ENTEROBACTER CLOACAE LIPOPOLYSACCHARIDE EXPORT SYSTEM PROTEIN (LPTC) GENE EXPRESSION VARIATION VIA EXPOSE TO BIOSYNTHESIZED ZINC OXIDE NANOPARTICLES

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ABSTRACT

Synthetic nanoparticles (NPs) have become more widely used in the disinfection and health sectors owing to their ability to effectively penetrate biological systems. Recent research has demonstrated the ability of microbial proteins and enzymes to function as reducing agents throughout the NP-production process, providing a different option to chemical and physical techniques. This method not only saves money and is effective, but also has the least possible environmental impact. This work used *Lactobacillus* spp., as the reducing and capping agent to create zinc oxide nanoparticles, (ZnO NPs). Numerous analytical techniques were employed to examine the generated ZnO NPs; these techniques include ultraviolet–visible spectroscopy (UV–vis), scanning electron microscopy (SEM), X-ray diffraction (XRD), and Fourier transform infrared spectroscopy (FT-IR). As an opportunistic pathogenic bacterium, *Enterobacter cloaca* possesses several virulence characteristics that enable it to infiltrate target tissues. However, bacteria can become resistant to antibiotics, which in turn makes treating bacterial infections becomes more difficult. With the development of science at the biotechnology level, using zinc oxide ZnO NPs on isolates of *Enterobacter cloacae*, we conducted research in the promising field of nanotechnology. The impact of three different concentrations of ZnO NPs on the gene expression of *Enterobacter cloaca* lipopolysaccharide transport (Lpt) proteins in isolates obtained from some patients suffering of various infections was investigated. Bacteria with the highest multidrug resistance were selected for further analysis. Expression of LptC gene was reduced significantly at concentration of 125 mg/mL ($P < 0.01$) for all the isolate that treated with ZnO NPs.

Keywords: *Enterobacter cloaca*; *LptC* Gene; Nanoparticles; Zinc Oxide; Antibacterial; Gene Expression

INTRODUCTION

Zinc oxide (ZnO) nanoparticles (NPs) have participated extensive interest because of their unique features which distinguish them from their individual components (**Jiang et al., 2018; Ong et al., 2018**). Lately, ZnO NPs have been utilized in many different products such as photo catalystis, cosmetics, medicines, and nutritious additives for animals. ZnO NPs can particularly increase the bioactivity of pharmacophores and are less toxic as well as biodegradable (**Mohd Yusof et al., 2019**). Recently, ZnO NPs have had a noticeable importance as antimicrobial factors in nutrient packaging, textiles, and wound plasters manufacturing (**Huang et al., 2018**). Various pathogenic bacteria have been used to investigate the in vitro antimicrobial effectiveness of ZnO NPs. The antibacterial activity of ZnO NPs is regularly determined by their size and formulas. ZnO NPs have high surface to volume ratios because it makes them more reactive, which enhances their antibacterial properties (**Seil, J.T. & Webster, T.J, 2012; Sun et al., 2018**). In a number of studies, ZnO NPs were tested against various strains of microbial pathogens, such as *Salmonella typhi, Enterobacter cloacae, Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Bacillus cereus, Pseudomonas aeruginosa, Klebsiella pneumonia, Proteus mirabilis, Candida albicans,* and *Streptococcus sp.* (**Shanmugam et al., 2023**). Due to the increase in pathogenic bacteria that are multidrug resistance (MDR), scientists are currently concentrating on the creation of NPs as an alternative to antibiotics (**Khajeh Bami et al., 2018**). Many chemical methods, such as solvent gel technique, solvent vaporisation, and microemulsion precipitation, have been proposed for ZnO NP production. Even so, these methods are not ECO friendly because they use of hard chemicals for stabilisation and reduction, which bond to ZnO NPs and direct their biological purpose (**Mohd Yusof et al., 2020**). Moreover, these techniques contribute to the creation of dangerous byproducts, causing secondary pollution. Therefore, investigations are being conducted to substitute conventional methods with more sustainable ones for the production of ZnO NPs (**Hasson et al., 2019**). Using microorganisms for production of metal NPs has recently gained popularity because it is low-cost, ECO friendly, and biocompatible. Many kinds of microorganisms like Yeasts, fungi, and bacteria are being tried because of their efficiency in the production of ZnO NPs and Zn^{+2} sorption. Many types of Bacteria produce ZnO NPs intracellular such as *Lactobacillus*. For extracellular production of ZnO NPs different types of fungi can use such as *Aspergillus aeneus* and *Pichia fermentans* (**Król et al., 2018**).

