Chlorine Resistance in Salmonella Typhimurium and Campylobacter jejuni Isolated from Poultry Processing Line

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Article

Chlorine Resistance in Salmonella Typhimurium and *Campylobacter jejuni* Isolated from Poultry Processing Line

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Abstract: Salmonella Typhimurium and *Campylobcater jejuni* are major food-borne pathogens that continue to persist in the poultry processing industry and cause health and economic burdens. Chlorine is commonly used to mitigate bacterial contamination in poultry processing. However, inducing of adaptive stress response mechanism in sub-lethal exposure limits the effectiveness of chlorine. In the present study, we determined the effect of pre-exposure to chlorine on chlorine tolerance in S. Typhimurium and *C. jejuni*. MIC and MBC were determined in caecal, neck skin, post–chill carcass washing, and the environmental isolates. The carcass washings were exposed to either 3-5 ppm, 20-30 ppm, or 40- 50 ppm of chlorine in the chill tank and MICs and MBCs were significantly higher ($p<0.05$). Notably, 60% of *C. jejuni* isolated from the carcasses in a 20-30 ppm chill tank showed the highest MBC of 160 ppm. Chlorine-resistant percentage in *S.* Typhimurium and *C. jejuni* of the carcass was 78.8% and 83% respectively, while 94.1% and 80% of resistance were detected in 20-30 ppm chill tank isolates. The highest resuscitation detected carcass washing isolates exhibited the sub-lethal injury. Chlorine resistance limits the space to work with concentration adjustments. Therefore moving to alternative chemical and novel multi-huddle interventions is crucial.

Keywords: *S.* Typhimurium ; *C. jejuni*; chlorine; resistance; MIC; MBC; resuscitation

1. Introduction

Foodborne infections are the main public health problem that remains a common cause of illness and death, worldwide. The public health impacts due to foodborne pathogens such as Salmonella and *Campylobacter* are enormous in addition to significant social and economic burdens [1]. According to the European Union One Health 2021 Zoonoses Report *Campylobacter* and Salmonella as the first and second most reported zoonoses in humans, accounting for >65 % and >29% of confirmed human cases respectively [2]. Globally, it is estimated that 93.8 million cases are caused by non-typhoidal Salmonella every year [3], While around 96 million bacterial gastroenteritis cases each year are caused by *Campylobacter* [4,5]. These two foodborne pathogens are colonized in the chicken gut and contaminate the poultry carcass during processing, especially in evisceration. The likelihood of an outbreak occurring is estimated to be 12% in slaughtering and 33.5 % in processing for Salmonella [6] and is estimated that chickens are responsible for up to 30% of human Campylobacteriosis cases [7]. Therefore, safe production and distribution of food across the farm-to-fork continuum are crucial to ensure that consumers receive wholesome food. As the poultry-processing environment is in optimal conditions to host the bacteria's growth, the persistence of Salmonella and *Campylobacter* throughout the processing line is significant. During processing, chicken carcasses get contaminated with bacteria from feathers, skin, and ruptured intestinal tracts [8]. Cross-contamination of carcasses can also occur during the different stages of processing such as chilling, portioning and packing [8,9]. Furthermore, the cross-contamination of the kitchen utensils and bench tops during cooking and, the refrigerator in storing is also significant [10,11]. While the farm interventions applied to prevent and reduce colonization of these pathogens in poultry are

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desirable, none have upheld adequate to eliminate the food-borne pathogens from poultry products [12,13]. Therefore, processing interventions such as chilling and post-chill dip are critical to reducing the contamination of poultry products before reaching the customer. USDA Food Safety and Inspection Service (FSIS) implemented a procedure and pathogen reduction performance standards; hazard analysis and critical control point (HACCP) to mitigate the contamination of food-borne pathogens in poultry meat [14]. This regulation resulted in additional water use by poultry processors to eliminate carcass contamination by adding inside/ outside carcass washers, sprays, chilling carcasses to 4 °C and, antimicrobial chemical decontamination [15]. USDA-FSIS has permitted of using antimicrobials in poultry production, such as chlorine, sodium hypochlorite (SH) acidified sodium chlorite (ASC), and peracetic acid (PAA) are referred to as GRAS (Generally Recognized as Safe. Due to the cost-effectiveness, easy availability, and easy application, chlorine has become the most common sanitizer used in carcass washing. There, immersion chilling in chlorinated water is the most common method of application [9]. Further, a previous study reported that 73% of processing plants used chlorine or a combination of chlorine and another antimicrobial to reduce the bacterial load on the carcasses during processing [15]. Chlorine is an oxidizing agent that has been shown to reduce the membrane permeability in both Gram-negative and Gram-positive bacterial species [16]. Although the European Union has banned the use of chlorine, the USA, Australia, and most of the Asian countries still use chlorine in the chill tank [17–19]. Due to the constant addition of organic material into the chill tank, chemical decontamination with chlorine represents a significant challenge. The efficacy of chlorine, however, is dependent on organic load and total bacterial load [20,21]. Increasing concentrations of organic matter content and contact time reduces the total free chlorine availability, which reduces the effectiveness of chlorine in pathogen reduction. Therefore chlorine tolerance levels of Salmonella and *Campylobacter* can be higher than the current chlorine concentrations used in the chill tank to reduce the bacterial load [21,22].The USDA permits the use of sodium hypochlorite at 50 ppm of free or available chlorine on chicken carcasses and 200 ppm of free chlorine on food-contact surfaces during sanitation [20]. The ability of resistance development against the biocidal also plays a major role in the persistence of foodborne pathogens in the food chain [23]. Salmonella and *Campylobacter* have shown the ability to survive and persist in poultry processing environments [24]. This can be credited to its microbial ability to adapt and develop/acquire tolerance and/or resistance to different antimicrobial agents including oxidizers such as chlorine [22,25]. Therefore, pathogens like *Campylobacter jejuni*, *Listeria monocytogenes*, and Salmonella enterica could adapt to diverse processing environment-related stressors, influencing their growth, survival, and persistence in the poultry processing line [26,27]. Notably, exposure to sublethal doses of chlorine can cause sub-lethal injury and can be resuscitated after removing the stress condition and providing an enriched environment [21]. This could be a continuous problem even the processors altered the chlorine concentrations in the chill tank to cope up the situation. Therefore, it is very crucial to determine the effective dosage of chlorine in reducing bacterial load and their resistance profiles to chlorine to optimize the current decontamination protocols.

