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Research article

Probiotic *Lactobacillus salivarius* mediated synthesis of silver nanoparticles (AgNPs-LS): A sustainable approach and multifaceted biomedical application

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ABSTRACT

Biogenic synthesis of silver nanoparticles (AgNPs) has emerged as an eco-friendly and sustainable approach with diverse biological applications. This study presents synthesis of AgNPs-LS using a probiotic strain *Lactobacillus salivarius* (*L. salivarius*) and explores their multifaceted biological activities, including antibacterial, antibiofilm, anti-quorum sensing, antifungal, antioxidant, anticancer, anticoagulant and thrombolytic properties. The biosynthesis of AgNPs-LS was successfully achieved using *L. salivarius* cell free supernatants, resulting in well-characterized nanoparticles as confirmed by UV–Vis spectroscopy, Fourier Transform Infrared (FTIR) spectroscopy, transmission electron microscopy (TEM) and dynamic light scattering (DLS) and zeta potential analysis. The AgNPs-LS demonstrated potent antibacterial activity against different pathogenic bacteria (*C. violaceum, P. aeruginosa, S. aureus*, *E. coli* and *S. marcescens*), emphasizing their potential in combating bacterial infections. Moreover, these AgNPs-LS were effective in inhibiting biofilm formation (>60 % at 1/2 MIC), a key mechanism of bacterial virulence, highlighting their utility in preventing biofilm-related infections. AgNPs-LS exhibited anti-quorum sensing activity, disrupting bacterial communication systems and potentially reducing virulence factor such as, violacein production in *C. violaceum,* pyocyanin production in *P. aeruginosa* and prodigiosin production in *S. marcescens*. Additionally, AgNPs-LS also exhibited notable antifungal activity towards a different pathogenic fungus (*F. proliferatum, P. purpurogenum, A. niger and R. stolonifer*). In terms of health applications, the AgNPs-LS displayed significant antioxidant activity, effectively scavenging $DPPH^{\bullet}$ (IC_{50} = 42.65 μg/mL) and $ABTS^{\bullet+}$ (IC_{50} = 53.77 μg/mL) free radicals. Furthermore, AgNPs-LS showed cytotoxicity against breast cancer cells (MCF-7) (IC_{50} = 52.29 μg/mL), positioning them as promising candidates for cancer

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therapy. Moreover, AgNPs-LS were also shown promising anticoagulant and thrombolytic activities under practical conditions. Therefore, the biogenic synthesis of AgNPs-LS using *L. salivarius* offers a sustainable and cost-effective route for producing AgNPs with an array of biological activities. These AgNPs-LS have the potential to address various challenges in healthcare, ranging from antimicrobial, anticancer applications to biofilm inhibition, antioxidant therapy, anticoagulant and thrombolytic agents.

1. Introduction

Silver nanoparticles (AgNPs) are nanomaterials that have received considerable attention for their unique physical, chemical and biological properties [1–3]. There has been extensive use of AgNPs in a variety of fields, such as medicine, biotechnology, catalysis and electronics [4–7]. Among the various applications of AgNPs, their antimicrobial activity is one of the most promising and important. AgNPs can limit the growth and proliferation of various pathogenic microorganisms, including bacteria, fungi and viruses. Moreover, AgNPs can overcome the problem of antibiotic resistance, which is a major challenge for treating infectious diseases [8–11].

However, chemical or physical methods of synthesizing AgNPs often require toxic reagents and high energy consumption, which may pose environmental and health risks [12–14]. Therefore, there is a need for developing green and eco-friendly methods for the synthesis of AgNPs using biological sources, such as plants, algae, fungi and bacteria [5,9,10]. Among these sources, bacteria are particularly attractive because they are easy to culture, have high biomass yield and can produce AgNPs with different shapes and sizes [14,15].

Among the various bacteria that can synthesize AgNPs, probiotic bacteria have gained interest due to their health benefits and safety [16]. When administered in adequate amounts, probiotic bacteria confer beneficial effects on the host [17,18]. Probiotic bacteria can produce different biomolecules like exopolysaccharides, proteins, enzymes and metabolites that can reduce silver ions to form AgNPs [19]. Moreover, probiotic bacteria can enhance the biological activities of AgNPs by providing functional groups on their surface or by synergistic effects [20].

In recent years, probiotics have emerged as a focal point in both scientific research and commercial development due to their profound impact on human health and well-being. The exploration of probiotics extends beyond their traditional role in gut health, encompassing a wide array of potential applications in areas such as immunomodulation, disease prevention and even environmental remediation [21,22]. This surge in interest has propelled probiotics into the spotlight of interdisciplinary research, where their unique properties and mechanisms of action intersect with diverse fields, including nanotechnology. Probiotics offer distinct advantages as microbial factories for the synthesis of metallic nanoparticles, using their inherent biological capabilities such as enzymatic activity, surface binding and metabolic pathways [23–25]. These specialized properties enable precise control over nanoparticle synthesis, facilitating the production of nanoparticles with different characteristics such as size, shape and surface chemistry. By utilizing the bio-fabrication potential of probiotics, researchers can unlock innovative pathways for the sustainable and scalable synthesis of metallic nanoparticles, providing the way for novel applications in fields ranging from medicine to catalysis and beyond [23,24,26,27].

Lactobacillus salivarius is a probiotic bacterium that belongs to the lactic acid bacteria group. This Gram-positive, rod-shaped, facultative anaerobic bacterium is widely distributed in the mouth, gastrointestinal tract and vaginal cavity in humans and animals. Studies have shown that it improves oral health, intestinal health, immune system modulation and infection prevention [28,29]. It can also produce AgNPs by reducing silver ions which will be a simple, rapid and cost-effective process that does not require any additional stabilizing or capping agents. Therefore, the present study aimed to synthesize AgNPs via *L. salivarius* and to evaluate their antibacterial, anti-quorum sensing, antibiofilm, antifungal, antioxidant, anticancer, anticoagulant and thrombolytic properties.

2. Materials and methods

2.1. Material and reagents

Silver Nitrate ($AgNO_3$, ≥99.5 %, SRL) was procured from Sisco Research Laboratories Pvt. Ltd (India), De Man, Rogosa and Sharpe (MRS) Broth, Mueller Hinton Broth, Agar Powder, Luria Broth (LB), Phosphate - Buffered Saline (PBS), pH 7.0, Dimethyl Sulphoxide (DMSO), Chloroform, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Potato Dextrose Broth (PDB), Nystatin, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Hi-AR™ (MTT) and all other chemicals were procured from Himedia (India).

2.2. Biogenic synthesis of AgNPs using L. salivarius (AgNPs-LS)

A culture of *L. salivarius* MTCC-15009 was collected from the Microbial Type Culture Collection (MTCC) (IMTECH, Chandigarh, India) and cultured on De Man, Rogosa and Sharpe (MRS) agar plate (HiMedia®, Mumbai, India) at 37 °C. A single colony of *L. salivarius* was then transferred to sterile MRS broth and incubated for 48 h at 37 °C. The bacterial culture supernatant was obtained by centrifugation at 10,000 rpm for 10 min at 4 °C. The supernatant (10 mL) was mixed with silver nitrate solution (90 mL) and kept in the dark for 24 h at 30 °C. After 24 h of incubation, medium displayed a change in color [9] which indicated the synthesis of silver nanoparticles. The present approach utilizing the cell-free supernatant of *L. salivarius* for the synthesis of silver nanoparticles aligns with the principles of biogenic synthesis. In this method, the biological components present in the supernatant act as reducing and

stabilizing agents, facilitating the formation of silver nanoparticles. This biogenic approach connects the natural biochemical processes of the microorganism, making it an environmentally friendly and sustainable method for nanoparticle synthesis.

