The Role of Egg Yolk in Modulating the Virulence of Salmonella Enterica Serovar Enteritidis

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Contribution of food vehicles to pathogenicity of disease-causing microorganisms is an important but overlooked research field. The current study was initiated to reveal the relationship between virulence of Salmonella enterica serovar Enteritidis and egg yolk as a hosting medium. Mice were orally challenged with Salmonella Enteritidis cultured in egg yolk or tryptic soy broth (TSB). Additionally, mice were challenged with Salmonella Enteritidis cultured in TSB, followed by administration of sterile egg yolk, to discern the difference between pre-growth of the pathogen and its mere presence in egg yolk during infection. The pathogen’s Lethal dose 50 (LD50) was the lowest when grown in yolk (2.8×10^2 CFU), compared to 1.1×10^3 CFU in TSB, and 4.6×10^3 CFU in TSB followed by administration of sterile yolk. Additionally, mice that orally received Salmonella Enteritidis grown in egg yolk expressed a high death rate. These findings were supported by transcriptional analysis results. Expression of promoters of virulence-related genes (sopB and sseA) in genetically modified Salmonella Enteritidis reporter strains was significantly higher (p < 0.05) when the bacterium was grown in yolk. Sequencing of RNA (RNA-seq) revealed 204 differentially transcribed genes in Salmonella Enteritidis grown in yolk vs TSB. Yolk-grown Salmonella Enteritidis exhibited upregulated virulence pathways, including type III secretion systems, epithelial cell invasion, and infection processes; these observations were confirmed by RT-qPCR results. The transcripomic analysis suggested that upregulation of virulence machinery of Salmonella Enteritidis grown in egg yolk was related to increased iron uptake, biotin utilization, flagellar biosynthesis, and export of virulence proteins encoded on Salmonella pathogenicity island 1, 2, 4, and 5. These biological responses may have acted in concert to increase the virulence of Salmonella infection in mice. In conclusion, growth in egg yolk enhanced Salmonella Enteritidis virulence, indicating the significance of this food vehicle to the risk assessment of salmonellosis.

Keywords: Salmonella Enteritidis, virulence, transcriptomic analysis, mouse model, stress response, RNA sequencing
1 INTRODUCTION

Shell eggs are frequently associated with salmonellosis (CDC, 2010; CDC, 2016; CDC, 2018a; CDC, 2018b) and Salmonella enterica serovar Enteritidis is often linked to these outbreaks (Braden, 2006). It was estimated that among approximately 65 billion eggs produced annually in the United States, 3.25 million (0.005%) are contaminated with Salmonella Enteritidis (Schroeder et al., 2005). Shell eggs become contaminated with S. enterica through a horizontal or vertical transmission route (Howard et al., 2012). During horizontal contamination, the pathogen is gradually internalized from the surface of the eggshell, whereas in vertical contamination, pathogen cells are transferred from the infected reproductive organs into the forming egg. In both transmission routes, S. enterica can migrate into the nutrient-rich egg yolk and proliferate into a large population (Gantois et al., 2009).

Fitness of hens and their eggs as hosts for Salmonella serovars, prior to human infection, has been identified in several studies. Salmonella Enteritidis seems to have an exceptional genetic advantage to colonize a hen’s oviduct during systemic infection, allowing the pathogen’s access to the egg through the vertical transmission route (Guard-Petter et al., 1997; Parker et al., 2001; Parker et al., 2002; Lu et al., 2003; Buck J et al., 2004; Morales et al., 2005; Mizumoto et al., 2005; Gantois et al., 2008; Shah et al., 2017). Wang et al. (2018) also confirmed that genetic repertoires in two Salmonella Enteritidis strains isolated from poultry resulted in notable differences in their survivability in egg white. However, Shah (Khan et al., 2021) showed that genetically homogeneous Salmonella Enteritidis strains could vary phenotypically in their virulence under the same experimental conditions, and the researchers concluded this was caused by differences in the strains’ ability to express virulence and stress response genes.

Egg yolk may serve as an ideal medium for Salmonella Enteritidis prior to infection of a susceptible host. Although Baron et al. (2017) concluded that egg white did not induce the expression of Salmonella Enteritidis virulence, a recent study provided evidence that Salmonella Typhimurium in egg yolk, but not in egg white or on egg shell, showed increased transcription of virulence genes (Khan et al., 2021). Other researchers demonstrated that Salmonella Enteritidis grown in egg yolk displayed elevated ability to colonize intestines and express disease markers in a mouse model of human colitis (Moreau et al., 2016); however, the mechanism behind this increased pathogenicity is still unclear.

Multiple studies demonstrated the impact of food on the infectious ability of Salmonella serovars. For instance, food components may contribute to bacterial survival against the first line of defense in the human gastrointestinal tract; stomach acidity (Waterman and Small, 1998; Koseki et al., 2011; Aviles et al., 2013). Additionally, stress exerted by certain food components onto pathogens could increase their survival in the gastrointestinal tract and virulence (Yuk and Schneider, 2006; Oliveira et al., 2011; Aviles et al., 2013). Considering that several salmonellosis outbreaks were associated with consumption of egg, it is necessary to investigate whether egg components, particularly yolk, induce virulence in Salmonella Enteritidis. Additionally, exploring the underlying mechanism by which egg yolk shapes the pathogenicity of this bacterium is imperative.

Epidemiological investigations predicted a wide range of infectious doses (1.1 \times 10^5 to 7.3 \times 10^5 CFU) of Salmonella Enteritidis in egg and egg-related products (Levy et al., 1996; WHO/FOA, 2002; Teunis et al., 2010). Currently, the Lethal dose 50 (LD_{50}) of Salmonella Enteritidis in egg yolk is only broadly defined. Considering that the infectious dose is one of the primary factors to consider in risk assessment of foodborne pathogens, it is critical to determine with greater accuracy the LD_{50} of Salmonella Enteritidis in the egg environment. Therefore, the current study was initiated to determine Salmonella Enteritidis LD_{50} in mice fed Salmonella Enteritidis that was cultured in different media, including egg yolk. It should be noted that death of the animal is defined here as meeting the early removal criteria (ERC) for removal of animals from the study by humane euthanasia. Molecular mechanisms underlying differential pathogenicity were then evaluated by assessing transcriptional changes in Salmonella Enteritidis virulence genes when the pathogen was grown in egg yolk.

