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Bio-synthesized Silver Oxide Nanoparticles are Effective Anti-Uro-pathogenic *Pseudomonas aeruginosa* and *Proteus mirabilis* Isolated from Cattle

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Abstract:

Background:

Urinary tract infections in cattle represent a significant veterinary concern, impacting animal health, welfare, and economic productivity. The frequently diverse bacterial pathogens isolated from bovine UTIs, posing particular challenges due to their inherent virulence factors and increasing antimicrobial resistance profiles.

Aims:

In this study, an alternative controlling protocol was used to counteract the antimicrobial resistance of the bacterial isolates *Proteus mirabilis* (PM14) and *Pseudomonas aeruginosa* (PA59) using biosynthesize silver oxide nanoparticles (AgONPs) by cell-free supernatants of a mixed species of: *Bacillus subtilis*, *B. amyloliquefaciens*, *B. atrophaeus*).

R**esults**:

The findings of VITEK system showed that PM14 was sensitive to ceftazidime/avibactam, amikacin, ciprofloxacin, meropenem, and gentamycin, while PA59 was resistant to most of the tested antibiotics. Regarding to agar well diffusion, the growth inhibition zones of bacterial isolates were 19 and 21mm when 100 µg/ml of AgONPs was applied against PM14 and PA59, respectively. Moreover, bacterial growth of both PM14 and PA59 was inhibited at 15.6 µg/mL of AgONPs, as an MIC with a bacterial survival of (0.3 - 0.6 %), compared to the control group, which was growing without treatment.

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Conclusions:

The promising efficacy of AgONPs in inhibiting the proliferation of uropathogenic species PM14 and PA59, isolated from bovine subjects, suggests their potential as a viable and potent antimicrobial agent for veterinary therapeutic applications.

Keyword:

Silver oxide nanoparticles; Biosynthesis; *Bacillus* mix; Antibacterial activity; Urinary tract infection; *P. aeruginosa*; *P. mirabilis*

Introduction:

Nanotechnology is a promising field for the development of new types of small-sized materials for various applications, including biomedicine (Zain *et al.,* 2022). Physical and chemical methods are more commonly used to synthesize nanoparticles, however, using toxic chemicals in these approaches severely limits their biomedical applications in the clinical settings. Nanoparticles range in size from 1 to 100 nanometers and compose of organic matter, metal, metal oxides, or carbon (Hasan, 2015). Nanoparticles have unique physical, chemical, and biological properties compared to larger-sized particles. This phenomenon can be attributed to increased surface area, chemical reactivity, mechanical strength, and other factors (Ealia and Saravanakumar,2017). Recently, nanoparticles have implicated in a variety of biomedical applications, including cancer therapy, cell labeling, drug delivery, antimicrobial agents, diagnostics, cosmetics, biomarkers, cell biology, ointments, antioxidants, and wound healing (Zulfiqar *et al.,*2024) The emergence of drug-resistant microorganisms is a huge challenge to control infections. Developing effective bactericidal agents to counteract drug-resistant bacteria is an urgent public demand (Allahverdiyev *et al.,*2011). Therefore, metal oxide nanoparticles could be taken in consideration as important potential and alternative antimicrobial agents (Shume *et al.,*2020). Microbial-mediated nanoparticle synthesis is a green chemistry approach that combines nanotechnology and microbial biotechnology. Bacteria, actinomycetes, fungi, yeasts, and viruses have been shown to synthesize gold, silver, gold-silver alloy, selenium, tellurium, platinum, palladium, silica, titania, zirconia, quantum dots, magnetite, and uraninite nanoparticles (Narayanan and Sakthivel,2010). Urinary tract infection (UTI) is a relatively minor condition that can lead to serious health problems for cows, affecting productivity and overall health. These infections are caused by the presence of various pathogenic bacteria in the urinary tract. Among these bacteria, *Pseudomonas aeruginosa* and *Proteus mirabilis* are important as etiological agents of UTIs in cows. According to several studies, *P. aeruginosa* is one of the most antibiotic-resistant bacteria and capable of forming biofilms, increasing its ability to survive in the urinary tract environment and causing chronic infections (Sharma *et al*.,2023). This bacterium is prevalent in agricultural environments and farms, and it is easily transmitted to livestock through