2.3. Characterization of AgNPs-LS

2.3.1. Ultraviolet–visible (UV–vis) spectra

UV–vis spectrophotometry was used to monitor the reduction of silver ions to AgNPs-LS. The scanning range was from 300 to 700 nm at a resolution of 1 nm (UV-1800, Spectrophotometer, Shimadzu, Kyoto, Japan). As a blank, distilled water was used [30].

2.3.2. Fourier transform infrared (FT-IR) spectroscopy analysis

FT-IR spectroscopy (Bruker®, Billerica, MA, USA) was used to determine the possible interactions between *L. salivarius* culture supernatant and $AgNO_3$. The spectra were taken from 500 to 4000 cm^{-1} [31].

2.3.3. Dynamic light scattering (DLS) and zeta potential analysis

The hydrodynamic diameter and zeta potential of the AgNPs-LS were evaluated using a Malvern Zetasizer Nanosystem (Worcestershire, UK). Initially, the synthesized AgNPs-LS were suspended in an aqueous solution and filtered through a 0.22 μm syringe-driven filter unit to ensure uniform dispersion. Subsequently, the size distribution of the AgNPs-LS was determined using DLS technique, which involved the utilization of a Malvern Zetasizer Nano series compact scattering spectrometer.

2.3.4. TEM analysis

The size and shape of AgNPs-LS were measured using TEM analysis. A JEM-1400 Plus, Jeol (India) instrument was utilized in this analysis. The AgNPs-LS sample was applied onto a carbon-coated copper grid and then dried in a vacuum dryer for 1 h before applying it to the grid [32].

2.4. Antibacterial activity

2.4.1. Cup/well diffusion method

The antibacterial potential of *L. salivarius* cell free supernatants (CFSs) and synthesized AgNPs-LS was evaluated against *C. violaceum* (MTCC-2656), *S. marcescens* (MTCC-97), *S. aureus* (MTCC-96), *E. coli* (MTCC-9537) and *P. aeruginosa* (MTCC-741) derived from the Microbial Type Culture Collection (IMTECH, Chandigarh, India) using agar cup diffusion as per standard methods [33]. Firstly, 20 mL of molten and cooled MH agar was poured into sterile Petri dishes. Using a sterilized swab, bacteria were spread for each strain, which was grown overnight, onto the MH agar plates individually. A sterile cork borer was used to make wells and fill them with *L. salivarius* CFSs and AgNPs-LS. The test plates were incubated for 24 h at 37 °C. After overnight incubation at 37 °C, the diameter of the zone of inhibition (in millimetres) was measured. As an antibacterial standard, streptomycin was used against all pathogens. The experiment was repeated three times.

2.4.2. Determination of minimum inhibitory concentration (MIC)

The standard broth micro-dilution method [34] was utilized to determine MIC values of AgNPs-LS as per the Clinical Laboratory Standards Institute (CLSI). Dilutions of AgNPs-LS (1000, 800, 400, 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.5, 0.78, 0.39 and 0.19 μg/mL) were performed in Mueller–Hinton broth with appropriate controls. The overnight grown bacterial culture (0.5 McFarland standard) was inoculated into each tube and tubes were incubated at 37 °C for 24 h. The bacterial growth was completely inhibited at the lowest concentration measured as the MIC. In order to validate the MIC test, loopfuls of inoculums from tubes that showed no growth after incubation were spread onto MHB agar plates.

2.5. Determination of anti-biofilm activity

Determination of the anti-biofilm potential of *L. salivarius* CFSs and AgNPs-LS was carried out using glass test tubes. Within the glass tubes, *S. marcescens*, *C. violaceum* and *P. aeruginosa* was grown in LB medium (3 mL) consisting of 500 μL of *L. salivarius* CFSs and AgNPs-LS (sub-MICs). All the tubes were incubated for 72 h at 37 °C in a shaking condition at 100 rpm. As soon as the incubation was completed, the planktonic cells were removed from the tubes and the biofilm that had developed was washed twice with PBS. Following that, developed biofilm was stained with crystal violet for 5 min and then excess stain was removed by washing with PBS. After washing, tubes were allowed to dry for another 5 min. At last, stained biofilm was dissolved in glacial acetic acid and absorbance of the solution was measured at 595 nm using UV spectrophotometer. Individual bacterial strain in LB medium without any AgNPs-LS served as a control [35]. The percentage inhibition of biofilm was calculated as:

$$[(\text{OD of control - OD of treatment}) \ / \ \text{OD of control}] \times 100$$

2.6. Determination of quorum sensing (QS) inhibition

The *L. salivarius* CFSs and AgNPs-LS was assessed for its ability to inhibit QS in *C. violaceum* using a well-diffusion assay [36]. Overnight-grown bacterial culture was spread on LB agar plates and wells were created by gel punctures. The *L. salivarius* CFSs and

AgNPs-LS (50 μL) was inoculated into the wells and at 30 °C, plates were incubated for overnight. After incubation, presence of an inhibitory effect around the well indicated successful interference with QS.

2.7. Determination of violacein pigment production in C. violaceum

In accordance with the previously described procedure, a spectrophotometer-based method was used to measure violacein pigment production [36]. Overnight-grown *C. violaceum* culture was inoculated into LB broth, both in presence and absence of *L. salivarius* CFSs and AgNPs-LS (sub-MICs). The tubes were then incubated for 24 h at 30 °C. After incubation, 2 mL of the bacterial culture was centrifuged for 5 min at 10,000 rpm and supernatant was discarded. Next, violacein pigment from the pellet was extracted via dissolving it in 1 mL of DMSO. The mixture underwent another centrifugation step to settle the cells. Finally, optical density of the supernatant was measured using DMSO as a blank at 585 nm via spectrophotometer. The inhibition of violacein production via AgNPs-LS was calculated as [(OD of control - OD of treatment)/OD of control] × 100.

2.8. Determination of pyocyanin pigment production in P. aeruginosa

In accordance with the previously described procedure, pyocyanin pigment production was measured spectrophotometrically [37]. Overnight-grown *P. aeruginosa* culture was inoculated into LB broth, both with and without *L. salivarius* CFSs and AgNPs-LS (sub-MICs). The tubes were then incubated for 24 h at 30 °C. After incubation, 1.5 mL of bacterial culture was extracted with 3 mL of chloroform. The organic phase obtained was re-extracted in 0.2 N HCl (1.2 mL). At 520 nm, optical density of pink color aqueous phase was measured. Using the same formula as described above, determination of pyocyanin inhibition was carried out.

2.9. Determination of prodigiosin pigment production in S. marcescens

In accordance with the previously described procedure of Slater et al. [38], prodigiosin pigment production was measured spectrophotometrically. Overnight-grown *S. marcescens* culture was inoculated into LB broth, both with and without *L. salivarius* CFSs and AgNPs-LS (sub-MICs). The tubes were then incubated for 24 h at 37 °C. After incubation, centrifugation of the bacterial culture was carried out to collect cell pellets which was redissolved using an acidified ethanol solution (96 mL ethanol + 4 mL 1 M HCl). Next, centrifugation at 10,000 rpm for 10 min was further performed and absorption of the supernatant (which contained prodigiosin) was measured via a spectrophotometer at 534 nm. Using the same formula as described earlier, determination of prodigiosin inhibition was carried out.

