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FOOD MICROBIOLOGY - RESEARCH PAPER

Antimicrobial and antibiofilm activity of silver nanoparticles against *Salmonella* Enteritidis

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Abstract

Salmonella enterica serotype Enteritidis is one of the main pathogens associated with foodborne illnesses worldwide. Biofilm formation plays a significant role in the persistence of pathogens in food production environments. Owing to an increase in antimicrobial resistance, there is a growing need to identify alternative methods to control pathogenic microorganisms in poultry environments. Thus, this study aimed to synthesize silver nanoparticles (AgNPs) and evaluate their antibiofilm activity against poultry-origin *Salmonella* Enteritidis in comparison to a chemical disinfectant. AgNPs were synthesized, characterized, and tested for their minimum inhibitory concentration, minimum bactericidal concentration, and antibiofilm activity against *S.* Enteritidis strains on polyethylene surfaces. The synthesized AgNPs, dispersed in a liquid medium, were spherical in shape with a mean diameter of 6.2 nm. AgNPs exhibited concentration-dependent bactericidal action. The bacterial reduction was significantly higher with AgNPs (3.91 $\log_{10}$ CFU • cm^{-2}) than that with sanitizer (2.57 $\log_{10}$ CFU • cm^{-2}). Regarding the time of contact, the bacterial count after a contact time of 30 min was significantly lower than that after 10 min. The AgNPs exhibited antimicrobial and antibiofilm activity for the removal of biofilms produced by *S.* Enteritidis, demonstrating its potential as an alternative antimicrobial agent. The bactericidal mechanisms of AgNPs are complex; hence, the risk of bacterial resistance is minimal, making nanoparticles a potential alternative for microbial control in the poultry chain.

Keywords Antibiofilm activity · Biofilm · Poultry · *Salmonella* · Silver nanoparticles

Introduction

Salmonella spp. are among the most common foodborne pathogens, affecting numerous people annually [1]. *Salmonella enterica* serotype Enteritidis is one of the main bacteria causing most foodborne illnesses worldwide [2, 3]. In Brazil, between 2009 and 2017, approximately 35% of the foodborne outbreaks linked to bacterial agents were caused by *Salmonella* spp. [4]. Poultry products are recognized as the main source of *Salmonella* infection in humans [5].

The survival and persistence of pathogens in the environment, particularly in poultry processing plants, are significant risk factors, which contribute to their dissemination through the food chain [6]. Biofilm formation plays a significant role in the survival of pathogens in food production environments [7]. This represents a risk to consumer health and results in economic losses for the industry [8]. *Salmonella* Enteritidis has the ability to adhere to surfaces commonly found in the poultry processing industry over a wide temperature range [9–12]. Due to the increasing number of antimicrobial-resistant strains, it is necessary to identify alternative methods to control pathogenic microorganisms in poultry environments [13]; the use of nanoparticles is a potential alternative to antimicrobial agents [14].

Nanoparticles are materials with at least one dimension (1–100 nm) in the nanometric scale or whose basic unit in

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the three-dimensional space is in this range [14]. Nanoparticles have several applications, including in drugs and medications, manufacturing and materials, environment, electronics, energy harvesting, and mechanical industries [15].

Metal nanoparticles, such as those of silver, copper, titanium, gold, and zinc, usually exhibit antimicrobial activity, with differences in potencies and spectra of activity [16]. Silver nanoparticles (AgNPs) are known for their wide range of applications and low cytotoxicity in mammalian cells. Their antimicrobial activity is associated with the generation of reactive oxygen species [17]. Previous studies have shown the antimicrobial effect of AgNPs against foodborne pathogens such as *Salmonella* serotypes, *Escherichia coli*, and *Listeria monocytogenes* [18, 19].

However, whether AgNPs have the potential to prevent and remove *S.* Enteritidis biofilms from surfaces found in poultry environments is still unclear. Therefore, this study aimed to synthesize AgNPs and evaluate their antibiofilm activity against poultry-origin *Salmonella* Enteritidis.

