



Evaluation The Antibacterial Activity of Biosynthesis Silver Nanoparticles by *Lactobacillus Gasseri* Bacteria

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Abstract

Biosynthesis of AgNPs is a new approach in the field of nanotechnology with optimistic implementation in medicine, food control, and pharmacology. In this study, the silver nanoparticles were produced by *Lactobacillus gasseri* filtrate. The production of AgNPs was confirmed by the color change from yellow to brown. Using UV visible spectrophotometer at 424 nm wavelength, and X-ray diffraction, it was found that the size of the synthesized particles was 58.5 nm after applying Scherrer's equation. The inhibitory activity of silver nitrate on the growth of some pathogenic isolates was studied *Staphylococcus haemolyticus* Gram positive, and *Klebsiella pneumoniae* Gram negative. The highest inhibitory diameter was 14.6 mm at 100% concentration (stock) against *Staphylococcus haemolyticus* bacteria was followed by that of *Klebsiella pneumoniae* bacteria with an average inhibition zone diameter reached 13.6 mm at 100% concentration. The highest effect was of AgNPs on the growth of *Staphylococcus haemolyticus* bacteria, as it found the average diameter of the inhibition zone reached to 29.3 mm, followed by *Klebsiella pneumoniae* with the average diameter of the inhibiting zone it was 22.6 mm at 100% concentration (stock). This study showed AgNPs have more antibacterial activity against Gram positive bacteria than Gram negative bacteria. The importance of this study lies in testing the effectiveness of by *Lactobacillus gasseri* bacteria in the biosynthesis of silver nanoparticles and studying their antibacterial activity on pathogenic bacteria.

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1. Introduction

The problem of antibiotic resistance has become a major concern for clinicians and for all in their quest to treat and prevent infections caused by microorganisms that were previously thought to be eliminated with antimicrobials. These organisms are back in new forms that are resistant to almost all clinically important antimicrobials. *K.pneumoniae* is one of those clinically important organisms that have gained much resistance to antibiotics. *K.pneumoniae* is an Enterobacteriaceae that is one of the opportunistic pathogens that cause a broad spectrum of diseases and exhibit increasingly frequent acquisition of antibiotic resistance [1]. *K.pneumoniae* is responsible for most Gram negative infections such as cystitis fibrosis, pneumonia, urinary tract infections, surgical wound infections, endocarditis, and septicemia [2]. High mortality rates and extended hospitalization are often associated

with infection caused by this organism [3]. *S.haemolyticus* is a commensal microorganism of humans, often found on the skin and mucous membranes, and is a member of the coagulase-negative staphylococci group [4]. This bacterium is known to be an opportunistic pathogen and one of the coagulase-negative staphylococci groups most frequently isolated in clinical cases, notably from bloodstream infections related to invasive devices. The ability to forming a biofilm is an important aspect of virulence-associated with adherence to medical devices and it is a common phenotype in *S.haemolyticus* clinical strains [5, 6].

Lactobacillus spp. are facultative anaerobic, catalase-negative, Gram positive, non-spore forming rods that often grow better under microaerophilic conditions. When stained with Gram stain they appear in various forms, including short, plump rods, long, slender rods, in chains or palisades. Their colonies vary in shape from small to medium gray colonies that usually show alpha hemolysis on blood agar. Lactobacilli grow on a variety of media including MRS (Man, Rogosa, Sharpe agar) where they grow as white colonies, usually, mucous Lactobacillus consists of more than 170 species and 17 subspecies that have been properly propagated and have a good standing in the nomenclature. In humans, they are naturally present in the vagina and gastrointestinal tract but can be opportunistic pathogens occasionally like *L.rhamnosus*, *L.fermentum*, *L.paracasei*, *L.oris*, *L.gasseri*, *L.iners*, and *L.salivarius* [7].