2 MATERIAL AND METHODS

2.1 Ethics Statement

The animal study was reviewed and approved by the Ohio State University Institutional Animal Care and Use Committee (Protocol OSU 2009A0035). Mice were carefully inspected daily and once their body weight loss or disease symptoms met the early removal criteria (ERC), they were removed immediately. Carbon dioxide asphyxiation (replacement of air in a cage with 100% CO2) was then applied followed by cervical dislocation (applying pressure to the neck and disarticulating the cervical vertebrae from the skull) was used for euthanizing the mice.

2.2 Bacterial Strains

Salmonella Enteritidis ODA 99-30581-13 (Perry et al., 2008; Perry et al., 2011; Perry and Yousef, 2013), isolated originally from shell eggs and provided by the Ohio Department of Agriculture (Reynoldsburg, OH, USA), were used in the current study. Two reporter strains were generated in this study; these were derivatives of Salmonella Enteritidis ODA 99-30581-13. Two strains, Salmonella Typhimurium pBA409 and pRG49, were used for plasmids retention. All strains were streaked from frozen stocks, held at -80°C in tryptic soy broth (TSB; Bacton, Dickson and Company, Franklin Lakes, NJ, USA) containing 15% glycerol (Sigma-Aldrich, Burlington, MA, USA), onto tryptic soy agar (TSA; Bacton, Dickson and Company). Inoculated agar plates were incubated at 37°C for 18 h and single colonies were sub-cultured onto fresh TSA plates, which were subsequently incubated at 37°C for 18 h. Single colonies from the sub-cultures were inoculated into TSB tubes (5 mL each) and incubated at 37°C for 18 h. Reporter strains were prepared under similar conditions except that all media were supplemented with 10 μg/mL tetracycline (Sigma-Aldrich).
2.3 Salmonella Inoculation in Yolk and Microbiological Media

Unfertilized washed Grade AA large shell eggs were obtained, 5 to 10 days after laying, from Salmonella-free flock on the farms of Hertfeld farms (Grand Rapids, OH, USA). The eggs were stored at 4°C and used in experiments within a week of storage. Egg surfaces were decontaminated by submerging intact eggs in ethanol (70% v/v) for 1 min. Eggs were removed and excess ethanol was flame. The yolk was separated from the egg using a sterile stainless-steel yolk separator and placed in a homogenizer bag (Fisher Scientific, Fair Lawn, NJ, USA). The sterility of the yolk was ensured by spreading 100 μL of the yolk onto a TSA plate which was incubated at 37°C for 24 h. The yolk of three eggs was collected and homogenized for 1 min before 15 mL (approximately equivalent to the volume of the yolk of one egg) were transferred into the sterile 50-mL conical centrifuge tube. Meanwhile, 15 mL of TSB were also transferred to another 50-mL conical centrifuge tube. Salmonella Enteritidis cell suspension was prepared by separating the cells in the TSB overnight culture by centrifugation and washing the resulting pellet three times using sterile saline (0.85% NaCl) solution (Fisher Scientific). The resulting cell suspension was diluted 1:10,000 in the saline solution before 10 μL of which was added to the yolk or TSB (final population of approximately 10^3 CFU per 15 mL egg yolk or TSB). After proper mixing, the inoculated media were incubated at 30°C before use in further experiments; this incubation temperature mimics the worst-case scenario for storage of naturally-contaminated eggs (Perry and Yousef, 2013).

2.4 LD₅₀ and Survival Curves of Infected Mice

To determine if presence of Salmonella Enteritidis in egg yolk increases its virulence, an in vivo study was conducted using female C57BL/6 mice obtained at 6 to 8 weeks of age (The Jackson Laboratory, Bar Harbor, ME, USA). The study was performed at the Veterinary Medicine Complex at The Ohio State University abiding by protocols approved by The Ohio State University Institutional Animal Care and Use Committee (IACUC; OSU 2009A0035). The mice were inoculated via intragastric gavage and received either Salmonella grown in TSB (Baxon, Dickson and Company), Salmonella grown in yolk, or Salmonella grown in TSB followed by sterile yolk; within each treatment group, each cage of five mice was challenged with one of the 10-fold dilutions of Salmonella from 10^7 CFU to 10^2 CFU, the same growth medium was used as diluent. Control groups included mice treated with sterile TSB, sterile yolk and sterile TSB followed by sterile yolk. Body weight and ERC were closely monitored to determine euthanizing point. The experiment was repeated twice.

2.5 Genetic Transformation

2.5.1 Construction of Salmonella sopB and sseA Reporter Strains

Plasmids pBA409 and pRG49 are derived from pSB401 (Winson et al., 1998) which encodes p15A, a tetracycline resistance marker and a promoterless luxCDABE operon. For pBA409, the promoter fragment of pSB401 was replaced with the regulatory region of the gene encoding inositol phosphate phosphatase from Salmonella Typhimurium SPI-1 effector, sopB (Goodier and Ahmer, 2001). To construct pRG49, the promoter region of the Salmonella Typhimurium SPI-2 gene, sseA, was amplified with PCR (using primers GGCGACGTACGGATCCCGCAAGGATCCGGCGCAGGGAAGGATCCGCAGCAA and CTGACGGTATCTCCACCGGGGGTTTG), cloned into pCR-blunt II Topo, removed with EcoRI, and ligated to pSB401 that had also been digested with EcoRI, thus replacing the original promoter fragment. pBA409 and pRG49 were then electroporated into Salmonella Enteritidis ODA 99-30581-13 (Table 1).

2.5.2 Luciferase Activity

Egg yolk was inoculated with reporter Salmonella strains as previously mentioned. Luciferase activity of reporter strains was measured at 10, 12, 14, 16, 18, 20, and 24 h during Salmonella growth in egg yolk at 37°C to indicate the expression of the virulence genes. At each sampling point, aliquots (200 μL) were distributed into 96-well microplates (Corning, Fisher Scientific) and the luminescence of each well was measured using a multilabel counter (Wallac Victor 3; PerkinElmer Life and Analytical Sciences, Shleton, CT, USA). The bacterial population was also determined at each sample by spread plating onto TSA supplemented with 10 μg/mL of tetracycline and the luciferase activity was normalized against Salmonella population. Each experiment was repeated three times.

2.6 Transcriptomic Analysis Using RNA Sequencing

2.6.1 Determining Salmonella Growth Curve and Stage of RNA Extraction

Inoculated TSB or yolk was sampled after 0, 1, 2, 4, 7, 10, 13, 16, 24, and 48 h incubation at 30°C and the Salmonella Enteritidis population at each time point was measured by spread-plating technique using TSA medium. The growth curves, from three independent repetition, were fit into the modified Gompertz model (Juneja et al., 2009).

