

ORIGINAL RESEARCH ARTICLE

Chlorine-resistant *Salmonella* Typhimurium and *Campylobacter jejuni* in the poultry processing lineGayani Weerasooriya^{*}^{id}, H.M. Thiwanka Dulakshi, P. Shyamali de Alwis, M.A. Roshan Priyantha^{id}

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Abstract

Salmonella Typhimurium and *Campylobacter jejuni* are major foodborne pathogens that persist along the poultry processing line, posing public health risks and significant economic burdens. Chlorine is commonly used in spin chill tanks to wash poultry carcasses and mitigate carcass contamination during processing. Although chlorine concentrations vary across the poultry industry, the prevalence of foodborne pathogens in poultry remains significant. This study determined the chlorine resistance in *S. Typhimurium* and *C. jejuni* isolated from caecal, neck skin, carcass washings, and environmental samples using minimum inhibitory concentration/minimum bactericidal concentration assays. Regardless of the chlorine concentration in the chill tank, higher tolerance levels were observed in *S. Typhimurium* and *C. jejuni* isolated from carcass washings. The lowest tolerance level was observed in environmental isolates that had not been previously exposed to chlorine. Notably, 60% of carcass-washing *C. jejuni* pre-exposed to 40 ppm chlorine in the chill tank showed a higher tolerance level of 160 ppm chlorine, while 80% of *S. Typhimurium* pre-exposed to 20 ppm showed an 80 ppm tolerance level. Notably, 94.1% of *S. Typhimurium* and 80% of *C. jejuni*, isolated from the carcass washing, were resistant to chlorine in the present study. Similarly, the highest resuscitation rates were detected in the same isolates at 52.4% and 58.5%, respectively. The lowest resistance and the highest resuscitation rate were observed in the environmental isolates. Pre-exposure to chlorine during processing has increased the chlorine resistance of both pathogens. The observed resistance may limit the effectiveness of mitigation protocols that rely on concentration adjustments in the chill tank. Therefore, an alternative chemical with a novel disinfection protocol is crucial to mitigate bacterial contamination in poultry processing.

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1. Introduction

Foodborne infections are a major public health problem that remains a common cause of illness and death worldwide. The public health impacts of foodborne pathogens such as *Salmonella* and *Campylobacter* are enormous, as are the significant social and economic

burdens. According to the European Union One Health 2021 Zoonoses Report, *Campylobacter* and *Salmonella* are humans' first and second most reported pathogens, accounting for >65% and >29% of confirmed human cases, respectively.¹ Globally, it is estimated that 93.8 million gastroenteritis cases are caused by non-typhoidal *Salmonella* every year², while around 96 million are caused by *Campylobacter*.³ These two foodborne pathogens are colonized in the chicken gut and contaminate the poultry carcass during processing. Chickens are responsible for 30% of human outbreaks, while the likelihood of an outbreak is estimated at 12% in slaughter and 33.5% in processing.^{4,5} Therefore, the safe production and distribution of food across the farm-to-fork continuum are crucial to ensure that consumers receive wholesome food. Chicken carcasses become contaminated with feathers, skin, and ruptured intestines during processing and chilling, and portioning and packing further cross-contaminate the product.^{6,7} The cross-contamination of the kitchen utensils, bench tops during cooking, and other food items in the refrigerator during storage is also significant.^{8,9} The United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) procedures and pathogen reduction performance standards resulted in additional water use by poultry processors to eliminate carcass contamination, including adding inside/outside carcass washers, spraying, chilling carcasses to 4 °C, and antimicrobial chemical decontamination.¹⁰ USDA-FSIS has permitted the use of Generally Recognized as Safe (GRAS) chemicals in poultry processing, such as chlorine, sodium hypochlorite (SH), acidified sodium chlorite (ASC), and peracetic acid (PAA). Due to its cost-effectiveness, availability, and ease of application, chlorine has become the most commonly used chemical in carcass washing.⁷ Studies have shown that 73% of processing plants use chlorine or a combination of chlorine and another antimicrobial to reduce bacterial load on carcasses during processing.¹⁰ Chlorine is an oxidizing agent that increases membrane permeability and causes nucleic acid damage and leakage of intracellular biomolecules in both Gram-negative and Gram-positive bacterial species.^{11–13} The European Food Safety Authority (EFSA) and other bodies raised concerns that chlorinated chicken could mask underlying deficiencies in farm-level hygiene, potentially contributing to antimicrobial resistance or leaving chemical residues. Given the potential risks to consumer health and environmental safety, the European Union banned the use of chlorine in carcass washing in 1997.^{14,15} However, the USA, Australia, and most Asian countries still use chlorine in chill tanks.¹⁶ Chemical decontamination using chlorine is a significant challenge due to the constant addition of organic matter into the chill tank, which reduces the

effectiveness by lowering total free chlorine availability.¹⁷ Therefore, chlorine tolerance levels of *Salmonella* and *Campylobacter* can be higher than the actual chlorine concentrations used in the chill tank.^{18,19} Given the health risks, USDA permits the use of SH at 50 ppm free available chlorine on chicken carcasses and 200 ppm free chlorine on food-contact surfaces during sanitation.¹⁷ The ability of resistance development against the sanitizers also plays a major role in the persistence of foodborne pathogens in poultry processing environments and the food chain.^{20,21} This can be credited to its microbial ability to adapt and develop resistance to different sanitizers, including oxidizers such as chlorine.^{19,22} Foodborne pathogens, such as *Campylobacter jejuni*, *Listeria monocytogenes*, and *Salmonella enterica*, can adapt to diverse processing-related stressors, influencing their growth, survival, and persistence in the food chain.^{23,24} Notably, exposure to sub-lethal doses of chlorine can cause sub-lethal injury and can be resuscitated after removing the stress condition and by providing an enriched environment.¹⁸ Therefore, it is highly crucial to determine the maximum tolerance levels of chlorine and their resistance profiles to optimize the current decontamination protocols to mitigate foodborne infections.

