Research Article

Risk Mitigation of *E. coli* O_{157} and its Intimin (*eaeA*) and Shiga Toxin (*stx2*) Gene Expression in Chilled Chicken Fillets Using Chitosan Nanoparticle Loaded with Lysozyme

Aalaa S. Saad[1]*, Amira A. Elokle[2], Dalia Y. Youssef[2], Neven M. Omara[2], Samr Kassem[3]

[1]Biotechnology Department, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Egypt; [2]Reference Lab for Examination of Food of Animal Origin, Food Hygiene Department, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Egypt; [3]Nanomaterials Research and Synthesis Unit, Animal Health Research Institute (AHRI), Agricultural Research Center (ARC), Giza, Egypt.

Abstract | Chitosan nanoparticles loaded with lysozyme (CS-LZ-NPs) are gaining popularity as an alternative approach for inhibiting foodborne pathogens. This study aimed to evaluate the antibacterial activity of synthesized Chitosan nanoparticles (CS-NPs) and CS-LZ-NPs against *E. coli* O_{157} in chilled chicken fillets and relative *eae*A (Intimin), *stx*2 (Shiga toxin) expression. The average particle size was 250 nm and 281.4 nm for CS-NPs and CS-LZ- NPs and the PDI (polydispersity index) was 0.09 and 0.58, respectively. TEM imaging showed uniformity and ranging in size from 13.1 to 17.6 nm and 15.6 to 19.5 nm for CS-NPs and CS-LZ-NPs, respectively. In the SRB (sulforhodamine B) assay, CS-NPs, and CS-LZ-NPs showed no toxicity at concentrations ranging from 50 to 450 µg/mL for both nanoparticles. The MIC of CS-NPS and CS-LZ-NPS were 2.5 mg/mL and 0.312 mg/mL, respectively. As well, the number of viable *E. coli* O_{157} in fillets with CS-NPs and CS- LZ-NPs gradually decreased significantly ($P<0.05$) on chilling than the control. Moreover, *eae*A and *stx*2 relative gene expression decreased until the 10th day, reaching 0.3 and 0.41 in the CS-LZ-NPs group and 0.5 and 0.48 in the CS-NPs group. Also, the treatment with either CS-NPS or CS-LZ-NPS did not affect the average sensory score of the chicken fillets.

Keywords | Chicken fillet, Chitosan nanoparticles, *E. coli* O_{157}, Gene expression, Lysozyme, Toxins

Received | December 01, 2023; **Accepted** | January 27, 2024; **Published** | February 14, 2024
***Correspondence** | Aalaa S. Saad, Biotechnology Department, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Egypt; **Email:** alaa.samir87@yahoo.com
Citation | Saad AS, Elokle AA, Youssef DY, Omara NM, Kassem S (2024). Risk mitigation of *E. coli* O_{157} and its intimin (*eae*A) and shiga toxin (*stx*2) gene expression in chilled chicken fillets using chitosan nanoparticle loaded with lysozyme. Adv. Anim. Vet. Sci., 12(4):603-613.
DOI | https://dx.doi.org/10.17582/journal.aavs/2024/12.4.603.613
ISSN (Online) | 2307-8316

INTRODUCTION

Chicken meat is popular in the consumer market due to its quick and easy preparation and affordable prices, making it a preferred choice for consumers adapting to modern lifestyles. The consumption of poultry meat, especially chicken, continues to rise globally, particularly in developing countries where cultural or religious restrictions do not exist (Kralik *et al.*, 2018). However, chicken carcasses promote the growth of foodborne microorganisms, including Salmonella, *E. coli*, Campylobacter, and Staphylococcus aureus, which are significant causes of human foodborne outbreaks (Bhaisare *et al.*, 2012).

E. coli O_{157} is a major foodborne pathogen responsible for outbreaks and severe diseases such as hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Abuladze *et al.*, 2008). Shiga toxin is the main virulence component

of Shiga toxin-producing *E. coli* (STEC), which causes immunopathologies such as HC and HUS. However, without the adherence elements, *stx*2 alone is unlikely to cause severe disease. It has been discovered that STEC strains possess multiple genes that enable attachment, colonization, production, and secretion of toxin proteins. The *eae* gene, located on the pathogenicity island LEE (locus of enterocyte effacement), encodes the intimin protein, which is crucial for colonization in most STEC strains (Yang *et al*., 2020).

Therefore, microbial contamination during poultry meat processing is essential to the procedures used in processing. When processing poultry, Poultry carcasses and cuts have been found to effectively reduce contamination with food-borne pathogens through the use of various decontamination techniques, including physical, chemical, and biological methods. As consumer awareness of the dangers of man-made chemicals in food has grown, the food industry is keenly interested in using natural compounds that have the potential to produce foods that are extremely safe. A new challenge in poultry processing is to provide effective food preservative materials to meet consumer demand for natural and organic products, especially in the area of microbiological safety (El-Khawas *et al*., 2020; Abd El-Fatahand Saad, 2020). Previous studies found *E. coli* O_{157} in chicken fillets (Ali *et al*., 2010).

Lysozyme belongs to the hydrolase family and is a small, monomeric protein that remains unaffected by a wide range of pH values and temperatures. Besides its antibacterial activities, lysozyme is widely distributed and excreted by plants and animals and possesses anti-inflammatory, anti-cancer, and analgesic properties. Numerous studies have shown that the enzyme lysozyme is a peptide glycan N-acetyl muramoyl hydrolase, commonly known as muramidase, which can lyse bacterial cell walls (Ahmed and Al-Mousawi, 2021).

Research carried out *in vivo* has revealed the antimicrobial characteristics of lysozyme. Li *et al*. (2014) found that adding lysozyme to nano-fibrous mats effectively inhibited the growth of *S. aureus* and *E. coli*. Because of its biological activity, lysozyme and its complex are widely used in food packaging, food preservatives, and wound dressing (Yuan *et al*., 2013).

