



Ensuring egg safety: review of microbial control in the supply chain

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Foreword

Salmonella Enteritidis and *Salmonella* Typhimurium have dominated the epidemiology of *Salmonella* and are the most common causes of human salmonellosis. This review is focused on the management of *Salmonella* in the egg supply chain and the effects of various egg storage temperatures on the *Salmonella* serovars. The role of refrigeration in *Salmonella* virulence is also reviewed.

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This report is an addition to Australian Eggs Limited's range of peer reviewed research publications and an output of our R&D program, which aims to support improved efficiency, sustainability, product quality, education, and technology transfer in the Australian egg industry.

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Executive Summary

Microbial contamination of eggs is an important food safety issue of significance, not only to producers, but also to food and health regulatory authorities. Contamination with *Salmonella* is one of the most significant food safety issues associated with eggs. Worldwide, contaminated eggs and egg products are commonly identified as the source of *Salmonella* during traceback investigations. In Australia and New Zealand, *Salmonella* Typhimurium (ST) is the leading cause of foodborne salmonellosis. *Salmonella* Enteritidis is seldom isolated from commercial egg farms in Australia, apart from recent occurrences in some states on the East Coast. Most of the locally reported *Salmonella* Enteritidis (SE) human infections are attributed to overseas travel. The frequency of *Salmonella* on retail eggs in Australia is low. The most recent reported prevalence of *Salmonella* on Australian retail eggs was 3.5%. An estimate of the odds for the presence of *Salmonella* (all species, not just pathogenic) on eggs is greater than 1 in 20,000. Considering the estimated production of eggs in Australia and per capita consumption, the risk of foodborne illness in general is very low for humans consuming eggs. Current quality assurance, animal husbandry, and biosecurity practices recommended by Australian Eggs have been adopted by egg producers nationwide to mitigate *Salmonella* on farms and subsequently through the supply chain. Although currently there is no registered *Salmonella* Enteritidis vaccine in Australia, pen trials have been conducted to test the currently available *Salmonella* Typhimurium vaccines against *Salmonella* Enteritidis.

Globally, storage requirements for whole eggs vary widely. In Australia, egg storage temperatures vary between states. Although most states have embraced the Food Standards Australia New Zealand (FSANZ) standards there are discrepancies in how these recommendations are practised. Refrigeration of eggs below 7.2°C is linked with the inhibition of *Salmonella* growth and subsequently a decrease in the risk of causing infection in humans. Yet, there are conflicting data regarding the impact of storage temperatures on *Salmonella*, which may contribute to the disparity between international recommendations for egg storage. The virulence of *Salmonella* spp. in humans is influenced by various factors including temperature, which has a crucial role in the transfer of bacteria across hosts and is one of the main variables controlling their metabolic, growth, and pathogenic gene expressions. The survivability of *Salmonella* in egg contents was found to be enhanced at 5°C, since the expression levels of survival and stress response-related genes, *yafD*, *prop*, and *rpoS*, were significantly greater at 5°C compared to 25°C. Despite bacterial survival, a greater expression of virulence-related genes at 5°C may not lead to an increased risk of *Salmonella* infection or a more severe disease. Warmer temperatures for egg storage may lead to an increased potential of *Salmonella* multiplication in the yolk, whose nutrients may cause the expression of certain virulence genes via biochemical linkage. There is not sufficient scientific evidence to suggest that storing eggs at less than 7°C will influence the virulence of bacteria. Although lower temperature reduces the rate of *Salmonella* multiplication in egg contents, bacteria can still survive at lower temperatures such as 7°C. Despite industry recommendations, eggs can be exposed to various temperatures in the supply chain. Further studies are essential to understand the relationship between the storage temperature and bacterial virulence.

1 Introduction

The adoption of cold storage in food supply chains first began in the 1870s when ice became widely used in the shipping and transport of beef and pork (Heitz, 1929; Craig & Holt, 2017). Later in the 1880s, mechanical refrigeration became more widespread allowing a larger number of food commodities including dairy and eggs to be transported greater distances (Craig & Holt, 2017). Before the twentieth century, eggs were predominantly seasonal as peak production occurred primarily in the spring and early summer (Jenkins, 1919). In addition, eggs are fragile and perishable, thus the sale of eggs was limited to restricted geographical regions close to farms (Jenkins, 1919; Heitz, 1929; Craig & Holt, 2017). The use of reduced temperatures in the egg supply chain enhanced the preservation of egg quality factors and allowed for longer storage and wider distribution (Craig & Holt, 2017).

Internal egg quality factors include assessment of yolk height, yolk colour, albumin viscosity, and Haugh units, while external quality measures are shell thickness, size, and cleanliness (Coutts & Wilson, 2007). Internal quality factors decrease over time but can be a function of storage conditions including temperature, humidity, and handling (Bennion, 1940; Kamel et al., 1980; Grashorn et al., 2016). The relationship between egg quality factors and storage conditions, in particular temperature, has been studied extensively and is widely known to decrease more rapidly at higher storage temperatures ($\geq 25^{\circ}\text{C}$) compared with lower storage temperatures ($\leq 15^{\circ}\text{C}$) (Jones & Musgrove, 2005; Lee et al., 2016; Jones et al., 2018). This link between storage temperature and the maintenance of quality over time was one of the original driving factors for the cold storage of eggs through the food supply chain (Craig & Holt, 2017).

Microbial contamination of eggs, however, is an important food safety issue associated with eggs, and over the last 40 years has become of significant importance not only to producers but food and health regulatory authorities. Contamination with *Salmonella* is one of the most significant food safety issues associated with eggs. Worldwide, eggs are commonly identified as the source of *Salmonella* during traceback investigation, with over 75% of the 1.2 million cases of salmonellosis each year linked to the consumption of contaminated shell eggs (Hester, 2017). This review follows the journey of eggs from lay to consumption, and will evaluate how environmental conditions influence *Salmonella* survivability and subsequent virulence during this process.

1.1 *Salmonella* and eggs

Salmonellosis causes considerable economic losses worldwide. In Australia, the annual cost of gastroenteritis-related expenses, including treatment, loss of productivity, deaths, and government monitoring has been estimated at approximately \$811 million (Simpson et al., 2018). A steady increase in *Salmonella* infections was seen in Australia from 2000 to 2013 (Ford et al., 2016), although it has been decreasing from 2019 to 2021 (National Notifiable Disease Surveillance System (NNDSS), 2023). There has been a decline in egg related *S. Typhimurium* cases nationally and overall, and there has been a 40% decline in human Salmonellosis from a peak in 2016 (McLure et al., 2022). As the number of reported cases continues to increase, activities promoting awareness of these foodborne diseases are an important public health issue. This issue is further exacerbated in many countries, where minimal refrigeration is available to store, transport, and handle eggs before they are sold to consumers (Almonacid et al., 2007).

Salmonella spp. is one of the most common causes of bacterial foodborne gastroenteritis in humans. Infection with *Salmonella* is generally self-limiting but is associated with cramping, diarrhea, and vomiting resulting in significant morbidity, mortality, and economic losses. Humans can acquire *Salmonella* through the consumption of a variety of contaminated foods, but raw eggs and foods

containing raw eggs are among the most common sources of this bacterial species (Humphrey, 1994; EFSA, 2007; Kirk et al., 2015). In Australia, a total of 213 foodborne outbreaks were associated with *Salmonella* from 2013 to 2015, with 124 of them being attributed to the consumption of eggs and egg-based products (OzFoodNet, 2021).

Over 2600 *Salmonella enterica* serovars have been characterised to date (Guibourdenche et al., 2010); some more frequently isolated from egg farm environments than others (Snow et al., 2007; Denagamage et al., 2016; Moffatt et al., 2017). Historically, *Salmonella* (S.) Enteritidis and S. Typhimurium have been the two most frequently reported serovars identified during outbreaks worldwide (EFSA, 2011b; Threlfall et al., 2014; Havelaar et al., 2015). In Australia and New Zealand, S. Typhimurium is the leading cause of foodborne salmonellosis (Whiley et al., 2016)

Until recently, S. Enteritidis was seldom isolated from commercial egg farms, and human infections attributed to the serovar S. Enteritidis were typically linked to overseas travel (MPI, 2011; OzFoodNet, 2012; Moffatt et al., 2016).