A recent prevalence study demonstrated that the contamination rates of *Campylobacter* and Salmonella on raw post-slaughter meat is 80.66% and 68.66 %, respectively, in Sri Lanka [28]. As in other Asian countries, 90% of the poultry processing plants use chlorine in spin chill tanks. Due to the unavailability of a standard protocol or guideline, three different chlorine concentrations are used; 3-5 ppm, 20-30 ppm, and 40-50 ppm, in the processing plants in Sri Lanka [28]. Further during pre-chill carcass washing usually 2-3 ppm of chlorine is used in inside – outside carcass washers. Therefore foodborne pathogens such as Salmonella and *Campylobacter* are exposed to different chlorine concentrations during processing. Studies conducted to explore the resistant profiles of food-borne pathogens for biocidal are limited. The present study was designed to understand the chlorine tolerance level of Salmonella Typhimurium and *Campylobacter jejuni* for chlorine during chemical decontamination in the chill tank. Further, we determined the effect of previous exposure to different chlorine concentrations on chlorine resistance in Salmonella Typhimurium and *Campylobacter jejuni* isolated from poultry processing line.

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2. Materials and Methods

2.1. Preparation of Campylobacter and Salmonella Inoculums

Salmonella Typhimurium and *Campylobacter jejuni* isolates, isolated from poultry processing plants in a previous study were used in the present study [28]. These food-borne pathogens were isolated from different stages of the processing line. The environmental pooled samples were collected from the unloading area of the processing plant. Caeca and neck skin samples were collected from the carcasses after going through inside/outside carcass washers (2-3 ppm of chlorine in potable water). Post-chill carcass washers were collected after being exposed to chlorine in the chill tank. Here, the chlorine concentrations used by the poultry processing plants in their chill tanks were 3-5 ppm, 20-30 ppm, and 30-50 ppm (Table 1). The chlorine contact time in the chill tank was similar in all the processing plants, where samples were collected. S. Typhimurium and *C. jejuni* isolates were stored at -80 in Brilliant Heart Infusion Broth with 50 % glycerol. In preparation for MIC/MBC experiments, S. Typhimurium was subcultured into Columbia sheep blood agar (SBA) and incubated at 37°C for 24 h, while *C. jejuni* was subcultured onto Columbia sheep blood agar (SBA) and incubated at 42°C in 10% CO_2 for 48 h.

Table 1. Sampling points and isolation number of S.Typhimurium and *C. jejuni.*

Sample point	**Isolation number (*n*)**	
	S.Typhimurium	*C. jejuni*
Ceaca	16	11
Neck skin	12	15
Carcass washing (3-5 ppm)	15	13
Carcass washing (20-30 ppm)	17	25
Carcass washing (3-5 ppm)	29	15
Environmental samples	22	17
Total isolate number	**111**	**96**