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**TABLE 1** | Characteristics of Salmonella Enteritidis strains constructs in the current study.

| Strain | Source or description | GenBank accession/[Reference] |
|--------|-----------------------|------------------------------|
| Salmonella Enteritidis ODA 99-30581-13 | Isolated from chicken egg | NZ_JACTGY010000040.1/ (95–97) |
| Salmonella Typhimurium pBA409 | Containing plasmid sopB::luxCDABE | (Goodier and Ahmer, 2001) |
| Salmonella Typhimurium pRG49 | Containing plasmid sseA::luxCDABE | (Current study) |
The ratio of 260 and 280 was determined a spectrophotometer (NanoVue; GE Healthcare Life Sciences, instructions. The RNA quality and quantity were determined using 5m i nan d th R N Aw a sp u ri Eppendorf, Hauppauge, NY, USA) at 12,000 × gation kit (RNeasy mini; Qiagen) according to manufacturer cation system (Beckman Coulter, Carlsbad, CA, USA) was used according to the manufacturer’s protocol.

\[ N = a + (b - a)e^{-c(t-d)} \]

where “N” is Salmonella Enteritidis population (log CFU/mL), “a” is the lower asymptote (log CFU/mL), “b” is the upper asymptote (log CFU/mL), “c” is the growth rate (log CFU/mL/ h), “d” is the inflection point (h), and “t” is time (h). The model’s mathematical parameters (a, b, c, and d) were determined using statistical software (JMP Pro 14; SAS Institute Inc., Cary, NC). Late log phase, when the growth of Salmonella Enteritidis in the TSB reached 1.5 × 10^8 CFU/mL, was selected for RNA extraction. By plugging the desired final population into the Salmonella growth curve model, the time to reach that population could be calculated to determine the RNA extraction point for yolk and TSB.

2.6.2 RNA Extraction and Quality Assessment
To facilitate RNA extraction, Salmonella cells were captured from inoculated and incubated egg yolk and TSB using magnetic beads coated with Salmonella-antibody (Dynabeads anti-Salmonella; Life Technology, Carlsbad, CA, USA). Magnetic beads (200 μL) were added to 1 ml of egg yolk that was diluted with 9 mL of saline. The mixture was homogenized on the sample mixer (Dynabeads Rotator Mixer 947-01; Invitrogen, Carlsbad, CA, USA) for 15 min. After the beads were recovered by attaching the tube to the magnetic plate (Dynal Magnetic Particle Concentrator-6 120-02D; Invitrogen) for 3 min, the supernatant was discarded, and the beads were resuspended in saline. This washing step was repeated two additional times before the beads were resuspended in 100 μL of saline which was later transferred to the 2-mL screw-capped tube (Fisher Scientific) containing a mixture of 400 mg of 0.1 mm and 400 mg of 0.5-mm glass beads each (BioSpec Products, Inc., Bartlesville, OK, USA) and 600 μL of Buffer RLT (RNeasy mini kit; Qiagen, Germantown, MD, USA) supplemented with 6 μL of β-mercaptoethanol (Bio-Rad, Hercules, CA, USA). The mixture was treated in the homogenizer (4-Place Mini Bead Mill Homogenizer, VWR, Montigny le Bretonneux, France) at 5 m/s for three 60-s intervals to release the RNA from the bacterial cells. The tubes were cooled on ice for 1 min between homogenization intervals. The homogenized samples were centrifuged (5415R centrifuge; 15 min. After the beads were recovered by attaching the tube to the magnetic plate (Dynal Magnetic Particle Concentrator-6 120-02D; Invitrogen) for 3 min, the supernatant was discarded, and the beads were resuspended in saline. This washing step was repeated two additional times before the beads were resuspended in 100 μL of saline which was later transferred to the 2-mL screw-capped tube (Fisher Scientific) containing a mixture of 400 mg of 0.1 mm and 400 mg of 0.5-mm glass beads each (BioSpec Products, Inc., Bartlesville, OK, USA) and 600 μL of Buffer RLT (RNeasy mini kit; Qiagen, Germantown, MD, USA) supplemented with 6 μL of β-mercaptoethanol (Bio-Rad, Hercules, CA, USA). The mixture was treated in the homogenizer (4-Place Mini Bead Mill Homogenizer, VWR, Montigny le Bretonneux, France) at 5 m/s for three 60-s intervals to release the RNA from the bacterial cells. The tubes were cooled on ice for 1 min between homogenization intervals. The homogenized samples were centrifuged (5415R centrifuge; Eppendorf, Hauppaug, NY, USA) at 12,000 × g and 4°C for 5 min and the RNA was purified from the supernatant using a purification kit (RNeasy mini; Qiagen) according to manufacturer’s instructions. The RNA quality and quantity were determined using spectrophotometer (NanoVue; GE Healthcare Life Sciences, Buckinghamshire, UK). The ratio of 260 and 280 was determined to assess the RNA purity. The integrity of RNA was determined using bioanalyzer (Agilent 2100, Agilent Technologies, Santa Clara, CA, USA) and only RNA preparations with integrity number greater than 7 were used for subsequent experiments.

2.6.3 Library Construction and RNA Sequencing
The library construction and sequencing were conducted by Novogene Co. (Davis, CA, USA) from 250 ng input of each RNA sample. To remove rRNA from total RNA, a commercial kit (Ribo-zero rRNA removal kit (Bacteria); Illumina, San Diego, CA, USA) was used according to the manufacturer’s protocol. The strand-specific mRNA library was subsequently constructed with the NEBNext Ultra Directional RNA Library Prep Kit (New England BioLabs Inc., MA, USA). In brief, enriched mRNA was fragmented using divalent cations (RNA fragmentation reagent, Life Technology, CA, USA) in buffer (NEBNext First Strand Synthesis Reaction Buffer; New England BioLabs Inc.). First strand cDNA was synthesized using random hexamer primer and RNA-dependent DNA polymerase, Moloney Murine Leukemia Virus Reverse Transcriptase (M-MuLV), prior to second strand cDNA synthesis, during which dUTP, dATP, dCTP, and dGTP were applied. The exonuclease and polymerase were used to repair the DNA overhangs and adenylate the 3’ ends of the DNA fragments. The library fragments were multiplexed using the index primer kit (NEBNext Multiplex Oligos for Illumina kit; New England BioLabs Inc.). Hairpin loop structured NEBNext Adapters were ligated to the DNA fragments, which were cleaned up by bead-based AMPure XP purification system (Beckman Coulter, Beverly, USA) to select for cDNA fragments that were 150 to 200 bp in length. Uracil excision through uracil-specific excision enzyme (USER, New England BioLabs Inc.) and PCR enrichment through DNA polymerase (Phusion High-Fidelity DNA polymerase, Fisher Scientific), universal PCR primers and Index (X) Primer were performed on the purified cDNA fragments to create indexed libraries. The barcoded cDNA fragments were purified again using AMPure XP system before the library quality was assessed on the Agilent Bioanalyzer 2100 (Agilent Technologies). The libraries were clustered on the cBot Cluster Generation System using HiSeq PE Cluster Kit cBot-HS (Illumina, CA, USA) based on manufacturer’s instruction and sequenced on the Illumina HiSeq 2500 platform (Illumina; paired-end, 150 bp per read). Three biological repeats were analyzed for both the control group and the treatment group (3 × 2) rendering six samples in total.

