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Microbiological survey of commercial egg layer farms in New Zealand for the presence of *Salmonella*

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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

Microbiological survey of commercial egg layer farms in New Zealand for the presence of *Salmonella*

Epidemiological evidence suggests *Salmonella* on New Zealand eggs is not an important pathway for human salmonellosis. However, robust nationally representative data for *Salmonella* contamination of eggs is not available to support this. Informative surveys of eggs at retail are expensive and produce highly uncertain results due to low prevalence of contaminated eggs. This survey of New Zealand egg layer environment does not provide data on eggs contaminations, but, producing data on *Salmonella* prevalence in the layer farms and packhouses environment, shows the likelihood that commercial eggs are exposed to *Salmonella*.

The overall prevalence of *Salmonella* in the New Zealand layer environment was lower than found in studies of similar environmental samples in Australia (where rates of egg-associated salmonellosis is high). The survey results showed the highest prevalence of *Salmonella* was in layer shed pooled dust samples, followed by boot/manure belt swabs, pooled faeces, and on packhouse egg contact surfaces.

Findings of *Salmonella* on packhouse egg contact surfaces only occurred in the farms with the highest prevalence of *Salmonella*-positive layer shed samples. As the isolates obtained from layer sheds and packhouse samples were genetically related indicating a direct association between layer shed prevalence of *Salmonella* spp. and egg contact surface prevalence. Whole genome Single Nucleotide Polymorphism analyses further supported that persistent resident populations are present in the layer sheds and farm environments, rather than from multiple sporadic contaminating events.

All of the *Salmonella* serotypes isolated in this survey have been commonly detected in other New Zealand environment surveys. Importantly, the more prevalent serotypes from this survey are rarely associated with human infections. The most clinically relevant serotype, *S*. Typhimurium, was isolated in only 14% of positive samples. The absence of *S.* Enteritidis from the isolates found in this survey reinforces the conclusion that this serotype is not endemic in poultry in New Zealand.

A total of twelve out of twenty eight surveyed farms had at least one Salmonella-positive sample, with many of these twelve farms having a high level of biosecurity and cleaning practices. This finding illustrates the challenge of eliminating *Salmonella* from the egg production environment, and underlines the importance of maintaining high hygiene standards along the whole supply chain from production to consumers.

The absence of human salmonellosis outbreaks attributed to eggs in New Zealand indicates that relevant controls are generally good. Still, the survey results will help MPI and the industry to optimise practices intended to minimise likelihood of *Salmonella* presence on eggs.

Microbiological survey of commercial egg layer farms in New Zealand for the presence of *Salmonella*

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New Zealand has a very low reported incidence of egg associated salmonellosis although contaminated eggs are a significant cause of foodborne salmonellosis overseas, including Australia. The risk from *Salmonella* contamination is a key factor influencing decisions about egg storage and shelf life.

To better understand the risk posed by *Salmonella* on eggs in New Zealand, and allow comparisons with Australian data, this study performed a baseline survey of *Salmonella* prevalence in the egg production environment. A survey was carried out of twenty-eight commercial chicken egg layer farms throughout New Zealand that comprised different production sizes and practices. Samples were taken for microbiological testing to assess the prevalence and serotypes of *Salmonella* present in the egg production environment including:

- farm-level feed, as a potential source of contamination of hens and eggs;
- the egg layer environment, which has the potential to contaminate hens and eggs (pooled faeces, pooled dust, manure belt/boot swabs); and,
- egg contact surfaces in the packhouse (egg conveyor, candler, grading equipment, egg suction cups, egg wash cloth).

Key findings for *Salmonella* prevalence in the egg layer environment were as follows:

- Overall prevalence of *Salmonella* in New Zealand layer environment was lower than found in studies testing similar samples in Australia (manure belt/boot swab and faeces samples). This is consistent with lower rates of egg-associated outbreaks in New Zealand.
- A total of 12/28 farms had at least one *Salmonella*-positive sample. Four of the twelve positive farms had only one *Salmonella* positive sample, three of them were pooled dust samples. 21/67 farm sheds and 3/26 packhouses had at least one *Salmonella*-positive sample.
- Of the 43/323 *Salmonella*-positive samples, pooled dust samples had the highest prevalence (19/67), followed by boot/manure belt swabs (11/67), pooled faeces (7/67), packhouse egg contact surfaces (5/87), and one feed sample tested positive (which may have been contaminated from the shed) (1/33).
- A significantly higher prevalence of *Salmonella*-positive layer shed samples was observed from caged (colony and conventional cages) systems (33/75), compared with cage-free (free-range and barn) systems (4/126). However, this comparison needs to be interpreted cautiously. Multiple practices differed between these types of laying systems, which could all contribute to differences; such as a higher average flock size and density in caged sheds, and 60% of cage sheds have multi-aged flocks while cage-free flocks were more often of a single age.
- Farms with *Salmonella*-positive packhouse samples also had the highest numbers of positive layer shed samples, consistent with a high laying shed prevalence increasing the likelihood of egg surface contamination. This suggests that cross contamination between contaminated and uncontaminated eggs via packhouse surfaces may occur although eggs were not analysed.

Five serotypes were identified among the isolates, including *S*. Infantis, *S*. Thompson, *S*. Typhimurium, *S*. Anatum and *S*. Mbandaka. All of these serotypes are commonly isolated

from the environment in New Zealand, and are amongst the most common identified on egg layer farms world-wide. All *S*. Typhimurium isolates were closely related to phage type 56 variant isolates. At the time of the survey, the 56 variant phage type was one of the most commonly isolated phage types from various New Zealand environmental samples and animal types including birds, raising the possibility that wild birds may be the source of *S.* Typhimurium isolates in this survey. All of these serotypes have been regularly isolated from reported salmonellosis cases and environmental sources in New Zealand in the last three years. The absence of *S.* Enteritidis from the isolates found in this survey reinforces the conclusion that this serotype is not endemic in poultry in New Zealand.

Whole genome Single Nucleotide Polymorphism (wgSNP) analyses demonstrated that *S*. Thompson and *S.* Infantis isolates were typically more closely related to other isolates from:

1. the same layer shed than from a separate shed,
2. the same farm than from a separate farm, and
3. packhouse egg contact surface isolates were related to shed isolates from the same farm.

These results support the presence of resident, persistent populations in the shed and farm environments rather than multiple sporadic contamination events. Results also support that egg packhouse isolates may have arisen in the laying sheds.

Strong biosecurity practices to reduce introduction events, combined with rigorous cleaning procedures to eradicate persistent populations, should control *Salmonella* contamination. However, *Salmonella* was detected on farms deemed to have a high level of biosecurity and cleaning practices. This illustrates the challenge of eliminating *Salmonella* from the egg production environment, and underscores the importance of maintaining practices to mitigate *Salmonella* egg contamination risks to consumers.

Results of this survey establish a useful benchmark of *Salmonella* prevalence in the egg production environment. This benchmark could serve as a point of reference for assessing the impact on *Salmonella* prevalence resulting from changes to regulations or practices, as well as comparing the effectiveness of the wide range of current practices in the management of *Salmonella*.

2. INTRODUCTION

In 2015, 143 commercial egg producers operated in New Zealand, 18 of which produced 85% of the approx. 1 billion eggs produced per year [5]. Commercial egg layer farms are distributed throughout New Zealand.

New Zealand has a very low reported incidence of egg associated salmonellosis although contaminated eggs are a significant cause of foodborne salmonellosis overseas, including Australia. *Salmonella* contamination of eggs is a key risk factor influencing decisions about egg storage and shelf life.

To better understand the risk posed by *Salmonella* on eggs in New Zealand, and allow comparisons with Australian data, this study performed a baseline survey of *Salmonella* prevalence in the egg production environment, and assessed practices relevant to the control of *Salmonella* in the production environment and on eggs.

Foodborne non-typhoidal salmonellosis has a considerable impact on human health worldwide and a significant proportion of cases overseas have been associated with the consumption of contaminated eggs [1-3]. New Zealand has a very low reported incidence of egg-associated salmonellosis [4], with strong evidence for egg consumption accounting for only four of 204 salmonellosis outbreaks over the 2000-2009 period, and two of 106 salmonellosis outbreaks over the 2010-2014 period [5, 6]. However, the majority of reported salmonellosis cases are sporadic and transmission routes are usually not identified [5].

Reported rates of salmonellosis linked to egg consumption have increased significantly in Australia over recent years, with 166 outbreaks, 3200 cases, 650 hospitalisations and at least four deaths recorded over the 2001-2011 period [1].

Two main pathways exist by which egg contents become contaminated by *Salmonella*:

1. vertical (trans-ovarian) transmission, when *Salmonella* colonizing hen ovaries contaminates eggs prior to shell formation; or

2. horizontal (trans-shell) transmission, by direct faecal contamination of the egg as it is laid, or post-laying contamination from the external environment.

S. Enteritidis is the predominant serotype capable of ovarian colonization of hens and vertical transmission to eggs. It is the major serotype found in egg-laying chickens and attributed to egg-associated salmonellosis in Europe and North America [7-9]. However, *S*. Enteritidis is not currently considered endemic in poultry in Australia and New Zealand [10], and although other *Salmonella* serotypes may be able to be internalised in eggs, the source of most egg contaminations in Australia and New Zealand is thought to be external.

Multiple risk factors have been identified for *Salmonella* survival on, or internalisation into eggs. These risk factors include the type and motility of the *Salmonella* strain(s) present, the degree of faecal contamination of the egg, damage to the integrity of the egg cuticle, shell and/or membrane, the time and temperature of storage, and the presence of moisture (discussed in detail by [5, 10, 11]).

A 2007 survey assessing the presence of *Salmonella* in and on 3,710 cage-laid, free-range, and barn-laid eggs obtained from Auckland and Christchurch retail outlets identified *Salmonella* contamination on nine (1.8%) egg shell surfaces, but not within eggs [12]. All contaminated eggs were cage-laid (3.6% of cage-laid eggs). All isolates comprised *Salmonella* Infantis, which is endemic to New Zealand and commonly isolated from New Zealand salmonellosis cases. Four of the nine contaminated eggs were considered “dirty” (obvious contamination of shell with faecal, feather or other organic material). An Auckland

study in 2001 isolated *Salmonella* (*S*. Thompson, *S*. serotype 6,7 : k : - and *S*. Infantis) from the surfaces of eggs in 13/93 samples (14%) (six eggs in each sample), but not within eggs [4]. Finally, a 1995 South Island study did not detect *Salmonella* on 2,046 egg surfaces or from 2,037 egg contents [13]. Therefore, there is no evidence for internal egg contamination by *Salmonella* in New Zealand, but the prevalence of surface contamination of eggs in the 2007 survey was higher than reports from Australia, the United Kingdom and Northern Ireland [10, 14-16].

Minimising the presence of *Salmonella* on or in eggs involves reducing the risk of contamination during production and having control strategies post-collection, during storage, transport and food handling. Understanding the prevalence and risks associated with *Salmonella* in the egg production environment is an important prerequisite for establishing efficient *Salmonella* control and management procedures. The weak evidence for salmonellosis attributed to eggs from human health surveillance data, the low incidence of *Salmonella* contamination of egg surfaces, and the absence of evidence of internal contamination of eggs, suggest all that the risk posed by *Salmonella* from eggs in New Zealand is low.

Although testing for *Salmonella* is carried out on some of the larger farms in New Zealand, particularly those that export eggs, there is no information to date on the prevalence of *Salmonella* in the egg production environment. Therefore, this research was carried out to gain a better understanding of the current risk posed by *Salmonella* spp. on eggs in New Zealand. The research objectives of this survey were to determine the prevalence of *Salmonella* spp. on New Zealand layer farms, the potential sources of *Salmonella* spp. contamination and whether there was a correlation between the on-farm prevalences and egg contact surfaces in packhouse prevalences as to indicate a potential cross-contamination route to eggs.

The previously reported low rate of surface contamination of eggs by *Salmonella* in New Zealand indicated that testing of a large number of eggs would be required to achieve statistically valid results, as small numbers of positives may generate large uncertainty intervals [17]. This rationale for testing the laying environment instead of eggs was also adopted in recent Australian (New South Wales and Queensland) baseline surveys [26, 27].

Environmental testing of faeces and dust in the egg production environment has been shown to strongly correlate with the within-flock prevalence of *Salmonella* and forms the basis of most monitoring programs for *Salmonella* in the poultry industry [18-21]. A correlation has also been found between the prevalence of positive environmental samples and the number of contaminated eggs produced [25, 43, 44]. European Union (EU) sampling programs incorporate pooled faecal samples (cage flocks) or two pairs of boot swabs (barn and free-range flocks), which also pick up dust, food, and other detritus. Some EU sampling programs replace a faecal sample or boot swab with a dust sample collected from different areas of the shed, or swabs of surfaces with visible dust present. The two recent Australian egg layer farm surveys used pooled faecal samples and boot swabs [26, 27]. The sensitivity of pooled faecal samples has been reported to increase with an increasing number of droppings in the sample [19], with 60 pinch samples of individual faeces predicted to reliably detect 5% flock prevalence of *Salmonella* [7]. Dust samples have been found to be more sensitive than faecal samples for *Salmonella* [19], likely due to the organism being better-able to survive in dry conditions, and thus out-compete, other Enterobacteriaceae [34]. Furthermore, surveys using a combination of both faecal and dust samples have been found to be the most sensitive at detecting *Salmonella* than either sample type individually [18, 19, 35].

