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# Invasive potential of sub-lethally injured *Campylobacter jejuni* and *Salmonella* Typhimurium during storage in chicken meat juice

Gayani Weerasooriya, Samiullah Khan, Kapil K. Chousalkar *, Andrea R. McWhorter

*School of Animal and Veterinary Sciences, University of Adelaide, Roseworthy, South Australia, 5371, Australia*

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ABSTRACT

*Campylobacter jejuni* and *Salmonella* Typhimurium are major foodborne pathogens that cause gastrointestinal infections in humans. Humans can acquire these bacteria through the consumption of contaminated poultry meat. In many countries, during poultry meat processing, chicken carcasses are often treated with sanitizers to reduce the load of food spoilage and foodborne pathogens. The European Union, however, currently does not permit the use of sanitizers for chicken meat processing. The survivability and virulence of sub-lethally injured bacteria after exposure to chemical decontamination, however, is poorly understood. Here, we investigated the survivability and invasion potential of *Campylobacter jejuni* and *Salmonella* Typhimurium following exposure to chlorine and acidified sodium chlorite (ASC) during storage in chicken meat juice (CMJ). *Campylobacter jejuni* and *Salmonella* Typhimurium were stored in CMJ under refrigeration (5 °C) following exposure to either chlorine or ASC. Bacterial culturability, motility, and invasive capacity were subsequently investigated. Changes in the expression of *Campylobacter* and *Salmonella* specific stress response and virulence genes were also investigated. The results revealed that CMJ facilitated the survival of both *Campylobacter* and *Salmonella* following exposure to chlorine but not ASC. Both the chlorine and ASC reduced bacterial invasiveness, motility, and culturability of *Campylobacter jejuni* but not *Salmonella* Typhimurium. Bacterial stress response and virulence genes in *Campylobacter jejuni* (*rpoB*, *sodB, flaG*, *flaA*, *cadF*, *racR*) and *Salmonella* Typhimurium (*rpoH, rpoS, hilA*, *fimH, spvR*, *avrA*) were upregulated over time, indicating an increase in virulence potential. This study suggested that sub-lethally injured *Campylobacter jejuni* and *Salmonella* Typhimurium in CMJ remain a significant risk in the food chain due to the likelihood of cross-contamination while handling chicken meat. Furthermore, the greater bactericidal effects of ASC can reduce the risk of contamination.

## 1. Introduction

Foodborne gastrointestinal disease is a major public health issue with significant socioeconomic impacts in terms of loss of productivity and expenses related to healthcare (WHO, 2013, pp. 9–11). Worldwide, among bacterial food-borne illnesses, *Campylobacter* has been linked with the highest annual number of cases (95,613,970), followed by *Salmonella* (78,707,591) (WHO, 2015). *Campylobacter jejuni* (*C. jejuni*) and *Salmonella enterica* serotype Typhimurium (*S.* Typhimurium) are major foodborne pathogens and are the most common enteropathogenic bacteria reported during chicken meat related human infections (EFSA, 2016). Infection with either of these bacteria typically includes self-limiting gastroenteritis, with diarrhoea, fever, and severe abdominal pain (Acheson & Allos, 2001; Chen et al., 2013).

*C. jejuni* and *S.* Typhimurium colonise the intestine of broilers and high loads are often detected in feces that can contribute to carcass contamination during processing (Antunes et al., 2016; Byrd et al., 1998). Despite established standard protocols to mitigate bacterial load during processing (FSANZ, 2013; USDA, 1995, pp. 54450–54457), contamination of chicken meat in the supply chain can be significant (EFSA, 2010). Many countries use "generally recognized as safe" (GRAS) chemicals, such as chlorine, acidified sodium chlorite (ASC), or peracetic acid (PAA) to mitigate the bacterial load on chicken meat (Trachoo & Frank, 2002; Walsh et al., 2018). It should be noted that the European Union has banned the use of most sanitizers used in food processing for health and safety reasons (EFSA, 2012). Some of the most significant challenges associated with chemical decontamination are the reduced efficacy due to the constant presence of organic material (Virto et al., 2005) as well as non-injured or sub-lethally injured bacteria remaining on carcasses.

* Corresponding author. School of Animal and Veterinary Science, The University of Adelaide, Roseworthy, 5371, SA, Australia.
*E-mail address:* kapil.chousalkar@adelaide.edu.au (K.K. Chousalkar).

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Sub-lethally injured bacteria following exposure to sanitizers can recover and may subsequently be resuscitated under favourable conditions (Muhandiramlage et al., 2020; Ridgway, 1982). This population of bacteria represents a significant public health risk. The survivability of sub-lethally injured *C. jejuni* and *S.* Typhimurium during refrigerator conditions, chilling, and or freezing may increase the risk of cross contamination (Birk et al., 2004; El-Shibiny et al., 2009). Furthermore, bacteria in meat juice from thawed or raw poultry packages also represent a significant risk (Burgess et al., 2005). Bacteria in chicken meat juice could potentially contaminate ready-to-eat foods or combined with poor hygiene lead to cross contamination in the kitchen (Chen Fur et al., 2011; Donelan et al., 2016). Prolonged viability of *Campylobacter* and *Salmonella* in chicken meat juice (Birk et al., 2004; Karki et al., 2019) may increase the risk of human infections (Keener et al., 2004).

Post-processing, chicken meat is stored refrigerated until it is cooked. The ability of chemically stressed bacteria to survive in cold storage in the presence of chicken meat juice has not been extensively investigated. Bacteria typically respond to stress by altering their cellular morphology, membrane composition, and cellular metabolism (Mitchell & Silhavy, 2019). Stress adapted bacteria produce a range of proteins that frequently provide cross-protection against other stressors. Enhanced survival of stressed *Campylobacter* and *Salmonella* throughout the food processing chain (Keener et al., 2004) may lead to altered virulence (Guillén et al., 2021) representing a significant public health risk.

In this study, we investigated the culturability, *in-vitro* invasion potential, and virulence of stressed *C. jejuni* and *S.* Typhimurium isolates stored at refrigeration temperature in the presence of chicken meat juice. To mimic the field condition of chicken meat in the retail market, we inoculated *C. jejuni* and *S.* Typhimurium exposed to either chlorine or ASC into chicken meat juice and stored at refrigeration temperature. Bacterial culturability as well as *in-vitro* virulence were characterised.

## 2. Materials and methods

### 2.1. Bacteria

*Campylobacter jejuni* (*C. jejuni*) and *Salmonella* Typhimurium (*S.* Typhimurium) isolates were isolated from chicken meat as part of a separate study (Chousalkar et al., 2019). The isolates were maintained at −80 °C in a 50:50 mixture of brain heart infusion broth and 100% glycerol. Twelve *C. jejuni* isolates were selected for this study from a pilot study, that was done to compare the invasive capacity of *Campylobacter* strains into cultured human intestinal epithelial cells. A pilot *in-vitro* invasion assay experiment was conducted. From this experiment, 6 *C. jejuni* isolates exhibiting $>10^5$ CFU/mL cell invasion were considered high invasive (C1–C6), and 6 that showed $< 10^2$ CFU/mL were considered low invasive (C7–C12) were selected for the present study. Six *S.* Typhimurium isolates were also selected randomly as they did not exhibit variation in invasiveness in the pilot study. *C. jejuni* isolates were resuscitated from the culture stock on Columbia sheep blood agar (SBA) (Thermo Fisher Scientific, Australia) and incubated at 42 °C in 10% $CO_2$ for 48 h. *S.* Typhimurium isolates were cultured on nutrient agar (Thermo Fisher Scientific, Australia) and incubated at 37 °C for 12–18 h.

### 2.2. Chicken meat juice

Chicken meat juice (CMJ) was prepared as described previously with modifications (Brown et al., 2014). Briefly, fresh whole carcasses were sourced from two processing plants in South Australia and kept at 5 °C for 2–3 h to collect the juicy liquid. The CMJ was then centrifuged at 4300×*g* for 20 min. The supernatant was filter sterilized using a 0.2 μm membrane filter (ValCap 90 PF Filter Unit (Life Sciences, Australia) and stored at −20 °C. The sterility of CMJ was confirmed before each experiment by plating onto SBA and incubating at either 37 °C or 42 °C in 10% $CO_2$.

### 2.3. Inoculum preparation

Concentrations of chlorine and acidified sodium chlorite (ASC) used in this study were selected according to the standard protocol for decontamination in processing plants in Australia (FSANZ, 2005). Chlorine at 8 ppm (4% Sodium hypochlorite, Sigma-Aldrich, USA) was prepared in sterile water. To prepare the ASC, sterile water was acidified to pH 2.4–2.5 using 4 M citric acid (Sigma –Aldrich, USA). Sodium chlorite (31%, Chem-Supply, Australia) was added to obtain a concentration of 900 ppm.