As in other Asian countries, 90% of Sri Lanka's poultry processing plants use chlorine in spin chill tanks. Due to the lack of a standard protocol or guideline, chlorine concentrations in chill tanks vary from 5 ppm to 40 ppm across different poultry companies in Sri Lanka.²⁵ However, even with the higher chlorine concentrations, the contamination rates of post-chill carcass washings with *Campylobacter* and *Salmonella* are 80.66% and 68.66%, respectively.²⁵ Studies have not been conducted in Sri Lanka to explore the chlorine resistance profiles of common foodborne pathogens found in the poultry processing plants. The present study was designed to understand the current situation of chlorine resistance in *Salmonella* Typhimurium and *Campylobacter jejuni* acquired in the poultry processing environment. Furthermore, we determined the effect of pre-exposure to chlorine on the chlorine tolerance levels of *S. Typhimurium* and *C. jejuni* during processing and on their resuscitation potential.

2. Materials and methods

2.1. Preparation of *Campylobacter* and *Salmonella* inoculum

S. Typhimurium and *C. jejuni*, used in the present study, were isolated from poultry processing plants in a previous study conducted in 2024–2025.²⁵ The samples were collected from nine ($n = 9$) large-scale poultry processing plants in Sri Lanka. According to the recorded data, three different

chlorine concentrations: 5 ppm, 20 ppm, and 40 ppm, have been used in the chill tanks in different processing plants. The processing plants were included in each chill tank chlorine concentration category. The chlorine contact time in the chill tank was similar in all the processing plants selected for the study, and the samples collected on the day of sampling originated from the same flock to minimize the strain heterogeneity or prior environmental adaptation. Ten neck skin samples ($n = 10$) and ten caeca samples ($n = 10$) were collected from the carcass at the evisceration section of each processing plant. Caeca were collected during evisceration, and neck skin samples were collected from the carcasses after going through the inside/outside carcass washers. Ten ($n = 10$) carcass washing samples at the post-spin chill were collected. The carcass washings were collected by rinsing and massaging the carcasses in 200 mL of sterile distilled water, as described in a previous study.²⁵ Free chlorine concentrations in the chill tanks were recorded at the time of sampling. Fecal droppings were included in the environmental samples, and pooled samples were collected from the unloading area of the processing plant. Altogether, ninety ($n = 90$) neck skin samples, ninety ($n = 90$) carcass washing samples, and thirty ($n = 30$) environmental pooled samples were collected. Isolation and identification of *Salmonella* and *Campylobacter* were performed in the laboratory according to the method described by Chousalkar *et al.*¹⁶, and ISO 6579-1:2017 guidelines.²⁶ Here, the isolates from environmental samples and caecal samples have not been exposed to chlorine previously. The isolates from the neck skin samples have been exposed to 3 ppm chlorine in potable water during passage through the inside–outside carcass washers. The isolates from post-chill carcass washings have been exposed to 5 ppm, 20 ppm, or 40 ppm chlorine, as chlorine concentrations in chill tanks vary across the poultry industry (Table 1). The bacterial isolates

were grouped according to the chlorine concentration in the chill tank. Only *C. jejuni* was selected using quantitative polymerase chain reaction, and *S. Typhimurium* was selected via serotyping to minimize strain variation. The isolated *S. Typhimurium* and *C. jejuni* were stored at -80°C in brain heart infusion broth containing 50% glycerol. In preparation for minimum inhibitory concentration (MIC)/minimum bactericidal concentration (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 SBA and incubated at 42°C in 5% O_2 , 10% CO_2 , 85% N_2 for 48 h.

2.2. Determination of minimum inhibitory concentrations of chlorine

To determine the bacteriostatic effect of chlorine, the MICs were determined. *S. Typhimurium* ($n = 111$) and *C. jejuni* ($n = 96$) were used in the MIC assay according to the method used previously, with some modifications.²⁷ Briefly, the microbroth dilution method for MIC determination was performed according to the Clinical and Laboratory Standards Institute standards.²⁸ Chlorine solutions (10% SH, SIGMA-ALDRICH, USA) were prepared at concentrations ranging from 2.5 ppm to 160 ppm in nutrient broth No. 2 (NB2; Oxoid, UK), in accordance with the World Health Organization industrial standards for the chicken meat industry. Chlorine levels were measured using a digital chlorine meter (LOVIBOND, Germany). Prepared chlorine solutions (90 μL ; 2.5–160 ppm) were added to the 1st–10th wells of round-bottom 96-well microtiter plates. *S. Typhimurium* and *Campylobacter jejuni* inoculums were prepared in sterile distilled water to obtain 10^8 colony-forming units (CFUs)/mL using a 0.5 McFarland Standard. Subsequently, 10 μL of the prepared inoculum was added to the wells with 90 μL of chlorine dilutions to obtain 10^7 CFU/mL. The NB2 without chlorine