Silver metal was known since about 4000 B.C. and it was used in many medical uses, even before it was realized that microorganisms are the main cause of infection [8]. With the Appearance of nanotechnology, it became possible to produce nanoscale silver [9, 10]. It is used as a more effective antibacterial compared with antibiotics, as it provides a good bond with the bacteria through the cell membrane and thus penetrates the membrane into the bacterial cell, and it does not have any activity in its normal state, but by ionizing it with the presence of water or tissue fluids, it leads to the release of bioactive silver ions. This compound has a strong affinity for the sulfur groups in the cell membrane. There are no side effects on human health if it is in low concentrations as it depends on the shape, size, stability, and biocompatibility of nanoparticles, but long exposure to silver causes severe diseases such as argyrosis [11]. Silver nanoparticles can be synthesized using traditional or unconventional methods, using two different approaches: top-down and down-top, and there are many traditional methods used to obtain Silver nanoparticles, such as chemical reactions / Photochemical, thermal decomposition of various silver compounds, electrochemical, radiation, and the use of microwave-assisted methods [12]. Unconventional methods of creating these particles depend on the use of microorganisms such as bacteria, fungi, marine algae, and yeasts or various alcoholic or aqueous plant extracts, as they are considered as a reducing or inhibitory agent [13], Green synthesis methods are considered to be the best in preparing silver nanoparticles due to their many advantages: low cost, environment friendly and does not require high pressure, energy, or the use of chemical reagents [14, 15]. The aim of this study performs biosynthesis of AgNPs using *L.gasseri* bacterial filtrate with silver nitrate solution and study the inhibitory effect against pathogenic bacteria (*S.haemolyticus* and *K.pneumoniae*), comparing the antibacterial activity of silver nitrate and silver nanoparticles.

2. Materials and Methods

2.1. Collection of Bacterial Isolates

Twenty isolates from the vagina were collected for *Lactobacillus sp.*, twenty isolates from urine samples for *S.haemolyticus*, and twenty isolates from stool samples for *K.pneumoniae* from the Microbiology Laboratory at Al-Olwiya Teaching Hospital for Children in Baghdad, where they were collected, diagnosed with VITEK device from 2020/10/11 to 2020/12/25. Biochemical tests were carried out to confirm the diagnosis of the bacteria, and then the isolates were planted on the surface of the slanted bed medium formed from Brain Heart Infusion Agar medium and MRS agar then incubated and kept at 4°C until use.

2.2. Morphological and Microscopic Examination

Isolates were grown on MacConkey agar, to observe the colonies' shape and ferment ability for lactose and glucose and on solid blood medium to identify their color, size, and ability to degrade hemolysis and Mannitol salt agar and MRS agar, and then stained with Gram Stain [16].

2.3. Preparation of Silver Nitrate Solution

The silver nitrate solution was prepared at a concentration of 5 mM by dissolving 0.0849 g of silver nitrate, in 100 ml of distilled deionized water. It was prepared and kept in dark conditions to prevent its oxidation, and was used in the synthesis of silver nanoparticles [17].

2.4. Biosynthesis and Purification of Silver Nanoparticles by Using *L. Gasseri* Bacteria

The bacteria were seeded in MRS agar medium for 48 hours, after which the growth medium was discarded by a 4000 rpm centrifuge for 10 minutes, the sediment was removed and the filtrate sterilized with filters of 0.22µm pore dimension and the filtrate was preserved until its use [18]. The silver nanoparticles were synthesized using a filtrate of *L.gasseri* bacteria; 10ml of culture filtrate was mixed with 90 ml of 5 mM silver nitrate solution and incubated at room temperature for 48 hours. The main discovery of synthesized silver nanoparticles was done in the reaction mixture by detecting the color alteration of the medium from pale yellow to brown as well as observed optical density [18]. For purification of AgNPs the aqueous solution containing AgNPs was placed in test tubes and then placed in a centrifuge at a speed of 10,000 rpm, for 10 minutes. One minute for 10 minutes and the process was repeated 3 times until the filtrate became devoid of any color, and the concentrated precipitate containing silver nanoparticles was placed in an hour bottle, and it was dried in an electric oven at a temperature of 50 °C (the thermal drying method) so that the excess water was removed and then collected The precipitate after drying and preserving until use [19].

2.5. Characterization of Silver Nanoparticles

1 ml of the solution containing silver nanoparticles and add 9ml of deionized water to it. The aqueous solution was then measured to reveal the nanoparticles formed at wavelengths ranging from 300 to 600 nm. The appearance of the highest absorption (λ max) after the wavelength of 400 nm indicates the formation of silver nanoparticles [20]. XRD was used to characterize nanoparticles structure. Thin-film of AgNPs has been prepared by dropping 1mL of AgNPs on glass slides with 1 cm² in dimension and dried by heating on a hot plate. The grain size of NPs was calculated using Scherrer's equation [21].

$$D = K \lambda / \beta \cos \theta$$

where: D is crystallite size, K is the shape factor, λ is the X-ray wavelength, β is the line broadening at half the maximum intensity FWHM, and (θ) is the Bragg angle. This system supplied with Cu-k alpha radiation at a wavelength of ($\lambda = 1.5406$ nm) produced at 40 kv. The samples were scanned over a range of 10°-70° at room temperature [22].

