

## Shelf-life extension and *Campylobacter jejuni* inhibition by application of zinc oxide nanoparticles in chicken fillets



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### ABSTRACT

**Objective:** study the effect of Zinc oxide nanoparticles on shelf life extension of chicken meat fillets and elimination of *Campylobacter jejuni*.

**Design:** Experimental study.

**Samples:** Nine kg of fresh chilled chicken fillets divided into 45 chicken fillets (each sample weight 100-120 g) were collected from various poultry shops in Benha City.

**Procedures:** *Campylobacter jejuni* was intentionally inoculated into broiler breast fillets, followed by the application of zinc oxide nanoparticles (ZnO NPs) suspension with varying concentrations (5 mM and 10 mM) with 3 different sizes (25 nm, 50 nm, and 75 nm) for each concentration and storage at 4 °C.

**Results:** The results showed that ZnO suspensions had a strong inhibitory effect on *Campylobacter jejuni* growth after 8 days in the refrigerator at 4 °C. Among the concentrations used of 5 and 10 mM ZnO NPs, the higher antibacterial activity with a subsequent marked reduction in *Campylobacter jejuni* was concentration-dependent. Additionally, ZnO NPs had a significant impact on the virulence genes of *Campylobacter jejuni* (*cdtA*, *cdtB*, and *cdtC*), especially at diameters of 25 and 50 nm on one side and 10 mM on the other side. ZnO NPs have the strongest impact due to their tiny size.

**Conclusion and clinical relevance:** ZnO NPs can eliminate *Campylobacter jejuni* from chicken meat and extend the shelf life of chicken fillets without affecting its sensory characteristics.

**Keywords:** Broiler; breast fillets; ZnO nanoparticles; *Campylobacter jejuni*; virulence gene

### INTRODUCTION

Chicken meat comprises a substantial source of high-quality protein source in most countries. Chicken is rich in essential amino acids along with vitamins and minerals. Lean chicken contains more protein than the same amount of lean roast beef and the prices of chicken meat are lower than those of beef, pork, or mutton [1].

Chicken is a white meat when compared with darker meat, it comes with less fat and more protein. As a result, people looking for a healthy diet decide to consume poultry meat. Breasts are the best parts of chicken because they are quite low in fat. Further, chicken breasts or fillets are easy to cut, chop, and slice as well as they don't require a lengthy or complex preparation before cooking. Besides, chicken breasts also help to boost our energy, and since they are not heavy, we can perform more productively, whether at work or when practicing a sport. Therefore, chicken breasts are an ideal food item because not only they are healthy, but they are convenient, too [2].

Chicken fillets act as a potential source of hazards as consumers stored it in the refrigerator for many days and continuously use it for several days so the aim of our work is to search for natural, safe preservatives as nanoparticles to extend the shelf life and safety of the product for human consumption. We apply our experiment for improving and extending shelf life and the possibility of

reducing the number of *Campylobacter jejuni* as a major pathogen contaminating the chicken fillets.

*Campylobacter* species are motile, Gram-negative, slender curved or spiral rods, appearing vibroid, and are microaerophilic, best growing in a gaseous atmosphere of approximately 5–10% oxygen, 10% carbon dioxide and 85% nitrogen. Although many animal species harbor *Campylobacter* in their intestinal tract, wild birds and domestic poultry are the most important reservoirs and considered as the largest potential source of *Campylobacter* for human and the high optimum growth temperature of *C. jejuni* and *C. coli* could be an adaptation to the higher body temperature of birds [3]. High levels of *Campylobacter* isolation from retail chicken have been previously reported in both industrialized and developing countries [4-6]. The most prevalent cause of diarrhea is campylobacteriosis which is caused by a bacterial *Campylobacter jejuni* infection in the human body. Gram-negative spiral bacterium *Campylobacter* causes damage to the small intestine and colon. Bloody diarrhea, vomiting, abdominal pain, and fever are all symptoms of this pathogenic bacterium [14].