Due to the potential for hen feed to act as a contamination source for both hens and eggs [22-25], testing the farm level food supply is also often incorporated into environmental surveys [26, 27]. The MPI Risk Management Programme (RMP) template for eggs incorporates requirements to ensure *Salmonella* and other hazards are minimised in feed [30]. Compared with the farm-level supply source, feed at the point of consumption has a

higher likelihood for *Salmonella* presence due to cross-contamination from hen faeces, dust and litter making it difficult to attribute the source of contamination [27].

Egg collection and packing areas are also important potential reservoirs for external contamination of egg shells, with overseas studies isolating *Salmonella* from a high proportion of egg contact packhouse surfaces [24, 28, 29]. The presence of *Salmonella* on egg contact surfaces in the packhouse environment acts as an indicator that *Salmonella* could also be present on eggs.

3. MATERIALS AND METHODS

3.1 SELECTION OF LAYER FARMS FOR SAMPLING

Farms for which data were available were sorted into six geographic regions (TABLE 1)[1]. To eliminate regional bias, the survey aimed to achieve a proportionate number of farms from the different egg-laying regions. Farms from each region were in part randomly selected by random number generation using the standard Microsoft Excel random number generator (RANDBETWEEN) function.

TABLE 1. Regional breakdown of New Zealand egg layer farms. Numbers are based on egg producer farms for which data were available at the time of selection (137 out of a total 143 farms).

DESIGNATED REGION	REGIONS	NUMBER OF FARMS IN REGION	PECENTAGE OF FARMS IN NZ	NUMBER OF FARMS IN SURVEY	PERCENTAGE OF FARMS IN SURVEY
1 North NI	Northland, Auckland	40	29.2	8	28.6
2 Mid NI	Waikato, Bay of Plenty, Gisborne, Hawke's Bay	29	21.2	3	10.7
3 South NI	Taranaki, Manawatu-Wanganui, Wellington	24	17.5	8	28.6
4 North SI	Tasman, Marlborough	7	5.1	2	7.1
5 Mid SI	West Coast, Canterbury	16	11.7	3	10.7
6 South SI	Otago, Southland	21	15.3	4	14.3
Total		*137*	*100*	*28*	*100*

In addition, selection was also influenced by farm practices. In particular, the survey sought to include a representation of the criteria listed below:

- All laying systems. The majority of hens in New Zealand are conventionally caged (67%), with an additional 14% colony-caged systems, and 19% free-range or barn-layer systems. In general, although the number of cage-free farms is greater, caged system farms and sheds house more birds and flocks than cage-free sheds.
- All egg washing practices; including no egg washing, washing only dirty eggs, and washing all eggs.
- Single and multi-aged flocks.
- All production sizes. The survey aimed to achieve approximately 50% each of farms defined as high production farms (>20,000 birds), and small production (≤20,000 birds). Selected farms contained from 500 to 405,800 birds.

Using the above criteria, a total of forty-seven farms were invited to participate in the survey. Based on the willingness of businesses to participate, as well as statistical considerations, twenty-eight farms were then selected for the survey. (See TABLE 2 for farm practices of New Zealand layer farms and selected farms, and Section 3.5 for statistical considerations). The twenty-eight farms represented 20% of the total egg producers, and contained 46.0% of total laying hens (1.60 million of 3.48 million), in New Zealand.

[1] To minimise the ability for individual farms to be identified, the regions of specific farms have not been mentioned in the report.

TABLE 2. Layer farm data for New Zealand farms and selected farms in this survey (28 farms).

		% NZ FARMS[1]	% FARMS IN SURVEY[1]	% PACKHOUSES IN SURVEY
LAYER SYSTEM	Conventional cage	17.2	35.7	
	Colony cage	3.9	21.4	
	Free-range	67.7	60.7	
	Barn	11.0	17.9	
	No data	16.5		
FLOCK AGE	Single-age	46.5	75.0	
	Multi-age	33.9	46.4	
	No data	29.1		
FLOCK SIZE	Large >20,000	27.6	53.6	
	Small ≤20,000	58.3	46.4	
	No data	14.2		
EGG WASHING	None	22.8		38.5
	When dirty	44.1		38.5
	All non-cracked eggs	6.3		23.1
	No data	26.8		

[1]numbers add up to >100 as some farms have more than one parameter.

3.2 FARM VISITS

Farms visits were performed in order to collect samples of the layer environment.

Farm visits were conducted by two people; with one surveyor visiting North Island farms, and the other, South Island farms. Both surveyors conducted the first farm visit to standardise sampling methodology. The surveyors were accompanied at all times during the visit, typically by the farm owner and/or manager. The entire visit lasted from between two to four hours, depending on the size of the production operation. Because the *Salmonella* status of a flock may be influenced by seasonality and associated environmental factors such as humidity [31, 32], to improve comparability all farm visits were conducted within a two month period between October to December, 2016. Environmental conditions during the visits were noted. Temperatures ranged from 9-24°C, and the weather was raining during seven visits.

3.3 ENVIRONMENTAL SAMPLING

Sample types and methodology

The sampling plan was structured to generate data on *Salmonella* prevalence amongst New Zealand commercial egg layer flock and to identify the nature and importance of the potential sources of *Salmonella* contamination of flocks and/or egg environments. Samples included inputs (feed), pooled faecal material, pooled dust samples, boot swabs (cage-free systems) or manure swabs (caged systems), and samples from grading/packing sheds.

Whenever possible, the farm-level food supply was tested. Where samples from the feed silo could not be obtained, samples were taken from the shed hopper, or if also not available, the feed trough. Because the samples from the feed trough came from a different source than those from the farm level source, the results could not be directly compared.

Within the grading shed and packhouse, egg contact surfaces were swabbed, including conveyors, rollers, candlers, grading equipment, tables, brushes, suction cups. Where present, egg wash cloths were sampled.

The sampling methodology is described for each type of sample in TABLE 3.

TABLE 3. Sample types and sampling methodology.

SAMPLE	SAMPLE METHODOLOGY
Feed	Approx. 500 g sample from farm storage source. When a sample from farm source/silo could not be obtained, a sample was acquired from shed hopper, or when not available, feed trough. Tested one sample/farm, or more when sheds tested used different feed types.
Faecal material	Barn/free-range systems: approx. 200 g or 60 pinches of moist faecal material was collected from different areas of the floor or nesting boxes. Caged systems: approx. 200 g or 60 pinches of moist faecal material was collected from ends of manure belts. Where possible, the farmer was asked to run manure belt prior to sampling for fresh faecal material. Samples were collected into sample bags using gloved hands or sterile tongue depressor applicators. Tested up to three laying sheds/farm.
Dust sample	Approx. 100 g/250 ml of dust material was collected from ~20 surfaces throughout shed with visible dust presence e.g. air exhaust baffles, ledges, horizontal beams, surfaces of nest boxes (barn and free-range), egg belts and cage ledges (caged systems). Samples were collected into sample bags using sterile tongue depressor applicators. Tested up to three laying sheds/farm.
Boot swab	Barn/free-range sheds: 1 pair of boot swabs, pre-wetted with skim milk (Hardy Diagnostics), were placed on boots over plastic boot covers (Nasco, Hardy Diagnostics) and ~100 paces taken covering ~50% of bird access area during process of other sampling. Tested up to three laying sheds/farm.
Cage swab	Caged sheds: Sponges pre-wetted with buffered peptone water (BPW) (World Bioproducts) swabbed on ends of manure belts of multiple tiers and cage lines. Tested up to three laying sheds/farm.
Packhouse egg contact surfaces	For each egg packhouse, tested up to six sites where appropriate, including swabs of: 1. egg accumulator/conveyor/roller OR reusable egg collection trays 2. candler/candler rollers 3. egg grading equipment/table 4. egg suction cups 5. egg roller brushes 6. egg wash cloth Swabs prewetted with BPW (World Bioproducts) were swabbed over running equipment for 2 minutes, or ~1 m^2 area, as appropriate.

Sample size determination

The number of sheds surveyed per farm was determined by the number of sheds present, as well as flock size. Similar to criteria from the recent Queensland 2014 egg layer survey [26], 1-2 sheds from farms designated as “small” (≤20,000 birds) and up to 3 sheds from larger farms (>20,000) birds) were sampled. In systems where >2 (small farms) or >3 (large farms) sheds were present, shed selection was based on capturing different variables present within the farm. In particular, sheds were selected based on flock age (youngest and oldest flock age where two sheds were tested; youngest, oldest and median flock age where three sheds were tested). In addition, differing layer systems were sampled when present within the same farm. The total number of sheds sampled (67) contained 25.0% (0.87 million of 3.48 million) of all laying birds in New Zealand.

The desired number of each sample type to be obtained was calculated based on the number of farms surveyed and the number of sheds sampled per farm (TABLE 4). No historical *Salmonella* prevalence data was available for New Zealand layer farms. Therefore, the average predicted prevalence was calculated using prevalence values obtained from published surveys from other countries (TABLE 4). Average prevalence over all samples for

Australian surveys was 18.6% [26, 27]. Assuming the across-industry prevalence in New Zealand is ≤18% for each sample type, it was calculated that at least 62 samples of each sample type would be required to provide 90% confidence of the sampling prevalence being within 5% of the true prevalence. Calculations, using a one-tailed, binomial distribution, exact test, and a power of 0.8, with G-power 3.1 software, determined the independent chance of *Salmonella* presence in each single sample. Based on the predicted number of samples of each sample type that we would obtain from a given number of farms, sampling at least 28 farms was found sufficient to achieve this statistical power.

TABLE 4. Predicted sample number and *Salmonella* prevalence for each sample type proposed in this study.

SAMPLE TYPE	SAMPLE NUMBER[1]				AVG PREDICTED PREVALENCE[2]	LOCATION	REFERENCE
	20 farms	**25 farms**	**28 farms**	**30 farms**			
faeces	45	56	63	68	23	Australia (NSW, QLD)	[26, 27]
dust	45	56	63	68	51[3]	Europe	[18]
farm level feed	20	25	28	30	5.5	Australia (NSW, QLD)	[26, 27]
boot/cage swabs	45	56	63	68	32	Australia (NSW, QLD)	[26, 27]
packhouse	100	125	140	150	25[3]	England, Wales	[28]
total	*255*	*318*	*357*	*384*			

[1] Sample numbers were calculated based on 3 sheds each being sampled on 50% of the farms, 2 sheds on 25% of farms, 1 shed on 25% of farms, and an average of 5 packhouse samples per farm.

[2] The reported *Salmonella* prevalence for different sample types was calculated from recent studies that utilised similar testing parameters to those proposed here, and where possible, were from Australia, where *S.* Enteritidis is not currently endemic.

[3] Prevalence from countries where *S.* Enteritidis is endemic.

3.4 MICROBIOLOGICAL ANALYSIS

Samples were sent on ice to the ESR Public Health Laboratory, Christchurch, stored overnight, and tested the following day. Testing for *Salmonella* spp. was performed according to the standardised methods currently used in the European Union (ISO 6579:2002 for feed samples and ISO 6579:2002/Amd.1:2007 for faecal and dust samples, boot, cage and packhouse swabs).

Briefly, homogenous samples were added to BPW at a 1 to 10 dilution (25 g aliquots of feed, dust or faeces, swabs in 10 ml BPW, boot swabs, and egg wash cloths were added to 225, 90, 200 and 300 ml BPW, respectively) and incubated for 37°C ± 1°C for 18 h ± 2 h for pre-enrichment.

For feed sample enrichment in selective media, 0.1 ml of BPW enrichment was added to 10 ml Rappaport-Vassiliadis with soya (RVS) broth (Fort Richard Laboratories, Auckland, New Zealand) and incubated at 41.5 ± 1°C for 24 ± 3h. In addition, 1 ml of BPW was added to 10ml Muller-Kauffmann tetrathionate novobiocin (MKTTn) broth (Oxoid; Thermofisher, Auckland, New Zealand) and incubated at 37 ± 1°C for 24 ± 3h.

For non-feed sample enrichment in selective media, three 33 µl volumes of BPW broth were plated to a modified semi-solid Rappaport-Vassiliadis (MSRV) agar plate (Oxoid; Thermofisher), and plates were incubated at 41.5 °C ± 1°C for 24 h ± 3 h. 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, to ensure that enrichment on MSRV medium was sufficiently robust to select for

Salmonella isolates present, pre-enrichments from the first eight farms (representing 92 samples) were also inoculated into MKTTn broth, incubated at 37°C± 1°C for 24 h ± 3 h. As complete concordance between MKTTn broth and MSRV plates for presumptive *Salmonella* presence/absence was observed, subsequent enrichments were plated to MSRV medium only.

