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Egg surface survival and internalisation by New Zealand egg-associated Salmonella isolates as affected by storage temperature

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Scientific Interpretative Summary

This SIS is prepared by MPI risk assessors to provide context to the following report for MPI risk managers and external readers

Egg surface survival and internalisation by New Zealand egg-associated *Salmonella* isolates as affected by storage temperature

Under the Animal Products Act 1999 primary processors of eggs in New Zealand operate under a registered risk management programme (RMP). The current MPI Egg RMP template includes the following storage and shelf life options for eggs:

• 21 days where the storage/holding temperature may exceed 15˚C;

• 35 days if stored or held at 15˚C or less.

The question has been raised as to whether these options are too restrictive given the lack of epidemiological evidence that in New Zealand eggs are an important pathway for human salmonellosis; that the strain, which commonly internalise eggs, *Salmonella* Enteritidis, is not prevalent in New Zealand; that incidence of *Salmonella* contamination on the shells of eggs in New Zealand is low and there is no evidence of internal contamination of New Zealand eggs with *Salmonella*.

A review of recent international studies suggested that the modelling approach of the 2015 study “Horizontal transfer and growth of *Salmonella enterica* in chicken (*Gallus gallus*) eggs in New Zealand” is inappropriate for assessing risk salmonellosis to consumers of New Zealand eggs. However, international data were considered insufficient to assess the effect of New Zealand storage temperatures on the ability of New Zealand serotypes to internalise eggs, survive in albumen, and/or grow in egg yolk.

The purpose of this project was to investigate the survival of *Salmonella* on eggs and the ability to penetrate into eggs using the *Salmonella* serotypes identified from the recent egg layer farm survey in New Zealand and to estimate the validity of modelling utilised in the previous reports that support the current standards for egg shelf life.
Experimental results showed that New Zealand *Salmonella* egg-associated isolates die-off more rapidly on egg surfaces at the higher storage temperature, which is consistent with international reports for survival of non-Enteritidis serotypes. However, faecal contamination of egg shells increased survival of *Salmonella* on the surface emphasizing the need to ensure that eggs are clean. *Salmonella* was not detected in contents (albumen or yolk) of uncracked eggs during 35 days storage at both 15°C and 22°C.

It is therefore concluded that:

- *Salmonella* that might contaminate the egg surfaces during egg production at layer farms will not increase in numbers over storage time.
- For New Zealand consumers 15°C storage of clean, uncracked eggs does not reduce exposure to *Salmonella* compared with storage at room temperatures.
- Cleanness and integrity of the egg shell are critical factors for shell egg safety.

This review will be used by MPI to inform a review of options for egg shelf life and storage conditions in the Egg RMP Template.

Egg surface survival and internalisation by New Zealand egg-associated *Salmonella* isolates as affected by storage temperature

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This project reviewed documentation and experimental findings regarding *Salmonella* survival on the shell and internalisation in chicken eggs in the context of the New Zealand processing and retail environment. The review focused on addressing the question "Is storage of shell eggs at 15°C or less for a shelf life of 35 days necessary to protect consumers of New Zealand eggs from salmonellosis?".

The first section of this document reviews new data on *Salmonella* survival on the shell and internalisation since the 2015 risk assessment document "Horizontal transfer and growth of *Salmonella enterica* in chicken (*Gallus gallus*) eggs in New Zealand". The 2015 report recommended that eggs should be held at 15˚C or less for shelf life of 35 days. This recommendation was based on level of external contamination of eggs surveyed in 2007, research published up to 2011 and yolk mean time (YMT) model calculations.

Research published since 2011 pertaining to New Zealand processing and retail environment, and *Salmonella* serotypes relevant to the New Zealand egg layer environment were evaluated.

Based on the new evidence the validity of the YMT approach to evaluate New Zealand egg shelf life was questioned because of the following:

- Due to the absence of *S.* Enteritidis (which can internally colonise the hen), the main route for *Salmonella* contamination of eggs in New Zealand is via cross-contamination to egg surfaces. Therefore, the effect of storage temperature on survival of *Salmonella* on egg surfaces may be a more important consideration than internal contamination processes. Literature supports that *Salmonella* presence on eggshells does not increase as storage times increase, and most likely declines over time. Thus, the risk from *Salmonella* present on egg shells does not increase by prolonging storage times. Storage temperatures, which delay the loss of viability, will increase the time during which cross-contamination or trans-shell penetration could occur. In some studies, higher storage temperatures (e.g. 25°C compared with 4°C or 12°C) led to a faster reduction in *Salmonella* viability on eggs. However, available data were variable and were deemed insufficient to assess the effect of New Zealand-recommended storage temperatures (≤15°C and ~20-25°C) on the survival of New Zealand-relevant serotypes on egg surfaces.
- The YMT model used in the earlier 2015 MPI Risk Assessment was based on inoculation of egg albumen with high (500 Colony Forming Units (CFU)) of *S*. Enteritidis, a serotype that has not been identified in the New Zealand egg layer environment. Based on results from international studies, some non-Enteritidis, New Zealand-relevant serotypes are able to internalise and survive in egg albumen. However, data were considered insufficient to assess the effect of New Zealand-relevant storage temperatures on the ability of New Zealand-relevant serotypes to internalise eggs, survive in albumen, and/or grow in egg yolk.

Experimental studies were undertaken to fill the identified gaps in knowledge and better inform on the question of whether storage at 15°C or less for a shelf life of 35 days necessary to protect consumers of New Zealand eggs from salmonellosis. Experiments were designed to address:

- The survival over time of New Zealand-relevant *Salmonella* serotypes on eggs at New Zealand-relevant storage temperatures (focusing on 15°C and 22°C).
- The effect of the same storage temperatures on the ability of the New Zealand-relevant *Salmonella* serotypes to internalise and survive in egg contents (albumen and yolk).
- The effect of faecal contamination on the survival of *Salmonella* in and on eggs

Key experimental findings of the study were as follows:

- Survival of *Salmonella* on egg surfaces was higher following incubation at 15°C (31% relative humidity [RH]) compared with 22°C (45% RH) after both 21 and 35 days of incubation. Reduced survival of New Zealand *Salmonella* egg-associated isolates on egg surfaces at the higher storage temperature and higher RH is consistent with some earlier reports from non-Enteritidis serotypes in international studies.
- *Salmonella* present on visibly clean eggshell surfaces declined in viability over time at both storage temperatures, and was virtually undetectable from eggs stored at 22°C for 35 days.
- A substantially higher concentration of viable *Salmonella* was recovered from eggs contaminated with chicken faeces. The contribution of faeces to *Salmonella* survival on eggs was particularly dramatic on eggs stored at 15°C for 35 days (2.38 log higher CFU recovery on eggs containing faeces than those without).
- No *Salmonella* was detected in egg contents (albumen or yolk) at any incubation temperature or time point, regardless of the presence of faeces.

One caveat is that eggs used in these experiments were “best-case scenario” in that they were unwashed (i.e. cuticles were intact), visibly spotless (except those with faeces artificially added), not cracked, and shells contained no visible deformities. Therefore, experimental results may not equally apply to eggs that have micro-cracks.

Overall, findings from this study suggest that:

- The risk of *Salmonella* cross contamination from externally contaminated egg surfaces will not increase over storage time.
- For clean, uncracked eggs, 15°C storage does not provide better protection for consumers compared with room temperatures.
- Cleanness and integrity of the egg shell are critical factors for egg safety.

2. INTRODUCTION

New Zealand has a very low reported incidence of egg-associated salmonellosis (Chousalkar *et al.*, 2017;Lake *et al.*, 2004). However, a significant proportion of foodborne nontyphoidal salmonellosis infections have been attributed to the consumption of contaminated eggs internationally (European Food Safety Authority (EFSA), 2007;Kirk *et al.*, 2015;Moffatt *et al.*, 2016).

To minimise the risk of egg-associated salmonellosis, the Ministry for Primary Industries (MPI) recommends that layer farm operators operating under a Risk Management Programme (RMP) apply one of following storage and shelf life options to their eggs:

21 days where the storage/holding temperature may exceed 15˚C,

35 days if stored or held at 15˚C or less, or

Another combination to be specified, and justified by the producer.

These recommendations were supported by the 2015 MPI Risk Assessment report "Horizontal transfer and growth of *Salmonella enterica* in chicken (*Gallus gallus*) eggs in New Zealand"[1] (Ministry for Primary Industries, 2015). The report noted that the only New Zealand survey (2007) of *Salmonella* on eggs showed a higher level of external contamination than available at that time surveys of Australian eggs. Assessment of growth potential of *Salmonella* during the storage of eggs utilised yolk mean time (YMT) calculations based on *Salmonella* Enteritidis behaviour inside eggs assuming that *Salmonella* was already present inside eggs at the start of storage.

However, there is no evidence of internal contamination of New Zealand eggs with *Salmonella*. MPI was concerned that overseas studies based on *S.* Enteritidis may not be relevant to *Salmonella* serotypes/strains found in New Zealand and assumptions made in the 2015 report do not reflect the real life situation.

An MPI-funded microbiological survey conducted by ESR detected *Salmonella* in the environment of egg layer farms. In some farms, *Salmonella* was ubiquitous in the processing environment suggesting that despite the implementation of risk management programmes, contamination on the outside of eggs is possible. Subsequent processing, storage and transport conditions may determine if *Salmonella* is able to survive on the outside of the eggs, internalise and grow within the egg.

This project reviewed in the context of New Zealand processing and retail environment new data on *Salmonella* survival on the shell and internalisation since the 2015 paper. With the aim on updating the 2015 Risk Assessment the review focused on addressing the question "Is storage of shell eggs at 15°C or less for a shelf life of 35 days necessary to protect consumers of New Zealand eggs from salmonellosis?". The assessment of new evidence was based on:

New epidemiological data on salmonellosis incidence and egg-associated *Salmonella* outbreaks in New Zealand and Australia from MPI and Australian Government Department of Health online published reports.

New data (post-2011) from the international scientific literature on the prevalence, survival and penetration of *Salmonella* on / in eggs and how this is influenced by temperature and serotype. Attention was also paid to inhibitory properties of egg albumen for the survival and growth of *Salmonella*. Choice of focal literature was in part guided by MPI,

[1] Referred to in this report as the "2015 MPI Risk Assessment report"

as well as relevant papers assessed by the 2016 Risk Profile (update): *Salmonella* (non typhoidal) in and on eggs (Rivas *et al.*, 2016), and a search of the PubMed database for primary references (examples of search term combinations; "*Salmonella*" and "egg", "albumen", "lysozyme" or "yolk mean time").

The 2017 report on "Microbiological survey of commercial egg layer farms in New Zealand for the presence of *Salmonella* and assessing on-farm practices for egg production and handling in relation to *Salmonella* control." ((Kingsbury, 2018) in preparation). This report has provided new insights into the serotypes and prevalence of *Salmonella* in the environment on New Zealand egg layer farms, and actual farm practices in place relevant to *Salmonella* mitigation on eggs.

Discussions with experts from Australian institutes. Discussions included unpublished prevalence survey data for *Salmonella* in and on eggs, biofilm formation on eggs, and methodologies for testing of *Salmonella* in and on eggs.

The review of existing evidence identified gaps of knowledge and led to designing new experiments necessary to address the risk question. Experimental work was designed and implemented to provide data on the ability of the types of *Salmonella* associated with eggs in New Zealand to:

Survive on egg shell surfaces under the recommended cool storage and ambient temperatures (15˚C and room temperature/22˚C, respectively) during the shelf life of the eggs (21 and 35 days), and

Penetrate and survive in the internal contents of eggs under the same New Zealand storage conditions.

Survive on egg shells and penetrate inside the egg in the presence and absence of chicken faeces on egg shells.

3 HORIZONTAL TRANSFER AND GROWTH OF SALMONELLA ENTERICA IN CHICKEN (GALLUS GALLUS) EGGS IN NEW ZEALAND: AN UPDATE

For a general background describing *Salmonella enterica* biology, survival characteristics, serotypes, pathogenesis, and contamination in and on eggs, please refer to the "Risk profile (update): *Salmonella* (non typhoidal) in and on eggs" (Rivas *et al.*, 2016).

3.1 *SALMONELLA* SOURCE ATTRIBUTION TO EGGS IN NEW ZEALAND AND AUSTRALIA

3.1.1 New Zealand

Every year the EpiSurv database, administered by ESR, collects data on salmonellosis cases, outbreaks and sources of exposure in New Zealand. Data relating to foodborne transmission is compiled into an annual foodborne annual disease report.

The 2015 MPI Risk Assessment document (Ministry for Primary Industries, 2015) summarised data from reported *Salmonella* outbreaks in New Zealand from 2000-2009 where eggs were implicated. Of the 204 outbreaks of salmonellosis, eggs were only implicated three times.

Table 1 summarises data from New Zealand *Salmonella* outbreaks from 2010-2016 where eggs were implicated. Over this period, there were four outbreaks where eggs were reported as an ingredient in a suspected vehicle. However, in all but one outbreak, the evidence linking the food as a source of infection was weak. One outbreak was considered to have a strong link to the suspected food. In this food eggs were mixed with other ingredients that may have been the source.

Therefore, as concluded in the previous risk assessment document (Ministry for Primary Industries, 2015), there is still minimal evidence for eggs as a transmission vehicle for human salmonellosis in New Zealand.

Table 1. Summary of 2010-2016 New Zealand *Salmonella* outbreaks where eggs were suspected as a source (Foodborne annual disease reports[2]).

YEAR	PREPARATION AND/OR EXPOSURE SETTING	SUSPECTED VEHICLE	NUMBER ILL (CONFIRMED, C; PROBABLE, P)
2010	Restaurant/café	Chocolate mousse cake - uncooked egg whites	10C, 11P
2010	Home	Spanish cream - uncooked eggs	4C
2013	Restaurant/cafe/bakery	Boiled egg and ham sandwich[b,c]	10C
2016	Supermarket/delicatessen	Eggs, raw peppers, soft brie cheese[a,c]	3C

[a] weak evidence linking outbreak to food
[b] strong evidence linking outbreak to food
[c] multiple foods linked

3.1.2 Australia

In Australia OzFoodnet is the national network for foodborne disease surveillance and response. It has conducted surveillance for enteric and foodborne disease outbreaks since 2000 (Kirk *et al.*, 2008).

[2] http://www.foodsafety.govt.nz/science-risk/human-health-surveillance/foodborne-disease-annual-reports.htm; accessed 30-11-2017

As discussed in the previous risk assessment document (Ministry for Primary Industries, 2015), there has been a significant increase in salmonellosis outbreaks in Australia linked to the consumption of raw or lightly cooked eggs since 2005. That document presented egg-associated salmonellosis data from 2001-2008. Table 2 summarises Australian egg-associated salmonellosis outbreak data from 2009-2014. Over this period, the number of egg-associated outbreaks has continued to rise (although a decrease was observed in 2013). A lack of knowledge of the risk of *Salmonella* on eggs by food operators, together with consumer and food service industry practices allowing cross-contamination, consumption of raw or lightly cooked eggs, and poor temperature control of egg products, have been reported as contributing to the egg associated cases (Glass *et al.*, 2016;Moffatt *et al.*, 2016).

Table 2. Egg-associated outbreaks of Salmonellosis, Australia, 2009-2017 (OzFoodNet[a]).

YEAR	SALMONELLOSIS NOTIFICATIONS / CASES PER 100,000[b]	FOODBORNE SALMONELLOSIS OUTBREAKS	EGG-ASSOCIATED OUTBREAKS (CONFIRMED OR SUSPECTED)	SEROTYPES OF EGG-ASSOCIATED OUTBREAKS
2009	9,430 / 43.5	59	18	*S.* Typhimurium, *S.* Singapore, *S.* Virchow. *S.* Saintpaul
2010	11,817 / 53.6	36	21	*S.* Typhimurium, *S.* Singapore
2011	12,198 / 54.6	45	26	*S.* Typhimurium
2012	11,165 / 49.1	65	29	*S.* Typhimurium
2013	12,724 / 55.0	50	23	*S.* Typhimurium
2014	16,272 / 69.3	96	42	*S.* Typhimurium
2015	16,954 / 71.2	ND [c]	ND	ND
2016	18,059 / 74.7	ND	ND	ND
2017	16,416 / 66.8	ND	ND	ND[i]

[a] For the years 2009-2011, data were sourced from OzFoodNet annual reports. For the years 2012-2014, data were summarised from OzFoodNet quarterly reports. Annual information was not available for 2015 onward. All reports are available at http://health.gov.au/internet/main/publishing.nsf/Content/cdna-ozfoodnet-reports.htm#annual

[b] *Salmonella* notifications were sourced from http://www9.health.gov.au/cda/source/rpt_3.cfm, accessed 07-08-2018

[c] ND No data; accessed 4-10-2018.

3.2 NEW ZEALAND AND INTERNATIONAL COMMERCIAL EGG LAYING FARM SURVEYS

3.2.1 *Salmonella* prevalence on New Zealand and international egg layer farms

A 2016 risk profile for *Salmonella* (non-typhoidal) in and on eggs (Rivas *et al.*, 2016) highlighted that there were no data on *Salmonella* prevalence in New Zealand layer flocks or layer farm environments. The report recommended a separate study to gather information on the prevalence of *Salmonella* and the potential for *Salmonella* to contaminate eggs, via environmental sampling of New Zealand layer farms.

In response to the recommendations of the risk profile, a recent study surveyed the prevalence of *Salmonella* in the New Zealand commercial egg layer environment (farm-level feed, laying shed dust, fresh faeces, boot/manure belt swabs and packhouse egg contact surfaces) (Kingsbury, 2018). Egg collection and packing areas were also sampled since these are important potential sites for external contamination of egg shells (Davies and Breslin, 2003;Dewaele *et al.*, 2012;Utrarachkij *et al.*, 2012).

Previous international microbiological surveys reported that the presence of *Salmonella* in faeces and dust in the egg production environment strongly correlated with the within-flock prevalence of *Salmonella*. (Arnold *et al.*, 2010;Arnold *et al.*, 2011;Carrique-Mas *et al.*, 2008;Wales *et al.*, 2007). These observations form the basis of most monitoring programs for *Salmonella* in the poultry industry. A correlation has also been found between the prevalence of *Salmonella*-positive environmental samples, particularly faeces, and contaminated eggs produced at the same farm (Arnold *et al.*, 2014;Dewaele *et al.*, 2012;Gole *et al.*, 2014c).

The previously reported low rate of surface contamination of eggs by *Salmonella* indicated that testing a large number of eggs to achieve statistically valid results would be necessary,

and small numbers of positives may generate large uncertainty intervals (Carrique-Mas and Davies, 2008). Consequently, eggs were not sampled in the 2018 New Zealand survey.

