Research Article

Silver Nanoparticles as an Antibacterial Candidate for Poultry: An Alternative to Synthetic Antibiotics

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Abstract | This study aimed to determine the concentration of silver nanoparticles that can inhibit gram-positive and negative bacteria. It was done so that the silver nanoparticles could be evaluated as an antimicrobial agent, and there was also the possibility that they could be used as a feed additive. This study assesses microbial inhibition and the minimum inhibitory concentration (MIC) in a laboratory setting. The substances utilized included silver nanoparticles, nutrient agar medium, Man's Rogosa Sharpe Agar, Man's Rogosa Sharpe Broth, bacterial cultures of *Lactobacillus acidophilus* ATCC 4356, and *Lactobacillus* sp. FNCC 0020, *Salmonella typhimurium* ATCC 700720, *Escherichia coli* FNCC 0091, tetracycline, and 70% alcohol. Antibiotic sensitivity testing employed the Kirby-Bauer and optical density 600 techniques to assess microbial growth inhibition and the minimum inhibitory concentration (MIC). The research data was examined for comparative statistics using a completely randomized design. Concentrations between 10 and 50 ppm in the inhibition zone test successfully inhibited the growth of *Salmonella typhimurium* and *Escherichia coli* while not affecting the viability of *Lactobacillus acidophilus, Lactobacillus* sp. The MIC for gram-negative bacteria such as *Salmonella typhimurium* and *Escherichia coli* was 6.25 ppm, and the optical density at 600 was approximately 0.09.

Keywords | Antibacterial, Antibiotic, Minimum inhibitory concentration, *Nanotechnology*, Optical density, Silver nanoparticles

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INTRODUCTION

The emergence of antibiotic-resistant bacteria is an urgent global problem. Antibiotic resistance occurs when bacteria evolve and develop mechanisms to survive the drugs and kill the bacteria. This poses a major challenge to public health as it limits the effectiveness of antibiotics, making infections more difficult to treat and increasing mortality. Antibiotic-resistant bacteria such as *Escherichia coli* (Poirel *et al*., 2018), *Salmonella* sp. (Pławińska-Czarnak *et al*., 2022), *Pseudomonas aeruginosa* (Kunz *et al*., 2022), *Staphylococcus aureus* (Lee *et al*., 2018), *Streptococcus pyogenes* (Cattoir, 2022) and other bacterial strains are increasingly problematic globally. The bacteria cause serious inflammation of the gastrointestinal tract, the dermis, and other cell tissues (Popova and Ignatov, 2023). *Nanotechnology* has the potential to bring significant advances in various fields of medicine as a replacement for synthetic antibiotics against pathogenic bacteria. *Nanotechnology*, or nanoparticles, is a new scientific

discipline related to the ability to measure, manipulate, and shape materials at the nano-size level (Calipinar and Ulas, 2019). Srivastava and Bhargava (2022) explain that nanotechnology is the conversion of larger molecules to nanometer size and the changing the physico-chemical properties of cell matrices in terms of human or animal welfare on a size scale of 1-100 nanometers.

