Refrigeration of eggs influences the virulence of Salmonella Typhimurium

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Salmonella Typhimurium is a human pathogen associated with eggs and egg-derived products. In Australia, it is recommended that eggs should be refrigerated to prevent condensation that can enhance bacterial penetration across the eggshell. Except for the United States, the guidelines on egg refrigeration are not prescriptive. In the current study, in-vitro and in-vivo experiments were conducted to understand the role of egg storage temperatures (refrigerated vs ambient) on bacterial load and the virulence genes expression of Salmonella Typhimurium. The in-vitro egg study showed that the load of Salmonella Typhimurium significantly increased in yolk and albumen stored at 25 °C. The gene expression study showed that ompR, misL, pefA, spvA, shdA, bapA, and csgB were significantly up-regulated in the egg yolk stored at 5 °C and 25 °C for 96 h; however, an in-vivo study revealed that mice infected with egg yolk stored at 25 °C, developed salmonellosis from day 3 post-infection (p.i.). Mice fed with inoculated egg yolk, albumen, or eggshell wash stored at refrigerated temperature did not show signs of salmonellosis during the period of the experiment. Data obtained in this study highlighted the importance of egg refrigeration in terms of improving product safety.

Egg associated human salmonellosis is a significant economic burden on global public health systems. It is generally recommended that eggs should be refrigerated to prevent condensation and subsequent bacterial penetration across the eggshell; however, condensation has not been significantly linked with Salmonella Enteritidis penetration level. Guidelines for egg storage have been mentioned in documents, such as FAO guide, HACCP and ISO 22000, UNECE Standard Egg-1, Chinese National Standard GB 2749–2015; however, it is not compulsory to refrigerate eggs in Australia. In Europe, the EC Regulation No. 589/2008 prevents eggs from refrigeration before sale to consumers. In several countries, including Australia, table eggs are commonly stored at ambient temperature on supermarket shelves. Consequently, there is a continuing debate on whether to refrigerate or not refrigerate eggs. The principle justification for eggs refrigeration is to restrict the growth of pathogenic organisms within the edible components.

Eggs can be contaminated with Salmonella through vertical and horizontal routes during egg formation in the layer hen or handling in the supply chain. In Australia, Salmonella Typhimurium is the major food-borne pathogen of human salmonellosis, which is often associated with the consumption of contaminated eggs or egg-based products. However, other serotypes such as S. Infantis and S. Enteritidis also have been implicated. Washing is an effective strategy for controlling Salmonella on table eggs, but it can cause damage to the egg cuticle. Once Salmonella enters the egg internal contents, it can replicate at ambient temperature. In many countries, including Australia, supermarkets and grocery shops have no prescriptive guidelines on egg storage. Therefore, it is important to understand the role of storage temperatures on survivability and growth of Salmonella Typhimurium in eggs and its subsequent virulence factors in causing salmonellosis in humans. Relevant research on the behaviour of Salmonella in eggs about storage temperatures were either focused on the penetration of bacteria across the eggshell or using mainly Salmonella Enteritidis as a model organism. The bacterial load on the eggshell surface is inversely affected by storage time and temperature, while an increase in relative humidity supports the bacterial survivability as shown for Salmonella Enteritidis. The survival behaviour of Salmonella Enteritidis both in an egg and culture media is different from other serotypes including Salmonella Typhimurium. Therefore, studies are required to understand the behaviour of Salmonella Typhimurium in eggs and the subsequent development of salmonellosis through the consumption of the contaminated egg components.

In the host (as shown for mouse), upon ingestion, Salmonella Typhimurium survives in the acidic environment of the stomach and migrates to the intestine, where it invades the intestinal epithelia and triggers inflammation and onset of clinical disease. Salmonella uses Type III secretory systems encoded by SPI (Salmonella Proteins of Invasion and Effector).
Salmonella \( n = 6 \) were dipped in the inoculum were dipped for 90 s in BPW (prepared in 1 L sterile beaker) that contained 1 × 10⁹ CFU/mL of shed environment has shown to be variable¹⁹. Therefore, in the current study, the inoculum dose for Salmonella Typhimurium grown in egg components was not adjusted for mice infection.

Various studies have investigated the effects of storage temperatures on the growth and survivability of Salmonella in eggs¹⁰,¹¹,²⁰, however, no subsequent investigations on the development of salmonellosis through the consumption of the contaminated egg contents have been conducted. The growth kinetics of Salmonella Typhimurium is regulated by temperature. For example, the survivability of Salmonella Typhimurium on eggshell is better at 22 °C, while bacterial load decreases with storage time at 4 °C²¹. Salmonella Typhimurium grows well in the yolk at either 15 °C or 22 °C, whereas its growth in albumen is temperature dependent²¹. A study has linked the up-regulation of yafD and xthA with the Salmonella Enteritidis survival in albumen²².