2.10. Determination of antifungal activity

The antifungal efficacy of *L. salivarius* CFSs and AgNPs-LS was assessed through agar well diffusion, targeting *Aspergillus niger, Penicillum purpurogenum, Rhizopus stolonifer* and *Fusarium proliferatum*. PDA plates supported the growth of all fungal strains, cultivated for 3–5 days at 30 °C [39]. Following growth, a fungal suspension was created in sterile PBS (pH - 7.0), and the inoculum was standardized to 10^7 spores/mL post-counting in a cell counter chamber. This suspension (1 mL) was evenly spread on PDA plates. Wells (8 mm) were made using a sterile cork-borer and 100 μL of *L. salivarius* CFSs and AgNPs-LS were added to each well, allowing a 2 h incubation at 4 °C. Nystatin served as the standard antifungal, and subsequent to a 72 h incubation at 30 °C, inhibition zones were measured.

2.11. Determination of DPPH free radical scavenging activity

The scavenging activity of DPPH$^{\bullet}$ by *L. salivarius* CFSs and AgNPs-LS was evaluated according to a modified method of Brand-Williams et al. [40]. AgNPs-LS stock solutions (100 μg/mL) in methanol was used to prepare working solutions with different concentrations (5–100 μg/mL). A solution of DPPH$^{\bullet}$ (0.2 mM) was also prepared in methanol. To each working solution of AgNPs-LS (100 μL), an equal volume of DPPH$^{\bullet}$ solution was added and mixed well. For 30 min, the mixtures were kept at room temperature in the dark. The colour change from violet to yellow represents DPPH$^{\bullet}$ reduction. ELISA plate reader (BioTek, USA) was used to measure the absorbance of samples at 517 nm. The percentage of DPPH$^{\bullet}$ scavenging was determined by applying the formula below:

$$\text{DPPH scavenging activity} = ((\text{Abs control - Abs sample}) / \text{Abs control}) \times 100$$

Ascorbic acid was used as the reference and DPPH$^{\bullet}$ as the control. An IC_{50} was calculated, which is the concentration of samples required to reduce 50 % of DPPH$^{\bullet}$.

2.12. Determination of ABTS radical scavenging activity

A modified method from Re et al. [41] was utilized to determine scavenging ability of *L. salivarius* CFSs and AgNPs-LS against ABTS free radicals. The oxidant ABTS was produced by persulfate oxidation of 2,2-azinobis (3-ethylbenzoline-6-sulfonic acid)-($ABTS^{2-}$). After mixing ABTS solution (7 mM) with 2.45 mM ammonium persulphate, the radical cation ($ABTS^{\bullet+}$) was formed in 12–16 h at room temperature in the dark. A 96-well plate was filled with *L. salivarius* CFSs and different concentrations of AgNPs-LS (5–100 μg/mL) and

200 μL of $ABTS^{\bullet+}$ solution. A 10 min incubation at RT in the dark was performed on the plate. ELISA plate readers (BioTek, USA) were used to measure the absorbance at 750 nm for decolorization. The following formula was used to calculate the percentage of $ABTS^{\bullet+}$ scavenging:

$$ABTS^{\cdot+} \text{ scavenging activity } (\%) = \left[1\text{-}\left(Abs_{sample}\right) / \left(Abs_{control}\right)\right] \times 100$$

where, $Abs_{control}$ is the absorbance of ABTS radical + methanol and Abs_{sample} is the absorption of the ABTS + sample.

2.13. Determination of cytotoxicity

The cytotoxicity study of *L. salivarius* CFSs and AgNPs-LS on MCF-7 cell lines was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After plating the MCF-7 cells in 96-well tissue culture plates at 1.5×10^4 cells per well, the cells were allowed to attach for 24 h. After the incubation and attachment of cells, treatment with *L. salivarius* CFSs and different concentrations (5–100 μg/mL) of AgNPs-LS was applied to the cells and further incubated for 24 h at 37 °C under humid conditions at 5 % CO_2. Then, 100 μL of MTT (5 mg/mL) solution was added into each well and further incubated for 4 h at 37 °C under humid conditions at 5 % CO_2 for the development of purple color precipitate. Then, the content of the wells was carefully removed and 150 μL of DMSO was added to dissolve the developed purple precipitates. At last, absorbance was measured at 570 nm using ELISA plate readers (BioTek, USA). The experiment was carried out in triplicates and the concentration required for 50 % inhibition of cell viability (IC_{50}) was determined [42]. The cell viability (%) was calculated using the following formula:

$$\% \text{ Cell viability} = \text{Absorbance at 570 nm of treated cells} / \text{Absorbance at 570 nm of control cells} \times 100$$

2.14. Determination of anticoagulant and thrombolytic property

The effect of AgNPs-LS on blood coagulation was assessed using fresh human blood at room temperature. A 100 μL solution of AgNPs-LS (100 μg/mL) was mixed with 2 mL of blood in a vial, while another vial of blood without any addition served as a control. The two samples were monitored for an hour at room temperature for any changes. The ability of AgNPs-LS to break down blood clots was also examined. A thin layer of fresh blood was applied on a glass slide and allowed to clot. Then, 50 μL of the AgNPs-LS solution (100 μg/mL) was added to the clot. The clot dissolution process was observed with the naked eye for 60 min to study the thrombolysis [43].

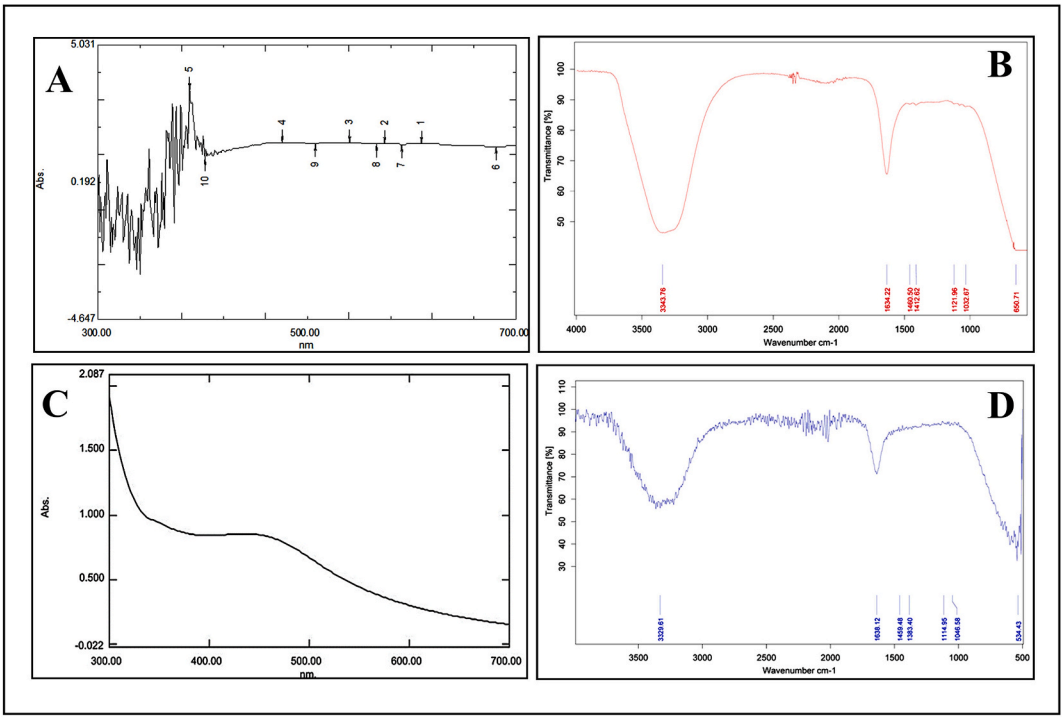

Fig. 1. – Characterization of AgNPs-LS via UV–Vis and FTIR analysis (A). The UV–visible absorption spectra of *L. salivarius* CFSs, (B). The FTIR analysis of *L. salivarius* CFSs, (C). UV–Vis absorption spectrum of AgNPs-LS showing the surface plasmon resonance peak, (D). FTIR spectrum of AgNPs-LS indicating the functional groups involved in the synthesis and stabilization of silver nanoparticles.

