



Biogenic Silver Nanoparticles: An Antibacterial and Antibiofilm Approach to Control Carbapenem-Resistant *Klebsiella pneumoniae*

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Abstract

The increasing resistance of *Klebsiella pneumoniae* to antibiotics, particularly carbapenems, underscores the urgent need for alternative antimicrobial strategies. This study aimed to evaluate the antimicrobial and antibiofilm activity of biogenic silver nanoparticles (Bio-AgNPs), synthesized using *Trichilia catigua* extract and to investigate their mechanism of action on bacterial cells. The antimicrobial efficacy of Bio-AgNPs was assessed by determining the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and through susceptibility assays, time-kill analysis, flow cytometry, and electron microscopy. Bio-AgNPs exhibited strong antibacterial activity against all tested *K. pneumoniae* strains, with MICs ranging from 0.49 to 15.62 µg/mL. Eradication of biofilms required concentrations 16 to 64 times higher than those effective against planktonic cells, highlighting the protective nature of biofilm matrix. Time-kill assays showed a concentration-dependent bactericidal effect, with 4xMIC rapidly eliminating viable cells. Flow cytometry indicated membrane destabilization, increased fluidity at lower concentrations, and extensive structural damage at higher concentrations. Although lipid peroxidation and reactive oxygen species (ROS) were detected, they were not statistically significant, suggesting that membrane disruption is the primary bactericidal mechanism. Electron microscopy corroborated the structural disintegration of both bacterial cells and biofilms after treatment. Bio-AgNPs demonstrated potent antimicrobial and antibiofilm effects against multidrug-resistant *K. pneumoniae*, with membrane damage as the main mechanism of action. These results support the potential application of Bio-AgNPs as a therapeutic alternative for biofilm-associated infections. This study highlights the antibacterial and anti-biofilm potential of biogenic silver nanoparticles (Bio-AgNPs) as an effective tool against multidrug-resistant *Klebsiella pneumoniae*, with efficacy against both free-living cells and structured biofilms.

Introduction

Klebsiella pneumoniae is an increasingly relevant pathogen due to its association with serious infections in hospital settings and resistance to multiple antibiotics, including carbapenems [1]. A member of the *Enterobacteriaceae* family, this species was included in the World Health Organization (WHO)'s first list of priority pathogens in 2017 due to its carbapenem resistance [2]. In 2024, carbapenem-resistant *K. pneumoniae* remained on the updated WHO list, reinforcing global concerns about infections caused by multi-drug-resistant pathogens and the ongoing impact of these organisms on public health [3, 4]. The continued presence on the list underscores the urgency of developing new antibacterial agents and interventions to contain the spread of these high-priority pathogens.

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The relevance of *K. pneumoniae* as a priority hospital pathogen is, in part, due to its multiple virulence factors that enhance its ability to cause severe and persistent infections [5]. Protected by a polysaccharide capsule, the bacteria resist phagocytosis and readily adhere to host surfaces and cells, facilitating colonization [6]. Additionally, the lipopolysaccharides (LPS) present in the cell wall act as endotoxins, triggering significant inflammatory responses that can progress to sepsis in more severe cases [6]. Another critical factor is the production of biofilm, an extracellular matrix whose primary function is to protect bacterial cells from the immune system and antibacterial agents [7–9]. These virulence mechanisms make the control of multidrug-resistant *K. pneumoniae* a major challenge [10].

In this context, nanotechnology has emerged as an innovative field and is recognized as one of the pioneering sciences of the 21st century. It has enabled significant advancements, including the development of nanometer-scale materials characterized by high reactivity and a favorable surface area-to-volume ratio, which are particularly advantageous for biomedical applications [11, 12]. Additionally, green nanotechnology has gained considerable attention within the scientific community due to its environmentally friendly and cost-effective nature [13].

The biosynthesis of silver nanoparticles (AgNPs) using fungi, bacteria, and plants adheres to the principles of green chemistry and support sustainable development. Besides being more economical and eco-friendly, this approach produces nanoparticles with high bioactivity and reduced toxicity [11, 14]. Biogenic silver nanoparticles (Bio-AgNPs) have emerged as a promising alternative antibacterial agent, with diverse applications in sanitizers [15, 16], veterinary medicine [17] and the treatment of bacterial infections [18, 19]. Research into these biogenic nanoparticles may offer novel strategies for combating high-priority pathogens. Therefore, the aim of this study was to evaluate the antibacterial and antibiofilm activities of Bio-AgNPs synthesized from *Trichilia catigua* extract against KPC-producing *Klebsiella pneumoniae* isolates.

Materials and Methods

Synthesis of Biogenic Silver Nanoparticle (Bio-AgNP)

The biogenic silver nanoparticles (Bio-AgNPs) used in this study were synthesized as previously described (BR 1020190117605-2019; NanoVerdeAg®), using a plant-based green synthesis approach with *Trichilia catigua* extract as the reducing and stabilizing agent. Briefly, silver nitrate was employed as the metallic precursor at a final

concentration of 10 mM, prepared by dissolving 1.7 g of AgNO₃ in 1 L of sterilized distilled water. The *T. catigua* extract, obtained from the plant bark and rich in bioactive compounds, was added to the silver nitrate solution at a ratio of 1:100 (10–50 mL of extract per liter of precursor solution), acting both as a reducing agent and as a source of organic compounds that coat and stabilize the nanoparticles. The reaction mixture was incubated at 30 °C, without agitation, and protected from light for 24 to 72 h to allow the reduction of ionic silver to metallic silver nanoparticles, as evidenced by a visible color change. After synthesis, the nanoparticle suspension was stored at 4 °C under light-protected conditions. The method allows the production of Bio-AgNPs in both colloidal and micellar forms. Nanoparticle formation and physicochemical properties were confirmed by Scanning Electron Microscopy (SEM) for size and morphology analysis, Energy Dispersive Spectroscopy (EDS) for elemental composition, and zeta potential measurements to assess surface charge and colloidal stability.

Bacterial Strains

Twenty-eight clinical isolates of *Klebsiella pneumoniae* were previously analyzed for their genetic profiles, antibiotic resistance, biofilm-forming capacity, and phylogenetic relationships [20]. Based on these analyses, clonal strains were excluded, and seven non-clonal clinical isolates of KPC-producing *K. pneumoniae* (KPC-Kp) were selected. These isolates, designated Kb17, Kb19, Kb22, Kb23, Kb24, Kb27, and Kb28, are maintained in the biorepository of the Laboratory of Bacteriology and Bioassays (LaBBio) at the Federal University of Pelotas (UFPel, Pelotas, RS, Brazil), and were kindly provided by the Microbiology Laboratory of the University Hospital, UFPel. Additionally, the reference strain *K. pneumoniae* ATCC® 700603™ was included in this study and was provided by the Oswaldo Cruz Foundation (FIOCRUZ, Rio de Janeiro, RJ, Brazil).