Salmonella serotypes specific differences in survivability in egg albumen¹⁴,²³ suggest that Salmonella Typhimurium may regulate its transcriptional machinery differently. To answer this question, we hypothesized that the virulence of Salmonella Typhimurium is enhanced at 25 °C compared with refrigeration temperature and, therefore, if consumed, contaminated eggs stored at ambient temperature will cause clinical disease in mice. The present study had three main objectives: (1) Understand the growth and survivability of Salmonella Typhimurium in eggs at 5 °C and 25 °C. (2) Determine the effects of storage temperatures on Salmonella Typhimurium gene expression in yolk, albumen and on the eggshell surface. (3) Study the virulence of Salmonella Typhimurium cultured in raw egg components in BALB/c mice.

Methods

Salmonella growth and survivability kinetics in eggs at different temperatures. Eggs preparation and inoculation with Salmonella Typhimurium. For both the in-vitro and in-vivo experiments, a pure culture of Salmonella Typhimurium definitive type 9 stored at −80 °C in 50% glycerol was revived on nutrient agar (NA; ThermoFisher Scientific, Australia) and subcultured in Luria Bertani (LB; ThermoFisher Scientific, Australia) broth until the optical density (OD) at 600 nm reached approximately 1. Fresh eggs directly obtained from a 33-week old cage layer hen flock were sanitized by dipping in 75% ethanol for 90 s and air dried completely in a class II biosafety cabinet. To study the survivability of bacteria on the eggshell surface, individual eggs \((n = 6\) in each treatment group) were dipped for 90 s in 1 × 10⁶ CFU/mL of Salmonella suspension prepared in LB broth. To confirm bacterial deposition on the eggshell surface, an extra set of eggs \((n = 6\) were dipped in the inoculum and processed immediately for the recovery of Salmonella. For studying Salmonella growth or survivability in the albumen and yolk, individual eggs received a 0.1 mL inoculum of 1 × 10⁷ CFU per egg directly injected into the egg albumen or yolk and the injection holes were sealed. This dose was selected to mimic a field scenario where a low load (1.71 log₅⁰ MPN per egg) of Salmonella on an egg has been reported¹⁹. All the treatment groups were incubated at 5 °C or 25 °C for either 96 h or 28 days. The 5 °C treatment eggs were put in a 55 L plastic container and stored in a cool room and the 25 °C condition eggs were stored in an incubator. Two separate hygrometers were used to monitor the relative humidity (%) levels of the two treatment groups. These two temperature conditions were selected as most likely during storage eggs are exposed to an ambient temperature close to 25 °C. The control eggs in each treatment group \((n = 4\) per treatment) were treated in sterile LB and processed in the same way as of the Salmonella inoculated eggs.

Quantitative recovery of Salmonella from the inoculated eggs. For estimation of Salmonella survivability on the eggshell surface, individual eggs from both the treatment groups \((5 °C and 25 °C) were washed for 2 min in 10 mL of buffered peptone water (BPW; ThermoFisher Scientific, Australia) in Whirl–Pak sample bags, the rinsate was serially diluted (10 times) in phosphate buffered saline (PBS) and plated onto xylose lysine deoxycholate (XLD; ThermoFisher Scientific, Australia) agar plates for overnight incubation at 37 °C. Salmonella culturability in albumen and yolk of the stored eggs was also determined. Eggs were opened into a sterile 90 mm petri dishes, then poured into sterile bags for thorough mixing, and using a syringe, 0.1 mL of either albumen or yolk was added to 0.9 mL of BPW and mixed. Serial tenfold dilutions were prepared in PBS and plated onto XLD agar plates. The plates were incubated overnight at 37 °C and Salmonella colonies were enumerated to determine the total number of bacteria in each sample. The egg contents of the 5 °C stored samples were enriched for the qualitative assessment of Salmonella following a previously described method¹⁹. Briefly, 1 mL samples of yolk or albumen of individual eggs were enriched in 9 mL of BPW and incubated overnight at 37 °C. From the incubated samples, 100 μL was added to 10 mL of Rappaport–Vassiliadis soya peptone (ThermoFisher Scientific, Australia) broth and incubated overnight at 42 °C. The samples were streaked onto XLD, incubated overnight at 37 °C and read as positive or negative for characteristic Salmonella colonies. To rule out the chances of external contamination, control groups were processed in the same manner. The growth of Salmonella was recorded as CFU/mL of albumen or yolk or eggshell surface rinsate. The in-vitro egg experiments were repeated for a total of three times.

Role of temperature and storage time in regulation of genes of Salmonella in egg components. To obtain good quality RNA from Salmonella inoculated egg components, albumen and yolk were inoculated with 1 × 10⁶ CFU/mL of Salmonella, while for the eggshell surface inoculation, intact individual eggs were dipped for 90 s in BPW (prepared in 1 L sterile beaker) that contained 1 × 10⁶ CFU/mL of Salmonella. Based on the protocol of TRIzol Reagent (Invitrogen, Australia), at least 1 × 10⁷ cells of bacterial origin are required to extract sufficient RNA. Our pilot experiment showed that 1 × 10⁶ CFUs of Salmonella per mL of egg content was not sufficient for quality RNA extraction.
Preparation of egg components. Intact eggs were sanitized in ethanol and allowed to dry thoroughly as described previously. As the volume of albumen and yolk vary from egg to egg; therefore, for precise measurement of per ml volume for Salmonella inoculation, egg contents were separated in sterile petri dishes. Maximum albumen (both the colloidal and watery parts) or yolk from individual eggs was aspirated using separate syringes and decanted into 50 mL Falcon tubes. The quantity of albumen or yolk in the tubes was adjusted based on Salmonella inoculum (1 × 10^8 CFU/mL of yolk or albumen). There were 3 biological replicates for each of the egg components.