Silver nanoparticles are the most popular metal group nanoparticles to be researched in the last decade because they are effective as antimicrobial agents and as substitutes for synthetic antibiotics. Silver is a safe inorganic antibacterial agent that kills many pathogenic microorganisms through its ability to ionize in solution (Mohamed *et al*., 2020). Positively charged silver ions (Ag^+) interact with the structure of microorganisms, both inside and outside the cell, causing their inhibition or destruction (Yin *et al*., 2020). In addition, silver ions can disrupt cell membrane integrity, interfere with cellular processes, and damage important biomolecules. These mechanisms of action make silver nanoparticles a powerful tool in the fight against microbial infections. Siddiqi *et al*. (2018) explain that silver nanoparticles have antimicrobial activity against various infectious and pathogenic microorganisms, including bacteria resistant to various drugs. The potential of silver nanoparticles is also explained by Cheng *et al*. (2016) that silver nanoparticles can be used as antibiotics because they are associated with various mechanisms of action against microorganisms in various structures at one time and provide the ability to kill various types of bacteria. Similarly, Natan and Banin (2017) found that silver nanoparticles have antifungal properties, which makes them effective in treating fungal infections. Naumenko *et al*. (2023) conducted a study demonstrating the antiviral properties of silver nanoparticles, highlighting their potential in fighting viral infections. Antimicrobial studies in the field of poultry related to the administration of silver nanoparticles can reduce *Escherichia coli* in the digestive tract of broiler chickens (Kumar and Bhattacharya, 2019). In addition, Sawosz *et al*. (2007) found that the administration of silver nanoparticles at 25 mg/kg into drinking water was able to increase the population of lactic acid bacteria in the quail gut and improve gut health. These studies provide strong evidence regarding the effectiveness of silver nanoparticles in combating various types of pathogens. These advantages make silver nanoparticles an attractive option to address the challenge of antibiotic resistance. In this study, we aimed to determine the effectiveness of liquid silver nanoparticles against the growth of *Escherichia coli*, *Salmonella typhimurium*, and lactic acid bacteria and test the minimum concentration of inhibitors using optical density 600 (OD600).

MATERIALS AND METHODS

Preparation of silver nanoparticles

Silver nanoparticles were prepared by combining 10 mL of noni leaf extract as a bioreductor with 80 mL of a solution containing one mM $AgNO_3$. The samples were then cooled for twenty-four hours after being incubated at 90°C for 120 minutes (Sathishkumar *et al*., 2012). After that, the particle size analyzer was used to perform the analysis. The particle size distribution value was found to be 84.78 ± 1.54 nm, the diversity index value was found to be 0.23 ± 0.015, and the zeta potential was found to be -22.03 mV.

Microbial inhibition assay

The activity of silver nanoparticles was tested using the Kirby-Bauer assay method. The study utilized Gram-negative bacteria *Salmonella typhimurium* ATCC 700720 and *Escherichia coli* FNCC 0091, as well as Gram-positive bacteria *Lactobacillus acidophilus* and *Lactobacillus* sp. A series of dilutions of the test compound, a parent solution of colloidal silver nanoparticles synthesized from the most effective treatment, were required to perform the antimicrobial activity tests. The samples tested included a positive control consisting of 45 ppm tetracycline, noni leaf extract, and silver nanoparticles. Each sample was replicated a total of six times. The following treatments were tested as follows: KN= negative control; KP= 45 ppm tetracycline; P1= 10 ppm noni leaf extract; P2= 20 ppm noni leaf extract; P3= 30 ppm noni leaf extract; P4= 40 ppm noni leaf extract; P5= 50 ppm noni leaf extract; P6= 10 ppm silver nanoparticles; P7= 20 ppm silver nanoparticles; P8= 30 ppm silver nanoparticles; P9= 40 ppm silver nanoparticles; P10 = 50 ppm silver nanoparticles.

Rejuvenation of bacterial pure culture (Wahyudi *et al*., 2011)

The four solid pure culture colonies were *Salmonella typhimurium* ATCC 700720, *Escherichia coli, Lactobacillus acidophilus* ATCC 4356, and *Lactobacillus* sp. *FNCC 0020* was taken as one sterile ose from the pure culture and placed into a test tube that contained 7 mL of nutrient agar solution. After that, the test tube was inoculated with NA medium and MRSA slant, and the mixture was then placed in an incubator at 37°C for twenty-four hours.