Minimum Inhibitory and Bactericidal Concentrations (MIC and MBC)

The antimicrobial activity of the silver nanoparticles was evaluated using the standard broth microdilution method, following the Clinical and Laboratory Standards Institute (CLSI, 2020) guidelines. Serial dilutions of the nanoparticles were prepared to achieve final concentrations ranging from 0.24 to 125 µg/mL. Bacterial suspensions were adjusted to a 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL). The MIC determination was conducted in Mueller-Hinton Broth (MHB; Sigma®, St. Louis, USA). Negative controls consisted of inoculated broth without nanoparticles. All microplates were incubated at 36 °C for

24 h. Following incubation, 20 μL of 0.4% resazurin solution (Sigma[®], St. Louis, USA) was added to each well, and the plates were incubated for an additional 2 h at 36 °C. The MIC was defined as the lowest concentration at which no color change from blue to pink was observed, indicating bacterial viability inhibition. To determine the MBC, aliquots (10 μL) from wells showing no color change were plated onto Brain Heart Infusion (BHI) agar (Kasvi[®], Italy) and incubated at 36 °C for 24 h. The MBC was defined as the lowest concentration that completely inhibited visible bacterial growth. All assays were performed in triplicate for each bacterial strain.

Biofilm Susceptibility Tests

To determine the minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC), the methodology described by [21] and adapted by [22] was followed. Bacterial suspensions (1.5×10^8 CFU/mL) were prepared from fresh 18–24 h cultures, and 20 μL of each suspension was added to 180 μL of Tryptic Soy Broth (TSB) in 96-well microplates. Plates were incubated at 36 °C for 24 h to allow biofilm formation. Following incubation, non-adherent cells were removed by washing the wells three times with a sterile 0.9% saline solution. Subsequently, TSB containing the same serial dilutions of Bio-AgNP used in the MIC and MBC assays were added to each well, and the plates were incubated under the same conditions. MBIC was defined as the lowest nanoparticle concentration that inhibited visible bacterial growth in the wells, assessed by the absence of turbidity. To determine the MBEC, 5 μL from each well showing no visible growth was plated onto BHI agar and incubated at 36 °C for 24 h. MBEC was defined as the lowest concentration that completely prevented bacterial regrowth following treatment, indicating biofilm eradication. Based on the MBIC and MBEC results, two additional approaches were employed to evaluate nanoparticle efficacy [23]; (i) preventive treatment, in which bacterial suspensions were incubated with specific dilutions (0.24 $\mu\text{g}/\text{mL}$ to 125 $\mu\text{g}/\text{mL}$) of nanoparticles in TSB for 24 h at 36 °C; and (ii) pre-formed biofilm treatment, in which bacterial suspensions were initially incubated in TSB for 4 h at 36 °C to allow early-stage biofilm development. Afterward, wells were washed with phosphate-buffered saline (PBS, 0.1 M), and fresh TSB containing Bio-AgNP at MIC, 2×MIC, and 4×MIC, followed by incubation for another 24 h at 36 °C. After incubation, wells were washed, fixed, stained (crystal violet), and the remaining biofilm biomass was quantified by measuring absorbance at 540 nm. A two-way ANOVA was performed using GraphPad Prism 8.0.1, followed by Dunnett's post-test to identify statistically significant differences between the negative control and the different treatments.

Time-Kill Assay

The time-kill assay was conducted following the Clinical and Laboratory Standards Institute (CLSI, 2012) guidelines, with minor modifications. The assay was performed using the reference strain *K. pneumoniae* ATCC[®] 700603[™] and one clinical isolate (Kb17). Bacterial suspensions ($\sim 10^6$ CFU/mL) were exposed to Brain Heart Infusion (BHI) broth alone (negative control) and BHI supplemented with Bio-AgNP at concentrations corresponding to the MIC, 2×MIC, and 4×MIC. At predetermined time points (0 h, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, and 24 h), 100 μL aliquots were collected, serially diluted (1:10) in sterile 0.9% saline solution, and plated in triplicate on BHI agar. Plates were incubated at 36 °C for 24 h, after which the number of colony-forming units (CFU) was determined. Time-kill curves were constructed by plotting the $\log_{10}$ CFU/mL versus time and compared to the negative control. Bactericidal activity was defined as a $\geq 99.9\%$ reduction ($\geq 3 \log_{10}$) in the initial CFU/mL count. Triplicate assays were analyzed using GraphPad Prim 8.0.1. Data were subjected to two-way ANOVA with Dunnett's post-test to determine statistical differences.

Flow Cytometry Analysis

Flow cytometry was conducted to evaluate the cellular damage induced by silver nanoparticles in *K. pneumoniae* ATCC[®] 700603[™] and the clinical isolate Kb17. Bacterial suspensions were prepared from fresh cultures, adjusted to a 0.5 McFarland standard, and inoculated into microtubes containing TSB broth. Bio-AgNPs were added at concentrations corresponding to MIC, 2×MIC, and 4×MIC, as determined in previous assays. Untreated bacterial cultures served as negative controls. Following 24 h of incubation at 36 °C, fluorescent probes were added to assess various physiological parameters. Membrane fluidity was evaluated using Merocyanine 540 (Sigma-Aldrich, USA) and YO-PRO[™]-1 (Thermo Fisher Scientific, USA), while membrane integrity was assessed with 5(6)-carboxyfluorescein (Sigma-Aldrich, USA) and propidium iodide (PI). Lipid peroxidation (LPO) was analyzed using BODIPY[™] 581/591 C11 (Thermo Fisher Scientific, USA), and intracellular reactive oxygen species (ROS) production was detected using CM-H₂DCFDA (Thermo Fisher Scientific, USA) in combination with PI. Incubation times for each probe followed the respective manufacturers' protocols. After staining, cultures were washed three times with PBS, and the resulting bacterial pellets were resuspended in PBS containing 4% formaldehyde. Samples were analyzed using Attune Cytometric Software v2.1. All assays were conducted in triplicate. Statistical analysis was performed using GraphPad Prism 8.0.1.

Table 1 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Bio-AgNP against clinical isolates of *K. pneumoniae*

Isolate	MIC ($\mu\text{g/mL}$)	MBC ($\mu\text{g/mL}$)
ATCC [®] 700603 TM	0.49	0.49
Kb17	0.97	7.81
Kb19	15.62	31.25
Kb22	3.90	15.62
Kb23	0.97	15.62
Kb24	0.97	15.62
Kb27	0.97	7.81
Kb28	0.97	15.62

One-way ANOVA followed by Brown-Forsythe post-test was used for multiple comparisons.