Following selective enrichments, broths and opaque growth zones from MSRV plates were streaked onto Xylose Lysine Deoxycholate agar (XLD), Hektoen Enteric agar (HE) and Bismuth Sulphite agar (BSA) plates. Presumptive *Salmonella* isolates were sub-cultured, and 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.

3.5 WHOLE GENOME SEQUENCE ANALYSES

Purified isolates confirmed as *Salmonella* spp. were further genotyped using whole genome sequence analysis. One *Salmonella* isolate per positive sample was selected and DNA was extracted using the Bioline Isolate II Genomic DNA kit according to the manufacturer's instructions (Bioline, Total Lab Systems, Auckland, New Zealand). DNA libraries were prepared using the TruSeq Nano DNA Library Preparation Kit (Illumina) and sequenced using an Illumina MiSeq platform with MiSeq V2 chemistry, and 2x150 paired-end runs. Sequence coverage was typically ≥40x. Sequence quality checks were performed using Nullarbor and FastQC software. Sequence reads were trimmed using Trimmomatic 0.33 (Illumina) to remove adapter sequences and bases below a quality score 10 (Phred33 scale). Contaminating PhiX sequences were removed by aligning to a PhiX reference genome using Bowtie 2 software. Sequence reads below 50 bases were discarded. *De novo* assembly was performed on processed reads using SPAdes 3.9 software (settings --only-assembler --careful -k 21,33,55,77,99,127).

Serotypes were assigned using both the *Salmonella* In Silico Typing Resource (SISTR) [36] and online SeqSero algorithms [37] from assembled sequence or paired-end sequence read sets (the algorithms were in agreement for all assignments).

Within-serotype comparisons were performed using whole genome sequence analysis tools within BioNumerics 7.6 (Applied Maths). Whole genome single nucleotide polymorphism (SNP) analysis of paired-end sequence read sets were mapped against the genome assembly of one of the isolates included in the comparison. The published, high quality *S*. Typhimurium LT2 genome (NCBI accession numbers NC_003197 and NC_003277; [38]) was selected as a default reference genome for wider genomic comparisons. For comparisons between more closely related isolates, reference genomes were selected based on having a high genomic coverage and high N50 value (*S*. Thompson isolate 16PH0683-001, farm 14, feed; *S*. Infantis isolate 16PH0644-009, farm 4, shed 3, dust; *S*. Typhimurium isolate 16PH0752-003; farm 24, shed 1, dust sample). SNP filtering was set at strict (parameters: inter-SNP distance, minimum 12 bp between SNPs; non-informative SNPs, remove non-informative SNP positions; absolute coverage, total 5, forward 1, reverse 1; ambiguous bases, remove positions with at least one ambiguous base; unreliable bases, remove positions with at least one unreliable base; gaps, remove positions with at least one gap). Cluster analysis trees were constructed using the BioNumerics 7.6 Neighbour Joining Tree algorithm.

4. RESULTS

This survey aimed to determine the prevalence and potential contamination sources of *Salmonella* spp. on commercial New Zealand layer farms.

4.1 *SALMONELLA* DETECTION ON FARMS

Laboratory results (presence/absence of *Salmonella*) were provided to farmers within three weeks of farm visits, with an offer of assistance from MPI or an industry avian veterinarian to review farm practices, where required.

Data are summarised in TABLE 5. In total, *Salmonella* was detected in 43 of the 323 samples tested. Of the twenty-eight farms sampled, twelve had at least one *Salmonella*-positive sample, although only eight had more than one positive sample. At least one *Salmonella*-positive sample was obtained in 21 of the 67 sheds. Furthermore, 3 of the 26 packhouses sampled had at least one positive sample. *Salmonella* was isolated from farms in all six regions of New Zealand.

FIGURE 1 depicts the observed prevalence of *Salmonella* in feed (farm-level input), egg layer (faeces, boot/manure belt swabs, dust), and packhouse environments (egg conveyor/accumulator/roller/reusable egg collection trays, candler/candler rollers, egg grading equipment/table, egg roller brushes, egg suction cups, egg wash cloth). Overall, the highest prevalence of *Salmonella* was observed in dust samples from the layer shed environment.

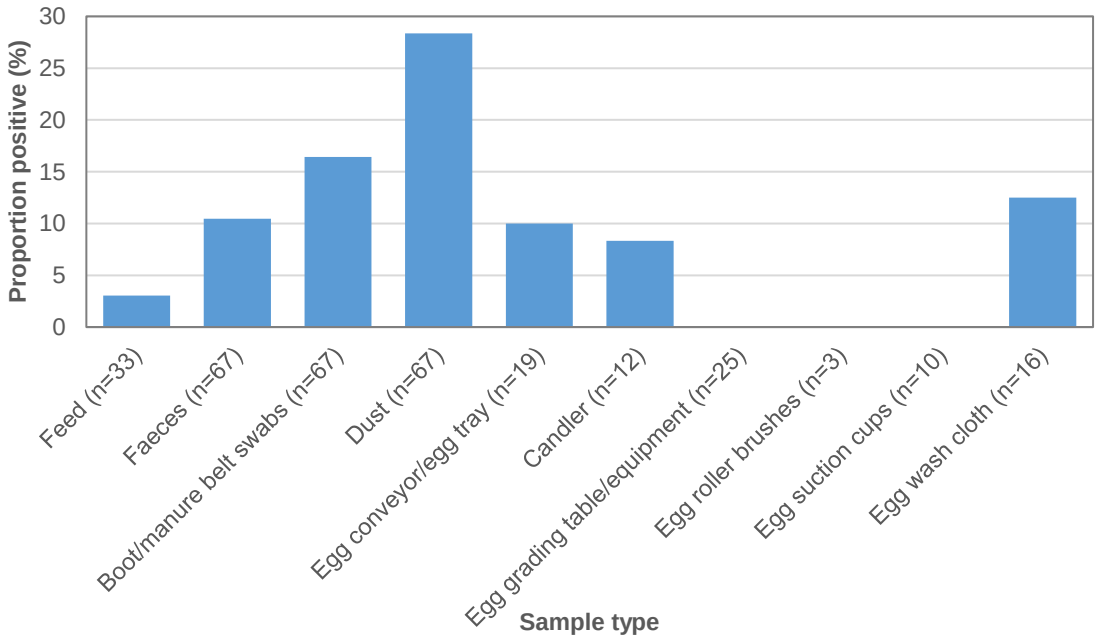

FIGURE 1. *Salmonella* prevalence in feed, egg layer environment and packhouse samples.

TABLE 5. Summary of *Salmonella* prevalence for each farm in the survey[1].

FARM	LAYER SYSTEM	RESULTS BY SAMPLE TYPE					RESULTS BY SAMPLE TYPE						TOTAL POSITIVE SAMPLES
		SHED 1	SHED 2	SHED 3	POSITIVE SAMPLES	POSITIVE SHEDS	FEED	DUST	FAECES	BOOT/ MANURE BELT	PACK HOUSE	OTHER	
1	Free-range	0/3	0/3	0/3	0/9	0/3	0/2	0/3	0/3	0/3	0/3	0/1	0/15
2	Colony cage	0/3	0/3		1/9	1/3	0/1	0/2	0/2	0/2	0/4		**1/14**
	Conventional cage			1/3				1/1	0/1	0/1			
3	Conventional cage	1/3			2/9	2/3	0/1	1/1	0/1	0/1	0/4	0/1	**2/15**
	Colony cage		0/3	1/3				1/2	0/2	0/2			
4	Colony cage	3/3			7/9	3/3	0/1	1/1	1/1	1/1	2/4		**9/14**
	Conventional cage		3/3	1/3				2/2	1/2	1/2			
5	Free-range	0/3	0/3		0/6	0/2	0/1	0/2	0/2	0/2	0/2		0/9
6	Conventional cage	3/3	3/3		7/9	3/3	0/2	2/2	2/2	2/2	1/4		**8/15**
	Colony cage			1/3				1/1	0/2	0/2			
7	Free-range	0/3	1/3	0/3	1/9	1/3	0/1	1/3	0/3	0/3	0/3		**1/13**
8	Free-range	0/3	0/3	0/3	0/9	0/3	0/1	0/3	0/3	0/3	0/4		0/14
9	Free-range	0/3	1/3		1/6	1/2	0/1	0/2	0/2	1/2	0/1		**1/8**
10	Colony cage	0/3			0/9	0/3	0/1	0/1	0/1	0/1	0/4		0/14
	Conventional cage		0/3	0/3				0/2	0/2	0/2			
11	Free-range	0/3			0/3	0/1	0/1	0/1	0/1	0/1	0/4		0/8
12	Barn	0/3	0/3		0/6	0/2	0/1	0/2	0/2	0/2	0/3		0/10
13	Barn	0/3			0/9	0/3	0/1	0/1	0/1	0/1	0/4		0/14
	Free range		0/3	0/3				0/2	0/2	0/2			
14	Conventional cage	3/3	2/3	1/3	6/9	3/3	1/1	2/3	2/3	2/3	2/5		**9/15**
15	Free-range	0/3			0/3	0/1	0/1	0/1	0/1	0/1	0/2		0/6
16	Conventional cage	3/3			3/9	1/3	0/2	1/1	1/1	1/1	0/5		**3/16**
	Barn		0/3					0/1	0/1	0/1			
	Organic free -range			0/3				0/1	0/1	0/1			
17	Free-range	0/3	0/3		0/6	0/2	0/1	0/2	0/2	0/2	0/4		0/11
18	Barn	0/3	0/3	0/3	0/9	0/3	0/1	0/3	0/3	0/3	0/4		0/14
19	Free-range	0/3			0/6	0/2	0/1	0/1	0/1	0/1	0/4		0/11
	Barn		0/3					0/1	0/1	0/1			
20	Conventional cage	2/3			2/3	1/1	0/1	1/1	0/1	1/1	0/3		**2/7**
21	Colony cage	2/3	0/3		4/9	2/3	0/1	1/1	0/1	1/1	0/2		**4/12**
	Conventional cage			2/3				1/2	0/2	1/2			
22	Free-range	0/3	0/3	0/3	0/9	0/3	0/2	0/3	0/3	0/3			0/11
23	Conventional cage	0/3	1/3		1/6	1/2	0/1	1/2	0/2	0/2	0/2		**1/9**
24	Free-range	1/3	1/3		2/6	2/2	0/2	2/2	0/2	0/2	0/1		**2/9**
25	Free-range	0/3	0/3		0/6	0/2	0/1	0/2	0/2	0/2	0/3		0/10
26	Free-range	0/3	0/3		0/6	0/2	0/1	0/2	0/2	0/2			0/7
27	Organic free -range	0/3	0/3		0/6	0/2	0/1	0/2	0/2	0/2	0/5		0/12
28	Free-range	0/3	0/3		0/6	0/2	0/1	0/2	0/2	0/2	0/3		0/10
Total					**37/201**	**21/67**	1/33	19/67	7/67	11/67	5/87	0/2	**43/323**

[1] Sheds were considered positive if at least one sample was positive.

- Purple arrows indicate "high-prevalence" farms with >50% positive results.
- Colour coding:
  - light grey: when there are no samples in the corresponding category
  - orange: positive result for sheds
  - green: positive samples per sample type
  - blue: total positive samples

Feed. Only 1 out of 33 feed samples tested positive for *Salmonella* (FIGURE 1), and this was a sample obtained from the trough in the shed.

Layer shed samples. The highest prevalence of *Salmonella* detection was from dust samples (19/67), followed by boot/manure belt swabs (11/67), and faecal samples (7/67) (FIGURE 1). The majority of the sheds (9/11) for which boot/manure, belt swabs or faeces tested positive also had a positive dust sample.

For six farms, a single shed tested positive, while for five farms, all sheds sampled (from one to three) tested positive. The number of positive samples per shed ranged from eleven sheds with just one positive sample (which comprised dust in 10/11 sheds), four sheds each with two positive samples, and six sheds with three positive samples (TABLE 5). The total number of positive shed samples per farm ranged from 1 to 7 (although the sample number was influenced by the numbers of sheds sampled, determined by farm and total flock size) (TABLE 5). Farms in which the highest number of shed samples tested positive were arbitrarily designated as "high-prevalence" farms for the purpose of this survey. Three "high-prevalence" farms were identified, farms 4 and 6 (7/9 positive shed samples) and 14 (6/9, positive shed samples).

Packhouse samples. *Salmonella* was isolated from 5/87 packhouse egg contact surfaces. Importantly, the only three farms with positive packhouse samples were those designated "high-prevalence" farms (farms 4, 6 and 14), and in which the highest number of positive layer shed samples were obtained (7/9, 7/9, and 6/9).

4.2 PREVALENCE OF *SALMONELLA* BASED ON PRODUCTION SYSTEM, FLOCK SIZE, SINGLE/MULTI-AGE FLOCK MANAGEMENT, AND FLOCK AGE

The *Salmonella* prevalence in shed samples was analysed in relation to the type of layer production system, flock size, single/multi-age flock management, and flock age variables. Because certain comparisons included arbitrary designations, and due to the small sample size relevant to certain criteria, statistical calculations to determine significance of observations were not made.