For each of the 12 *Campylobacter* and 6 *Salmonella* isolates, a 10 mL suspension was prepared in 0.9% saline to obtain OD600 of 0.2 to obtain the final bacterial concentration of $10^9$ CFU/mL. The bacterial suspension was centrifuged at 4300×*g* for 15 min, and the pellet was resuspended in 10 mL of either chlorine (8 ppm) for 2 min or ASC (900 ppm) for 1 min, at room temperature. The exposure time was determined according to results obtained during our previous studies (Muhandiramlage et al., 2020; Weerasooriya et al., 2021). Bacterial cultures prepared in 0.9% saline were used as the control. Chemically treated bacterial cultures and controls were centrifuged at 4300×*g* for 15 min, subsequently, the pellets were washed by resuspending in 1 mL of 0.9% saline and were centrifuged at 4300×*g* for 15 min. The washed pellet was resuspended in 3 mL sterile CMJ and incubated at 5 °C for 12 days. There were 3 biological replicates for each isolate.

### 2.4. Bacterial culturability in chicken meat juice

Bacterial culturability was determined on days 0, 2, 5, 7, 9, and 12 post inoculation of the CMJ. The bacterial counts were obtained by preparing 10-fold serial dilutions in 0.9% saline and drop plating 10 μL. *C. jejuni* isolates were plated onto SBA plates and incubated at 42 °C in 10% $CO_2$ for 48 h. *S.* Typhimurium isolates were plated onto xylose lysine deoxycholate (XLD) (Thermo Fisher Scientific, Australia) and incubated at 37 °C for 12–18 h. Bacterial counts were $Log_{10}$ transformed and reported as CFU/mL of the CMJ.

### 2.5. Gentamicin protection assay

The human intestinal epithelial cell line, Caco-2 (ATCC HTB-37), was used for the gentamicin protection bacterial invasion assay as described previously (McWhorter & Chousalkar, 2015). Briefly, 150 μL DMEM with no supplements was added followed by 100 μL of bacteria in CMJ to the Caco-2 cells at each time point of storage (Day 0, 3, 5, 7, 9 and 12). The initial concentration (Day 0) of the inoculum of untreated control was $10^8$ CFU/mL and the bacterial concentration of treatment groups reflected the bacterial count remaining after the treatment to mimic the natural conditions during storage. Invasion test controls included *S.* Typhimurium infected cells were incubated for 1 h at 37 °C with 5% $CO_2$, while *C. jejuni* infected cells were incubated for 4 h under the same conditions as *C. jejuni* takes a longer time to invade into Caco-2 cells (Friis et al., 2005). The number of *C. jejuni* that had invaded was determined by preparing serial 10-fold dilutions in 0.9% saline and spread plating 100 μL onto mCCDA agar (Thermo Fisher Scientific, Australia). Plates were incubated at 42 °C in 10% $CO_2$ for 48 h. *S.* Typhimurium counts were obtained by drop plating 10 μL of dilutions onto XLD agar and incubated at 37 °C for 18 h. Bacterial colonies were enumerated and percent invasion was calculated. Invasion assays were conducted at day 0, 2, 5, 7, 9 and 12 post inoculation of the CMJ. Experiments were performed in duplicate and were repeated three times.

To determine if bacteria had invaded but were not culturable after direct plating, any negative samples were added to resuscitation media. Briefly, 100 μL of cell lysate of all the replicates of control and chemical treated groups in the invasion assay were inoculated into 900 μL of

broth. *C. jejuni* was incubated in the Preston broth (nutrient broth number 2 with *Campylobacter* selective supplement) (Oxoid, Australia) at 42 °C in 10% $CO_2$ for 48 h, while *S.* Typhimurium was added to the nutrient broth at 37 °C for 18 h and the *C. jejuni* and *S.* Typhimurium growth was recorded as positive or negative.

### 2.6. Bacterial motility

Motility experiments were performed with *C. jejuni* and *S.* Typhimurium stored in the CMJ. Bacterial motility was tested on day 0, 6, and 12 post inoculation. Motility plates were prepared by adding 0.3% bacteriological agar no.1 (Oxoid, Australia) to the brain heart infusion broth (Oxoid, Australia). One microliter of each CMJ treatment group was stab inoculated into the center of the motility agar. *C. jejuni* plates were incubated at 42 °C in 10% $CO_2$ for 48h and *S.* Typhimurium was incubated at 37 °C for 20 h. The distance migrated (diameter in mm) was measured using a digital caliper. Experiments were performed with duplicates and repeated twice.

### 2.7. Bacterial viability

Intracellular ATP production was determined using the BacTiter-Glo Microbial Cell Viability Assay (Promega, Australia) according to the manufacturer's instruction. Bacterial intracellular ATP production was measured at day 0, 6, and 12 post-inoculation of the CMJ. Luminescence was read using a ClarioStar microplate reader (BMG, Australia) with the gain set to 3000. Heat-treated (95 °C for 25 min) *C. jejuni* and *S.* Typhimurium at a concentration of $10^7$ CFU/mL were used as dead control.

### 2.8. qRT-PCR

Gene expression of *C. jejuni* and *S.* Typhimurium following exposure to chlorine or ASC and stored in the CMJ was conducted at day 0, 6, and 12 time points. A high (C5) and low (C10) invasive *C. jejuni* isolates and two *S.* Typhimurium isolates (S1 and S2) were selected for gene expression experiments. Each two isolates of both the *C. jejuni* and *S.* Typhimurium stored in the CMJ without exposed to sanitizers were used as the control in this study. Bacterial RNA was extracted at day 0, 6, and 12 time points as previously described (Weerasooriya et al., 2021).

Primers for candidate target and reference genes for *C. jejuni* and *S.* Typhimurium (Table 1) were designed using the National Centre for Biotechnology Information (NCBI) software. Genes were selected based on their ontologies associated with motility, adhesion, cell invasion, and stress response as previously described (Wieczorek et al., 2018; Yang et al., 2014). Primers were optimized for target specificity using qPCR and melt curve analysis. Products were visualized by gel electrophoresis. To determine the amplification efficiency of individual primers, qPCR was performed on 8 different 5-fold serial dilutions of *C. jejuni* and *S.* Typhimurium cDNA.

cDNA was prepared from all *C. jejuni* and *S.* Typhimurium RNA samples. Approximately 1000 ng was reverse transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Australia) as per the manufacturer's protocol. For qPCR, using SensiFast Sybr Hi-Rox Kit (Bioline, Australia), the master mix was prepared as described previously (Weerasooriya et al., 2021). Thermocycling conditions in QuantStudio 6 (ABI, Australia) thermal cycler were: 95 °C for 3 min, 40 cycles of denaturation at 95 °C for 10 s, annealing at 56 °C for 30 s and extension at 72 °C for 30 s. A melting curve step was included to assess the specificity of amplification. The relative change in the gene transcription ratio (fold change) for each candidate target gene was normalized against the expression to the reference gene *16S rRNA*. qPCR data were expressed as $Log_2$ fold change. For assessing the regulation of genes of *C. jejuni* and *S.* Typhimurium affected by chlorine and ASC, the untreated control group was used as a reference at each time point. Three biological replicates were used in qPCR and all the samples were run in duplicates.

### 2.9. Statistical analysis

Bacterial survival, cell invasion, motility, and ATP assay data were analysed in GraphPad Prism Version 9 (GraphPad Software, Inc., USA) using one- and two-way Analysis of Variance (ANOVA) with Tukey's multiple comparisons test. The relative expression data of individual genes were analysed by $2^{-\Delta\Delta Cq}$ method from quantitation cycle (Cq) values using the reference gene *16S rRNA*. All treatment groups were compared with control groups using ANOVA and student's t-test at every time point. P-values $< 0.05$ were considered statistically significant.

## 3. Results

### 3.1. Survivability of C. jejuni and S. Typhimurium in chicken meat juice

To determine the long term effects of sanitizer exposure, *C. jejuni* and *S.* Typhimurium isolates were exposed to either chlorine or ASC and stored in CMJ at 5 °C. Bacterial culturability was determined over 12 days (Fig. 1). Most of the *C. jejuni* isolates in the untreated group had an average 2 log reduction at day 12, except C3, C10, C11, and C12 that had an average of 3 log reduction (Fig. 1A). Exposure to chlorine caused a significant reduction in culturability ($p < 0.001$), with the exception of C2 and C12, which were culturable up to day12. Significant variation ($p < 0.01$) in bacterial culturability was observed following chlorine exposure (Fig. 1B). The reduction of culturable bacteria over time was also significant ($p \leq 0.0001$) for all *C.jejuni* isolates. Greater culturability was observed for most of the "high" invasive *C. jejuni*, which were culturable for up to day 7 except C4, which was unculturable after day 2. Most of the "low" invasive isolates (C7, C8, C9, and C10) exhibited a significant 4–5 log reduction ($p \leq 0.0001$) following chlorine exposure at day 0 compared to the untreated group. These isolates exhibited lower culturability and were unculturable after day 5 (except C8). Following treatment with ASC, none of the *C. jejuni* isolates were culturable (Fig. 1C) from day 0.