The prevalence of *Salmonella* in the New Zealand egg production environment was lower compared with the similar Australian or international studies (Table 3).

Table 3. Prevalence of *Salmonella* in egg production environments in New Zealand and internationally

Region/Country	Year	Reference	Prevalence % (n)				
			Farm level feed	Laying shed dust	Laying shed faeces	Laying shed boot/manure belt swabs	Packhouse egg contact surfaces
New Zealand	2016	(Kingsbury, 2018)	3 (33)	28 (67)	10 (67)	16 (67)	6 (87)
Australia (Queensland)	2014	(Cuttell *et al.*, 2014)	0 (21)	NT	29 (53)	38 (53)	NT
Australia (New South Wales)	2013	(New South Wales Food Authority, 2013)	11 (27)	NT	17 (90)	27 (99)	NT
Europe	2010	(Arnold *et al.*, 2010)	NT	51	NT	NT	NT
England, Wales	2003	(Davies and Breslin, 2003)	NT	NT	NT	NT	25

NT not tested

Salmonella prevalence was also lower at the New Zealand layer shed (31.3%) and farm level (42.9%) compared with the New South Wales (49.6%-positive sheds, 44.9%-positive farms) and Queensland egg layer surveys (43.4%-positive sheds, 57.1%-positive farms) (Cuttell *et al.*, 2014;Kingsbury, 2018;New South Wales Food Authority, 2013). The lower farm and shed prevalence from the New Zealand survey was even more striking considering that 16.4% of positive sheds and 14.3% of positive farms in this study were based on positive dust samples only, which was not directly sampled in the Australian surveys.

While the overall prevalence of *Salmonella* on New Zealand layer farms was low relative to international surveys, *Salmonella* was detected on egg contact surfaces from the egg packhouse of three farms. Once contaminated, these surfaces would be a source of further contamination on additional eggs processed on the same surfaces. Therefore, despite implementation of Risk Management Programmes (RMP) to minimise the risk, contamination on the outside of the egg may occur.[3]

3.2.2 *Salmonella* serotypes present on New Zealand and international egg layer farms

The serotypes identified from the New Zealand egg layer farm survey and from Australian surveys are shown in Table 4. Five serotypes were identified in the New Zealand survey, all of which are commonly isolated from the environment in New Zealand[4], and are amongst the most common types identified on egg layer farms world-wide (with the exception of *S*. Thompson which is not commonly documented). *S*. Enteritidis was not identified in this survey, consistent with previous reports stating that is this serotype is not endemic in New Zealand flocks (Ministry for Primary Industries, 2015).

S. Typhimurium is the most common serotype isolated from clinical cases in both New Zealand[5] and Australia, and is the most common serotype contributing to egg-associated salmonellosis in Australia (Chousalkar and Gole, 2016;Moffatt *et al.*, 2016;Moffatt *et al.*, 2017). It is also the serotype most commonly associated with laying hens and eggs in non-European countries (Singh *et al.*, 2010), and the second most common in Europe after *S*.

[3] Risk management programmes, including controls for *Salmonella*, have been required on layer farms since 2004.
[4] https://surv.esr.cri.nz/enteric_reference/nonhuman_salmonella.php; accessed 19-02-2018
[5] http://www.foodsafety.govt.nz/science-risk/human-health-surveillance/foodborne-disease-annual-reports.htm; accessed 30-11-2017

Enteritidis (European Food Safety Authority (EFSA) Panel on Biological Hazards (BIOHAZ), 2014)[6].

In the recent New Zealand survey, in contrast to the most of Australian surveys, *S*. Typhimurium was not a dominant strain (Table 4). The serotype was typically only isolated once per farm, and it was not isolated from faecal samples (Kingsbury, 2018). This is consistent with sporadic introduction of the serotype onto New Zealand farms rather than evidence for the presence of a resident population and/or or carriage by flocks.

Table 4. *Salmonella* serotypes identified from egg layer farm surveys in New Zealand and Australia

REGION/COUNTRY	YEAR	REFERENCE	PREVALENCE OF SEROTYPES PRESENT
New Zealand	2016	(Kingsbury, 2018)	*S*. Infantis (44%), *S*. Thompson (35%), *S.* Typhimurium (14%), *S*. Anatum (5%), *S.* Mbandaka (2%) (N=43)
Australia (Queensland)	2014	(Cuttell *et al.*, 2014)	*S*. Typhimurium (20%), *S.* Infantis (12%), *S*. Agona (12%), *S.* Corvallis (9%), *S*. Senftenberg (6%), *S*. Montevideo (6%), *S.* Alachua (6%), *S.* Heidelberg (6%), *S*. Anatum (6%), *S*. Zanzibar (6%), *S.* Amsterdam (3%), *S*. Give (3%), *S*. Liverpool (3%), *S.* Virchow (3%) (N=35)
Australia (New South Wales)	2013	(New South Wales Food Authority, 2013)	*S*. Typhimurium (30%), *S.* Infantis (19%), *S*. Senftenberg (14%), *S.* Montevideo (8%), *S.* Singapore (5%), *S*. Havana (4%), *S*. Orion (4%), *S*. subs 1 ser rough (3%), *S.* Agona (2%), *S.* Give (2%), *S.* Livingston (2%), *S*. subs 1 ser 4 (2%), *S*. Tennessee (2%), *S*. Virchow (2%), *S*. Wangata (2%), *S*. Bredeney (1%), *S*. Johannesburg (1%) (N=130)
Australia	2014	(Gole *et al.*, 2014c)	*S*. Oranienburg (77%), *S.* Typhimurium (12%), *S*. Worthington (8%), *S*. Agona (3%), *S.* Serotype 4,5,12:-:- (2%), *S.* serotype rough:g,s,t:- (1%) (N=130)
Australia	2017	(Gole *et al.*, 2017)	*S.* Mbandaka (65%), *S*. Typhimurium (24%), *S.* Agona (6%), *S*. Infantis (1%), *S.* Anatum (1%), *S*. Worthington (1%), *S.* Singapore (0.5%), *S.* serotype 4,5,12:i:- (0.5%), and *S.* serotype 1 rough:f,g,s:- (0.5%) (N=209)

3.2.3 *Salmonella* prevalence in and on eggs in New Zealand and internationally

Data available during the preparation of the 2015 MPI Risk Assessment document suggested that there was a high level of contamination (1.8%) on the outside of New Zealand eggs compared with Australia, based on data from the 2007 New Zealand survey and Australian data available at that time (Ministry for Primary Industries, 2015). However, small numbers of positive samples can generate large uncertainty intervals. Although there are no new studies on *Salmonella* prevalence on eggs in New Zealand, new surveys of Australian prevalence data have been published since the report (included in Table 5). While two studies did not detect *Salmonella* on eggs (0% prevalence; (Chousalkar *et al.*, 2010;Symes *et al.*, 2016)), four other studies reported a higher prevalence of *Salmonella* on egg shells than in the 2007 New Zealand study (Chousalkar and Roberts, 2012;Fearnley *et al.*, 2011;Gole *et al.*, 2013;Gole *et al.*, 2014c).

Consistent with previous studies, no recent Australian surveys of retail eggs have identified *Salmonella* in the egg contents (Table 5). However, *S.* Typhimurium and *S*. Infantis were both detected from internal contents of eggs sampled directly from Australian egg farms at levels of no more than 1 Most Probable Number (MPN)/ml (Helen Crabb, University of Melbourne, pers. comm., 2018, Table 5). The ability to detect *Salmonella* from egg contents in this study, but not previous studies, may be due to the age and *Salmonella*-shedding status of the laying hens from which the eggs were derived, testing of a larger number of eggs by the recent study, or differences in testing methodology between studies (Helen Crabb, University of Melbourne, pers. comm., 2018). In the 2018 study freshly laid eggs were typically tested on the day of laying). Presence of *Salmonella* in contents of freshly laid eggs does not inform on whether these isolates/serotypes are able to survive in egg albumen during storage. An inability to survive in albumen could account for detection in freshly laid eggs in this study but absence in eggs at retail from other Australian studies.

[6] https://www.food.gov.uk/sites/default/files/acmsf-egg-reportv1.pdf; accessed 6-12-2017

Serotypes present on New Zealand eggs and layer farms (*S*. Infantis, *S*. Typhimurium and *S.* Mbandaka) have also been isolated from egg contents in other international studies (Martelli and Davies, 2012).

Table 5. Extent of contamination by *Salmonella* serotypes on and in eggs at retail or directly from layer farms in New Zealand and Australia (adapted from MPI, 2015). Eggs were from retail sources unless specified.

COUNTRY	YEAR/REPORT DATE	NUMBER OF SAMPLES (EGG NUMBER)	SAMPLE	*SALMONELLA* PREVALENCE: SAMPLES POSITIVE, PERCENTAGE POSITIVE (MICROBIAL LOAD/CONTAMINATED EGG WHERE REPORTED)	SEROTYPE	REFERENCE
New Zealand	1994	341[a] (2,046)	Shell	0%		(Johnson, 1995)
		339[a] (2,037)	Contents	0%		
New Zealand	2001	93[a] (~558)	Shell	13; 14.0%	*S*. Infantis, *S*. Thompson, *S*. serotype 6,7:k:-	(Lake *et al.*, 2004)
		700	Contents	0%		
New Zealand	2007	514[a] (3,710)	Shell	9; 1.8% (<5 MPN/egg on 8 eggs, 44 MPN/egg on 1 egg)	*S*. Infantis	(Wilson, 2007)
		3,710	Contents	0%		
Australia	2003 (report 2006)	6,476	Shell	0%		(Thomas *et al.*, 2006)
		20,000	Contents	0%		
Australia	2008 (report 2011)	199[b] (2,388)	Shell	7; 3.5%	*S*. Infantis, *S*. Typhimurium, *S*. Johannesburg, *S*. Livingstone	(Fearnley *et al.*, 2011)
		199[b] (2,388)	Contents	0%		
Australia	2010	500	Shell	0%		(Chousalkar *et al.*, 2010)
		500	Shell pores, internal membrane	0%		
		500	Contents	0%		
Australia	2012	260[a] (1,560)	Shell	7; 2.7%	*S*. Infantis, *S*. serotype 4,12:d	(Chousalkar and Roberts, 2012)
		260[a] (1,560)	Shell pores, internal membrane	0%		
		260[a] (1,560)	Contents	0%		
Australia	2013	310[a] (1,860)	Shell	14; 4.5%	*S*. Infantis, *S*. serotype 4,12:d	(Gole *et al.*, 2013)
		310[a] (1,860)	Shell pores, internal membrane	0%		
		310[a] (1,860)	Contents	0%		
Australia	2014	521[d]	Shell	20; 3.8%	*S*. Worthington, *S*. Oranienburg, *S*. Typhimurium	(Gole *et al.*, 2014c)
		521	Contents	0%		
Australia	2016	668[a] (4,008)	Contents	0%		(Symes *et al.*, 2016)
Australia	2018	3,238[c,d] (9,714)	Egg pools (shell and content)	68, 2.1% 6, 0.2% 16, 0.5%	All *Salmonella* *S*. Typhimurium *S*. Infantis	H. Crabb, University of Melbourne, pers. comm. 2018
		260 (for each component)	All samples	9, 3.4% (0.579 MPN/ml/contaminated egg)	All *Salmonella*	
			Shell	4, 1.4%	*S*. Infantis, *S*. Typhimurium	
			Shell, internal membrane	3, 1%	*S*. Infantis	
			Contents (yolk plus white)	2, 0.7%	*S*. Typhimurium, *S*. Infantis	

[a] tested in groups of six, i.e. one sample is six or at least six eggs
[b] tested in groups of twelve, i.e. one sample is twelve eggs
[c] tested in groups of three, i.e. one sample is three eggs
[d] eggs were obtained from layer farm

3.3 SURVIVAL OF *SALMONELLA* ON EGGS AS A FACTOR OF TEMPERATURE

3.3.1 Background

S. Enteritidis is not associated with chickens and eggs in New Zealand, and internal contamination of eggs has not been reported in New Zealand surveys. As such, cross-contamination from contaminated egg shells during food preparation is thought to be the main risk factor for foodborne *Salmonella* infections (Moffatt *et al.*, 2016). Contamination of egg shells at time of food preparation depends on *Salmonella* survival and, potentially, growth on the egg shell during storage time.

Growth of *Salmonella* by itself on clean egg shell surfaces is not expected to occur. However, chicken faeces on the surface of eggs may have a protective effect and act as a source of nutrients for any *Salmonella* present (Park *et al.*, 2015;Schoeni, 1995). Although there is no set definition for "dirty egg" in the RMP Template for Eggs (NZFSA, 2007), New Zealand eggs sold at retail are required to be visibly clean. In the recent survey of New Zealand layer farms, responses from egg producers varied considerably for what they considered a "dirty egg" (Kingsbury, 2018). Furthermore, four of the nine retail eggs contaminated with *Salmonella* in the 2007 survey of New Zealand retail eggs were considered "dirty" (obvious contamination of shell with faecal, feather or other organic material) (Wilson, 2007). If present, faecal matter on retail egg shell surfaces provides potential for growth of *Salmonella.*

The following sections review literature on the effect of storage temperature on the survival and possible growth of *Salmonella* on egg shells, and the relevance of biofilm formation and *Salmonella* serotype to this. These inter-related topics were not covered in the 2015 MPI Risk Assessment report.

3.3.2 *Salmonella* load on egg shells

The total number of *Salmonella* (the load) present on eggs at the time of contamination will affect the likelihood and extent of both cross-contamination and trans-shell penetration. Loads present are influenced by the source of contamination, egg handling practices on and off the farm, and time and storage conditions since contamination (Chousalkar *et al.*, 2017). Reports from different studies of concentrations of *Salmonella* on externally contaminated eggs are mixed:

- Natural contamination was reported to rarely exceed 10^2 Colony Forming Units (CFU)/eggshell (Humphrey, 1994).
- The level of *Salmonella* on positive egg shells from an Australian free-range farm were 1.7±0.1 MPN per egg (Gole *et al.*, 2017).
- The survey of New Zealand eggs at retail found loads of <5 MPN/egg on eight externally contaminated eggs and 44 MPN/egg on the ninth contaminated egg (Wilson, 2007).

3.3.3 Biofilm formation

The ability of *Salmonella* to form biofilms on eggshell surfaces was recently demonstrated (Pande *et al.*, 2016b). Biofilm is comprised of interacting cells embedded in an extracellular matrix. The matrix is produced by the cell and comprised of curli, fimbriae and cellulose polymers, which promote linkage between the *Salmonella* cells. The formation of a biofilm allows *Salmonella* to better-survive harsh physical and environmental stressors, contributing to its persistence on a wide range of biotic and abiotic surfaces (reviewed by (Steenackers *et al.*, 2012)).

A total of 145 Australian egg layer farm isolates comprising seven serotypes were tested for biofilm-relevant phenotypes, biofilm-relevant gene expression, and biofilm formation *in vitro* and on eggs. Phenotypes were tested at both at 22 and 37°C, while biofilm formation on

eggs was tested at 22°C only (Pande *et al.*, 2016b). Biofilm-relevant phenotypic characteristics were enhanced at 22°C compared with 37°C. Biofilm formation ability on eggs was ranked as follows: *S.* Anatum > *S.* Worthington > *S*. Agona > *S*. Oranienburg > *S*. Typhimurium > *S*. Mbandaka > *S*. Infantis.

The ability to form a biofilm on eggs may represent an increased food safety risk, making biofilm-forming strains more difficult to eradicate from eggs, and potentially increasing their on-shell survival. However, the relevance and extent by which biofilms are able to form on eggs in the egg production environment, and how this is affected by egg washing procedures, is not known. It is also not known if there are differences in biofilm formation at 15°C compared with 22°C, because 15°C was not included in the experiments above.

3.3.4 Influence of temperature on *Salmonella* survival on eggs

Some earlier data suggested that survival of *Salmonella* on eggs was enhanced by storage at low temperatures, with an inverse relationship also seen between survival and relative humidity (RH) (reviewed by (European Food Safety Authority (EFSA) Panel on Biological Hazards (BIOHAZ), 2014)). Data from publications covering the 2011-2016 period which assessed the effect of storage temperature and time on *Salmonella* survival on eggs were summarised in the 2016 risk profile document (Rivas *et al.*, 2016). These data, together with additional information, are included in Table 6, which summarises the effect of the incubation temperature on the survival of *Salmonella* on eggs.

S. Infantis survival on eggs during storage at 5.5°C and 25.5°C was compared over time for up to ten weeks (Lublin *et al.*, 2015). Following two weeks of incubation, a greater decline was observed at 25.5°C than 5.5°C, as measured by both the percentage of eggs on which *Salmonella* was detected, and the concentration of *Salmonella* (CFU) present. However, *S*. Infantis persisted on egg surfaces, and survival was similar at both temperatures following ten weeks of incubation.

S. Typhimurium (egg-associated outbreak isolate) survived better on eggs at 22°C compared with 4°C after one week of incubation. Conversely, an *S.* Sofia isolate (isolated from a chicken carcass, and not associated with eggs) survived slightly better at 4°C. Note that the RH also differed between the incubation temperatures, which could also have influenced survival (McAuley *et al.*, 2015). Neither serotype was recovered from eggs after storage at either temperature for four weeks. Serotype-dependent differences in survival were observed at earlier time points, with better survival by *S*. Sofia than *S*. Typhimurium at both temperatures.

Salmonella serotype- and incubation temperature-dependent effects on survival on egg surfaces were also reported in Pasquali *et al* (2016). After incubation for four weeks, *S*. Enteritidis survived better at 8 and 20°C compared with 4°C, *S*. Typhimurium survived better at 4°C than 8 and 20°C, while no decline in recoverable CFU of *S*. Tennessee from egg surfaces was observed at any temperature. However, all data included large confidence intervals, and, as seen in Table 6, survival data fluctuated for some serotypes and conditions.

A study examined the survival of a cocktail of *Salmonella* serotypes (*S*. Enteritidis, *S*. Typhimurium, *S*. Heidelberg, *S*. Hartford and *S*. Newport) on eggs stored at different relative humidities (43 and 85%) and temperatures (4, 12 and 25°C), and with or without inoculated faeces (Park *et al.*, 2015). *Salmonella* on eggshells declined more rapidly at 25°C compared with 4°C and 12°C, in all conditions (RH, +/- faeces). The best survival following incubation for 21 days was on eggshells inoculated with faeces at 4°C with 43% RH. Conversely, *Salmonella* survival was the lowest on eggs without faecal inoculation, incubated at 25°C and 85% RH, and in these conditions, the bacteria were not detectable after 21 days.