2.2. Minimum Inhibitory Concentrations (MIC) Determination to Chlorine

To determine the bacteriostatic effect of chlorine Minimum inhibitory concentrations (MIC) test was performed. S.Typhimurium (*n*=111) confirmed by serotyping and *C . jejuni* (*n*=96) confirmed by qPCR used in MIC and MBC assays [28]. The broth micro-dilution method of MIC determination was done according to the CLSI standards [29]. Chlorine (10% Sodium hypochlorite, SIGMA-ALORICH, USA) concentrations were prepared to range from 2.5 ppm to 160 ppm in Nutrient Broth No. 2 (NB2-Oxoid, UK) considering the WHO recommended industrial standards for the chicken meat industry. Chlorine levels were measured using a digital chlorine meter (LOVIBOND, Germany). Ninety micro-liters (90 µL) of chlorine was added into round bottom 96-well microtiter plates S. Typhimurium and *C. jejuni* inoculums were prepared in sterile distilled water to obtain 10^8 CFU/mL using 0.5 McFarland Standard. Subsequently 10 µL of the prepared inoculum was added into the wells with 90 µL of chlorine dilutions to obtain 10^7 CFU/mL The NB2 without chlorine was considered as the positive control (growth control) at the 11^{th} well and the NB2 without either chlorine or bacterial inoculum was the negative control (sterility control) at the 12^{th} well in the test. All the isolates and the negative and positive controls were tested in duplicates. S. Typhimurium plates were incubated at 37 °C for 20 h while *Campylobacter jejuni* plates were incubated at 42°C in 10% CO_2 for 20 h. The lowest concentration, which does not give visible bacterial growth in 96 well plates, was defined as the MIC value. Salmonella Typhimurium ATCC 133111 and *Campylobacter jejuni* ATCC 33291 strains was used as a control strain.

2.3. Minimum Bactericidal Concentrations (MBC) Determinations to Chlorine

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Minimum bactericidal concentration (MBC) is the lowest concentration of antibiotics that kills 99.9% of the inoculum. The MBC of S.Typhimurium and *C. jejuni* were conducted to determine the bactericidal effect of chlorine in the MIC assay. Subsequently, 10µl of broth from each growth inhibited well in the MIC plates was drop plated on sheep blood agar. S.Typhimurium plates were incubated at 37 °C for 24 h, and *C . jejuni* plates were incubated at 42°C in 10% CO_2 for 48 h. The MBC was determined as the lowest concentration of chlorine required to reduce viable cell numbers below the detection limit. The resistance of S.Typhimurium and *C . jejuni* to chlorine considered the MIC and MBC values higher than the highest concentration used in the chill tanks in the processing plants in Sri Lanka of 40 ppm.

2.4. Resuscitation Assay

Resuscitation assay was conducted to determine the bacterial recovery after the growth inhibition in MIC/MBC assay [21]. While doing the MBC assay 50 µL of bacterial growth-inhibited wells up to 160 ppm were inoculated into 150 µL of enriched broth for S.Typhimurium and *C. jejuni* resuscitation. S.Typhimurium inhibited wells were inoculated into Nutrient broth and incubated at 37°C for 24 h and for *C. jejuni*, inoculated into Preston broth (nutrient broth number 2 with *Campylobacter* selective supplement) (Oxoid, UK) and incubated at 42°C in 10% CO_2 for 24 h. After incubation, 10 µL was drop-plated onto SBA to determine the recovery of *S.*Typhimurium and *C. jejuni* isolated from different sample types from exposure to chlorine.

2.5. Statistical Analysis

The MIC /MBC data were presented as percentages. The prevalence data were analyzed using Fisher's exact test. To determine the significant differences in the effect of chlorine on S.Typhimurium and *C. jejuni* along with the previous exposure of chlorine in different steps in the processing line was analysed by One-way Analysis of Variance (ANOVA) and Two-way ANOVA followed by Turkey's multiple comparison tests by. All statistical analyses were performed using GraphPad Prism Version 8 (GraphPad Software, Inc., United States). In all cases, a P-value of < 0.05 was considered statistically significant.

3. Results

3.1. Salmonella Typhimurium and Campylobacter Jejuni Susceptibility to Chlorine: MIC and MBC Determination

The results were analyzed to understand the chlorine tolerance levels of S.Typhimurium (Table 2) and C. jejuni (Table 3) isolated from different steps in the processing line.

Table 2. Percentage of Salmonella Typhimurium isolates at different MIC and MBC levels.

Chorine Concentrations (ppm)	Minimum inhibitory concentration (MIC) Isolate percentage (%)				Minimum bactericidal concentration (MBC) Isolate percentage (%)			
	Carcass wash	Neck skin	Caeca	Environment	Carcass wash	Neck skin	Caeca	Environment
2.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5	0.0	0.0	0.0	11.7	0.0	0.0	0	4.5
10	1.6	0.0	0.0	11.7	0.0	0.0	0	4.5
20	3.3	0	6.2	35.2	3.2	11.2	6.25	36.3
40	29.5	62.5	50.0	41.1	18.0	44.4	56.25	54.5

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80	65.6	37.5	43.8	0.0	68.9	44.4	37.5	0.0
160	0.0	0	0.0	0.0	3.3	0	0	0.0
>160	0.0	0	0.0	0.0	6.6	0	0	0.0

The majority of S.Typhimurium (65.6%) isolated from carcass washing exhibited MIC at 80 ppm and it was significant ($p<0.05$). And, 68.95% of isolates exhibited MBC at 80 ppm for chlorine. Notably, 9.9% of whole carcass S.Typhimurium required ≥160 of chlorine to have bactericidal effect. When considered the MIC and MBC values of S.Typhimurium , isolated from neck skin samples, 62.5% of isolates showed 40 ppm of MIC level and it was significant ($p<0.05$). The MBC at 40 ppm and 80 ppm was observed in 44.4% of the population. S.Typhimurium isolated from the environmental samples exhibited similar MIC and MBC values for chlorine, while the highest proportion was exhibited at 40 ppm and it was 41.1% and 54.5% respectively. When we compared the MIC values of S.Typhimurium, the lowest MIC values were detected in the isolates from the environmental samples.