2 Global supply chain requirements

The link between eggs and *Salmonella* has precipitated many countries to develop standards for mitigating this bacterial species throughout the egg supply chain. The implementation of rigorous egg safety regulations has been linked to the decline in the number of salmonellosis cases in the UK and the US (Chousalkar et al., 2018). In Australia, however, the incidence of egg-related salmonellosis has been gradually increasing until 2014 (Ford et al., 2016; Moffatt et al., 2016) but declined during 2017–2019 due to tighter regulations and *Salmonella* vaccinations in layer flocks ((McLure et al., 2022).

2.1 Egg storage and temperature regulatory requirements

Globally, storage requirements for whole eggs vary widely. The United States Food and Drug Administration (FDA) Code of Federal Regulations Part 118, for example, stipulates that eggs must be held and transported at temperatures no greater than 7.2°C (45°F) 36 hours post-lay (7 CFR Part 56, 2023) and that retail establishments must maintain this temperature when eggs are stored or displayed (Part 115).

In Canada, similar temperature requirements are also stipulated (CFIA, 2023). In the UK, the British Lion Code of Practice indicates that eggs must be held at a constant temperature that can range between 5 and 20°C (British Egg Industry Council, n.d.). European recommendations, however, are in striking contrast with US requirements. The European Commission's (EC) Regulation 589/2008 dictates that eggs must not be refrigerated, and eggs held at 5.0°C for 24 hours for transport or 72 hours for retail must be downgraded (European Union, 2008). Cold storage also does not occur in Chile (Almonacid et al., 2007). The criteria for food safety, hygiene, and handling in the Primary Production and Processing (PPP) Standards apply to primary production and processing enterprises but the standards do not cover biosecurity, animal welfare, or market access hazards (NSW Food Authority, 2022).

In Australia, egg storage temperatures vary between states. Although most states have embraced the Food Standards Australia New Zealand (FSANZ) standards there are discrepancies in how these recommendations are practised. In New South Wales, the egg storage requirements for whole eggs are based on the Australian Eggs recommendation of less than 15°C for egg processors (NSW Food Authority n.d.). In Victoria, it is recommended that eggs should be kept below 20°C during storage and transport, and that measures should be implemented to prevent excessive temperature fluctuations until consumption (DEECA, 2024). Limitations, however, are placed on processed egg products that must be negative for *Salmonella* in five, 25-gram samples.

Each Australian jurisdiction has a biosecurity regulation. The New South Wales Department of Primary Industries has recently established the Biosecurity (*S. Enteritidis*) Control Order 2019, which imposed minimum biosecurity requirements for the poultry and egg sectors, and licensed egg farmers are expected to conduct routine *S. Enteritidis* testing as part of a new regulatory measure implemented in mid-2020 (NSW Food Authority, 2022).

The Australian egg industry also has an Egg Standards of Australia (ESA) program that is voluntary for egg producers in Australia. This program offers a set of compliance standards that have been reviewed against current regulatory and retail requirements. This program is based on the Hazard Analysis Critical Control Points that address various aspects of food safety. Although this is voluntary, the program structure offers multiple levels of certifications for wider adoption. In addition to this, the National *Salmonella* Enteritidis Monitoring and Accreditation Program (NSEMAP) is also available to all egg producers in Australia exporting eggs to overseas markets. NSEMAP is coordinated by the New

South Wales Department of Primary Industries and includes regular surveillance for SE on farms.

3 The egg supply chain: from farm to table

Refrigeration of eggs below 7.2°C (7 CFR Part 56, 2023) is linked with the inhibition of *Salmonella* growth and subsequently a decrease in the risk of causing infection in humans (Gast & Holt, 2000). Yet, there are conflicting data regarding the impact of storage temperatures on *Salmonella*, which may contribute to the disparity between international recommendations for egg storage. Refrigeration at 4°C, for example, has been shown to reduce *Salmonella* Enteritidis penetration through the shell membrane (Miyamoto et al., 1998). Cooling, however, can extend the survivability of *Salmonella* spp. on the eggshell and cannot reduce the amount of bacteria already present inside the egg (EFSA, 2014).

The conditions on a dry eggshell may induce a low metabolic state in *Salmonella* that may enable bacteria to survive for longer periods at low temperatures (Radkowski, 2002; Messens et al., 2006). The low temperature at which eggs may be stored poses a risk of contamination spreading from eggshell surfaces to other food items when eggs are handled. Nevertheless, it is important to note that *Salmonella* Enteritidis can be found within the internal contents of eggs, and storing eggs at low temperatures has been demonstrated to decrease bacteria growth in egg internal contents (Gast et al., 2010). Thus, how eggs are handled (including exposure to higher temperatures) throughout the egg supply chain can affect food safety. The egg supply chain is explained in Figure 1.

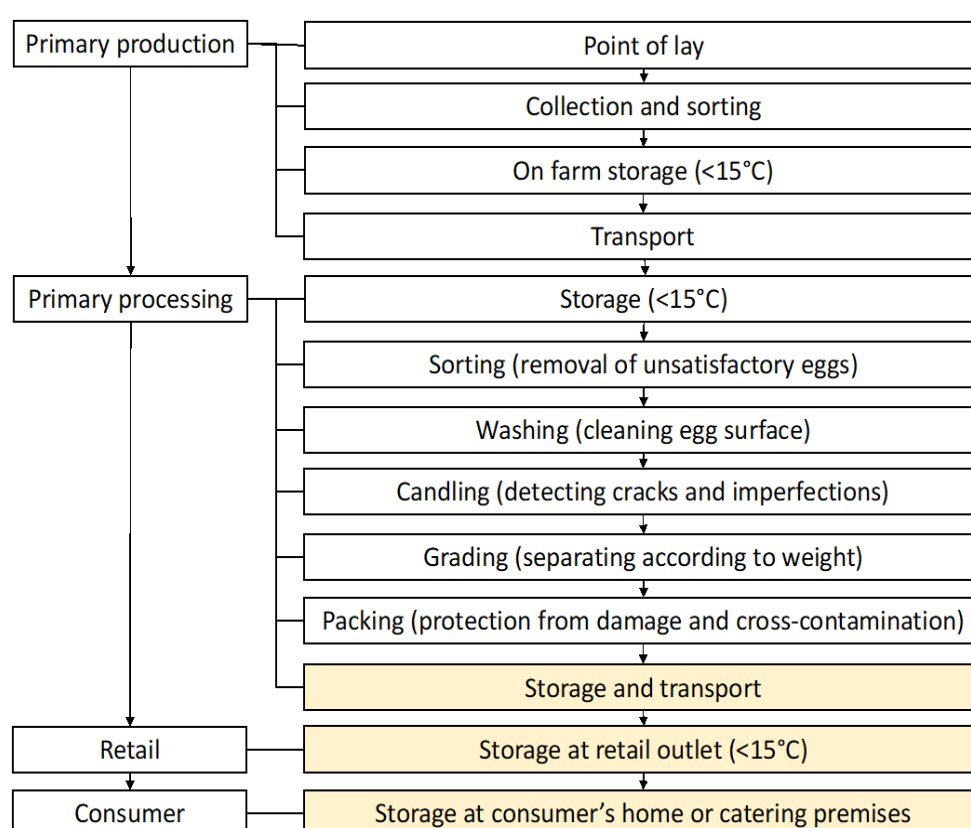


Figure 1 Flowchart for shell egg production and processing in Australia.

It was summarised based on FSANZ in Public health and safety of eggs and egg products in Australia (FSANZ, 2009).

3.1 Epidemiology and control of *Salmonella* on layer hen farms

The first point of *Salmonella* control is on a farm. Different *Salmonella* serotypes are routinely detected in the layer hen farm environment and can enter poultry farms in a variety of ways, including vehicles, feed, water, humans, wild birds, rodents, reptiles, or insects (Foley et al., 2008; Wales et al., 2013). According to a statewide cross-sectional survey (2013–2018) conducted by the New South Wales Government Food Authority, approximately 62% of the environmental samples from Australian farms were positive for *Salmonella* spp. (NSW Food Authority, 2022). Although detected in the environment, *Salmonella* contamination on eggs is low in cage egg farms and can be further reduced by egg washing (McWhorter & Chousalkar, 2020). Low levels of *Salmonella* were also detected in the free range production system, which implies that flock and farm management has an impact on the level of *Salmonella* contamination (McWhorter & Chousalkar, 2019). Eggshells taken from the cage front have been shown to have bacterial titres as high as 10^5 CFU/egg (Gole et al., 2014a). There is a correlation between environmental *Salmonella* contamination, eggshell contamination, and human illness, which is of importance to the industry (Moffatt et al., 2017).