2.15. Statistical analysis

Results are presented as the mean ± SD of the number of experiments performed. The significance of the results was determined for the treatments using an ordinary one-way ANOVA followed by Dunnett's multiple comparisons test at $p \leq 0.05$. The analyses were carried out using the Graph Pad Prism software 8.0.

3. Results

3.1. Biogenic synthesis and characterization of AgNPs-LS

The culture supernatant of *L. salivarius* was used for the biosynthesis of AgNPs-LS. The synthesis of AgNPs-LS was confirmed by observing the color change of the culture supernatant from watery yellow to deep brown. The control sample without *L. salivarius* supernatant remained colorless under the same conditions. The UV–Vis absorption spectrum of AgNPs-LS showed a peak at around 438 nm, indicating the surface plasmon resonance of AgNPs (Fig. 1A and Fig. 1C). The FTIR spectrum of AgNPs-LS (Fig. 1B and Fig. 1D) exhibited several important peaks, such as 3329.61 cm^{-1}, 1638.12 cm^{-1}, 1459.48 cm^{-1} and 1046.58 cm^{-1} corresponding stretching vibration of O-H, C=O, C-O-H and C-C. These functional groups may derive from the biomolecules in the culture supernatant and acted as capping material for the AgNPs-LS, enhancing their stability and preventing their aggregation. In TEM analysis, AgNPs-LS were sized and shaped. AgNPs were spherical and polyhedral in shape and averaged 20–100 nm in size as revealed by TEM images (Fig. 2C). Additionally, Fig. 2A illustrates the histogram representing the particle size distribution of AgNPs-LS. According to the DLS analysis, AgNPs-LS exhibited an average diameter of 79.47 nm in the aqueous colloidal solution. The presence of multiple peaks in the particle dimension suggests that the produced particles vary in both shape and size. The polydispersity index was measured at 0.60. Furthermore, the zeta potential serves to characterize the surface charge and stability of AgNPs-LS. As depicted in Fig. 2B, the synthesized AgNPs-LS displayed a negative charge, with a zeta potential value of −14.53 mV. This value falls within the range of −20 to −30 mV, indicating moderate stability. Thus, it is evident that the synthesized AgNPs-LS exhibited moderate stability.

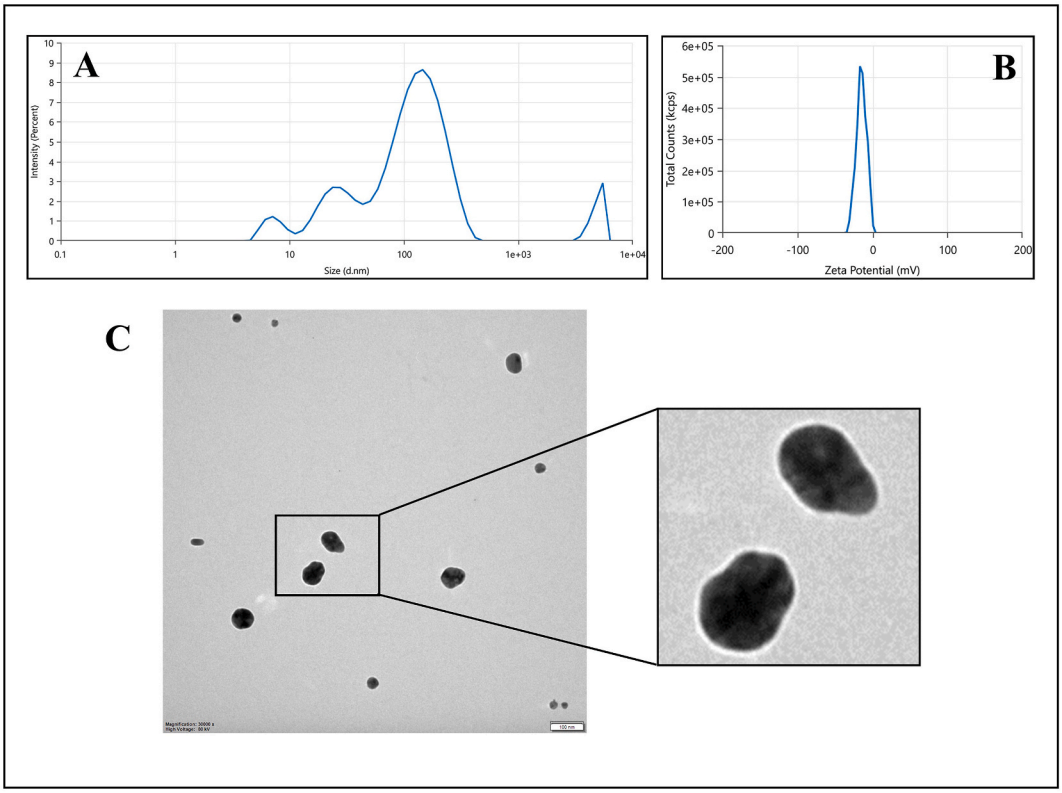

Fig. 2. – Characterization of AgNPs-LS via DLS, zeta potential and TEM analysis. (A&B). DLS and zeta potential analysis spectrums obtained from AgNPs-LS. The size distribution (maximum intensity at 8.70 nm) and stability (−14.53 mV) of the synthesized AgNPs-LS. **(C).** The TEM analysis of AgNPs-LS shows the morphology and size range of AgNPs-LS, which are mostly spherical and polyhedral in shape.

3.2. Antibacterial potential of AgNPs-LS

To determine the anti-bacterial property of *L. salivarius* CFSs and AgNPs-LS against different Gram-positive and Gram-negative bacteria, agar cup diffusion method was used. The results are shown in Fig. 3. Against all the bacteria tested, *L. salivarius* CFSs and AgNPs-LS exhibited antibacterial activity. Among the five bacteria, *C. violaceum* was found to be the most sensitive, followed by *P. aeruginosa, S. aureus*, *E. coli* and *S. marcescens.* Moreover, synthesized AgNPs-LS has higher anti-bacterial activity compared to *L. salivarius* CFSs. The MIC value of AgNPs-LS was determined to be 50 μg/mL for *C. violaceum*, *S. aureus*, *E. coli* and *P. aeruginosa* whereas 100 μg/mL towards *S. marcescens*. A concentration of AgNPs-LS below MIC (sub-MICs) was used to test their efficacy against formation of biofilm and QS-regulated virulence factors.