Preparation of Salmonella inoculum. A stock culture of Salmonella Typhimurium was revived on NA media plate and a single colony was subcultured in LB broth until the OD (measured at 600 nm) of the culture reached approximately 1. The inoculum dose for the egg surface was prepared in LB, while for the egg internal contents, Salmonella culture was pelleted by centrifugation (4000 × g for 15 min). The pellet was resuspended in 1 mL of PBS and its OD at 600 nm was read as described earlier. The culture was diluted to obtain 1 × 10^8 CFU/mL of Salmonella and a 0.1 mL of this inoculum was added into either 0.9 mL of yolk or albumen. Thus, the final dose of Salmonella was 1 × 10^8 CFU/mL of egg content. For the positive control group, Salmonella Typhimurium was incubated (with 3 biological replicates) in a shaking incubator at 37 °C in LB broth for 12 h and 1 × 10^8 CFU/mL was processed for RNA extraction.

Salmonella RNA extraction from egg components and pure culture of bacteria. To recover Salmonella from the egg surface for RNA extraction, individual eggs (n = 3) at each incubation time-point (12, 72, and 96 h) from each treatment group (5 °C and 25 °C) was solubilized in 1 mL of PBS and its OD at 600 nm was read as described earlier. The culture was diluted to obtain 1 × 10^8 CFU/mL of Salmonella and a 0.1 mL of this inoculum was added into either 0.9 mL of yolk or albumen. Thus, the final dose of Salmonella was 1 × 10^8 CFU/mL of egg content. For the positive control group, Salmonella Typhimurium was incubated (with 3 biological replicates) in a shaking incubator at 37 °C in LB broth for 12 h and 1 × 10^8 CFU/mL was processed for RNA extraction.

Primer, cDNA synthesis and Fluidigm PCR. A total of 95 primers for targeted genes of Salmonella Typhimurium involved in stress response, virulence, survivability, metabolism, and host colonization were designed using NCBI as described in our previous publications and were synthesized by Merck, Australia. The main aim behind selecting these genes was to understand their regulations in egg components about storage temperature and their subsequent role in causing clinical salmonellosis in mice. Using PCR (see Supplementary material Text S1) and 2% agarose gel electrophoresis, all the primers were tested for target specificity in cDNA synthesized from Salmonella Typhimurium extracted RNA. To cross check the issue of genomic DNA contamination, RNA samples were also included in the PCR run. cDNA was synthesized from the extracted RNA as per the protocol of the QuantiTect Reverse Transcription Kit (Qiagen, Australia). Briefly, 1 μg of RNA per sample was subjected to genomic DNA removal in a 14 μL total reaction volume for 3 min at 42 °C as per the protocol of the kit. For the reverse transcription step, the samples (in 20 μL reaction volume) were incubated for 25 min at 42 °C and to inactivate the QuantiScript reverse transcriptase, a final incubation step for 3 min at 95 °C was executed. The cDNA samples were stored at –80 °C until used for cDNA synthesis and Fluidigm PCR.

Effect of Salmonella inoculated egg components on clinical disease in mice. Animal ethics. The experimental protocol was approved by the Animal Ethics Committee at The University of Adelaide under approval number S-2018-009. Specific pathogen free 6–8 week-old, female BALB/c mice were sourced from the Laboratory Animal Services at The University of Adelaide. The mice were fed ad libitum a commercial diet free from Salmonella following the guidelines specified in the "Australian Code for the care and use of animals for scientific purposes, 8th edition (2013)."

Ethics approval and consent to participate. The University of Adelaide, Animal Ethics Committee under Approval Number No., approved the experimental setup. S-2018-009. All animal experiments complied with the...
ARRIVE guidelines and were also carried out in accordance with the guidelines -specified in Australian Code for the Care and Use of Animals for Scientific Purposes 8th edition 2013.

Mice inoculation with egg components containing Salmonella. Mice were divided into 16 treatment groups with 7 mice per group (Table 1). Yolk, albumen, and eggshell wash used as inoculums were prepared as described for the in-vitro studies above. Briefly, the inoculated eggs were stored at either 5 °C or 25 °C for 96 h and individual mice received through oral gavage 0.1 mL of shell wash, yolk, or albumen that had either been inoculated or non-inoculated with Salmonella Typhimurium. The positive control groups received through oral gavage 1 × 10^3 CFU/0.1 mL of Salmonella Typhimurium in LB broth stored at either 5 °C or 25 °C (inoculum adjusted) for 12 h. Further detail of the inoculum that individual mice in each treatment group received is outlined in Table 1.