2.6.4 Read Alignment and Analysis of Gene Transcription Data
Initial sequencing quality was assessed with metrics including GC content and the proportion of reads with average base call scores exceeding quality score of 20 (Q20) and Q30. Adaptors were trimmed and low quality base calls were removed using Trimmomatic (Bolger et al., 2014) with parameters ILLUMINACLIP:[adapter.fa]:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36. Trimmed fastq files were aligned to Salmonella Enteritidis strain P125109 using Subread 1.5.0 (Liao et al., 2013) and aligned bam files were sorted and duplicate reads marked using Picard 2.3.0 (Picard Tools - By Broad Institute). Reads were then counted using FeatureCounts (Liao et al., 2014) and analyzed for differential expression using DESeq2 1.24.0 (Love et al., 2014). Differentially expressed genes (DEGs) were identified as those with padj < 0.05. DEGs were analyzed against Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) database to annotate their main biological functions and pathways, and the Search Tool for Retrieval of Interacting Genes/proteins (STRING) analysis (Szklarczyk et al., 2019) was performed to
identify connections between different pathways. Gene Set Enrichment Analysis (GSEA (Mootha et al., 2003; Subramanian et al., 2005),) was also conducted to identify significantly up- or downregulated pathways from a ranked list of all sampled genes. Gene sets (pathways) analyzed included KEGG pathways listed under the Salmonella Enteritidis P125109 (KEGG genome ID T00776) that contain > 5 genes.

2.6.5 Preparation of cDNA for Reverse Transcription Quantitative PCR (RT-qPCR) Analysis
DNA was removed from the extracted RNA by DNase of a commercial kit (Turbo DNase kit, Invitrogen, Vilnius, Lithuania) following the kit manufacturer’s instructions. The DNase-treated samples were measured for final RNA concentration (NanoVue spectrophotometer) and stored in the -80°C freezer until further use. The RNA was transcribed to cDNA using cDNA reverse transcription kit (High-Capacity cDNA, Applied Biosystems, Waltham, MA, USA) set to run the following sequence: 25°C for 10 min, 95°C for 5 min. The cDNA synthesis was performed using PCR thermocycler (GeneAmp PCR System 2400, Applied Biosystems, Waltham, MA, USA) set to run the following sequence: 25°C for 10 min, 95°C for 2 min (stage 1); 95°C for 7 min (stage 2); 95°C for 15 s, 60°C for 1 min (stage 3, repeated 40 times). Each reaction was triplicated within each RT-qPCR run and the RT-qPCR experiment for each reaction was independently repeated three times.

2.6.6 RT-qPCR Analysis
RT-qPCR was performed to assess the differential transcription of selected genes, spanning Salmonella pathogenicity islands (SPI) 1 to 5, using the specific primers reported in Table 2. A comprehensive panel of virulence genes (up to 23), with putative functions as regulators for SPIs and effectors or secretions apparatus, were investigated. Before RT-qPCR, the primers were tested on conventional PCR to ensure their specificity and to optimize the annealing temperature (Mundi et al., 2013). For each 20-μL reaction, 5 μL of cDNA was added to the 10 μL SYBR Select PCR master mix (Applied Biosystems), 500 nM of each of the forward and reverse primers (Table 2; Millipore Sigma, Danvers, Massachusetts) and proper volume of water. Using real-time PCR system (7900HT; Applied Biosystems), PCR cycles were conducted in sequence as following: 50°C for 2 min (stage 1); 95°C for 7 min (stage 2); 95°C for 15 s, 60°C for 1 min (stage 3, repeated 40 times). Each reaction was triplicated within each RT-qPCR run and the RT-qPCR experiment for each reaction was independently repeated three times.

2.7 Statistical Analysis
Survival curves were generated by a statistical software (GraphPad Prism 9.0.0; GraphPad Software, San Diego, CA, USA) and the data from two repeats were combined and compared using Gehan-Breslow-Wilcoxon test, which was adjusted by Tukey-Kramer test. LD₅₀ was calculated by combining the data from two repeats and fitting the dose-response curve. The fit of all three LD₅₀ curves was compared as a whole model by GraphPad Prism 9.0.0 using a least squares regression model and significance was determined at a p-value of < 0.05. The parallelism F-test and

