Potential control mechanisms for *Salmonella enterica* serovar Typhimurium contamination of eggs and raw egg products

By

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Abstract

Salmonellosis is a foodborne gastrointestinal illness caused by *Salmonella*, with outbreaks in Australia commonly linked to eggs and raw egg products. Globally, *S.* Enteritidis is the primary cause of salmonellosis outbreaks linked to eggs and raw egg products; however, in Australia *S.* Typhimurium is the strain most frequently identified. Previous research has focused on the control of *S.* Enteritidis; however, there is a need for more research investigating potential control mechanisms for *S.* Typhimurium to inform Australian food safety guidelines and public health interventions. This research project focused on, the risk factors associated with keeping chickens in the backyards and the role this may play in the spread of salmonellosis and exploring control mechanisms for *S.* Typhimurium in eggs and raw egg products.

Keeping poultry in domestic backyards is increasing in popularity. This could potentially increase the spread of zoonotic bacterial species such as *Salmonella*, *Campylobacter* and *Shigella* as these bacterial species are often isolated from poultry and poultry products. In this study, chicken faeces were collected from 82 backyards and 15% were either positive for *Salmonella* or *Campylobacter* but none were positive for *Shigella*. Following the questionnaires collected from the backyard chicken owners, washing dirt off eggs, storing the eggs at room temperature, consumption of raw egg products, spreading poultry faeces in the backyard and having flocks with more than 10 birds were identified as risky behavioural patterns. This demonstrates the need for better education as these risk factors could increase the likelihood of foodborne diseases linked to pathogens present in the backyard chicken flocks.

Temperature and pH are commonly used to control replication and survival of pathogenic bacteria in food. The Food Standards Australia New Zealand (FSANZ) recommends the preparation, processing, transporting and storage of raw egg products such as mayonnaise to be conducted under a temperature less than 5°C. The guidelines further state that the pH of the mayonnaise should be adjusted to less than 4.6 or 4.2. Despite these guidelines salmonellosis outbreaks have increased during the past decade in Australia. This was the first study to focus on exploring the survival of *S.* Typhimurium in raw egg mayonnaise, with the aim of informing Australian food safety guidelines.

The results of this research contradict the current guidelines, as a higher survival rate of *S.* Typhimurium was observed at 4°C. The findings from this research suggested that storing mayonnaise (pH adjusted to 4.2) at room temperature for at least 24 h prior to consumption could reduce the incidence of salmonellosis linked to eggs and raw egg products in Australia.

In order to reduce the incidence of salmonellosis outbreaks related to eggs and raw egg products, the current food safety guidelines should be revised and modified according to the novel scientific data. The guidelines involving the preparation of raw egg products should be amended to inform the environmental health officers (EHOs), chefs and food handlers to leave the pH adjusted (pH 4.2) mayonnaise at room temperature for at least 24 h before consumption. Adaptation of the developed decontamination method prior to the preparation of raw egg products will reduce cross contamination in the kitchen and further ensure food safety.

Foodborne illnesses often occur due to inappropriate food handling. Competent policy modification and effective policy implementation along with educational programmes for the general public, EHOs, chefs and food handlers could reduce the public health burden of gastrointestinal diseases linked to eggs and raw egg products in Australia.

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Signed: Thilini Keerthirathne

Date: 04/03/2020

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Publications

Keerthirathne, T.P., Ross, K., Fallowfield, H. and Whiley, H. (2016). A review of temperature, pH, and other factors that influence the survival of *Salmonella* in mayonnaise and other raw egg products. *Pathogens*, *5*(4), p.63. https://doi.org/10.3390/pathogens5040063

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CHAPTER 1. Introduction

1.1 *Salmonella* species

Salmonella species are ubiquitous (WHO, 2018) facultative, Gram-negative, rod-shaped bacteria which belong to the Enterobacteriaceae family (D'Aoust and Maurer, 2007). The genus *Salmonella* consists of zoonotic bacterium species and is normally transmitted through contaminated food or water (Uzzau et al., 2000). The *Salmonella* genus contains two species, *S. enterica* and *S. bongori* (Brenner et al., 2000). The species *S. enterica* is divided into 6 subspecies, *enterica* (subspecies I), *salamae* (subspecies II), *arizonae* (subspecies IIIa), *diarizonae* (subspecies IIIb), *houtenae* (subspecies IV), and *indica* (subspecies VI) (Brenner et al., 2000). The sub species *enterica* is further divided into *S. enterica* serovar Choleraesuis, *S. enterica* serovar Enteritidis, *S. enterica* serovar Typhimurium, *S. enterica* serovar Paratyphi and *S. enterica* serovar Typhi (Ryan et al., 2017).

The serovars of *Salmonella* are also broadly classified into typhoidal and non-typhoidal *Salmonella* (NTS) (**Figure 1.1**). NTS serovars include Typhimurium and Enteritidis which are pathogens with a wide range of host specificity, whereas *S. enterica* serovars, Typhi, Sendai, and Para typhi A, B, and C (typhoidal *Salmonella* serovars) are highly adapted to the humans as a host and are the causative agents of enteric fever (Gal-Mor et al., 2014). There are more than 2,500 *Salmonella* species identified, although it is estimated that only 100 are pathogenic to humans (CDC, 2017).
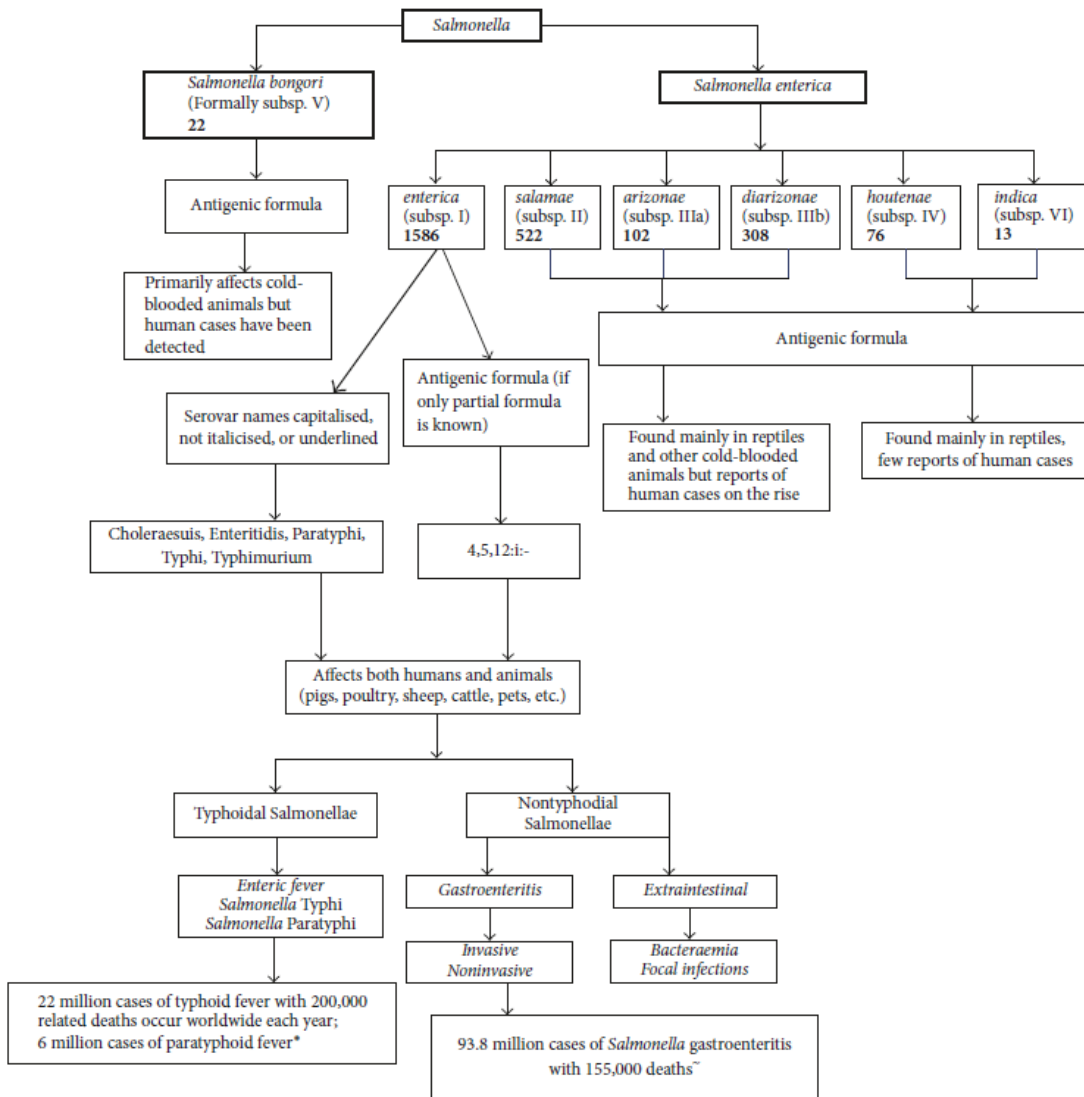
Figure 1.1: Nomenclature of the _Salmonella_ genus (Ryan et al., 2017)

1.2 Foodborne illnesses

Foodborne diseases are one of the most serious health problems affecting public health and development (Macholz, 1985). In the US the annual cost spent for foodborne illnesses is between US$ 6.5-34.9 billion (Buzby and Roberts, 1997) and it is estimated that foodborne illnesses cost around AUD$ 1.2 billion in Australia each year (Angulo et al., 2008).

Salmonellosis is one of the commonly recorded foodborne illnesses caused by non-typhoidal *Salmonella* species (Acheson and Hohmann, 2001). An infected person will develop diarrhoea, fever and vomiting (Rodríguez et al., 2017). In the United States it is estimated that there are 1.4 million illnesses and 600 deaths due to salmonellosis each year. The most common serotypes isolated from human specimens are *S.* Typhimurium and *S.* Enteritidis (Mead et al., 1999). In 2017, 91,662 human cases of salmonellosis were reported in the European Union (EU) (EFSA, 2019). In Europe from 2000 to 2002, the main cause of salmonellosis was *S.* Enteritidis, although cases of *S.* Typhimurium, *S.* Hadar, *S.* Virchow, and *S.* Infantis were also reported (Palli, 2011).

1.2.1 Salmonellosis in Australia

Currently *Salmonella* spp. is one of the most commonly reported causes of community gastroenteritis in Australia (Sinclair et al., 2002). According to the Australian National Notifiable Disease Surveillance System, the incidence of salmonellosis has been significantly increasing since the 1990 (**Figure 1.2**) with the number of reported cases doubling from 6,992 in 2008 to 14,158 in 2018 (**Table 1.1**) (NNDSS, 2019). During the period of 3 months from October to December in 2015, 32% of the foodborne illnesses were caused by *S.* Typhimurium. Similarly, in 2014 during the same period 41% of the gastroenteritis infections were caused due to *S.* Typhimurium (OzFoodNet, 2019). The highest number of *S.* Typhimurium cases (664 cases) were reported in South Australia (OzFoodNet, 2015) (**Figure 1.3**).

Even though food items like peanuts, beef, pork and chicken have been connected with the outbreaks of salmonellosis, eggs and food products prepared using eggs are the most frequent foods that are associated with the disease (De Oliveira Elias et al., 2015). Eggs and egg-based food products play an important role in the number of reported salmonellosis outbreaks. During the 15 years from 2001 to 2016, 50% of the reported salmonellosis outbreaks were due to food products containing raw or under cooked eggs and 95% of these outbreaks were due to the contamination of *S.* Typhimurium (Ford et al., 2018).
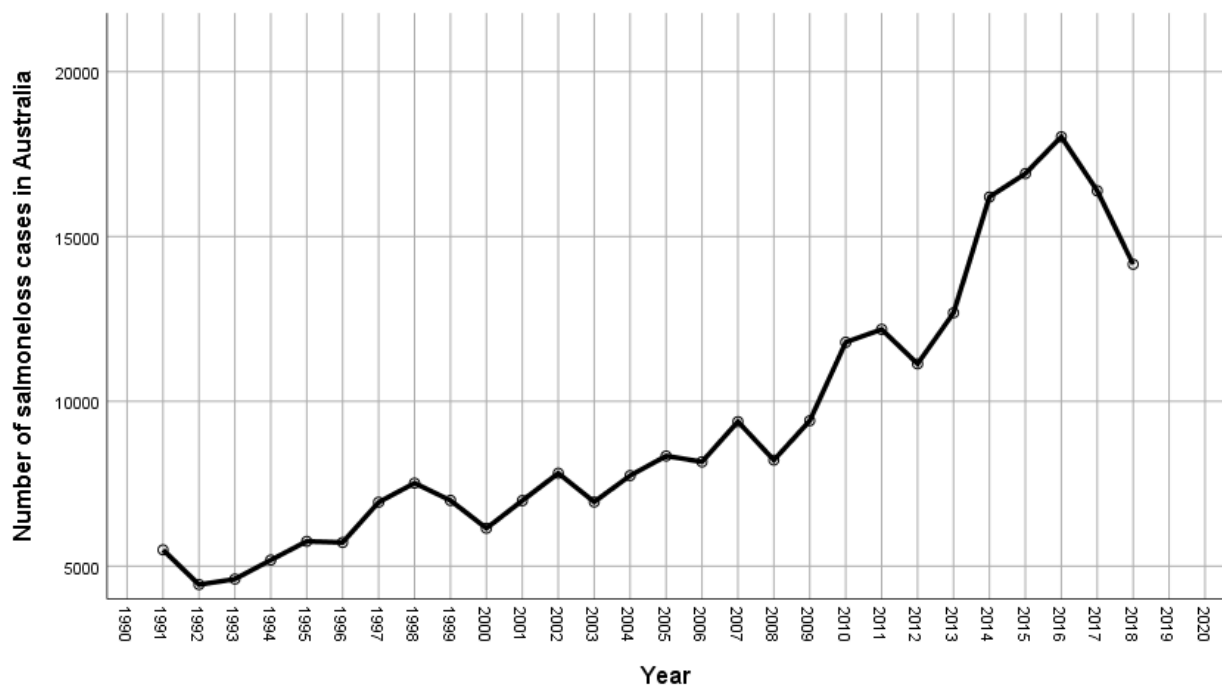
Figure 1.2: The number of salmonellosis cases recorded from 1990 to 2018 in Australia (NNDSS, 2019). Rise in the number of salmonellosis cases in 2016 were due to the increased number of *S*. Saintpaul and *S*. Bareilly which was not commonly seen during previous years.

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Table 1.1: Number of notified cases of salmonellosis per 100,000 population in Australia in 2008 and 2018 according to the National Notifiable Disease Surveillance System (NNDSS, 2019).

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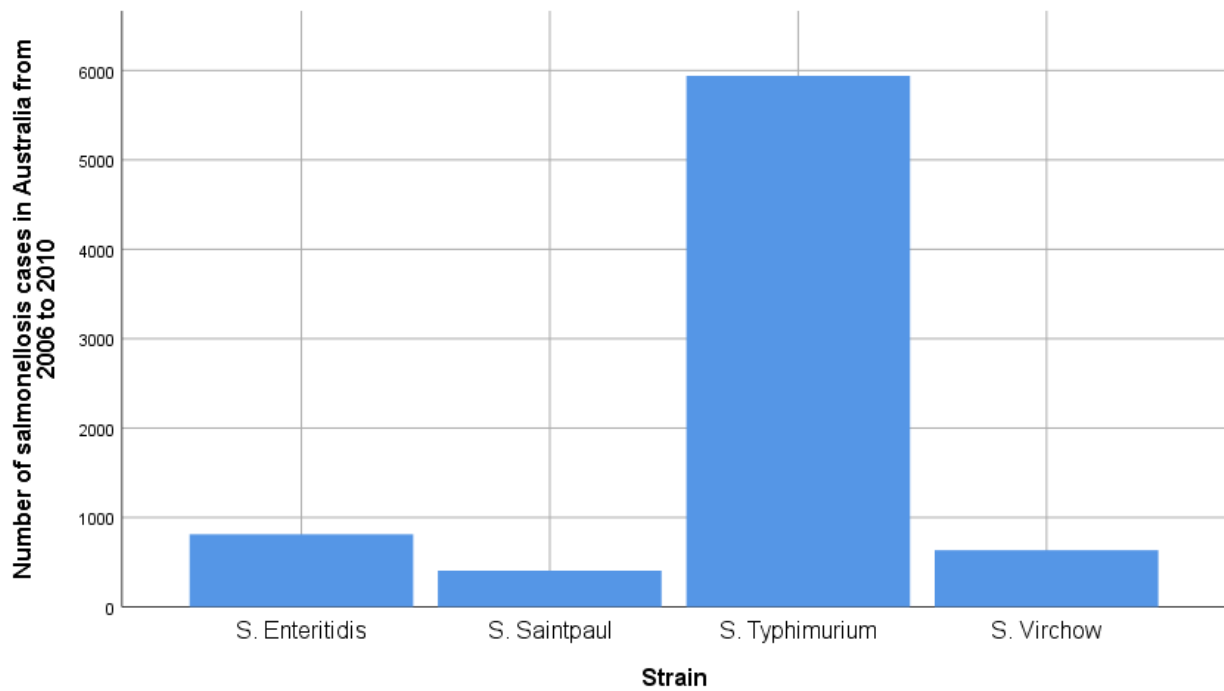
Figure 1.3: *S.* **Typhimurium was predominantly reported in Australia during the years 2006 to 2011 (OzFoodNet, 2015)**

1.3 Salmonellosis and poultry

Poultry play an important role in spreading zoonotic diseases such as salmonellosis (Antunes et al., 2003). Poultry can acquire *Salmonella* infection through direct contact with the contaminated faeces of reptiles (lizards and snakes), rodents (Meerburg and Kijlstra, 2007), cockroaches (Kopanic Jr et al., 1994), litter beetles (Skov et al., 2004), other wild and domestic birds (Hernandez et al., 2003), or through indirect contact with contaminated environmental sources. Poultry may contain *Salmonella* in their feathers, feet and beaks (CDC, 2019) and can shed *Salmonella* through their faeces contaminating the environment (Van Immerseel et al., 2005). According to Im et al. (2015) 50.7% of surveyed poultry flocks were positive for *Salmonella* in Korean layer farms. Another study conducted in Canadian layer farms found that 52.9% of the flocks from layer farms were infected with *Salmonella* (Poppe et al., 1991). Soil, shoes, hands and clothes of people can also be contaminated with *Salmonella* during handling poultry and eggs (Hale et al., 2012, CDC, 2019). In the USA, an estimated 3.2 million cases of gastrointestinal illnesses are caused by improper handling techniques of domesticated animals, with 11% attributed to *Salmonella* spp. (Hale et al., 2012).

1.3.1 Prevention strategies for *Salmonella* in poultry

Broad spectrum antibiotics such as penicillin and tetracyclines (NRC, 1980) were widely used in the feed for the prevention of bacterial diseases in poultry in the past to maintain healthy flocks and to prevent and control *Salmonella* and other bacterial infections (Kawano et al., 1996). Though effective in most instances, the use of antibiotics has been reduced with the emergence of the antibiotic resistant strains of bacteria (Wray and Davies, 2000).

Specific non digestible oligosaccharides, polysaccharides, lipids and proteins are used as prebiotics, as an alternative to antibiotics, to improve the gut microbiome of poultry to reduce colonisation of *Salmonella* (Hajati and Rezaei, 2010). Common, natural substances such as *Cichorium intybus* (chicory), *Allium cepa* (onion), *Allium sativum* (garlic)*, Allium ampeloprasum* (leek)*, S. lycopersicum* (tomato) can be used as prebiotics to improve the gut health of poultry (Ganguly, 2013). A study conducted by Oyarzabal and Conner (1995) demonstrated that *Salmonella* spp. are unable to

metabolise fructooligosaccharide as a carbon source, which is used as a prebiotic in the poultry feed. Another study demonstrated that providing fructooligosaccharides in the feed could reduce the colonisation of *S.* Typhimurium in the gastrointestinal tract (Bailey et al., 1991). According to a review by Ricke (2015) the concentration and the time period of the fructooligosaccharides provided in the feed, and other environmental stresses could impact the effectiveness of the prebiotics.

Apart from antibiotics and the prebiotics, probiotics are also used to maintain healthy flocks (Jin et al., 1997). *Lactobacillus* spp., *Bacillus subtilis*, *Enterococcus faecium*, *Streptococcus thermophilus*, *Enterococcus faecalis*, *Aspergillus oryzae*, *Saccharomyces cerevisiae*, and *E. coli* are commonly used as probiotics (Khan and Naz, 2013). Probiotics aid in maintaining gut pH and increase the competition for nutrients, which prevents the growth of the pathogenic bacteria (Alagawany et al., 2018). An *in vitro* study showed that *S.* Typhimurium was sensitive to amylase and phytase produced by the *Bacillus* spp. extracted from chicken faeces, which can be used as a potential probiotic (Hosseini et al., 2018). According to Akbari et al. (2008) chickens supplemented with a commercial preparation of *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, and *Enterococcus faecali* prior to the *S.* Typhimurium infection reduced *Salmonella* colonisation.

There are different types of *Salmonella* vaccines developed to control *Salmonella* infections in poultry. These include live-attenuated vaccines, inactivated vaccines, subunit vaccines, nucleic acid vaccines and vectored vaccines (Desin et al., 2013). The Australian Egg Corporation reported that live-attenuated vaccines of *Salmonella* can be used to eliminate *S.* Typhimurium from the layer flocks (Groves, 2011). While results are promising with layer flocks (Desin et al., 2013), the vaccine is ineffective on the young poultry because of the under-developed immune system.

1.3.2 Domestication of poultry

The domestication of poultry has been practiced even before the sixth millennium BC (West and Zhou, 1989). The proportion of the population that have domesticated poultry in backyards is increasing with the increased interest in self-sustainability projects (Lerman et al., 1994) and the increased demand for high quality fresh food products (Hellin et al., 2015). The US Department of Agriculture estimated that within the metro areas of Denver, Los Angeles, Miami, and New York, 0.8% of the population had domesticated poultry in their backyards (USDA, 2013). The ability to use poultry droppings as manure and their role in pest control has made poultry husbandry more popular (Alders and Pym, 2009). Additionally, people consider poultry as pets (Lee et al., 2006). A study conducted in the US by Elkhoraibi et al. (2014) found that 57.4% of the study population (n=1,487) considered backyard poultry as pets.

Increasing numbers of the population with poultry in backyards also increases the likelihood of zoonotic diseases such as *Salmonella* (Antunes et al., 2003), *Listeria* (Jay, 1996), *Campylobacter* (Vellinga and Van Loock, 2002) and *Escherichia coli* (Blanc et al., 2006, Doyle and Schoeni, 1987). *Salmonella* is one of the most prevalent bacterial species spread through poultry (Griggs and Jacob, 2005, Bryan and Doyle, 1995), which poses an increased risk to public health (Leveque et al., 2003). In a study in India, *Salmonella* infection in backyard poultry was 6.1% (n=360) (Samanta et al., 2014). According to a report published by the CDC in 2018, in the US, 334 people were diagnosed with salmonellosis infection due to backyard poultry (CDC, 2018). Currently, there are limited studies conducted in Australia for the identification of *Salmonella* spp. infecting backyard poultry. A study conducted with 30 backyard flocks in South Australia reported that 4 backyard chicken flocks were positive for *Salmonella* spp. (Manning et al., 2015). McDonagh et al. (2019) reported that the 2% of the tested backyard poultry were positive for *Salmonella* (n=53). Another study conducted by Jafari et al. (2007) of 35 backyard flocks reported that 5 (14%) samples were positive for *Salmonella.* Another study conducted in Paraguay (n=50) reported that 3.5% were positive for *Salmonella* (Leotta et al., 2010).

The prevalence of *Salmonella* infection in poultry can vary due to different environmental factors such as the weather, the feed, poultry species and the condition of the coop (Weerasooriya et al., 2017). Different *Salmonella* spp. have been isolated from different backyard chicken flocks. This includes *S*. Agona, *S*. Bovismorbificans (Manning et al., 2015), *S*. Bareilly, *S*. Mbandaka, *S*. Rissen (Im et al., 2015), *S*. Heidelberg, *S*. Infantis, *S*. Hadar, and *S*. Schwarzengrund (Poppe et al., 1991). The difference in the infection rates could be due to factors such as the virulence, the shedding rates and the expanse of colonisation in the chicken gut. The limited number of studies of backyard poultry means that the true prevalence of a flock being infected is not known.

1.4 Seasonal variation of salmonellosis

An increase in the incidence in salmonellosis has been reported during the warm summer months, which is a similar pattern seen in other foodborne illnesses (Slutsker et al., 1997). Higher environmental temperatures increase the replication of bacteria in manure (Miyatake and Iwabuchi, 2005) and enhances the transmission between animals (Petersen et al., 2007). According to the Australian Welfare National Hospital Morbidity Database, hospital admissions for *Salmonella* infection show a similar seasonal pattern (AIHW, 2019).

1.5 Eggs, raw egg products and salmonellosis

Well known food products which use raw eggs, for example, foods like homemade ice cream, mayonnaise, eggnog, special diet or health drinks, mousse and hollandaise sauce, pose a risk of *S.* Enteritidis and *S.* Typhimurium infection (Braden, 2006).

The eggs can be contaminated during different stages of their formation, processing and packaging (Gantois et al., 2009). Vertical or transovarian contamination of eggs occur during the formation of the egg when ovaries of the hen are infected, which contaminates the internal contents of the egg. Horizontal transmission takes place if the eggs are contaminated by means of a contaminated environment (De Reu et al., 2006). The contamination in the internal contents of the egg due to *S.* Enteritidis infection in the ovaries and the oviduct of poultry is well documented (Wales and Davies, 2011). But information about *S.* Typhimurium infection in the reproductive organs is limited and typically eggs are only externally contaminated with *S.* Typhimurium (Pande et al., 2016).

According to previous studies, there is an increased potential for whole eggs with low shell quality to be penetrated by *Salmonella* spp. (Sauter and Petersen, 1974). Flock age also influences the ability of *Salmonella* spp. to penetrate the shell and the membranes of the eggs (Berrang et al., 1998) due to the thinning of the egg shell when the birds in the flock become older (Bruce and Johnson, 1978). It has been found that there is an increased incidence of egg shell penetration at the beginning of lay until end of lay by *S.* Enteritidis (Nascimento et al., 1992).

S. Enteritidis is the strain found in most parts of the world including the US (Patrick et al., 2004) and the European Union (Gantois et al., 2009). Though *S.* Enteritidis is the most commonly identified strain in poultry layer farms, this strain is not commonly reported in Australian layer farms (Chia et al., 2009, Sexton et al., 2007, Sumner et al., 2004). In contrast, the strain *S.* Typhimurium is commonly found in Australia (Ford et al., 2018).

S. Typhimurium and S. Enteritidis are commonly linked with outbreaks caused by eggs or raw egg products. In September 1994 there was an outbreak of *S.* Enteritidis in the United States due to consumption of ice cream which was distributed to all 48 states through a household-delivery

system. During the investigation of the outbreak *Salmonella* contamination of the ice cream plant was not detected and it was found that there had been cross-contamination of pasteurized ice cream premix during the transportation in tanker trailers that had previously transported non-pasteurized liquid eggs contaminated with *S.* Enteritidis (Braden, 2006). There was another outbreak in the United States from ice cream when contaminated unpasteurized egg yolks, which were not cooked during manufacturing, were used to produce the ice cream (Armstrong et al., 1970). Ice cream served at a birthday party, made with raw eggs were also involved with an *S.* Enteritidis outbreak in London in 1998 (Dodhia et al., 1998).

In contrast, another investigation conducted in London in 1988 to determine the cause of a food poisoning outbreak found that the infection was *S.* Typhimurium. It was determined that mayonnaise used at a private lunch was the mode of infection since the infection was linked with consumption of the foods that contained mayonnaise. It was confirmed that the source of infection was contaminated eggs because the same strain was isolated from the poultry faeces at the egg farm (Mitchell et al., 1989).

In Australia, most salmonellosis cases are linked to eggs and raw egg products and these outbreaks are commonly caused by *S.* Typhimurium (Fearnley et al., 2011) in contrast with the US and Europe, where egg related salmonellosis cases are commonly caused by *S.* Enteritidis (Vo et al., 2010). In 2001 to 2009, 90% of the salmonellosis outbreaks related to eggs and raw egg products were caused by *S.* Typhimurium in Australia (Moffatt, 2011). Another study conducted by Stephens et al. (2008) reported that in 2005 *S.* Typhimurium was the main cause of egg related outbreaks in Tasmania. OzFoodNet (2006) also identified that food products containing eggs or raw eggs such as sandwiches, desserts, cakes, sauces and gravy were the main cause of salmonellosis outbreaks within Australia in 2005. Moreover, during the period of 2010 – 2011, in Australia, 1000 individual salmonellosis cases related to eggs and raw egg products were recorded (Moffatt and Musto, 2013). Ford et al. (2018) also identified that egg-based dishes such as tiramisu, fried ice cream, mousse, custard, mayonnaise, aioli, hollandaise sauce, tartare sauce, which contained raw or undercooked eggs accounted for 49% of the salmonellosis cases (n=990) in the years, 2001 to 2016 in Australia.

Among these salmonellosis cases 95% of these egg related outbreaks were due to *S*. Typhimurium contamination.

Hence, there is a need for effective, reliable and potential control mechanisms to reduce the incidence of salmonellosis outbreaks linked to eggs and raw egg products in Australia in relation to *S*. Typhimurium.

1.6 Research question and the objectives of the study

Aim 1: Investigating the potential role backyard chicken may play in the spread of salmonellosis, campylobacteriosis and shigellosis in Australia

There are limited studies conducted in Australia to identify the potential risk factors associated with domesticated, 'backyard', poultry. The increasing popularity of keeping poultry in the backyards could contribute to the increase in salmonellosis, campylobacteriosis and shigellosis outbreaks and could also increase the potential of spreading these pathogens to neighbouring poultry farms. The lack of knowledge of the general public in proper handling of poultry could pose a threat to children, elderly and immune compromised people.

Therefore, the aim of this study is to identify the prevalence of *Salmonella, Campylobacter* and *Shigella* in backyard poultry. A survey (page number 330) was also conducted to assess the knowledge of the general public on domestication and handling of poultry. Information on the different types of feed used, conditions of the poultry houses, history of clinically proven salmonellosis infections, storage of eggs, discarding and washing of eggs, control measures for *Salmonella*, consumption of raw eggs and disposal of poultry droppings and other domesticated animals were assessed, as these factors could increase the risk of *Salmonella, Campylobacter* or *Shigella* infection and contamination in the household.

Aim 2: Develop a whole egg decontamination method that can eliminate *S*. Typhimurium without impacting egg properties and can be conducted using common commercial kitchen equipment

Eggs and raw egg products are the main cause of non-typhoidal *Salmonella* outbreaks in Australia (Fearnley et al., 2011) and the majority of these outbreaks are caused by *S*. Typhimurium (Moffatt, 2011). It is currently not possible to guarantee egg produced in Australia are free from *Salmonella* contamination (Whiley and Ross, 2015). Therefore, the aim of this research project was to develop an effective, rapid and simple decontamination method for whole shell eggs externally contaminated with *S*. Typhimurium. To ensure the application of the developed method it must be able to be conducted in a commercial kitchen and cannot affect the usability or quality of the egg. Decontamination of eggs immediately prior to the preparation of raw egg food products will reduce the risk of salmonellosis.

Aim 3: Identify the optimum pH and temperature combination for the control of *S*. Typhimurium during the production of raw egg mayonnaise

Temperature and pH are commonly used to control microorganisms in food. However, evaluating the effectiveness of pH and temperature as a control method is complicated by the intra-cellular mechanisms that aid the development of tolerance within *Salmonella* species to ensure survival under various pH conditions. Most of the research focuses on *S*. Enteritidis strain and there are a very few studies conducted on *S*. Typhimurium. According to the food safety guidelines published by the government of South Australia raw egg products including mayonnaise should be prepared, transported and stored below 5°C and the pH should be below 4.2 (SA Health, 2019). But according to research conducted with the *S*. Enteritidis strain, survival of *Salmonella* at acidic pH conditions is increased at lower temperatures (Xiong et al., 1999, Radford and Board, 1993). So, pH and temperature combinations need to be better understood for effective control of *S.* Typhimurium in raw egg products. Hence, the aim of this study is to identify the most effective pH and temperature combination for the control of *S.* Typhimurium in raw egg mayonnaise.

1.7 Research benefits

This study investigated the most effective combination of pH and temperature for the control of *Salmonella* in mayonnaise. Further, the developed rapid and simple egg decontamination method can be used just before the preparation of raw egg products to minimize the number of salmonellosis outbreaks. Identification of the prevalence of *Salmonella* in backyard poultry could aid in the implementation of proper control measures and to bring awareness to the general public and health authorities.

The outcomes of this project can be used to minimize the salmonellosis outbreaks related to eggs and raw egg products. Moreover, identifying the risk factors associated with backyard poultry could reduce the incidence of foodborne illnesses in humans and improve public health.

1.8 Structure of the thesis

The thesis contains 5 chapters. The first introduction chapter is followed by the 2nd chapter which explains the analysis conducted to investigate the potential role backyard chicken may play in the spread of salmonellosis in Australia (Aim 1). The 3rd chapter contain the experiments conducted to develop an egg decontamination method that can eliminate *S.* Typhimurium without impacting egg properties and can be conducted using common commercial kitchen equipment (Aim 2). The identification of the optimum pH and temperature combination for the control of *S.* Typhimurium during the production of raw egg mayonnaise (Aim 3) is explained in the 4th chapter followed by the general discussion of the project which is included in the 5th chapter. There may be some repetition throughout the thesis as it consists of published journal articles.

CHAPTER 2. Investigating the potential role backyard chicken may play in the spread of salmonellosis, campylobacteriosis and shigellosis in Australia

2.1 Overview of the chapter

This chapter describes the prevalence of *Salmonella* spp., *Campylobacter* spp. and *Shigella* spp. infection in poultry kept in the backyards. The knowledge of the general public on handling poultry and fresh eggs was analysed using a survey (Appendix, page number **330**) that asked questions from backyard chicken owners. Additionally, the risk factors that may contribute to the spread of pathogenic bacterial species were identified. This chapter consists of a scientific paper titled "Identification of *Salmonella*, *Campylobacter* and *Shigella* in backyard chicken flocks and associated risk factors" (page number **44**). This chapter also contains preliminary experiments that led to the method development which were not included in the paper. Ethical clearance for the study was obtained from the Flinders University Social and Behavioural Research Ethics Committee (project number 7737) (Appendix, page number **328**).

2.2 Method development

Zoonotic gastrointestinal diseases such as salmonellosis, campylobacteriosis and shigellosis are commonly linked with poultry and poultry products. As the number of people keeping chickens in their backyards is increasing (Conan et al., 2012), the incidence of these zoonotic infections can increase and could pose a threat to public health. To assess this, the prevalence of *Salmonella* spp., *Salmonella* Typhimurium, *Campylobacter* spp. and *Shigella* spp. was determined using probe based real time PCR assays as described in the scientific paper (page number **44**). The PCR assays were optimised using known positive and negative controls.

2.2 Optimizing the probe based real time PCR assay for the detection of *Salmonella* spp. and *S.* Typhimurium

DNA extracted from faeces can inhibit the PCR reactions due to the presence of inhibitory compounds (Nsubuga et al., 2004). Therefore, the presence of inhibitory compounds in the DNA extract were assessed according to the experiment outlined below.

2.2.1 Methods

S. Typhimurium DNA extracted from a pure culture was used as the control (neat). DNA was also extracted from a known negative chicken faeces sample (negative control). The negative control was spiked with *S.* Typhimurium DNA to obtain 1:10 and 1:100 dilutions. The reaction conditions are presented in the scientific paper sections 2 (**Table 2.2**).

2.2.2 Results

No inhibition was observed in the PCR reactions conducted using the *Salmonella* spp. specific primers (**Figure 2.1**) and *S.* Typhimurium specific primers (**Figure 2.2**). The Ct value of the sample containing *S.* Typhimurium DNA in the concentration of 1:10 was higher than the 1:100 dilution. Therefore, it was assumed that there are no PCR inhibitory compounds in the DNA extracts.
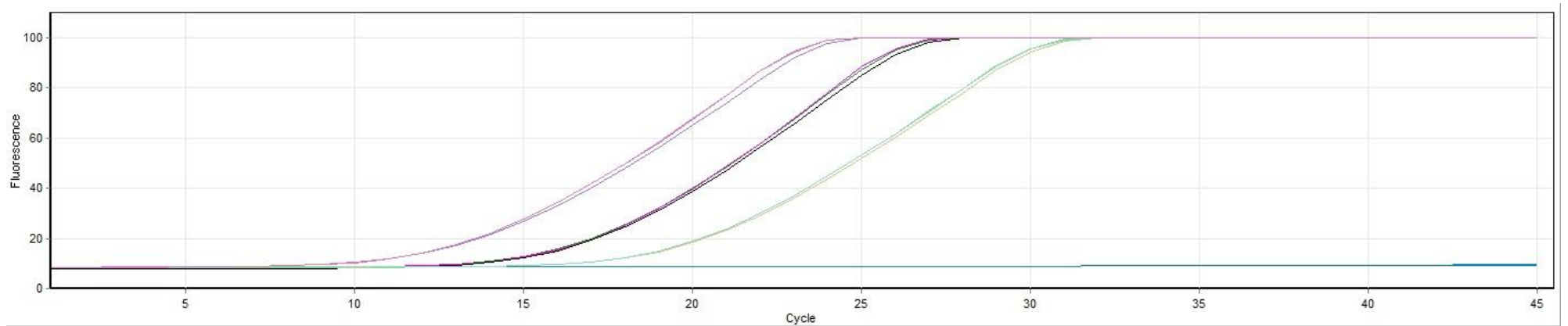
Figure 2.1 Checking for inhibitory compounds in the PCR reaction for the *Salmonella* spp. primers; A – (neat) ST DNA extracted from *S.* Typhimurium culture, B- 1/10 dilution (DNA extracted form a known negative chicken faeces sample spiked with *S.* Typhimurium DNA and C – 1:100 dilution (DNA extracted from a known negative chicken faeces sample spiked with *S.* Typhimurium DNA)
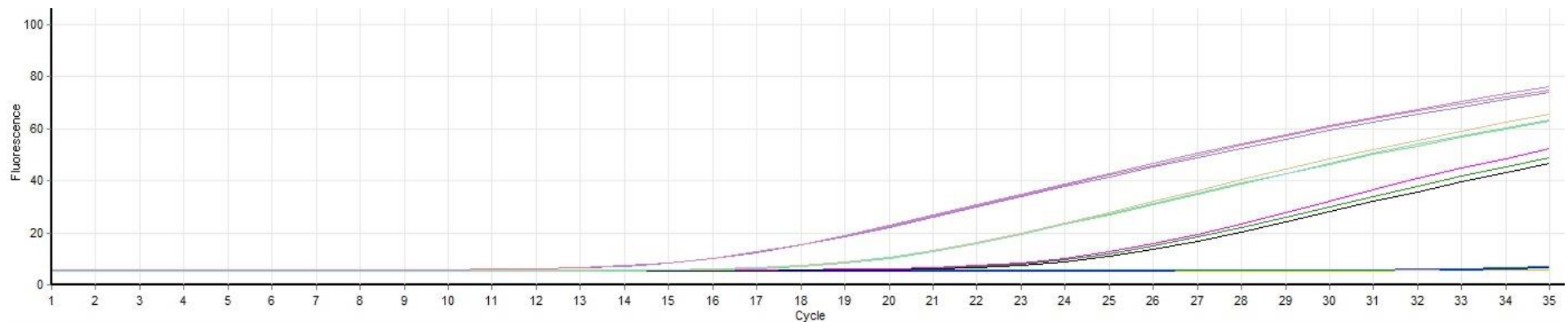
Figure 2.2: Checking for inhibitory compounds in the PCR reaction for the ST primers; A – (neat) ST DNA extracted from *S*. Typhimurium culture, B- 1/10 dilution (DNA extracted from a known negative chicken faeces sample spiked with *S*. Typhimurium DNA and C - 1/100 dilution (DNA extracted form a known negative chicken faeces sample spiked with *S*. Typhimurium DNA)

2.3 Optimizing the probe based real time PCR assay for the detection of *Shigella* spp.

As *Shigella* spp. are occasionally identified in poultry and poultry products the collected chicken faeces were analysed for the presence of *Shigella* spp.

