

ADAPTATION OF *SALMONELLA* WELTEVREDEN AND *SALMONELLA* TYPHIMURIUM TO DIFFERENT FOOD MATRICES AND TEMPERATURE CONDITIONS

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ABSTRACT

Foodborne pathogens like *Salmonella* pose a significant threat to public health due to their ability to survive across diverse temperatures and food matrices. This study investigated the survival and virulence gene expression of *Salmonella* Weltevreden and *Salmonella* Typhimurium in four food matrices (fish, shrimp, chicken, and sprouts) stored at 37°C, 4°C, and -20°C. Samples were spiked with both serovars, and their growth and gene expression were monitored over three days using quantitative real-time PCR. Both strains demonstrated the ability to survive and adapt across the tested conditions. Notably, *S. Weltevreden* exhibited significantly more virulence gene expression in shrimp under cold storage than in other food matrices, suggesting enhanced pathogenic potential in specific food environments. Despite its limited duration, this study highlights the resilience of *Salmonella* in varied storage conditions and underscores the need for stringent food safety protocols and further research into long-term survival and gene regulation.

Keywords: Nontyphoidal *Salmonella*, Food matrices, Virulence gene expression, Temperature conditions, Pathogenicity, Survival in food matrices

INTRODUCTION

Foodborne infections are a leading cause of human illnesses worldwide, resulting from the consumption of food contaminated with pathogenic microorganisms or their toxins. These microbes significantly impact public health and the economy due to their pathogenic potential. Bacterial pathogens contribute substantially to the global burden of foodborne diseases, with a disproportionately higher burden reported in developing countries (Abebe *et al.*, 2020). Prominent food-borne pathogens include nontyphoidal *Salmonella*, *Campylobacter*, *Listeria*, and Shiga toxin-producing *Escherichia coli* (Adley and Ryan, 2016). Among these, nontyphoidal *Salmonella* has been implicated in numerous recent multi-country outbreaks, predominantly involving *Salmonella* Typhimurium and *Salmonella* Enteritidis (CDC, 2020a). Additionally, *Salmonella* Weltevreden has emerged as a regionally important serovar, particularly in Southeast Asia and Europe, where it is frequently associated with seafood, aquatic environments, broilers in China, and vegetables in Malaysia (Ren *et al.*, 2016; Deekshit *et al.*, 2012; Salleh *et al.*, 2003). According to the Centres for Disease Control and Prevention (CDC), *Salmonella* accounts for approximately 1.35 million illnesses, 26,500 hospitalisations, and 420 deaths annually in the United States (Ranjan *et al.*, 2026). About one million of these foodborne illnesses are linked to foods like eggs, cucumbers, chicken, pre-cut melon, raw tuna, pistachios, sprouts, and others (CDC, 2020b). Although salmonellosis is often underreported in India, a sporadic outbreak associated with contaminated food have been reported including an outbreak of *S. Enteritidis* linked to chicken shawarma consumption, which was recently reported in Kerala (Deepanjali *et al.*, 2022). The prevalence and survival of *Salmonella* are influenced by physicochemical parameters such as temperature, pH, nutrient availability, contact surface, and water activity in food matrices or adaptation to the different food matrices and temperate conditions (Finn *et al.*, 2013; Diaz-Torres *et al.*, 2020; Sun *et al.*, 2022). Temperature is a critical factor influencing growth, and stress adaptation and has become a critical concern due to its impact on *Salmonella* survival. Pathogenicity islands in *Salmonella*, which harbour multiple gene cassettes, contribute to virulence (Van Asten and Van Dijk, 2005), intracellular survival and environmental adaptation. (Hensel *et al.*, 1997). *Salmonella* can persist even after prolonged freezing, posing significant challenges to food safety during production, processing, storage, and improper cooking (Muller *et al.*, 2012). Given these challenges, understanding the interplay between food matrices and *Salmonella* survival, replication, and virulence is crucial. This

study investigated the survival, replication, and differential expression of virulence genes in two major *Salmonella* serovars, *S. Weltevreden* and *S. Typhimurium*, across different food matrices under varying temperature conditions. We hypothesise that *S. Weltevreden* and *S. Typhimurium* exhibit different survival and virulence gene expression across food matrices and temperature conditions, reflecting serovar-specific adaptive mechanisms that influence their persistence and pathogenic potential.

MATERIAL AND METHODS

Bacterial strains

Stock cultures of nontyphoidal *Salmonella* (*Salmonella* Typhimurium ST14028 ATCC strain and *Salmonella* Weltevreden (SW9), an environmental strain isolated from seafood) were obtained from the institutional repository of Nitte University Centre for Science Education and Research (NUCSE). The glycerol broth preserved strain at -80°C was revived using Luria Bertani (LB) broth (HiMedia Laboratories Pvt. Ltd., India). The overnight grown culture was streaked onto selective media plates Hektoen Enteric agar (HEA) (HiMedia Laboratories Pvt. Ltd., India) for phenotypic confirmation.