The primary mode of *Salmonella* transmission is oral-faecal, and infection typically does not cause clinical symptoms in birds (Andino & Hanning, 2015). *Salmonella* can survive and persist in the farm environment for extended periods, which can lead to long-term infection of birds and the build-up of *Salmonella* in the shed environment. Therefore, measures are taken to control the infections of birds in poultry farms. Vaccination of laying birds is one of the most extensively utilised control strategies. The *Salmonella* Enteritidis control programs in the UK include implementing vaccination in parent birds and laying hens, use of feed additives hygiene and biosecurity practices. This has resulted in the decline of *S. Enteritidis* cases in humans (Cogan & Humphrey, 2003).

3.2 Epidemiology of *Salmonella* in vaccinated flocks

The British Lion Code of Practice mandate that all poultry flocks are vaccinated for SE has been linked with a substantial drop in the number of documented human salmonellosis cases (Cogan & Humphrey, 2003). In the US, the total rate of *Salmonella* infections has not decreased in the last 25 years, but the widespread vaccination of hens against ST has recently led to a decrease in this serotype (CDC, 2023).

In Australia, there are two live, attenuated *S. Typhimurium* vaccines available for commercial use. Vaxsafe® ST (Bioproperties Pty Ltd, Ringwood, VIC, Australia) and Poulvac® ST (Zoetis, Australia) are both mutant *S. Typhimurium* strains that have been attenuated through the disruption of the *aroA* gene (Bachtir et al., 2003). Several laboratory-based trials have been conducted to evaluate the efficacy of these live, attenuated *S. Typhimurium* vaccines. Birds administered these vaccines develop humoral immunity to *Salmonella* (Groves et al., 2016; McWhorter & Chousalkar, 2018) and the use of live, attenuated strains has been shown to provide cross-protection against other *Salmonella* serotypes (Muniz et al., 2017). The co-administration of a commercial *S. Typhimurium* vaccine and a killed prototype *S. Enteritidis* vaccine resulted in a cross-protective immune response (Scott et al., 2021). However, there have been limited trials conducted on farms demonstrating vaccine efficacy on a production scale. A recent Australian study evaluated the effects of vaccination of layers against *S. Typhimurium* on the prevalence of the organism in the shed environment (Sharma et al., 2018). Because of other farm management practices, the prevalence of *Salmonella* Typhimurium in the sheds participating in the study was low, making it difficult to ascertain the contribution of vaccination against *Salmonella* Typhimurium (Sharma et al., 2018).

Between 2017 and 2019, 75% of NSW commercial layer flocks were vaccinated with Vaxsafe® ST, and a reduction in salmonellosis cases was observed in the human population (McLure et al., 2022). This decrease was, however, likely multi-factorial. Along with the vaccinations, the use of feed additives,

such as organic acids, may have contributed to the reduction of *Salmonella* in these flocks as well as improvements in biosecurity. **Additionally, during this period, the NSW Food Authority invested resources in the training of food handlers, increasing awareness of egg safety.** Nevertheless, the evidence suggests that vaccination of hens significantly reduces the quantity and persistence of intestinal colonisation, which contributes to a reduction of bacteria on farms (EFSA, 2004). In experimental settings, *Salmonella* vaccination was correlated with a reduction in shell egg contamination (Woodward et al., 2002). Thus, vaccinating layer hens, together with other preventative measures, such as cleaning and disinfecting, the inclusion of feed additives (such as organic acids), strict hygiene, biosecurity, and rodent control all contribute to the reduction of *Salmonella* in poultry flocks (Desin et al., 2013; O'Brien, 2013; Groves et al., 2016).

3.3 *Salmonella* contamination routes of eggs

The oral-faecal route of transmission is most common for *Salmonella*. Layer hens can become infected with *Salmonella* and intermittently shed bacteria in their faeces contaminating the shed environment. This can lead to a build-up of *Salmonella*, which can contribute to the re-infection of hens. Eggs can become contaminated either vertically or horizontally. Vertical transmission occurs when bacteria contaminate eggs before oviposition, originating from an infection of reproductive organs with *Salmonella* (Timoney et al., 1989; Keller et al., 1995; Miyamoto et al., 1997; Gantois et al., 2009). Vertical transmission is most associated with *S. Enteritidis*, which has a high tropism for the layer hen oviduct (De Buck et al., 2004; Gantois et al., 2009). Vertical transmission has been reported for other serovars, such as *S. Typhimurium* (Keller et al., 1997; Okamura et al., 2001; Gast et al., 2011). These laboratory trials, however, used high inoculum doses or atypical routes of bacterial administration that are not an accurate reflection of the farm environment.

Horizontal transmission occurs when eggs become contaminated with *Salmonella* from the environment (De Reu et al., 2006). Eggshells of birds infected with *S. Typhimurium* were consistently positive for bacteria but internal contents were negative (Pande et al., 2016). Similar results were observed during long-term longitudinal studies investigating *S. Typhimurium*-positive egg farms (Gole et al., 2014a; Gole et al., 2017). **These results indicate that the contamination of egg internal contents with *S. Typhimurium* is uncommon, and that horizontal transmission is the likeliest route for this serotype.** Therefore, it is crucial to minimise the contamination of shell eggs at all points of production, from laying on the farm to handling by the consumer before consumption. Improper handling of eggs can lead to secondary contamination of other slightly cooked or uncooked egg-containing products, such as mayonnaise and ice cream, which may be potentially hazardous and lead to outbreaks involving large numbers of people (Lublin & Sela, 2008).

In Australia, there is a low frequency of *Salmonella*-contamination amongst commercially produced eggs (DeWinter et al., 2011; Gast & Guraya, 2013), with 3.5% *Salmonella* positive samples isolated from external of the retail eggs (Fearnley et al., 2011). Despite this, a significant number of human cases of salmonellosis occur each year. Contamination of eggshells may occur either on or off the farm, along the supply chain, or during egg handling (Chousalkar & McWhorter, 2016). In Australia, primary processing for shell eggs includes sorting, washing, candling, grading, packing, and storage (FSANZ, 2009) (Figure 1). According to the voluntary Code of Practice by Australian Eggs (2010), egg collection should be carried out no less than once daily and stored at temperatures below 15°C ($\pm$ 3°C).

3.4 Egg washing

Egg washing is used by many commercial farms to minimise bacteria on the eggshell surface. However, washing chemicals may change the eggshell surface and harm the cuticle layer, which may facilitate the migration of *Salmonella* through eggshell pores (Gole et al., 2014b). Egg washing is therefore prohibited in the UK and the rest of the European Union because of the risk of *Salmonella* replication

within eggs and shell membranes (British Egg Industry Council, 2013).

Egg washing has been adopted by many countries and it can result in a significant decrease in the mean percentage of *Salmonella*-positive eggs (McWhorter & Chousalkar, 2020). Moreover, it has been shown that egg washing does effectively decrease microbial contamination on the egg surface, which reduces the likelihood of *Salmonella* penetrating the egg contents (Hutchison et al., 2004; Messens et al., 2011). Regardless of storage temperature, the presence of faeces on eggs will increase the chance of *Salmonella* from egg surfaces becoming cross-contaminated, thus washing is an important step in the primary processing of eggs (Kingsbury et al., 2019). Therefore, shell eggs are recommended to be washed before transporting them to the retail premises (Australian Eggs, 2010). Eggs with a high amount of faecal material are deemed 'unacceptable' and are not included in the supply chain. These eggs are removed prior to egg washing to minimise the contamination of wash water.

The most prevalent method of egg washing in Australia is wet egg washing, which includes a prewash with detergent, followed by sanitising, rinsing, and drying (Chousalkar & McWhorter, 2016). Australian Eggs (2010) suggests that during prewash, the water temperature is maintained between 41°C and 44°C, and egg detergent is used. It is important to maintain a temperature during washing to avoid the ingress of bacteria from the eggshell through the pores to the internal contents (Chousalkar & McWhorter, 2017). To sanitise eggs, it is recommended that the water used is 3–4°C warmer than the prewash step, and an egg-safe and approved sanitiser is used. A temperature differential can lead to a negative pressure environment facilitating the movement of *Salmonella* through the pores.

