For *S.* Typhimurium, the average log CFU/egg shell (pores) after 24 hours of storage was 3.4±0.40 and the average log CFU/egg shell (pores) after 72 hours of storage was 3.3±0.20 (Figure 3.3). Samples from the group 1, 5°C storage treatment group were positive for both *S.* Typhimurium and *S.* Hessarek after further enrichment in RVS broth.

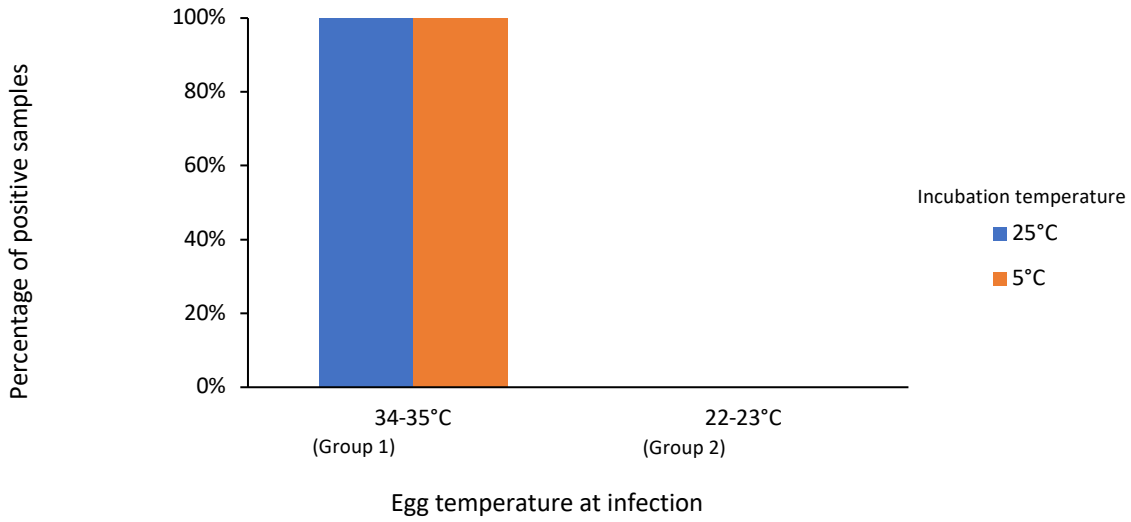

Figure 3. 2 Egg shell penetration results for egg shell crush (pores). Group 1 contained eggs infected immediately after lay and group 2 contained eggs completely cooled before infection. The results were the same for both *S.* Typhimurium and *S.* Hessarek at each time point (24 and 72 hours). There was no penetration of bacteria through the egg shell pores into the egg contents. Figure shows the percentage of positive samples from each treatment group at each storage temperature.

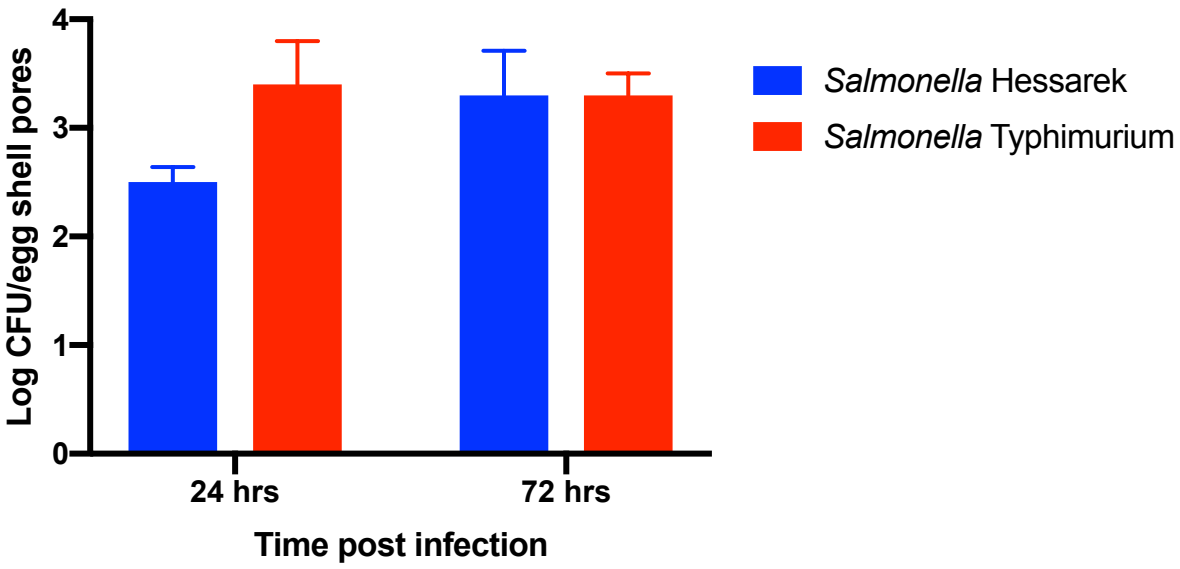

Figure 3. 3 Salmonella counts in egg shell pores (Log CFU/egg shell pores) 24 and 72 hours post infection. Eggs were stored at ambient temperature (25°C) after infection.

3.3.3 Inoculation of egg contents

3.3.3.1 Inoculation with *S*. Typhimurium

There was a substantial increase in bacterial load from infection to day 4 P.I. (post infection) in eggs stored at 25°C. By day 4 P.I. the number of bacteria in the egg components (yolk and albumen) had increased to around 10^7-10^8 CFU/mL (figure 3.4a and 3.4b). After 4 days P.I., bacterial growth remained constant in both yolk and albumen at 25°C. In the albumen of eggs stored at 25°C there was a significant effect of days P.I. ($P=\leq 0.01$) and treatment ($P=\leq 0.01$). In the yolk of eggs stored at 25°C there was a significant effect of treatment ($P=\leq 0.01$). This shows the growth of bacteria in both the albumen and yolk were significant, regardless of the treatment, with days P.I. also being a significant factor for the albumen. The results indicated there was no growth of bacteria at 5°C after direct plating regardless of whether yolk or albumen had been infected. After further enrichment, some 5°C stored eggs were positive for

Salmonella (see figures 3.4c and 3.4d for exact proportions of positives for each treatment group). After enrichment of the eggs stored at 5°C there was a significant effect of days P.I. (P=≤0.01) for albumen and a significant effect of treatment (P=≤0.01) for yolk.

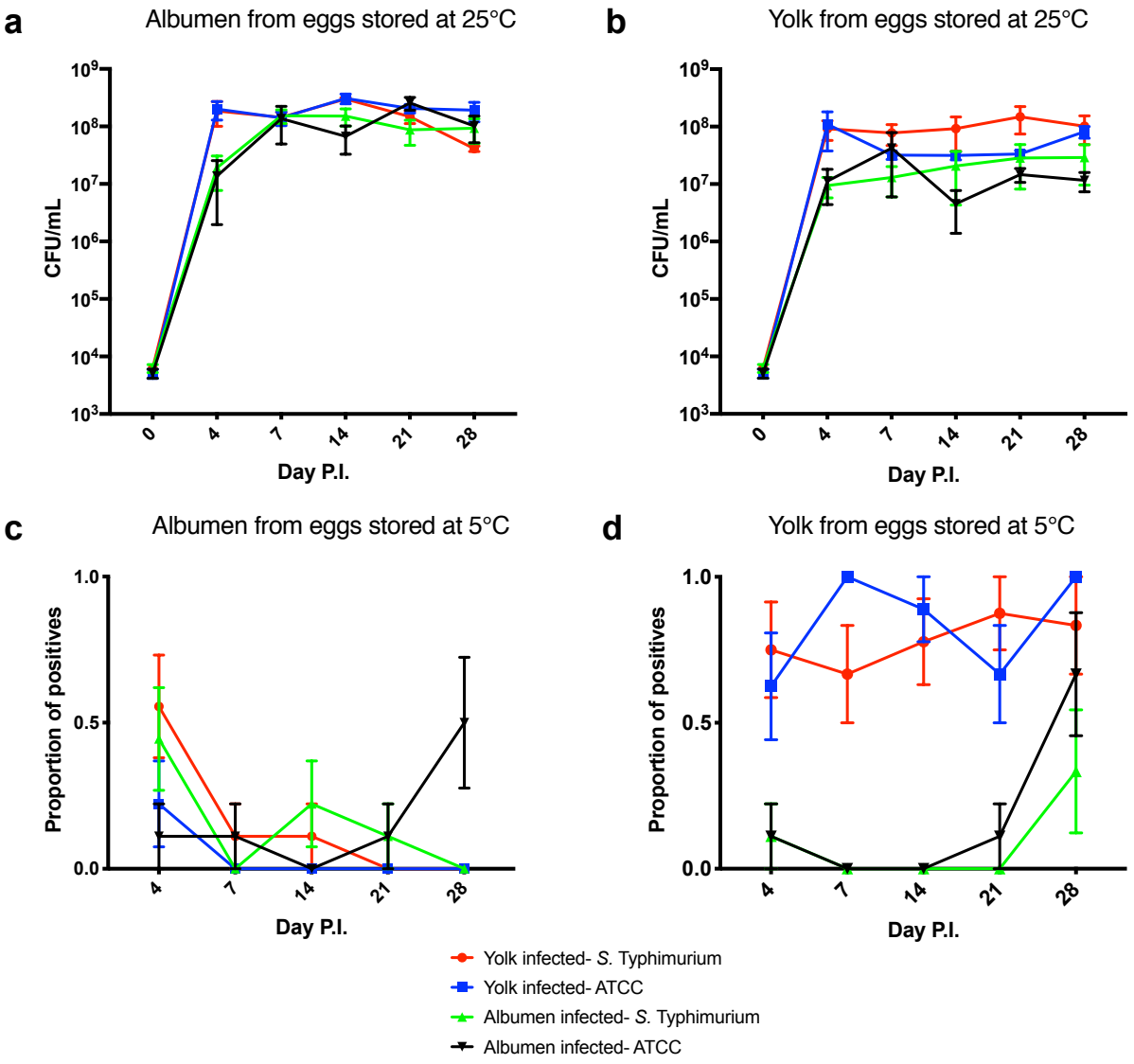

Figure 3. 4 Bacterial growth in eggs infected with S. Typhimurium. **a.** Bacterial growth in the albumen of infected eggs stored at 25°C. Significant effect of days P.I. (P=≤0.01) and treatment (P=≤0.01). **b.** Bacterial growth in the yolk of eggs stored at 25°C. Significant effect of treatment (P=≤0.01). **c.** Bacteria positive albumen samples from eggs stored at 5°C after RVS enrichment presented as a proportion of positives. Significant effect of days P.I. (P=≤0.01). **d.** Bacteria positive yolk samples from eggs stored at 5°C after RVS enrichment presented as a

proportion of positives. Significant effect of days P.I. and treatment (P=≤0.01). In all graphs; red line= eggs that received *S.* Typhimurium inoculum directly in yolk, blue line= eggs that received ATCC inoculum directly in yolk, green line= eggs that received *S.* Typhimurium inoculum directly in albumen and black line= eggs that received ATCC inoculum directly in albumen. Day P.I.= day post infection. CFU/mL= colony forming units per millilitre.