The leftover inoculums from the treatment groups were maintained on ice, serially diluted and plated on XLD agar to confirm the actual dose of Salmonella Typhimurium that mice received. The experiment was conducted over 21 days. During the post-infection period (day 0 to 21), the mice were routinely observed for clinical signs of non-typhoidal salmonellosis (e.g. lethargy, hunching, ruffled fur) and mortality. Adhering to the Animal Ethics Committee Guidelines, mice suffering from the clinical disease with a score of five or above were humanely euthanized using carbon dioxide and the collected organs were processed for the quantification of Salmonella.

Salmonella detection in mice feces. From all the treatment groups, fecal samples were collected on day 3, 6, 9, 12, 15, and 18 post- infection and processed for Salmonella isolation. The cage and bedding materials were changed at every fecal sampling to minimize the risk of carryover. For the qualitative assessment of Salmonella, 1 g of fecal samples was mixed into 9 mL of BPW, vortexed and incubated overnight at 37 °C. The samples were processed following the RVS enrichment method previously described. The plates were then examined to determine if the samples were positive (scored as 1) or negative (scored as 0) for Salmonella Typhimurium.

Salmonella load in organs. At the point of cull, segments of liver, spleen, ileum and cecum were collected and homogenized in tubes containing 0.5 mL of 0.9% saline and stainless-steel beads (2–8 mm). Homogenates were serially (tenfold) diluted and processed for the quantitative and qualitative assessment of Salmonella. For the quantitative assessment, 0.1 mL of the homogenate was directly plated onto XLD, incubated overnight at 37 °C and colonies were counted. The CFU data were expressed as log_{10} load of Salmonella per gram of the organ.

Statistical analysis. The Salmonella load data were analyzed in GraphPad Prism version 8.0 using one- and two-way ANOVA with Tukey’s multiple comparison test for determining the level of significance (P < 0.05). The relative gene expression fold change was calculated by 2^ΔΔCq method and LB 12 h was used as a reference control. The log, fold change data between the 12, 72 and 96 h were analyzed in StatView version 5.0.1 by repeated measure analysis and the level of significance was determined by PLSD (P < 0.05).

Results

Growth kinetics and survivability of Salmonella in egg components. Post-inoculation with Salmonella, the relative humidity at both 5 °C and 25 °C varied from 76 to 82% over the 96 h egg storage experiment. The in-vitro experiment showed that Salmonella Typhimurium inoculated into egg yolk grew significantly to log_{10} 7.88 CFU/mL at day 4 of storage at 25 °C, whereas its growth plateaued from day 4 until day 28 of storage (Fig. 1A). This showed that after 96 h of storage, the load of Salmonella Typhimurium increased by log_{10} 7.88 CFU/mL at day 4 of storage at 25 °C.

| Treatment | Inoculum |
|-----------|----------|
| Negative control 5 °C | LB broth |
| Negative control 25 °C | LB broth |
| Shell wash control 5 °C | BPW |
| Albumen control 5 °C | Albumen |
| Yolk control 5 °C | Yolk |
| Shell wash control 25 °C | BPW |
| Albumen control 25 °C | Albumen |
| Yolk control 25 °C | Yolk |
| Positive control Salmonella in LB broth stored at 5 °C | 10^3 CFU |
| Salmonella from shell wash stored at 5 °C | 10^3 CFU |
| Salmonella in albumen stored at 5 °C | 1 CFU |
| Salmonella in yolk stored at 5 °C | 10^3 CFU |
| Positive control Salmonella in LB broth stored at 25 °C | 10^3 CFU |
| Salmonella from shell wash stored at 25 °C | 10^3 CFU |
| Salmonella in albumen stored at 25 °C | 10^3 CFU |
| Salmonella in yolk stored at 25 °C | 10^3 CFU |

Table 1. Treatment groups and inoculum dose used in the mice study.
3.88 CFU/mL in the yolk. The data showed that when inoculated in egg albumen, by day 4 post-inoculation, *Salmonella Typhimurium* migrated from albumen and grew to $\log_{10} 5.70$ CFU/mL in the yolk (Fig. 1A). Load of *Salmonella Typhimurium* in the albumen of albumen-inoculated eggs remained constant from day 0 to day 4 of storage and then increased to $\log_{10} 6.84$ CFU/mL (Fig. 1B). Overall, these data showed that the growth of *Salmonella Typhimurium* significantly increased in the yolk within 96 h of storage at 25 °C of the inoculated eggs. In the qualitative assessment, 75.6% of the yolk and 15.6% of the albumen samples of the eggs stored at 5 °C were positive for *Salmonella* after the enrichment process (Supplementary Fig. S1A), demonstrating that *Salmonella Typhimurium* survived better in yolk than albumen over the 28 days of storage (Supplementary Fig. S1B).