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contaminated water or surfaces. Its pathogenicity is attributed to a diverse array of virulence factors, including exotoxins, proteases, and flagella (Ali and Alsayeqh,2022). The Gram-negative bacterium *P. mirabilis* is well-known for producing the urease enzyme, which raises pH and causes the formation of urinary calculi by hydrolyzing urea in urine into ammonia (Al-Ezzy *et al*.,2023). This process exacerbates inflammation. Additionally, it can migrate to the upper urinary tract, where in extreme cases, it can cause kidney and bladder inflammation. Both *P. aeruginosa* and *P. mirabilis* are serious risky pathogens due to their resistance to the conventional therapies and their high prevalent in cattle with urinary tract infections (Pereira *et al*.,2025; Al-Ezzy *et al.*,2021). The current study aims to investigate the efficacy of AgONPs as antibacterial agents against *P. aeruginosa* and *P. mirabilis* species isolated from cattle suffering from UTIs. Evaluation of AgONPs antimicrobial activity *in vitro*, and exploring their mechanisms of action, could pave the way for establishing the scientific basis for recommending AgONPs as a safe, effective, and alternative therapeutic antimicrobial in veterinary applications. The potential benefits of AgONPs extend beyond their direct antimicrobial effects; their ability to penetrate biofilms and their relatively low toxicity to mammalian cells at effective concentrations make them attractive candidates for veterinary therapeutics (Iqbal *et al.,*2019; Frippiat *et al*.,2025). Furthermore, the development of novel antimicrobial agents is crucial for reducing reliance on conventional antibiotics, thereby mitigating the development and spread of antimicrobial resistance in livestock populations and, by extension, in the broader ecosystem (Woolhouse *et al*.,2015). This research contributes to evolving our understanding on nanotechnology in veterinary medicine and offers a viable option for addressing the critical challenge of bacterial infections in cattle.

Materials and Methods:

Sources of Probiotic Bacilli

The commercial products of probiotic bacilli (Nutrition Formulators, FLUSA Exclusively for Vita Range LLC. Miami, FL, USA) were purchased and maintained in the microbiology laboratory of Veterinary Medicine College/University of Diyala, Iraq. The mixed bacilli product was first resuspended in 10 ml of skimmed milk, and incubated for 48 hours. After incubation, 10 ml of bacterial growth was inoculated into MRS agar (Oxoid, England) and incubated aerobically for 24 hours at 37°C (Algburi *et al*.,2016). The three species of probiotics were initially identified using morphological features and some biochemical tests, including; IMViC,tests, sugar fermentation, H_2S and CO_2 production. Then, the identification was confirmed using VITEK® 2 Compact system (bioMérieux, Lyon, France) (Bergey, 1994 and Kareem *et al*., 2022).

Isolation, Identification and Antibiotic Susceptibility of Uropathogenic of Bacterial Species

Uropathogenic *P. aeruginosa* (PA59) and *P. mirabilis* (PM14) were isolated from urine samples of cattle. These samples were collected by a specialist veterinarian using a sterile tube

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during the urination flow The initial PA59 and PM14 identification involved inoculating samples on enrichment and selective culture media; blood, and MacConkey agars (HiMedia, Maharashtra, India) (Filius et al.,2003). The VITEK® 2 compact system was then used to identify bacterial samples based on their biochemical reactions. The identification of bacillimixed species was also identified using VITEK® 2 Compact system. In addition to bacterial identification, the VITEK system was used to assess bacterial susceptibility to the tested antibiotics.

Preparation of Cell-Free Supernatant from *Bacillus* Mix Species

Cell-free supernatants (CFS) of the selected probiotic bacilli were prepared following (Algburi *et al*., 2020), with minor modifications. Briefly, the bacilli mix was inoculated into MRS broth and incubated for 24-36 hours under aerobic conditions at 37°C. Following incubation, the bacterial cells were precipitated by centrifugation (EBA 200, Andreas Hettich, Tuttlingen, Germany) at (250 rpm at 4 °C for 30 minutes). The CFS was separated and filtered through a 0.45 µm syringe filter (Millipore Sigma, Burlington, MA), then kept at 4°C for later use (Arrioja-Bretón *et al*., 2020).