Through the gram-negative facultative anaerobes bacteria that are widely found in environment are *Enterobacter cloacae*, which belong to the *Enterobacter genus*. Recently, there have been rising bother related *Enterobacter* species because of their developed association with nosocomial infections, especially in staff workers with impaired immune systems. ESKAPE microorganisms, which include *Enterobacter* spp. which is among the species that can result in opportunistic infection, are credited to be the main global reason of nosocomial infections. Commonly in patients needing mechanical ventilation (**Cabral et al., 2017**). The *Enterobacter cloacae* complex (ECC) consists of several species and its taxonomic class has been constantly updated. Over the past several decades, the ECC has been linked with MDR and has become an important nosocomial microbe. *E. cloacae* can easily obtain several genetically mobile elements that are extremely pathogenic and include genes for virulence and resistance. Because they generate constituent AmpC β-lactamase, these species have intrinsic impedances to ampicillin, amoxicillin, first-generation cephalosporins, and cefoxitin. They are also extended spectrum β-lactamase producers. Consequently, throughout therapy, Enterobacter spp. can become resistant to antibiotics, thereby reducing the number of available treatments (**Santajit, S. & Indrawattana, N, 2016**).

Lipopolysaccharide (LPS) acts as a permeability barrier that shields gram-negative bacteria from medication. Cells generate lipopolysaccharides (LPS), which are carried to the outer membrane (OM) by seven lipopolysaccharide transport (Lpt) proteins (LptA–LptG). Of the seven Lpt proteins, LptC works with LptA to move LPS from the inner membrane (IM) to the outer membrane (OM), and LptD/LptE facilitates assembly. Since Lpt proteins are crucial for the assembly of LPS, new antibiotics may target them because of their interaction with one another, which is required for LPS production (**Dai et al., 2022**).

Because they are easily handled and classified as food-grade microorganisms, lactic acid bacteria (LAB) are microorganisms of particular interest and are "widely acknowledged as protected" (**Kerry et al., 2018**) during the manufacture and preservation of food. Furthermore, particular LAB strains have probiotic features, thus enhancing their ability to promote health when eaten by people or

animals (**Kerry *et al.*, 2018**). In addition, many searches have conformed the effectiveness of LAB in facilitating the production of ZnO NPs (**Suba *et al.*, 2021**). LAB have intensive cell walls that are consists of polysaccharides, collagen, peptidoglycans, and lipoteichoic acid which play a significant role in metal ion biosorption and bioreduction processes by drawing metal cations to induce NP synthesis because of their negative electrokinetic features (**Garmasheva *et al.*, 2016**). In this study, probiotic Lactobacillus spp. cell biomass was used to analyse the production of ZnO NPs. Here, we demonstrate how the biomass of *Lactobacillus* cells can react with zinc acetate to create ZnO NPs. Various physicochemical descriptions were employed to confirm ZnO NP production. The antibacterial activity and expression of the *LptC* gene before and after ZnO treatment were also studied.

MATERIALS AND METHODS

Preparation of zinc solution

To prepare a stock solution (1 M) from Zinc acetate ($ZnC_4H_6O_4$) powder was dissolved in deionised water (DW).