3.3.3.2 Inoculation with *S*. Hessarek

There was a substantial increase in bacterial growth from infection to day 4 P.I. in eggs stored at 25°C. By day 4 P.I. the number of bacteria in the albumen had increased to around 10^6-10^8 CFU/mL and the number of bacteria in the yolk had increased to around 10^7-10^8 CFU/mL (figures 3.5a and 3.5b). After 4 days post inoculation, bacterial growth remained constant in both yolk and albumen at 25°C. In the albumen of eggs stored at 25°C there was a significant effect of days P.I. (P=≤0.01) and treatment (P=≤0.01). In the yolk of eggs stored at 25°C there was a significant effect of days P.I. (P=≤0.01). This shows the growth of bacteria in both the albumen and yolk were significant, with days P.I. being the most significant factor for growth in yolk and both days P.I. and treatment significantly affecting growth in the albumen. The results indicated there was no growth of bacteria at 5°C after direct plating regardless of whether yolk or albumen had been infected. After further enrichment, some 5°C stored eggs were positive for *Salmonella* (see figures 3.5c and 3.5d for exact proportions of positives for each treatment group). After enrichment of the eggs stored at 5°C there was a significant effect of treatment (P=≤0.01) for both the yolk and albumen.

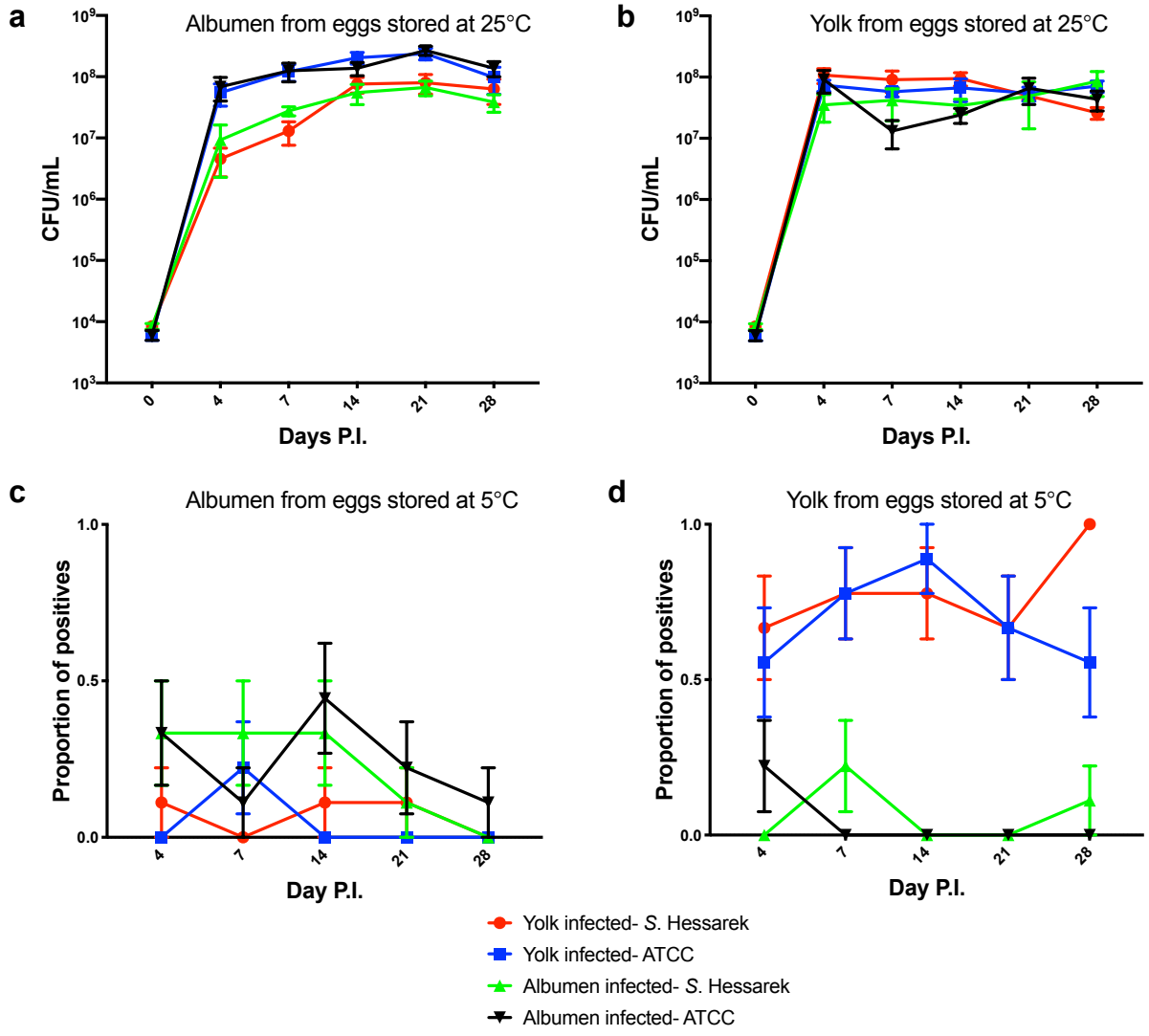

Figure 3. 5 Bacterial growth in eggs infected with S. Hessarek. **a.** Bacterial growth in the albumen of eggs stored at 25°C. Significant effect of days P.I. (P=≤0.01) and treatment (P=≤0.01). **b.** Bacterial growth in the yolk of eggs stored at 25°C. Significant effect of days P.I. (P=≤0.01). **c.** Bacteria positive albumen samples from eggs stored at 5°C after RVS enrichment presented as a proportion of positives. Significant effect of treatment (P=≤0.01). **d.** Bacteria positive yolk samples from eggs stored at 5°C after RVS enrichment presented as a proportion of positives. Significant effect of treatment (P=≤0.01). In all graphs; red line= eggs that received *S.* Hessarek inoculum directly in yolk, blue line= eggs that received ATCC inoculum directly in yolk, green line= eggs that received *S.* Hessarek inoculum directly in albumen and black line= eggs that received ATCC inoculum directly in albumen. Day P.I.= day post infection. CFU/mL= colony forming units per millilitre.

3.4 Discussion

The initial egg cooling observational study found it took approximately 50 minutes for the eggs to cool completely to shed temperature, which is consistent with previous work undertaken (Mortola and Gaonac'h-Lovejoy, 2016). Determining the length of time for an egg to cool completely was important for the egg shell penetration study because eggs need to be completely cooled for those corresponding treatment groups. All yolk and albumen samples were negative for all treatment groups for both *S.* Typhimurium and *S*. Hessarek infection, indicating the bacteria was not able to penetrate from the egg shell pores into the egg contents. Eggs which were completely cooled before infection, had no bacterial growth in the egg shell pores. After enrichment, all of the egg shell pore samples from the group 1, 25°C storage treatment group were positive for *Salmonella* for both *S.* Typhimurium and *S*. Hessarek infection. These results were the same for 24 hour and 72 hour storage for both *S.* Typhimurium and *S*. Hessarek infection. The results indicate the bacteria were not able to penetrate into the contents of the egg, even though they penetrated into the shell pores of the eggs. This could be due to another mechanical barrier the egg possesses against bacterial penetration, the shell membrane. The shell membrane is comprised of the outer and inner shell membranes (Lifshitz et al., 1964). Due to its structure, the shell membrane is tough, flexible and functions as a mechanical barrier against bacterial penetration. The shell membranes also act as a filter and are more impenetrable to bacteria than the shell (Garibaldo and Stokes, 1958). During this experiment, eggs were tested 24 and 72 hours post infection. Typically, the turnaround time for eggs from farm to supermarket shelf is four to five weeks. There is a possibility that the structure of egg shell membranes could be affected during this storage time (Chen et al., 2005) and bacterial penetration may be enhanced. In eggs infected with *S*. Hessarek the average log CFU/egg shell (pores) after 24 hours of storage was 2.5 ±0.14

and the average log CFU/egg shell (pores) after 72 hours of storage was 3.3±0.41. For *S*. Typhimurium, the average log CFU/egg shell (pores) after 24 hours of storage was 3.4±0.40 and the average log CFU/egg shell (pores) after 72 hours of storage was 3.3±0.2. There was an increase in *S*. Hessarek count in shell pores, but this increase in bacterial count was not significant. Information is scarce on the multiplication of *Salmonella* spp. in egg shell pores and given these results, it needs to be explored further. The difference in results could be attributed to the serovar difference. Of the eggs which were positive for multiplication of *S.* Typhimurium in the pores after 24 hours, the bacterial count was highly variable. Egg shell pore samples from the group 1, 5°C storage treatment group, were positive for both *S.* Typhimurium and *S.* Hessarek after further enrichment in RVS broth after being negative by direct plating. The results indicated that if the egg was infected immediately after lay, before the egg cooled and the cuticle completely hardened, both *S*. Hessarek and *S*. Typhimurium were able to penetrate into the egg shell pores, but not through to the egg contents. The cuticle of the egg does not mature for a period of time after laying, therefore some of the shell pores may still have be open, allowing bacteria to penetrate into the pores. Cooling of eggs may create negative pressure inside the egg enhancing bacterial penetration across the egg shell (Miyamoto et al., 1998). It has been concluded that *S*. Typhimurium is able to penetrate across the shell through to egg contents after three weeks post infection (Gole, et al., 2014b), however in this study eggs were processed three days post infection. Had we left infected eggs for three weeks, penetration in egg contents would have been observed.

Few trials have been conducted examining *S.* Typhimurium growth within the egg contents and limited research has been conducted in general looking at *S.* Hessarek infection in eggs. Given that *S*. Hessarek has been involved in an increased number of egg related outbreaks, it is essential to understand its replication abilities in egg contents. Considerably more research

has been undertaken looking at *Salmonella* Enteritidis (*S.* Enteritidis) infection of eggs. The results of the egg inoculation trial indicated that after storage at 5°C, there was no growth of bacteria after direct plating of egg contents, whereas there was a significant increase in bacterial growth from infection to day 4 P.I. in eggs stored at 25°C, in both the yolk and albumen. These results were similar for both *S.* Typhimurium and *S.* Hessarek. It has been shown previously that time and temperature play an important role in bacterial growth. It has been demonstrated that storing eggs below 7°C prevents bacteria from growing (Fajardo et al., 1995). Albumen has been reported as being growth restricting for *Salmonella* because of its antimicrobial properties (Gantois et al., 2009), but this didn't seem to be the case in the 25°C stored samples in this trial, where the bacteria were found to grow rapidly post infection until day four. This has also been found to be the case in some previous *Enteritidis* studies, as summarized by Gantois et al., 2009. By day 4 P.I. the number of bacteria in both egg components had increased to around 10^6-10^8 CFU/mL. The number of bacteria has previously been found to increase rapidly in the first few days after infection in *S.* Enteritidis infected eggs (Gast and Holt, 2000b), much like what occurred during this trial. In the initial growth phase, bacteria consume iron reserves. Once these are consumed, the bacteria enter a lag phase where in most cases there is little or no change in the number of *Salmonella* present (Baron et al., 1997). During this trial, after 4 days post inoculation, bacterial growth remained constant in both yolk and albumen at 25°C. As shown in figures 3.4a and 3.5a, in eggs that were albumen infected, the bacteria migrated from the albumen into the yolk and multiplied to similar levels as in the albumen in all 25°C stored eggs. This occurs so the bacteria can reach the nutrient rich yolk, survive and multiply rapidly (Gantois et al., 2009; Gast and Holt, 2000b). After further enrichment, some 5°C stored eggs were positive for *Salmonella* (see figures 3.4c, 3.4d, 3.5c and 3.5d for exact proportions of positives for each treatment group). Given that after enrichment some of the 5°C were positive, this means

bacteria were unable to multiply at 5°C but survived in both yolk and albumen. While the proportion of positive albumen samples were quite low, the proportion of positive yolk samples from yolk infected eggs was much higher. So, while the lower storage temperature obviously affected the bacteria's ability to multiply, the nutrient rich environment of the yolk aided the bacteria's survival in many of the samples and the antimicrobial properties of the albumen plus lower temperature minimised the number of positive albumen samples.