*S.* Typhimurium isolates in the untreated and chlorine treated groups were culturable throughout the experimental period. The untreated group exhibited an increase in bacterial count over time achieving an average 2 log increase by day 12 (Fig. 1D). A significant variability in chlorine sensitivity was observed between isolates. For S1 and S4, culturability was an average of 5 log lower compared with the untreated isolates at day 0. Following chlorine treatment, the other *S.* Typhimurium isolates exhibited an average of 2.5 log reduction. Chlorine treated *S.* Typhimurium isolates exhibited relatively stable culturability (Fig. 1E). Culturable *S.* Typhimurium was not observed following exposure to ASC (Fig. 1F).

### 3.2. Invasiveness of Campylobacter and Salmonella

The *in-vitro* invasiveness of *C. jejuni* (Fig. 2) and *S.* Typhimurium (Fig. 3) in CMJ was investigated to determine the effects of sanitizer treatment and cold storage on the aspect of bacterial invasion. On average, $10^5$ CFU/mL of untreated *C. jejuni* invaded Caco-2 cells. The invasive capacity of untreated bacteria remained stable over the entire experiment (Fig. 2A). A significant difference ($p \leq 0.0001$) was observed between "high" and "low" invasive *C. jejuni* isolates (except for isolates C7 and C8). The percent recovery of the untreated group was significantly higher ($p \leq 0.0001$) at day 9, and day 12 compared to the early days of incubation (Fig. 2B). The percent recovery of the isolates in the "low" invasive group also increased with time. Chlorine treatment resulted in a significant reduction of invasiveness for all *C. jejuni* isolates ($p \leq 0.0001$) (Fig. 2C). Significant variation in invasive capacity was observed between *C. jejuni* isolates after chlorine exposure. Most of the invasive isolates exhibited reduced invasiveness over time. *C. jejuni* isolates were not invasive after exposure to the ASC.

**Table 1**
Primer sequence and validation in qPCR for *C. jejuni* and *S.* Typhimurium.

| Gene symbol | Function | Primer sequence (5′-3′) | Fragment size (bp) | NCBI Accession number | Annealing (°C) | qPCR efficiency (%) | Correlation coefficient ($R^2$) | Slope |
|---|---|---|---|---|---|---|---|---|
| C. jejuni | | | | | | | | |
| *16S rRNA** | Reference gene | F:TGC TAG AAG TGG ATT AGT GG<br>R:GTA TTA GCA GTC GTT TCC AA | 91 | NR_041834.1 | 56 | 98 | 0.992 | −3.37 |
| *racR* | Adhesion and cell invasion | F:AGCGATAAAGTTGTGGGGCT<br>R:CAGCGACTTACAAGCTGTTCTC | 287 | NC_002163.1 | 56 | 107.9 | 0.998 | −3.146 |
| *flgA* | Motility and cell invasion | F:ACCACAGCTTTAATACGCCGA<br>R:TTTGCACGAATCCTTTCGCC | 122 | NC_002163.1 | 56 | 105.8 | 0.995 | −3.189 |
| *flaA* | Adhesion and cell invasion | F:ATGAGTAGCGCAGGAAGTGG<br>R:ATCACAGCCATAGCGCCTTT | 257 | NC_002163.1 | 56 | 111.5 | 0.999 | −3.073 |
| *cadF* | Adhesion and cell invasion | F:ACATTATGGCGCGGGTGTAA<br>R:GGAGTTGCACGAGTATCAGCA | 183 | NC_002163.1 | 56 | 103.4 | 0.999 | −3.242 |
| *sodB* | Oxidative stress resistance | F:TCAAAGGCGCTACAGGAGTT<br>R:GCGTGCATTGCGATGATCTA | 182 | NC_002163.1 | 56 | 101.2 | 0.999 | −3.296 |
| *rpoB* | Targeting gene for VBNC | F:CGCGAATGCATGGAAAGAGG<br>R:CCACGAACCACGATCAGGAA | 309 | NC_002163.1 | 56 | 104.2 | 0.997 | −3.227 |
| ***S.* Typhimurium** | | | | | | | | |
| *16S rRNA** | Reference gene | F:CAGAAGAAGCACCGGCTAAC<br>R:GACTCAAGCCTGCCAGTTTC | 167 | NC_003197.2 | 56 | 98.8 | 0.996 | −3.37 |
| *rpoS* | A regulator of the general stress response | F:GCCGTATGCTTCGTCTCAAC<br>R:TATCCCAGCAGACCGAAACG | 220 | NC_003197.2 | 56 | 91.8 | 0.956 | −3.146 |
| *rpoH* | A regulator of the heat shock response | F:GTCTCACCTGCGCTTTGTTG<br>R:AACGATACGCCAGTTACGCA | 208 | NC_003197.2 | 56 | 93.5 | 0.999 | −3.486 |
| *spvR* | Host defense mechanism and inducing lysis of macrophages | F:GAGAGCCGTTAACAGCCAAA<br>R:ATATCAGGTTTGCCGCAGAG | 171 | NC_003197.2 | 56 | 101 | 0.997 | −3.189 |
| *hilA* | A regulator controls the transcription of invasion genes within PI.1 | F:ATTAAGGCGACAGAGCTGGA<br>R:GCAGAAATGGGCGAAAGTAA | 134 | NC_003197.2 | 56 | 103.6 | 0.995 | −3.073 |
| *fimH* | Promote binding to the target receptor and induce colonization and invasion | F:GCCGCAAAACTGTGAAGTGA<br>R: ATTATCGGACACCATCGCCT | 223 | NC_003197.2 | 56 | 110 | 0.995 | −3.097 |
| *avrA* | Promote the intestinal invasion in vivo and activate the inflammatory response | F:GAGCTGCTTTGGTCCTCAAC<br>R:AATGGAAGGCGTTGAATCTG | 173 | NC_003197.2 | 56 | 106.6 | 0.998 | −3.242 |

Candidate reference (*) and target genes in expression studies by qPCR. To calculate the amplification efficiency of individual primers, a standard curve was generated using a 5-fold dilution of cDNA amplified in QuantStudio 6 (ABI, Australia) thermocycler real-time system. The standard curve was obtained by plotting the Cq values against the starting quantity of the template for each dilution.