As the samples stored at 5 °C did not show growth of *Salmonella* after the direct plating; therefore, in the subsequent in-vitro experiments, inoculated eggs were stored only for 96 h. In contrast to 25 °C, *Salmonella Typhimurium* counts remained constant for yolk and albumen samples stored at 5 °C for 96 h (Fig. 2A). However, the load of *Salmonella Typhimurium* was significantly higher in the yolk compared with the albumen. *Salmonella Typhimurium* survived numerically higher on the eggshell surface stored at 5 °C compared with the 25 °C (Fig. 2B). For the eggshell treatment groups, on average, each egg had $9.7 \times 10^5$ (or 5.99 on $\log_{10}$ scale) CFU of *Salmonella*, which was just under 1 mL of inoculum that contained $1 \times 10^6$ CFU/mL of LB broth and stored at 5 °C or 25 °C for 96 h. The stored eggs were processed for the recovery of *Salmonella* and the load was presented as $\log_{10}$ CFU/mL of albumen, yolk or shell rinsate. Within each storage temperature, different superscripts show significant differences (P < 0.05). Values are mean of $\log_{10}$ CFU ± S.D.

**Figure 1.** Load of *Salmonella Typhimurium* in yolk or albumen inoculated eggs stored at 25 °C. (A) Load in yolk of yolk and albumen inoculated eggs. (B) Load in albumen of yolk and albumen inoculated eggs. At each sampling time-point, 3 eggs from each treatment group were processed for the quantification of *Salmonella Typhimurium* load through direct plating on XLD media plates. At day 0, each egg was inoculated with $10^3$ CFU/0.1 mL of *Salmonella Typhimurium* directly injected to either yolk or albumen.

**Figure 2.** Survivability of *Salmonella Typhimurium* in eggs stored for 96 h. (A) Yolk and albumen inoculated eggs stored at 5 °C. (B) Eggshell inoculated eggs stored at 5 °C or 25 °C. Individual eggs in each of the yolk and albumen treatment groups received $1 \times 10^3$ CFU/0.1 mL, and the eggs were stored at 5 °C for 96 h. For the shell treatment groups, intact eggs were dipped in *Salmonella* inoculum containing $1 \times 10^6$ CFU/mL of LB broth and stored at 5 °C or 25 °C for 96 h. The stored eggs were processed for the recovery of *Salmonella* and the load was presented as $\log_{10}$ CFU/mL of albumen, yolk or shell rinsate. Within each storage temperature, different superscripts show significant differences (P < 0.05). Values are mean of $\log_{10}$ CFU ± S.D.

*Salmonella transcription profile is affected by egg storage temperature.* To understand the effects of storage temperatures on the gene expression of *Salmonella Typhimurium* in the yolk, albumen and on eggshell, Fluidigm PCR was performed on *Salmonella* RNA extracted from inoculated egg contents whereas
RNA obtained from Salmonella grown in LB for 12 h at 37 °C acted as a control for the relative gene expression analysis. Overall, several genes were up- or down-regulated in different egg components and storage temperatures; therefore, only the genes significantly up-regulated at least at one storage time point or involved in pathways that include Salmonella pathogenicity islands are presented in this manuscript. Many of the key Salmonella genes involved in virulence and colonization in the mammal host were downregulated in albumen and on egg surface of the samples that were stored at 5 °C and 25 °C.

**Stress response, host colonization, virulence and invasion.** Among the genes involved in host colonization, aggregation and infection, the expression levels of bapA, cadB, cadC, misL, ompR, shdA, spvA and spvC in at least one egg content and storage time-points were up-regulated (Fig. 3). Among them, bapA is involved in biofilm formation and host colonization, and the deletion of bapA has shown reduced colonization potential in the gut of mice.\(^{26}\) bapA was significantly up-regulated in the yolk and its mean expression was significantly higher in the 25 °C stored yolk compared with the 5 °C (Fig. 3A). However, in albumen and on the egg surface, the expression of bapA was not consistent, where it was down-regulated on the egg surface of the samples stored at 25 °C. cadB, cadC and misL were up-regulated in the yolk stored both at 5 °C and 25 °C, while in albumen, these genes were up-regulated at 96 h of incubation in the samples stored at 5 °C (Fig. 3B–D).

Among the genes involved in stress response, ompR was the only up-regulated gene across all the samples at the stored conditions (Fig. 4A). For albumen and egg surface samples, the mean fold change of ompR was significantly higher at 5 °C compared with the 25 °C stored samples. shdA was not consistently regulated in albumen and on egg surface, while in yolk, it was consistently upregulated both at 5 °C and 25 °C at the storage time-points (Fig. 4B). The expression of shdA is involved in colonization as shown in mice challenged with Salmonella Typhimurium.\(^{27}\) spvA and spvC were up-regulated in yolk but were down-regulated in albumen and on egg surface stored at 5 °C and 25 °C for 96 h (Fig. 4C,D).