PCR for the detection of virulence genes

The DNA was extracted from the two isolates using the CTAB (Cetyl Trimethyl Ammonium Bromide) method described by Ausubel *et al.*, (1992) and was subjected to PCR. A reaction mixture of 30µl containing 3µl of 10X buffer (100mM of Tris-HCl (pH 9), 1.5mM of MgCl₂, 500mM of KCl and 0.1% gelatin), deoxyribonucleotides (dATP, dTTP, dGTP and dCTP) each at a concentration of 200µM, ten picomoles each of both forward and reverse primers (Table 1) and 1.0 U of Taq DNA polymerase (HiMedia Laboratories Pvt. Ltd., India), with 2.0 µl of template DNA, was set up. The reactions were carried out using a thermal cycler (Eppendorf, Germany). The virulence genes were selected based on their involvement in the survival and replication of *Salmonella*.

Table 1 Oligonucleotide primers used in this study

Primer	Primer Sequence (5'→3')	Target Gene	Annealing temperature	References
invA-F	GTGAAATTATCGCCACGTTCTGGGCAA	<i>invA</i>	63 °C	(Rahn et al., 1992)
invA-R	TCATCGCACCGTCAAAGGAACC			
stn-F	TGTGCCGTCAGCACTAAATC	<i>Stn</i>	54 °C	
stn-R	TGGTGTATCTGCCTGACCA			
rpoE-F	GGTAGTTCTTCGCGGTATTGAC	<i>rpoE</i>	54 °C	
rpoE-R	ATAAAGTGGCGAGTCTGGTTTC			
icdA-F	TGGTATCGGTGTTGATGTCACCTC	<i>icdA</i>	54 °C	This study
icdA-R	ATCCTGGCCGTAAACCTGTGTG			
rpoS-F	ATACGCAACCTGGTGGATTC	<i>rpoS</i>	54 °C	
rpoS-R	TCGTTGAGACGAAGCTACG			
ompR-F	CGTTATCGCGTTCGGTAAGT	<i>OmpR</i>	54 °C	(Kotian et al., 2020)
ompR-R	AGATCTGGACGTCGATGGAG			

Preparation of different food matrices

The four food matrices selected for this study were fish (Indian mackerel), shrimp (Indian prawn), chicken (boneless broiler chicken), and sprouts (green gram), all purchased from a local market in Mangalore, Karnataka. Each food matrix was divided into 50 g portions using a sterile scalpel, washed three times with distilled water, and placed in sterile glass Petri plates. To remove background flora, the food matrices were exposed to UV light (254 nm) in a laminar flow biosafety cabinet for 30 minutes, except for sprouts, which were treated for 45 minutes. UV-treated food samples were then plated on LB agar and incubated at 37 °C overnight to confirm the absence of background flora. Additionally, all food samples were screened for the presence of *Salmonella*, and only *Salmonella*-negative samples were included in the study.

Preparation of inoculum for spiking study

Revived cultures were inoculated into 50 mL of sterile LB broth for the spiking study in a 100 mL conical flask. The flask was then incubated at 37 °C in a shaker incubator at 120 rpm until an absorbance of 0.5 OD₆₀₀ was obtained. An aliquot of the 0.5 OD₆₀₀ culture was taken based on the calculations obtained for ST14028 and SW9 to obtain the desired cell concentration of 2.5×10⁵ CFU/mL and 2.5×10⁶ CFU/mL, respectively, which was then used to spike the food samples. This desired cell count was obtained by plating 0.5 OD₆₀₀ culture on an LB plate.

Examination of artificially contaminated food matrices

The food matrices (50g each) were spiked with *Salmonella* Weltevreden (2 × 10⁷ CFU/400g) and *Salmonella* Typhimurium (2 × 10⁷ CFU/400g) separately (Kumar et al., 2015). Food matrices without spiking were considered as a control. Both sets of spiked food matrices were further subjected to 4 different temperatures, viz 37 °C, RT (ambient temperature 27° ±2 °C), 4 °C, and -20 °C and analysed for three consecutive days starting from day zero. The temperatures of 37 °C and RT were used solely for comparative purposes and not as a food safety consideration. The experiment was performed in triplicate.

Growth rate analysis using plate count

Growth of both the *Salmonella* serovars in various food matrices was analysed by plate count in Hektoen Enteric Agar (HEA) since HEA allows the growth of *Salmonella* over other microorganisms. On the 0th day, surface sampling was done using a sterile cotton swab with 0.85% physiological saline content. After that, the mixture was serially diluted and plated onto HEA using the spread plate method. The plates were incubated at 37 °C for 24 h. Colonies were enumerated by considering the dilution factor of the sample and were calculated as colony-forming units per gram (CFU/g). The same protocol was followed for 1, 2, and 3-day intervals for different temperatures on all four food matrices.