2.6. Antimicrobial Activity of Silver Nitrate and Silver Nanoparticles against Bacterial Isolates

Muller Hinton Agar was prepared, the plates were swabbed with *S.haemolyticus* and *K.pneumoniae*, well were punched into the gar using a sterilized well cutter. The well was loaded with 100µl of different concentrations of 5mM AgNO₃ (12.5%, 25%, 50%, and 100%) of the silver nitrate solution and AgNPs for both types of bacteria, deionized water was used as a controlling factor. The dishes were incubated at 37 ° C for 24hrs. After the incubation period is over, the inhibition zone was measured [23].

3. Results and Discussion

3.1. Bacterial Isolation and Identification

To identify the bacterial isolates of *L.gasseri*, *S.haemolyticus*, and *K.pneumoniae*, in addition to diagnosing the isolates with the Vitek system, the isolates were culture in four culture media. For the isolation of pathogenic Staphylococci sp. *S.haemolyticus* grows on Mannitol salt agar (as a selective medium) colonies appear yellow with yellow zones. *S.haemolyticus* can use mannitol as a food source and produces acidic fermentation byproducts that lower the pH of the media the phenol red in the agar will turn yellow due to the acidity of the media, as shown in Figure1 (A), and grow on blood agar Figure 1(B). *L.gasseri* bacteria grow on selective MRS agar media and produced round shapes, off-white to cream color, and shiny colonies, as shown in Figure 1(C). *k.pneumoniae* grows on MacConkey agar and ferments lactose, produces pink and mucoid colonies as shown in Figure 1(D).

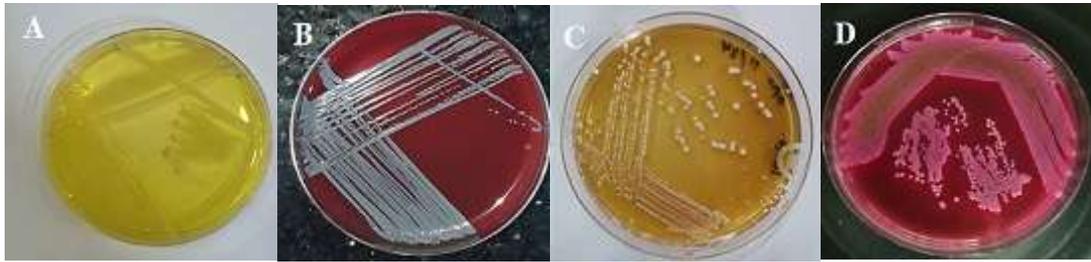


Figure 1: Shows bacterial isolates growing on culture media. (A), (B) *S.haemolyticus* on Mannitol salt agar, Blood agar, (C) *L.gasseri* on MRS agar, and (D) *K.pneumoniae* on MacConkey agar.

3.2. Microscopic Examination

This was done using a Gram stain and the cells were examined with a light microscope to identify the shapes of the cells, their colors, and their sizes. The results are as shown in (Figure 2).

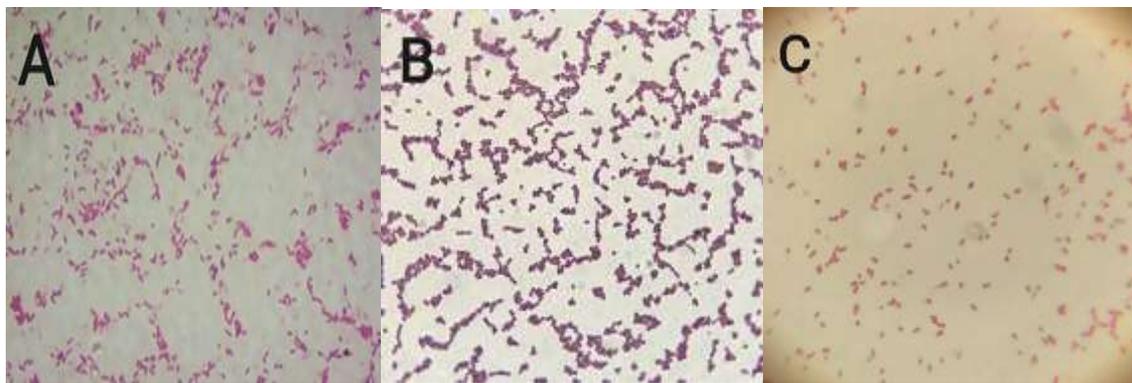


Figure 2: Show some microorganisms with gram stain ($\times 100$), (A) *L.gasseri*, (B) *S.haemolyticus*, (C) *K.pneumoniae*.