*Campylobacter jejuni* is a disease-causing bacterium that produces a cytolethal distending toxin, which prevents cells from dividing and activates the immune system *Campylobacter jejuni* is able to avoid the small intestine and colon as a result of this. Consumption of Raw or

undercooked poultry, unpasteurized dairy, polluted water, food, and post-cooking contaminated chicken products and animal or human excrement can all spread *Campylobacter* [15].

the forerunner of a surge of developments that could rock the agro-food business in the coming years. The creation of materials with new qualities for use as antimicrobial agents has benefited greatly from the introduction of nanotechnology, which involves the fabrication and usage of materials with sizes of up to around 100 nm in one or more dimensions has brought great opportunities for the development of materials with new properties for use as antimicrobial agents [16].

The increase in relative surface area that happens as particle size decreases down to the Nanoscale leads to these innovative and enhanced material characteristics. When compared to the identical material at the macro or micro scale, nanoscale materials are also more physiologically active [17]. Inorganic and organic materials can be used to make nanomaterials, although nanosized inorganic compounds have high antibacterial action at low concentrations [18].

ZnO has emerged as a viable antimicrobial alternative among antibacterial inorganic compounds. In addition, ZnO has an advantage over all other metal oxides, such as titanium oxide, in that its activity is not photo-activated and it has a long shelf life [19]. Zinc oxide nanoparticles (ZnO NPs) are non-toxic, biocompatible, and biosafe, and have been found in a variety of biological applications in everyday life, including medicine delivery, cosmetics, and medical equipment [20].

When a material's size is reduced from a micrometer to a nanometer, it exhibits improved qualities such as improved diffusivity and chemical reactivity, as well as improved biological properties. In general, NPs can be added to food directly as an addition or indirectly through food packaging [21]. Only a few publications have been published on the antibacterial properties of ZnO NPs in food. As a result, the primary goal of this research is to use ZnO NPs of various sizes and concentrations to increase the shelf life of chicken meat while also lowering *Campylobacter jejuni* which represents a Gram-negative bacterium that can contaminate such food. The effect of ZnO NPs on virulence genes (*cdtA*, *cdtB*, and *cdtC*) was also investigated.

## 2. MATERIAL AND METHODS

### 2.1. Collection of samples

Accurately, 9 Kg of fresh chilled chicken fillets divided into 45 chicken fillet (each sample weight 100-120 g) were collected from various poultry shops in Benha City, Kalyobia Governorate, Egypt. The experimental work was applied to determine the bactericidal effect of ZnO NP untreated control group and ZnO NPs treated samples using different concentrations (5% and 10%) and sizes (75nm, 50nm, and 25nm).

### 2.2. *Campylobacter jejuni* strain preparation

The food isolates of *Campylobacter jejuni* was obtained from the Food Analysis Center Faculty of Veterinary Medicine, Benha University. *Campylobacter* cefax agar was used to culture the strain. Five colonies of the tested strain were carefully selected and inoculated into tubes containing 0.1 percent sterile peptone water (5 ml), which were then cultured at 37 °C for 24 hours before being used to make dilutions up to 10<sup>8</sup>. Furthermore, the cell concentration was determined by cultivating the dilutions on *Campylobacter* cefax plates. By using the tube dilution procedure, the cell count was adjusted to 5 x10<sup>6</sup> CFU/ml [22].

### 2.3. Preparation of Zinc Oxide nanoparticles

Actually, ZnO nanoparticles with sizes of 25 nm, 50nm, and 75 nm were purchased from NanoTech Egypt for Photo-Electronics according to the NT-ZONP brand with a certificate of analysis. ZnO nanoparticles are white powder, spherical shape, and stable colloid in a mixture of methanol, chloroform, and water. To obtain a homogenous solution of nanoparticles at different concentrations (5mM and 10 mM), 150 ml distilled water were added to each concentration of nanoparticles in glass containers. Then, the resulting homogenous suspensions were autoclaved for 30 minutes to be sterilized [23].