RNA extraction by TRIzol method

The surface sampling from the food matrices (Fish, Shrimp, Chicken and Sprouts) was collected in 900 µl of 0.85% saline was centrifuged at 5000 rpm for 5 min. The supernatant was discarded, and the pellet was resuspended in 750 µl of TRIzol reagent (Invitrogen, USA). After a brief vortex, the mixture was incubated at room temperature in dark conditions for 15 minutes. 200 µl of chloroform (HiMedia Laboratories Pvt.Ltd, India) was added and mixed vigorously by vortexing, followed by incubation at room temperature for 15 minutes in the dark. The mixture was then centrifuged at 12000 rpm for 15 minutes at 4°C. The obtained supernatant containing the RNA was carefully transferred into a fresh RNase-free microcentrifuge tube. An equal volume of ice-cold isopropyl alcohol (Merck Lifesciences) was added and incubated at -80 °C overnight. It was then centrifuged at 12000 rpm for 15 minutes at 4 °C. The supernatant was discarded, and 600 µl of 75% ethanol was added to the pellet, followed by centrifugation at 5000 rpm for 7

minutes at 4 °C. The supernatant was discarded, and the pellet was air-dried. The pellet was then resuspended in 30 µl of RNase-free water. The extracted RNA was subjected to DNase treatment using a kit (TaKaRa, Japan) per the manufacturer's instructions. The concentration of the DNase-treated RNA was measured using a spectrophotometer (Implen, Nanophotometer, Germany) and stored at -80 °C until further use.

cDNA synthesis

cDNA synthesis was done using a commercially available cDNA synthesis kit (Prime Script 1st strand cDNA synthesis kit, Takara Bio, Japan) following the manufacturer's protocol. The reaction mixture consisted of 1 µl of random hexamers (50 µM) and 1000 ng of RNA, with RNase-free water used to make up the volume to 11.5 µl. The mixture was initially incubated at 65 °C for 5 minutes to facilitate primer annealing, after which it was immediately placed on ice for 5-10 minutes. To the above reaction mixture, 4 µl of Prime Script Buffer (5 X), 1 µl of dNTP mixture (10 mM each), 1 µl of Prime Script Reverse Transcriptase (200 U/µl), 0.5 µl of RNase inhibitor (40 U/µl) and RNase-free water was added to make a total volume of 20 µl. The thermal cycling conditions were 42 °C for 60 minutes of reverse transcription and 70 °C for 10 minutes of inactivation, ending with a 4 °C hold. The obtained cDNA was stored at -20 °C for further use.

Real-time PCR for quantification of gene expression

Real-time PCR was used to quantify the expression level of virulence genes, viz *rpoE*, *stn*, *invA*, *ompR*, *rpoS*. An appropriate housekeeping gene, i.e., *icdA*, was selected as an internal standard for the normalisation of data. Uninoculated food matrices for each group were also included as a control for the normalisation of data. The quantitative real-time PCR (qPCR) was performed using TAKARA TB Green® Premix Ex Taq™ RT II (TliRNaseH Plus) kit. The optimised primer concentrations for all the virulence genes were determined and used in the subsequent steps. Melt-curve analysis was performed for the entire gene to check for the amplification of untargeted fragments. The real-time qPCR reaction mixture contained 5µl of TB Green Premix Ex Taq II (TliRNaseH Plus) (2X), and optimised volumes of both forward and reverse primers, 0.2 µl ROX Reference Dye (50X) and RNase-free water were used to make up the reaction volume to 9µl. This reaction mixture was dispensed into a 96-well real-time PCR plate. 1µl of cDNA was used as a template to get a final reaction volume of 10 µl. The assay was performed in QuantStudio™ 5 Real-Time PCR detection system (Applied Biosystems, ThermoScientific, USA). Data analysis was conducted using the 2^{-ΔΔCt} method to determine the fold change in gene expression. All the obtained expression values are presented as heat maps using GraphPad Prism software version 8.0. For real-time PCR, two biological replicates were included in the study

