Salmonella enterica isolates from layer farm environments are able to form biofilm on eggshell surfaces

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ABSTRACT
This study examined the eggshell biofilm forming ability of Salmonella enterica isolates recovered from egg farms. Multicellular behaviour and biofilm production were examined at 22 and 37°C by Congo red morphology and the crystal violet staining assay. The results indicated that the biofilm forming behaviour of Salmonella isolates was dependent on temperature and associated with serovars. Significantly greater biofilm production was observed at 22°C compared with 37°C. The number of viable biofilm cells attached to eggshells after incubation for 48 h at 22°C was significantly influenced by serovar. Scanning electron microscopic examination revealed firm attachment of bacterial cells to the eggshell surface. The relative expression of csgD and adrA gene was significantly higher in eggshell biofilm cells of S. Mbandaka and S. Oranienburg. These findings demonstrate that Salmonella isolates are capable of forming biofilm on the eggshell surface and that this behaviour is influenced by temperature and serovar.

KEYWORDS
Biofilm; eggshell; Salmonella

Introduction
Members of the bacterial species Salmonella enterica are common causes of human gastroenteritis, a disease characterised by gut inflammation and diarrhoea (Winter et al. 2010). It is estimated that gastroenteritis caused by Salmonella spp. accounts for 93.8 million cases and 155,000 deaths worldwide each year (Majowicz et al. 2010). In Australia, contaminated food products of animal origin, particularly egg and egg products, are frequently associated with outbreaks of human salmonellosis (OzFoodNet Working Group 2015, 2012).

The formation of a biofilm is one of the mechanisms Salmonella spp. utilise for survival in harsh physical and chemical environments (Costerton et al. 1999). A biofilm is a community of interacting bacterial cells attached to biotic or abiotic surfaces, embedded in a self-produced extracellular polymeric matrix (Costerton et al. 1999). The extracellular matrix of a biofilm is predominantly comprised of curli, fimbriae and cellulose, which promote linkage and interaction between bacterial cells (Steenackers et al. 2012). Differential multicellular behaviour contributes to the formation of distinct colony morphotypes on Congo red agar plates supplemented with Coomassie brilliant blue (Romling et al. 2003; Seixas et al. 2014).

Salmonella within a biofilm displays higher resistance to environmental stressors, antibiotics and disinfectants compared to planktonic counterparts, thus making the eradication of this bacterium extremely difficult from surfaces commonly used in food and the poultry industry (Joseph et al. 2001; Steenackers et al. 2012).

Biofilm formation is a complex process and regulated by several sets of genes involved in extracellular matrix production and adhesion. csgD, a transcriptional regulator of the LuxR superfamily, positively regulates the expression of Salmonella biofilm associated extracellular matrix components, including curli, fimbriae and cellulose (Gerstel & Römling 2003; White et al. 2008; Grantcharova et al. 2010). csgD stimulates curli production through transcriptional activation of the csgBAC operon, which encodes the major curli subunit CsgA as well as the nucleator protein CsgB. csgD also indirectly regulates cellulose synthesis by activating transcription of adrA. Being a member of the GGDEF protein family, AdrA synthesises c-di-GMP as diguanylate cyclase, and its transcription is regulated by csgD (Liu et al. 2014). BapA, a large surface protein involved in biofilm formation and the expression of bapA, is coordinated with that of genes encoding curli, fimbriae and cellulose, through the action of csgD (Latasa et al. 2005; Wang et al. 2016).
Biofilm formation is influenced by several factors including environmental conditions, e.g., the type of culture medium and the surface material, as well as strain origin and serovar (Vestby et al. 2009; Castelijn et al. 2012; Lianou & Koutsoumanis 2012; Schonewille et al. 2012; De Oliveira et al. 2014). The ability of *Salmonella* to attach and form a biofilm represents a significant public health risk for many industries, including those involved in food production and processing. Biofilm on food preparation surfaces and equipment could serve as a persistent source of cross contamination, compromising the safety of food products and human health (Shi & Zhu 2009).