**Flagella, fimbrae and biofilm formation.** In biofilm formation, both curli and cellulose synthesis are co-regulated by a complex regulatory network in which cgD (agpD) acts as a global regulator in the expression of cgB and cgA.\(^{28}\) In the yolk stored both at 5 °C and 25 °C, cgB, fimH, pefA and pefB were up-regulated across all the time-points, while their up-regulation was consistent in albumen and on the surface of eggs across the storage time-points (Fig. 5A–D). In the albumen stored at 5 °C, cgB, cgD, fimH, pefA were significantly up-regulated at only 96 h. The expression of bapA is up-regulated in the formation of curli and cellulose through the involvement of cgD.\(^{28}\) This indicates that Salmonella on the egg surface at 5 °C resisted well to the low temperature. Genes such as yaiC (adrA), fimA, fliA and fliC were up-regulated at least in one egg component at one storage time-point.
Glycolysis, purine and pyrimidine metabolism. Multiple genes involved in cell metabolism were up-regulated in the yolk stored both at 5 °C and 25 °C for 96 h (Fig. 6A,B). Among the investigated genes, carA, carB, purD, pyrB, pyrD and pyrE were up-regulated in the yolk stored both at 5 °C and 25 °C across all the storage time-points, while the up-regulations of purE, purG and pyrC were inconsistent (Fig. 6A,B). Unlike samples stored at 5 °C, there were not many Salmonella genes significantly up-regulated in albumen stored at 25 °C. In the albumen stored at 5 °C, adk and yaiC (adrA) were significantly up-regulated at 96 h, while aroC, fbp, pgm, ptsG, purA, purD, purE, purF, pyrB, pyrC, pyrD and pyrE were up-regulated both at 72 and 96 h. At the surface of eggs stored...
at 5 °C, carA, carB and purF were significantly up-regulated at 12, 72 and 96 h. Apart from the genes involved in purine and pyrimidine metabolism, aroC catalyses the pathway of chorismate that serves as the starting substrate in the synthesis of aromatic amino acid biosynthesis. The up-regulation of aroC confirms that cell integrity was compromised by the refrigerated temperature in Salmonella in albumen and on the egg surface. hisD was the only gene up-regulated in yolk, albumen and on the egg surface across all the storage time-points except on the surface of the eggs stored at 25 °C. These data indicate that multiple pathways involved in cell metabolism were activated by Salmonella Typhimurium in response to temperature and egg component with pathways being more consistently up-regulated in the yolk samples.

Two-component system and Salmonella pathogenicity islands. Our data showed that the phoP and phoQ involved in the two-component system were significantly down-regulated across all the sampling time-points in egg components, except the albumen stored for 72 and 96 h at 5 °C (Fig. 6A). This positively correlated with the expression of other invasion genes such as prgH. All of the investigated genes involved in the regulation of type III secretory systems, such as ssaB, ssaC, ssaD, ssaE, ssaG, ssaH, ssaI, ssaJ, ssaK, ssaL, ssaM, ssaN and ssaP were significantly down-regulated in the egg components stored at 5 °C and 25 °C for 12, 72 and 96 h except the ssaA that was up-regulated on the egg surface at 5 °C. hilA, the transcriptional activator of SPI1 and co-activators such as hilC and hilD were all down-regulated in the egg components stored for 96 h both at 5 °C and 25 °C.

Other genes that inconsistently upregulated in the yolk, albumen and on egg surface were proP, narL, narX, rpoS, xthA and yafD. In brief, bapA, csgB, fimH, misL, ompB, pefA, pefB, purD, pyrD, pyrE, shdA, spaA and spaC consistently up-regulated across all the three storage time-points in the yolk are implicated in maintaining the virulence of Salmonella in yolk. Interestingly, these genes were not consistently up-regulated in the albumen and on the egg surface.

Salmonella Typhimurium invasiveness in mice and development of clinical disease. The treatment groups that received Salmonella inoculated yolk stored at 25 °C for 96 h and the positive control group that received Salmonella in LB broth stored at 25 °C for 12 h were culled at day 6 and day 8 post-infection, respectively, due to the development of clinical signs with a score of 5 and above. The percent survival rate of the clinically sick mice was very low compared with all other treatment groups that survived until culled at day 21 p.i. (Supplementary Fig. S2A). There was no significant difference in the percent survivability in the treatment groups that received yolk and LB inoculated groups.

Fecal shedding profile of Salmonella inoculated mice and Salmonella load in organs. Prior to the challenge with Salmonella Typhimurium, feces were collected from the mice and tested for the presence of Salmonella. All mice were Salmonella negative prior to commencing the infection challenge. Salmonella was not detected in the feces...
of control mice or mice infected with egg contents stored at 5 °C at any time-point during the experiment. Feces collected from mice inoculated with LB or yolk stored at 25 °C were positive for *Salmonella* at day 3 and 6 post-infection. Mice inoculated with shell wash and egg albumen stored at 25 °C containing *Salmonella Typhimurium* were culture negative for the bacteria in their feces at day 3, 6, 9, and 12 post-infection. At day 15 and 18 post-infection, however, fecal samples collected from mice in these two treatment groups were culture positive for *Salmonella Typhimurium* (Fig. 7A).