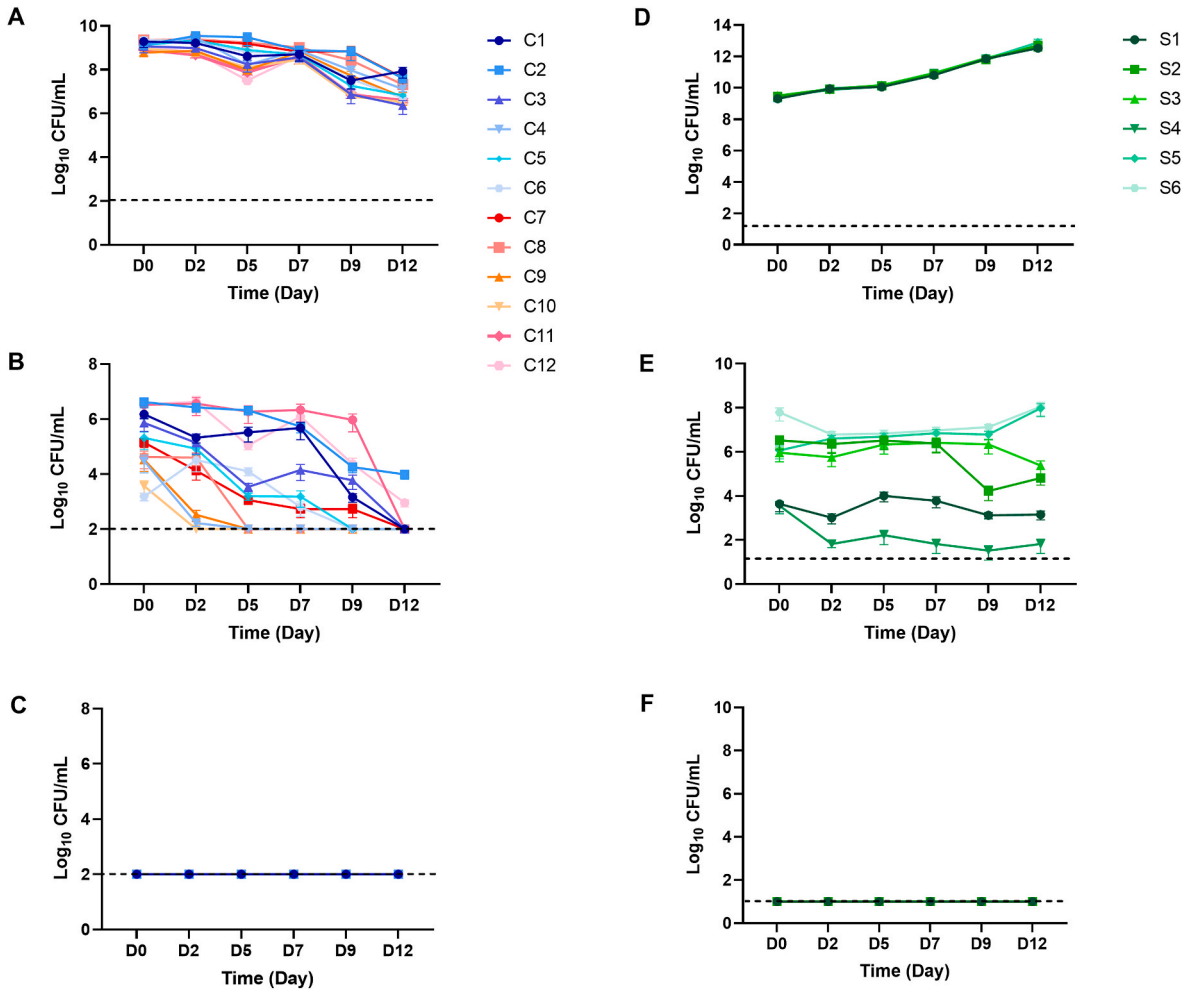


**Fig. 1.** Culturability of *C. jejuni* and *S.* Typhimurium. *C. jejuni* "high" invasive (C1–C6, blue shades), "low" invasive (C7–C12, red shades), and *S.* Typhimurium (S1–S6, green shades) isolates were exposed to either chlorine or ASC and inoculated into CMJ and stored at 5 °C. Culturability of *C. jejuni* untreated (A) chlorine treatment (B), ASC treatment (C), and *S.* Typhimurium untreated (D) chlorine (E), ASC (F) was tested. Data are presented as mean $\log_{10}$ transformed CFU/mL chicken meat juice ± the standard error of the mean.

Untreated *S.* Typhimurium exhibited consistent invasiveness over the course of the experiment (Fig. 3A). No significant difference was observed between the isolates. The percent recovery significantly declined ($p \leq 0.0001$) for all isolates from day 7 onwards (Fig. 3B). All the isolates were invasive over the remainder duration of the experiment following chlorine exposure (Fig. 3C), except the S4 lost invasive capacity from day 0. Interestingly, the percent recovery for chlorine treated S1, S2, S3, S5 was significantly higher ($p \leq 0.0001$) than the untreated group (Fig. 3D). Higher variability in percent recovery was observed in between the isolates after being exposed to chlorine. *S.* Typhimurium was not invasive after exposure to ASC in this experiment.

Bacteria that were not cultured by direct plating during the invasion assay were added to resuscitation media (Table 2). The resuscitation data of untreated groups are not shown as all bacteria were culturable following invasion.

Most of the non-culturable chlorine exposed *C. jejuni* isolates were resuscitated in Preston broth. At day 9 and 12, none of the *C. jejuni* isolates treated with chlorine prior to the invasion experiment could be resuscitated. At all-time points, no resuscitation was observed for *C. jejuni* isolates treated with ASC. All chlorine treated *S.* Typhimurium isolates were resuscitated at all time points. Although ASC treated *S.* Typhimurium isolates were not culturable by direct plating during the invasion experiment, some isolates were resuscitated.

### 3.3. Motility of *C. jejuni* and *S. Typhimurium* in chicken meat juice

*C. jejuni* and *S.* Typhimurium are flagellated bacterial species and motility plays an important role in host cell invasion. The motility of bacterial isolates was measured at day 0, 6, and 12. A significant reduction ($p \leq 0.0001$) in motility was observed over time for both the *C. jejuni* untreated (Fig. 4A) and chlorine treated groups (Fig. 4B). At day 0, *C. jejuni* isolates exposed to chlorine did not exhibit a significant difference compared to untreated bacteria. At day 6, however, a significant difference was detected between the two treatment groups ($p < 0.001$). "High" invasive *C. jejuni* isolates exhibited significantly ($p < 0.001$) greater motility than the "low" invasive group - except C7 and C8. All *C. jejuni* isolates were non-motile following exposure to ASC.

Motility of *S.* Typhimurium isolates was not affected by exposure to chlorine. No significant difference was detected between untreated (Fig. 4C) or chlorine treatment groups (Fig. 4D). *S.* Typhimurium treated with ASC did not exhibit motility at day 0 or day 6 but minimal motility was observed at day 12 for all the isolates (Fig. 4E).

### 3.4. Viability of *C. jejuni* and *S. Typhimurium* in chicken meat juice

To determine the viability of *C. jejuni* and *S.* Typhimurium during storage in CMJ at 5 °C, the intracellular ATP production was measured. In both the untreated and chlorine treated groups, ATP production of *C. jejuni* declined significantly ($p \leq 0.001$) over time (Fig. 5A). At day 0,

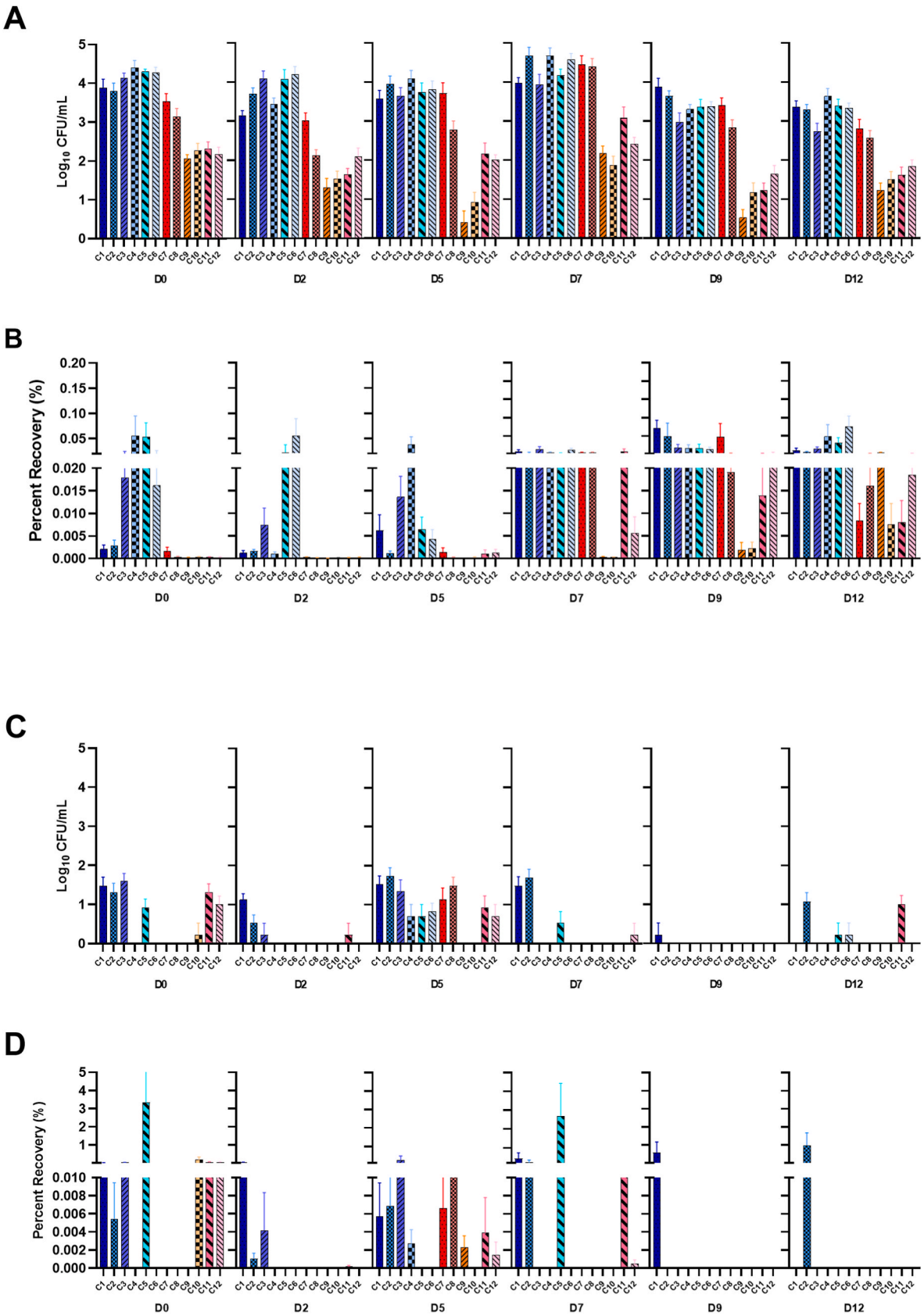


**Fig. 2.** Invasiveness of *C. jejuni* into Caco-2 cells. Six "high" (blue shades) and six "low" (red shades) invasive *C. jejuni* isolates were exposed to either chlorine or ASC and inoculated into CMJ and stored at 5 °C. The number of bacteria invaded into Caco-2 cells in untreated (A), chlorine treatment (C), and the percent recovery of *C. jejuni* in untreated (B), and chlorine treatment (D) were tested. Data are presented as mean $\log_{10}$ transformed CFU/mL cell lysate ± standard error of the mean. Percent recovery is presented as mean percentage ± standard error of the mean.