Finally, eggs should be air-dried or mechanically dried after washing in clean water (Australian Eggs, 2010). Increasing the temperature at each step is necessary to prevent bacterial ingress across the shell during the washing step (Galiş et al., 2013). However, it has been observed that egg-washing chemicals may have the potential to change the eggshell surfaces and may harm the protective cuticle layer. Increased penetration of *S. Typhimurium* in washed eggs was observed compared with unwashed eggs (Gole et al., 2014c), which could be due to the damage to the cuticle. The presence of a positive temperature differential, along with moisture, creates an optimal environment for bacteria to penetrate the eggshell (Berrang, 1999). Accordingly, appropriate processing and handling after washing should be implemented to minimise the potential for contamination.

Following washing, it is necessary to undergo a candling and visual check process to detect any cracks or imperfections in the eggs to ensure that they are of good quality (FSANZ, 2009; Australian Eggs, 2010). The next step is to feed eggs onto either a manual or automatic egg grading machine. The eggs are then graded and packed according to their weight, and labelled with a best-before date. During transportation, it is crucial to prevent contamination and the growth of any surviving microorganisms to ensure the safety and quality of the eggs. Proper measures must be taken to transport the eggs from the farm to the retail outlet in a manner that ensures this. Thus, it is recommended that shell eggs be stored below 15°C ($\pm 3^\circ\text{C}$) during all stages, including at the farm, during transport, and at the retail outlet (Australian Eggs, 2010). It is also important to avoid large temperature fluctuations at each stage until eggs reach the consumer. Temperature variation can cause condensation on the surface of eggshells and increase the risk of contamination through ingress of bacteria through the shell pores (Bradshaw et al., 1990; Australian Eggs, 2010). Decisions regarding egg storage and shelf life are influenced by the effect of storage temperature on *Salmonella* contamination inside and on the surface of eggs (Kingsbury et al., 2019).

Storage temperature along the supply chain can influence egg quality as well as the growth and persistence of *Salmonella* on and in the eggs' internal contents. *Salmonella* replication has been observed at temperatures between 7 and 48°C, but reduced rates of replication were observed at temperatures lower than 10°C (Chen et al., 2005). Similar results were obtained by Gast and Holt (2000)

showing that rapid proliferation in the egg yolk occurred at 17.5 and 25°C, however, the growth of *Salmonella* at temperatures below 10°C was reduced. Post-lay, the internal temperature known to inhibit *Salmonella* growth in eggs cannot be achieved immediately. Moreover, the validity of *Salmonella* inhibition at low temperatures is dependent on the site (eggshell versus internal contents) as well as the level of contamination (Gast & Holt, 2000). Storage of eggs at room temperature speeds up the degradation of the vitelline membrane, while the integrity of the vitelline membranes showed better preservation at 4°C, and to some extent at 10°C (Chen et al., 2005). Vitelline membrane is a protective barrier for the egg, and degradation can enhance *Salmonella* penetration across egg contents to the egg yolk.

It should be emphasised that limited studies have investigated *Salmonella* survivability on the surface of eggs stored at 15°C or lower, which is the current Australian practice. While rapid multiplication of *Salmonella* Enteritidis has been observed in the yolk of shell eggs stored at 16°C (Bradshaw et al., 1990), there is limited investigation that has been conducted using serovars commonly found in the Australian egg industry.

3.5 Storage in the domestic kitchen and retail premises

The rise in human cases of *Salmonella* infection associated with eggs has prompted egg producers to implement on-farm intervention approaches and increase recommendations for consumers regarding the safe handling and consumption of eggs and egg products. Therefore, eggs must be properly stored not only at retail premises but also at consumers' homes to maintain their quality and safety. Australian Eggs (2010) recommends that eggs should be stored under 15°C for retailers and wholesalers and at refrigeration temperature (5°C or less) for the public. However, it is common for eggs to get stored at ambient temperatures in retail settings or small grocery shops across Australia. Eggs are also often left on the bench at room temperature for several hours before use. Several studies found slow or negligible growth of *Salmonella* in egg yolk was observed at 7°C or below (Kim et al., 1989; Bradshaw et al., 1990; Clay & Board, 1991; Saeed & Koons, 1993; Lublin & Sela, 2008). *Salmonella* can multiply rapidly in egg yolks stored at room temperature, leading to significant increases in population. For instance, studies have shown that *Salmonella* in egg yolks can have replication times as short as 35 min at 37°C, and 3.5 hours at 15.5°C (Bradshaw et al., 1990). Compared to storage at 8°C and 20°C, a better inhibition effect has been found at 4°C (Gross et al., 2015; Pasquali et al., 2016). Yet, even if cold storage is applied for the public, at farms or during transport eggs might have been exposed to temperatures allowing *Salmonella* to grow before purchase. In such cases, refrigeration at the consumer stage may not effectively reduce the risk of *Salmonella* contamination (Cardoso et al., 2021). Besides, *Salmonella* on eggshell surfaces can lead to cross-contamination in the kitchen. During processing, bacterial contamination of food items might be facilitated by bacterial adherence to inert equipment surfaces (Fletcher & Floodgate, 1973), and the adhesion capability is influenced by temperature, pH, bacterial properties, and nutrient availability (Keevil et al., 1987).

Nevertheless, shell eggs are still displayed at ambient temperature in retail premises. Also, consumers are not aware of the significance of egg storage temperature on food safety, which may influence the risk of salmonellosis associated with the consumption of eggs and egg products at every stage of the food journey, from shopping to serving. Most previous studies were conducted at constant temperatures; however, constant temperatures may not fully replicate real-world scenarios. It has been found that a significant proportion of refrigerators have temperatures higher than 7°C, and it was observed that the highest temperature within the refrigerator was typically found near the door (Cardoso et al., 2021). Frequent door opening may result in temperature fluctuations and condensation on the eggshell, and temperature fluctuations can lead to increased *Salmonella* levels within the egg (Okamura et al., 2008). However, storing eggs at a temperature above 7°C is commonly

observed, which facilitates the growth of *Salmonella*, from the time they are laid until they are purchased. As a result, eggs with high levels of *Salmonella* can be introduced into the processing chain and ultimately reach consumers (Cardoso et al., 2021). Time and temperature are known to be crucial factors in controlling the growth of *Salmonella*; consumers typically have limited control over these factors before retail. Moreover, consumers are often not provided with information regarding the specific time and temperature conditions that eggs have been exposed to earlier in the supply chain. Therefore, the food regulations regarding shell egg storage can be important in controlling the level of *Salmonella* in eggs, and thus the risk of *Salmonella* infections.

4 *Salmonella* survival and invasion in eggs

A common recommendation for reducing the microbial risk associated with eggs is refrigeration, which inhibits microbial growth. It has also been shown that egg storage at 4°C resulted in greater retention of weight and volume, and a relatively lower albumen pH (Chen et al., 2005). The pH value of egg whites observed a quicker increase at higher storage temperatures due to the carbon dioxide released via eggshell pores, from 7.6 to 9.3 within a few days (Baron et al., 2016). The heterogeneous structure of egg white has a viscosity that is 35 times higher than that of water, which limits the movement of bacteria in egg white, thus restricting the availability of nutrients needed for bacterial growth (Baron et al., 2016). It is also interesting to note that the bactericidal effect of albumen under room temperature is multifactorial. On one hand, the pH value increases faster at room temperature compared with refrigerated temperatures and creates an unfavourable condition for bacteria (Tranter & Board, 1984; Kang et al., 2006), and the alkaline environment promotes the antibacterial effect that further limits the acquisition of iron (Tranter & Board, 1984; Okamoto et al., 2004). On the other hand, the gel structure in albumen can be disrupted as the pH increases, and thus decreases the viscosity, which creates more favourable conditions for microorganisms to penetrate and to proliferate within the eggs, and lysozyme activity is significantly affected by higher temperatures and pH (Venkataramani et al., 2013).

Although egg storage requirements may not be in complete agreement with the results of previous studies, these findings serve as a scientific foundation for the existing temperature requirements for egg storage and transportation set by the USDA (United States Department of Agriculture) and FDA (Food and Drug Administration), which further emphasise the significance of maintaining low temperatures as a crucial measure in controlling and preventing the growth of *Salmonella* in eggs upon lay (Chen & Thesmar, 2008). Messens et al. (2004) also suggest that, even when albumen is contaminated, chilling practices are implemented soon after lay to prevent *Salmonella* from developing in eggs. In brief, the initial degree and location of contamination, the likelihood of nutrient or microbial migration within eggs during storage, and the pace at which growth-restricting temperatures are attained all affect how well egg refrigeration works to prevent the replication of *S. Enteritidis*.

