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Antibacterial Effect of Silver Nanoparticles along with Cell Wall Synthesis-Inhibiting Antibiotics on *Staphylococcus aureus* Isolated from Ewe's Mastitis

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Abstract | This research was conducted to isolate *Staphylococcus aureus* from mastitis-affected sheep that are resistant to most antibiotics and aimed to study the synergistic effect of antibiotics combination along with AgNPs nanoparticles. The results obtained were the isolation of 18 isolates. Representing (18%) were found *S. aureus*, 10(10%) were seen as *E. coli*. Most isolates from mastitic sheep in the current study shared high resistance to the antimicrobial drugs used. Their resistance to Ampicillin (100%), Cefepime (100%), Gentamicin (100%), and Sulfamethoxazole (100%). In contrast, their resistance to other antibiotics (Ceftriaxone, Ceftazidime, and Norafloxacin) varied in degree. The minimum inhibitory concentrations (MICs) of the silver nanoparticles tested against *S. aureus and E. coli* isolates were determined by using a microdilution method. The results show that a MIC concentration of 0.15µg/ml inhibited the growth of *S. aureus* isolates, while a concentrations 0.07 µg /ml inhibited visible growth for *E. coli* isolates. The findings indicated that amalgamating Ampicillin, Cefepime, and Gentamicin with AgNPs exhibits a superior antimicrobial effect on synthesizing bacterial cell walls when investigated against *S. aureus and E. coli*. It is more isolated from sheep mastitis than silver nanoparticles alone. The antimicrobial effect of AgNPs increased with higher concentrations of AgNPs and showed a maximum synergistic antimicrobial effect. When AgNPs were used in combination with any of the antibiotics (Ampicillin, Cefepime, Gentamicin), the reaction mixture exhibited antimicrobial activity, as indicated by the minimum inhibitory concentration (MIC) of Ag. Ampicillin against *S. aureus* inhibited the growth of isolates at a concentration of 0.03µg/ml. In contrast, concentrations of 0.3µg/ml were inhibitory for *E. coli* isolates, while the combination of cefepime and nanoparticles showed a synergistic effect against *S. aureus* and *E. coli*. The concentration 0.03µg/ml inhibited the growth of *S. aureus,* while concentrations 0.07 µg /ml were inhibitory for *E. coli,* while Gentamicin showed an MIC value for Both *S. aureus and E. coli* were at a concentration of 0.07 µg/ml.

Keywords | Silver nanoparticles, *Staphylococcus aureus*, Ewes' mastitis

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INTRODUCTION

Mastitis is significant disease that impacts the safety and productivity of animals due to its widespread occurrence and the large substantial economic losses it entails. There are many pathogenic causes of mastitis, including bacterial, fungal, and mycoplasma infections. Due to the multiplicity of pathogens, especially bacteria,

it is not easy to control (Eberhart *et al*., 1987; Capurro *et al*., 2010; Yaseen *et al*., 2025; Obaid *et al*., 2025). Research in nanotechnology has unveiled numerous new avenues, yielding innovative, practical, and occasionally unforeseen applications (Nafea *et al*., 2025; Yaseen *et al*., 2024; Obaid *et al*., 2020). Bacterial mastitis is a widespread condition, often caused by *Staphylococcus aureus*. In dairy ruminants, *Staphylococcus aureus* is an opportunistic pathogen that can cause mastitis in these animals. It is also present in healthy carriage. Numerous mastitis control programs have been implemented to address the issue, yet they have not consistently demonstrated efficacy (Judge *et al*., 1997; Obaid *et al*., 2020; Kareem and Hussain, 2023; Altemeemi *et al*., 2021; Ali *et al*., 2024; Al-Sailawi *et al*., 2024). Antibiotic resistance is prevalent in many countries (Jasim *et al*., 2025; Al-Khuzaay *et al*., 2024). Stressed the necessity of developing comprehensive control programs for the disease and limiting its spread in fields, as it depends on early and rapid diagnosis of all infected udder quarters in the early stages of the disease, as its spread leads to the infection of other animals without visible symptoms appearing on them. It is not easy to diagnose except by relying on field and laboratory tests. Due to the indiscriminate use of antibiotics in recent years, many bacterial isolates have developed resistance to most of these antibiotics. In addition to the responsibility of antibiotics for causing many side effects, mainly if they are used in high and incorrect doses, some researchers have resorted to finding alternatives to these drugs (Jasim *et al*., 2021; Kadhim *et al*., 2024; Yahya *et al*., 2024). Indicated for treatment with plant extracts and medicinal herbs because they contain many active substances that have proven their effectiveness.

This research was designed to investigate the isolation and characterization of *S. aureus* bacteria from milk samples from sheep suffering from mastitis. Also, we aim to assess the antibiotic sensitivity and determine the minimum inhibitory concentration value. Finally, we aimed to investigate the synergistic effects of antibiotics administered individually and in combination with AgNPs.

MATERIALS AND METHODS

The California Mastitis Test (CMT) was conducted on milk samples to identify clinical and subclinical mastitis. One hundred milk samples were gathered from sheep farms located in Baghdad Governorate. After cleaning and disinfecting with 70% alcohol, the sheep breasts were dried with sterile cotton and throwaway towels. Ten milliliters of milk were obtained in a sterilized BHI container after initial milking of the teat was discarded. All samples were transported and stored at 4 °C. Straight to the lab for a follow-up assessment, the samples were shaken well for bacteriological culture first, and the remaining part of each milk sample was used to carry out chemical tests (Mellenberger, 2001; Hameed *et al*., 2025).

Staph aureus isolation and identification

A 100 µl milk specimen was inoculated onto mannitol salt agar (Oxoid, England)and incubated for 24 hours at 37°C. *S. aureus* related colonies were chosen and moved to 5% sheep blood agar (Himedia India). All isolates were presumed to be determined through Gram staining, cultural characteristics, and coagulase testing using fresh rabbit plasma (tube method) (Omran *et al*., 2024; Abd El-Rahmana *et al*., 2024). Eight isolates of *S. aureus* were examined from mastitis milk samples of 100 sheep.

S. aureus molecular diagnosis

DNA of *S. aureus* was extracted using the manufacturer's protocol for a DNA purification kit (Intron Korea) from a 24-hour bacterial culture in BHI medium. PCR was used to amplify the *16S rRNA* gene for accurate identification of *S. aureus* (Yang *et al*., 2007). For 16S rRNA gene amplification, the F primers (5'-GCGATTGATGGTGATACGGGT-3') and R primers(5'-AGCCAAGCCTTGACGAACTAAAGC-3') were utilized. A PCR reaction was performed using the CinnaGen PCR Master Kit, with a final volume of 25µL. The composition consisted of 12.5µl of mix master (2X concentration), 0.4 mM of each (F and R) primer, and 2 µl of DNA sample. The total volume of the mixture was adjusted to 25 µl using deionized distilled water. DNA was extracted and utilized as the positive control, and sterile water was used as the negative control. DNA replication was carried out using the following protocol: 35 heat cycles, each lasting one minute at 94°C for denaturation, 30 seconds at 55°C for the annealing stage, and one minute at 72°C for extension. The last stage involved completing the reaction for 3.5 minutes at 72°C. For every isolate of *S. aureus*, an identical-sized PCR result was produced. The dimensions of the PCR products were determined utilizing 1.2% agarose gel electrophoresis alongside a 100 bp DNA ladder marker.