3.3. Effect of AgNPs-LS on biofilm formation

The *L. salivarius* CFSs and AgNPs-LS were evaluated for their antibiofilm activity using the crystal violet staining method, which measures the amount of biofilm biomass attached to the surface of a glass tube. In Fig. 4, results are shown and the results showed that AgNPs-LS exhibited higher and a dose-dependent antibiofilm effect of AgNPs-LS at respective sub-MIC concentrations on all the tested bacterial pathogens than *L. salivarius* CFSs. According to the results, biofilm inhibition occurred at sub-MIC concentrations of AgNPs-LS of 79.65, 54.51 and 24.61 % against *C. violaceum*, 73.32, 49.81 and 22.53 % against *P. aeruginosa* whereas 65.30, 47.96 and 34.72 % against *S. marcescens* (Fig. 4).

3.4. Effect of AgNPs-LS on quorum-sensing inhibition

The anti-quorum sensing activity of *L. salivarius* CFSs and AgNPs-LS against different bacterial pathogens was evaluated by measuring the inhibition of quorum sensing-regulated pigments, such as violacein, prodigiosin and pyocyanin. The results are presented in Fig. 5A–C. The results showed that *L. salivarius* CFSs and AgNPs-LS exhibited higher and a dose-dependent anti-quorum sensing effect on all the tested bacterial pathogens than *L. salivarius* CFSs. The pigment inhibition percentage increased significantly as the concentration of AgNPs-LS increased. At sub-MIC levels of AgNPs-LS, *C violaceum*, *P aeruginosa* and *S. marcescens* produced less violacein, pyocyanin and prodigiosin pigments, respectively. The reduction for violacein production was 80.27, 50.69 and 29.38 % (Fig. 5A), for pyocyanin 66.95, 52.32 and 41.72 % (Fig. 5B) whereas for prodigiosin were 74.23, 52.17 and 25.79 % at 1/2 MIC, 1/4 MIC and 1/8 MIC, respectively (Fig. 5C).

3.5. Antifungal potential of AgNPs-LS

In the present investigation, the agar well diffusion method was employed to assess the antifungal efficacy of *L. salivarius* CFSs and AgNPs-LS against various fungal strains. The results revealed high antifungal properties of AgNPs-LS than *L. salivarius* CFSs across all examined fungal strains. At a concentration of 100 μg/mL, the AgNPs-LS demonstrated the capacity to hinder the growth of *F. proliferatum, P. purpurogenum, A. niger* and *R. stolonifer* resulting in inhibition zones measuring 20 mm, 17 mm, 15 mm and 12 mm, respectively (Fig. 6).

3.6. Antioxidant potential of AgNPs-LS

To determine the antioxidant activity of *L. salivarius* CFSs and AgNPs-LS, the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radical scavenging assay was

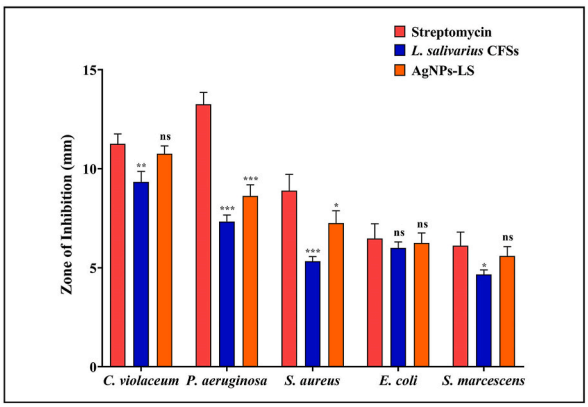

Fig. 3. The antibacterial potential of *L. salivarius* CFSs and AgNPs-LS against different Gram-positive and Gram-negative bacterial pathogens. The results are expressed as the mean ± SD of three replicates. Significance; ns > 0.05, $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$ with respect to Streptomycin.

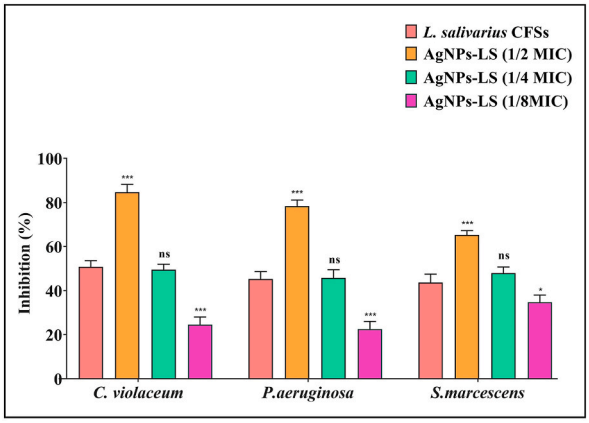

Fig. 4. The percentage inhibition of biofilm production of different bacterial strains by *L. salivarius* CFSs and AgNPs-LS (at different sub-MICs). The data are shown as the mean ± SD of three independent experiments. The superscript letters indicate significant differences at $p \leq 0.05$ with respect to the control. Significance; ns > 0.05, $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$ with respect to *L. salivarius* CFSs.

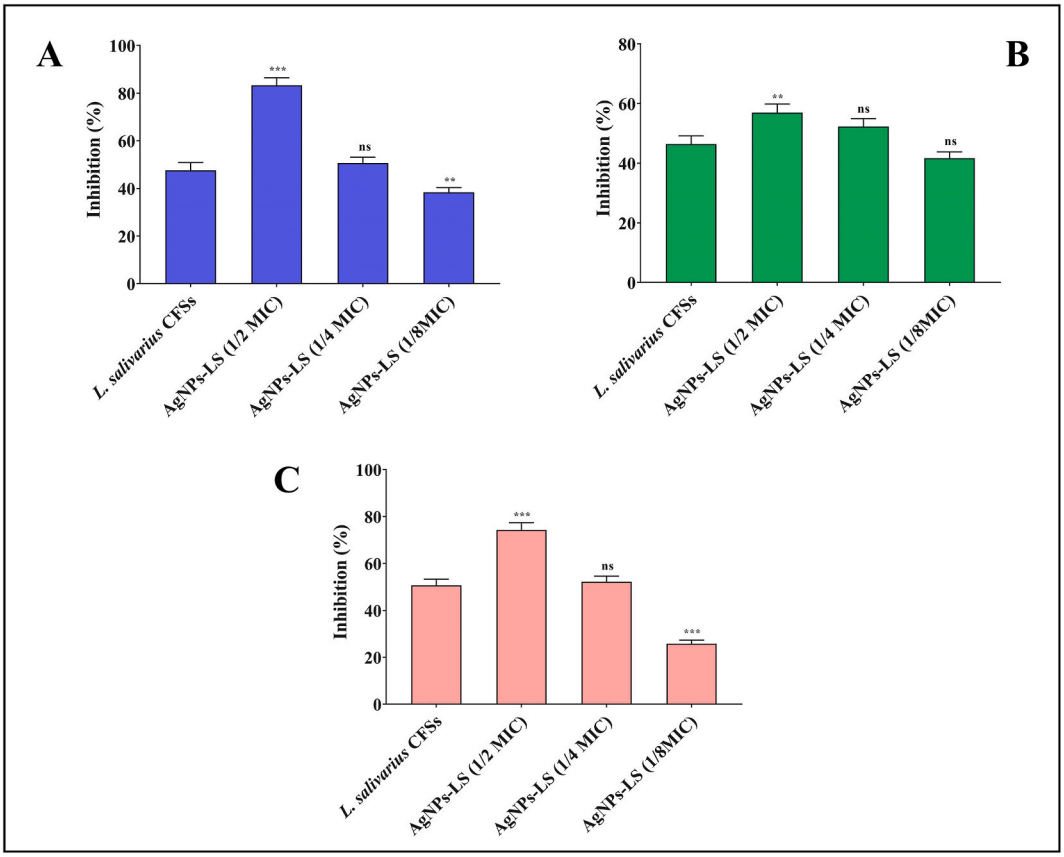

Fig. 5. The percentage inhibition of QS-mediated production of pigments of different bacterial strains by *L. salivarius* CFSs and AgNPs-LS (at different sub-MICs). **(A).** The percentage inhibition of violacein production in *C. violaceum* by *L. salivarius* CFSs and AgNPs-LS. **(B)**. The percentage inhibition of pyocyanin production in *P. aeruginosa* by *L. salivarius* CFSs and AgNPs-LS. **(C).** The percentage inhibition of prodigiosin production in *S. marcescens* by *L. salivarius* CFSs and AgNPs-LS. The data are shown as the mean ± SD of three independent experiments. The superscript letters indicate significant differences at $p \leq 0.05$ with respect to the control. Significance; ns > 0.05, $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$ with respect to *L. salivarius* CFSs.