Inhibition testing of samples against test bacteria

Salmonella typhimurium ATCC 700720 and *Escherichia coli* FNCC 0091 were tested on nutrient agar. Nutrient agar was prepared by measuring 20 grams in 1000 milliliters of sterile water. However, *Lactobacillus acidophilus* ATCC 4356 and *Lactobacillus* sp. FNCC 0020 was tested by introducing 68.2 grams of MRSA dissolved in 1000 milliliters of Aqua fresh. After 15 minutes at 80°C, each

medium was thoroughly mixed. It was then sterilized for fifteen minutes at an autoclave pressure of 1.5 atm and a temperature of 121°C. The medium was disinfected at 35°C with 10 milliliters of test bacteria, aseptically poured into a 12-milliliter petri dish, and allowed to solidify (Mansur and Hidayat, 2019). An aseptic paper disc was moistened with a solution of silver nanoparticles and a concentration of noni leaf water extract and then placed on a petri dish containing *Salmonella typhimurium* ATCC 700720, *Escherichia coli* FNCC 0091, *Lactobacillus acidophilus* ATCC 4356, and *Lactobacillus* sp. FNCC 0020. The medium was then evaluated for its ability to inhibit bacterial growth. It was also cultured for twenty-four hours at 37°C. An automated colony counter model Scan 500 (Scan 500, Interscience, Saint Nom, France) was used to determine the size of the silver nanoparticles inhibition zone.

Minimum inhibitory concentration (MIC) evaluation

The MIC test was conducted following the CLSI (2020) method in a 96-well microtiter plate using a 200 ml Eppendorf Reference 2 pipette from Camlab, Cambridge, USA, and the nutrient broth (NB) microdilution technique. The purpose of analyzing the mic is to obtain the minimum AgNPS concentration in inhibiting bacteria. Bacterial cultures of *Salmonella typhimurium* ATCC 700720 and *Escherichia coli* FNCC 0091 were standardized to an optical density of 0.5 McFarland standard, equivalent to a cell colony count of around 1.5 x 10^8 CFU/mL. Three sample groups were assessed on a single microplate: the positive control group (KP), the AgNPs group, and the negative control group (KN). Each well of the microplate was filled with NB media and 100 µL of the test solution. AgNPs solution was added to wells in volumes up to 100 µL from column 2 to column 11. Column 2 of the microtiter plate contains the highest concentration of silver nanoparticles (AgNPs), while column 11 contains the lowest concentration. Column 12 was used as the negative control containing only the medium, while column 12 served as the positive control containing both the medium and the bacterial inoculum. Each well was then cultured for 24 hours at 37°C with 50 µL of the bacterial suspension, according to Sandasi *et al.* (2010). The antimicrobial efficacy of AgNPs was evaluated by quantifying the optical density (OD) at a wavelength of 600 nm using a UV-VIS spectrophotometer (Multiskan Sky Thermoscientific, Bydgoszcz, Poland). An OD600 value of ≤ 0.1 indicates inhibition of bacterial growth, while an OD value of ≤ 0.2 indicates turbid bacterial growth (Santos *et al.*, 2010; Beal *et al.*, 2020).

Statistial analysis

Data collected from the observations were analyzed using a fully randomized design. If the test had a significant effect at the 5% probability level ($P < 0.05$), the test was further conducted using Duncan's New Multiple Range Test (DMRT). Software used for data analysis included SPSS version 21 and Graphpad™ Prism version 9 for descriptive data (Hummel *et al.*, 2021; Simon *et al.*, 2021).