Sequences of isolates

The *16s rRNA* gene from eight *Staphylococcus aureus* isolates was analyzed and sequenced as part of the experiment. Sequence alignment results obtained through BLAST and BioEdit were compared with data from the GenBank, available online at NCBI.

Antimicrobial susceptibility testing

Bauer *et al*. (1966) have stated that antimicrobial susceptibility testing was performed on all *Staphylococcus aureus* using the disk diffusion technique. Bacterial samples were cultivated at 37°C in Mueller Hinton

broth medium (Oxoid, England) for 24 hours as the culture medium (Abdula *et al.*, 2024). Following growth, the samples were compared using a 0.5 McFarland turbidity tube. The 48 microplate wells, four wells. The Disk Agar Diffusion method was employed on Muller Hinton Agar to determine per row were considered the resistance patterns of isolates against Amoxicillin (30µg), Ceftriaxone (10µg), Ceftazidime (30 µg), Cefepime (10µg), Imipenem (10 µg), Meropenem (10µg), Gentamycin (10µg), Amikacin (10 µg), Sulfamethoxazole (25µg), and Norfloxacin (30 µg). After incubation, the diameter of the inhibition zones was measured and categorized as sensitive, resistant or intermediate using the guidelines provided by (The Clinical and Laboratory Standards Institute, 2021).

Preparation of AgNPs

The synthesis of AgNPs utilized included silver nitrate (p.a., Fagron), ammonium (28–30% [w/w], p.a., Sigma–Aldrich), sodium hydroxide as the solvent (p.a., Lach–Ner), and D-maltose (p.a., Sigma–Aldrich) (Gokulakrishnan *et al.*, 2012). Selected multi-resistant bacterial strains were used to assess the synergistic effects of AgNPs in combination with six antibiotics including amoxicillin, ceftriaxone, ceftazidime, cefepime, imipenem, and meropenem.

AgNP synthesis and characterization

The tollens technique, which has been previously documented and involves reducing the complex cation [Ag(NH3)2]+ by D-maltose in alkaline conditions, was followed in the synthesis of AgNPs for this investigation (Kvitek *et al.*, 2009). The concentrations of each component involved in the reaction were as follows: Ammonia at 5·10–3 mol· dm^3, sodium hydroxide at 9.6·10–3 mol·dm–3, silver nitrate at 1·10–3 mol· dm^3 (mass concentration equal to 108 mg/L of Ag), and D-maltose as a reducing agent at 1·10^2mol· dm^3. A magnetic stirrer was used to stir the reaction mixture continuously. AgNPs were synthesized at room temperature, or roughly 21 °C. AgNPs were additionally stabilized during production by adding gelatin to the dispersion at a final concentration of 0.05%, which helped prevent partial aggregation by introducing a stabilizing agent into the culture medium. Using the 90 Plus Particle Size Analyzer (Brookhaven Instruments Co.), dynamic light scattering was used to measure the particle diameter of the prepared AgNPs.

Transmission electron microscopy (TEM) was used to confirm the nanodimensions of the AgNPs using the JEM 2010 apparatus (Jeol, Japan). Using a Specord S 600 spectrophotometer (Analytik Jena, Germany), the ultraviolet-visible (UV-Vis) light spectra and surface plasmon resonance of AgNP dispersions diluted 10-fold were recorded.

Preparation of antimicrobial agents and assessment of (MIC) and (MBC) for silver nanoparticles

In 1.5 ml micro-centrifuge tubes, a stock solution of pure Ag nanoparticles was made by dissolving in DW to a final concentration of 10 mg/ml and filtering with a 0.22 µm Millipore filter. Two-fold serial dilutions were performed from the stock solution to achieve concentrations ranging from 5 mg/ml to 0.009 mg/ml. The stock solution was diluted to 2.5 mg/ mL by aseptically transferring 100µL into microtiter plate A1, along with 100 µL aliquots of sterile Mueller-Hinton broth (MHB). Once the contents of each well have been sufficiently mixed, one hundred µl aliquots of A1 will be transferred to the equivalent wells in B1, which also contain 100 µl aliquots of MHB. This will result in another 50% antimicrobial dilution (1.25 mg/ml). The procedure was repeated for each row to generate the following dilutions: 0.6 mg/mL, 0.3 mg/mL, 0.15 mg/mL, 0.07 mg/mL, 0.03 mg/mL, 0.01 µg/mL, and 0.009 mg/Ml.

Preparation of inoculum

Bacteria were cultured overnight on agar to yield the isolates. Three to five well-isolated colonies of the same morphological type were selected from the agar plate culture to prepare the inoculum for the MIC test. Each colony's tip was brushed with a sterile loop, and the growth was then transferred into a tube holding four to five milliliters of MHB. The colony was then incubated at 35 °C for approximately two hours, or until it reached the 0.5 McFarland Standard. Normal saline or sterile MHB was used to regulate the turbidity actively developing broth culture. After that the suspension was diluted (100 µL of bacterial suspension added to 9,900 µL of MHB) to achieve a bacterial cell count of approximately 10^8 CFU/mL. The optical density measurement of a bacterial cultivation solution with varying concentrations of silver nanoparticles after 24 hours was used to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of silver nanoparticles against *S. aureus*, thereby assessing the antimicrobial effectiveness of silver nanoparticles on bacterial growth. Each experiment was performed thrice on distinct days.

Determination of MIC and MBC for silver nanoparticles in conjunction with antibiotics

The minimum concentration of an antimicrobial agent that can inhibit visible bacterial growth without causing cell death is referred to as the MIC. The broth micro-dilution utilizing a 96-well polystyrene plate is the most suitable approach for determining MIC values. This method was employed for quantitative assessment to investigate the in vitro antimicrobial activity of Ag nanoparticles against

bacterial isolates, following the guidelines established by the Clinical and Laboratory Standards Institute (CLSI), as detailed by Balouiri *et al.* (2016) and Sabee *et al.* (2020). To ascertain the MIC and MBC of silver nanoparticles in conjunction with antibiotics, 100 µl of a standardized bacterial suspension at a concentration of 1× 10^8 CFU/mL was introduced into each well containing 100 µl of diluted AgNPs previously prepared. This resulted in an overall volume of 200 µL per well. To assess the sensitivity of the bacterial isolates, a positive control was performed in column 11 of the microplate, which included broth, the solvent for antimicrobial agents, and bacterial inoculation.