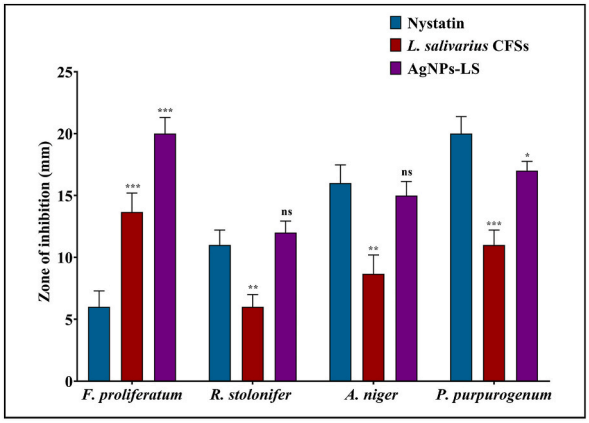

Fig. 6. Antifungal activity *L. salivarius* CFSs and AgNPs-LS against different fungal pathogens. The results are expressed as the mean ± SD of three replicates. Significance; ns > 0.05, *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$ with respect to Nystatin.

performed. The assay involved the measurement of the reduction in the absorbance of both free radicals in the presence of *L. salivarius* CFSs and AgNPs-LS. A high antioxidant activity was observed by AgNPs-LS against both free radicals than *L. salivarius* CFSs. The AgNPs-LS effectively scavenged the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ free radicals, leading to a reduction in the absorbance in a dose-dependent manner. The IC_{50} value (the concentration of AgNPs-LS required to scavenge 50 % of free radicals) was found to be 42.65 μg/mL against $DPPH^{\bullet}$ (Fig. 7A) and 53.77 μg/mL against and $ABTS^{\bullet+}$ (Fig. 7B).

3.7. Cytotoxic potential of AgNPs-LS

The *L. salivarius* CFSs and AgNPs-LS were further evaluated for their potential anticancer properties via MTT assay against breast cancer cells (MCF-7). Upon treatment with *L. salivarius* CFSs, cell viability was found to decrease slightly whereas after the treatment of AgNPs-LS, cell viability was found to decrease significantly in a dose-dependent manner. In the presence of higher concentrations of AgNPs-LS, MCF-7 cells showed a significant reduction in viability. AgNPs-LS had an IC_{50} value of 52.29 μg/mL against MCF-7 breast cancer cells (Fig. 8).

3.8. Anticoagulant and thrombolytic property of AgNPs-LS

The assessment of anticoagulant and thrombolytic efficacy of AgNPs-LS involved their introduction into human blood samples, followed by careful observation. In the control, coagulation commenced within 10 min of incubation, progressing to a gradual thickening of the blood sample, ultimately ending in the formation of a substantial blood clot after 60 min (Fig. 9A). Conversely, the blood sample treated with AgNPs-LS exhibited no discernible alterations, and notably, no evidence of coagulation was observed even after 60 min of incubation Fig. 9B). In the evaluation of the thrombolytic characteristics, the introduction of AgNPs-LS was made onto an already formed blood clot, facilitating subsequent observation (Fig. 9C). Upon immediate addition, a progressive liquefaction of the blood clot on the glass slide was initiated, reaching complete dissolution within 30 min, as depicted in Fig. 9D.

4. Discussion

Silver nanoparticles (AgNPs) are nanomaterials that have attracted much attention for their diverse applications in medicine and biotechnology, especially for their different types of biological activities [4,6,7]. Numerous approaches exist for synthesizing AgNPs, including chemical, physical and biological methods. Among these, biological approaches stand out for their eco-friendliness, cost-effectiveness and compatibility with living systems, making them a preferred choice over other methods [5,9]. Probiotic bacteria are one of the promising sources for the biosynthesis of AgNPs, as via producing metabolites, they can reduce silver ions to AgNPs and stabilize them as well [18,19]. Hence, in the present study, AgNPs-LS were synthesized from probiotic *L. salivarius*, characterized via different bio-physical methods and evaluated for their different biological activities.

Different techniques were used to characterize the biosynthesized AgNPs-LS, including UV–Vis spectroscopy, FTIR, TEM, DLS and zeta potential analysis. Using UV–Vis spectroscopy, a peak was observed around 438 nm, indicating that AgNPs-LS are forming. AgNPs-LS were found to have a spherical and polyhedral shape as shown by TEM images. In the FTIR analysis, several important functional groups were detected such as O-H, C=O, C-O-H and C-C in the solution of AgNPs-LS, possibly responsible for AgNPs-LS reduction and capping. According to the DLS analysis, AgNPs-LS exhibited an average diameter of 79.47 nm in the aqueous colloidal solution with polydispersity index at 0.60. Furthermore, the zeta potential displayed a negative charge of AgNPs-LS with a zeta potential value of −14.53 mV. Many previous studies have described the synthesis and characterization of AgNPs using various probiotic lactic acid bacteria strains with a similar approach [16,44–46].

The antimicrobial activity of AgNPs is one of their most notable characteristics. Microorganisms, including bacteria, fungi, and