Another study compared the survival of *S*. Typhimurium on the shells of washed eggs stored at different temperatures (4°C, 14°C, 23°C and 35°C) and relative humidities (95%, 70%, 40% and 20%, respectively) for up to four weeks (Whiley *et al.*, 2016). A rapid decline in recoverable CFU was observed after one week storage at all temperature/humidity combinations, with the least decline observed at 4°C. However, surprising results were observed after four weeks incubation at all temperatures, where recoverable CFU was observed to increase back to inoculum levels or higher. The authors attributed this increase to an increased permeability of the eggshell over time allowing for a transfer of nutrients, presumably to the eggshell surface, supporting growth. This explanation would assume that inhibitory components also present in albumen (discussed in Section 3.5) were not also transferring to the eggshell surface. An increase (~2.5-log from week 3 to week 4) was even observed at 4°C, a temperature which does not support the growth of *Salmonella*[7]. To our knowledge, this increase following an initial decline in CFU has not been reported elsewhere. Following 28 days incubation, there was no significant difference between the four incubation temperatures on *Salmonella* viability on eggshells. However, the high level of variability of recoverable CFU observed at previous time points makes it difficult to draw firm conclusions from these experiments for any effect of incubation temperature on *S.* Typhimurium survival on eggs.

Two further studies compared the survival of *Salmonella* egg farm isolates comprising either four different serotypes (Singapore, Adelaide, Worthington and Livingstone) (Gole *et al.*, 2014b), or five different *S*. Typhimurium isolates (Gole *et al.*, 2014a) on washed and unwashed eggs following three weeks incubation at 20°C and 37°C. Data for these studies were not included in Table 6 because the starting concentration per egg was not reported, and thus log reduction could not be determined. Findings were as follows:

- Survival on unwashed eggs of serotypes *S.* Singapore, *S.* Adelaide and *S*. Worthington (inoculated with 10^5 CFU/ml in Phosphate Buffered Saline (PBS)) was significantly higher at 20°C than 37°C. At the latter temperature, no *Salmonella* were detected after the three weeks. However, for *S*. Livingstone, survival was similar at both temperatures (Gole *et al.*, 2014b).
- Survival on unwashed eggs of some, but not all, *S.* Typhimurium isolates (inoculated with 10^5 CFU/ml in PBS) was significantly higher at 20°C than 37°C (Gole *et al.*, 2014a).
- The degree of survival of *Salmonella* on both washed and unwashed eggs inoculated with 10^5 CFU/ml at 20°C was serotype-dependent. Relative survival included: *S*. Livingstone > *S*. Worthington > *S.* Adelaide > *S.* Singapore (Gole *et al.*, 2014b).
- The degree of survival of *S.* Typhimurium on both washed and unwashed eggs inoculated with 10^5 CFU/ml at 20°C varied by isolate (Gole *et al.*, 2014a).

[7] http://www.foodsafety.govt.nz/elibrary/industry/non-typhoid-salmonellae.pdf; accessed 28-03-2018

Table 6. Influence of storage temperature and serotype on the survival of *Salmonella* on egg shell surfaces (studies published since 2011; adapted from Rivas *et al.* 2016).

SALMONELLA SEROTYPE	INOCULUM CONCENTRATION CFU/ML OR CFU/EGG (LOG)	STORAGE CONDITIONS	CHANGE IN CONCENTRATION COMPARED WITH INOCULUM (LOG_{10} CFU/G OR /CM^2)[a]	REFERENCE
Infantis	5.7 (in peptone water)	72% RH: 5.5°C, 2 weeks 5.5°C, 4 weeks 5.5°C, 6 weeks 5.5°C, 10 weeks 69% RH: 25.5°C, 2 weeks 25.5°C, 4 weeks 25.5°C, 6 weeks 25.5°C, 10 weeks	 ↓ 0.6 ↓ 2.2 ↓ 2.1 ↓ 2.2[b] ↓ 2.0 [b] ↓ 1.9 ↓ 1.4 ↓ 1.6	(Lublin *et al.*, 2015)
Enteritidis (3 strains)	7.0 (in peptone water)	7°C, 4 weeks	↓ 1.4	(Jin *et al.*, 2013)
Enteritidis	5 (in saline)	4°C, 8 days 4°C, 15 days 4°C, 3 weeks 4°C, 4 weeks 8°C, 8 days 8°C, 15 days 8°C, 3 weeks 8°C, 4 weeks 20°C, 8 days 20°C, 15 days 20°C, 3 weeks 20°C, 4 weeks	↓ 1.75 ↓ 2.75 ↓ 3.25 ↓ 4.0 ↓ 1.25 ↓ 1.75 ↓ 2.5 ↓ 2.25 ↓ 2.0 ↓ 2.0 ↓ 2.0 ↓ 3.0	(Pasquali *et al.*, 2016)
Typhimurium	5 (in saline)	4°C, 8 days 4°C, 15 days 4°C, 3 weeks 4°C, 4 weeks 8°C, 8 days 8°C, 15 days 8°C, 3 weeks 8°C, 4 weeks 20°C, 8 days 20°C, 15 days 20°C, 3 weeks 20°C, 4 weeks	NC NC NC NC ↓ 0.75 ↓ 0.75 ↓ 1.75 ↓ 2.0 ↓ 1.25 ↓ 1.0 ↓ 0.75 ↓ 2.0	(Pasquali *et al.*, 2016)
Tennessee	5 (in saline)	4°C, 8 days 4°C, 15 days 4°C, 3 weeks 4°C, 4 weeks 8°C, 8 days 8°C, 15 days 8°C, 3 weeks 8°C, 4 weeks 20°C, 8 days 20°C, 15 days 20°C, 3 weeks 20°C, 4 weeks	↓ 1.5 NC NC NC NC ↓ 0.75 NC ↓ 0.75 ↑ 0.75 NC ↑ 1.25 NC	(Pasquali *et al.*, 2016)
Typhimurium	6 (in buffered peptone water)	88-100% RH: 4°C, 1 week 4°C, 2 weeks 4°C, 4 weeks 38-55% RH: 22°C, 1 weeks 22°C, 2 weeks 22°C, 4 weeks	 ↓ ND ↓ ND ↓ ND ↓ 2.63 ↓ ND ↓ ND	(McAuley *et al.*, 2015)
Sofia	6 (in buffered peptone water)	88-100% RH: 4°C, 1 week 4°C, 2 weeks 4°C, 4 weeks 38-55% RH: 22°C, 1 weeks 22°C, 2 weeks 22°C, 4 weeks	 ↓ 2.37 ↓ 3.41 ↓ ND ↓ 2.99 ↓ 3.45 ↓ ND	(McAuley *et al.*, 2015)
Cocktail: Enteritidis, Typhimurium, Heidelberg, Hartford, Newport	5.6 (in saline)	85% RH: 4°C, 3 weeks 12°C, 3 weeks 25°C, 3 weeks	 ↓ >5.6 ↓ >5.6 ↓ ND	(Park *et al.*, 2015)
Cocktail: Enteritidis, Typhimurium,	6.0 (in PBS + sterile faeces)	85% RH: 4°C, 3 weeks	 ↓ 1.3	(Park *et al.*, 2015)

Heidelberg, Hartford, Newport		12°C, 3 weeks 25°C, 3 weeks	↓ 1.4 ↓ 3.5	
Cocktail: Enteritidis, Typhimurium, Heidelberg, Hartford, Newport	5.6 (in PBS)	43% RH: 4°C, 3 weeks 12°C, 3 weeks 25°C, 3 weeks	 ↓ 1.6 ↓ 2.1 ↓ 4.5	(Park *et al.*, 2015)
Cocktail: Enteritidis, Typhimurium, Heidelberg, Hartford, Newport	6.0 (in PBS + sterile faeces)	43% RH: 4°C, 3 weeks 12°C, 3 weeks 25°C, 3 weeks	 ↓ 0.5 ↓ 1.0 ↓ 1.5	(Park *et al.*, 2015)
Typhimurium	4 (in PBS)	95% RH: 4°C, 1 week 4°C, 2 weeks 4°C, 3 weeks 4°C, 4 weeks 70% RH: 14°C, 1 week 14°C, 2 weeks 14°C, 3 weeks 14°C, 4 weeks 40% RH: 23°C, 1 week 23°C, 2 weeks 23°C, 3 weeks 23°C, 4 weeks 20% RH: 35°C, 1 week 35°C, 2 weeks 35°C, 3 weeks 35°C, 4 weeks	 ↓ 1.0 ↓ 1.25 ↓ 2.25 NC ↓ 3.0 NC NC ↑0.75 ↓ 3.25 ↓ 2.0 ↓ 3.5 NC ↓ 2.5 ↓ >4.5 ↓ 1.75 ↑0.75	(Whiley *et al.*, 2016)

[a] ↓ = decreased by >0.5 log; ↑ = increased by >0.5 log; NC = no change (change ≤0.5 log); ND, not detected. Data are reported from text, if provided, or estimated from graphs.
[b] The prevalence at 26°C also decreased from 100% at day 0 to 30% eggs positive by 2 weeks. At 6°C the prevalence was 90% at 2 weeks.

3.3.5 **Summary/data gaps**

The survival of *Salmonella* on egg shells, as well as the formation of biofilm on eggs, are temperature dependent. There was a wide variability in data between experiments and thus, it is difficult to make any predictions for survival on eggs at storage conditions of interest. In general, a decline in *Salmonella* viability on eggshells over time is expected. A number of experiments showed a greater decline in recoverable numbers of non-Enteritidis serotypes at room temperature (~20-25°C) compared with refrigeration (~4°C) storage temperatures. While a reduction in recoverable numbers on the egg shell surface is likely due to a reduction in viability, migration of cells into the egg shell has also been suggested as a mechanism for reduced numbers (Pasquali *et al.*, 2016).

Salmonella may persist over several weeks on eggshells, especially in lower RH.

There were few direct comparisons which assessed survival at both storage temperatures relevant to New Zealand storage temperatures (<15°C (~10-15°C) or room temperature (~20-25°C). In addition, no reports assess the survival at 35 days (5 weeks), which is the New Zealand storage time for eggs stored at <15°C.

Serotype- and strain-type influence the survival and biofilm formation of *Salmonella* on eggshells at different temperatures. However, no data were found that assessed the survival of some New Zealand-relevant serotypes (*S*. Thompson, *S*. Mbandaka and *S*. Anatum) or phage types (DT 56 variant[8]) on eggs. Importantly, *S*. Thompson, was the second most widely isolated serotype from New Zealand egg layer environments and has been isolated from New Zealand eggs at retail (see section 3.2).

[8] Although phage typing was not performed in the 2016 NZ *Salmonella* egg layer survey, *S*. Typhimurium isolates were highly related to other DT 56 variants by SNP analysis

3.4 PENETRATION INTO EGGS BY *SALMONELLA* AS A FACTOR OF TEMPERATURE

3.4.1 Background

An overview of egg penetration by *Salmonella* is provided in the 2015 MPI Risk Assessment report and 2016 risk profile reports (Ministry for Primary Industries, 2015;Rivas *et al.*, 2016). In brief, there are two main pathways by which egg contents become contaminated by *Salmonella*; vertical (trans-ovarian) transmission, when *Salmonella* colonising hen ovaries contaminates eggs prior to shell formation; or horizontal (trans-shell) transmission, by faecal contamination of the egg as it is laid or from the external environment post-laying.

S. Enteritidis is the predominant serotype capable of ovarian colonization of hens and vertical transmission to eggs (European Food Safety Authority (EFSA) Panel on Biological Hazards (BIOHAZ), 2010, 2014;Martelli and Davies, 2012). Levels of *S*. Enteritidis deposition in eggs via this route typically involve very small numbers of organisms (<10-20 CFU per egg) and likely preferentially occurs within the albumen or yolk membrane[9] (European Food Safety Authority (EFSA) Panel on Biological Hazards (BIOHAZ), 2014). Occasional colonisation of the hen's reproductive tract by strains of non-Enteritidis serotypes relevant to the New Zealand egg industry, e.g. *S*. Infantis and *S*. Typhimurium, has been demonstrated (McWhorter and Chousalkar, 2016). Earlier reports of colonisation and/or vertical transmission into eggs by non-Enteritidis serotypes have been reviewed elsewhere (Martelli and Davies, 2012; Ministry for Primary Industries, 2015; Samiullah, 2013). However, egg contamination by this route is seldom, and horizontal/trans-shell transmission is considered the most likely source of contamination of egg contents by these serotypes (Pande *et al.*, 2016a).

Penetration into eggs by *Salmonella* has been shown to be influenced by egg-dependent factors (cuticle deposition and maintenance, shell quality, shell porosity, shell membranes, micro-cracks), environmental factors (temperature differential between the egg and the external environment, moisture) and bacterial factors (strain/serotype and bacterial load). The penetration into eggs by *Salmonella* is most likely to occur immediately after laying when the egg cuticle is immature and offers less protection against bacterial penetration. In addition, the temperature differential of laid eggs and the cooler environment can result in any *Salmonella* present close to egg shell pores being sucked into the egg by the negative pressure, particularly if eggs are damp. Various reports have detected *Salmonella* internalisation within 3-30 min following exposure (Al-Bahry *et al.*, 2012;Berrang *et al.*, 1999;Cason *et al.*, 1993;Javed *et al.*, 1994).

3.4.2 Effect of temperature on *Salmonella* penetration into eggs

Recent experiments, which focus on dependence of *Salmonella* penetration into eggs on temperature, and with a focus on non-Enteritidis serotypes, are summarized in Table 7. Detection of egg internalisation involved either the use of xylose lysine deoxycholate (XLD) agar-filled eggs ("agar eggs"; internalisation by *Salmonella* results in blackening of the interior eggshell), detection from whole egg contents using Polymerase Chain Reaction (PCR) or culture (the latter also requires survival of *Salmonella* in the egg albumen).

The ability of five different strains of *S.* Typhimurium or different *Salmonella* serotypes (*S*. Singapore, *S*. Adelaide, *S*. Worthington, *S*. Livingstone) to penetrate eggs at 20°C was compared (Gole *et al.*, 2014a;Gole *et al.*, 2014b). All strains were able to penetrate eggs according to experiments with agar eggs. However, only *S*. Typhimurium strains and *S*. Adelaide were detected in egg internal contents using a direct plating method of egg contents. Internalisation of the five *S*. Typhimurium isolates were compared at 20 and 37°C; internalisation was higher for one isolate at 20°C although the effect of temperature on internalisation by the other isolates was not significant (Gole *et al.*, 2014a).

[9] https://www.food.gov.uk/sites/default/files/acmsf-egg-reportv1.pdf; accessed 12-12-2017

Table 7. Influence of storage temperature and serotype on the internalisation of *Salmonella* in eggs

Salmonella serotype	Experimental setup	Storage/experimental conditions	Results	Reference
Typhimurium (5 isolates assessed separately)	Washed eggs, immersed in 5 log CFU/ml PBS inoculum	20°C, 3 weeks	Sets of internalised egg pools (5 pools, 2 eggs/pool): 0/5 to 4/5 (direct agar culture) 3/5 to 5/5 (PCR detection)	(Gole *et al.*, 2014a)
	Unwashed eggs, immersed in 5 log CFU/ml PBS inoculum	20°C, 3 weeks	Sets of internalised egg pools (5 pools, 2 eggs/pool): 0/5 to 4/5 (direct agar culture) 1/5 to 4/5 (PCR detection)	
Singapore	Unwashed eggs immersed in 5 log CFU/ml PBS inoculum	20°C, 3 weeks	Detection in 0/10 egg contents (direct agar culture)	(Gole *et al.*, 2014b)
	Washed and unwashed XLD-agar filled eggs immersed in 5 log CFU/ml PBS inoculum	20°C, 3 weeks	Internalised 32/40 eggs	
Adelaide	Unwashed eggs immersed in 5 log CFU/ml PBS inoculum	20°C, 3 weeks	Internalised 1/10 eggs (direct agar culture)	
	Washed and unwashed XLD-agar filled eggs immersed in 5 log CFU/ml PBS inoculum	20°C, 3 weeks	Internalised 36/40 eggs	
Worthington	Unwashed eggs immersed in 5 log CFU/ml PBS inoculum	20°C, 3 weeks	Internalised 0/10 eggs (direct agar culture)	
	Washed and unwashed XLD-agar filled eggs immersed in 5 log CFU/ml PBS inoculum	20°C, 3 weeks	Internalised 33/40 eggs	
Livingstone	Unwashed eggs immersed in 5 log CFU/ml PBS inoculum	20°C, 3 weeks	Internalised 0/10 eggs (direct agar culture)	
	Washed and unwashed XLD-agar filled eggs immersed in 5 log CFU/ml PBS inoculum	20°C, 3 weeks	Internalised 25/40 eggs	
Infantis	Washed and unwashed XLD-agar filled eggs immersed in 5 log CFU/ml PBS inoculum	4°C, 3 days 20°C, 3 days 37°C, 3 days	Eggs internalised: 2/10 7/10 7/10	(Chousalkar *et al.*, 2010)
Infantis	5.7 log CFU per egg inoculated on egg surface	5.5°C, 72.1% RH 2 weeks 4 weeks 6 weeks 10 weeks 25.5°C, 68.7% RH: 2 weeks 4 weeks 6 weeks 10 weeks	Eggs internalised (detection following egg content enrichment): 7/10[a] 1/10 0/10 6/10 0/10[a] 1/10 2/10 4/10	(Lublin *et al.*, 2015)
Infantis	Unwashed eggs immersed in 5 log CFU/ml	20°C, 3 weeks 37°C, 3 weeks	Eggs internalised (PCR detection): 5/10 6/10	(Samiullah *et al.*, 2013)
Typhimurium	Unwashed eggs immersed in ~7 log CFU/ml PBS inoculum	4°C, 95% RH: 1 week 2 weeks 3 weeks 4 weeks 14°C, 70% RH: 1 week 2 weeks 3 weeks 4 weeks 23°C, 40% RH: 1 week 2 weeks 3 weeks 4 weeks 35°C, 20% RH: 1 week 2 weeks 3 weeks 4 weeks	Eggs internalised (detection by plating) 0/12 2/12 1/12 3/12 2/12 2/12 0/12 6/12 3/12 10/12 7/12 12/12 9/12 12/12 11/12 12/12	(Whiley *et al.*, 2016)

[a] significant difference between 5.5 and 25.5°C (P<0.002)

Using an agar egg method, the serotype *S*. Infantis penetrated more eggs at 20°C and 37°C compared with 4°C (Chousalkar *et al.*, 2010). Internalisation by *S*. Infantis at 4°C was only observed using the highest inoculum (10^7 CFU), while internalisation at 20°C and 37°C was

also observed when lower inoculum levels (10^3 CFU) were tested. However, it is unclear whether results at 4°C reflect reduced internalisation or reduced detection given that the minimum temperature for growth of *Salmonella* is 7°C[10]. In another publication, a similar frequency of internalisation of unwashed eggs by *S*. Infantis at 20°C and 37°C were reported (Samiullah *et al.*, 2013).