Chitosan (CS), a linear polysaccharide derived from chitin through demineralization and deproteinization, has a wide range of applications in both medicine and agriculture. The exceptional biological properties of CS, such as its biocompatibility, biodegradability, non-toxicity, and antibacterial activity, have attracted significant attention in various fields (Chandrasekaran *et al*., 2020) and attracted interest in several disciplines. Compared to their original counterparts, the physical properties of nanoparticles (NPs) have significantly changed due to their smaller size. CS-NPs combine the advantages of NPs, such as their small size, increased surface area, quantum size effects, and the properties of CS (Divya *et al*., 2017).

Reverse transcription polymerase chain reaction (RT-PCR) is the preferred method for analyzing mRNA expression from various sources due to its high sensitivity (Kim *et al*., 2021). It can determine gene expression from a known amount of cDNA or RNA and fold changes. RNA must be converted to cDNA to be detectable in qPCR since only DNA is detectable in PCR. Furthermore, cDNAs are more stable during the dilution phase compared to RNA. However, this phase is susceptible to quantification errors of unknown magnitude due to the possibility of varying reverse transcription efficiency between samples. In comparison, cDNA is superior to RNA for qPCR results in terms of quantification accuracy (Lu *et al*., 2012).

This study aimed to synthesize chitosan nanoparticles (CS-NPs) and load them with lysozyme (CS-LZ-NPs). Moreover, it evaluated the antibacterial activity of the synthesized CS-NPs and CS-LZ-NPs against *E. coli* O_{157} in chilled chicken fillets and the relative expression of *eaeA* and *stx*2. To our knowledge, this is the first study to determine the antimicrobial activity of CS-NPs and CS-LZ-NPs against *E. coli* O_{157} in preserved chicken fillets under refrigerated conditions and the expression of target toxin genes (intimin and Shiga).

MATERIALS AND METHODS

Bacterial strains

The strain of *E. coli* O_{157} (AHRI/301) used in this experiment was obtained from the Department of Bacteriology, AHRI, ARC. It was identified using the automated VITEK® 2 system and antisera and stored at -80 °C. The frozen (glycerol stock) *E. coli* O_{157} culture was activated by inoculation into Tryptic Soya Broth (TSB; Biolife) and incubated overnight at 37 °C for 24 h to reach a final concentration of approximately 10^8 CFU/ml, as determined by plating serial dilutions on Sorbitol MacConkey Agar (LAB M). Serial dilution was performed on sterile PBS to give approximately 10^6 CFU/ml in the inoculated solution.

Preparation of CS-NPs and chitosan nanoparticles loaded with lysozyme (CS-LZ-NPs)

Chitosan, with a molecular weight of 100-300 kDa and a degree of deacetylation of 93–95%, was obtained from Lanxess, Germany. Sodium tripolyphosphate (TPP), anhydrous and ultra-pure, was obtained from Oxford, India. Egg white lysozyme (20,000 U/mg protein) was

purchased from Bio Basic Inc., Canada. Sodium hydroxide (1 M) was obtained from Sigma Aldrich, USA. CS-NPs were prepared as described by (Piras *et al*., 2014) with modifications. For empty CS-NPs, CS (1 mg/ml) was dissolved in acetic acid (1% V/V), and TPP (1 mg/ml) was dissolved in deionized water. Nanoparticles were obtained directly after dropwise titration of 20 ml of TPP to 50 ml of CS solution with continuous magnetic stirring for 2 h, pH adjustment to 4 using Na OH (1 M), and then centrifugated for 30 min. For CS-LZ-NPs, different concentrations (0.5, 1, 1.5, 2, and 2.5 mg/ml) of lysozyme were mixed and titrated with TPP in CS solutions using the same method to synthesize and purify empty CS nanoparticles.

Characterization of synthesized NPs

The particle size and surface charge of prepared bare CS-NPs and CS-LZ-NPs (2.5 mg/ml) were characterized by a NANOTRAC-WAVE II Zeta-Sizer (MICROTRAC, USA). The morphological investigation of synthesized NPs was performed using TEM (JEM-2100, JEOL). The UV analysis was performed using a UV-visible spectrophotometer (SHIMADZU-2600i, USA).

Cytotoxicity assay

An SRB assay using oral epithelial cells (OEC) was used to assess cell viability after treatment with various concentrations of prepared empty CS-NPs and CS-LZ-NPs (50–450 µg/ml), according to the method described by (Allam *et al*., 2018).

Encapsulation efficiency (EE%) and *in vitro* release study

The loading capacity and EE percentage of CS-LZ-NPs were measured at pH 4 with different lysozyme concentrations (0.5, 1, 1.5, 2, and 2.5 mg/ml), according to (Wu *et al*., 2017). The UV-visible spectrophotometer was used to measure the lysozyme concentration in the supernatant of the solution, and EE% was calculated as the amount of lysozyme retained in the CS-LZ-NPs compared to the total amount of lysozyme used in the synthesis protocol. The percentage of in vitro release in phosphate-buffered saline (PBS, pH 7.4) was determined by CS-LZ-NPs prepared with an enzyme concentration of 2.5 mg/ml at pH 4, following the method described by (Wu *et al*., 2017). A UV spectrophotometer was used to measure the release over time within 350 min.

Antimicrobial activity of CS-NPs and CS-LZ-NPs

The broth microdilution assay was performed to determine the MIC values of CS-NPs and CS-LZ-NPs, following the method described by CLSI. Two-fold serial dilutions of the CS-NPs and CS-LZ-NPs (up to 5 concentrations ranging from 5 to 0.312 mg/ml) with an enzyme concentration of 2.5 mg/ml in CS-LZ-NPs were set up in a microdilution plate. Each microwell received an equal volume of bacterial culture at a final concentration of 1×10^5 CFU/ml. Identical amounts of bacterial culture were added to saline as a positive control with no additive. The plate was kept at 37 °C for 24 h, and 100 µL of each well was plated on the correct agar medium. Colonies were counted after incubation.

Preparation of chicken fillets

The chicken breast meat, which was examined and found to be free of *E. coli* O_{157}, was sliced, and the chicken fillets were divided into six groups as illustrated in detail in Figure 1.