Scanning Electron Microscopy (SEM)

SEM was employed to assess alterations in bacterial cell morphology and biofilm formation following treatment with Bio-AgNPs. For morphological analysis, bacterial cells were exposed to Bio-AgNPs at MIC-determined concentrations, centrifuged, washed three times with PBS, and deposited onto sterile coverslips. After air drying, cells were fixed in sterile 2% glutaraldehyde at 36 °C for 18 h. The samples were subsequently dehydrated through a graded ethanol series (30%, 50%, 70%, and 100%). For biofilm analysis, sterile coverslips were placed at the bottom of 6-well polystyrene plates, and the wells were filled with culture medium supplemented with Bio-AgNPs at concentrations corresponding to the MBIC and MBEC values. After 24 h of incubation at 36 °C, the coverslips were gently washed with a 0.9% saline solution, dehydrated using the same ethanol gradient, and fixed with methanol. All samples were sputter-coated with a thin layer of gold using a Denton Vacuum Desk V system (Gold and Carbon Sputtering), and surface morphology was examined with a JEOL JSM-6610LV Scanning Electron Microscope.

Results

Effect of Bio-AgNPs on Planktonic Cells and Biofilm Formation of *K. pneumoniae*

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values are summarized in Table 1. MIC values for Bio-AgNPs ranged from 0.49 to 15.62 $\mu\text{g/mL}$, with the reference strain *K. pneumoniae* ATCC[®] 700603TM being the most susceptible. In general, clinical isolates exhibited higher MIC values, and MBCs were consistently equal to or higher than the corresponding MICs, ranging from 7.81 to 31.25 $\mu\text{g/mL}$.

Table 2 Minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC) of Bio-AgNP against clinical isolates of *K. pneumoniae*

Isolate	MBIC ($\mu\text{g/mL}$)	MBEC ($\mu\text{g/mL}$)
ATCC [®] 700603 TM	31.25	125
Kb17	31.25	> 125
Kb19	31.25	> 125
Kb22	15.62	> 125
Kb23	15.62	125
Kb24	15.62	125
Kb27	15.62	> 125
Kb28	31.25	125

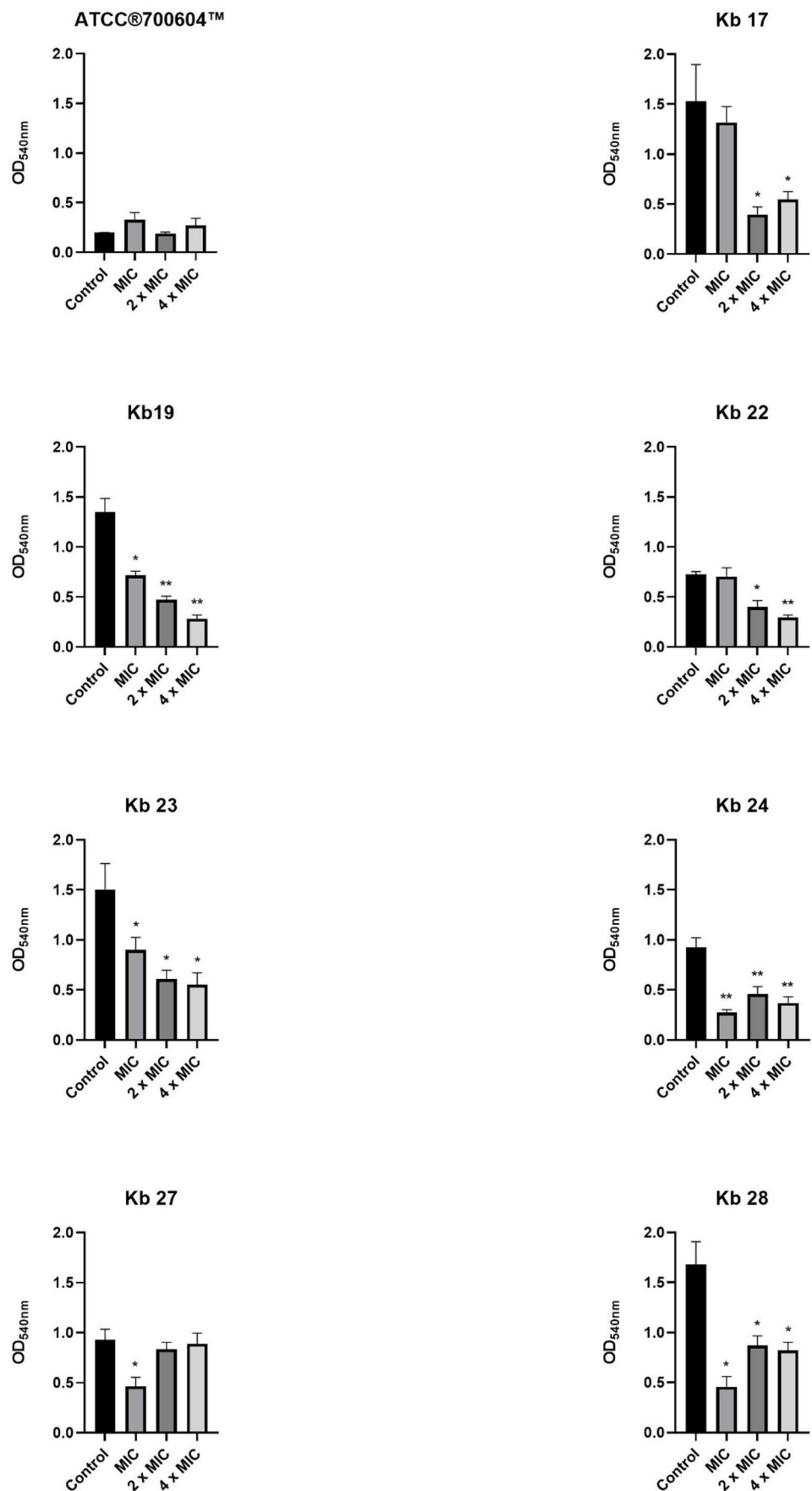
Biofilm susceptibility assays demonstrated that Bio-AgNPs exhibited antibiofilm activity against all *K. pneumoniae* strains tested (Table 2). Minimum biofilm inhibitory concentrations (MBICs) ranged from 15.62 to 31.25 $\mu\text{g/mL}$, indicating variable susceptibility among the isolates. In contrast, established biofilms showed greater tolerance, as complete biofilm eradication (MBEC) was achieved only for a subset of strains at the highest concentration tested (125 $\mu\text{g/mL}$), while the remaining isolates did not exhibit total eradication, highlighting the increased resistance of mature biofilms to Bio-AgNP exposure.