RESULTS AND DISCUSSIONS

The statistical analysis in this study shows significant differences ($P < 0.01$) in the diameter of the inhibition zone among various treatments, as presented in Table 1. The use of 10 ppm silver nanoparticles (P6), 20 ppm silver nanoparticles (P7), 30 ppm silver nanoparticles (P8), 40 ppm silver nanoparticles (P9), and 50 ppm silver nanoparticles (P10) results in a larger inhibition zone diameter ($P < 0.01$) compared to the use of noni leaf extract concentrations ranging from 10 to 50 ppm and lower ($P < 0.01$) compared to tetracycline 45 ppm (KP) against *Escherichia coli* and *Salmonella typhimurium*. The effect of this inhibition is due to the interaction of silver nanoparticles with the bacterial cell membrane, which disrupts cell function. Silver nanoparticles display bacteriostatic characteristics by interacting with the cell membrane of bacteria, hindering their normal function. This interaction causes the nanoparticles to act. The accumulation of silver nanoparticles on the bacterial cell membrane and their binding to the membrane disrupts its function (Figure 1). The electrostatic attraction between the positively charged silver ions and the negatively charged cell membrane is responsible for this phenomenon. The electrostatic attraction between the positively charged silver ions and the negatively charged cell membrane is responsible for this phenomenon. It is possible to find carboxyl groups, phosphate groups, and amino groups in lipopolysaccharides, which are the components responsible for forming the cell membrane. It is responsible for the modification of the structure of the cell membrane. The presence of porins in gram-negative bacteria makes it possible for silver nanoparticles to pass through the outer membrane of these bacteria. It may cause damage to the membrane, rendering it less effective. Because of this action, the membrane may be damaged, which will reduce its permeability (Zheng *et al.*, 2018). The interchange between nanoparticle silver and the cell membrane, which occurs after the nanoparticle silver has entered the cell membrane, damages the nanoparticle silver. Nanoparticle silver penetrates the cell membrane upon entry, causing damage to the internal components of the cell (Figure 1). It inhibits membrane-bound enzymes and proteins by binding to disulfide bonds and obstructing active sites. Furthermore, nanoparticle silver can harm DNA components (Singh *et al.*, 2021).

In a recent study by Menichetti *et al.* (2023), they were proposed that Gram-negative bacteria might be more vulnerable than Gram-positive bacteria because of their thin cell membrane, which is about 8-12 nm thick, and the existence of negatively charged lipopolysaccharides that aid in the attachment of nanoparticle silver (Figure 1). Gram-positive bacteria possess a denser membrane, ranging from 20-80 nm, with negatively assessed peptidoglycans that can impede the penetration of silver nanoparticles (Fröhlich and Fröhlich, 2016; Slavin *et al.*, 2017). Silver nanoparticles can damage cell membranes by generating reactive oxygen species (ROS) internally and externally in bacteria and by interacting with cell wall components. The results of this investigation are consistent with previous research conducted by Hwang *et al.* (2008), which indicated that nanoparticle silver had a more significant effect impact on Gram-negative bacteria than compared to Gram-positive bacteria. The negative charge of bacterial cell membranes in Gram-negative bacteria is due to carboxyl, phosphate, and amino groups in lipopolysaccharides. The negative charge leads to membrane damage and stimulates the generation of reactive oxygen species (ROS). Higher levels of ROS impact the deactivation of respiratory enzymes, hinder the production of adenosine triphosphate, and interfere with DNA and protein synthesis (Qing *et al.*, 2018; Tyagi *et al.*, 2023).

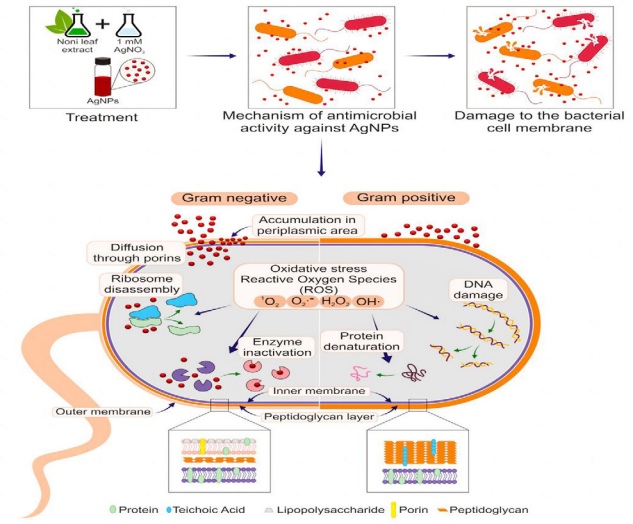

Figure 1: Antimicrobial mechanism of silver nanoparticle.