Inoculation procedures

E. coli O_{157} cells were grown in a stationary phase in tryptic soy broth (TSB; Biomark Laboratories). The chicken fillets were inoculated with an *E. coli* O_{157} solution.

Group 1 fillets were inoculated with *E. coli* O_{157}, immersed in CS-NPs, and stored in the refrigerator (chill preservation).

Group 2 fillets were inoculated with *E. coli* O_{157}, treated with CS-LZ-NPs, and preserved in the refrigerator (chill preservation).

Group 3 fillets were inoculated with *E. coli* O_{157} and stored in the refrigerator (chilled preservation) without CS-NPs or CS-LZ-NPs (control).

Group 4 fillets were treated with CS-NPs but not inoculated with *E. coli* O_{157}.

Group 5 fillets were treated with CS-LZ-NPs but not inoculated with *E. coli* O_{157} for sensory evaluation of product technology.

E. coli O_{157} viable counts

Fillets from each group were sampled at 2 h on day 0, then every 3 days for all groups till spoilage signs. All tests were performed in triplicate. A total of 25 g of each sample was homogenized using a Stomacher (Seward Stomacher 80 Biomaster, England) with 225 ml of saline (CHEM LAB NV) to achieve a 1:10 dilution. Ten-fold serial dilutions of the homogenate were prepared using 0.1% sterile peptone water (LAB M). One milliliter of the sample suspension was aseptically transferred to the dried surface of three sorbitol MacConkey agar plates (LAB M) and spread by a sterile curved glass spreader stick. The plates were held in an upright position until the inoculum was absorbed by the agar and then incubated at 37 °C for 24 h to determine the total number of *E. coli* O_{157} (by summing the *E. coli* O_{157} in the three TBX-Agar (Biolife) and multiplying by the sample dilution factor). The reduction percentages (R%) were calculated according to the following equation:

$$R\% = (\text{control count} - \text{treatment count}/\text{control count}) \times 100$$

(Elsherif and Shrief, 2021)

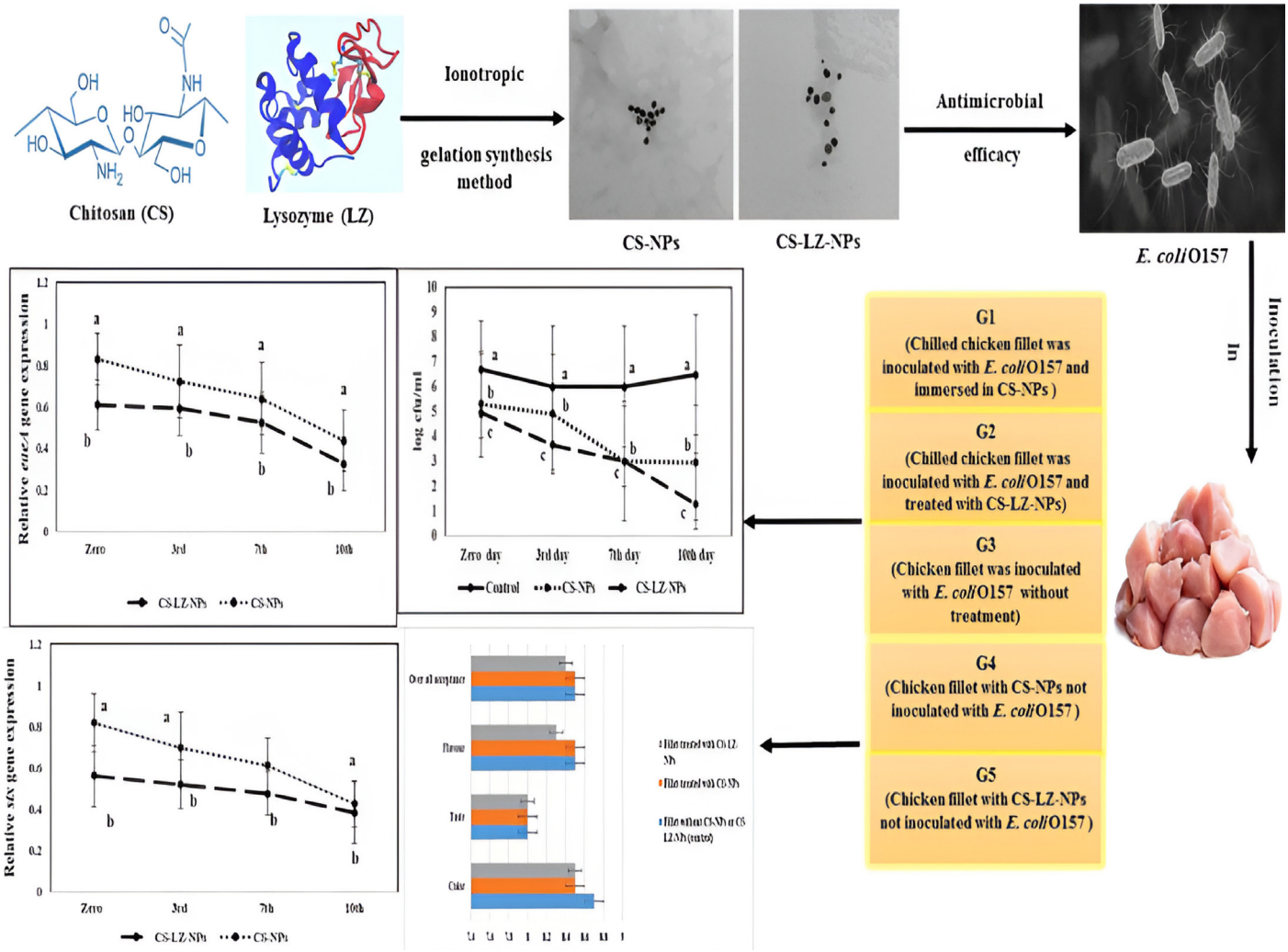

Figure 1: Schematic illustration of the Experimental design.