Biosynthesis of AgONPs Using cell-free Supernatant of Probiotic Bacilli

The anhydrous silver nitrate ($AgNO_3$) with molecular weight169.88 g/mol and purity > 99%, was purchased from HiMedia., India. According to Das et al. (2017), 0.017 g of $AgNO_3$ was dissolved in 100 ml of distilled water and mixed with 20 ml of CFS of the tested bacilli species. The mixture was exposed directly to the sunlight for 10 minutes, when the temperature was 40-43°C. After sunlight exposure, the color of the reaction mixture quickly changed from yellow to reddish-brown, indicating AgONPs production (Belaiche *et al*., 2021). The control groups that were used in this assay include MRS broth, $AgNO_3$ solution, and CFS of bacilli, each one was used individually. After 15 minutes of reaction, the nanoparticles were accumulated in the bottom of the test bottle. The solution mixture alongside the control groups was centrifuged, separately at (250 rpmat 4 °C for 20 minutes) to participate AgONPs. The nanoparticles were washed three times with distilled water to remove any remaining impurities that might interfere with their NPs properties. The nano products were dried using oven at 40°C one day and left stored at room temperature for two weeks to dry completely. The products were ground into a fine powder using physical crush for further analysis (Al-Saadi *et al*.,2015).

Characterization of AgONPs

For characterization of AgONPs, ultraviolet –visible spectrophotometer (UV-VIS spectrophotometer), transmission electron microscopy (TEM) and X-ray diffraction (XRD) were used to study the features of the AgONPs (Zaheer, 2012), the nanoproduct was analyzed using a spectrophotometer to identify $AgNO_3$ reduction processes. Then, 2 mL of the reaction mixture was measured for absorbance spectra (190-1100 nm) using a UV-1800 Spectrophotometer (Shimadzu

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Corporation, Kyoto, Japan). The shape and the size distribution histogram of AgONPs were determined by TEM using ZEISS LEO 912 AB Transmission Electron Microscope (Zeiss, Oberkochen, Germany), by accelerating the voltage (100 kV). X-ray diffraction (XRD) (X-ray diffraction, Shimadzu-7000 powder Corporation, Japan) was used to characterize the crystalline structure and phase composition of the produced AgONPs. The nanoparticles product was subjected to XRD-7000 to study the phase purity, crystal structure, and approximate size of the nanocrystallites using the powder XRD technique-ray diffraction patterns and monochromatic high-intensity CuKα radiation (Lambda λ=1.54060 A0) and a diffraction mode instrument (Dakhil, 2017).

Antibacterial Activity of AgONPs

The antibacterial activity of AgONPs was evaluated using agar well diffusion, and micro-dilution methods to determine minimum inhibitory concentration (MIC) of AgONPs against isolated uropathogenic PA59 and PM14 (CLSI, 2021), with some modifications. Regarding the agar well diffusion method, 100 µL of the overnight cultures of the selected *P. aeruginosa* and *P. mirabilis* was adjusted to cell density of 1.5×10^6 CFU/mL (using Macferland NO 0.5) and spread on Mueller Hinton agar plates (Chavez-Esquivel *et al.*, 2021). Wells were made in the agar using a sterile gel borer. Then, 100 µL of 100 µg/mL of AgONPs were added into the wells, and the agar plates were incubated under aerobic conditions for 24 hours, at 37 °C. After incubation, the zones of bacterial growth inhibition were measured using a vernier digital caliper. Negative and positive controls were used in this assay. Broth microdilution method was then used to determine the MIC of AgONPs bacteria were grown aerobically in Brain-Heart Infusion broth (HiMedia, India) at 37°C overnight, then diluted into Mueller-Hinton broth to achieve $5x10^6$ CFU/mL. Then, a 100 µL of the tested PA59 and PM14 were added separately into wells of 96 well-microplates containing varying concentrations of AgONPs (0.9, 1.9, 3.9, 7.8, 15.6, ,31.3, 62.5, 125, 250, 500 µg/mL). The microplate was incubated at 37°C for 24 hours. Negative and positive controls were used in this assay (broth and bacterial cells, respectively) and negative (broth only). This experiment was performed in triplicates. The MIC was determined according to CLSI (Wayne, 2011), as the lowest concentration of antimicrobial agents that inhibit bacterial growth by the naked eye.