*Salmonella* spp. are able to form biofilm on a large number of abiotic surfaces including stainless steel, plastics, cement, rubber and glass (Steenackers et al. 2012). Biofilm formation on the surfaces of eggshells, however, has to date not been documented for any bacterial species. Worldwide egg and eggshell contamination by *Salmonella* is a major concern for poultry industries. For its survival and growth on the outer surface of an egg *Salmonella* must overcome low nutrient availability and temperature stress (Gantois et al. 2009). Over the past decade, the number of foodborne salmonellosis cases traced to egg or egg products has substantially increased (OzFoodNet Working Group 2012). This suggests that *Salmonella* spp. are able to attach and or form biofilm on the eggshell surface, and remain viable, resulting in the cross contamination of a variety of food products. Food safety and public health impacts associated with biofilm forming foodborne pathogens emphasises the importance of understanding eggshell biofilm formation by *Salmonella* spp. The main objective of the present study was to determine the biofilm forming ability of egg farm related *Salmonella* serovars on the eggshell surface. In addition, the impact of temperature and serovar variation on biofilm formation was also investigated.

**Materials and methods**

**Bacterial strains and serotyping**

One hundred and forty-five *Salmonella* isolates of seven different serovars: S. Agona (*n*=6), S. Anatum (*n*=6), S. Infantis (*n*=16), S. Mbandaka (*n*=30), S. Oranienburg (*n*=30), S. Typhimurium (*n*=26), and S. Worthington (*n*=31) were used to study biofilm formation. All *Salmonella* isolates used in this study were previously isolated in the authors’ laboratory from 33 caged layer flocks across 13 different egg farms during epidemiological studies (Chousalkar & Roberts 2012; Gole et al. 2014a, 2014b) and were serotyped at the Australian *Salmonella* Reference Centre, Microbiology and Infectious Diseases, SA Pathology, Adelaide, South Australia.

**Biofilm formation assay**

**Phenotypic characterisation by Congo red morphology**

The colony morphology of *Salmonella* isolates (*n*=145) was determined on Congo red agar plates for curli, fimbriae and cellulose production, as described previously (Castelijn et al. 2012) with some modifications. Briefly, stock *Salmonella* cultures were grown on nutrient agar plates at 37°C overnight. Single colonies of *Salmonella* were grown in 5 ml of Luria-Bertani (LB) broth (10 g bacto tryptone) (Oxoid, Adelaide, Australia), 5 g yeast extract (Oxoid), 10 g sodium chloride (Fischer Chemicals, Melbourne, Australia) with shaking (110 rpm) at 37°C for 6 h. Each *Salmonella* isolate was plated (3 μl) onto LB agar without sodium chloride, supplemented with Congo red (40 μg ml⁻¹, Sigma Aldrich, St Louis, MO, USA) and Coomassie brilliant blue (20 μg ml⁻¹, Sigma Aldrich). The inoculated plates were incubated at 22 and 37°C for 96 h and colonies were visualised macroscopically. *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 was used as a positive control strain during this study.

**Quantitation of biofilm formation by crystal violet staining assay**

*Salmonella* isolates (*n*=145) were grown on LB agar plates overnight at 37°C. Single bacterial colonies were inoculated in 10 ml of LB broth without sodium chloride and incubated at 37°C overnight. Twenty μl of overnight grown bacterial culture were mixed with 180 μl of LB broth without sodium chloride in polystyrene round bottom 96 well plates (Sarstedt, Adelaide, Australia). Negative control wells contained 200 μl of LB broth only. Inoculated plates were incubated statically at either 22 or 37°C for 96 h. After incubation, the plate contents were decanted and wells were washed gently three times with sterile distilled water to remove loosely bound bacteria. Plates were air dried and stained with 200 μl of 0.1% (w/v) crystal violet for 30 min at room temperature. Following staining, wells were gently washed three times with sterile distilled water and air dried. Bound crystal violet stain in the wells was resuspended with ethanol-acetone (70:30) for 10 min at room temperature. The absorbance of each well was measured at 590 nm (A_{590}) using a microplate spectrophotometer (Benchmark plus, Biorad, Hercules, CA, USA). The mean absorbance of negative controls was subtracted from the absorbance values obtained from experimental wells. All experiments were conducted in duplicate and repeated twice.