The results of statistical analysis (Table 1), the treatment of 10 ppm silver nanoparticles (P6), 20 ppm silver nanoparticles (P7), 30 ppm silver nanoparticles (P8), 40 ppm silver nanoparticles (P9) and 50 ppm silver nanoparticles (P10) had no inhibition zone diameter (P>0.05) compared to tetracycline 45 ppm (KP) against Gram-positive bacteria, namely *Lactobacillus acidophilus* and *Lactobacillus* sp. Several factors influence the susceptibility of Gram-positive bacteria, such as *Lactobacillus*, to silver nanoparticles. One factor is the production of bacteriocins,

Table 1: Inhibition zone diameter of silver nanoparticles against gram-negative (*Escherichia coli*, *Salmonella typhimurium*) and gram-positive (*Lactobacillus acidophilus*, *Lactobacillus* sp.) bacteria.

Treatments	Gram-negative		Gram-positive	
	Escherichia coli	*Salmonella typhimurium*	*Lactobacillus acidphopilus*	*Lactobacillus* sp.
KN	0,00^{e}	0,00^{e}	0,00^{b}	0,00^{b}
KP	27,93^{a}	22,53^{a}	30,13^{a}	28,57^{a}
P1	0,00^{e}	0,00^{e}	0,00^{b}	0,00^{b}
P2	0,00^{e}	0,00^{e}	0,00^{b}	0,00^{b}
P3	0,00^{e}	0,00^{e}	0,00^{b}	0,00^{b}
P4	0,00^{e}	0,00^{e}	0,00^{b}	0,00^{b}
P5	0,00^{e}	0,00^{e}	0,00^{b}	0,00^{b}
P6	18,33^{d}	7,33^{d}	0,00^{b}	0,00^{b}
P7	18,57^{d}	7,67^{d}	0,00^{b}	0,00^{b}
P8	18,93^{d}	8,57^{c}	0,00^{b}	0,00^{b}
P9	19,73^{c}	8,63^{c}	0,00^{b}	0,00^{b}
P10	20,60^{b}	9,37^{b}	0,00^{b}	0,00^{b}
SEM	1,79	1,11	1,45	1,34
P-value	0,001	0,001	0,001	0,001

Note: ^{a,b,c,d,e} Different superscripts in the same column indicate significant differences (P<0,05). KN = negative control; KP= positive control (tetracycline 45 ppm); P1 = 10 ppm noni leaf extract; P2 = 20 ppm noni leaf extract; P3 = 30 ppm noni leaf extract; P4 = 40 ppm noni leaf extract; P5 = 50 ppm noni leaf extract; P6 = 10 ppm silver nanoparticles; P7 = 20 ppm silver nanoparticles; P8 = 30 ppm silver nanoparticles; P9 = 40 ppm silver nanoparticles; P10 = 50 ppm silver nanoparticles.

antimicrobial peptides produced by bacteria group *lactobacillus* (Sharma *et al.*, 2023). Bacteriocin synthesis could enhance bacterial resistance to nanoparticle silver by offering an extra protective mechanism against the antimicrobial properties of nanoparticle silver. Furthermore, the sturdy cell wall composition of *Lactobacillus*, measuring approximately 20–80 nm in thickness, provides defense against external substances, such as nanoparticle silver (Godoy-Gallardo *et al.*, 2021). The robust cell wall structure limits the connection between silver nanoparticles and the bacterial membrane, reducing the affinity of nanoparticle silver to the bacterial surface and thus lowering susceptibility to the antimicrobial properties of nanoparticle silver. The enzymatic functions or metabolic processes of lactic acid bacteria, including the production of lactic acid and other compounds, can influence the sensitivity to nanoparticle silver. The metabolic processes may create an environment that is less conducive to the antimicrobial effects of nanoparticle silver, thus contributing to the reduced inhibitory effect of nanoparticle silver on the bacteria group *Lactobacillus* (Vavřiník *et al.*, 2021). Other researchers (Lara *et al.*, 2010) reported that the cell wall composition of *Lactobacillus*