Ethics approval:

The Scientific Ethical Committee of the College of Veterinary Medicine, University of Diyala, Iraq, approved this study (Approval no: Vet Medicine (206); June 2025, S and A).

Results:

Characterization of AgONPs

The Initial characterization of AgONPs products were carried out using a UV-vis spectrophotometer. UV-vis spectra (Figure 1) showed a strong peak at 420 nm, which confirms the formation of AgONPs. The phase purity and crystalline nature of AgONPs were examined by XRD

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(Figure 2 and Table 1). The XRD showed more intense peaks at 2θ values at 32.5734º- 40.6504º- 44.0435º- 46.564º- 55.1855º- 67.2519º- 74.9025º- 79.0605º (200) (004) (114) (132) (224) (402) (242) (206) with average crystalline size 29.453. The presence of strong peaks in the diffraction pattern confirms that the synthesized materials are highly crystalline. The TEM picture of the AgONPs is shown in Figure 3 in which the surface morphology of AgONPs indicated a smooth surface with a spherical shape and a particle size of 16 nm. However, some large particles were noticed, possibly due to some aggregation during sample preparation.

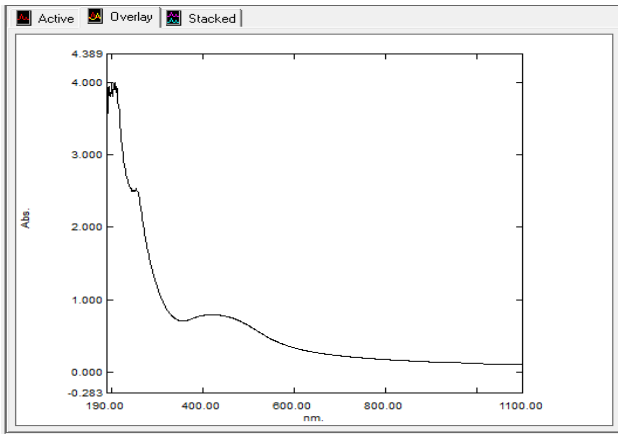
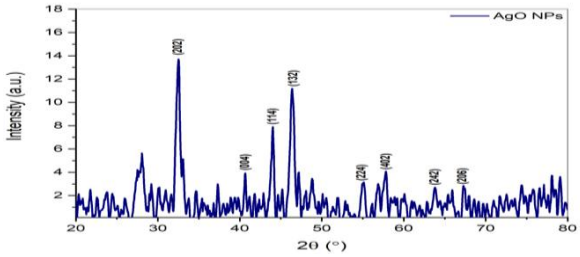


Figure 1: UV-vis spectra of AgONPs biosynthesized using bacilli mix species. A pronounced broad peak is produced at 420 nm.



No.	2 theta (degree)	hkl	FWHM (deg)	2 theta (Rad.)	FWHM (Rad)	D (nm)	Matched by
1	32.5734	202	0.2716	0.22632	0.005	30.005	01-084-1108
2	40.6504	004	0.1811	0.22632	0.003	45.008	
3	44.0435	114	0.2716	0.181056	0.005	29.726	
4	46.564	132	0.4526	0.22632	0.008	18.003	
5	55.1855	224	0.5432	0.181056	0.009	14.863	
6	67.2519	402	0.3621	0.271584	0.006	22.765	
7	74.9025	242	0.1811	0.271584	0.003	45.529	
8	79.0605	206	0.2716	0.181056	0.005	29.726	
					Average	29.453	