| Gene | Gene function | Forward primer | Reverse primer | Reference |
|------|---------------|----------------|----------------|-----------|
| gapA | Housekeeping gene used for normalization | GGTGTTAGCTAGTGGCTGAA | AGGTTGGAACGATGTCCTG | (Finn et al., 2013) |
| hIC | Regulatory genes for SPI-1 | CCACTTTTGACTCAAGTCACTG | CACCCGCAATGCTACA | (Kollanoor Johny et al., 2017) |
| hID | Important SPI-1 effector genes | GGCACTCTGCTGATGTTAC | GGCACTCTGCTGATGTTAC | (Upadhyaya et al., 2013) |
| ivnF | Important SPI-4 effector genes | CGCAATGATTAGTGGGAAC | CGCAATGATTAGTGGGAAC | (Choi et al., 2007) |
| sipA | SPI-1 effector genes involved in lipid metabolism | TCGTCTTTTTCGCCAATC | AGATAAAACGCTCTGCCAAT | (Kollanoor Johny et al., 2017) |
| sopB | Important SPI-1 effector genes | GGGCTGTTTATGCAGCTTAAC | GGGCTGTTTATGCAGCTTAAC | (Upadhyaya et al., 2013) |
| sopE | Regulatory genes for SPI-2 | AAACACTTTTAACAACACAC | AAACACTTTTAACAACACAC | (Choi et al., 2007) |
| sopF | SPI-2 effector genes involved in lipid metabolism | CGATTTGTGCTGATGTTAC | CGATTTGTGCTGATGTTAC | (Upadhyaya et al., 2013) |
| sopG | Important SPI-2 effector genes | TTAGGCTTTGACGTGAC | TTAGGCTTTGACGTGAC | (Lee et al., 2012) |
| sseJ | SPI-3 effector genes involved in lipid metabolism | ACAGATGATGATGATGATG | ACAGATGATGATGATGATG | This study |
| sseL | Important SPI-3 effector genes | GGCACTCTGCTGATGTTAC | GGCACTCTGCTGATGTTAC | This study |
| marT | Regulatory genes for SPI-3 | TCGAGCTTACATGCTGCTGCTG | TCGAGCTTACATGCTGCTGCTG | This study |
| mgtB | Important SPI-4 effector genes | AGTGAAGTCGTGTGAC | AGTGAAGTCGTGTGAC | (Upadhyaya et al., 2013) |
| ssiE | Important SPI-4 effector genes | CCATTTTGACGTGAC | CCATTTTGACGTGAC | This study |
| pipB | Important SPI-5 effector genes | GGTTGCTGCTGCTGCTGCTG | GGTTGCTGCTGCTGCTGCTG | This study |
| svpR | Regulatory genes for Salmonella virulence plasmid | CCCATGATGATGATGATG | CCCATGATGATGATGATG | This study |
| svpA | Important effector genes on Salmonella virulence plasmid | GGTTGCTGCTGCTGCTGCTG | GGTTGCTGCTGCTGCTGCTG | This study |
| svpB | virulence plasmid | GGTTGCTGCTGCTGCTGCTG | GGTTGCTGCTGCTGCTGCTG | This study |
| fhd | Regulatory gene for flagellar biosynthesis | GGTTGCTGCTGCTGCTGCTG | GGTTGCTGCTGCTGCTGCTG | This study |
parallelism Chi-square test were used to compare the growth model of *Salmonella* in the yolk and TSB, using a statistical software (JMP Pro 14). To demonstrate the transcription level of virulence genes, luminescence was normalized against the bacterial population and normalized luminescence levels were statistically compared using two-way ANOVA (two factors: time and treatment). RT-qPCR data were analyzed using ΔΔCt method (Pfafl, 2001) using *gapA* as the housekeeping gene to compare the expression level of *Salmonella* virulence genes in yolk compared to TSB. Data with ≥ 2-fold changes were considered as significant. A cutoff at 5% of false discovery rate (FDR) was used for determining significance of KEGG, GO, and STRING analysis and for GSEA analysis, a cutoff at 0.05 of normalized *p* value was used. Except for the RT-qPCR data and differentially expressed gene analysis, all statistical analysis with a *p*-value of < 0.05 was considered as significant.

3 RESULTS

3.1 Growth in Egg Yolk Changes *Salmonella* Enteritidis Virulence in Mice

Mice were fed, through oral gavage, with *Salmonella* Enteritidis that had been grown in egg yolk (yolk group), grown in TSB (TSB group), or grown in TSB with subsequent administration of sterile egg yolk (TSB+yolk group), and results are shown in Figure 1A. Significantly different (*p* = 0.0094) LD₅₀ values were observed among these three groups. The LD₅₀ of *Salmonella* Enteritidis was the lowest (2.8×10² CFU) in mice of the yolk group, compared to that in the TSB group (1.1×10³ CFU) or the TSB+yolk group (4.6×10³ CFU). It is apparent from these results that growth in egg yolk, but not mere presence of the pathogen in sterile yolk in the gut, increased the virulence of *Salmonella* Enteritidis.

Mice survival curves representing individual *Salmonella* Enteritidis doses (10² to 10⁷ CFU), pre-grown in egg yolk or TSB, were constructed (Supplementary File S1). Based on these curves, mice in the yolk group met the ERC faster, particularly at the higher *Salmonella* Enteritidis doses, compared to mice in two other groups. Survival curves for the three mice groups, combined at all dose levels, were constructed and compared statistically (Figure 1B). Mice survival curve for the yolk group was significantly different than that for the TSB group (*p* = 0.0002) or TSB+yolk group (*p* < 0.0001). However, survival curves corresponding to TSB and TSB+yolk mice groups were not significantly different (*p* = 0.987). The median survival ratio and hazard ratio for mice given *Salmonella* in yolk vs. TSB, calculated using Mantel-Haenszel model group, were 0.89 and 1.57, respectively.

3.2 Expression of Virulence Determinants When *Salmonella* Enteritidis Was Grown in Egg Yolk

To determine how virulence of *Salmonella* Enteritidis in mice might be enhanced by pre-growth in egg yolk, expression of selected virulence genes was compared when the pathogen was cultured in egg yolk and the microbiological medium, TSB. This was preceded with an experiment to determine the optimum phase of *Salmonella* growth for completing this analysis.

3.2.1 Characteristics of *Salmonella* Enteritidis Growth in Egg Yolk

The stage of growth is known to have a profound effect on gene expression in bacteria (Klumpp et al., 2009). To compare the expression of virulence genes of *Salmonella* Enteritidis in egg yolk and TSB, it was important to culture the pathogen in both media to the same stage of growth. Monitoring the growth of *Salmonella* Enteritidis in these media and modeling the resulting curves (Figure 2A) helped to define the time points suitable for harvesting RNA under these two different conditions. The predicted growth model in the TSB was
where \( N \) is *Salmonella Enteritidis* population (log CFU/mL) and \( t \) is the incubation time in hours. The performance measure, \( r^2 \), for both models was 0.99, indicating a good fit of data by the model. The parallelism F-test and Chi-square test suggested that the overall shape of the two modeled growth curves was not significantly different (\( p > 0.05 \)). However, when different stages of growth in both curves were compared, the predicted growth parameters for
Salmonella Enteritidis in TSB and egg yolk were as follow: populations at lag phase were 1.6 ± 0.2 and 1.1 ± 0.2 log CFU/mL, populations at stationary phase were 9.4 ± 0.1 and 9.5 ± 0.2 log CFU/mL, growth rates were 0.30 ± 0.02 and 0.23 ± 0.02 log CFU/mL/h, and curve inflection points were 6.0 ± 0.3 and 5.9 ± 0.4 h, respectively. Based on these growth curves, it took Salmonella Enteritidis 13.0 h and 14.9 h to grow from < 10^7 CFU/mL to 1.5 × 10^9 CFU/mL in TSB and egg yolk, respectively. Preliminary data confirmed that the Salmonella Enteritidis population must reach 10^8 CFU/mL, at least, to produce RNA with acceptable quality and quantity for conducting the transcriptional analysis. Therefore, the two time points mentioned previously (13.0 h and 14.9 h for incubation in TSB and egg yolk, respectively) were selected for harvesting cells to be used in the transcriptional analysis.