2.3.1 Methods

A DNA strand complementary to the *Shigella flexneri* ipaH gene (**Figure 2.3**) which consisted of the primer and probe sequences was commercially designed (IDT integrated DNA technologies) and was used as the positive control. A real time PCR reaction was carried out to determine the DNA concentration of the positive control that should be used in the probe-based PCR reaction. The desiccated DNA strand was dissolved in sterile MilliQ® water (10 ng/mL) and a serial dilution was carried out to obtain 1 pg/µL, 10 pg/µL and 100 pg/µL concentrations of the positive control. These dilutions were used in the PCR reactions. The PCR conditions are as presented in the scientific paper (page number **48**)

```
>M76445.1 Shigella flexneri ipaH gene, 5'end

ATGAAACCGATCAACAATCATTCTTTTTTTCGTTCCCTTTGTGGCTTATCATGTATATCT
CGTTTATCGGTAGAAGAACAGTGTACCAGAGATTACCACCGCATCTGGGATGACTGGGCT
AGGGAAGGAACAACAACAGAAAATCGCATCCAGGCGGTTCGATTATTGAAAATATGTCTG
GATACCCGGGAGCCTGTTCTCAATTTAAGCTTACTGAAACTACGTTCTTTACCACCACTC
CCTTTGCATATACGTGAACTTAATATTTCCAACAATGAGTTAATCTCCCTACCTGAAAAT
TCTCCGCTTTTGACAGAACTTCATGTAAATGGTAACAACTTGAATATACTCCCGACACTT
CCATCTCAACTGATTAAGCTTAATATTTCATTCAATCGAAATTTGTCATGTCTGCCATCA
TTACCACCATATTTACAATCACTCTCGGCACGTTTTAATAGTCTGGAGACGTTACCAGAG
CTTCCATCAACGCTAACAATATTACGTATTGAAGGTAATCGCCTTACTGTCTTGCCTGAA
TTGCCTCATAGACTACAAGAACTCTTTGTTTCCGGCAACAGACTACAGGAACTACCAGAA
TTTCCTCAGAGCTTAAATATTTGAAGGTAGGTGAAAATCAACTACGCAGATTATCCAGA
TTACCGCAAGAACTATTGGCTCTGGATGTTTCCAATAACCTACTAACTTCATTACCCGAA
AATATAATCACATTGCCCATTTGTACGAATGTTAACATTTCAGGGAATCCATTGTCGACT
CGCGTTCTGCAATCCCTGCAAAGATTAACCTCTTCGCCGGACTACCACGGCCCGCAGATT
TACTTCTCCATGAGTGACGGACAACAGAATACACTCCATCGCCCCCTGGCTGATGCCGTG
ACAGCATGGTTCCCGGAAAACAAACAATCTGATGTATCACAGATATGGCATGCTTTTGAA
CATGAAGAGCATGCCAACACCTTTTCCGCGTTC<mark>CTTGACCGCCTTTCCGATA</mark>CCGTCTCT
GCACGCAATACCTCCGGATTCCGTG<mark style="background:#0f0">AACAGGTCGCTGCATGGCTGGAA</mark>AAACTCAGTGCC
TCTGCGGAG<mark>CTTCGACAGCAGTCTTTCGCT</mark>GTTGCTGCTGATGCCACTGAGAGCTGTGAG
GACCGTGTCGCGCTCACATGGAACAATCTCCGGAAAACCCTCCTGGTCCATCAGGCATCA
GAAGGCCTTTTCGATAATGATACCGGCGCTCTGCTCTCCCTGGGCAGGGAAATGTTCCGC
CTCGAAATTCTGGAGGACATTGCCCGGGATAAAGTCAGAACTCTCCATTTTGTGGATGAG
ATAGAAGTCTACCTGGCCTTCCAGACCATGCTCGCAGAGAAACTTCAGCTCTCCACTGCC
GTGAAGGAAATGCGTTTCTATGGCGTGTCGGGAGTGACAGCAAATGACCTCCGCACTGCC
GAAGCTATGGTCAGAAGCCGTGAAGAGAATGAATTTACGGACTGGTTCTCCCTCTGGGGA
CCATGGCATGCTGTACTGAAGCGTACGGAAGCTGACCGCTGGGCGCAGGCAGAAGAGCAG
AAGTATGAGATGCTGGAGAATGAGTACTCTCAGAGGGTGGCTGACCGGCTGAAAGCATCA
GGTCTGAGCGGTGATGCGGATGCGCAGAGGGAAGCCGGTGCACAGGTGATGCGTGAGACT
GAACAGCAGATTTACCGTCAGCTGACTGACGAGGTACTGGCCTGAACCGCCCCGGGAATC
CTGGAGACTAAACTTCCTGAGAAAGAGGTAAACAGGATGACTAAAAATACTCGTTTTTCC
CCCGAAGTCCGTCAACGGGCAGTCCGTATGGTTCTGGAAAGTCAGGGCGAATATGACTCA
CAATGGGCGACAATTTGTTCCATTGCTCCAAAGATTGGCTGTACGCCGGAGACTCTGCGT
GTCTGGGGTCGCCAGCATGAGCGGGATACCGGGGGCGGTGATGGAGGGCTCACCACCGCT
GAACGTCAGCGT
```

Figure 2.3: The primer and probe sequences located on the *Shigella flexneri* ipaH gene. A nucleotide strand with 157 bp containing the probe and primer sequences was commercially designed and was used as the positive control for the real time PCR to detect *Shigella* spp.

2.3.2 Results

The DNA concentration used for the PCR reaction for the identification of *Shigella* spp. was selected as 1 pg/µL as the fluorescence was detected after 19 cycles (**Figure 2.4**)
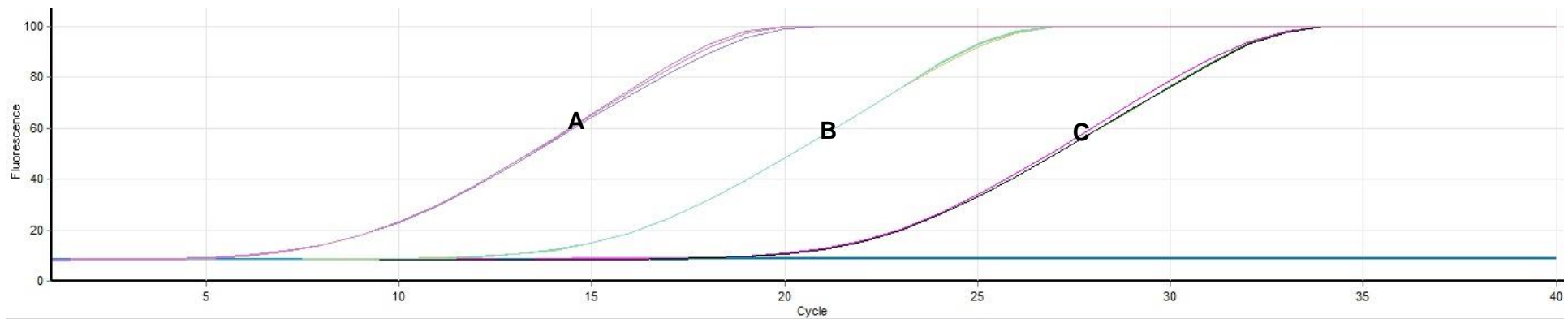
Figure 2.4: As the positive control was commercially designed for the detection of *Shigella* spp a real time PCR reaction was carried out to determine the DNA concentration of the positive control to be used in each PCR reaction. Dilutions used in the PCR reaction were 1 pg/µL (C), 10 pg/µL (B) and 100 pg/µL (A)

2.4   Sample collection

Chicken faeces samples were collected from 82 different backyards (**Figure 2.5**). Samples were collected over 9 months (**Figure 2.6**).

Figure 2.5: A typical backyard chicken flock; a multi- breed and a multi-aged chicken flock
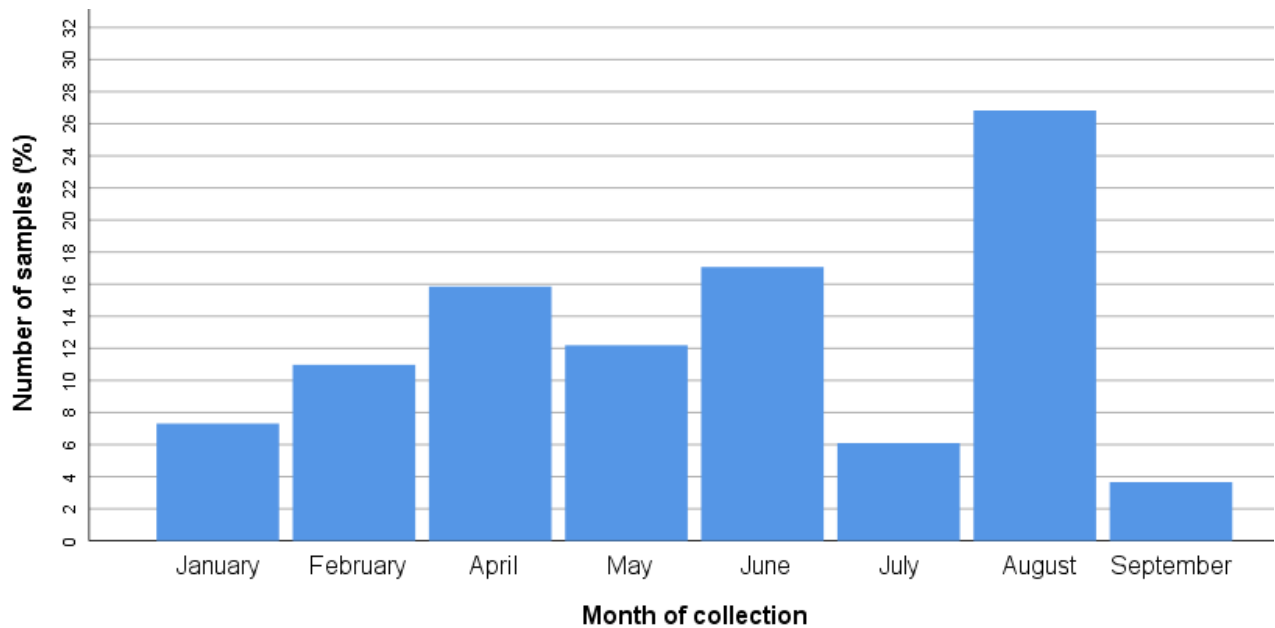
Figure 2.6: The percentage of chicken faeces samples collected from January 2019 to September 2019 from backyards from January 2019 to September 2019. A higher percentage of positives were recorded during the warmer month of February (33%) and colder and wetter month of July (40%) even though a smaller number of samples collected during those months

2.5   Analysis of the data collected from the survey

A survey was designed to assess the knowledge of the general public on handling fresh eggs and poultry and to identify potential risk factors that may contribute to the increased incidence of gastrointestinal infections.

2.5.1   Methods

The survey (page number **330**) consisting of 16 questions was given to the backyard chicken owners and the results were assessed using the SPSS software.

2.5.2   Results

Most of the participants had 3-5 birds in their flocks (**Figure 2.7**). Most of the chicken flocks contained multi-aged birds (**Figure 2.8**), 4% of the study population was diagnosed with clinically proven *Salmonella* and a significant percentage had no control measures for *Salmonella*. Some of the backyard chicken owners had the chickens confined to one specific area while others allowed the chickens to roam around the backyard with no restrictions (**Table 2.1**). The poultry droppings were used as manure or spread on site (**Figure 2.9**). Most of the backyard chicken owners did consume under cooked eggs or raw egg products, washed the eggs if the eggs contained any dirt, consumed cracked eggs and stored eggs on the shelf (**Figure 2.10** and **Figure 2.11**). These factors were identified as potential risk factors as they could increase the bacterial load on the edible potion of the egg if the eggs were already contaminated. Sixteen percent (16%) of the samples were positive for a pathogen.
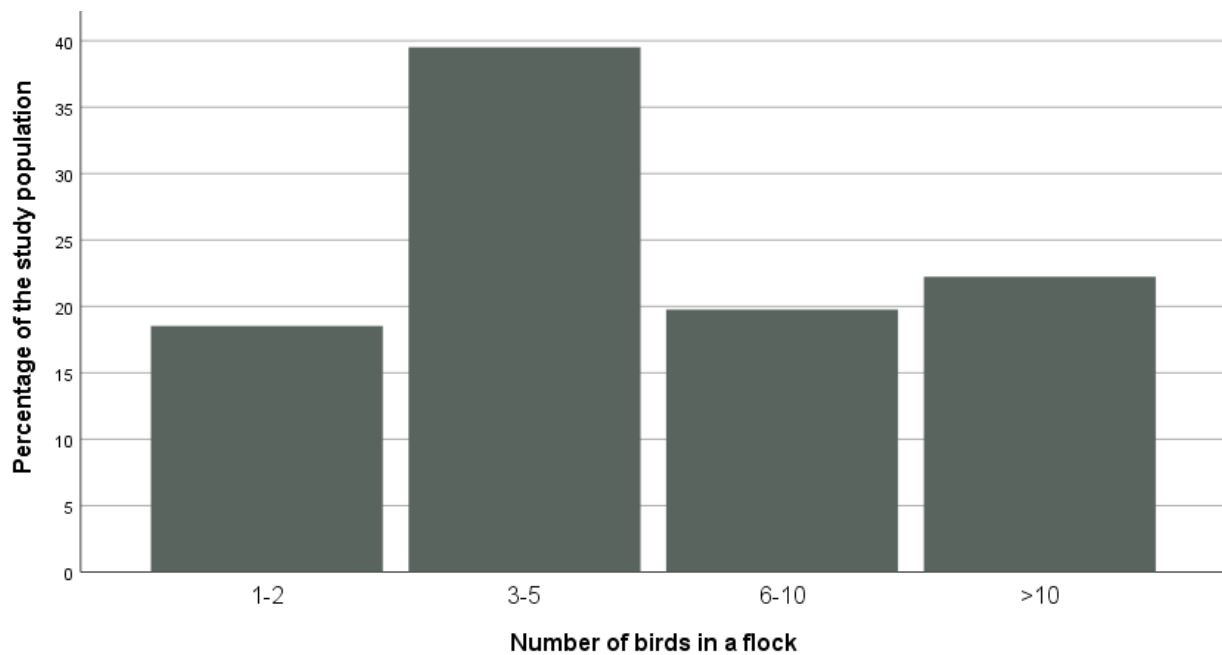
Figure 2.7: The percentage of the study population against the number of birds in a flock.
Most of the participants had small flocks with 3-5 birds and more than 20% had larger flocks
which contained more than 10 birds. Thirty-two Participants (n=32) had 3-5 birds in their
flocks and 19% (n=5 out of 15 flocks) was positive for a pathogen. Among the flocks which
contained more than 10 birds 38% were positive for either *Salmonella* spp. or
***Campylobacter* spp. but none of the flocks with 2-3 birds was infected.**
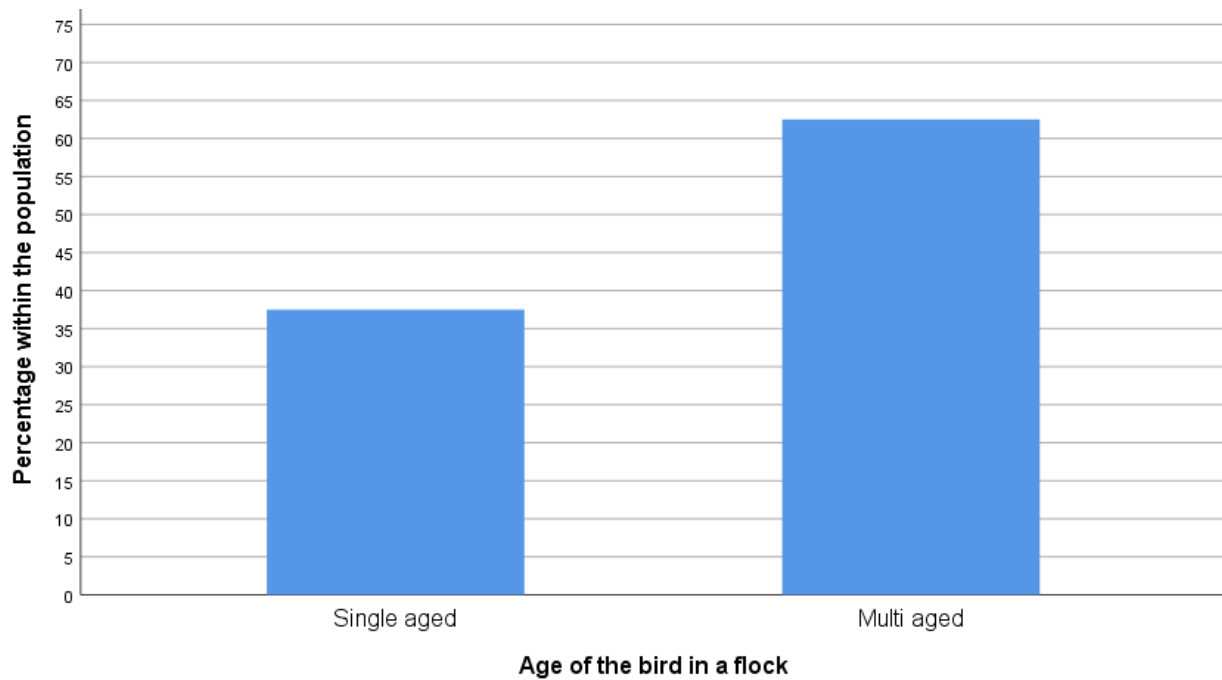
Figure 2.8: The percentage of the study population against the age of the birds within a flock. Most of the participants had multi aged birds in a flock. Often backyard chicken flocks with more than 10 bird contain multi-aged birds. Only 3% of the single aged flocks were detected with an infection whereas 24% of the multi-aged flocks were positive for one of the pathogens tested. As older bird are more susceptible to infections they could be infecting the younger birds in a multi-aged flocks (Hester, 2005)
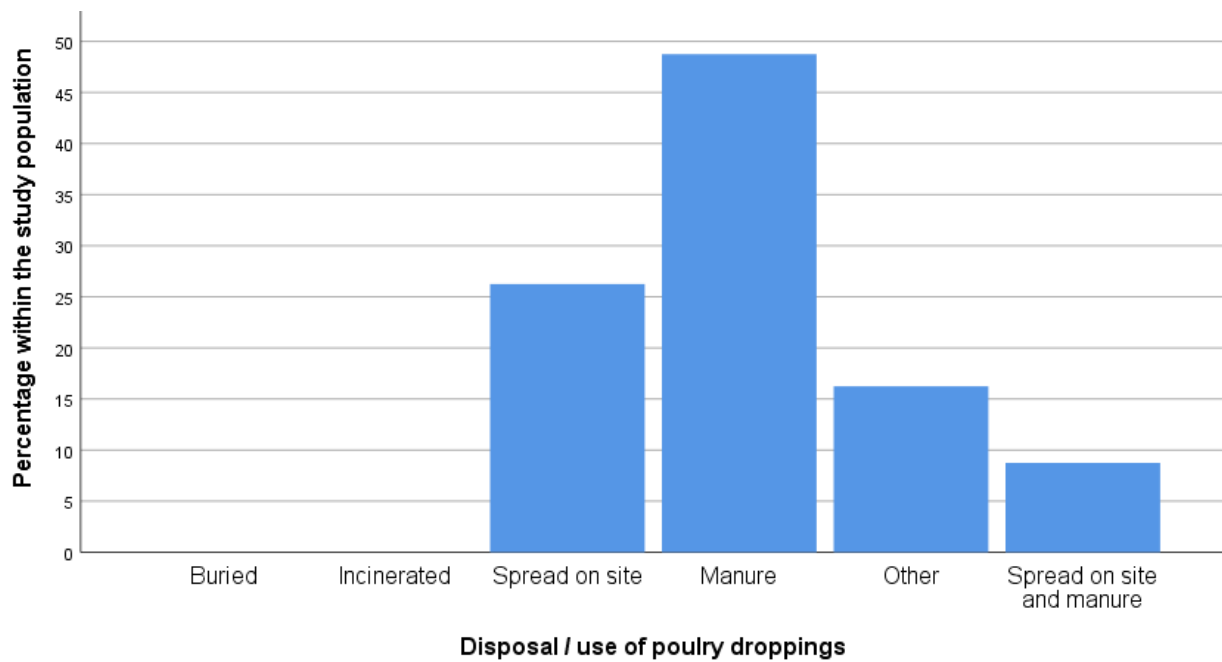
Figure 2.9: A significant number of participants used the faeces as manure while others spread the faeces on site. None of them buried or incinerated the faeces. Spreading the faeces onsite could increase the risk of infection
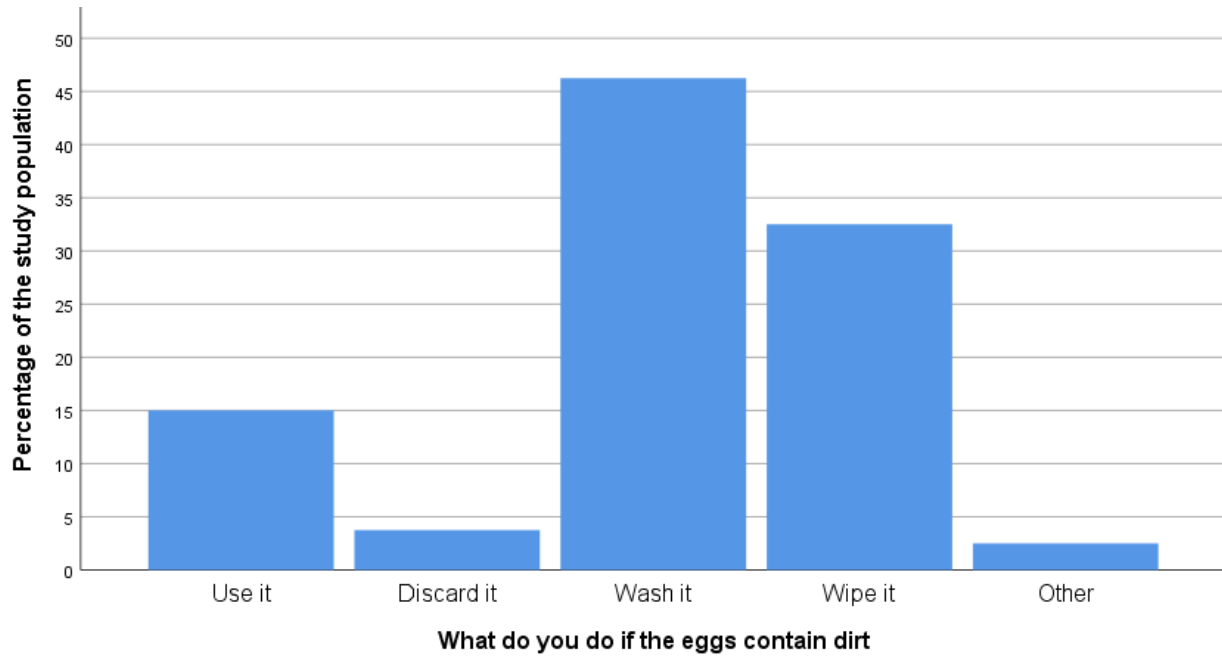
Figure 2.10: The way the general public handle dirt on eggs at the point of collection. Most of the study population washed the eggs if the eggs were dirty, and a significant amount of the study population wiped, discarded or fed them to their pets (other). Washing the eggs could increase the risk of contamination if the eggs are consumed raw
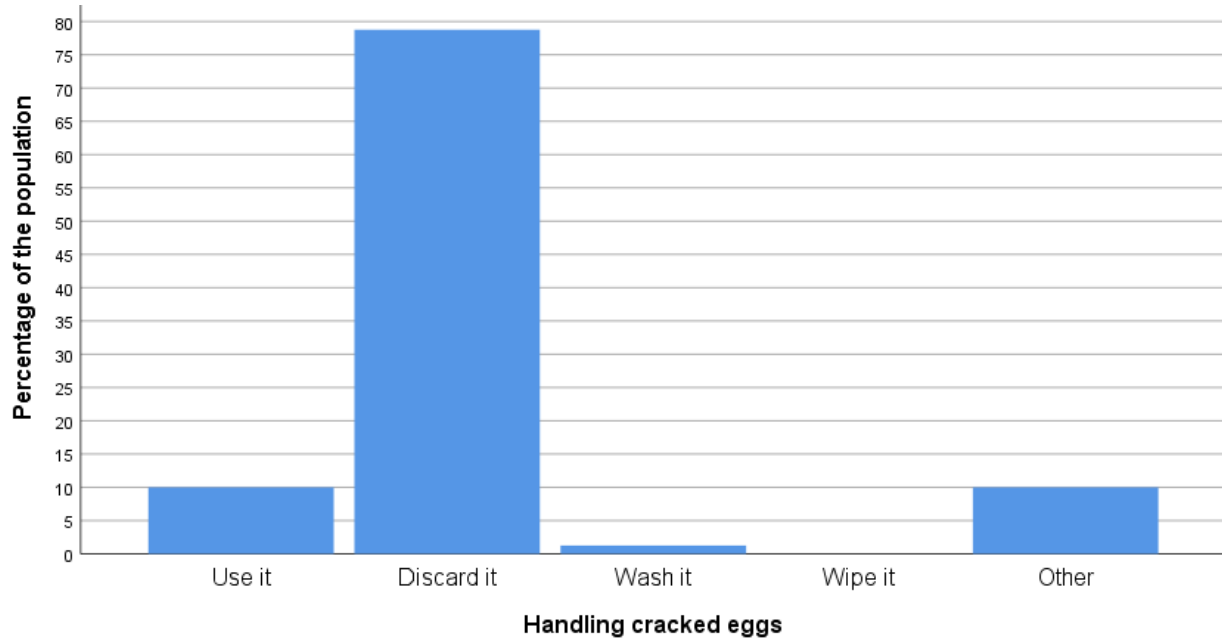
Figure 2.11: The way the general public handle cracked eggs. Consumption of cracked eggs was practised by 10% of the population while more than 75% of the study population discarded cracked eggs. A few washed the cracked eggs. Consumption of cracked eggs could increase the risk of bloodborne illness as the internal contents of the egg could be contaminated

Table 2.1: The percentage of the study population which engaged in risky behaviour. These factors can spread the zoonotic diseases and increase the bacterial load on the edible potion of the egg if the eggs are already contaminated

Data collected	Percentage of the study population
Participants who did not have chickens confined into a specific area	82
Participants who consumed raw or under cooked eggs	26
Participants who stored eggs at room temperature	19
Participants who washed the dirt off eggs	46

2.6 Identification of *Salmonella* in chicken faeces samples using culturing techniques

Salmonella colonies are often identified by the distinctive colonies produced on XLD agar which are red colonies with a black centre (Kenner and BA Kenner, 1974).

2.6.1 Methods

Isolation of *Salmonella* colonies using culture methods are described in the scientific paper (page number **48)**.

2.6.2 Results

Some of the isolates identified as *Salmonella* isolates did produce red colonies with black centres (**Figure 2.12**) while some of the isolates did not produce the black centre but appeared as red colonies on XLD agar (**Figure 2.13**).
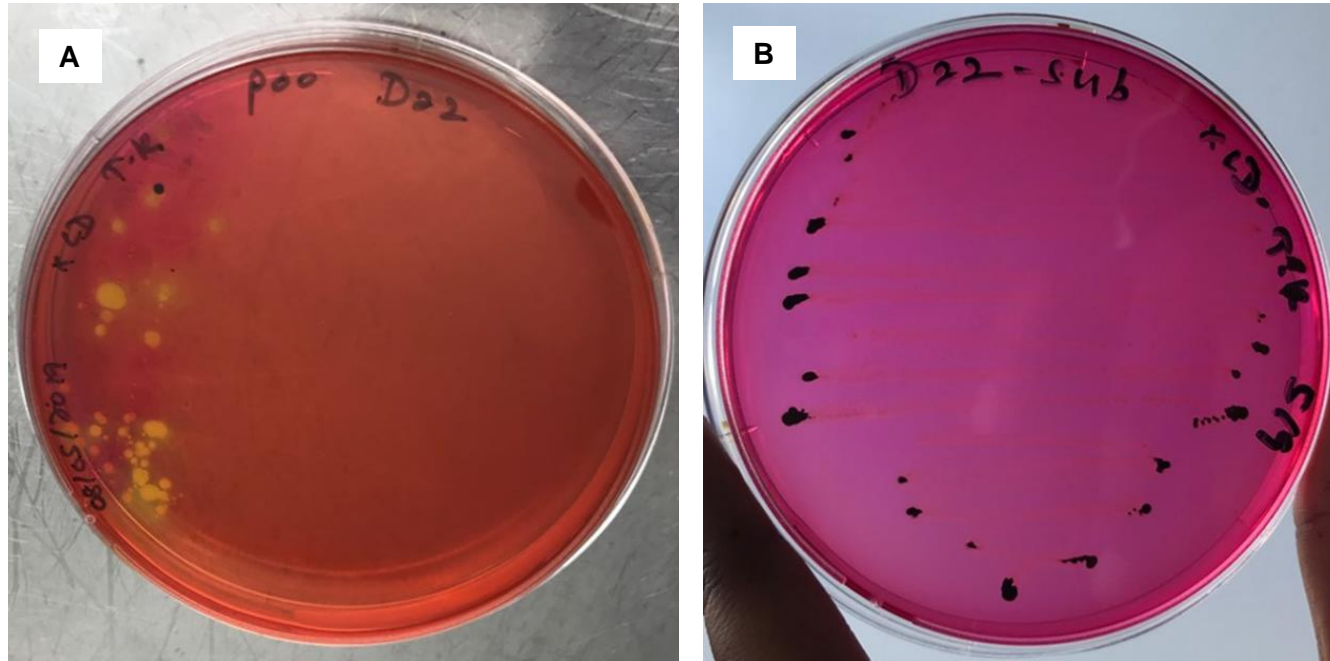
Figure 2.12: Isolation of pure cultures of *Salmonella*-like colonies from chicken faeces A: culture isolates on XLD agar chicken from chicken faeces and BPW mixture of the sample D22 B: Pure sub-culture of *Salmonella* on XLD agar isolated from the sample D22
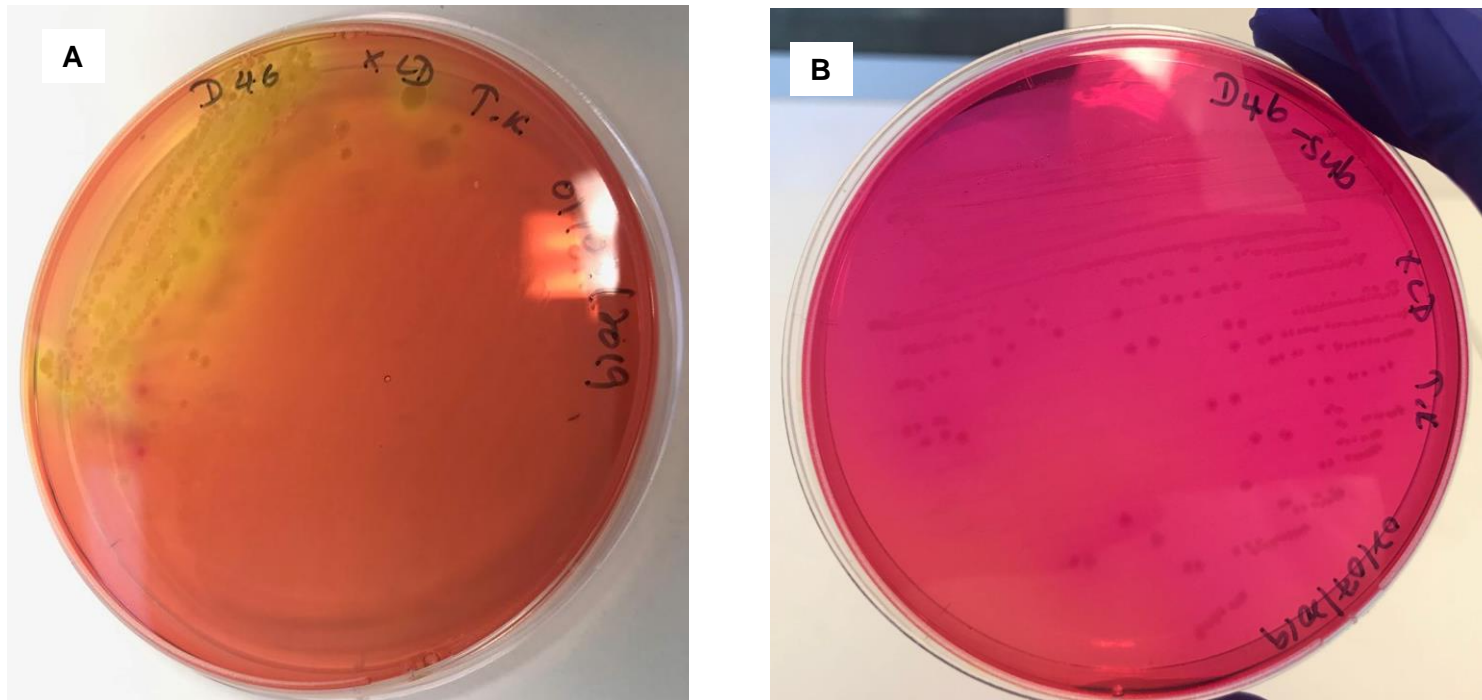
Figure 2.13: Isolation of pure cultures of *Salmonella*-like colonies from chicken faeces A: culture isolates on XLD agar chicken from chicken faeces and BPW mixture of the sample D46 B: Pure culture of *Salmonella* on XLD agar isolated from the sample D46

2.7 Identification of pathogens in chicken faeces samples using molecular techniques

Probe based real time PCR assays were conducted to identify the presence of *Salmonella Campylobacter* and *Shigella* in the collected chicken faeces samples.

2.7.1 Methods

The methods used are described in the scientific paper, section 2 (page number **48**).

2.7.2 Results

Among the collected samples there were *Salmonella* spp. positive samples (n=5) (**Figure 2.14** and **Figure 2.15**), *Campylobacter* positive samples (n=8) (**Figure 2.16**) and one *S.* Typhimurium positive sample (**Figure 2.17**). None of the samples were positive for *Shigella* spp. (**Figure 2.18**).

Figure 2.14: Real time PCR assay for the sample D22 using *Salmonella* spp. primers (PC- Positive control). The collected chicken faeces sample was processed and cultured on to XLD ager and a pure culture was obtained by subculturing followed by DNA extraction and PCR foe the identification of the culture isolate. PCR reaction for each isolate was conducted in triplicate to conform the results
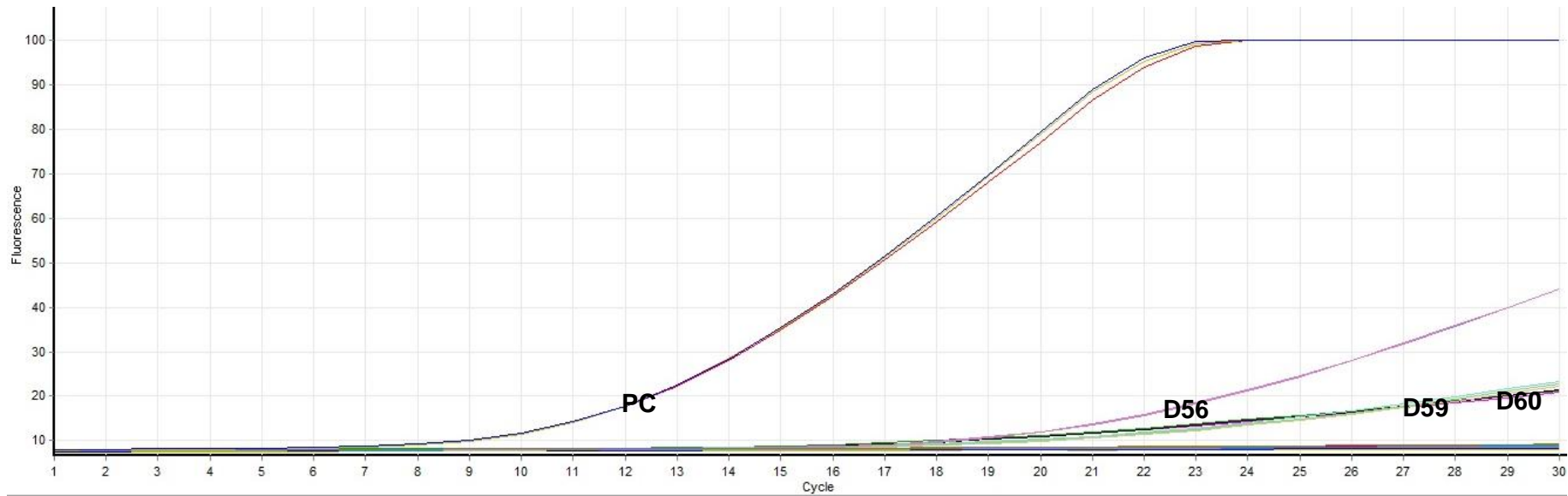
Figure 2.15: Real time PCR for the culture isolates from the samples D56, D59 and D60 using *Salmonella* spp. primers. The PCR was conducted for the culture isolates isolated from chicken faeces following DNA extraction. PCR reaction for each isolate was conducted in triplicate to conform the results (PC – positive control)
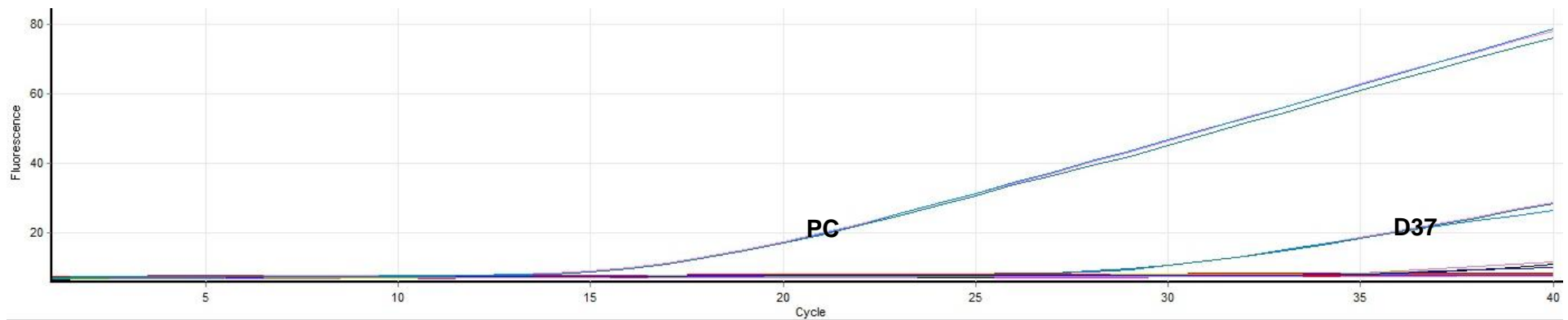
Figure 2.16: Real time PCR for the sample D37 using *Campylobacter* spp. primers. The PCR was conducted for the DNA extracted from chicken faeces. PCR reaction for each isolate was conducted in triplicate to conform the results (PC – positive control)
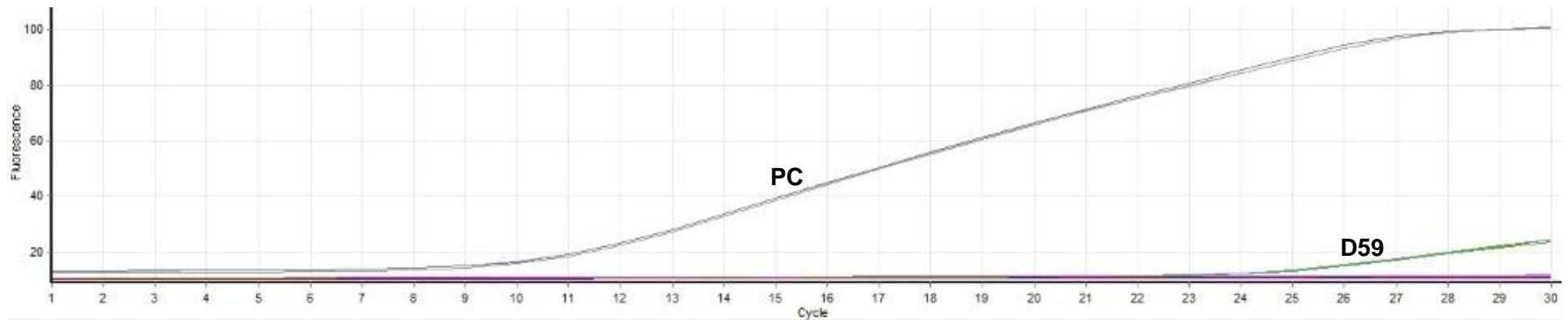
Figure 2.17: Real Time PCR for the identification of *S*. Typhimurium. The PCR reaction was conducted for the DNA isolated from the *Salmonella* spp. positive culture isolates. Only 1 sample (D59) among the 5 *Salmonella* spp. positive culture isolates were identified as *S*. Typhimurium. (PC- Positive control). PCR reaction for each isolate was conducted in triplicate to conform the results
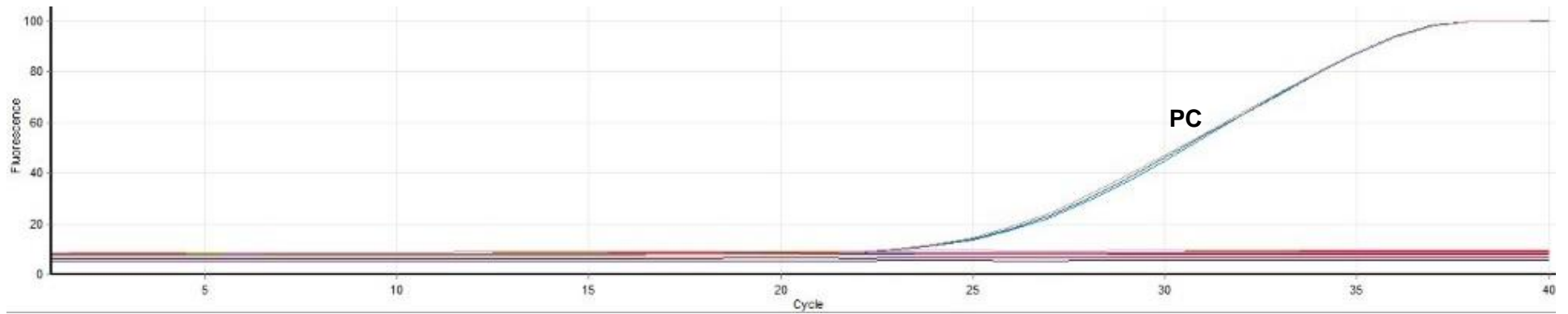
Figure 2.18: Real time PCR was conducted for the identification *Shigella* **spp. in chicken faeces samples.** *Shigella* **spp. specific primers were used for the reaction and none of the samples were positive for** *Shigella* **spp. (PC- Positive control). PCR reaction for each isolate was conducted in triplicate to conform the results**

CITATION

Identification of *Salmonella*, *Campylobacter* and *Shigella* in backyard chicken flocks and associated risk factors

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Abstract

Worldwide, foodborne illness is a significant public health issue in both developed and developing countries. Salmonellosis, campylobacteriosis and shigellosis are common foodborne gastrointestinal illnesses caused by the bacteria *Salmonella* spp., *Campylobacter* spp. and *Shigella* spp. respectively. These zoonotic diseases are frequently linked with eggs and poultry products. The aim of this study was to investigate the presence of *Salmonella* spp., *Campylobacter* spp. and *Shigella* spp. in Australian backyard chicken flocks. Chicken faeces samples were collected from 82 different backyard flocks. DNA was extracted from the faeces and real time PCR was conducted for the identification of *Salmonella* spp., *Campylobacter* spp. and *Shigella* spp. separately. A questionnaire was given to the backyard chicken owners and data were analysed using the SPSS software. The questionnaire was designed to assess the knowledge of the general public in handling poultry and eggs and also to identify the potential risk factors that may contribute to foodborne illness outbreaks. Six percent (6%) of the samples were positive for *Salmonella* spp., 10% were positive for *Campylobacter* spp. and none were positive for *Shigella* spp. Washing and storing eggs on the shelf could increase the risk of gastrointestinal diseases. According to the survey, 46 % of the participants washed their eggs and 19% stored their eggs at room temperature. This highlights the need for greater education for backyard chicken owners regarding the proper handling of eggs to reduce the increase in the incidence of gastrointestinal diseases.