*Salmonella* was enumerated from all the organs that were sampled from mice culled during the trial. The organs collected from the treatment groups that did not shed *Salmonella* in the feces were also negative for *Salmonella*, after the enrichment culture method. *Salmonella* load was higher (P < 0.05) in cecum, ileum, liver and spleen of the mice fed with *Salmonella* in LB and yolk compared with the mice received *Salmonella* in albumen and on shell wash stored at 25 °C (Fig. 7B).

**Discussion**

The main objective of this study was to understand the temperature driven changes in *Salmonella Typhimurium* inoculated into table eggs. The in-vitro study showed that *Salmonella Typhimurium* grew significantly in yolk by day 4 of storage at 25 °C and from day 4 to day 28, the growth plateaued. A significantly higher load in the albumen inoculated eggs stored at 25 °C on day 7 compared with day 4 showed that *Salmonella Typhimurium* grew in albumen at a slower rate. The slower growth rate of *Salmonella Typhimurium* in albumen compared with yolk could be due to its antimicrobial properties as shown for *Salmonella Enteritidis*. The increased load of *Salmonella Typhimurium* in the yolk of albumen inoculated eggs confirmed that yolk favoured its growth.

*Salmonella* was enumerated from all the organs that were sampled from mice culled during the trial. The organs collected from the treatment groups that did not shed *Salmonella* in the feces were also negative for *Salmonella*, after the enrichment culture method. *Salmonella* load was higher (P < 0.05) in cecum, ileum, liver and spleen of the mice fed with *Salmonella* in LB and yolk compared with the mice received *Salmonella* in albumen and on shell wash stored at 25 °C (Fig. 7B).

Of control mice or mice infected with egg components containing *Salmonella Typhimurium*. (A). Qualitative assessment of feces for *Salmonella*. (B). Load of *Salmonella* in caecum, ileum, liver and spleen of challenged mice on day of cull. Except of four, the rest of the treatment groups (Table 1) did not shed *Salmonella* in the feces until the termination of the trial on day 21 p.i. Treatment groups that received *Salmonella* in yolk and LB were euthanized on day 6 and day 8 respectively due to the development of clinical salmonellosis. *Salmonella* was not recovered from any of the organs collected from the remaining treatment groups that did not shed *Salmonella* in the feces. Within each organ, different superscripts show significant differences (P < 0.05). In Panel (A) of the figure, values are proportion of *Salmonella* positive fecal samples, while in Panel (B), values are the mean of log_{10} CFU ± S.D.
that eggs should be stored at refrigerated temperature to reduce the growth rate of _Salmonella_. It is important to note that a different inoculum dose was used on egg surface compared to egg internal contents. This was to mimic the field conditions (real-life scenario) where _Salmonella_ infected floc can lay eggs with up to 10⁶ CFU/egg. In order to get an inoculum of 10⁸ for mouse challenge from the egg surface, egg surface was inoculated with 10⁶ CFU, as eggshells have numerous pores and it is possible that _Salmonella_ Typhimurium can penetrate through pores. Given that _Salmonella_ Typhimurium penetration across the eggshell pores was not tested in this study, further investigation is necessary. Overall, the data in the in-vitro whole egg experiment confirmed that within 4 days of inoculation _Salmonella_ Typhimurium grew at a faster rate in the yolk of both the yolk and albumen inoculated eggs stored at 25 °C. Therefore, in the subsequent in-vitro and in-vivo experiments, eggs were stored until day 4 post _Salmonella_ Typhimurium inoculation.

The Fluidigm PCR data showed that _Salmonella_ Typhimurium up-regulated genes involved in cell metabolism, fimbriae formation, stress response, virulence and survival in egg. However, the expression pattern varied with temperature and egg component. An in-vitro study with egg albumen showed that when incubated below 30 °C for a maximum of 24 h, egg albumen exhibited bacteriostatic activity against _Salmonella_ Enteritidis. Differences in the survival ability of two genetically similar strains of _Salmonella_ Enteritidis in an egg show that _Salmonella_ adopts a wide range of strategies that include nucleotide deletion or insertion. Differences in the gene expression pattern observed in the current study could be due to the variable behaviour of the bacteria, as _Salmonella_ Typhimurium expresses its pathogenicity islands differently.

The csg region in _Salmonella_ spp., encodes protein polymers known as curli fimbriae, which promote community behaviour and host colonization. In low temperature, curli are important for cell aggregation, adhesion to surfaces and biofilm formation. The up-regulation of csgB both at 5 °C and 25 °C in the yolk, albumen and at 5 °C at the egg surface shows that _Salmonella_ expressed the curli protein to maintain the physiology of survival and aggregation. Interestingly, in this study, the non-consistent regulation of csgD with csgB showed their independent regulations in the experimental conditions. yafD in _Salmonella_ Enteritidis provides resistance to albumen, and in the current study, a condition dependent up-regulation of expression in the albumen stored at 5 °C for 12 and 72 h was observed. The consistent up-regulation of ompR across all the sampling time-points in the egg components stored both at 5 °C and 25 °C shows its role in the survivability of _Salmonella_ in an egg. The data showed that ompR played a greater role in _Salmonella_ survival at 5 °C, as it acts as a central regulator in reprogramming the _Salmonella_ transcriptome in a stressful environment. Among the genes involved in osmotic stress response, the up-regulation of rpoS both in albumen and yolk at 5 °C shows that _Salmonella_ diverted the egg component and temperature driven stress mainly through this gene. However, it is important to note that not all the genes involved in osmotic stress response were included in the current study.

