

Effect of ozone treatment on thermo tolerant *Campylobacter* contaminated poultry products retailed at Baghdad markets/Iraq

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ABSTRACT

Campylobacter is the primary cause of bacterial gastrointestinal diseases in humans. While *Campylobacter coli* (*C. coli*) is responsible for around 5% of cases, *Campylobacter jejuni* (*C. jejuni*) accounts for more than 90% of cases. Human campylobacteriosis can occur mostly by handling raw chicken carcasses improperly or eating undercooked chicken. There is still a significant rate of *Campylobacter* detection in carcasses after chilling or freezing, even though there are controls in place to reduce cross-contamination. To determine whether or not ozone (0.5 ppm) at refrigeration temperature for 1/2 and 1 hr effectively killed or reduced *Campylobacter* on chickens, this investigation was conducted. Thirty samples tested positive for *Campylobacter* and were exposed to ozonated water. After treatment, the percentage of negative samples was 10% after 30 minutes and 63.33% after 60 minutes, according to the data. From a public health perspective, the decline in positive percentage samples was quite noteworthy. The results demonstrated that after 60 minutes at refrigeration temperature, the bacterial counts decreased by 2-4 Log₁₀ (CFU/mL). Also, treatments with ozone for 30 and 60 minutes were tested for their ability to kill *Campylobacter*. The results showed that 63.33% and 100% of the samples were negative after the treatments. Importantly, this proves that ozone effectively killed *Campylobacter* even when organic material concentrations were high, which has important implications for public health.

Keywords: Ozone, *C. jejuni*, *C. coli*, Chicken meat, Refrigeration temperature

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INTRODUCTION

According to **Kanaan (2024)**, a zoonotic disease is one that spreads from animals or animal products to humans. Due to the thermotolerant *Campylobacter* species, *Campylobacter* enteritis is the only kind of campylobacteriosis that is of significant public health concern globally. Species of *C. jejuni* and *C. coli* predominate (**Kanaan, and Khashan, 2018**). While *C. coli* accounts for 5-10% of human illnesses in the US, *C. jejuni* accounts for the vast majority (**Hadi Ghaffoori et al., 2022**). While there are a number of ways in which *Campylobacter* spp. can infect people, the most prevalent one is by eating infected chicken (**Kanaan, 2024**). Additionally, tainted raw meat and prepared meals can cross-contaminate each other (**Hoelzl et al., 2013**). Large outbreaks of campylobacteriosis are typically linked to contaminated drinking

water or raw or tainted milk (**Kanaan and Mohammed, 2020**), however, this is not the sole dietary carrier for *Campylobacter*. The presence of harmful germs in internal organs, skin, and utensils makes meat vulnerable to contamination during processing procedures. Contamination with feathers/skin and fecal matter from the birds' internal organs ruptures during processes like scalding, plucking, and evisceration (**Kanaan et al., 2021; Kanaan, 2021; Kanaan et al., 2022; Kanaan et al., 2023; Kanaan, 2023**). Cooling, vapor-vacuum systems, and vapor pasteurization are among the processing methods used to remove bacterial contamination from chicken meat (**A EL DAHSHAN et al., 2013**). There are a number of chemicals that are utilized for decontamination, including organic acids, ozone, peracetic acid (PAA), trisodium phosphate, and chlorine and chlorine compounds (**Kanaan, 2018**). The sanitation of chicken operations frequently makes use of chlorine. From both an environmental and food safety perspective, chlorine produces a number of hazardous byproducts (**Hecer et al., 2007**). Scientists in the food industry are exploring potential new cleaning and sanitizing chemicals that can prevent food spoilage and harmful microorganisms without harming people or the environment. The chicken business is interested in these efforts to find chlorine replacements for use in poultry plants, especially chillers (**A EL DAHSHAN et al., 2013**). Natural ozone (O₃) has piqued the interest of food scientists looking for a substitute for traditional disinfectants (**Muhlisin et al., 2015**). The food sector makes extensive use of this gas for a variety of sterilizing purposes, including but not limited to: bacterial and viral inactivation, deodorization, bleaching (discoloration), organic matter breakdown, mycotoxin destruction, and many more (**Cárdenas et al., 2011; Cho et al., 2014; Muhlisin et al., 2016**). Despite the fact that it breaks down quickly into oxygen, it does not leave any dangerous residues in or on treated items (**Kanaan, 2018**). Consequently, O₃ is thought to be the best choice for eliminating potentially harmful bacteria from food (**Gabler et al., 2010**). Chicken meat is widely consumed in Iraq and is a major vector for campylobacteriosis. This study has been carried out to evaluate the impact of O₃ treatment on the microbiological safety of chicken meat contaminated with thermotolerant *Campylobacter* that was found in Baghdad governorate.