Table 3. Percentage of *Campylobacter jejuni isolates* at different MIC and MBC levels.

Chorine Concentrations (ppm)	**Minimum inhibitory concentration (MIC)**				**Minimum bactericidal concentration (MBC)**			
	Isolate percentage (%)				**Isolate percentage (%)**			
	Carcass wash	Neck skin	Caeca	Environment	Carcass wash	Neck skin	Caeca	Environment
2.5	0.0	0	0.0	0.0	0.0	0.0	0.0	0.0
5	0.0	5.3	9.0	11.7	0.0	0.0	0.0	11.7
10	5.7	21.1	45.5	23.5	0.0	5.3	18.2	11.7
20	32.1	36.8	45.5	23.5	0.0	42.1	27.3	35.2
40	52.8	36.8	0.0	41.1	17.0	42.1	54.5	41.1
80	9.4	0.0	0.0	0.0	34.0	10.5	0.0	0.0
160	0.0	0.0	0.0	0.0	39.6	0.0	0.0	0.0
>160	0.0	0.0	0.0	0.0	9.4	0.0	0.0	0.0

In *C . jejuni* In *C. jejuni*, the highest percentage (52,8%) of the population from whole carcass washing exhibited MIC at 40 ppm , while the MBC was 160 ppm in 39.6% of the population. Further, in whole carcass *C. jejuni* detected higher MBC at ≥160 ppm in the 49% of the population. Interestingly, there was a significant ($p<0.01$) difference, in between the MIC and MBC values of *C. jejuni* isolated from the carcass washings.

There 36.6% of *C. jejuni* isolated from neck skin exhibited 20 ppm and 40 ppm of MIC values, while a similar MBC pattern was observed with 42.1% in either 20 or 40 ppm MBC. A similar percentage of *C. jejuni* (41.1%) isolated from the environment exhibited 40 ppm of MIC for chlorine.

3.2. Chlorine Resistance Profiles of S. Typhimurium and C. jejuni

Considering the highest chlorine concentration (40 ppm) used in the chill tank as a cut-off point, the chlorine resistant of S.Typhimurium and *C . jejuni* was analysed (Figure 1).

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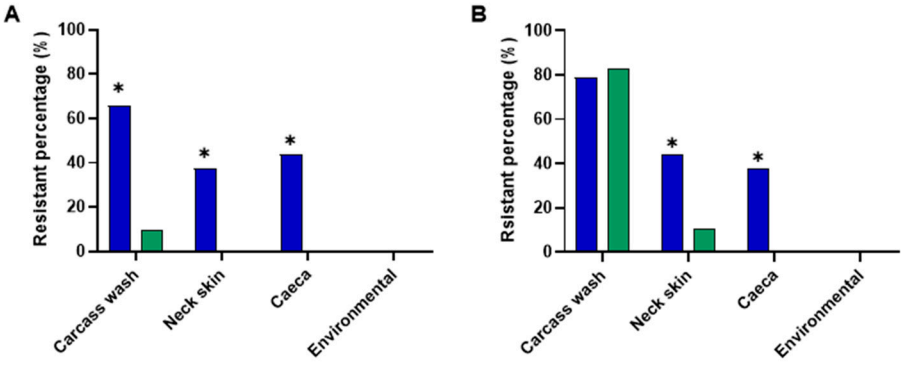

Figure 1. Resistant percentage of S.Typhimurium (Blue), *C. jejuni* (Green), according to the MIC (A) and MBC (B) data in different isolation points in the processing plant. * Denotes statistically significance ($p < 0.05$).

According to the MIC (Figure 1A), 65.5 % of S.Typhimurium isolates from whole carcass washing were resistant to chlorine. While chlorine resistant was observed in 78.8% of isolates when considering the MBC values (Figure 1B). S.Typhimurium isolated from neck skin and caecal samples, detected 37.5% and 43.8 % resistant respectively. In *C. jejuni* 9.4 % of isolates were resistant to chlorine when considering the MIC of from carcass wash isolates, while it was 83% when considered the MBC. The Resistant percentage of *C. jejuni* isolated from neck skin and caeca was 44.4% and 37.5% respectively.