### 3.2.2 Expression of Selected Virulence Genes at Various Growth Stages

Salmonella Enteritidis sopB and sseA reporter strains were grown in TSB or egg yolk and expression levels of sopB and sseA were used to indicate changes in the pathogen’s virulence capability. The expression of sopB and sseA was significantly higher when Salmonella Enteritidis was grown in egg yolk (p = 0.0019 and p = 0.0306, respectively), compared to its growth in TSB (Figures 2B, C). The expression of the virulence genes, sopB and sseA, was measurable after incubation of Salmonella Enteritidis for 12 to 14 h and 14 to 16 h in TSB and yolk, respectively (Figure 2) and both time ranges were within the late exponential phase of growth. The difference in lag times, between the start of incubation and the detection of expression, was possibly related to the fact that the late exponential phase came later in yolk than in TSB (Figure 2).

### 3.2.3 Global Transcriptomic Analysis

RNA-seq data provided a global overview of the differences in gene expression between Salmonella Enteritidis grown in egg yolk and TSB. The total number of transcribed genes in both growth media was 4343 while only 34 and 49 genes were expressed solely when the pathogen grew in yolk and TSB, respectively (Figure 3A). Of the 4343 transcribed genes, 204 were differentially transcribed (padj < 0.05); these included 74 downregulated and 130 upregulated genes (Figure 3B). Among the upregulated genes, the two largest groups were involved in virulence and stress response associated with membrane stress, respectively (Figure 3B). For instance, baeS, one of the membrane-stress regulators (Baron et al., 2017), and ftsH, encoding a metalloprotease to maintain membrane protein integrity were significantly upregulated (Figure 3B). The phage shock protein (Psp) operon, which contains a set of genes induced under dissipation of proton motive force due to the impaired inner membrane integrity (Engl et al., 2011), and multiple heat stress response genes (dnaK, dnaJ, clpB, ibpB, and ibpA) that are commonly involved in bacterial membrane repair, as the cell envelope is the main target of heat injury (Aljarallah and Adams, 2007; Hews et al., 2019), were also upregulated (Figure 3B). MutM, which is involved in base excision repair of DNA damaged under stressed condition, was also included in the set of DEGs (Figure 3B). The formate dehydrogenase system (fdnG, fdnI, fdoI, and fdhE) that utilizes formate as an electron donor to regenerate proton motive force by forming redox loop, was upregulated, whereas the formate hydrogenlyase complex (hycI, hycG and hycF), which oxidizes formate to CO₂ with the concomitant reduction of protons to H₂, was repressed (Figure 3B) (Jormakka et al., 2002; Huang et al., 2008). Another set of genes among the downregulated DEGs included the arn operon and the regulator of the operon, pmrAB (Figure 3B). Those genes are related to the antimicrobial resistance against positively charged antimicrobial peptides, such as polymyxin, by masking the negative charge of the cell membrane and resulting in a more hydrophobic cell membrane (Huang et al., 2020; Park et al., 2021).

### 3.2.4 Functional Enrichment and Pathway Analysis of Differentially Expressed Genes (DEGs)

Using gene ontology (GO), DEGs were functionally analyzed to identify enriched functional categories in the Salmonella Enteritidis transcriptome. Significantly enriched terms in each...
GO category are displayed in Figure 4 and Supplementary File S2. Among the biological categories, the upregulated DEGs were enriched in biotin biosynthetic processes and protein transport and localization, such as secretion by type III secretion system and protein localization to extracellular region (Figure 4). DEGs encoding type III secretion apparatus included sipB, sipC, sipD, invA, invI, prgH, prgL, prgK, spaO, spaP, spaQ, orgA, and orgB on SPI-1 and sseB, sseC, ssaG, and ssaN on SPI-2 while DEGs encoding effectors secreted through the type III secretion system included sipA, sipB, sipC, sipD, and sopB on SPI-1, sopD2, sseL on SPI-2, and pipB and pipB2 on SPI-5. Sulfur compound metabolic processes, including hydrogen sulfide biosynthesis, were the most represented in the downregulated DEGs (Supplementary File S2). The enriched molecular function GO terms included protein binding in the upregulated DEGs (Figure 4), and sulfate transporter activity in the downregulated DEGs (Supplementary File S2). No downregulated DEGs were found significant by the enriched cellular component GO terms but for the upregulated DEGs, ribosome and type III secretion system-related cellular components were enriched (Figure 4 and Supplementary File S2).

Kyoto encyclopedia of genes and genomes (KEGG) database mapping revealed several significantly enriched pathways (Figure 5). The majority of the enriched pathways in the upregulated DEGs were related to Salmonella virulence factors, such as “bacterial secretion system,” “Salmonella infection,” and “bacterial invasion of epithelial cells,” whereas three pathways were enriched in the downregulated DEGs and those were associated with sulfur and amino sugar metabolism, and antimicrobial peptide resistance.

Network analysis was constructed for the DEGs using STRING tool (Figure 6). In the network analysis, functionally related genes are clustered together; this is obvious considering that DEGs encoding Salmonella infection process and type III secretion system (e.g., sipA, sipB, sipC, sipD, and sopB), and Salmonella invasion (e.g., invA, ssaG, ssaJ, ssaN, and spaO) were connected (Figure 6). The main connector in the network is hilA, which is a regulator gene for SPI-1 (Figure 6). It was noticed that htpG, a gene encoding heat shock protein, was co-expressed with other stress response genes (e.g., dnaJ, dnaK, ibpB, clpB) (Figure 6). Moreover, htpG interacts with virulence genes via sopE, which encodes an effector protein on SPI-1. The ribosome biosynthetic protein genes (e.g., rplE, rplK, rplN, rplQ, rplX, rpsD) are connected to Salmonella virulence genes via sseL, which encodes an effector protein, or via rpoA, which encodes RNA polymerase, and lastly connected to the protein translocase gene secA, which is involved in the translocation of Salmonella effector/invasion proteins into the host cell (Figure 6). Overall,
the network analysis indicates that the transcription of Salmonella virulence genes was enriched and co-expressed with some stress response genes. The elevated expression of all these genes requires the ribosomal machinery to be upregulated.