3.3. Biosynthesis of Silver Nanoparticle

The results of the biosynthesis process were shown by using *L.gasseri* bacteria filtrate with AgNO_3 solution at a concentration of 5 mmol, at a pH of 6.5 and a room temperature of 37°C for 48 hours on the synthesis of AgNPs, and the color change of the mixture to a brown color was evidence of positive the synthesis process (Figure 3).

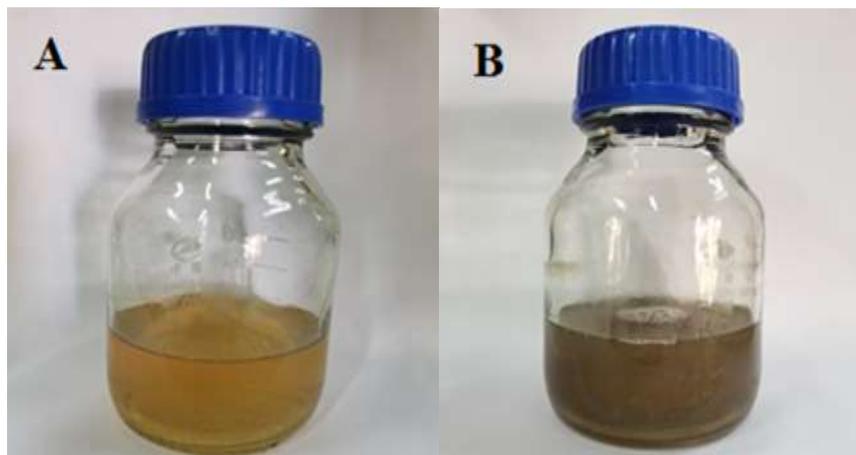


Figure 3: Stages of the synthesis of silver nanoparticles, (A) at zero time, (B) after 48 hours.

The color change of the solution explains the formation of the silver nanoparticles by the enzymes present, such as the nitrate reductase enzyme, which the bacteria secrete out of the cell and which helps in reducing the silver nitrate to nanoparticles [24], this enzyme is secreted by many types of bacteria such as *P.aeruginosa*, *E.coli*, and *B.subtilis* [25]. This color difference is due to the difference in the electronic density of the nanoparticles, which is due to the difference in the size of the nanoparticles [26].

3.4. Characterization of Silver Nanoparticle

3.4.1. Characterization by UV Visible Spectrophotometer (UV)

The characterization of the silver nanoparticles created using a UV visible spectrophotometer showed the highest absorbance (λ_{max}) at the wavelength of 424 nm (Figure 4).

