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Research Article

Evaluating the effect of ZnO NPs synthesized by ginger (*Zingiber officinale*) on Escherichia coli biofilm gene using real-time PCR

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Abstract
Biofilm formation in microorganisms is one of the most aspects of the food industry, which can be a source of food spoilage and foodborne illness. This study assessed the inhibition activity of green synthesis zinc oxide nanoparticles (ZnONPs) inferred from an extract of ginger (*Zingiber officinale*) on the biofilm formation gene of *Escherichia coli* isolated in chicken meat and the expression level of the *YjaA* gene. The characteristic of the prepared NPs was confirmed by a UV-Visible spectrophotometer, which showed maximum absorption peaks at 370nm. X-Ray Diffraction (XRD) and Fourier Transform Infrared (FTIR) were used to identify the phytosynthesis purity and morphological properties of NPs. Three isolates of *E. coli* were isolated from clinical samples and formed a strong biofilm in Congo red media. Then, they were subjected to different concentrations of ZnONPs (0.5, 1, and 1.5mg/ml) to determine the effect of ZnO NPs on *YjaA* gene expression. A two-step (RTqPCR) analysis was used and the results revealed a significant inhibitory effect on gene expression (P>0.05). The mean of gene folding decreased from 1±00 in control to 0.192±0.006 and 0.132±0.006 for 1 and 1.5mg/ml, respectively.

Keywords: Biofilm, ZnONPs, *Zingiber officinale*, gene, Real time PCR.
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Introduction

Plant extracts were used for the first time in the biosynthesis of metallic nanoparticles as reductants and stabilizer agents. Green synthesis of NPs is a single-step method, non-pathogenic, low energy-consuming, generates a large number of metabolites, and a low-cost-effective (Akintelu et al. 2020). It has more advantages than using microorganisms and is an eco-friendly approach. Ginger, *Zingiber officinale* (Zingiberaceae) had terpenes and phenolic compounds. Carbohydrates and lipids are the main components of the ginger rhizome (Stoilovaa et al. 2007; Ansari et al. 2022).

Escherichia coli, most foodborne pathogens, can adhere and colonize surfaces, forming biofilms. They are pathogens in food production, especially in chicken meat (Abdul Wahid et al. 2017; Bokov et al. 2022). These bacteria are indicators in the manufacturing materials and surfaces in food processing (Gardea-Torresdey et al. 2003; Huldani et al. 2022). Biofilm formation in *E. coli* is a major factor in 60% of infections and antimicrobial resistance because the bacteria's extracellular matrix protects them from antimicrobial agents, leading to chronic infections and treatment problems (Rode et al. 2007). In EHEC, the *yjaA* gene encodes genes that regulate cellular responses to hydrogen peroxide and acid stress (Smanthong et al. 2015) to reduce bacteria's pathogenicity and drug resistance, inhibiting or destroying biofilm formation.

Zinc oxide nanoparticles (ZnO NPs) have shown antibacterial and anti-biofilm formation activity against important foodborne pathogens such as *E. coli* (Lee 2011). Nanoparticle-based therapeutic

approaches are cost-effective against biofilm-associated bacterial infection due to their unique chemical and physical properties. Furthermore, because of the importance of these bacteria to public health, ZnO nanoparticles can be used as a cleaning agent for surfaces, apparatus, and production lines in food plants to prevent the formation of foodborne bacterial biofilms. Therefore, this study assessed the inhibition activity of green synthesis ZnO NPs inferred from an extract of ginger on the biofilm formation gene of *E. coli* isolated in chicken meat and the expression level of the *YjaA* gene.

Materials and methods

Preparation of ginger extract: The ethanolic extract of *Z. officinale* was prepared based on Liu et al. (2009). For this purpose, fresh ginger was purchased from a local market and dried for 72 hours at room temperature (25°C) before ground into powder. A 50g powder was soaked in 500ml of 70% ethanol solution in an amber glass container and shaken twice daily for five days. Whatman No. 1 filter paper filtrated the resultant extracts under decreased pressure. A rotary evaporator was used to evaporate the hydro-ethanolic solvent at 60°C. To evaporate the residual ethanol, then kept at 37°C for three days (Dziedzic et al. 2013).