Lactic acid bacterial isolation

Lactic acid bacteria were isolated from local cow yogurt using the de Man Rogosa Sharpe (MRS) medium. The sample sources were stirred at 150 rpm for 24 hours at 37 °C while being inoculated in MRS broth in 250 millilitre flasks. Following successive dilution and plating on MRS agar, the bacterial culture was incubated for 24 hours at 37 °C. Single colonies of various morphologies were created using the streak-plate method. For all isolates, the cell shape, Gram staining characteristics, and catalase reactions were observed and documented. MRS broth include 25% (v/v) glycerol was used to save the bacterial isolate that were gram-positive and catalase-negative for additional processing at -20°C (**Abdul-Husin *et al.*, 2016**).

Biosynthesis of ZnO NPs

Production of ZnO NPs was done through biosynthesis as described by **Markus *et al.*, 2016**. *Lactobacillus* bacteria inoculums were cultured in MRS broth, incubated for 24 hours at 37 °C with 150 rpm stirring. The metal salts, which is zinc acetate was dissolved in deionized water (DI), then the bacterial extract added to the metal salts solution and placed in a shaking incubator at room temperature at a speed of 150 rpm. The filtrate was separated by centrifuge, then ethanol was added to the precipitate and placed in a centrifuge. This process was repeated twice to ensure that the particles were washed from any residue of the extract and Acetate. The precipitate was taken an oven at 60°C to dry.

Characterization of the synthesized ZnO NPs

The optical characteristics of the ZnO NPs were examined at wavelengths ranging from 300 to 700 nm using UV vis absorption spectroscopy (Hitachi U-2001). The crystalline structures of the ZnO NPs were assessed by X-ray diffraction (XRD). The ZnO NP crystallisation and phase purity were determined using XRD (XRD Modelle – D8 Advance, BRUKER, Germany). The functional groups of the NPs were examined using Fourier transform infrared spectroscopy (FTIR; Jascov- 650 Spectrophotometre). In addition, the ZnO NPs were studied morphologically by field-emission scanning electron microscopye (FE-SEM, Hitachi model s-3400n).

Evaluation of antibacterial activity

The agar well diffusion method was used to measure antibacterial activity. Tests were conducted to determine the antibacterial activity of the synthetic NPs against *Enterobacter cloacae* strains. Sterilised Petri dishes were filled with Mueller Hinton Agar-Hi Media (250 mL). The bacterial inoculum was distributed equally over a sterile Petri dish with MH agar using a sterile cotton swab. The agar was then punctured with 6 mm diameter wells for each of the concentrations—31, 62, 125, 250, and 500 µg/mL and one well for the control group (ZnO NP supernatant as the control) using a cork borer. The plates were then incubated for 24 hours at 36 ± 1^0C under aerobic conditions. After incubation, confluent bacterial growth was observed. The inhibition of bacterial growth was expressed in millimetres. A time-kill kinetics test was used to assess the degree of ZnO NP exposure in the cultures. As a test, new cultures were cultivated in NP containing medium, and as a control, other cultures were cultivated without the addition of NPs.

Purification of RNA and generation of cDNA

Three tubes with 5 mL of nutritional broth were infected with *Enterobacter cloacae*, and the tubes were then incubated for 24 hours at 37°C with 100 rpm. Following growth of the bacteria, 125 µL of NPs was added and the mixture was incubated for a further eight hours. Following treatment, 4000 xg centrifugation was used for 10 minutes at 4°C to extract the bacterial cells (**AL-Saad *et al.*, 2020**). RNX Plus kit (The CinnaGen Co.) was used to extract RNA according to the

manufacturer's instructions. After DNase I treatment, reverse transcription using 200 U of MMLV reverse transcriptase (Thermo Scientific) was performed to generate cDNA after the concentration of the collected RNA had been measured using a spectrophotometre (JENWAY6305, England). After being incubated for 60 minutes at 42°C, the RT mix was incubated for 10 minutes at 70°C. The cDNA stock was stored at −20°C until further analysis.