Production system. The prevalence of *Salmonella*-positive sheds and shed samples based on the shed layer system (caged systems including conventional cage and colony cage, and cage-free systems including free-range and barn) is shown in FIGURE 2 and FIGURE 3. Conventional cage sheds had the highest proportion of sheds with at least one positive sample (13/16), followed by colony cage sheds (4/9), then free-range sheds (4/34); no *Salmonella*-positive samples were obtained from barn sheds. This difference applied across all sample categories. Three free-range sheds were also classified as organic, and two cage-free sheds were classified as an aviary/multi-tier system; no *Salmonella*-positive samples were obtained from these sheds.

When shed sample numbers were combined, there was a significantly higher prevalence of *Salmonella*-positive shed samples from caged system sheds (33/75) than from cage-free sheds (4/126) ($p<10^{-12}$, Pearson's chi-squared test). Nine of the ten farms in which caged system sheds were sampled had at least one positive *Salmonella* sample. Finally, all three farms designated "high-prevalence", and from which *Salmonella* was detected in packhouse samples, were caged-system sheds and farms. (Note that when different layer systems were present on one farm, up to three systems were tested. Therefore, based on our results, the proportion of caged system sheds tested could influence whether farms were designated "high-prevalence".)

In addition to the different shed infrastructure, multiple interconnected practices differ between caged and cage-free systems; for example, flock size, and single versus multi-age

flock management, making it difficult to assess the contribution of any one variable on *Salmonella* prevalence. Possible associations of each practice relative to *Salmonella* prevalence are discussed in the following categories.

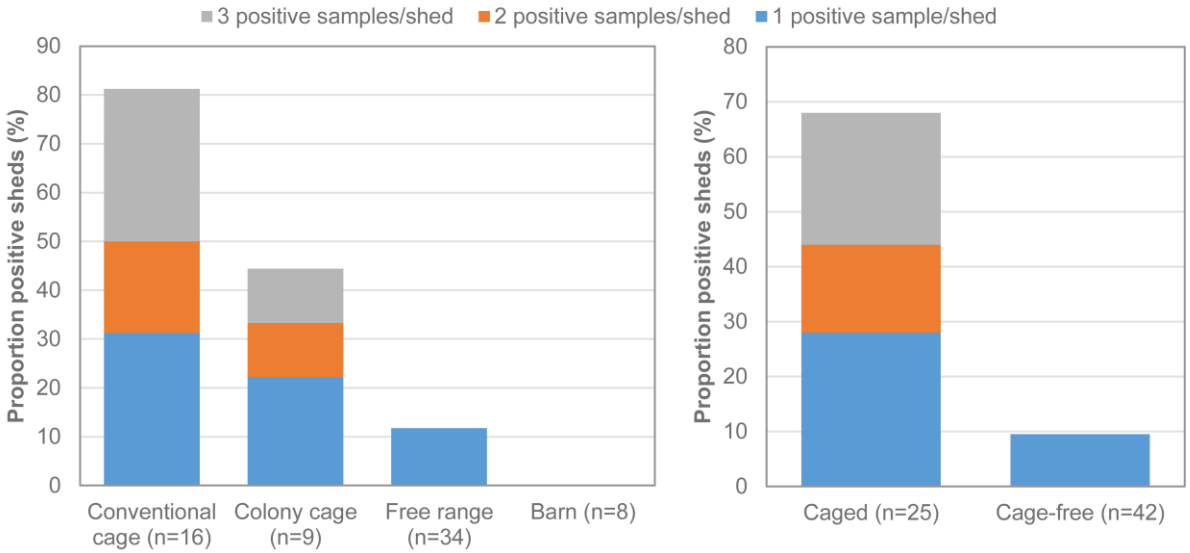

FIGURE 2. *Salmonella* prevalence in egg layer sheds based on production system. n = total number of sheds.

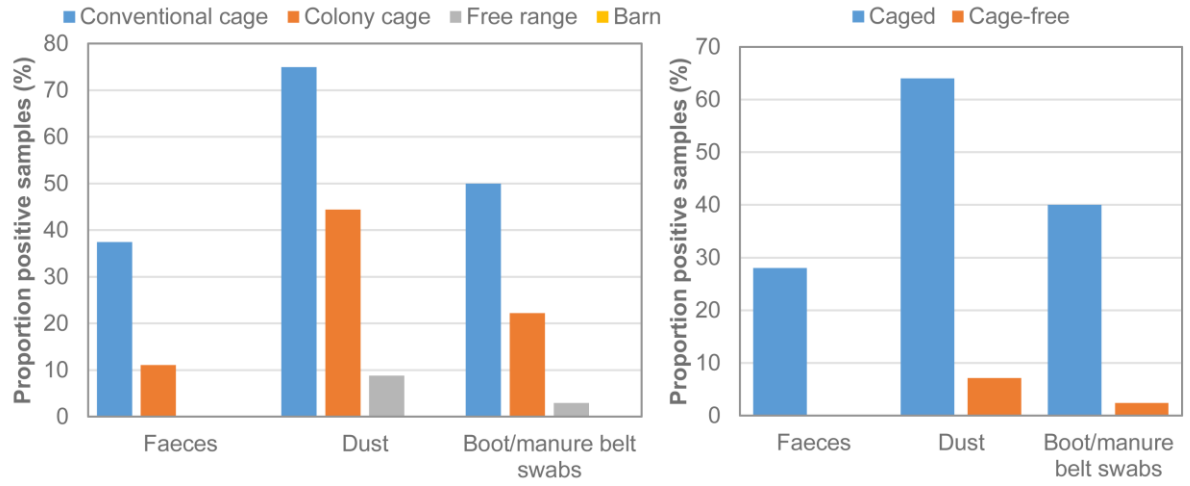

FIGURE 3. *Salmonella* prevalence in egg layer environmental samples based on production system.

Flock size. The number of birds housed in conventional or colony cage sheds sampled in this survey were on average higher than flock numbers in cage free sheds (FIGURE 4A). The prevalence of *Salmonella*-positive sheds increased as flock numbers per shed increased, ranging from 13.8% (4/29) for sheds housing ≤5,000 birds, to 75.0% (6/8) for sheds housing >30,000 birds (FIGURE 4B). For positive sheds with ≤5,000 birds, only a single sample was positive per shed, but for larger flock sizes, 50% of positive sheds had at least two positive samples.

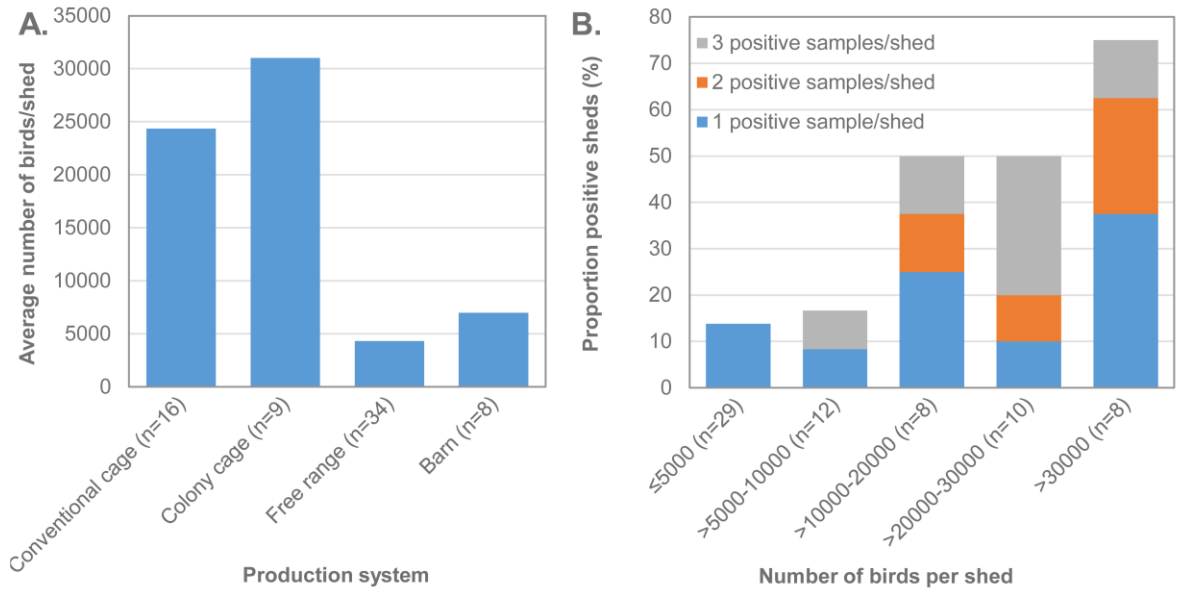

FIGURE 4. Flock size dynamics for (A) production systems and (B) *Salmonella* prevalence in egg layer sheds. n = total number of sheds.

Single versus multi-age flock management. Multi-age flock management is more prevalent in caged systems than cage-free systems, as was also noted in this survey (FIGURE 5A). A 3.4-fold higher percentage of multi-aged sheds were *Salmonella*-positive (12/19) compared with single-aged sheds (9/48; FIGURE 5B).

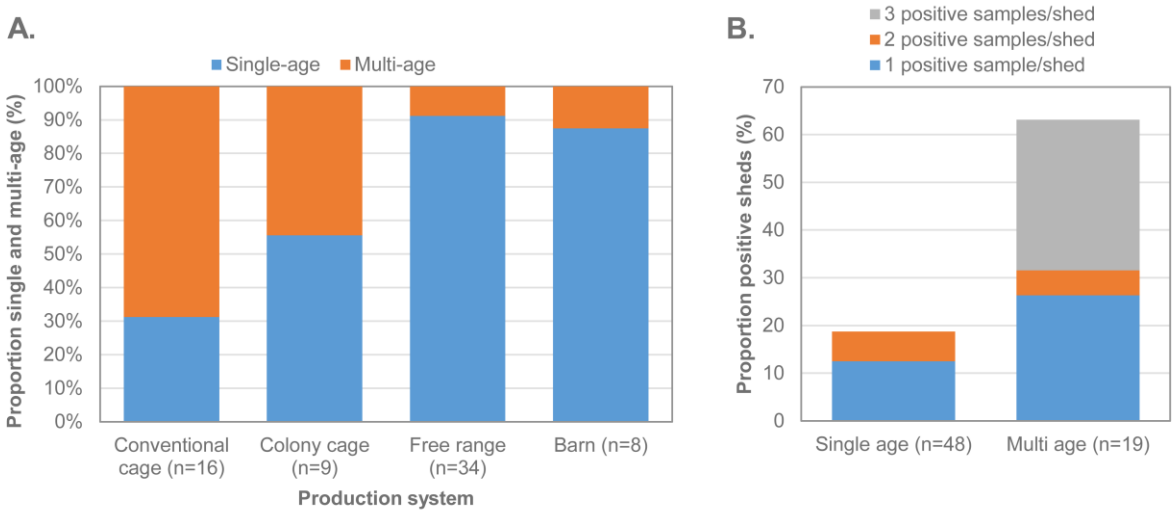

FIGURE 5. Single and multi-age flock dynamics for (A) production systems and (B) *Salmonella* presence in egg layer sheds. n = total number of sheds.

Flock age. The influence of flock age on *Salmonella* prevalence of sheds was considered. The ages of flocks surveyed in this survey ranged from 18 to 81 weeks. Ages for single-aged flocks were arbitrarily divided into three groups, each containing 22-23 week intervals (early lay, 15-37 weeks; mid-lay, 38-60 weeks; and late-lay, >61 weeks). The highest *Salmonella* prevalence was for late-lay flocks, followed by early age, with zero positive single-age sheds for mid-lay flocks (FIGURE 6).

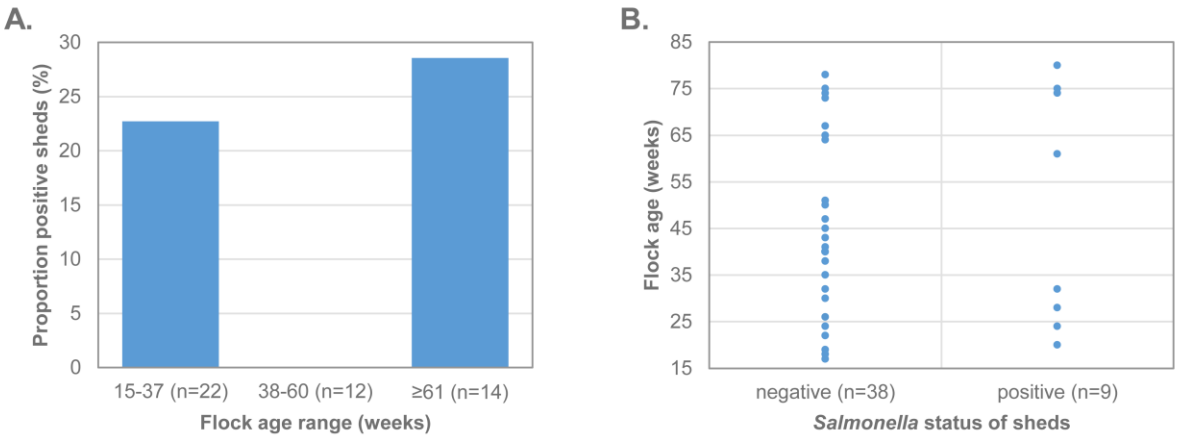

FIGURE 6. *Salmonella* presence in egg layer sheds based on flock age of single aged sheds. (A) Proportion of *Salmonella*-positive sheds in arbitrary age designations. (B) Scatter plot of flock age versus *Salmonella*-positive/negative status of layer shed. n = total number of sheds for each variable.