Gene expression assay

Extraction

The EasyPure® RNA kit was used to extract the mRNA, following the instructions provided in the pamphlet. Specifically, 200 µL of the prepared sample was mixed with 20 ml of proteinase K. Then, 200 ml of binding buffer 5 containing carrier RNA was applied and stirred for 15 s. The mixture was then incubated at 56 °C for 15 min, and 250 ml of absolute ethanol was added. The sample was then vortexed for 15 s and incubated at ambient temperature (15–25 °C) for 15 min. The mixture was then transferred to a spin column tube and centrifuged at 12,000 g for 60 s, with the flow-through liquid being discarded. The column was washed with 500 ml of washing buffer, and the process was repeated. The spin-column tubes were then transferred to a clean 1.5 ml micro centrifuge tube with 20 ml RNase-free water added to the center of the column. The sample was then incubated for 60 s at ambient temperature and centrifuged for 60 s at 12,000 rpm. The obtained RNA was stored at -80 °C until use.

Complementary DNA synthesis

Using EasyScript® FirstStrand cDNA Synthesis SuperMix, 5 µg of the extracted RNA was mixed with 1 µL of anchored oligo (dT) 18 primer, 10 µL of 2XES reaction mix, 1 µL of EasyScript® RT/RI enzyme mix, and RNase-free water until the volume reached 20 µL. The mixture was then incubated for 15 min at 42 °C.

Real-time PCR

The oligonucleotide primers used in this study (Table 1) were synthesized by Metabion® International AG. Data acquisition and analysis were performed using real-time PCR version 2.2.2 (Applied Biosystems). The amplification for Rt-PCR was carried out with TransStart Green qPCR SuperMix in a total volume of 20 µL, consisting of 1 µL of template DNA, 0.4 µL of each forward and reverse primer, 10 µL of 2X TransStart® Green qPCR SuperMix, 0.4 µL of passive reference dye (50X), and finally, 7.8 µL of double-distilled water. The cycle thresholds of the target genes (*eae*A and *stx*2) belonging to *E. coli* were obtained from samples containing CS-NPs, CS-LZ-NPs, and blanks (control) and compared and normalized to the housekeeping gene (GAPDH). The fold changes in the target genes were determined using the $2^{-\Delta\Delta Ct}$ method of the formula, as described by (Pfaffl, 2001).

Table 1: Primers used for the real-time polymerase chain reaction.

Primer	Sequence (5-3)	Cycling conditions	
GAPDH	AAGTTTGCACTTCCGTGTTC	one cycle at 95°C for 2 min, 40 cycles at 95°C for 5 s, and 60°C for 10 s.	Khatib *et al.*, 2017
	CGTTGTCGTACCAGGATACC		
Stx2	CTGTGGCCGTTATACTGAAT		
	GTCGCCAGTTATCTGACATT		
*eae*A	TGCTGGCATTTGGTCAGGTC		Chassagne *et al.*, 2009
	CGCTGA(AG)CCCGCACCTAAATTTGC		

Sensory evaluation of chicken fillets containing CS-NPs and CS-LZ-NPs

The investigators in this study were 14 specialists from the staff of the Animal Health Research Institute's Food Hygiene Department, ARC. The study evaluated ready-to-eat chicken fillets with CS-NPs, ready-to-eat chicken fillets with CS-LZ-NPs, and blank (control) samples that did not contain CS-NPs or CS-LZ-NPs. The most frequently cited descriptors included color, taste, aroma, and acceptance. The specialists were asked to rank samples according to their intensity for each of the four descriptors. There was a 9-point hedonic scale where 9 indicated an extreme aversion to fillets compared to fillets without chitosan or lysozyme (Kim *et al.*, 2015). The examiner considered the sample rejected if the scores were less than five.

Statistical analysis

All analyses were performed in triplicate. Data were analyzed using statistical Excel (Microsoft, 2016). Significant differences between the results were examined by a Student's t-test at $p \leq 0.05$. The data were expressed as means ± SD. To assess the comparative expression of target genes, the 2–ΔΔCT method was utilized in comparison with the control group.

RESULTS AND DISCUSSION

Characterization of CS-NPs and CS-LZ-NPs

Zetasizer determined particle size, polydispersity index (PDI), and surface charge (Fig. 2A1, A2) at pH 4. The average particle size was 250 nm, and 281.4 nm for CS-NPs (5 mg/ml) and CS-LZ-NPs (lysozyme concentration 2.5 mg/ml), and the polydispersity index (PDI) for CS-NPs and CS-LZ-NPs was 0.09 and 0.58, respectively. Surface charges were +20.3 and +29.2 mV, respectively. The ionotropic gelation technique using tripolyphosphate (TPP) as a crosslinker was employed to synthesize CS-NPs due to its perfect gelation ability and non-toxic nature (Koukaras *et al.*, 2012). The chitosan to TPP concentration of 1:1 was used to control the physicochemical properties of the prepared nanoparticles. The interaction between cationically charged chitosan and TPP, which has a high-density negative charge, leads to gel formation with improved mechanical resistance. The concentration of chitosan to TPP affects the mechanical strength of the formula and the size distribution of the nanoparticles due to the electrostatic interaction between both molecules (Calvo *et al.*, 1997). The nanoparticles obtained in this study exhibited a narrow size distribution, increasing as the concentration percentage increased (Deng *et al.*, 2006). The positive charge observed in the Zetasizer analysis indicated the presence of positive amino groups on the particle surface (Wu *et al.*, 2017). TEM imaging further confirmed the uniformity and small size of the nanoparticles (Fig. 2B1, B2). The CS-NPs and CS-LZ-NPs appeared as spherical, solid, consistent particles without agglomeration, ranging in size from 13.1 to 17.6 nm and 15.6 to 19.5 nm, respectively. UV analysis of CS-NPs, CS-LZ-NPs, and lysozyme was performed to study the complex pattern between CS-NPs and lysozyme (Fig. 2C). The absorption peaks of CS-LZ-NPs and lysozyme were recorded at 285 and 280 nm, respectively. In comparison, the CS-NPs showed a weak absorption peak at 280 nm. The absorption peak at 280 nm is usually related to the amino acid composition of the lysozyme, which is present in the presence of CS-NPs at 285 nm. This higher excitation revealed binding between lysozyme and CS-NPs, resulting in an increase in optical density compared to free lysozyme (Wu *et al.*, 2017).