Conversely, A broth and an antibacterial solution without inoculum functioned as the negative control in column 12 of the microplate. The microtiter plates have been incubated overnight at 37 ºC for 18 to 24 hours. Following incubation, MIC values were assessed visually by adding 30 mL of Alamar blue dye to microplate wells and incubating at 37 ºC for 1 hour. Alamar Blue dye served as an indicator, utilizing a resazurin-based solution that quantitatively assesses cell viability through the reducing capacity of living cells. Resazurin, the active component of Alamar Blue reagent, is a non-toxic, cell-permeable substance that appears blue and is nearly non-fluorescent. Resazurin is reduced to resorufin upon entering living cells, resulting in a red, highly fluorescent compound. The minimum inhibitory concentration (MIC) was defined as the minimal concentration of each fraction that demonstrated no visible growth. MIC values were ascertained in duplicate to guarantee precision (Sabee *et al.*, 2020).

RESULTS AND DISCUSSION

Phenotypic characterization

As shown in Table 1, *Staphylococcus aureus* (8 %) was identified in 100 samples taken from 40 clinical and 60 subclinical mastitic sheep. Additionally, several mixed infections were found (Table 2).

Table 1: Clinical, subclinical, and total mastitis incidence.

	Milk samples	Number of positive Staph aureus	%
Clinical mastitis	40	2	31.81
Subclinical mastitis	60	6	8.30
Total No.	100	8	14.16

Table 2: Types of bacteria isolated from the milk of sheep suffering from mastitis and their percentages.

%	No.	Isolates
8%	8	*Staphylococcus aureus*
10%	10	*Escherichia coli*

The diagnosis is based on the results of bacterial culture and microscopic examination. Each type was classified based on the shapes of the colonies, lysis on blood agar, and biochemical tests, as shown. Every bacteriological analysis aligns with identifying the *Staph* genus and its species. These findings align with data regarding the isolation of *S. aureus* species as documented in (Quinn *et al.*, 2004).

The most important principles used in diagnosing these germs are based on Quinn *et al.* (2004). The colonies are resembled to those of *Staphylococcus* spp., which appear on nutrient agar within 24 hours of incubation at 37°C. They were circular, convex, shiny, and opaque, with a smooth circular non-toothed edge resembling oil droplets and colors ranging from white to dark orange, they were positive for coagulase yeast. *S.aureus* spp. produce distinct colonies on selective media that. grow on Manitol salt agar plates (Figure 1A) shows the characteristic golden colonies Microscopically, they appeared as Gram-positive cocci and formed clusters. While colony morphology of isolates of *S. grew* after 24 hours of incubation on blood agar under aerobic conditions at 37°C, the culture exhibited shiny, convex, hemolytic white colonies measuring 2-3 mm in diameter after 48 hours (Figure 1B).

Figure 1: Colony characteristics of *Staph aureus* on selective media (A) mannitol salt agar, (B) Blood agar.

Molecular study

As shown in Figure 1, the bacteriological isolation of *Staphylococcus aureus* from the milk samples obtained from mastitic sheep revealed the presence of *S. aureus* organisms in them. Every fecal sample that tested positive for bacteria had its positivity verified by PCR, which revealed the amplification of 370 bp fragments, as shown in Figure 2.

Sequencing the isolates

The sequencing analysis results were promptly submitted to GenBank with accession numbers MZ429309.1 and MZ429310.1. A compilation of *S. aureus* kDNA sequences, comprising two *S,aureus* records, was acquired from GenBank for phylogenetic analysis. The positive sample in this study was closely associated with Taiwan.

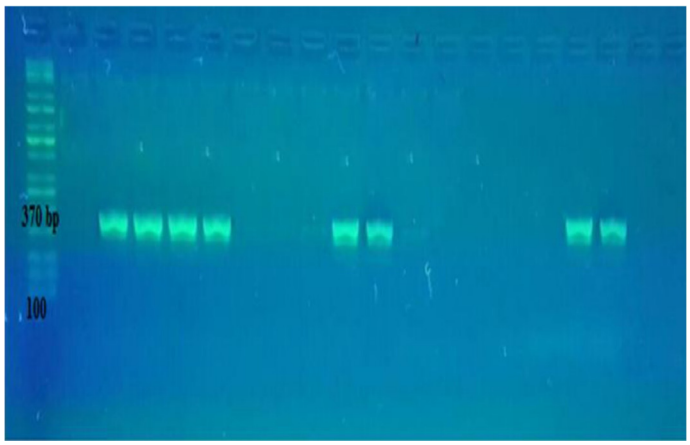

Figure 2: PCR product, with a band size of 370 bp for 16S rRNA. The product was electrophoresed on 1.5% agarose at 70 volt/cm2—1x TBE buffer for 1:30 hours. N: DNA ladder (100), lane 2-4, 8, 9, 15, 16 represents *Staph aureus*. PCR product of band size 370bp, visualized under UV light.

Staph aureus kDNA with accession number ON041097.1 and it matched with *Staph aureus* from ChinaON222796.1 Japan AP025693.1and USA CP064772.1, Nigeria CP051191.2, CanadaCP078521.1, Australia CP093933.1, India OM936855.1 Egypt OM920074.1 China: Guangzhou: CP088157.1:

This was determined by phylogenetic analysis.

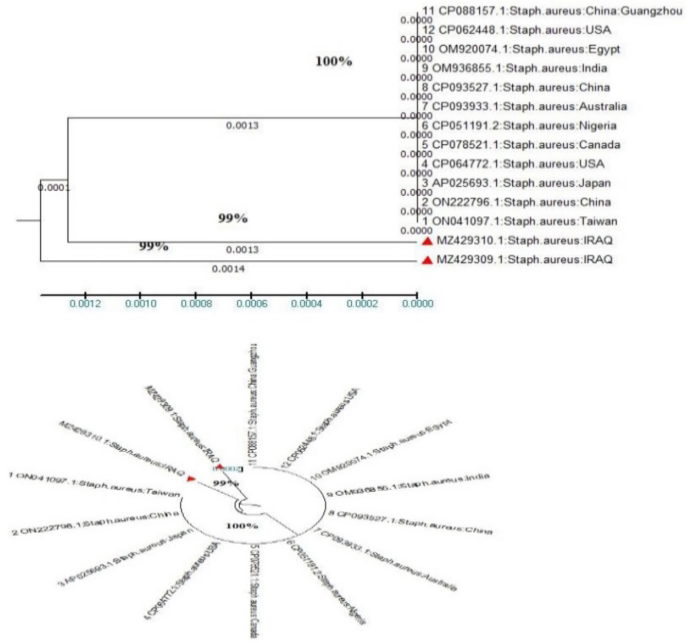

Figure 3: Phylogenetic analysis indicating genetic diversity between isolates based on kDNA sequence analysis.