4.3 *SALMONELLA* SEROTYPES ISOLATED

Salmonella strains isolated from the layer farm environment grouped into five serotypes; the most common was *S*. Infantis (19 isolations), followed by *S*. Thompson (15 isolations), *S*. Typhimurium (6 isolations), *S.* Anatum (2 isolations) and *S*. Mbandaka (isolated once) (TABLE 6). *S.* Enteritidis was not identified in any of the samples.

None of the *S*. Typhimurium isolates from this survey were identified as the attenuated MeganVac®1 vaccine strain. Unlike the environmental isolates in this survey, the vaccine strain differs in biochemical profile (weak H_2S reaction, and types as *Hafnia alvei* using rapid identification systems [39]). In addition, the vaccine strain has mutations in *cya* (adenylate cyclase) and *crp* (cAMP receptor protein) genes [40-42]. However, both genes were intact in the annotated genomes of each isolate from this survey, and a Basic Local Alignment Search Tool (BLAST) search of the annotated genes against the GenBank database confirmed that the annotation of these genes was also correct.

TABLE 6. Serotypes of layer farm *Salmonella* isolates.

ISOLATE NUMBER	FARM NUMBER	LAYER SYSTEM	SHED/ PACKHOUSE	SAMPLE TYPE	SEROTYPE
16PH0632-009	2	Conventional cage	Shed 3	Dust	Typhimurium
16PH0633-003	3	Conventional cage	Shed 1	Dust	Anatum
16PH0633-009	3	Colony cage	Shed 3	Dust	Anatum
16PH0644-002	4	Colony cage	Shed 1	Faeces	Infantis
16PH0644-003	4	Colony cage	Shed 1	Dust	Infantis
16PH0644-004	4	Colony cage	Shed 1	Manure belt swab	Infantis
16PH0644-005	4	Conventional cage	Shed 2	Faeces	Infantis
16PH0644-006	4	Conventional cage	Shed 2	Dust	Infantis
16PH0644-007	4	Conventional cage	Shed 2	Manure belt swab	Infantis
16PH0644-009	4	Conventional cage	Shed 3	Dust	Infantis
16PH0644-011	4	Conventional/colony cage	Packhouse	Egg accumulator/conveyor/roller	Infantis
16PH0644-014	4	Conventional/colony cage	Packhouse	Egg wash cloth	Infantis
16PH0657-002	6	Conventional cage	Shed 1	Faeces	Thompson
16PH0657-003	6	Conventional cage	Shed 1	Dust	Thompson
16PH0657-004	6	Conventional cage	Shed 1	Manure belt swab	Thompson
16PH0657-005	6	Conventional cage	Shed 2	Faeces	Thompson
16PH0657-006	6	Conventional cage	Shed 2	Dust	Thompson
16PH0657-007	6	Conventional cage	Shed 2	Manure belt swab	Thompson
16PH0657-009	6	Colony cage	Shed 3	Dust	Thompson
16PH0657-015	6	Conventional/colony cage	Packhouse	Egg wash cloth	Thompson
16PH0658-006	7	Free-range	Shed 2	Dust	Typhimurium
16PH0663-006	9	Free-range	Shed 2	Boot swab	Typhimurium
16PH0683-001	14	Conventional cage	Shed 1	Feed	Thompson
16PH0683-002	14	Conventional cage	Shed 1	Faeces	Thompson
16PH0683-003	14	Conventional cage	Shed 1	Dust	Thompson
16PH0683-004	14	Conventional cage	Shed 1	Manure belt swab	Thompson
16PH0683-005	14	Conventional cage	Shed 2	Faeces	Infantis
16PH0683-007	14	Conventional cage	Shed 2	Manure belt swab	Thompson
16PH0683-009	14	Conventional cage	Shed 3	Dust	Infantis
16PH0683-011	14	Conventional cage	Packhouse	Egg accumulator/conveyor/roller	Thompson
16PH0683-012	14	Conventional cage	Packhouse	Candler/candler rollers	Thompson
16PH0682-002	16	Conventional cage	Shed 1	Faeces	Infantis
16PH0682-003	16	Conventional cage	Shed 1	Dust	Infantis
16PH0682-004	16	Conventional cage	Shed 1	Manure belt swab	Infantis
16PH0712-003	20	Conventional cage	Shed 1	Dust	Infantis
16PH0712-004	20	Conventional cage	Shed 1	Manure belt swab	Infantis
16PH0743-003	21	Colony cage	Shed 1	Dust	Mbandaka
16PH0743-004	21	Colony cage	Shed 1	Manure belt swab	Infantis
16PH0743-009	21	Conventional cage	Shed 3	Dust	Infantis
16PH0743-010	21	Conventional cage	Shed 3	Manure belt swab	Infantis
16PH0746-006	23	Conventional cage	Shed 2	Dust	Typhimurium
16PH0752-003	24	Free-range	Shed 1	Dust	Typhimurium
16PH0752-006	24	Free-range	Shed 2	Dust	Typhimurium

4.4 GENOTYPIC COMPARISONS OF *SALMONELLA* ISOLATES

Whole genome SNP analyses were employed to compare the relatedness between:

- *S*. Typhimurium isolates with clinical isolates of different NZ-relevant phage types.
- Other serotypes were also compared to historical clinical isolates.
- Isolates of the same serotypes (including *S*. Typhimurium) originating from the same and different farms.

Comparison of *S.* Typhimurium isolates with NZ-relevant phage types. Genomes from the six *S.* Typhimurium isolates from this survey were compared with those of 90 New Zealand clinical *S.* Typhimurium isolates for which genomic data was available (data provided by ESR). The 90 clinical isolates comprised 14 commonly isolated phage types in New Zealand over the last three years[2] and included amongst the most common phage types from similar Australian egg layer surveys [26, 27].

Certain phage types, for example definitive phage types (DT) 108/170 and DT42, were distributed into more than one different phylogenetic cluster (FIGURE 7).

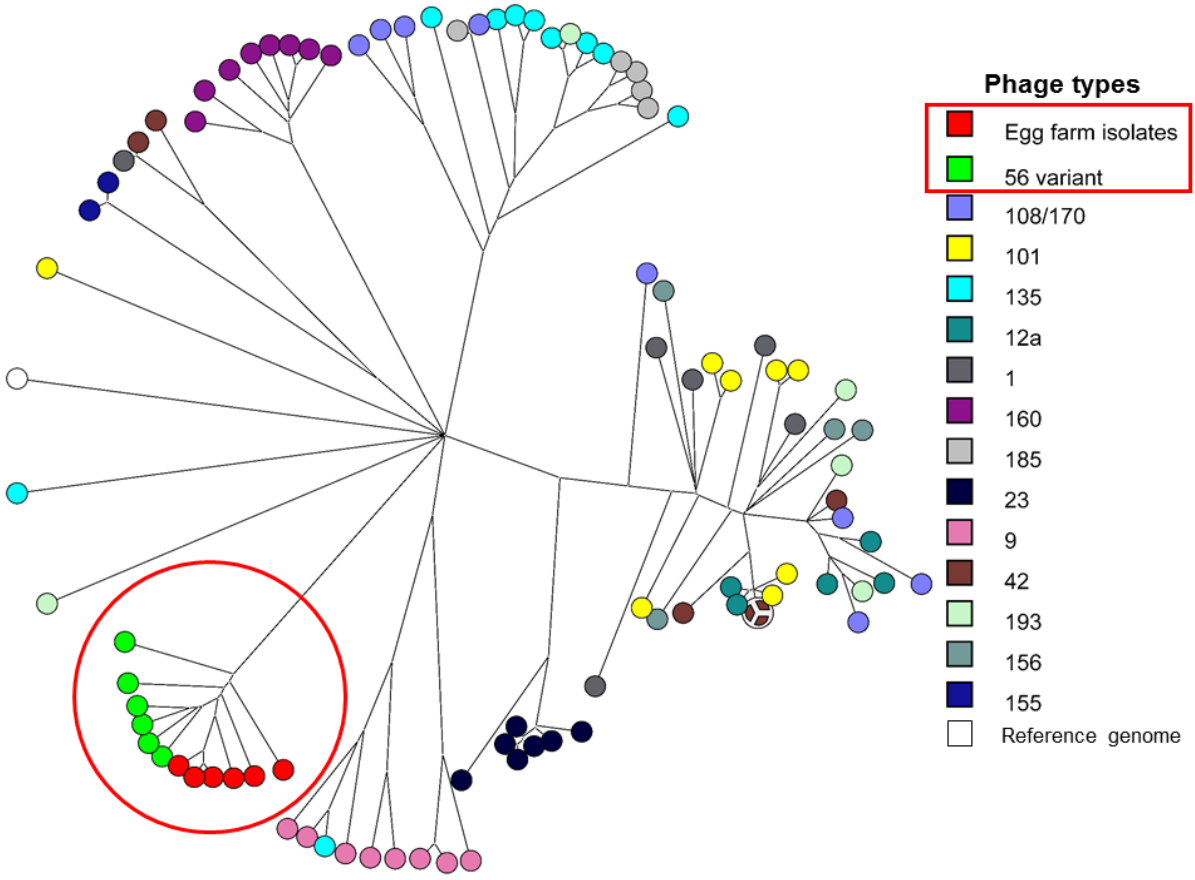

FIGURE 7. Representation of the genetic relatedness between *S.* Typhimurium isolates from this survey and 90 clinical isolates belonging to 14 phage-types, using single nucleotide polymorphism (SNP) analysis. Each dot represents a different isolate, colour-coded by phage type as per the key. Branch lengths are proportional to the number of SNP differences between isolates; i.e. the shorter the branch lengths, the more closely related the isolates.

[2] https://surv.esr.cri.nz/enteric_reference/human_salmonella.php accessed 16-06-2017

All six *S*. Typhimurium egg farm isolates clustered most closely to the six DT56 variant clinical isolates included in the comparison (FIGURE 7). Results support that all six isolates may comprise, or are closely related to, members of this phage type included in the comparison (stronger evidence could be obtained with the inclusion of additional DT56 variant isolates in the comparison, but this was outside the scope of the survey). As few as 30 SNP differences separated the most closely related of the egg layer and DT56 variant strains (prior to 2013, reported as RDNC May 2006) (FIGURE 8). In addition, 2 isolates from 2 different farms (Farm 24 and Farm 2) differed by only 12 SNPs.

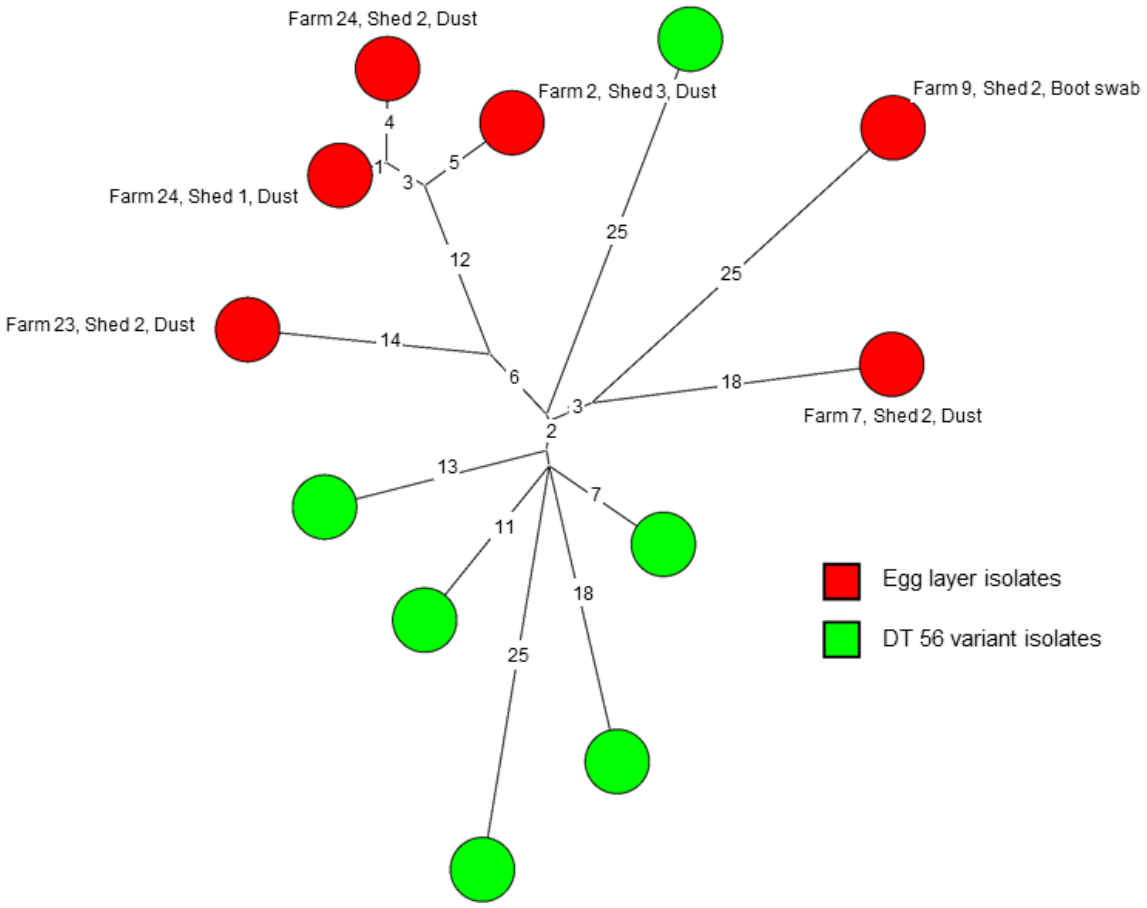

FIGURE 8. SNP differences between *S*. Typhimurium isolates from this survey (n=6) and phage type 56 variant historical isolates (n=6) (also included in FIGURE 7). Each dot represents a different isolate, colour-coded as per the key. Branch length and numbers indicate the number of SNP differences between isolates.