3.2.5 Gene Set Enrichment Analysis
In contrast to GO enrichment and KEGG analysis, gene set enrichment analysis (GSEA) identifies patterns of up- or downregulation of pre-defined gene sets from ranked lists of all analyzed genes. Among 87 defined gene sets (based on KEGG pathways), 11 were significantly upregulated and 24 significantly downregulated (Figure 7). The significantly upregulated pathways included ribosome, Salmonella virulence related pathways (including bacterial secretion system, salmonella infection, flagellar assembly, bacterial invasion of epithelial cells, and protein export), biotin metabolism, homologous recombination, biosynthesis of siderophore group non-ribosomal peptides, RNA degradation, and fatty acid biosynthesis (Figure 7A). A set of core enriched genes involved in "homologous recombination," a pathway important to DNA damage repair, included ssb1, recA, recF, recG, recN, recO, recR, ruvA, ruvC, dnaE, dnaX, holB-D, priA, and priB. Within the significantly downregulated pathways, the majority are involved in sulfur, carbohydrate, and amino acid metabolism (Figure 7B).

3.2.6 Verification of RNA-Seq Results Using RT-qPCR
RT-qPCR was performed on selected Salmonella virulence-related genes, using glyceraldehyde-3-phosphate dehydrogenase A gene, gapA, as a reference. When Salmonella Enteritidis was grown in egg yolk, genes tested within its pathogenicity islands SPI-1, SPI-2, SPI-4, and SPI-5, were significantly upregulated (>2-fold), compared to genes from cells grown in the TSB (Figure 8). Genes of SPI-3 (mgtBC) were downregulated and the genes spvR and spvB of Salmonella virulence plasmid were upregulated in the yolk (Figure 8C). Additionally, flhD, a gene that encodes the regulator for flagellar biosynthesis [which indirectly regulates SPI-1 genes via activation of the flagellar gene flf (Fabrega and Vila, 2013)], was also upregulated (Figure 8C). Within SPI-1, major regulators (hiIC and hiILD), a transcription regulator of type III secretion system (invF), as well as effector genes involved in bacterial internalization (sipA and sipB) (Fabrega and Vila, 2013) were highly upregulated (>10-fold). SopB, co-regulated with SPI1 and the protein product of which is secreted by type III secretion system on SPI-1, was also strongly upregulated. The major regulators of SPI-2 (ssrA and ssrB) were significantly upregulated but not as highly transcribed as the major regulators in SPI-1; however, sseL and sseJ, the two effector genes functioning to change host cell physiology and enhance bacterial survival in host cell (Uchiya et al., 2004; Uchiya and Nikai, 2004; Arena et al., 2011; Jennings et al., 2017) were noticeably upregulated. SPI-5 genes encoding for PipB, which is involved in accumulation of lipid rafts and the alternation of phospholipid structure of host cell membrane (Subramanian and Qadri, 2006; Shivcharan et al., 2018), were also appreciably upregulated. PipB is co-regulated with SPI-2 and its protein product is secreted by type III secretion system on SPI-2. These results confirmed the observations from RNA-sequencing analysis.
4 DISCUSSION

Based on the mouse study, *Salmonella Enteritidis* grown in egg yolk showed the infectious pattern of faster disease onset and lower infectious dose, in comparison to *Salmonella* grown in a common microbiological medium, TSB (Figure 1B). Other researchers also found that mice orally challenged with *Salmonella Enteritidis* in the yolk displayed a significantly higher degree of pathogen intestinal colonization, fecal shedding, and dissemination in liver and spleen, and inflammation of liver and ceca, compared to mice orally challenged with *Salmonella Enteritidis* in LB, another common microbiological medium (Moreau et al., 2016). In previous studies, researchers argued that food characteristics could have a protective effect for the pathogens. For example, high viscosity could delay gastric acid emptying, proteins may have a buffering effect against gastric acids, and fat might form a protective layer around the pathogens (Ponder, 2017). These proposed effects of the *Salmonella*-carrying medium cannot explain the results of the current study because mice challenged with *Salmonella* grown in TSB, which was immediately followed by administering sterile egg yolk, did not show the same disease severity as the mice challenged with *Salmonella* grown in egg yolk. Therefore, the increase in *Salmonella* virulence, as observed in the current study, cannot be attributed to the protective effect of the yolk medium.

4.1 *Salmonella Enteritidis Grown in Egg Yolk Induced Virulence Genes Transcription*

When *Salmonella* invades macrophage, SPI-2 plays a fundamental role in pathogen survival and cell-to-cell spread, whereas SPI-1 and flagella-related genes are strongly downregulated (Eriksson et al., 2003; Hautefort et al., 2008). However, when *Salmonella* invades epithelial cells, not only SPI-
FIGURE 7 | Pathways that are upregulated (A) or downregulated (B) based on gene set enrichment analysis (GSEA). Each pathway is represented by a dot; the size, color, and enrichment score value of the dot represents the total number of genes within the pathway, normalized p value, and proportion of the genes in the pathway considered as core enrichment, respectively.

FIGURE 8 | Changes in the transcription of virulence genes within Salmonella pathogenicity island (SPI)-1 (A), SPI-2 (B) and other virulence components (C), when the pathogen was grown in yolk compared to its growth in tryptic soy broth. The fold change was calculated through \( \Delta \Delta Ct \) method using gapA, a housekeeping gene, as a reference. Error bars represent standard deviation of three independent repeats.
1 but SPI-2 and flagella are also strongly upregulated (Hautefort et al., 2008). In the current study, growth of Salmonella Enteritidis in egg yolk upregulated many genes including virulence genes in SPI-1, SPI-2 and flagella genes (Figures 2, 4, 5, and 7A). Thus, it is likely that Salmonella spread and reproduce in egg yolk in a manner similar to that during its invasion of epithelial cells. The key metabolic status of Salmonella in egg yolk was in concert with the signature metabolic status of the pathogen during epithelial invasion. For instance, Hautefort et al. (2008) reported that when Salmonella is inside epithelial cells, its transcription of genes involved in biosynthesis of biotin (an enzyme cofactor for fatty acid biosynthesis) was induced 10-fold, compared to that for Salmonella inside a macrophage. Biotin biosynthesis was the top-ranked upregulated pathway in the current study (Figure 7A). Additionally, biosynthesis of siderophore was a significantly upregulated pathway (Figure 7A). Studies supported that siderophores are important for Salmonella fitness in the gastrointestinal tract (Pi et al., 2012; Saha et al., 2017; Saha et al., 2019).