Susceptibility of isolated bacteria to antibiotics

After using standard strains to evaluate the effectiveness of antibiotic tablets, the efficiency of these tablets was noted before they were used for this test. Then the sensitivity of bacteria isolated from cases of clinical mastitis to antibiotics was tested, as shown in (Tables 1 and 2), and their sensitivity was recorded. Their resistance to Ampicillin (100%) was higher than that of Cefepime (100%), Gentamicin (100%), and Sulfamethoxazole (100%), while their resistance to other antibiotics (Ceftriaxone, Ceftazidime, and Norfloxacin) varied. Figure 4 illustrates that Ceftazidime, Amikacin, and Ceftriaxone exhibited moderate sensitivity towards the majority of *Staph aureus* isolates.

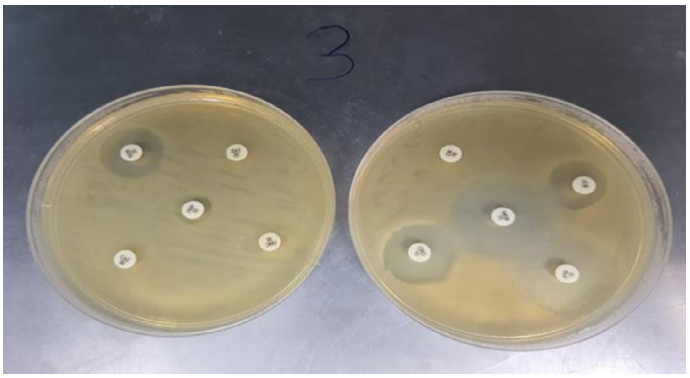

Figure 4: Antibiotic susceptibility testing of *Staphylococcus aureus* isolates from sheep on Mueller-Hinton agar.

Characterization of the Ag nanoparticles

We were employed to create AgNPs. AFM confirms that this technique yields AgNPs. It was found that the average diameter was about 21 nm, with a very narrow size distribution (Figure 5). Additionally, the average diameter was found to be about 56.00 nm.

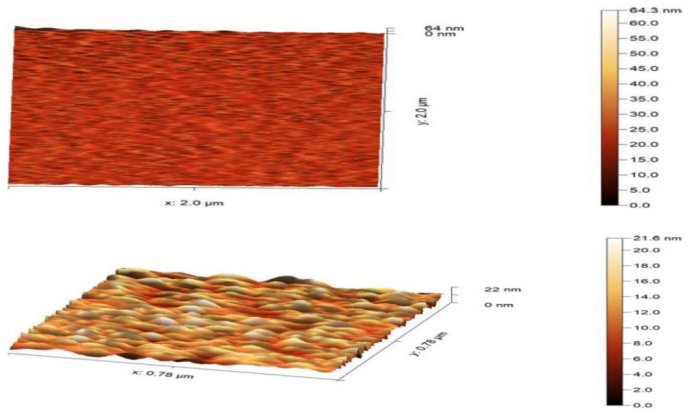

Figure 5: (A and B) showing atomic force microscopy image of AgNPs after stirring for 20min at 75 °C.

Scanning electron microscopy (SEM)

The results indicate that nanoparticle morphology exhibits significant variability in size and shape. The surface-deposited silver nanoparticles are visible at a magnification of 100,000 X. The SEM image indicates a high density of silver nanoparticles, confirming the formation of silver nanostructures. Figure 6 presents the SEM analysis of the synthesized silver nanoparticles. The synthesized particle

exhibited an average size of 43 nm, consistent with the findings of Jithesh (2013). The nanoparticles exhibited both spherical and irregular shapes, consistent with the findings of Promy (2018), which also noted these morphological characteristics. Davi *et al.* (2013) noted that the AgNPs synthesized from Sargassum longifolium exhibited a cubical morphology. The morphology of metal nanoparticles significantly alters their optical and electronic properties (Xu and Kall, 2002). A comparable phenomenon was documented by Chandran *et al.* (2006).

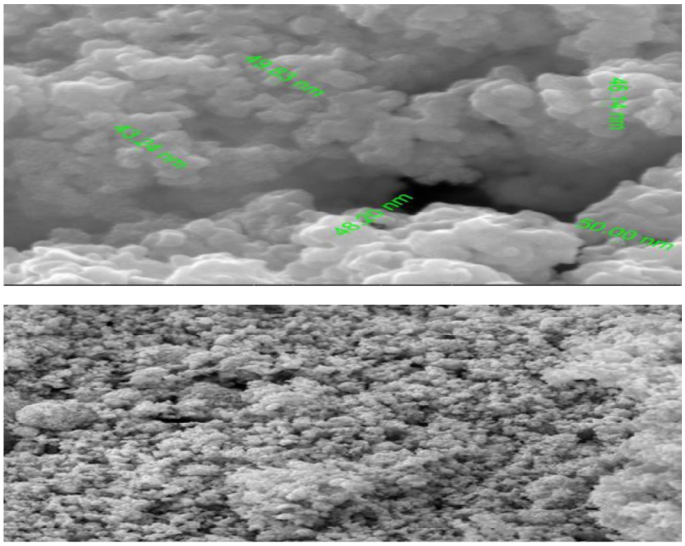

Figure 6: SEM image of AgNPs synthesized after stirring for 20min at 75 °C at 100000X magnification.

Antibacterial activity of silver nanoparticles (Ag.NP) against *S. aureus* and *E. coli*

The antimicrobial activity of the synthesized AgNPs was tested against *S. aureus* and *E. coli*. The observations indicate that the inhibition was close in concentration, resulting in reduced visible growth in both *S. aureus* (lane 1). The concentration of 0.15 µg/ml inhibited the growth of *S. aureus* isolates in 50% (2/4) of the cases. In comparison, concentrations of 0.07 µg/ml were inhibitory to visible growth for isolates of *E. coli*. These concentrations destroyed pathogenic strains of *E. coli*, as well as multiple-drug-resistant strains of *S. aureus*. Silver nanoparticles (AgNP) without antibiotics exhibited antimicrobial activity against several species of bacteria, including Gram-positive bacteria, such as *S. aureus* (Dung *et al.*, 2009; Aljeboury *et al.*, 2019) Gram-negative bacteria have been identified as an effective bactericidal agent (Rai *et al.*, 2012; Kim *et al.*, 2008; Lara *et al.*, 2010; Chaudhari *et al.*, 2012; Sondi, 2004). The results are shown in Figure 7.