Green-synthesis of ZnO NPs: Green-synthesis of ZnO nanoparticles was prepared using ginger root extracts (Clermont et al. 2013). For this purpose, deionized water was used to prepare 50mL of 0.01M Zinc acetate dihydrate. 500ml of the extract was slowly added in a magnetic stirrer by continuous stirring. 1.0M sodium hydroxide was used to maintain pH at 12. The mixture was stirred for 2 hours until a white precipitate formed, after which it was centrifuged for 10min at 10,000rpm. The pellets were washed in deionized water and dried overnight in a hot air oven at 200°C. The resulting white powder was carefully collected and sent to be characterized. UV, X-ray direction (XRD), and Fourier-transform infrared (FTIR) techniques were used on the synthesized NPs. ZnO nanoparticles were prepared by adding a measured volume of dimethyl sulfoxide (DMSO) organic solvent to a measured volume of ZnO nanoparticles (0.5, 1, and 1.5g/mL). For a few minutes, the mixture was allowed to sonicate.

Preparation of bacterial inoculum: *Escherichia coli* isolates collected from meat samples were examined for biofilm formation using the Congo Red Agar (CRA) method and then subjected to PCR to detect biofilm *yjaA* gene amplified at 211bp. Subsequently, a bacterial isolate was cultured on tryptic soy broth (TSB) for 24 hours at 37°C with 2% glucose and subject to the concentration of 0.5, 1, and 1.5mg/ml of biological ZNONPs and incubated aerobically. A spectrophotometer was used to adjust the bacterial cultures to a viable cell count of 10^7CFU/mL (optical density of 0.5) at 630nm (Shenzhen, China).

Real-time PCR of *YjaA* gene expression: The effect of ZnO NPs on *E. coli YjaA* Biofilm gene expression was investigated using a two-step reverse transcription real-time PCR analysis. *lsrA* gene was used as housekeeping gene. The RNA prep Bacteria Kit (Total RNA Extraction Kit (AccuZol 1M) Bioneer, Korea) to extract total RNA and cDNA synthesis were performed using a spectrophotometer to measure absorbance. The ratio of absorbance at 260 and 280nm was used to determine the purity of the extracted RNA with a final volume of 20ml and a period of 2hr. The *yjaA* gene expression was measured using 5ng of cDNA and specific primers. SYBR green PCR Master Mix (AB, USA) with primer was used for real-time PCR (Table 1). The reactions were run for 10min at 95°C in an Applied Biosystems 7300 real-time PCR system, followed by 40 cycles of denaturation at 95°C for 10sec, annealing, and extension at 60°C for 1min.

Results and Discussion

Characterization of Gn-ZnONPs: FTIR detected the functional groups of biomolecules in the ginger extract responsible for ZnO nanoparticle reduction

Table 1. Primers used in the study.

Primer		Primer sequence (5'-3')	Amplicon size (bp)	
YjaA	F	GACGCTGTGAAGTGTCAGGA	211bp	(10)
Biofilm	R	ATGGAGAATGCGTTCCTCAAC		