Significantly higher internalisation of eggs by *S*. Typhimurium at higher temperatures (23°C and 35°C compared with 4°C and 14°C) following incubation for 4 weeks has also been reported (Whiley *et al.*, 2016). Overall rates of internalisation were high (100% of eggs stored at 23°C and 35°C for 4 weeks) despite only a small proportion of the contents of each egg (300 µl; ~1% of total egg contents) being sampled by plating. As discussed by the authors, egg penetration may have been promoted by the methodology used. In particular, the study used washed eggs (which have a reported higher internalisation (Gole *et al.*, 2014a; Gole *et al.*, 2014b)), a high inoculum concentration (7 log CFU/ml), and inoculation involved immersion of eggs in the inoculum. The egg immersion inoculation method was also used by other studies described here (Table 7). A possible mechanism by which this methodology might promote internalisation is by creating a temperature differential between the egg and the inoculum.

In contrast to reports showing a higher internalisation at higher temperatures, a further study showed significantly higher internalisation by *S*. Infantis at refrigeration temperatures (5.5°C) compared with room temperature (25.5°C) after two weeks incubation following surface inoculation of eggs and detection by enrichment of egg contents (Lublin *et al.*, 2015). Differences between temperature effects among reports may be due to methodological or other experimental differences such as RH, incubation time and detection methods.

3.4.3 Summary/data gaps

There are temperature- and serotype-dependent effects for *Salmonella* internalisation of eggs. However, there was not sufficient data to directly assess the effect of New Zealand-relevant storage temperatures and storage times on internalisation. While no data were available for internalisation by some serotypes present in New Zealand, all non-Enteritidis serotypes assessed in Table 7 were capable of internalisation of eggs. It is likely that most, if not all, motile serotypes can migrate across the egg shell and membrane.

3.5 SURVIVAL OF SALMONELLA IN ALBUMEN AS A FACTOR OF TEMPERATURE

3.5.1 Albumen defence mechanisms

Once *Salmonella* have penetrated the egg cuticle, shell and shell membrane, the albumen (egg white) forms the next layer of defence.

The egg white is composed of four distinct layers:

- The chalaze layer is comprised of spiral filaments ranging from the egg yolk to the two ends of the egg, and maintains central suspension of the egg yolk within the egg;
- The external liquid egg white in direct contact with the shell membrane;
- The thick egg white is fixed to the ends of the egg and is more gelatinous than the liquid egg white;
- The internal liquid egg white, located between thick egg white and egg yolk.

Components of the albumen include:

[10] https://www.foodsafety.govt.nz/elibrary/industry/non-typhoid-salmonellae.pdf; accessed 28-09-2018

- Proteins (10.6%): 50% of total protein comprises ovalbumin, as well as various proteins and peptides with predicted or confirmed antimicrobial activity (Table 8, reviewed by (Baron *et al.*, 2016)). Of these, the antimicrobial activity of lysozyme and ovotransferrin are well documented and are discussed separately below.
- Carbohydrates (0.9%): 50% of which comprise glucose.
- Minerals (0.5%): Particularly, chloride, sodium, sulphur and potassium.
- Vitamins: Group B vitamins but not fat-soluble vitamins (A, D, E and K).

Table 8. Egg white proteins or peptides with predicted or confirmed antimicrobial activity against *Salmonella*. Adapted from (Baron *et al.*, 2016).

TYPE OF ACTIVITY	PROTEIN / PEPTIDE NAME	CONCENTRATION (G/L)	ANTIMICROBIAL ACTIVITY CONFIRMED OR PREDICTED
Hydrolase	Lysozyme	3.5	Confirmed
Iron-chelating protein	Ovotransferrin	13	Confirmed
Vitamin-chelating protein	Avidin	0.05	Confirmed
	Riboflavin-binding protein	0.8	Predicted
	Thiamine-binding protein	NR	Predicted
Protease inhibitors	Ovostatin	0.5	Confirmed
	Cystatin	0.05	Confirmed
	Ovoinhibitor	1.5	Predicted
	Ovomucoid	13	Predicted
	Ovalbumin related protein X	0.5	Predicted
	Ovalbumin related protein Y	5-7	Predicted
	Heparin cofactor	NR	Predicted
	Similar to pancreatic secretory trypsin inhibitor	NR	Predicted
	Similar to Kunitz-like protease inhibitor	NR	Predicted
Bactericidal permeability-increasing proteins (BPI)	Tenp	NR	Predicted
	BPIL2	NR	Predicted
	Similar-to-BPI	NR	Predicted
Defensins	AvBD11	NR	Confirmed
	Gallin	NR	Confirmed
Lipocalin	Gallin EX-FABP	NR	Confirmed

NR not recorded

Lysozyme: Lysozyme is present at high concentrations (3.5 g/L). Lysozyme hydrolyses the glycosidic (1-4) β-linkage between the N-acetylglucosamine and N-acetylmuramic acid residues in Gram-positive bacterial peptidoglycan. Because the peptidoglycan layer is important for structural integrity of the cell, its degradation can result in cell swelling and lysis in conditions of low osmotic strength. Gram-negative bacteria such as *Salmonella* are generally resistant to lysis by lysozyme due to the presence of an additional, protective outer membrane, which prevents access of lysozyme to the peptidoglycan layer.

Some Enterobacteriaceae have also been reported to express lysozyme inhibitors. For example, *S*. Enteritidis expresses PliC (periplasmic lysozyme inhibitor of c-type lysozyme) (Callewaert *et al.*, 2008). Mutation of *pliC* eliminates the production of lysozyme inhibition activity. The *pliC* gene has also been identified in *S*. Typhimurium, and the PliC protein crystal structure determined (Leysen *et al.*, 2011).

The functionality of lysozyme in the albumen over time, at different incubation temperatures, and under alkaline conditions, is unclear. Concentrations of lysozyme present in the albumen were shown to remain constant over time in experiments carried out using fertilized hens eggs incubated at 37°C for five days. However, the optimum pH range for hens egg lysozyme lytic activity is 5.0-7.0 (Maidment *et al.*, 2009), being inactivated at high pH (Ibrahim *et al.*, 1996); while the pH of the egg albumen may reach at least >9 after several days (Rehault-Godbert *et al.*, 2010;Scott and Silversides, 2000). Consistent with this, lysozyme activity was found to decrease by approximately half (30,397 to 15,035 U/mg) following two days incubation of eggs at 37°C, and pH increased to over 9.5 . Over time, pH reduced again to near-neutral levels, and lysozyme activity increased.

In addition, because of the presence of the protective outer membrane and *pliC*, lysozyme may not be effective against *Salmonella* in albumen. Consistent with this, no effect was observed following incubation of *S*. Enteritidis with increasing concentrations of lysozyme (107-1007 units/mg protein) in pasteurised egg white (Jakočiūnė *et al.*, 2014). Furthermore, lysozyme did not inhibit *S*. Enteritidis or *S*. Typhimurium in growth media at the concentrations used (Facon and Skura, 1996;Hughey and Johnson, 1987). No information was found on the effect of lysozyme on other serotypes of *Salmonella*, although fine differences in the lipopolysaccharide structure could result in differing sensitivities between serotypes. Indeed, this has been shown to account for differences in binding of lysozyme to *S*. Typhimurium and *S*. Enteritidis, the latter of which also survives better in egg albumen (Clavijo *et al.*, 2006;Coward *et al.*, 2013;Coward *et al.*, 2012).

There may also be a temperature-dependent effect on the survival of different serotypes in hen albumen. A recent review summarised reports where lysozyme adhered less effectively to the lipopolysaccharide of *S*. Enteritidis than that of *S*. Typhimurium at hen body temperature (41.5°C) although there was little difference in adherence at room temperature[11]. No information was found on the survival of *Salmonella* in lysozyme at 15°C versus 22°C.

Ovotransferrin: Ovotransferrin is present at high concentrations in egg albumen at 13 g/L (1.7 mM). Ovotransferrin is a metal-binding transport protein with a high affinity for iron, binding two Fe^{3+} ions per molecule with high affinity, and can also chelate multivalent ions such as zinc, copper and manganese (reviewed by Baron *et al*. (Baron *et al.*, 2016)). Due to the low concentration of iron in egg albumen (25 µM) and high concentration of ovotransferrin, all iron present in the albumen is expected to be chelated to ovotransferrin. The antibacterial (bacteriostatic) activity of ovotransferrin is due to this iron-deficient environment inhibiting bacterial growth as iron is an important cofactor for bacterial proteins. Antimicrobial activity may also arise from direct binding of ovotransferrin and the bacterial membrane. In addition, ovotransferrin may have a bacteriocidal effect via binding of divalent cations which are important for bacterial membrane integrity. To date, a bacteriocidal effect has been noted for Gram-positive but not Gram-negative bacteria.

The concentration of ovotransferrin and associated iron-binding activity in egg albumen is not static. A significantly increased concentration of both ovotransferrin and iron-binding capacity over five days of incubation of fertilized eggs at 38°C has been demonstrated (note that changes observed in fertilised and unfertisilised eggs likely differ) (Fang *et al.*, 2012). Conversely, iron is released from the yolk over time as the vitelline membrane breaks down (Gantois *et al.*, 2009), which could counter any effects from increased ovotransferrin over time.

Minimal data were found on the effect of storage temperature (15°C versus 22°C) on ovotransferrin activity of albumen. However, there is evidence that ovotransferrin antimicrobial activity is higher at incubation temperatures 34-36°C than at ambient temperatures (Tranter and Board, 1984).

No data were found for any differential sensitivity of *Salmonella* serotypes to ovotransferrin. However, as for lysozyme, differences in lipopolysaccharide between *Salmonella* serotypes has been speculated to affect binding to ovotransferrin (Clavijo *et al.*, 2006). Any differences in the ability of different serotypes or isolates to chelate and transport iron would also likely affect sensitivity to ovotransferrin.

[11] https://www.food.gov.uk/sites/default/files/acmsf-egg-reportv1.pdf; accessed 6-12-2017

3.5.2 Temperature-dependent effects of *Salmonella* survival in albumen

As discussed above, the temperature at which eggs are stored affects various properties of albumen, which in turn, affects *Salmonella* survival within albumen. Most information on *Salmonella* survival in albumen relates to *S*. Enteritidis. Some reports have suggested that *S*. Enteritidis is better-adapted to survive in egg albumen, and various genes or genome islands which are absent from most other serotypes have been found to benefit *S*. Enteritidis survival in albumen (Shah *et al.*, 2012).

The potential of albumen from eggs that had been stored at 4°C, 20°C and 37°C for up to 30 days prior to inoculation, to inhibit the growth of *S*. Enteritidis was examined (Rehault-Godbert *et al.*, 2010). The authors found that anti-*Salmonella* activity of freshly laid eggs was low. The anti-*Salmonella* activity of egg whites increased after a few days of storage at 20°C and 37°C, and this rate of increase was higher at 37°C. After the 30-day incubation period, the antimicrobial activity was the highest at 20°C storage compared with the other temperatures. Therefore, the extent by which *Salmonella* is inhibited by albumen will depend on the time following incubation at which internal contamination of eggs occur.

Using incubation conditions pertinent to New Zealand egg storage conditions, two isolates of *S* Typhimurium and one *S*. Sofia isolate were tested for the ability to survive and grow in egg components including albumen separated from eggs, at 15°C and 22°C, as well as 37°C (McAuley *et al.*, 2015). First, no significant differences were observed between serotypes/strains for albumen survival/growth in this study. The isolates were found on average to not only maintain viability, but also grow at some temperatures, following inoculation into albumen and incubation up to 35 days (Table 9, inoculum of 5×10^4 CFU/ml). Minimal growth in albumen occurred at 15°C (and in some experimental replicates, a decline in viability was observed). Of note, *Salmonella* growth in the albumen significantly increased with increasing temperature (15°C < 22°C < 37°C) (P<0.001).

Salmonella levels inoculated into albumen by McAuley *et al.* (2015) (and others) are orders of magnitude higher than what would usually be encountered following natural internalisation of eggs and it remains possible that the high CFU used in this experiment could dilute any antimicrobial effects due to a stoichiometric effect with albumen components. Indeed, at the incubation temperature of 15°C when a lower inoculum was used (5×10^2 CFU/ml compared with 5×10^4 CFU/ml; note that both concentrations are likely higher than would be encountered in natural contamination of eggs), viability of all three isolates decreased to near or below the limit of detection after 35 days. The lower inoculum was not tested for incubation in albumen at 22°C and 37°C. None-the-less, results as presented support that survival in albumen at 15°C storage temperature is lower than at 22°C.

Using experimental conditions where low concentrations of *Salmonella* inoculum were exposed to albumen (because exposure required internalisation), results from another study were consistent with albumen having antimicrobial activity against *S*. Typhimurium (Al-Bahry *et al.*, 2012). Eggs were inoculated by dipping for three minutes into 10^6 CFU/ml inoculum and egg components were sampled at 30, 60, 90 and 120 min intervals post-inoculation by serial dilution plating. Experiments were performed at 37°C (which would be relevant to egg temperatures following laying, but not during typical egg storage conditions present in New Zealand; other temperatures were not tested). *S*. Typhimurium was detected in the albumen at all time points. Concentrations in albumen peaked at 90 min (~10-15 CFU/ml) and declined significantly (P<0.05) at 120 min (~5-10 CFU/ml). However, no data were available for viable counts over longer periods, such as 21 or 35 days, which are relevant to the MPI-recommended egg storage times.

Table 9. Influence of storage temperature on the survival and growth of non-Enteritidis *Salmonella* serotypes in egg contents

Salmonella serotype	Experimental setup	Storage/experimental conditions	Results[a]	Reference
Typhimurium (egg isolate)	Egg yolk: 5 x 10^2 CFU/ml (in BPW) inoculated into separated, mixed egg yolk, incubated up to 44 days	15°C 22°C 37°C	Max. growth rates (CFU/ml/h) 0.115 0.323 0.865	(McAuley *et al.*, 2015)
	Egg albumen: 5 x 10^4 CFU/ml (in BPW) inoculated into separated, mixed egg albumen, incubated up to 35 days	15°C 22°C 37°C	Max. growth rates (CFU/ml/h) -0.007 0.023 0.076	
	Whole egg: 5 x 10^2 CFU/ml (in BPW) inoculated into separated, mixed egg contents, incubated up to 44 days	15°C 22°C 37°C	Max. growth rates (CFU/ml/h) 0.099 0.238 0.614	
Sofia	Egg yolk: 5 x 10^2 CFU/ml (in BPW) inoculated into separated, mixed egg yolk, incubated up to 44 days	15°C 22°C 37°C	Max. growth rates (CFU/ml/h) 0.116 0.324 0.821	(McAuley *et al.*, 2015)
	Egg albumen: 5 x 10^4 CFU/ml (in BPW) inoculated into separated, mixed egg albumen, incubated up to 35 days	15°C 22°C 37°C	Max. growth rates (CFU/ml/h) 0.005 0.022 0.095	
	Whole egg: 5 x 10^2 CFU/ml (in BPW) inoculated into separated, mixed egg contents, incubated up to 44 days	15°C 22°C 37°C	Max. growth rates (CFU/ml/h) 0.093 0.211 0.613	
Infantis	Direct injection of 3.7 log CFU (in peptone water) into egg yolk in whole eggs, incubated up to 10 weeks	5.5°C: 2 weeks 4 weeks 6 weeks 10 weeks 25.5°C: 2 weeks 4 weeks 6 weeks 10 weeks	Change in concentration (log_{10} CFU/g)[b] NC ↓<1 ↓<1 ↓<1 ↑ ~4 ↑ ~5 ↑ ~5 ↑ ~5	(Lublin *et al.*, 2015)

[a] Result output differed by publication

[b] ↓ = decreased by >0.5 log; ↑ = increased by >0.5 log; NC = no change (change ≤0.5 log). Data estimated from graphs

3.5.3 Summary/data gaps

Storage temperature affects both the antimicrobial properties of the albumen and the extent of growth and/or inhibition on *Salmonella*. Of the albumen components, ovotransferrin is likely to be more important than lysozyme for *Salmonella* growth inhibition.

Information on *Salmonella* growth/survival in albumen is often derived from studies using inoculation of isolated and homogenised albumen. Although this is a convenient system, it is an imperfect model for testing growth/survival in intact albumen within eggs. For example, albumen pH influences *Salmonella* survival, yet the changes in albumen pH over time differ between the experimental isolated albumen and albumen within intact eggs (Rehault-Godbert *et al.*, 2010).

To achieve statistically significant data on *Salmonella* growth/survival in albumen, most data is derived from albumen inoculated with orders of magnitude higher levels of *Salmonella* than would likely be encountered during a natural contamination event. This may result in an over-estimate of growth/survival due to titration of antibacterial components by high numbers of bacteria. Conversely, because higher numbers of cells would utilise any limiting nutrients and growth factors available (for example, the growth-limiting concentrations of iron available), fewer growth generations might be observed when using high compared with low inoculum concentrations.

There are likely serotype- and strain-dependent differences in albumen survival. Despite the paucity of information available for the survival and/or growth of non-Enteritidis serotypes in egg albumen, the detection of New Zealand-relevant serotypes from egg contents of table

eggs in international surveys (Martelli and Davies, 2012), and the ability to detect viable inoculum of some serotypes from egg contents in egg invasion studies (Table 7), indicate that at least some non-Enteritidis serotypes may survive in egg albumen.

3.6 SURVIVAL AND GROWTH OF *SALMONELLA* IN EGG YOLK AS A FACTOR OF TEMPERATURE

In contrast to the growth inhibitory properties of the albumen, the nutrient-rich egg yolk supports bacterial growth. The egg yolk (vitelline) membrane acts as a barrier which prevents *Salmonella* penetration into the yolk, and diffusion of yolk nutrients into the albumen (Ministry for Primary Industries, 2015). However, yolk membrane permeability increases over time. The rate of increase in permeability is also temperature dependent. Growth of *Salmonella* within eggs is assumed to be suppressed while the egg yolk membrane remains intact, but temperature-dependent growth is predicted following either migration across the vitelline membrane, encountering the yolk as the vitelline membrane degrades over time, or when eggs are broken and their contents released.