A comparative analysis between MIC/MBC and MBIC/MBEC values demonstrated that *K. pneumoniae* isolates required higher concentrations of Bio-AgNPs to inhibit or eradicate biofilms than to suppress planktonic growth. For example, the ATCC[®] 700603TM strain showed MIC and MBC values of 0.49 $\mu\text{g/mL}$, while its MBIC and MBEC values were substantially higher at 31.25 $\mu\text{g/mL}$ and 125 $\mu\text{g/mL}$, respectively. A similar pattern was observed among clinical isolates: while most presented MIC values of 0.97 $\mu\text{g/mL}$ and MBC values ranging from 7.81 to 15.62 $\mu\text{g/mL}$, their MBICs were consistently higher (15.62 to 31.25 $\mu\text{g/mL}$), and MBECs, when detectable, reached up to 125 $\mu\text{g/mL}$.

Bio-AgNP Activity in the Disruption and Inhibition of *K. pneumoniae* Biofilms

The biofilm disruption assay was performed to assess the activity of Bio-AgNPs against established biofilms (Fig. 1). Based on MIC and MBC values, treatments were applied at MIC, 2×MIC, and 4×MIC concentrations. Significant biofilm disruption was observed in isolates Kb17, Kb19, Kb22, Kb23, Kb24, Kb27 and Kb28. Notably, the treatment at MIC exhibited a strong disruption effect against isolates Kb19, Kb23, Kb24, Kb27, and Kb28. Treatment with 2×MIC significantly reduced biofilm biomass in isolates Kb17, Kb19, Kb22, Kb23, Kb24, and Kb28, while 4×MIC showed activity against Kb17, Kb19, Kb22, Kb23, Kb24, and Kb28.

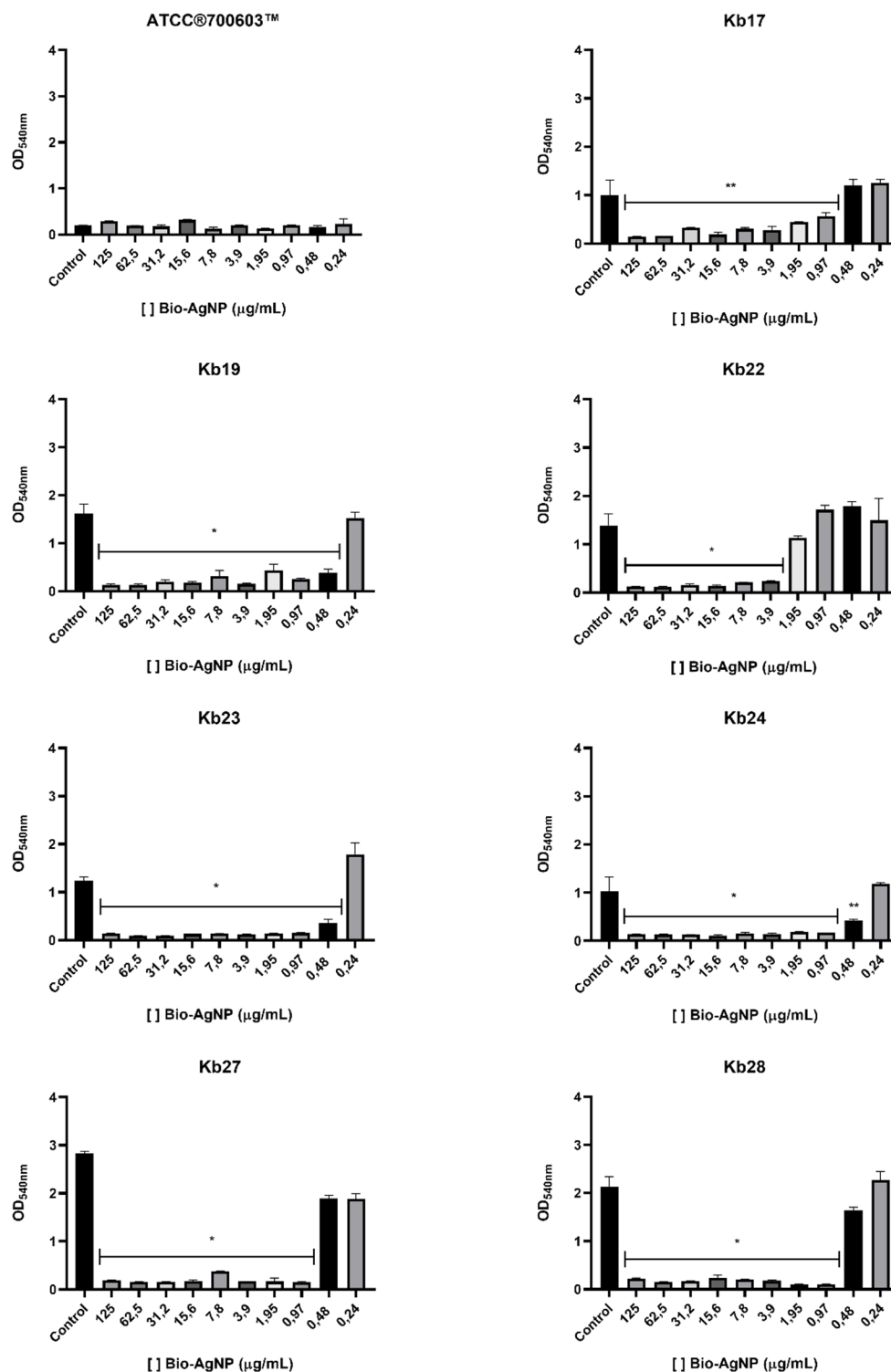
Fig. 1 Graphical representation of the biofilm disruption activity of the Bio-AgNP in *K. pneumoniae* isolates. Results presented as mean $\pm$ SD for each treatment. Significance was determined by two-way ANOVA followed by Dunnett's multiple comparison test. * $p < 0.01$, ** $p < 0.0001$



The biofilm inhibition assay was performed using concentrations based on the MIC and MBC results. Biofilm formation was evaluated in the presence of Bio-AgNPs at concentrations ranging from 0.24 $\mu\text{g/mL}$ to 125 $\mu\text{g/mL}$, as shown in Fig. 2. Bio-AgNP significantly inhibited biofilm formation in all tested clinical isolates when compared to untreated controls ($p < 0.0001$ and $p < 0.001$). At 0.48 $\mu\text{g/mL}$

mL, a statistically significant reduction in biofilm formation was observed in 37.5% (3/8) of the isolates (Kb19, Kb23, and Kb24). Increasing the concentration to 0.97 $\mu\text{g/mL}$ enhanced the inhibitory effect, with 75% (6/8) of the isolates (Kb17, Kb19, Kb23, Kb24, Kb27, and Kb28) exhibiting significant antibiofilm activity. This concentration demonstrated the most consistent inhibition across most isolates.