Figure 2 and Table 1: The XRD findings of the AgONPs

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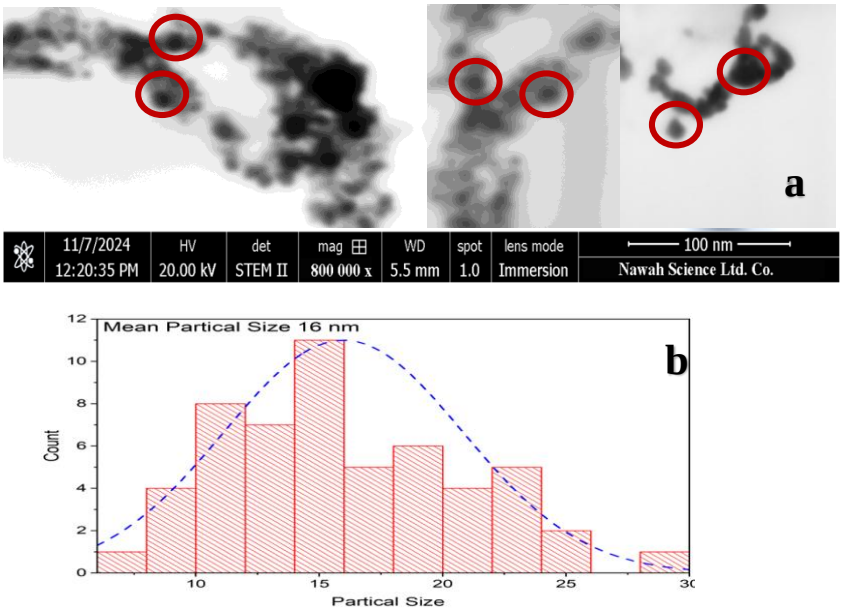

Figure 3: (a) Morphological and elemental analysis of AgONPs. (b) TEM analysis indicates a spherical shape and size distribution based on the TEM

Antibacterial Activity of AgONPs

The current study determined the antibacterial potency of AgONPs against uropathogenic PA59 and PM14 isolated from cattle, respectively. The antibiotic susceptibility of PM14 and PA59 were tested using the VITEK® 2 compact system. Following antibiotic sensitivity testing, the selected PA59 isolates were resistant to 8 antibiotics, specifically: ampicillin/sulbactam, Ceftazidime Ceftazidime/Avibactam, Ceftolozane/Tazobactam Cefepime, Imipenem, Meropenem, Ciprofloxacin. In regards to *P. mirabilis* PM14 isolated, the bacterial isolate was resistant to 7 antibiotics, specifically: ampicillin/sulbactam, piperacillin/tazobactam, ceftazidime, ceftolozane/tazobactam, imipenem, tigecycline, colistin. In our study, agar well diffusion assay showed that 100 µg/mL of AgONPs inhibited the growth of PA59, with a zone of inhibition was 21 mm. While, the zone of PM14 inhibition was narrower, in compare to PA59 zone of inhibition was 19 mm using the same concentration of AgONPs with (Table 2). This finding suggests that PA59 exhibits a higher inhibition rate by AgONPs than PM14.

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Table 2: Antibacterial activity of 100 µg/mL AgONPs against multi-drug-resistant PA59 and PM14 isolated from cattle. The experiment was performed in quadruplicate.

Bacterial species	Bacterial growth inhibition zones (mm)
P. aeruginosa PA59	21
P. mirabilis PM14	19

Determination of Minimum Inhibitory Concentrations (MICs) of AgONPs

The MICs of AgONPs were determined using broth-micro dilution method to identify the lower concentration that inhibits the growth of the isolated bacterial pathogens PA59 and PM14. Various concentrations of AgONPs were prepared. Figure 4 shows that PA59 growth was inhibited at AgONPs of 3.9µg/mL and 7.8 µg/mL, as MICs at which the growth survival % were 4.2 ± 0.8 and 3.5 ± 0.5, respectively. While no survival growth % was observed at 15.6 µg/mL compared to the control group. In regards to PM14, the bacterial growth was inhibited at AgONPs of 3.9 µg/mL and 7.8 µg/mL, at which the growth survival % were 59.1 ± 1.9 and 52.0 ± 0.2, respectively. Also, survival growth % was 0.6 ± 0.2 observed at 15.6 µg/mL compared to the control group. These findings confirm the data of agar well diffusion assay that PM14 was more resistant than PA59. These findings indicate that AgONPs possess a potential antimicrobial activity.