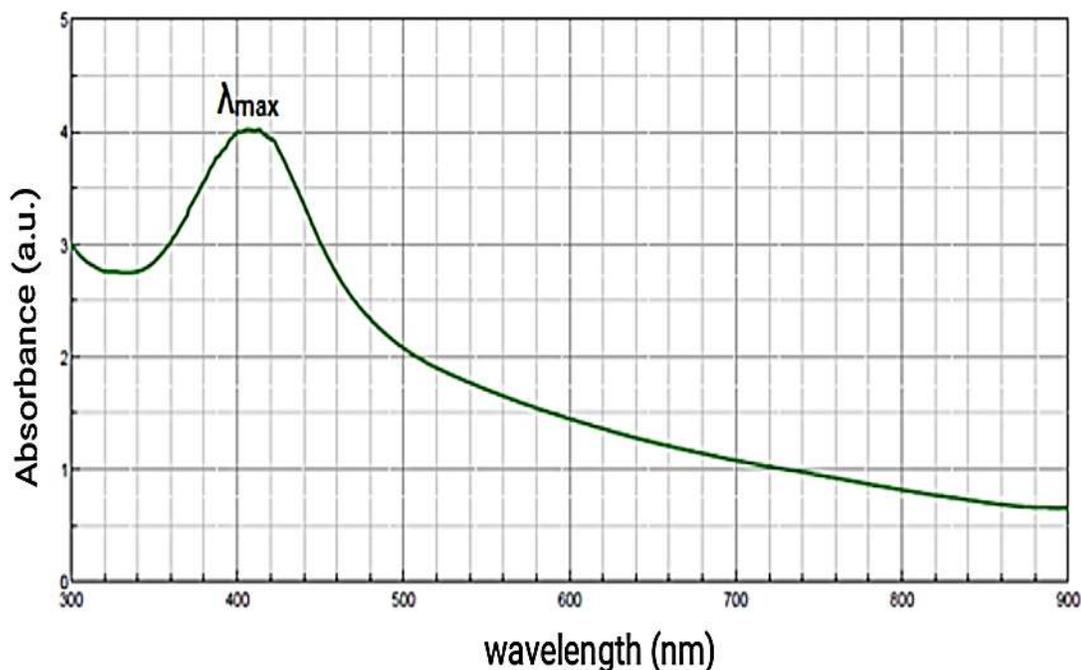


Figure 4: Characterization of silver nanoparticles using a UV visible spectrophotometer.

The characterization of AgNPs using a UV visible spectrophotometer is one of the most important tests used to verify the formation of silver nanoparticles, and they are detected depending on the extent of the optical absorption between wavelengths (300-900) nanometers to investigate the size of the nanoparticles, which ranges between (2-100) nanometers, as metals of nanoscale have free electrons, and these electrons give (SPR) depending on their size, which is formed as a result of the vibrations of the metal's electrons compared to light waves. And covered by showing (SPR) after the wavelength of 400 nanometers in the process of synthesizing silver nanoparticles using *L.gasseri* filtrate and agree with both [27, 28].

3.4.2. Characterization by X-ray Diffraction

XRD shows there are four peaks related to Ag nanoparticles at angle 2θ , 38.48, 44.74, 65.09, and 79.80 corresponding's to (111), (200), (220), and (311) planes respectively when comparing with JCPDS File no. 04-0783, (Figure 5). According to the strongest peak (111), the crystallite size is 58.5 nm using Scherrer's equation. Also, there are no other unknown peaks agree with both [28, 29]. Nanoparticles synthesized by bacteria are often spherical [30].

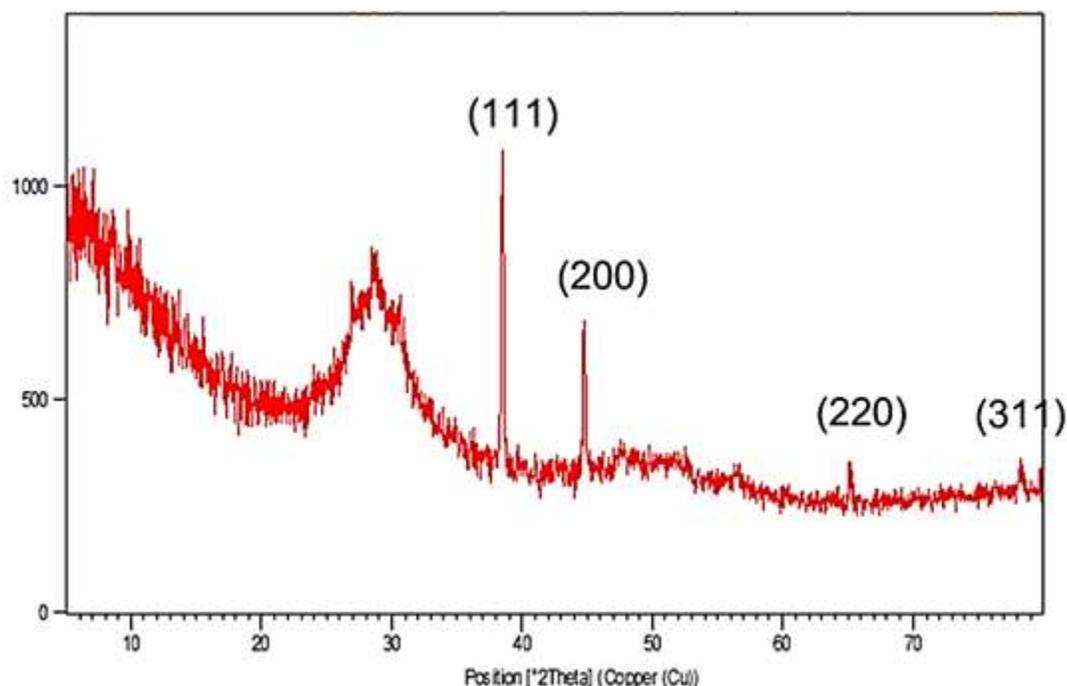


Figure 5: Characterization of silver nanoparticles using an X-Ray diffraction.