Fig. 2 Graphical representation of the inhibitory capacity of silver nanoparticle on biofilm formation in *K. pneumoniae* isolates. Results are presented as mean $\pm$ SD for each treatment. Significance was determined by two-way ANOVA followed by Dunnett's multiple comparison test and $*p < 0.0001$ $**p < 0.001$



Time-kill Assay

The time-kill assay results are presented in Fig. 3. For the reference strain *K. pneumoniae* ATCC® 700603™, exposure to Bio-AgNP at the MIC concentration exhibited bacteriostatic activity between 30 min and 6 h, as indicated by a reduction in colony counts compared to the untreated control. After 24 h, bactericidal activity was observed. When treated with 2×MIC and 4×MIC, bacteriostatic activity was observed from 30 min to 6 h and 30 min to 2 h, respectively, followed by bactericidal effects at 8 h and 4 h of exposure. For the clinical isolate Kb17, treatment with Bio-AgNP at MIC resulted in bacteriostatic activity from 15 min to 4 h, with a progressive decline in viable counts until complete bacterial elimination was observed at 24 h. Treatment at 2×MIC maintained bacteriostatic activity for up to 8 h, followed by bactericidal activity at 12 h. At 4×MIC, bactericidal activity was recorded after 6 h of exposure.

Fig. 3 Time-kill curves of *K. pneumoniae* clinical isolate and reference strain treated with biogenic silver nanoparticles (Bio-AgNPs). Kb17: *K. pneumoniae* clinical isolate; ATCC® 700603™: *K. pneumoniae* reference strain. For Kb17, MIC=0.975 µg/mL; 2×MIC=1.95 µg/mL; 4×MIC=3.9 µg/mL. For ATCC® 700603™, MIC=0.4875 µg/mL; 2×MIC=0.975 µg/mL; 4×MIC=1.95 µg/mL. * $p < 0.0001$ compared to untreated control

Flow Cytometry Analysis

The effects of Bio-AgNP on the cell membrane of *K. pneumoniae* were evaluated using flow cytometry, focusing on membrane fluidity, membrane selectivity, lipid peroxidation, and reactive oxygen species (ROS) production. A significant increase in membrane fluidity was observed at MIC and 2×MIC concentrations, suggesting destabilization of the lipid bilayer. Conversely, at 4×MIC, membrane fluidity was markedly reduced, indicating extensive structural damage and potential loss of membrane function (Fig. 4a). Membrane selectivity remained relatively stable across all treated groups, while greater variability was detected in the untreated control (Fig. 4b), suggesting that Bio-AgNPs primarily alter membrane dynamics rather than permeability to ions. Lipid peroxidation levels were elevated in the MIC-treated group, although the increase was not statistically significant, potentially indicating early oxidative damage to membrane lipids (Fig. 4c). Similarly, ROS production

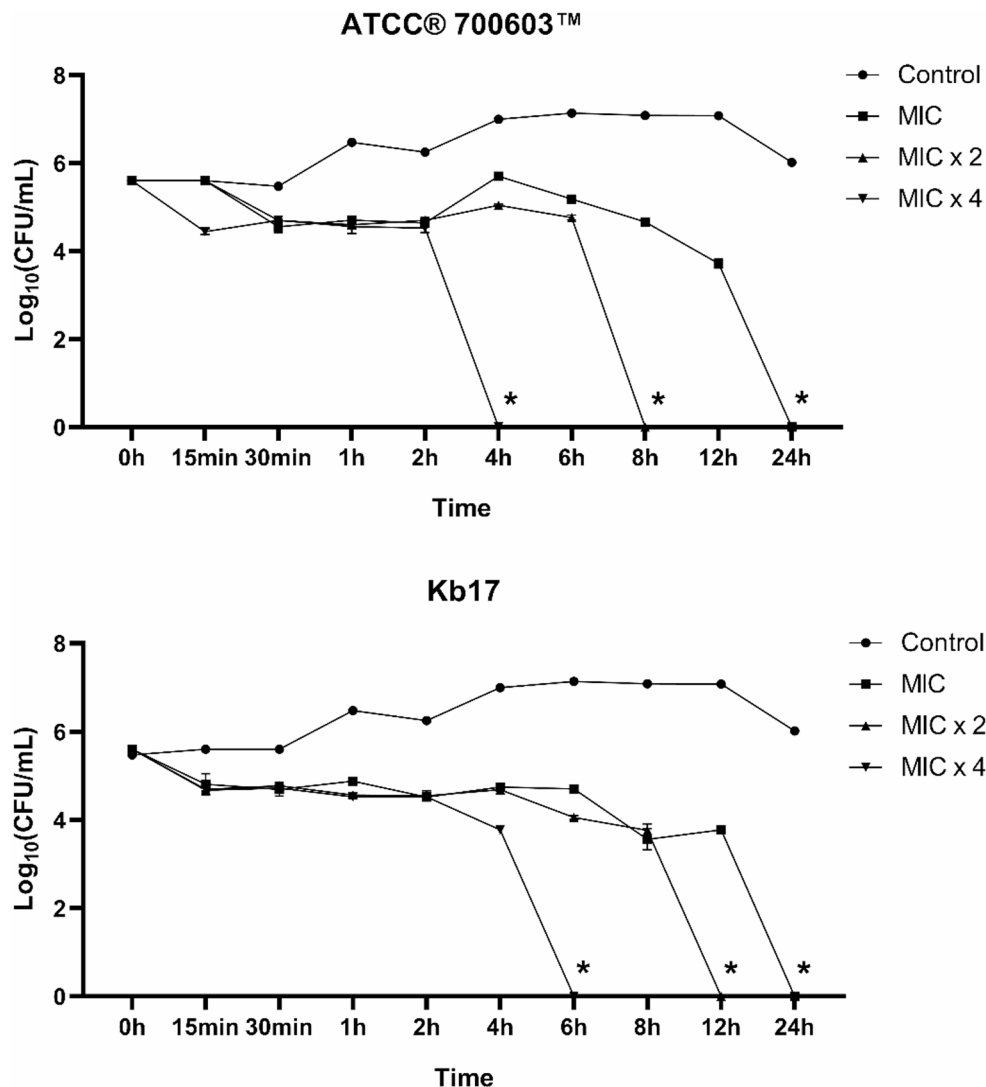
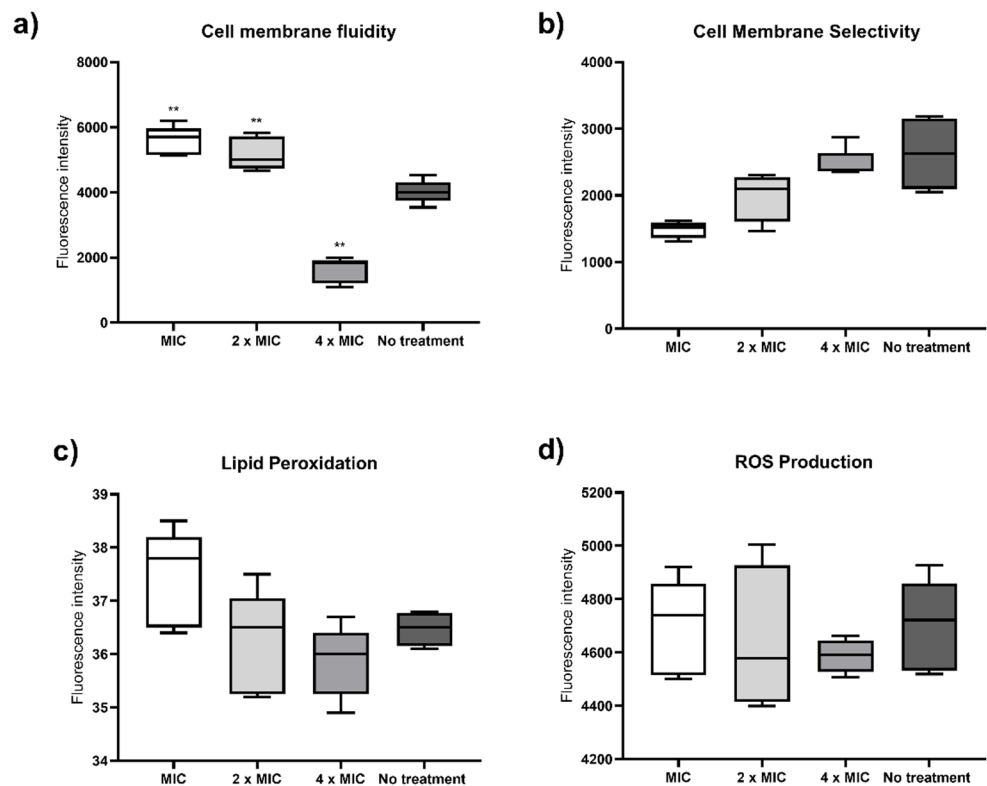