3.5 Conclusion

To conclude, when eggs were infected immediately after lay, before the eggs cooled, *S*. Typhimurium DT9 and *S*. Hessarek were able to penetrate into the shell pores, but not through to the contents. Although the replication of *S*. Hessarek was not significant, further studies are required to investigate the multiplication mechanism of *S*. Hessarek in shell pores. Infected eggs that were stored at 5℃ were negative after direct plating, whereas eggs stored at 25℃ were positive after direct plating during the entire experiment. These results indicate an obvious difference in the effect of storage temperature on bacterial growth within the egg. Recommendations for best storage practice would be to store eggs at refrigerator temperatures as soon as possible, after lay. Bacterial growth in the eggs increased rapidly by day 4 P.I. and remained constant, demonstrating how quickly bacterial growth can escalate at ambient temperatures.

4.0 Examining the effects of different storage temperature of eggs infected with *Salmonella* Typhimurium when fed to mice.

4.1 Introduction

Salmonella Typhimurium (*S*. Typhimurium) is a major cause of non-typhoidal salmonellosis, a worldwide foodborne infectious disease (Chaudhuri et al., 2013; Ford et al., 2016; Majowicz et al., 2010; Okuno et al., 2019) In Australia, the vast majority of egg and egg product related outbreaks can be attributed to *S.* Typhimurium (Moffatt et al., 2016). Contamination of eggs can occur at any point in the production chain (Fajardo et al., 1995) and while it is recommended eggs be stored below 15 °C on farm (Australian Egg Corporation Limited, 2010), there are no regulatory requirements for the storage of eggs once they leave the farm. Given that the occurrence of *Salmonella* infection has increased over the last decade (Ford et al., 2016), there is a clear need to control the spread and incidence of this pathogen in the food chain. There are clear public health benefits and economic gains to be made by decreasing the incidence of outbreaks of *Salmonella* infections (Humphrey, 2004). Given the survival and persistence of *Salmonella* in the environment, it is challenging to eliminate it entirely from the food supply chain. There are certain measures which could be put in place to mitigate its effect (Humphrey, 2004), right from farm to fork. It is thought that the storage temperature of eggs may play an important role in minimising bacterial growth within the egg and therefore reducing the risk of Salmonellosis (Fajardo et al., 1995; Schoeni et al., 1995). Estimates of the infective dose of *Salmonella* in food products has been variable, but it is typically anticipated that a dose of 10^3-10^8 colony forming units (CFU) would cause disease in humans. However, infective dose may be affected by the bacteria's pre-infection environment, meaning the infective dose may be lower (Acheson, 2003; Humphrey, 2004).

Consumption of raw egg and partially cooked eggs are often the cause of Salmonellosis (Humphrey, 2004; Australian Egg Corporation Limited, 2010). It has been demonstrated that storing eggs below 7°C prevents bacteria from replicating (Fajardo et al., 1995), however as mentioned, there are no regulatory requirements on the storage temperature of eggs in

Australia. Limited research has been conducted using an *in vivo* model to study the effect of storage temperature on bacterial growth in eggs or egg contents and the subsequent effects when consumed. This trial aimed to examine the effect of storage temperature; ambient temperature (25°C) versus refrigerated temperature (5°C), of eggs infected with *S.* Typhimurium when consumed. An *in vivo* mouse model was utilised and it was hypothesised that an ambient storage temperature (25°C) would give rise to favourable conditions for bacterial growth within the egg, influence the bacterial virulence resulting in clinical symptoms and illness in the mice, in comparison to eggs infected and stored at refrigerated temperature (5°C).

4.2 Materials and methods

4.2.1 Bacterial suspension

Bacteria used in this trial was *Salmonella* Typhimurium DT9 (MLVA type 03 15 07 11 550). The isolate was sourced from a layer farm environment during a previous trial conducted within our research group (Gole, et al., 2014a). Bacterial suspensions were prepared as outlined in 2.1.1.

4.2.2 Egg shell inoculation

Freshly laid eggs were obtained from a commercial caged flock (aged between 35-40 weeks) and inoculated with the bacterial suspension at a dose rate of $1x10^6$ CFU/mL of *S.* Typhimurium. Inoculation occurred as described in 2.2. Uninfected groups were also stored under the same conditions.

4.2.3 Inoculation of egg contents

Freshly laid eggs were obtained from a commercial caged flock (aged between 35-40 weeks) and inoculated with the bacterial suspension at a dose rate of $1x10^3$ CFU/mL of *S.*

Typhimurium. Egg contents were infected as outlined in 2.3. Uninfected control groups were also stored under the same conditions.

4.2.4 Positive controls

Prepared as described in 2.1.3.

4.2.5 Mouse infection

Mouse infection occurred as described in 2.5. Mouse weight ranged from 14.6-23.4 grams at infection. In this trial, the seven mice from each treatment group received 100µl of egg shell wash, yolk or albumen, via oral gavage as per each treatment group (table 4.1).

Table 4. 1 Treatment groups for the infected egg mouse trial. For each treatment group there was an infected and uninfected group stored at 5°C and 25°C. All infected groups and positive control were infected with the same *S.* Typhimurium strain.

Treatment group	**Storage temperature**			
Yolk	5◦C (infected)	25◦C (infected)	5◦C (not infected)	25◦C (not infected)
Albumen	5◦C (infected)	25◦C (infected)	5◦C (not infected)	25◦C (not infected)
Shell wash	5◦C (infected)	25◦C (infected)	5◦C (not infected)	25◦C (not infected)
Positive control	5◦C (infected)	25◦C (infected)		
Negative control	5◦C (not infected)	25◦C (not infected)		

4.2.6 Sample processing

Faecal samples were processed as outlined in 2.6.1 and organ samples were processed as outlined in 2.6.2.

4.2.7 Statistical analysis

All statistical analyses were performed using GraphPad Prism version 8.0. one-way and 2way

ANOVA with Tukey's Multiple comparisons test was used to determine any significance in the bacterial colonisation of the organs. P-values less than 0.05 were considered significant.

4.3 Results

During this experiment egg contents were infected with 10^3 CFU/mL of *Salmonella*. As described in chapter 3, by day 4 P.I. the number of bacteria in the egg components (yolk and albumen) stored at 25°C had increased to around 10^7-10^8 CFU/mL. Prior to mouse infection, when testing the bacterial growth within the egg after 4 days P.I., growth within the egg yolk ranged from $4x10^7$ – $3x10^8$ CFU/mL. These results show the growth within the yolk is consistent after four days of storage at 25°C. The egg shell treatment group was inoculated with 10^6 CFU/mL of *Salmonella.* After 4 days P.I. though, no bacteria was recovered, even after storage at 25°C. This was also the case with all treatment groups stored at 5°C. For these samples, it was then difficult to determine how much bacteria actually infected the mice. Inoculum checks did confirm the egg contents (yolk and albumen) were infected with 10^3 CFU/mL and egg shell was infected with 10^6 CFU/mL of bacterial suspension.

4.3.1 Survival

Two treatment groups culled during the trial (figure 4.1), due to a clinical score of five or more, were positive control 25°C and infected yolk 25°C. All other groups survived until the end of the trial. All animals were culled from the positive control 25°C group by day 8 P.I. and all animals were culled from the Yolk 25°C group by day 6 P.I. There was a statistically significant difference in survival between the animals that were culled during the trial and those that survived until the end ($P \leq 0.01$).

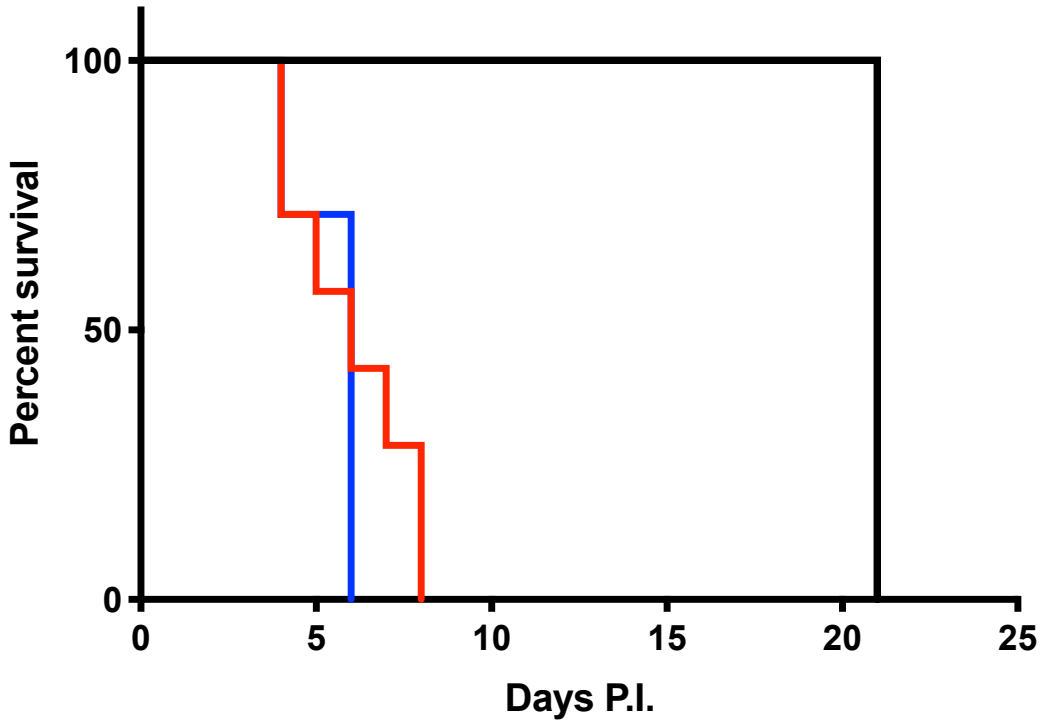

Figure 4. 1 Survival curve for mice fed egg components infected with Salmonella Typhimurium. Two groups were culled during the trial; positive control stored at 25°C (red line) and infected yolk stored at 25°C (blue line). All other groups (black line) survived until the end of the trial.

4.3.2 Faecal samples

Only four treatment groups of the total 16 treatment groups shed *Salmonella* in their faeces during the trial. Positive control 25°C and yolk 25°C groups shed bacteria from the first faecal sampling (day 3 P.I.) until the entire group was culled. Shell wash 25°C and albumen 25°C began shedding at day 15 P.I, but both groups showed no clinical symptoms.

4.3.3 Organ samples

Salmonella was enumerated from all organs that were sampled from mice culled during the trial. The average number of bacteria enumerated from the liver was $5.67 \times 10^8 \pm 8.09 \times 10^8$ cfu/g tissue (figure 4.2a) and the average number of bacteria enumerated from the spleen was $5.2 \times 10^7 \pm 3.35 \times 10^8$ cfu/g tissue (figure 4.2b). The average number of bacteria enumerated

from the caecum was $5.3x10^9 \pm 1.01x10^8$ cfu/g tissue (figure 4.2c) and the average number of bacteria enumerated from the small intestine was $4.27x10^8 \pm 9.51x10^6$ cfu/g tissue (figure 4.2d). The bacterial load was similar in the organs from the Yolk 25°C group in comparison to the bacterial load present in the organs from positive control 25°C group. There was no statistically significant differences between the bacterial colonisation of each of the organs.

All surviving mice were culled at the conclusion of the trial. *Salmonella* was enumerated from organs from two of the surviving groups, shell wash stored at 25°C and albumen stored at 25°C (figure 4.3). All other groups were negative for bacterial colonisation. The bacterial load in organs from the mice culled day 21 P.I. was less than those culled during the trial. There were no statistically significant differences between the bacterial colonisation of each of the organs. The average number of bacteria enumerated from the organs was $6.3270x10^2 \pm 2.0759x10^2$ CFU/g of tissue.