Within-serotype comparisons of egg layer farm isolates. Whole genome SNP comparisons of *S*. Typhimurium, *S*. Infantis and *S*. Thompson isolates are individually depicted in FIGURE 8, FIGURE 9 and FIGURE 10. In addition, three genomes from historical New Zealand egg-associated isolates were included in the comparisons. *S*. Infantis isolates from an egg shell surface [12] and a patient in which egg consumption was implicated in illness, had 68 and 29 SNP differences, respectively, compared with some isolates from this survey FIGURE 9. Interestingly, a historical *S*. Thompson strain isolated from egg shell rinse clustered closely together with isolates from farm 14, differing by only four SNPs from two shed 1 isolates FIGURE 10. It is not known if there were any

connections between the source of the three historical isolates and farms included in this survey.

Isolates from the same layer sheds were often more closely related to other isolates from the same shed than to other isolates from other sheds in the same farm or from an independent farm. Most noteably, no SNP differences were observed between *S*. Infantis isolates from farm 21 shed 3, dust and manure swab samples (FIGURE 9), or between *S*. Thompson isolates from farm 14, shed 1, dust and faeces samples (FIGURE 10).

Furthermore, isolates acquired from within the same farm typically clustered more closely to each other than to isolates from a separate farm. Exceptions included divergent *S*. Infantis isolates from farm 16 shed 1 manure belt compared to the other shed 1 isolates; and farm 14 isolates. Two different serotypes were also isolated from farms 14 and 21, suggesting multiple contamination events on these farms.

Isolates from packhouse egg contact surfaces were also closely related to shed isolates within the farm. For example, for farm 4, there was only one SNP different between the *S*. Infantis isolates from the packhouse egg accumulator/conveyor/roller and shed 3 dust samples. Also, only three SNPs differentiated the packhouse egg washcloth (used to clean dirty eggs) and shed 2 faecal isolates.

Isolates of *S*. Typhimurium, *S*. Infantis, and *S*. Thompson arose from three, five and two regional locations, respectively. Therefore, there was no evidence for a regional relationship with the type of *Salmonella* found.

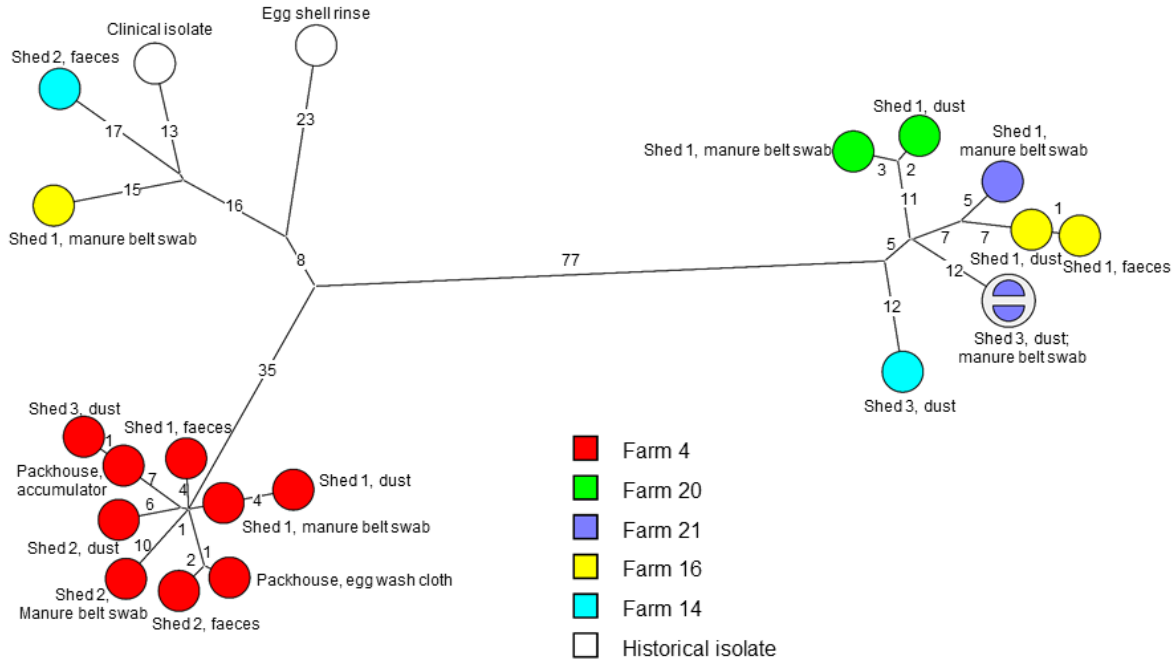

FIGURE 9. SNP differences between *S.* Infantis isolates from this survey. Each dot represents a different isolate, colour-coded by the farm of isolation, as per the key. Branch lengths and numbers represent SNP differences between isolates. Isolates with no SNP differences are represented as "pie sectors".

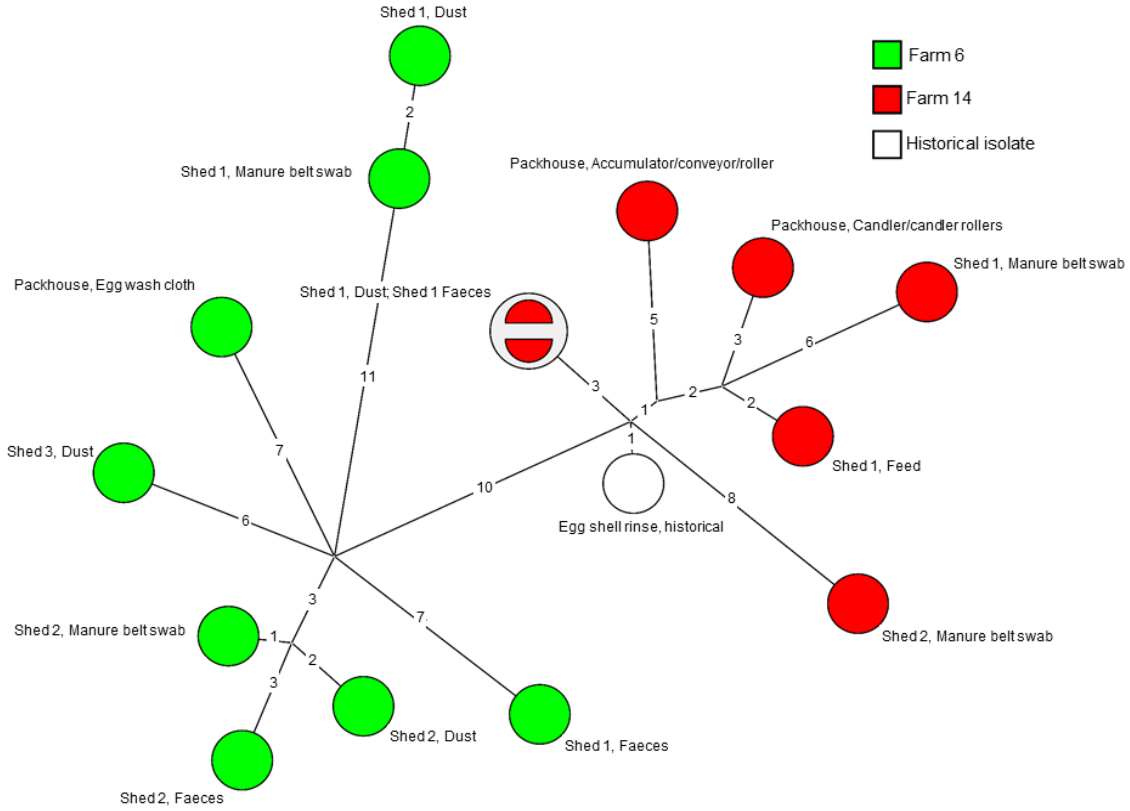

FIGURE 10. SNP differences between *S.* Thompson isolates from this survey mapped against isolate 16PH 0683-001 (farm 14, feed sample). Each dot represents a different isolate, colour-coded by the farm of isolation, as per the key. Branch lengths represent SNP differences are represented as "pie sectors".

5. DISCUSSION

5.1 *SALMONELLA* DETECTION ON FARMS

Overall, the survey provided a baseline of the prevalence of *Salmonella* serotypes and genotypes present in the New Zealand layer egg environment.

Feed. In this study, a single feed sample tested positive for *Salmonella*. The sample was from a farm that produces its own feed, and did not supply other farms surveyed. Seven other farms in this survey also produced their own feed. Self-produced feed has been reported to have a higher risk of *Salmonella* contamination than dedicated feed mills due to higher quality control and biosecurity procedures during manufacture at the feed mills [54].

While the survey aimed to access feed samples from the silo where possible, the sample that tested positive was obtained from the trough in the shed. This also happened to be on one of the farms with the highest prevalence of layer shed detections. Therefore, it was equally likely that this sample could have been cross-contaminated from the shed environment. Consistent with this, all three shed samples from this farm (faeces, dust, and manure belt swab) also tested positive for *Salmonella*. Regardless of whether the source of the feed contamination was from the shed or the shed contamination was from the feed, the detection of *Salmonella* from the feed represents a potential source for re-infection of hens not already harbouring *Salmonella*.

The *Salmonella*-positive feed type was mash, which was also used by most of the farms in the survey (15/28), while twelve farms used crumbles (including three farms that also used mash), and four farms used pellets. *Salmonella*-inhibitory heat-treatment, which is a part of the feed pelleting/crumble manufacture process, was not used on any mash feeds in this survey. For this reason, other studies have found mash feed more likely to be contaminated than pellet feed [22, 55]. However, a *Salmonella* inhibitor was added to most mash feed in this survey (including the positive sample source; for some farms, addition was only when deemed necessary). Four farms used feed that, to their knowledge, did not undergo *Salmonella*-inhibitory treatments; however, all farms indicated feed was tested for *Salmonella* by the manufacturer.

Layer shed samples. *Salmonella* prevalence was found to be the most abundant in layer shed dust samples, followed by manure belt/boot swabs, and faeces samples. *Salmonella* present in dust may have arisen from either faecal shedding during *Salmonella* carriage by flocks (past or current), or from contamination of the layer shed from an external source. Presence of *Salmonella* in faeces indicated *Salmonella* carriage by flocks, although it is possible that *Salmonella* populations residing in dust may have contaminated the manure belt or faeces directly, or may have been the source of the *Salmonella* infection of hens. Because faecal samples only come from ~60 birds while dust samples likely arise from a much larger proportion of the birds in the shed, dust samples also have the potential to detect a low within-flock prevalence and the presented results support previous studies showing that dust is the most sensitive sample for detecting *Salmonella* in layer shed environments [19]. Confirmation of positive current carriage by flocks could be further substantiated by cloacal swabbing of a subset of hens in sheds in which positive pooled faeces samples were obtained, but was outside of the scope of this survey. Regardless of the source, contaminated dust, faeces and litter are all potential sources for cross-contamination of *Salmonella* to eggs.