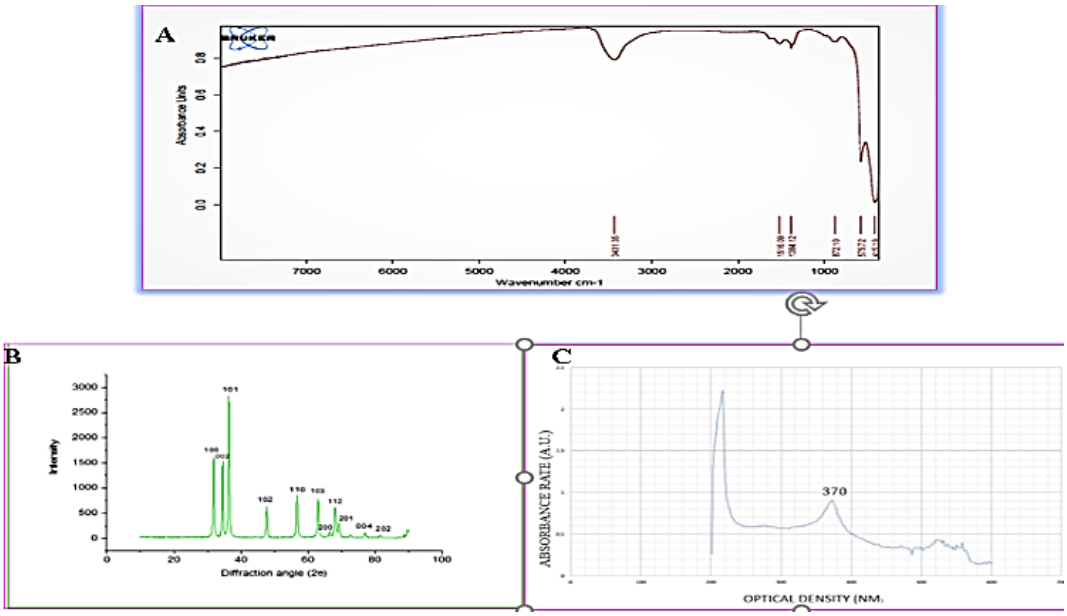

Fig.1. Primers used in the study.

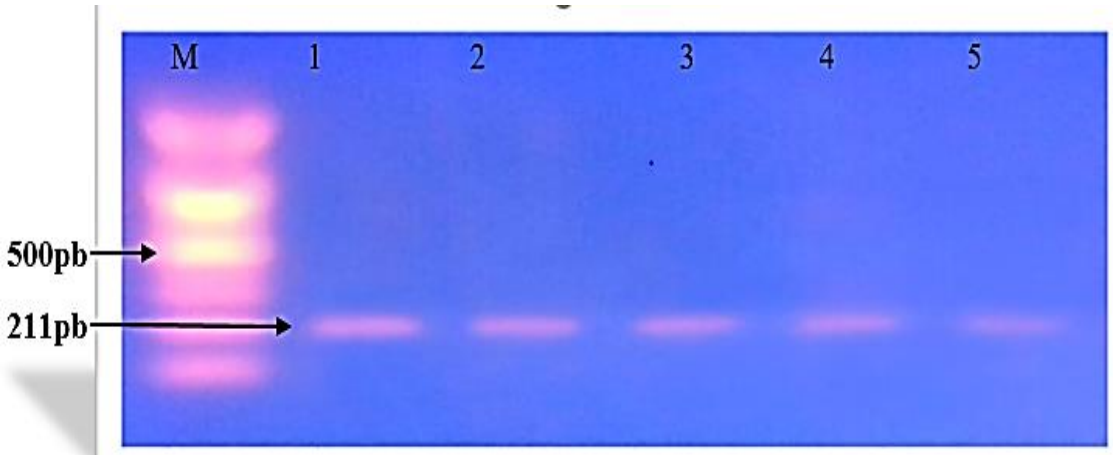

Fig.2. Agarose gel electrophoresis of PCR assay shows positive Biofilm results of *E. coli* biofilm *YjaA* gene in lane M, DNA marker 100-1000bp, and lane 1-5 positive (211bp).

and stabilization (Fig. 1A). The peaks at 3430, 415, 1516, 1384, and 872cm^{-1} correspond to N–H (Amine) or amide groups, Zn-O bond, and C=O stretch, and ketones, respectively. (Ni et al. 2005; Sharma et al. 2016).

The X-ray diffraction pattern (XRD) of ZnO-NPs showed definite line broadening of peaks, indicating nanoscale particles of 31.79°, 34.44°, 36.27°, 47.53°, 56.57°, 62.82°, 66.32°, 67.90°, 69.03°, 72.51°, 76.88°, 81.30°, and 89.50°. Bragg reflections with two values of 31.79°, 34.44°, 36.27°, 47. (100), (0 02), (1 0 1), 102, (110), (103), (20 0), (112), (201), (004), (202), (104), and (203) are beyond the diffractions of ZnO nanoparticles to hexagonal

Table 2. Means of gene inhibition of *E. coli yjaA* gene after subject to green ZN0-NPs.