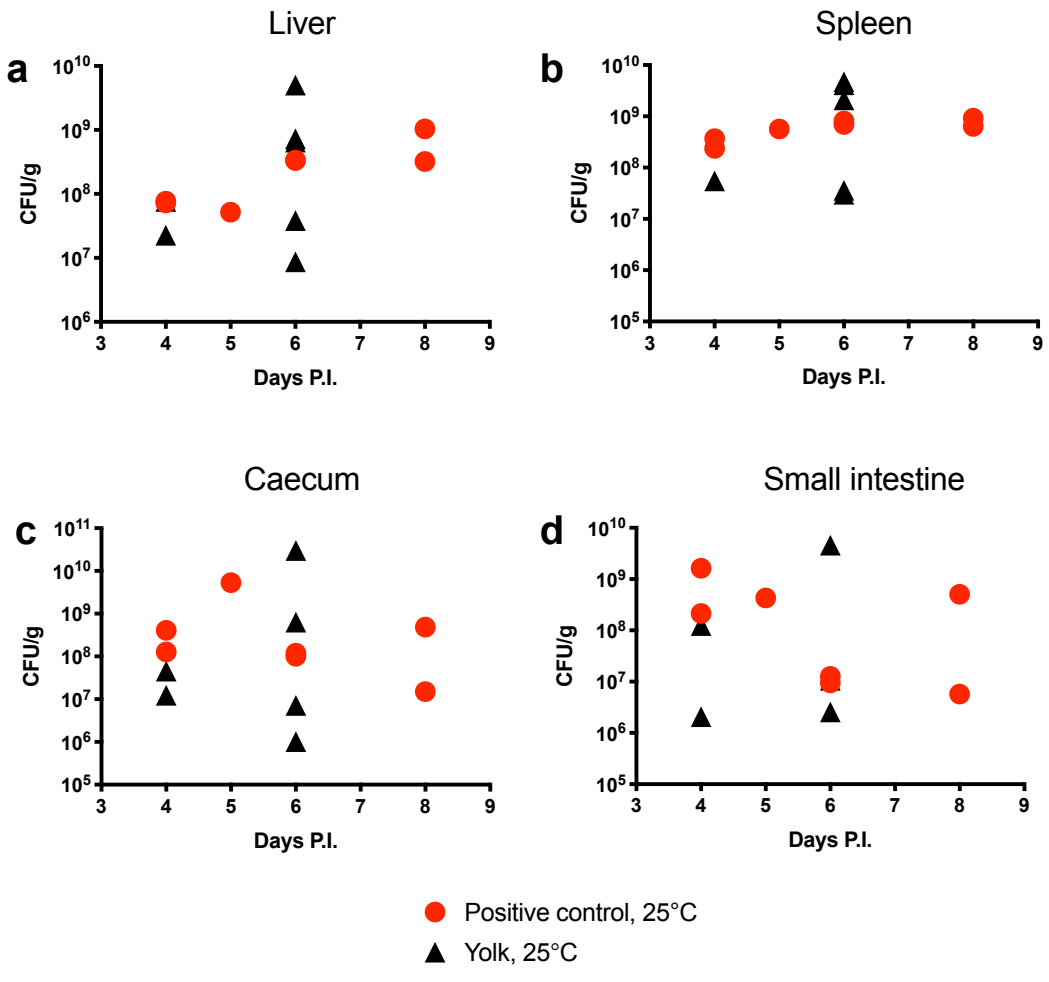

Figure 4. 2 Bacterial colonisation of the liver, spleen, caecum and small intestine of mice culled during the egg trial. Red circles represent positive control stored at 25°C and black triangles represent infected yolk stored at 25°C **a.** bacteria enumerated from the liver. **b.** Bacteria enumerated from the spleen. **c.** Bacteria enumerated from the caecum. **d.** Bacteria enumerated from the small intestine. No statistically significant differences between the organ samples.

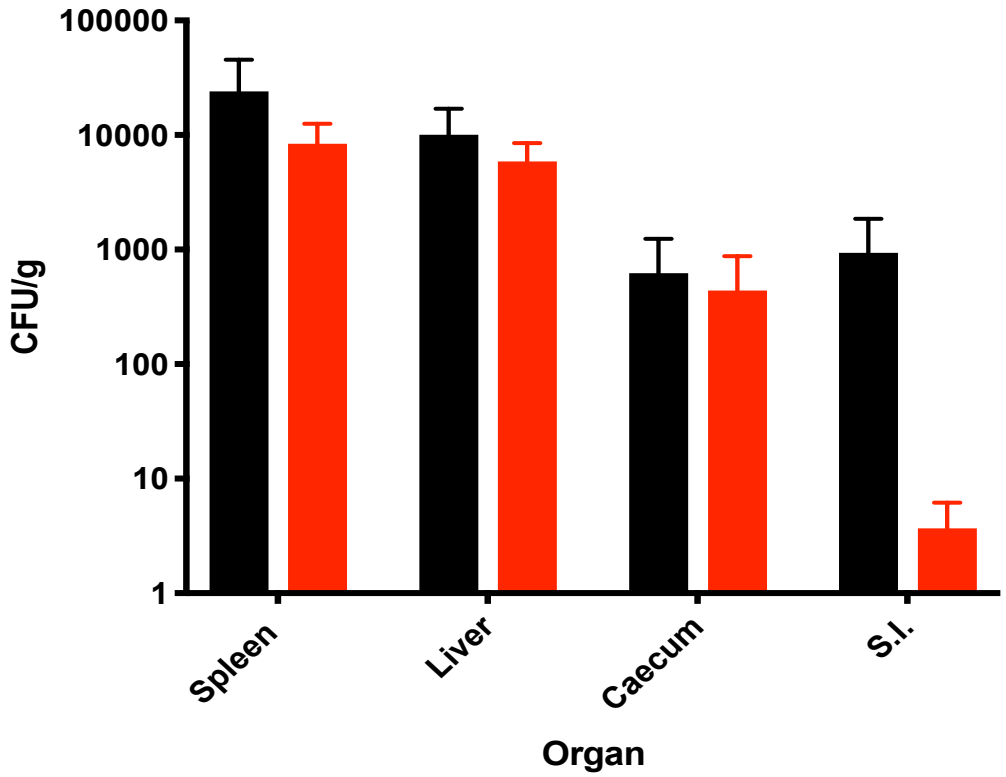

Figure 4. 3 Bacterial colonisation of the liver, spleen, caecum and small intestine of surviving mice inoculated with shell wash stored at 25°C and albumen stored at 25°C. Shell wash stored at 25°C (black column) and albumen stored at 25°C (red column) were culled at the conclusion of the trial. No statistically significant differences between the organ samples.

Average bacterial colonisation of the liver, spleen, caecum and small intestine of all mice with positive organ samples is compared in figure 4.4. There were no statistically significant

differences between the organ samples. It is obvious though, the difference in the average bacterial colonisation of organs culled during the trial and those culled at the conclusion of the trial.

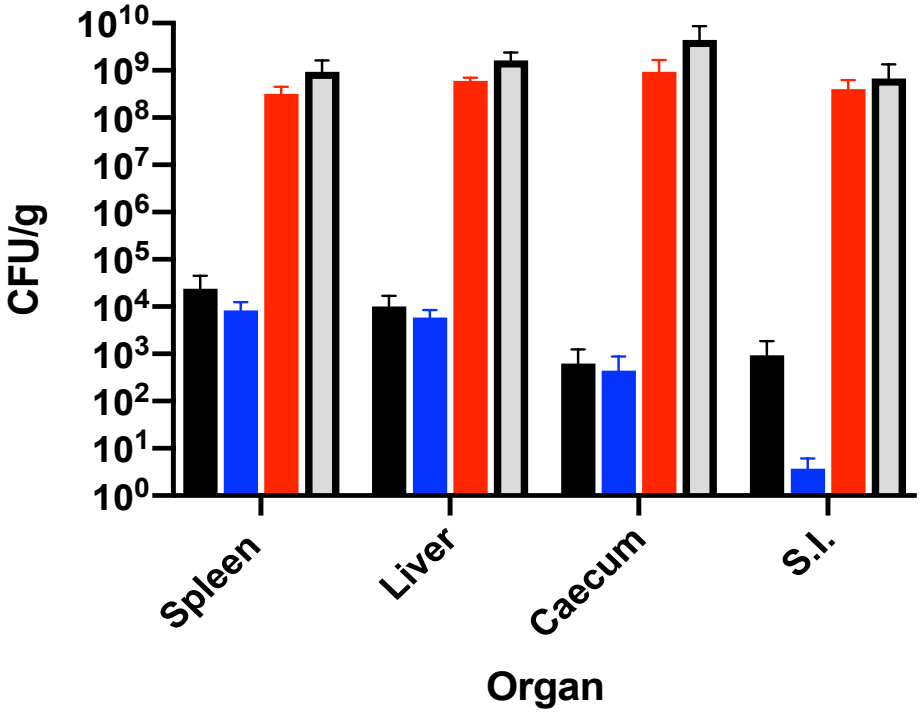

Figure 4. 4 Bacterial colonisation of the liver, spleen, caecum and small intestine of all mice with positive organ samples from the egg trial. Shell wash stored at 25°C (black column), albumen stored at 25°C (blue column), positive control stored at 25°C (red column) and yolk stored at 25°C (grey column). No statistically significant differences between the organ samples.

4.4 Discussion

The egg contents were initially infected with $1x10^3$ CFU/mL, as previous *in vitro* egg trials conducted (see chapter 3) showed the number of bacteria present in the egg increased substantially in the four days post infection. Therefore, in this trial the same dosage was utilised so the number of bacteria in the egg contents was potentially around the 10^6-10^8

CFU/mL when infecting the mice, which is thought to be a typical infective dose of *Salmonella* in food products (Acheson, 2003; Humphrey, 2004). The egg shell infection groups were dipped in a bacterial suspension containing $1x10^6$ CFU/mL, so the mice received an infective dose similar to those fed infected egg contents. No bacteria were recovered from infected eggs stored at 5°C, so it was not possible to accurately determine how much bacteria were present before inoculating them to mice. Two treatment groups were culled during the trial, positive control 25°C and infected yolk 25°C. At least one animal began showing clinical symptoms from both groups by day 2 P.I. All animals were culled from the positive control 25°C group by day 8 P.I. and all animals were culled from the yolk 25°C group by day 6 P.I. This time frame for disease progression was similar to previous *S.* Typhimurium infection work (McWhorter and Chousalkar, 2015). All other groups survived until the end of the trial. There was a statistically significant difference between the animals that were culled during the trial and those that survived until the end ($P \leq 0.01$).