3.6.1 Temperature-dependent effects of *Salmonella* growth in egg yolk

Literature was assessed for the temperature-dependent effects on the growth of non-Enteritidis *Salmonella* serotypes in egg yolk (both in isolated yolk and via direct injection in whole egg yolks) (Table 9).

The growth of one *S*. Sofia and two *S*. Typhimurium isolates (strains that were also assessed in studies described in sections 3.3.4 and 3.5.2) were compared following inoculation of either mixed yolk, or the entire egg contents (mixed yolk and albumen), and incubation at 15°C, 22°C and 37°C (McAuley *et al.*, 2015). Significant growth was observed for all samples, with all reaching stationary phase (10^8-10^9 CFU/ml) from a starting inoculum of 5 x 10^2 CFU/ml during the course of the experiment; occurring within three days at 15°C, 26 h at 22°C, and 9-10 h at 37°C in egg yolk. Maximal growth rates occurred at 37°C followed by 22°C. Growth at 15°C was slower. Significantly faster growth was observed in inoculated yolk alone compared with mixed yolk and albumen, consistent with albumen having inhibitory effects. No significant differences were observed between the three isolates.

Significant growth of *S*. Infantis was also demonstrated following direct injection (3.7 log CFU) into yolks of whole eggs and incubation at room temperature (25.5°C) (Lublin *et al.*, 2015). A significant 4-log increase in CFU was reported after a 2-week incubation at this temperature, while a decline in viability was seen for eggs incubated at 4°C. Other temperatures were not tested.

Previous studies have reported that rapid growth in the egg yolk is possible at temperatures above 10°C (Thomas *et al.*, 2006).

3.6.2 Yolk mean time (YMT) model: relevance for New Zealand serotypes at New Zealand-relevant storage temperatures

YMT is an arbitrary definition based on the storage time at a given temperature whereby the vitelline membrane degrades, allowing *Salmonella* present in the albumen to reach the yolk and grow to levels of concern in 20% of eggs (proposed by (Whiting *et al.*, 2000)). The United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS), Health Canada, the Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO), the Australian Egg Corporation Limited (AECL) and New Zealand MPI have all used the assumption that no significant growth is possible until the yolk membrane has deteriorated when modelling *Salmonella* growth within eggs as a function of temperature (European Food Safety Authority (EFSA) Panel on Biological Hazards (BIOHAZ), 2014; Ministry for Primary Industries, 2015; Thomas *et al.*, 2006).

Original YMT modelling by Whiting *et al*. used growth based on high concentrations of *S*. Enteritidis inoculated directly into the egg albumen to calculate YMT, as well as *S*. Enteritidis growth rates in the yolk (Whiting *et al.*, 2000). Conditions to support growth at a given temperature in an individual egg may vary substantially; thus, large confidence intervals are associated with the YMT predictions. Citing earlier literature, which measured the time until *S*. Typhimurium growth when injected into eggs, Thomas *et al.* suggested that YMT does not necessarily reflect the behaviour of non-Enteritidis serotypes (Cogan *et al.*, 2004;Thomas *et al.*, 2006).

Accordingly, Thomas *et al*. used calculations based on predicted *S*. Typhimurium growth rates to assess egg storage times relevant to Australian egg production conditions and serotypes. Growth rates (days until 1 log growth of *Salmonella* in the egg content once growth is supported) were based on *S.* Typhimurium growth on chicken meat. The limited data from *S*. Typhimurium growth rates in eggs were consistent with the model. This model assumed that the YMT proposed by Whiting *et al.* was the same for *S*. Enteritidis and non-Enteritidis serotypes.

Based on the model predictions, Thomas *et al.* reported that storage at 16°C in the retail environment may allow growth of *Salmonella* in contaminated eggs in as little as 18 days after the end of on-farm storage under median industry practices (Thomas *et al.*, 2006). This estimate reduces to 10 days if eggs are stored at 22°C.

The 2015 MPI Risk Assessment report modelled storage times and temperatures based on YMT calculations using an equation from Whiting *et al.* It was assumed that contamination involved a single cell, that contamination occurred post-laying, and used growth rate predictions based on Thomas *et al*. (Ministry for Primary Industries, 2015;Thomas *et al.*, 2006;Whiting *et al.*, 2000). Based on the calculations, the total number of days until 20% of contaminated eggs permitted growth of *Salmonella* was predicted to be 45.9, 28.1, 17.2, 10.5 and 6.5 for storage temperatures of 10, 15, 20, 25 and 30°C, respectively. After this time, logarithmic growth would result in any *Salmonella* present in the egg rapidly reaching levels likely to deliver an infectious dose in a temperature-dependent manner. Based on these calculations, the report recommended that retaining the current recommendations for New Zealand storage times and temperatures would be prudent.

A number of assumptions inherent with the YMT model are listed below (in italics). The relevance of these for assessing New Zealand egg storage times with New Zealand-relevant serotypes are discussed in light of new information available since the risk assessment.

Assumption 1: The YMT model estimates time until growth occurs in 20% of eggs. Both the Whiting and Thomas models use the same calculation for breakdown of the vitelline membrane and access to yolk content, based on data from *S*. Enteritidis. As discussed above, YMT was calculated following inoculation of a high concentration of *S.* Enteritidis directly into the egg albumen, so the results may not be applicable for other serotypes or for lower concentrations of *Salmonella*. YMT is not an absolute number and as discussed, there are wide confidence intervals for this estimation. The time for any individual egg to support growth at a given temperature could vary substantially based on, for example, the condition of the egg.

Assumption 2: Contamination of egg contents by Salmonella occurs in early stages following laying. Unless cracks are present, internal contamination of the egg is most likely to occur during oviposition or shortly after laying, when cross-contamination from faeces is most likely to occur and the cuticle is not yet mature. In addition, the source for internal contamination (*Salmonella* present on egg shell surface), likely declines in viability over time (assuming that faecal matter is not present). None-the-less, the potential for internal contamination remains as long as the external contamination source remains. In one study, the percentage of externally contaminated eggs in which viable *Salmonella* was detected in egg contents

increased over time during storage at 25.5°C (Lublin *et al.*, 2015). If egg internalisation into the albumen occuring later during egg storage and nearer YMT, a greater opportunity may exist for the invading bacterium to access the growth-supporting yolk, because protection offered by intrinsic albumen properties may decrease with time.

Assumption 3: Non-Enteritidis serotypes also internalise eggs and survive in albumen until YMT at a similar rate to S. Enteritidis. As discussed (Section 3.4), non-Enteritidis serotypes are able to internalise eggs, but internalisation is influenced by serotype and is a rarer occurrence than *S.* Enteritidis internalisation. Survival rate in the albumen is also serotype- and strain-specific, and lower for many serotypes than for *S.* Enteritidis (Section 3.4).

Assumption 4: Salmonella cells will not reach the yolk or proliferate while the egg yolk membrane remains intact. Contrary to Assumption 4, a single report demonstrated rapid penetration of *Salmonella* from inoculated shells to the yolk (30-120 min), despite insufficient time for breakdown of the vitelline membrane to occur (Al-Bahry *et al.*, 2012). However, these findings are unusual, and might instead be due to yolk cross-contamination by the methodology employed. Also contrary to Assumption 4, papers have reported a low level of proliferation of *S.* Enteritidis and other serotypes in the albumen, which was temperature-dependent and varied depending on the age/storage time of the egg (Fang *et al.*, 2012;McAuley *et al.*, 2015). A potential underestimation of the *Salmonella* proliferation prior to the expiry of the YMT using the YMT approach has also been discussed elsewhere (Thomas *et al.*, 2006).

Assumption 5: Upon reaching the egg yolk, all serotypes and strains will grow at the same rates as those modelled for S. Typhimurium. However, no information is available for the growth rate in egg yolks of New Zealand-relevant serotypes such as *S*. Thompson, *S*. Anatum or *S*. Mbandaka; or for any New Zealand egg environment-associated isolates. In addition, the population growth modelling used by Thomas *et al.* 2006 was based on growth rates of *S.* Typhimurium on ground chicken meat starting from a high initial inoculum, rather than in egg yolk starting from a single cell inoculum. A model based on data from a particular experiment is valid only in conditions similar to that experiment (although some data for *Salmonella* growth in whole eggs or yolks fell within the confidence intervals of the model).

Assumption 6: Risk assessment based on YMT model considers only risk posed by potential presence of Salmonella inside the egg and ignores a risk of cross-contamination of food due to a potential presence of Salmonella on the egg surface. Salmonella present either on the surface of eggs or within the contents may both act as a sources for foodborne illness. However, contamination from egg surfaces has been considered to be the most relevant for egg-associated salmonellosis in New Zealand and Australia (Glass *et al.*, 2016;Moffatt *et al.*, 2016;Rivas *et al.*, 2016). As discussed (Section 3.3.4), some evidence is available whereby certain *Salmonella* serotypes survive less well on eggs at higher temperatures (e.g. ~20-25°C relative to 4°C); yet, YMT calculations predict that these higher temperatures pose a greater risk for any *Salmonella* present within eggs. Because studies indicate that *Salmonella* declines in number on egg shells over storage at all temperatures, risk from cross-contamination decreases at any storage temperature over time; however, conditions that prolong survival (e.g. low RH or presence of faecal matter) on eggs extend the time for which cross-contamination or internalisation of cells present on eggs can occur. (Note, any effects on storage conditions on biofilm formation on eggs and the relevance of this for cross-contamination or internalisation remain to be determined.)

3.7 CONCLUSIONS AND PERSPECTIVES

- Incidence of egg-associated salmonellosis is low in New Zealand, but it is increasing in Australia. Consistent with this, the prevalence of *Salmonella* in the New Zealand egg layer farm environment was low relative to international comparisons including

Australia. However, *Salmonella* was isolated from packhouse egg contact surfaces in New Zealand; thus, contamination on the outside of the egg might occur.

- While all new data related to prevalence in and on eggs were considered, attention was paid to data that related to the serotypes identified on New Zealand egg layer farms (*S*. Typhimurium, *S*. Infantis, *S*. Thompson, *S*. Anatum and *S*. Mbandaka). *S*. Enteritidis was not isolated from the New Zealand egg production environment, and this serotype is not considered to be associated with New Zealand chickens or eggs.
- *Salmonella* may form biofilms on eggs at ambient temperatures. However, the relevance of this to survival on eggs, cross-contamination to food contact surfaces, or internalisation of eggs at New Zealand-relevant storage temperatures and times is not known.
- The viability of *Salmonella* declines on egg surfaces over storage at most temperatures, although survival is highly dependent on the serotype present, temperature, RH and presence of faeces. Under some conditions, certain isolates could remain viable on egg surfaces for the duration of the shelf life. At least in some reports, certain serotypes survived less well on eggs at higher temperatures; for example, 22°C compared with 15°C. However, there were inconsistencies in the available data, and between reports. Furthermore, no information was available for some New Zealand-relevant serotypes such as *S*. Thompson, which was the second most prevalent serotype isolated from New Zealand egg layer environments, including from egg contact surfaces, and has been isolated from egg surfaces.
- Based on the available data for *Salmonella* survival on egg shells, storage of shell eggs at 15°C or less for a shelf life of 35 days might not be necessary to protect consumers of New Zealand eggs from salmonellosis given that both:
    - viability declines on egg shell surfaces over storage time at all storage temperatures, which is likely. Assuming that the decline in viability increases with increasing storage temperature, as some studies suggest, storage at room temperature would in fact be preferable than storage at 15°C or less.
    - cross-contamination from contaminated egg shells rather than egg contents is the most relevant transmission route for egg-associated salmonellosis in New Zealand. This assumes that internalisation, survival and growth of New Zealand-relevant serotypes within eggs is minimal or does not occur.
- *Salmonella* has not been isolated from egg contents in existing New Zealand studies. Yet, trans-shell internalisation, survival within egg albumen, and/or growth in the yolk has been observed overseas in laboratory studies for some New Zealand-relevant serotypes such as *S.* Typhimurium. (Note that most of the studies assessed internalisation, survival in albumen, and growth in egg yolk in independent experiments).
- A high level of uncertainty associated with the published data does not allow reliable predictions for the risk associated with *Salmonella* survival and growth in egg contents.

The best way to fill data gaps is an examination of the survival of *Salmonella* isolates from the New Zealand egg production environment in and on eggs at New Zealand-relevant storage temperatures (15 and 22˚C) and times (21 and 35 days).

4 *SALMONELLA* SURVIVAL IN AND ON EGGS AT NEW ZEALAND-RELEVANT STORAGE TEMPERATURES: EXPERIMENTAL STUDY

4.1 INTRODUCTION

The experimental study was designed to address the following questions:

- How well do New Zealand-relevant serotypes and strains survive on visibly clean egg surfaces, at New Zealand-relevant storage times and temperatures (15°C compared with ~22°C[12]; for 0, 21 and 35 days)?
- Are New Zealand-relevant serotypes and strains able to internalise and survive in egg contents at New Zealand-relevant storage times and temperatures?
- What is the effect of faecal contamination of egg shells on *Salmonella* survival on and internalisation into eggs at New Zealand-relevant storage times and temperatures?

4.2 MATERIALS AND METHODS

4.2.1 Pilot experiments to elucidate egg inoculation and sampling methodology

This section describes pilot experiments, which were set up prior to the main experimentation to help determine the methodology.

Correlation between cell concentration and optical density (OD): The approximate CFU of *Salmonella* contained in a PBS suspension with an optical density at 600 nm (OD_{600nm}) of 0.5 was determined prior to egg inoculation. A 1 ml volume of overnight *Salmonella* Brain Heart Infusion Broth (BHI) culture was pelleted by centrifugation, and re-suspended in 5 ml PBS to obtain an OD_{600nm} of 0.5. The solution was well-vortexed and serially diluted 10-fold. Volumes of 100 µl from the 10^{-5} and 10^{-6} dilutions were spread-plated onto Tryptic Soy Agar (TSA) plates in triplicate, and plates were incubated at 37°C for ~18 h. Colonies arising on TSA plates were counted and used to estimate the concentration of CFU present in the starting suspension. It was calculated that an OD_{600nm} suspension of 0.5 resulted in approximately 4 x 10^8 CFU/ml. Therefore, for the main experiment and the pilot experiments, *Salmonella* suspensions were made at OD_{600nm} of 0.25 (aiming for 2 x 10^8 CFU/ml) for egg inoculum preparation.

Determining detection methodology for egg shell enumeration and inoculum concentration: The first trial experiment sought to compare MPN and surface rinsate methodologies for enumeration of *Salmonella* present on shell surfaces. Nine supermarket-purchased cage-laid eggs were inoculated at five demarcated shell locations with 10 µl volumes of *S*. Typhimurium strain 16PH0752-003 suspension (~1x10^3 CFU per egg). Three eggs were tested one hour following drying of inocula on eggs, and the other six eggs were tested following incubation for one week at 15°C and 22°C (three eggs per temperature). Testing involved addition of 10 ml BPW to eggs in Whirl-Pak bags, massaging the inoculated regions through the plastic (20 massages per spot), incubation of eggs at room temperature

[12] 22°C was chosen because this is the ambient temperature in the ESR laboratory when air conditioning is used, and is a temperature commonly used in the literature to represent egg storage in ambient conditions with air conditioning (for example, Thomas et al. 2006).

for one hour to loosen *Salmonella* from shell surfaces, followed by massaging the inoculation spots again prior to plating. Salmonella present in the surface rinsate was detected by either:

- Direct plating: Six volumes of 333 µl rinsate were plated onto XLD plates and colonies were counted following incubation for ~24h at 37°C.
- Nine-tube MPN method: The same BPW egg surface rinsate was tested by nine-tube, three-dilution MPN (3 tubes of 1 ml rinsate in 9 ml BPW, 3 tubes of 0.1 ml in 9.9 ml BPW, 3 tubes of 0.01 ml rinsate in 10 ml BPW). After 18 h pre-enrichment, cultures were enriched in RVS and MKKTn broth for 24 h, and plated to HE and XLD.

Very low recovery of *Salmonella* was observed from eggs sampled on the day of inoculation (an average of 15 CFU/egg by direct plating; ~100-fold lower than the inoculum concentration). Furthermore, *Salmonella* was not detected from egg surfaces by direct plating or MPN methods after one week of incubation.

Recovery limit of Salmonella from egg shells: Based on the findings of the first pilot experiment, eggs were inoculated with a range of concentrations of a *Salmonella* cocktail (all strains that will be used in the main study, in the event that earlier findings were specific to the single strain used; Table 10). Specifically, eggs (two per inoculum concentration) were inoculated with the following concentrations of the *Salmonella* cocktail: 1.05×10^3, 1.05×10^4,1.05×10^5, 1.05×10^6 CFU per egg. Once spots had dried, eggs were added to BPW, surface rinsate was obtained as described in the previous pilot experiment, and three volumes of 333 µl were plated to XLD. Colonies were counted following incubation of plates. As observed in the first experiment, a 100-200-fold reduction of colonies recovered relative to the inoculum concentration added to eggs was observed for all inoculum concentrations. As such, an inoculum concentration of 10^6 CFU/egg was deemed necessary to detect a several log decline in recoverable CFU on eggs over time and to compare the effect of different incubation temperatures on *Salmonella* recovery from egg surfaces.

Egg shell recovery method assessment: A qualitative trial was performed to assess whether alternative methodologies described in the literature may allow for better recovery of *Salmonella* from egg shell surfaces relative to the rinsate method used in the earlier trial experiments. A total of 24 eggs were inoculated with approximately $1x10^5$ CFU of *S.* Typhimurium strain 16PH0752-003. Twelve eggs were tested on the day of inoculation after the inoculum had dried and the remaining twelve were tested following six days incubation at 22°C. Two eggs each were tested by the following methods:

a) Surface rinsate (as described in previous pilot experiments)
b) Surface rinsate followed by sonication in water bath sonicator (for 1 or 5 minutes; Bandelin Sonorex, RF 100H, 50-60 kHz)
c) Eggshell crush. Eggs were cracked, contents were drained, and the remaining albumen on shell interiors was rinsed off with 2 volumes of 25 ml BPW. Inoculated regions on the shells were excised and added to 10 ml BPW. Shells were crushed using a glass rod (100 crushes per sample) and vortexed for 1 min to release *Salmonella* from shells.
d) Eggshell crush (as above) with 1-minute sonication in water bath sonicator
e) Eggshell crush (as above) with 5-minute sonication in water bath sonicator
f) Eggshell crush (as above) with sonication using ultra-sonic probe (Sonics Vibra-cell). Samples were placed on ice and treated for 9 sec on, 9 sec off for a total of 1 min on (i.e. also 1 min off; with a total run time of 2 mins), at 30% power.