4.1 Natural egg defence

The egg is equipped with several physical barriers, including the shell, cuticle, and membranes (Figure 2), which act as a defence against microorganisms and other substances that may penetrate the egg contents. The cuticle, the primary barrier against bacterial invasion, plays a crucial role in protecting the egg's contents from microorganisms. It is formed after mineralisation, which is on the surface of the egg, and it covers and fills the shell pores (Dunn, 2004). The cuticle allows for gas exchange while preventing microorganisms from entering the egg (Sparks & Board, 1984). As eggs age, the cuticle becomes dehydrated, causing it to shrink and exposing pores to a higher risk of bacterial penetration (Mayes & Takeballi, 1983). It has been demonstrated that improved deposition of the cuticle will enhance the egg's inherent ability to resist bacterial invasion (Bain et al., 2013), which may decrease the transmission of diseases vertically and horizontally (EFSA, 2011a).

Albumen, more commonly known as egg white, possesses various antimicrobial activities (Julien et al., 2019), including high pH inhibiting bacterial growth (Sharp & Whitaker, 1927), high viscosity limiting the movement of microbes (Schneider & Doetsch, 1974; Yadav & Vadehra, 1977), and osmotic stress caused by high osmolality (Clavijo et al., 2006). Egg white contains several antibiotics, particularly lysozyme, which is around 3.5 g/L (Baron et al., 2016). Lysozyme is a protein that can hydrolyse the 1,4-glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine in the polysaccharide

layer of the Gram-positive bacterial cell walls (Baron et al., 2016; Ferraboschi et al., 2021), and accounts for 3.4–5.8% of the total proteins in egg white (Wilcox & Cole, 1957; Sauter & Montoure, 1972). It has been regarded as an endogenous antibiotic that can disrupt cell walls (Fleming, 1922), whereas the antibacterial effect is noticeably reduced on Gram-negative bacteria because the lipopolysaccharides layer covering the outer membrane provides a protective effect (Ferraboschi et al., 2021).

In addition to the physio-chemical characteristics of egg white, ovotransferrin (oTf), a substance in egg white that restricts the availability of iron which is essential for bacterial growth (Garibaldi, 1970; Andrews et al., 2003), can offer additional protection against pathogens. Due to the low concentration of iron (25 $\mu\text{mol/L}$) and high concentration of oTf (1.7 mmol/L) in albumen, it may be assumed that all the iron is chelated by oTf, given the protein's high affinity for iron and capacity to chelate two iron atoms per molecule (Baron et al., 2016). The bacteriostatic activity of oTf against Gram-negative bacteria appears to be mediated by separate processes, according to the available information. First, there's the well-known iron-deprivation effect, which essentially explains why egg white has bacteriostatic properties towards *S. Enteritidis*. It is also possible for oTf to directly interact with *S. Enteritidis*' outer membrane, allowing the outer layer to permeate and have bactericidal effects as a result (Baron et al., 2016). To contaminate the egg contents, pathogens need to penetrate the eggshell, shell membrane, and undesirable growth conditions of albumen.

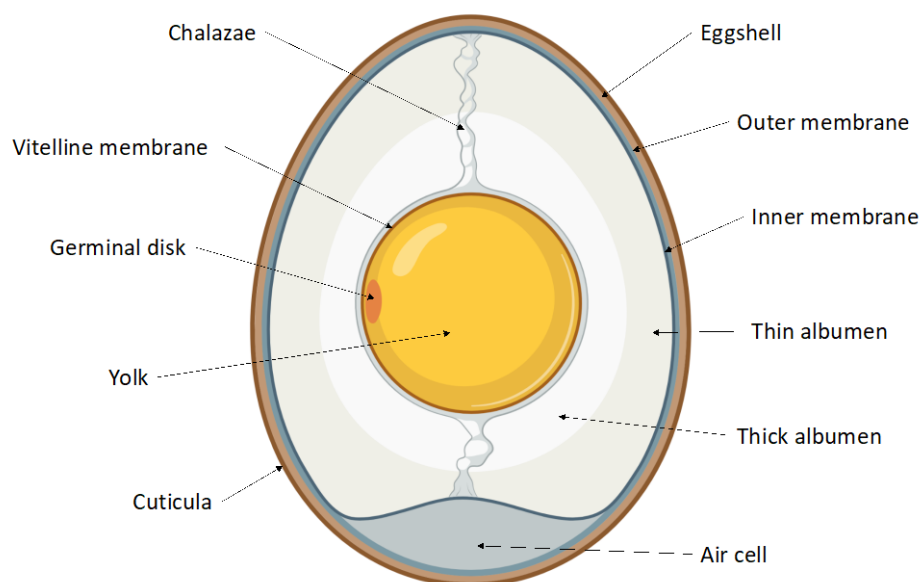


Figure 2 The composition and structure of an egg

4.2 Effect of time and temperature on the survivability of *Salmonella* on and in eggs

The survivability and invasiveness of *Salmonella* on and in eggs are influenced by various factors, such as the quality and integrity of the eggshell (De Reu et al., 2006; Chousalkar et al., 2010; Gole et al., 2014c; Gole et al., 2014b), the environmental conditions in which eggs are stored (Lublin et al., 2015; Whiley et al., 2016), and the serotypes involved (Gast et al., 2007). Although *S. Enteritidis* is mainly associated with vertical transmission (De Buck et al., 2004; Gantois et al., 2009), non-Enteritidis *Salmonella* serotypes can internalise and survive within egg contents despite the antimicrobial properties, particularly of the albumen. Isolates of *S. Typhimurium* have been found to contain genes conducive to *Salmonella* survival in egg albumen (McWhorter & Chousalkar, 2015; McWhorter et al.,

2015). Yet, instead of direct infection of egg contents it is thought that cross-contamination of food, or food contact surfaces, with non-Enteritidis *Salmonella* strains is the primary method of transmission to humans (Glass et al., 2016). As a result, rather than its impact on internal contamination processes, considering the effect of storage temperature on *Salmonella* survival on external egg surfaces may be more crucial to consider (Kingsbury et al., 2019). The surface used for egg breaking should also be regarded as a potential source of cross-contamination, as well as the potential hazards posed by crushed eggshells (Whiley et al., 2016). *Salmonella* found on the eggshell highlights the significance of handling eggs properly in the food industry and in the kitchen to prevent cross-contamination of other food products (Chousalkar et al., 2018).

Storage conditions on the survivability of *Salmonella* on and in eggs have been intensively studied but there is considerable variation in the outcomes of bacterial survival and egg storage. *Salmonella* on the eggshell surface reduces with time rather than increasing (Lublin et al., 2015; McAuley et al., 2015; Park et al., 2015; Pasquali et al., 2016), and some studies claimed that higher storage temperatures, such as 25°C compared to 12 or 4°C, led to a quicker decline in *Salmonella* viability on eggs, and thus the reduced risk of *Salmonella* infection on eggshell surfaces was observed at room temperature (25°C) rather than when stored at 12°C or 4°C (Park et al., 2015). The longer survival of bacteria in cold environments was due to the metabolic activities that were suppressed at refrigerated temperatures, as the temperature-dependent enzyme-catalysed process is significantly diminished at low temperatures (Jay et al., 2005). However, the decreased viability of *Salmonella* on eggshells does equate to a reduced risk of *Salmonella* infection. *S. Hessarek*, for example, can migrate from the eggshell surfaces to the eggshell pores and survive at both 5°C and 25°C for 72 h, while the ability to penetrate and survive in eggshell pores and internal contents was significantly influenced by storage temperature, and was more commonly found in eggs kept at 25°C (Lin et al., 2021). Several studies have demonstrated that when internal egg contents were contaminated with *Salmonella*, increased survival and rapid replication were seen at room temperature (Appendix Table 1) (Oliveira & Silva, 2000; Chen et al., 2005; Okamura et al., 2008; Gurtler & Conner, 2009; Gast et al., 2010; Lublin et al., 2015; Kingsbury et al., 2019). Only a small number of cells (10^2 CFU) is required to infect susceptible individuals (Fabrega & Vila, 2013), and if contaminated eggs are not kept under refrigeration, these would pose an increased risk throughout storage (Hammack et al., 1993); if they are refrigerated from the time they are laid, a significantly reduced rate of bacterial penetration into the egg contents will be observed (Lin et al., 2021).