### 2.4. Inoculation of chicken fillets with the *Campylobacter jejuni*

The fillet samples were dipped in 150 ml of sterile peptone water (0.1%) contaminated with *C. jejuni* at a concentration of 5 x10<sup>6</sup> CFU/ml for 15 minutes at room temperature (25°C). The fillet samples were left at room temperature for 30 minutes after dipping to allow bacteria to be attached and absorbed [24].

### 2.5. Application of ZnO Nanoparticles

Each tested sample was dipped for 15 min in the zinc oxide nanoparticles (ZnO NPs) suspension with varying concentrations (5 mM and 10 mM) with 3 different sizes (25 nm, 50 nm, and 75 nm) then drained well for 5 min on a sterile stainless wire mesh screen. The control group was dipped in sterile distilled water. The inoculated samples with known *Campylobacter jejuni* count (5 x10<sup>6</sup>) were divided into 7 groups (100 gm of each) as follows:

Group 1: 100g chicken fillet + 150 ml Distilled water (Control)

Group 2: 100g chicken fillet + 150 ml of 5 mM ZnO NPs (25 nm)

Group 3: 100g chicken fillet + 150 ml of 5 mM ZnO NPs (50 nm)

Group 4: 100g chicken fillet + 150 ml of 5 mM ZnO NPs (75 nm)

Group 5: 100g chicken fillet + 150 ml of 10 mM ZnO NPs (25 nm)

Group 6: 100g chicken fillet + 150 ml of 10 mM ZnO NPs (50 nm)

(Group 7 100g chicken fillet + 150 ml 10 mM ZnO NPs) (75 nm)

The previous control and treated chicken fillet samples were labeled and each single sample was separately packaged in polyethylene bags and stored at 4°C till analysis.

All tested samples of such groups either control or treated were subjected to sensory and chemical assessment at zero time (within 2 hours after treatment) then periodically every 2 days until decomposition appear in each group (zero, 2, 4, 6 and 8 days). The scheme was replicated for 5 times.

### 2.6. Sensory examination

The examined samples of chicken fillets were analyzed for the quantification of the final sensory profile according to procedures of World's Poultry Science Association [25]. Five trained panelists applied the proposed organoleptical method of raw chicken meat analysis. The different attributes were quantified on a rating scale from 1 to 3. The sensorial analyzed attributes including external aspect, odor, color and muscular elasticity and the overall acceptability was determined. Further, the overall acceptability as well as *C. jejuni* count was conducted at 0, 2, 4, 6 and 8 days.

### 2.7. Bacteriological examination:

Enumeration of *C. jejuni* was performed using *Campylobacter* cefax plates according to the technique [22].

### 2.8. Polymerase Chain Reaction (PCR)

Application of multiplex PCR for identification and characterization of cytological distending toxins as virulence genes of *C. jejuni* represented by *cdtA*, *cdtB* and *cdtC* was carried out using the primer sets described in **table (1)**.

#### 2.8.1. DNA Extraction using QIA amp kit

Boiling extraction method [27] was used to extract DNA from *Campylobacter* isolates. Briefly, a few colonies, taken from pure cultures were transferred into Eppendorf tubes containing 300µl ultrapure distilled water. Equal amounts of TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)] were added to the tubes and the suspension was boiled at 100°C for 10 minutes and centrifuged for 10 min at 12000 rpm afterwards. The supernatant fluid was used as a PCR template and frozen at -20 °C until needed further use.