NPs cytotoxicity evaluation

The cytotoxicity of the synthesized CS-NPs and CS-LZ-NPs was assessed using an OEC cell line by SRB assay at concentrations ranging from 50 to 450 µg/ml. In the SRB assay, CS-NPs and CS-LZ-NPs showed no toxicity at concentrations ranging from 50-450 µg/ml for both nanoparticles, indicating that cells had the same characteristics as control cells. For CS-NPs, cell viability ranged from 99.82% to 92.5% at concentrations ranging from 50 to 450 g/ml. However, in the case of CS-LZ-NPs, cell viability ranged from 101.82% to 95.23% at concentrations ranging from 50 to 450 g/ml, as shown in Figure 3A. The higher survival rates of both nanoparticles are related to the biocompatibility and biodegradability of chitosan and the non-toxic property of lysozyme (Wu *et al.*, 2017), which exhibits multiple beneficial biological activities. Lysozyme, derived from egg white, has also been

recognized by the US Food and Drug Administration (FDA) and approved for food preservation as safe for human health (Khorshidian *et al.*, 2022).

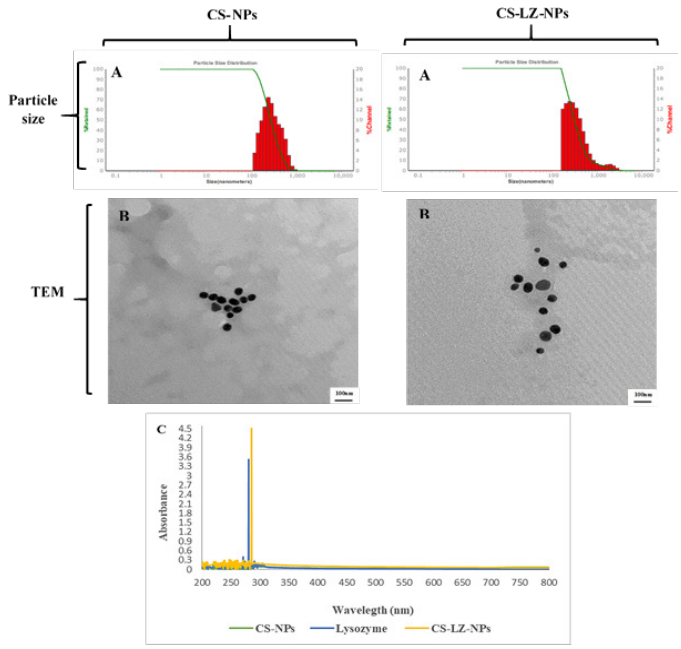

Figure 2: Characterization of blank CS-NPs and CS-LZ-NPs. (A1 and A2) particle size pattern of synthesized NPs showing (A1) size distribution of CS-NPs of 250 nm, (A2) size distribution of CS-LZ-NPs of 281 nm. (B1 and B2) TEM imaging of blank CS-NPs (B1) and CS-LZ-NPs (B2) showed consistent solid spherical nanoparticles without agglomeration and size ranged from 13.1 to 17.6 nm and 15.6 to 19.5 nm, Scale bar = 100 nm. (C) UV analyses of synthesized chitosan, chitosan lysozyme nanoparticles and lysozyme at wavelength range of 200-800 nm.

Encapsulation efficiency (EE%) and *in vitro* release study

The EE% of CS-LZ-NPs at pH 4 was measured with different concentrations of lysozyme (0.5, 1, 1.5, 2, and 2.5 mg/ml) (Fig. 3B) in the supernatant of the solution by a UV-visible spectrophotometer. The present study has shown that, in agreement with that of (Wu *et al.*, 2017), there is a direct correlation between the concentration of loaded lysozyme and EE% at pH 4. The maximum EE% value of 85% was observed at a lysozyme concentration of 2.5 mg/ml rather than at lower concentrations of loaded lysozyme. CS-LZ-NPs formula with an enzyme concentration of 2.5 mg/ml, prepared at pH 4, was selected for the in vitro detection of percent release in PBS (pH 7.4) by UV spectrophotometer for 350 min. The current results showed that the maximum release percentage was 18% after 300 min and dropped to only 16% after 350 min, indicating 82% and 84% retention of lysozyme in the CS-LZ-NPs formula, respectively, for 350 min (Fig. 3C). These results demonstrated the ability of CS-LZ-NPs to