The process of action of AgNPs remains inadequately defined. Researchers have proposed various mechanisms for the antibacterial effect of silver ions (Yoon *et al.*, 2007; Li *et al.*, 2005; Razooki *et al.*, 2019). The antimicrobial properties of nanomaterials are attributed to the photocatalytic production of reactive oxygen species (ROS), which harm cellular and viral structures and weaken bacterial cell walls and membranes. Moreover, these mechanisms involve disrupting energy transduction, inhibiting enzymatic activity, and interfering with DNA synthesis (Huang *et al.*, 2008; Brett, 2006; Al-Maliki *et al.*, 2025). It is posited that silver nanoparticles initially adhere to the surface of the cell membrane and subsequently infiltrate the bacteria and cytoplasm, resulting in cellular destruction as the AgNPs permeate the cell. The chosen clinical bacterial isolates demonstrated resistance to conventional antibiotics, including Ampicillin, Cefepime, and Gentamicin (Aljeboury *et al.*, 2019; Razooki *et al.*, 2025).

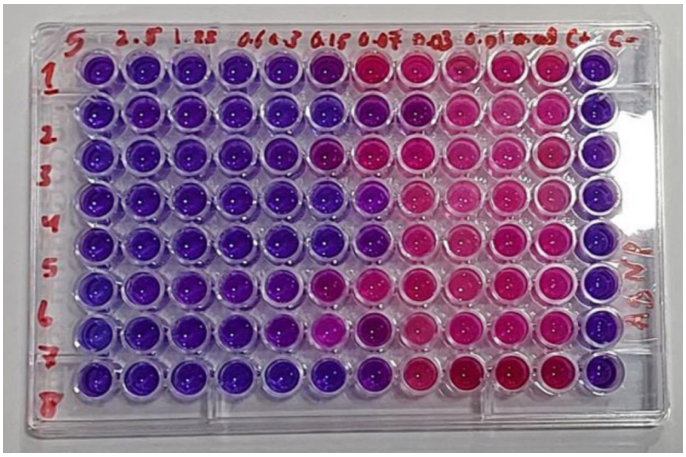

Figure 7: The results of the broth microdilution method for determining minimum inhibitory concentrations (MIC) are presented. (C-) Negative control (broth only), (C+) Positive control (bacteria in broth). Wells exhibiting blue coloration showed no growth or inhibited growth, whereas wells displaying red coloration.

Determination of minimum inhibitory concentrations (MIC) of the synergistic effect of ampicillin with nanoparticles against *S. aureus* and *E. coli*

The antibacterial activities of Ampicillin were augmented in the presence of AgNPs. However, the highest synergistic effect was observed with Ampicillin. The observations indicated that the reaction mixture demonstrated antimicrobial activity in the MIC by combining Ag.NP and Ampicillin against *S. aureus*. The concentration of 0.03 µg/ml inhibited the growth of isolates (lane 2), while the concentration of 0.3 µg/ml was inhibitory for *E. coli* isolates (lane 6). The hypothesis proposed that nanoparticles create a complex with Ampicillin, disrupting peptidoglycan in the cell wall (Fayaz *et al.*, 2010). Due to their positive charge, they interact with the negative charges of transmembrane proteins, potentially compromising the integrity of the cell membrane and obstructing transport channels (Davies and Etris 1997). They might be able to enter the bacteria

and interfere with processes such as protein synthesis, transportation, and nucleic acid function (Yamanaka and Hara, 2005; Razooki *et al*., 2020). Additionally, our study aligns with Birla *et al*. (2009), who reported an increase in the efficacy (percentage) of antibiotics, such as Ampicillin, when combined with AgNPs against *S. aureus* and *E. coli*.

Determination of minimum inhibitory concentrations (MIC) of synergistic effect of cefepime with nanoparticles against *S. aureus* and *E. coli*

When tested together, the combination of Cefepime and nanoparticles worked effectively, and the combination showed a synergistic effect against *S. aureus* and *E. coli*. The concentration of 0.03 µg/ml inhibited the growth of *S. aureus* isolates (lane 3). In contrast, 0.07 µg/ml concentrations were inhibitory for *E. coli* isolates in lane 7 (Figure 7). Cefepime (CEF) is classified as an extended-spectrum cephalosporin, also known as a fourth-generation cephalosporin, which exhibits a broader range of antimicrobial activity than third-generation cephalosporins. CEF exhibited significant efficacy against both gram-negative and gram-positive bacterial strains (Afsaneh *et al*., 2021).

Determination of minimum inhibitory concentrations (MIC) of the synergistic effect of gentamicin with nanoparticles against *S. aureus* and *E. coli*

The concentration of gentamicin tested on both *S. aureus* and *E. coli* ranged from 5 to 0.009 µg/ml, consistent with previous studies. The data indicate that the MIC value for both *S. aureus* and *E. coli* is 0.07 µg/ml, as observed in lanes 4 and 8, respectively (Figure 7). The antibacterial activities of Gentamicin and AgNPs were enhanced, as evidenced by the inhibition of visible growth when the antibiotics were combined with the nanoparticles. Gentamicin is a protein synthesis inhibitor. Gentamicin works by irreversibly binding to the 30S subunit of the bacterial ribosome, thereby interrupting protein synthesis. The precise mechanism of action remains under investigation; nonetheless, multiple mechanisms have been suggested. Reports indicate that the combination of antibiotic and AgNPs complexes releases Ag+ at a higher rate compared to AgNPs alone. Additionally, it has been suggested that the conjugation of both molecules occurs through the active groups of antibiotics, such as hydroxyl and amine groups. This will enhance the effective concentration of the antibiotic at a targeted location (Sharma *et al*., 2016; Dixit *et al*., 2017).

The combination of gentamicin with biologically synthesized AgNPs resulted in increased susceptibility in the examined bacteria. The synergistic effect of silver nanoparticles (AgNPs) and antibiotics, such as gentamicin, was significantly demonstrated against *E. coli* and *S. aureus*.

These results align with the findings of Birla *et al*. (2009), who reported increased efficacy percentages of antibiotics such as gentamicin and Ampicillin when combined with AgNPs against *S. aureus* and *E. coli*. Fayaz *et al*. (2010) evaluated the antimicrobial properties of biologically synthesized AgNPs in comparison to commercially available antibiotics against both Gram-negative and Gram-positive bacteria.