Faecal shedding is commonly used as a tool for tracking disease incidence and progression in *Salmonella* infection trials (Bearson and Bearson, 2009; Lawley et al., 2008; McWhorter and Chousalkar, 2015). During this trial, altogether four treatment groups shed *Salmonella* in their faeces during the trial. Positive control 25°C and yolk 25°C groups shed bacteria from the first faecal sampling (day 3 P.I.) until the entire group was culled. The disease progression was quite similar between the positive control 25°C and yolk 25°C groups. Yolk has been highlighted as a high-risk food product (Denehy et al., 2011; SA Health, 2012). Yolk contains high levels of iron, which is an important requirement for bacterial growth (Humphrey and Whitehead, 1993; Kortman et al., 2012). Growth of *S.* Typhimurium has been shown to increase in response to the presence of iron. Moreover, adhesion of *S.* Typhimurium to epithelial cells increases when the bacteria is pre-incubated in higher iron environments (Kortman et al., 2012). The results of this trial show the yolk is a favourable environment for

bacterial growth and the yolk environment positively affected the bacteria's ability to colonise the digestive tract of the mice, to then be shed in their faeces. Shell wash 25°C and albumen 25°C began shedding at day 15 P.I, but both groups showed no clinical symptoms. If the trial had been allowed to continue on past 21 days P.I., it is possible these animals would have begun to show clinical symptoms of salmonellosis. The delay in shedding could be attributed to the bacteria being initially stressed due to the growth environment (shell wash and albumen). Albumen has been reported as being growth restricting for *Salmonella* because of its antimicrobial properties (Gantois et al., 2009), so this may have contributed to a delay in clinical symptoms the albumen 25°C treatment on the mice. Once the shell wash 25°C group was infected, it was stored at 25°C to emulate sitting at an ambient temperature, similar to sitting on the shelf in a retail store. Being dipped in the bacterial suspension for infection and then being left to dry on the surface of the egg for four days prior to infection, may have been a stressful event for the bacteria which could have contributed to the delay in effects of the shell wash 25°C treatment on the mice. There are also natural barriers on the egg shell to prevent bacterial penetration into the egg. The cuticle of the shell plays an important role, as previous studies have indicated that the extent of cuticle cover is related to the ease of microbial penetration into the internal contents of the egg (De Reu, 2006; Gole, et al., 2014a). When the cuticle matures after the egg is laid, it is a good barrier against water diffusion across the shell and prohibits bacteria passing into the egg by closing the pores (Board, 1982). With the shell pores filled with cuticle, this only leaves the surface of the shell for the bacteria to inhabit. Stress of the surrounding environment (shell wash and albumen in this case) coupled with the exposure to the stressful environment of the digestive tract of the mice (Rychlik and Barrow, 2005) could have caused a delay in disease progression. There were no clinical signs or bacterial shedding in the mice from the treatment groups that were stored at 5°C. It has been demonstrated that storing eggs below 7°C prevents bacteria from replicating (Fajardo et

al., 1995). This is likely the main reason for the 5°C treatment groups not having an effect on the health of the mice.

Mouse models have long been used as an extremely productive model to investigate *S.* Typhimurium pathogenesis (Bearson and Bearson, 2009). The effects of the infection are similar to those experienced by humans (Mathur et al., 2012), with the exception of some gastrointestinal symptoms, as *S.* Typhimurium causes systemic infection in mice (Chaudhuri et al., 2018). Organs commonly examined in *Salmonella* mouse infection trials, include the liver, kidney, spleen, heart, intestines (ileum), caecum and colon (Mathur et al., 2012; Ren et al., 2009). When mice become infected with *S.* Typhimurium, they generally show signs of intestinal inflammation (Lawley et al., 2008). This was the case with all the animals culled during this trial. On visual inspection, the intestines were inflamed. The liver and spleen were enlarged as expected (Chaudhuri et al., 2018). These effects of the disease were not as obvious in the organs of mice culled at the conclusion of the trial. Innate immune response is the first line of defence when a host becomes infected with a pathogen and along with adaptive immunity, they ensure clearance of the pathogen (Mathur et al., 2012). *S.* Typhimurium infection is usually self-limiting in humans, but given the systemic infection it causes in mice, this isn't usually the case (Chaudhuri et al., 2018). *Salmonella* was enumerated from all organs that were sampled from mice culled during the trial (positive control 25°C and yolk 25°C). Similar levels of bacteria were present in the liver and spleen ($5.67x10^8 \pm 8.09x10^8$ cfu/g tissue and $5.2x10^7 \pm 3.35x10^8$ cfu/g tissue respectively) in the mice. When testing the bacterial growth within the egg after 4 days P.I., growth within the egg yolk ranged from $4x10^7 – 3x10^8$ CFU/mL. A previous mouse infection study that involved mice infected via oral gavage with 10^7 and 10^8 CFU of *S*. Typhimurium produced varied results when it came to colonisation of the organs, however colonisation of the spleen and liver of some of the mice did reach $10^6 – 10^7$ CFU/g (Chaudhuri et al., 2018). As stated, yolk is a favourable environment for bacterial

growth (Denehy et al., 2011; SA Health, 2012) prior to infection, which may explain the higher rate of colonisation of organs in this trial. The bacterial colonisation was comparable in the caecum and small intestine in the mice. The average number of bacteria enumerated from the caecum and small intestine was $5.3x10^9 \pm 1.01x10^8$ cfu/g tissue and $4.27x10^8 \pm 9.51x10^6$ cfu/g tissue respectively. A previous study conducted by Ren *et al.* 2009, examining the effects of age of mice and bacterial colonisation of mouse organs found the level of bacteria had increased during the 4 days P.I. in the ileum, a section of the small intestine, in mice infected with 10^6 and 10^8 CFU. Another study demonstrated that colonisation of the caecal contents ranged from $10^6 – 10^{10}$ CFU/g in mice infected with 10^8 CFU of *S.* Typhimurium (Barthel et al., 2003). Both of these studies implemented the use of a streptomycin pre-treated mouse model which emulates many aspects of colitis in an infected human (Barthel et al., 2003). The bacteria present in the organs from the yolk 25°C group behaved similar to the bacteria present in the organs from positive control 25°C group. This again reaffirms the bacteria were not stressed or behaved differently when infecting the host, in comparison to the positive control, highlighting yolk as a positive environment for bacterial growth. There was no statistically significant differences between the bacterial colonisation of each of the organs.

All surviving mice were culled at the conclusion of the trial. *Salmonella* was enumerated from organs from two of the surviving groups, shell wash 25°C and albumen 25°C. As previously discussed, these groups had begun to shed *Salmonella* in their faeces from day 15 P.I. All other groups were negative for bacterial colonisation. Less bacteria colonised organs from the mice culled day 21 P.I. than those culled during the trial. The average number of bacteria enumerated from the organs was $6.3270x10^2 \pm 2.0759x10^2$ CFU/g of tissue (figure 4.3). If these mice continued past day 21 P.I. they may have begun to show clinical signs of salmonellosis and the level of bacterial colonisation of the organs may have increased. There was no statistically significant differences between the bacterial colonisation of each of the organs in

these mice. The treatment group is likely to be the cause of the delayed colonisation/shedding of the bacteria. The albumen is considered a hostile environment for bacterial growth (Tranter and Board, 1984). It has a number of chemical components which provide microbial defence. The alkaline state of the albumen has a negative effect on bacterial growth and increases the chelating (bond of ligand to a metal ion) effect of ovotransferrin, a chemical component of albumen (De Reu, 2006). It is also thought that ovotransferrin and the alkaline nature of the albumen are primarily responsible for the inability of bacteria to grow in the albumen (Tranter and Board, 1984).

In comparing the average bacterial colonisation of the liver, spleen, caecum and small intestine of all mice with positive organ samples. There were no statistically significant differences between the organ samples. It is obvious though, the difference in the average bacterial colonisation of organs culled during the trial ($1.59x10^9 \pm 3.14x10^8$ CFU/g tissue) and those culled at the conclusion of the trial ($6.3270x10^2 \pm 2.0759x10^2$ CFU/g of tissue). As discussed, only mice fed with infected egg components stored at 25℃ exhibited signs of disease or had bacteria isolated from their organs once culled at the conclusion of the trial. All mice fed infected egg components stored at 5℃ were negative for bacterial colonisation. These results highlight the impact that storing eggs at a refrigerated temperature has on the growth and survivability of *Salmonella* in the egg, which in turn has an impact on the incidence of Salmonellosis when eggs are consumed.

4.5 Conclusion

To conclude, mice fed infected yolk stored at 25℃ began to develop disease symptoms 2 days P.I. Bacteria was enumerated from the organs sampled in all animals culled during the trial. The results from the yolk 25℃ group and the positive control 25℃ group were quite similar, indicating the yolk is a favourable environment for bacterial growth. Although it took

some time for the animals infected with egg albumen and shell wash stored at 25℃ to begin showing any indication of bacterial colonisation, they did begin shedding *Salmonella* in their faeces day 15 P.I. and bacteria was enumerated from some of the organs when the animals were culled at the conclusion of the trial, although in lower quantities in comparison to the positive control 25℃ and yolk 25℃ groups. All other groups were negative for *Salmonella* shedding and colonisation of the organs tested. These results indicate an obvious difference in the effect of storage temperature on bacterial growth/survival within the egg prior to infection and the subsequent occurrence of salmonellosis. This also shows the need to store eggs at a refrigerated temperature to reduce the risk of *Salmonella* infection, thereby highlighting the need for prescriptive regulations surrounding the storage eggs at the retail level and increased education for the public on correct storage of eggs.

5.0 Investigating the effects of feeding *Salmonella* Typhimurium infected aioli, stored over different time periods at different storage temperatures, to mice.

5.1 Introduction

Salmonella Typhimurium is an important pathogen to the Australia egg industry, as it is associated with the majority of outbreaks relating to eggs and egg products (Moffat et al., 2016). In Australia, the number of incidents of salmonellosis is second only to campylobacteriosis (Denehy et al., 2011). The egg production chain has many aspects to it, however, the end point of the chain is most often the kitchen, where improper handling, improper storage and cross contamination occurs (Humphrey, 2004). Undercooked or raw egg products have often been shown to be the cause of *Salmonella* outbreaks in humans, when consumed (Denehy et al., 2011; Humphrey, 2004; Stephens et al., 2008). Egg products that have been frequently implicated in human Salmonellosis include desserts such as, tiramisu and mousse, drinks such as protein drinks containing raw eggs and eggnog and sauces and dressings such as mayonnaise and aioli (SA Health, 2012). Aioli is of particular interest because the main ingredient is egg yolk, which has been shown to be a high-risk food ingredient if contaminated (Denehy et al., 2011). If eggs used to make aioli are contaminated with *Salmonella*, these bacteria can also contaminate the end aioli product. Common food safety issues identified in restaurants and kitchens include poor hygiene by not washing hands after handling eggs, inadequate cleaning of the mixer used to make aioli and failure to monitor the temperature of the end product (Stephens et al., 2008), are all concerns in regard to aioli production. With safety recommendations often not being followed while handling aioli, such as not preparing a new batch daily, not discarding at the end of the day, not storing it below 5°C and storing it for more than 24 hours (SA Health, 2012), it is important to examine what effects this mismanagement may have on the food safety of aioli when consumed. Limited research has been conducted using an *in vivo* model to study the effect of storage temperature on bacterial growth/survival in raw egg products such as aioli, and the subsequent effects when consumed. This study aimed to investigate the effects of feeding

Salmonella infected aioli to mice when aioli was stored over time at different storage temperatures. An *in vivo* mouse model was utilised, and it was hypothesised that an ambient storage temperature (25°C) would give rise to favourable conditions for bacterial growth/survival, resulting in clinical symptoms of Salmonellosis in the mice, in comparison to infected aioli stored at a refrigerated temperature (5°C).

5.2 Materials and methods

5.2.1 Bacterial suspension

Bacteria used in this trial was *Salmonella* Typhimurium DT9 (MLVA type 03 15 07 11 550). The isolate was sourced from a layer farm environment during a previous trial conducted within our research group (Gole, et al., 2014a). Bacterial suspensions were prepared as outlined in 2.1.2.

5.2.2 Preparation of aioli

Commercial eggs were purchased from a supermarket for the preparation of aioli. Eggs were sanitised in 75% ethanol and left to air dry. Aioli was then prepared in a food processor using the following recipe; four egg yolks mixed until yolk colour starts to lighten. Mix in four cloves of freshly crushed garlic and one tablespoon of Dijon mustard. Add 30mL of lemon juice and 15mL of white vinegar. Check the pH to ensure it is between 3.5 and 4.0. While mixing, slowly add 300mL of canola oil and then 300mL of extra virgin olive oil. Mix until aioli thickens. Aioli was then weighed and aliquoted into 250mL containers containing 20g of aioli and stored in the refrigerator until inoculation.