serves as a defense mechanism by limiting the interaction between nanoparticles and the membrane of the bacteria. It reduces adhesion. It reduces the binding of nanoparticle silver to the bacterial surface and lowers the vulnerability to decrease susceptibility to the antimicrobial properties' effects of nanoparticle silver. Likewise, Matei *et al.* (2020) discovered that the enzymatic functions and metabolic mechanisms of lactic acid bacteria, such as the generation of lactic acid and other substances, could impact the resistance to nanoparticle silver. Sidhu and Nehra (2020) noted that lactic acid bacteria's capacity to generate bacteriocins, antimicrobial peptides, could aid in their resistance to nanoparticle silver. This investigation showed a remarkable effect of tetracycline at this concentration, which is highly efficient at a concentration of 45 ppm in inhibiting the proliferation of both bacterial strains. Another investigation (Landoni and Albarellos, 2015) discovered that tetracycline concentrations between 45 and 100 ppm can inhibit protein synthesis, resulting in bactericidal and bacteriostatic effects on pathogenic and non-pathogenic bacteria. Oliva *et al.* (1992) stated that tetracycline is a broad-spectrum antibiotic that hinders cell protein production by attaching aminoacyl tRNA (aa-tRNA) to the A site of the 30S subunit, thus focusing on ribosomes. Pioletti *et al.* (2001) found that tetracycline disrupts protein synthesis in Gram-negative and Gram-positive bacteria by interfering with binding aminoacylated tRNA to ribosomes. Inhibiting the synthesis of proteins is accomplished by tetracycline by interfering with the binding of aminoacylated tRNA to ribosomes in bacteria. The elongation phase of protein synthesis is the phase that is most significantly impacted by this interference. It does this by preventing aminoacyl tRNA from binding to ribosomal subunits.

Incapability to inhibit bacteria is because the levels of metabolites present in the noni leaf extract at concentrations of 10, 20, 30, 40, and 50 ppm (P1, P2, P3, P4, P5) are insufficient to produce the desired antimicrobial effect. Therefore, a higher extract concentration is required to achieve antimicrobial effects. Zhang *et al.* (2016) studied the antimicrobial effects of noni leaf extract. Concentrations ranging from 50% to 100% did not impact gram-negative bacteria (*Escherichia coli*). The suppressive effect was observed at a concentration of 200% of the noni leaf extract. A study by Halimah *et al.* (2019) demonstrated that noni leaf extract concentrations ranging from 2.55% to 10% did not exhibit antimicrobial activity against *Salmonella typhimurium* bacteria, as indicated by the lack of inhibition zone formation. This finding supports the results of the study, which also demonstrated no inhibitory effect at the concentrations examined.

The MIC values of silver nanoparticles, *Escherichia coli*, and *Salmonella typhimurium* ranged from 6.25 to 50 ppm. Bacterial growth was detected when the treatment turbidity and OD600 values ranged from 1.24 to 0.15 at concentrations lower than 3.125 ppm. With a concentration of 6.25 parts per million (ppm) of silver nanoparticles, the OD600 measurement was approximately 0.09, suggesting bacterial growth was inhibited (Figures 2, 3). At a concentration of 6.25 ppm silver nanoparticles, the MIC against both bacteria resulted in an OD600 value of around 0.09, demonstrating inhibition of bacterial growth. The findings align with the study, showing that bacterial growth is suppressed when the OD600 value is below 0.1 and stimulated when it surpasses 0.1. Zarei *et al.* (2014) found that the MIC ranged from 3.125 to 6.25 ppm for silver nanoparticles against *Salmonella typhimurium* and *Escherichia coli.* Begum *et al.* (2022) and Erjaee *et al.* (2017) found that at 7.8 ppm, the minimum inhibitory concentration (MIC) of silver nanoparticles effectively inhibited the growth of *Escherichia coli* and *Salmonella typhimurium* bacteria. The results indicate that silver nanoparticles can efficiently suppress the growth of gram-negative bacteria. An elevated concentration of silver nanoparticles at around 50 ppm led to a notable reduction in the growth of gram-negative bacteria (*Salmonella typhimurium, Escherichia coli*) with OD600 values dropping