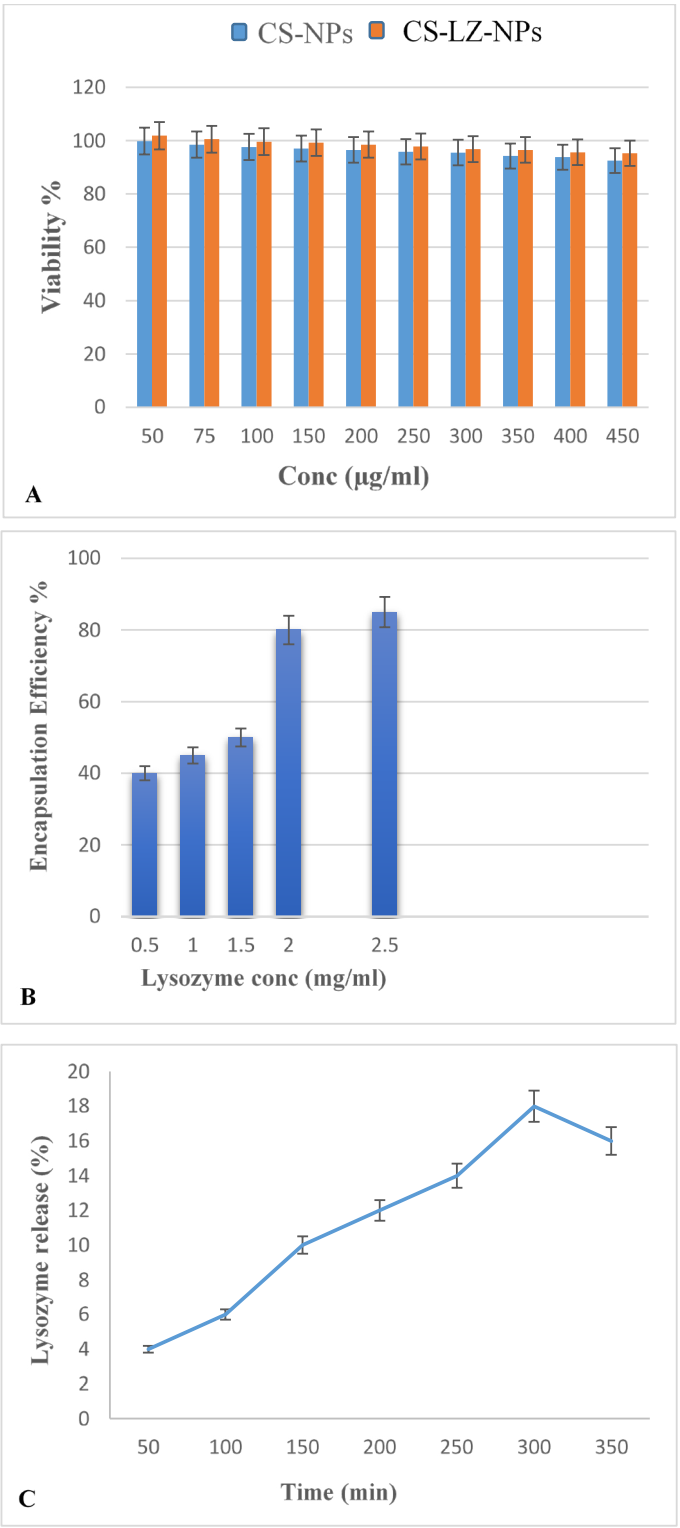

Figure 3: (A) SRB cytotoxicity assay of blank CS-NPs and CS-LZ-NPs at different concentrations ranged from 50 to 450 µg/ml. (B) Encapsulation efficiency percent of CS-LZ-NPs at pH 4 with different concentration of lysozyme (0.5, 1, 1.5, 2 and 2.5 mg/ml). (C) In vitro release study of CS-LZ-NPs with lysozyme concentration of 2.5 mg/ml prepared at pH 4.

prolong the release percentage and to be entrapped in the mixture for an extended period, which might be beneficial for antibacterial efficacy when used in the field of food

preservation. The percentage of the release of lysozyme from CS-NPs is influenced by many factors, such as the concentration of lysozyme, the size of the CS-NPs, and the pH of the release medium (Wu *et al*., 2017; Dragan *et al*., 2016).

Antimicrobial efficacy of CS-NPs and CS-LZ-NPs on *E. coli* O_{157}

The bactericidal activities of CS-NPs and CS-LZ-NPs against the *E. coli* O_{157} strain were recorded. CS-LZ-NPs with an enzyme concentration of 2.5 mg/ml showed bactericidal activities against *E. coli* O_{157} at concentrations of 2.5, 1.25, 0.625%, and 0.312 mg/ml each. CS-NPs with a CS concentration of 5 mg/ml exhibited bactericidal activities only at 5 and 2.5 mg/ml concentrations. These concentrations were selected based on the results reported by (Wu *et al*., 2017), who observed complete basement membrane damage with irregularity of *E. coli* cell shape and leakage of cytoplasmic contents at a concentration of 1 mg/ml CS-Lys-NPs. The minimum inhibitory concentration (MIC) values of CS-NPs were 2.5 mg/ml. However, the CS-Lys-NP concentration of 0.312 mg/ml was sufficient to elicit complete inactivation of *E. coli*, indicating the increased sensitivity of *E. coli* when lysozyme has been integrated into CS-NPs. These values are consistent with various studies confirming the antimicrobial activity of CS nanoparticles against *E. coli* at lower concentrations (Younes *et al*., 2014). A complete reduction (R%) in *E. coli* O_{157} occurred on the third day with CS-LZ-NPs and on the seventh day with CS-NPs (Table 2).

Table 2: Reduction percentages (R %) of *E. coli* O157 after inoculation in chicken fillets during refrigerated storage.

Time of storage	CS-NPs	CS-LZ-NPs
Zero day	0.96	0.98
3rd day	0.92	1.00
7th day	1.00	1.00
10th day	1.00	1.00

Legend: The numbers indicate the R%.

Experimental outcomes

Figure 4 shows the influence of CS-NPs and CS-LZ-NPs on the viable count of *E. coli* O_{157} in chicken fillets at chilled preservation, expressed as log CFU/g. The initial counts (after 2 hours on day 0) of CS-NP-treated chicken fillets (Group 1), CS-LZ-NP-treated chicken fillets (Group 2), and control (Group 3) were 5.3 log CFU/g, 4.95 log CFU/g, and 6.7 log CFU/g, respectively. Consequently, the number of viable *E. coli* O_{157} in fillets treated with CS-NPs and CS-LZ-NPs gradually decreased significantly ($p < 0.05$) during chilling compared to the control to 2.9 log CFU/g in the CS-NP group and 1.2 log CFU/g (19 CFU/g) in the CS-LZ-NP group on the tenth day of preservation. Intriguingly, CS-NPs and CS-LZ-NPs not only increased the shelf life of the chicken fillet but also reduced the risk of *E. coli* O_{157} below the infectious dose (50-100 colony forming units) (CFU/g or ml), as mentioned by (O'flynn *et al*., 2004). Additionally, there was a significant difference between the CS-NPs treated group and the CS-LZ-NPs treated group on all experimental days ($p < 0.05$). Naturally sourced nanoparticles have received significant attention in food preservation and pharmaceutical products. Our study used CS-LZ-NPs as an antimicrobial agent against *E. coli* O_{157} at a 5 mg/ml dose with a lysozyme concentration of 2.5 mg/ml and showed higher potency than CS-NPs. The antimicrobial effect of chitosan depends on various factors, such as the electrostatic interaction between the cationically charged chitosan particles and the anionically charged bacterial cell wall, the degree of deacetylation, the molecular weight, and pH of the media (Yilmaz, 2020). The antimicrobial effect of lysozyme is referred to as the enzymatic hydrolytic role of lysozyme on the glycosidic bonds of peptidoglycan chains between NAM (N-acetylmuramic acid) and NAG (N-acetylglucosamine); the non-enzymatic role is due to the electrostatic interaction between positively charged lysozyme and negatively charged teichoic acids in the cell membrane of Gram-positive bacteria and the phospholipids of the bacterial membrane (Primo *et al*., 2018).