5.2.3 Aioli infection

Bacterial suspensions were prepared as per 5.2.1 at specific intervals to ensure suspension was ready to inoculate aioli from the treatment groups. The details regarding the treatment group and specific time points of aioli infection, prior to mouse consumption, are described in table 5.1. Based on results from previous *S.* Typhimurium infected aioli trials conducted within our research group, each treatment group of aioli was inoculated with $1x10^9$ CFU/mL. After inoculation, infected aioli was stored at 5°C or 25°C until mouse infection. Uninfected groups were also stored under the same conditions. Prior to infection of mice, each treatment group was plated on Xylose Lysine Deoxycholate (XLD) agar (Oxoid, Australia) to check for the survival of bacteria.

Table 5. 1 Treatment groups for the infected aioli mouse trial. pH of the aioli used in this study was 4.0. For each treatment group there was an infected and uninfected group stored at 5°C and 25°C. All infected groups and positive control were infected with the same *S.* Typhimurium strain.

Treatment group	**Storage temperature**			
Aioli- 12 hours	5◦C (infected)	25◦C (infected)	5◦C (not infected)	25◦C (not infected)
Aioli- 24 hours	5◦C (infected)	25◦C (infected)	5◦C (not infected)	25◦C (not infected)
Aioli- 36 hours	5◦C (infected)	25◦C (infected)	5◦C (not infected)	25◦C (not infected)
Aioli- 48 hours	5◦C (infected)	25◦C (infected)	5◦C (not infected)	25◦C (not infected)
Aioli- 72 hours	5◦C (infected)	25◦C (infected)	5◦C (not infected)	25◦C (not infected)
Positive control	5◦C (infected)	25◦C (infected)		
Negative control	5◦C (not infected)	25◦C (not infected)		

5.2.4 Positive controls

Prepared as described in 2.1.3.

5.2.5 Mouse infection

Mouse infection occurred as described in 2.5. Mouse weight ranged from 14.0-20.6 grams at infection. In this trial, the seven mice from each treatment group received 100µl of aioli stored for different periods of time at 5°C or 25°C, via oral gavage as per each treatment group (table 5.1).

5.2.6 Sample processing

Faecal samples were processed as outlined in 2.6.1 and organ samples were processed as outlined in 2.6.2.

5.2.7 Statistical analysis

All statistical analyses were performed using GraphPad Prism version 8.0. 2way ANOVA with Tukey's Multiple comparisons test was used to determine any significance in the bacterial colonisation of the organs. P-values less than 0.05 were considered significant.

5.3 Results

5.3.1 Survival

Two groups of mice were culled during the trial; positive control stored at 25°C and positive control stored at 5°C (figure 5.1). One mouse was culled from the infected 12 hour aioli stored at 5°C and two mice were culled from the infected 24 hour aioli stored at 5°C. All other groups survived until the end of the trial. All animals were culled from the positive control stored at 5°C group by day 4 P.I. and all animals were culled from the positive control stored at 25°C group by day 5 P.I. The one mouse culled from the infected 12 hour aioli stored at 5°C and the two mice were culled from the infected 24 hour aioli stored at 5°C were culled day 7 P.I. There was a statistically significant difference between the positive control groups that were culled

during the trial and the rest of the mice (P≤0.01).

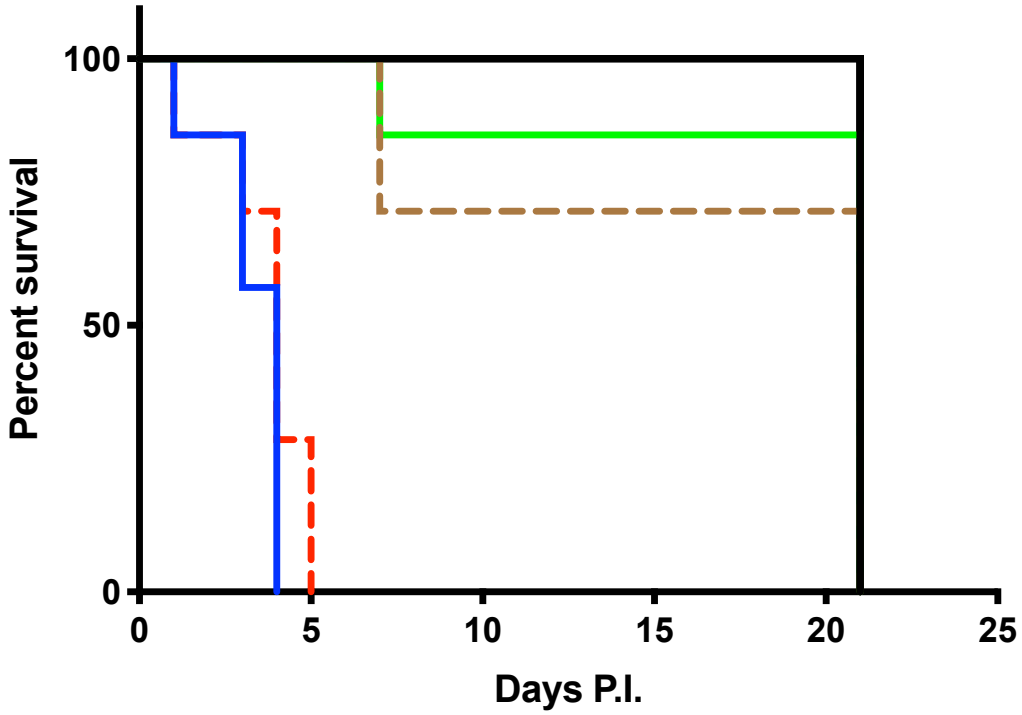

Figure 5. 1 Survival curve for mice fed with aioli infected with *Salmonella* Typhimurium. Two groups were culled during the trial; positive control stored at 25°C (dotted red line) and positive control stored at 5°C (blue line). One mouse was culled from infected 12 hour aioli stored at 5°C (green line) and two mice were culled from the infected 24 hour aioli stored at 5°C (dotted brown line). All other groups (black line) survived until the end of the trial.

5.3.2 Faecal samples

Four treatment groups shed *Salmonella* in their faeces during the trial. Positive control 5°C and positive control 25°C groups shed from the first faecal sampling (day 3 P.I.) until the entire group was culled. Infected aioli 12 hour 5°C and infected aioli 24 hour 5°C began shedding at day 3 P.I., but both groups ceased shedding after mice exhibiting clinical signs were culled from the group. One mouse was culled from the 12 hour 5°C group and two from the 24 hour 5°C group. Shedding in these groups did not occur again during the trial.

5.3.3 Organ samples

Salmonella was enumerated from all organs that were sampled from mice culled during the trial. The average number of bacteria enumerated from the liver was $4.73x10^7 \pm 6.38x10^7$ cfu/g tissue (figure 5.2a) and the average number of bacteria enumerated from the spleen was $2.37x10^8 \pm 1.26x10^8$ cfu/g tissue (figure 5.2b). The number of bacteria that colonised the caecum (figure 5.2c) and small intestine (figure 5.2d) varied fractionally more than the liver and spleen; however, the number of bacteria isolated was similar in the caecum and small intestine in the mice. The average number of bacteria enumerated from the caecum was $3.76x10^8 \pm 3.77x10^5$ cfu/g tissue (figure 5.2c) and the average number of bacteria enumerated from the small intestine was $5.83x10^7 \pm 8.62x10^5$ cfu/g tissue (figure 5.2d). All surviving mice were culled at the conclusion of the trial and none of the organs sampled were positive for *Salmonella*.

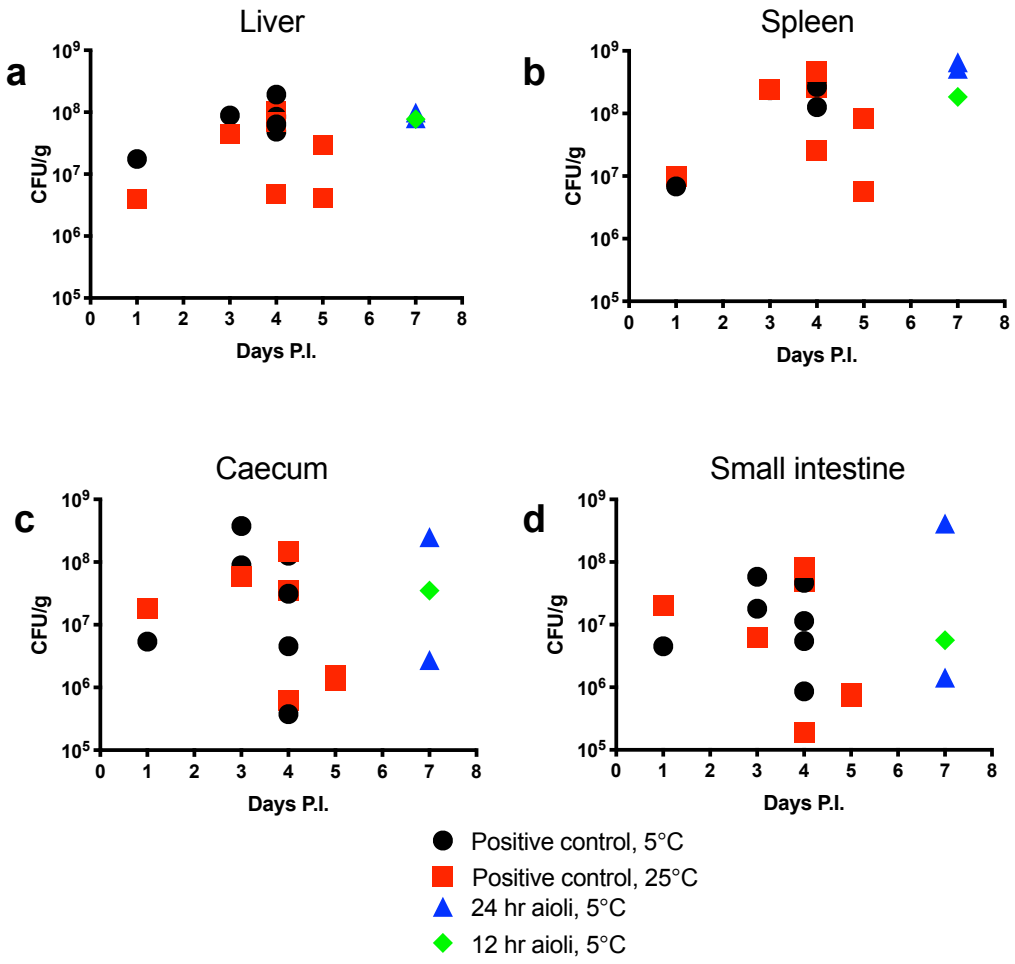

Figure 5. 2 Bacterial colonisation of the liver, spleen, caecum and small intestine of mice culled during the aioli trial. Black circles represent positive control stored at 5°C, red squares represent positive control stored at 25°C, blue triangles represent infected aioli 24 hour stored at 5°C and green diamond represents infected aioli 12 hour stored at 5°C. **a.** Bacteria enumerated from the liver. **b.** Bacteria enumerated from the spleen. **c.** Bacteria enumerated from the caecum. **d.** Bacteria enumerated from the small intestine.

There was a significant effect of organ ($P \leq 0.01$) and treatment ($P \leq 0.01$) on the bacterial colonisation of the organs from the culled mice (figure 5.3). The average bacterial count of the spleen samples from the infected aioli 24 hour 5°C group were significantly different from all liver, caecum and small intestine samples and the spleen samples from the positive control 5°C and positive control 25°C groups. The average bacterial colonisation of the spleen samples from the positive control 5°C group was also significantly different to the small intestine samples from the positive control 5°C and positive control 25°C groups.