1 Introduction

Globally, there are around 93.8 million cases of foodborne illnesses recorded each year (Majowicz et al., 2010). *Salmonella* (Hale et al., 2012)*, Campylobacter* (El-Tras et al., 2015) and *Shigella* (Bachand et al., 2012) are zoonotic bacterium species that commonly cause gastrointestinal illnesses in humans*.* These bacterial species are frequently isolated from poultry and poultry products (Keener et al., 2004, Antunes et al., 2003, Sackey et al., 2001). Predominantly, keeping poultry in backyards is practised to obtain fresh eggs for domestic use (Orji et al., 2005). On the other hand, the increasing number of field to fork restaurants (Diaz, 2018) with their own poultry production could increase the risk of foodborne diseases due to cross contamination, food preparation errors (temperature and pH), poor hygiene and sanitation within the restaurant (Angulo et al., 2006). The poultry droppings are used as manure (Orji et al., 2005) and also for composting (Reza, 2016), which increases the risk of cross contamination if the flock is infected with a potential human pathogen. According to Hale et al. (2012), an estimated 3.2 million cases of gastrointestinal illnesses were caused by improper handling techniques of domesticated animals in the US.

The *Salmonella* infection in poultry is a significant public health concern (Lalsiamthara and Lee, 2017, Sadeyen et al., 2004). According to the CDC (2018), 334 cases of salmonellosis infections were linked to backyard poultry in the US. *Salmonella* can colonize the gastrointestinal tract of poultry and will be shed through faeces, contaminating the environment and the entire flock (Turner et al., 1998, Behravesh et al., 2014). The wide range of host adaptability and the ability to persist in the environment for longer periods of time makes controlling salmonellosis complicated (Chousalkar et al., 2016). Studies conducted in Korean (Im et al., 2015) and Canadian (Poppe et al., 1991) layer farms found that more than 50% of the chickens were infected with *Salmonella* which emphasise transmission of the zoonotic pathogens through contaminated environments. Studies conducted in Massachusetts, Iran (Jafari et al., 2007), Paraguay (Leotta et al., 2010) and in India (Samanta et al., 2014) involving 53, 35, 50 and 360 backyard chicken flocks respectively, confirmed *Salmonella* infection in chickens; with the percentage of positive samples varying from 2% to 14 %.

Campylobacter infection in poultry is common in farms as well as backyards. The review presented by Keener et al. (2004) reported that 50% to 70% of poultry were infected with *Campylobacter* and the increased infection rates could be due to elevated body temperature of the birds. Atanassova and Ring (1999) found that 41% (n= 509) of poultry flocks were positive for *Campylobacter* in Germany. Studies conducted in the US, Japan, Spain and Ireland confirmed *Campylobacter* sp. contamination in retail chicken meat (Murphy et al., 2006). Anderson et al. (2012) recorded that 58% (n=291) of the tested backyard chicken flocks were positive for *Campylobacter* in New Zealand. In Finland 45% (n=51) of the tested backyard chicken flocks were positive for *Campylobacter*. According to OzFoodNet (2006b), in 2005, 78 *Campylobacter* cases were recorded in Australia; among which 19% were linked to chicken based dishes.

In addition to *Salmonella* and *Campylobacter*, *Shigella* infection in poultry is also identified as a risk factor for public health and development mainly because of the low infectious dose (Cetinkaya et al., 2008). Humans can acquire shigellosis through cross contamination with poultry (Shi et al., 2014). A study conducted in Nigeria reported that 6% (n=360) of eggs tested positive for *Shigella* and could pose a threat to public health when eggs are consumed raw or under cooked.

Currently, there are very few studies conducted on backyard poultry in Australia (Chousalkar and Gole, 2016). Attitudes, behaviours, customs and beliefs, (Powell et al., 2011) cultural backgrounds, lack of knowledge, lack of awareness on new scientific developments (Ovca et al., 2017) and lack of motivation to change behavioural patterns (Cabana et al., 1999) on handling eggs, raw egg products and poultry in backyard chicken owners could increase the risk of foodborne illnesses. This paper describes the presence of *Salmonella, Campylobacter* and *Shigella* in domesticated poultry and provides insight into the practices and knowledge on handling poultry and eggs by the general public. Testing environmental samples for the presence of these organisms will help identify possible risk factors of keeping poultry in the backyards.

2 Materials and methods

2.1 Ethical clearance

Ethical clearance was obtained from the Flinders University Social and Behavioral Research Ethics Committee (Project ID 7737) (page number **328**).

2.2 Sample collection

Sample collection was carried out for 8 months from January to September 2019. Eighty-two samples were collected from 82 different backyard poultry flocks. Four samples were collected from Victoria and one each from New South Wales (NSW) and Tasmania. The other remaining samples were collected from South Australia (SA). The samples which were from Victoria, New South Wales, Tasmania and two samples from South Australia were collected as DESS (dimethyl sulfoxide, disodium EDTA, and saturated NaCl) (Beknazarova et al., 2017) preserved samples. Other poultry faeces samples were collected into sterile containers. All samples were transported into the Flinders University Environmental Health laboratory PC2 facility.

2.3 DNA extraction from culture isolates for the identification of *Salmonella* app

A gram of the faeces sample was mixed with 9 mL of buffered peptone water (Oxoid Ltd, Basingstoke, Hampshire, England) and allowed to stand for 10 min. A loop full of the mixture was inoculated on to an XLD agar (Oxoid Ltd, Basingstoke, Hampshire, England) plate. The plates were incubated overnight at 37°C (Memmert Incubator IN30). Following incubation subculturing was carried out to obtain pure cultures of the *Salmonella* like colonies. The DNA was extracted from a single colony of the pure culture according to the method stated in the section 2.4.

2.4   DNA extraction from poultry faeces

The DNA was extracted from the samples within a week of sample collection and was stored in the fridge (-8°C) until used. DNA was extracted from 0.25 g of the sample using the Qiagen DNeasy Power Soil kit (MO BIO Laboratories, Inc., USA) according to the manufacturer's instructions. Briefly, the sample was added to the power bead tube provided and was gently vortexed. The solution C1 was added followed by centrifugation and separation of the supernatant. C2 and C3 solutions were added sequentially? and were vortexed and incubated at 4^{0}C for 5 min separately. At the end of these steps the supernatant was separated. The C4 solution was mixed and added to the sample and the C4-sample mix was added to the provided spin column followed by centrifugation. The flow-through was discarded, and the column was washed with the C5 solution. The DNA was eluted from the column by adding the solution C6. The extracted DNA was stored at -20^{0}C until used.

2.5   Positive, negative and the non-template control for the PCR reactions

DNA extracted from chicken faeces negative for *Salmonella* was used as the matrix for spiking. The positive control for the identification of *Salmonella* spp. and *S.* Typhimurium contained DNA extracted from *Salmonella* negative poultry faeces spiked with DNA extracted from a *S.* Typhimurium (ATCC 53647) culture. For the identification of *Campylobacter* spp*.,* the positive control contained DNA extracted from a pure culture of *C. jejuni* (ATCC 33291).   The positive control for the identification of *Shigella* spp. contained a 157 bp oligonucleotide strand which contained the primer and the probe sequence (IDT integrated DNA technologies) (5'CCAACACCTTTTC CGCGTTCCTTGACCGCCTTTCCGATACCGTCTCTGCACGCAATACCTCCGGATTCCGTGAACA GGTCGCTGCATGGCTGGAAAAACTCAGTGCCTCTGGAGCTTCGACAGCAGTCTTTCGCTGTT GCTGCTGATGCCACTGA 3'). The negative control for the identification of these bacteria contained DNA extracted from a poultry faeces sample collected from a farm where routine testing is being

carried out and known to be negative for these organisms. The non-template controls were carried out for each reaction with double sterilized water.

2.6   Detection of *Salmonella* spp. using real time PCR

The real time PCR was carried out (Rotor-Gene Q, Qiagen real-time PCR cycler, France) using primers and probes specific for *Salmonella* spp. (**Table 2.2**). The specificity of the primers was checked using the BLAST search in the NCBI database. The *Salmonella* spp. target probe contained a fluorescent dye 6-carboxyfluorescein (FAM) at the 5' end and the 3' end contained the quencher. The amplification process consisted of 30 cycles with the following steps, activation, denaturation, annealing and extension with temperature ramping from 95^0C for 10 min, 95^0C for 10 s, 64^0C for 1 min, 64^0C for 1 min and 64^0C for 10 min respectively. A 20 µL PCR reaction contained, 10 µL probe super mix (SsoAdvanced Universal Probes Supermix), 1 µL *Salmonella* spp. primer mix, 4 µL double distilled water and 5 µL template DNA. A non-template control, a negative control and a positive control was used for the reaction. Each reaction was conducted in triplicate. The Ct values less than 25 but higher than 6 was considered as positive *Salmonella* isolates.

2.7   Detection of *S.* Typhimurium using real time PCR

S. Typhimurium specific primers were used for the analysis (**Table 2.2**). The samples positive for *Salmonella* spp. were screened for the presence of *S.* Typhimurium. The amplification process consisted of 30 cycles with the following steps, activation, denaturation, annealing and extension with temperature ramping from 95^0C for 10 min, 95^0C for 10 s, 64^0C for 1 min, 64^0C for 1 min and 64^0C for 10 min respectively. A 20 µL PCR reaction contained, 10 µL probe super mix (SsoAdvanced Universal Probes Supermix), 1 µL *S.* Typhimurium primer mix, 4 µL double distilled water and 5 µL template DNA. A non-template control, a negative control and a positive control was used for the reaction. Each reaction was conducted in triplicate. The Ct values less than 29 but higher than 6 was considered as positive *S. Typhimurium* isolates.

2.8   Detection of *Campylobacter* spp. using real time PCR

The real time PCR assay was conducted using the primers and the probes mentioned in **Table 2.2**. Each reaction contained 10 µL of the probe super mix (SsoAdvanced Universal Probes Supermix), 1 µL *Campylobacter* spp*.* primer mix and 4 µL of double distilled water. The total volume of the reaction was 20 µL which contained 5 µL template DNA. Each PCR run was conducted in triplicate and the amplification consisted of 40 cycles with the temperatures ramping from 95^0C for 15 s and 60^0C for 30 s with an initial denaturation step for 95^0C for 3 min. The Ct values less than 40 but higher than 7 was considered as positive *Campylobacter* isolates.

2.9   Detection of *Shigella* spp. using real time PCR

The probes and primers used are presented in **Table 2.2**. The real time PCR reaction contained an initial hold at 95°C for 10 min followed by 40 cycles of 95°C for 30 s and 60°C for 30 s. The initial denaturation step was for 20 s at 95°C. The total volume of each reaction was 20 µL which contained 5 µL template DNA, 10 µL probe super mix (SsoAdvanced Universal Probes Supermix), 1 µL *Shigella* spp. primer mix and 4 µL double distilled water. Each PCR run was conducted in triplicate. The Ct values less than 37 but higher than 6 was considered as positive *Shigella* isolates.

2.10 Analysis of the data collected from the questionnaire

The questionnaire contained 16 questions (page number **330**), which included the suburb and the postcode, how long the domestication of poultry was practised in the current location, the breed of poultry, the number of birds in the flock, whether the birds were single or multi aged, diagnosis of clinically proven salmonellosis cases in family members, feed, any control measures for *Salmonella*, whether the poultry were caged or free range, how the poultry droppings were disposed of, main purpose of domestication, consumption of raw or under cooked eggs, whether dirty eggs were used, washed or wiped, storage of eggs, and the presence of other domesticated animals. The data were analysed using chi-square analysis on the SPSS software.

Table 2.2: Primers and probes used for the real time PCR assay for the identification of *Salmonella* spp., *S.* Typhimurium, *Campylobacter* spp. and *Shigella* spp.

Species	Primers and probes	Product size (bp)	Target gene	Reference
Salmonella spp.	5' AGGCCTTCGGGTGTAAAGT 3' 5' GTTAGCCGGTGCTTCTTTG 3' 5' AACCGCAGCAATTGACGTTACCC 3'	97	16s rRNA	(Lee et al., 2009)
S. Typhimurium	5' TGCAGAAAATTGATGCTGCT 3' 5' TTGCCCAGGTTGTAATAGC 3' 5' ACCTGGGTGCGGTACAGAACCGT 3'	100	*fliC*	(Lee et al., 2009)
Campylobacter spp.	5'GAATGAAATTTTAGAATGGGG 3' 5'GATATGTATGATTTTATCCTGC 3' 5' TTTAACTTGGCTAAAGGCTAAGGCT 3'	358	*VS1*	(Yang et al., 2003)
Shigella spp.	5' CTTGACCGCCTTTCCGATA 3' 5' AGCGAAAGACTGCTGTCGAAG 3' 5' AACAGGTCCTGCATGGCTGGAA 3'	117	*ipaH*	(Ma et al., 2014)

3 Results

The questionnaire which was designed to assess the knowledge and the behavioural patterns of the backyard chicken owners discovered that only 1 participant domesticated chickens for meat and eggs while all the other participants domesticated poultry only for eggs. Within the study population, only 26% consumed raw or under cooked eggs. None of the participants or their family members were diagnosed with salmonellosis during the time they had poultry in their backyard. None of the samples were positive for more than one pathogen but 6% (n=5) and 10% (n=8) were positive for *Salmonella* spp. and *Campylobacter* spp. respectively.

The majority of the participants (96%) had no control measures for *Salmonella*. Only 18% had the birds confined in to one specific area in the backyard while the other participants (83%) allowed the poultry to roam around in the entire backyard. Among the samples that were positive for either *Salmonella* spp. or *Campylobacter* spp., 92% were allowed to roam around the backyard while only 8% of the chickens who were confined into a specific area were recorded as positive for a pathogen.

Higher temperatures (Namata et al., 2008) and wet weather conditions (Van Hoorebeke et al., 2010) could increase the amount of positive bacterial infections in hens. Similarly, as Australia is located in the Southern Hemisphere a higher percentage of positives were recorded during the warmer month of February (33%) and colder and wetter month of July (40%). Most of the participants (n=32) had 3-5 birds in their flocks and the positive percentage for a pathogen was 19% (n=5 out of 15 flocks). A significant percentage (38%) of positives were recorded in the flocks with more than 10 birds (n=5 out of 13 flocks) but none of the flocks with 1-2 birds had a positive infection (n=15). The majority of the backyard chicken flocks (63%) were multi-aged and had different species of chickens including Isa Brown, White Leghorn, Barnevelder, Australorp, Light Sussex, Silver Dorking, Rhode Island, Bantam, Heritage, Silkie, Araucana, Welsummer, Wyandottes, Plymouth rocks, Cochin and French Maran.

Poultry faeces were either spread on site (26%), used as manure (49%), disposed of in the green waste bin (for municipal waste collection) or just left. Some backyard chicken owners used poultry

droppings as manure or were spread on site (9%). Most of the participants (46%) washed their eggs if they were dirty and only 33% of the participants wiped the dirt off the eggs. A few participants (4%) discarded dirty eggs but 15% just used the eggs without washing or wiping the dirt off. If the eggs were cracked 79% discarded and 10% used the eggs, but a few participants (1%) washed the eggs. Most of the participants (81%) stored the eggs in the fridge while 19% stored the eggs at room temperature.

According to the data collected 26% of the backyard chicken owners consumed raw eggs or raw egg products. A considerable proportion (24%) of the study population both washed the eggs and stored at room temperature, and 47% of the population consumed raw egg products prepared with the eggs stored at room temperature. Another considerable proportion of the population (26%) washed the eggs and consumed raw egg products. These factors are all risk factors that increase the risk of gastrointestinal infection in humans.

Samples (n=82) were collected from domesticated backyard chicken flocks located in 73 different suburbs in Australia. Direct PCR for the DNA isolated from the collected faeces samples confirmed that 10% (n=8) were positive for *Campylobacter* spp. Identification of *Salmonella* by culturing methods followed by PCR was more sensitive than PCR carried out for the faeces samples due to the low detection limit. Direct PCR for the DNA extracted from the faeces resulted in 4% (n=3) positive samples while isolating the *Salmonella* like cultures followed by PCR resulted in 6% (n=5) positive samples. One of the culture isolates were identified as *S.* Typhimurium, and none were positive for *Shigella* spp. Fifteen percent of the samples (n=13) were positive for either *Salmonella* spp. *or Campylobacter* spp.

4 Discussion

Poultry, eggs and raw egg products are frequently linked with zoonotic human infections. The main cause of salmonellosis in humans has been identified as eggs and raw egg products (Cowden et al., 1989, White et al., 1997). Eggs can be contaminated via a contaminated environment which is characterized as the horizontal transmission of *Salmonella,* which is primarily seen with the *S.* Typhimurium species. However, *S.* Enteritidis is commonly recorded in the US and in Europe, but *S.* Typhimurium is the strain commonly recorded in Australia (Ford et al., 2018). Moreover, poultry and poultry products are also commonly linked with *Campylobacter* outbreaks (Hansson et al., 2018) and there are some studies demonstrating *Shigella* infection in poultry (Obi and Ike, 2018, Elmanama et al., 2018) and poultry meet (Uddin et al., 2019). None of the samples were positive for more than one pathogen. *Salmonella* and *Campylobacter* utilize the same colonization sites in the chicken gastrointestinal tract (Zhang et al., 2007). Competition for nutrients and the binding sites on the mucous membrane (Patterson and Burkholder, 2003) could be influencing the colonization of only one human pathogen in the gut environment of the chickens. Additionally, gut colonization of pathogenic bacteria in poultry can vary among different poultry species (Weerasooriya et al., 2017).

Contamination of the internal contents of an egg can take place with the movement of pathogenic bacteria through the porous egg shell into the internal contents, increasing risk of gastrointestinal diseases (Vlčková et al., 2018). Penetration of *S.* Typhimurium into the egg through the intact shell can also occur due to the thinning of the egg shell with the age of the hens (Crabb et al., 2019). Washing the eggs can damage the cuticle covering the porous egg shell further facilitating bacterial penetration (Gole et al., 2014). The majority of the participants washed the eggs, which could increase the penetration of bacteria into the egg. A considerable number of participants (19%) stored their eggs at room temperature. Storage of eggs at room temperature enable replication of bacteria inside the egg (Humphrey, 1994, Whiley et al., 2015). Washing eggs and storage at room temperature can increase the risk of infection if the eggs are consumed raw.

Competition for food and water can contribute towards decreased nutrition in poultry, leading to decreased immunity (Cheville, 1979). According to the results the flocks that contained 2-3 birds were not diagnosed with any of the pathogens tested, but 38% of the flocks that contained more than 10 birds were positive for either *Salmonella* or *Campylobacter*. This could be due to larger backyard flocks containing multi-aged birds including older birds. Older birds are more susceptible to infections and could increase infection rates as older birds can infect the younger birds (Hester, 2005). Data from this study support this, with higher infection rates recorded in multi-aged flocks (24% or n=12 out of 50 flocks) compared with the single-aged flocks (3% or n=1 out of 30 flocks).

The increasing popularity of domesticating poultry could contribute to the increase in salmonellosis, campylobacteriosis and shigellosis outbreaks. The lack of knowledge of the general public on proper handling of poultry and eggs could pose a risk, particularly to children, elderly and immunocompromised people. Therefore, it is important to introduce and implement new mechanisms to educate the general public on handling eggs, raw egg products and poultry to reduce the risk of infection and cross contamination of ready-to-eat food products. Backyard poultry could also potentially spread pathogenic bacteria to the neighbouring poultry farms. Thus, identification and characterization of bacterial strains that infect poultry could help recognize the potential mechanisms that could transfer pathogens to commercial chicken farms from backyards.

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Conflicts of Interest

The authors declare no conflict of interests.

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2.8 Concluding observations of the chapter

The experiments included in this chapter were conducted to identify the presence of zoonotic pathogens in backyard chickens. The risk factors associated with keeping poultry in domestic settings was also assessed. Washing eggs, storage of eggs at room temperature, consumption of raw eggs and having multi aged birds in the flock were identified as potential risk factors which could increase the risk of gastrointestinal illnesses. Spreading chicken faeces of infected hens on site as manure was identified as a potential risk factor that could contribute to the spread of these zoonotic pathogens. Effective educational approaches and strategies should be implemented to educate the general public on handling poultry and fresh eggs to reduce the incidence of gastrointestinal diseases linked to backyard poultry.

CHAPTER 3. A whole egg decontamination method using common commercial kitchen equipment to eliminate *S.* Typhimurium while maintaining egg properties

3.1 Overview of the chapter

The aim of this chapter was to develop a whole egg decontamination method to decontaminate eggs externally contaminated with *S*. Typhimurium. The method needed to preserve the usability and quality of the eggs and be easy to implement in a commercial kitchen.

This chapter consists of a published literature review titled "'Reducing risk of salmonellosis through egg decontamination processes" (Keerthirathne et al., 2017) (page number **68**) and a scientific paper titled "A successful technique for the surface decontamination of *Salmonella enterica* serovar Typhimurium externally contaminated whole shell eggs using common commercial kitchen equipment" (Keerthirathne et al., 2019b) (page number **120**) . This chapter also contains preliminary experiments and method development not included in the research paper.

CITATION

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Reducing risk of salmonellosis through egg decontamination processes

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Abstract

Eggs have a high nutritional value and are an important ingredient in many food products. Worldwide foodborne illnesses, such as salmonellosis linked to the consumption of eggs and raw egg products, are a major public health concern. This review focuses on previous studies that have investigated the procedures for the production of microbiologically safe eggs. Studies exploring pasteurization and decontamination methods were investigated. Gamma irradiation, freeze drying, hot air, hot water, infra-red, atmospheric steam, microwave heating and radiofrequency heating are all different decontamination methods currently considered for the production of microbiologically safe eggs. However, each decontamination procedure has different effects on the properties and constituents of the egg. The pasteurization processes are the most widely used and best understood; however, they influence the coagulation, foaming and emulsifying properties of the egg. Future studies are needed to explore combinations of different decontamination methods to produce safe eggs without impacting the protein structure and usability. Currently, eggs which have undergone decontamination processes are primarily used in food prepared for vulnerable populations. However, the development of a decontamination method that does not affect egg properties and functionality could be used in food prepared for the general population to provide greater public health protection.

1 Introduction

Egg yolk is an extremely nutritious (Jaekel et al., 2008), excellent food supplement providing most of the essential amino acids, vitamins A, B_3 (Powrie and Nakai, 1986), B_{12}, B_2, E (Surai and Sparks, 2001), folate and micronutrients including choline (Powrie and Nakai, 1986). Eggs are considered to be rich in fatty acids which influence the metabolism of the body and are consumed by a huge portion of humans (Surai and Sparks, 2001). Eggs are also considered as an important ingredient in several food products (Jaekel et al., 2008) because of their ability to produce and stabilize emulsions, their frothing constancy and their thermal gelation which are important factors during the preparation of food products (Gallegos and Franco, 1999, Dev et al., 2008).

The temperature-dependent pseudo-plastic rheological behaviour of eggs is a significant factor in their commercial applications (Telis-Romero et al., 2006). These properties of the egg are dependent on its protein structure, which is highly heat sensitive. Thus, the pasteurization of eggs should be carefully considered in order to prevent the denaturation of proteins when heated (Dev et al., 2008).

2 Is Decontamination of Eggs Essential?

Worldwide, salmonellosis is a major public health concern in developed as well as developing countries (Pires et al., 2014). In 2011, the Centers for Disease Control and Prevention reported that annually there are one million cases of salmonellosis in the USA (Mølbak et al., 1999). Even though several food items such as peanuts (Kirk et al., 2004), beef (Davies et al., 1996), pork (Mølbak et al., 1999) and chicken (Gieraltowski et al., 2016) have been associated with outbreaks of salmonellosis, the primary cause is raw eggs and egg products (Painter, 2013, Moffatt et al., 2016). According to Painter (Painter, 2013), in the United States from 1998 to 2008, 57% of *S.* Enteritidis outbreaks were linked to food products prepared using eggs. In Australia, the incidence of salmonellosis has increase from 57.7% from 2005 to 2015 with up to 45% of foodborne salmonellosis outbreaks being linked to eggs (Moffatt et al., 2016, Department of Health, 2016). In the UK, from 2008 to 2009 there were approximately 38,600 cases of salmonellosis (Quick et al., 2015). However, across the

European Union there has actually been a 50% decrease in the incidence of salmonellosis from 2005 to 2009 due to extensive control procedures carried out along the food chain (Schuman et al., 1997). This includes the British Lion Code of Practice which has significantly improved British egg safety and resulted in a major reduction in the risk of salmonellosis from eggs (Cao et al., 2009). Despite this reduction, in 2014 there was still one death and 286 reported incidences of salmonellosis associated with a hospital canteen and three restaurants in the UK which were linked to eggs from a German producer (Inns et al., 2015).

Raw eggs are used in many food products such as homemade ice cream, mayonnaise and cold desserts (Duguid and North, 1991). Contaminated eggs which have not undergone heat treatment or other decontamination processes may present a potential risk to public health (Reynolds et al., 2010, Moffatt et al., 2012, Dyda et al., 2009). Eggs can be contaminated during different stages of their formation, processing and packaging (De Reu et al., 2006, James et al., 2002). Vertical or transovarian contamination of eggs can happen during the formation of the egg when the ovaries of the hen are infected and horizontal contamination can occur if the eggs come into contact with a contaminated environmental source (De Reu et al., 2006).

In many countries it is not presently feasible to guarantee that eggs are produced free of *Salmonella* contamination and post-harvest control methods including pasteurization are essential for reducing the risk of foodborne illness (Whiley and Ross, 2015). Decontamination of eggs can be done using several means such as irradiation (Seregély et al., 2006), acidic electrolyzed water (Huang et al., 2006) and high pressure carbon dioxide processing (Garcia-Gonzalez et al., 2009), which are all non-thermal methods (Seregély et al., 2006). Additionally, pasteurization methods include freeze drying (Jaekel et al., 2008), hot air, hot water, infra-red, and atmospheric steam (James et al., 2002), microwave heating and radiofrequency heating (Kannan et al., 2013). These pasteurization methods all utilize different temperature conditions (**Table 3.1**) (Dev et al., 2008).

Table 3.1: The effect of the pasteurization and decontamination methods and the effect of the method on *Salmonella* spp.

Method	Effect on Egg Properties	Methods Used to Determine the Quality of the Egg	Reduction of *Salmonella* Cells	Reference
Microwave oven power 9 for 15 s.	No effect on egg quality	Interior quality of eggs was determined by Haugh unit, albumen index, yolk index. Functional property was determined by foam volume and foam stability. Albumen and yolk viscosity were determined using a Brookfield viscometer. Sensory evaluation for appearance, texture, flavor and overall acceptability was performed using seven-point Hedonic scale ranging from 7 (like very much) to 1 (dislike very much).	From 10^8 cells/mL to 0.08 colony forming units CFU/mL on intact whole table eggs (1.2 log CFU/mL)	(Shenga et al., 2010)
Hot air temperature: 550–650 °C; 10 m/s. Cold air temperature: 20–25 °C; 40 m/s; 32 s. Revolving frequency of the eggs: 1.2 Hz.	No negative effects on the main quality traits of egg	The albumen pH was measured by a pH meter. Thermocoagulation of albumen (turbidity of albumen) was determined by the transmission measured by a spectrophotometer at 600 nm and turbidity of the albumen was calculated.	From 2.8×10^8 CFU/mL to 0.65×10^8 CFU/mL on intact whole table eggs	(Pasquali et al., 2010)
Hot air oven/55 °C for 2 h and moist heat 57 °C for 15 min.	No adverse effects on egg quality or sensory properties	Egg albumen pH, measuring thiobarbituric acid (TBA) value in egg yolk, albumen protein solubility were determined by the procedures adapted from previous literature.	Inoculated *S*. Typhimurium cells (10^8 cells/mL) were brought down to 0.3	(Shenga et al., 2010)

		Interior quality of eggs was measured by the Haugh unit, albumen index, yolk index. Functional property was measured using foam volume and foam stability. Albumen and yolk viscosity were determined directly, using a brookfield viscometer.	CFU/mL in intact whole table eggs	
Hot dry air (hot air oven, 55 °C/180 min) and water bath (57 °C/25 min).	Overall functionality of pasteurized intact whole table eggs are acceptable under the heating conditions defined in this study	Haugh unit was used to measure the Interior quality of eggs. The pH of the egg white was measured by a pH meter. Viscosity was measured with a Brookfield digital viscometer. Turbidimetric measurements were done on a spectrophotometer at 600 nm. The yolk index reflects were used spherical shape of egg yolk.	From 10^6–10^7 CFU/mL to 0.8 CFU/mL (7 log) reductions on intact whole table eggs	(Hou et al., 1996)
Moist heat treatment of 50–57 min at 58 °C and 65–75 min at 57 °C.	Yolk and albumen pH were unaffected following treatment; no difference in the sensory and functional properties	Yolk index was used to determine the shape of the yolk, Haugh unit values were used to determine the properties of albumen.	From 3×10^8 CFU of *S.* Enteritidis to *Salmonella* free intact whole table eggs	(Schuman et al., 1997)
A study conducted using RF (10 MHz–3 GHz) heating at	Though egg shell and shell membrane were highly transparent to RF with increase in	Viscosity was measured with a Brookfield digital viscometer. The amount of protein coagulation was measured by the spectrophotometer.	Not given Performed on intact whole table eggs	(Kannan et al., 2013)

temperatures 5 °C–56 °C.	the heating rate, viscosity and foam stability decreased while turbidity and coagulation increases.			
2 min of treatment with slightly acidic electrolysed water containing 4 mg/L of chlorine, in the pH 6.3–6.5.	AEW did not significantly affect albumen height or eggshell strength; however, there were significant effects on cuticle presence	Not given.	From 10^8 CFU/mL to 0.7 CFU/mL (4.9–5.0 log CFU/mL) on intact whole table eggs	(Cao et al., 2009)
0.5 kGy of gamma radiation.	No effects to the egg quality	The contents of moisture, total protein, ash, total lipids, protein solubility, free sulfhydryl, pH, total carotenoids, quantitative determination of amino acids, free fatty acids and peroxide value was determined according to the previous literature. Sensory evaluation was conducted for their appearance, color and odor.	Elimination of *S.* Enteritidis in liquid egg white and yolk	(Badr, 2006)
Microwave oven power 9 for 15 s.	No effect on egg quality	Interior quality of eggs was determined by Haugh unit, albumen index, yolk index. Functional property was determined by foam volume and foam stability. Albumen and yolk viscosity were determined using a Brookfield Viscometer	From 10^8 cells/mL to 0.08 CFU/mL in intact whole table eggs (1.2 log CFU/mL)	(Shenga et al., 2010)

		Sensory evaluation for appearance, texture, flavor and overall acceptability was performed using seven-point Hedonic scale ranging from 7 (like very much) to 1 (dislike very much)		
Hot air temperature: 550–650 °C; 10 m/s. Cold air temperature: 20–25 °C; 40 m/s; 32 s. Revolving frequency of the eggs: 1.2 Hz.	No negative effects on the main quality traits of egg	The albumen pH was measured by a pH meter. Thermocoagulation of albumen (turbidity of albumen) was determined by the transmission measured by a spectrophotometer at 600 nm and turbidity of the albumen was calculated.	From 2.8×10^8 CFU/mL to 0.65×10^8 CFU/mL on intact whole table eggs	(Pasquali et al., 2010)
Hot air oven/55 °C for 2 h and moist heat 57 °C for 15 min.	No adverse effects on egg quality or sensory properties	Egg albumen pH, measuring thiobarbituric acid (TBA) value in egg yolk, albumen protein solubility were determined by the procedures adapted from previous literature. Interior quality of eggs was measured by the Haugh unit, albumen index, yolk index. Functional property was measured using foam volume and foam stability. Albumen and yolk viscosity were determined directly, using a Brookfield Viscometer.	Inoculated $S.$ Typhimurium cells (10^8 cells/mL) were brought down to 0.3 CFU/mL on intact whole table eggs	(Shenga et al., 2010)
hot dry air (hot air oven, 55 °C/180 min) and water bath (57 °C/25 min).	Overall functionality of pasteurized intact whole table eggs are acceptable under the	Haugh unit was used to measure the interior quality of eggs. The pH of the egg white was measured by a pH meter.	From 10^6–10^7 CFU/mL to 0.8 CFU/mL (7 log) reductions.	(Hou et al., 1996)

heating conditions defined in this study	Viscosity was measured with a Brookfield digital viscometer.
	Turbidimetric measurements were done on a spectrophotometer at 600 nm.
	The yolk index reflects were used spherical shape of egg yolk.

3 Pasteurization of Eggs

Heating food products using microwaves is an easy and accessible method (Salazar-González et al., 2012). However, the distribution of the absorbed energy is affected by the shape and composition of the food, surface area and equipment used for the processing (Fu, 2004). Additionally, when using microwaves for heating eggs, it was found that at higher power levels, the eggs tended to burst. However, this could be avoided by using low power levels and slower heating (Dev et al., 2010).

According to a study done by Dev et al. (Dev et al., 2008), the eggshell and shell membrane showed great transparency to microwaves. Pasteurization of intact whole table eggs was achieved by heating the eggshell until the yolk reached 61.1°C (ensuring the broad end of the eggs were placed facing upwards). Microwaves are more effective in producing high quality pasteurized eggs in a short time period when compared with water bath pasteurization (Dev et al., 2008). This is supported by another study conducted in 2010, which described that microwaves (microwave oven/power 9 for 15 s) were able to artificially reduce inoculated *S.* Typhimurium cells from a concentration of 10^7 cells/100 μL to 1.2 log CFU/mL in intact whole table eggs (Shenga et al., 2010). However, further investigations need to be conducted in order to explore the effectiveness of microwaving with regard to confounding factors such as egg geometry, dielectric properties and size on the interior heating process (Dev et al., 2008). For instance, according to Denys et al. (Denys et al., 2004), the natural convection formed by the albumen of the egg, which is of relatively low viscosity, has an increased influence on the distribution of temperature while pasteurizing the intact eggs by the means of steam or water. Thus, pasteurization of eggs should take place under low temperatures and requires multifaceted optimization (Denys et al., 2004). According to Guilmineau and Kulozik (Guilmineau and Kulozik, 2007), there is a gap between understanding the impact of pasteurization conditions on the egg yolk and the properties of the final product when the emulsions are concerned because it can affect the protein functions in a negative manner.

According to the recommendations of the United States Department of Agriculture, pasteurization of egg yolk, egg white and filtered liquid whole egg using heat should be done at a minimum temperature and holding time of 60 °C for 6.2 min, 55.6 °C for 6.2 min and 60 °C for

3.5 min, respectively (USAD, 1969). Pasquali et al. (2010) showed that pasteurization of intact whole table eggs can be achieved by heating to 60 °C for 8 s with the use of hot air generators while the eggs were rotating and rolling by mechanical means, followed by a treatment of cold air (20–25 °C) for 32 s. This treatment showed a significant reduction in *S.* Enteritidis on the shells of the eggs while the quality of the eggs was not affected.

There have been several studies comparing the effects of pasteurization of eggs using either dry heat or water bath (Shenga et al., 2010, Hou et al., 1996). In 2010, Shenga et al. (Shenga et al., 2010) investigated dry heat (hot air oven/ 55°C for 2 h) and moist heat (circulating water bath/57 °C for 15 min) for the pasteurization of shell eggs. Following the pasteurization process of the artificially inoculated eggs (*S.* Typhimurium 6 log CFU /mL), the cell counts were reduced to 2.1 log CFU/mL and 2.0 log CFU /mL by dry heat and moist heat pasteurization, respectively. Another study by (Hou et al., 1996) presented that intact whole table eggs can be pasteurized by heating in a circulating water bath (57 °C/25 min) to reduce *S.* Enteritidis (ATCC 13076) cells from 10^6–10^7 CFU/mL to 10^3–10^4 CFU/mL. However, only a 10^1–10^2 CFU/mL reduction of *S.* Enteritidis was observed using hot air (hot air oven, 55 °C/180 min). Combining the two methods (57 °C for 25 min moist heat treatment and 55 °C for 60 min of hot-air heating) provided a 7 log reduction in *S.* Enteritidis cells.

According to James et al. (James et al., 2002), surface decontamination of eggs can be done by immersing them in water heated to 95 °C for 10 s without the eggs being cracked. Muriana et al. (Muriana et al., 1996) states that exposure to temperatures over 70 °C for less than 1.5 s results in a 6 log reduction of *S.* Enteritidis in liquid eggs.

Furthermore, Shenga et al. (Shenga et al., 2010) used internally inoculated egg yolks (10^7 cells/100 μL) and applied dry heat for 2 h (55°C) and moist heat for 57°C (15 min), and suggested that the moist heat pasteurization method is superior as it reduced the *S.* Typhimurium cells in a shorter period of time compared to dry heat pasteurization and did not affect the properties of the egg albumen.

Gaseous ozone is another decontamination method (Perry et al., 2011, Donner, 2011). However, according to Perry et al. (2011), the treatment of intact whole table eggs with moist heat (56 ± 0.1

°C/10 min), followed by a treatment of gaseous ozone, was found to be less effective on albumen when compared with the conventional heat pasteurization process.