Conc. Of NPs	Mean of folding
Control	1±0[a]
0.5	1.37±0.11[b]
1	0.192±0.006[c]
1.5	0.132±0.006[b]
LSD	0.202

Means with a different small letter in the same column are significantly different (P<0.05).

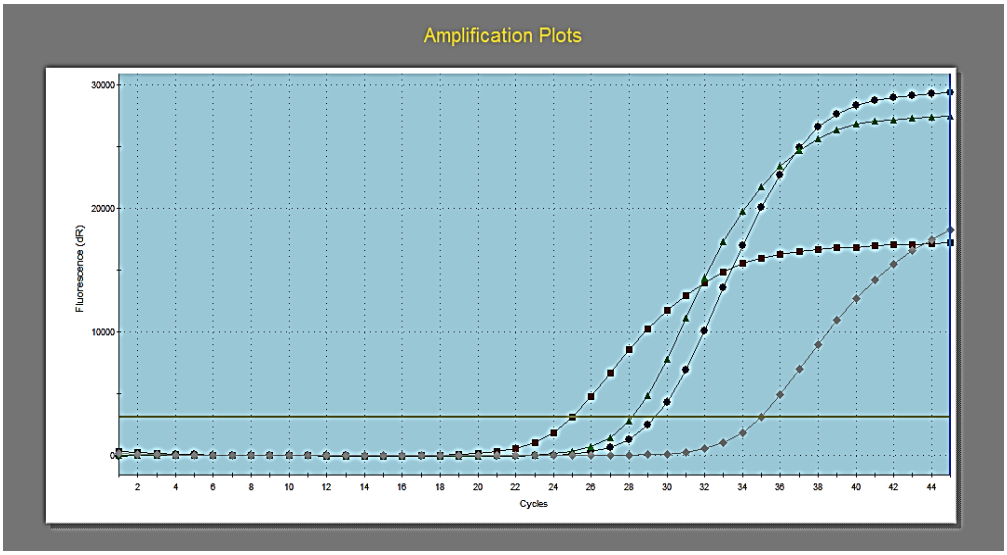

Fig.3. RT-qPCR amplification biofilm formation genes (*yjaA* gene) in treated and untreated (control) *E. coli* isolate (red blot = T1, blue plot = T2, green plot =T3, and gray plot = control).

planes (Fig. 1B). The capping agent stabilizing the nanoparticle may have caused these sharp Bragg peaks. All of the peaks matched the CPDF Card No. 01-079-2205 (Kalpana et al. 2018). The average crystallite size of the synthesized ZnO nanoparticles was estimated as 24.36nm using Scherrer's equation from sharp diffraction peaks (100, 002, and 101).

UV-visible absorption spectrum: ZnO NPs was confirmed by a significant absorption band with a maximum wavelength of 370nm (Fig. 1C). This sharp peak indicates that the particles are Nanosized, according to the spectrum, which is identical to what has been reported for ZnO NPs (Khitam et al. 2018).

Molecular identification of biofilm formation *YjaA gene*: The PCR molecular assay was performed to confirm the presence of biofilm genes in *E. coli* isolates. The isolates were subjected to PCR analysis, which revealed a 211bp band for the *yjaA* gene (Table 1, Fig. 2). These findings were consistent with previous studies (Cramaton et al. 2001; Wang et al. 2013).

Quantitative real-time PCR analysis:

Effect of green ZnO-NPs on the expression level of the *yjaA* gene: *Escherichia coli* is a common foodborne pathogen that can be transmitted through contaminated poultry products (King et al. 2018). Nanotechnology provides new antibacterial and antibiofilm techniques. Green synthesis ZnO NPs have the potential to be used as an alternative to conventional antibiotics. Reverse Transcription Quantitative Real-Time PCR (RTq PCR) was used to analyze expression levels of biofilm encoding *YjaA* gene in *E. coli* isolates compared to their expression levels before and after treating the isolates with ZnO-NPs. As a result, gene expression was measured using CT values to test target gene compared with housekeeping genes and determine the fold change of gene expression (Flemming & Wingender 2018).