Materials and methods

Preparation of AgNPs

AgNPs were synthesized via a chemical reduction method using hydrogen. Synthesis was performed at the Molecular Catalysis Laboratory (Institute of Chemistry, Universidade Federal do Rio Grande do Sul), according to the method described by Redel et al. [20]. Briefly, 100 mg of silver oxide (Ag_2O) and 240 µL of butylimidazole were dispersed and dissolved in 3 mL of an ionic liquid (1-ethyl-3-methylimidazolium methanesulfonate) in a Fisher-Porter reactor for 15 min at room temperature (± 25 °C). The system was then charged with 4 atm of hydrogen and stirred for 2 h at 80 °C. Following this, the reactor was evacuated at 50 °C for 1 h. Ultrapure water (50 mL) was then added to this colloidal solution.

Characterization of AgNPs

AgNP synthesis was confirmed, using UV–Vis spectroscopy, by the presence of a plasmonic band at approximately 400 nm, which is characteristic of this type of a nanoparticle. Cary 50 Conc spectrophotometer (Varian, Brentwood, USA) was used, and the measurements were performed by absorbance scanning in the wavelength range of 300–800 nm using the average scan mode.

The obtained nanoparticles were also analyzed using transmission electron microscopy (TEM), to determine their size, shape, composition, and dispersion [21–23]. TEM analysis was performed using an EM 208S microscope (Philips, Beaverton, USA) operating at 100 kV. A small amount of nanoparticles was dispersed in isopropanol. A drop of this solution was then added to a carbon grid/plate (300 mesh) for reading. Analyses were performed using a JEM 1200 ExII electron microscope (Jeol, Tokyo, Japan), operating at 120 kV. Size and distribution of the nanoparticles were determined at a resolution of 470 pixels/cm to obtain more accurate measurements. A histogram was generated by counting approximately 200–300 particles and grouping them in terms of size. The particle diameters in the micrographs were measured using Origin software (OriginLab, Northampton, USA).

Bacterial strains

Ten strains of *Salmonella* Enteritidis were randomly selected from our stock collection. All the strains had been isolated from poultry sources (Table 1) and were previously tested for their ability to produce biofilms at 28 °C in polystyrene microplates. The strains were stored at − 80 °C in brain heart infusion (BHI) broth (Oxoid, Hampshire, England) supplemented with 25% glycerol (Synth, Diadema, Brazil). For reactivation, the isolates were seeded on xylose lysine deoxycholate (XLD) agar (Merck; Darmstadt, Germany) and

Table 1 *Salmonella* Enteritidis strains: identification, source, and year of isolation

Strain identification	Source of isolation	Year of isolation
330	Poultry product involved in human salmonellosis outbreaks	2008
217	Drag swab	2000
311	Poultry product involved in human salmonellosis outbreaks	2011
344	Poultry product involved in human salmonellosis outbreaks	2007
329	Poultry product involved in human salmonellosis outbreaks	2008
338	Poultry product involved in human salmonellosis outbreaks	2008
275	Poultry product involved in human salmonellosis outbreaks	2004
224	Poultry viscera	2000
230	Drag swab	2001
282	Poultry product involved in human salmonellosis outbreaks	2007

incubated at 37 °C. After 24 h, colonies that were morphologically consistent with those of *Salmonella* spp. (blackish-centered colonies) were identified; of these, one colony was selected and inoculated into BHI broth under the same incubation conditions.

Determination of minimum inhibitory concentration and minimum bactericidal concentration of AgNPs

Inoculum preparation

The strains were cultivated on Luria–Bertani (LB) agar (Kasvi, São José dos Pinhais, Brazil) at 37 °C for 24 h. After incubation, the colonies were inoculated into LB broth (Kasvi, São José dos Pinhais, Brazil) to adjust the LB culture turbidity to an optical density (OD) of 0.030–0.040 using a spectrophotometer, operating at a wavelength of 595 nm (SP-22; Biospectro; São Paulo, Brazil), which corresponds to a standard inoculum of 10^7 colony-forming units (CFU)/mL. Serial dilution in LB broth was performed to obtain a culture of 10^5 CFU/mL, which was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of AgNPs [24].