Fig. 4 Flow cytometry analysis of cytotoxic effects on *K. pneumoniae* ATCC® 700603™ cells after treatment with Bio-AgNP. (a) Cell membrane fluidity; (b) Cell membrane selectivity; (c) Lipid peroxidation; (d) ROS production. Data are presented as the mean $\pm$ standard error of the mean (SEM) for each treatment. Significance was determined by Brown-Forsythe and Welch analysis of variance (ANOVA). The experiments were carried out with at least three replications. * $p < 0.05$ and ** $p < 0.0001$, in comparison to non-treated strain



increased following treatment with MIC and 2×MIC concentrations, but these changes were not statistically significant (Fig. 4d).

Exposure of the clinical isolated *K. pneumoniae* (Kb17) to Bio-AgNP revealed notable alterations in membrane properties. Membrane fluidity increased significantly at MIC and 2×MIC concentrations, suggesting initial destabilization of the lipid bilayer, followed by a marked reduction at 4×MIC, likely due to extensive structural damage (Fig. 5a). Membrane selectivity remained stable across treated groups, whereas the untreated control exhibited high variability, reflecting natural heterogeneity among bacterial cells (Fig. 5b). Lipid peroxidation was significantly elevated at MIC and 2×MIC, indicating oxidative damage to membrane lipids; at 4×MIC, greater dispersion in the data suggests more severe damage affecting subpopulations of cells (Fig. 5c). ROS production also increased at MIC and 2×MIC concentrations; however, at 4×MIC, ROS levels returned to baseline, comparable to the control group, possibly indicating that cells were too severely damaged to sustain oxidative responses (Fig. 5d). These findings indicate that Bio-AgNP compromises bacterial membrane integrity by promoting fluidity changes and oxidative stress at lower concentrations, while higher concentrations lead to irreversible membrane damage and potential cell death.

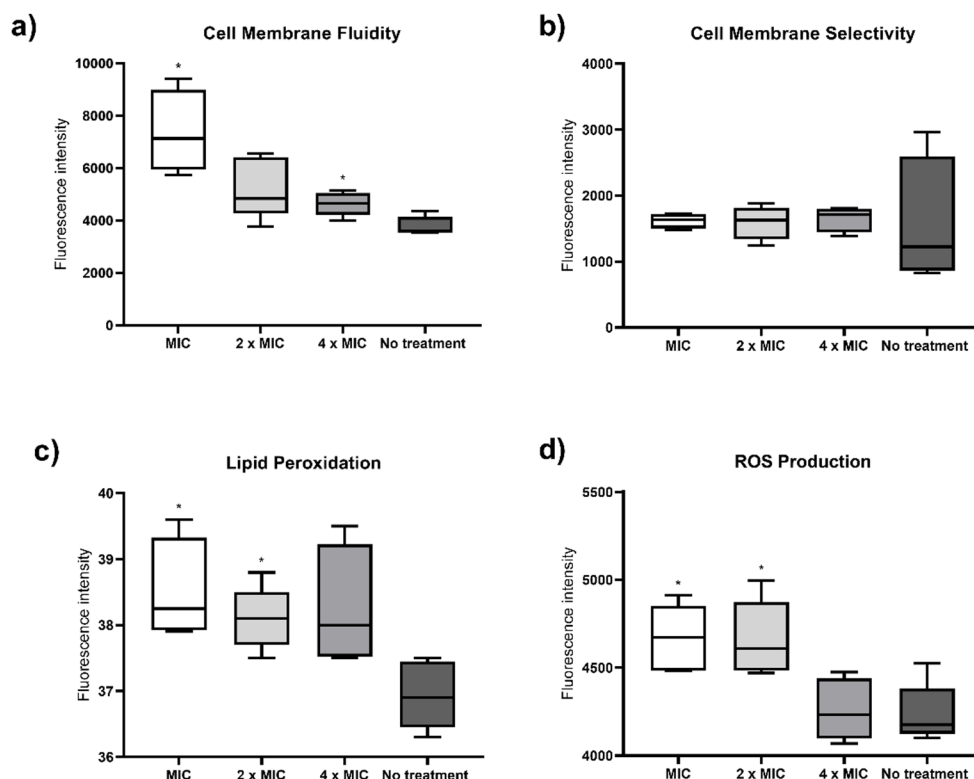
Scanning Electron Microscopy (SEM)

Figure 6 presents representative SEM images of untreated (Fig. 6a) and Bio-AgNP-treated *K. pneumoniae* cells (Fig. 6b). Untreated cells displayed smooth, intact membranes with preserved cellular morphology. In contrast, cells exposed to Bio-AgNPs showed clear signs of structural damage, including membrane disorganization, surface roughness, and deformation, indicative of nanoparticle-induced cellular destabilization and loss of membrane integrity. Figure 6c and d present SEM images illustrating the effect of Bio-AgNP on biofilm formation. In the untreated control (Fig. 6c), a dense biofilm structure is observed, characterized by an abundance of extracellular matrix that obscures individual bacterial cells and reflects typical mature biofilm organization. In contrast, Fig. 6d, corresponding to the Bio-AgNP-treated sample, reveals a substantial reduction in biofilm density. The bacterial cells appear more distinct and isolated, with preserved morphology and reduced extracellular matrix, indicating a significant inhibitory effect of Bio-AgNP on biofilm development.