When the resistant levels of S. Typhimurium and *C. jejuni* compared according to the MICs , S.Typhimurium resistant percentage was significantly higher (P<0.01) in carcass wash, neck skin, and caecal isolates compared to those of *C. jejuni*. Interestingly, when the MBC of *C. jejuni* considered, isolates from carcass wash showed higher resistance compared to that of S.Typhimurium. Notably in S.Typhimurium, the chlorine-resistant percentage was more or less similar in all types of Sample isolates. However, the resistant percentage was significantly (p< 0.05) higher, in carcass washing isolates in *C. jejuni*. Notably, no chlorine resistance was observed in the environmental isolates in both S. Typhimurium and *C. jejuni*.

3.3. Effect of Chlorine Concentration in the Chill Tank on MIC/MBC Profiles of S. Typhimurium and C. jejuni

The MICs and MBCs of S. Typhimurium and *C. jejuni* isolated from the whole carcass washings were analyzed according to the chlorine concentrations in the chill tanks, where the samples were collected. The effect of differences in chlorine concentrations in the chill tank on the tolerance levels of S. Typhimurium (Figure 2) and *C . jejuni*. (Figure 3) was determined.

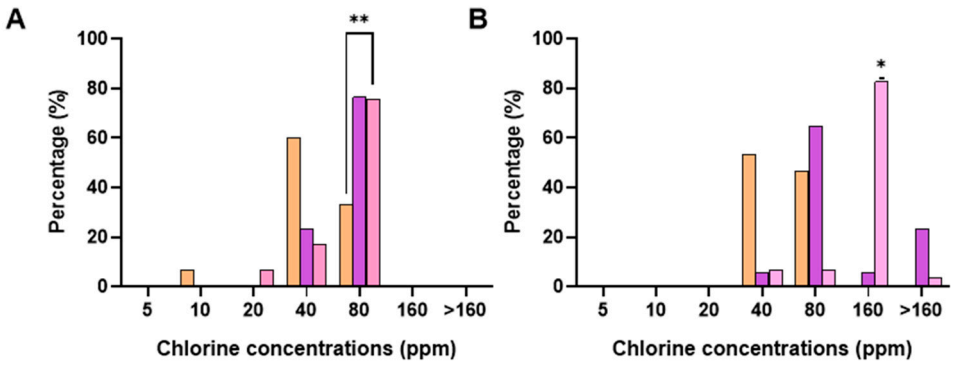

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Figure 2. Percentage of *S.* Typhimurium, given different chlorine tolerance levels in MIC (A) and MBC (B) assay. The isolates were collected from the chill tanks treated with 3-5 ppm (Orange), 20-30 ppm (Purple), and, 40-50 ppm (Pink) chlorine concentrations. * Denotes statistically significance ($p < 0.05$). ** Denotes statistically significance ($p < 0.001$).

A significantly high (p<0.001),percentage of S. Typhimurium was detected at 80 ppm of MICs / MBCs from carcass wash isolates where 20-30 ppm and 40- 50 ppm chlorine was used. Interestingly 100% S. Typhimurium isolated from the chill tank with 3-5 ppm chlorine showed ≥40 ppm of MBC value, which is 8 times higher than the actual chlorine concentration used in the chill tank. Further, 80% of S. Typhimurium from 40-50 ppm chlorine chill tanks detected MBC at 160 ppm and it was significantly high (p<0.05).

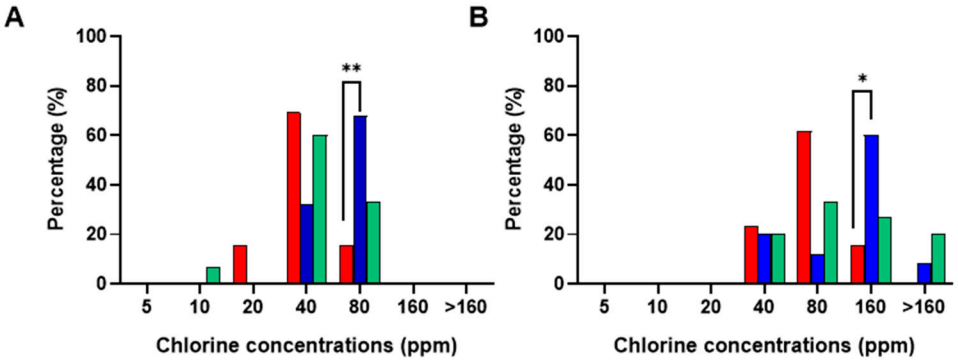

Figure 3. Percentage of *C . jejuni*, given different chlorine tolerance levels in MIC (A) and MBC (B) assay. The isolates were collected from the chill tanks treated with 3-5 ppm (Green), 20-30 ppm (Red), and, 40-50 ppm (Blue) chlorine concentrations. * Denotes statistically significance ($p < 0.05$). ** Denotes statistically significance ($p < 0.001$).

The highest percentage of *C. jejuni* (69.2%, 60%) was detected MIC at 40 ppm, in the isolates isolated from 3-5 ppm and 40-50 ppm chill tanks, while the highest MBC percentage was exhibited at 80 ppm. Notably the highest *C. jejuni* percentage (68%) showed MIC at 80 ppm with chlorine treatment at 20-30 ppm in the chill tank, while the MBC was 160 ppm in most of the population (60%).