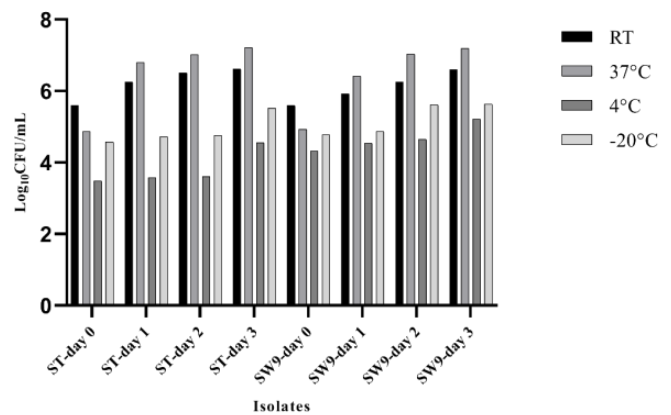
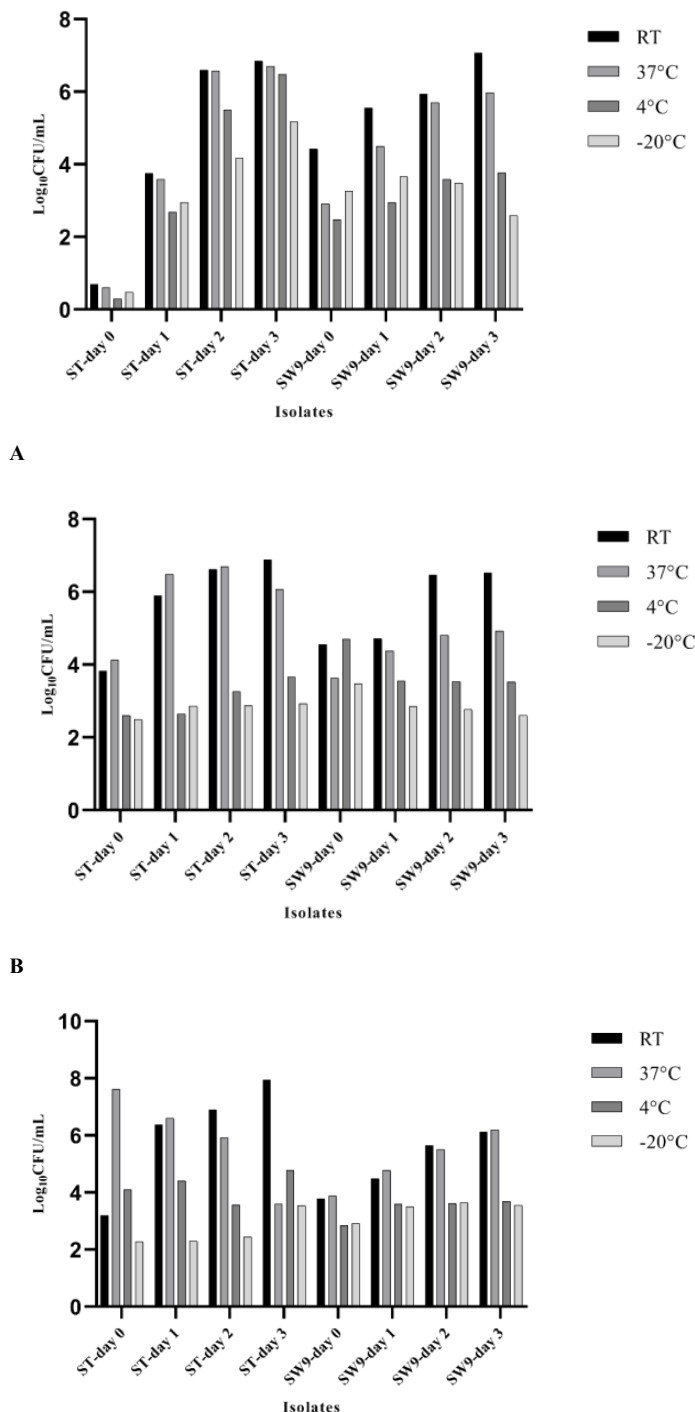
RESULTS

Growth and survival ability of *Salmonella* on different food matrices

Salmonella Typhimurium (ST14028) and *Salmonella* Weltevreden (SW9) were recovered by taking surface sampling from all four food matrices, viz. Fish, Shrimp, Chicken and Sprouts, at different temperatures from day 0 to day 3 and enumerated as CFU/g using agar plate method on Hektoen Enteric agar. In the case of ST spiked in fish and shrimp matrix, we observed that over the period from day 0 to day 3, there was an increase in the count of *Salmonella* at all temperatures. Cell count increased from 3.8 log₁₀ CFU/g to 6.8 log₁₀ CFU/g at RT, and at 4 °C, a rise from 2.6 log₁₀ CFU/g to 3.6 log₁₀ CFU/g was observed. At -20 °C, a slight variation, i.e., 2.4 log₁₀ CFU/g to 2.9 log₁₀ CFU/g, was noted. While at 37 °C, a 2-log increase was observed.

Recovery of ST in chicken showed a sharp increase in the cell count at RT, that is, 3.2 log₁₀ CFU/g to 7.9 log₁₀ CFU/g, while a slight variation from 2.2 log₁₀ CFU/g to 3.5 log₁₀ CFU/g was observed during the three consecutive days at -20

°C. No variation was observed at 4 °C. In sprouts, an increase in ST count was observed irrespective of temperature variation from day 0 to day 3. Similarly, in SW9 spiked onto fish, a continual elevation pattern from day 0 to day 3 in all temperatures that are 4.4 log₁₀ CFU/g to 7.0 log₁₀ CFU/g at RT, 2.9 to 5.9 log₁₀ CFU/g at 37 °C, 2.4 to 3.7 log₁₀ CFU/g at 4 °C and 3.2 to 2.5 log₁₀ CFU/g at -20 °C was noted. In the case of chicken and sprouts, an increase in the cell counts from day 0 to day 3 in all the exposed temperatures (RT, 4 °C, -20 °C, 37 °C) was observed. While in shrimps, the RT and 37 °C promoted the growth of SW9 from day 0 to day 3, while 4 °C and -20 °C temperature exposure showed a reduction in the cell count. ST in fish and shrimp showed an increase in cell count in all the temperatures, while SW9 showed a decrease in cell count at lower storage temperatures. Chicken and sprouts as matrices promoted the growth of both isolates at all temperatures. However, in the case of chicken, lower temperatures showed a slight variation in cell count from day 0 to day 3 (Fig. 1). All the experiments were replicated in triplicate.