3.5. Study of the Antimicrobial Activity of Silver Nitrate and Silver Nanoparticles against Bacterial Isolates

The results of studying the effect of silver nitrate by agar well diffusion method, when using different concentrations of 5mM AgNO₃ (12.5%, 25%, 50%, 100%) in Tables 1 and Figure 6. showed that they had a slight effect on *S.haemolyticus* and *K.pneumoniae* bacteria the highest inhibitory diameter in the current study was 14.6 mm at 100% concentrations (stock) against *S.haemolyticus* bacteria was followed by that of *K.pneumoniae* bacteria with an average inhibition zone diameter of 13.6 mm at 100% concentrations (stock) and that the minimum inhibition diameter in the current study was 11 mm at a dilution (12.5%) against *K.pneumoniae* bacteria, The inhibition zone diameter showed that silver nitrate was more effective against *S. haemolyticus* in comparison to *K.pneomoniae* bacteria , these results are in line with the finding of the previous study that reported AgNO₃ was more effective against Gram positive bacteria in comparison to Gram negative bacteria [31].

Table 1: Antibacterial activity of Silver nitrate and biosynthesized silver nanoparticles against two types of pathogenic bacteria.

M.O	Inhibition zone in diameter of different concentration			
	Silver nitrate			
	D1	D2	D3	stock
<i>S.haemolyticus</i>	12.66±0.58	13.16±0.58	13.66±0.58	14.66±0.58
	Silver nanoparticles			
	18.33±0.58	19.33±0.58	22.33±1.15	29.33±0.58
<i>K.pneumoniae</i>	Silver nitrate			
	11.00±1.0	11.66±0.58	13.33±0.58	13.66±0.58
	Silver nanoparticles			
	19.33±1.15	20.66±0.58	21.66±0.58	22.66±0.58

D1= 12.5%, D2= 25%, D3= 50%, stock=100% concentrations

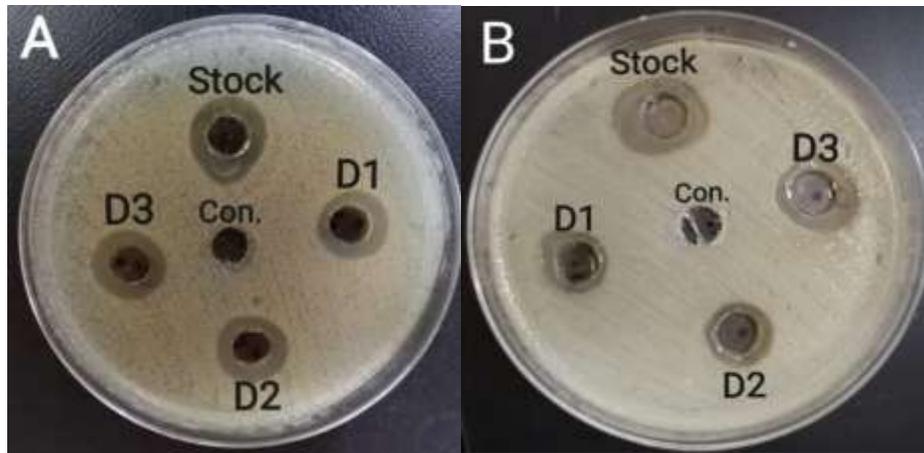


Figure 6: The inhibitory effect of Silver nitrate against bacterial isolates. (A) *S. haemolyticus*, (B) *K. pneumoniae*, D1= 12.5%, D2= 25%, D3= 50%, stock=100% concentrations, cont.=control

The results of studying the effect of nanoparticles by the agar well diffusion method showed that they have a high inhibitory effect compared to silver nitrate. The highest effect was of silver nanoparticles synthesized by *L. gasseri* bacteria on the growth of *S. haemolyticus* bacteria, as it found the average diameter of the inhibition zone reached 29.3 mm, followed by *K. pneumoniae* with an average diameter of the inhibiting zone It was 22.6 mm at 100% concentration (stock). The concentration of 12.5% was the lowest inhibitory concentration, with a minimum inhibitory diameter of 18.3 mm against *S. haemolyticus* bacteria. (Table 1 and Figure 7).