**Studies on biofilm formation on eggshell**

**Salmonella isolates and egg source**

Four *Salmonella* isolates from each *Salmonella* serovar: S. Agona, S. Anatum, S. Infantis, S. Mbandaka,
S. Oranienburg, S. Typhimurium and S. Worthington, isolated from layer farms, were used to investigate their biofilm forming ability on the eggshell. All isolates were examined for their potential to survive and form biofilm on eggshell surfaces at 22°C for 48 h. Based on the results of biofilm formation at 22°C, ambient temperature was selected to study eggshell biofilm formation. In this study, fresh unwashed eggs were obtained from the cage front of a 39-week-old caged layer flock housed at Roseworthy campus, The University of Adelaide.

Biofilm formation on eggshell

To ensure that a defined surface area was covered by bacterial culture, an individual egg was marked with a pencil (1.2 cm² area) and disinfected with formaldehyde fumigation (Samberg & Meroz 1995). Eggshell surfaces were further sanitised by immersing the eggs in 70% ethanol for 2 min, allowing them to dry in a class II biosafety cabinet, and then immediately used for experimentation. Biofilm formation on the eggshell was assessed at 22°C on individual eggs in triplicate for each isolate of representative serovar. All experiments were performed in duplicate.

Stock Salmonella cultures were grown on nutrient agar plates at 37°C overnight. Single colonies of Salmonella were grown in 20 ml of LB broth with overnight incubation at 3°C. Eggs were placed horizontally in Whirlpack bags (Fischer Scientific, Melbourne, Australia) containing 5 ml of overnight grown bacterial culture and 45 ml of LB broth without sodium chloride (10 g bacto tryptone and 5 g yeast extract, Oxoid) (1:10), ensuring the marked surface area was covered with bacterial suspension. The bags were then incubated statically at 22°C for 48 h. Negative control bags contained LB broth only. The cell concentration (CFU) in working bacterial suspensions was calculated by plating 10-fold serial dilutions on nutrient agar. The number of bacterial cells contained within the eggshell biofilm was enumerated by the plate count method, as described earlier (Castelijn et al. 2012) with some modification. Following incubation at 22°C for 48 h, eggs were aseptically removed from the Whirlpack bags using sterile forceps and kept in Petri plates. The eggshell (1.2 cm² area) was rinsed thrice with sterile 0.85% normal saline solution to remove unbound cells. Attached cells were collected with a sterile cotton swab to remove biofilm-associated bacteria. The cotton swab was placed into a microcentrifuge tube containing 0.85% normal saline and vortexed with acid washed glass beads (710–1180 μm, Sigma-Aldrich) at full speed for 1 min. Serial dilutions were prepared in 0.85% normal saline and plated on xylose lysine deoxycholate (XLD) agar plates (Thermo Scientific, Adelaide, Australia). Plates were incubated at 37°C overnight and colonies were counted to determine the number of colony forming units (CFU). The results were expressed as log₁₀ CFU cm⁻². In this study, three biological replicates of each Salmonella isolate were used to ensure reproducibility of the results and the experiments were conducted in duplicate.

RNA extraction and quantitative real time PCR (RT-PCR)

The biofilm cells on the eggshell surface were removed as described above, cotton swabs were suspended in 1 ml of RNAlater® Stabilisation Solution (Ambion™, Austin, TX, USA) and cells were harvested by centrifugation at 5,000 g for 10 min. RNA was extracted from triplicate independent cultures of each Salmonella isolate from each serovar using a RNeasy mini kit (Qiagen, Melbourne, Australia) according to the manufacturer’s guidelines. The concentration of RNA was analysed using a NanoDrop™ 1000 spectrophotometer (Thermo Fischer Scientific, Wilmington, DE, USA) and samples were stored at −80°C.