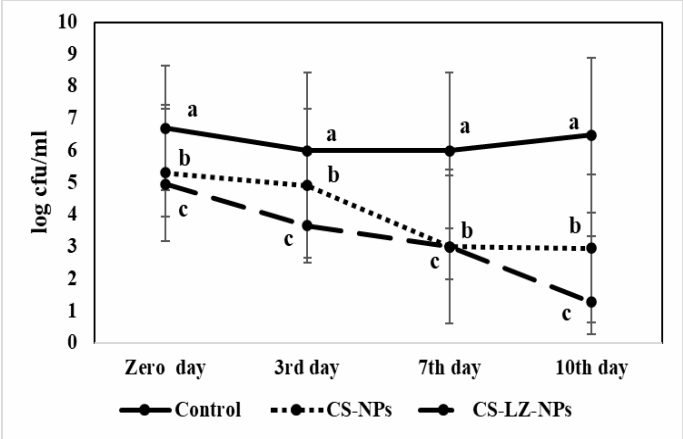

Figure 4: *E. coli* O_{157} viable count (log CFU/g) of chicken fillet treated with CS-NPs and CS-LZ-NPs and the control (untreated) at chilling preservation. Values are expressed as the mean ±SD. There are significant differences between points that have different letters on the same day of preservation.

The effect of lysozyme on Gram-negative bacteria is weak due to the presence of a thin peptidoglycan layer in the cell membrane (7-8 nm) in the case of *E. coli*, the absence of teichoic acids, and the presence of phospholipids, lipopolysaccharides, and lipoproteins in the outer membrane, which act as a barrier restricting the ability of lysozymes to target cellular peptidoglycan (Khorshidian *et al*., 2022). Modifying lysozyme through

denaturation or conjugation with other compounds, mainly polysaccharides, is advisable to enhance lysozyme's antimicrobial function against gram-negative bacteria (Vilcacundo *et al*., 2018). The synergistic effect between one of the most abundant linear polysaccharides from a natural source, such as chitosan and lysozyme, is effective against *E. coli* O_{157} at a low 1 mg/ml concentration, as reported by (Wu *et al*., 2017). Chitosan enhances the hydrolytic effect of lysozyme on peptidoglycan chains and increases the permeability of lysozyme, resulting in cell membrane disruption depending on the higher positive charge on the particle surface with the higher PDI of synthesized CS-LZ-NPs, which opens the cell barriers to lysozyme, optimizing the antimicrobial efficacy up to complete cell lysis and death (Khorshidian *et al*., 2022).

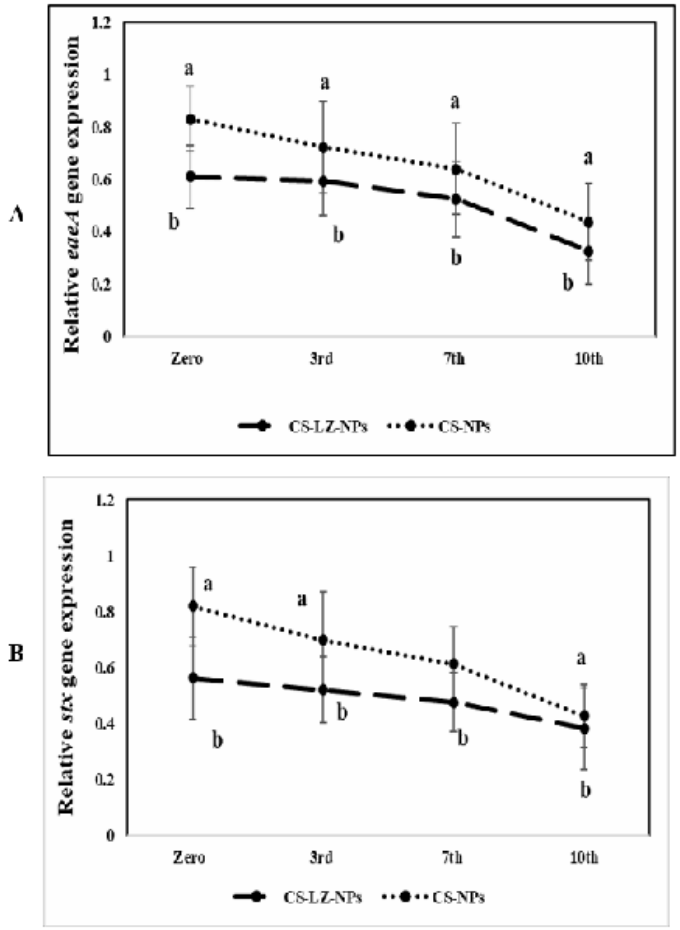

Figure 5: (A). Relative *eae*A gene expression of *E. coli* O_{157} in chicken fillet treated with CS-NPs and CS-LZ-NPs at chilling preservation. Values are expressed as the mean ±SD. There are significant differences between points that have different letters on the same day of preservation. (B). Relative stx gene expression of *E. coli* O_{157} in chicken fillet treated with CS-NPs and CS- LZ-NPs at chilling preservation. Values are expressed as the mean ±SD. There are significant differences between points that have different letters on the same day of preservation