The two-component signal transduction system is involved to modify the cellular output in response to environmental signals both in-vitro and in-vivo conditions. The down-regulation of phoP and phoQ in the egg components show that _Salmonella_ was unable to up-regulate its two-component system in the conditions applied in this study. The current study confirms that _Salmonella_’s persistence in yolk, albumen and on the egg surface leads to the downregulation of type III secretory system. Overall, _Salmonella_ Typhimurium regulated its transcriptional machinery differently in eggs stored at 5 °C and 25 °C, whereas the temperature driven changes affected the in-vivo virulence capacity of _Salmonella_ in the murine model.

The findings in the in-vitro egg studies correlated with the in-vivo mice trial, where the data demonstrated that _Salmonella_ Typhimurium in the yolk stored at 25 °C showed the invasive potential that resulted in the development of clinical salmonellosis. _Salmonella_ load in the 25 °C stored albumen slightly increased by day 4 of storage; however, the infected mice treatment group that received 10⁴ CFU in albumen did not start shedding _Salmonella_ in feces until day 15 p.i. The yolk contains high levels of iron, which is an important requirement for bacterial growth. Growth of _Salmonella_ Typhimurium has been shown to increase in response to the presence of iron, and adhesion of _Salmonella_ Typhimurium to epithelial cells increases when the bacteria are pre-incubated in higher iron environments. Therefore, this study confirmed that egg yolk can become a higher risk food of iron, and _Salmonella_ Typhimurium grew at a faster rate in the yolk of both the yolk and albumen inoculated eggs stored at 25 °C. Therefore, in the subsequent in-vitro and in-vivo experiments, eggs were stored until day 4 post _Salmonella_ Typhimurium inoculation.

In the current study, no significant increase in _Salmonella_ load in feces until day 15 p.i. The yolk resulted in fecal shedding from day 3, while the latter group did not shed the bacteria until day 15 p.i. Shell wash at 25 °C and albumen at 25 °C inoculated mice began shedding _Salmonella_ from day 15 p.i. with no clinical signs of salmonellosis during the trial period. This delay in shedding could be attributed to the _Salmonella_ being initially stressed due to the storage environment (shell wash and fresh egg albumen). The stress of the treatment group environment (shell wash and albumen) coupled with the exposure to the stressful environment of the digestive tract of the mice could have caused a delay in the development of salmonellosis and fecal shedding. Although _Salmonella_ survived in the egg components at 5 °C storage, the inoculum did not result in the fecal shedding of _Salmonella_ in mice during the trial period. Surprisingly, this was the case for the _Salmonella_ stored LB in the 5 °C treatment group as well. Observing gross pathological lesions during dissection in the _Salmonella_ in yolk and LB (stored at 25 °C) inoculated groups confirmed that these mice developed salmonellosis. However, these findings were not observed.
in the mice from the albumen or shell rinsate inoculated or any other group that did not shed *Salmonella* in the feces. In Australia, upon collection from the layer sheds, eggs are stored at temperatures ≤ 15 °C on farms. This temperature is then maintained during grading steps at farms. Further studies are necessary to investigate the effect of farm storage temperature on *Salmonella* virulence.

**Conclusions**

Overall, the growth and survivability of *Salmonella Typhimurium* in egg affected by ambient temperature. The temperature influenced the virulence of *Salmonella Typhimurium* and the storage of inoculated eggs at ambient temperature resulted in salmonellosis. In the in-vitro study, the panel of genes assessed for their functions in maintaining the virulence of *Salmonella* showed that genes involved in metabolism, stress response, virulence, and colonization were down-regulated in the albumen and on the egg surface. In the in-vivo experiment, mice infected with egg wash and albumen containing *Salmonella* stored at ambient temperature started shedding *Salmonella* in feces on day 15 p.i. shows that egg components coupled with storage temperature affected the virulence of the bacteria. The data provide evidence that eggs stored at refrigerated temperature significantly reduces the risk of salmonellosis.

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**Author contributions**
K.C.C., A.M. and T.M.: conceptualisation and experimental work. S.K.: Gene expression work, data analysis and writing the manuscript. K.C.C. and A.M.: editing the manuscript.

**Funding**
Tália S. Moyle was supported during her research by an Australian Government Research Training Program Scholarship.

**Competing interests**
The authors declare no competing interests.

**Consent for publications**
All authors have approved the submission of the manuscript.

**Additional information**
**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-97135-4.

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