Quantitative PCR was performed using Rotor gene 600 real time thermal cycler (Corbett Research, Qiagen) with QuantiFast SYBR® Green RT-PCR Kit (Qiagen) as per the manufacturer’s recommended protocol. PCRs were performed in a total reaction volume of 20 μl and contained 10 μl of 2× QuantiFast SYBR Green RT-PCR master mix, 2 μl (1 μM) each of the forward and reverse primers, 0.2 μl of QuantiFast RT Mix, 2.8 μl of RNase-free water and 5 ng of template RNA. Real time PCR was performed using the following cycling conditions: 10 min reverse transcription at 50°C, 5 min initial PCR activation step at 95°C followed by 40 cycles of denaturation at 95°C for 10 s and combined annealing and extension at 60°C for 30 s. Each RNA sample was analysed in duplicate and each PCR reaction included a non-template control. Primer sets synthesised by Geneworks (Adelaide, Australia) were used to amplify *adrA* bapA, *csgB* and *csgD* genes (Supplementary material Table S1). To generate a standard curve, the serially diluted RNA standard (1,000 ng to 0.001 ng) was quantified in each quantitative RT-PCR run. The slopes of the standard curves were used to measure the amplification efficiency for each of these primers. Quantitative RT-PCR results were analysed by comparative threshold cycle (Ct) method and an internal calibrator was used to normalise the transcription levels (Livak & Schmittgen 2001). The relative gene expression was calculated by the 2^ΔΔCt formula, where ΔCt is equivalent to the Ct of the internal calibrator subtracted from the Ct of the target gene.

Scanning electron microscopy (SEM)

SEM was performed to examine the attachment of bacterial cells and cell density on the eggshell during biofilm formation. For SEM, following incubation at 22°C for 48 h, eggs were aseptically removed from Whirlpack bags and the eggshell (1.2 cm² area) was rinsed three times with...
sterile 0.85% normal saline solution to remove unbound cells. Eggshell samples were removed carefully and fixed in a solution containing 1.25% glutaraldehyde, 4% paraformaldehyde, and 4% sucrose in phosphate buffered saline (PBS) (pH 7.2). Samples were rinsed once in wash buffer containing 4% sucrose in PBS, post fixed in 2% osmium tetroxide (OsO4) for 30 min and dehydrated in ascending grades of ethanol. Samples were immersed in a 1:1 mixture of 100% ethanol and hexamethyldisilizane, (ProSciTech, Townsville City, QLD, Australia), then in two changes of 100% hexamethyldisilizane before drying in a fume hood. Samples were mounted on aluminium stubs and coated with platinum for observation under a scanning electron microscope (Philips XL30 FEGSEM) at Adelaide Microscopy, the University of Adelaide.

**Statistical analysis**

For all experiments, the data are presented as mean ± the standard errors of the mean. To determine the significant mean differences between Salmonella serovar, data of biofilm formation and attachment of viable biofilm cells on the eggshell was analysed by one-way analysis of variance (ANOVA) followed by Turkey’s post hoc multiple comparison test.

Significant differences in the expression levels of genes between Salmonella serovars were analysed statistically using the Kruskal–Wallis test. All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, Inc. La Jolla, CA, USA). In all cases, a p-value of < 0.05 was considered statistically significant.

**Results**

**Colonies morphology on Congo red agar plates**

In order to determine curli, fimbriae and cellulose production, the multicellular behaviour of all Salmonella isolates was examined on Congo red agar plates after incubation for 96 h at 22 and 37°C. Two major colony morphotypes were observed in this study: (1) red, dry and rough (rdar), indicative of curli, fimbriae and cellulose production, and (2) smooth and white (saw), indicating a lack of both curli, fimbriae and cellulose production (Figure 1). At 22°C, rdar and saw morphotypes were displayed by 91.03% (132/145) and 8.96% (13/145) Salmonella isolates respectively (Table 1). In contrast, 100% of the Salmonella isolates (145/145) displayed saw morphology on Congo red agar plates at 37°C (Table 1).

**Crystal violet staining assay**

To quantify biofilm production formed by Salmonella isolates at 22 and 37°C after incubation for 96 h, the crystal violet staining assay was used. Overall, the amount of biofilm formation was significantly influenced by temperature (Figure 2A) and representative isolates of Salmonella serovar (Figure 2B and C). Biofilm formation was significantly higher (p < 0.05) at 22°C (OD590 = 2.29 ± 0.02) compared with 37°C (OD590 = 0.21 ± 0.01). Among the seven serovars, isolates of S. Anatum (OD590 = 2.71 ± 0.05) produced significantly more (p < 0.05) biofilm at 22°C compared to S. Agona (OD590 = 2.27 ± 0.04); S. Infantis (OD590 = 2.03 ± 0.05); S. Mbandaka (OD590 = 1.98 ± 0.03); S. Oranienburg (OD590 = 2.21 ± 0.03); S. Typhimurium (OD590 = 2.41 ± 0.06) and S. Worthington isolates (OD590 = 2.43 ± 0.04) (Figure 2B). At 37°C, biofilm formation was weak and increased variability was observed between serovars when compared with 22°C (Figure 2C). At 37°C, only S. Oranienburg (OD590 = 0.72 ± 0.05) and S. Typhimurium (OD590 = 0.21 ± 0.02) formed significantly more (p < 0.05) biofilm compared with the isolates of other serovars: S. Agona (OD590 = 0.05 ± 0.01); S. Anatum (OD590 = -0.01 ± 0.01); S. Infantis (OD590 = 0.02 ± 0.01); S. Mbandaka (OD590 = 0.06 ± 0.01) and S. Worthington (OD590 = 0.04 ± 0.01) (Figure 2C).