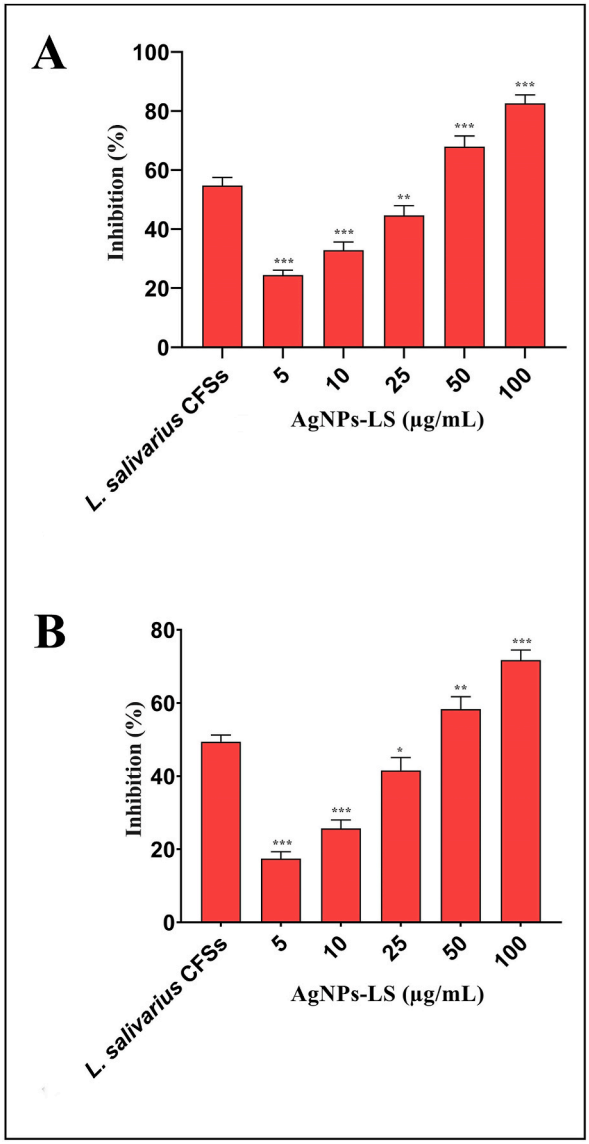

Fig. 7. Antioxidant activity of *L. salivarius* CFSs and AgNPs-LS. **(A).** DPPH$^{\bullet}$ radical scavenging activity, **(B).** ABTS$^{\bullet+}$ radical scavenging activity. The data are shown as the mean ± SD of three independent experiments. The superscript letters indicate significant differences at $p \leq 0.05$ with respect to the control. Significance; ns > 0.05, $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$ with respect to *L. salivarius* CFSs.

viruses have been reported to be susceptible to AgNPs [47]. Due to AgNPs ability to disrupt cell membranes and inhibit vital cellular processes, microorganisms are reported to killed by AgNPs [48]. The biosynthesized AgNPs-LS exhibited antibacterial activity against both Gram-positive and Gram-negative bacteria in this study, such as *C. violaceum*, *S. marcescens*, *S. aureus*, *E. coli* and *P. aeruginosa*. The antibacterial activities of AgNPs derived from different probiotic bacteria represent a promising avenue in combating foodborne pathogens. The AgNPs synthesized from these probiotic strains exhibit potent antibacterial properties against a wide range of food-borne pathogens, including *Salmonella*, *Escherichia coli* and *Listeria monocytogenes* [49]. These nanoparticles function by disrupting the cellular integrity of the pathogens, interfering with essential metabolic processes, and ultimately leading to their inhibition or death [46]. Furthermore, the use of probiotic derived AgNPs offers a dual advantage such as, the inherent probiotic properties of the bacteria alongside the antimicrobial effects of the nanoparticles, potentially providing a synergistic approach for enhancing food safety and preservation [16]. Ongoing research in this area aims to further elucidate the mechanisms underlying the antibacterial action of probiotic-derived AgNPs and explore their practical applications in food industries and healthcare settings.

Many previous studies have shown that AgNPs synthesized from LAB strains possess antibacterial activity. For instance, AgNPs from *L. acidophilus* showed cytolysis and membrane disruption of *Klebsiella pneumoniae* [50]. AgNPs from *Brevibacterium linens* inhibited *E. coli* and *S. aureus* (multidrug-resistant strains), as reported by Nithya et al. [49]. *B. subtilis*, *S. aureus*, *P. aeruginosa*, *K. pneumoniae* and *E. coli* were reported to suppressed by AgNPs from *L. amylophilus* GV6, as studied by Kumar et al. [51]. The AgNPs

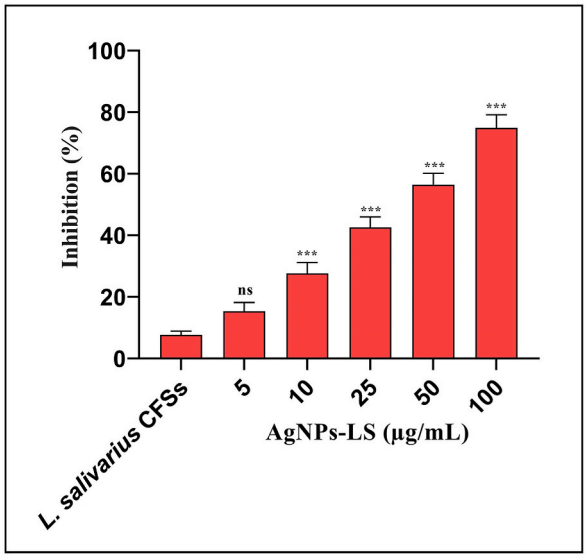

Fig. 8. The effect of *L. salivarius* CFSs and AgNPs-LS on the viability of MCF-7 breast cancer cells was assessed by MTT assay. The data are shown as the mean ± SD of three independent experiments. The data are shown as the mean ± SD of three independent experiments. Different superscript letters indicate significant differences at $p \leq 0.05$ with respect to the control. Significance; ns > 0.05, $*p < 0.05$, $**p < 0.005$, $***p < 0.0005$ with respect to *L. salivarius* CFSs.

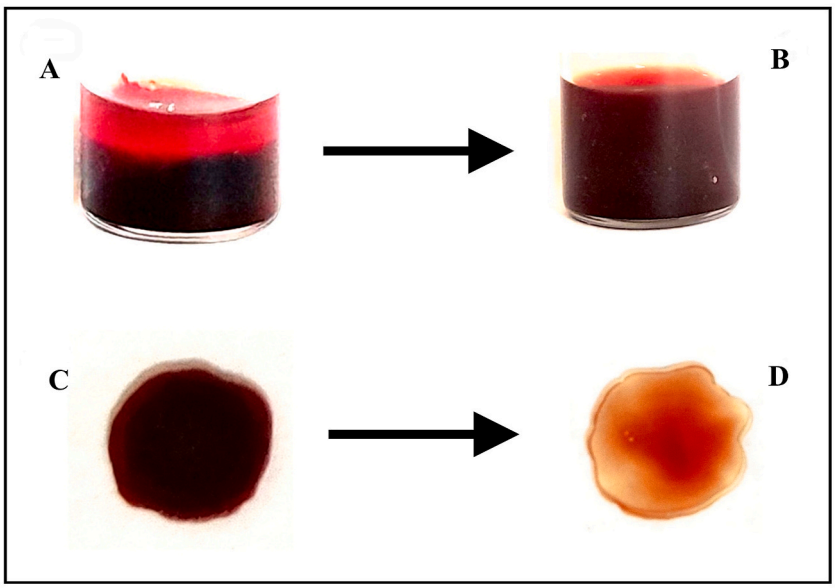

Fig. 9. Anticoagulant and Thrombolytic activity of AgNPs-LS. (A). Control of anticoagulant activity after 60 min (B). Test of anticoagulant activity after 60 min (C). Control of thrombolytic activity (D). Test of thrombolytic activity.

derived from *L. plantarum* 92T and *L. acidophilus* 58p reported for their antibacterial activity against *E. coli*, *S. epidermidis*, *S. flexneri*, *K. pneumonia* and *S. sonnei* by Garmasheva et al. [52]. The AgNPs derived from *L. bulgaricus* were effective against *S. aureus*, *S. epidermidis* and *S. typhi*, as reported by Naseer et al. [46]. AgNPs derived from four different LAB strains such as, *L. plantarum* F22, *L. pentosus* S6, *L. paraplantarum* KM1 and *L. crustorum* F11 showed antibacterial activity against *L. monocytogenes, B. cereus* and antibiotic-resistant *S. aureus*, as reported by Sharma et al. [16]. Therefore, AgNPs-LS holds great potential for the development of novel antimicrobial agents to combat drug-resistant pathogens.