Minimum inhibitory concentration (MIC)

The MIC was determined using the broth microdilution method, according to the method described by Duffy et al. [24]. Briefly, the AgNP stock solution (1 mg/mL) was diluted in LB broth, and the AgNP concentrations of 17, 18, 20, 25, 50, and 100 µg/mL were tested. Sterile 96-well flat-bottomed polystyrene microplates (Kasvi, São José dos Pinhais, Brazil) were used for MIC determinations. In columns 1 to 6, 50 µL of LB broth, 100 µL of AgNPs (one concentration for each column), and 50 µL of bacterial suspension at 10^5 CFU/mL were added. As a negative control, 100 µL of LB broth and 100 µL of each AgNP concentration were added to three wells in each column. As a positive control, 150 µL of LB broth and 50 µL of bacterial suspension at 10^5 CFU/mL were added to column 12. The plates were then incubated under aerobic conditions at 37 °C for 24 h. The OD was measured using a microplate reader at a wavelength of 595 nm (Biotek, Winooski, VT, USA).

Minimum bactericidal concentration (MBC)

The MBC was determined from the same 96 well plates used for the MIC by counting the bacteria in wells with no or very little visible growth. Bacterial counts were performed on XLD medium using the drop plate technique [25]. MBC is defined as the lowest concentration that kills 99.9% of the initial bacterial population [24].

Removal of formed biofilms from polymeric surfaces

High-density polyethylene (HDPE) coupons were considered for this assay because of their widespread use in poultry production, particularly in transportation crates. The coupons were produced with dimensions of 1 cm (width) × 1 cm (length) × 0.1 cm (thickness).

The strains were reactivated in BHI broth for 18–24 h at 37 °C. After incubation, the strains were cultured on trypticase soy agar (Oxoid, Basingstoke, UK) plates for 24 h at 37 °C. One colony of each strain was suspended in 5 mL of trypticase soy broth without glucose (TSB) (BD Biosciences, Franklin Lakes, NJ, USA) for 18 h at 37 °C. Turbidity of the TSB culture was adjusted to 1 on the McFarland scale, corresponding to the standard inoculum of 10^8 CFU·mL^{-1}, with 0.224–0.300 optical density (OD) in a spectrophotometer at a wavelength of 620 nm (SP-22; Biospectro; São Paulo, Brazil).

To evaluate the ability of the nanoparticles to remove biofilms formed by *S.* Enteritidis, 3 mL of each bacterial suspension was inoculated into three wells of a 12-well flat-bottomed polystyrene plate (Kasvi, São José dos Pinhais, Brazil). Coupons were tested in triplicates and individually added to the wells. The plates were incubated at 28 °C for 24 h. After incubation, the coupons were individually removed using sterile tweezers and washed with 5 mL of 0.1% buffered peptone water (BPW) (Merck, Darmstadt, Germany), to remove planktonic cells. Furthermore, the coupons were transferred to another microplate containing the following three treatments: (1) 3 mL of AgNPs at 200 µg/mL; (2) 3 mL of a polyhexamethylene biguanide hydrochloride-based sanitizer associated with a quaternary ammonium compound at 200 ppm; and (3) 3 mL of sterile distilled water (control group). The selected concentrations are those recommended for food contact surfaces (FDA, 2021). Plates containing coupons were incubated at 28 °C for 10 min (AgNPs and sanitizer) or 30 min (AgNPs). For the sanitizer, the maximum contact time at the highest concentration recommended by the manufacturer was 10 min. After contact (10 or 30 min), the coupons were removed using sterile tweezers and immersed for 5 min in 5 mL of 0.1% BPW containing neutralizing agents, 2% polysorbate (Dinâmica, Diadema, Brazil), 0.25% sodium thiosulfate (Dinâmica, Diadema, Brazil), and five glass beads (1 mm). The coupons were homogenized on a vortex shaker (Kasvi, São José dos Pinhais, Brazil) for 1 min to release sessile cells.