Radio frequency (RF) heating is another encouraging application in food processing because of the rapid and uniform heat dissemination, increased penetration, as well as low energy consumption (Piyasena et al., 2003). A study conducted using RF (10 MHz–3 GHz) heating at temperatures of 5–56 °C indicated that the eggshell and shell membrane were highly transparent to RF and further studies on egg decontamination using RF should be conducted (Kannan et al., 2013).

Despite pasteurization being the best understood and most widely available decontamination method, it may have an impact on the important properties of the eggs such as coagulation, foaming and emulsifying (Unluturk et al., 2008). The protein denaturation process in an egg is dependent on the temperature and the heating time (Raikos et al., 2007). Gel formation and aggregation of the proteins is a multi-stage, complex process dependent on protein concentration, ionic strength, pH as well as interactions with other surrounding components present in the environment (Yasuda et al., 1986). Thus, alterations of these factors can affect the egg proteins and the rheological properties (Raikos et al., 2007). A study conducted by Raikos et al. (Raikos et al., 2007) reported that sugar and salt can increase the temperature transition of the egg proteins and increased the temperature in which they were gelatinized.

Liquid yolks were freeze dried in a series of various temperature conditions (6.3 °C/2 h, 45 °C/1 min, −25 °C/5 h) and the study reported that there were no changes in the properties of the thawed yolk when compared to fresh yolk (Jaekel et al., 2008).

4   Non-Thermal Treatments for the Decontamination of Eggs

Acidic electrolyzed water can be used in the food processing industry (Reynolds et al., 2010). A study conducted by Cao et al. [39] reported that treatment of intact whole table eggs with slightly acidic electrolyzed water containing 4 mg/L of chlorine at pH 6.3–6.5 inactivated the pure *S.* Enteritidis cultures within 2 min. Another egg decontamination method is irradiation (Donner, 2011b). Farkas (Perry et al., 2011) states that the sensory and functional properties of the eggs are radiation sensitive. However, as an alternative for heat pasteurization, irradiation has been used to control *Salmonella* from egg products (Huang et al., 1997). According to a study done by Badr (Badr, 2006), the optimum dose for treating liquid egg white and yolk without affecting the chemical and sensory properties is 3 kGy of gamma irradiation under room temperature followed by storage at 4 ± 1 °C. Additionally, in 1997 it was also concluded that elimination of *S.* Enteritidis from the surface of the eggshell could be achieved with a minimal dosage of 0.5 kGy without any noteworthy effects to the egg quality (Serrano et al., 1997). According to Neal (Neal, 1965), radiation is better suited for the decontamination of frozen eggs and egg albumin than pasteurization. Moreover, Serrano et al. (Serrano et al., 1997) also stated that a 4 $\log_{10}$ reduction of *Salmonella* can be obtained by a 1.5 kGy dosage of irradiation in intact whole table eggs and liquid eggs without any effect on the colour and thermal characteristics. Interestingly, it is also stated that irradiation at the dose of 0.5 and 5 kGy had no effect on the viscosity of the yolk-egg products, but with the irradiation dosage of 2 kGy, the viscosity was found to be decreasing. However, minor degradations of higher molecular weight proteins except for albumin were found (Pinto et al., 2004). Nevertheless, irradiation could increase the production of free radicals due to the lipid peroxidation, and as eggs are high in poly-unsaturated fatty acids, the production of increased lipid peroxidation products could decrease the consumer acceptability (Du et al., 1999). However, damage to the microorganisms at a sub-lethal level could be attained during irradiation, thus increasing their susceptibility to the environmental stresses (Farkas, 1998). Moreover, oxygen-free packaging for the foods that are sensitive to radiation could reduce the oxidation, consequently improving the possibility of the radiation treatment while retaining the flavour (Farkas, 1998).

Another alternative for the pasteurization is high pressure carbon dioxide processing (13.0 MPa, 45 °C, 50% working volume ratio and 400 min^{-1} stirring speed during 10 min), which has been demonstrated to increase the shelf life of liquid egg (Garcia-Gonzalez et al., 2009).

5 Conclusions

Currently, decontaminated eggs are commonly used to protect high-risk populations from salmonellosis, for example in food prepared for aged care facilities or hospitals. However, raw eggs and egg products are still a major cause of foodborne illness; as such, the use of decontaminated eggs should be more widely considered. This review presents the currently available methods for egg pasteurization and decontamination. Further studies should be performed to identify the precise dosage of irradiation as well as the possible temperature gradients that could be commercialized to obtain *Salmonella*-free eggs. Additionally, combinations of different pasteurization and decontamination methods need to be explored to potentially produce microbiologically safe eggs without impacting their usability.

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3.2 Method development

Prior to testing the decontamination method using artificially contaminated eggs (reported in the paper "A successful technique for the surface decontamination of *Salmonella enterica* serovar Typhimurium externally contaminated whole shell eggs using common commercial kitchen equipment" (Keerthirathne et al., 2019b).

Experiments were conducted to determine the survival of *S.* Typhimurium standard strain (ATCC 53647) and an infectious strain (wild type) *S.* Typhimurium strain when placed in water at 57°C. The optimum method to artificially contaminate eggs with *S.* Typhimurium was also assessed.

3.3 Investigating the survival of S. Typhimurium (ATCC 53647) after heat treatment at 57 °C

The ability of the *Salmonella* strains to withstand heat is a significant concern within the food industry (Podolak et al., 2010). Therefore, the survival of *S.* Typhimurium (ATCC 53647) was determined in water heated to 57°C before testing the decontamination method against artificially contaminated eggs.

3.3.1 Methods

An overnight *S.* Typhimurium (ATCC 53647) broth culture was centrifuged at 1,400 g for 15 min. The pellet was washed by resuspending in peptone water and re-centrifuging. Optical density at 600 nm (OD_{600}) was adjusted to 1 to obtain a culture concentration ~10^9 CFU/mL. The culture was then serially diluted using peptone water to obtain a culture at a concentration of 10^5 CFU/mL which was used for the experiment.

Five mL of the culture was added to a zip lock bag and was placed in a temperature-controlled water bath (Sunbeam® Duos Sous-Vide & Slow Cooker) for 10 s, 30 s, 1, 2, 3 and 3.5 min at 57°C. Following the heat treatment, the broth cultures were spread plated on to XLD agar (100 µL) and incubated overnight at 37°C. All the steps were conducted in triplicate.

3.3.2 Results

S. Typhimurium (ATCC 53647) was not able to be cultured after 30 s at 57°C indicating higher sensitivity to temperature than the *S.* Typhimurium infectious strain (**Figure 3.1**).

3.4 Investigating the survival of *S.* Typhimurium infectious strain after heat treatment at 57 °C

3.4.1 Methods

The *S.* Typhimurium infectious strain (wild type) in the stationary phase (10^5 CFU/mL) was used for the experiment (the concentration was adjusted as described above). Five mL from the culture was added to a zip lock bag. The bag with the culture was placed in sous-vide cooking appliance with water heated to 57°C for 30 s, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, and 25 min (21 different time periods). This step was conducted in triplicate.

Growth on XLD agar

Hundred µL from the heat-treated broth cultures for 30 s, 1, 1.5, 2, 2.5 and 3 min was plated on XLD agar.

Resuscitation of *Salmonella*

 From the 3 min onwards 1 mL was added in to 9 mL peptone water and was incubated overnight at 37°C which was also conducted in triplicate to check for resuscitation of *Salmonella* cells.

Confirmation of *Salmonella* growth

The broth cultures with growth was were plated on XLD following overnight incubation to confirm the growth of *Salmonella*.

3.4.2   Results

The culturability of the infectious strain was observed following 3 min at 57°C (**Figure 3.1**). Though the culturability was lost resuscitation of the cells were observed following 10, 11, 12 and 13 min at 57°C (**Table 3.2**).

Table 3.2: The resuscitation of _Salmonella_ cells in peptone water following heat treatment at 57°C when a broth culture of the infectious strain was placed in water heated to 57°C in a zip lock bag. Resuscitation was observed following 13 min at 57°C, but no resuscitation was seen following 14 min till 25 min at 57°C

Time	Resuscitation/ enrichment Bag 1			Resuscitation/ enrichment Bag 2			Resuscitation/ enrichment Bag 3		
	Broth culture 1	Broth culture 2	Broth culture 3	Broth culture 1	Broth culture 2	Broth culture 3	Broth culture 1	Broth culture 2	Broth culture 3
10 min	R	-	-	-	-	-	R	-	-
11 min	-	-	-	-	-	-	-	-	-
12 min	R	R	-	-	-	-	-	-	R
13 min	-	-	-	-	-	-	R	-	-
14 min	-	-	-	-	-	-	-	-	-
15 to 25 min	-	-	-	-	-	-	-	-	-

***R**- Positive resuscitation / **-** - No resuscitation
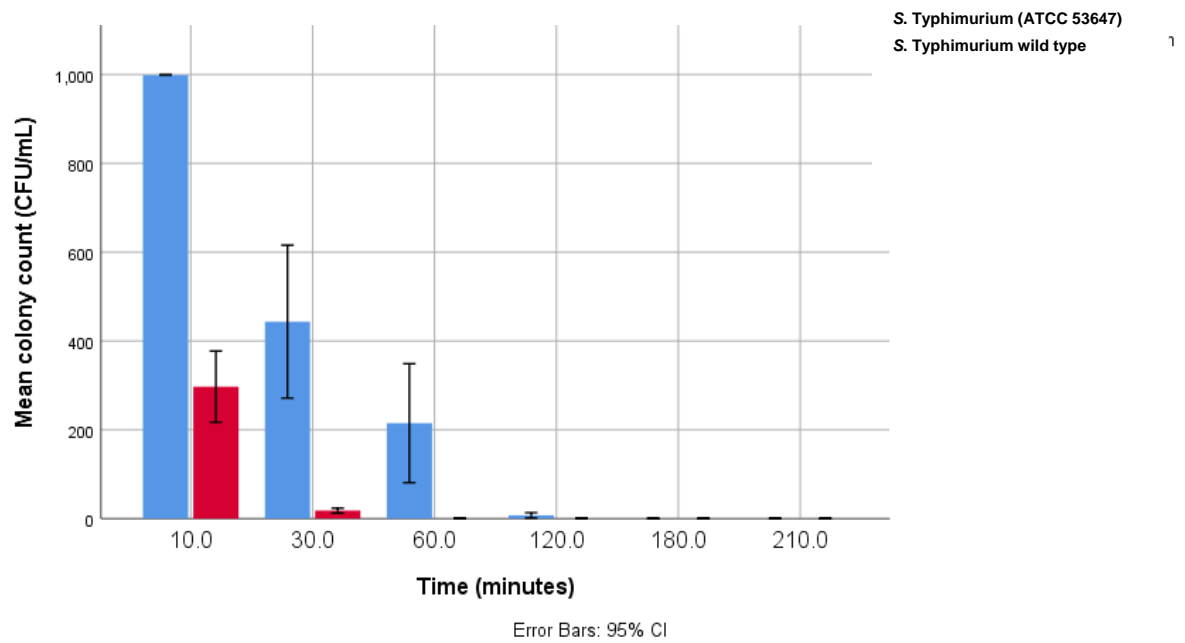
Figure 3.1: Survival of *Salmonella* following heat treatment at 57ºC when a broth culture of *Salmonella* was placed in water heated to 57ºC in a zip lock bag. The infectious strain culturability was more persistent than the *S*. Typhimurium (ATCC 53647) of the on XLD agar persisted longer than the standard strain at 57ºC

Though the culturability of the standard strain was lost following 30 s at 57°C the culturability of the infectious strain lasted for 3 min at the same temperature (**Figure 3.1**), demonstrating that the infectious strain was more heat tolerant than the standard strain.

3.5 Determining the optimum method to artificially contaminate the surface of whole eggshells with *Salmonella*

The following experiment was conducted to detect the effect of decontaminating the eggshell with 70% ethanol prior to the experiments.

3.5.1 Methods

S. Typhimurium (ATCC 53647) culture in the exponential phase was centrifuged at 1,400 g for 15 min and the pellet was washed with peptone water. The spectrophotometric reading was adjusted to (OD_{600}) 1 (~10^9 CFU/mL). This culture was serially diluted using peptone water to obtain a concentration 10^5 CFU/mL which was used for the experiment.

Six commercially available extra-large caged eggs were used for the experiment. Eggs with cracks or dirt were discarded and clean uncracked eggs were used for the study. Three eggs were sterilized by placing the eggs 70% ethanol for 30 s. The eggs were dried at room temperature under aseptic conditions and placed in the *Salmonella* culture for 1.5 min to artificial inoculate the eggs. The remaining 3 eggs were not treated with 70% ethanol prior to the inoculation. The contamination of the eggs was carried out by placing the eggs in the *S.* Typhimurium (ATCC 53647) culture (10^5 CFU/mL) for 1.5 min.

The intact shell, internal contents and the crushed shells were processed in peptone water according to the methods described in the paper "A successful technique for the surface decontamination of *Salmonella enterica* serovar Typhimurium externally contaminated whole shell eggs using common commercial kitchen equipment" (page number **120**) (Keerthirathne et al., 2019b)

3.5.2 Results

The treatment with the 70% ethanol increased the permeability of the eggshell and increased the penetration of the *Salmonella* cells (**Figure 3.2**). The eggshell cuticle is a proteinaceous layer which protects the egg from bacterial contamination, spoilage and loss of moisture and freshness (D'Alba et al., 2017, Hutchison et al., 2004). Ethanol can denature the properties of the cuticle allowing bacteria to easily penetrate the egg. Therefore, the experiments conducted to develop the whole shell egg decontamination procedure omitted sterilizing the eggshell with 70% ethanol prior to artificial contamination with *Salmonella*. A negative control (eggs with no artificial contamination) was used during the experiments to determine if the eggs were contaminated prior to the experiments.
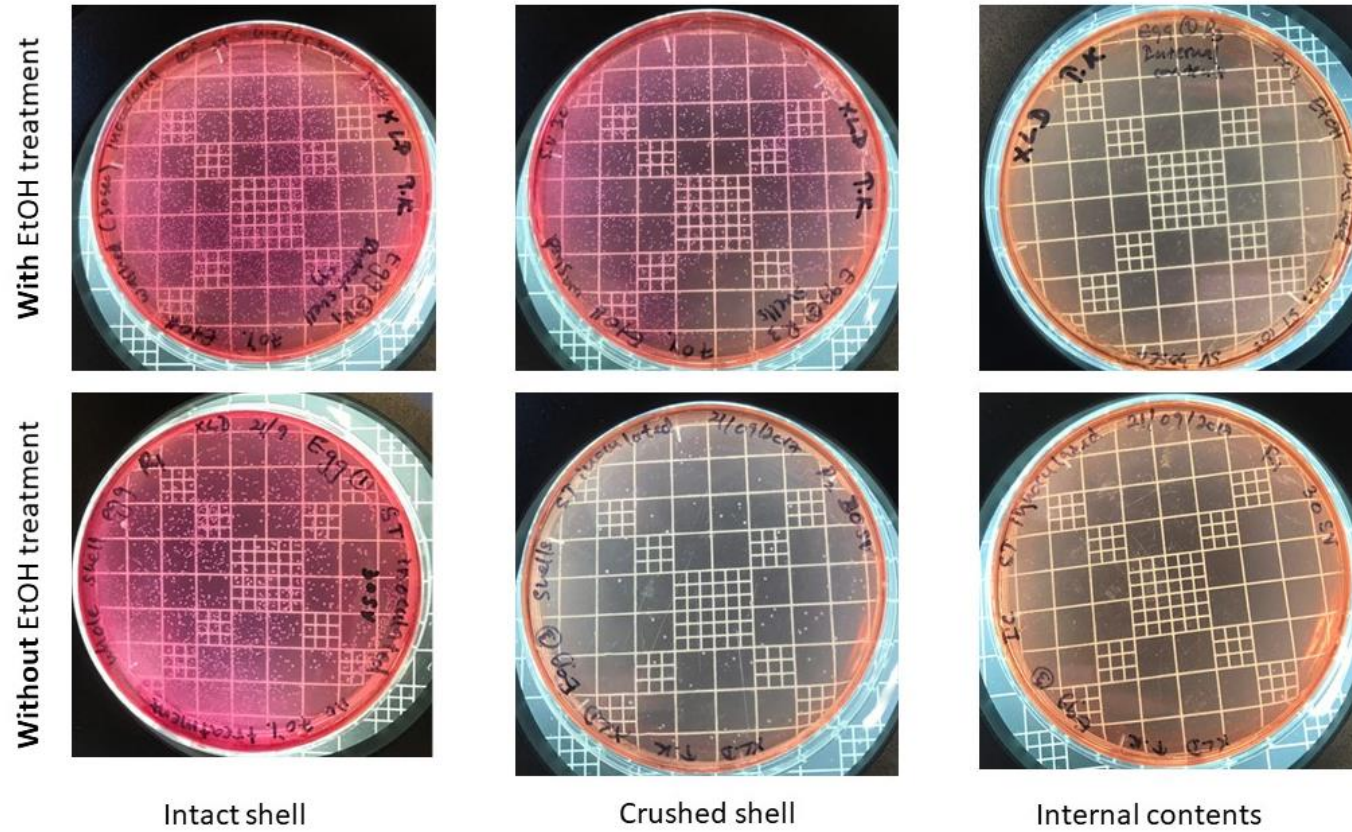
Figure 3.2: The growth of *Salmonella* colonies observed with and without the 70% ethanol treatment of the eggs prior to the experiments. The number of *Salmonella* colonies observed in the eggshell membrane and the internal contents of the 70% ethanol treated eggs were higher than the eggs without the ethanol treatment. Pre-treating the eggs with 70% ethanol may have disrupted the eggshell cuticle which allowed facilitated the penetration of *Salmonella* cells into the eggshell membrane and the internal contents

3.6 Measuring the internal temperature of the whole egg during incubation

The egg yolk and the plasma are sensitive to heat (Le Denmat et al., 1999) and denaturation of the egg albumen could impact the usability of the eggs (Campbell et al., 2003). According to Hou et al. (1996) immersing eggs in water heated to 57°C for 25 to 30 min could denatured the egg albumen. As heat has been shown to impact the physical properties of eggs, the internal temperature of the egg during the treatment in the 57°C sous-vide water bath was measured.

3.6.1 Methods

The internal temperature of the egg was measured using a Hobo temperature logger (U12 4-External Channel Outdoor/Industrial Data Logger, Onset Computer Corp., Bourne, MA). A hole through the eggshell was drilled (**Figure 3.3**), and the thermistor was inserted into the egg and sealed using hot, quick drying glue (**Figure 3.4**). The egg was placed in water heated to 57°C and the internal temperature measured and recorded every minute until the internal temperature of the egg reached 57°C.

Figure 3.3: Eggs were drilled to insert the temperature rod of the HOBO temperature logger to observe the internal temperature changes during treatment in the sous-vide water bath at 57°C

Figure 3.4: The egg sealed with hot, quick dying glue after placing the thermistor into the egg prior to placing the eggs with the thermistor in the sous-vide water bath

3.6.2 Results

The changes in the internal contents of the eggs were tested, it took 28 min for the internal temperature of the egg to reach 57°C and the internal temperature reached 50°C following 9 min at 57°C (**Figure 3.5**).
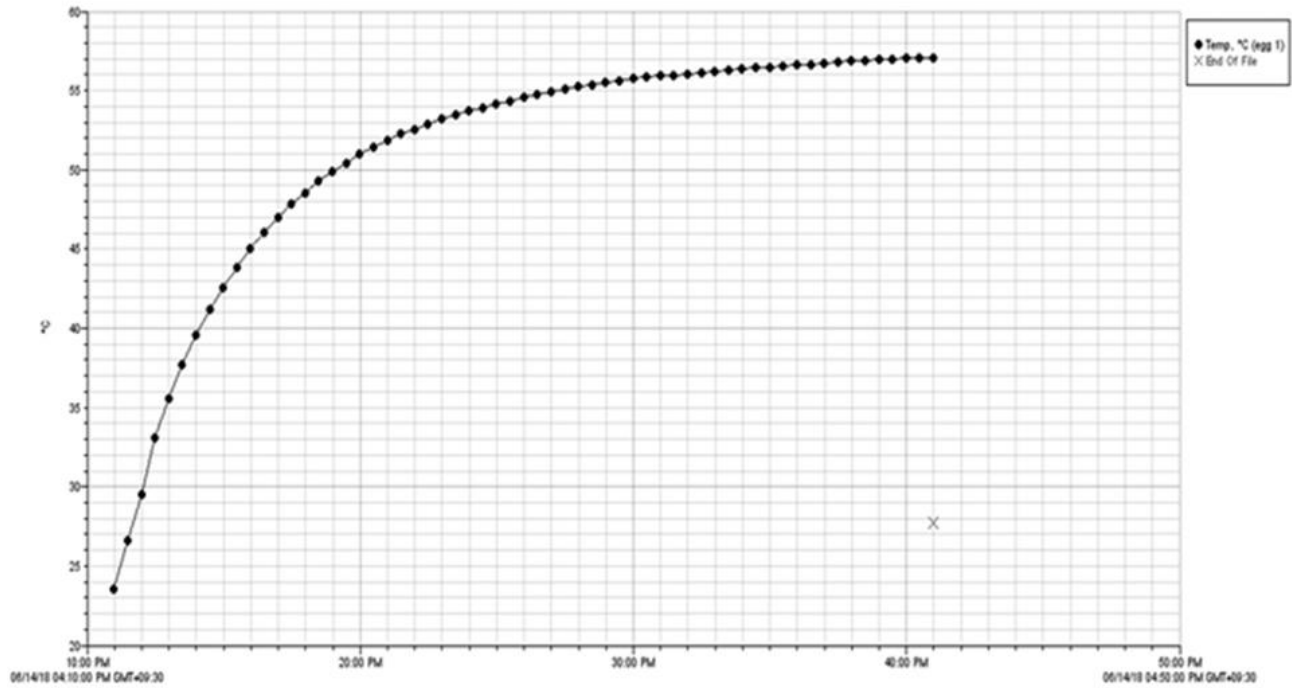
Figure 3.5: The graph generated using the HOBO software which shows the internal temperature change in the egg when placed in the sous-vide water bath heated to 57°C

3.7 Surface decontamination of whole shell eggs externally contaminated with *S.* Typhimurium

S. Typhimurium is the strain commonly associated with outbreaks of *Salmonella* linked to eggs and raw egg products (De Buck et al., 2004, Firestone et al., 2007, OzFoodNet, 2009). *S.* Typhimurium commonly contaminate the shells of the eggs (Martelli and Davies, 2012, De Buck et al., 2004). Therefore, the decontamination method was developed to decontaminate the eggshell surface.

3.7.1 Methods

The methods are explained in detail in the paper "A successful technique for the surface decontamination of *Salmonella enterica* serovar Typhimurium externally contaminated whole shell eggs using common commercial kitchen equipment" (page number **120**) (Keerthirathne et al., 2019b).

The method was validated with two strains of *S.* Typhimurium a standard strain (ATCC 53647) and the wild type, infectious *S.* Typhimurium isolate. A sous-vide cooking appliance was used for the method (**Figure 3.6**). The eggs were contaminated by immersing them in *S.* Typhimurium culture for 90 s (**Figure 3.7**), air dried and placed in the at 57°C (**Figure 3.8**).

Figure 3.6: The sous-vide cooking appliance used for the experiments
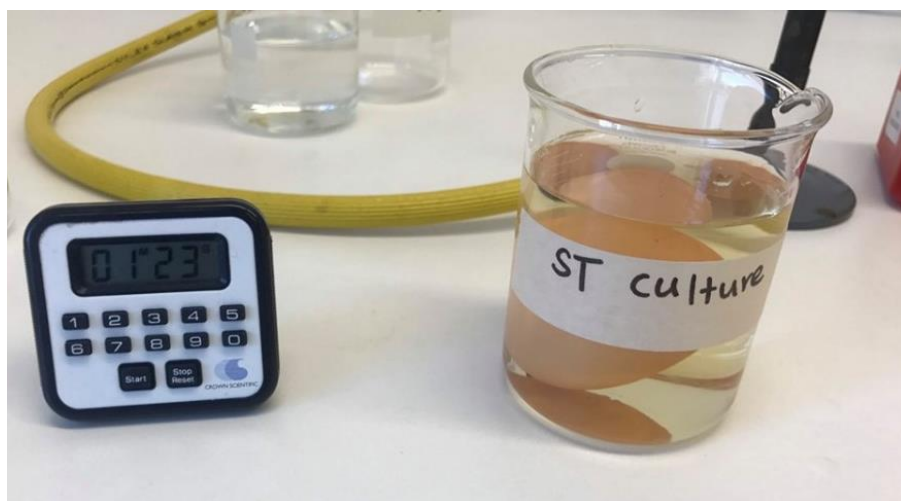
Figure 3.7: Egg immersed in the *Salmonella* culture for 90s to artificially inoculate the eggshell with *Salmonella*

Figure 3.8: Eggs immersed in a commercial sous-vide water bath at 57°C to decontaminate the eggshell artificially contaminated with *Salmonella*

Following the heat treatment, the intact whole egg was placed in a zip lock bag (**Figure 3.9**) and massaged in 10 mL buffered peptone water for 1 min followed by spread plating on XLD agar. The shells were crushed in 10 mL buffered peptone water followed by spread plating on XLD agar (**Figure 3.10**). The eggs were removed, and the internal contents were homogenized and 2 mL of this solution was added to 8 mL buffered peptone water and spread plated on XLD agar (**Figure 3.11**). An enrichment step was carried out to increase the limit of detection and to detect possible resuscitation of *Salmonella* cells (**Figure 3.12**).

Figure 3.9: The heat-treated egg was placed in a zip lock bag containing 10 mL buffered peptone water and massaged for 1 min to dislodge the *Salmonella* cells attached to the eggshell surface
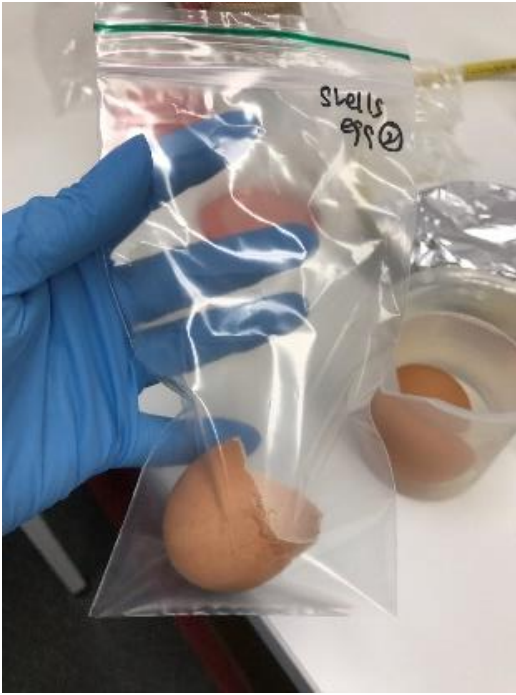
Figure 3.10: The shells were placed in a zip lock bag containing buffered peptone water and were crushed to dislodge the *Salmonella* cells in the eggshell membrane

Figure 3.11: The internal contents of the eggs were placed in a zip lock bag and were mixed to obtain a uniform suspension. This suspension was mixed with peptone water and was plated on XLD agar to detect any surviving *Salmonella* cells in the internal contents of the egg
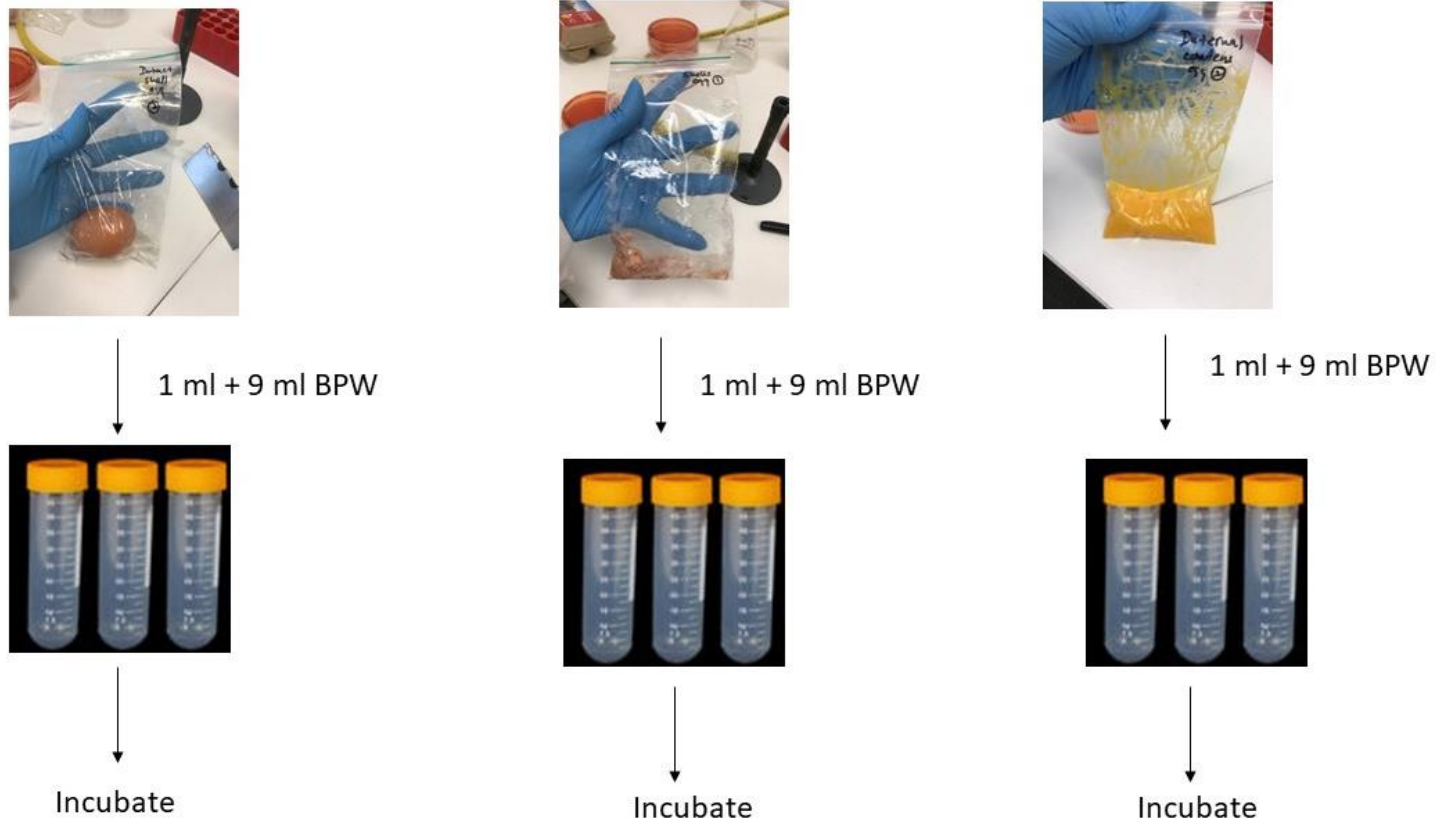
Figure 3.12: Increasing the limit of detection and observing possible resuscitation of *Salmonella*. An enrichment step was conducted following the heat treatment using buffered peptone water (BPW) to increase the limit of detection to 1 CFU/mL and to observe possible resuscitation of *Salmonella* cells

3.7.2 Results

No growth of the standard strain was observed following incubation for 6 min at 57°C (**Figure 3.13**) but culturability was lost after 3.5 min. The culturability of the infectious strain was lost after heat treating the eggs for 9 min at 57°C (**Figure 3.14**).
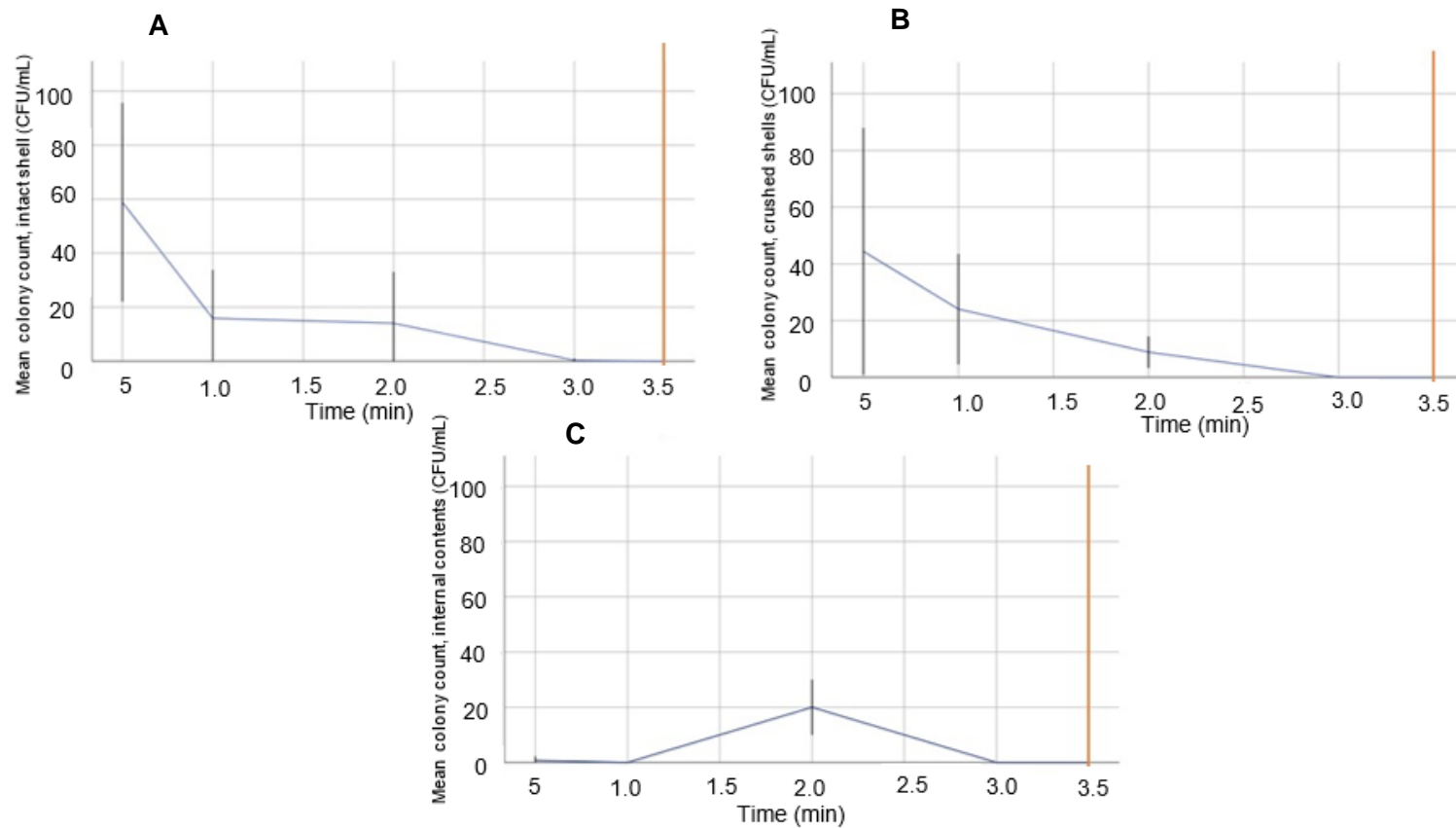
Figure 3.13:The number of *Salmonella* cells (ATCC 53647 strain) detected on XLD agar following the heat treatment of the eggs at 57°C. No growth of *Salmonella* was observed on XLD agar when the intact shell (A), crushed shells (B)and the internal contents (C) were analysed after 3.5 min at 57°C indicating loss of culturability. Error bars indicate 95% confidence intervals
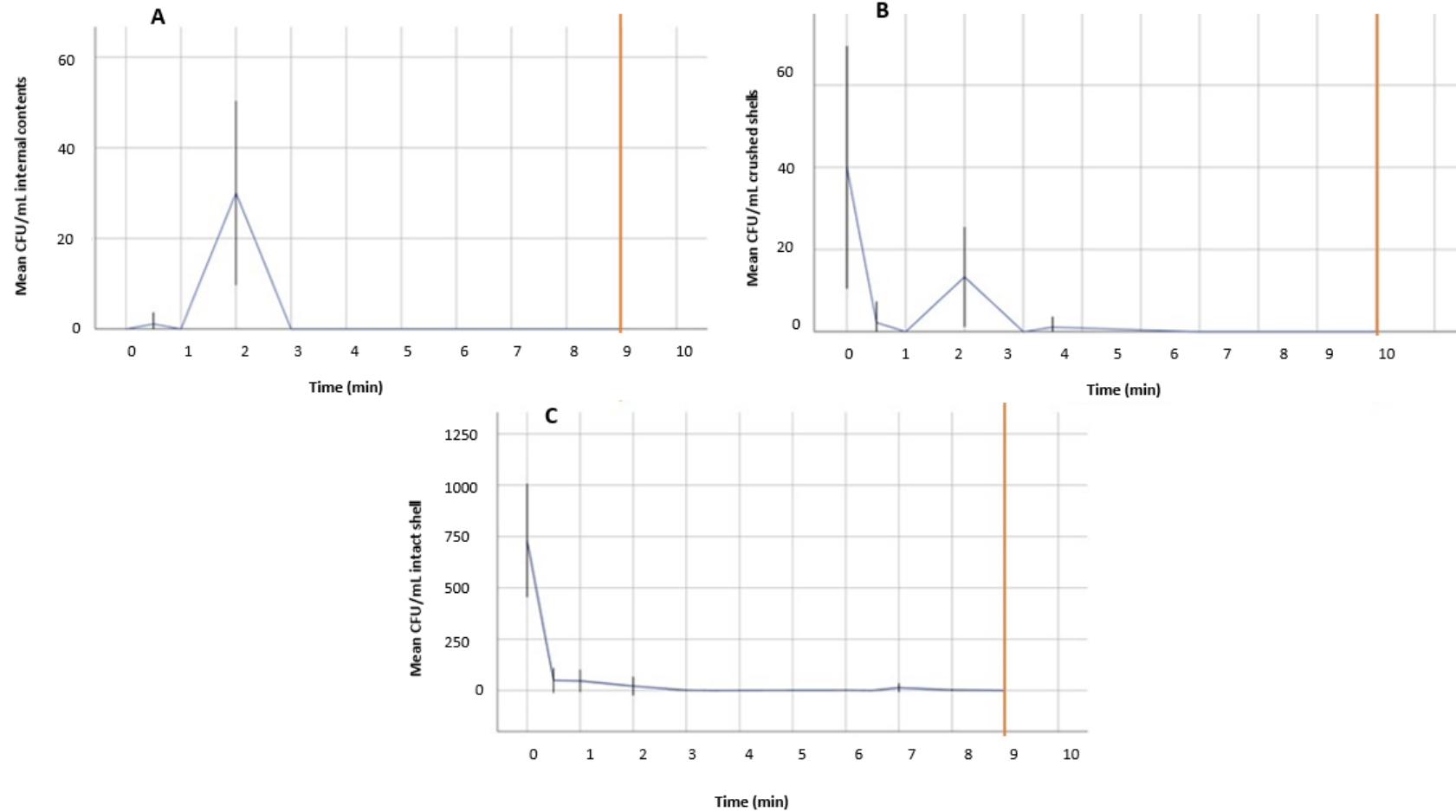
Figure 3.14: The number of *Salmonella* cells (infectious strain) detected on XLD agar following the heat treatment of the eggs at 57°C. No growth of *Salmonella* was observed on XLD agar when the internal contents (A), crushed shells (B) and the intact shell (C) were analysed after 9 min at 57°C. Error bars indicate 95% confidence intervals

3.8 The usability and quality of the treated eggs

The egg quality was checked following the heat treatment. The foaming ability, foam stability, protein denaturation, albumen pH, Haugh unit, yolk index and the difference in weight of the egg before and after the heat treatment at 57°C were assessed.