4.3 Egg spoilage and egg quality

Eggs can deteriorate during storage either microbially or non-microbially. In addition to *Salmonella*, Gram-positive bacteria from the genera *Arthrobacter*, *Bacillus*, *Micrococcus*, and *Staphylococcus*, and Gram-negative bacteria of the genera *Pseudomonas*, *Proteus*, *Alcaligenes*, *Aeromonas*, and coliforms, are the most common contaminants of eggs (Erkmen & Bozoglu, 2016). Eggs can also become spoiled by mould, which is typically referred to as spots, because mycelial growth forms on the egg (Erkmen & Bozoglu, 2016). In terms of non-microbial spoilage, the yolk membrane weakens and albumen gets thinner and watery as the egg ages, although the low temperature and low humidity can prohibit the growth of microorganisms, they may lead to a quicker water loss in eggs and cause undesired quality change (Erkmen & Bozoglu, 2016). Therefore, maintaining the egg quality during storage is important for the extension of shelf life.

According to current industry standards, eggs should be stored for no more than 35 days after being laid (FSANZ, 2018). This recommendation was determined based on a yolk mean time modelling approach, which evaluated the duration for which eggs could be kept at a specific temperature before the internal conditions of 20% of those eggs would become favourable for the growth of *Salmonella* (Whiting et al., 2000; Thomas et al., 2006; MPI, 2019). Various factors can affect the risk of acquiring salmonellosis from eating eggs, such as the source of the eggs, the type of food, and the temperature

and duration of storage (Mokhtari et al., 2006; Thomas et al., 2006).

Storage conditions influence the eggs' internal quality. Albumen quality is often determined by Haugh units (HU), which measure the height of the air cell and the height of the thick albumen (Haugh, 1937) and are an important parameter during the egg grading process. *Salmonella* may grow at 16°C in 18 days, in 10 days if kept at 22°C, and in 4.6 days if stored at 30°C (Thomas et al., 2006). It has been observed that the permeability of the vitelline membrane and viscosity of albumen change throughout storage time, allowing *S. Enteritidis* to penetrate the yolk (Chen et al., 2005). The vitelline membrane is made up of a layer of glycoproteins on top of a collagenous matrix (Clay & Board, 1991). The porosity of the membrane was increased as glucose in the albumen interacted with these proteins if eggs were stored higher than 6°C, and yolk contents could be released into the albumen after 21 days at 20°C, supporting the growth of bacteria (Clay & Board, 1991; Humphrey & Whitehead, 1993; Messens et al., 2004). Therefore, egg quality can be better retained in cool storage, and as a result, bacterial penetration and growth are reduced. However, viable cells may still exist even though refrigeration may prevent proliferation. To enhance food safety, refrigeration storage may need to be mandated from shortly after the eggs are laid until just before preparation and consumption.

5 *Salmonella* virulence in human post-consumption of eggs or egg products

5.1 Clinical disease

In the EU, characteristics are evaluated to determine if a *Salmonella* serovar is of significant public health concern, including the likelihood that it may cause human salmonellosis, the frequency with which it affects animal populations, the speed with which it spreads, and indications of enhanced virulence (Moffatt et al., 2017). Like many other infectious illnesses, the immune system of the host and the inoculation dosage affect how the infection develops and progresses (Sanderson & Demerec, 1965).

Nontyphoidal salmonellosis is the most prevalent cause of hospitalisations and mortality among foodborne infections. Symptoms including nausea, vomiting and diarrhea are normally self-limiting, and persist for around seven days (Andino & Hanning, 2015). The most common *Salmonella* serotypes that cause salmonellosis in the US are *S. Typhimurium* and *S. Enteritidis* (Altekruse et al., 1997). Patients with immunological weaknesses have been shown to experience a greater incidence of bacteremia (Sperber & Schleupner, 1987).

5.2 *Salmonella* infection of the human gut

An infection typically requires 10^6 bacterial cells to occur, but it has been demonstrated that lower doses of bacteria can also lead to the onset of disease, particularly in more susceptible people such as the elderly, infants, and patients with chronic gastrointestinal diseases (Giannella et al., 1972; Arvanitakis, 2010). Upon consumption, many bacteria are killed by the acid in the stomach, but some *Salmonella* can develop the mechanism to survive under this lethal acidity (Foster, 1991), and pass through the stomach and reach the small intestine. *Salmonella* then attaches to the epithelial cells of the intestinal mucosa through fimbriae, and endotoxins are released. These endotoxins activate intracellular cyclic adenosine monophosphate (cAMP), leading to the secretion of water and electrolytes from the bloodstream into the intestinal lumen. This process results in secretory diarrhea characterised by the rapid loss of a large volume of fluids due to the immediate action of toxins (Arvanitakis, 2010). Another important aspect of *Salmonella* enteric infection is the bacterial infiltration of the intestinal mucosa and the subsequent acute inflammation. Fever and diarrhea are clinical symptoms of this bacterial invasion and inflammation (Arvanitakis, 2010).

5.3 Virulence factors

The virulence of *Salmonella* spp. is influenced by various factors that involve the different stages of *Salmonella* infection. These factors include the production of toxins such as lipopolysaccharide (LPS) endotoxin, enterotoxin, and cytotoxin; the mechanisms for colonisation, adhesion, and invasion of host cells; and the ability to survive inside host cells (Madigan & Martinko, 2007). For instance, *hila* encodes a transcriptional regulator belonging to the OmpR/ToxR family, which is required for *Salmonella* invasion (Bajaj et al., 1995). Environmental factors include temperature, pH, oxygen tension, and osmolarity. One of the most significant stresses on *Salmonella* spp. is the temperature (Castelijn et al., 2012; Yang et al., 2016), which has a crucial role in the transfer of bacteria across hosts, and is one of the main variables controlling their metabolic, growth, and pathogenic gene expressions (Sirsat et al., 2011; Shah et al., 2013). *S. Enteritidis* that may be present in eggs is more heat-susceptible when exposed to low temperatures prior to heating (Humphrey, 1990b) so there is a higher likelihood

that these microorganisms can be effectively eliminated through cooking. On the contrary, the survivability of *Salmonella* in egg contents was found to be enhanced at 5°C, since the expression levels of survival and stress response-related genes, *yafD*, *prop*, and *rpoS*, were significantly greater at 5°C compared to 25°C (Lin et al., 2021). Additionally, *Salmonella*'s survival rate is enhanced by synthesising the cold shock proteins (CSPs) when experiencing a temperature decline from 30°C to 10°C (Andino & Hanning, 2015), which also allows the resumption of replication later (Craig et al., 1998; Jeffreys et al., 1998; Kim et al., 2001).

From the overall perspective, increased expression of virulence-related genes at 5°C may not lead to an increased risk of *Salmonella* infection or a more severe disease. Warmer temperatures of egg storage may lead to increased potential of *Salmonella* multiplication in the yolk, whose nutrients may cause the expression of certain virulence genes via biochemical linkage (Morales et al., 2005). It has also been found that the virulence of *S. Enteritidis* was enhanced when grown in egg yolk. Mice that were fed egg yolk infected with *S. Enteritidis* had enhanced colonisation of *S. Enteritidis* in the intestines, increased faecal shedding, a higher extent of organ damage, and displayed more severe illness symptoms than those fed *S. Enteritidis* infected Luria Bertani broth or faeces (Moreau et al., 2016). Investigation into the virulence of *Salmonella* grown in albumen is also important, as egg white is a relatively harsh environment for it to survive. It was observed that the virulence of *S. Enteritidis* was not impacted by pre-incubation in a medium that resembled egg white from mouse and chicken models (Baron et al., 2004), and a decreased virulence was observed of *S. Enteritidis* grown in an alkaline medium from a mouse model (McDermid et al., 1996). Baron et al. (2004) illustrated that egg white conditions may not cause adequate or long-lasting changes to provide cells a benefit or a disadvantage during infection. Motility is also an important virulence factor for *S. Enteritidis* to grow in eggs, as evidenced by the inability of non-motile *Salmonella* serovars Gallinarum and Pullorum to proliferate under these circumstances, which may be prevented since the lack of flagella limits passage through the albumen towards the nutrient-rich yolk (Cogan et al., 2004). The curli fimbriae are also related to bacterial movement, the production of which plays a key role in enabling frequent yolk invasion. Interestingly, the production of curli fimbriae is suppressed below 20°C, thus may contribute to *S. enterica* serovar's reduced ability to proliferate in eggs at lower temperatures (Cogan et al., 2004).