Table 1. Sample type, number of isolates, and exposed chlorine level during processing

Sample type	Chlorine exposure in the processing line (ppm)	Isolation number (n)	
		<i>S. Typhimurium</i>	<i>C. jejuni</i>
Ceaca	0	16	11
Neck skin	3	12	15
Carcass washing 1	5	15	13
Carcass washing 2	20	17	25
Carcass washing 3	40	29	15
Environmental samples	0	22	17

was considered as the positive control (growth control) at the 11th well, and the NB2 without either chlorine or bacterial inoculum was the negative control (sterility control) at the 12th well. All the isolates and the negative and positive controls were tested in duplicates. The assays with *S. Typhimurium* were incubated at 37 °C for 20 h, while *C. jejuni* assays were incubated at 42 °C in 5% O₂, 10% CO₂, 85% N₂ for 20 h. The lowest concentration that did not yield visible bacterial growth in 96-well plates was defined as the MIC. *S. Typhimurium* (ATCC 13311) and *C. jejuni* (ATCC 33291) were used as test control strains.

2.3. Determination of minimum bactericidal concentrations for chlorine

The MIC values for chlorine were confirmed by MBC assay in the present study. MBC is the lowest concentration of chlorine that kills 99.9% of the inoculum. MBCs of *S. Typhimurium* and *C. jejuni* were determined to assess the bactericidal effect of chlorine. After reading the MIC values from the MIC assay, 10 µL of broth from each growth inhibited well in the MIC plates was drop-plated onto sheep blood agar. *S. Typhimurium* plates were incubated at 37 °C for 24 h, and *C. jejuni* plates were incubated at 42 °C in 5% O₂, 10% CO₂, 85% N₂ for 48 h. The MBC was defined as the lowest chlorine concentration required to reduce viable cell numbers below the detection limit. All the duplicates in the MIC assay were tested in MBC. The maximum level of chlorine that can be used in chill tanks has not yet been documented in Sri Lanka. However, there are currently no widely accepted quantitative criteria for determining whether a microorganism is resistant to chlorine. According to the previous study, the highest chlorine concentration detected in the chill tank was 40 ppm.²⁵ This parameter was considered the cut-off point in the determination of the resistance to chlorine in the present study. MIC and MBC values above 40 ppm were considered resistant to chlorine in *S. Typhimurium* and *C. jejuni*. The MIC and MBC assays were conducted in duplicate and repeated twice.

2.4. Resuscitation assay

Resuscitation assay was conducted to determine sub-lethal injury of *S. Typhimurium* and *C. jejuni* in exposure to chlorine. The inhibited bacteria exposed to chlorine in the MIC assay were recovered by providing an enriched environment, as previously described, with some modifications.¹⁸ While the MBC assay was conducted, the resuscitation assay was subsequently conducted. Briefly, 50 µL of bacterial growth-inhibited wells of *S. Typhimurium* in the MIC assay were inoculated into 150 µL nutrient broth (Oxoid, UK) and incubated at 37 °C for 24 h. Similarly, 50 µL of inhibited *C. jejuni* were inoculated into 150 µL

Preston broth (NB2 with Preston *Campylobacter* selective supplement-SR0117E) (Oxoid, UK) and incubated at 42 °C in 5% O₂, 10% CO₂, 85% N₂ for 24 h. After incubation, 10 µL from the enriched broth was drop-plated onto SBA. *S. Typhimurium* was incubated at 37 °C for 24 h, and *C. jejuni* was incubated at 42 °C in 5% O₂, 10% CO₂, 85% N₂ for 48 h to determine the recovery after exposure to chlorine. Here, the resuscitation is considered when the bacteria recover at the MBC value.

2.5. Statistical analysis

The MIC/MBC and resuscitation data were presented as percentages and were analyzed using Fisher's exact test. Here, we assumed that pre-exposure to chlorine affects chlorine resistance. To determine differences in chlorine tolerance levels of *S. Typhimurium* and *C. jejuni*, and the effect of chill tank chlorine concentration, the MIC and MBC values of each isolate were analyzed against the chill tank chlorine concentration using two-way analysis of variance followed by Tukey's multiple-comparison tests. 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. *S. Typhimurium* and *C. jejuni* susceptibility to chlorine: minimum inhibitory concentration and minimum bactericidal concentration determination

The MIC/MBC were analyzed to determine the chlorine tolerance levels of *S. Typhimurium* (Figure 1) and *C. jejuni* (Figure 2) isolated from the processing line. The results revealed that a significantly higher (*p* < 0.05) percentage (65.6%) of *S. Typhimurium* isolated from carcass washing showed MIC of 80 ppm (Figure 1A), while the MBC was 80 ppm (Figure 1B) in the present study. Notably, based on the MBC values obtained, 9.9% of *S. Typhimurium* in the carcass wash required 160 ppm chlorine to exert a bactericidal effect. Among *S. Typhimurium* isolates from neck skin samples, 62.5% showed a 40 ppm MIC (Figure 1A), which was significant (*p* < 0.05). However, the MBC (Figure 1B) values of those isolates differed from the MIC values, with the majority, 44.4%, showing MBC at 80 ppm. The MIC and MBC values were similar for *S. Typhimurium* isolated from environmental samples, with the highest proportions at 40 ppm: 41.1% and 54.5%, respectively. When we compared the MIC/MBC values of *S. Typhimurium*, the lowest MIC/MBC values were observed in isolates from environmental samples.