#### 2.8.2. Amplification of the selected virulence genes:

Actually, 40 µl of PCR mixture were prepared. All reactions contained appropriate concentrations of 3 primer sets, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 1 × Ex *Taq* DNA polymerase buffer, and 1.0 U of Ex *Taq* DNA polymerase in a 40-mL reaction volume. The PCR cycling protocol [28] was applied as following; An initial

denaturation at 94°C for 60 sec, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. Finally, 5 µl of each amplicon was electrophoresed in 2 % agarose gel stained with ethidium bromide and visualized on UV transilluminator. A 100 bp DNA ladder was used as a marker for PCR products. The obtained results were statistically evaluated by application of Analysis of Variance (ANOVA) test according to [29].

## 3. RESULTS

Overall acceptability, as recorded in **Table (2)**, of chicken fillets treated with 5 mM Zinc oxide nanoparticles of different nano-sizes during cold storage at 4°C results revealed spoilage of the untreated control group on the 4<sup>th</sup> day of refrigeration; while the treated groups with ZnO nanoparticle (75, 50 and 25 nm size) showed fair, fair and medium organoleptic score with mean values of 4.0, 4.7 and 5.2 at the 8<sup>th</sup> day of the refrigeration. Indicating the lower nano-size gave better sensory quality over the experimental period.

On the other hand, the overall acceptability, as recorded in **Table (3)**, of chicken fillets treated with 10 mM Zinc oxide nanoparticles of different nano-sizes during cold storage at 4°C results revealed spoilage of the untreated control group on the 4<sup>th</sup> day of refrigeration; while the treated groups with ZnO nanoparticle (75, 50 and 25 nm size) showed medium, medium and good organoleptic score with a mean value of 5.2, 5.7 and 6.0 at the 8<sup>th</sup> day of the refrigeration. Indicating the increasing concentration of the used nanomaterial gave better sensory quality over the experimental period.

Regarding the inhibitory effect of ZnO nanoparticles on the experimentally inoculated *Campylobacter jejuni* ( $5 \times 10^6$  CFU/g) in chicken fillet samples; recorded results in Tables (4 and 5) showed greater reduction percent with increasing ZnO nanoparticle concentration of lower nano-size; where the mean counts of *Campylobacter jejuni* after addition of 5 mM nano-ZnO with nano-size of 75, 50 and 25 nm 8<sup>th</sup> day of refrigeration were  $1.8 \times 10^5 \pm 1.6 \times 10^5$ ,  $1.4 \times 10^5 \pm 0.5 \times 10^5$  and  $7.3 \times 10^4 \pm 1.6 \times 10^4$  CFU/g with reduction percent of 96.4, 97.2 and 98.5%. Moreover, the mean counts of *Campylobacter jejuni* after the addition of 10 mM nano-ZnO with nano-size of 75, 50, and 25 nm on the 8<sup>th</sup> day of refrigeration were  $9.9 \times 10^4 \pm 2.1 \times 10^4$ ,  $8.1 \times 10^4 \pm 1.9 \times 10^4$  and  $2.7 \times 10^4 \pm 0.4 \times 10^4$  CFU/g with reduction percent of 98.0, 98.4 and 99.5%, respectively.

Referring to the molecular identification of some *Campylobacter jejuni* virulent genes (*cdtC*, *cdtB* and *cdtA*) in response to 10mM nano-ZnO treatment of various sizes. The evaluated samples treated with nano-ZnO of 25 nm demonstrated the absence of the examined genes after treatment, demonstrating that nano-ZnO has a direct inhibitory effect on the pathogenic genes of *Campylobacter jejuni*.