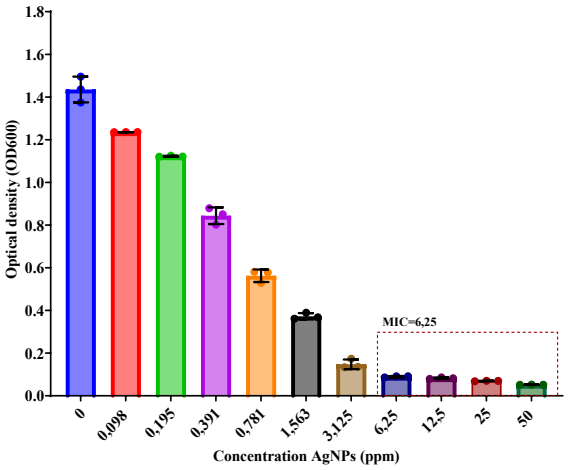

Figure 2: MIC analysis results using OD600 on *Escherichia coli.*

below 0.1 after 25 hours of incubation (Saxena *et al.*, 2010). The results of this study have potential implications for further research that silver nanoparticles can be used as antimicrobial antibiotics and feed additives to improve digestive efficiency, immunity, and performance in livestock and poultry because they have antimicrobial effects on pathogenic bacteria and do not interfere with the growth of lactic acid bacteria. Michalak *et al.* (2022) reported that nanoparticles positively affect animal performance, productivity, and carcass quality by preserving blood homeostasis and intestinal microflora, controlling oxidative damage, and improving immune reaction. Furthermore, Dosoky *et al.* (2021) explained that silver nanoparticles

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could be used as a relatively safe feed supplement for broilers at a concentration of 4 ppm due to their anti-inflammatory, antimicrobial, and immunostimulatory properties, while a concentration of 8 ppm causes mild inflammatory reactions and immunosuppression in the bursa, thymus, and spleen.

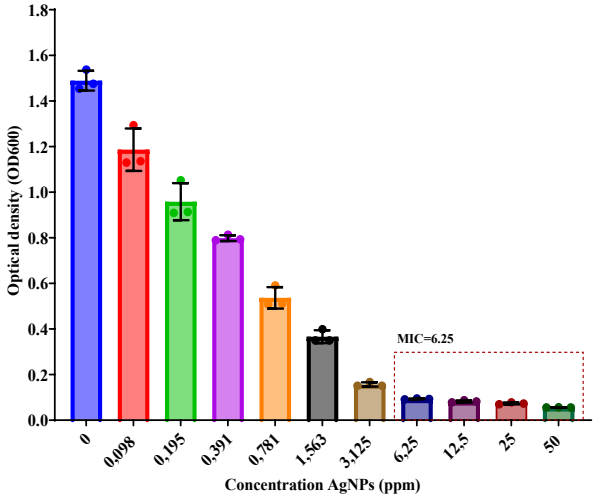

Figure 3: MIC analysis results using OD600 on *Salmonella typhimurium*.

CONCLUSION

Silver nanoparticles effectively inhibited *Salmonella typhimurium* ATCC 700720 and *Escherichia coli* FNCC 0091 without affecting *Lactobacillus acidophilus* ATCC 4356, and *Lactobacillus* sp. FNCC 0020, which can act as antibiotics, with the lowest concentration of silver nanoparticles being 6.25 ppm.

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NOVELTY STATEMENT

We explored silver nanoparticles as a substitute for synthetic antibiotics in poultry farming because of concerns about resistance. Our study compared their antibacterial efficacy and mechanism to that of conventional antibiotics. This approach targets bacterial infections without causing resistance or harm to lactic acid bacteria. Improving poultry health and productivity supports global efforts to reduce the use of synthetic antibiotics, enhance food safety, and promote public health.

AUTHOR'S CONTRIBUTION

MH conducted experiments, performed laboratory analysis, analyzed the data, and wrote the manuscript. NDD supervised the experiment and revised the manuscript. BA experimented and authored the manuscript. Z crafted and revised the manuscript. All authors were responsible for reviewing and approving the final manuscript.

Conflict of interests

The authors have declared no conflict interest.

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