The mixture of antibiotics with AgNPs demonstrated significant efficacy in bacterial inhibition; should bacteria develop resistance to one agent, the alternative bactericidal agent remains effective in eliminating the bacteria. The bactericidal effect in synergism is amplified through the interaction of active groups, such as hydroxyl and amino groups in these antibiotics, with AgNPs via chelation. An antibiotic–AgNP combination is formed, consisting of an AgNP core embraced by antibiotic molecules. Integrating antibiotics with metal nanoparticles may enhance the efficacy of antibiotics against resistant pathogens (Li *et al*., 2005; Jasim *et al*., 2025). Additionally, nanoparticle-antibiotic conjugates decrease the required dosage of both components, thereby minimizing toxicity and enhancing antimicrobial efficacy. The conjugates exhibited effectiveness against resistant bacteria. This conjugation led to increased antibiotic concentrations at the site of antibiotic-microbe interaction, thereby enhancing the binding between microbes and antibiotics. Factors including particle size, dosage, duration of use, shape, temperature, and pH influence the synergistic effects of silver nanoparticles. However, limited research has explored the role of these synergistic effects in conjunction with antibiotics. Recent strains of bacteria, such as *S. aureus,* have emerged with significant levels of resistance. Addressing bacterial resistance requires measures that can prevent the emergence and dissemination of multidrug-resistant bacterial strains, as well as the development of novel antimicrobial agents.

CONCLUSION

The mixture of antibiotics with AgNPs demonstrated significant efficacy in bacterial inhibition and nanoparticle-antibiotic conjugates decrease the required dosage of both components, thereby minimizing toxicity and enhancing antimicrobial efficacy.

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NOVELTY STATEMENT

Treating mastitis ewes with an antibiotic medication using nanotechnology opens up new therapeutic alternatives for animals.

AUTHOR CONTRIBUTION

Abdulameer Jawad Zayier: Bacterial isolation, identification, culture, and preservation were managed, he has also planned the study, carried out PCR amplification, sequencing, data analysis, correspondence, paper writing. Ahmed Flayyih Hasan: Data curation, data recording, and isolate preservation.

Funding

This study was carried out independently of any sponsored organization, health department, or institution.

Ethics approval

The Biotechnology Research Center's Ethical Committee of Al-Nahrain University provided approval to conduct this study.

Availability of data and material

The data, samples, and genetic materials used in this work were obtained from Al-Nahrain University's Biotechnology Research Center.

Generative AI or AI-assisted Technology Statement

The author(s) declare that no Genrative AI was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Abd El-Rahmana HA, Hasanb AF, Alyasiric T, El-Wahshd HM, Althubyanie SA, Basyonyf MA, Mahmodf DH (2024). Co-treatment with cranberry and vitamin-c mitigates reproductive toxicities induced by phenobarbital in male rats. Cell Physiol. Biochem., 58: 722-738. https://doi.org/10.33594/000000745

Abdula AM, Mohsen GL, Jasim BH, Jabir MS, Rushdi AI, Baqi Y (2024). Synthesis, pharmacological evaluation, and *in silico* study of new 3-furan-1-thiophene-based chalcones as antibacterial and anticancer agents. Heliyon, 10(11). https://doi.org/10.1016/j.heliyon.2024.e32257

Afsaneh Najafi, Pegah Khosravian, Majid Validi, Mohammad Porgham Daryasari, Fatemeh Drees, Abolfazl Gholipour, (2021) Antimicrobial action of mesoporous silica nanoparticles loaded with cefepime and meropenem separately against multidrug-resistant (MDR) Acinetobacter baumannii, Journal of Drug Delivery Science and Technology, Volume 65, 102757,.

Ali AS, Kadhim NA, Rasool EMA, Al-Erjan M, Lahhob QR, Mudhafar M (2024). Harnessing CRISPR-Cas9 gene editing for the eradication of inherited retinal diseases in purebred dogs: A path to preservation and health. J. Anim. Health Prod., 12(Special Issue 1): 145–156.

Aljeboury GH, Risan MH, Algafari RN (2019). Effect of probiotic extracted from *Lactobacillus* sp. antagonist helicobacter pylori of human stomach ulcer. J. Glob. Pharma. Technol., 11(5): 731-737.

Al-Khuzaay HM, Al-Juraisy YH, Hasan AF, Tousson E (2024). Antitumor activity of β-glucan isolated arom date fruits щn cancer cells *in vivo*. Opera Med. Physiol., 11(3): 41-48.

Allahverdiyev AM, Kon KV, Abamor ES, Bagirova M, Rafailovich M (2011). Coping with antibiotic resistance: Combining nanoparticles with antibiotics and other antimicrobial agents. Expert. Rev. Anti Infect. Ther., 9: 1035–1052. https://doi.org/10.1586/eri.11.121

Al-Maliki NS, Jumaah YH, Hameed HM, Khudhair OE, Hadid MA, Hasan AF (2025). Evaluation of miRNA-155 as a biomarker for cancer stem cells and its role in chemotherapy resistance in Iraqi patients with acute myeloid leukemia. Opera Med. Physiol., 12(1): 30-37.

Al-Sailawi HA, Hadi AA, Raheem HA, Mudhafar M, Dhahi SJ, Lahhob QR (2024). Impact of serratiopeptidase vs. N-acetyl cysteine (NAC) on skin grafting healing in albino male rabbits. Adv. Anim. Vet. Sci., 12(10): 1941-1947. https://doi.org/10.17582/journal.aavs/2024/12.10.1941.1947

Altemeemi AS, Kadhim NK, Nsaif GS (2021). Changes in interleukins and follicle stimulating hormone in toxoplasmosis male patients. Indian J. Forensic Med. Toxicol., 15(1): 803-807.

Balouiri M, Sadiki M, Ibnsouda SK (2016). Methods for *in vitro* evaluating antimicrobial activity: A review. J. Pharma. Anal., 6(2): 71-79. https://doi.org/10.1016/j.jpha.2015.11.005

Bauer AW (1996). Antibiotic susceptibility testing by a standardized single disc method. Am. J. Clin. Pathol., 45: 149-158.

Bauer, A.W., Kirby, W.M.; Sherris, J.C. and Turck, A. (1966). Antibiotic susceptibility testing by a standardized single disc method. Am. J. Clin. Pathol., 45(5): 493 – 465.

Birla SS, Tiwari VV, Gade AK, Ingle AP, Yadav AP, Rai MK (2009). Fabrication of silver nanoparticles by *Phoma glomerata* and its combined effect against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Lett. Appl. Microbiol., 48(2): 173-179. https://doi.org/10.1111/j.1472-765X.2008.02510.x

Boerlin P, Kuhnert P, Hussy D, Schaellibaum M (2003). Methods for identification of *Staphylococcus aureus* isolates in cases of bovine mastitis. J. Clin. Microbiol., 41(2): 767-771. https://doi.org/10.1128/JCM.41.2.767-771.2003

Brett DW (2006). A discussion of silver as an antimicrobial agent: Alleviating the confusion. Ostomy/wound Manage., 52(1): 34-41.