The highest percentage (52.8%) of *C. jejuni* isolated from carcass washing exhibited MIC (Figure 2A) at 40

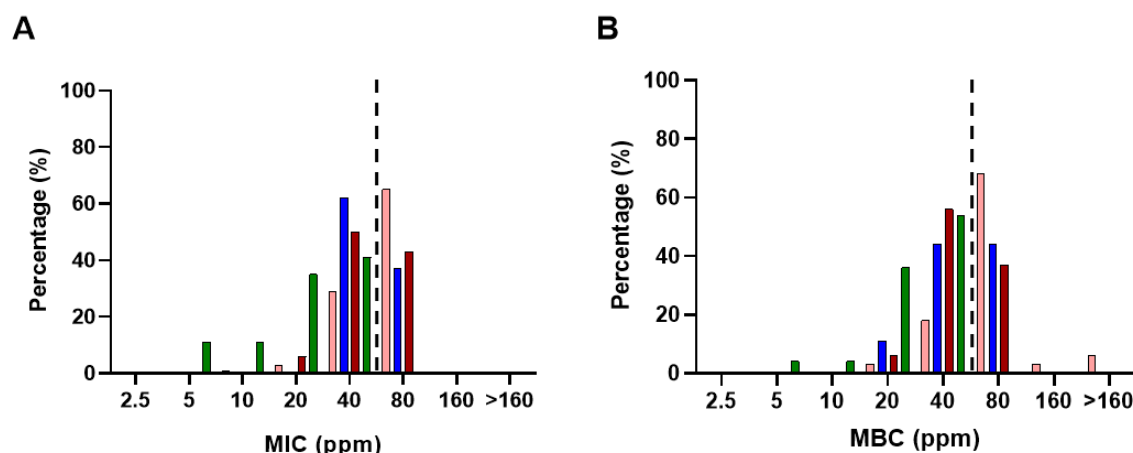


Figure 1. The minimum inhibitory concentration (MIC) (A) and minimum bactericidal concentration (MBC) (B) of *Salmonella* Typhimurium isolated from carcass washings (pink), neck skin (blue), caeca (brown), and environment samples (green). The black dashed line denotes the highest chlorine concentration in the chill tanks, 40 ppm, during the study.

ppm, while the MBC (Figure 2B) of 39.65% of the isolates was 160 ppm. Furthermore, 49% of *C. jejuni* from carcass washings detected higher MBC at ≥ 160 ppm in the present study. Interestingly, there was a significant ($p < 0.01$) difference between the MIC (Figure 2A) and MBC (Figure 2B) values of *C. jejuni* isolated from the carcass washings. The highest percentage (36.6%) of *C. jejuni* isolated from neck skin exhibited MICs of 20 ppm and 40 ppm, and a similar MBC pattern was observed, with 42.1% in both 20 ppm and 40 ppm. The highest percentage of *C. jejuni* (41.1%) isolated from the environment exhibited an MIC of 40 ppm, which was similar to the MBC.

3.2. Chlorine resistance profiles of *S. Typhimurium* and *C. jejuni*

Using 40 ppm as the cut-off point for the chill tank, the chlorine resistance of *S. Typhimurium* and *C. jejuni* was analyzed (Figure 3). The chlorine resistance was analyzed using both MIC (Figure 3A) and MBC (Figure 3B), and the results were compared to understand the differences between MIC and MBC in determining resistance. According to the MIC (Figure 3A), 65.5% of *S. Typhimurium* isolates from carcass washing were resistant to chlorine, whereas 78.8% were resistant when the MBC was considered (Figure 3B). The chlorine-resistant percentage of *S. Typhimurium* isolated from neck skin and caecal samples was 37.5% and 43.8%, respectively, according to MIC values, and was very similar to the resistant parentage when considering MBC, which was 44.4% and 37.5%, respectively.

Notably, in *C. jejuni*, while 9.4% of isolates from carcass washings were resistant to chlorine according to the MIC (Figure 3A), 83% were resistant when considering the MBC (Figure 3B). None of the isolates from neck skin, caeca, or environmental samples were resistant according to MIC, whereas only 10.2% of *C. jejuni* isolates from neck skin were resistant according to MBC. When the resistance levels of *S. Typhimurium* and *C. jejuni* were compared using MIC, the *S. Typhimurium* resistance percentage was significantly higher ($p < 0.01$) in carcass wash, neck skin, and caecal isolates than in *C. jejuni* isolates. Interestingly, when the MBC (Figure 3B) of *C. jejuni* is considered, isolates from carcass washing and neck skin showed higher resistance than those of *S. Typhimurium*. In *S. Typhimurium*, the chlorine resistance percentage was similar across all sample types, whether measured as MIC or MBC. However, according to MBC, the resistance percentage was significantly ($p < 0.05$) high in *C. jejuni* isolated from carcass washings. Furthermore, no chlorine resistance was observed in either *S. Typhimurium* or *C. jejuni* isolated from the environmental samples.