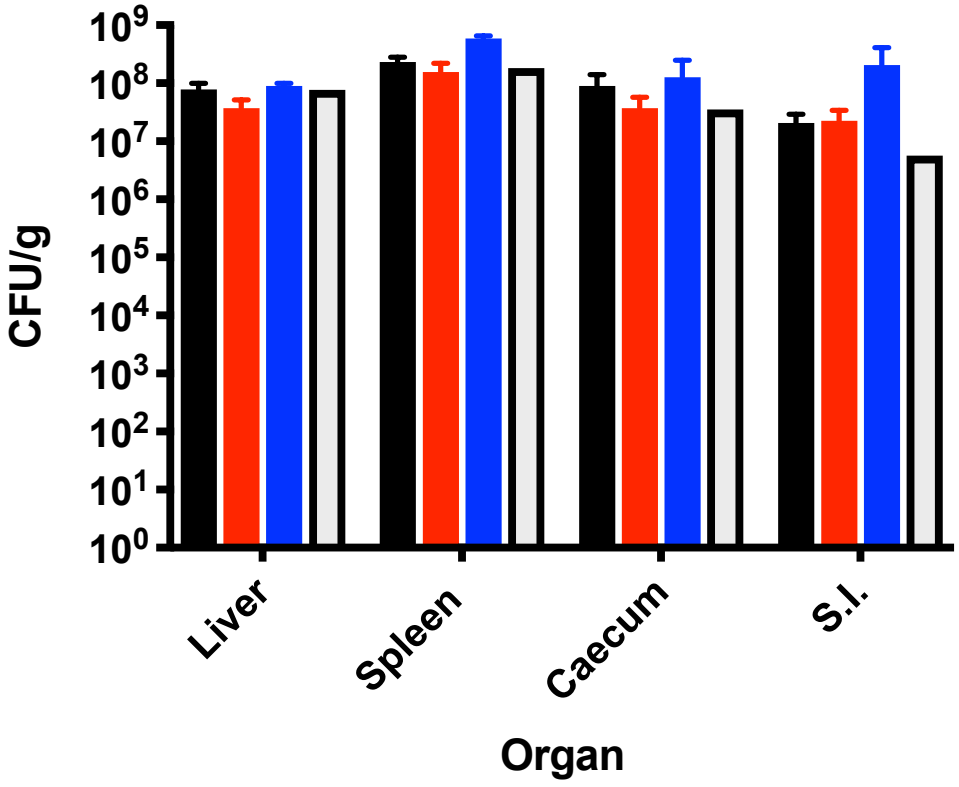

Figure 5. 3 Bacterial colonisation of the liver, spleen, caecum and small intestine of all mice culled during the aioli trial. Positive control stored at 5°C (black column), positive control stored at 25°C (red column), infected aioli 24 hour stored at 5°C (blue column) and infected aioli 12 hour stored at 5°C (grey column). Significant effect of organ (P=≤0.01) and treatment (P=≤0.01).

5.4 Discussion

Given the novelty of this trial, there is limited previous published literature to compare these results to. When mice become infected with *S.* Typhimurium, disease symptoms usually appear within a short period of time. Two groups that were culled during the trial were positive control 25°C and positive control 5°C. Disease symptoms began in both the positive control groups from day 1 P.I. All animals were culled from the positive control stored at 5°C group by day 4 P.I. and all animals were culled from the positive control stored at 25°C group by day 5 P.I. This was not unexpected given these were the positive controls. Only one mouse was culled from the infected 12 hour aioli 5°C group and two mice were culled from the infected 24 hour aioli 5°C group on day 7 P.I. All other groups survived until the end of the trial. These limited results were surprising given the high infection dose in the aioli. Disease progression was slightly slower in these animals in comparison to the control groups and this is likely due to the source of the infection. However, the time frame for disease progression for all the animals culled was similar to previous *S.* Typhimurium infection work (McWhorter and Chousalkar, 2015). The treatment environment (aioli) coupled with the exposure to the stressful environment of the digestive tract of the mice (Rychlik and Barrow, 2005), could have caused a delay in disease progression. These factors were also the likely cause of how few mice succumb to disease during the trial. Clinical symptoms appeared quite rapidly in the affected mice, which is most likely due to the high infection dose in the treatment groups. A

high infection dose of 10^9 CFU/mL was chosen based on results from previous *S.* Typhimurium infected aioli trials conducted within our research group. There was a statistically significant difference between the positive control groups that were culled during the trial and the rest of the mice (P=≤0.01).

Four treatment groups shed *Salmonella* in their faeces during the trial. Positive control 5°C and positive control 25°C groups shed from the first faecal sampling (day 3 P.I.) until the entire group was culled. Given these were the positive controls, these results were to be expected, especially given the high infection dose. Tracking faecal *Salmonella* shedding during an experiment is common practice to monitor disease progress (Bearson and Bearson, 2009; Lawley et al., 2008). Infected aioli 12 hour 5°C and infected aioli 24 hour 5°C began shedding at day 3 P.I. Both groups ceased shedding after the one mouse exhibiting clinical signs was culled from the infected 12 hour aioli 5°C group and two mice exhibiting clinical signs were culled from the infected 24 hour aioli 5°C group. Shedding in these groups did not occur again during the trial. These results although limited, because only three aioli infected mice had to be culled during the trial due to clinical disease, indicated the infection in these mice was similar to what occurred in the positive control mice. After mice become infected with *Salmonella,* the bacteria penetrate the epithelial layer of the intestine, colonises Peyer's patches and mesenteric lymph nodes and spreads to other organs in the body (Barthel et al., 2003; Muotiala and Makela, 1993). Mice can also persistently shed *Salmonella* in their faeces if they are infected. This Persistence is what enables the faecal-oral route of transmission (Swearingen et al., 2012) and cause other mice to become infected. Given that after the mice that were shedding from the infected 12 hour aioli 5°C group and infected 24 hour aioli 5°C group were culled, no other animals shed *Salmonella* in their faeces, we can assume these animals were not infected via the faecal-oral route. Preparation of the aioli is likely to have an effect on subsequent bacterial capabilities if the aioli is contaminated with bacteria. The pH

of the aioli used to infect the mice in this trial was 4.0, and it has been shown that a pH of 4.2 and below has better protective properties against *Salmonella* survival in raw egg products (Keerthirathne et al., 2016). Vinegar and lemon juice are both used when preparing aioli and other raw egg-based sauces such as mayonnaise, as a means of minimising the risk of bacterial growth by acidification (Perales and Garcia, 1990). It is suggested *Salmonella* that has been pre-exposed to acidic conditions, such as found in mayonnaise or in this case aioli, may be better equipped to withstand the subsequent exposure to the acidic conditions of the gut. A high fat environment (in the case of mayonnaise and aioli, vegetable and olive oil are added to the emulsion) is also thought to protect *Salmonella* against the highly stressful environment of the gut (Humphrey, 2004). Studies in *S.* Enteritidis infected mayonnaise have shown that the bactericidal effects of acidification is enhanced at temperatures over 25°C, such as storing the mayonnaise at an ambient temperature, whereas storing the mayonnaise below 5°C allowed the bacteria to persist for longer (Lock and Board, 1994; Perales and Garcia, 1990; Zhu et al., 2012). These results are consistent with the infected mice culled in this trial. No mice from the 25°C stored aioli groups were culled. Only mice from 5°C stored aioli groups had to be culled due to clinical disease.

It is common for mouse models to be utilised to investigate *S.* Typhimurium pathogenesis (Bearson and Bearson, 2009). *S.* Typhimurium causes systemic infection in mice (Chaudhuri et al., 2018) therefore liver, kidney, spleen, heart, intestines (ileum), caecum and colon are commonly examined in *Salmonella* mouse infection trials (Mathur et al., 2012; Ren et al., 2009). Intestinal inflammation was observed in the mice that were culled due to exhibiting clinical signs, which is a common occurrence when mice become infected with *S.* Typhimurium (Lawley et al., 2008). The liver and spleen were also enlarged as expected (Chaudhuri et al., 2018). *S.* Typhimurium infection is usually self-limiting in humans, but given the systemic infection it causes in mice, this isn't usually the case (Chaudhuri et al., 2018).

Salmonella was enumerated from all organs that were sampled from mice culled during the trial. Similar levels of bacteria were present in the liver and spleen in the mice. The average number of bacteria enumerated from the liver and spleen was $4.73x10^7 \pm 6.38x10^7$ cfu/g tissue and $2.37x10^8 \pm 1.26x10^8$ cfu/g tissue respectively. Chaudhuri et al. 2018 conducted a mouse study where mice were orally infected with 10^7 and 10^8 CFU of *S*. Typhimurium. Bacterial colonisation of the organs varied, however colonisation of the spleen and liver of some of the mice did reach $10^6 – 10^7$ CFU/g (Chaudhuri et al., 2018). These results are comparable to this study, although the slightly higher colonisation of the organs may be attributed to the higher infection dose in this study. The number of bacteria that colonised the caecum and small intestine varied more than the liver and spleen; however, the average number of bacteria enumerated was similar in the caecum and small intestine in the mice. The average number of bacteria enumerated from the caecum and small intestine was $3.76x10^8 \pm 3.77x10^5$ cfu/g tissue and $5.83x10^7 \pm 8.62x10^5$ cfu/g tissue respectively. Previous study has demonstrated the level of bacteria increases during the 4 days P.I. in the ileum, a section of the small intestine, in mice infected with 10^6 and 10^8 CFU (Ren et al., 2009). Barthel et al. 2003 demonstrated that colonisation of the caecal contents ranged from $10^6 – 10^{10}$ CFU/g in mice infected with 10^8 CFU of *S.* Typhimurium. The bacteria present in the organs from the infected aioli 24 hour 5°C and infected aioli 12 hour 5°C behaved similar to the bacteria present in the organs from positive control 5°C group, indicating the infection source had little effect on the bacteria's pathogenicity in those animals. All surviving mice were culled at the conclusion of the trial and none of the organs sampled were found to be positive for the presence of *Salmonella*. There was a significant effect of organ ($P=\leq 0.01$) and treatment ($P=\leq 0.01$) on the bacterial colonisation of the organs from the culled mice. The average bacterial colonisation of the spleen samples from the infected aioli 24 hour 5°C group were significantly different from all liver, caecum and small intestine samples and the spleen

samples from the positive control 5°C and positive control 25°C groups. The average bacterial colonisation of the spleen samples from the positive control 5°C group was also significantly different was the small intestine samples from the positive control 5°C and positive control 25°C groups. Even though the average bacterial colonisation of the spleen samples from the infected aioli 24 hour 5°C group were significantly different from almost all other samples, there were only two mice culled from the group during the trial. This may have skewed the result, which may have been different if more mice were culled. Given the limited results of this trial, further research needs to be conducted focusing on the short-term storage of aioli to confirm the best method of storage prior to service to consumers.

5.5 Conclusion

To conclude, no infection occurred in mice fed infected aioli stored at 25℃, whereas some mice from the infected aioli 24 hour 5°C and 12 hour 5°C groups did succumb to salmonellosis. The bacteria enumerated from the organs of these mice was comparable to the number of bacteria enumerated from the control mice that were culled. The bacteria present in the organs from the infected aioli 24 hour 5°C and infected aioli 12 hour 5°C behaved similar to the bacteria present in the organs from positive control 5°C group, indicating the infection source had little effect on the bacteria's pathogenicity in those animals. Given how few mice from infected aioli groups were affected by the *Salmonella,* it would be beneficial to conduct further research to determine if the results were accurate. Based on these results and others examining *S.* Enteritidis infection in mayonnaise (Lock and Board, 1994; Perales and Garcia, 1990), it may be best practice to prepare aioli using acidifying agents (lemon juice and vinegar) and store it at ambient temperature for up to 24 hours prior to serving. However, it must be kept in mind that there are other food spoiling bacteria that may be more active at an ambient temperature. These bacteria could reduce the shelf life of aioli.