Results from the assay on the relative expression of *E. coli* O_{157} *eae*A and *stx*2 genes in chicken fillets containing sub inhibitory levels of CS-NPs and CS-LZ-NPs, when preserved by chilling, are presented in Figure 5. The expression of both genes was significantly reduced ($p < 0.05$) in the treated groups compared to the untreated (control) group. Furthermore, the CS-LZ-NPs group showed more significant downregulation of gene expression than the CS-NPs group ($p < 0.05$). Notably, on day 0, the relative expression of the *eae*A gene was 0.82 and 0.6 in the CS-NPs and CS-LZ-NPs groups, respectively, while on the tenth day, the expression levels were 0.5 in the CS-NPs group and 0.3 in the CS-LZ-NPs group. On day 0, the relative expression of the *stx*2 gene in the CS-NPs group was 0.81, while in the CS-LZ-NPs group, it was 0.56. It further decreased until the tenth day, reaching 0.48 in the CS-NPs group and 0.41 in the CS-LZ-NPs group. The mechanism by which CS affects gene expression in bacterial cells is via its ability to cross the membrane and adsorb onto DNA molecules, thereby preventing RNA transcription from DNA. Therefore, the physical properties of CS, such as its molecular weight and degree of deacetylation, play a crucial role in its antimicrobial activity (Benhabiles *et al*., 2012).

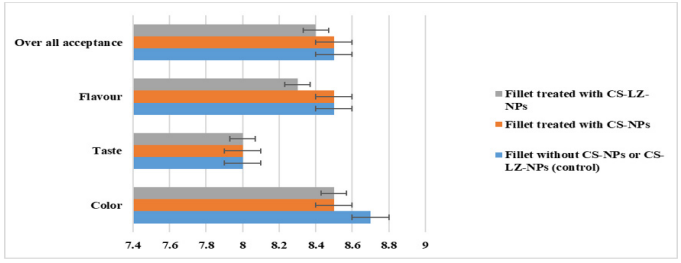

Figure 6: The average score of taste, color, flavor, and overall acceptability of chicken fillets without CS-NPs and or CS-LZ-NPs, chicken fillets with CS-NPs, and chicken fillets with CS- LZ-NPs.

The results of the sensory evaluation of chicken fillets treated with CS-NPs and CS-LZ-NPs are presented in Figure 6. The evaluations included color, taste, aroma, and overall acceptability, with high scores recorded for chitosan-treated fillets (8.5, 8, 8.5, and 8.5, respectively) and chitosan and chitosan lysozyme-treated fillets (8.5, 8, 8, and 8, respectively). The treatment of chicken fillets with either CS-NPs or CS-LZ-NPs did not significantly affect the average score of taste, color, aroma, and overall product acceptability compared to chicken fillets without chitosan and lysozyme. This finding is consistent with that reported by (Eldaly *et al*., 2018), who found that the application of chitosan coating on chicken fillets improved sensory properties during refrigerated storage, and (Kijowski *et al*., 2013), who reported that chicken samples exposed to lysozyme and stored under cold storage conditions did not differ qualitatively from fresh samples. Incorporating LZ into CS-NPs increased down-regulation activity compared

to CS-NPs without lysozyme. The average zeta potential of CS-Lys-NPs increased due to the cooperation between lysozyme and CS-NPs via the NH and OH groups and the consumption of lysozyme in CS-NPs. Due to their higher surface charge density and more significant affinity for bacterial membranes, CS-Lys-NPs are more effective in down-regulating the activity of bacterial gene expression against *E. coli* (Rabea *et al*., 2003).

CONCLUSION AND RECOMMENDATION

The results showed that the nanoparticles could extend the shelf life of chicken fillets for ten days and reduce the bacterial counts of *E. coli* O_{157} to below the infectious dose. This was achieved by downregulating the relative expression of the *eae*A and *stx*2 genes, each by 0.8 in the CS-LZ-NPs group on day 10 of preservation. Overall, this study's findings suggest that using CS-LZ-NPs can positively impact the food industry and food hygiene by extending the shelf life of chilled chicken fillets and controlling microbial contamination. Therefore, we recommend the use of CS-LZ-NPs for preserving chicken fillets in the refrigerator.

Based on the findings of this study, it can be concluded that the use of CS-LZ-NPs can have a positive impact on the food industry and food hygiene. By extending the shelf life of chilled chicken fillets and controlling microbial contamination, CS-LZ-NPs can help preserve chicken fillets in the refrigerator. Therefore, we recommend the application of CS-LZ-NPs for this purpose.

Limitations and future directions

In the present study, the antimicrobial activity of CS-NPs and CS-Lys-NPs was evaluated against *E. coli* O_{157} and their activity on the relative expression of the *eae*A and *stx*2 genes.

We look forward to investigating the activity of CS-Lys-NPs on the relative expression of other toxin genes, such as *fim*A, *omp*A, etc. As well, additional research is necessary to ensure that CS-Lys-NPs is considered a healthy product and does not compromise consumer health.

ACKNOWLEDGMENTS

Not applicable.

NOVELTY STATEMENT

To our knowledge, this is the first study that has investigated the antimicrobial activity of CS-NPs and CS-LZ-NPs against *E. coli* O_{157} in preserved chicken fillets under refrigerated conditions and the expression of target toxin genes (intimin and Shiga). The effectiveness of lysozyme against gram-negative bacteria is limited due to the presence of a thin layer of peptidoglycan in the cell membrane, the absence of teichoic acids, and the presence of phospholipids, lipopolysaccharides, and lipoproteins in the outer membrane. These factors act as a barrier that limits the ability of lysozymes to reach the cellular peptidoglycan. To enhance the antimicrobial function of lysozyme against gram-negative bacteria, it is recommended to conjugate lysozyme with other compounds such as polysaccharides.

AUTHOR'S CONTRIBUTION

AS: Designed the experiment and the gene expression calculation.
AE, DY and NO: Performed the bacteriological work of the investigation.
SK: performed the preparation and characterization of nanoemulsions.
All authors participated in writing and revising the entire manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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