3.3. Effect of chlorine concentration in the chill tank on minimum inhibitory concentration and minimum bactericidal concentration profiles of *S. Typhimurium* and *C. jejuni*

To understand the effect of pre-exposure to chlorine on their chlorine tolerance levels and resistance profiles, the MICs and MBCs of *S. Typhimurium* (Figure 4) and *C. jejuni* (Figure 5) isolated from the carcass washings were

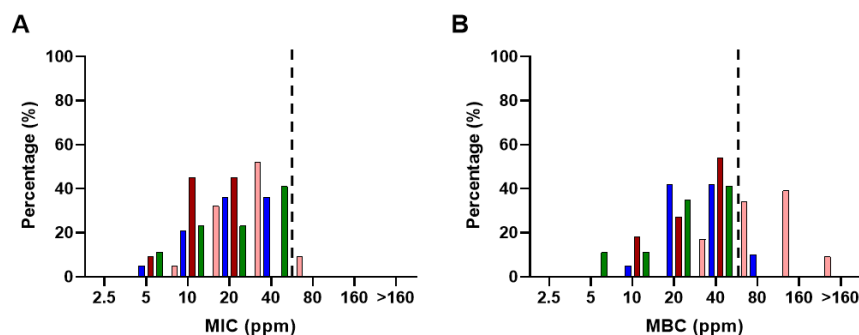


Figure 2. The minimum inhibitory concentration (MIC) (A) and minimum bactericidal concentration (MBC) (B) of *Campylobacter jejuni* isolated from carcass washings (pink), neck skin (blue), caeca (brown), and environment samples (green). The black dashed line denotes the highest chlorine concentration in the chill tanks, 40 ppm, during the study.

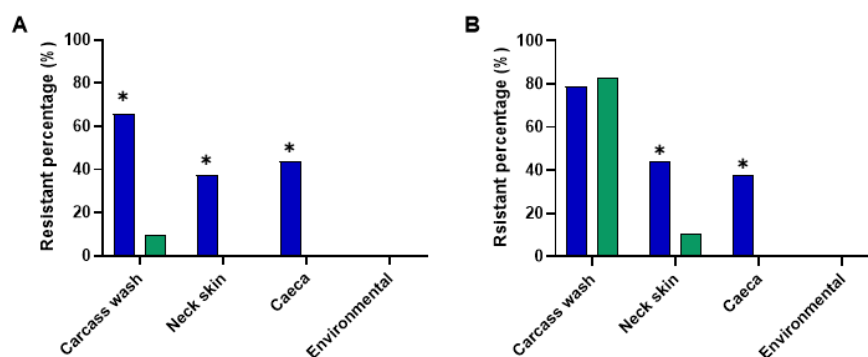


Figure 3. Resistant percentage of *Salmonella Typhimurium* (blue) and *Campylobacter jejuni* (green), according to the minimum inhibitory concentration (MIC) (A) and minimum bactericidal concentration (MBC) (B) data in different isolation points in the processing plant.

Note: * $p < 0.05$.

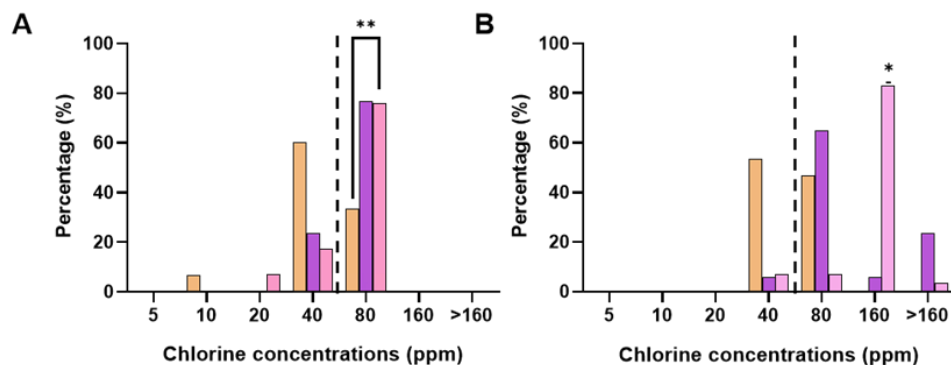


Figure 4. Percentage of *Salmonella Typhimurium*, given different chlorine tolerance levels in minimum inhibitory concentration (MIC) (A) and minimum bactericidal concentration (MBC) (B) assay. The isolates were collected from the chill tanks treated with 5 ppm (orange), 20 ppm (purple), and 40 ppm (pink) chlorine concentrations. The black dashed line denotes the highest chlorine concentration in the chill tanks, 40 ppm, during the study.

Notes: * $p < 0.05$; ** $p < 0.001$.