Biofilm formation and quorum sensing are important mechanisms utilized by bacteria to promote survival and virulence [8]. Unlike conventional antibiotics, biofilms are complex communities of microorganisms that are encased in an organic matrix that produces its own protective barrier [53]. Currently, antibiotic resistance in biofilms is a major concern in the medical field [54]. A number of

studies have shown that AgNPs prevent the formation of biofilms and disrupt those that have already formed. Despite being able to penetrate biofilm matrix, AgNPs are capable of directly interacting with bacterial cells, explaining their antibiofilm activity. AgNPs can disrupt the extracellular polymeric substances (EPS), which are responsible for biofilm stability, thus preventing further biofilm development [55–57]. Additionally, AgNPs can interfere with bacterial cell signaling, leading to the inhibition of biofilm-associated genes and enzymes involved in biofilm formation [4,58]. The process of quorum sensing involves bacteria communicating with each other in response to a population density [59]. An important role of QS is to contribute to biofilm formation, virulence, and antibiotic resistance [60]. AgNPs produced by LAB have been found to possess anti-QS activity, inhibiting QS-regulated phenotypes in various bacterial species. The anti-QS mechanism of AgNPs involves interference with the production and reception of autoinducer molecules, which are essential for QS signaling. AgNPs can bind to autoinducers, preventing their interaction with QS receptors and thereby disrupting the communication network within bacterial communities. Consequently, this disruption leads to the downregulation of QS-controlled genes, inhibiting biofilm development and reducing bacterial virulence [61,62]. In the present study, in addition to their anti-bacterial properties, AgNPs-LS have demonstrated antibiofilm and anti-QS activity against *P. aeruginosa, C. violaceum* and *S. marcescens*. The antifungal activity of AgNPs-LS was also observed against *F. proliferatum, P. purpurogenum, A. niger,* and *R. stolonifer*. At higher concentrations, AgNPs in solution may adhere to and saturate fungal hyphae, leading to the destruction of fungal cells. This inhibitory effect is attributed to Ag^{+} ions, primarily affecting membrane-associated enzymes in the respiratory chain. Additionally, Ag^{+} ions may influence the expression of microbial proteins and enzymes, disrupting DNA replication. Competitive inhibition is another mechanism through which AgNPs interact with substrates, inactivating enzymes and hindering the production of essential products for cell activity [39,63].

Antioxidants play a vital role in protecting the body against oxidative stress and associated diseases [64]. The antioxidant activity of AgNPs synthesized by lactic acid bacteria can be attributed to their ability to scavenge reactive oxygen species (ROS) [65]. A variety of ROS are produced as a result of the metabolism of cells, such as superoxide anion, hydrogen peroxide and hydroxyl radicals. An excess of ROS in the body can cause oxidative stress in the cells, which can damage their components, causing various diseases to develop as a consequence [66]. AgNPs possess the ability to neutralize these ROS, thereby exhibiting antioxidant activity. The antioxidant mechanisms of AgNPs involves multiple pathways. AgNPs can directly scavenge ROS, preventing their harmful effects on cellular components [67]. Further, AgNPs can increase antioxidant enzyme expression, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). The role of these enzymes in cellular defense against oxidative stress is crucial. Endogenous antioxidants, such as glutathione (GSH), can be enhanced by AgNPs, which further contribute to their antioxidant properties [68]. In the present study, AgNPs-LS have been found to have higher antioxidant activity against both $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals.

Anticancer agents are substances that can induce apoptosis (programmed cell death) or necrosis (uncontrolled cell death) in cancer cells without affecting normal cells [69]. Cancer is a disease characterized by uncontrolled proliferation and invasion of abnormal cells that can evade apoptosis and resist conventional therapies [70]. AgNPs can act as anticancer agents by generating ROS or releasing silver ions that can trigger various cellular responses such as DNA damage, mitochondrial dysfunction, cell cycle arrest and apoptosis in cancer cells [71,72]. AgNPs-LS derived from *L. salivarius* have been shown to have cytotoxicity against breast cancer (MCF-7).

The anticoagulant and thrombolytic activities of AgNPs-LS were also evident in our study. While the theoretical foundations of these activities remain unclear, a plausible biochemical mechanism can be inferred by considering the thrombolysis process. Biogenic silver nanoparticles may engage in inhibiting enzymes responsible for generating blood clotting proteins [73]. It is likely that nano-silver impedes the conversion of prothrombin into thrombin, a crucial step in the production of insoluble fibrin strands and the catalysis of other coagulating factors [74]. Additionally, silver nanoparticles may participate in activating enzymes that generate plasmin, facilitating the breakdown of cross-links in fibrin molecules and the dissolution of blood clots [75]. The findings of this study align with the reported thrombolytic activity of biochemically mediated silver nanoparticles by Ref. [76]. The obtained results clearly depict the complete dispersion of blood clots achieved by AgNPs-LS. While blood coagulation is essential to prevent excessive bleeding, the timely dissolution of clots is equally crucial to mitigate thrombosis [77]. Traditional antithrombotic treatments, such as streptokinase, face limitations due to their short half-life, susceptibility to neutralization by foreign agents, and the potential for excessive bleeding [78]. Despite the limited information in the literature regarding the use of nano-silver as a thrombolytic agent, this study claims to have demonstrated the efficacy of AgNPs-LS as both anticoagulant and thrombolytic agents in the management of thrombosis. The demonstrated potential of AgNPs-LS in this study may find practical applications in the clinical domain for preventing thrombosis and addressing related disorders. Therefore, the present study in which AgNPs-LS derived from probiotic *L. salivarius* have multiple biological activities that make them promising candidates for various biomedical applications.

5. Conclusion

This study provides a comprehensive exploration of the multifaceted properties of silver nanoparticles (AgNPs-LS) synthesized from probiotic *L. salivarius*. The synthesized AgNPs-LS exhibited notable antibacterial and antibiofilm activities, showcasing their potential as effective agents against bacterial pathogens. Moreover, the anti-quorum sensing properties suggest a role in disrupting bacterial communication mechanisms, further contributing to their antibacterial efficacy. The antifungal activity observed against various fungal strains indicates a broad spectrum of action, enhancing the versatility of these nanoparticles. The demonstrated antioxidant and anticancer properties highlight the potential therapeutic applications of these silver nanoparticles. Their ability to scavenge free radicals and inhibit cancer cell proliferation suggests a promising avenue for further investigation in cancer treatment strategies. Additionally, the anticoagulant and thrombolytic activities underscore their potential in managing thrombotic disorders. The environmentally friendly synthesis of silver nanoparticles from probiotic *L. salivarius* adds to their appeal, aligning with the growing emphasis on sustainable and green nanotechnology. This study contributes valuable insights into the diverse applications of

silver nanoparticles, providing the way for future research and potential biomedical advancements.

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Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Abdelmushin Abdelgadir: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **Mohd Adnan:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis. **Mitesh Patel:** Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis, Conceptualization. **Juhi Saxena:** Validation, Resources, Investigation, Formal analysis, Data curation. **Mohammad Jahoor Alam:** Visualization, Validation, Software, Methodology, Formal analysis. **Mohammed Merae Alshahrani:** Validation, Methodology, Investigation, Formal analysis, Data curation. **Ritu Singh:** Writing – review & editing, Validation, Methodology, Investigation, Data curation. **Manojkumar Sachidanandan:** Validation, Methodology, Investigation, Formal analysis. **Riadh Badraoui:** Writing – review & editing, Visualization, Validation, Resources, Formal analysis. **Arif Jamal Siddiqui:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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