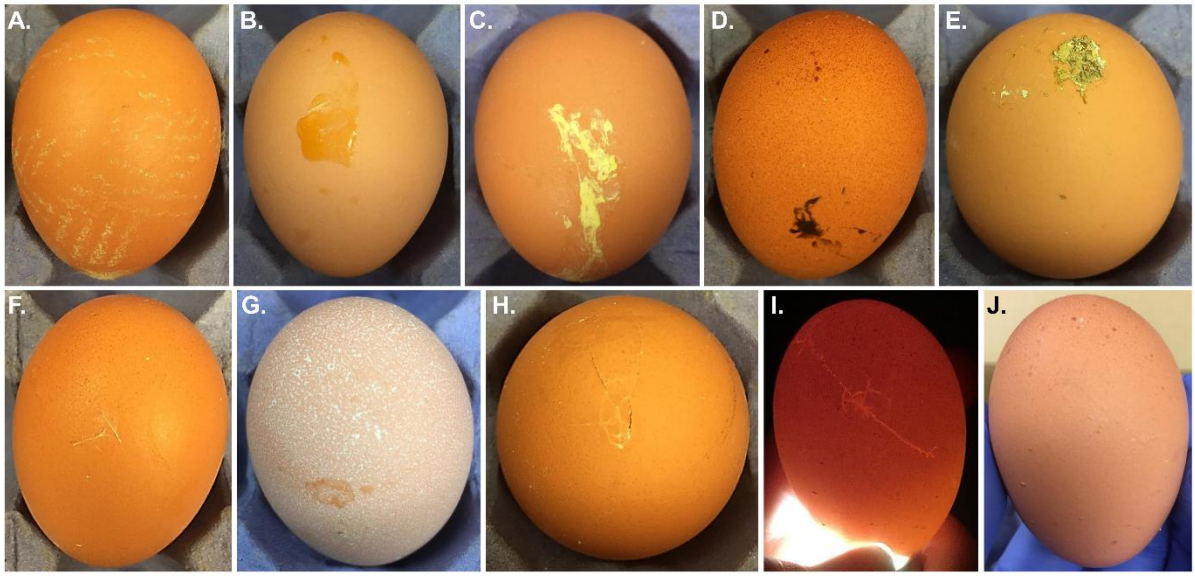
Rinsate (333 µl and 100 µl) from each of the methods was plated onto XLD, and colonies were counted following incubation of plates. The best recovery was deemed to be from methods a) surface rinsate and c) eggshell crush. Based on these findings, surface recovery of *Salmonella* from eggs that had been incubated for six days at 22°C were tested by both

methods (six eggs per treatment). Approximately 3-4-fold increased recovery was obtained using the surface rinsate method, which was implemented for the main experimentation.

4.2.2 Egg selection and preparation

To ensure that all eggs were of the same age (laid at the same time) and at the beginning of their shelf life, packaged eggs were obtained directly from a local farm following the daily grading, on the day prior to egg inoculation. All eggs were of the same grade and size (standard, size 6, ~53 g). Eggs were cage-laid by 56 and 70-week old vaccinated chickens, which had never been treated with antibiotics. Because egg washing procedures have been reported to affect the protective egg cuticle and promote internalisation (Gole *et al.*, 2014a;Gole *et al.*, 2014b), unwashed and untreated eggs were used. Upon receipt at the laboratory, eggs were checked to ensure that all were visibly clean, free from obvious deformities (e.g. unusual shape, rough-shelled, overly speckled, overly calcified or wrinkled eggs) and cracks (gross and hairline cracks, observed using bright light/candling). Examples of rejected eggs are shown in Figure 1 below.

Figure 1. Examples of eggs rejected from use in the study. A-G. dirty eggs, B-C. contamination of shells by egg content, D-E. faecal contamination of shells; F. feathers adhering to shell, G. dirty shell and rough surface, H. visible crack, I. hairline crack visible during candling, J. the same egg as shown in I. viewed under normal light.



4.2.3 Bacterial strain used in the study

Ten *Salmonella* isolates listed in Table 10 were selected to include:

- All five serotypes isolated from the recent *Salmonella* egg layer study (*S*. Infantis, *S*. Thompson, *S*. Typhimurium, *S*. Anatum and *S*. Mbandaka) (Kingsbury, 2018),
- Isolates within a serotype that were genetically distinct were included, as within serotype differences in survival in and on eggs have been reported (Gole et al., 2014a).
- An *S*. Infantis egg shell wash isolate from an earlier survey of commercial New Zealand eggs (Wilson, 2007),
- Two clinical isolates of *S*. Typhimurium comprising different phage types (DT 155, 160), from outbreaks in New Zealand where consumption of raw eggs was a risk factor.

In addition, *Salmonella* Menston NZRM 383 and *Escherichia coli* NZRM 916 were included as positive and negative controls for media, respectively.

Table 10. *Salmonella* egg-associated isolates included in egg inoculum cocktail.

NAME	SEROTYPE	SAMPLE TYPE	REFERENCE/SOURCE
16PH0752-003	*S.* Typhimurium DT 56 variant[1]	Farm 24, free range, shed 1, dust	(Kingsbury, 2018)
ERL10-3996	*S.* Typhimurium DT 160	Clinical isolate. Otago 2010 outbreak associated with consumption of Spanish cream made with raw eggs.	(Rivas *et al.*, 2016)
ERL10-3039	*S.* Typhimurium DT 155	Clinical isolate. South Canterbury 2010 outbreak associated with consumption of chocolate mousse cake made with raw egg served at a café/delicatessen.	(Rivas *et al.*, 2016)
16PH0644-014	*S.* Infantis	Farm 4, packhouse egg washcloth	(Kingsbury, 2018)
16PH0743-010	*S.* Infantis	Farm 21, conventional cage Shed 3, manure belt swab	(Kingsbury, 2018)
ERL06-1174	*S.* Infantis	Egg shell wash	(Wilson, 2007)
16PH0657-015	*S.* Thompson	Farm 6, packhouse, egg washcloth	(Kingsbury, 2018)
16PH0683-002	*S.* Thompson	Farm 14, conventional cage, shed 1, faeces	(Kingsbury, 2018)
16PH0633-003	*S.* Anatum	Farm 3, conventional cage, shed 1, dust	(Kingsbury, 2018)
16PH0743-003	*S.* Mbandaka	Farm 21, colony cage, shed 1, dust	(Kingsbury, 2018)

[1] Assignment of phage type 56 variants was presumptive and based on a high degree of genetic similarity to other phage type 56 variant isolates, rather than by conventional phage typing.

4.2.4 Inoculum preparation, egg inoculation and incubation

On the day prior to egg inoculation, single colonies of *Salmonella* isolates that had been plated onto CBA and XLD agar, were inoculated into individual 10 ml BHI broth cultures and incubated for ~18 h at 37°C. The following day, the OD_{600nm} of each culture was determined. Approximately 1 ml volumes of each of the ten strains (adjusted based on OD_{600nm} readings so that the equivalent CFU of each strain was added) were aliquoted into a single centrifuge tube and cells were pelleted by centrifugation (2000 x *g*, 10 min, room temperature). The medium was discarded and the cell pellet was washed with 1 mL PBS. Following the second centrifugation, the pellet was resuspended in 1 ml PBS, which was used to inoculate a 5 ml volume of PBS to a density of OD_{600nm} of 0.25 (approximately 2 x 10^8 CFU/ml). The suspension was well-vortexed and serially diluted to obtain 10 ml *Salmonella* cocktail/PBS inoculum containing ~2x10^7 and ~2x10^4 CFU/ml. To determine the exact inoculum concentration, the ~2x10^4 CFU/ml dilution was further diluted 10-fold (1 ml in 9 ml PBS) and volumes of 50 µl, 100 µl and 333 µl were plated onto TSA plates in triplicate. Plates were incubated at 37°C overnight and colonies arising were counted.

A chicken faeces/PBS slurry was prepared on the day prior to egg inoculation, as follows. A sample of pooled, fresh faeces arising from caged chickens that had not been treated with antibiotics, was acquired from the same local commercial laying farm as the eggs for this study. A 140 ml volume of PBS was added to 70 g faeces and the solution was mixed to homogeneity. The faecal slurry was sterilised by autoclaving for 15 min at 121°C. Sterility of the slurry was tested by streaking the slurry onto CBA, TSA, MacConkey Agar and XLD plates, followed by incubation at 37 for 24 h; all plates tested negative following incubation. The faecal slurry was applied to demarcated spots on eggs prior to *Salmonella* inoculation using a 1 µl inoculating loop. Due to the heterogeneous nature of the faecal slurry, the exact amount of faeces/PBS per inoculation spot differed, but was on average, approximately 18 µg per spot. The amount of faeces per application spot was typically similar-to-less than that observed in Figure 1D and E; thus, levels added realistically approximated what might be present.

A schematic for inoculating and testing eggs is presented in Figure 2. Eleven or twelve eggs per experimental treatment/time were placed in egg cartons that had been sterilised by ultraviolet light for 30 minutes in a laminar flow hood. Each egg was handled with gloved hands and marked around the blunt end (avoiding the top, so as to avoid the egg air sac) with circles at five separate locations to represent inoculation sites. Eggs were inoculated at distinct sites rather than dipping eggs in inoculum because real-world contamination likely only occurs at distinct spots on the egg.

- 58 eggs were inoculated onto five demarcated sites on the egg with 10 µl each *Salmonella*/PBS suspension to achieve ~10^6 CFU *Salmonella*/egg (shell surface enumeration).[13]
- 58 eggs were inoculated onto five demarcated sites on the egg with 10 µl each *Salmonella*/PBS suspension to achieve ~10^3 CFU *Salmonella*/egg (yolk presence/absence, albumen MPN).
- 58 eggs were inoculated onto five demarcated sites on the egg with faeces/PBS and 10 µl each *Salmonella*/PBS suspensions to achieve ~10^6 CFU *Salmonella*/egg (shell surface enumeration).
- 58 eggs were inoculated onto five demarcated sites on the egg with faeces/PBS and 10 µl each *Salmonella*/PBS suspensions to achieve ~10^3 CFU *Salmonella*/egg (yolk presence/absence, albumen MPN).
- 14 eggs were inoculated with 5 x 10 µl PBS only (controls for *Salmonella*/PBS-inoculated eggs).
- 14 eggs were inoculated with faeces/PBS only (controls for *Salmonella*/faeces/PBS-inoculated eggs).

Inoculated eggs were allowed to dry in a laminar flow hood at ambient temperature and eggs that were not being tested on the day of inoculation were transferred to 15°C or 22°C incubators. A data logging device (Temprecord^{TM}) was alternated between incubators weekly to monitor RH.

Table 11. Testing regimes for egg treatments.

EGG TREATMENT	INOCULUM CONCENTRATION (FINAL CFU/EGG)	INCUBATION TIME (DAYS POST INOCULUM)	EGG INCUBATION TEMPERATURE	NUMBER OF EGGS/TEST	*SALMONELLA* TESTING[1]
Salmonella cocktail / PBS	10^6	0, 21, 35	15, 22°C	10 eggs	Egg surface enumeration
	10^3			10 eggs	Yolk: presence/absence Albumen: 7 tube MPN
Salmonella cocktail / PBS / faeces	10^6	0, 21, 35	15, 22°C	10 eggs	Egg surface enumeration
	10^3			10 eggs	Yolk: presence/absence Albumen: 7 tube MPN
PBS control	0	0, 21, 35	15, 22°C	1 egg	Egg surface: presence/absence
	0			1 egg	Yolk: presence/absence Albumen: presence/absence
PBS / faeces control	0	0, 21, 35	15, 22°C	1 egg	Egg surface: presence/absence
	0			1 egg	Yolk: presence/absence Albumen: presence/absence

[13] Although higher than is typically observed on eggs, inoculum levels were selected so that statistically significant data could be obtained for monitoring a decline in viability, as has been the approach taken in most literature assessed (limit of detection per egg surface is 5 CFU/egg).

Figure 2. Enumeration and detection methods for *Salmonella* in and on eggs. (Note that additional eggs were inoculated than were tested, in the event of breakage.)

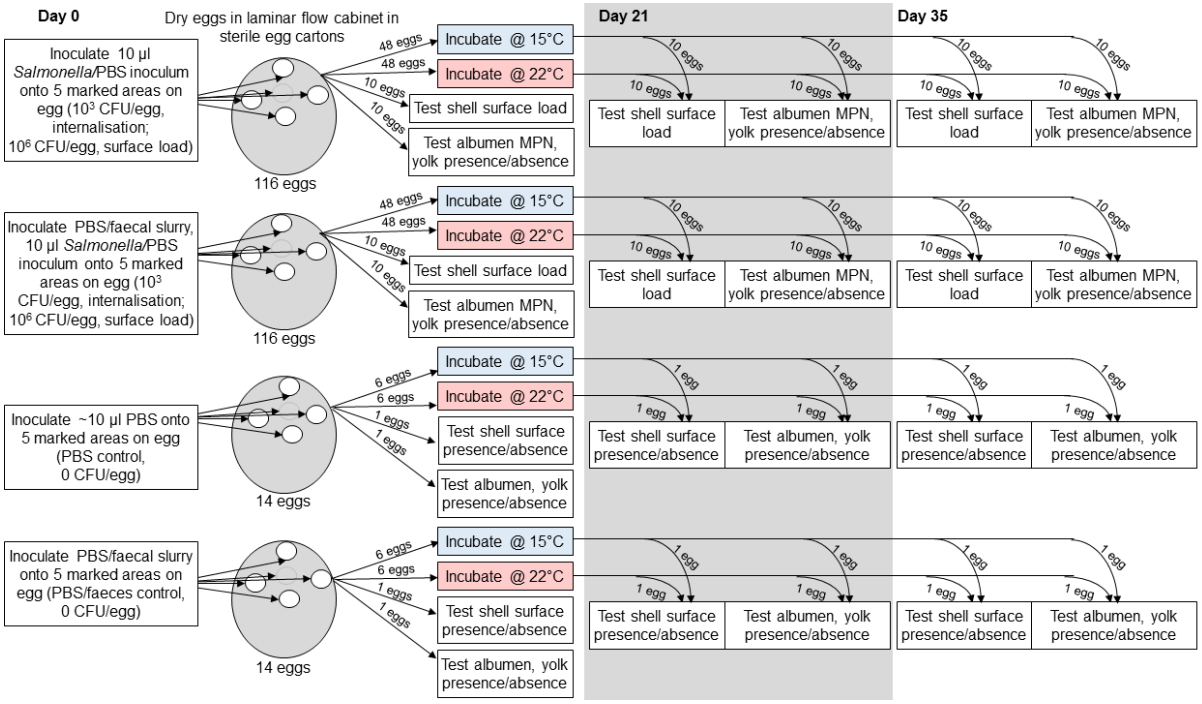
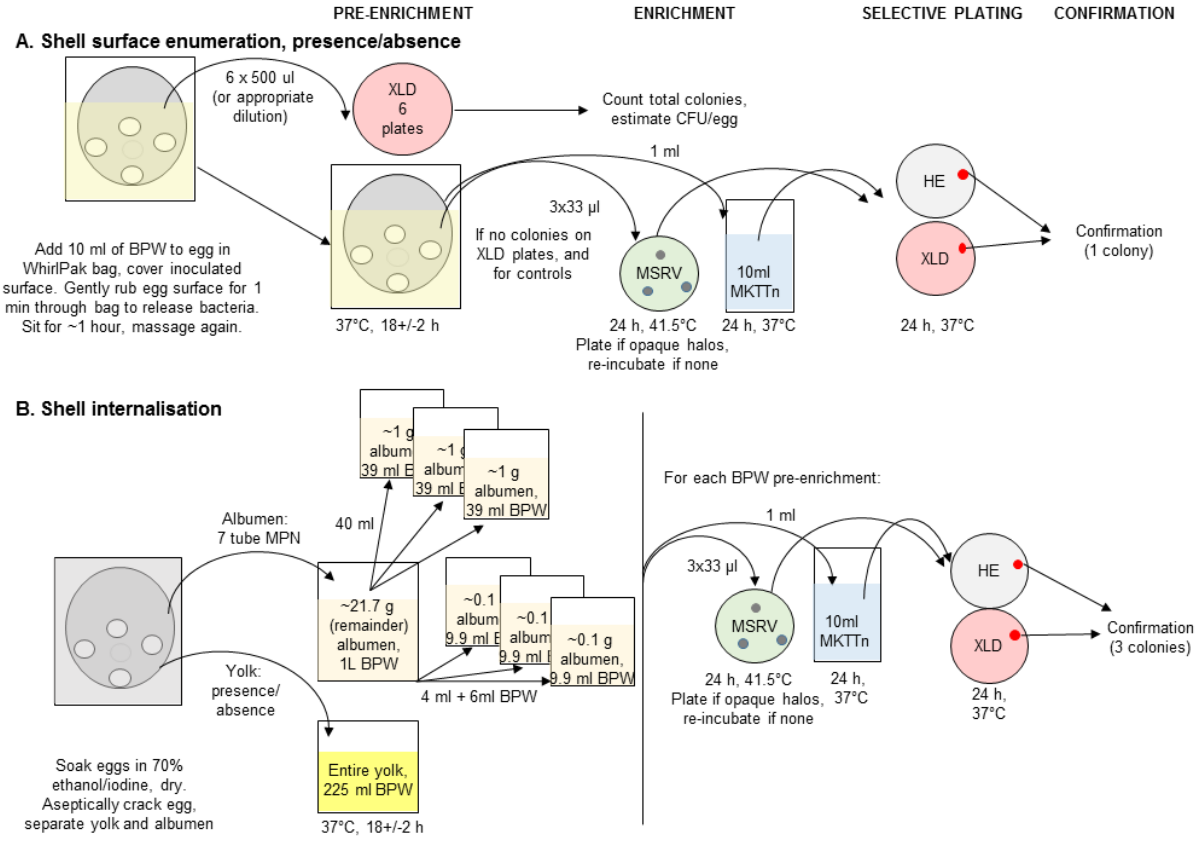


Figure 3. Overview of methodology used to test egg surfaces and contents for *Salmonella*.