Similarly, significantly higher (p<0.05) MIC (80 ppm) and MBC (160 ppm) were determined in *C. jejuni* , when the chill tank chlorine concentrations were 20-30 ppm and 40-50 ppm compared to 3-5 ppm.

3.4. Chlorine Resistance Profiles of S. Typhimurium and C. jejuni Isolated from Carcass Wash

Considering the highest chlorine concentration (40 ppm) used in the chill tank as a cut-off point, the chlorine resistant of S.Typhimurium and *C. jejuni* from carcass washings was analyzed (Figure 4).

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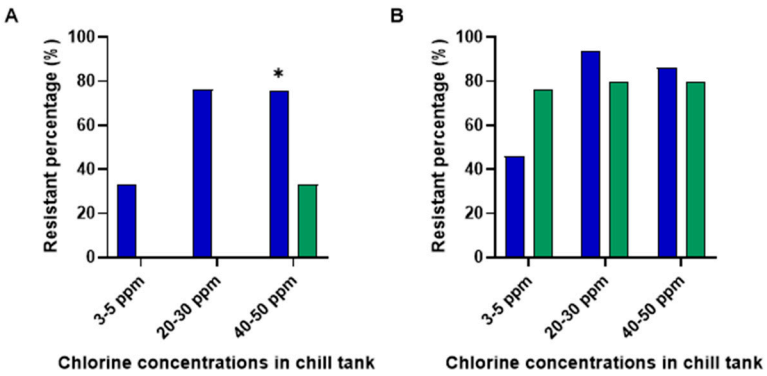

Figure 4. Resistant percentage of S.Typhimurium (Blue), and *C. jejuni* (Green), according to the MIC (A) and MBC (B) data in different isolation points in the processing plant. * Denotes statistically significance ($p < 0.05$).

According to the MIC /MBC data, the highest resistant percentage for S. Typhimurium was detected in carcass wash isolates from 20-30ppm chlorine in the chill tank, which was 76.5% and 94.1% respectively. According to the MIC and MBC data 75.8% S. Typhimurium isolated from 40-50 ppm chlorine also showed a higher resistance. Interestingly, according to MICs, no resistance was detected in *C. jejuni* at 3-5 ppm and 20-30 ppm chlorine, but a significantly higher ($p<0.001$) percentage (80%) was detected as resistant when considered the MBCs. Notably, a significant difference ($p <0.01$) was observed in resistant profiles of *C. jejuni* between the MIC and MBCs.

3.5. Recovery of S. Typhimurium and C. jejuni after Exposed to Chlorine

A resuscitation assay was conducted to determine the sub lethal damage to S. Typhimurium and *C. jejuni* in exposure to chlorine (Table 4)

Table 4. Percentage of resuscitated S.Typhimurium and *C. jejuni* after expose to chlorine in MIC/MBC assay.

Isolated Sample	S.Typhimurium recovery percentage (%)	*C . jejuni* recovery percentage (%)
Carcass wash	52.4% (32/61)	58.5 (30/51)
Neck skin	50 % (4/8)	55.5 % (5/9)
Caeca	33.3 % (5/15)	16.6 % (1/6)
Environmental	16.6 % (2/12)	14.2 % (1/7)

The lowest resuscitation percentage was observed in both S. Typhimurium and *C. jejuni* was observed in caecal isolates. While the highest recovery rate was observed in carcass wash isolates. The bacterial resuscitation of S. Typhimurium and *C. jejuni* in carcass wash and Neck skin samples was around 50%. The lowest resuscitation of S. Typhimurium and *C. jejuni* was observed in environmental isolates.

4. Discussion

Human salmonellosis and campylobacteriosis are major foodborne infections, attributed to the consumption of contaminated poultry meat and meat by-products. Despite the measures taken to minimize cross-contamination during processing, the persistence of these two pathogens in the food

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processing chain is significant. With the presence of organic matter content pathogens get exposed to sub-lethal doses of chlorine-inducing resistance in Salmonella and *Campylobacte*r [22,25]. In the present study, we investigated the tolerance levels of food-borne pathogens in exposure to chlorine. Due to the unavailability of the guidelines to interpret the resistant levels, both MIC and MBC values were taken to determine the chlorine resistance in the present study.