Capurro A, Aspàn A, Unnerstad HE, Waller KP, Artursson K (2010). Identification of potential sources of *Staphylococcus aureus* in herds with mastitis problems. J. Dairy Sci., 93(1): 180-191. https://doi.org/10.3168/jds.2009-2471

Chandran SP, Chaudhary M, Pasricha R, Ahmad A, Sastry M (2006). Synthesis of gold nanotriangles and silver nanoparticles using Aloevera plant extract. Biotechnol. Prog., 22(2): 577-583. https://doi.org/10.1021/bp0501423

Chaudhari PR, Masurkar SA, Shidore VB, Kamble SP (2012). Antimicrobial activity of extracellularly synthesized silver nanoparticles using Lactobacillus species obtained from VIZYLAC capsule. J. Appl. Pharma. Sci., (Issue), pp. 25-29.

Clinical and Laboratory Standards Institute, (2021). Performance standards for anti- microbial susceptibility testing; Twenty – sixth informational supplement. CLSI docu-ment M100-S26. Wayne, PA: Clinical and Laboratory Standards Institute.

CLSI C (2016). Performance standards for antimicrobial susceptibility testing. Clin. Lab Stand. Inst., 35(3): 16-38.

Davies RL, Etris SF (1997). The development and functions of silver in water purification and disease control. Catalysis Today, 36(1): 107-114. https://doi.org/10.1016/S0920-5861(96)00203-9

Devi JS, Bhimba BV, Peter DM (2013). Production of biogenic silver nanoparticles using Sargassum longifolium and its applications.

Dixit A, Das S, Jyoti A, Kaushik S (2017). Biogenic synthesis of silver nanoparticles and its potential application in prevention of acute ear infections. J. Pharma. Sci. Res., 9(1): 14.

Dung T, Buu N, Quang D (2009). Synthesis of nanosilver particles by reverse micelle method and study of their bactericidal properties. J. Phys., 187(6): 1-8. https://doi.org/10.1088/1742-6596/187/1/012054

Eberhart RJ, Harmon RJ, Jasper DE, Natzke RP, Nickerson SC, Reneau JK, Spencer SB (1987). Current concepts of bovine mastitis. The National Mastitis Council, Arlington, Virginia, pp. 47.

Fayaz AM, Balaji K, Girilal M, Yadav R, Kalaichelvan PT, Venketesan R (2010). Biogenic synthesis of silver nanoparticles and their synergistic effect with antibiotics: A study against gram-positive and gram-negative bacteria. Nanomed. Nanotechnol. Biol. Med., 6(1): 103-109. https://doi.org/10.1016/j.nano.2009.04.006

Ghydaa HA, Sameer NM (2020). Evaluation of antagonisticpotentialo flactobacillus isolates against phytopathogenic fungi and pathogenic bacteria *in vitro*. Syst. Rev. Pharma., 11: Issue12.

Gokulakrishnan R, Ravikumar S, Raj JA (2012). *In vitro* antibacterial potential of metal oxide nanoparticles against antibiotic resistant bacterial. https://doi.org/10.1016/S2222-1808(12)60089-9

Huang Z, Zheng X, Yan D, Yin G, Liao X, Kang Y, Hao B (2008). Toxicological effect of ZnO nanoparticles based on bacteria. Langmuir, 24(8): 4140-4144. https://doi.org/10.1021/la7035949

Jasim BH, Ali EH (2021). Isolation, extraction, purification, and characterization of fibrinolytic enzyme from *Pseudomonas aeruginosa* and estimation of the molecular weight of the enzyme. Arch. Razi Inst., 76(4): 809.

Jasim BH, Mohammed KR, Mohammed SA, Sulaiman SD, Hasan AF (2021). Extraction, optimization, and purification of anti-cancer l-glutaminase enzyme from bacillus subtilis B7.

Jasim BH, Sattar RJ, Kamel MD, Ali EH, Jabir MS, Saddi MH, Fadhil ZA (2025). Thrombolytic activity and antibacterial activity optimize staphylokinase enzyme production from *Staphylococcus aureus*. Indones. J. Biotechnol., 30(1): 26-33. https://doi.org/10.22146/ijbiotech.95686

Jithesh. (2013) Biosynthesis of Silver Nanoparticles using Trigonella Foenum Graecum and the Determination of their Antimicrobial Acti vity. International Journal of Science and Research (IJSR), India Online ISSN: 2319-7064

Jones GM, Bailey TL (2009). Understanding the basics of mastitis.

Judge LJ, Erskine RJ, Bartlett PC (1997). Recombinant bovine somatotropin and clinical mastitis: Incidence, discarded milk following therapy, and culling. J. Dairy Sci., 80(12): 3212-3218. https://doi.org/10.3168/jds.S0022-0302(97)76294-5

Kadhim AS, Jasim BH, Ghadir GK (2024). Antibacterial activity of *Klebsiella pneumoniae* isolated from pneumonia patients. J. Emerg. Med. Trauma Acute Care, 2024(6): 18. https://doi.org/10.5339/jemtac.2024.absc.18

Kareem KN, Hussein ZA (2023). The GGC medium reduces the DNA fragmentation of human spermatozoa via *in vitro* activation. Arch. Razi Inst., 78(2): 709-714.

Kim KJ, Sung WS, Moon SK, Choi JS, Kim JG, Lee DG (2008). Antifungal effect of silver nanoparticles on dermatophytes. J. Microbiol. Biotechnol., 18(8): 1482-1484.

Kvitek L, Vanickova M, Panacek A, Soukupova J, Dittrich M, Valentova E, Zboril R (2009). Initial study on the toxicity of silver nanoparticles (NPs) against *Paramecium caudatum*. J. Phys. Chem. C, 113(11): 4296-4300. https://doi.org/10.1021/jp808645e

Lara HH, Ayala-Núnez NV, Ixtepan TLDC, Rodríguez PC (2010). Bactericidal effect of silver nanoparticles against multidrug-resistant bacteria. World J. Microbiol. Biotechnol., 26: 615-621. https://doi.org/10.1007/s11274-009-0211-3

Le AT, Tam PD, Huy PT, Huy TQ, Van Hieu N, Kudrinskiy AA, Krutyakov YA (2010). Synthesis of oleic acid-stabilized silver nanoparticles and analysis of their antibacterial activity. Mater. Sci. Eng. C, 30(6): 910-916. https://doi.org/10.1016/j.msec.2010.04.009

Li P, Li J, Wu C, Wu Q, Li J (2005). Synergistic antibacterial effects of β-lactam antibiotic combined with silver nanoparticles. Nanotechnology, 16(9): 1912. https://doi.org/10.1088/0957-4484/16/9/082

Obaid MR, Tareq YF, Kareem KN, Hameed SD, Tarq SZ, Sahib AD, Hasan AF (2025). Changes in the level of lipid profile in diabetes mellitus in samples patients. J. Biosci. Appl. Res., 11(1): 331-336. https://doi.org/10.21608/jbaar.2025.350090.1134

Mellenberger R (2001). Cinical mastitis during lactation. Br. Vet. J., 151: 197.