4.2.5 Determination of *Salmonella* egg shell surface enumeration, presence/absence

A schematic for enumerating and detection of *Salmonella* present on egg shell surfaces is shown in Figure 3A. The bacterial load (enumeration or presence/absence) of *Salmonella* on egg surfaces was determined for the egg treatments indicated in Table 11. Specifically, a 10

ml volume of BPW was added to single eggs in Whirl-Pak bags, ensuring that the inoculated areas were covered. Following addition, inoculated regions on eggs were massaged through the bag plastic (20 rubs per spot), eggs were soaked for 1 hour at room temperature, and the inoculated regions were again massaged in the same manner to detach bacteria from the shell surface. Volumes of 500 µl of BPW rinsate were plated onto six XLD plates (i.e. 3 ml plated in total, containing 3/10 of total egg bacterial load; i.e. limit of detection was ~3.3 CFU/egg). Lower volumes and dilutions were plated at 0 days post inoculation (333 µl and 100 µl of undiluted rinsate and 100 µl of 10-fold-diluted rinsate). Plates were incubated at 37°C, and the following day, black-centred colonies (due to hydrogen sulphide production indicative of *Salmonella* strains; all isolates used in the inoculum were hydrogen sulphide-positive) and a lightly transparent zone of reddish colour (due to the colour change of the indicator) were counted to determine the bacterial load per egg.

The remainder of BPW (7 ml for experimental eggs or entire 10 ml for control eggs) was pre-enriched with the intact egg in the event that no colonies were recovered during enumeration and/or to detect *Salmonella* that remained attached to the shell (limit of detection, 1 CFU/egg). The BPW enrichments were incubated at 37°C or 18+/-2 h.

For each pre-enrichment, three 33 µl volumes were plated onto a Modified Semi-solid Rappaport-Vassiliadis (MSRV) agar plate (Oxoid; Thermofisher), and incubated at 41.5 ± 1°C for 24 ± 3h (selective enrichment). Plates that remained negative following 24 h incubation (characterised by the absence of a grey-white, turbid zone extending out from the inoculated drop) were incubated for a further 24 h. In addition, a 1 mL volume of the pre-enriched BPW culture was transferred to a tube containing 10 ml of Müller-Kauffmann tetrathionate/novobiocin (MKTTn) broth, and incubated at 37°C ± 1°C for 24 ± 3h (selective enrichment).

Following selective enrichment, all MKTTn broths and opaque growth zones from MSRV plates were streaked onto XLD and Hektoen Enteric Agar (HE) selective plates to obtain well-isolated colonies. Plates were incubated at 37°C for 24h ± 3h and examined for the presence of typical and atypical *Salmonella* colonies. Typical colonies of *Salmonella* grown on XLD agar are described above, and those grown on HE have blue-green coloured colonies with black centres. For each sample, up to five suspicious colonies were re-streaked for purification. Presumptive confirmation for *Salmonella* spp. was determined using standard biochemical (MacConkey Agar, Triple Sugar Iron slant (TSI), Lysine Iron Agar (LIA), urea, indole peptone, oxidase), microgen, and serology agglutination tests. Confirmation tests were performed on one isolate, and further isolates were tested in the event that the first isolate tested negative.

4.2.6 Determination of *Salmonella* internalisation into eggs

A schematic for detection of *Salmonella* present in egg albumen and yolk contents is shown in Figure 3B.

Because albumen has not been reported to be conducive for *Salmonella* growth and only low numbers of *Salmonella* cells (if any) were expected to internalise egg shells, an MPN method was considered the most appropriate method to capture and enumerate any *Salmonella* present in the albumen. At the time of testing, experimental eggs were surface-sterilised by dipping for at least 15 seconds in a solution consisting of three parts of 70% ethanol to one part iodine/potassium iodide solution prepared as described (Cox *et al.*, 2015), and allowed to dry. Eggs were aseptically cracked and separated into yolk and albumen components. The albumen MPN was determined using seven tubes/enrichments (1 x 21.7ml, 3 x 1 ml and 3 x 0.1 ml albumen) and a modified ISO 6579:2017 method (International Organization for Standardization, 2017a, 2017b), as follows. A 25 ml volume of the albumen was added to 1 L BPW, and mixed thoroughly. Three 40 ml volumes of the albumen/BPW pre-enrichment were added to individual Whirl-Pak bags, each containing 1

ml albumen. A further three 4 ml volumes of the initial albumen/BPW pre-enrichment were added to additional tubes containing 6 ml BPW (each containing 0.1 ml albumen; leaving the initial enrichment with 21.7 ml albumen). The albumen MPN was tested using the MPNcalc software. Entire albumen from control eggs was added to 1L BPW, and no further dilutions were tested (presence/absence).

Egg yolks were tested for presence/absence of *Salmonella*. At the first time point (0 days incubation), yolks were separated and collected using egg separators. Yolks were carefully rinsed with 50 ml BPW to wash off the majority of adhering albumen, and the rinsate was discarded. At later time points, egg yolks were found to be more fragile, and frequently cracked, making separation with an egg separator not feasible. Therefore, components were carefully separated, retaining the yolk within the egg shell. Excess albumen was removed from the yolk using a pipette. As such, yolk samples also contained some adhering albumen, but albumen samples did not contain any yolk component. The entire yolk (~25g) was added to a 250 ml volume of BPW in Whirl-Pak bag. The yolk was broken and mixed gently but thoroughly with the BPW, and incubated at 37°C for 18+/-2 h.

Albumen and yolk pre-enrichment cultures were plated onto XLD and HE agar and confirmation was performed as previously described for egg shell pre-enrichments (section 4.2.6).

4.2.7 Control experiment: Salmonella enumeration on petri dish surfaces

The same *Salmonella* cocktail inoculum that was applied to eggs for surface enumeration was also applied to five marked areas on sterile, empty petri dishes, in the presence and absence of faeces application. Three petri dishes were tested for each treatment condition and time point. *Salmonella* from inoculation spots was assayed by adding 5-10 ml BPW (depending on the time point) to petri dishes. The inoculation regions were swabbed 20 times with a sterile cotton swab, and re-rubbed after one hour to loosen any Salmonellae attached to the plate surface. Dilutions of rinsate were plated to XLD to determine the viable/recoverable CFU present.

4.2.8 Statistical analysis

Data from individual eggshell enumeration were $\log_{10}$-transformed prior to all statistical analyses. To allow log transformation of data when no colonies were detected following direct plating (limit of detection 3.33 CFU/egg when 3 ml was plated) but *Salmonella* was detected following enrichment of the entire egg (limit of detection of 1 CFU/egg), a count of 1.67 CFU/egg (50% of direct plating limit of detection) was assigned. When there was no detection by either method, a count of 0.5 CFU/egg (50% of total egg enrichment limit of detection) was assigned. A similar approach has been adopted elsewhere (Messens *et al.*, 2006).

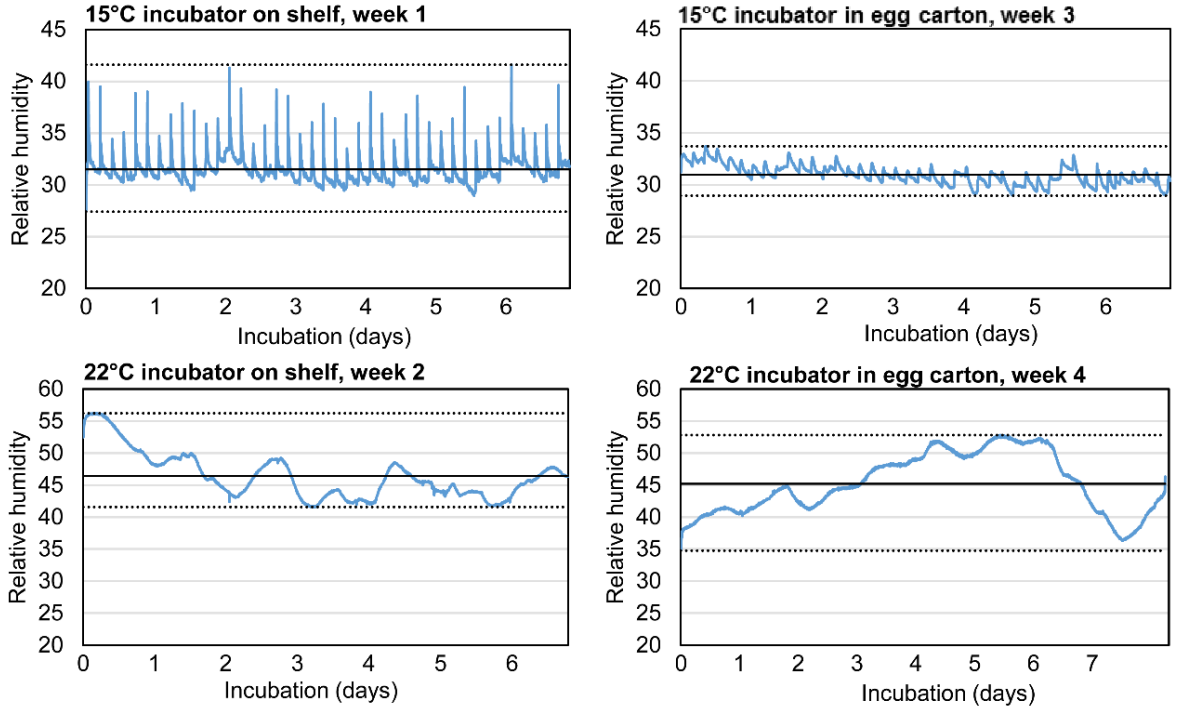
4.3 RESULTS

4.3.1 Relative humidity during egg storage

The RH inside an empty egg carton and on the incubator shelf monitored over one-week time periods for both of the egg storage incubators is shown in Figure 4. The RH in the 15°C incubator averaged 31.5 ranged from 27.4 - 41.6 on the incubator shelf (week 1 of egg incubation) and there was a cyclic pattern to the RH increases and decreases. Values were also cyclic when recordings were taken inside an empty egg carton (week 3 of egg incubation), with a similar mean (30.96) but a narrower range of RH values (28.93 - 33.7). The RH on the 22°C incubator shelf (week 2 of egg incubation) averaged 46.43, and ranged from 41.59 - 56.25. A similar mean RH of 45.22 was recorded inside the empty egg carton in the 22°C incubator (week 4 of egg incubation), and ranged from 34.76 - 52.84. Therefore, the RH was higher at 22°C than 15°C, and was considered along with incubation

temperature for the effect of *Salmonella* survival in and on eggs. Average RH values recorded inside egg cartons were used in tables 12-14.

Figure 4. Relative humidity (RH) readings recorded in 15°C and 22°C incubators on the shelf and in empty egg containers during egg incubation. The black intact line represents the mean and the dotted lines represent the minimum and maximum RH values recorded during each incubation period.



4.3.2 Enumeration of *Salmonella* on eggshell surfaces as affected by temperature/relative humidity, storage time and the presence of faeces

Results of direct enumeration of *Salmonella* on egg surfaces are shown in Figure 5 and Table 12 (Appendix).

The exact inoculum concentration applied to egg surfaces was calculated to be 5.98 $\log_{10}$ (9.45 x 10^5) CFU/egg, as determined by plating of the diluted *Salmonella* cocktail inoculum. The initial sampling time point of inoculated eggshell surfaces (plating of eggshell rinsate) occurred approximately 2 h following egg inoculation. At the initial sampling point, an average reduction relative to the applied inoculum of 2.2 and 1.48 $\log_{10}$ CFU was recovered from eggs inoculated with *Salmonella* cocktail in the absence and presence of faeces, respectively.

Salmonella was recovered at higher levels from eggs incubated at 15°C (31% average RH) compared with 22°C (45% average RH) after both 21 and 35 days incubation. In the absence of faeces, there was a 1.04 and 1.25 $\log_{10}$-higher recovery of *Salmonella* CFU from eggs stored at 15°C than from those stored at 22°C, at 21 and 35-day time points, respectively. In the presence of faeces, there was a 1.98 and 2.62 $\log_{10}$-higher recovery of *Salmonella* CFU from eggs stored at 15°C than from those stored at 22°C, at 21 and 35-day time points, respectively.

Higher levels of *Salmonella* were recovered from egg surfaces in the presence compared with the absence of faeces. The highest concentrations of *Salmonella* were recovered from eggs that were artificially contaminated with faeces and were incubated at 15°C. An average 1.78 and 2.38-higher $\log_{10}$ CFU was recovered from these eggs than eggs stored at 15°C without faeces after 21 and 35 days of incubation, respectively. At 22°C an average 0.84

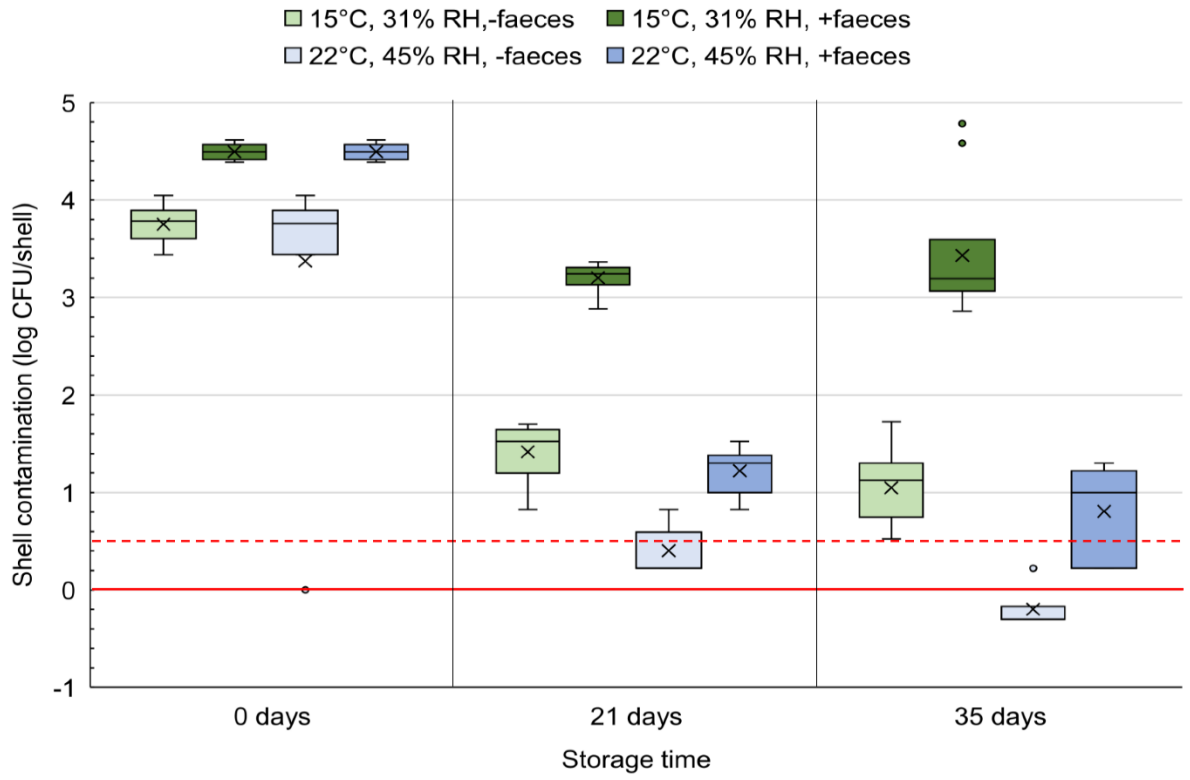
and 1.00-higher $\log_{10}$ CFU was recovered from faeces-contaminated compared with faeces-free eggs after 21 and 35 days of incubation.

Following 21 days of incubation of inoculated eggs, a reduction in recoverable CFU was recorded for all egg treatments. Viable CFU recovered from eggs continued to decline at 35 days of incubation for all conditions except for eggs containing faeces stored at 15°C. Indeed, after 35 days of incubation, *Salmonella* CFU levels from two of these eggs (see outlier values, Figure 5) were similar to levels from the 0-day sampling period. These levels were particularly striking when compared with data from eggs stored at 22°C in the absence of faeces at the same time point. By five weeks of incubation, no recoverable CFU were detected following direct plating of rinsates from the latter eggs, and *Salmonella* was only detected from 2/10 eggshells following enrichment of entire eggs.

No *Salmonella* was detected on the surface of any control eggs at any incubation temperature, egg treatment or storage time point.

Similar to the observations on egg surfaces, higher levels of *Salmonella* were recovered from inoculated petri dishes with faeces compared with dishes without faeces evaluated during the control experiment (Table 13; Appendix). Higher levels were also observed from dishes incubated at 15°C compared with 22°C. After 35 days of incubation, no CFU were recovered from petri dishes stored at 22°C, regardless of the presence of faeces (note, rinsates were not enriched). At the same time point, and average 0.6 $\log_{10}$ CFU per dish were recovered from dishes stored at 15°C in the absence of faeces. In dramatic contrast, an average of 3.6 $\log_{10}$ CFU per dish was recovered from dishes stored at 15°C in the presence of faeces.

Figure 5. Box and whisker plot showing enumeration of *Salmonella* on eggshells as a function of egg storage temperature and the presence/absence of faeces. Ten eggs were sampled per variable. The solid line within the box marks the median, while the X marks the mean. The box borders represent the 25th and 75th percentiles. Whiskers above and below the box indicate the maximum and minimum values assuming no outliers are present. The red dotted and intact lines indicate limits of detection by plating of egg rinsate and egg enrichment, respectively. Values below these limits indicate no detection by the respective detection method.



4.3.3 Presence of *Salmonella* in egg albumen and yolk as affected by temperature, storage time and presence of faeces on the shells

The exact inoculum concentration applied to egg surfaces for internalisation experiments was calculated to be 9.45 x 10^2 CFU/egg, as determined by plating of the diluted *Salmonella* cocktail inoculum.

No *Salmonella* was detected in the albumen of eggs at any storage temperature, sampling time or treatment condition (Table 14, Appendix; <0.04 MPN/ml albumen for each egg). Note that the MPN procedure involved testing the majority of the albumen (25 ml of ~30 ml entire albumen); thus, there was a reasonable likelihood that low level contamination with viable *Salmonella* would have been captured at each time point if any had been present. *Salmonella* was also not detected in the entire albumen components of any control, uninoculated eggs.

Table 14 also shows the presence/absence results for *Salmonella* in entire egg yolks as a factor of incubation temperature/RH, incubation time and the presence of faeces on egg surfaces. Note that egg yolks also may have included a minimal amount of attached albumen (see Materials and Methods). No *Salmonella* was detected in any egg yolks at any time point or treatment variable. In addition, *Salmonella* was not detected in the yolks of any control eggs.