MATERIALS AND METHODS

Isolation, identification and Confirmation of *Campylobacter*

Twenty *C. coli* and ten *C. jejuni* samples, totaling thirty, were taken from prior research (**Kanaan and Khashan, 2018**). The samples were then exposed to O₃ treatment (0.5 ppm) for ½ and 1 hr at 4°C to see if the bacteria could be reduced or eliminated from the chicken flesh. Colony isolation and identification followed standard bacteriological protocols as previously described (**Ghaffoori, 2017; Kanaan and Khashan, 2018; Hadi Ghaffoori et al., 2022**), as part of the experiment, which involved analyzing the organism's shape and movement using a microscope, testing its catalase and oxidase activity, creating hydrogen sulfide (H₂S), growing it in an aerobic environment at different temperatures, and hydrolyzing hippurate. To tell *Campylobacter* isolates apart from other Gram-negative bacteria, they were put through more biochemical tests with the Oxoid Biochemical Identification System (O.B.I.S.) Campy (Oxoid, ID0803M, UK) system, which can find L-alanyl amino peptidase and has Gram lysis built in. A multiplex PCR experiment was used to confirm the species of the isolates, as mentioned earlier (**Kanaan, and Khashan, 2018**).

Ozone treatment

Determination of ozone concentration (ppm) using Ozone CHE Mets® Kit (U.S.A), (K-7404/R-7404: 0-0.6&0.6-3ppm)

After filling the empty sample cup with five drops of A-7400 activator, the concentration of ozone (in parts per million) was measured using the technique outlined by **Mohammed *et al.* (2022)**. The amount of material to be examined was added to the sample cup until it reached the 25 ml level. The next step was to snap the top of the ampoule and insert it through the sample cup. We used a sample solution to fill the ampoule. In order to blend the ampoule, it was turned upside down many times, letting the bubble go from one end to the other. After drying, the ampoule was given a minute to acquire its color.

The relevant comparator was used to reach the result:

- A- Place the flat end of the ampoule into the comparator, which is part of the A-Low Range Comparator. From its lowest position, the comparator was held up to a light source. To find the optimal color match, the comparator was spun around.
- B- In order to find the optimal color match, with the help of the high range comparator, the ampoule was placed between the color standards (**Mohammed *et al.*, 2022**).

Calculation of ozone concentration output (ppm/in water) of the ozone generator

We used the CHE-Mets-Kit to test the ozone concentration (ppm) in the water generated by the ozone generator. The 1.5 liters of tap water were placed in a plastic container and sealed with its lid. A hole in the lid of the plastic container was used to insert the aeration stone. A total of four exposure durations five, ten, fifteen, and thirty minutes were selected. Replacement of the tap water and flushing of the plastic container with new tap water followed each exposure time, and the process was then repeated. Of the four intervals used, 15 and 30 minutes produced the greatest concentration in water, at 0.5 ppm (**Mohammed *et al.*, 2022**).