Quantitative analysis of *LptC* gene expression by qPCR

Primer3 was used to design the forward primer (5'AAGACTGGTCGCCAGCATCG'3') and the reverse primer (5'TGAGGCCAAAGGGCAATCG'3'), both were used for real-time PCR to amplify the 576-base-pair fragment of the *LptC* gene. The cDNA was quantified using Rotor-Gene 6000 real-time PCR equipment (Corrbet Research Australian Model RG2072D. A total of 100 ng of cDNA template, 7.5 µL of SYBR® Green PCR Master Mix dye (QIAGEN), 10 pmol of each primer, and 4.5 µL of DI water were added to each 15 µL PCR reaction. The amplification regimen involved 40 cycles of 95°C for 15 s and 60°C for 40 s, separated by 5 min at 95°C. The 16S rRNA gene was used as a reference for data normalisation. Each sample was examined in triplicate, including the no-template and no-RT controls. The $2^{-\Delta\Delta CT}$ technique was used to analyse the data, where $\Delta\Delta C_T = \Delta C_T$ (treated sample) - ΔC_T (untreated sample), $\Delta C_T = C_T (LptC\ gene) - C_T (16S\ RNA)$, and C_T is the amplified gene's threshold cycle value (**Al-Mousawi *et al.*, 2022**).

Statistical Analysis

All antibacterial activity experiments were performed in triplicate and SPSS (version 20) software was used for the statistical analysis. A normality test was performed to determine the normality of the data. Each dataset had a normal distribution. One-way analysis of variance (ANOVA) was employed to statistically analyse the differences between various NP concentrations. $P < 0.05$ was the threshold for statistical significance. The average gene expression of *LptC* was compared between the control group and the groups treated with ZnO NPs using Duncan's and Tukey's tests in the SAS 9/13 program, which was accessible in real-time PCR (Rotor-Gene 6000 series software 1.7).

RESULTS

### ZnO NP production and characterization

Each examined strain of *lactobacillus* could biosynthesize ZnO NPs in varying amounts. When all of the ZnO NPs samples were decanted, a white precipitate was observed at the bottom of the flask. Following drying, the resulting white precipitates were used to calculate the concentration of biosynthetic ZnO NPs per gram of powder, as shown in Fig. (1).
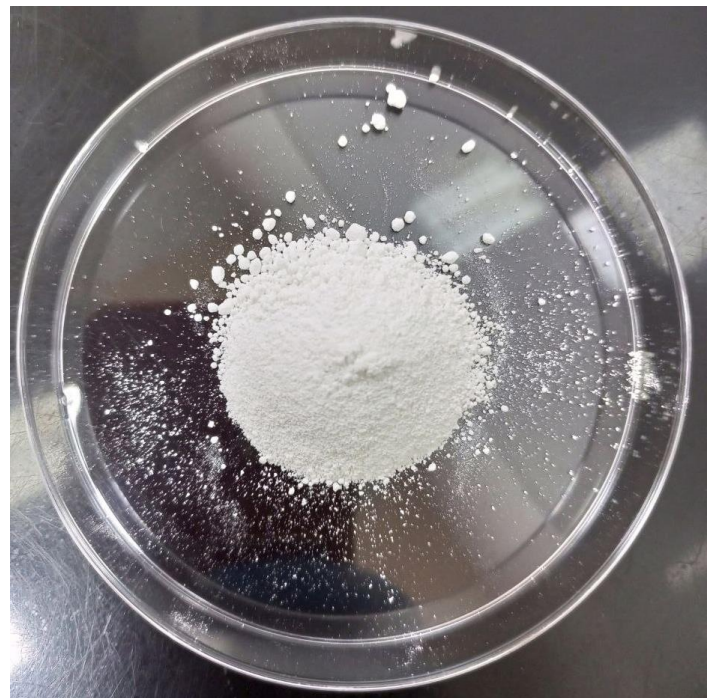
Figure 1 ZnO NPs that had been collected were subjected to several washings with distilled water, ethanol, and overnight drying to produce a white powder.