4.4 DISCUSSION

4.4.1 Effect of storage temperature and time on Salmonella presence on eggs

Previous studies have demonstrated a temperature-dependent effect on *Salmonella* survival on egg shell surfaces. However, data between studies were conflicting, did not include some of the *Salmonella* serotypes and phage types that have been isolated from the New Zealand egg layer environment, and/or did not directly compare the storage temperatures relevant to New Zealand egg storage recommendations.

In this study, the survival of New Zealand *Salmonella* isolates that had been inoculated onto visibly clean, fresh, unwashed egg surfaces, was greater following storage at 15°C compared with 22°C at both 21 and 35 days of storage. *Salmonella* declined in viability at both temperatures over time, and was not detected from surfaces of most (8/10) visibly clean eggs stored at 22°C for 35 days. Taken together, results do not support that storage of shell eggs at 15°C is more protective than storage at 22°C when considering *Salmonella* on egg surfaces.

The average and range of RH also differed in the two storage temperature incubators (averages of 31% and 45% RH at 15°C and 22°C inside egg cartons, respectively). While it is unclear if these differences in RH are sufficient to influence *Salmonella* survival, results from a previous study showed better survival of *Salmonella* on eggs at a lower RH (43% compared with 85%) and at a lower temperature when RH was controlled (12°C compared with 25°C; RH 43%) (Park et al., 2015) (**Table 6**). Therefore, the lower storage temperature and lower RH may both play roles in the increased survival of *Salmonella* on eggs stored at 15°C.

Some studies have discussed that a reduction in *Salmonella* recovery from eggs over time may be due to penetration of *Salmonella* cells from the shell surface into the eggs (Pasquali *et al.*, 2016;Whiley *et al.*, 2016). Our study demonstrated an incubation temperature-dependent reduction of *Salmonella* recovery in control experiments involving the *Salmonella* cocktail inoculated onto smooth petri dish surfaces instead of eggs. This suggests that adhesion or inactivation cause the low recovery, rather than adsorption into the porous egg shell surface.

4.4.2 Effect of chicken faeces on *Salmonella* survival on eggs

An improved recovery of *Salmonella* was observed from eggs that were artificially contaminated with sterile faeces compared with faeces-free eggs incubated at the same temperature. Differences were particularly dramatic for eggs stored at 15°C, and at the 35-day sampling point, two inoculated eggs containing faeces had approximately the same CFU levels as those detected 2-hours post-inoculation. These results are consistent with those reported previously (Table 7, (Park *et al.*, 2015)). Faeces has been reported to have a protective effect and act as a source of nutrients for any *Salmonella* present. Note that although the experimentally-applied faeces was sterile, microbiota present in faeces may also influence *Salmonella* survival on naturally contaminated eggs. To our knowledge, no data is available on the growth rate of *Salmonella* in faeces (either sterile or non-sterile) at different temperatures. Higher numbers of *Salmonella* were recovered from eggs inoculated with faeces at the initial sampling point, only approximately 2 hours post inoculation. This suggests that the presence of faeces may improve recovery from the shell.

A review of data from studies that used varied methods for recovery of *Salmonella* from shell surfaces suggests that improved recovery in the presence of faeces is not unique to our study or methodology used (Table 15, Appendix). The discrepancy between inoculation and recovery numbers was not addressed in the literature assessed. The phenomenon does not appear to be due to an immediate transit of *Salmonella* into the egg pores in faeces-free eggs making it recalcitrant to the rinsing method, because similar results were also observed on the smooth surfaced petri dish controls. It is possible that *Salmonella* deposition on faeces, which is easily washed off the eggs and suspended into solution, facilitates recovery. If this is the case, the same rationale would mean that presence of faeces will also facilitate cross-contamination from egg surfaces.

The mechanisms by which salmonellae survive desiccation stress, as would occur on egg surfaces, are poorly understood (Li *et al.*, 2013). Three of the strains present in the inoculum cocktail were isolated from dust, and thus, may be better-adapted for resistance to desiccation stress. (Note, no analyses were performed here to ascertain which isolates survived best on egg surfaces). Changes in outer-membrane LPS, curli, cellulose formation and filaments have been observed in *Salmonella* cells growing in low water activity (also related to biofilm formation, Section 3.3.3) (White and Surette, 2006). Fatty acid metabolism, accumulation of osmoprotective solutes and upregulation of transporters were also observed in *Salmonella* under desiccation conditions. Therefore, comparing the cell morphology, physiology, metabolome and the transcription response of *Salmonella* on eggs may provide interesting insights into the mechanisms by which incubation temperature and faeces affect survival during desiccation stress.

Results from this study highlight that any faecal presence on eggs will elevate the risk of cross-contamination of *Salmonella* from egg surfaces, regardless of the storage temperature. Findings reinforce the importance of rigorous quality control measures to either reject dirty eggs or channel these for egg washing according to the strict RMP guidelines, to ensure that faecally contaminated eggs do not reach the retail market.

4.4.3 Internalisation and survival of New Zealand Salmonella isolates in eggs

Experiments performed in this study were designed to mimic as much as possible a natural egg contamination event. Some isolates may be naturally better-adapted for internalisation and survival in eggs. Therefore, the experimental inoculum composition was designed to include ten isolates that were genetically distinct to cover a range of genotypic and phenotypic diversity. Egg outbreak-associated *Salmonella* strains were included in the inoculum in the event that they may better internalise, survive or grow in eggs than non-outbreak strains. In addition, the inoculum size was chosen to approximate upper limits of what might be experienced during natural contamination, based on observations from the literature (discussed in Section 3.3.2). Furthermore, while some studies only sampled a

proportion of the albumen or yolk, the bulk of the albumen (25 ml of ~30 ml) and the entire yolk were tested in this study to increase the likelihood of capturing any viable *Salmonella*, if present inside the egg. Nevertheless, no *Salmonella* was detected in either albumen or yolk, at any incubation temperature, or sampling time point. Furthermore, *Salmonella* was also not detected from contents of eggs that were also externally contaminated with faeces, which was shown to increase the surface survival of *Salmonella*.

Results support that internalisation of New Zealand *Salmonella* isolates into unwashed, uncracked eggs and survival in albumen for the duration of egg storage, is a rare event. If the event of internalisation occurs, survival of *Salmonella* cells is unlikely. Our data are consistent with previous surveys, none of which detected *Salmonella* in the contents of eggs at retail in New Zealand or Australia (as discussed in Section 3.2.3 (Johnson, 1995;Lake *et al.*, 2004;Symes *et al.*, 2016;Wilson, 2007)).

Some serotypes of *Salmonella* that are present in New Zealand have been shown in international studies to be able to internalise eggs (discussed in Section 3.3.2, Table 7). Differences observed between studies and our results are likely, at least in part, to be due to differences in experimental egg inoculation. Our study employed a more natural inoculation approach of application of *Salmonella* in low inoculation volumes at five sites on the egg. In contrast, most studies listed in Table 7 inoculated eggs by immersion of the egg into the inoculum (Chousalkar *et al.*, 2010;Gole *et al.*, 2014a;Gole *et al.*, 2014b;Samiullah *et al.*, 2013;Whiley *et al.*, 2016). Such an approach may facilitate *Salmonella* internalisation, particularly if there is a temperature differential between the inoculum and the egg, or if eggs take time to dry. Other studies also used a higher inoculum concentration, for example, Lublin *et al.* (2015) inoculated eggs with 2.7-log higher CFU per egg. Detection of *Salmonella* in egg contents in this study required the ability of *Salmonella* to penetrate across the shell and survive in egg albumen. However, detection of internalisation in other studies was determined using an agar egg technique or PCR, neither of which would confirm that *Salmonella* detected in the albumen was viable (Chousalkar *et al.*, 2010;Gole *et al.*, 2014a;Gole *et al.*, 2014b;Samiullah *et al.*, 2013).

The absence of detectable internalisation by *Salmonella* at any temperature, time or condition in this study, and the absence of detection in previous New Zealand studies, all call into question the relevance of using YMT model, which assumes that internalisation is occurring, to base egg storage guidance. However, as a cautionary note, eggs were rigorously screened for the experiments described here to be visibly spotless (except when faeces was artificially applied), uncracked, with no visible deformities on shells, and were unwashed. Not all eggs at retail may meet these conditions. Certain egg treatments have been shown to facilitate *Salmonella* internalisation into eggs, such as egg washing which removes the protective egg cuticle and, if performed incorrectly, may create a large differential in temperature between the inside and outside of the egg, or insufficient drying of eggs following washing. The conclusion that *Salmonella* is highly unlikely to internalise retail eggs in New Zealand assumes strict adherence to egg handling guidelines and rigorous quality control processes to eliminate eggs with compromised shells, all the more critical.

4.5 CONCLUSIONS

This experimental study was designed to provide evidence to inform risk management decisions regarding egg storage times and temperatures with respect to *Salmonella* control in and on New Zealand eggs at retail. The following findings indicate that storage at 15°C or less for a shelf life of 35 days is not necessary to protect consumers of New Zealand eggs from salmonellosis:

1. The survival of New Zealand egg-relevant *Salmonella* isolates on visibly clean eggshells was substantially lower when stored at 22°C compared with 15°C (although the differential effects of RH at the two temperatures may also affect viability). Results

support a lower risk for *Salmonella* cross-contamination from egg surfaces (or internalisation) from eggs that have been stored at 22°C relative to 15°C.

2. The survival of *Salmonella* declined at both temperatures over time in the absence of faeces, and was almost undetectable at 22°C after 35 days. Results support that the risk from cross-contamination by *Salmonella* present on egg surfaces will not increase after 21 days at either temperature, particularly for eggs stored at 22°C relative to 15°C.

3. Viable *Salmonella* were not detected in egg contents at any storage time or condition. Therefore, this study found no evidence to support that a YMT-type model is appropriate to assess egg storage conditions for New Zealand. However, while not detected in these experiments, it cannot be completely ruled out that New Zealand-relevant *Salmonella* cells might penetrate and survive in egg contents if a greater sample size was used, or in eggs of a poorer quality (e.g. dirty or with a compromised shell).

4. Experiments demonstrating substantially enhanced *Salmonella* survival in the presence of chicken faeces emphasise the importance of maintaining and enforcing current regulations that require eggs sold at retail to be visibly clean.

Consumer protection and risk assessment would also consider exposure and dose-response. However, these experiments can inform risk management at the production and retail level.

GLOSSARY

BHI	Brain Heart Infusion Broth
BPW	Buffered peptone water
CBA	Columbia Blood Agar
CFU	Colony forming units
EPF	Egg Producers Federation of New Zealand
ESR	Institute of Environmental Science and Research Ltd (NZ)
HE	Hektoen Enteric Agar
ISO	International Organisation for Standardisation
LIA	Lysine Iron Agar
MKTTn	Müller-Kauffmann tetrathionate/novobiocin
MPI	Ministry for Primary Industries
MPN	Most Probable Number
MSRV	Modified Semisolid Rappaport-Vassiliadis Soya Peptone Agar
OD_{600nm}	Optical density at 600 nm
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RH	Relative humidity
RMP	Risk Management Programme
RVS	Rappaport-Vassiliadis Soya Peptone broth
SEM	Scanning Electron Microscopy
TSA	Tryptic Soy Agar
TSI	Triple Sugar Iron slant
YMT	Yolk Mean Time
XLD	Xylose lysine deoxycholate

APPENDIX A: SUPPLEMENTARY TABLES

Table 12. Survival of *Salmonella* cocktail on the surface of eggs as determined by direct plating of egg surface rinsate: comparison between incubation temperature, incubation time and presence of faeces.

INCUBATION TEMPERATURE, AVERAGE RELATIVE HUMIDITY (% RH)	EGG TREATMENT	MEAN LOG_{10} CFU/EGG (STD DEV)[1]		
		0 DAYS INCUBATION[2]	21 DAYS INCUBATION	35 DAYS INCUBATION
15°C, 31% RH	*Salmonella*/PBS	3.75 (0.20)	1.42 (0.28)	1.05 (0.38)
15°C, 31% RH	*Salmonella*/PBS/faeces	4.50 (0.08)	3.20 (0.14)	3.43 (0.67)
22°C, 45% RH	*Salmonella*/PBS	3.75 (0.20)	0.38 (0.28)[3]	-0.20 (0.22)[4]
22°C, 45% RH	*Salmonella*/PBS/faeces	4.50 (0.08)	1.22 (0.22)	0.81 (0.46)[5]

[1] The limit of detection by direct plating was 3.33 CFU per egg. The limit of detection for enrichment of the intact egg was 1 CFU per egg. When there was no detection by direct plating but detection by enrichment, an arbitrary CFU of 1.67 was assigned to enable log_{10} transformation. When there was no detection by either method, an arbitrary CFU of 0.5 was assigned.
[2] Eggs at Time 0 days of incubation had by definition not been incubated at either 15 or 22°C temperatures. Therefore, the same eggs were used for the Time 0 calculations for both incubation temperatures.
[3] No *Salmonella* was detected on three eggs by direct plating, but these were positive following enrichment of the intact egg.
[4] No *Salmonella* was detected on any eggs by direct plating, but 3/10 were positive following enrichment of the intact egg.
[5] No *Salmonella* was detected on three eggs by direct plating, but these were positive following enrichment of the intact egg.

Table 13. Survival of *Salmonella* cocktail on petri dish surfaces as determined by direct plating of dish surface rinsate: comparison between incubation temperature, incubation time and presence of faeces.

INCUBATION TEMPERATURE, AVERAGE RELATIVE HUMIDITY (% RH)	PETRI DISH TREATMENT	LOG_{10} CFU/PETRI DISH		
		0 DAYS INCUBATION[1]	21 DAYS INCUBATION	35 DAYS INCUBATION
15°C, 31% RH	*Salmonella*/PBS	3.64	0.37	0.62
15°C, 31% RH	*Salmonella*/PBS/faeces	4.64	3.51	3.56
22°C, 45% RH	*Salmonella*/PBS	3.64	0.37	ND[2]
22°C, 45% RH	*Salmonella*/PBS/faeces	4.64	0.47	ND

[1] Petri dishes at Time 0 days of incubation had by definition not been incubated at either 15 or 22°C temperatures. Therefore, calculations from the same dishes were used for the Time 0 calculations for both incubation temperatures.
[2]ND no detection

Table 14. Most Probable Number (MPN) of *Salmonella* in albumen and presence/absence in egg yolk: comparisons between incubation temperature, incubation time and presence of faeces

INCUBATION TEMPERATURE	EGG TREATMENT	0 DAYS INCUBATION[1]			21 DAYS INCUBATION			35 DAYS INCUBATION		
		AVE MPN/ML ALBUMEN[2]	EGGS WITH DETECTION IN ALBUMEN (%)	EGGS WITH DETECTION IN YOLK (%)	AVE MPN/ML ALBUMEN	EGGS WITH DETECTION IN ALBUMEN (%)	EGGS WITH DETECTION IN YOLK (%)	AVE MPN/ML ALBUMEN	EGGS WITH DETECTION IN ALBUMEN (%)	EGGS WITH DETECTION IN YOLK (%)
15°C, 31% RH	*Salmonella*/PBS	<0.04	0/10 (0%)	0/10 (0%)	<0.04	0/10 (0%)	0/10 (0%)	<0.04	0/10 (0%)	0/10 (0%)
15°C, 31% RH	*Salmonella*/PBS/ faeces	<0.04	0/10 (0%)	0/10 (0%)	<0.04	0/10 (0%)	0/10 (0%)	<0.04	0/10 (0%)	0/10 (0%)
22°C, 45% RH	*Salmonella*/PBS	<0.04	0/10 (0%)	0/10 (0%)	<0.04	0/10 (0%)	0/10 (0%)	<0.04	0/10 (0%)	0/10 (0%)
22°C, 45% RH	*Salmonella*/PBS/ faeces	<0.04	0/10 (0%)	0/10 (0%)	<0.04	0/10 (0%)	0/10 (0%)	<0.04	0/10 (0%)	0/10 (0%)

[1] As with surface enumeration calculations, eggs at Time 0 days of incubation had by definition not been incubated at either 15 or 22°C temperatures. Therefore, values from the same eggs were used for the Time 0 calculations for both incubation temperatures.
[2] The limit of detection per egg was 1 MPN in 25 ml (the majority of the albumen volume), and 1 viable cell in the entire egg yolk (presence/absence).

Table 15. Literature was assessed to establish differences observed experimentally between *Salmonella* inoculum concentration and amount recovered from inoculated egg surfaces.

Salmonella inoculum	cfu detected per egg (Time 0)	Change between inoculum cfu and cfu detected on egg at time 0	Method of surface load detection	Reference
5.7 log CFU/egg in 50 µl peptone water spread on ~1cm spot on blunt end of egg	~3.6 log CFU/egg	~2.1 log reduced detection on eggs compared with inoculum concentration	The contaminated eggshell region was excised 10x volume BPW added, homogenised and stored overnight at 4°C. The BPW was then diluted and plated onto Brilliant Green Agar, and resulting colonies counted.	(Lublin *et al.*, 2015)
5 log CFU (in saline) dispensed in 100 µl volume onto egg	~3.8-5 CFU/g (~per egg)	0-1.2 log reduced detection compared with inoculum concentration (although it is unclear if CFU/g correlated to CFU/egg)	Egg contents were removed. Eggshells were added to 10x volume of saline (0.9% NaCl), diluted and plated to Brilliant Green Agar, and resulting colonies counted.	(Pasquali *et al.*, 2016)
2 × 50 μl aliquots of ~6 log CFU dispensed onto 1 cm^2 areas of egg	2.88-3.89 log MPN/cm^2 (it was not clear whether this means per cm^2 of egg surface)	2.1-3.1 log reduced MPN detection compared with inoculum concentration (assuming that MPN/cm^2 relates to the cm^2 of inoculated egg surface)	Eggs placed in stomacher bag containing 10 ml BPW, held at 4°C for 16h to soften cuticle material and subjected to abrasion with a sterile swab to assist in removing bacterial cells from the egg surface. The BPW was dispensed as a five tube MPN over a 5 log dilution range.	(McAuley *et al.*, 2015)
A 20 µl volume of 9 log CFU/ml (in PBS), with or without 20 µg sterile faeces, was applied per egg; i.e. 7.3 log CFU/egg.	5.6 log CFU (no faeces present) or 6 CFU (faeces present)	~1.4 log (no faeces present) or ~1 log (faeces present) reduced detection on eggs compared with inoculum concentration	Egg placed in a Whirlpak bag containing 10 ml of BPW and rubbed for 1 min. Rinsate was serially diluted and plated onto XLD and resulting colonies counted.	(Park *et al.*, 2015)

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