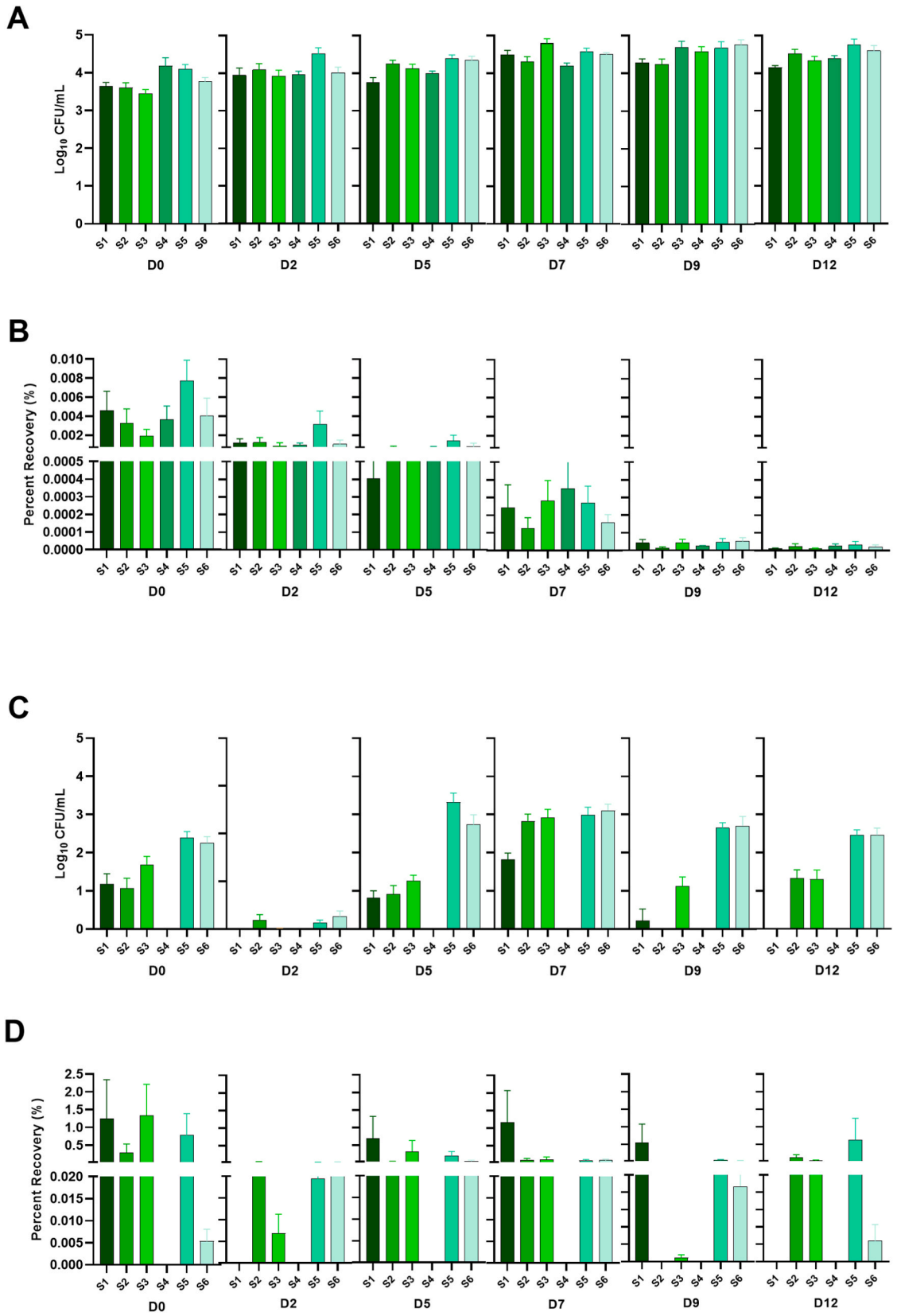


**Fig. 3.** Invasiveness of *S.* Typhimurium into Caco-2 cells. Six *S.* Typhimurium isolates were exposed to either chlorine or ASC and inoculated into CMJ and stored at 5 °C. The number of bacteria invaded into Caco-2 cells in untreated (A), chlorine treatment (C), and the percent recovery of *S.* Typhimurium in untreated (B), and chlorine treatment (D) are shown. Data are presented as mean $\log_{10}$ transformed CFU/mL cell lysate ± standard error of the mean. Percent recovery is presented as mean percentage ± standard error of the mean.

**Table 2**
Resuscitation of chlorine and ASC treated *C. jejuni* and S. Typhimurium invaded into Caco-2 cell lines.

| Timepoint (Days) | Chlorine | | ASC | |
|---|---|---|---|---|
| | Unculturable Isolates | Resuscitated Isolates | Unculturable Isolates | Resuscitated Isolates |
| *C. jejuni* (***n*** = **12**) | | | | |
| **0** | 5/12 | 5/5 | 12/12 | 0/12 |
| **2** | 8/12 | 4/8 | 12/12 | 0/12 |
| **5** | 2/12 | 1/2 | 12/12 | 0/12 |
| **7** | 8/12 | 4/8 | 12/12 | 0/12 |
| **9** | 10/12 | 0/10 | 12/12 | 0/12 |
| **12** | 8/12 | 0/8 | 12/12 | 0/12 |
| *S.*Typhimurium (***n*** = **6**) | | | | |
| **0** | 0/6 | 0/0 | 6/6 | 1/6 |
| **2** | 3/6 | 3/3 | 6/6 | 1/6 |
| **5** | 1/6 | 1/1 | 6/6 | 1/6 |
| **7** | 1/6 | 1/1 | 6/6 | 2/6 |
| **9** | 2/6 | 2/2 | 6/6 | 3/6 |
| **12** | 2/6 | 2/2 | 6/6 | 2/6 |