**Enumeration of Salmonella biofilm cells on eggshell**

To determine whether Salmonella isolates are able to attach and form biofilm on eggshell, the bacterial cells were removed by swabbing and enumerated by plate count and the results are shown in Figure 3. Control eggs cultured for Salmonella were negative. The numbers of viable biofilm cells attached to the eggshell varied significantly between representative isolates of Salmonella serovars. The number of viable cells recovered from eggshells inoculated with S. Anatum

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**Table 1. Congo red agar morphotypes from various Salmonella serovars after incubation for 96 h at 22 and 37°C.**

| Morphotypes/Temperature | S. Agona (n=6) | S. Anatum (n=6) | S. Infantis (n=16) | S. Mbandaka (n=30) | S. Oranienburg (n=30) | S. Typhimurium (n=26) | S. Worthington (n=31) | Total (n=145) |
|-------------------------|----------------|-----------------|-------------------|-------------------|----------------------|----------------------|----------------------|--------------|
| rdar (22°C)             | 6 (100%)       | 6 (100%)        | 16 (100%)         | 30 (100%)         | 29 (96.66%)          | 26 (100%)           | 19 (61.29%)          | 132 (91.03%) |
| saw (22°C)              | 0              | 0               | 0                 | 0                 | 1 (3.33%)            | 0                    | 12 (38.71%)          | 13 (8.96%)   |
| rdar (37°C)             | 0              | 0               | 0                 | 0                 | 0                    | 0                    | 0                    | 0            |
| saw (37°C)              | 6 (100%)       | 6 (100%)        | 16 (100%)         | 30 (100%)         | 30 (100%)           | 26 (100%)           | 31 (100%)           | 145 (100%)   |

Abbreviations: rdar – red, dry and rough; saw – smooth and white.
To obtain detailed information on the architecture of biofilm formation following incubation for 48 h at 22°C by representative isolates of *Salmonella* serovars, the eggshells were visualised by SEM. SEM revealed that the bacterial cells were firmly adhered to the eggshell surface and had formed a dense multilayer. A characteristic extracellular matrix was observed after incubation for 48 h, indicating (6.65 ± 0.12 log₁₀ CFU cm⁻²) were significantly (*p* < 0.05) greater than *S. Agona* (5.95 ± 0.15 log₁₀ CFU cm⁻²), *S. Infantis* (5.58 ± 0.09 log₁₀ CFU cm⁻²), *S. Mbandaka* (5.69 ± 0.11 log₁₀ CFU cm⁻²), *S. Oranienburg* (5.88 ± 0.08 log₁₀ CFU cm⁻²) and *S. Typhimurium* isolates (5.72 ± 0.21 log₁₀ CFU cm⁻²). Eggs inoculated with *S. Worthington* (6.27 ± 0.13 log₁₀ CFU cm⁻²) had significantly more (*p* < 0.05) viable biofilm cells than eggs treated with *S. Infantis* isolates (5.58 ± 0.09 log₁₀ CFU cm⁻²).

**Figure 1.** Colony morphology on Congo red agar plates. Three μl of overnight grown culture in LB broth, without sodium chloride, were plated on Congo red agar plates, incubated at either 22 or 37°C and visualised after 96 h. Differences in colony morphotypes were evident between the two temperatures. The red, dry and rough (rdar) morphotype was observed at 22°C for both experimental isolates (A) and the control strain *S. Typhimurium* ATCC 14028 (B). Smooth and white (saw) colonies on Congo red agar plates at 37°C were observed for all experimental isolates (C) as well as *S. Typhimurium* ATCC 14028 (D). Images in this figure are representative samples of the rdar and saw morphotypes observed at 22 or 37°C.