D

Figure 1 Growth and survival of *S. Typhimurium* (ST) and *S. Weltevreden* (SW9) on A. Fish B. Shrimp C. Chicken and D. Sprouts food matrices. The values obtained are converted into Log₁₀ and plotted as a bar graph.

ST and SW9 RNA quantification on different food matrices

All the isolates were checked for virulence genes and found to harbour those genes. The RNA concentration in the case of the fish matrix for ST at RT increased from day zero (21 ng/μl) to day 3 (413 ng/μl), respectively. The concentration of RNA was surprisingly higher at -20 °C than at 4 °C in the fish matrix on all the days. In the case of shrimp for ST, the RNA concentration was increased by about 13 to 596 ng/μl, 54 to 165 ng/μl, 6 to 74 ng/μl, and 3 to 65 ng/μl from zero to the 3rd day at RT, 37 °C, 4 °C and -20 °C, respectively. Similarly, in the case of chicken for ST, the RNA concentration was around 57 to 720 ng/μl, 26 to 42 ng/μl, 10 to 20 ng/μl, 1 to 12 ng/μl from zero to 3rd day at RT, 37 °C, 4 °C and -20 °C respectively. In sprouts, the concentration of RNA was about 20 to 81 ng/μl, 112 to 212 ng/μl, 1 to 18 ng/μl, 13 to 113 ng/μl from zero to the 3rd day at RT, 37 °C, 4 °C and -20 °C, respectively.

The concentration of SW9 RNA was comparatively better than ST in all the food samples. The fish matrix recovered the highest quantity of SW9 RNA compared to any other food matrix. It was found to be 222 to 448 ng/μl, 113 to 295 ng/μl, 48 to 371 ng/μl, 71 to 126 ng/μl from zero to 3rd day at RT, 37 °C, 4 °C and -20 °C, respectively. The lowest concentration of RNA from the SW9 spiked sample was recovered from the chicken matrix, with 31 to 325 ng/μl, 71 to 147 ng/μl, 1 to 7 ng/μl, and 1 to 7 ng/μl from zero to 3rd day at RT, 37 °C, 4 °C and -20 °C respectively. However, in this study, RNA contamination from food matrices was not considered.

A drastic reduction in the RNA concentration of both ST and SW9 was observed at lower temperatures on all the days (4 °C and 20 °C) in the chicken matrix compared to other food matrices.

Differential expression of virulence genes

Real-time PCR was carried out to determine the virulence gene expression of *rpoE*, *stn*, *invA*, *rpoS* and *ompR* on various food matrices at three temperature conditions, viz 37 °C, 4 °C and -20 °C. In the fish matrix, at 37 °C, ST and SW9 showed upregulation of *rpoE* with a 3-fold increase on day 3. While *invA* showed downregulation in both isolates on all 3 days of the study. However, *stn*, *rpoS* and *ompR* showed variable expression. Similarly, at -20 °C, both the serovars showed variable expression of most of the genes tested. While at 4 °C, all the genes were upregulated except *ompR* in ST, and *invA* was consistently upregulated in both the serovars on all three 3 days (Fig. 2A).

In the case of shrimp, all the genes were consistently upregulated from day 0 to day 3 in ST at 37 °C with high expression of *stn* and *invA* genes. However, in SW9, most genes showed variable expression on days 2 and 3 except *invA*. However, at 4 °C, variable gene expression was observed in both the serovars on all the days. At -20 °C, except for *invA*, the remaining genes showed upregulation in both the serovars (Fig. 2B). In the case of the chicken matrix, the expression of *invA* was found to be consistently high at 37 °C, 4 °C and -20 °C in both the serovars on all three 3 days. On day 2 at 37 °C, maximum upregulation of 12-fold in ST and 8-fold in SW9 was observed. An upregulation of *rpoE* was also noted at 4 °C for both the serovars. While other genes showed variable expression at different temperatures (Fig. 2C).

In sprouts, lower temperature triggered the expression of *rpoE*, while a complete downregulation of *rpoE* was observed at 37 °C. *stn* showed downregulation at 37 °C and 4 °C, while it is upregulated at -20 °C. The invasion gene (*invA*) showed downregulation at 37 °C and upregulated at 4 °C in both the serovars (Fig. 2D).