6 *Salmonella* mitigation practices on egg farms in Australia

The frequency of *Salmonella* on retail eggs in Australia is low. The most recent reported prevalence of *Salmonella* on Australian retail eggs (outside of the egg) was 3.5% (Fearnley et al., 2011). Current quality assurance, animal husbandry, and biosecurity practices recommended by Australian Eggs have been adopted by producers nationwide to mitigate *Salmonella* on farms and subsequently through the supply chain. Below is a summary of some of the common Australian egg producer practices used to reduce *Salmonella* in the farm environment.

Intermittent shedding of *Salmonella* in the faeces of a persistently infected layer can contribute to the contamination of the shed environment as well as equipment. Reducing the load of *Salmonella* in birds' various intervention strategies contributes to minimising egg contamination. Rodents and insects can transmit *Salmonella* to layer hens. To combat this, Australian producers follow rodent control practices to minimise pests on farms.

Other on-farm mitigation strategies target the reduction of faeces on egg contact surfaces. An egg laid in an environment highly contaminated with faecal material is at risk of becoming contaminated with *Salmonella*. Thus, nest boxes and egg belts are to be kept clean and free of faeces. Eggs covered in faeces are classified by Food Standards Australia New Zealand as 'unacceptable' and cannot be sold. Cracked eggs also represent a risk for *Salmonella* as bacteria can migrate through the shell into the egg internal contents. Cracked or damaged eggs can also lead to the contamination of egg-handling equipment. Yolk has been shown to support the growth of *Salmonella*, which can lead to contamination of equipment. Routine cleaning of egg belts, rollers, and suction cups also contributes to the control of *Salmonella*. Cleaning of the egg grading floor and equipment also contributes to *Salmonella* control.

The implementation of the ESA program for egg producers in Australia has also played a vital role in *Salmonella* mitigation in the egg industry. The National *Salmonella* Enteritidis Monitoring and Accreditation Program (NSEMAP) is also available to all egg producers in Australia exporting eggs to overseas markets. As part of this program, egg farms that export eggs are involved in regular *Salmonella* surveillance on-farm.

Strict biosecurity practices also contribute to the mitigation of *Salmonella* on poultry farms. Training of staff in areas of food safety and biosecurity is an important component of risk mitigation. Control of vehicles on the farm and avoiding the sharing of vehicles and other equipment minimises the transmission of *Salmonella* between farm properties. Staff and visitor login records requiring details on prior exposure to poultry, illness, and international travel are also important biosecurity measures.

In addition to biosecurity practices, there are several interventions such as use of probiotics (Khan & Chousalkar, 2021), organic acids (Thompson & Hinton, 1997) and prebiotics (Markazi et al., 2018), which have been tested for their efficacy in reducing the *Salmonella* level on-farm. Previous studies recorded that a continuous feeding of probiotic supplement can significantly reduce the level of *Salmonella* in layer flocks (Khan & Chousalkar, 2020).

7 Conclusion

At a national level, the number of egg-related *Salmonella* outbreaks in humans declined over the past 5 years. This reflects the current on-farm intervention strategies as well as strategies implemented by food regulators for food handlers. There were human *S. Enteritidis* cases that were linked to egg farms but, those have been contained with mass culling and disinfection of infected properties. In Australia, on farms, freshly collected eggs stored in egg storage rooms that are generally set at a temperature of $< 16^{\circ}\text{C}$, and this temperature control chain is carefully maintained/monitored in the egg grading floor settings. Some Australian supermarkets store eggs at refrigeration temperature on the shelf, however, this practice is not consistent in many retail outlets or grocery stores across Australia. It is important to note that *Salmonella* can still survive at lower temperatures and replicate if exposed to a favourable environment. The results of studies investigating *Salmonella* survival in different parts of eggs stored at refrigeration or room temperature were variable and strain dependent.

The virulence of *Salmonella* serovars in egg contents stored at refrigeration versus room temperature is yet to be understood. Before the implementation of any policies on egg refrigeration in Australian conditions, robust scientific evidence is required to study the behaviour of Australian *Salmonella* strains in terms of their multiplication, viability, and virulence in egg contents, stored at supply chain-relevant temperatures. This needs further investigation.

8 Research gaps and recommendations

8.1 On-farm control of *Salmonella*

In Australia and overseas, there is a large body of literature on *Salmonella* epidemiology, the risk factors, and the level of egg contamination. Several studies have evaluated the effectiveness of *Salmonella* intervention strategies on-farm including vaccination, use of in-feed organic acids, and probiotics. In Australia, many egg farmers follow a combination of the above-mentioned intervention strategies to reduce the risk of *Salmonella*. Many large-scale Australian egg farms have started vaccinating their flocks against *Salmonella*. While none of these interventions eliminate *Salmonella* spp., there is also a challenge of emerging *Salmonella* serovars. Hence, ongoing monitoring of *Salmonella* in vaccinated flocks provides valuable information. Although some jurisdictions *Salmonella* reference labs can provide the *Salmonella* serovar specific information and the sample type/source, there is a lack of scientific evaluation on such information. Moreover, decisions about *Salmonella* testing is influenced by the budget, veterinary advice, and quality assurance (QA) practices adopted by farms.

Recommendation 1: Ongoing *Salmonella* surveillance in *Salmonella*-vaccinated layer flocks

Salmonella vaccination is now widely practiced in New South Wales and other states across Australia. There are also not many studies that investigated the duration of the immunity during the rearing after *Salmonella* vaccination. Longitudinal studies are required to screen *Salmonella* shedding and its prevalence in *Salmonella*-vaccinated flocks housed in different production systems.

Recommendation 2: Ongoing workshops/information sessions on food safety for egg farmers and/or farm employees

On-farm shedding and the prevalence of *Salmonella* are influenced by management practices adopted on egg farms. Given the consumer demand for cage-free eggs, the number of new small-scale egg farms (farm owners with variable farming experience) is on the rise. These small-scale egg farms may not have access to biosecurity-related resources. Hence, continuous extension activities for farm managers, farm owners, and QA managers is necessary. It is also important to have information sessions for regulators so that they are aware of the practical limitations of the industry. The continuous dialogue (in the form of a workshop or round table discussion) between the industry and regulators is essential. Such dialogue enables joint discussions and decisions on future research that could address both industry and regulatory issues. Previous studies of egg storage temperatures indicate that storing eggs at refrigeration temperatures was more effective in controlling *Salmonella* levels and better at maintaining egg quality. Eggs, however, are mainly held approximately at 15°C throughout the Australian egg supply chain. At this temperature, increased survivability on the eggshell compared to ambient temperatures, and rapid multiplication in the yolk was observed (Kim et al., 1989; Humphrey, 1990a; Bradshaw et al., 1996; Kingsbury et al., 2019), which indicated higher risks of *Salmonella* infection. Also, little has been done on the subsequent virulence of *Salmonella* in eggs stored at 15°C in causing salmonellosis. Despite the clear potential public health advantages that may arise from storing eggs at refrigeration temperatures, concerns have been raised that the ability of *Salmonella* to survive through later cooking and processing may be increased (Chapman et al., 1988). Our preliminary pilot study in mice indicated that there was no significant difference in *Salmonella* virulence when mice were fed contaminated egg yolk stored at 5°C vs 15°C.

Recommendation 3: Further studies are required on the comparative virulence of *Salmonella* spp. in eggs stored at < 7°C vs 15°C

Although lower temperature reduces the rate of *Salmonella* multiplication in egg contents, bacteria can still survive at lower temperatures such as 7°C. Studies on the comparative virulence of *Salmonella* spp. in eggs stored at < 7°C vs 15°C are necessary. Additionally, most studies have been conducted using *S. Enteritidis*, the most prevalent serovar in salmonellosis worldwide (EFSA, 2007; Threlfall et al., 2014; Havelaar et al., 2015). *Salmonella* Typhimurium, however, is the leading cause of human salmonellosis in Australia and New Zealand (Whiley et al., 2016). Moreover, *S. Hesse* is an emerging serotype within South Australia (Chinello et al., 2020), but very limited studies have investigated this serotype and its survivability on and in eggs at various storage temperatures. It is interesting to know whether there are metabolic variations across serovars in shell eggs and their behaviours at different storage temperatures, as this may account for the severity and spread of the disease. Therefore, the temperature of egg storage on the farm and during egg transportation (15°C) is of great significance.