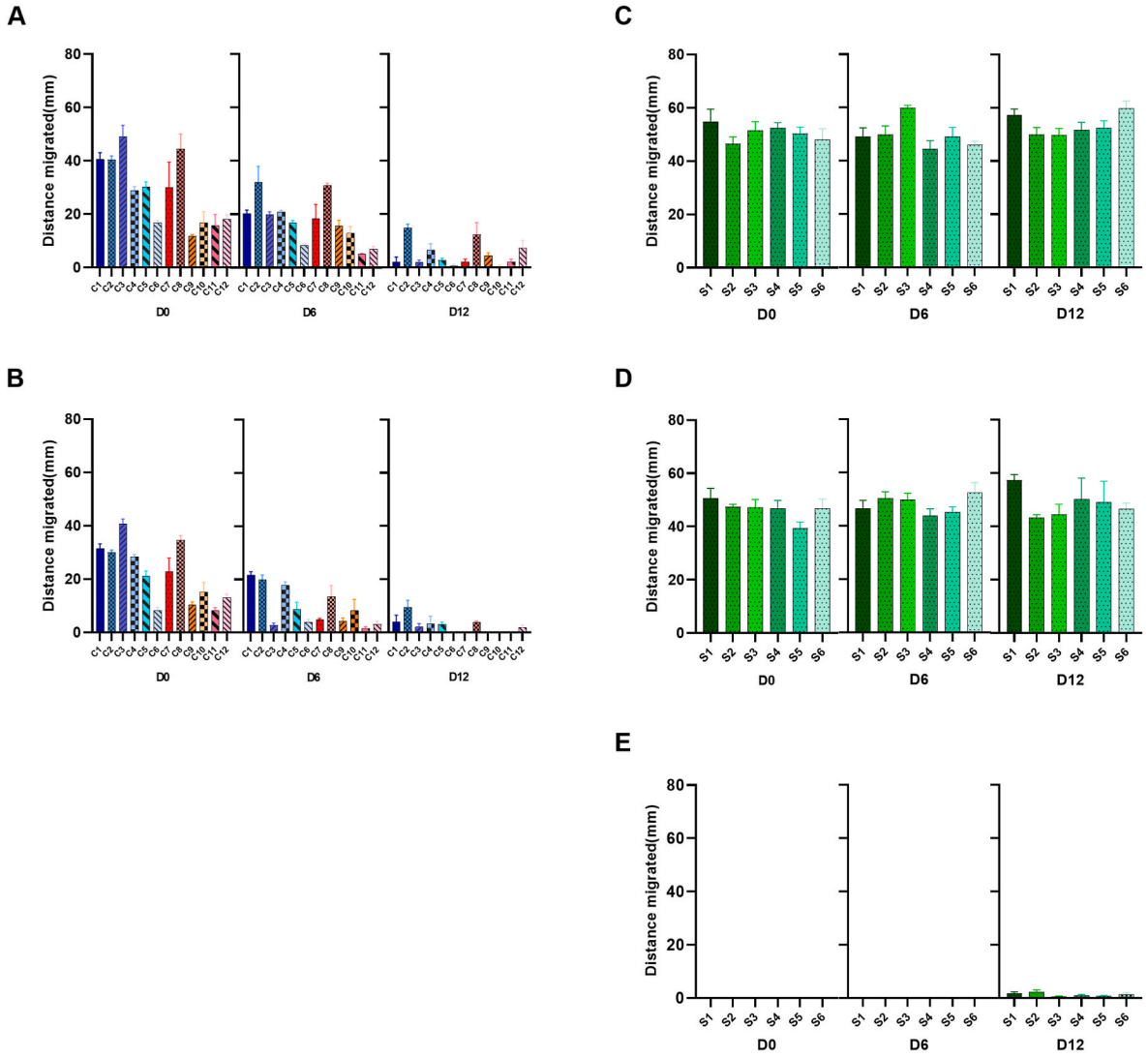


**Fig. 4.** Motility of *C. jejuni* and *S.* Typhimurium after exposure to chlorine and ASC during incubation in CMJ. Motility was characterized for untreated (A, C) as well as bacteria treated with either chlorine (B, D) or ASC (E). Six "high" (blue shades) and six "low" invasive (red shades) *C. jejuni* (A, B) and six *S.* Typhimurium (C, D, E) were included in these experiments. Data are presented as distance migrated (mm) ± the standard error of the mean.

ATP production of all isolates was significantly affected in exposure to either chlorine ($p < 0.05$) or ASC ($p \leq 0.0001$). Over time, the mean ATP production of the untreated group was not significantly different from the chlorine treatment. Treatment with ASC affected bacterial viability and ATP production in this group was significantly lower ($p \leq 0.001$) than either the untreated or chlorine groups. No significant difference

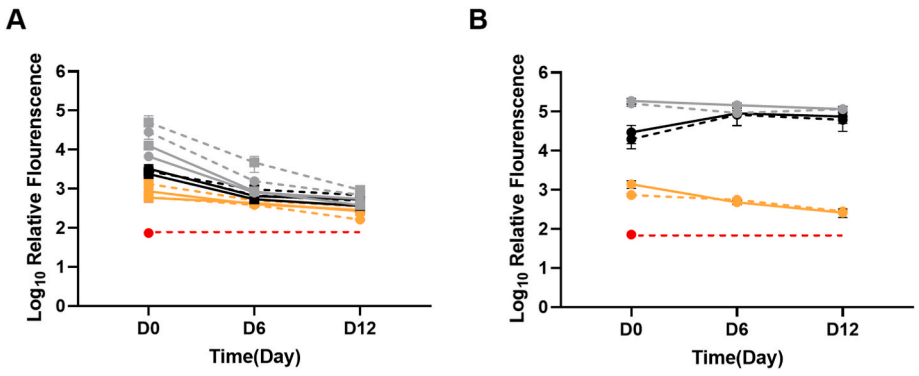


**Fig. 5.** ATP production of *C. jejuni* in exposure to chlorine and ASC. Intracellular ATP was determined for two *C. jejuni* "high" (solid line) and two "low" (hash line) invasive isolates (A) and two *S.* Typhimurium S1 (solid line) and S2 (hash line) (B). Bacteria were either untreated (grey), or exposed to chlorine (black) or ASC (orange). Heat treated (95 °C for 25 min) bacterial cultures were used as the "dead" control (red hash line). Data are presented as $\log_{10}$ relative fluorescence ± the standard error of the mean.

between the ATP levels of "dead" control and *C. jejuni* exposed to ASC at day12 was observed.

ATP concentration of untreated *S.* Typhimurium isolates was stable over the time (Fig. 5B). Interestingly, a sudden decline in ATP production was observed following chlorine exposure but recovered to levels similar to the untreated group. Following ASC treatment, ATP production was significantly lower ($p \leq 0.0001$) at all time-points compared to untreated and chlorine treated groups. ATP production by *S.* Typhimurium exposed to either chlorine ($p \leq 0.0001$) or ASC ($p \leq 0.01$) was significantly higher than the "dead" control throughout the experiment.

### 3.5. Gene expression of *C. jejuni* and *S. Typhimurium* in chicken meat juice

The expression of genes linked with bacterial stress response and cell invasion were investigated for both *C. jejuni* (Fig. 6) and *S.* Typhimurium (Fig. 7). Generally, the selected genes for the expression study in *C. jejuni* exposed to chlorine (Fig. 6A) were highly upregulated than for the ASC treatment group (Fig. 6B). The genes *rpoB* (stress response) and *flaG* (flagella) were significantly ($p \leq 0.001$) upregulated following exposure to chlorine at day 0, compared with oxidative stress (*flaA* or *sodB*) or virulence (*racR*) genes. Notably, *cadF* (virulence) was downregulated. Expression levels of all the genes were significantly higher ($p \leq 0.001$) at day 6 compared to day 0. Except for *flaA*, the C10 *C. jejuni* isolate exhibited gene upregulation by > 3 $\log_2$ fold change. Lower expression

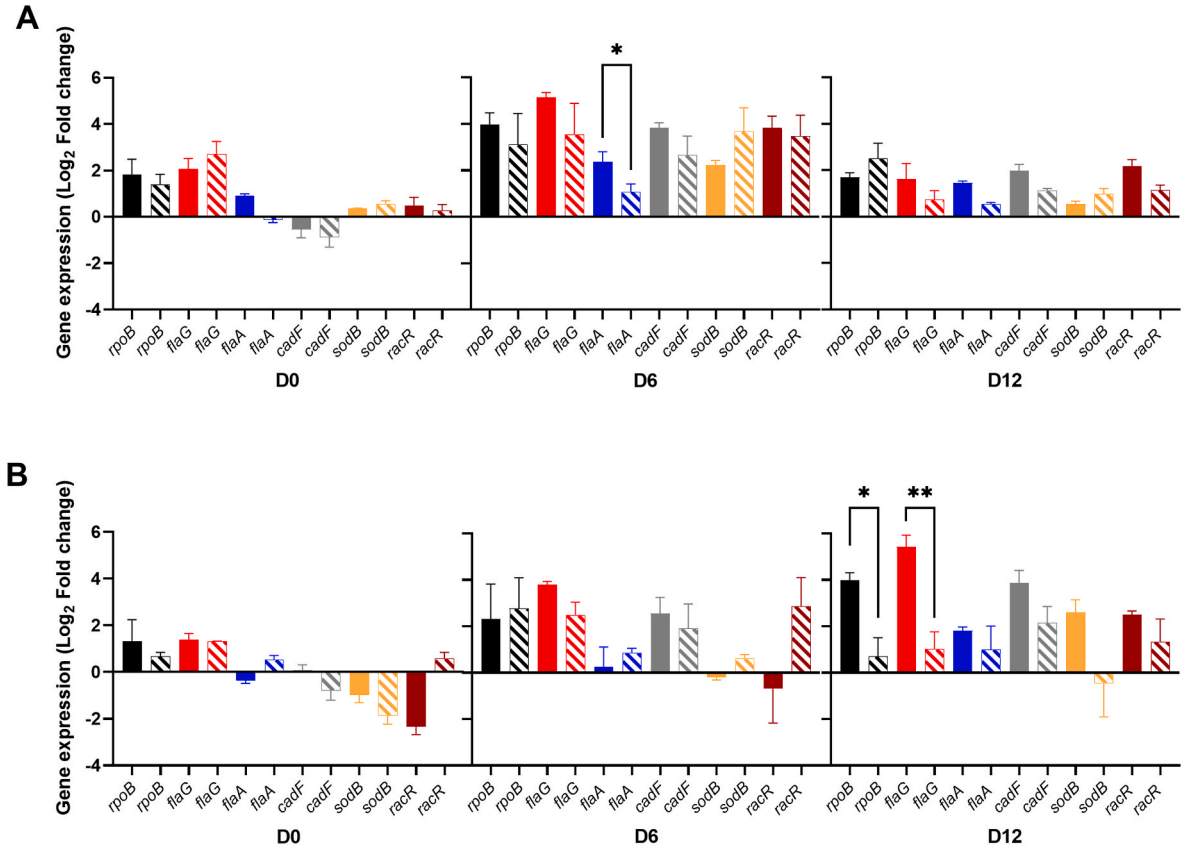


**Fig. 6.** Regulation of genes of *C. jejuni* in chicken meat juice. *C. jejuni* "high" invasive C5 (solid bar) and "low" invasive C10 (striped bar) isolates were exposed to either chlorine (A) or ASC (B) and inoculated into CMJ and stored at 5 °C. Gene expression of *rpoB*, *flaG*, *flaA*, *cadF*, *sodB*, and *racR* was determined by qRT-PCR. For assessing the regulation of genes of *C. jejuni* affected by chlorine and ASC, the untreated control group was used as a reference at each time point. The data are presented in $\log_2$ fold change ± SE. Asterisk * shows $P < 0.01$, while ** shows $P < 0.001$.