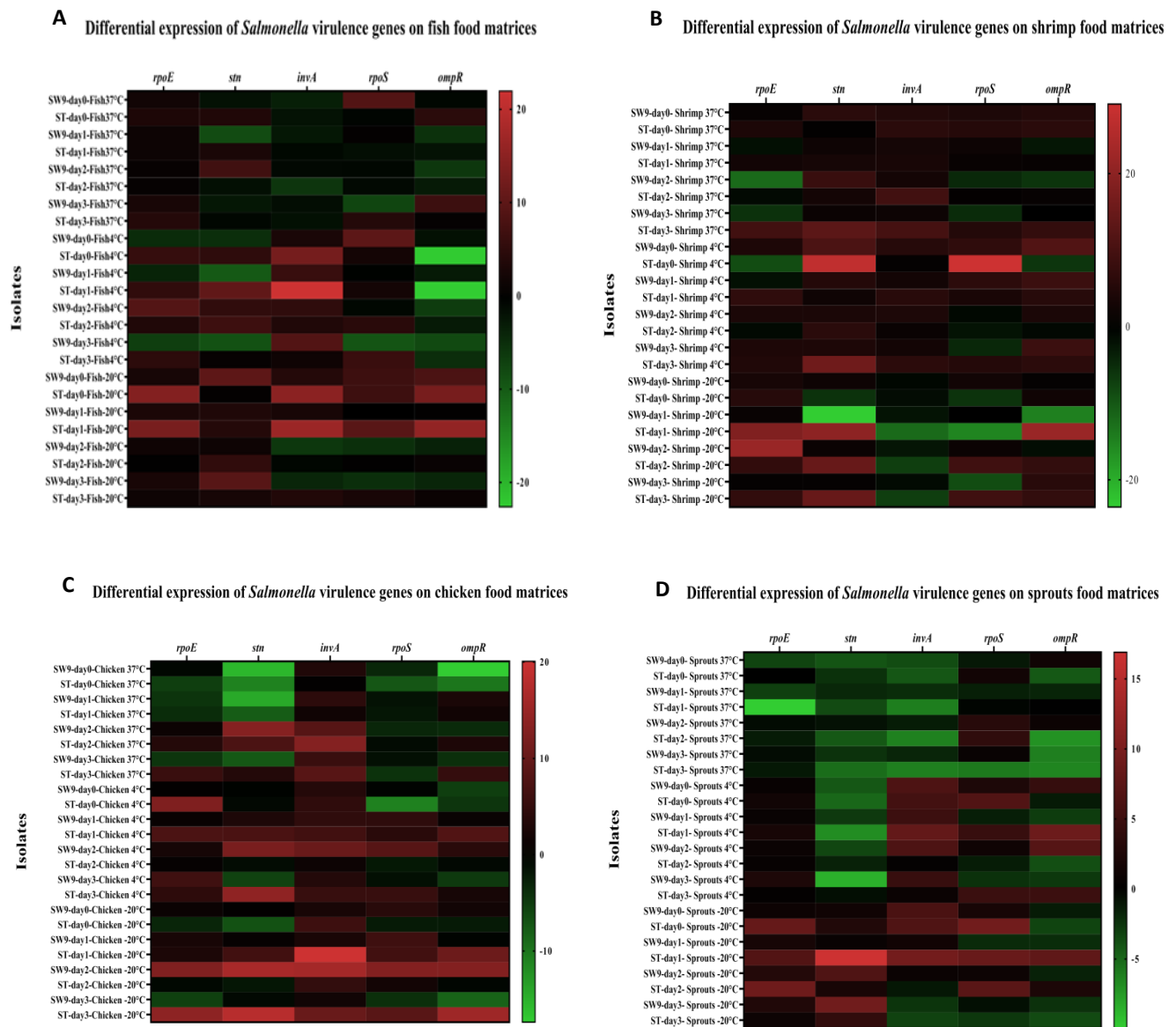


Figure 2 The expression levels of virulence genes of *S. Typhimurium* and *S. Weltevreden* on different food matrices **A.** Fish **B.** Shrimp **C.** Chicken and **D.** Sprouts food matrices are presented using fold change values transformed to control. The log₂ (fold change values) and the colour scale are shown right on the heat map.

DISCUSSION

The incidence of *Salmonella* in seafood and poultry is commonly reported worldwide (Kumar et al., 2009; Guerin et al., 2004). It is well-known that food matrices are nutrient-rich and promote bacterial growth and multiplication. Apart from nutrients, factors like pH, storage temperature, water activity and surface area of the matrix are also known to affect their growth and survival (Nissen et al., 2001; Ingham et al., 2005; Ahmed et al., 2023; Morasi et al., 2022; Mkgangara et al., 2023; Asish et al., 2023). However, temperature is a critical factor that affects the cellular and metabolic activities of bacteria and plays a vital role in the survival, replication, and biofilm formation of bacteria on different food matrices (Francis et al., 2001; Huang et al., 2015; Sudarshana et al., 2008; Luo et al., 2009; Francis et al., 2012; Huang et al., 2019). This study mainly focused on understanding the growth dynamics and virulence gene expression of *S. Weltevreden* (SW9) and *S. Typhimurium* (ST14028) on different food matrices at RT (only for growth kinetics study), 37 °C, 4 °C and -20 °C, observed over a period of 3 days. ST in fish and shrimp showed an increase in cell count in all the temperatures, while SW9 showed a decrease in cell count at lower storage temperatures. However, none of the temperature conditions had a bactericidal effect on *Salmonella*. This contradicts the study of Kumar et al., (2015), wherein -20 °C storage temperature showed a decline in *Salmonella* population compared to 4 °C when seafood samples were spiked (Kumar et al., 2015). Nevertheless, *Salmonella* is known to survive at frozen temperatures on shrimp for more than 8 weeks (Thushani et al., 2003). We could thus observe variation in the survival pattern in different serovars. Chicken and sprouts as matrices promoted the growth of both isolates at all temperatures. However, in the case of chicken, lower temperatures showed a slight variation in cell count from day 0 to day 3. This could