3.8.1 Methods

Eggs were broken onto a levelled glass plate and the height of the egg albumen surrounding the egg yolk (**Figure 3.15**) and the height of the egg yolk was measured using a micrometer (**Figure 3.16**). The viscosity of the egg albumen was also measured as an indication for the usability of the eggs (**Figure 3.17** and **Figure 3.18**).
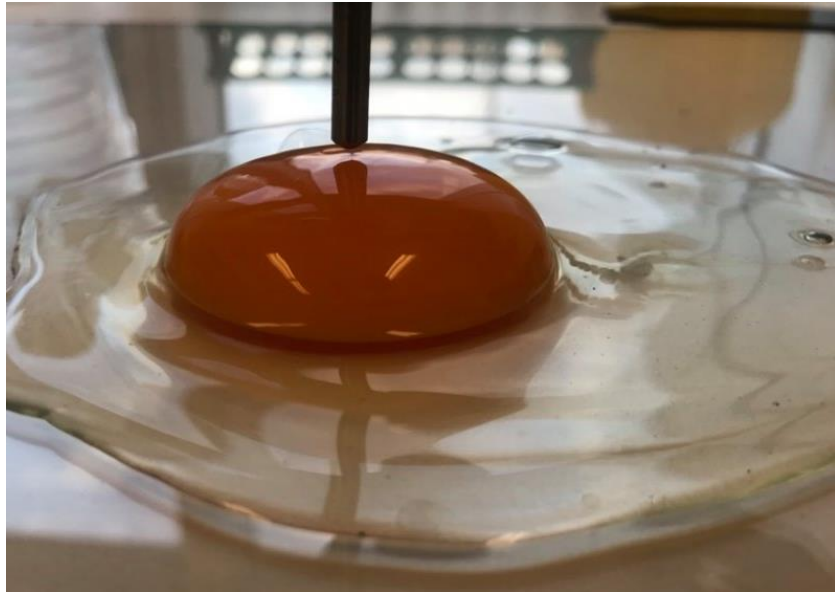
Figure 3.15: The height of the egg yolk was measured using a micrometer to calculate the egg yolk index to determine the quality of the egg following the heat treatments

Figure 3.16: The height of the egg albumen was measured using a micrometer to calculate the Haugh unit to determine the quality of the egg following the heat treatments

Figure 3.17: The viscosity of the eggs was observed to determine the effect of the heat treatment on the usability of the eggs. After 9 min at 57ºC the viscosity of the eggs was not affected

Figure 3.18: The viscosity of the eggs was completely lost following 28 min at 57°C indicating damage to the egg proteins

3.8.2   Results

There was no significant difference between the yolk index, Haugh unit or height of foam in the control eggs and the eggs treated for 9 min, although after 28 min at 57°C the viscosity of the eggs was lost.

CITATION

Keerthirathne, T. P., Ross, K., Fallowfield, H. and Whiley, H. 2019. A successful technique for the surface decontamination of *Salmonella enterica* serovar Typhimurium externally contaminated whole shell eggs using common commercial kitchen equipment. *Foodborne Pathogens and Disease* (available online ahead of print).

A successful technique for the surface decontamination of *Salmonella enterica* serovar Typhimurium externally contaminated whole shell eggs using common commercial kitchen equipment

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Abstract

Eggs are a highly nutritious food source used in a wide range of food products. In Australia, eggs are a frequent source of foodborne salmonellosis outbreaks, associated with eggshell contamination with *Salmonella enterica* serovar Typhimurium (ST). Despite their potentially hazardous nature, raw eggs are often used and consumed in mayonnaise, mousse, ice cream and eggnog. The aim of this study was to develop a shell egg decontamination method that removed ST contamination from the outside of an egg without impacting its usability. The decontamination method was developed by the adaptation of a temperature-controlled water bath (commonly present in kitchens and associated with the sous-vide technique) for the surface decontamination of eggs. The outside of whole eggs was artificially inoculated with two ST strains. The eggs were decontaminated by placing in a sous-vide cooker with the water heated to 57^0C. The remaining viable ST present on the whole shell egg, crushed shells, internal eggs contents and sous-vide water were enumerated over time by culturing onto XLD agar. The quality of the uncontaminated heat-treated eggs was determined by measuring the Haugh unit, yolk index, albumen pH, thermocoagulation and stability of foam. A blind control study was conducted to assess the acceptability and usability of the treated eggs by chefs and food handlers for the preparation of mayonnaise. Complete decontamination of ST was achieved by treating eggs for 9 min in the sous-vide cooker (57^0C). No statistically significant difference was observed in the quality of treated eggs compared with non-treated eggs using the quality measurements and acceptability score from chefs. This method provides a simple approach that can be adopted by chefs and food handlers to obtain safe eggs prior to the preparation of raw egg products.

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3.9 Concluding observations of the chapter

S. Typhimurium is the strain most commonly found in Australia to contaminate the eggshell surface. Therefore, the aim of this chapter was to develop a method to decontaminate the eggshell surface from *S.* Typhimurium given the Australian circumstances. The HACCP based critical control points of this method is described in the **Figure 3.21**. This "Egg safe" method had no or very little effect on the functional properties of the egg and successfully decontaminated the eggs. Therefore, this simple and a rapid method can be adopted to decontaminate eggs before the preparation of raw egg products in commercial or domestic kitchens, to reduce the incidence of salmonellosis linked to eggs and raw egg products.

```
┌─────────────────────────────────────────────────────────────────────┐
│ Method – egg decontamination prior to the preparation of raw egg products │
│                                                                       │
│        Eggs to be fully immersed in water heated to 57 °C for 9 min   │
└─────────────────────────────────────────────────────────────────────┘
                                    ↓
            ┌─────────────────────────────────────────────┐
            │           Critical control points           │
            │                                             │
            │        Eggs should be fully immersed        │
            │                                             │
            │      Water should be heated to 57 °C        │
            │                                             │
            │        Eggs to be treated for 9 min         │
            └─────────────────────────────────────────────┘
                                    ↓
      ┌─────────────────────────────────────────────────────────────┐
      │                          Monitor                            │
      │                                                             │
      │                   Check the water level                     │
      │                                                             │
      │ Check for fluctuations of temperature using a temperature logger 57 °C │
      │                                                             │
      │      Measure the time the eggs are to be treated using a stopwatch │
      └─────────────────────────────────────────────────────────────┘
                                    ↓
      ┌─────────────────────────────────────────────────────────────┐
      │                      Corrective action                      │
      │                                                             │
      │   Make sure not to cross contaminate the eggs with non-treated eggs │
      └─────────────────────────────────────────────────────────────┘
```

Figure 3.21: The HACCP based critical control points which can be adopted to produce *Salmonella* free raw egg products

CHAPTER 4. The optimum pH and temperature combination for the control of *Salmonella* Typhimurium during the production of raw egg mayonnaise

4.1 Overview of the chapter

The aim of this chapter was to identify the optimum pH and temperature conditions for the control of *Salmonella* Typhimurium in raw egg products such as mayonnaise. The chapter describes the survival of *S.* Typhimurium under different pH and temperature conditions. This chapter consists of a published literature review titled "A review of temperature, pH, and other factors that influence the survival of *Salmonella* in mayonnaise and other raw egg products" (Keerthirathne et al., 2016) (page number **148**) and a peer reviewed journal publication titled "The combined effect of pH and temperature on the survival of *Salmonella enterica* serovar Typhimurium and implications for the preparation of raw egg mayonnaise" (Keerthirathne et al., 2019a) (page number **212**). This chapter also contains the preliminary experiments and method development that were not included in the published research paper.

CITATION

A review of temperature, pH, and other factors that influence the survival of *Salmonella* in mayonnaise and other raw egg products

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Abstract

Salmonellosis is one of the main causes of foodborne illnesses worldwide, with outbreaks predominately linked to contamination of eggs and raw egg products, such as mayonnaise. This review explores previous studies that have investigated *Salmonella* control mechanisms utilized in the production of raw egg mayonnaise and other food products. Apart from the use of pasteurized eggs, the main control mechanism identified is the pH of the raw egg products, which plays an important role in the consistency and stability while affecting the survival of *Salmonella* spp. However, currently there is no consensus regarding the critical pH limit for the control of *Salmonella.* The effectiveness of pH as a control mechanism is influenced by the type of acid used; with the effectiveness of lemon juice compared with vinegar highly debated. Additionally, *Salmonella* susceptibility to pH stresses may also be influenced by storage temperature (in some studies refrigeration temperatures protected *Salmonella* spp. from acidulants) and is further complicated by the development of *Salmonella* cross tolerance induced responses, pH homeostasis achieved by the cellular anti-port and symport systems and acid tolerance response (ATR). These mechanisms provide *Salmonella* with an added advantage to ensure survival under various pH conditions. Other confounding factors include the fat content, and the addition of NaCl, garlic and plant essential oils (PEOs) from mint, cinnamon, cardamom and clove.

1 Introduction

Worldwide, foodborne illness is one of the most serious health problems affecting public health and development (Newman et al., 2015). In the USA, it was estimated that the annual incidence of foodborne illness was 48 million cases and the economic burden estimate ranges from US $ 51.0-77.7 billion (Scharff, 2012). In Canada annual estimates of foodborne illness range from 3.1-5.0 million cases (Thomas et al., 2013) and in Australia the incidence is estimated at 5.4 million cases costing AUD$1.2 billion annually (Hall et al., 2005). Worldwide it is estimated that *Salmonella* is responsible for 80.3 million cases of foodborne illness (Majowicz et al., 2010).

Salmonella spp. are members of the family Enterobacteriaceae (Janda and Abbott, 2015) and are facultative anaerobic rods (Batista et al., 2015). There are more than 2600 known serovars (Gal-Mor et al., 2014) and within a serovar the strains could be different in virulence (Rabsch et al., 2002). The serovars of *Salmonella* are broadly classified into typhoidal and non-typhoidal *Salmonella* (NTS). Nontyphoidal *Salmonella* (NTS) serovars include Typhimurium and Enteritidis which are pathogens with a wide-range of host specificity but *S. enterica* serovars, Typhi, Sendai, and Paratyphi A, B, and C are highly adapted to the humans as a host and are the causative agents of enteric fever (Gal-Mor et al., 2014). While there are over 2,600 identified serotypes of *Salmonella,* the majority of clinical *Salmonella* cases can be attributed to 20 serotypes (CDC, 2007) but there are instances where infection occurs due to uncommon serotypes of *Salmonella* (Wilson et al., 2016).

One of the main sources of foodborne salmonellosis is eggs and raw egg products (Whiley and Ross, 2015). Outbreaks of *Salmonella* serotype Enteritidis have been repeatedly associated with the consumption of raw and undercooked eggs (Mishu et al., 1994, Delarocque-Astagneau et al., 1998). The eggs can be contaminated during different stages of their formation, processing and packaging. Vertical or transovarian contamination of eggs can happen during the formation of the egg when the ovaries of the hen are infected. Horizontal transmission can take place if the eggs are contaminated by means of a contaminated environment (De Reu et al., 2006). According to previous studies, there is an increased probability of whole eggs with low shell quality being able to be penetrated by *Salmonella* spp. (Sauter and Petersen, 1974) and egg weight and flock age also influence the ability of *Salmonella* spp. to penetrate the shell and the membranes of the eggs (Berrang et al., 1998). Moreover, it is found that there is an increased incidence of eggshell penetration from the beginning to the end of lay by *S.* Enteritidis (Nascimento et al., 1992) and there is an increased risk of *Salmonella* contamination when the flock becomes older (Bruce and Johnson, 1978).

Even though several food items like peanuts, beef, pork and chicken have been connected with the outbreaks of salmonellosis, eggs and food products prepared using eggs seem to be the most frequent foods that are involved with the disease (De Oliveira Elias et al., 2015). Raw eggs are used in many food products like pastries, homemade ice cream, and mayonnaise. Contaminated eggs that have not undergone heat treatment pose a significant

risk to public health and outbreaks of *S.* Enteritidis have been repeatedly associated with raw or undercooked eggs (CDC, 1996).

In 2008 the OzFoodNet, conveyed that 5.4 million cases of foodborne disease, occur in Australia annually, costing an estimated amount of $1.2 billion per year among which 8,310 cases were found to be *Salmonella* infections at a rate of 39 cases per 100,000 population (Hall et al., 2005). While improved sanitation and water supplies have made a significant contribution towards the decrease in incidence of enteric diseases, *Salmonella* spp., and *Campylobacter* spp. are commonly reported pathogens of community gastroenteritis (Sinclair et al., 2002).

Within raw egg products, homemade mayonnaise prepared with raw eggs is one of the most common foods linked to salmonellosis outbreaks (Scallan et al., 2011). Mayonnaise is a methodically prepared, semi-solid (stabilized oil-water emulsion) dressing, which is a combination of raw eggs, vinegar, oil and spices and is perhaps one of the oldest and most widely used in the world (Depree and Savage, 2001, Di Mattia et al., 2015). Currently, in many countries it is not feasible to produce eggs guaranteed to be free from *Salmonella* contamination and hence the control mechanisms utilized post collection and during food handling are essential for protecting public health (Whiley and Ross, 2015).

In the USA it is estimated that there are around 1.4 million illnesses and 600 deaths caused by salmonellosis annually, with the most common serotypes identified as *S.* Typhimurium and *S.* Enteritidis (Mead et al., 1999). In fact from 1998 to 2008 there were a total of 403

outbreaks of foodborne salmonellosis and 112 were linked to eggs and raw egg products (Jackson et al., 2013). In 2009, it was estimated that there were a total of 6.2 million cases of salmonellosis in the European Union and the incidence rate correlated with the prevalence of *S.* Enteritidis in laying hens (Havelaar et al., 2013). In Australia in 2011, 45% of all salmonellosis outbreaks were linked to eggs or egg related products (OzFoodNet., 2015). Globally, there have been numerous published outbreaks of salmonellosis that have been caused by raw egg mayonnaise (Mitchell et al., 1989) In the UK, a large outbreak of *Salmonella* Typhimurium definitive type 49 was linked to eggs with raw egg mayonnaise identified as the likely cause of infection (Mitchell et al., 1989). Another outbreak of salmonellosis was also reported from the UK in December 2000 which identified egg mayonnaise sandwiches as the vehicle of infection (Mason et al., 2001). Moreover, in Brazil potato salad made with homemade mayonnaise and stored at unsuitable temperatures was associated with foodborne infection and *S.* Enteritidis was identified as the infecting organism (Carneiro et al., 2015). Additionally, in 2010 buffet dishes which contained mayonnaise was associated with a salmonellosis outbreak in Germany (Von Wissmann et al., 2012).

This review explores studies investigating different mechanisms for controlling *Salmonella* in raw egg mayonnaise. The *Salmonella* serovar, country of study and control mechanism investigated are presented in **Table 4.1**.

2 pH/ Acid tolerance and Temperature

The pH of mayonnaise plays an important role in its structure and the stability (Depree and Savage, 2001). Mayonnaise is an emulsion (**Figure 4.1**) stabilized by denatured proteins that form a network that can be impacted by the isoelectric pH of the egg yolk protein. When the charge on the proteins is minimized the viscoelasticity and stability of the mayonnaise is at its highest (Depree and Savage, 2001).

Food safety guidelines published online by the Government of New South Wales (NSW), Australia, suggest that a pH at or below 4.2 has shown to be effective in controlling *Salmonella* in raw egg products, however there are numerous factors that influence the bactericidal efficiency such as the type of acid used (Jung and Beuchat, 2000, Zhu et al., 2012), temperature (Yang et al., 2014), water activity (Mattick et al., 2000), garlic, ginger and pepper (Indu et al., 2006).

Many bacterial species induce responses to environmental stress (Alvarez-Ordonez et al., 2015). When *Salmonella* spp. are exposed to a stress this can produce cross-tolerance to many or various stresses (Hiramatsu et al., 2005). Gruzdev *et al*. reported that following carbon starvation, *Salmonella* spp. demonstrated greater tolerance to low pH, hyperosmolarity, heat, polymyxin B, and peroxides. (Gruzdev et al., 2011). Another study conducted by Leyerand and Johnson (1993) demonstrated that exposure of *Salmonella* to mild acids (pH 5.8) could induce adaptation to lower pH, heat, NaCl (2.5 M), crystal violet,

and polymyxin B. Additionally, subjecting *S. enterica* cells to an initial acid shock or pH 5.8 or 4.5 before inoculating mayonnaise (pH 4.2 to 4.5) increased the survival rate and persistence of the organism at 4°C (Leuschner and Boughtflower, 2001).

Salmonella can also achieve pH homeostasis, which is when the intracellular pH is maintained compared with the environmental pH (Krulwich et al., 2011). Homeostasis is facilitated by cellular proton pumps, potassium/proton and sodium/proton antiport systems (Booth, 1985). The ability of *Salmonella* to decrease proton extrusion and membrane proton conductance enables the cell to be protected against acid stress (Foster and Hall, 1991). Additionally, *S.* Typhimurium has a regulated response to further protect from acid stress which is called the acid tolerance response (ATR) (Foster and Hall, 1990). The ATR protects *Salmonella* spp. at pH levels of 3.0-4.0 but is activated when environmental pH values are between 6.0 and 5.5 and when pH homeostasis fails (Foster and Hall, 1990). These pH conditions are referred to as the post shock stage and the pre shock stage respectively(Foster and Hall, 1990). During the post shock stage stimulation of 43 acid shock proteins takes place in order to prevent and repair the damage done to macromolecules by the acids (Foster and Hall, 1991).

In contrast, studies conducted by Álvarez-Ordóñez *et al.* (2010) and Samelis *et al.* (2003) suggest that *S.* T*yphimurium* vulnerability to acid stress is dependent on growth temperature. *S. Typhimurium growth was observed* in the temperature range of 25–37^{0}C at pH 4.5.(Álvarez-Ordóñez et al., 2010, Samelis et al., 2003). Alali *et al.*(2012) proposed that lowering the pH of the mayonnaise based homemade salads decreased the rate of survival

of *Salmonella* regardless of the temperature. According to a study conducted by Koutsoumanis *et al.* (2004) the minimum pH value that permitted the growth of *S.* Typhimurium was 3.94 within the temperature range 25 ^{0}C to 35 ^{0}C.

3 Vinegar vs. lemon juice

Jung and Beuchat (2000) found that citric acid (lemon juice) was more effective at controlling *S.* Typhimurium compared with acetic acid (vinegar/ 8.3 M), lactic acid (2 M) and malic acid (2 M) at an equivalent acid concentration of pH 5.4, 4.4, and 3.7. This is supported by work done by Zhu *et al.* (2012) which demonstrated that lemon juice was more effective than commercial wine vinegar at controlling *Salmonella* in mayonnaise spiked with either a mixture of *S.* Enteritidis (phage 4, 8 and 13) or a mixture of *S.* Typhimurium, *S.* Heidelberg and *S.* Enteritidis (untypeable phage type). It was also shown that both mixtures of *Salmonella* survived longer at 4°C compared to 25°C. The different bactericidal effects observed at different temperatures could be explained by more efficient cross membrane migration of the organic acids at higher temperatures (Yang et al., 2014).

However, these findings differ from a study done by Perales and Garcia (1990) that demonstrated that homemade mayonnaise made with vinegar had a greater bactericidal effect on *S.* Enteritidis compared with homemade mayonnaise made using lemon juice at the same pH (pH ranging from 3.6 to 5 (Perales and Garcia, 1990). This study was supported by Lock and Board (1995a) who also demonstrated that the number of *S.* Enteritidis PT4

organisms spiked into mayonnaise made with vinegar declined within six days of storage at 20^0C, but the same result was not observed when the mayonnaise made to the same pH using lemon juice. Roller *et al.* (2000) concluded that chitosan added to mayonnaise containing acetic acid or lemon juice could be used as a preservative against the normal flora (Roller and Covill, 2000).

4 Addition of NaCl and Reduction of water activity

Several cellular mechanisms of bacterial cells are involved in osmoregulation which regulates the osmolality of the cell protecting physical and chemical properties of the intracellular environment in response to environmental stress (Yang et al., 2014). It is achieved by accumulation of electrically neutral, low molecular weight compounds such as osmoprotectants (e.g., proline, glycine-betaine, or ectoine) inside the cell (Csonka and Hanson, 1991). It is clear that *Salmonella* is adapted to endure prolonged starvation and desiccation periods (Podolak et al., 2010). It has been demonstrated that during the early stages of starvation *Salmonella* can upregulate the osmoprotectant transporters (proP, proU, and osmU) ensuring the survival of this bacteria under low and intermediate moisture conditions (Finn et al., 2013b).

Salt is a common preservative used in food products (Rahman, 2015). The sodium ions associate with water molecules to reduce the amount of unbound water in foods, making it

difficult for the microorganisms to grow (Henry and Taylor, 2010). Salt can stimulate osmotic shock in microbial cells affecting the growth and promoting cell death (Davidson et al., 2013).

It has been shown that the permeability of the *S.* Typhimurium cells is altered by heat and this allows the sodium ions to penetrate the cell in to the cytoplasm interfering the cell metabolism (Manas et al., 2001). Although there are limited studies investigating the effect of salt concentration on *Salmonella* contamination of mayonnaise there have been studies demonstrating that *Salmonella* spp. are capable of enduring extended starvation and desiccation stresses (Finn et al., 2013a). Even though reducing the amount of available water in food is a long-established method for controlling bacterial growth (Mattick et al., 2000), there have been outbreaks of salmonellosis linked to foods with low water activity (a_w), such as peanut butter (Podolak et al., 2010). Water activity is defined as the ratio of water vapor pressure (P_{wv}) in a food system to the saturation water vapor pressure (P_{swv}) at the temperature of the food system (**Figure 4.2**). Optimal growth of *Salmonella* spp. occurs when the a_w is 0.99 but there is evidence that *Salmonella* may develop increased tolerance that allows for survival under low a_w conditions for longer periods of time (i.e. 43 days) (Mattick et al., 2000). According to Mattick et al. (2000),*Salmonella* spp. are found to have an increased heat tolerance at low a_w.

5 Garlic (Allium sativum)

According to the scripts found on ancient Egyptian pyramids, garlic has been used for medicinal purposes as well as a spice since ancient times (Kumar and Berwal, 1998). In 1822, the antibacterial properties of onion and garlic were observed and recorded (Johnson and Vaughn, 1969). According to the literature, garlic contains strong anti-bacterial compounds that are effective against *Salmonella* and often used as a spice for fermented fish (Bernbom et al., 2009). Use of garlic in food products could increase the shelf life as well as decrease the potential for food poisoning (Kumar and Berwal, 1998). Garlic, ginger and pepper are known to contain bactericidal agents against *Salmonella* (Indu et al., 2006). Traditional medicine uses the extract of these plants in order to treat infections caused by enteric bacteria like *S. typhi* (Ekwenye and Elegalam, 2005). It has been demonstrated that addition of 1% garlic to the mayonnaise inoculated with acid tolerance induced *S.* Enteritidis reduced counts of the organism by 10-fold after 2 days compared with the control mayonnaise batch incubated without garlic (Leuschner and Zamparini, 2002).

6 Oils

Plant essential oils (PEOs) are well-known as antibacterial agents that could be used to control foodborne diseases (Bajpai et al., 2008). These plant secondary metabolites are hydrophobic in nature and can be added to mayonnaise, which is advantageous because they can interact with the cell membranes of the bacteria subsequently causing the cell components to flow out from the cell (Lambert et al., 2001). According to Wendakoon *et al*. (1995) some PEOs can hinder the enzymatic reactions through the inhibition of the proteins of bacteria (Wendakoon and Sakaguchi, 1995). PEOs from mint, cinnamon, cardamom and clove were found to reduce the bacterial count of *S.* Enteritidis in milk products, yogurt and cucumber (Tassou et al., 1995). In a study conducted by Dabbah *et al.* (Dabbah et al., 1970) PEOs from orange, lemon and grapefruit reduced the *Salmonella* count in milk. According to Valverde *et al.,* (Valverde et al., 2010) cinnamon bark oil in the concentration of 7000 ppm can be used to decrease *Salmonella* sp. in liquid whole eggs. Additionally, it has also been found that the antimicrobial activity of oregano essential oils (OEO) is improved when combined with ethylenediaminetetraacetic acid (EDTA) or nisin (Da Silva et al., 2016). The inhibitory activity of nisin can be increased when combined with EDTA which alters the bacterial outer membrane enabling nisin to access the cytoplasmic membrane (Stevens et al., 1992).

7 Fat content

Juneja and Eblen (2000) reported that increased fat content in food decreased the water activity, which could lead to poor heat conductivity, increasing the survival rate of the pathogen *S.* Typhimurium in beef. This is supported by a study that showed the fastest decrease in *S.* Typhimurium was detected in fat free mayonnaise (at pH 2.6) compared with full fat mayonnaise at the same pH (Lock and Board, 1994).

8 Conclusions

Salmonella contamination of raw egg products, such as mayonnaise, is a major public health concern as a causative agent of salmonellosis outbreaks. This review paper explores studies that have investigated *Salmonella* control mechanisms within raw egg mayonnaise. One of the main factors influencing the survival of *Salmonella* in mayonnaise, whilst also affecting the appearance and stability, is pH. Some studies indicate that *Salmonella* susceptibility to pH stresses may be dependent on the growth temperatures; however, this is still debated. Additionally, the effectiveness of pH as a control mechanism is influenced by the type of acid used. The effectiveness of lemon juice compared with vinegar as a control mechanism is also still debated, with current studies producing contradicting results. Additionally, evaluating the effectiveness of pH as a control mechanism is further complicated by the development of *Salmonella* cross tolerance induced responses, pH homeostasis achieved by the cellular anti-port and symport systems and ATR which provides *Salmonella* with an added advantage to ensure survival under various pH environments. There have also been

a few studies investigating the effectiveness of additive such as salt, garlic and PEOs from mint, cinnamon, cardamom and clove to inhibit the growth of *Salmonella* spp.

Currently, it is not possible to guarantee that raw egg mayonnaise will not be contaminated with *Salmonella.* Therefore, there is an urgent need for further research to continue to explore the potential control mechanisms discussed in this paper. This will inform protocols for food handling and mayonnaise preparation to reduce the risk of foodborne salmonellosis.

Table 4.1: *Salmonella* serovar, country of study and control mechanism investigated

Country	*Salmonella* spp.	Food	Control mechanisms	Comments	Reference
U.K.	*S.* Enteritidis	Mayonnaise	pH	20 mL vinegar (6% w/v acetic acid) per fresh egg yolk, 40 ml per fresh egg white or 60 ml per fresh whole egg was used. Should be held at 20°C or above for at least 48 h before refrigeration or consumption	(Xiong et al., 2000)
U.K.	*S.* Enteritidis	Mayonnaise based shrimp salad	pH/ preservatives chitosan and acetic acid	Chitosan could be useful as a preservative combined with acetic acid	(Roller and Covill, 2000)
China/ U.S.	*S.* Typhimurium *S.* Heidelberg *S.* Enteritidis	Home-Style Mayonnaise	pH commercial wine vinegar, lemon juice, and acetic or citric acid	*Salmonella* counts in acid solutions at 4^0C were reported as significantly higher than those in samples at 25^0C. Viability of *Salmonella* decreased as the amounts of vinegar and lemon juice in mayonnaise increased	(Zhu et al., 2012)
France	*S.* Typhimurium	Reduced calorie mayonnaise	pH /temperature	Higher temperature with a low pH, greater the inactivation of the organism	(Membre et al., 1997)
U.K.	*Salmonella*	Mayonnaise based potato salad,	pH, NaCl and temperature	Decreased pH and increased the bactericidal activity irrespective of sodium concentration or storage temperature	(Alali et al., 2012)

		macaroni salad, and coleslaw		Sodium concentrations had little or no effect on the behavior of *Salmonella* when stored at 4 or 10^{0}C for up to 27 days.	
U.K.	*S.* Enteritidis	Mayonnaise	pH, acetic acid (vinegar)	Mayonnaise made with vinegar to a pH of 4.1 or less controlled *S.* Enteritidis Storage of mayonnaise at refrigeration temperatures protected *Salmonella* spp. from acidulants and therefore a holding time of 24 h at 18-22°C was advised before refrigeration.	(Radford and Board, 1993)
Spain	*S.* Enteritidis	Home-made Mayonnaise	pH/ temperature	Vinegar was used as an acidulant to achieve a pH of 3.6 - 4 and storage in a warm place is recommended.	(Perales and Garcia, 1990)
U.S.	*S. Senftenberg*	Egg salads	pH/ temperature	Significant decrease in *Salmonella* numbers, particularly during storage at room temperature (22°C) at the acidity ranging from pH 4.25 – 4.30 was recorded	(SIMMONS et al., 1979)
Brazil	*S.* Enteritidis	Mayonnaise	oil oregano essential oil	Natural antimicrobial to reduce the *S.* Enteritidis growth	(Da Silva and De Melo, 2012)
Spain	*S.* Enteritidis	Egg mayonnaise	oil (**v**irgin olive oil**)**	Egg mayonnaise made with virgin olive oil required more than 48 h to reduce the number of microorganisms to an undetectable level.	(Roller and Covill, 2000)

			temperature		
U.K.	*S.* Enteritidis	Home-made Mayonnaise	pH oils (olive oil with garlic, basil, soya, grapeseed, rapeseed, groundnut, sunflower, hazelnut)	The death rate of the *S.* Enteritidis differed with the various oils	(Lock and Board, 1995b)
Brazil	*S.* Enteritidis	Mayonnaise	oil oregano essential oils (OEO1 / OEO2) Nisin EDTA	Antimicrobial activity of OEO against *S.* Enteritidis was improved when combined with Nisin or EDTA.	(Da Silva et al., 2016)
U.K.	*S.* Enteritidis	Mayonnaise	garlic	Garlic (1%) reduced the viable cells of *S.* Enteritidis in mayonnaise by a factor of 10	(Leuschner and Zamparini, 2002)
Greece	*S.* Enteritidis	Mayonnaise based Aubergine salad	sorbic/ benzoic acids	Addition of preservatives decreased the pathogen survival	(Tassou et al., 2009)
U.K.	*S.* Enteritidis	Mayonnaise	pH	Within the pH 4.2-4.5 *S.* Enteritidis remained stable for 4 weeks at 4 ^{0}C	(Leuschner and Boughtflower, 2001)
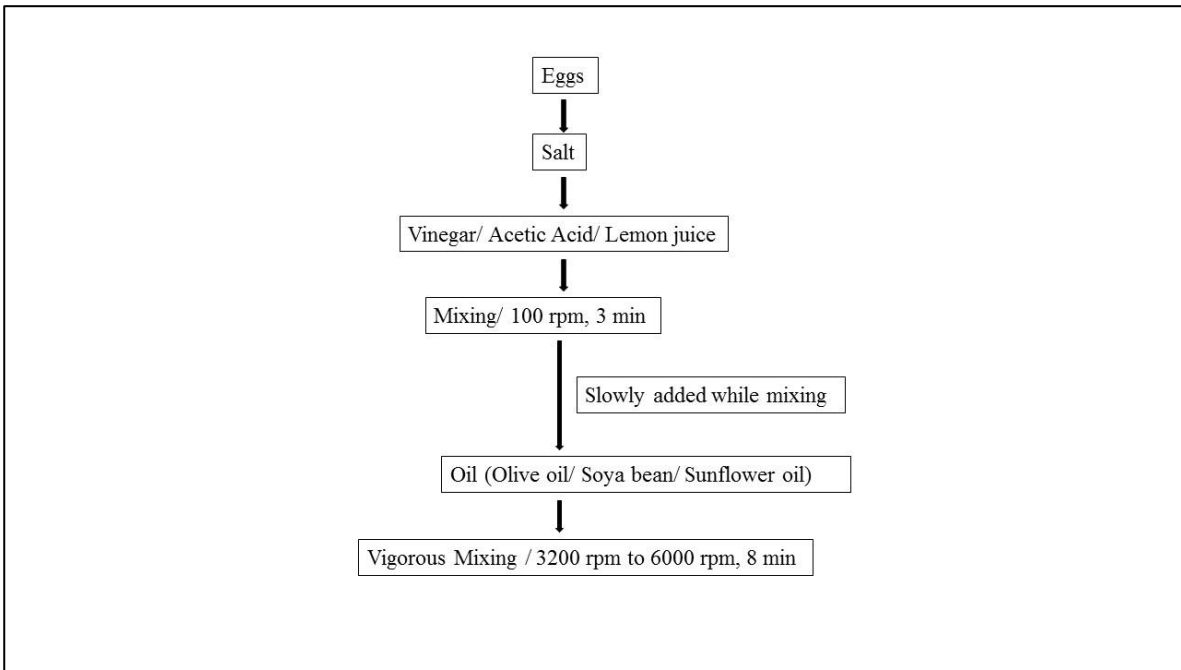
Figure 4.1: Preparation of Mayonnaise (Giacintucci et al., 2016, Da Silva et al., 2016, Da Silva and de Melo, 2012, Medina et al., 2007, Lock and Board, 1995b)

Figure 4.2: Definition of Water activity

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4.2 Method development

Characterizing the growth curves is beneficial to understand the metabolic features of an organism (Yus et al., 2009). Growth curve models with different pH, temperature and water activity are used to predict the shelf life of food products (McMeekin et al., 2013) and to determine the critical steps in the line of production (Zwietering et al., 1990) as undesirable numbers of pathogenic microorganisms in food could lead to foodborne outbreaks (Koutsoumanis and Lianou, 2013).

Internal and external pH have the ability to control the biological as well as physiological processes of a bacterial cell (Choi and Groisman, 2016). Moreover, pH plays an important role in the structure and the stability of mayonnaise and can affect the visco-elasticity and stability of mayonnaise (Depree and Savage, 2001).

Among other factors, pH and temperature are commonly used key factors to control *Salmonella* in eggs and raw egg products. The following experiments were conducted to determine the influence of pH on the growth of *S.* Typhimurium (ST) which is identified as the main source of foodborne salmonellosis related to eggs and raw egg products in Australia (Fearnley et al., 2011).

The objectives of the following experiments were to model the growth of *Salmonella enterica* serovar Typhimurium (ATCC 53647) when plate to broth transfer was practised, observe the effect of pH on the survival of *S.* Typhimurium when incubated at 37°C, to observe the survival of *S.* Typhimurium at 37°C when the culture was in different stages of the growth curve and to observe the survival of *S.* Typhimurium at 37°C at higher inoculum concentration at low pH conditions.

4.2.1 *Salmonella* Strains

Two S. Typhimurium strains were used for the experiments; a standard strain ATCC 53647 (genotype gyrA1816 deltacya-3 deltacrp-2 and incapable of producing functional adenylate cyclase and cyclic AMP receptor protein) and an infectious strain. (isolated from a faecal sample of an infected individual and was provided by the *Salmonella* Reference Laboratory (Adelaide, Australia). *S.* Typhimurium ATCC 53647 strain stock cultures were maintained at −80°C in glycerol solutions. Prior to use, the frozen culture was retrieved from -80°C storage on XLD agar (OXOID Ltd, Basingstoke, UK) using the streak plate method (**Figure 4.3**). The plates were incubated at 37°C overnight. A single colony was re-streaked on to the XLD agar plates followed by overnight incubation at 37°C to obtain a pure culture.
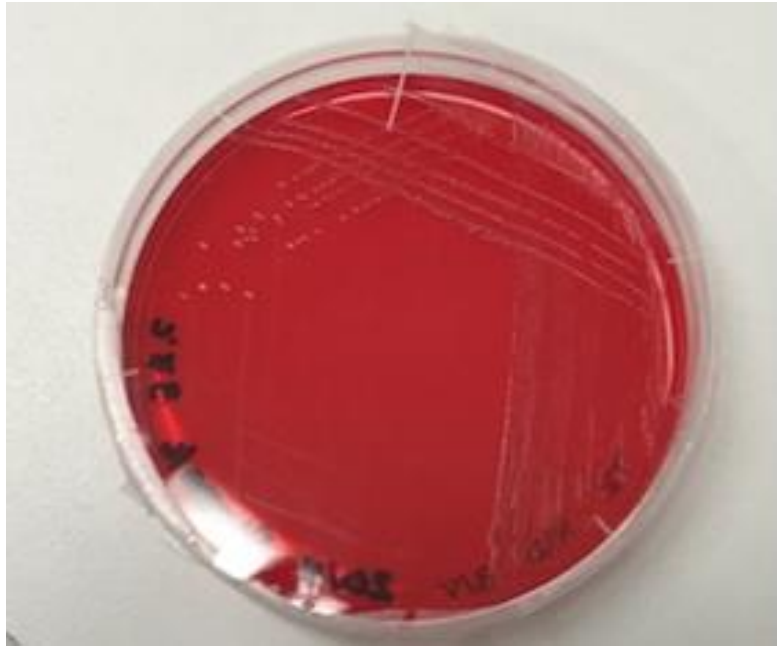
Figure 4.3: *S.* Typhimurium (ATCC 53647) on XLD agar following overnight incubation at

37°C

Prior to obtaining the infectious strain a bio-safty training was completed for working with microbiologicals of risk group 2 and for working in a PC2 facility (Appendix; page number **326** and **327**).The *S.* Typhimurium infectious strain was transported in to the physical containment 2 (PC2) facility (Health Sciences Building, Flinders University) and the experiments involving the infectious strain was carried out in a bio-safty cabinet (Safemate Vision Class II 1.2; EuroClone S.p.A). The initial culture was sub-cultured on XLD agar medium followed by incubation at 37°C overnight. Unlike the standard strain, the infectious strain produced red colonies with black centres (**Figure 4.4**) . A single colony was re-streaked on to the XLD agar plates followed by overnight incubation at 37°C to obtain a pure culture.
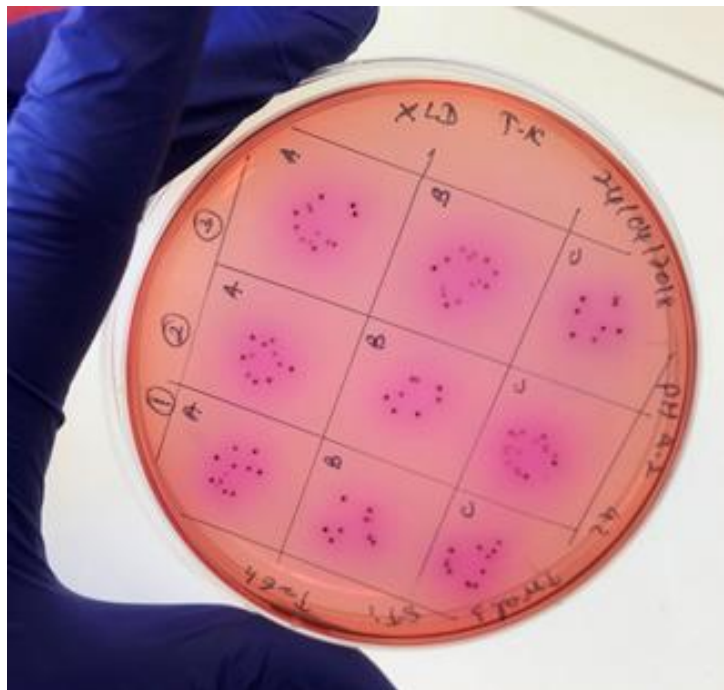
Figure 4.4: Red *Salmonella* colonies with black centres (infectious strain) on XLD agar media following overnight incubation at 37ºC

4.2.2   Growth of *Salmonella* in peptone water

Peptone water was prepared according to the manufacturer's instructions by adding 15 g powdered media in to 1 L distilled water followed by sterilizing at 121°C for 15 min. The glassware and the cotton plugs were sterilised by autoclaving at 121°C for 30 min. All the experiments were conducted under aseptic conditions. A single colony from an overnight culture of *S.* Typhimurium on XLD agar was introduced in to 100 mL peptone water (pH 7.0) (Oxoid Ltd, Basingstoke, UK) and was incubated at 37°C in an orbital mixing incubator (200 rpm) to obtain a broth culture of *S.* Typhimurium (**Figure 4.5**).
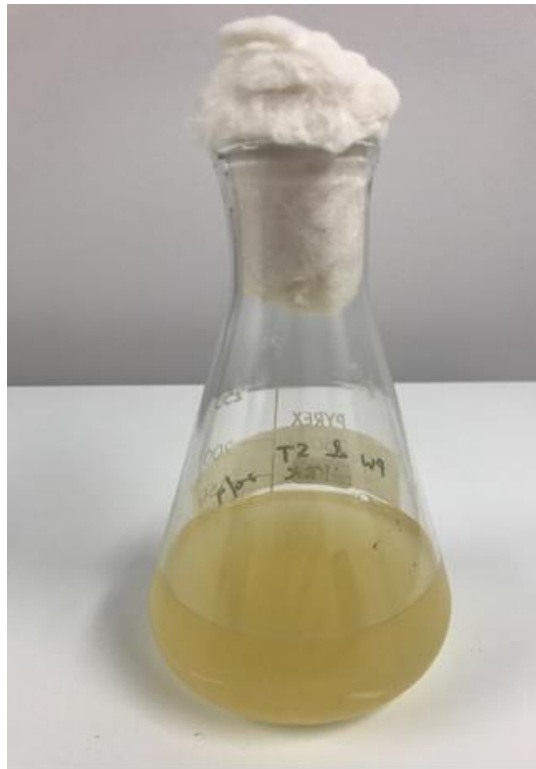
Figure 4.5: Broth culture of *Salmonella* in peptone water following overnight incubation at 37°C in an orbital mixer incubator

4.2.3   Storage of *Salmonella* cultures

The cultures were stored in a 1:1 glycerol and water suspension. Glycerol and water were mixed and sterilized by autoclaving for 15 min at 121°C. One and a half mL 1:1 glycerol and water solution was added to 2 mL sterile cryogenic vials. *Salmonella* cultures from XLD agar were introduced into the cryogenic vials containing the glycerol and water solution and stored at -80°C until required.