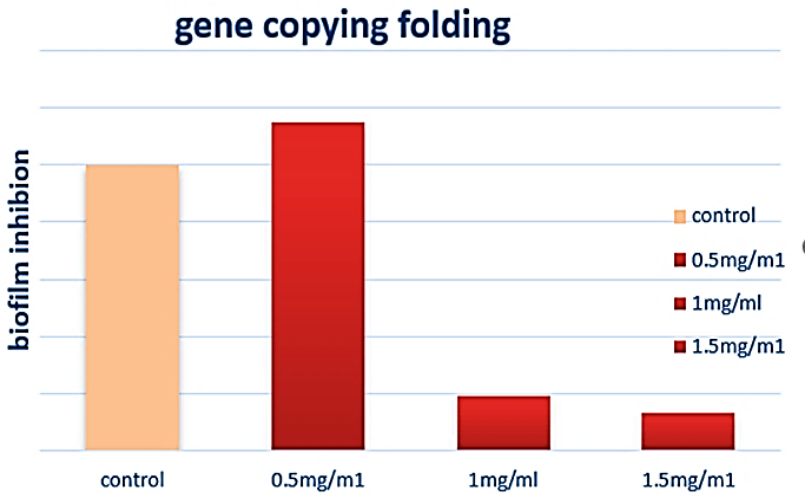

Fig.4. Effect of GnZnO NPs on relative genes expression: the fold change decrease in 1 and 1.5mg/ml while increasing 0.5mg/ml in the biofilm genes expression of *E. coli* isolates after being treated with green ZnONPs.

Three experimental isolates of *E. coli* that formed biofilm Congo Red Media were chosen for RT-qPCR assessments of *yjaA* gene expression. *Escherichia coli* isolates were cultured in the negative control medium (broth of biofilm bacteria with 1% glucose and without ZNONPs treatment) and medium containing three different concentrations of ZnO-NPs with biological 0.5, 1 and 1.5mg/ml for 24 hours. The level of *yjaA* gene expression decreased compared to the level of gene expression in control isolates.

Biofilm development is inhibited by increasing NPs concentrations, as the cell count to NPs ratio decreases with increasing NPs concentrations (Fig. 4). In the presence of zinc nanoparticles at 1 and 1.5mg/ml, these concentrations were enough to kill all of the cells seeded in the culture. This prevented the cells from growing and forming biofilms. While the addition of 0.5mg/ml ZnO NPs to the broth increased gene biofilm expression, similar to previous reports (Applerot et al. 2012; Musarrat et al. 2015), indicating that biofilm gene expression will be increased because of exposure to trace amounts of ZnO nanoparticles leading to cell damage. However, a small percentage of dead bacteria in a bacterial population will provide nutrients to the living cells, such as released intracellular DNA and RNA, lipids, proteins, and polysaccharides (Janak et al. 2015).

Bacteria's response to ZnO NPs is largely unknown at the gene expression level. The antibacterial mechanism of ZnO NPs is not fully understood. However, the best antibacterial activity displayed so far is when the concentration of ZnO NPs is higher and the surface area of particles is larger (Brayner et al. 2006). However, other reasons include the type of NPs used, the used bacterial strain, NPs concentration, quantification methods, and the biofilm culture environment (Chendong et al. 2016). Biofilm formation is aided by an increase in several viable cells over time.

As cell growth recovers, biofilm development will be accelerated. In many bacteria, biofilms are linked to multi-drug resistance (Moroboshi et al. 2007). As a result, reducing or destroying biofilm formation can be a good way to reduce bacterial antibiotic resistance and pathogenicity.

Conclusions

The inhibitory effect of zinc oxide nanoparticles was demonstrated in this study. After treating the isolates with ZnO-NPs at concentrations of 1 and 1.5mg, levels of gene expression were significantly reduced to suppress *YjaA* gene biofilm formation. There was a significant difference in the gene expression of the biofilm of *E. coli*. ZnO nanoparticles can be used as a cleaning agent for surfaces, apparatus, and production lines in food plants to prevent the

formation of, which is important for public health.

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