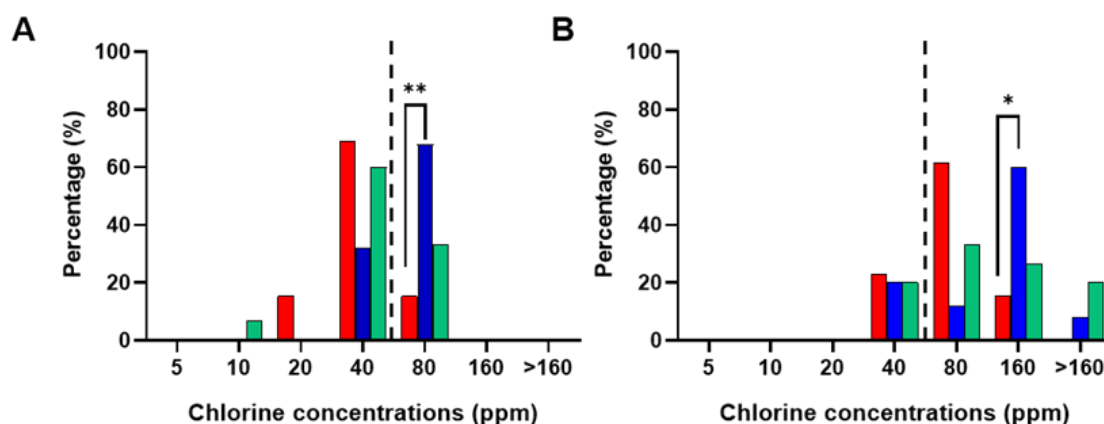


Figure 5. Percentage of *Campylobacter jejuni*, given different chlorine tolerance levels in the minimum inhibitory concentration (MIC) (A) and minimum bactericidal concentration (MBC) (B) assay. The isolates were collected from the chill tanks treated with 5 ppm (green), 20 ppm (red), and 40 ppm (blue) chlorine concentrations. The black dashed line denotes the highest chlorine concentration in the chill tanks, 40 ppm, during the study. Notes: * $p < 0.05$; ** $p < 0.001$.

analyzed. A significantly high ($p < 0.001$) percentage of *S. Typhimurium*, isolated from the carcass washings from the 5 ppm chill tank, exhibited high tolerance levels (MICs/MBCs at 40 ppm and 80 ppm, respectively). When the chlorine concentration of the chill tank was 20 ppm, around 80% of the isolates detected MIC/MBC at 80 ppm. At a 40 ppm chlorine concentration in the chill tank, a significantly higher ($p < 0.05$) percentage (80%) of *S. Typhimurium* showed the highest tolerance level at 160 ppm in MBC.

In the present study, the highest percentage of *C. jejuni* (69.2%) exposed to 5 ppm chlorine in the chill tank demonstrated MIC at 40 ppm (Figure 5A), while a significant percentage (65%) showed tolerance to 80 ppm in the MBC assay (Figure 5B). Interestingly, *C. jejuni* exposed to 5 ppm in the chill showed tolerance levels of 40–160 ppm, with similar percentages. Notably, when the chill tank chlorine concentration was 40 ppm, both the MIC and MBC of *C. jejuni* were significantly higher ($p < 0.05$), reaching 80 ppm and 160 ppm, respectively.

3.4. Chlorine resistance profiles of *S. Typhimurium* and *C. jejuni* isolated from carcass wash

Using the highest chlorine concentration (40 ppm) in the chill tank as a cut-off point, the chlorine resistance of *S. Typhimurium* and *C. jejuni* from carcass washings was analyzed (Figure 6). The highest resistance percentage among *S. Typhimurium* isolates was detected in carcass-washing isolates from the 20 ppm chill tank. It was 76.5% according to MIC levels (Figure 6A) and 94.1% according

to MBC levels (Figure 6B). When the chill tank chlorine concentration was 40 ppm, 75.8% of *S. Typhimurium* were found to be resistant in the MIC assay. Moreover, the resistance percentage was 82% based on MBC values. Interestingly, when determining resistance in *C. jejuni*, MIC values (Figure 6A) indicated that they were not resistant to chlorine isolated from carcass washings, either at 5 ppm or 20 ppm in chill tanks. However, when considering the MBC values, a significantly high ($p < 0.001$) percentage (80%) was detected as resistant (Figure 6B). Notably, a significant difference ($p < 0.01$) was observed in the resistant profiles of *C. jejuni* when MICs and MBCs were analyzed separately.

3.5. Resuscitation of *S. Typhimurium* and *C. jejuni* after exposure to chlorine in the minimum inhibitory concentration assay

A resuscitation assay was conducted to determine sub-lethal injury to *S. Typhimurium* and *C. jejuni* following exposure to chlorine (Table 2).

The highest resuscitation rate was observed in carcass washing and neck skin isolates of both *S. Typhimurium* and *C. jejuni*. Further, the resuscitation percentage was higher in *C. jejuni* than in *S. Typhimurium* isolates from carcass washing and neck skin. The resuscitation percentage was significantly lower in both *S. Typhimurium* and *C. jejuni* isolated from caeca and environmental samples compared to carcass washings and neck skin isolates. The lowest resuscitation rate was observed in *S. Typhimurium* and *C. jejuni* isolated from environmental samples, at 16.6% and 14.2%, respectively.

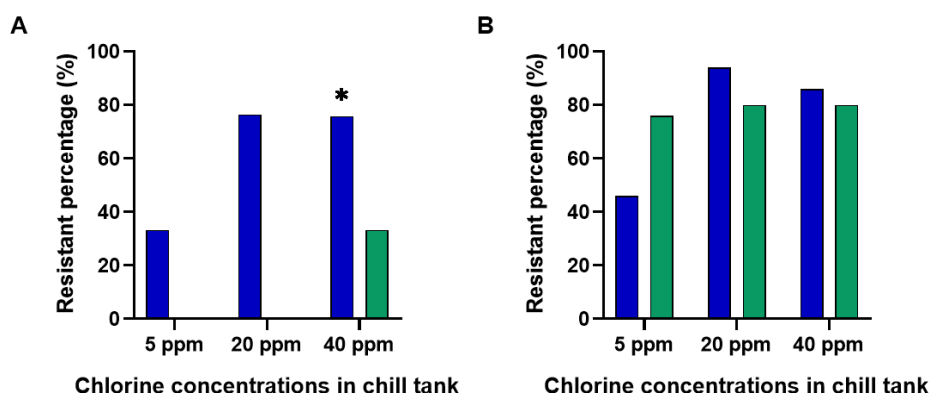


Figure 6. Resistant percentage of *Salmonella* Typhimurium (blue), and *Campylobacter jejuni* (green), according to the minimum inhibitory concentration (MIC) (A) and minimum bactericidal concentration (MBC) (B) data in different isolation points in the processing plant.