The effect of ozonated water (0.5ppm) on *Campylobacter*

Every sample that tested positive was placed in ozonated water with a concentration of 0.5 ppm for 30 to 60 minutes at 4°C. This experiment made use of a plastic container. With the help of an aeration stone (diffuser) and one ozone gas entrance port on the plastic cover, the gas could be injected into the water and distributed uniformly (micro-bubbled). One liter of compressed air per minute (600 mg/hr) was used as feed gas for the ozone generator. The samples were placed in ozonated water after being thawed in the fridge at 4°C for the night. In order to determine the impact of ozonation on *Campylobacter*, the chicken flesh samples were exposed to the ozonated water for 30 and 60 minutes. Within three hours after collection, all samples were washed in 200 ml of BPW by vigorously shaking them in a sterile plastic bag for one minute. To clean the bag, we angled it so the rinse liquid could flow to one corner, held the sample back to disinfect the bottom back corner with 70% ethanol, and rinsed it with sterile water (**Kanaan, 2018**). After the carcass was aseptically sliced in half, 25 milliliters of the rinse were transferred to a clean bottle with 100 milliliters of Preston broth using a disposable syringe. The mixture was pre-enriched for 4 hours at 37°C in a microaerophilic jar, and then enriched for 24 hours at 42°C. Before being enriched at 42°C for 24 hours, 1 ml of ozonated water was inoculated into 5 ml of (double strength) Preston broth and left to incubate for 4 hours at 37°C in an anaerobe jar under microaerophilic conditions. To get individual colonies after enrichment, sterile loops were used to streak one loopful of media onto Preston agar in parallel. For 24 to 48 hours, each plate was placed in an anaerobic jar with microaerophilic conditions at 42°C. The bacteria were counted using the Miles and Mizra method (**Miles *et al.*, 1938**). This method is used to determine the number of colony forming units in a bacterial suspension, or homogenate. To do this, sterile BPW tubes were used to dilute a series of decimal ten dilutions of enrichment broths (1 mL of broth per 9 mL of Buffered Peptone Water (BPW)). The dilutions were made to a concentration of at least 10⁻⁸, and the mixture was thoroughly mixed using a vortex mixer for 30 seconds. For each dilution series, one plate of Preston agar was needed,

and the surface of the plates had to be dry enough for a 20 µl drop (0.02 ml) to be absorbed in 15-20 minutes. Each plate was marked with the dilution. The next step was to drop 5 × 20 µl of the suitable dilution onto the agar surface of each plate and let the drop spread organically. Prior to inversion and incubation in an anaerobic jar under microaerophilic conditions at 42°C for 24 - 48 hours, the plates were allowed to dry upright on the bench for 15-20 minutes. Every plate was examined for growth, and the drops with the most full-size, individual colonies were considered to have the most colonies. Typically, drops with 10–20 colonies were considered to have the maximum number of colonies. By adjusting for the low of Miles and Mizra (Miles et al., 1938), the microbial load log titers were calibrated.

CFU per ml = Average number of colonies for a dilution × 50 × dilution factor (Miles et al., 1938).

Data analysis

MedCalc Software BVBA version 22.019 (BE, USA) was used to analyze the data. The proportion was utilized as a descriptive statistic. To find out what the difference was between the percentages before and after treatment with aqueous ozone, two sets of Chi-square tests were used.

RESULTS AND DISCUSSION

Our results (Table 1) indicated that three samples (or 10% of the total) showed no bacterial growth on the agar surface after being treated with ozonated water (0.5 ppm/30 minutes) at refrigeration temperature, whereas twenty-seven samples (or 90% of the total) tested positive for *Campylobacter* (more than 100 colonies/plate). According to Table 1 and Figure 1, 19 samples (63.33%) tested negative for *Campylobacter* following treatment with ozonated water (0.5 ppm/30 minutes) at refrigeration temperature, whereas 11 samples (36.7%) tested positive. Statistically, there was a significant difference ($P \leq 0.05$) between the 30 and 60-minute ozone treatment efficacy ($P < 0.0001$; $X^2=18.065$) according to the data (Table 1).