## 4. DISCUSSION

#### 4.1. Overall acceptability of chicken fillets treated with 5 and 10 mM Zinc oxide nanoparticles of different sizes during cold storage at 4°C

Inorganic nanoparticles under extreme conditions such as high temperatures and pressures remain stable. Furthermore, the majority of them are non-toxic, furthermore, they include minerals that are necessary for human health [30]. The results of **Table (2)** indicated that the overall acceptability of control and treated chicken fillet samples with 5 mM ZnO NPs were 9 "excellent grade" at zero time of cold storage at 4 °C. On the 2<sup>nd</sup> day of chilling, the overall acceptability became 4 "fair", 7.1, 7.3 & 7.7 "very good" for control, 75, 50 & 25 nm-sized ZnO NPs, respectively. Signs of spoilage appeared after 4 days of cold storage of control chicken fillet samples. However, the shelf life of such samples extended till the 8<sup>th</sup> day of cold storage, but the results at the end day of storage (8<sup>th</sup> day) cleared that the overall acceptability was 4, 4.7 "fair" & 5.2 "medium" for treated samples with 5 mM ZnO NPs at sizes of 75, 50 & 25 nm, respectively.

Concerning the application of 10 mM ZnO NPs (**Table 3**), the obtained results proved their effectiveness in prolongation of chicken fillets shelf life through enhancement of their overall acceptability to become 5.2, 5.7 "medium" & 6 "good" after 8 days of cold storage at different sizes 75, 50 or 25 nm ZnO NPs, respectively. These findings were better, to some extent than those obtained by using 5 mM ZnO NPs (Table 3). In other words, the sensorial characteristics of treated chicken fillets were improved by decreasing the size and increasing their concentrations of ZnO NPs.

The changes in the shelf life of chicken fillets when utilizing varied-sized ZnO NPs at 5 or 10 mM ZnO NPs were significant ( $P < 0.05$ ). The findings are consistent with those published by [31-34]. The US Food and Drug Administration Agency has classified zinc oxide as "generally recognized as safe" [35]. In general, ZnO NPs have a high affinity for moisture, which allows them to be added to foods and absorb moisture, hence extending the shelf life of the item. Furthermore, ZnO NPs are known to be powerful antioxidants that inhibit lipid oxidation, hence improving the quality of some foods. As a result, raising the concentration and decreasing the size of ZnO NPs improves their antioxidant activity and deactivates free radicals, extending the shelf life of chicken flesh [36].

#### 4.2. Influence of application of 5 and 10 mM ZnO NPs of different sizes on *C. jejuni* ( $5 \times 10^6$ /g) inoculated into chicken fillets during cold storage at 4°C

Results of **tables 4** and **table (5)** indicated that after 8 days of cold storage at 4°C, the effect of using 5 mM and 10 mM ZnO NPs of different sizes on *Campylobacter jejuni* ( $5 \times 10^6$ /g) applied to chick fillets was clear. The reduction percent of *Campylobacter jejuni* count was 16 %, 96.4 %, 97.2 %, and 98.5 % for control and 75, 50, and 25 nm treated chicken fillets with 5 mM ZnO. The application of 10

mM ZnO, on the other hand, resulted in reduction percentages of 16%, 98 %, 98.4 %, and 99.5 %. As a result of the application of 5 mM and 10 mM ZnO NPs of varied sizes, significant variations ( $P < 0.05$ ) developed between the investigated samples of chicken fillets. These findings were very comparable to those reported previously [37, 38].

In fact, the antibacterial characteristics of ZnO NPs are heavily influenced by the concentration, charge, size, and crystal structure of the NPs, all of which influence how they interact with bacteria. Other important elements that determine the antibacterial properties of NPs include the bacterial strain and the exposure time [39].

It is critical to note that ZnO NPs have a larger effect on Gram-positive bacteria than Gram-negative bacteria, such as *C. jejuni* whose wall is made up of lipopolysaccharides, lipoproteins, and phospholipids, which form a penetration barrier that prevents certain NPs from entering. *Campylobacter jejuni* also possesses a modest negative charge on its cell wall, allowing it to expel (reject) NPs [39, 40]. The current investigation, on the other hand, demonstrated the efficiency of ZnO NPs for controlling *C. jejuni* inoculated into chicken fillets, particularly in greater concentrations and smaller sizes, as well as during the long exposure time.