4.3   Growth curve for *Salmonella enterica* serovar Typhimurium (ATCC 53647) in nutrient broth (pH 7)

This experiment was conducted to characterize the growth curve under standard growth conditions to understand when the cells were in exponential and stationary phases for the future experiments. The experiments were carried out using a pure culture of *S*. Typhimurium (ATCC 53647) often transferred from plate to broth. Therefore, the growth curve of *S*. Typhimurium was generated to observe the growth curve pattern when plate to broth transfer was practised. All the experiments were carried out under aseptic conditions.

4.3.1   Methods

A loopful of an overnight culture of *S.* Typhimurium from the XLD agar plate was introduced in to 100 mL of nutrient broth (pH 7.0) (OXOID Ltd, Basingstoke, UK) and was incubated at 37ºC in an orbital mixing incubator (200 rpm) and spectrophotometric readings (UV-1800, Shimadzu UV-spectrophotometer, Japan) were recorded in triplicates at 600 nm (OD_{600}) every 2 h for 24 h. The experiment was repeated 3 times (**Figure 4.6**).

The growth curve was plotted using the mean of the spectrophotometric readings at OD_{600} against time of incubation using the SPSS software. The time period required for the *Salmonella* culture to complete the growth curve and time taken at each stage (log, lag, stationery and death phase) were noted and calculated.
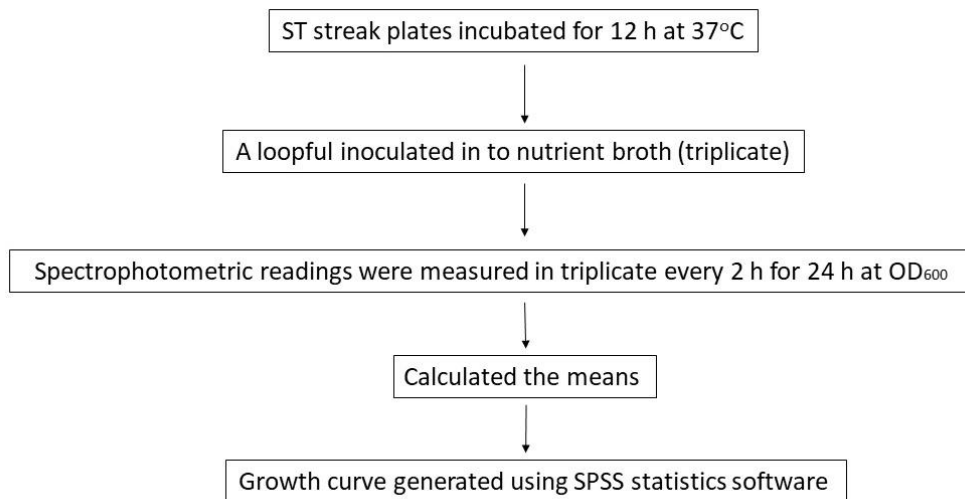
Figure 4.6: The experimental design followed to generate the growth curve for *S*. Typhimurium (ATCC 53647) in nutrient broth (pH 7)

4.3.2   Results

During this experiment the *Salmonella* culture was transferred from the XLD plate to nutrient broth. Because of plate to broth transfer the growth curve showed a longer lag phase than a culture transferred from nutrient broth to nutrient broth. The exponential phase lasted for 12 h following the long lag phase. The growth curve of *S.* Typhimurium (ATCC 53647) in nutrient broth indicates that *Salmonella* require a considerable time i.e. approximately 8 h to adapt to the changed environmental conditions. A constant stationary phase was observed for approximately 12 h (**Figure 4.7**).

A characteristic bacterial growth curve shows four discrete phases. The lag phase, exponential growth/ log phase and the stationary phase preceding the death phase (Rolfe et al., 2012). Growth curve models with different pH, temperature and water activity are used to predict the shelf life of food products (McMeekin et al., 2013) and the critical steps in the line of production to ensure the survival and growth of pathogenic microorganisms (Zwietering et al., 1990).

A long lag phase was observed in *S.* Typhimurium when the plate to broth transfer was practised. The log phase started after 10 h of incubation at 37°C and lasted proximately for 12 h. Although the lag phase is the earliest and the most poorly understood stage of the bacterial growth cycle (Rolfe et al., 2012) it is presumed that during the lag phase the bacterial cells undergo an adaptation process required for the bacteria to survive in new environmental conditions (Madigan et al., 1997b). The growth curve generated in nutrient broth to obtain the pre-inocula presented with a longer lag phase, but with an interesting exponential / log phase. This could be an indication regarding how *Salmonella* cells adapt to the external environment.
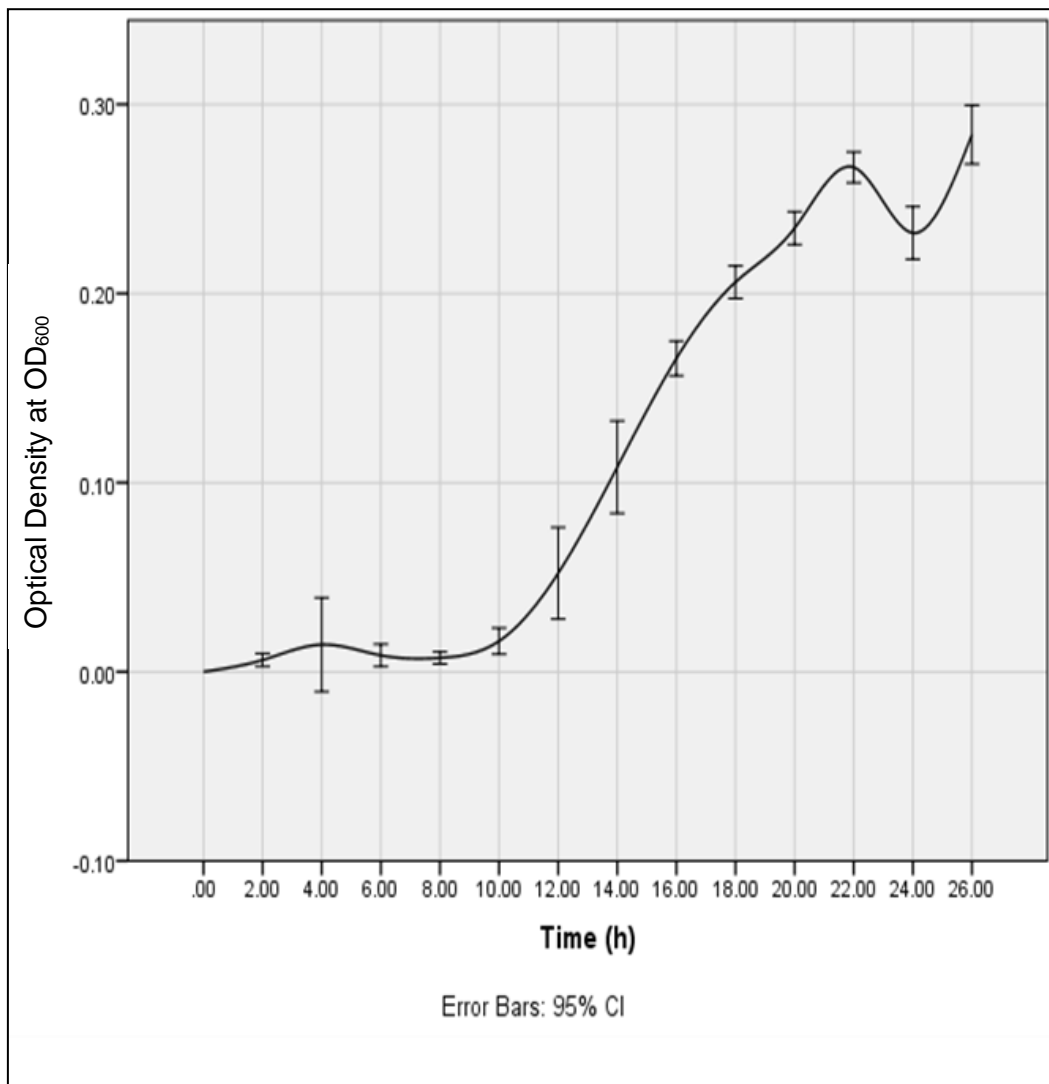
Figure 4.7: Growth curve of *S*. Typhimurium (ATCC 53647) indicating the longer lag phase when the plate to broth transfer is practised. The growth curve was plotted using the mean of spectrophotometric readings at OD_{600} obtained from the three trials conducted in triplicate against the time of incubation using the SPSS software

4.4 Effect of pH on the growth of *S.* Typhimurium American Type Culture Collection (ATCC) 53647 at 37°C

4.4.1 Methods

The effect of pH on *Salmonella* was determined at the optimum growth temperature, where all the biological processors, growth and replication are functioning at optimum temperature which is 37°C (Bronikowski et al., 2001).

The pH of the peptone water was adjusted using acetic acid (as an alternative to vinegar used in the preparation of mayonnaise) to 4.2, 4.4 and 4.6. The pH was measured using a pH meter. Peptone water at pH 7 was used as the control. A loopful of an overnight culture from the XLD agar plate was introduced in to 100 mL of peptone water and the culture was incubated at 37°C in an orbital mixing incubator with agitation at 200 rpm. Optical density of the broth culture was measured at OD_{600} using a UV spectrophotometer. The readings were recorded in triplicate every 2 h, 4 h, 1 day, 4 days, 5 days, 6 days, and 7 days. The experiment was repeated three times.

4.4.2 Results

Growth was observed only in the control (pH = 7) (**Table 4.2**). The growth of *Salmonella* (ATCC 53647) was inhibited at pH 4.2, 4.4 and 4.6 when incubated at 37°C (**Figure 4.8**).

Internal and external pH have the ability to control the biological as well as physiological processes of a bacterial cell (Choi and Groisman, 2016) with pH and temperature the main factors influencing the growth of *Salmonella* in food products. This experiment demonstrated that the acidic pH conditions tested were effective at preventing the growth of the *S.* Typhimurium strains used in this study.
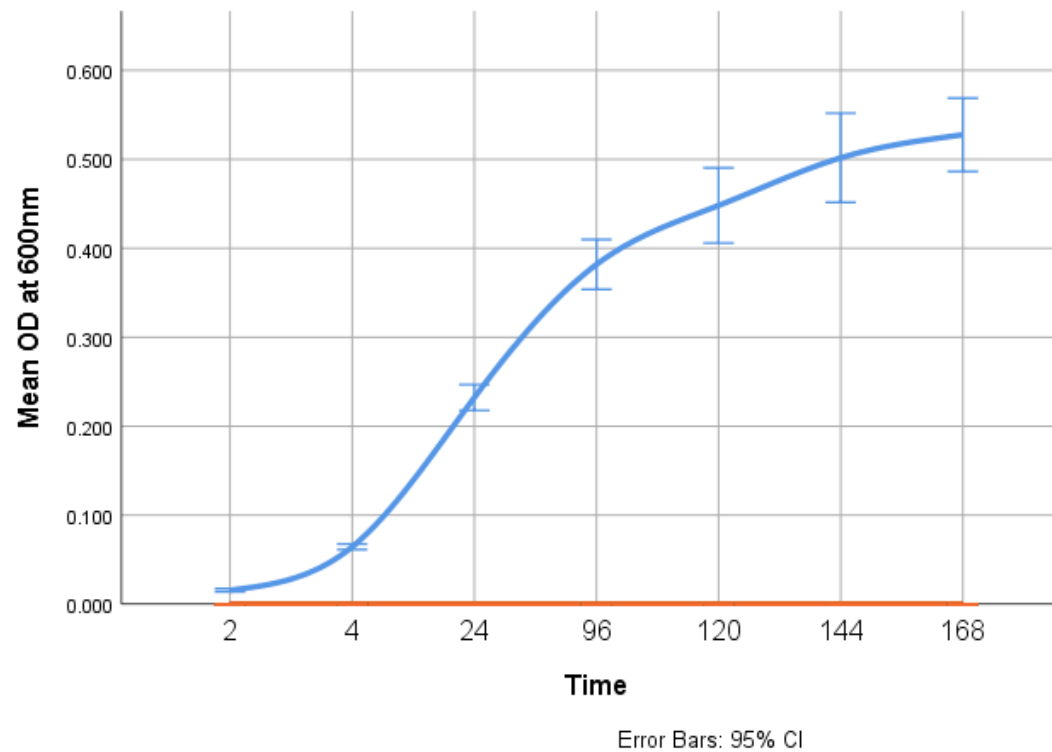
Figure 4.8:The effect of pH 4.2, 4.4 and 4.6 on the growth of *S.* Typhimurium (ATCC 53647) in peptone water at 37°C was compared to the growth of *S.* Typhimurium in the control (peptone water at pH = 7). No growth was observed at any of the tested acidic pH conditions, except in the control which is indicated in the blue line. The curve was plotted using the mean of spectrophotometric readings at OD$_{600}$ obtained from the three trials conducted in triplicate against the time of incubation in hours using the SPSS software

Table 4.2:The effect of pH on the growth of *S.* Typhimurium (ATCC 53647) following 24 h of incubation at 37°C. At 37°C, under acidic conditions (pH 4.2, 4.4 and 4.6) the growth of *Salmonella* was inhibited. Growth was only observed in peptone water (pH 7) which was used as the control

Trial	Temperature	pH	Time (h)	Growth
1A	37	4.2	24	-
1B	37	4.2	24	-
1C	37	4.2	24	-
1A	37	4.4	24	-
1B	37	4.4	24	-
1C	37	4.4	24	-
1A	37	4.6	24	-
1B	37	4.6	24	-
1C	37	4.6	24	-
1A	37	7	24	+
1B	37	7	24	+
1C	37	7	24	+

*+ indicates positive growth in broth media and – indicates no growth in broth media. pH 7 was used as the control

4.5 Effect of pH on the growth of *S.* Typhimurium using an inoculum from different stages of the growth curve

The following experiment was conducted to observe the growth of *S.* Typhimurium (ATCC 53647) at 37°C when the culture was in different stages of their growth curve. This part of the research was conducted as there was no standard method to determine the effect of the stage of growth of the inoculum on its acid tolerance.

4.5.1 Methods

For this experiment inocula in the lag phase (time = 2 h), log phase (time = 12 h), stationary phase (time = 24 h) and post stationary phase (time = 48 h) was used. A typical *Salmonella* colony from an overnight culture on XLD agar was introduced in to 100 mL nutrient broth and was incubated at 37°C (mother culture). When the culture was 2 h, 12 h, 24 h and 48 h old 1 mL from the mother culture was introduced in to 100 mL of pH adjusted (pH 4.2 or 4.6) peptone water. Peptone water at pH 7 was used as the control. The broth cultures were incubated at 37°C for 7 days in an orbital shaker incubator. Optical density readings were recorded in triplicates at OD_{600} every 2 h, 4 h ,6, 24 h and 7 days. The experiment was conducted in triplicate and was repeated three times (The experimental design followed to observe the effect of pH on the growth of *S.* Typhimurium in different stages of the growth curve **Figure 4.9**).
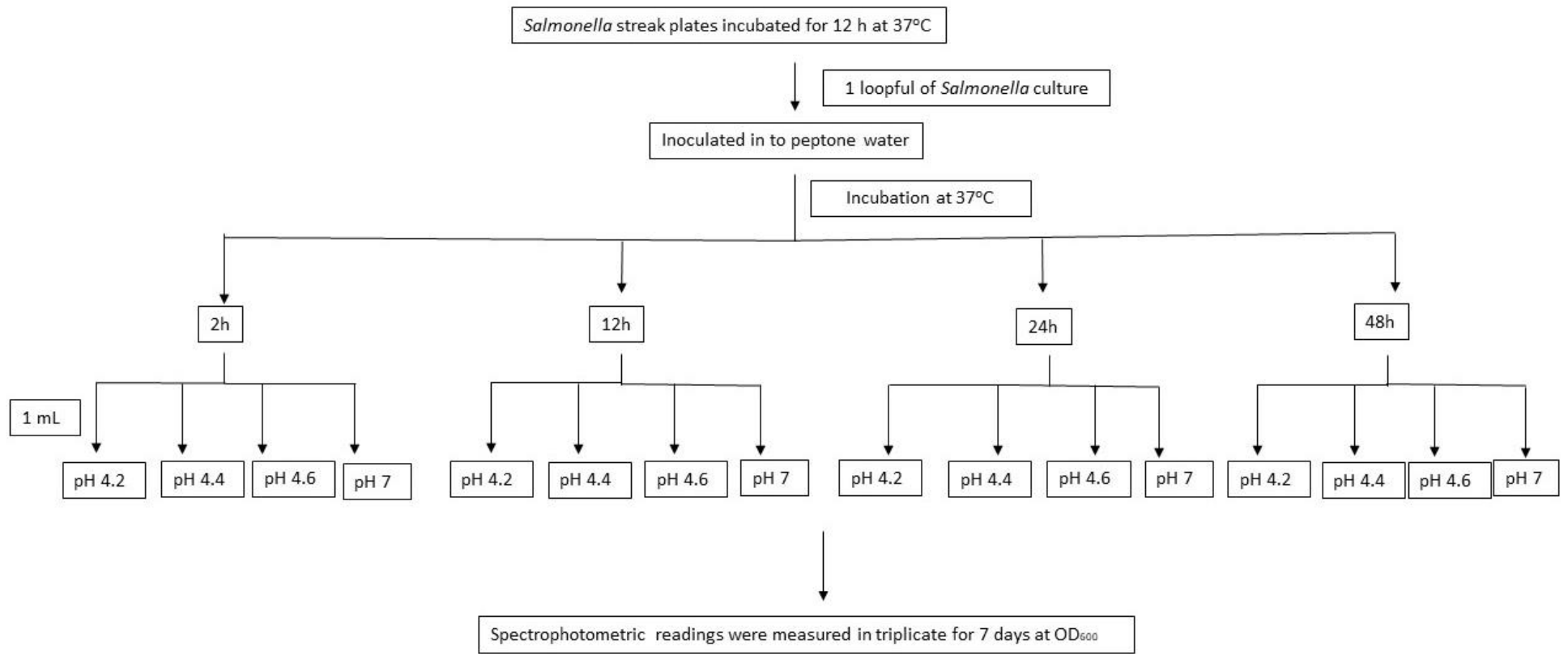
Figure 4.9: The experimental design followed to observe the effect of pH on the growth of *S*. Typhimurium in different stages of the growth curve

4.5.2   Results

No growth was observed in pH 4.2 or pH 4.6 in either 2 h, 12 h, 24 h and 48 h old cultures except for the controls (pH 7). The growth curves of the controls (pH 7.0) was not influenced by the growth stage. There was no notable lag phase in either of the control experiments. Thus, despite the stage of the inoculum, no growth was observed at 37°C.

4.6   Growth of *S.* Typhimurium at 37°C at higher inoculum concentration at acidic pH conditions

Though there was no growth observed at 37°C at acidic pH conditions during the previous experiments, another variable, the concentration of the inoculum on the growth of *Salmonella* was tested during this experiment.

4.6.1   Methods

Salmonella was incubated at 37°C in nutrient broth (pH 7.0) over night. Following incubation, the culture was centrifuged at 1,400 g for 15 min at room temperature. Cells were washed with sterile water and re-suspended in peptone water (pH 7.0) to obtain a higher concentration of the culture. The spectrophotometric reading at OD_{600} were recorded as 2.0.

One mL from the suspension was introduced in to 100 mL peptone water pH 4.2 and 4.6. pH 7.0 was used as the control during the experiment (**Figure 4.10**). The spectrophotometric readings were taken before the incubation and readings were recorded in triplicates at OD_{600} for 7 days.
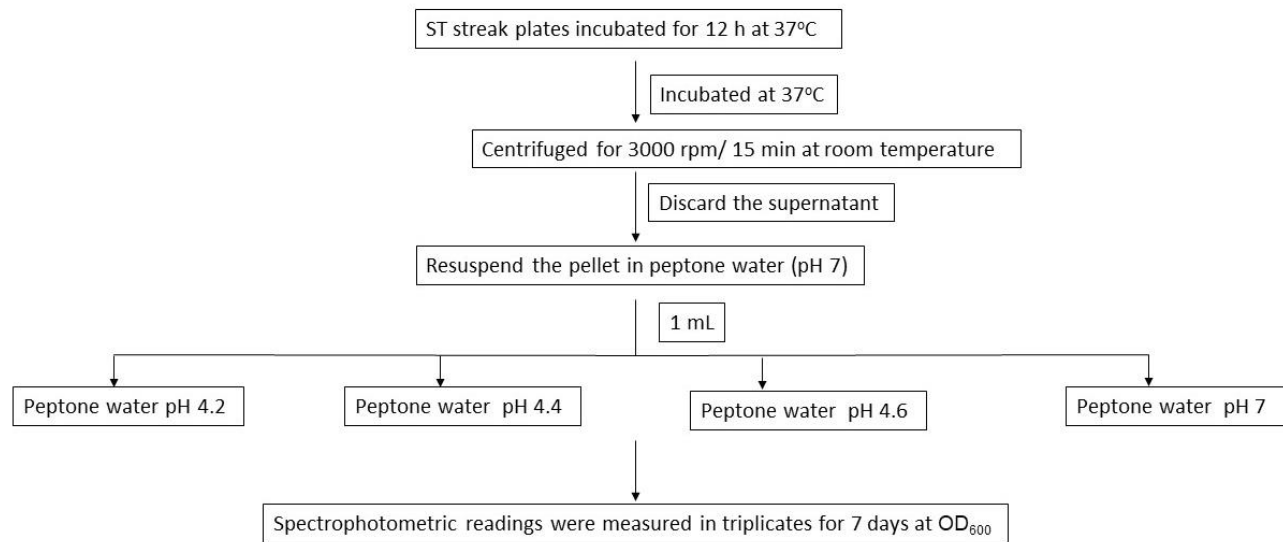
Figure 4.10: Experimental design illustrating the experiment conducted to observe the effect of acidic pH on the growth of *S*. Typhimurium when incubated at 37^O^C

4.6.2   Results

There was no growth observed in peptone water pH 4.2 and 4.6 though there was a high concentration of the initial inoculum. However, a notable growth of the organism was observed at pH 7 at 37℃ (control).

4.7 Combined effect of pH and temperature on the survival of *Salmonella* in different pH and temperature conditions in peptone water

The following experiment was focused on the survival of *Salmonella* in pH adjusted peptone water incubated at different temperature conditions. The survival of the S. Typhimurium infectious strain (wild strain) and the standard strain (ATCC 53647) in different pH (4.2, 4.4, 4.6) and temperature (37, 23 and 4°C) conditions was determined separately to understand the influence of temperature on the survival of *Salmonella* in acidic environments. The experiments were conducted in peptone water and the pH 7 was used as the control. The methodology was described in the paper "The combined effect of pH and temperature on the survival of *Salmonella enterica* serovar Typhimurium and implications for the preparation of raw egg mayonnaise" (Keerthirathne et al., 2019a) (page number **212**).

4.7.1 Methods

The experimental design is illustrated in the (**Figure 4.11**). Twelve different combinations of pH and temperature were tested during the experiment and the experiment was repeated 3 times (**Figure 4.12**) for each *Salmonella* strain. To increase the limit of detection and to observe possible resuscitation of *Salmonella* an enrichment step was conducted as described in the paper, section 2 (**Figure 4.13**).

4.7.2 Results

Higher temperatures increased the antibacterial activity of the organic acid in peptone water while the lower temperatures protected *Salmonella* (**Figure 4.16** and **Figure 4.17**)
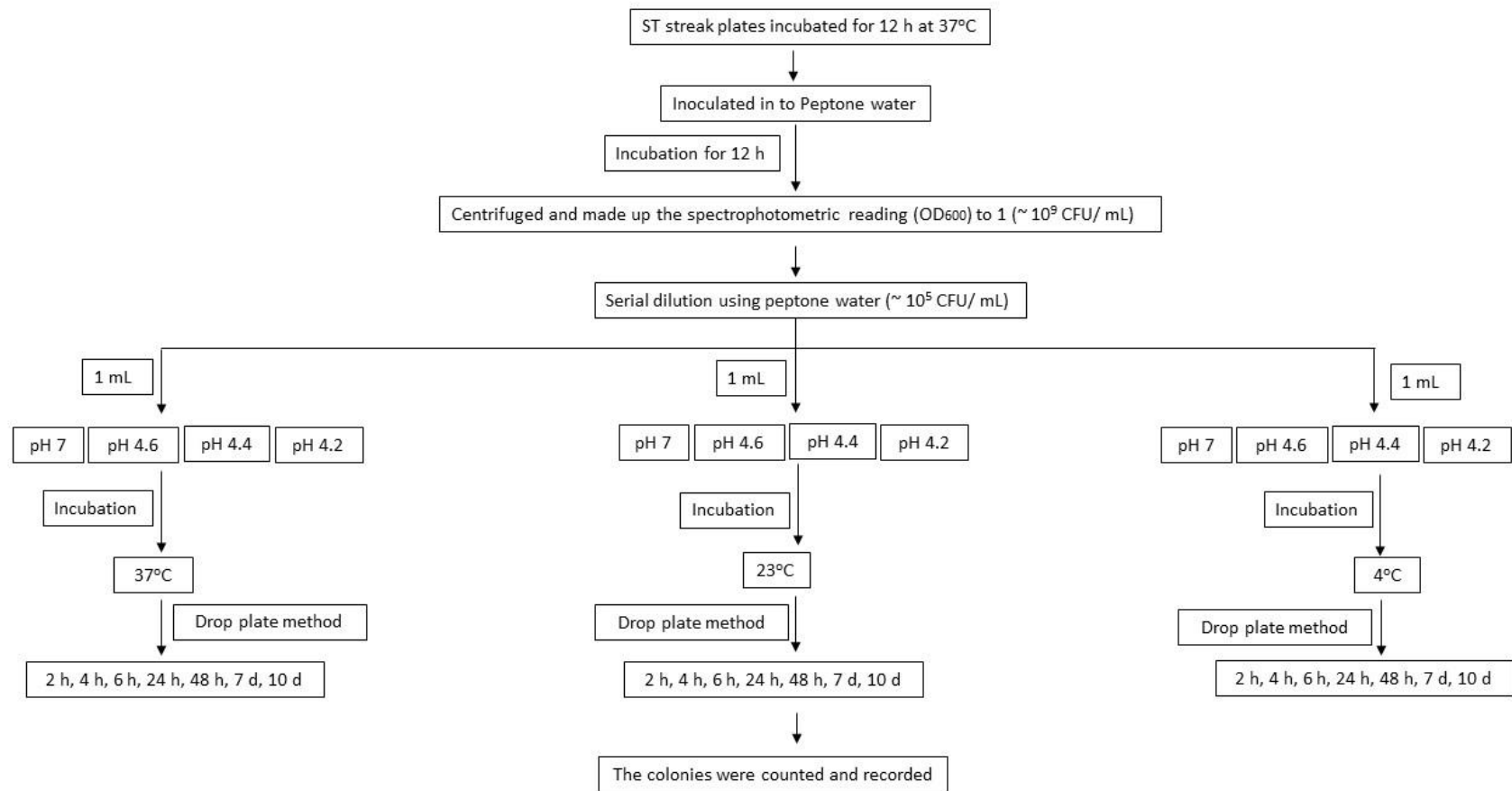
Figure 4.11: The experimental design followed to observe the combined effect of pH and temperature on the survival of *Salmonella*

Figure 4.12: Thirty-six (36) conical flasks were analysed per trial and 3 trials were conducted per *Salmonella* strain to determine the combined effect of pH and temperature on the survival of *Salmonella*
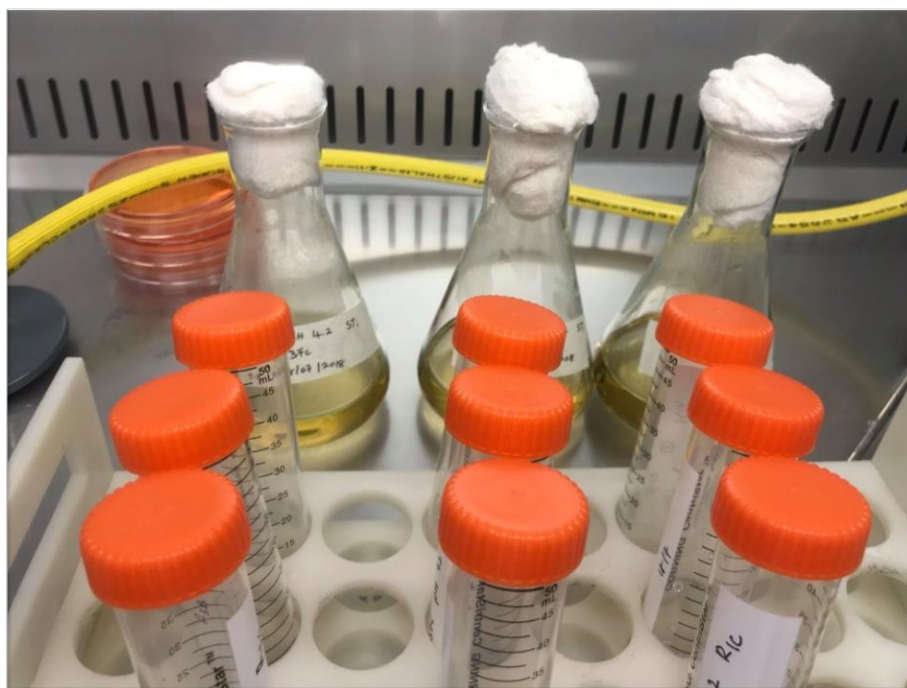
Figure 4.13: Increasing the limit of detection from 10 CFU/mL to 1 CFU/ mL or possible resuscitation of *Salmonella* cells. Each temperature and pH combination were tested with 3 broth cultures (triplicate) during each trial. The resuscitation/enrichment step was then conducted in triplicate for each broth culture by adding 1 mL from the broth culture to 9 mL sterile buffered peptone water
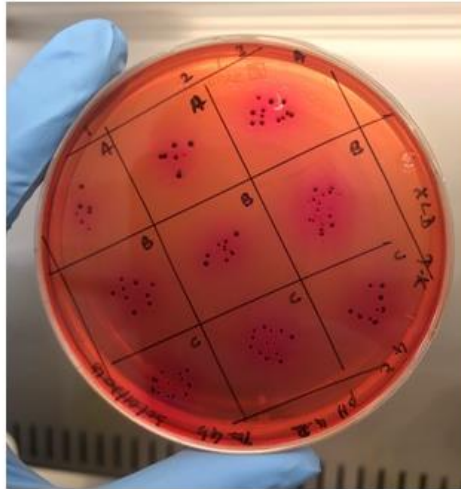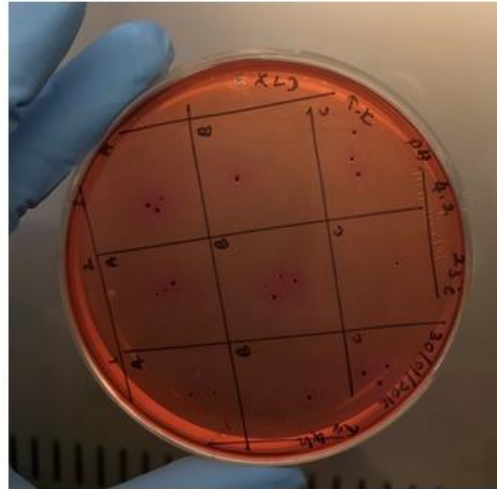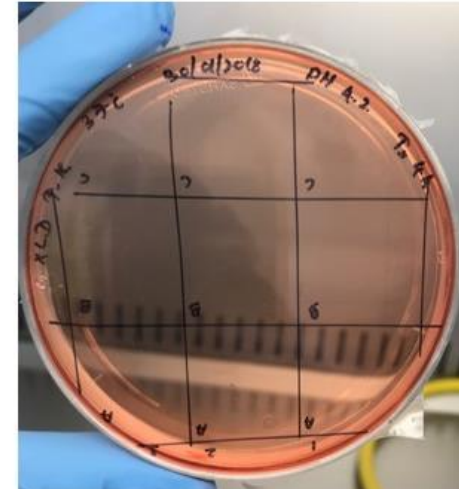
Trial 1; 4°C; T=4h ; pH 4.2   Trial 1; 23°C ; T=4h ; pH 4.2   Trial 1; 37°C ; T=4h ; pH 4.2

Figure 4.14: Drop plate method conducted on XLD agar. The survival of _Salmonella_ (infectious strain) at pH 4.2 at 4, 23 and 37°C. The highest survival was observed at 4°C while the viability of _Salmonella_ was rapidly lost at 37°C
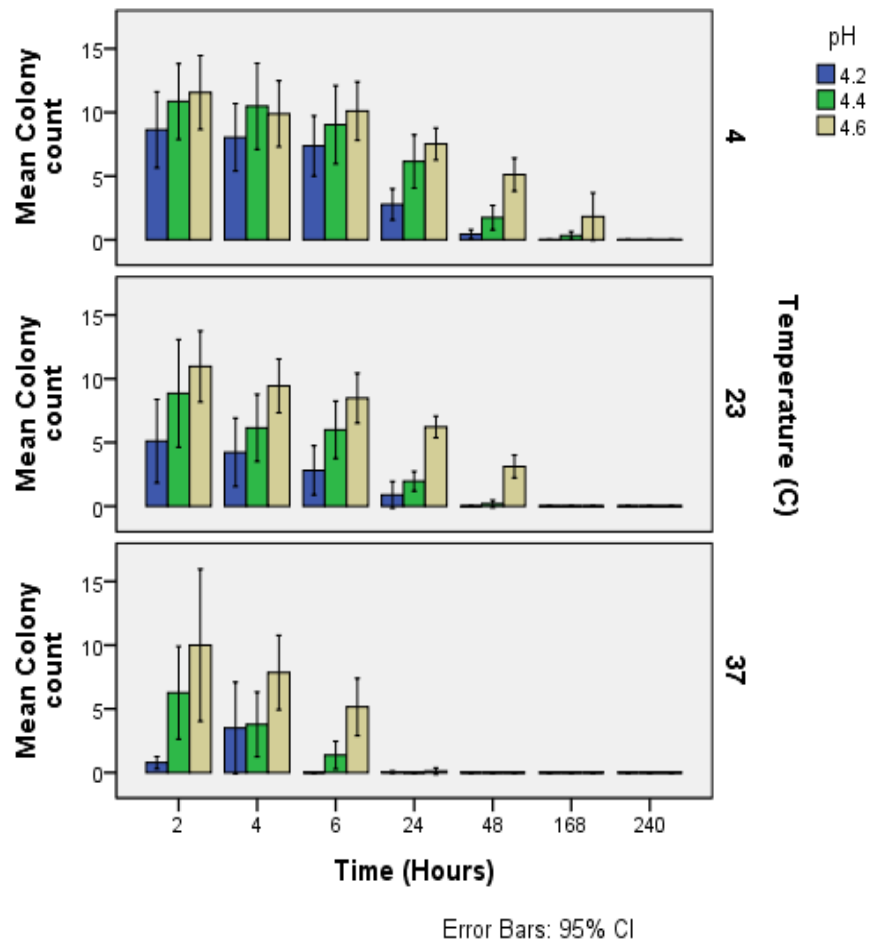
Figure 4.15: Survival of *S*. Typhimurium (standard strain) in peptone water. *Salmonella* survived for more than 7 days at 4ºC at acidic pH conditions. At 23 and 37ºC the antibacterial activity of the organic acids was increased. Hence a lower colony count (CFU/10 µL) was observed at higher temperature conditions. Peptone water with the pH adjusted to 7 was used as the control (not shown on the graphs as the number of colonies were uncountable)
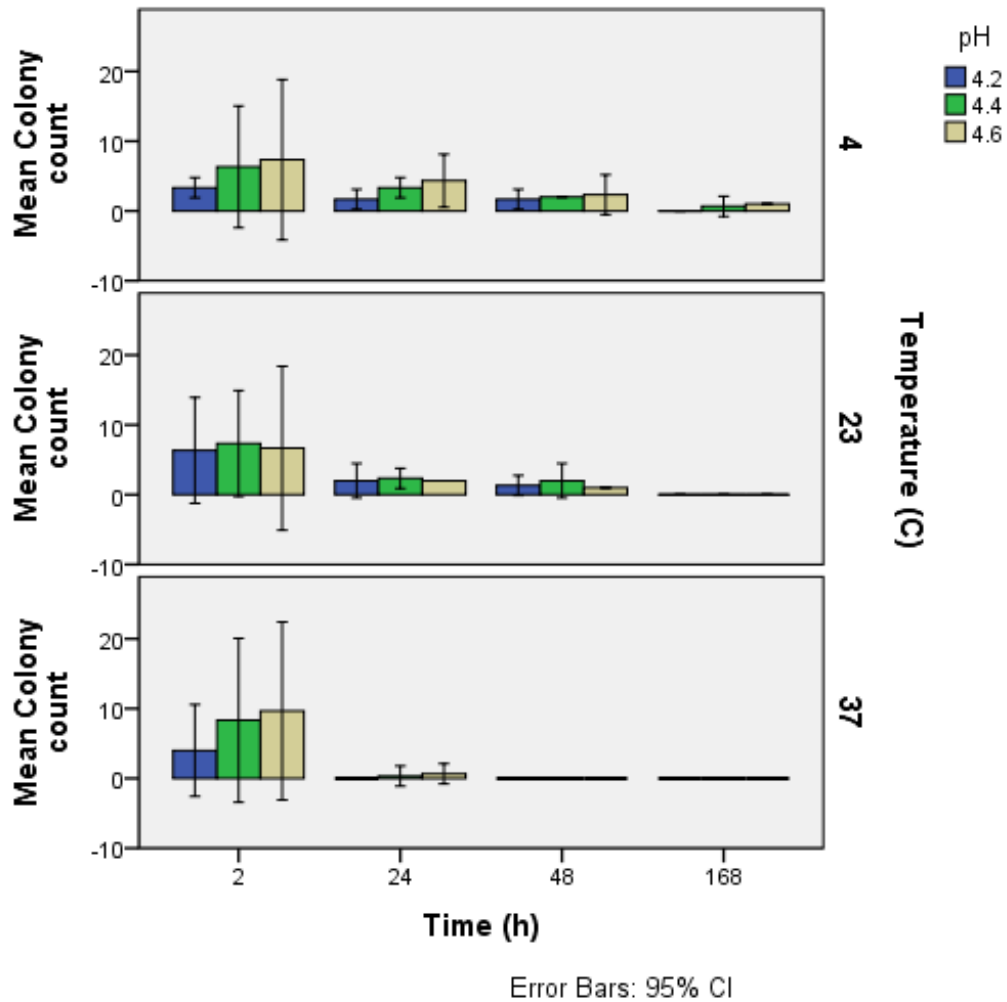
Figure 4.16: The mean colony count (CFU/10 μL) of the *S.* Typhimurium ATCC 53647 strain when incubated at different pH (4.2, 4.4 and 4.6) and temperature (4, 23 and 37°C) conditions in peptone water. Peptone water with the pH adjusted to 7 was used as the control (not shown on the graphs as the number of colonies were uncountable). An increased survival was seen at 4°C while the lowest survival was seen at 37°C

4.8 Combined effect of pH and temperature on the survival of *Salmonella* in mayonnaise at different pH and temperature conditions

The following experiment was conducted to observe the survival of *Salmonella* in raw egg mayonnaise.

4.8.1 Methods

Mayonnaise was prepared with egg yolks (**Figure 4.17**) and pH was adjusted to 4.2, 4.4 and 4.6 using vinegar. Unlike the experiments conducted with peptone water where the control was at pH 7, mayonnaise with pH adjusted to 5.6 was used as the control as a vinegar was added to prepare the emulsion (**Figure 4.18**). The prepared mayonnaise was inoculated with *Salmonella* (infectious strain) and incubated at 37, 23 and 4°C (**Figure 4.19**). The methodology was described in detail in the paper "The combined effect of pH and temperature on the survival of *Salmonella enterica* serovar Typhimurium and implications for the preparation of raw egg mayonnaise" (Keerthirathne et al., 2019a) (page number **212**). The experimental design is same as illustrated in the **Figure 4.11** which describes the experiments conducted with peptone water. Twelve different combinations of pH and temperature were tested during the experiment. The experiments were conducted in triplicate and was repeated 3 times.