Nafea MH, Kadhim AJ, Obayes KR, Jasim BH, Hasan AF (2025). Biosynthesis of TiO2 nanoparticles and evaluation of their antibacterial activities. J. Biosci. Appl. Res., 11(1): 260-269. https://doi.org/10.21608/jbaar.2025.419831

Najafi A, Khosravian P, Validi M, Daryasari MP, Drees F, Gholipour A (2021). Antimicrobial action of mesoporous silica nanoparticles loaded with cefepime and meropenem separately against multidrug-resistant (MDR) *Acinetobacter baumannii*. J. Drug Deliv. Sci. Technol., 65: 102757. https://doi.org/10.1016/j.jddst.2021.102757

Obaid RM, Yaseen FT, Mukhlif MY (2020). Blood cells depletion after chemotherapy in Iraqi women with breast cancer. Indian J. Forensic Med. Toxicol., 14(4): 3379-3382.

Obaid RM, Yaseen FT, Salim AK (2020). Correlation between

vitamin D3 (cholecalciferol) and thyroid diseases in Iraqi patients. Ann. Trop. Med. Publ. Health, 23: 231-603. https://doi.org/10.36295/ASRO.2020.231603

Omran ZH, Hussen AK, Yahya AM, Mohammed DA, Rawdhan HA, Ahmed HM, Sattar RJ, Hussen YA, Jasm BH and Sadoon NM (2024). Copper nanoparticles against two types of bacteria *Staphylococcus aureus* and *Escherichia coli*. J. Nanostruct., 14(3): 780-788.

Hameed HM, Razooki ZH, Hasan AF (2025). Therapeutic effect of essential oils (*Citrus sinensis*) against ehrlich ascites model induced renal toxicity in female mice. Agricultural Science Digest. 45(2): 317-322. doi: 10.18805/ag.DF-632

Pooloth J (2013). Biosynthesis of silver nanoparticles using *Trigonella foenum* graecum and the determination of their antimicrobial activity. Int. J. Sci. Res., 2(5): 2319-7064.

Promy V (2018). Antidiabetic activity of green gold-silver nanocomposite with *Trigonella foenum graecum* L. seeds extract on streptozotocin- induced diabetic rats. Pak. J. Zool., 50(2): 711-718. https://doi.org/10.17582/journal.pjz/2018.2.711.718

Quinn PJ, Carter ME, Markey BK, Carter GR (2004). Clinical veterinary microbiology. 6th ed., Mosby Edinburgh, pp. 49, 226-231, 345-348.

Rai MK, Deshmukh SD, Ingle AP, Gade AK (2012). Silver nanoparticles: The powerful nanoweapon against multidrug-resistant bacteria. J. Appl. Microbiol., 112(5): 841–852. https://doi.org/10.1111/j.1365-2672.2012.05253.x

Razooki ZH, Abed IJ, Al-Mashhadani MK (2019). Effect of the aqueous carbon source on growth rate of the microalgae *Coelastrella* sp. MH923012. Plant Arch., 19(2): 1420-1425.

Razooki ZH, Al-Mashhadani MK, Abed IJ (2020). Biomaterial composition of the microalga *Coelastrella* sp. (MH923012): Effect of carbon source. Mater. Today Proce., 20: 621-626. https://doi.org/10.1016/j.matpr.2019.09.200

Razooki ZH, Mohammed SA, Hameed HM, Hasan AF, El-Wahsh HM (2025). Prophylactic action of Moringa oleifera against cyclophosphamide-induced harmful effects in male mice. J. Anim. Health Prod., 13(2): 335-339. https://doi.org/10.17582/journal.jahp/2025/13.2.335.339

Sabee MMSM, Awang YBZA, Hamid A (2020). Gentamicin loaded PLA microspheres susceptibility against *Staphylococcus aureus* and *Escherichia coli* by Kirby-Bauer and micro-dilution methods. AIP Conf. Proc. 2267 (1): 020032. https://doi.org/10.1063/5.0017438

Sharma K, Kaushik S, Jyoti A (2016). Green synthesis of silver nanoparticles by using waste vegetable peel and its antibacterial activities. J. Pharm. Sci. Res., 8: 313–316.

Sondi I, Salopek-Sondi B (2004). Silver nanoparticles as antimicrobial agent: A case study on *E. coli* as a model for Gram-negative bacteria. J. Colloid Interface Sci., 275(1): 177–182. https://doi.org/10.1016/j.jcis.2004.02.012

Syring C, Boss R, Reist M (2012). Bovine mastitis: The diagnostic properties of a PCR-based assay to monitor the *Staphylococcus aureus* genotype B status of a herd, using bulk tank milk. J. Dairy Sci., 95(3): 3674-3682. https://doi.org/10.3168/jds.2011-4968

Xu H, Kall M (2002). Morphology effects on the optical properties of silver nanoparticles. J. Nanosci. Nanotechnol., 4: 254-259. https://doi.org/10.1166/jnn.2004.034

Yahya A, Adil OW, Mohammed HO, Hasan AF (2024). Histopathological and immunohistochemical studies on the effects of silver oxide nanoparticles (AgNPs) on male rats' liver. J. Biosci. Appl. Res., 10(3): 392-398.

Yamanaka M, Hara K, Kudo J (2005). Bactericidal actions of a silver ion solution on *Escherichia coli*, studied by energy-fltering transmission electron microscopy and proteomic analysis. Appl. Environ. Microbiol., 71(11): 7589–7593. https://doi.org/10.1128/AEM.71.11.7589-7593.2005

Yang Y, Xudong S, Yao-Wu Y (2007). Detection of *Staphylococcus aureus* in dairy products by polymerase chain reaction. Agric. Sci., 6(3): 857-862. https://doi.org/10.1016/S1671-2927(07)60122-9

Yaseen TF, Al-Jumaily KMR (2025). The impact of interleukin-21 and 23 serum level and gene expression in celiac disease among sample of Iraqi patients. Asian J. Dairy Food Res., 44(2): 234-239. https://doi.org/10.18805/ajdfr.DRF-476

Yaseen FT, Al-Jumaily RMK (2024). Evaluation of global DNA methylation, homocysteine and vitamin B12 levels among patients with celiac disease. Gastroenterology, 58(4): 258-263. https://doi.org/10.22141/2308-2097.58.4.2024.637

Yoon KY, Hoon BJ, Park JH, Hwang J (2007). Susceptibility constants of *Escherichia coli* and *Bacillus subtilis* to silver and copper nanoparticles. Sci. Total Environ., 373(2–3): 572–575. https://doi.org/10.1016/j.scitotenv.2006.11.007