Table 1: Effect of ozonated water treatment on *Campylobacter* isolates recovered from chicken meat

Ozonated water treatment (0.5 ppm)/ 4° C							
30 min				60 min			
No. of samples 10 (%)		No. of samples 20 (%)		No. of samples 10 (%)		No. of samples 20 (%)	
<i>C. jejuni</i>		<i>C. coli</i>		<i>C. jejuni</i>		<i>C. coli</i>	
+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
8 (80)	2 (20)	19 (95)	1 (5)	4 (40)	6 (60)	7 (35)	13 (65)
Total –ve samples for <i>Campylobacter</i>		3 (10)		19 (63.33)			
P value		P < 0.0001		$X^2=18.065$			
-ve = No bacterial growth on agar surface							
+ ve (30) = More than 100 colonies / plate							
+ ve (60) = Less than 20 colonies / plate							
<i>C. jejuni</i> = <i>Campylobacter jejuni</i>							
<i>C. coli</i> = <i>Campylobacter coli</i>							

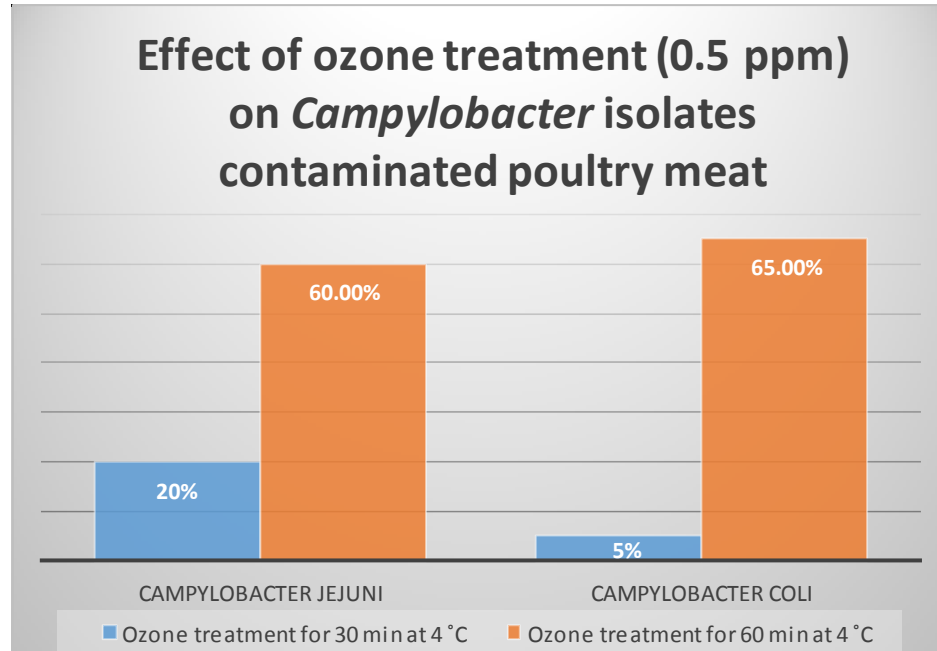


Figure 1. Effect of ozone treatment (0.5 ppm) on *Campylobacter* isolates contaminated poultry meat

In Table 2, we can see that after 60 minutes of treatment at 4°C, the number of bacteria dropped by 2–4 Log₁₀ (CFU/mL). This shows that ozonated water (0.5 ppm) is effective at killing bacteria because it lowers the *Campylobacter* count (Log₁₀/mL).

Table 2: Antimicrobial efficiency of ozonated water on the reduction of *Campylobacter* count (Log₁₀ / ml)

Positive samples	Log ₁₀ / ml count after ozone treatment (0.5 ppm)		Log ₁₀ Decreased
	30 min	60 min	
+Ve sample <i>C. jejuni</i>	1.2 × 10 ⁷	1.6 × 10 ⁵	2
+Ve sample <i>C. jejuni</i>	1.1 × 10 ⁶	1.1 × 10 ²	4
+Ve sample <i>C. coli</i>	1.2 × 10 ⁶	1.2 × 10 ²	4
+Ve sample <i>C. coli</i>	1.4 × 10 ⁶	1.1 × 10 ³	3

Campylobacter jejuni= *C. jejuni*; *Campylobacter coli*= *C. coli*

After treating the ozonated water with meat drip with chicken for 30 and 60 minutes, 63.33% and 100% of the samples tested negative for *Campylobacter*, which means there was no bacterial growth on the agar surface (Table 3).