Packhouse samples. *Salmonella* was only isolated from packhouse egg contact surfaces on farms which also had a high prevalence of *Salmonella* in the layer sheds. The three positive packhouses did not pack eggs from other farms. Therefore, results support that *Salmonella* isolates obtained in the packhouse originated in the laying shed, likely, via cross-

contamination of eggs. Moreover, laying shed isolates arising from the same farm were genetically related. A number of overseas studies have reported a correlation between the number of positive environmental samples and the proportion of positive eggs in a flock; thus, suggesting that prevalence of infection and on-farm hygiene are indeed directly related to the number of contaminated eggs produced [24, 56, 57]. In addition, the type of positive sample was also relevant, with one study reporting a 59 times higher likelihood of egg shell contamination when faecal samples were positive for *Salmonella*, and nine times higher likelihood when dust samples were positive [33]. Two out of three faecal samples tested positive on each farm in which positive packhouse isolates were obtained, and these comprised 6/7 of all positive faecal samples obtained from the farms in this survey. As packhouse contact surfaces are in direct contact with a large number of eggs, and contamination could have occurred from contaminated egg surfaces, positive packhouse samples may indicate possible egg surface contamination.

5.2 PRODUCTION SYSTEM

This survey aimed to determine the prevalence and potential contamination sources of *Salmonella* spp. on commercial layer farms. Differing farm logistics and practices meant that samples could not be completely standardised, although all reasonable steps were taken to control this. It was also not possible to assess the real effect of one production system factor over another given the interrelatedness between them and the limited number of samples analysed. Therefore, only simple correlations between microbiological results and production system factors are indicated here. This rationale was also employed by similar international studies [26,27].

Salmonella prevalence was found to be significantly higher in New Zealand caged layer shed systems relative to cage-free systems. Results are consistent with a previous New Zealand survey which only identified *Salmonella* on the surfaces of cage-laid eggs (3.6% of cage-laid eggs), but not from barn or free-range eggs [12]. A higher *Salmonella* prevalence in the layer environment samples or eggs from caged systems relative to cage-free sheds was previously reported in Germany, the United Kingdom, France, Belgium and Australia [26, 58, 59]. Conflicting results were found in other studies, where either no difference was found between systems, or a higher contamination of eggs from free-range systems was reported [60-62].

Flock size. A larger flock size (and associated higher number of birds per shed in caged system sheds) has previously been found to be of higher risk than smaller flock sizes for *Salmonella* carriage, attributed to higher levels of *Salmonella*-contaminated dust and dander being produced which can re-infect birds [3, 11, 33]. This is consistent with the findings in this survey.

It should be noted that the density of the flocks was not assessed; in particular the number of cages per shed, the size of the cages and the size of the sheds for either layer system, were not recorded.

The density may be more important than the number of birds itself because a higher number of birds per square meter will favour the transmission of pathogens between birds and will increase the amount of stress, lowering then their immune response (Gast, 2017).

Single versus multi-age flock management. Multi-age flock management is also more common for caged production systems than cage-free systems. Multi-age flock management has been found to be a risk factor for flock contamination by *Salmonella* because cleaning of sheds after depopulation of one flock becomes more difficult when birds from another flock still remain in the shed [21, 33, 63, 64]. For one of the “high-prevalence” farms at the end-of-lay, no additional cleaning was performed other than routine maintenance (daily floor sweeping and egg collection belt cleaning, weekly cleaning of fans and ducts). The other two

farms performed dry cleaning (no indication was given how dry cleaning differed from routine maintenance, except one farm dry-cleaned cages at end-of-lay). None of the three "high-prevalence" farms performed sanitation or fumigation of sheds following dry cleaning, which could result in persistence of *Salmonella* in the shed environment for subsequent flocks. A detailed assessment of the cleaning procedures for each "high-prevalence" farm could be of benefit to these farms. The efficacy of any recommended cleaning and sanitation changes could be monitored by sampling the environment directly before and after cleaning and sanitation.

Flock age. Studies have reported that the onset of lay represents a time of stress for hens, during which time the immune system is suppressed, resulting in increased shedding of *Salmonella*, and thus increasing the percentage of flock infection [8, 65, 66]. Others reported a trend toward an increase in contamination of the laying environment over time, which can then re-infect flocks [21, 24]. This is consistent with the finding that early and late-lay flocks had a higher *Salmonella* prevalence than mid-lay.

In summary, a large diversity of criteria has to be taken into account to explain the prevalence of *Salmonella* in specific production systems. In particular, the factors which increase the likelihood of positive *Salmonella* results are the high flock size and flock density, the multi-age management system, and the age of the flocks (early-lay and late-lay). Most of these conditions are also more likely to be found in cage layer shed systems compared to cage-free systems.

5.3 *SALMONELLA* SEROTYPES ISOLATED

The five serotypes isolated in this survey have been isolated from reported human cases of salmonellosis in New Zealand in each of the previous three years (2015-2017) (FIGURE 11, TABLE 7). The serotypes associated with the two most recent (2010 and 2013) egg-associated (chocolate mousse cake and boiled egg and ham sandwich) *Salmonella* outbreaks in New Zealand were *S*. Typhimurium and *S*. Infantis [5]. The evidence linking the outbreak to the food was considered "strong", but these are mixed foods and contamination could have come from another ingredient.

All five serotypes are also commonly isolated from non-clinical (the environmental, animal and animal feed) sources in New Zealand (TABLE 8)[3], although these data depend on submissions by other laboratories and do not constitute a representative sampling programme. Each serotype has also been isolated from the poultry (broiler and/or egg layer) environmental samples (feed, farm environment or product) in 2015-2017. The two most commonly identified serotypes in this survey, *S.* Infantis and *S.* Thompson, comprised two of the three serotypes isolated from New Zealand eggs in previous studies [4, 12].

Importantly, the serotype *S.* Enteritidis, which is the dominant serotype in European and North American flocks and the cause of the majority of egg-associated outbreaks in these countries, was not isolated in this survey. Consistent with our findings, *S*. Enteritidis is not currently considered endemic for the New Zealand egg layer sector, and has not previously been identified on New Zealand layer farms or in eggs [10].

Studies have shown that certain isolates of at least some of serotypes isolated here (*S*. Typhimurium and *S*. Infantis) are able to survive on egg surfaces from the point of lay to the time of consumption. These isolates can internalise eggs and will grow if they reach the yolk either through migration through the albumen or through breakdown of the vitelline membrane (reviewed in [5]). Survival on egg surfaces and invasion have been shown to be influenced by factors such as the bacterial load on eggs, the degree of faecal contamination, moisture and humidity, rapid temperature changes, the storage temperature and storage time. Therefore, determination of the actual risk posed by the egg production environmental

isolates would require further studies to assess the ability of these isolates to survive on, penetrate, and/or grow inside eggs at temperatures and storage times relevant to New Zealand storage practices. In addition, *in vitro* and *in vivo* invasion and pathogenicity profiles of isolates could be determined and similar studies have been performed for Australian egg-associated isolates [67]. Virulence phenotype information obtained could then be used for genetic association studies, also utilising the genomic sequence data obtained in this survey.

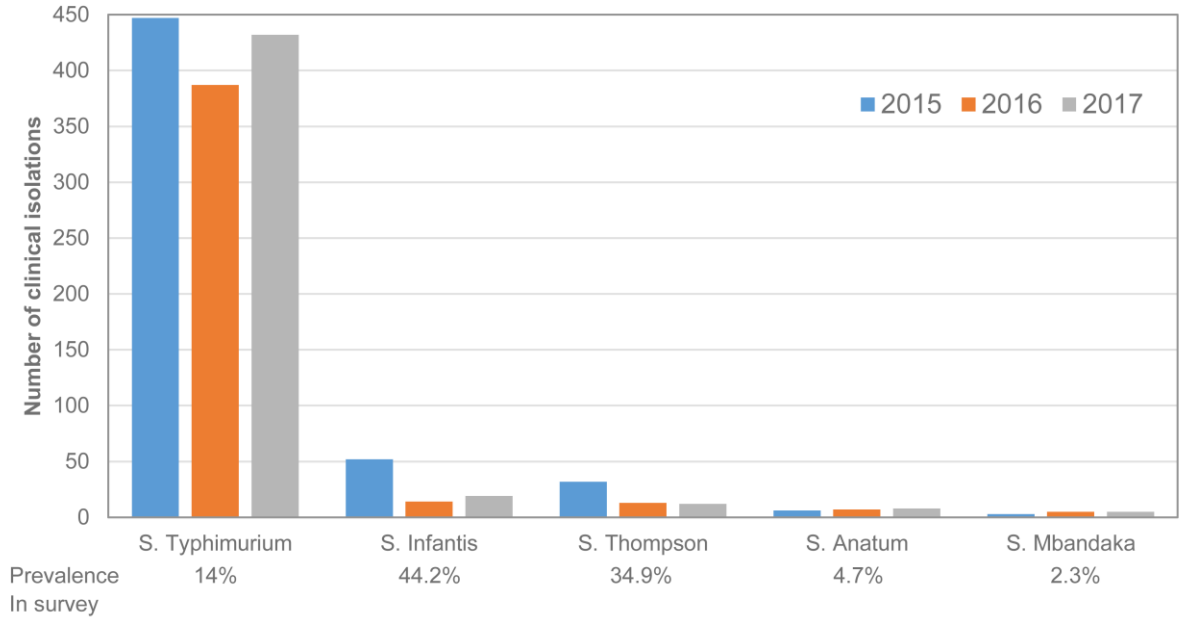

FIGURE 11. Prevalence of isolation of *Salmonella* serotypes relevant to this survey from humans in New Zealand (2015-2017)[3].

TABLE 7. Prevalence of isolation of *Salmonella* serotypes relevant to this survey from humans in New Zealand (2015-2017)[1].

SEROTYPE	PREVALENCE (2015)	PREVALENCE (2016)	PREVALENCE (2017)
S. Typhimurium (phage type 56 variant)	447 (96)	387 (64)	432 (117)
S. Infantis	52	14	19
S. Thompson	32	13	12
S. Anatum[2]	6	7	8
S. Mbandaka	3	5	5
Total isolates	*1133*	*1150*	*1217*

[1] Data source https://surv.esr.cri.nz/enteric_reference/human_salmonella.php (accessed 23-11-2018)
[2] S. Anatum numbers also include S. Anatum var. 15+

[3] https://surv.esr.cri.nz/enteric_reference/human_salmonella.php (accessed 23-11- 2018)

TABLE 8. Prevalence of isolation of *Salmonella* serotypes relevant to this survey from non-clinical sources (environment, animals, animal feed) in New Zealand (2015-2017)1,2,3.

SEROTYPE	PREVALENCE (2015)		PREVALENCE (2016)		PREVALENCE (2017)	
	Poultry	Total non-clinical	Poultry	Total non-clinical	Poultry	Total non-clinical
S. Typhimurium (phage type 56 variant)	17 (9)	258 (56)	13 (5)	249 (43)	11 (5)	371 (59)
S. Infantis	2	14	1	20	2	26
S. Thompson	0	1	0	2	0	1
S. Anatum	3	6	0	9	1	12
S. Mbandaka	2	10	0	6	1	9
Total isolates	*46*	*637*	*24*	*684*	*27*	*972*

[1] Data source https://surv.esr.cri.nz/enteric_reference/nonhuman_salmonella.php (accessed 23-11-2018)
[2] Poultry prevalence is from environmental, feed and miscellaneous sources.
[3] Prevalence of isolates reported to EpiSurv may not represent true environmental prevalence.

5.4 GENOTYPIC COMPARISONS OF *SALMONELLA* ISOLATES

S. Typhimurium

These results and those from other studies suggest that a wgSNP-based approach is a better tool to assess relatedness between isolates than phage typing and should be considered as a replacement methodology. However, whilst new WGS approaches and criteria are being established, comparing isolates of interest against historical isolates typed by conventional methods provides benefit for inferring phylogenetic relationships and population structure.

For example, FIGURE 7 shows that two phage types, DT 108/170 and DT 42, are distributed into different phylogenetic clusters. These results support that these phage types may have arisen independently on multiple occasions. Similar conclusions were recently published investigating relationships between a subset of these and other phage types using a wgSNP approach based on a fewer number of polymorphic sites [43].

The *S*. Typhimurium isolates identified in this survey were found to be closely related to the DT56 variant phage type. This phage type has also been commonly isolated from non-clinical sources (particularly, bovine, avian, equine and feline sources) over the 2014-2016 period in New Zealand (TABLE 8) and from patients in New Zealand over both this period (TABLE 7) and the 2010-2014 period (425 cases) [5]. Unlike phage types DT108/170 and DT42, S. Typhimurium DT56 variant isolates have been reported to be highly clonal [68], although data has not been published for New Zealand isolates.

In the United Kingdom, DT56 variants are considered to be host-adapted to wild birds, which may act as the primary reservoir for this phage type in United Kingdom [68, 69]. Therefore, it remains possible that wild birds are the source of the *S*. Typhimurium isolates from farms in this survey. Consistent with this, four of the six *S*. Typhimurium isolates from this survey were from free-range sheds, which provide greatly increased access to wild bird invasion. In addition, the presence of wild birds or activity in sheds and/or feed storage areas was observed for three of the five farms. Furthermore, the predominance of this phage type in the New Zealand environment in general at the time of this survey (based on EpiSurv isolation data, TABLE 8) may explain why all *S*. Typhimurium isolates from the study were closely related to this phage type, and also likely accounts also for the predominance of this phage type in the clinical setting (rather than any association with eggs).