Recommendation 4: Further studies are required to understand the relationship between fluctuating storage temperature and bacterial virulence

There is no sufficient scientific evidence to suggest how temperature fluctuations affect bacterial metabolism and virulence. Further studies are essential to understand the relationship between fluctuating storage temperature and bacterial virulence.

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10 Plain English Summary

Project Title:	Ensuring egg safety: review of microbial control in the supply chain
Australian Eggs Limited Project No	GROW020
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Objectives	To review the literature on <i>Salmonella</i> Enteritidis and <i>Salmonella</i> Typhimurium management/mitigation, and the role of temperature and refrigeration.
Background	Egg-associated human salmonellosis is a significant economic burden on global public health systems. It is generally recommended that eggs should be refrigerated to prevent condensation and subsequent bacterial penetration across the eggshell; however, condensation has not been significantly linked with <i>Salmonella</i> Enteritidis penetration level. Except for the United States, the guidelines on egg refrigeration in the supply chain in other countries are not prescriptive.
Research	A full review is produced.
Outcomes	Given that Egg Standards are under review by Food Standards Australia and New Zealand (FSANZ), the information generated by this review will provide evidence (in Australian settings) and guidance for future work. This evidence will be used by the industry, regulators at the state level, and FSANZ for implementing uniform practices around egg storage temperature during egg handling in supply chain.
Implications	The literature was reviewed and research gaps are identified in the Australian context.
Key words	Egg storage, <i>Salmonella</i> , Refrigeration
Publications	None to date

Appendix

Table 1 Overview of egg storage temperatures on the survivability of *Salmonella* serotypes

<i>Salmonella</i> Serotype	Temperatures (°C)	Results/findings from the study	Reference
<i>S. Hessarek</i>	5, 25	<i>S. Hessarek</i> penetrated eggshells more effectively at 25°C than 5°C. Refrigerating eggs immediately upon laying reduced the risk of internal <i>S. Hessarek</i> contamination.	Lin et al. (2021)
<i>S. Typhimurium</i> <i>S. Infantis</i> <i>S. Thompson</i> <i>S. Anatum</i> <i>S. Mbandaka</i>	15, 22	Greater <i>Salmonella</i> levels were found on the eggshells when eggs were stored at 15°C, after 21 and 35 days post-inoculation. The level of <i>Salmonella</i> from eggshell surfaces decreased over time at both storage temperatures.	Kingsbury et al. (2019)
<i>S. Typhimurium</i>	4, 14, 23, 35	Washed free range eggs should be stored at 4°C to reduce the <i>S. Typhimurium</i> penetration from external to internal contents.	Whiley et al. (2016)
<i>S. Enteritidis</i> <i>S. Typhimurium</i> <i>S. Heidelberg</i> <i>S. Hartford</i> <i>S. Newport</i>	4, 12, 25	<i>Salmonella</i> on eggshells declined at a faster rate at 25°C than at 4 or 12°C.	Park et al. (2015)
<i>S. Infantis</i>	5.5, 25.5	<i>Salmonella</i> on eggshells declined by 2 logs at both temperatures after 4 weeks of inoculation. At 25.5°C, <i>Salmonella</i> grew to 8 log CFU/mL in egg content after 4 weeks post-inoculation.	Lublin et al. (2015)
<i>S. Singapore</i> <i>S. Adelaide</i> <i>S. Worthington</i> <i>S. Livingstone</i>	20, 37	<i>Salmonella</i> load was higher on eggshell surfaces at 20°C than at 37°C.	Gole et al. (2014b)
<i>S. Enteritidis</i>	10, 15, 20, 25	The first day of unrefrigerated storage did not affect the ability of <i>S. Enteritidis</i> to migrate to the egg yolk. The migration of <i>S. Enteritidis</i> towards the yolk was driven by the inoculation site.	Gast et al. (2010)
<i>S. Enteritidis</i>	4, 20, 37	<i>S. Enteritidis</i> replicated better at 20°C than at 4°C, but eggs stored at 20°C prior to contamination better retained antimicrobial activity of egg white than did eggs stored at 4 or 37°C over 30 days.	Rehault-Godbert et al. (2010)
<i>S. Enteritidis</i>	7, 10, 23, 37	The growth of <i>Salmonella</i> was inhibited at 7°C after 8 days post-inoculation, while the load of <i>Salmonella</i> was significantly high in yolk and whole egg when eggs were stored at 10°C.	Gurtler and Conner (2009)
<i>S. Enteritidis</i>	Constant: 10, 20, 30 Changing: 22-30, 25-35	At constant temperatures, <i>S. Enteritidis</i> multiplied rapidly at 30°C, however, <i>S. Enteritidis</i> did not replicate at 10 or 20°C over 6 weeks.	Okamura et al. (2008)
<i>S. Enteritidis</i>	4, 10, 22	Storage of infected eggs at 4 and 10°C could effectively inhibit the growth of <i>S. Enteritidis</i> in eggs over three weeks post-inoculation, but storage at 22°C did not.	Chen and Thesmar (2008)

S. Enteritidis S. Virchow	6, 25	Serovar type, storage temperature, and time post-inoculation significantly affected <i>Salmonella</i> survival in eggs.	Lublin and Sela (2008)
S. Enteritidis S. Heidelberg	20, 30	<i>Salmonella</i> penetration and multiplication in egg yolks were significantly reduced at 20°C compared to 30°C.	Gast et al. (2007)
S. Enteritidis	15, 20, 25	<i>Salmonella</i> survival on eggshells decreased over time, with higher viability at lower temperatures and humidity. The penetration of <i>S. Enteritidis</i> was not affected by storage temperature or relative humidity.	Messens et al. (2006)
S. Enteritidis	7, 30	Refrigeration decreased the level of <i>S. Enteritidis</i> migration from vitelline membrane to yolk. A reduced frequency of <i>S. Enteritidis</i> penetration was seen when eggs were refrigerated.	Gast et al. (2006)
S. Enteritidis S. Typhimurium	37, 42	<i>Salmonella</i> level decreased significantly when eggs were incubated at 42°C.	Guan et al. (2006)
S. Enteritidis	4, 10, 22	Growth was inhibited in albumen when eggs were stored at 4 and 10°C, whereas <i>Salmonellae</i> thrived at 22°C. The egg quality was maintained when eggs were stored at 4°C.	Chen et al. (2005)
S. Enteritidis	4-8, 25	<i>S. Enteritidis</i> persisted on eggshells for 21 days of storage at 25°C and 4–8°C. Within 24 h of storage, <i>S. Enteritidis</i> migrated from the eggshell to the yolk. The refrigeration had no effect on <i>S. Enteritidis</i> migration to yolk.	Oliveira and Silva (2000)
S. Enteritidis	10, 17.5, 25	<i>S. Enteritidis</i> inoculated in yolks grew at 17.5 and 25°C, but bacteria replicated slowly at 10°C. When <i>Salmonellae</i> were inoculated from exterior surface, the bacterial cells could reach to a higher level within 1 day at 25°C, while a slower growth was observed at 17.5°C.	Gast and Holt (2000)
S. Enteritidis	4, 23	Minimal or no growth of <i>Salmonella</i> was recorded in eggs stored at 4°C for 2–3 days, while a significant growth at room temperature (23°C) was recorded within 3 days post-inoculation.	Saeed and Koons (1993)
S. Enteritidis S. Typhimurium	8, 10, 12, 15	None of the <i>Salmonella</i> strains grew in the yolk at 8°C during the three weeks of the inoculation period; bacteria grew slowly at 10°C, and relatively rapidly at 12°C and above.	Humphrey (1990a)
S. Enteritidis	2-8, 26	<i>S. Enteritidis</i> growth was minimal in eggs held in refrigerated temperatures for up to 16 days post-inoculation, but when eggs were stored at room temperature for the same period, the population of the organism rose by more than 8 log.	Hammack et al. (1993)
S. Enteritidis	7, 15.5, 37	No <i>Salmonella</i> growth in eggs was observed at 7°C after 94 days post-inoculation; a rapid <i>Salmonella</i> growth was recorded at 15.5 and 37°C.	Bradshaw et al. (1990)
S. Enteritidis	4, 10, 16, 21, 27	The load of <i>S. Enteritidis</i> increased significantly with the increase of egg storage temperature, and significantly decreased when eggs were stored at 4°C.	Kim et al. (1989)