Figure 4.17: Egg yolks were separated using an egg yolk separator in to a sterile beaker for the preparation of mayonnaise

Figure 4.18: The pH of the mayonnaise was adjusted using vinegar before the inoculation of

the *Salmonella* culture

Figure 4.19: Mayonnaise were prepared, and pH was adjusted to 4.2, 4.4, 4.6 and 5.6. The mayonnaises were inoculated with *Salmonella* and incubated at 37, 23 and 4ºC. Mayonnaise with a pH adjusted to 5.6 was used as the control

4.8.2 Results

The undissociated organic acids in vinegar act as an antibacterial agent in mayonnaise (Levine and Fellers, 1940) by penetrating the cell membrane and damaging the bacterial cells (Trček et al., 2015). Most of the previous studies were focused on observing the growth of *Salmonella* in mayonnaise (Baron et al., 1997, Sakha and Fujikawa, 2013). In circumstances where the raw egg product is already contaminated with *Salmonella*, identifying the most effective pH and temperature combination to reduce the viability of *Salmonella* is important to protect public health and development. Higher temperatures (37°C and 23°C) increased the antibacterial activity of the organic acid in mayonnaise while the lower temperatures (4°C) protected *Salmonella* (**Figure 4.20**). But, on the other hand if mayonnaise was not acidified according to the guidelines (pH 4.2) incubating the mayonnaise at room temperature could favor the replication and increase the risk of foodborne illnesses.
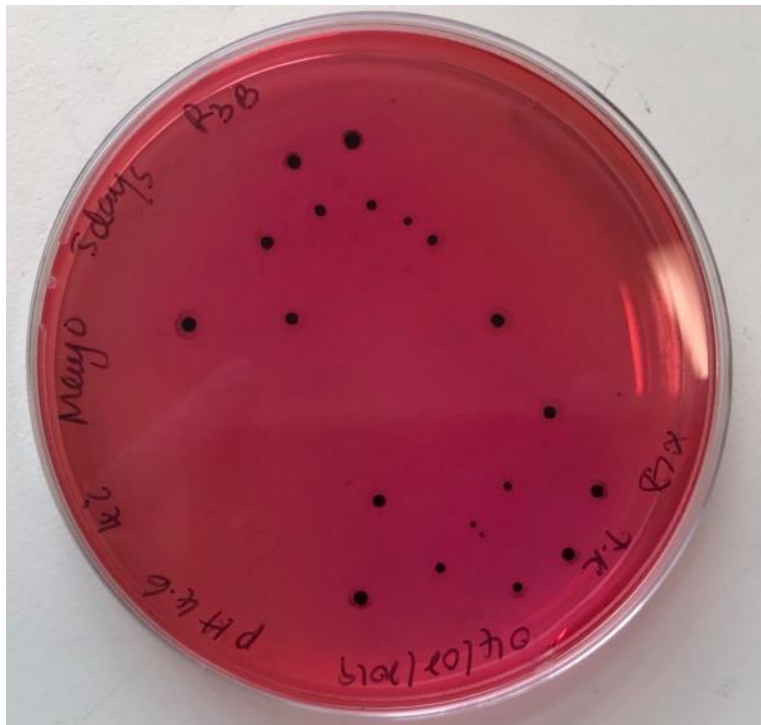
Figure 4.20: The survival of *Salmonella* in mayonnaise (pH 4.6) incubated at 4ºC after 5 days

CITATION

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Abstract

Raw egg products are often associated with salmonellosis. Australian guidelines recommend raw egg mayonnaise to be prepared and stored under 5°C and adjusted to a pH less than 4.6 or 4.2. Despite these guidelines, a significant amount of salmonellosis outbreaks are recorded annually in Australia. The aim of this study was to investigate the effect of pH and temperature on survival of *Salmonella* Typhimurium (ST) in peptone water (PW) and mayonnaise. The pH of PW and mayonnaise was adjusted to 4.2, 4.4 and 4.6 using acetic acid and vinegar respectively. The PW and mayonnaise were inoculated with ST and incubated at 37°C, 23°C and 4°C. The Survival of *Salmonella* was determined using the drop plate method. Survival was significantly ($p < 0.05$) improved at 4°C. In both mayonnaise and PW following 24 hours, there was no ST growth at pH 4.2. Resuscitation of ST was rapidly observed at 4oC while complete inactivation was observed at 37°C at pH 4.2, 4.4 and 4.6 in both PW and mayonnaise. Lower temperatures protected ST from the bactericidal effect of low pH. Preparation of mayonnaise at pH 4.2 or less and incubating it at room temperature for at least 24 h could reduce the incidence of salmonellosis

1 Introduction

Nontyphoidal salmonellosis is a gastrointestinal foodborne illness affecting public health (Newman et al., 2015). In Australia the incidence of salmonellosis has been increasing over the last decade (Ford et al., 2016) with the primary source of outbreaks identified as raw eggs and raw egg products (Louis et al., 1988, Chousalkar et al., 2017). According to Patrick et al. (2004) 80% of the 371 recorded outbreaks from 1985 to 1999 were related to eggs or egg products. Similarly in South Australia, from March 2017 to July 2018 Salmonellosis outbreaks were predominantly recorded due to eggs and raw egg products (Kenny et al., 2019). *Salmonella enterica* serovar Typhimurium (ST) is one of the commonly identified (Nguyen et al., 2014, Whiley et al., 2015).

Mayonnaise is a methodically prepared, semi-solid sauce, which is a combination of raw eggs, vinegar, oil and spices (Depree and Savage, 2001, Di Mattia et al., 2015). The most common control mechanisms for *Salmonella* in raw egg products involve various combinations of time, temperature and pH (Keerthirathne et al., 2016). According to the current guidelines in different states of Australia (New South Wales, Victoria and South Australia) raw egg products should be stored under 5^0C and the pH should be 4.2 or less than 4.6. Smittle (2000) states that as factory made mayonnaise is produced using pasteurized eggs; it isn't a threat to public health. On the other hand, on site manufactured mayonnaise has been linked with several salmonellosis outbreaks (Zhu et al., 2012a, Radford and Board, 1993) and is a growing public health concern as it is often prepared in cafes and restaurants.

The shelf life of a food product is often dependent on its pH, storage temperature and water activity (McMeekin et al., 2013). These factors are also important in controlling the growth and survival of pathogenic microorganisms in food products (Koutsoumanis and Lianou, 2013). There are guidelines for food handlers and chefs to follow while preparing raw egg products like mayonnaise. According to the Australia New Zealand Food Standards Code, the pH of the raw egg products should be adjusted to 4.2 or below and should be immediately refrigerated to a temperature below 5^0C (SA Health, 2013). The document further states that the raw egg product should be made in

small batches to minimize the time the raw egg product is at room temperature and all the processes in preparing the raw egg product such as "receipt, processing, storage and display" should be performed at a temperature below 5^0C.

The internal and external pH both have the ability to control biological and physiological processes of a bacterial cell (Foster and Hall, 1990) and unfavourable pH conditions can lead to loss of viability (Choi and Groisman, 2016). Interestingly, *Salmonella* utilizes mechanisms to survive unfavourable pH conditions and can achieve pH homeostasis (Krulwich et al., 2011). This is facilitated by the cellular proton pumps, potassium/proton and sodium/proton antiport systems (Booth, 1985), which enables the cell to be protected against acid stress (Foster and Hall, 1991). Additionally, the acid tolerance response (ATR) of *Salmonella* also acts as an additional protective barrier against the antibacterial activity of the acids (Foster and Hall, 1990). The ATR protects *Salmonella* spp. at low pH levels (pH 3 to 4), but is activated when environmental pH values are between 6.0 and 5.5 and when pH homeostasis fails (Foster and Hall, 1990). Due to these distinct characteristics, effectively controlling *Salmonella* has become a complex process. Moreover, pH also plays an important role in the structure and the stability of mayonnaise, which is an emulsion mainly consisting of eggs, oil and vinegar. The isoelectric pH of the egg yolk proteins is an essential quality to consider because it affects the viscoelasticity and stability of the mayonnaise by facilitating the emulsification process (Depree and Savage, 2001).

The interaction between storage temperature and pH are likely the main factors that influence the survival of *Salmonella* in on-site prepared raw egg mayonnaise. Despite the availability of numerous guidelines for the control of *Salmonella* in raw egg products, the incidence of salmonellosis outbreaks associated with these products in Australia is still increasing. This is the first study to investigate the combined effect of pH and temperature on the survival of *S.* Typhimurium in on-site prepared raw egg mayonnaise with the aim of informing the Australian food safety regulation.

2 Methods

2.1 Culture

Two strains of *Salmonella enterica* serovar Typhimurium (ST) were used for the study, a standard S. Typhimurium strain ATCC 53647 (STs) and a wild type *S*. Typhimurium (STi) strain which was isolated from a faecal sample of an infected individual.

2.2 Survival of *Salmonella* in acidic peptone water

For the experiment, pH of peptone water (PW) (Oxoid Ltd, Basingstoke, Hampshire, England) was adjusted using acetic acid to attain a pH of 4.2, 4.4 or 4.6. The PW suspensions were incubated (Memmert incubator, Germany) at 37^0C, 23^0C and 4^0C. PW at pH 7 incubated at 370C was used as the control. Each stage of the experiment was completed in triplicate and the experiment was repeated three times.

The *Salmonella* strains were retrieved from the -80^OC freezer by inoculating on to Xylose Lysine Deoxycholate (XLD) agar (Oxoid ltd, Basingstoke, Hampshire, England) plates using the streak plate method followed by incubation overnight at 37^OC. A typical *Salmonella* colony, with a black center, was introduced into 100 mL of PW pH 7 and was incubated at 37^OC overnight. The culture was centrifuged at 1,500 g for 15 min and the pellet was washed with PW and was resedimented by centrifugation at 1,500 g for 15 min.

The washed pellet was resuspended in PW and the optical density at 600 nm (OD600) was adjusted to 1 which was approximately equivalent to 10^9 CFU/mL (Chen et al., 2011) (UV-1800, SHIMADZU UV-spectrophotometer, Japan) and was subsequently serially diluted to a suspension of 10^5 CFU/mL which was used for the experiment. One (1) mL from this dilution was added to 100 mL of PW at pH 7, 4.6, 4.4 and 4.2. These cultures were each incubated at 37^OC, 23^OC, and 4^OC for 10 days. The drop plate method was used to plate 10 μL of each culture after every 2h, 4h, 6h, 1 day, 2 days, 7

days and 10 days to check the viability of the *Salmonella* on XLD agar. The number of representative colonies were recorded.

2.3 Resuscitation of *Salmonella* following acid treatment

The potential to resuscitate STi from a subset of incubations at different temperatures and pH conditions, was determined. After 1, 2, 7 and 10 days of incubation at each pH and temperature 1 mL from each of the samples was added to 9 mL of buffered peptone water (BPW) at pH 7 (Oxoid ltd, Basingstoke, Hampshire, England) which was incubated at 37°C overnight. Samples with increased turbidity were confirmed by spectrophotometric readings (UV-1800, Shimadzu UV-spectrophotometer, Japan) at 600 nm and confirmed to be *Salmonella* by plating on XLD agar.

2.4 Survival of STi in Mayonnaise

The recipe for the preparation of mayonnaise was adopted from the Certificate III Commercial Cookery, Student Recipe Learning guide from the tertiary and Further Education training provider, South Australia (TAFE SA) (TAFE SA., 2017). All the steps were carried out aseptically under a biosafety hood. The eggshells were disinfected by wiping with 70% ethanol and then broken and yolks separated using an egg yolk separator. Five mL vinegar was added to 6 egg yolks and was mixed in a sterile laboratory blender (Waring commercial Laboratory Blender, USA) until a uniform suspension was obtained. Sunflower oil was added gradually while blending.

The pH was tested (Hach Pocket Pro Multi 2, Colorado, USA) and was adjusted to pH 5.7 using vinegar. To adjust the pH further to 4.2, 4.4 and 4.6, white vinegar was gradually added to the mayonnaise. The mayonnaise samples from each pH (5.7, 4.6, 4.4, and 4.2) were inoculated with STi to achieve a final concentration of 10^7 CFU/g and was homogenized using a stomacher (Seward Laboratory blender Stomacher 400) for 2 min. Mayonnaise at pH 5.7 uninoculated with ST was prepared simultaneously and used as the negative control.

Fifty grams of mayonnaise at each pH, inoculated with ST and the uninoculated mayonnaise were incubated at 37°C, 23°C and 4°C. Following each incubation time interval (0, 1, 2, 5, 7 and 10 days) mayonnaise was homogenized in the stomacher for 2 min and 1 g of mayonnaise was mixed with 9 mL of 1% buffered peptone water (BPW). This step was performed in triplicate. From each of these triplicates, 10 µL was plated on to XLD agar using the drop plate method, which was also performed in triplicate. The colonies with typical *Salmonella* colony morphology on XLD agar were counted and recorded.

2.5 Resuscitation of *Salmonella* following incubation in mayonnaise

At each sampling time point BPW-mayonnaise mixtures were incubated at 37°C overnight (Reissbrodt et al., 2002). The incubated BPW-mayonnaise mixture was plated on XLD agar following overnight incubation and was incubated at 37°C overnight to check for resuscitation.

2.6 Statistical analysis

Repeated measures ANOVA was performed on SPSS software (IBM Corp. Released 2007. IBM SPSS for Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.) to analyze the results and generate the graphs.

3 Results

3.1 Acid tolerance of STs and STi in Peptone Water (PW) under different temperature conditions

Acid tolerance of the STs and STi were determined at different incubation temperatures. Incubation at 4°C significantly (p<0.05) increased the survival of both ST strains under all acidic pH conditions (pH 4.2, 4.4 and 4.6), compared to incubation at 23°C and 37°C. The bactericidal effect of the acidic pH was most effective after 24 h of incubation at 37°C. There were no viable STi at pH 4.2 at 37°C. Incubation at pH 4.6 was the least effective pH for inhibiting growth at all temperatures tested. STs and STi, both showed exponential growth in the control PW cultures (pH 7, 37°C or 23°C). There was no growth observed when STs or STi were incubated at 4°C at pH 7 but *Salmonella* survived at low temperature for more than 10 days in both PW and mayonnaise.

3.2 Resuscitation of *Salmonella* following incubation at acidic pH in PW

Although there were no observable colonies on the plates following incubation at 37°C, at pH 4.4 and 4.6; resuscitation of STi was observed at pH 4.4 during the first 24 h in PW. However, resuscitation of STi was comparatively reduced at 37°C (**Table 4.3**). Recovery of STi was observed following two days of incubation in pH 4.2 and pH 4.4 at 23°C. At 4°C, STi recovery was observed at all acidic pH conditions (pH 4.2, 4.4, 4.6) after 10 days of incubation indicating the maximum survival at 4°C. Complete and rapid inactivation of STi was observed at 37°C at pH 4.2 and resuscitation was not possible.

3.3 Survival and resuscitation of STi in mayonnaise

STi had a similar pattern of survival in mayonnaise (**Figure 4.21**) as seen in PW. Maximum survival was observed at 4°C in pH 4.6, and the viability was lost rapidly at 37°C at pH 4.2 with no resuscitation (**Table 4.4**). STi survived for more than 10 days at pH 5.7, and there was no growth of STi in the mayonnaise, used as the negative control. At 23°C, STi survived for more than 24 h at pH 4.6 with positive resuscitation after 48 h. No growth or resuscitation was observed at pH 4.2 and pH 4.4 following 24 h of incubation at 23°C. Similarly, there was no growth or resuscitation observed after 24 h at 37°C at pH 4.2, 4.4 or 4.6. In contrast, at 4°C resuscitation of STi was observed at 4.6 even after 10 days of incubation. Though culturability of STi was lost following 24 h of incubation at pH 4.2 at 37°C when incubated at 4°C resuscitation was observed even after 10 days.
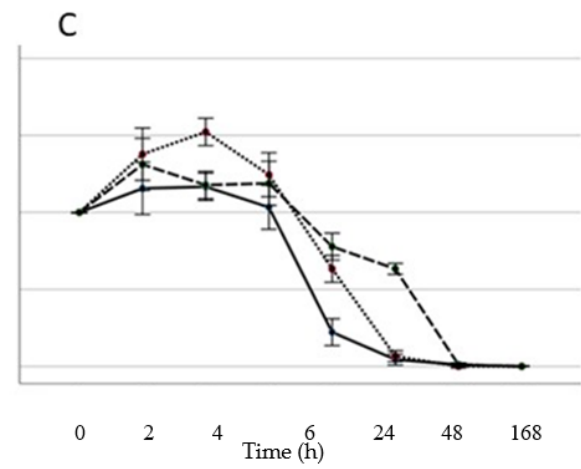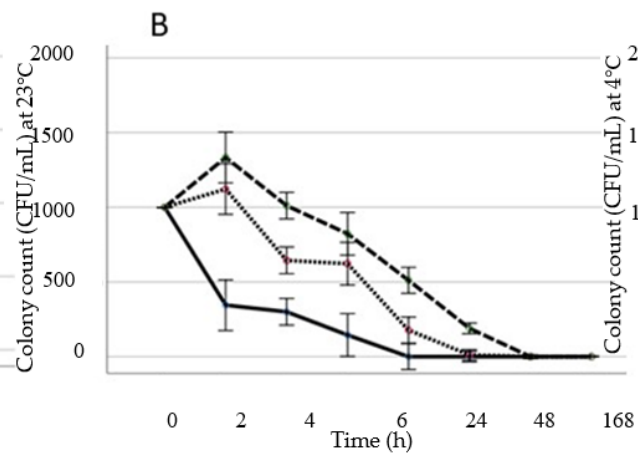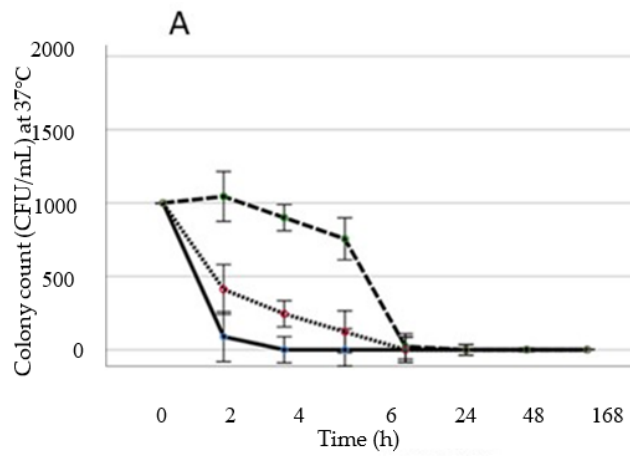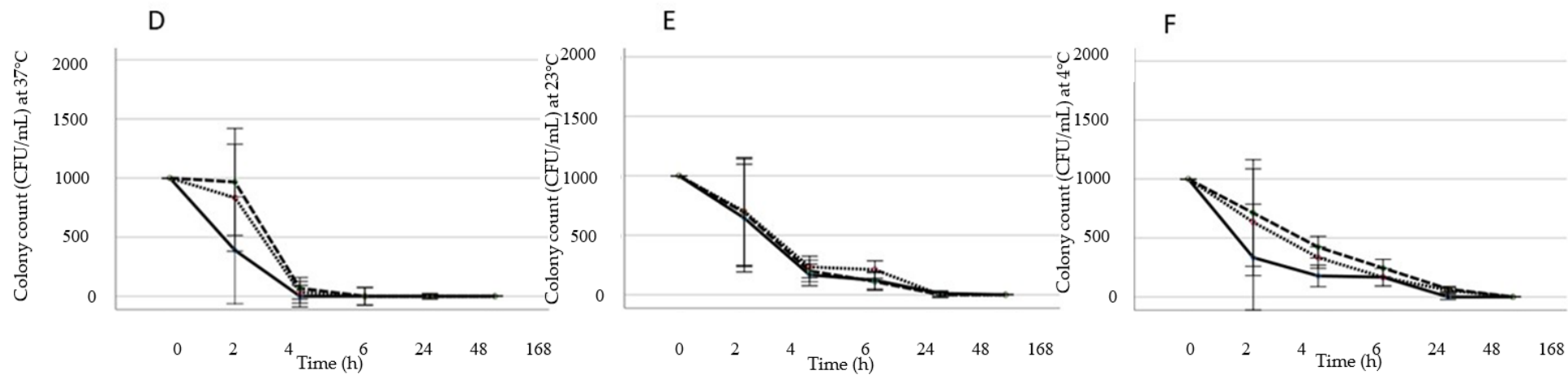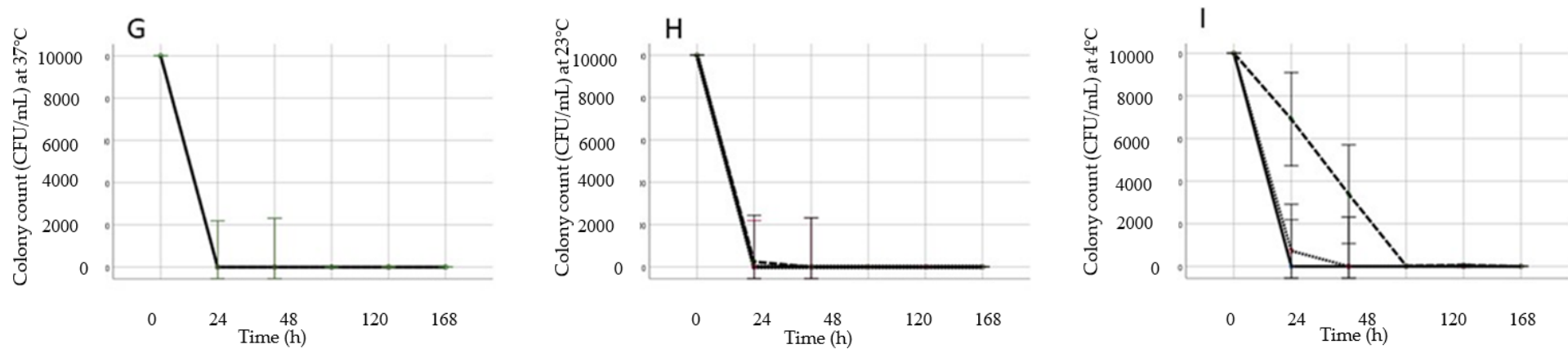
Figure 4.21: Survival of ST in PW at different pH and temperature conditions. A, B and C: Survival of STi in PW at 37⁰C, 23⁰C and 4⁰C in different pH conditions respectively. D, E and F: Survival of STs in PW at 37⁰C, 23⁰C and 4⁰C respectively. G, H and I: Survival of STi in mayonnaise at 37⁰C, 23⁰C and 4⁰C. Error bars at 95% confidence interval. (n=27 Three experiments, conducted in triplicate with triplicate drop plate analysis for each)

Table 4.3: Resuscitation (R) of wild type *S.* Typhimurium (STi) over time when incubated at different pH and temperatures in peptone water (PW)

Temperature (°C) [a]	Day	pH 4.2 Average colony count on XLD agar (CFU/mL)	pH 4.2 Resuscitation/ Growth	pH 4.4 Average colony count on XLD agar (CFU/mL)	pH 4.4 Resuscitation/ Growth	pH 4.6 Average colony count on XLD agar (CFU/mL)	pH 4.6 Resuscitation/ Growth
37	1	0	-	0	R	900	NA[a]
37	2	0	-	0	-	600	NA[a]
37	7	0	-	0	-	0	R
37	10	0	-	0	-	0	-
23	1	0	R	200	NA[a]	400	NA[a]
23	2	0	R	0	R	200	NA[a]
23	7	0	-	0	-	0	R
23	10	0	-	0	-	0	-
4	1	200	NA[a]	700	NA[a]	0	R
4	2	100	NA[a]	100	NA[a]	0	R
4	7	0	R	0	R	0	R
4	10	0	R	0	R	0	R

[a] If the Xylose Lysine Deoxycholate (XLD) agar plates had a positive growth for *Salmonella* the resuscitation/ enrichment step was not conducted. 0 = No culturable cells above the limit of detection (10 CFU/mL). R = resuscitation of viable but non culturable cells (VBNC) or enrichment to increase concentration above the limit of detection

Table 4.4: Resuscitation and survival of wild type *S*. Typhimurium (STi) over time when incubated at different pH and temperatures in mayonnaise

Temperature (⁰C) [a]	Day	pH 4.2 Number of positives for each of the three trials conducted in triplicate on XLD agar [a]	pH 4.2 Number of positives for each of the three trials conducted in triplicate in broth [b]/ R	pH 4.4 Number of positives for each of the three trials conducted in triplicate on XLD agar [a]	pH 4.4 Number of positives for each of the three trials conducted in triplicate in broth [b] /R	pH 4.6 Number of positives for each of the three trials conducted in triplicate on XLD agar [a]	pH 4.6 Number of positives for each of the three trials conducted in triplicate in broth [b] /R
37	0	27/27	NA	27/27	NA	27/27	NA
37	1	0/27	0/9	0/27	0/9	0/27	0/9
37	2	0/27	0/9	0/27	0/9	0/27	0/9
37	5	0/27	0/9	0/27	0/9	0/27	0/9
37	7	0/27	0/9	0/27	0/9	0/27	0/9
37	10	0/27	0/9	0/27	0/9	0/27	0/9
23	0	27/27	NA	27/27	NA	27/27	NA
23	1	0/27	0/9	0/27	0/9	9/27	8/9
23	2	0/27	0/9	0/27	0/9	0/27	3/9
23	5	0/27	0/9	0/27	0/9	0/27	0/9
23	7	0/27	0/9	0/27	0/9	0/27	0/9
23	10	0/27	0/9	0/27	0/9	0/27	0/9
4	0	27/27	NA	27/27	NA	27/27	NA
4	1	0/27	2/9	9/27	9/9	27/27	NA
4	2	0/27	6/9	0/27	5/9	19/27	9/9
4	5	0/27	5/9	2/27	6/9	14/27	7/9
4	7	0/27	0/9	0/27	3/9	9/27	4/9
4	10	0/27	1/9	0/27	0/9	0/27	4/9

[a] Three trials were conducted; each trial was done in triplicate and with triplicate drop plate analysis for each, NA = If there was growth observed on the drop plate then the resuscitation/enrichment step was not conducted, [b] When there were no culturable cells above the limit of detection a resuscitation step was conducted, There were three trials conducted in triplicate with three broth resuscitation analyses conducted for each R—Resuscitation (which could also be increased in concentration to above the limit of detection).

4 Discussion

Ensuring that eggs and raw egg products are prepared, transported and stored appropriately are crucial steps in reducing salmonellosis. For the preparation of raw egg mayonnaise, pH and temperature are key factors for the control of *Salmonella*. Results of this study indicate that the combined effect of pH 4.2 and the incubation temperature, 37^0C was the most effective at reducing *S.* Typhimurium; however, incubating at 23^0C was still more effective than 4^0C and may present a more practical approach. The STi strain survived longer than the STs strain in the pH adjusted PW at 4^0C. STi strain survived in pH 4.6 mayonnaise for more than 10 days at 4^0C, but viability was lost within 24 hours at the same pH when stored at 37^0C. The long-term survival of *Salmonella* at 4^0C under acidified conditions also demonstrates the potential risk posed by cross contamination in the refrigerator, cooking utensils, other ingredients etc. and the importance of sanitation and cleaning.

The lag phase is the earliest and the most poorly understood stage of the bacterial growth cycle (Rolfe et al., 2012). It is presumed that during the lag phase bacterial cells undergo an adaptation process required for survival in new environmental conditions (Madigan et al., 1997a). The metabolic functions of a bacterial cell are also influenced by the growth environment (Malik et al., 2015). These adaptive mechanisms as well as the ability to achieve pH homeostasis, the ATR and the presence of a capsule makes *Salmonella* more resistant to environmental stresses (Helander et al., 1997). Therefore, precisely engineered combinations of control mechanisms are needed to control the growth of *Salmonella*.

These results differ from the Australian guidelines for the preparation of raw egg mayonnaise, which state that the pH should be adjusted to 4.2 and the raw egg products should be stored below 5^0C (SA Health, 2013). The guidelines further indicate that mayonnaise should be made in small batches every day and the 2-hour/4-hour rule should be maintained. This study showed that *S.* Typhimurium present in acidified mayonnaise (pH <4.6) decreased over time and that older batches of mayonnaise may present a lower risk for salmonellosis compared with a batch made that day. On the other hand,

the advantage in making mayonnaise in small batches is that if a batch of mayonnaise is contaminated then fewer people will be exposed to that particular batch.

The pH of mayonnaise is reduced through the addition of vinegar and/or lemon juice (Levine and Fellers, 1940). The lipophilic nature of the undissociated acetic acid molecules penetrate the cell membrane and decrease the internal pH causing damage and death to the bacterial cells (Trček et al., 2015). The antibacterial activity of the acid also depends on the storage temperature and the water activity (a_w) of the egg-based sauce (Smittle, 1977). Higher temperatures rapidly reduced the viability of *Salmonella* when incubated at acidic pH. This is probably due to the increased cell membrane penetration of organic acids at higher temperatures (Zhu et al., 2012b). Other variables that can affect *S.* Typhimurium survival in mayonnaise include the type of oil, garlic, spices and plant essential oils (mint, cardamom, cinnamon, clove, orange, lemon and grapefruit) (Keerthirathne et al., 2017). Further research is needed to explore the combined effect of these different additions in recipes.

The risk of storing mayonnaise at 37°C is that if the pH is not correctly measured the warmer temperature will promote the growth of *S.* Typhimurium. The results of this study confirmed that at higher pH (i.e. pH 5.7 in mayonnaise and 7 in peptone water) there was rapid growth and increased cell numbers of *S.* Typhimurium at 37°C and at room temperature, whereas, no growth was seen at 4°C. This demonstrates the necessity for food handlers and chefs to confirm pH during the preparation of raw egg mayonnaise.

5 Conclusions

This study showed that the lower temperatures reduce the antibacterial activity of the organic acids which allowed *Salmonella* to survive at low pH for longer. preparing home-made mayonnaise at pH 4.2 and retaining at room temperature for at least 24 h effectively killed *S.* Typhimurium which could aid in reducing the prevalence of the salmonellosis outbreaks in Australia.

6 Further Research

Non-lethal but harsh environmental conditions could induce adaptive acid tolerance in *Salmonella*. Further studies into the development of acid tolerance of *Salmonella* in mayonnaise at different storage temperatures and pH conditions would be beneficial for further controlling salmonellosis outbreaks. As different species of *Salmonella* have different capacities to tolerate different environmental stressors, future research is needed to examine the survival of different sub-species of *Salmonella* in different pH and temperature conditions. The combined effect of garlic, salt, plant essential oils and spices in mayonnaise on the survival of *S.* Typhimurium should be assessed. There is also a need for further research to investigate the survival of other foodborne pathogens, such as *Campylobacter* spp., *Shigella* spp., and *E. coli* in raw egg mayonnaise under these conditions prior to making a change in food safety regulations.

Author Contributions

T.K.; Conducted the laboratory experiments, data analysis and drafted the manuscript. HW conceptualization, supervised, coordinated, edited and contributed to the manuscript. Kirstin Ross supervised, coordinated, edited and contributed to the manuscript. Howard Fallowfield supervised, coordinated, edited and contributed to the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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4.9 Concluding observations of the chapter

The ultimate control measure for the preparation on raw egg mayonnaise, particularly for vulnerable populations, is to use pasteurized eggs. However, the main barrier preventing the widespread use of pasteurized eggs is the impact on egg properties. The pH of the egg albumen, stability and the viscosity can be easily influenced by heat pasteurization techniques. Similarly, non-thermal pasteurization techniques such as irradiation can decrease the viscosity, increase the degradation of egg proteins and promote the production of free radicals in eggs (Keerthirathne et al., 2017). These factors decrease the consumer acceptability of the pasteurized eggs and prevent chefs and food handlers from using them. This highlights the importance of exploring the best control approaches which reduce the risk of salmonellosis linked to eggs and raw egg products and are still acceptable to the chefs, food handlers and consumers.

Despite the food safety guidelines for the preparation of raw egg products the incidence of salmonellosis linked to eggs and raw egg products is increasing in Australia. According to the guidelines, temperature and pH are commonly used control mechanisms to regulate the growth of pathogenic bacteria in food products such as mayonnaise. Therefore, we assessed the optimum pH and temperature combination for the control of *S.* Typhimurium in raw egg products. According to these results lower temperatures (4°C) protected the *Salmonella* cells against the antibacterial activity of the organic acids while the higher temperatures (23 and 37°C) rapidly reduced the survival of *Salmonella* at acidic pH conditions.

According to The Australian food safety guidelines, preparation, transport and storage of the raw egg products should be carried out below 5°C to reduce the incidence of salmonellosis. Contrastingly, according to the results the survival of *Salmonella* was increased at 4°C. Moreover, according to the results pH 4.2 at 37°C was the most effective combination to reduce the viability of *Salmonella*. However, pH 4.2 at 23°C may be a more practical approach. Leaving pH adjusted (pH 4.2) raw egg products at room temperature for at least 24 h before consumption will help reduce the incidence of salmonellosis outbreaks linked to raw egg products. The Australian food safety

guidelines should be transformed accordingly to protect public health and development. Accordingly, a Hazard Assessment Critical Control Point (HACCP) based protocol can be used to prevent salmonellosis outbreaks linked to eggs and raw egg products Figure 4.22.
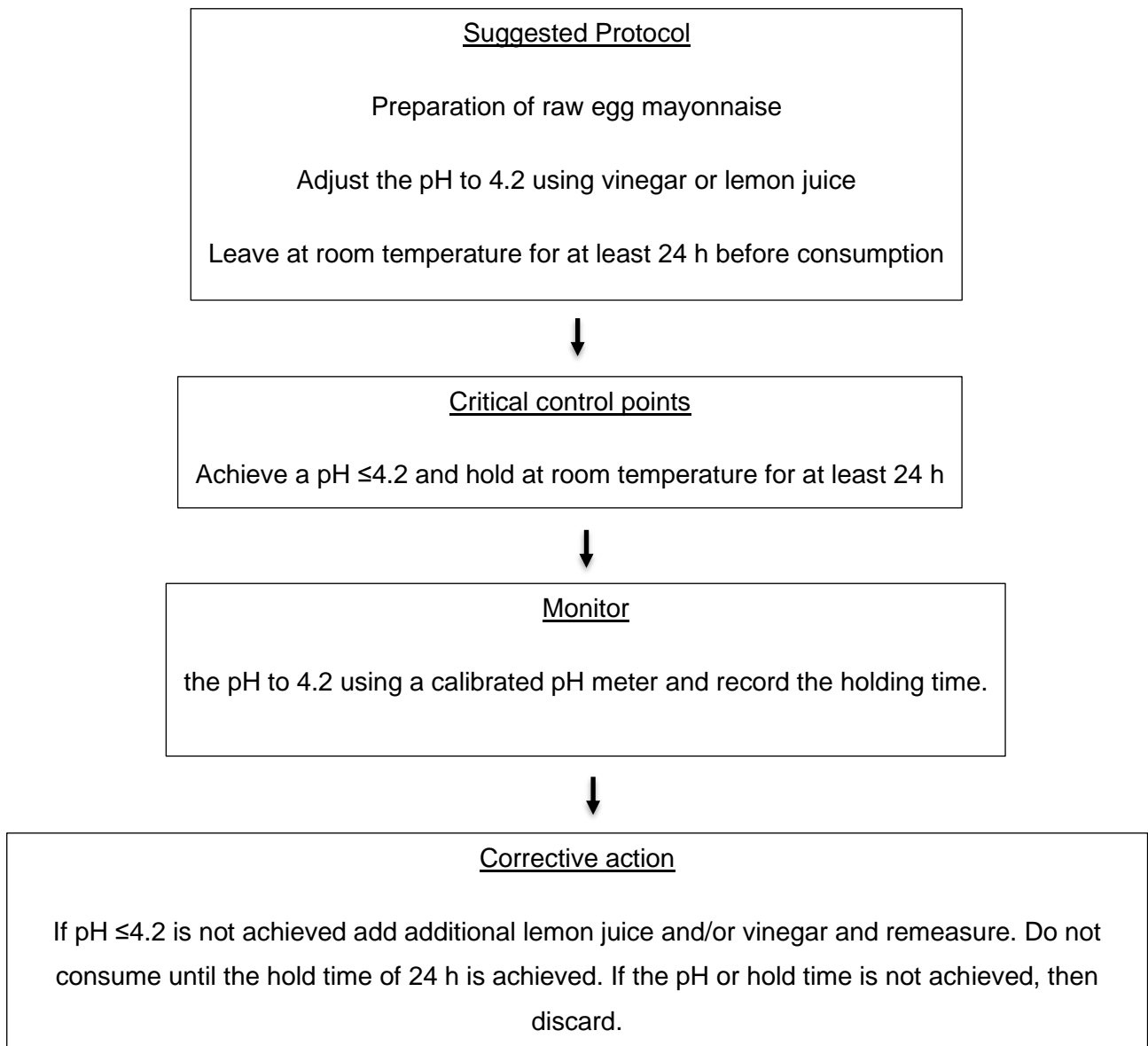
Figure 4.22: Application of the HACCP based principles could reduce the incidence of salmonellosis

5 Conclusion

S. Typhimurium and *S.* Enteritidis are common causes of salmonellosis outbreaks linked to eggs and raw egg products. *S.* Typhimurium is the strain most frequently identified in Australia linked to eggs and raw egg products (Ford et al., 2018, OzFoodNet, 2006). As *S.* Enteritidis is the strain commonly identified in other parts of the world, previous research has primarily focused on the control of *S.* Enteritidis. Therefore, there is a need for more research to identify the best control mechanisms for *S.* Typhimurium in Australia. This thesis addressed gaps in knowledge regarding the control of *S.* Typhimurium in eggs and raw egg products, with the aim of informing Australian public health and food safety regulations.

5.2   Objectives addressed throughout the project

5.2.1   Backyard chicken and zoonotic diseases

Salmonella (Hale et al., 2012)*, Campylobacter* (El-Tras et al., 2015) and *Shigella* (Bachand et al., 2012) are zoonotic bacterial species linked to gastrointestinal infections in humans. Previously there was only one study conducted on backyard poultry in Australia which assessed the prevalence of *Salmonella* infection in 30 domesticated backyard chicken flocks (Chousalkar and Gole, 2016). However, here presented, the first study to investigate the prevalence of *Salmonella* as well as the risk factors associated with keeping poultry in backyards and the knowledge of the general public on handling poultry. Chicken faeces collected from 82 backyards were screened for *Campylobacter* spp., *Salmonella* spp. and *Shigella* using qPCR. It was found that 15% of the bird faeces samples were positive for either *Campylobacter* spp. or *Salmonella* spp. and none were positive for *Shigella* spp. Traditional culturing methods on XLD agar followed by qPCR for the isolated cultures indicated that 4% (n=5) were positive for *Salmonella* spp. one of which was identified as *S.* Typhimurium.

There are many factors that decrease the immunity of poultry including, weather, age or competition for food and water. During this study larger poultry flocks containing multi-aged birds was identified as a risk factor, which could increase the incidence of salmonellosis and campylobacteriosis. In a multi-aged flock, the older immunocompromised birds could introduce pathogens to the younger birds. These factors might have contributed towards the higher infection rate of the larger flocks which contained more than 10 birds while the smaller flocks (1-3 birds) had the lowest infection rate. Lack of knowledge in the general public in handling fresh eggs and poultry could increase the risk of infection if the eggs are consumed raw or as a raw egg product such as mayonnaise. Washing the eggs, storage of eggs on the shelf, consumption of raw egg products, having flocks with more than 10 birds and having multi-aged flocks were identified as risk factors that could contribute to the increased incidence in gastrointestinal outbreaks.

5.2.2   The eggshell decontamination method

S. Enteritidis commonly contaminates the internal contents of the egg (Wales and Davies, 2011). In contrast, *S.* Typhimurium contaminates the outside of the eggshell (Martelli and Davies, 2012, De Buck et al., 2004). The review in Chapter 3 presented studies from around the world that focused on different egg decontamination methods. The review identified that, except for one study, all the studies were focused on *S.* Enteritidis (Hou et al., 1996, Stadelman et al., 1996, Schuman et al., 1997, Gast, 1993, Himathongkham et al., 1999). The only previous study that investigated the decontamination of *S.* Typhimurium from eggs was conducted by Himathongkham et al. (1999) and involved eggs that had been artificially internally contaminated. As *S.* Typhimurium is usually only found on the outside of the egg this research is not applicable to the Australian situation. Therefore, this is the first project that investigated a whole shell egg decontamination method by artificially inoculating the outside of the eggshell with *S.* Typhimurium, which is representative of the Australian situation.

Immersing eggs in water heated to 57°C for 9 min and 6 min effectively decontaminated the eggs inoculated with the infectious *S.* Typhimurium strain (wild type) and the standard *S.* Typhimurium strain (ATCC 53647) respectively. This decontamination method was proven to be successful at decontaminating whole shell eggs externally contaminated with *S.* Typhimurium without affecting the egg properties. This egg decontamination method was developed using an apparatus for sous-vide cooking. This was done to ensure easy implementation in a home or a commercial kitchen. Following the blind control study, the heat-treated eggs were acceptable to chefs and food handlers as there was no significant difference in the appearance, texture, smell, colour and stability of the mayonnaise prepared using the heat-treated eggs.