#### 4.3. PCR detection of *Campylobacter jejuni* virulence genes *cdtA*, *cdtB*, and *cdtC* and the influence of ZnO NPs application in their existence

Agarose gel electrophoresis of multiplex PCR for *Campylobacter jejuni* indicated the presence or absence of the virulence genes *cdtA* (631 bp), *cdtB* (714 bp), and *cdtC* (524 bp) as virulence genes of *Campylobacter jejuni* based on the treatment of the chicken fillets with ZnO NPs (**Figure 1 and Table 6**). Depict the effects of 10 mM ZnO NPs on *C. jejuni* virulence genes (cytological distending toxins) (6). The use of ZnO NPs with a size of 25 nm causes the gene expressions for *C. jejuni* virulence factors *cdtA*, *cdtB*, and *cdtC* to completely vanish. Using ZnO NPs with a diameter of 50 nm, the expressions of *cdtA* and *cdtB* were entirely removed. Furthermore, the use of ZnO NPs with a diameter of 75 nanometers reduced the activation of such genes. In control samples of chicken fillets, however, the expression of the *cdtA*, *cdtB*, and *cdtC* genes was obvious.

The antibacterial activity of ZnO NPs is directly proportional to their concentration, as larger concentrations of ZnO NPs are responsible for their antibacterial activity against specific diseases (Jeevanandam et al., 2018). Furthermore, tiny ZnO NPs can easily permeate the bacterial membrane, causing damage to the bacterial cell's protein, lipids, and DNA [41].

## Conclusion

Accordingly, the use of ZnO NPs at a concentration of 10 mM successfully increased the shelf life of chicken fillets while also destroying *Campylobacter jejuni* pathogen on one hand, and it had the ability to eliminate the serious

health effects caused by *Campylobacter jejuni* cytological distending toxins (cdtA, cdtB, and cdtC) especially at sizes of 25 nm and 50 nm on the other.

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### Conflict of interest

The authors declare that they have no conflict of interest.

**Table 1.** Oligonucleotide sequences of the examined virulent genes of *C. jejuni*.

Target gene	Oligonucleotide sequence (5' → 3')	Product size (bp)	References
cdtA (F)	5' AGGACTTGAACCTACTTTTC '3	631	[26]
cdtA (R)	5' AGGTGGAGTAGTAAAAAC '3		
cdtB (F)	5' ATCTTTTAACCTTGCTTTTGC '3	714	
cdtB (R)	5' GCAAGCATTAAAATCGCAGC '3		
cdtC (F)	5' TTTAGCCTTTGCAACTCCTA '3	524	
cdtC (R)	5' AAGGGGTAGCAGCTGTAA '3		

**Table (2).** Overall acceptability of chicken fillets treated with 5 mM Zinc oxide nanoparticles of different sizes during cold storage at 4°C.

Storage time	Control		Zinc oxide nanoparticles sizes					
	Score	Quality	75 nm Score	Quality	50 nm Score	Quality	25 nm * Score	Quality
Zero time **	9.0	E	9.0	E	9.0	E	9.0	E
2 days	4.0	F	7.1	VG	7.3	VG	7.7	VG
4 days	Spoiled		6.0	G	6.4	G	7.0	VG
6 days	Spoiled		5.3	M	5.7	M	6.1	G
8 days	Spoiled		4.0	F	4.7	F	5.2	M

E: Excellent VG: Very good G: Good M: Medium F: Fair; \* Significant changes ( $P < 0.05$ ) as a result of ZnO NPs size; \*\* Significant changes ( $P < 0.05$ ) as a result of storage time; \*\* As a result of storage duration, there were significant changes ( $P < 0.05$ ).

**Table 3.** Overall acceptability of chicken fillets treated with 10 mM Zinc oxide nanoparticles of different sizes during cold storage at 4 °C.