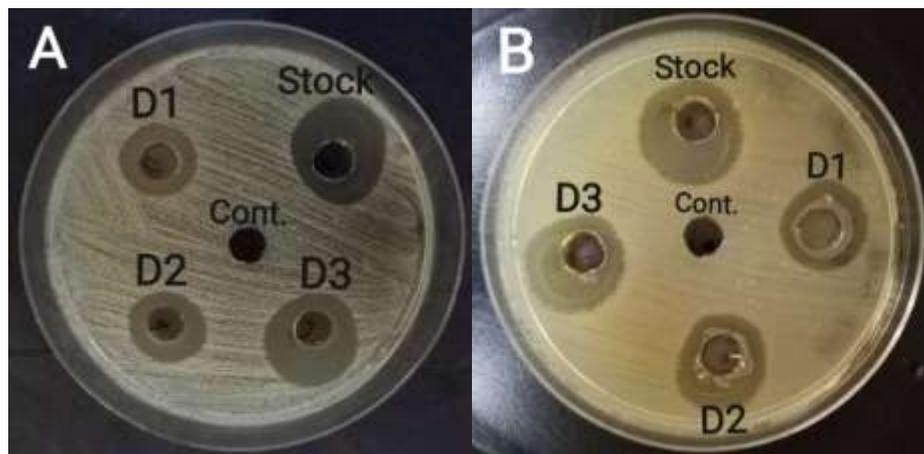


Figure 7: The inhibitory effect of AgNPs against bacterial isolates. (A) *S. haemolyticus*, (B) *K. pneumoniae*. D1= 12.5%, D2= 25%, D3= 50%, stock=100% concentrations, cont. =control

From the foregoing, it is clear that increasing the concentrations increases the rate of inhibition, and the antibacterial activity of AgNO_3 and AgNPs above, it is clear that AgNPs were more effective as growth inhibitor for *S. haemolyticus* bacteria and *K. pneumoniae* bacteria in comparison to AgNO_3 ; this is in agreement with previous studies [32, 33].

The reason for the high efficiency of silver nanoparticles is due to the size and surface area of these particles, which enable them to reach and contact the DNA of the microbial cell. Silver nanoparticles bind to the pores in the cell membrane and release silver ions, which in turn increase the permeability of the cell membrane and then cell dies [34]. In addition, these nanoparticles have a high surface area, which gives better contact and better interaction with

the microbial cell. Undoubtedly, the deadly effect on microbial cells is due to the release of silver ions from the silver nanoparticles, which remain in the cell membrane to change its structure and then increase the permeability of the cell membrane Until the cell dies and this is what he also indicated [35].

In a previous study, it was found that silver nanoparticles have a great tendency to interact with sulfur or phosphorous that contains soft bases such as RS, R-SH, or RS-RPR₃, so sulfur-containing proteins in the cell membrane or inside cells and elements containing phosphorous Such as DNA are likely to be preferred sites for the binding of silver nanoparticles [36].

Because of their biocidal and immunopotentiating properties, NPs are currently being explored as a possible alternative to antibiotics. Antimicrobial coatings on the surface of medical equipment using polymer-based nanoparticles and metal NPs could be used for a variety of biomedical purposes. When metallic NPs are combined with current medicines for bacterial infections, the amount of antibiotics that must be provided is reduced, lowering toxicity and the risk of resistance developing. There has been a paradigm shift toward green antibacterial NP synthesis that is both cost-effective and environmentally benign [37].

4. Conclusions

The present study demonstrated the synthesis of silver nanoparticles using locally *L. gasseri*. The synthesized silver nanoparticles were characterized by using UV-visible spectrophotometer, and X-ray diffraction. Antibacterial activity was observed against Gram positive (*S.haemolyticus*) and Gram negative (*K.pneumoniae*) pathogenic bacteria, biosynthesized AgNPs from biological sources represent potential biomedical applications for promising antibacterial agents.

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Conflict of Interest

There are no conflicts of interest regarding the publication of this manuscript.

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