5.2.3   The optimum pH and temperature combination for the control of *S.* Typhimurium

In Australia, raw egg products are one of the most common causes of salmonellosis outbreaks (Ford et al., 2018). Temperature and pH have been identified as the main factors that control the survival of *Salmonella* in mayonnaise. The mechanism, acid tolerance response, protects *Salmonella* against acid stress (Foster and Hall, 1990). The increased expression of the acid induced genes; cadA, cadB, adiA, and adiY at low pH aids the adaptation of *Salmonella* into low pH environments (Liu et al., 2017). According to Hu et al. (2018) virulence of *Salmonella* strains were increased with the increased expression of rfb gene under low pH conditions. These molecular mechanisms increase the survival of Salmonella in acidic environments.

Although there are many studies investigating the control of *S.* Enteritidis, there is a gap in knowledge in relation to the survival of *S.* Typhimurium in raw egg products. This is the first study to explore the survival and resuscitation of two different *S.* Typhimurium strains (an infectious strain and a standard strain) under 12 different combinations of temperature (37°C, 23°C and 4°C) and pH (4.2, 4.4 and 4.6) in both peptone water and in on-site prepared raw egg mayonnaise. Examining the survival of *S.* Typhimurium under acidic conditions (pH 4.2, 4.4 and 4.6) in peptone water and mayonnaise demonstrated that *S.* Typhimurium survived for longer at lower temperatures (4°C) compared with higher temperatures (23°C and 37°C). The most effective temperature and pH combination for controlling *S.* Typhimurium was 37°C at pH 4.2, indicating increased antibacterial activity at higher temperatures but holding the raw egg products at room temperature for 24 h (pH 4.2) might be the more practical approach. Therefore, for the preparation of raw egg products, which uses pH (vinegar or lemon juice) as a control mechanism (e.g. mayonnaise), should be changed from "immediately store under 5°C" to leave at room temperature for at least 24 h before consumption.

5.3 Food safety in commercial kitchens and implementation of new/modified policies

5.3.1 Improving food safety in commercial kitchens

Improper food handling techniques can lead to foodborne illnesses. Fostering a positive food safety culture is essential to reduce the incidence of foodborne illnesses in commercial settings. Food safety culture can be described as the collective procedures followed by the management system, chefs, food handlers and food delivery personnel to ensure safe food distribution to protect public health (Nyarugwe et al., 2016). Therefore, maintaining and assessing proper food safety culture within a premises, i.e. focusing on providing safe and quality food to minimize foodborne illnesses is important (Nayak and Waterson, 2017). An appropriate food safety culture can be achieved through regulated cooking procedures, proper storage temperature, proper hygiene and sanitisation techniques and by minimizing cross contamination in the kitchen (Mwamakamba et al., 2012).

Apart from chefs and food handlers, management should also be educated and willing to practice proper food handling and storage techniques (Powell et al., 2011). Communication and periodic food safety performance analysis are essential in reducing foodborne illnesses and preserving food safety culture (Griffith et al., 2010). In a food preparation premises, priority should be given to preserving food safety culture over saving money (Powell et al., 2011).

Increasing awareness and encouraging commercial food distribution facilities to follow food safety regulations could reduce the incidence of foodborne illnesses. Food safety regulations need to be practical, easy to follow and should be accepted by chefs and food handlers. According to SA Health food safety regulations raw egg products such as mayonnaise should be prepared daily in small batches (SAHealth, 2013). However, following these guidelines is not practical and very time consuming. This has resulted in negative responses, a lack of adoption and ultimately dishonest behaviour from chefs and food handlers (as illustrated by comments on social media (**Figure 5.1**). Furthermore, these current guidelines are not supported by this research and therefore should be

modified according to new scientific discoveries. According to the results of this research, preparing mayonnaise (at pH 4.2) and incubating it at room temperature for at least 24 h reduces the viability of *Salmonella*. These proposed guidelines are effective and easy to follow and therefore should be better accepted by chefs and food handlers.

Yesterday at 21:04

Would like to ask everyone's thoughts on a situation that had happened
recently, A health inspector about 2/3 months ago came in saying our
kitchen was immaculate, food storage and food handling etc etc at very high
standards which is what you want in any kitchen, until recently another
inspector visited picking out that our Aioli is supposed to be thrown out by
the end of the day. basically made daily, now we do understand the food
safety of raw eggs and correct storage. But this is the first time in 8 years as
a chef I have heard an inspector tell us we have to dump all our Aioli that is
only a day old since every restaurant I have worked in we made big batches
and no mention about it?? Thoughts on this are welcome, but no arguments
please. The inspector was practically telling us to buy in cheap aioli haha
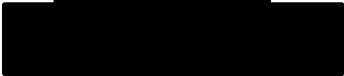
do what u do, tell em what they want to hear

Figure 5.1: Comments from chefs posted on a social media platform on requirements to make their raw egg products every day in small batches. The response on social media from chefs indicates that the current guidelines are impractical and therefore are not being followed

Improving food safety guidelines will help reduce the number of salmonellosis outbreaks linked to raw or undercooked eggs and raw egg products, which has continued to increase over the last decade in Australia. The environmental health offices (EHOs), the food handlers, and the chefs should be informed about the new scientific approaches on preparation of raw egg products. This can be efficiently achieved through competent food safety policy modification (**Figure 5.2**). The

successful implementation of a change in guidelines can be impeded by the presence of barriers. The specific barriers must be identified prior to implementation of a change in policy to ensure the correct approach is used (Diepeveen et al., 2013). Interpretation of policies can be influenced by the educational level of the person, cultural backgrounds and beliefs. Moreover, according to Cabana et al. (1999) "[l]ack of awareness, lack of familiarity, lack of agreement, lack of self-efficacy, low expectancy of favourable outcomes and lack of motivation" are barriers to successful policy implementation. A significant barrier to achieving food safety is the attitude and behaviours of food handlers. According to Powell et al. (2011), convincing food handlers to change their food handling techniques and attitudes in relation to new scientific findings remains a challenge (Ovca et al., 2017). The delay in transforming policies in accordance with the new scientific discoveries and techniques is also identified as a significant barrier in reducing the incidence of foodborne illnesses.

Successful implementation of guidelines can be achieved through strategic educational campaigns. Distributing the guidelines in simplified language to the general public will ensure accurate interpretation of the policies. Anti-smoking, AIDS, child immunization, road safety and blood donation campaigns conducted specifically for the targeted populations have successfully changed and improved behavioural patterns (Wakefield et al., 2010). According to Redmond and Griffith (2005), targeted food safety educational campaigns that focused on a specific group, for example chefs and food handlers, are more effective when introducing new strategies and techniques. Additionally, multiple or combined approaches have been successful in effective policy implementation. For an example, modifications such as tax increases on cigarettes along with mass campaigns have been shown to reduce cigarette consumption (Peterson et al., 1992) and according to Mytton et al. (2012) a 20% tax increase was effective in reducing the cigarette consumption. Cavill and Bauman (2004) identified that, lack of funding and expecting a rapid and positive outcome within a short time frame is one of the main difficulties in conducting a successful campaign. Setting specific goals within an achievable time period, measuring the progress and identifying the barriers and problems arising with policy implementation and modifying the implementation strategies accordingly could help in efficiently reducing the risk of foodborne diseases. Additionally, effectiveness of the new policies should be monitored over time to confirm the effect of the new guidelines on public health and

development as changing behavioural patterns is difficult and time consuming (Wooldridge et al., 1999). According to Elder et al. (2004) testing the campaign strategies within a target population prior to the mass campaign could help in identifying specific requirements, requests and changes, which could improve the optimal transmission of information. New educational and training programmes with updated scientific findings and new techniques should be easily accessible to the food handlers, chefs and EHOs to maintain and update their knowledge to achieve food safety (Ovca et al., 2018). Moreover, existing food safety evaluation tools should be modified, improved and transformed over time in accordance with the new scientific findings to minimize the risk of foodborne illnesses and to effectively identify relapse of hazardous behavioural patterns.

Introducing reward and promotion strategies will drive the need for "self-fulfilment", and motivate individuals "personal drive for achievement, recognition and promotion" (Wambugu and Ombui, 2013). Therefore, introducing specific reward strategies could influence positive behavioural change in people. For example the 'Scores on Doors' is a 5-star rating system which was introduced in 2004 in the UK and was based on a reward strategy (Worsfold and Worsfold, 2008). This scheme rewards food business on their food safety practices (SAHealth, 2019). Following the food safety inspection, the report will be converted to a 5-star rating system, where higher number of stars indicate higher food hygiene and vice versa (Worsfold and Worsfold, 2008). Furthermore, displaying the scores on the doors could provide the general public with a greater understanding the food safety status of the food businesses. Thus, the impact of this scheme could be maximised by educating the general public (Worsfold and Worsfold, 2007). A study conducted in the UK demonstrated that the availability of the food safety inspection report to the general public as the 5-star rating system encouraged the food businesses to improve food hygiene as it developed in to a customer attraction technique (Worsfold, 2006). Another study conducted in the US reported that grading the restaurants according to their food standards and displaying it to the general public decreased the incidence of hospitalizations due to foodborne illnesses by 20% in the year 2003 (Jin and Leslie, 2003).
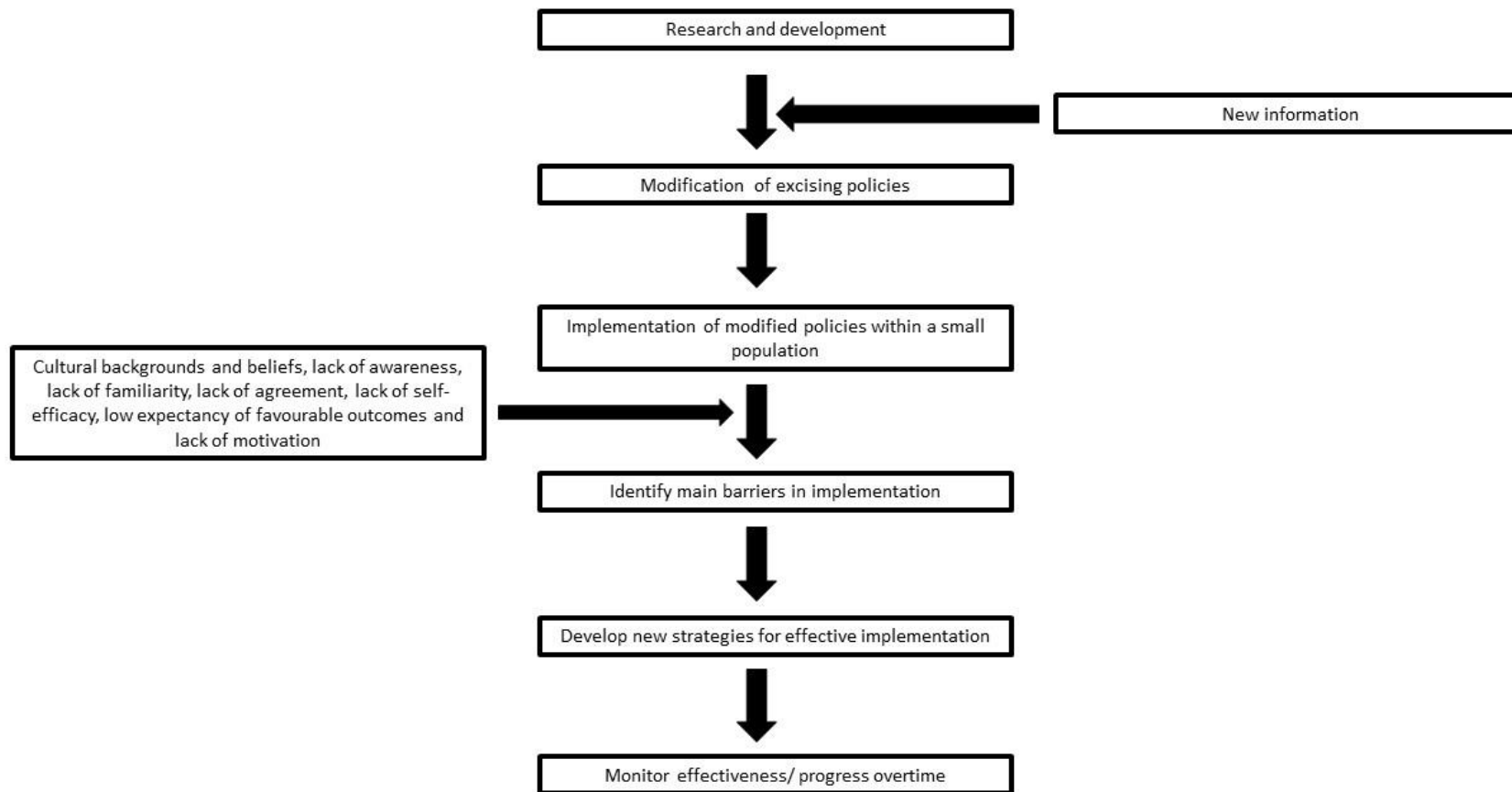
Figure 5.2: Workflow for the effective implementation of new or modified policies (Grol and Wensing, 2004)

5.4 Food safety in home kitchens and implementation of new/modified policies

5.4.1 Foodborne diseases and home kitchens

A significant proportion of foodborne illness can be attributed to improper food safety practices in the home (Redmond and Griffith, 2003). In the European Union more than half of all *Salmonella* outbreaks are linked to the home (Byrd-Bredbenner et al., 2013). Food handling errors in a home kitchen could pose a threat to the risk groups such as young, elderly and immunocompromised (Scott., 2003, Byrd-Bredbenner et al., 2013). According to the survey conducted during this study, a significant number of the participants (46%) washed their eggs and 19% stored their eggs on the shelf, which could increase the risk of campylobacterosis and salmonellosis infections. Lack of knowledge and lack of awareness amongst the general public in handling fresh eggs and poultry could increase the risk of infection if the eggs are consumed raw or as a raw egg product such as mayonnaise.

Home cooks should be aware of personal hygiene, the potential for cross contamination and the recommended cooking and storage temperatures to reduce the incidence of foodborne illnesses (Medeiros et al., 2001). Cross contamination can cause foodborne illnesses within the home kitchen. Research conducted by Josephson et al. (1997) on home kitchens investigated the contamination of counter tops, table tops, cutting boards, refrigerator handles, sink drains and microwave control panels and confirmed the presence of *E.coli, Salmonella* and *Campylobacter.* This research supports the work by Rusin et al. (1998) that recognized cutting boards as one of the common items which could facilitate cross contamination in a home kitchen. During this study, 15% of the backyard chicken faeces samples were found to be positive for either *Salmonella* spp. or *Campylobacter* spp. indicating that improper handling of both poultry and eggs could lead to foodborne illness. Another study that analysed food safety behaviour in the home found that young healthy individuals between 18 to 26 years old did not follow the recommended food storage temperatures and also used kitchen appliances that were in unsanitary conditions, which could promote the replication of pathogens (Byrd-Bredbenner et al., 2007). Educating the younger generation on safe food handling techniques

is important for reducing foodborne illnesses linked to households. Surveys conducted by Byrd-Bredbenner et al. (2010) identified that visual aids such as video games, photos and videos that emphasise the consequences of foodborne illnesses are effective in educational campaigns. Additionally, the paper further suggests that games focused on food safety, laboratory experiments, field trips, memorable mottos, group discussions and group work, songs and brief guest talks can also be used as successful campaign aids.

5.4.2 Improving food safety in home kitchens

Implementing new policies can be challenging when it is intended to change behavioural patterns. Reluctance to change attitudes and behaviours are a main barrier in achieving food safety. According to Powell et al. (2011), convincing individuals to change their food handling techniques and attitudes in relation to new scientific findings remains a challenge. Ovca et al. (2017) identified that the attitudes and behaviours of children are significantly influenced by parents and could influence the food safety habits. Thus, changing these behavioural patterns is difficult and time consuming. Additionally, lack of media attention (Zyglidopoulos et al., 2012), communication (Panday, 2007), motivation (Hardcastle et al., 2015), time, public participation and different attitudes are identified as major barriers in encouraging behavioural change. Hence, new strategies that could influence behavioural changes and beliefs should be implemented to introduce new food handling techniques.

Food safety can be achieved through education and well-organized policy implementation. Organizing social interactions or workshops, improved school curriculum to educate and inspire children, distributing and displaying information sheets and introducing social media blogs could be used to encourage food safety (Wakefield et al., 2010) (**Figure 5.3**). Media and educational campaigns have been used to positively change behaviours of people by targeting change in emotional responses (Rimal et al., 1999). To ensure maximum impact, food safety education campaigns should be targeted at informing children, elderly and immunocompromised as these are

the most vulnerable to foodborne diseases (Lund et al., 2011). Powell et al. (2011) identified "weekly media narratives" as one of the most effective methods which could influence the attitude and behaviours. Therefore, using media programs to introduce new scientific discoveries, changes in policy, new technologies and risk management could have a significant impact on improving public health and safety.
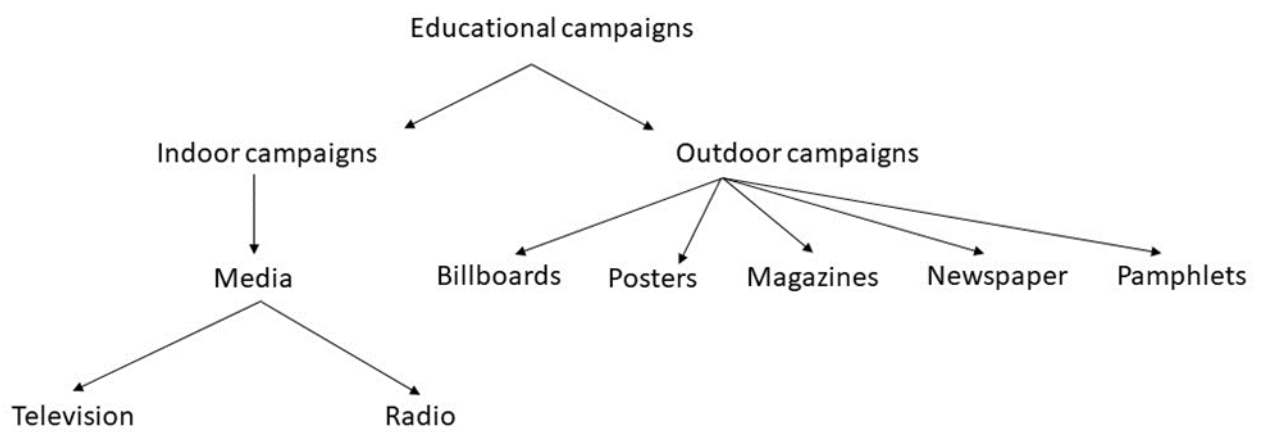
Figure 5.3: Different campaigns that could be used to deliver positive behavioural change in human health (Wakefield et al., 2010).

5.5 Proposed novel guidelines and implementation of policies to reduce the risk of salmonellosis related to eggs and raw egg products

Eggs and raw egg products are commonly linked to salmonellosis outbreaks in Australia. According to the Australian guidelines, raw egg mayonnaise should be prepared, processed, transported and stored under 5°C (FSANZ, 2016) and the pH should be maintained at or less than 4.6 (SAHealth, 2013) or 4.2 (NSW, 2016). However, the results presented in this thesis the guidelines should be changed from "immediately stored under 5°C" to leave at room temperature for at least 24 h before consumption and the regulatory emphasis should be placed on achieving the pH rather than temperature control. However, the effect of pH at room temperature on the survival of other possible infectious organisms such as *E. coli, Shigella* and *Campylobacter* should be assessed before the guidelines are changed. Moreover, the effect of other spoilage organisms and the effect on the consistency of mayonnaise which is incubated at room temperature should also be assessed.

Many chefs and food handlers believe that pasteurization affects the taste, quality and the usability of the eggs and the raw egg products (Nelson et al., 2008). Therefore, pasteurised eggs should be acceptable to the chefs, the food handlers and the protocol for pasteurisation should be easy and not time-consuming to follow. The developed egg decontamination method presented in this thesis successfully decontaminated the eggshells contaminated with *Salmonella* Typhimurium after placing in a sous-vide with water bath heated to 57°C for 9 min. This method did not affect the quality and the usability of the eggs, was rapid and simple and can be adopted in a home or a commercial kitchen. Therefore, the developed decontamination method can be used to decontaminate eggs before the preparation of raw egg products as an additional control barrier or when acid control is not used in raw egg products such as mousse, homemade ice-cream, egg-based health drinks and tiramisu.

Accurate modification of the food safety guidelines and effective implementation of the guidelines in commercial as well as home kitchens will help reduce the number of salmonellosis outbreaks linked to raw egg products, which has continued to increase over the last decade (NNDSS, 2019). Storage

temperature and pH of the raw egg products (mayonnaise) are the main factors that reduce the viability of the foodborne pathogens such as *Salmonella*. Therefore, the food safety guidelines for the preparation and storage of raw egg products in relation to pH and temperature should be updated in light of new scientific discoveries.

Educating EHOs, chefs and food handlers on changes to the policies can be done through food safety workshops, videos and pamphlets. A multidisciplinary campaign could be effective in reaching many individuals on new guidelines. The chefs and management in the commercial food facilities could be motivated to follow the guidelines by introducing a reward system such as the five-star rating system to ensure effective execution of the food safety culture. Additionally, the effectiveness of the campaigns should be monitored over time to ensure proper implementation of the novel guidelines (**Figure 5.4**).
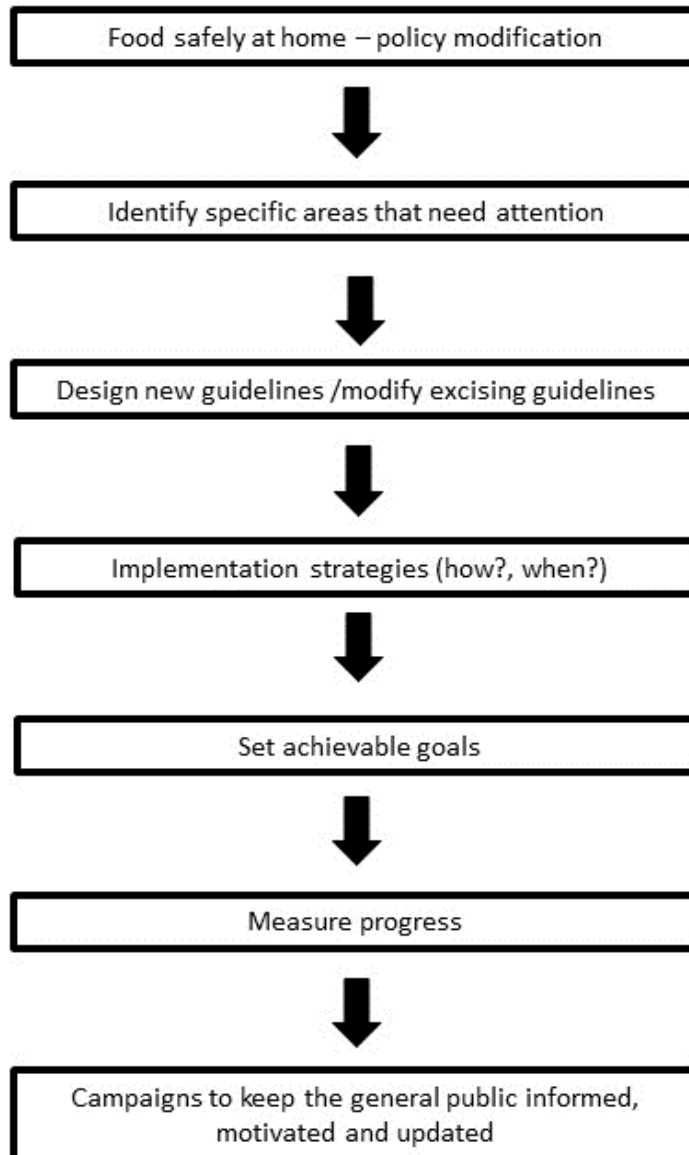
Figure 5.4 Implementation of new guidelines to increase food safety at home (Redmond and Griffith, 2005)

5.6 Proposed novel guidelines and implementation of policies to reduce the risk of foodborne illnesses related to backyard poultry

Poultry and eggs are commonly linked with zoonotic diseases. Therefore, lack of knowledge and lack of awareness among general public in the correct handling of fresh eggs and poultry could increase the risk of infection if the eggs are consumed raw or as a raw egg product such as mayonnaise. Increased popularity of keeping poultry in the backyard could lead to increased risk of salmonellosis, campylobacterosis and shigellosis. Therefore, educating backyard chicken owners on appropriate handling techniques and personal hygiene could help reduce the incidence of foodborne illnesses related to backyard chicken. Behavioural patterns such as reluctance to identify the risk (**Figure 5.5**) and lack of trust in novel discoveries and policy (**Figure 5.6**) are also potential risk factors. Washing the dirt off the eggs and storage of eggs at room temperature were identified as potential risk factors which could increase the penetration of *Salmonella* in to the eggs (Gole et al., 2014) and facilitate replication (Humphrey, 1994, Whiley et al., 2015) which could pose a threat of gastrointestinal infection if the eggs are consumed raw. Moreover, reluctance to change attitudes and behaviour (**Figure 5.7** and **Figure 5.8**) were also identified as barriers in changing behavioural patterns of the general public on handling poultry and fresh eggs in the backyards. These potential risk factors could contribute towards the increased incidence of foodborne illnesses among the backyard chicken owners.

According to the study conducted by Beam et al. (2013) awareness of the backyard chicken owners regarding the risks of *Salmonella* infection was limited. In the US, backyard chicken owners in urban, suburban and rural areas relied on information from the internet, veterinary clinics and agricultural organizations. A higher number of the population (87-90%) accessed their information via the internet. Only 16-24% and 7-13% accessed information through the veterinary clinics and agricultural organisations respectively (Elkhoraibi et al., 2014). Elkhoraibi et al. (2014) further suggests that educating and reaching backyard chicken owners through feed stores, hatcheries, through councils and at poultry shows could be an effective method for distributing information.

The policies could be modified to encourage compulsory registration of the backyard chicken owners at the councils as a potential mechanism to ensure educational material reaches backyard chicken owners. The education material such as pamphlets and information sheets could be handed over to the backyard chicken owners with information including washing and storage of eggs and handling poultry and fresh eggs. Flocks with more than 10 birds had a higher percentage (38%) of positives to either *Salmonella* spp. or *Campylobacter* spp. Therefore, having a chicken flock with less than 10 birds would reduce the risk of foodborne diseases linked to backyard poultry. The councils could take the initiative in informing the general public on having a limited number of birds and to have the birds confined in a specific, spacious area could also help reduce the incidence of foodborne illnesses.

Effective education techniques and strategies should be employed to educate the general public. Organising social interactions and workshops, improved school curriculum to educate and inspire children, distributing and displaying information sheets and introducing social media blogs could be used to encourage food safety. As "weekly media narratives" (Powell et al., 2011) and media programmes could have a significant impact on improving public health and safety, these can be used to introduce new scientific discoveries, changes in policy, new technologies and risk management.

Figure 5.5: Screen shots of social media describing an incident of backyard chickens linked to salmonellosis outbreaks. The comments from the general public indicate a lack of awareness of poultry handling hygiene

Figure 5.6: Screen shot of social media describing an incident of backyard chickens linked to salmonellosis outbreaks. The comments indicate lack willingness to identify the significance of the problem
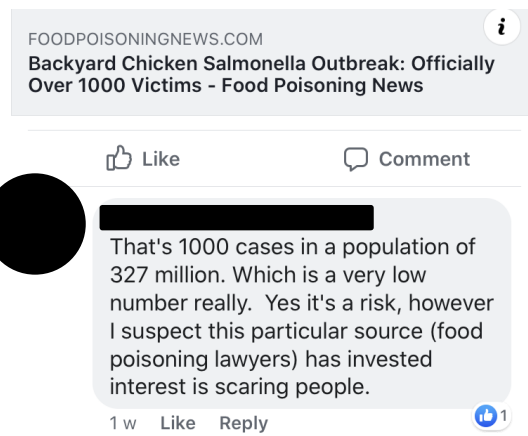
Figure 5.7: Screen shot of social media describing an incident of backyard chickens linked to salmonellosis outbreaks. The comments indicate indicated the lack of lack of trust in novel discoveries

Figure 5.8: Screen shot of social media describing an incident of backyard chickens linked to salmonellosis outbreaks. The comments indicate reluctance to change attitudes and behaviour
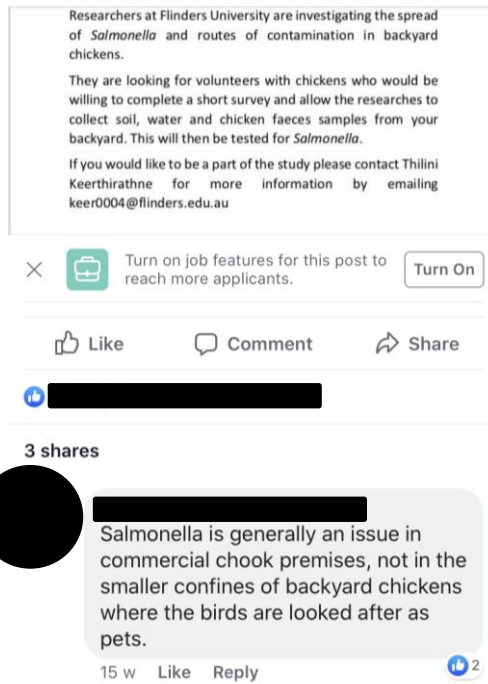
Figure 5.9: Screen shot of social media describing an incident of backyard chickens linked to salmonellosis outbreaks. The comments indicate a reluctance to change attitudes and behaviour

5.7 Future work

Reducing the incidence of foodborne diseases is often complicated by the complex nature of the microorganisms. Acid adaptation of *Salmonella* spp. through complex cellular mechanisms increases the survival of *Salmonella* in acidic pH conditions. Therefore, the effectiveness of pH as a regulatory mechanism for foodborne pathogens should be evaluated with acid tolerance induced strains of *Salmonella*. There are many recipes for the preparation of raw egg products with the optional use of salt, garlic, and plant essential oils from mint, cinnamon, cardamom, and clove. The combined effect of these spices, pH and temperature against *Salmonella* Typhimurium should be assessed for more effective control of salmonellosis linked to raw egg products. The egg decontamination method presented in this study was effective in decontaminating eggs contaminated with *S.* Typhimurium. However, this method should be tested with other *Salmonella* spp. particularly with the heat resistant strains of *Salmonella* and other foodborne pathogens that can contaminate eggs. The shelf life of the eggs and the effect of the heat on the eggshell cuticle should be assessed if the eggs are not used immediately following the decontamination.

According to the study, backyard chickens were a potential risk for campylobacteriosis and salmonellosis. It was also identified that there was a lack of knowledge among general public regarding the safe procedures for handling poultry and eggs. Therefore, future research should be conducted to design educational campaigns and evaluate their effectiveness. Research should also be conducted to assess the presence of other pathogens in the faeces of backyard chickens (virus, mycoplasma and fungi), which could pose a threat to public health and development. Studies should also be conducted to assess the impact that different food scraps added to the feed has on the presence of pathogens. These additives can improve the gut health of the chickens and have the potential to control the pathogen colonization in the chicken gut. Additionally, the gut colonization of the pathogenic bacteria differs among different chicken species. Studies should be designed to assess the chicken species less sensitive to gut colonization of foodborne pathogens and these

chicken species e introduced to backyard chicken owners to reduce the incidence of zoonotic foodborne diseases.

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APPENDIX

Conference abstract -1

Potential control of *Salmonella enterica* serovar Typhimurium in shell eggs using sous-vide pasteurisation

Salmonellosis is a foodborne illness of public health significance. In Australia the incidence of salmonellosis has been significantly increasing over the last decade with eggs and raw egg products identified as the main source of outbreaks. The primary cause of salmonellosis in Australia is *S.* Typhimurium. This study investigated the effectiveness of sous-vide pasteurisation to provide whole intact table eggs free from *Salmonella* contamination. During this study the optimum conditions for the control of *S.* Typhimurium and the influence on the eggs properties was explored. Whole shell eggs were inoculated with the *S.* Typhimurium in the concentration of 10^5 CFU/ml. Three eggs were tested at each time point and the experiment was repeated 3 times. The intact shell, internal contents and crushed shells were processed separately, and the remaining viable *Salmonella* were enumerated via culture on XLD agar. Egg Albumen pH, weight before and after heat treatment, thermocoagulation of proteins, Haugh unit, egg yolk index, volume of foam and volume of drainage was measured to test the egg quality following the pasteurization process. The optimum conditions for the control of *S.* Typhimurium and the influence on the egg's properties, including viscosity, colour, thermal coagulation and the egg protein quality was explored. This research demonstrates the potential for sous-vide to be used as a simple method for the preparation of microbiologically safe eggs, which can be used for the production of food containing raw eggs. The widespread use of pasteurised eggs would significantly reduce the incidence of salmonellosis.

Conference abstract- 2

Investigation into the effect of pH and temperature on the survival of *Salmonella* enterica serovar Typhimurium

The most common serotype linked with salmonellosis is *Salmonella* Enteritidis; however, in Australia it is *Salmonella* Typhimurium. Increasing incidence of salmonellosis is linked with eggs and raw egg products. Australian guidelines recommend mayonnaise preparation under 5°C at pH 4.6. The objective was to investigate the combined effect of pH and temperature on the survival of *S.* Typhimurium ATCC 53647. One mL log phase *Salmonella* cells (10^5 CFU/ mL) was added to 100 mL of nutrient broth with a pH of either 4.6, 4.4, 4.2, or 7.0 (adjusted with acetic acid) and was incubated at either 37, 23 or 4°C. Drop plate method on XLD agar was used to measure viable bacterial counts. Survival was significantly ($P<0.05$) improved when cultures were incubated at 4°C compared to 23°C and 37°C for all pH conditions tested. After 24 hours of incubation at 37°C, there were no viability in pH 4.2 and 4.4 but bacteria survived at 23°C and 4°C. The average count of survived bacteria after 7 days at 4°C at pH 4.4 was 10 CFU/ml while at 23°C it was 3 CFU/mL and no growth at 37°C. Average of 68 CFU/mL survived after 7 days at 4°C at pH 4.6 but only 3 CFU/mL and 0 CFU/mL were reported at 23°C and 37°C at pH 4.6 respectively. The maximum survival was at 4°C. In conclusion, lower temperatures protected *S.* Typhimurium from the bactericidal effect of low pH.

Conference abstract- 3

An effective method to decontaminate eggs using a common piece of kitchen equipment

In Australia, the incidence of salmonellosis has been increasing over the last decade, with eggs and raw egg products identified as the main source of outbreaks. The primary cause of salmonellosis in Australia is *Salmonella* Typhimurium. This study developed a decontamination method for whole shell eggs that does not affect the eggs' usability.

Eggs were inoculated with *S.* Typhimurium and placed in a *sous*-vide with water heated to 57°C. The optimum time needed for the complete removal of *Salmonella* at 57°C was assessed. Important factors affecting the quality and the consistency of the raw egg products such as the pH of the egg albumen, protein denaturation, freshness and quality of the eggs, volume and stability of foam were checked following the decontamination process. Complete removal of *Salmonella* was observed after 9 min at 57°C.

Treated eggs and untreated eggs were given to the chefs and food handlers who were asked to prepare mayonnaise. The consistency and the quality of the prepared mayonnaise was rated by the chefs and food handlers.

The heat treatment had no impact on the usability of the eggs and the chefs and food handlers could not detect a difference in the treated and untreated eggs.

This is a simple method that can be completed by food handlers prior to the preparation of raw egg products to reduce the risk of salmonellosis.

Biosafety training certificate - 2019

Removed due to copyright restrictions

Biosafety training certificate – 2017

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Ethics approval for the project number 7737

FINAL APPROVAL NOTICE

Project No.: 7737

Project Title: Investigation into the presence of Salmonella spp in environmental samples collected from domestic and commercial poultry houses and the associated risk factors

Principal Researcher: Ms Thilini Keerthiranthne

Email: keer0004@flinders.edu.au

Approval Date: 20 February 2018

Ethics Approval Expiry Date: 30 December 2022

Ethics approval for the project number 7795

FINAL APPROVAL NOTICE

Project No.: 7795

Project Title: Potential control mechanisms for Salmonella in eggs and raw egg products

Principal Researcher: Ms Thilini Keerthirathne

Email: keer0004@flinders.edu.au

Approval Date: 10 January 2018

Ethics Approval Expiry Date: 31 December 2021

The questionnaire which was used to collect data from the backyard

chicken owners

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OFFICE USE ONLY

ID:      _____

Date:  _____
```

Investigating the risk associated with *Salmonella* and egg production

Questioner – Domestic poultry

The questions below inquire information about the domestication of poultry. It is completely up to you whether to participate. You may withdraw at any time and you may skip questions you would prefer not to answer.

1 What is your residential Suburb and postcode?

Suburb	Postcode

2 How long have you kept poultry?

Years	Months

3 What breed of poultry do you have?

☐   Don't know

☐   Please specify…………………………………………………………………….

4 How many birds (poultry) do you have?

☐   1-2

☐   3-5

☐   6-10

☐   >10

inspiring achievement

5 Is the flock single or multi-aged?

☐   Singe aged

☐   Multi-aged

☐   Don't know

6 During the time you have kept poultry have you or anyone who lives in the household been diagnosed with clinically proven salmonellosis?

☐   No

☐   Yes, please provide details (who and when)

……………………………………………………

……

7 Where is the feed obtained from?

☐   Commercial, please specify

Brand…………………………………………………….

☐   Other, please specify…………………………………………

8 Do you use any control measures for *Salmonella*?

☐   Yes, please specify………………………………………………….

☐   No

9 Is the poultry caged or free range?

☐   Caged

☐   Free range

☐   Other, please specify…………………………………………………

10 How is the poultry droppings disposed/ used?

☐   Buried

☐   Incinerated

☐   Spread on site

☐   Used as manure

☐   Other, Please specify,

11 What is the main purpose of domestication? Indicate all relevant answers.

☐   Eggs for domestic use

☐   Eggs for selling

2

☐   Meat for domestic use

☐   Meat for selling

☐   Other, Please specify…………………………………………

12 Do you consume **raw / under cooked** eggs from the domesticated poultry?

    ☐ Yes

    ☐ No

13 If an egg is dirty, typically what do you do with it?

    ☐ Use it

    ☐ Discard it

    ☐ Wash it

    ☐ Wipe it

    ☐ Other_____ (please specify)

14 If an egg is cracked what do you do with it?

    ☐ Use it

    ☐ Discard it

    ☐ Wash it

    ☐ Wipe it

    ☐ Other_____ (please specify)

15 Where do you typically store your eggs?

    ☐ Fridge

    ☐ Shelf

16 Are there any other domesticated animals/Birds?

    ☐ Yes, please specify……………………………………….

    ☐ No

3

Survey which was used to access the acceptability and the usability of the heat-treated eggs (9-point Hedonic scale)

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<tr><td>Date:</td><td>_____</td></tr>
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Potential control mechanisms for *Salmonella* in eggs and raw egg products

Questioner – Chefs and food handlers

The questions below inquire information about the prepared mayonnaise.

Please complete the tables below according to your experience and opinion.

Egg batch A

	Appearance	Texture	Smell	Colour	Stability	Flavour	Taste	Overall acceptability
I like extremely								
I like very much								
I like moderately								
I like slightly								
I neither like or dislike								
I dislike slightly								
I dislike moderately								
I dislike very much								
I dislike extremely								

If you have any other comments please specify

……………………………………………………………………………………………

……………………………………………………………………………………………

……………………………………………………………………………………………

1

Egg batch B

	Appearance	Texture	Smell	Colour	Stability	Flavour	Taste	Overall acceptability
I like extremely								
I like very much								
I like moderately								
I like slightly								
I neither like or dislike								
I dislike slightly								
I dislike moderately								
I dislike very much								
I dislike extremely								

If you have any comments please specify

……………………………………………………………………………………………………

……………………………………………………………………………………………………

……………………………………………………………………………………………………

2

Egg batch C

	Appearance	Texture	Smell	Colour	Stability	Flavour	Taste	Overall acceptability
I like extremely								
I like very much								
I like moderately								
I like slightly								
I neither like or dislike								
I dislike slightly								
I dislike moderately								
I dislike very much								
I dislike extremely								

If you have any comments please specify

……………………………………………………………………………………………………

……………………………………………………………………………………………………

……………………………………………………………………………………………………

3