**Scanning electron microscopy**

To obtain detailed information on the architecture of biofilm formation following incubation for 48 h at 22°C by representative isolates of *Salmonella* serovars, the eggshells were visualised by SEM. SEM revealed that the bacterial cells were firmly adhered to the eggshell surface and had formed a dense multilayer. A characteristic extracellular matrix was observed after incubation for 48 h, indicating
Expression of biofilm forming genes by quantitative RT-PCR

Genes associated with curli, fimbriae and cellulose production can be used as a measurement of biofilm formation. Quantitative RT-PCR was used to measure the relative transcription levels of csgD, csgB, adrA and bapA genes in eggshell biofilm cells of S. Anatum, S. Agona, S. Infantis, S. Mbandaka, S. Oranienburg, S. Typhimurium and S. Worthington isolates after incubation for 48 h at 22°C.

The relative gene expression levels of csgD in eggshell biofilm cells of S. Mbandaka (1.517 ± 0.387) were significantly higher than the expression observed for S. Typhimurium isolates (0.287 ± 0.142, P = 0.028, Figure 5A). S. Oranienburg (0.303 ± 0.100) exhibited higher expression levels of adrA compared with S. Agona isolates (0.082 ± 0.031, \( p = 0.010 \), Figure 5C). No significant differences in the relative gene expression levels of either csgB or bapA genes in eggshell biofilm cells were observed between representative isolates of different Salmonella serovars (Figure 5B and D).

Discussion

This study investigated the ability of egg farm associated Salmonella serovars to form biofilm and the results demonstrate that biofilm formation was significantly influenced by both temperature and serovar. All the isolates tested during this study were found to possess the ability to form biofilm on the eggshell surface at 22°C. The ability to form a biofilm represents a significant risk not only to contamination of food items prepared with eggs but also in the kitchen environment.

At 22°C, the majority (91.03%) of isolates exhibited the rdar (red, dry and rough) morphotype that has been linked with curli, fimbriae and cellulose production. In contrast, all Salmonella isolates (100%) displayed the saw (smooth and white) morphotype at 37°C indicating the absence of curli, fimbriae and cellulose production. Similar morphotypes have been reported previously for different isolates of S. Typhimurium and S. Enteritidis (Romling et al. 1998, 2003; Castelijn et al. 2012; O’Leary et al. 2013). It has been proposed that bacteria expressing the rdar morphotype form tight attachments on abiotic surfaces and as a consequence are able to persist long-term in the environment (Romling et al. 1998; Vestby et al. 2009).

Findings of the crystal violet assay indicated that although there were significant differences between that a mature biofilm had formed (Figure 4B–H). All representative isolates of Salmonella serovars included in this study produced a dense layer of cells encapsulated by an abundant extracellular matrix over the observed eggshell surface area.
Salmonella serovars in biofilm formation, the Salmonella isolates exhibited dense biofilm production at 22°C compared with 37°C. The results presented here are consistent with the findings of a previous study that examined the biofilm formation capacity of 78 different isolates of eight Salmonella serovars and showed that biofilm formation is strongly dependent on serovar and that the greatest biofilm formation was observed at 20°C (Schonewille et al. 2012).

In Salmonellae, csgD (previously agfD) promoter is the key target for expression of multicellular behaviour (the rdar morphotype) and is regulated by environmental conditions. Expression of csgD positively regulates the production of polymers, thin aggregative fimbriae and cellulose that form the extracellular matrix of biofilm (Romling et al. 2000; Gerstel & Romling 2001; Liu et al. 2014). Previously it has been observed that the rdar morphotype and expression of thin aggregative fimbriae were restricted to low temperatures (below 37°C) (Römling et al. 1998; Grantcharova et al. 2010; Castelijn et al. 2013). Moreover, in the stationary phase of bacterial cell growth, nitrogen and phosphate depletion were found to induce csgD promoters that enhanced the rdar morphotype (Gerstel & Romling 2001). This could partially explain the higher biofilm formation at 22°C in this study. Faster depletion of nutrient availability and changes in oxygen tension and pH are among some of the factors responsible for causing weak biofilm production at 37°C (Gerstel & Romling 2001; Stepanović et al. 2003). Altogether, the data obtained from Congo red agar morphology and the crystal violet staining assay suggest that biofilm composition and regulation is dependent on temperature and vary between Salmonella serovars.