The optical characteristics of the ZnO NP were determined by UV–vis spectroscopy. As shown in Fig. (2), the absorption maximum of the ZnO NPs

occurred at 379 nm. **Vijayakumar *et al.*, 2018,** reported ZnO NP absorption spectra measured between 330 and 390 nm.
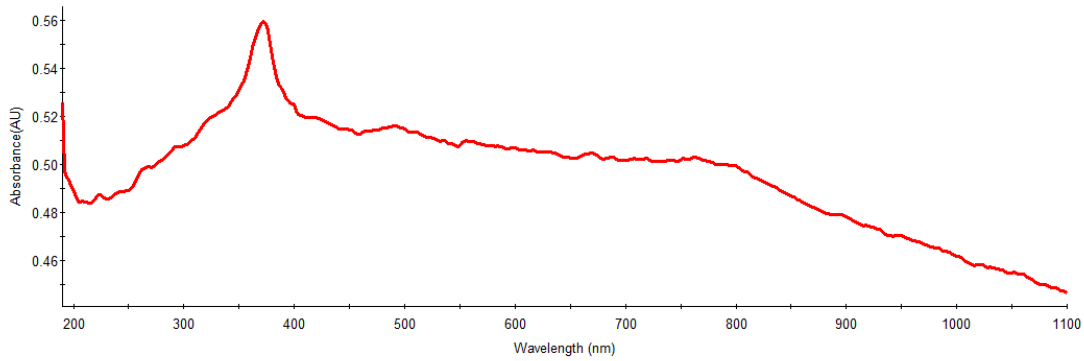
Figure 2 Spectrum of UV Vis absorption of ZnO NPs

As a result, the prepared biosynthesized ZnO NPs had a hexagonal crystalline structure based on the XRD pattern, which is in major agreement with this reference file. No other characteristic diffraction peaks were detected, indicating that the biosynthesized ZnO-NPs were free of other phase impurities and that their phase purity was high (Fig 3). Additionally, the narrow and strong diffraction peaks indicate that the produced ZnO NPs exhibited a crystalline structure. Furthermore, several XRD diffractograms reported for biosynthesized ZnO NPs are in reasonable agreement with the obtained results. According to the Debye Scherrer's equation, the average crystalline size of the NPs that corresponded to the strongest diffraction peak at $2\theta = 47.272°$ was 16 nm.
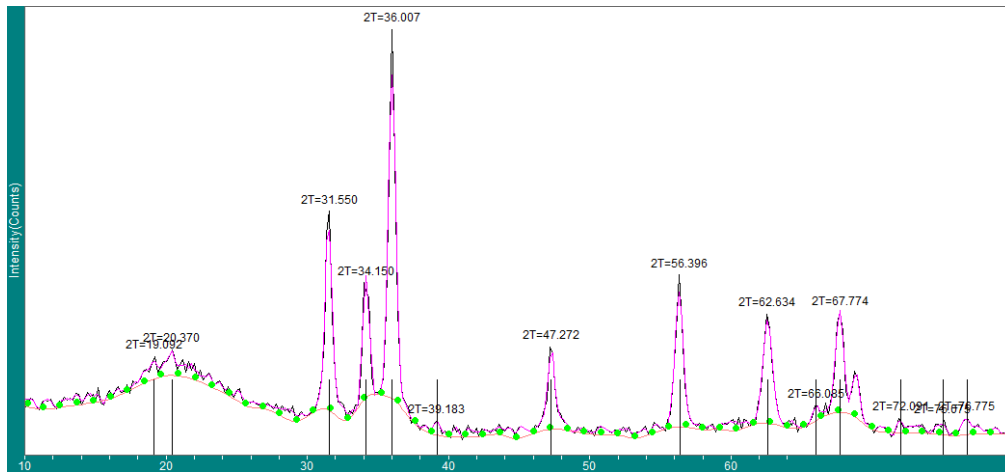
Figure 3 ZnO NPs analyzed using XRD