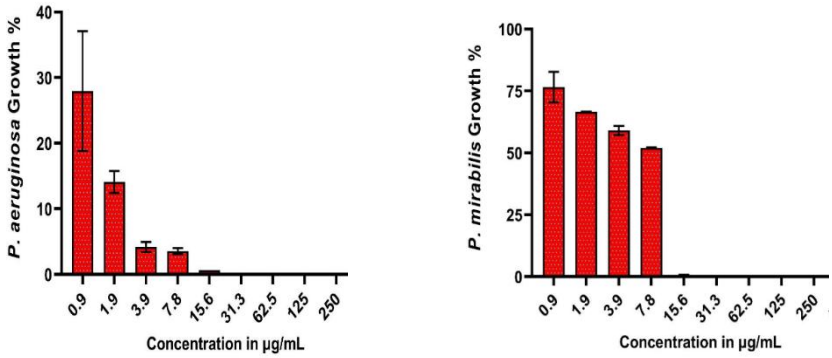

Figure 4: Minimum inhibitory concentrations of AgONPs against isolated *P. aeruginosa* and *P. mirabilis*

DISCUSSION

Currently, there is a public demand for the development of non-toxic and environmentally-friendly nanoparticles those having antibacterial properties and effectively counteract the emergence

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of drug-resistant microorganisms (Allahverdiyev *et al.,*2011; Shume *et al.,*2020). In this study, microbial-mediated nanoparticle synthesis, as a green chemistry approach, was used in which nanotechnology was combined with microbial biotechnology. Bacterial, extracellular, approach was used for preparation of AgNPs using biomass, cell-free supernatant, and derived components (Anand *et al*., 2022). Our findings showed that the addition of 20 mL of CFS of the mixed bacilli species to $AgNO_3$ and exposure to sunlight for 10 minutes led to a change in color of the reaction mixture from yellow to reddish-brown. The color changes occur due to the surface plasmon resonance of AgONPs in the visible region band at~420nm in the UV–vis spectrum clearly which reveals the formation of AgONPs (Kalimuthu *et al.*, 2008 and Rong H *et al*., 2002). The bio-reduction rate of Ag^{+2} was increased by incubating in sunlight the mixture at 40°C for 10 minutes. UV-Vis analysis showed the highest absorbance value after 10 minutes of incubation (Data not shown). Our data were coincided with the results of some previously published studies (Nguyen *et al*., 2021 and Arshad *et al*., 2022). In our work, using 20 mL CFS of the tested bacilli to bio-synthesize AgONPs was closed with the biogenic synthesis principles stated by several studies in which the produced AgONPs were characterized by TEM, XRD as a spherical shape with a particle size of 16-29 nm. The presence of strong peaks in the diffraction pattern confirms that the synthesized materials are highly crystalline. Our results of Figure 2 displayed the results of the XRD study of AgONPs which have a pure phase that matches the reported Bragg peaks. The absence of Bragg peaks for other chemical compounds indicated that biogenic AgONPs are pure and crystalline, with a uniform structure (Rahmanifar and Moradi Dehaghi 2014; Velsankar *et al*., 2020: El-Sheekh *et al*., 2022). In some recent studies, the ratio of $AgNO_3$ to CFS aqueous solution was 90:10 at which the size of the particles was between 30 and 50 nm. Using 10 mL CFS of probiotic and mixed with 90 mL of $AgNO_3$ solution produced spherical and averaged 20-100 nm size (Awadelkareem *et al*., 2023 and Abdelgadir *et al*., 2024). These results indicated that the catalyst activity of sunlight with 20 ml CFS was more effective in reducing and stabilizing AgONPs products. These biomolecules with particle size of 16-29 nm which were biologically prepared using an eco-friendly and sustainable approach could be followed easily (Alsamhary, 2020).