In Australia, Salmonella foodborne outbreaks are often associated with eggshell contamination and not due to bacterial colonisation of the egg internal contents (McAuley et al. 2015). There are no published data reporting the ability of Salmonella spp. to produce biofilm on an eggshell surface. Based on the data obtained from colony morphology and the crystal violet staining assay, further experiments were conducted to examine biofilm formation by Salmonella isolates on eggshell at 22°C. The attachment of Salmonella to the eggshell is the primary step in egg contamination and the biofilm formation process. The findings of this study demonstrated that all Salmonella serovars were able to attach to the eggshell surface but significant differences between representative isolates of various Salmonella serovars were observed. The survival of S. Typhimurium PT 135, an egg product related outbreak strain, was assessed on eggshells and the results indicated the persistence of this strain on the eggshell up to four weeks (McAuley et al. 2015). Previous findings from the authors’ laboratory showed that S. Typhimurium strains were able to survive on an eggshell for up to 21 days (Gole et al. 2014c). While the formation of biofilm was not assessed in either study, this could be one of the likely mechanisms that bacteria use to persist on eggshell. Previous studies on Salmonella Typhimurium isolates from poultry and pork production chains examined their ability to attach to stainless steel surfaces at 20 or 25°C for up to seven or 14 days. The results of these studies revealed long term survival of viable cells attached to the surface of stainless steel coupons. However, survival was influenced by different serovars (Castelijn et al. 2013), the condition of the medium and the incubation period (O’Leary et al. 2013; Wang et al. 2016).

Bacterial attachment to a surface is a complex process and influenced by several factors including temperature. The temperature of 22°C used in the present study to examine bacterial attachment makes it difficult to define the precise temperature range suitable for the attachment of Salmonella to the eggshell. Therefore, further studies at refrigeration temperature analysing the mechanism of survival and/or attachment of Salmonella to the eggshell are needed. The results of biofilm formation on the eggshell suggest that cleaning and disinfection in the food processing environment is necessary, as the persistence of biofilm cells could cross-contaminate other food products and increase the risk of food-borne outbreaks associated with eggs and egg related products.

The architecture of Salmonella attachment and biofilm development by representative isolates of different Salmonella serovars was visualised by SEM and the images showed a dense layer of closely attached cells encapsulated within an extracellular matrix. Such encapsulation of eggshell biofilm cells suggest that curli, fimbriae and cellulose

Figure 3. Enumeration of Salmonella biofilm cells from the eggshell after incubation for 48 h at 22°C. Data are expressed as mean log_{10} CFU cm⁻² ± SEM from two independent experiments. Bars marked with different lowercase letters indicate significant differences (p < 0.05, ANOVA).
Figure 4. SEM images showing biofilm formation by *Salmonella* serovars on eggshells after incubation for 48 h at 22°C. A dense layer of *Salmonella* cells surrounded by extracellular matrix components was observed on inoculated eggshells. Representative images of biofilm formation on the eggshell surface by the different *Salmonella* serovars used in this study. (A) Control; (B) *S*. Agona; (C) *S*. Anatum; (D) *S*. Infantis; (E) *S*. Oranienburg; (F) *S*. Mbandaka; (G) *S*. Typhimurium; (H) *S*. Worthington.
levels of only \textit{csgD} and \textit{adrA} genes were observed between different isolates of \textit{Salmonella} serovars. These data suggest the role of curli, fimbriae and cellulose in eggshell biofilm formation in a low nutrient medium at an ambient temperature of 22°C.