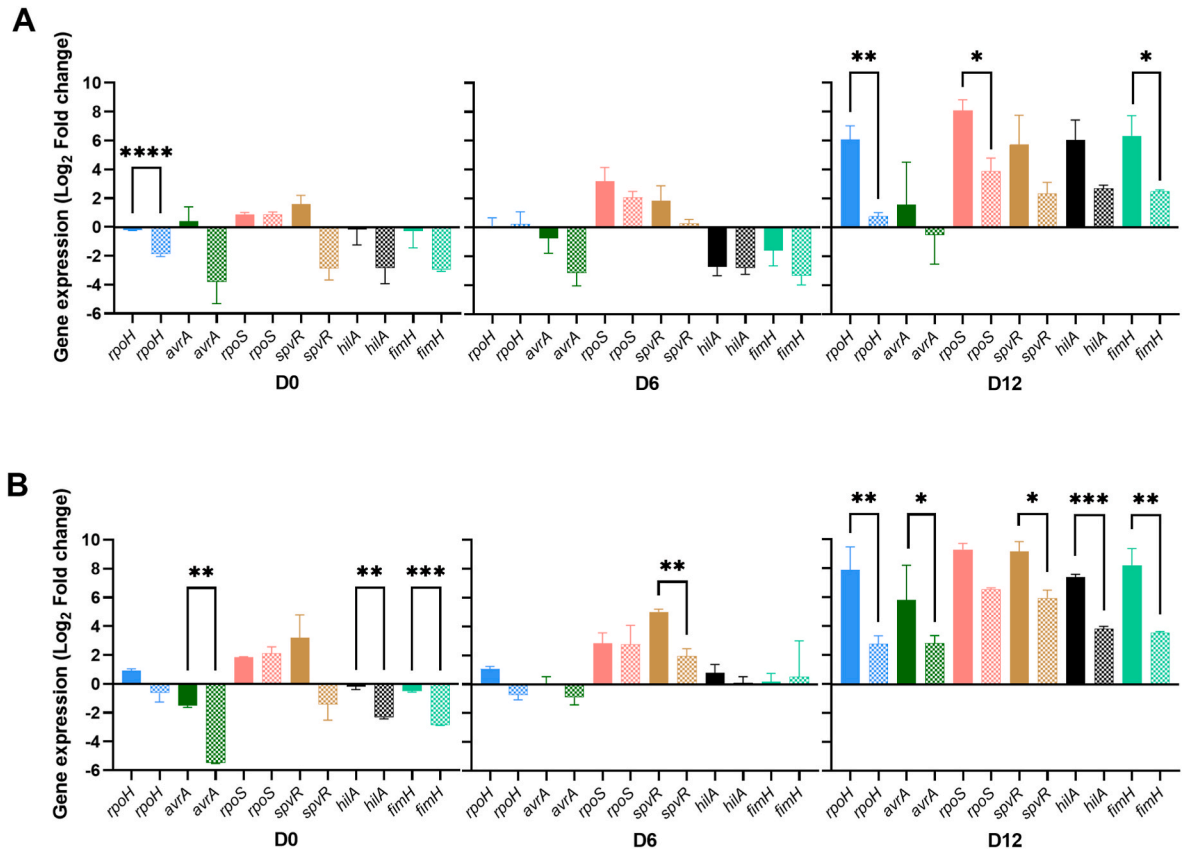


**Fig. 7.** Regulation of genes of *S*. Typhimurium in chicken meat juice. *S*. Typhimurium S1 (solid bar) and S2 (striped bar) isolates were exposed to either chlorine (A) or ASC (B) and inoculated into CMJ and stored at 5 °C. Gene expression of *rpoH*, *avrA*, *rpoS*, *spvR*, *hilA,* and *fimH* in *S*. Typhimurium exposed to chlorine (a) and ASC (b) was determined by qRT-PCR. For assessing the regulation of genes of *C. jejuni* affected by chlorine and ASC, the untreated control group was used as a reference at each time point. The data are presented in $\log_2$ fold change ± SE. Asterisk * shows $P < 0.01$, ** shows $P < 0.001$, and ***shows $P < 0.0001$.

levels were observed in almost all the genes of the "low" invasive C10 isolate compared to the "high" invasive C5.

In *C. jejuni* exposed to ASC, *rpoB* and *flaG* genes were highly upregulated in both the isolates, while *sodB* (C5, C10) and *racR* (C5) were highly downregulated at day 0. All the genes except *flaA* and *sodB* were significantly ($p \leq 0.001$) upregulated at day 6. In contrast to chlorine, the gene expression levels were significantly higher ($p \leq 0.001$) at day 12 compared to day 6, in the "high" invasive strain (C5). Interestingly, all the genes in high invasive C5 strain showed higher expression compared to "low" invasive C10 isolate.

Strain variation was also observed in gene expression between both *S*. Typhimurium isolates following exposure to either chlorine (Fig. 7A) or ASC (Fig. 7B). Generally, gene expression levels were significantly higher ($p < 0.01$) following exposure to ASC compared to chlorine. Following exposure to chlorine, except for *rpoS,* all the other genes *(rpoH, avrA*, *spvR, hilA,* and *fimH)* were significantly ($p < 0.001$) downregulated in S2 isolate, while slight upregulation was observed in *rpoS, avrA*, and *spvR* in S1 isolate at day 0. The expression levels of *rpoS* in either S1 or S2 were higher at day 6 compared to day 0. Although *rpoH* and *spvR* were downregulated at day 0 in S2, a slight upregulation was observed at day 6, all other genes (*avrA, hilA,* and *fimH)* were highly downregulated. Interestingly only *avrA* was downregulated in S1 at day 6, while all other genes were upregulated. Notably, all the genes were significantly upregulated ($p \leq 0.001$) in both S1and S2 at day12 compared to day 6 except *spvR* in S2, which was downregulated.

Gene expression profile of S2 in exposure to ASC was very similar to chlorine treated group at day 0 except the slightly higher expression levels in ASC treated group. The genes *rpoH*, *rpoS,* and *spvR* were upregulated, while *avrA, hilA,* and *fimH* were downregulated in S1 at day 0. At day 6, all the studied genes were upregulated in both S1 and S2, except, *rpoH* and *avrA* of S2, which were downregulated. Interestingly, all the genes in either S1 or S2 isolates were significantly ($p \leq 0.001$) upregulated at day 12 compared to day 0 and 6.

## 4. Discussion

Chemical decontamination is commonly used to control foodborne pathogens during commercial processing. Post-processing, poultry products are typically held under refrigeration to minimize bacterial growth and survival. Prolonged survival of *Campylobacter* and *Salmonella* in cold storage conditions in food matrix has previously been reported (Birk et al., 2004). In this study, isolates of *C. jejuni* and *S*. Typhimurium were treated with either chlorine or ASC and stored in chicken meat juice at refrigeration temperature. Untreated *C. jejuni* and *S*. Typhimurium were also stored in CMJ under the same conditions. Untreated *C. jejuni* exhibited a decline in culturability, but this is likely linked with cold stress experienced by bacteria under refrigerator conditions and is consistent with previous reports (Birk et al., 2004; Karki et al., 2019). Untreated *S*. Typhimurium, however, exhibited slow growth in chicken meat juice and is consistent with previous research (Guillén et al., 2021).

Following the chlorine exposure, a significant reduction in *C. jejuni* culturability was observed but this was strain dependent and is consistent with a previous report (Blaser et al., 1986). Sub-lethally injured *C. jejuni* can persist in a favourable environment, such as chicken meat juice, which provides a nutritious and favourable environment for bacteria during survival at low temperatures (Hazeleger et al., 1995). Following chlorine exposure, a significant reduction in culturability was observed for the *S*. Typhimurium isolates. As with *C. jejuni*, a difference in sensitivity between *S*. Typhimurium isolates was observed. Genetic

differences between strains may lead to differences in their ability to respond to stress (Guillen et al., 2020). No culturable *C. jejuni* or *S.* Typhimurium were observed following ASC exposure suggesting that it has a greater biocidal activity than chlorine. The bactericidal activity of ASC (Sexton et al., 2007) and the lethal damage caused to *C. jejuni* has been described earlier (Weerasooriya et al., 2021).