Discussion

Given the growing resistance of bacteria to conventional antibiotics, biogenic silver nanoparticles (Bio-AgNPs) have emerged as promising alternatives due to their enhanced

Fig. 5 Flow cytometry analysis of cytotoxic effects on *K. pneumoniae* clinical isolate (Kb17) cells after treatment with Bio-AgNP. (a) Cell membrane fluidity; (b) Cell membrane selectivity; (c) Lipid peroxidation; (d) ROS production. Data are presented as the mean $\pm$ standard error of the mean (SEM) for each treatment. Significance was determined by Brown-Forsythe and Welch analysis of variance (ANOVA). The experiments were carried out with at least three replications. * $p < 0.05$, in comparison to non-treated strain



biocompatibility, reduced toxicity, and environmentally friendly synthesis processes [24]. Recent studies have shown that silver nanoparticles synthesized from plant extracts exhibit potent antimicrobial activity against multidrug-resistant pathogens, including *K. pneumoniae* [25], *Staphylococcus aureus* [18], and *Acinetobacter baumannii* [26]. Additionally, these nanoparticles have demonstrated wound-healing properties in murine models, further supporting their therapeutic potential [27]. The green synthesis of silver nanoparticles not only reduces the use of hazardous chemicals but also incorporates bioactive compounds from plants, which may synergistically enhance antimicrobial activity [24, 28].

In this context, Bio-AgNPs represent a sustainable and effective strategy for the development of novel antibacterial agents. To evaluate their efficacy, we determined the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against clinical isolates and a reference strain. Our results showed that 62.5% of the isolates had a MIC of 0.975 $\mu\text{g/mL}$, while 50% exhibited an MBC of 15.625 $\mu\text{g/mL}$. The reference strain *K. pneumoniae* ATCC® 700603™ displayed a MIC and MBC of 0.4875 $\mu\text{g/mL}$. Considering that bacterial biofilms contribute substantially to resistance mechanisms; it is essential to assess not only the activity of Bio-AgNPs against planktonic cells but also their impact on biofilm formation and eradication.

The ability to form biofilms is a major bacterial resistance mechanism that enhances protection, enables intercellular

communication, and significantly reduces the efficacy of antibiotics [29, 30]. Biofilms play a crucial role in bacterial survival and persistence, consisting either of multiple bacterial species or a single species adhered to a surface [31]. Within this structure, bacterial cells are embedded in an extracellular matrix (ECM) composed of various biopolymers secreted by the bacteria, including exopolysaccharides (EPS), extracellular DNA (eDNA), proteins, and amyloidogenic components [32].

All clinical isolates used in this study are classified as strong biofilm producers [20], which presents a significant challenge in finding effective alternatives for controlling biofilm-related infections. Thus, our study evaluates the potential of Bio-AgNP in overcoming these limitations. The anti-biofilm activity of Bio-AgNP demonstrates both disruption activity (Fig. 1) and inhibition of biofilm formation (Fig. 2). Biofilm disruption assay revealed that concentrations of 2 $\times$ MIC and 4 $\times$ MIC were the most effective in disrupting these structures. Under these conditions, we observed that the Bio-AgNP concentration required for biofilm disruption was 16 to 64 times higher than that used against planktonic cells. This increase is attributed to the extracellular matrix of the biofilm, which provides protection to the bacterial community, reducing the effectiveness of the antimicrobial agent [33]. These findings are consistent with previous studies that have also reported a significant increase in the antimicrobial concentration required for biofilm eradication [18, 26, 34].