be due to freezing temperature, which causes damage to the cell wall, leading to cell death or reduced recovery of cells on plating (Kumar et al., 2015). Ambient temperature, i.e. RT and 37 °C, supported the growth of *Salmonella* in all matrices except in the chicken matrix for ST. We also noted that in the case of sprouts, the growth of bacteria was observed at all temperatures. Surprisingly, sprouts allowed the growth and multiplication of *Salmonella* more rapidly at -20 °C than at 4 °C. This is in contrast to the study of Alves et al., (2024), wherein vegetables stored at 7 °C showed a decreased count of *Salmonella* than at higher temperatures. Alfalfa sprouts have been associated with major *Salmonella* outbreaks (IFSN 2005; Food Safety News 2016, 2021). In addition, sprouts are eaten raw in many parts of the world, highlighting the necessity of safety measures to deliver them safely to consumers. The results obtained in our study point out that both the *Salmonella* serovars could grow in all four food matrices, such as fish, sprouts, chicken and shrimp. Lower room temperatures, like 4 °C and -20 °C, are usually known to slow the growth of bacteria, and hence, these temperatures are used in storage. However, in our study, we observed that both the *Salmonella* serovars survive and multiply in all the food matrices incubated at different temperatures, including low temperatures, though at a slower rate. This is similar to the study of Nissen et al., (2001), wherein an increase in the counts of *Salmonella* Enteritidis was observed in pork meat kept at 10 °C for 5 days. But in contrast to the study of Ingham et al., (2005), wherein no survival or growth of *Salmonella* in the frozen chicken samples and ground beef was observed. The high survival and replication rate of both *Salmonella* strains in sprouts in our study shows that food matrices play a very important role in the dissemination of food-borne pathogens such as *Salmonella* and might be the reason for a large number of outbreaks connected to those food matrices. The sprouting process increases the nutrient level at optimum water activity, pH and temperature, which may favour the growth of microorganisms like

Salmonella. This, in turn, highlights the role of the physicochemical properties of the food in the growth and survival of *Salmonella* (Huang et al., 2019). Therefore, to minimise food-borne illness, it is essential to store different food substances at a temperature that can control the growth of microorganisms without affecting the nutritional quality of the food matrices. Nevertheless, in our study, the growth and multiplication of both strains of *Salmonella* in all the food matrices and at different temperatures suggest the potential of these strains to survive on different food matrices at varied temperatures. Previous studies have shown that a major portion of RNA is constituted by rRNA, representing metabolically active bacterial cells or, in other words, bacterial load (Deutscher, 2006). In our study, RNA was used as a qualitative indicator of cellular growth and activity at various temperatures in the different food matrices. A correlation between RNA concentration and cell count was observed. The quantity of RNA steadily increased with the increase in cell count for up to 3 days at individual temperature conditions in all the food matrices. However, in the case of ST at 37 °C in the chicken matrix, though there was a decrease in cell count, no corresponding variation in RNA was seen. This is in agreement with reports of Kumar et al., (2015) wherein an increase in the total RNA was observed up to 3 days in seafood samples exposed to *Salmonella* Weltevreden, even though there was a decline in *Salmonella* count in seafood following incubation at RT and 45 °C. This could be due to the probable contamination of RNA from food samples or the stability that the food matrix offers to keep the RNA intact. In addition, the non-growth activity or dormant state of bacteria under stress conditions might also affect the rRNA concentration (Blazewicz et al., 2013). It is also noted that the concentration of RNA was higher in samples spiked with ST and SW9 at RT than in other temperature conditions, except in sprouts, wherein 37 °C showed higher RNA concentration for both ST and SW9. This variation might be due to the differential effect of different food matrices on bacterial survival, behaviour, virulence, biofilm formation and antimicrobial resistance at different temperature conditions (Chen et al., 2021). It is interesting to note that, though there is a reduction in RNA concentration at low temperatures, it showed an increasing trend of RNA concentration from day zero to day 3 in all the food matrices, including seafood. This is in contrast to the study of Kumar et al., (2015), wherein no RNA was detected beyond day one in the seafood matrix during the study.