6.0 General discussion

6.1 Major findings

Salmonella is a major cause of foodborne gastroenteritis world-wide (Ford et al., 2016). In Australia, *S.* Typhimurium is of particular importance to the Australian egg industry and subsequent food chain. This is because it is the most commonly identified serovar during human outbreaks of salmonellosis (Ford et al., 2016) and 90% of identified outbreaks relating to eggs in Australia are caused by S. Typhimurium (Moffatt et al., 2016). *S.* Typhimurium can survive in a broad range of environments due to adaptations and stress response mechanisms. This makes it difficult to determine the exact factors that enable it to survive in diverse conditions from farm to fork (Chaudhuri et al., 2013; Foley et al., 2013). *Salmonella* is able to survive and grow within eggs and one factor thought to play an important role in bacterial growth within infected eggs is, storage temperature. Storing eggs and egg products below 7°C can minimise bacterial growth within the egg therefore reducing the risk of Salmonellosis (Fajardo et al., 1995; Schoeni et al., 1995). Given in Australia, there are no strict regulations on the storage temperature of eggs once they leave the production farm, the main focus of this thesis was to investigate the effects of storage temperature on eggs and egg products infected with *S.* Typhimurium. In chapter 3 though, *S.* Hessarek was also included due to the recent increase in the occurrence of *S.* Hessarek in egg related outbreaks leading to concerns it is becoming an increased risk to public health in South Australia. It is unknown whether it has the same survival and egg penetration ability as *S.* Typhimurium, therefore this was investigated. In Chapter 4 the trial aimed to examine the effect of storage temperature (ambient temperature (25°C) versus refrigerated temperature (5°C) of eggs infected with *S.* Typhimurium when consumed by mice. Chapter 5 investigated the effects of feeding *Salmonella* Typhimurium infected aioli to mice. The aioli was stored for a period of time at different temperatures.

Chapter 3 utilised *in vitro* models to determine whether *S*. Typhimurium and *S*. Hessarek have

similar ability to penetrate the eggshell, what effects storage temperature may have on growth of *S*. Typhimurium and *S*. Hessarek within the egg and any differences in growth between *S.* Typhimurium and *S.* Hessarek in eggs at different temperatures. Neither *S.* Typhimurium nor *S*. Hessarek were able to penetrate from the egg shell pores into the egg contents at either 5°C (refrigerated temperature) or 25°C (ambient temperature) at any point during this study. However, eggs infected immediately after lay and stored at 25°C were positive for both strains of *Salmonella* in their egg shell pores after direct plating. Eggs infected and stored at 5°C were found to be positive only after further enrichment of the egg shell pore samples. These results highlight the inability of the bacteria to penetrate into the egg contents after 72 hours of infection, most likely due to other mechanical barriers eggs possess, such as shell membrane (Lifshitz et al., 1964). Lifshitz et al., 1964 also show ambient storage temperatures can facilitate bacterial penetration into the shell pores leaving the bacteria in a ready state for growth. When stored at 5°C the bacteria required an additional enrichment to induce a state for growth. This highlights the effect storage temperature has on minimising bacterial growth in eggs. Refrigeration appears to aid in preventing bacteria from penetrating into the egg shell pores. When eggs were inoculated and stored at 5°C, there was no growth of bacteria after direct plating of egg contents, whereas there was a significant increase in bacterial growth in eggs stored at 25°C. These results are in agreement with the thought that storing eggs below 7°C prevents bacteria from growing (Fajardo et al., 1995). After further enrichment, some 5°C stored eggs were positive for *Salmonella.* This means bacteria were unable to multiply at 5°C but survived in both yolk and albumen. While the proportion of positive albumen samples were quite low, the proportion of positive yolk samples from yolk infected eggs was much higher. Hence, the lower storage temperature affected the bacteria's ability to multiply, but the nutrient rich environment of the yolk aided the bacteria's survival. The antimicrobial properties of the albumen plus low storage temperature minimised the number of positive albumen samples when stored at 5°C.

Regardless, these results demonstrate the obvious differences in bacterial growth in eggs stored at ambient temperature in comparison to refrigerated temperature. *Salmonella* in infected eggs grows readily in unrefrigerated temperatures, whereas refrigeration limits *Salmonella* survival and growth. These results highlight the importance of regulating storage temperature after egg collection and in the supply chain. If an egg is infected and stored at an ambient temperature in a retail store, bacterial growth in the egg will likely occur. Implementing regulation on refrigeration of eggs at all stages of the supply chain, would help to minimise the risk of bacterial growth in eggs. While *Salmonella* can grow readily in infected eggs stored at ambient temperatures, it is important to examine what occurs when infected eggs are consumed and determine whether or not the storage temperature of infected eggs effects the bacteria's ability to induce salmonellosis *in vivo.* In Australia, eggs are stored below 15°C on farms before grading. This particular temperature was not included in this study, hence further studies are required to investigate the effect of farm relevant egg storage temperature on the penetration ability of S. Typhimurium.

In vivo trials commonly utilise mouse models for *Salmonella* research to observe disease progression, as the effects of infection are similar to those experienced by humans (Mathur et al., 2012). In chapter 4, mice were fed infected egg components, and some began to show clinical symptoms by day 2 P.I. Besides the positive control 25°C groups, the only other group that showed severe clinical disease during the trial was the group of mice fed with yolk stored at 25°C. These groups also shed *Salmonella* in their faeces from the first faecal sampling (day 3 P.I.) until they were culled. This indicates the bacteria was able to pass through to the gastrointestinal tract, after surviving the harsh environment of the stomach. Faecal shedding is commonly used as a tool for tracking disease incidence and progression in *Salmonella* infection trials (Bearson and Bearson, 2009; Lawley et al., 2008; McWhorter and Chousalkar,

2015). All results from the yolk 25°C group were quite similar to those obtained from the positive control 25°C group. Yolk has been highlighted as a high-risk food product (Denehy et al., 2011; SA Health, 2012). These results show the yolk is a favourable environment for bacterial growth and the bacteria was able to colonise the digestive tract of the mice, to then be shed in their faeces. Although delayed, mice from the treatment groups, shell wash 25°C and albumen 25°C began shedding at day 15 P.I, but both groups showed no clinical symptoms. The delay in shedding could be attributed to the bacteria being initially stressed due to the treatment environment such as egg shell surface and albumen. The treatment group environment coupled with exposure to the stressful environment of the digestive tract of the mice (Rychlik and Barrow, 2005) could have caused a delay in disease progression. There were no clinical signs or bacterial shedding in the mice from the treatment groups that were stored at 5°C. Similarly, once culled, there were no bacteria isolated from the organs from the 5°C groups. These results are not unexpected given the results of the egg *in vitro* trials in chapter 3, which demonstrated that infected eggs stored at 5°C were not positive for *Salmonella* after direct plating and required additional enrichment to recover any positive samples. The refrigerated temperature affects the bacteria's ability to survive in the egg contents (Fajardo et al., 1995), hence the lack of clinical disease occurrence in the infected mice from the 5°C storage temperature groups. Of the mice culled at the conclusion of the trial, *Salmonella* was enumerated from organs from two of the surviving groups, shell wash 25°C and albumen 25°C. The treatment group is likely to be the cause of the delayed colonisation/shedding of the bacteria, in particular the albumen given it is considered a hostile environment for bacterial growth (Tranter and Board, 1984). The results highlight the effects of storage temperature on bacterial growth/survival within the egg prior to infection and the subsequent occurrence of salmonellosis. As no *Salmonella* was enumerated from the organs of the mice infected with egg components stored at 5°C, it suggests that storing eggs at a refrigerated temperature prior to consumption reduces the risk of salmonellosis. It's not

only whole eggs which pose a food safety threat to consumers if infected. Raw egg products are often the cause of *Salmonella* outbreaks (Denehy et al., 2011; Humphrey, 2004; Stephens et al., 2008). Aioli is of particular interest, as the main ingredient is egg yolk, and as shown in chapters 2 and 3, *Salmonella* grows readily in the yolk environment. If eggs used to make aioli have been contaminated with *Salmonella*, these bacteria will also contaminate the end aioli product.

In chapter 5 the mouse model was again utilised, to look at the effects of storage time and storage temperature on aioli infected with *S.* Typhimurium when consumed. Besides the two positive control groups, one mouse was culled from the infected 12 hour aioli 5°C group and two mice were culled from the infected 24 hour aioli 5°C group. Faecal shedding was observed in these groups too until the animals were culled and the infected aioli 12 hour 5°C and infected aioli 24 hour 5°C groups ceased shedding after mice exhibiting clinical signs were culled from the group. Given only three aioli infected mice were culled during the trial due to clinical disease, the results are limited, but the infection in these mice was similar to what occurred in the positive control mice. This indicates the infection source had little effect on the bacteria's pathogenicity in those animals. All surviving mice were culled at the conclusion of the trial. No mice fed infected aioli stored at 25°C became ill and no *Salmonella* was enumerated from organ samples when the mice were culled. Acidification of raw egg products is commonly implemented to minimise the risk of bacterial growth (Perales and Garcia, 1990). Previous studies examining *S.* Enteritidis infected mayonnaise have shown that the bactericidal effects of acidification are enhanced at temperatures over 25°C, whereas storing the mayonnaise below 5°C allowed the bacteria to persist for longer (Lock and Board, 1994; Perales and Garcia, 1990; Zhu et al., 2012). Although only three mice fed infected aioli were culled during the trial due to exhibiting clinical symptoms of disease, results indicate

that infected aioli stored at refrigerated temperatures short term (12-24 hours), may pose food safety implications. These results are consistent with the results of this trial, in that no mice fed with infected aioli stored at 25°C exhibited clinical disease symptoms and infected mice that were culled were fed infected aioli stored at 5°C. The refrigerated temperature appears to have aided the bacteria to survive for a period of time in the aioli and such causing the mice to suffer from salmonellosis. Further research is required to determine the possible cellular changes and virulence of *Salmonella* Typhimurium in aioli stored at 5°C and 25°C.

6.2 Conclusions and future work

Overall, the results from the various experiments highlight the importance of storage temperature in controlling the risk of *Salmonella* in eggs and egg products. Storing eggs at 5℃ decreases the replication of *Salmonella* in the eggs in comparison to eggs stored at 25℃. At 25℃ bacterial growth increased rapidly in the egg. Similarly, mice fed infected egg components (particularly the infected yolk) stored at 25℃ experienced clinical disease and colonisation of the organs, whereas those fed with egg components stored at 5℃ did not exhibit clinical symptoms. This demonstrates the need to store eggs at a refrigerated temperature to reduce the risk of *Salmonella* infection in humans, highlighting the need for prescriptive regulations surrounding egg storage at the retail level and increased education for the public on correct storage of eggs. Acidification is a common practice implemented to minimise the risk of bacterial growth in raw egg products. Given the likelihood of the bactericidal effects of acidification being enhanced at temperatures over 25°C, best practice would be to prepare aioli using acidifying agents (lemon juice and vinegar) and store it at ambient temperature for up to 24 hours prior to serving. However, it is necessary to conduct further research or replicate the trial to determine if the results of this aioli trial are consistent. Further work may also be to examine the outcomes of different infection doses in whole eggs

and the degree of bacterial growth. Follow on work would then be to investigate different infectious doses in eggs during *in vivo* trials. Similar trials could also be conducted examining other important strains of *Salmonella* in the egg industry such as, *Salmonella* Enteritidis. Bacterial growth in eggs stored at 15°C also needs to be investigated, as this is the maximum temperature eggs are stored on farm. In this study, mice infection trials were conducted with *S*. Typhimurium only. The growth of *S.* Hessarek in eggs was comparable to *S*. Typhimurium. Hence, it could be deduced that after feeding mice with the *S*. Hessarek infected egg contents, the disease progression would be similar. However, further investigation is necessary.

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