Using network analysis, connections were observed among DEGs encoding ribosome biosynthetic proteins, Salmonella virulence proteins, and the translocation of Salmonella effector/invasion proteins into the host (Figure 6). This implied that Salmonella Enteritidis in egg yolk was programmed to actively produce virulence effector proteins through generous ribosomal activity even before the pathogen infects any animal host. During the intestinal internalization, pre-produced virulence effectors could assist rapid invasion and colonization in the host, whereas Salmonella from other sources may have to adapt to the host first, sense the signals from the host for transcription of virulence factors, and assemble virulence proteins before the invasion (Marcus et al., 2000; Hurley et al., 2014). Consequently, Salmonella Enteritidis grown in egg yolk is more apt to cause salmonellosis over the pathogen grown in other media, as supported by the current animal study results (Figure 1). With such remarkable protein secretion events, active metabolic pattern of Salmonella is required for combating the draining of bacterial energy levels (Hautefort et al., 2008). According to GSEA analysis (Figure 7A), upregulated metabolic pathways in egg yolk included nitrotoluene degradation, thiamine metabolism, glycerolipid metabolism, and lipoic acid metabolism. It is still unclear if these metabolic pathways worked together to support such a high level of energy demand or serve the essential metabolic needs that allow energy-efficient proliferation of Salmonella in the egg yolk.

4.2 Increased Virulence of Salmonella Enteritidis in Egg Yolk Is Potentially Related to Stress Responses Assisting Membrane and DNA Repair

The constructed network for DEGs using STRING analysis associated upregulated virulence factors to membrane-related stress response (Figure 6). This membrane stress can be related to the impaired inner membrane integrity leading to dissipation of proton motive force, consistent with the upregulation of the psp operon (Engl et al., 2011). To restore the proton motive force, Salmonella in egg yolk potentially utilized formate dehydrogenase system to regenerate protons and suppress the function of formate hydrogenlyase that transforms protons to H₂ (Figure 3B). Researchers have linked psp operon not only to heat shock response (Jovanovic et al., 2006) which was within the upregulated DEGs (Figure 3B), but also to virulence (Eriksson et al., 2003; Jovanovic et al., 2006; Faucher et al., 2006; Hautefort et al., 2008; Karlinsky et al., 2010; Wallrodt et al., 2013; Wallrodt et al., 2014). For example, the psp system is believed to be important for uptake, survival, and replication of Salmonella in macrophages (Eriksson et al., 2003; Faucher et al., 2006; Hautefort et al., 2008; Wallrodt et al., 2014). Beside these membrane stress related genes, genes involved in homologous recombination for DNA repair were also strongly upregulated (Figure 7A). It seems that Salmonella Enteritidis in egg yolk experience stress, which requires DNA and bacterial membrane repair. This also matched with the observation that it took Salmonella in egg yolk approximately 2 additional hours to enter the growth exponential phase, in comparison to Salmonella grown in TSB (Figure 2A). It seems that the upregulated cascade of DNA repair mechanisms reflects massive nucleotide alterations when Salmonella grew in egg yolk.

4.3 Salmonella Enteritidis Encounters Stresses in Egg Yolk Environment

Significant down regulation of nutrient metabolism, e.g., glycolysis, by Salmonella Enteritidis in egg yolk was observed (Figure 7B). It is possible that egg yolk sugars (e.g., glucose) interact tightly with the hydrophilic head of yolk phospholipids by forming hydrogen bonding (Pereira and Hünenberger, 2006); this interaction could limit the availability of this metabolizable sugar to Salmonella. Additionally, the largest fraction of egg yolk protein, lipovitellins, is non-covalently linked to nearly all the lipids at its large hydrophobic binding cavity forming non-water-soluble yolk granule (Hetta, 2008), limiting the availability of the protein source to Salmonella. Wang et al. (2021) showed that genes in the phase shock protein (psp family), DNA repair proteins, and heat stress response chaperones were upregulated during starvation stress of Escherichia coli incubated in saline for 24 h.

According to a preliminary experiment, viscosity of egg yolk was 2.1 Pa-s, which is equivalent to that of corn syrup, and egg yolk was 40 times more viscous than TSB. This high viscosity may have hindered the mobility of Salmonella and its nutrient uptake ability. In order to compensate for the limited movement in highly viscous medium, Salmonella could switch from swimming state (movement through liquids) to swarming state, a hyperflagellated state of bacterial cells allowing extra motility (Wang et al., 2004). This agrees with our finding of upregulation of flagella related genes when Salmonella was grown in egg yolk (Figures 6 and 7A). Additionally, Salmonella psp response is commonly accompanied with swarming (Wang et al., 2004; Erhardt and Dersch, 2015). In the context of virulence, the
increased flagellar biosynthesis accelerates the ability of the pathogen to approach epithelial cells during the adhesion stage (Stecher et al., 2004). As a matter of fact, in Enterobacter spp., flagellar development is often associated with the disruption of the Cyc protein family, which are related to sulfate reduction (Sturgill et al., 2004; Rossi et al., 2014; Hufnagel et al., 2018; Westerman et al., 2021); genes of this protein family were the most profoundly repressed group in the current study (Figure 7B). In order to maximize flagellar rotation during the swarming stage, Salmonella has to maintain a hydrated shell around the cell by altering its cell membrane structure significantly to decrease the hydrophobicity of the LPS (Toguchi et al., 2000). The downregulation of arr operon could possibly serve the function of producing a more hydrophobic LPS.

5 CONCLUSION

The current study demonstrated that growth in egg yolk significantly enhanced the virulence of Salmonella Enteritidis; this likely contributes to the risk of salmonellosis associated with consumption of contaminated eggs. Once grown in yolk to the late exponential phase, Salmonella not only replicated rapidly but also induced transcription of virulence factors. Therefore, the same pathogenic strain could show different pathogenicity in different food vehicles. Hence, the food vehicles that carry pathogens should be given considerable importance in foodborne disease assessment models and in disease mitigation policies. This study also identified potential mechanisms underlying the upregulation of Salmonella virulence in eggs. However, further work is needed to test the mechanisms proposed in this study.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author. The RNA sequencing data are publicly available in National Centre for Biotechnology Information (NCBI) sequence read archive (SRA) under accession number SRX14788580 and SRX14788579 for yolk and TSB, respectively.

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ETHICS STATEMENT

The animal study was reviewed and approved by the Ohio State University Institutional Animal Care and Use Committee (Protocol OSU 2009A0035).

AUTHOR CONTRIBUTIONS

The contribution of authors to the article in each item is listed in the order of last name. This article was conceptualized by AA, YX, and AY. BA and AY cooperated on funding acquisition. Experimental planning and methodology design were collaborated between AA, BA, AS-D, and YX. Experiments were executed by AA, AS-D, and YX while data curation was conducted by AA and YX. Formal data analysis was contributed by AA, MS, and YX. The original draft was written by AA and YX while reviewed and edited by AA, BA, AS-D, MS, YX, and AY. The entire process was supervised by BA and AY while the project was administrated, and resources were provided by AY. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.903979/full#supplementary-material

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