Salmonella adapts to a wide range of temperatures by regulating the expression of virulence and stress response genes. In this study, the response of invasion genes (*invA*), stress response genes (*rpoE*, *ompR* and *rpoS*) and *Salmonella* enterotoxin-related gene (*stn*) was analysed. As a part of the *Salmonella* pathogenicity island, the invasion gene *invA* plays a vital role in intracellular survival and replication of *Salmonella*. It was observed that *invA* was significantly upregulated at 4 °C in all the food matrices, supporting the survival of the bacteria. This is in contrast to the study of Kumar et al., (2015), wherein the expression of *invA* was significantly downregulated at 4 °C in seafood. It is also interesting to note that the expression of *invA* was higher in all the food matrices except in sprouts at 37 °C. Studies also showed that *Salmonella* enters the VBNC (Viable but Nonculturable) state at refrigerator and freezing temperatures while continuously expressing the virulence genes such as *invA* (Liu et al., 2026). Such temperature shifts may also trigger other virulence and stress-response genes, enhancing its pathogenic potential (Yang et al., 2014). An enterotoxin-related gene (*stn*), which is the causative agent of diarrhoea, was also analysed in the study (Chopra et al., 1994). This gene regulates the membrane composition and integrity of the bacterial cell wall. We observed downregulation of *stn* in sprout at 37°C and 4 °C, while it triggered the expression at -20 °C. Freezing temperature does not fully inactivate the bacteria; upon thawing, the stressed cells may regain their virulence and exhibit increased pathogenicity. *rpo* genes are usually expressed in stress conditions and are also known to have antioxidant defence by enhancing the expression of other *rpo* genes (Kumar et al., 2015). This could be an intrinsic function to protect the cell from low temperatures. However, in our study, *rpo* genes showed variable expression at all temperature conditions. In contrast, Kumar et al., (2015) showed downregulation of *rpoE* at all temperatures. Overall, the expression data obtained signifies the induction of virulence genes in *Salmonella* at various storage temperatures. This, in turn, highlights the ability of the bacteria to grow vigorously and show their pathogenicity in varied temperatures and food matrices. The survival and further high expression of virulence genes by both strains of *Salmonella*, even at low temperatures, highlights a growing concern for the food industry and public health. This resilience under cold storage conditions, commonly used to inhibit microbial growth, suggests that *Salmonella* is evolving into an increasingly formidable pathogen capable of persisting and potentially causing infection despite standard preservation methods. These adaptations of *Salmonella* enable contaminated food to remain infectious for a long period of time, complicating the process of detection and control. Further, improper storage and temperature abuse can increase foodborne outbreaks, possess a risk to vulnerable populations, which further highlights the need for strict storage temperature regulations in food safety systems (Khan et al., 2021).

CONCLUSION

In conclusion, the present study evaluated the survival, replication and differential expression of virulence genes of two major nontyphoidal *Salmonella* serovars, *Salmonella* Weltevreden and *Salmonella* Typhimurium, in different food matrices

at varying temperatures. The results indicated that both the *Salmonella* serovars were versatile for adapting to ambient and low temperatures and showed survivability and multiplication in all the food matrices. Differential expression of stress response genes and virulence genes highlights that storage temperature plays a significant role in viability, pathogenicity and food safety. Findings also highlight the serovar-specific expression of virulence and stress response genes of *Salmonella*. The gene expression data showed that low-temperature conditions, especially 4 °C, induced virulence gene expression in addition to ambient temperature. Though *Salmonella* Weltevreden has increasingly been reported among seafood samples, especially shrimps (Hounmanou et al., 2020), our study did not find any significant difference in the association of *Salmonella* Weltevreden with either shrimp or fish matrices compared to *S. Typhimurium*. Further studies on biofilm formation and the effect of the preservatives/sterilization process in different stages of food processing to reduce microbial load can help reduce the risk of *Salmonella* outbreaks.

Overall, the data obtained from this study highlight the induction of virulence genes in *Salmonella* at different storage temperatures and emphasize the pathogenicity and adaptability of this bacterium in diverse food settings. These findings have significant implications for food safety regulations, as they underscore the importance of implementing appropriate storage and processing conditions to mitigate the risk of food-borne illnesses caused by *Salmonella* contamination. Given that low-temperature storage does not reliably eliminate *Salmonella* risk, current food safety practices require review, particularly for high-risk products like seafood and sprouts.

Despite the crucial findings of the study, there are few limitations of the study, such as the short duration of the study (3 days), which may not fully capture long term adaptation, persistence or biofilm formation of *Salmonella* under prolonged storage conditions. The quality and integrity of the extracted RNA may have been influenced by inhibitors or contaminants from the different complex food matrices, affecting the qPCR accuracy and gene expression interpretation. The other parameter like physicochemical properties of the food matrices, which includes nutrient composition, water activity and surface characteristics, would have contributed to inconsistencies in bacterial growth and gene expression pattern and finally, the pH variation across the food matrices was not systematically measured or controlled, although pH is a known factor for influencing *Salmonella* survival and virulence gene regulation. Therefore, further studies can be done by incorporating controlled physicochemical parameters, extended time periods and improved RNA quality assessment are necessary to better understand the long-term survival dynamics and pathogenic potential of *Salmonella* in food systems.

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