Table 3: The potency of ozonated water contained meat drip against *C. jejuni* and *C. coli* isolates recovered from chicken meat

Samples	No. of samples tested	Potency of ozonated water (0.5 ppm)	
		-Ve after 30 min	- Ve after 60 min
+Ve samples with <i>C. jejuni</i>	10	7	10
+Ve samples with <i>C. coli</i>	20	12	20
Total		19/30 (63.33%)	30/30 (100%)

-Ve = No bacterial growth on agar surface; *Campylobacter jejuni*= *C. jejuni*;
Campylobacter coli= *C. coli*

Discussion

The oxidation process of the cellular membrane, which has been the principal target, may be seen in the killing of bacteria by ozone (Patil, 2010). According to Cárdenas *et al.* (2011), an ozone treatment at a concentration of 257 ppm O₃ / m³ had a negative effect on a circular beef sample taken from a carcass at 5°C on the day it was stored. The initial inoculation of 50 µL of 10⁸ CFU/mL had been decreased by 0.65 Log₁₀ CFU/g of *E. coli* O157:H7. Treatment of samples with ozonated water reduced the number of viable bacterial cells, as measured by CFU, according to this study's results. Also, at the same concentration (0.5 ppm), the number dropped as the exposure time to ozonated water increased. This might be because the inactivation rate of the microorganisms decreased as the exposure time increased since the ozone had more opportunity to come into contact with them. In any case, ozone treatments were carried out at refrigeration temperature, and as a general rule, ozone's antibacterial efficacy diminished as temperatures rose. The solubility and stability of ozone decrease with rising temperatures, and its breakdown rate also rises. Lowering the temperature of the liquid enhances the solubility and stability of ozone, which in turn increases its availability and efficacy (Patil, 2010). This study's fundamental findings were consistent with those of Jindal *et al.* (1995) when it came to the effectiveness of ozone treatment (0.44 - 0.54 ppm) at 1-3 °C in enhancing the microbiological safety and prolonging the storage life of broiler drumsticks during immersion chilling. On top of that, they found that ozone cut aerobic plate count, coliforms, and *E. coli* levels on broiler drumsticks by 95.5%. However, according to research by Al-Haddad *et al.* (2005), chicken carcasses stored at 7 °C with gaseous ozone applied for up to 30 minutes reduced the number of *Salmonellae* by 97%. The researchers suggested that this treatment could be used to lessen the contamination of chicken carcasses with *Salmonellae* and increase their shelf life. In another study, Hecer *et al.* (2007), compared the effectiveness of two antimicrobial treatments ozone at 1.5 ppm and chlorine at 30 ppm on broiler carcasses following evisceration. The results showed

that ozone had an average effect of 97.77% against *E. coli*, while chlorine had an effect of 57.9%. However, when it comes to the amount of *Staphylococcus/Micrococcus* bacteria, ozone has an average effect of 81.33% and chlorine has an effect of 50%. In their investigation, **Cho et al. (2014)**, found that when inoculated ground beef samples were exposed to ozone (214 ppm O₃/m³) at 4°C for three days, the initial concentrations of *E. coli* O157:H7 were reduced by 0.53 Log₁₀ CFU/g on 1 day, compared to the samples that were not exposed to ozone. Since most pathogen contamination starts on the surface of meat, they reasoned that ozone exposure would be an effective antibacterial treatment for meat items kept in the fridge, since it killed the *E. coli* O157:H7 bacteria there.