After serial dilutions (10^{-1} to 10^{-4}) performed using 0.85% (isotonic) sodium chloride solution as the diluent, a bacterial count was performed for each coupon using the drop plate technique [25] on XLD agar. The plates were incubated at 37 °C for 24 h under aerobic conditions to count the colony-forming units per square centimeter (CFU•cm^{-2}).

To determine the microbiological count, the surface area on both sides of the coupon and the side area were considered, as shown in the following formula [26, 27]:

$$CFU \bullet cm^{-2} = \left(\frac{VD}{VA}\right) \times AV \times \frac{D}{A}$$

where VD is the diluent volume used for rinsing (5 mL), VA is the aliquot volume used for plating (0.01 mL), Av is the average count recovered from the plates (CFU), D is the dilution used for counting, and A corresponds to the coupon area (2.4 cm^2). The results were expressed as log10 CFU • cm^{-2}.

Statistical analyses

All statistical analyses were performed using GraphPad Prism software (GraphPad, San Diego, CA, USA), with a significance level of 5%. One-way analysis of variance, followed by Tukey's honestly significant difference (HSD) test and *t* test were used to detect differences among the treatments.

Results

Characterization of AgNPs

From the electron microscopy images showed, it was found that the synthesized AgNPs were dispersed in a liquid medium and were mostly spherical in shape (Fig. 1A). The mean diameter was 6.2 ± 3.3 nm (Fig. 1B).

Determination of MIC and MBC of AgNPs

The values for MIC and MBC are shown in Fig. 2. Of the evaluated strains, 50% (5/10) were susceptible to 25 µg/mL AgNP, 20% (2/10) to 18 µg/mL AgNP, 20% (2/10) to 20 µg/mL AgNP, and 10% (1/ 10) to 50 µg/mL AgNP. There was no statistical difference ($p > 0.05$) between MIC (19.50 ± 2.32 µg/mL) and MBC (25.10 ± 9.26 µg/mL) mean values. AgNPs exhibited a statistically significant ($p < 0.05$) concentration-dependent bactericidal action (Fig. 3).

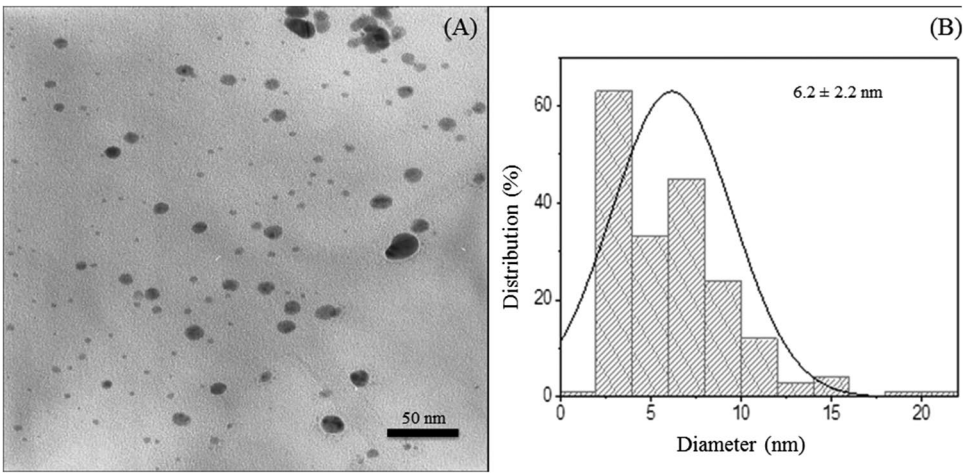

Fig. 1 Silver nanoparticles (AgNPs): **A** Micrograph of AgNPs: dispersion in liquid medium and spherical shape. **B** Distribution histogram of AgNPs by diameter (nm)