Note: * $p < 0.05$.

Table 2. Percentage of resuscitated *Salmonella* Typhimurium and *Campylobacter jejuni* after exposure to chlorine in the minimum inhibitory concentration assay

Isolated sample	Resuscitation percentage of <i>S. Typhimurium</i> (%)	Resuscitation percentage of <i>C. jejuni</i> (%)
Carcass washings	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)*

Note: * $p < 0.05$.

4. Discussion

Human salmonellosis and campylobacteriosis are major foodborne enteric infections worldwide, transmitted through the consumption of poultry meat. Despite measures taken to minimize cross-contamination during poultry processing, the persistence of *S. Typhimurium* and *C. jejuni* along the food processing chain remains significant. Although chlorine reduces bacterial levels, the organic matter content in the chill tank reduces free chlorine availability and causes sub-lethal injury, which can induce chlorine resistance in *Salmonella* and *Campylobacter*.^{19,22} In the present study, we investigated the MICs/MBCs of *S. Typhimurium* and *C. jejuni* and the effect of pre-exposure to chlorine in the chill tank on tolerance levels and resistance profiles. Higher chlorine tolerance at 80 ppm was detected in *S. Typhimurium* (65.6%) isolated from carcass washing, while 58.8% of *C. jejuni* showed a tolerance level at 40 ppm in the MIC assay. The observed higher tolerance in *S. Typhimurium* compared to *C.*

jejuni could explain the higher prevalence of *Salmonella* (80.66%) compared to *Campylobacter* (68.66%) in post-chill carcasses.²⁵ Furthermore, the observed differences in MICs/MBCs between the two pathogens can be due to the strain variation in the stress response mechanism.²⁹ However, according to MBC data, the higher chlorine tolerance level (160 ppm) in carcass washing for *C. jejuni* further explained that, although *C. jejuni* can be inhibited at 80 ppm, it requires a higher chlorine concentration (160 ppm) to be completely inactivated. This enhances the persistence of sub-lethally injured *C. jejuni* in the food chain.³⁰ The adaptive stress response mechanism of sub-lethally damaged *Campylobacter* limits the complete inactivation and further develops resistance.^{19,31} Both *S. Typhimurium* and *C. jejuni* isolates from carcass washing showed higher chlorine resistance than those from neck skin, caeca, and the environment. Although resistance rates were not different when MIC and MBC were considered separately in *S. Typhimurium*, there was a significant difference ($p < 0.01$) in resistant profiles

between *C. jejuni*. Therefore, the findings of the present study highlighted that MIC alone, without considering MBC, would not be accurate for determining the effective chlorine concentration. Similarly, a previous study reported that *Salmonella* required chlorine concentrations of 400 ppm and 800 ppm for complete inactivation.³² Similarly, another study reported a *Campylobacter* MIC of 128 ppm, although the chlorine concentration in the chill tank was 8 ppm.¹⁸ The present study further supports the evidence that common foodborne pathogens found in the poultry processing line can develop resistance to chlorine-based sanitizers.

The isolates used in the present MIC/MBC assay have been previously exposed to different chlorine concentrations. Sub-lethally injured foodborne pathogens exposed to chlorine induced an adaptive stress response and enhanced survivability.²² Further, stress-adapted bacteria frequently provide cross-protection against other stressors and enhance survival throughout the food processing chain.²¹ *S. Typhimurium* isolated from 20 ppm and 40 ppm chill tanks exhibited significantly high ($p < 0.001$) MIC/MBCs, which were two times higher than the chlorine concentration used in the chill tanks. Furthermore, 80% of *S. Typhimurium* isolated from the 40 ppm chill tank showed significantly higher tolerance at 160 ppm. Interestingly, 100% of *S. Typhimurium* isolates pre-exposed to 5 ppm of chlorine in the chill tank showed MIC/MBC of 40 ppm, which is almost eight times the actual chlorine limit. The revelation of 90% *Salmonella* contamination in a 5 ppm chill tank in a previous study further demonstrated the lesser effectiveness of chlorine at 5 ppm.²⁵ Here, we demonstrated that the increment of the exposed chlorine level increases the chlorine tolerance levels in MIC/MBC assays. Increasing chlorine resistance through pre-exposure to sub-lethal chlorine doses in the chill tank is a major limiting factor in eliminating *Salmonella* during poultry processing.³³ Similarly, a higher chlorine tolerance level of MIC > 256 mg/L was reported in a previous study for *S. Enteritidis*, *S. Kentucky*, and *S. Typhimurium*.³⁴ However, the maximum permissible limit for chlorine has been identified as 50 ppm to comply with the demand for cleaner food.³⁵ Previous studies have demonstrated diverse changes in initial MIC values and the MIC values after pre-exposure to sub-lethal doses of chlorine in different *Salmonella* strains. Further, it has been reported that MIC levels increase by 0.5- to 2-fold after sub-lethal chlorine stress.^{36–38} Similarly, higher MIC (40 ppm) and MBC (80 ppm) in *C. jejuni* pre-exposed to 5 ppm in the chill tank may be due to the chlorine-induced adaptive stress response in *C. jejuni*.¹⁹ Interestingly, 60% of *C. jejuni*, pre-exposed in a 40 ppm chill tank, revealed the highest MBC values at 160 ppm. Further, the highest resistance