The infrared graphs for the bacterial (ZnO NPs) synthesis were obtained. The peaks at 3389.15 and 2920.60 cm^{-1} demonstrated the extending O-H of the carbonyl group, and the overlapping of the -OH group's vibration mode revealed the extending NH of the amines. The medium band at 1538.77 cm^{-1} reflects bending of the diamine (NH), which intersected with acetate ions or amine groups, whereas the faint signal at 1958.07 cm^{-1} suggested stretching of the SH thiol group. The peak values for the effective generation of ZnO NPs varied from 500 to 667.59 cm^{-1}. The results verified the participation of many groups, including C=O, O-H, NH, and SH disulfide groups, involved in the reductive, encapsulating, and stabilising processes of the bacteria (Fig. 4).
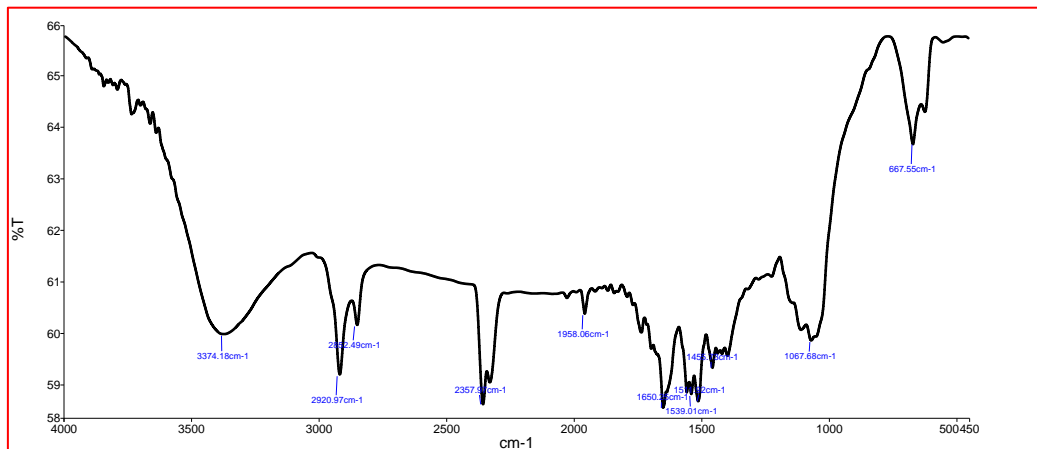
Figure 4 Analysis of ZnO NPs using FT-IR

SEM was used to examine the morphology of the organised ZnO NPs. The surface shapes of the biosynthesized ZnO NPs are shown in Fig. (5). The SEM images show that the majority of the biosynthesized ZnO NPs were spherical and that the ZnO molecules evolved slowly, formed hexagonal structures, and accumulated as bullets. However, no extracellular NPs were seen.
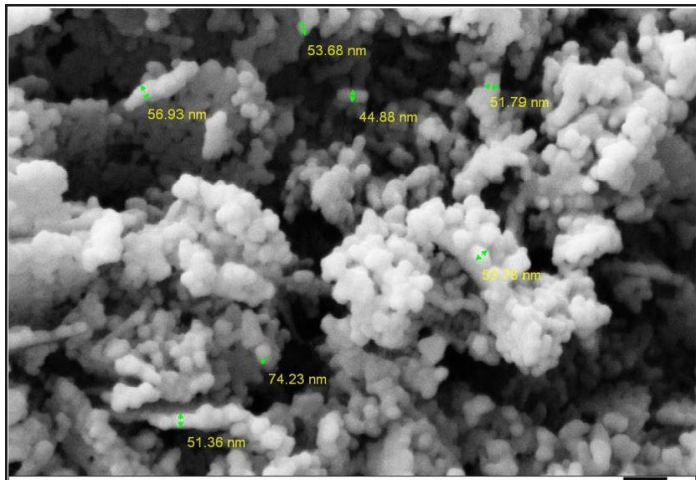
Figure 5 SEM picture of the prepared ZnO NPs