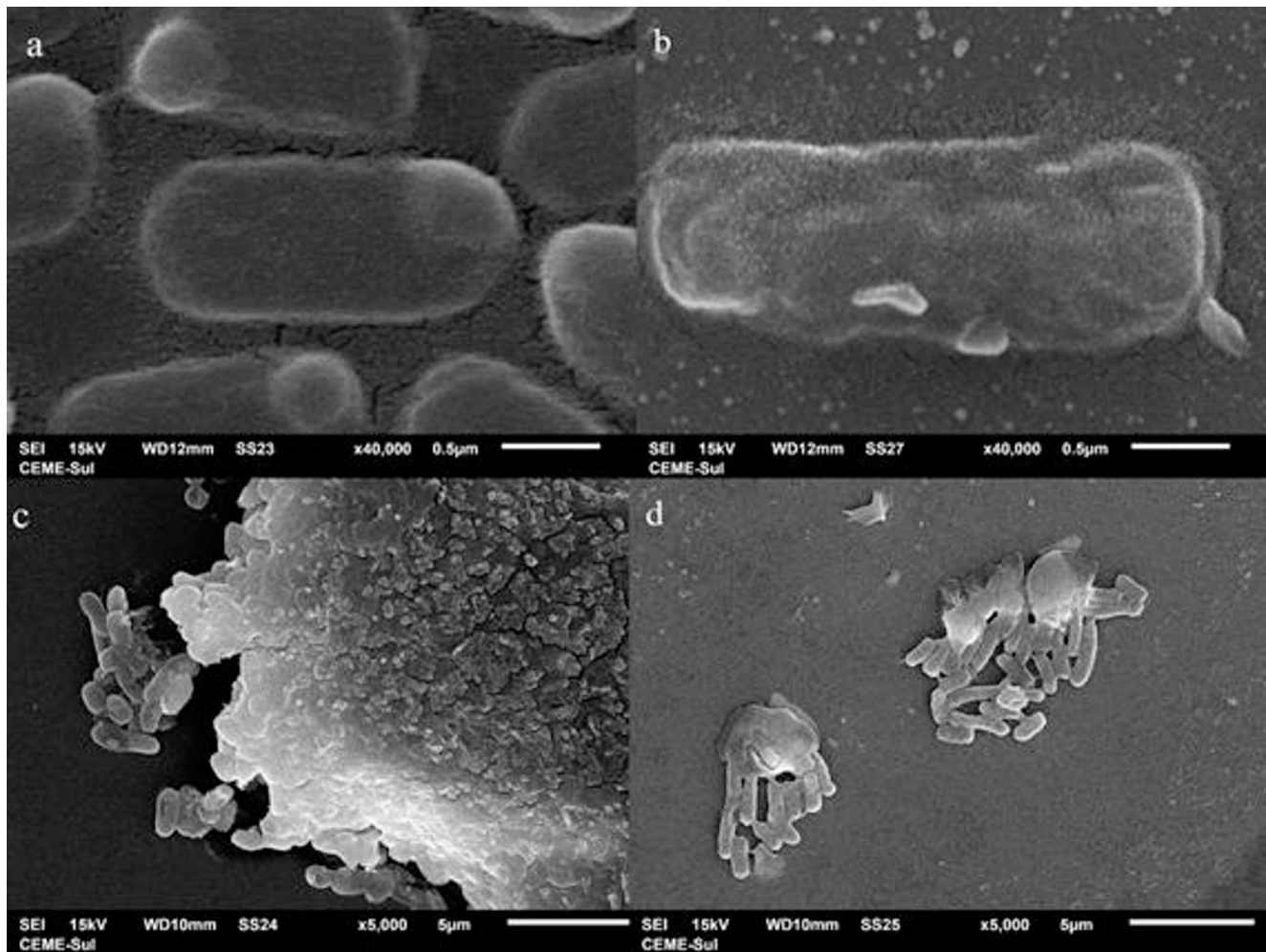


Fig. 6 Scanning electron microscopy (SEM) images of *K. pneumoniae* cells and biofilms following treatment with Bio-AgNP. (a) Untreated *K. pneumoniae* ATCC® 700603™ cells exhibiting intact and smooth membranes (40,000× magnification). (b) *K. pneumoniae* ATCC® 700603™ cell after treatment with Bio-AgNP, showing evident membrane damage and surface disorganization (40,000× magnification).

In terms of inhibition, Bio-AgNP demonstrated superior performance. The tests conducted in our study showed activity at all tested concentrations, with 0.97 µg/mL standing out, as 75% of the samples exhibited a significant effect. The ability of Bio-AgNP to inhibit biofilm formation may be attributed to alterations in structural and physicochemical properties, such as surface adhesion, hydrophobicity, cell motility, and phagocytosis, as well as the generation of reactive oxygen species [35, 36]. Its high activity is linked to its small particle size and increased surface area, which enhance interaction with bacterial cells [36]. This direct contact improves access to the intracellular environment, inhibiting biofilm development and impacting the polymeric matrix, which facilitates nanoparticle fusion. Additionally, Bio-AgNP can trigger protein denaturation and lipid bilayer disruption, allowing deeper penetration

(c) Untreated biofilm-forming *K. pneumoniae* clinical isolate Kb17, with dense cellular arrangement and intact morphology (5,000× magnification). (d) Biofilm-forming *K. pneumoniae* Kb17 after Bio-AgNP treatment, displaying disrupted biofilm structure and damaged bacterial cells (5,000× magnification)

into the biofilm and increasing antimicrobial efficacy [36, 37]. These findings are further supported by transmission electron microscopy analysis, which reveals a clear reduction in biofilm formation and structural integrity in treated samples (Fig. 6d) compared to untreated controls (Fig. 6c). The observed disruption in biofilm architecture aligns with the proposed mechanisms of Bio-AgNP action, reinforcing its potential as an antibiofilm agent.

The destabilization of the lipid bilayer and increased nanoparticle penetration not only enhance antimicrobial efficacy but also influence the speed at which bacterial cells are eliminated [38]. To assess this, we evaluated the bacterial killing time in response to Bio-AgNP exposure, providing insights into its rapid bacterial activity. The time-kill assay demonstrated a concentration- and time-dependent antimicrobial effect of Bio-AgNP, with

higher concentrations accelerating bacterial elimination. The observed bacteriostatic phase at MIC and its progressive reduction until complete eradication suggests a gradual accumulation of cellular damage. At higher concentrations, 2×MIC and 4×MIC, the faster bactericidal effect likely results from increased oxidative stress, membrane destabilization, and enhanced nanoparticle-cell interactions [32, 36, 39], leading to rapid cellular disruption. Similar findings have been reported in previous studies, where silver nanoparticles exhibited a dose-dependent bactericidal effect, with prolonged bacteriostatic activity at MIC and accelerated bacterial elimination at higher concentrations [15, 18, 26]. Differences between the standard strain and the clinical isolates may reflect variations in resistance mechanisms, such as differences in membrane composition, which can influence nanoparticle interaction and uptake [40]. To gain deeper insights into these effects, we assessed key cellular parameters using flow cytometry, including oxidative stress induction, membrane fluidity, selective permeability, and lipid peroxidation, allowing a broader assessment of Bio-AgNP effects at the cellular level.

The observed killing time correlates with structural damage and oxidative stress detected by flow cytometry. The increase in membrane fluidity at MIC and 2×MIC (Fig. 4a) suggests that Bio-AgNPs interact directly with the lipid bilayer, causing structural destabilization [36]. However, at 4×MIC, the marked reduction in fluidity indicates extensive membrane damage (Fig. 4a), which may lead to a loss of function and cellular collapse [40]. Interestingly, despite these changes in membrane dynamics, ionic selectivity remained relatively stable, suggesting that Bio-AgNP primarily disrupts membrane organization rather than directly compromising ion transport mechanisms (Fig. 4b). This is consistent with previous studies indicating that AgNPs can alter lipid packing and membrane curvature without necessarily inducing uncontrolled permeabilization [18, 26, 35, 38]. Furthermore, the slight increase in lipid peroxidation at MIC and 2×MIC (Fig. 5d) may indicate early oxidative damage, which could contribute to membrane destabilization [36, 39, 40]. However, the absence of a significant increase in ROS in the ATCC® 700603™ strain suggests that oxidative stress may not be the primary mechanism of Bio-AgNP action. In contrast, the significant increase in ROS at MIC and 2×MIC in the clinical isolate Kb17 (Fig. 5d) suggests that, under these conditions, oxidative stress may contribute to antimicrobial activity, possibly acting synergistically with membrane destabilization. The direct interaction with membrane lipids and the resulting biophysical alterations could play a more central role in bacterial cell death. These findings highlight the importance of evaluating structural membrane damage as a key factor in Bio-AgNP action, which can be further explored using transmission

electron microscopy for direct visualization of these effects (Fig. 6a and b).

Taken together, the findings of this study reinforce the potential of Bio-AgNPs as effective antimicrobial agents, particularly due to their ability to destabilize bacterial membranes and compromise cell viability. These insights open new avenues for the development of Bio-AgNP-based antimicrobial formulations, either as standalone agents or in combination with conventional disinfectants and antibiotics, to enhance efficacy and mitigate resistance development. Future studies should focus on optimizing Bio-AgNP formulations, evaluating their performance in real-world applications, and investigating their synergy with existing antimicrobial strategies. Expanding research on their biocompatibility and potential applications in healthcare settings will further establish their role as promising alternatives for controlling multidrug-resistant pathogens.

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Data Availability The datasets generated and/or analyzed during the current study are available from the corresponding author upon request.

Declarations

Competing Interests The authors declare no competing interests.

Ethical approval Not applicable.

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