From a public health perspective, the large drop in Log₁₀ (2-4) is warranted since even tiny quantities of *C. jejuni* (less than 500 cells) can cause widespread sickness if an infected product is sold. This study's findings corroborated those of **Wongsadee et al. (2008)**, who investigated the bacteriocidal effects of ozone on the survival of *C. jejuni* ATCC 29428 and found that *C. jejuni* exposed to ozone treatment for 0.5, 1, 2, 3, 5, and 7 minutes at concentrations of (0.03, 0.06, 0.12, 0.18, 0.30, and 0.42) mg/L in water reduced the survivals of *C. jejuni* by 0.5, 1.4, 4.3, 4.6, 5.1, and 5.6 Log₁₀ CFU/mL, respectively. The results showed that after 9 minutes of exposure to ozone at a concentration of 0.54 mg/L, the number of *C. jejuni* bacteria had decreased to less than the detection limit (1 Log₁₀ CFU/mL), a decrease of 7 Log₁₀ CFU/mL compared to the starting count (8 Log₁₀ CFU/mL). Since ozonated water was used as an intervention against *C. jejuni* contaminated chicken carcasses, the presence of organic matter in the water influences the disinfection rate as microorganisms compete with them for disinfectant. This could explain why the bacterial reductions found in this study were lower. Therefore, compared to demand-free systems, ozone inactivation of microbes in organic matter required longer contact durations and higher dosages. In line with the findings of **Potts et al. (2011)** who came to the conclusion that the plasma ozone system shows significant potential for large-scale food treatment, this study also found very encouraging results, especially for skin-off chicken breasts exposed to different concentrations of ozone, such as 1.2 and 3 mg for 30 seconds. *C. jejuni* was found to be 0.5 and 1.0 Log₁₀, respectively, following ozone treatment of 1.2 mg of skinless breasts and breasts with skins. Following 30 seconds of exposure to 3 mg of ozone, *C. jejuni* was completely killed (1.2 and ≥ 2.5 Log₁₀, respectively), while all other species showed significant decreases, including a total viable count decrease of 99.995% and a drop of 4.3 Log₁₀ in the number of organisms.

This study demonstrated that aqueous ozone (0.5 ppm) was successfully eliminated. The presence of *Campylobacter* in carcass drip increases the reactivity of ozone, which makes it more effective at sterilizing water from all types of bacteria and viruses. Another benefit, if proper microbiological controls are in place, is that the water that has been disinfected with ozone can be reused for initial cleaning stages, either directly or after re-ozonation, to achieve the desired quality. **Chang and Sheldon (1989)**, found that ozonation and diatomaceous earth (DE) filtration were excellent ways to prepare poultry process fluids for recycling. In addition, these reconditioning practices can effectively reduce the organic loads of wastewater that are sent to treatment facilities. They discovered that ozonation produced the best water quality, with a total microbial load including a 99.9 percent reduction and significant reductions in total solids. Consistent with previous research, this study found that ozonated water with a concentration of 4-12 ppm and 30 minutes of cooling in an immersion chiller with 4 ppm of ozone effectively reduced the total bacterial count and eliminated all pathogens from eviscerated chickens. The

researchers concluded that properly filtered chiller water can be safely recycled, reducing the need for refrigeration energy and water. **Graham et al. (2002)** also found that chiller overflow water pre-washed with 4-8 ppm ozone could be reconditioned after filtering, and they showed that pathogens like *Campylobacter*, *Clostridium perfringens*, and *Staphylococcus* could be eliminated after ozone treatment and chiller water filtration. Thus, the results of this study concur with their findings. In addition, they mentioned that using 1/4 gallon of makeup water per bird kept the pilot chiller bath water at 4°C and 2-4 ppm ozone, which was comparable to a commercial three-stage chlorinated chiller in terms of microbial counts. They also mentioned that using tap water with 6-8 ppm ozone for the final rinse of the birds after they came out of the chiller further reduced microbial counts. Furthermore, it was discovered by **Perkins et al. (2005)**, that treated water from poultry processing plants can be beneficially reused after removing solids, fats, oils, treated effluent greases, animal proteins, and pathogenic organisms. This treated water can also be used as bird chiller makeup or sent to other locations for maximum volumetric reuse (**Kanaan and Abdullah, 2021**).

CONCLUSION

The results show that under the conditions of this study, O₃ at a concentration of 0.5 ppm and an exposure time of 60 minutes significantly reduced the number of *Campylobacter* positive samples and the bacterial counts of the meat rinse samples by 2-4 Log₁₀ (CFU/mL). From a public health standpoint, this decrease is really noteworthy. Furthermore, after 30 and 60 minutes of treatment, ozonated water (0.5 ppm) successfully eradicated *Campylobacter* isolates from chicken carcasses, and drip had no influence on its effectiveness.

Competing Interests

The writers have been clean about any potential conflicts of interest.

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