in *C. jejuni* was also reported in the same pre-exposed group. A significant difference exists in resistance rates when MICs and MBCs are considered separately. These findings further showed that although chlorine can inhibit the growth of *C. jejuni* at 40 ppm, it can induce a viable but non-culturable state, requiring a higher concentration for complete inactivation. This explains the results of a recent study, which demonstrated that 30 ppm of chlorine in the chill tank reduced *Salmonella* and *Campylobacter* contamination by only 56.8% and 12.8%, respectively.³⁸

As the neck skin samples were collected from the carcass after passing through the inside/outside carcass washers, the isolates were already exposed to 3 ppm chlorine. Therefore, the tolerance levels of pre-exposed *S. Typhimurium* were considerably higher at 40 ppm, although the percentages were lower than the carcass washing isolates. The lower MIC/MBC values compared to those of carcass-washing isolates may be due to lower resistance in those isolates, as chlorine exposure is low. Furthermore, compared with the carcass-washing isolates, the neck-skin isolates exhibited very low resistance rates for both *S. Typhimurium* (44.4%) and *C. jejuni* (10.5%). Significantly lower resistance to chlorine in caecal isolates of *S. Typhimurium* and no chlorine resistance in *C. jejuni* may be due to lower exposure to chlorine in the processing line. The lowest tolerance level at 40 ppm was detected in both environmental *S. Typhimurium* and *C. jejuni*, while neither *S. Typhimurium* nor *C. jejuni* was resistant to chlorine in the present study. The environmental isolates were not pre-exposed to chlorine and were not exposed to various stresses, such as temperature and osmotic stress. This result further demonstrates that prior exposure to chlorine induces chlorine resistance in foodborne pathogens.

Sub-lethally injured foodborne pathogens can be resuscitated under appropriate enrichment conditions.^{18,39} The lowest resuscitation percentage in the present study was observed in both *S. Typhimurium* (16.6%) and *C. jejuni* (14.2%) isolated from environmental samples, while the highest resuscitation rate was observed in carcass-washing isolates, at 52.4% and 58.5%, respectively. The environmental isolates were not previously exposed to chlorine, and they might not have developed adaptive stress tolerance compared to isolates that were already exposed. The highest percentage of both environmental *S. Typhimurium* and *C. jejuni* were not resistant to chlorine in the present study. Therefore, a lower chlorine concentration would be sufficient to cause lethal cell injury. The lethally injured bacteria could not be resuscitated, even under favorable conditions.¹⁸ However, the isolates from carcass washings have previously been exposed to chlorine

in the chill tank and may be sub-lethally injured. It has been shown that, with increased chlorine concentration, resuscitation can also be increased in the presence of high organic matter content.¹⁸ The resuscitation rate of *C. jejuni* is higher than that of *S. Typhimurium* in carcass washing isolates. This may be due to strain-dependent variation in the stress response mechanism.²⁰ The induced chlorine resistance limits the clearance of bacterial load and allows the persistence of *S. Typhimurium* and *C. jejuni* in the food chain.^{20,25} The studies have shown that the incidences of foodborne disease are mostly linked with the consumption of chicken meat and the potential for the emergence of SH-resistant foodborne pathogens.⁴⁰ Interestingly, the present study demonstrated that higher resistance was detected following pre-exposure to higher chlorine levels in the chill tank. Therefore, it was highlighted that increasing chlorine concentration would not be effective in mitigating foodborne pathogen contamination. Moreover, higher chlorine concentration in the chill tank increases residual chlorine levels in chicken meat, posing a major public health risk.¹⁵ Furthermore, sub-lethally injured foodborne pathogens in post-spin-chill carcasses might exhibit greater stress tolerance and virulence, including increased intestinal invasiveness, posing a serious public health risk.^{25,41} Moreover, exposure to chlorine might induce cross-protection to acid tolerance and heat tolerance. Furthermore, prolonged exposure to disinfectants or excessive use of chlorine disinfectants poses a global risk of antibiotic-resistant bacteria proliferation, endangering human health.^{31,42,43}

5. Conclusion

To our knowledge, this is the first study to demonstrate chlorine tolerance levels and chlorine resistance profiles in *S. Typhimurium* and *C. jejuni* in the processing line. The present study revealed that the resistance to chlorine in *S. Typhimurium* and *C. jejuni* is significantly high. Pre-exposure to high chlorine concentrations has further increased the chlorine resistance and resuscitation. Environmental isolates were highly susceptible to chlorine and showed the lowest resuscitation. The results of the current study highlighted the major limiting factor of using chlorine in the elimination of *S. Typhimurium* and *C. jejuni* from the poultry processing line. Future research on the effectiveness of alternative chemicals, such as PAA and ASC, with the risk assessment is crucial to reduce the persistence of foodborne pathogens in the poultry processing line.

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Conflict of interest

The authors declare they have no competing interests.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Further disclosure

The paper has been uploaded to or deposited in a preprint server (Preprints.org; doi: 10.20944/preprints202501.0734.v1).

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