Antimicrobial efficiencies

The antimicrobial activity of the ZnO NP was tested using the agar well diffusion method for *Enterobacter cloacae* growth at different concentrations as shown in Fig. (6). The antibacterial results indicated that the MIC of *Enterobacter cloacae* growth is inhibited by 125 µg/mL ZnO NP. *Enterobacter cloacae* clinical isolates had MICs of ZnO NPs ranging from 31.25 to 500 µg/mL. As ZnO NP concentration increased, the antibacterial activity increased. The MBCs of the NPs that killed 50% and 100% of the isolates were 250 and 500 µg/ml, respectively.

Figure 6 Show (Inhibition Zones) of biosynthesis ZnO NPs by *lactobacillus* on *Enterobacter cloacae* growth.

Study of *LptC* Gene Expression

The effects of ZnO NPs on gene expression and real-time PCR quantification were utilised to investigate *LptC* gene expression. Following the late-log phase, the bacteria were treated with 125µg of ZnO NPs for 8 hours. The comparative critical threshold ($\Delta\Delta CT$) technique was then used to examine the data and quantify the expression of the *LptC* gene using RT-qPCR. The standard deviations of the three replicates are presented as error bars. When ZnO treatments were applied, the expression level of the *LptC* gene was downregulated, in contrast to the housekeeping gene 16S rRNA, which served as control (Table 1).

Table 1 Show The Gene Expression of *LptC gene* Before and After Treatment with ZnO NPs

No. of isolates	Effect of Lactobacillus ZnO NPs on *E. clocae*	Ct (*LptC*) of Target gene	Ct of reference (HK) gene	ΔCt Target (Ct (*LptC*) - Ct (HK))	$\Delta\Delta Ct$ (ΔCt – Ave ΔCt)	$2^{-\Delta\Delta Ct}$ Fold of gene expression	P-value
E1	Untreated	14.81	14.92	-0.11	-0.548	1.46 ±0.24	0.0061 **
	Treated	19.01	14.92	4.09	3.60	0.082 ±0.02	
E2	Untreated	14.87	14.88	-0.01	-0.448	1.36 ±0.12	0.0077 **
	Treated	17.65	14.87	2.78	2.297	0.20 ±0.07	
E3	Untreated	14.86	14.81	0.05	-0.388	1.30 ±0.14	0.0085 **
	Treated	18.06	14.03	4.03	3.547	0.085 ±0.04	
E4	Untreated	14.72	14.77	-0.05	-0.488	1.40 ±0.12	0.0078 **
	Treated	18.11	14.25	3.86	3.377	0.096 ±0.02	
E5	Untreated	14.70	14.80	-0.1	-0.538	1.45 ±0.13	0.0059 **
	Treated	18.24	14.67	3.57	3.087	0.117 ±0.03	
E6	Untreated	14.74	14.73	0.01	-0.428	1.34 ±0.14	0.0074 **
	Treated	18.29	14.54	3.75	3.267	0.103 ±0.07	
E7	Untreated	14.69	14.71	-0.02	-0.458	1.37 ±0.16	0.0068 **
	Treated	18.41	14.22	4.19	3.70	0.076 ±0.008	

** ($P \leq 0.01$).

A statistically significant difference was observed (P<0.01). Thus, ZnO NPs have a major effect on the lipopolysaccharide transport system in bacteria, which in turn affects the LptC-LptA complex, leading to the inhibition of the translocation of LPS from the inner membrane to the outer membrane.