\textit{csgD}, a transcriptional regulator of the LuxR superfamily, positively regulates the expression of \textit{Salmonella} biofilm associated extracellular matrix components, including curli and cellulose (Gerstel & Römling 2003; Fabrega et al. 2014). \textit{csgD} also indirectly regulates cellulose synthesis by activating transcription of \textit{adrA} (Romling et al. 2000; Gerstel & Römling 2003; Castelijn et al. 2012). In this study, the relative gene expression of \textit{csgD} was significantly higher in eggshell biofilm cells of \textit{S. Mbandaka} isolates when compared with \textit{S. Typhimurium} isolates. Interestingly, the higher expression of \textit{csgD} and \textit{adrA} genes observed in \textit{S. Mbandaka} and \textit{S. Oranienburg} isolates, respectively, was not associated with their enhanced eggshell biofilm formation. It is interesting to note that a
parallel difference in terms of csgD gene expression and eggshell biofilm formation between S. Mbandaka and S. Typhimurium isolates was not observed. Similarly, the expression of adrA was significantly higher in S. Oranienburg compared with S. Agona isolates. adrA is an important gene required for cellulose production and its expression in S. Typhimurium biofilm cells has been found to be influenced by the type of growth medium (Wang et al. 2016). The increased expression levels of csgD and adrA genes observed in S. Mbandaka and S. Oranienburg isolates, respectively, could be the result of early development of mature biofilm in these serovars, whereas the low expression levels of these genes in S. Typhimurium and S. Agona isolates could be the result of a developing phase in biofilm formation. Although differences in the gene expression pattern between representative isolates of serovars were observed, interestingly only S. Typhimurium has frequently been reported from egg product related human food poisoning outbreaks in Australia (OzFoodNet Working Group 2015). It is also important to note that other serovars such as S. Oranienburg, S. Mbandaka and S. Agona were detected in eggs or egg products but they were not reported frequently in human outbreaks in Australia (Taylor et al. 1998; Food Standards Australia New Zealand 2009).

The understanding of differential gene regulation during Salmonella biofilm formation is well defined and previous studies have examined this feature on various abiotic surfaces. However, to the authors’ knowledge this is the first study which examined the expression of the key genes involved during Salmonella biofilm formation on the eggshell. Thus, the results of this study cannot be compared with the earlier investigations. Previously, significant variations between time and the expression levels of biofilm-associated genes were observed in S. Typhimurium biofilm formed on stainless steel coupons using trypticase soy broth (Wang et al. 2016); and Castelijn et al. (2012) showed that the expression levels of csgD and adrA were significantly increased in Salmonella biofilm cells in a low nutrient medium at 25°C compared with 37°C.

Although biofilm formation on eggshells at 22°C appears to be serovar related in this study, comparison between serovars using four representative isolates may not be adequate to define the serovar specific differences in biofilm formation on eggshell. Hence, further studies screening a larger number of Salmonella isolates of various serovars are required. Moreover, the expression levels of biofilm genes were analysed at a single temperature and time point. Future studies at different temperatures and time intervals investigating the kinetics of gene expression and biofilm formation on eggshell by different Salmonella serovars are required.

Complete elimination of Salmonella from the poultry and food industry environment is challenging. Overall food safety improvement is possible through adequate hygienic measures, along with antimicrobial intervention strategies at all levels of the food production chain. To reduce eggshell contamination, egg washing with sanitisers is a common method worldwide, including in Australia (Chousalkar et al. 2015). Given the variation in egg washing protocols, it is unclear whether the current sanitisers, their concentration, and exposure time are able to prevent or completely remove biofilm cells on the eggshell. Further research examining the anti-biofilm effects of egg washing agents is required. The ability of Salmonella serovars to form eggshell biofilm observed in this study is of particular interest to the poultry and food industry, as survival and biofilm formation on eggshells at ambient temperature may increase the risk of food contamination and human Salmonella infections. The findings of this study have improved knowledge of eggshell biofilm formation by Salmonella serovars and the information should be useful in implementing anti-biofilm strategies against foodborne infections associated with Salmonella contamination.

In conclusion, this study has demonstrated that Salmonella isolates recovered from a layer farm environment are able to form biofilm on eggshells. The behaviour of biofilm formation was affected by temperature and representative isolates of various Salmonella serovars. In addition, it has been demonstrated that at ambient temperature, Salmonella isolates are able to attach and form biofilm on eggshells and genes associated with curli, fimbriae and cellulose production contribute to biofilm formation. However, differences between representative isolates of Salmonella serovars were evident in eggshell biofilm formation. Further, biofilm formation on the eggshell observed in this study could be a food safety and public health concern, as the persistence of such biofilm producing Salmonella strains would make their eradication more difficult, and enable them to develop resistance to antimicrobials and common disinfectants.

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Disclosure statement

All authors declare no conflict of interest.
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