Other serotypes:

Importantly, fine-detail SNP-based genomic comparisons between *S.* Infantis and *S*. Thompson isolates revealed that isolates were most closely related to those in the same layer shed than from other sheds on the same farm. Also, isolates from the same farm were more closely related to each other than from another farm. Isolates from the packhouse egg contact surfaces were also closely related to shed isolates within the farm. Therefore, these results indicate that there is a common contamination source between sheds, rather than multiple sporadic contaminating events occurring overtime, and/or that the presence of resident, persistent populations from one shed may be transported to other sheds on the farm, and to packhouse egg contact surfaces via contaminated eggs (although less likely, the possibility of cross-contamination from the packhouse back to the laying sheds, can not be ruled out).

In consequence, biosecurity procedures, such as changing personal protective equipment between sheds, consistent use of boot dips by personnel, improving cleaning and sanitation procedures to eliminate populations within sheds, may be areas in which to pay attention to reduce cross-shed contamination, and would likely be beneficial in controlling *Salmonella* on these farms.

Of the nine hatcheries supplying the farms in this survey, two hatcheries/rearing operations supplied the majority of the farms. S. Typhimurium was isolated from farms supplied by five different hatcheries/suppliers; and thus, no linkage between this serotype with a specific hatchery/rearing operation was found. One single operation (designated hatchery/rearing operation A, situated at two separate locations) supplied chickens to all five farms in which *S.* Infantis was isolated. This operation also supplied seven other farms involved in the survey, including three farms in which other serotypes were isolated (including both farms that contained S. Thompson), and four farms in which no *Salmonella* was isolated. Any linkage between hatcheries and *Salmonella* on layer farms would require further investigation, but the inconsistent prevalence data, and the closer genotypic linkage between isolates on individual farms than between farms, argues against a link between hatcheries and presence of *Salmonella* on farms.

5.5 COMPARISONS WITH AUSTRALIA AND OTHER COUNTRIES

The prevalence and serotypes of *Salmonella* from this survey were considered in an international context. The low prevalence of *Salmonella* isolation from feed observed in this study is consistent with findings from a recent survey of New Zealand processed animal feeds, which did not detect *Salmonella* in poultry feed [70]. A low prevalence (0%, n=21) was also reported in the previous Queensland 2014 egg layer survey; while feed prevalence was somewhat higher in the New South Wales 2010/2011 survey (farm level prevalence 11% (n=21); point-of-consumption-level prevalence (17% (n=101)) [26, 27] (FIGURE 12).

The prevalence of *Salmonella*-positive pooled faeces (10.5%) and faeces/manure belt swab samples (16.4%) in this survey were lower than those reported for equivalent sample types from recent baseline surveys of New South Wales and Queensland layer sheds (TABLE 4, FIGURE 12) (17 and 29% prevalence of *Salmonella* in NSW and QLD faecal samples; and 28 and 38% prevalence in NSW and QLD boot/manure belt swabs) [26, 27]. Importantly, dust, which accounted for the majority of *Salmonella*-positive samples in this survey, was not tested in those surveys (but dust prevalence was lower than in European surveys (Error! eference source not found.4)). *Salmonella* prevalence in this survey was lower at the 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) [26, 27]. The comparison between prevalence from this and Australian egg

layer surveys 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.

The serotypes found in this study are not unusual, and their relative proportions do not allow definite conclusions from such a small survey. *S.* Typhimurium is the serotype most commonly associated with laying hens and eggs in non-European countries [71], and the second most common in Europe (after *S*. Enteritidis, by a substantial margin) [8]. In this survey, *S*. Infantis was the most common serotype, followed by *S*. Thompson and then *S*. Typhimurium. In the Australian studies *S*. Typhimurium was most common, followed by *S*. Infantis [26, 27] (FIGURE 13). *S*. Infantis is amongst the most commonly found serotypes by the Australian egg industry and is one of the most commonly isolated serotypes worldwide [8, 45, 72, 73]. *S*. Anatum was isolated twice (6% of isolates) in the Queensland 2014 egg layer survey. *S*. Thompson and *S*. Mbandaka were not observed in the New South Wales 2010/2011 or Queensland 2014 egg layer surveys, but *S*. Mbandaka was common in other egg layer studies worldwide [3, 8, 31, 73, 74]. Importantly, S. Enteritidis, which causes the majority of egg-associated outbreaks in European and North American countries, was not identified in this study and is not considered endemic in New Zealand poultry.

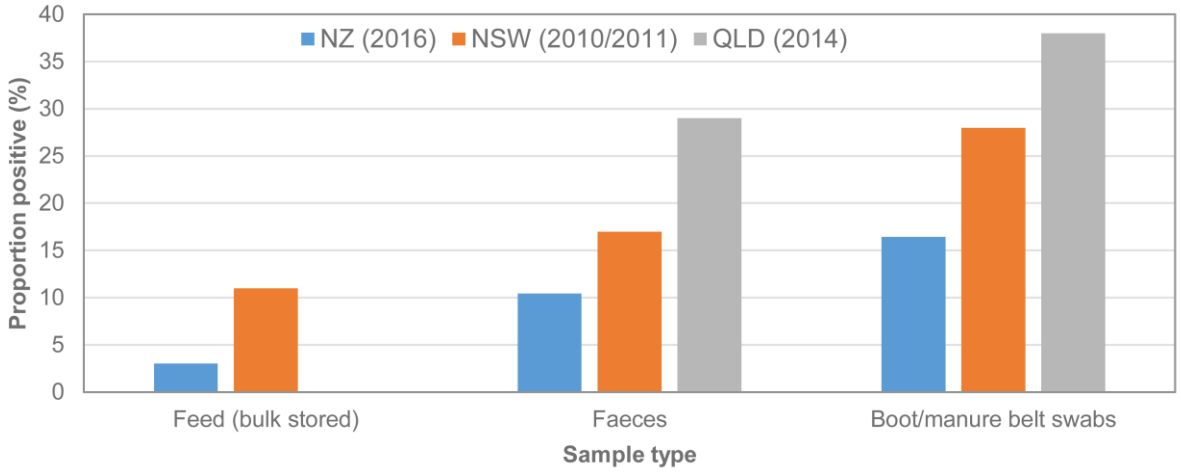

FIGURE 12. *Salmonella* prevalence in feed and egg layer environments compared with baseline studies from New South Wales (2010/2011) AND Queensland (2014) [29, 30]. (Note, the New South Wales and Queensland studies did not survey dust).

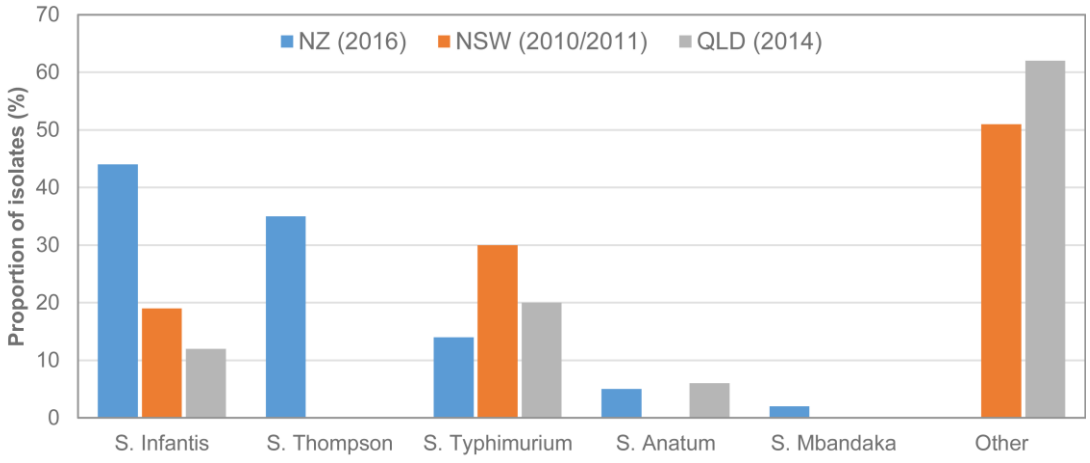

FIGURE 13. Comparison of *Salmonella* serotypes isolated in this survey with proportion of the same serotypes from similar New South Wales (NSW) [30] and Queensland (QLD) [29] egg layer farm surveys.

6. CONCLUSION

This survey is the first to evaluate the prevalence and types of *Salmonella* present in the New Zealand chicken egg laying and production environment. While conventional caged systems currently account for the overwhelming majority of eggs produced in New Zealand, legislative changes require the phasing out of conventional cages by 31 December 2022. The findings of this survey provide a useful baseline from which to gauge the impact of these and any other such changes to layer system practices on *Salmonella* prevalence. In addition, should future egg-associated salmonellosis outbreaks arise, the information garnered from this survey regarding the *Salmonella* serotypes and genotypes associated with egg production facilities, would provide useful data for comparing against human *Salmonella* isolates in source attribution studies.

Finally, this survey provides data useful to assist in the development and review of food safety standards related to management of the risk from *Salmonella* in and on eggs.

- The prevalence of *Salmonella* in the New Zealand egg production environment was lower in this survey compared with prevalence from equivalent samples from similar Australian studies (boot/manure belt swabs, pooled faeces). Findings are consistent with a low reported prevalence of *Salmonella* contamination of egg shells, and a low reported incidence of salmonellosis attributed to egg consumption in New Zealand.
- Caged systems, which produce the majority of eggs in New Zealand, had a higher *Salmonella* prevalence than cage-free layer systems (conventional cage > colony cage > free-range > barn). However, data should be viewed with caution due to the multiple interrelated risk factors associated with different laying system types.
- *Salmonella* was only isolated from packhouse egg contact surfaces from farms with the highest prevalence of *Salmonella*-positive shed samples, and isolates obtained from sheds and packhouse samples were genetically related. Therefore, results indicate an association between on-farm prevalence of *Salmonella* spp. and egg contact surface prevalence, and may provide an indicator for egg surface contamination.
- Consistent with previous New Zealand studies, no *S.* Enteritidis was isolated. The serotypes that were found are commonly isolated from the New Zealand environment and have also been isolated from reported cases of salmonellosis in New Zealand.

7. OPTIONS FOR FUTURE RESEARCH

- To provide insight about whether layer environment prevalence correlates with egg surface prevalence, further studies could target *Salmonella* testing of eggs from the "high-prevalence" farms versus "zero-prevalence" farms. However, because flock prevalence can change over time, testing of eggs would be best performed at the same or similar time to environmental sampling.
- Further studies would be beneficial to assess the ability of the isolates found in this survey to survive on, penetrate, and/or grow inside eggs at temperatures and storage times relevant to New Zealand storage practices. In addition, *in vitro* and *in vivo* invasion and pathogenicity profiles of isolates could be determined.
- A future survey could assess the impact of production system changes, particularly phasing out of conventional caged systems.

GLOSSARY

Aviary	Cage-free housing system featuring multi-tiered laying shed (either barn or free-range system). System consists of a raised slatted area providing perching and access to food / water at each level.
Barn	Cage-free housing system where birds remain inside shed. Shed can be fixed or moveable.
BLAST	Basic Local Alignment Search Tool.
BPW	Buffered peptone water.
BS	Bismuth Sulphite agar.
Caged	Comprises conventional-caged and colony-caged systems.
Cage-free	Comprises free-range and barn laying systems.
Colony cage	Cages contain minimum of 750 m^2 per bird, can house up to 60 birds, and contain scratching, nesting and perching areas. Also referred to as enriched or furnished cages.
Conventional cage	Cages contain 2-9 birds, and do not contain scratching, nesting, or perching areas.
DNA	Deoxyribonucleic acid.
DT	Definitive phage type (DT).
Egg production environment	Includes laying sheds and packhouse.
EPF	Egg Producers Federation of New Zealand.
ESR	Institute of Environmental Science and Research Ltd (NZ).
Farm level feed	Feed from feed storage area / silo, before access to feeding troughs.
Free-range	Cage-free housing system with outside range access for hens. The housing shelter may be fixed or moveable, such as a shed, aviary, perchery or ark.
HE	Hektoen Enteric agar.
ISO	International Organisation for Standardisation.
LIA	Lysine Iron Agar.
Litter	Material used as bedding / shed floor covering in cage-free systems. Also contains faeces, feathers, dust and any spilled feed.
MKTTn	Muller-Kauffmann tetrathionate novobiocin.
MLST	Multi Locus Sequence Typing.
MPI	Ministry for Primary Industries.
MSRV	Modified semi-solid Rappaport-Vassiliadis.
Packhouse	Building where eggs are processed, which may involve sorting, candling (crack detection), grading, washing, and packing.
Range	Outdoor area, usually pasture, used by free-range hens.
RMP Template for Eggs	June 2007 Risk Management Programme Template for Eggs
RVS	Rappaport-Vassiliadis with soya.
TSI	Triple Sugar Iron slant.
wgSNP	Whole genome Single Nucleotide Polymorphism.
XLD	Xylose lysine deoxycholate.

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