Storage time	Control		Zinc oxide nanoparticles sizes					
	Score	Quality	75 nm Score	Quality	50 nm Score	Quality	25 nm * Score	Quality
Zero time **	9.0	E	9.0	E	9.0	E	9.0	E
2 days	4.7	F	7.5	VG	7.7	VG	8.3	VG
4 days	Spoiled		6.1	G	7.0	VG	7.7	VG
6 days	Spoiled		5.5	M	6.3	G	6.7	G
8 days	Spoiled		5.2	M	5.7	M	6.0	G

**Table 4.** Influence of 5 mM ZnO NPs of different sizes on *C. jejuni* ( $5 \times 10^6$ /g) inoculated into chicken fillets during cold storage at 4 °C.

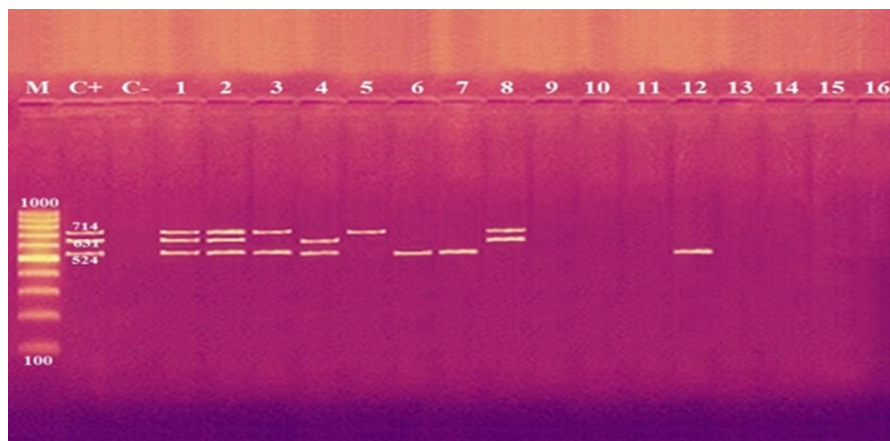
Storage time	Control		Zinc oxide nanoparticles sizes					
	Count	R%	75 nm Count	R%	50 nm Count	R%	25 nm * Count	R%
Zero time **	$5.0 \times 10^6$	-	$5.0 \times 10^6$	-	$5.0 \times 10^6$	-	$5.0 \times 10^6$	-
2 days	$4.8 \times 10^6$ $\pm 1.3 \times 10^6$	4	$2.9 \times 10^6$ $\pm 0.6 \times 10^6$	42	$2.6 \times 10^6$ $\pm 0.7 \times 10^6$	48	$1.9 \times 10^6$ $\pm 0.3 \times 10^6$	62
4 days	$4.7 \times 10^6$ $\pm 1.1 \times 10^6$	6	$2.3 \times 10^6$ $\pm 0.5 \times 10^6$	54	$1.1 \times 10^6$ $\pm 0.2 \times 10^6$	78	$8.5 \times 10^5$ $\pm 2.0 \times 10^5$	83
6 days	$4.5 \times 10^6$ $\pm 1.0 \times 10^6$	10	$6.7 \times 10^5$ $\pm 1.4 \times 10^5$	86.6	$5.0 \times 10^5$ $\pm 0.9 \times 10^5$	90	$2.8 \times 10^5$ $\pm 0.4 \times 10^5$	94.4
8 days	$4.2 \times 10^6$ $\pm 0.9 \times 10^6$	16	$1.8 \times 10^5$ $\pm 1.6 \times 10^5$	96.4	$1.4 \times 10^5$ $\pm 0.5 \times 10^5$	97.2	$7.3 \times 10^4$ $\pm 1.6 \times 10^4$	98.5

R%: Reduction %; \* Significant changes ( $P < 0.05$ ) as a result of ZnO NPs size; \*\* Significant changes ( $P < 0.05$ ) as a result of storage time

**Table 5.** Influence of 10 mM ZnO NPs of different sizes on *C. jejuni* ( $5 \times 10^6$ /g) inoculated into chicken fillets during cold storage at 4 °C.