In our study, agar well diffusion assay showed that the bacterial growth inhibition zone of *P. aeruginosa* (PA59) when AgONPs applied was larger in comparison to *P. mirabilis* (PM14) inhibition zone. These data indicated that *P. aeruginosa* is more susceptible to reactive oxygen species (ROS) produced by the nanoparticles or have a greater uptake of AgONPs due to bacterial comparatively thinner peptidoglycan, compared to the permeable outer membrane proteins than *P. mirabilis* (Lara *et al*.,2010). Ag+ penetration is improved and membrane integrity can be broken more easily due to the increased membrane permeability.

The antibiotic susceptibility of PA59 and PM14 were tested using the VITEK® 2 compact system. The MIC of each antibiotic was calculated as explained according to (Ling *et al*., 2001), indicated that PM14 was more resistant than PA59. The recent research studies show that *Proteus mirabilis* isolates from cattle showed a steady rise in antibiotic resistance, due to a combination of

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environmental and genetic factors. Environmental pressures and the indiscriminate use of antibiotics in animal production systems, particularly on cattle farms, create strong selective pressure that promotes the spread of strains resistant to traditional drugs like ampicillin, tetracycline, streptomycin, and fluoroquinolone (Sarwar *et al*., 2025). *P. aeruginosa* has a variety of defense mechanisms, including the ability to form biofilms, which serve as a natural barrier to antibiotic penetration and greatly diminish their efficacy (Moradali *et al*.,2017). Furthermore, these bacteria have a variety of resistance genes, such as those that encode carbapenemases and extended-spectrum beta-lactamases (ESBLs), which enable them to inactivate a broad spectrum of antibiotics, especially β-lactams and carbapenems (Oliver *et al*.,2015). The spread of the phenomenon in animal settings is accelerated by mobile genetic elements like integrons and plasmids, which are also essential for the transfer of resistance genes between bacterial populations.

In comparison to the other studies, higher concentrations of AgNPs ranging from 12-400 µg/ml were required to inhibit the growth of Gram-negative bacteria **(**Li *et al*., 2013; Rajesh *et al*., 2015; Alsharari *et al*., 2024). The data variations between our and other published studies could be associated with experiment conditions and the protocols of nanoparticles synthesis. The antimicrobial activity of the AgONPs is attributed to their mechanisms, such as cell membrane disruption, protein denaturation, and oxidative stress via reactive oxygen species (ROS) which are enhanced by bioactive capping agents from biological sources, which may improve stability and target specificity (Khan *et al*., 2016).The interest of using cell-free supernatant of probiotics is growing, primarily because of the antibacterial properties attributed to the organic acids they produce, which lower the pH of their surrounding medium. Additionally, it has been noted that bioactive compounds released by probiotics, such as bacteriocins and hydrogen peroxide, contribute significantly to their antimicrobial activity (Drider et al., 2016)

Conclusions:

The biologically synthesized AgONPs represent a promising frontier in addressing antibiotic resistance and infectious diseases, offering a sustainable, safe, and eco-friendly alternative to traditional chemical and physical methods. The biological components in the supernatant serve as reducing and stabilizing agents, when exposed to solar irradiation, Ag ions are converted to Ag atoms and, then AgONPs are produced. The bacterial "green" synthesis, using cell-free supernatants of probiotics lactic acid bacteria, minimizes the toxic byproducts, reduces energy consumption, and utilizes renewable biological resources, aligning with global sustainability goals. Biologically synthesized AgONPs exhibit potent antibacterial activity against multidrug-resistant *P. aeruginosa* and *P. mirabilis* isolated from cattle with UTI. Future investigation is required to shed ~~the~~ light on the combination of AgONPs and the commercially used antibiotics and/or naturally derived antimicrobials. These combinations could amplify the antimicrobial effects and enhance therapeutic potential. However, standardization and scalability of the prepared AgONPs still challenge issues impacting consistency in antibacterial performance. Therefore, research must prioritize elucidating

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structure-activity relationships, optimize synthesis protocols, and conduct rigorous in vivo trials to ensure safety and efficacy.

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Authors Contributions:

All the authors contributed to the study's idea, design and writing, in addition to material preparation, data collection, and analysis.

Disclosures

The authors declare no conflicts of interest.

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