The invasive capacity of *C. jejuni* and *S.* Typhimurium stored in chicken meat juice was investigated using the human intestinal epithelial cell line, Caco-2. Although the total bacterial load decreased over time in the untreated group, *C. jejuni* invasiveness remained consistent throughout the experiment. The strain dependent invasiveness of *C. jejuni* could be due to the genetic variation in attachment and invasion (Koolman et al., 2016). Untreated *S.* Typhimurium isolates exhibited consistent invasive capacity over the course of the experiment. Following chlorine treatment, the *C. jejuni* isolates exhibited decreased invasive capacity. Interestingly, the percent recovery of some isolates in the chlorine treated group were higher than in the untreated group. This could be due to the upregulation of flagella gene expression as a response to oxidative stress. Following chlorine exposure, a significant strain variation in invasiveness between *C. jejuni* isolates was observed. This could be due to the strain dependent stress responses of sub-lethally injured cells. Most of the invasive isolates lost their invasiveness between days 9 and 12, which could be linked with reduced numbers of culturable bacteria. The invasiveness of the *S.* Typhimurium isolates was also reduced following exposure to chlorine. This could be due to the genetic variation of stress response mechanism regarding virulence (Wang et al., 2010). Chlorine treated *C. jejuni* isolates that were not invasive into Caco-2 cells could be resuscitated by further incubation in nutrient media. Following exposure to ASC, none of the *C. jejuni* was either invasive or is resuscitated.

Resuscitation was conducted to recover *S.* Typhimurium not detected by direct plating. All the chlorine treated *S.* Typhimurium invaded into Caco-2 cells were resuscitated. This suggests that sub lethally injured *C. jejuni* and *S.* Typhimurium isolates, following the exposure to chlorine during the processing may retain the invasive ability if the right enrichment conditions are provided. An *in-vivo* experiments are required to test the virulence and pathogenicity of sub-lethally injured foodborne pathogens. Similar to *C. jejuni*, *S.* Typhimurium treated with ASC was not invasive. The oxidative capacity and acidity of ASC likely cause significant damage to *S.* Typhimurium. Reduced invasiveness into Caco-2 cells of *S.* Typhimurium exposed to acidic stress has been reported earlier (McWhorter et al., 2021).

Motility contributes to virulence by enabling bacteria to come into proximity to host intestinal epithelial cells, facilitating invasion (Saini et al., 2011). We observed a significant reduction ($p < 0.001$) in all *C. jejuni* isolates motility over time. Not surprisingly, the "high" invasive *C. jejuni* strains were more motile compared with "low" invasive isolates (except isolates C7 and C8). Following exposure to chlorine, *C. jejuni* isolates exhibited reduced motility that is likely a consequence of morphological changes and loss of flagella (Muhandiramlage et al., 2020). After exposure to ASC, *C. jejuni* did not show motility in the present experiment. This could be due to the reduced flagella activity in oxidative damage induced in exposure to ASC (Weerasooriya et al., 2021).

Neither untreated nor chlorine treated *S.* Typhimurium exhibited a reduction in motility during storage in CMJ. This indicates that chlorine exposure has a limited effect on *S.* Typhimurium motility. Higher motility observed for *S.* Typhimurium compared with *C. jejuni* could be due to the flagellar genetic variation as well as differences in bacterial responses to stress (C. Murphy et al., 2006). Motility was not observed at day 0 or 6 in ASC treated *S.* Typhimurium. This could be due to the effect of oxidative stress and the acidic stress caused by ASC. At low pH, the *Salmonella* PhoPQ signal transduction system negatively regulates the expression of flagella resulting in reduced bacterial motility (Adams et al., 2001). Interestingly *S.* Typhimurium isolates regained motility at day 12, suggesting that they could recover from the stress caused by ASC exposure.

Intercellular ATP was measured to determine the cell viability of chemically treated *C. jejuni* and *S.* Typhimurium. Untreated *C. jejuni*, exhibited a significant reduction of ATP that could be a consequence of a cold temperature, induced metabolic shift. *C. jejuni* exhibited a significant depletion of intracellular ATP after exposure to either chlorine or ASC that further reduced during storage. A significantly lower ($p < 0.001$) amount of ATP was detected in the *C. jejuni* isolates following ASC exposure. This could be a result of its higher oxidative capacity. Cellular respiration and vital cell metabolic pathways in bacteria are inhibited due to high oxidative stress (Weerasooriya et al., 2021). *S.* Typhimurium isolates did not exhibit a decline in ATP over time in either the untreated or chlorine treatment groups. This is consistent with a previous study showing that ATP production was observed for *S.* Typhimurium even after the chlorine treatment (Hazeleger et al., 1999). Following exposure to ASC, however, ATP was significantly reduced. This finding is consistent with the results obtained for bacterial survivability and motility data.

Both *Campylobacter* and *Salmonella* possess stress response mechanisms that facilitate their persistence in an environment. In the present study, *C. jejuni* upregulated the stress response gene, *rpoB* and, genes encoding the function of flagella and motility, *flaG,* and *flaA* following exposure to chlorine and ASC. Following exposure to chlorine, genes encoding proteins related to cell invasion motility and virulence (*flaG, flaA, cadF, racR)* were upregulated. This upregulation increased with the length of storage in chicken meat juice following exposure to chlorine. Induced expression of the oxidative stress response gene, *sodB,* and flagella genes in *C. jejuni* following exposure to chlorine has been reported earlier (Weerasooriya et al., 2021). Interestingly, most of the genes were downregulated following exposure to ASC at day 0 and the upregulation of genes at day 6 was lower compared to exposure to chlorine. This could be due to the higher oxidative damage caused by ASC in *C. jejuni*. Interestingly, the stress response (*rpoB*, *sodB*) and virulence genes (*cadF*, *racR*, *flaG*, *flaA*) were significantly upregulated at day 6 and 12 during storage in chicken meat juice. Although ASC induces significant oxidative damage (Weerasooriya et al., 2021), a proportion of bacteria from the population could have remained as sub-lethally injured and prolonged incubation in chicken meat juice may support the recovery of *C. jejuni* but this requires further investigation. Increases expression of genes involved in quorum sensing and o-linked flagellin glycosylation in *C. jejuni* exposed to chicken meat juice has been demonstrated earlier (Ligowska et al., 2011). Higher gene expression in *C. jejuni* exposed ASC, could be due to enhanced expression of virulence determinants as well as bacterial responses to oxidative, cold-shock, and acid stress responses (Caroline Murphy et al., 2003).

In *S.* Typhimurium, most of the genes were downregulated following chlorine or ASC exposure. Following exposure to chlorine, downregulation of flagellin biosynthesis and assembly genes, as well as virulence genes, have been previously described (Wang et al., 2010). The stress response genes, *rpoS,* and *rpoH*, however, were upregulated after chlorine treatment and could be linked with the oxidative stress response of *S.* Typhimurium in presence of CMJ. Interestingly, stress response (*rpoS* and *rpoH),* flagella and flagellin activity (*hilA, FimH*), and virulence (*spvR, avrA*) genes were significantly upregulated at day12. The finding of the present study indicated the virulence potential of sub-lethally injured *S.* Typhimurium following exposure to chlorine. Following ASC treatment, *S.* Typhimurium cell invasion and virulence genes (*hilA, FimH, spvR, avrA*) were downregulated immediately but all the genes were significantly upregulated over time. Preparation of ASC requires acidification to pH 2.5. The combined oxidative and acid stress, induced in *S.* Typhimurium may be linked with this observation. Exposure to an acidic environment activates the acid tolerance response in *Salmonella* and acid adapted cells are resistant to a variety of other environmental stresses (Alvarez-Ordonez et al., 2011). Additionally, the general stress response mechanism induced in *Salmonella* activates cross-protection to other various stress conditions (Wesche et al., 2009).

Furthermore, *rpoS and rpoH* are sigma factors whose expression is induced to promote survival under extra cytoplasmic stress (Fang et al., 1992). This general stress response mechanism might enable *S*. Typhimurium to recover and maintain virulence during storage.

## 5. Conclusions

We have demonstrated that *C. jejuni* and *S*. Typhimurium in refrigerated chicken meat juice remain culturable and retain virulence capacity. Treatment with disinfecting agents used in the food industry, however, can induce injury and stress that can affect the invasion and virulence potential of foodborne pathogens during prolonged storage. The present study demonstrated that ASC had greater bactericidal effects than chlorine which if implemented in processing plants could lead to reduced contamination of the downstream food supply. The observed increase in expression of the invasion gene suggests that *S*. Typhimurium might be a greater human health risk than *C. jejuni* during long term storage. Further *in-vivo* studies are needed to establish the pathogenicity for sub-lethally stressed bacteria in a host. Reduced chicken meat juice while packaging, and increased public awareness of cross contamination in the kitchen might help to reduce the public health risk, related to C. *jejuni* and *S*. Typhimurium in the chicken meat.

## Declaration of competing interest

The authors declare that they have no competing interests.

## CRediT authorship contribution statement

**Gayani Weerasooriya:** Investigation, Data curation, Software, Writing – original draft. **Samiullah Khan:** Conceptualization, Project administration, Resources, Software, Supervision, Writing – review & editing, Authorship. **Kapil K. Chousalkar:** Conceptualization, Funding acquisition, Project administration, Resources, Software, Supervision, Writing – review & editing, Authorship. **Andrea R. McWhorter:** Conceptualization, Funding acquisition, Project administration, Resources, Software, Supervision, Writing – review & editing, Authorship.

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