It was revealed that the highest percentage of S.Typhimurium (65.6%) isolated from carcass washing has MIC at 80 ppm, while it was 40 ppm in *C. jejuni* (58.8%). Required higher chlorine concentration in growth inhibition of S. Typhimurium compared to *C. jejuni* can be due to the strain variation in the stress response mechanism of S. Typhimurium, which increases the survivability in chlorine exposure [30]. Higher MICs in S. Typhimurium can cause higher carcass contamination in the chill tank, which was detected in a previous study with a higher prevalence of *Salmonella* (80.66%) compared to *Campylobacter* (68.66%) in carcass washings [28]. When considering the MBCs, the chlorine tolerance of *C. jejuni* was reported as higher at 160 ppm. These results revealed that, although *C. jejuni* can be inhibited at 80 ppm, it requires a higher chlorine concentration to kill the bacteria, which allows the persistence of sub-lethally injured *C. jejuni* in the food chain [31]. The adaptive stress response mechanism cause sub lethal damage in *Campylobacter*, which limits the complete inactivation and develop resistance [22,32]. The chlorine resistance was significant ($p < 0.05$) in *C. jejuni* isolated from carcass washings, when considering the MIC (78.8%) and MBC (83%) data. Interestingly the resistant percentages in *C. jejuni* were significantly different ($p<0.01$) when considering the MIC and MBC separately. Therefore the present findings highlighted that determining only MIC would be not enough when deciding the effective chlorine concentrations for foodborne pathogens. The inconsistent effectiveness of chlorine reported in recent years can be due to the developed resistance to chlorine as the pathogens are exposed to various stressors during processing. Stress-adapted bacteria frequently provide cross-protection against other stressors and enhance the survival of stressed *Campylobacter* and Salmonella throughout the food processing chain [24]. Further, presence of organic matter increases the resistant to chlorine in foodborne pathogens [16]. A previous study reported that Salmonella required chlorine concentrations as high as 400 ppm and 800 ppm to have complete inactivation [33]. Also, a recent study conducted in Australia, reported that the MIC level of *Campylobacter* is 128 ppm, although the permitted chlorine concentration in the chill tank is 8 ppm [21]. The findings of the present study confirmed that common food-borne pathogens found in broiler processing have developed a greater tolerance to chlorine-based antimicrobials.

In Sri Lanka, three different chlorine concentrations are used in the chill tank; from 3-5 ppm, 20-30 ppm, and 40-50 ppm. Therefore the isolates used in MIC/MBC assay have been previously exposed to different chlorine concentrations in the chill tank. There we determined the effect of exposure to different chlorine concentrations in the chill tank on chlorine tolerance levels of S. Typhimurium and *C. jejuni.*

S. Typhimurium isolated from chill tanks with 20-30 ppm and 40- 50 ppm chlorine m, exhibited significantly high ($p<0.001$) MIC and MBC values of 80 ppm, which is 4 times and 2 times higher than the actual chlorine used in the chill tanks respectively. Further, we observed that 80% of S. Typhimurium isolated from 40-50 ppm chill tank detected 160 ppm MBC value and it was significantly high ($p<0.05$). This shows the limitation of chlorine in eliminating Salmonella during processing [34]. A previous study has reported S. Enteritidis, S. Kentucky, and S. Typhimurium isolates with higher chlorine tolerance levels (MIC > 256 mg/L) in 90.9%, 95.5%, and 94.4%, respectively [35]. However, to comply with the demand for cleaner food and less use of chemicals in processing, the maximum permissible limit for chlorine is now 50 ppm [36].

Interestingly 100% of S. Typhimurium isolates exposed to 3-5 ppm of chlorine in the chill tank detected ≥40 ppm of MIC/ MBC, which is almost 8 times of actual chlorine limit. A recent study has shown that 90% of carcass contamination of Salmonella in the chill tank where used 3-5 ppm of chlorine [28]. Previous tudies have demonstrated that diverse changes in chlorine tolerance in different Salmonella strains after sub lethal exposure as expressed by higher MICs compared to the

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initial MIC before exposure to chlorine [37–40]. Further, it has reported an increment of 0.5-fold to 2-fold MIC levels after sublethal chlorine stress. The present study has given similar results and further confirms that sublethal exposure to chlorine induces chlorine resistance in S. Typhimurium.

Similarly higher MIC (40 ppm) and MBC (80 ppm) in *C. jejuni*, exposed to 3-5 ppm of chlorine in the chill tank could be due to the chlorine-induced adaptive stress response mechanism of *C. jejuni* [22]. Interestingly 60% of the population showed the highest MBC value of 160 ppm, which was previously exposed to 20-30 ppm of chlorine in the chill tank. This can be because even 20-30 ppm chlorine becomes sublethal to *C. jejuni* and induces the highest stress response. In addition, a change in cell structure including morphology induces in Salmonella and *Campylobacter* as a response to chlorine stress, which allows the bacterium to become more tolerant to higher chlorine concentrations and other antimicrobials [21,25]. Further, we observed the highest resistance in *C. jejuni*, which were previously exposed to 20-30 ppm of chlorine in the chill tank. Notably, MICs of *C . jejuni* showed either less or no resistance to chlorine, while significantly higher resistance was detected in MBCs (80%). This highlighted although 20-30 ppm chlorine can inhibit the growth of *C . jejuni* , it requires a higher chlorine concentration to have a biocidal activity. The present study shows the development of chlorine resistance in *C . jejuni,* when they have previously been exposed to chlorine. In a study investigating the efficacy of chlorine in chilling applications of carcasses, 30 ppm of chlorine reduced the percentage of positive isolates by only 56.8% and 12.8% for Salmonella and *Campylobacter*, respectively [41].