DISCUSSION

Nanotechnology can be thought of as an antibiotic replacement. Controlling antibiotic-resistant bacteria may be possible using nanotechnology. The antibacterial effect was more successfully established using ZnO NPs with a higher surface-to-volume ratio. Using *Enterobacter cloacae* in this study, we notice that these NPs had a highly antibacterial effect. Multidrug resistance bacteria can result in various conditions leading to some chronic diseases (**Ghorbani *et al.*, 2020**). ZnO NPs are a group of NPs that are safe for the environment for their activities, and their component ionises after a certain period of time (**Moradian *et al.*, 2018**). Due to their high surface-to-volume ratio and small size, the ions generated by NPs straightly act on bacterial membrane (**Hasson *et al.*, 2021**). The mechanisms of the antibacterial activity of NP still unclear, despite reports of their antibacterial effects

from chemical synthesis. ROS (Reactive oxygen species) are thought to be created when the surface of NPs interacts with bacterial cell wall and membrane, damaging cell membrane, increasing cell permeability, and disrupt intracellular leaks. There are my kinds of reactive form such as superoxide anion (O_2), Hydroxide (OH^-), and hydrogen peroxide (H_2O_2). Because these kinds are internalised into the bacterial cell membrane, they are hazardous because they breakdown cellular molecules such as proteins, lipids, and nucleic acids. Despite this, there is great disagreement among scientists regarding ROS and the extent of their antibacterial effect. Moreover, the properties of ZnO NPs allow it to electrostatically adhere to the surfaces of bacterial cells and cause damage to their surfaces (**Bhutiya *et al.*, 2018**).

However, the activity depends on the size of the NPs. The antibacterial effectiveness of smaller NPs is higher than that of larger NPs owing to their larger surface area and higher reactivity. According to Khatami et al., biosynthesized ZnO NPs with a 2.8 nm size exhibited better inhibitory action against *S. aureus* and *E. coli*, with a lower MIC value of 2.0µg/mL. Furthermore, Abdul-Husin and colleagues showed that the produced ZnO NPs suppress *E. coli* development at a significantly lower concentration of 50µg/mL, and they proposed that the

antibacterial action of ZnO NPs is size-dependent **(Abdul-Husin *et al.*, 2016)**. In contrast to our founding's, the results of other studies on the biosynthesized ZnO NPs generated were significant and yielded higher MIC values. According to **Hanif** and his colleagues, after ZnO NPs adhere to the bacterial cell wall, ions are released to rupture the cell membrane and cause the cytoplasm to leak out **(Hanif *et al.*, 2019)**.

This study showed that the expression of *lptC* gene in *Enterobacter cloacae* was significantly affected after exposure to ZnO NPs. Since bacteria have the ability to respond to environmental changes, which is controlled by regulatory interactions in the cell. Therefore, the main goal of ZnO NPs is to stop the effect of these regulatory interactions. It is hypothesised that surface hydrophobicity and surface charge affect bacterial adhesion and are beneficial in promoting bacterial surface adherence. There may be non-limited physicochemical interactions connected to the adhesion of LPS membranes **(Ravichandran *et al.*, 2020)**. ZnO NPs cause oxidative stress in bacteria and inhibit their growth, thereby lowering the synthesis of catalase, an antioxidant enzyme that protects bacteria from oxidative stress **(Abdo *et al.*, 2021)**. Although further research is needed to determine how ZnO NPs and ROS interact, the main focus of this work was to determine how ZnO NPs affect the expression of genes that create the structures of the LPS transport system.

CONCLUSION

ZnO NPs were shown to exhibit antibacterial activity against *Enterobacter cloacae*. This suggests the possibility of using this material in the manufacture of medicinal materials (antimicrobial agents). ZnO NPs decrease the prevalence of acquired resistance in pathogens resulting from incorrect or excessive use of antibiotics. Assessment of various NP concentrations revealed that 125 µg/mL was highly successful at altering bacterial gene expression.

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