Storage time	Zinc oxide nanoparticles sizes							
	Control		75 nm		50 nm		25 nm *	
	Count	R%	Count	R%	Count	R%	Count	R%
Zero time **	$5.0 \times 10^6$	-	$5.0 \times 10^6$	-	$5.0 \times 10^6$	-	$5.0 \times 10^6$	-
2 days	$4.8 \times 10^6$ $\pm 1.3 \times 10^6$	4	$2.4 \times 10^6$ $\pm 0.5 \times 10^6$	52	$2.0 \times 10^6$ $\pm 0.3 \times 10^6$	60	$1.5 \times 10^6$ $\pm 0.2 \times 10^6$	70
4 days	$4.7 \times 10^6$ $\pm 1.1 \times 10^6$	6	$1.2 \times 10^6$ $\pm 0.3 \times 10^6$	76	$9.4 \times 10^5$ $\pm 2.1 \times 10^5$	81.2	$6.9 \times 10^5$ $\pm 1.7 \times 10^5$	86.7
6 days	$4.5 \times 10^6$ $\pm 1.0 \times 10^6$	10	$4.5 \times 10^5$ $\pm 1.0 \times 10^5$	91	$3.6 \times 10^5$ $\pm 0.8 \times 10^5$	93.8	$1.1 \times 10^5$ $\pm 0.3 \times 10^5$	97.8
8 days	$4.2 \times 10^6$ $\pm 0.9 \times 10^6$	16	$9.9 \times 10^4$ $\pm 2.1 \times 10^4$	98	$8.1 \times 10^4$ $\pm 1.9 \times 10^4$	98.4	$2.7 \times 10^4$ $\pm 0.4 \times 10^4$	99.5

R%: Reduction %; \* Significant changes ( $P < 0.05$ ) as a result of ZnO NPs size; \*\* Significant changes ( $P < 0.05$ ) as a result of storage time



**Figure 1.** Agarose gel electrophoresis of multiplex PCR for *Campylobacter jejuni* virulence genes *cdtA* (631 bp), *cdtB* (714 bp), and *cdtC* (524 bp) as virulence genes of *Campylobacter jejuni*. Lane M: 100 bp ladder as molecular size DNA marker. Lane C+: Control positive *C. jejuni* for *cdtA*, *cdtB* and *cdtC* genes. Lane C-: Control negative. Lanes 1 & 2: Positive *C. jejuni* for *cdtA*, *cdtB* & *cdtC* genes. Lane 3: Positive *C. jejuni* for *cdtB* and *cdtC* genes. Lane 4: Positive *C. jejuni* for *cdtA* and *cdtC* genes. Lane 8: Positive *C. jejuni* for *cdtA* and *cdtC* genes. Lanes 6, 7 & 12: Positive *C. jejuni* for *cdtC* gene. Lane 5: Positive *C. jejuni* for *cdtB* gene. Lanes 9, 10, 11, 13, 14, 15 & 16: Negative *C. jejuni* for *cdtA*, *cdtB* & *cdtC* genes.

**Table 6.** Occurrence of virulence genes of *C. jejuni* strains isolated from untreated and treated chicken fillets with 10 mM ZnO NPs.

Chicken fillets	No. of tested strains	Virulence genes					
		<i>cdtA</i>		<i>cdtB</i>		<i>cdtC</i>	
		NO	%	NO	NO	NO	%
Untreated (Control)	4	3	75	3	75	4	100
10 mM ZnO NPs of 75 nm size treatment	4	1	25	2	50	2	50
10 mM ZnO NPs of 50 nm size treatment	4	0	0	0	0	1	25
10 mM ZnO NPs of 25 nm size treatment	4	0	0	0	0	0	0

*cdtA*, *cdtB* & *cdtC*: cytological distending toxin A, B & C genes

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