In the present study, the neck skin samples were taken from the carcass after going through inside/outside carcass washers. The isolates were only exposed to 2-3 ppm of chlorine as they get washed in potable water. Even though a higher S. Typhimurium percentage showed 40 ppm of MIC (62,5%) and MBC (44.4%0), still a considerable percentage required 80 ppm for bacterial inhibition. We observed similar MIC/MBC data in C . *jejuni* neck skin isolates. Comparative to the carcass washing isolates, the neck skin isolates exhibited very low resistant percentages for both S. Typhimurium (44.4%) and *C . jejuni* (10.5%). This can be due to the chlorine concentration and the exposure time is very low in carcass washers, compared to the chill tank.

Further, the MIC/MBCs of caecal S. Typhimurium were at 40 ppm, while in *C . jejuni* although the MIC was at 20 ppm, the MBC was 40 ppm. In caecal isolates, chlorine resistance of S. Typhimurium was significantly low ($p<0.05$), while no chlorine-resistant *C. jejuni* was detected. As the isolates came from caecal contents, they had not been exposed to chlorine previously. Hence, they have not developed resistance as carcass wash isolates. Similarly, the environment S. Typhimurium and *C. jejuni* exhibited low MIC/ MBC values in the present study. Both S. Typhimurium and *C. jejuni* exhibited 40 ppm of MIC/MBC value in the present study. Notably, neither S. *Typhimurium* nor *C . jejuni* isolated from the environment were resistant to chlorine in the present study. This can be due to the environmental S. Typhimurium and *C . jejuni* have not been exposed to chlorine previously. This result further proves the previous exposure to chlorine-induced chlorine resistance in foodborne pathogens.

Sublethally injured S. Typhimurium and *C . jejuni* can be resuscitated after giving appropriate enrichment conditions [21,42]. The lowest resuscitation percentage was observed in both S. Typhimurium (16.6%) and *C. jejuni* (14.2%), which were isolated from environmental samples, while the highest recovery rate was shown in carcass wash isolates, and it was 52.4% and 58.5 respectively. As the environmental isolates were not resistant to chlorine, the exposed chlorine levels might be lethal to bacteria and they might undergone irreversible cell death, which could not be resuscitated [21]. However, the whole carcass wash isolates were sub-lethally injured due to stress response mechanism induced by previously exposed sublethal chlorine dosage. The resuscitation of *S.* Typhimurium and *C. jejuni* after exposure to chlorine suggested that it is possible to revive bacteria after a sublethal injury caused by chlorine exposure [21]. The persistence of S. Typhimurium and *C . jejuni* in the food chain creates a major public health risk [23,28].

The present study revealed that increasing concentrations of chlorine is not an option in poultry processing to mitigate food borne pathogen contamination. A study has shown that the incidence of

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foodborne disease linked with the consumption of chicken meat and the potential for the emergence of sodium hypochlorite resistant food borne pathogens [43]. Moreover, increased chlorine concentration increases the risk of having chlorine residues in chicken meat. Therefore the risk of persistence of either resistant or sub-lethally injured foodborne pathogens in the post-spin chill carcasses is significant and they might have more stress tolerance levels and higher virulence which could be a serious public health risk. The virulence potential of sub-lethally injured *S. Typhimurium and C. jejuni* following exposure to chlorine has been reported [28,44]. Even more concerning are the correlations between chlorine resistance and multidrug-resistant foodborne pathogens [32]. Therefore optimizing the current chemical decontamination protocols in poultry processing is timely important.

5. Conclusions

To our knowledge, this is the first study to demonstrate the chlorine resistance in S. Typhimurium and *C. jejuni* which were previously exposed to different chlorine concentrations in the chill tank. The present study revealed that the chlorine tolerance levels of S. Typhimurium and *C. jejuni* are significantly high. Pre exposure to chlorine, further increases the chlorine tolerance level. This could be the reason for the lower effectiveness of current chlorine concentrations used in the poultry industry, which is unable to eliminate S. Typhimurium and *C. jejuni*. Despite having an arsenal of interventions available for poultry processing, new food safety challenges are presented by Salmonella and *Campylobacter*. Oxidative stress due to chlorine can induce the formation of biofilms and the develop cross protection to other stresses such as acid tolerance heat tolerance and oxygen tolerance and, antimicrobial resistance in S. Typhimurium and *C. jejuni*. To overcome these emerging challenges poultry industry, moving to an alternative chemical and novel multi-huddle interventions is crucial.

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Abbreviations

The following abbreviations are used in this manuscript:

USDA	United States Department of Agriculture
HACCP	Hazard Analysis and Critical Control Point
MIC	Minimum inhibitory concentrations
MBC	Minimum bactericidal concentrations
ASC	Acidified Sodium Chlorite
PAA	PerAcetic Acid
SH	Sodium Hypochlorite
GRAS	Generally Recognized as Safe
FSIS	Food Safety and Inspection Service

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