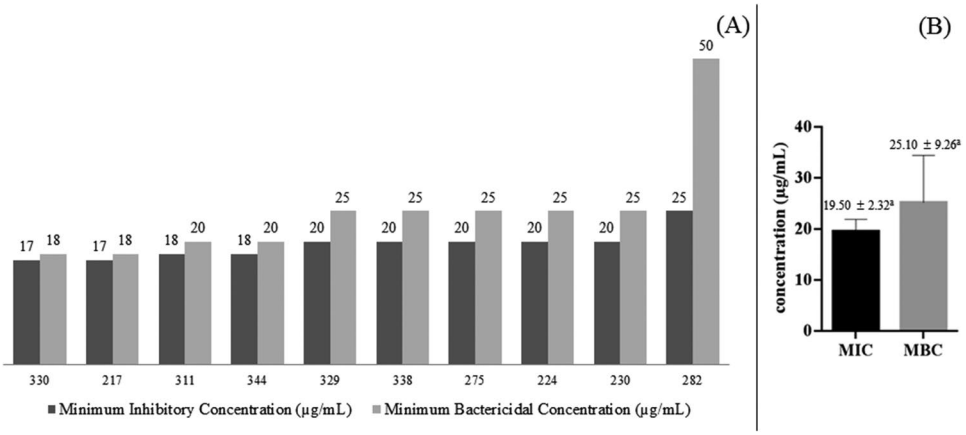

Fig. 2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of silver nanoparticles (AgNPs): **A** individual results. **B** Mean ± standard deviation

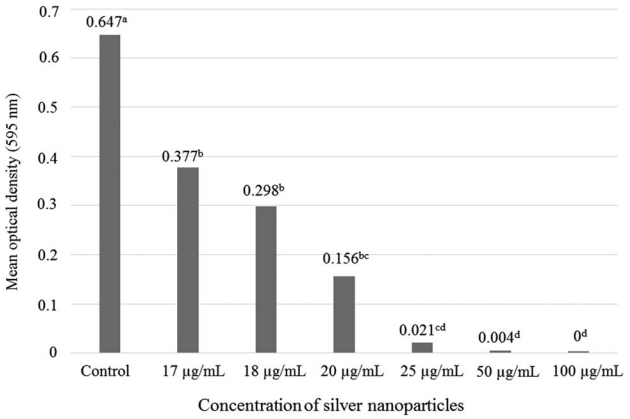

Fig. 3 Mean optical density (OD) reduction in *Salmonella* Enteritidis strains measured after incubating for 24 h at 37 °C to determine the minimum inhibitory concentration. Different letters indicate significant difference (Tukey test; $p < 0.05$) among optical density (OD) observed for different concentration of silver nanoparticles. Mean of three replicates per sample

Removal of formed biofilm on polymeric surfaces

The individual bacterial counts after treatment with AgNPs and sanitizer are shown in Fig. 4. Significant differences ($p < 0.05$) in bacterial counts were observed after 10 min of contact for both the treatment groups compared with those in the control group. The reduction was significantly ($p < 0.05$) higher for AgNPs (3.91 $\log_{10}$ CFU • cm^{-2}) than that for the sanitizer (2.57 $\log_{10}$ CFU • cm^{-2}). After 30 min of contact, AgNPs (1.17 $\log_{10}$ CFU • cm^{-2}) presented a significant reduction ($p < 0.05$) in bacterial count compared to the control (6.84 $\log_{10}$ CFU • cm^{-2}). Based on contact time, the bacterial count after 30 min was significantly lower ($p < 0.05$) than that after 10 min for AgNPs.

Discussion

Salmonella Enteritidis is linked to outbreaks of foodborne diseases worldwide, and poultry products are the main source of contamination [1, 5]. Food-processing plant environments present ideal surfaces for biofilm formation, mainly because of the large amount of available nutrients [10]. In addition, to economic losses, these structures are also a risk to consumer health, because rupture of biofilms is associated with the release of pathogens in food facilities, favoring food contamination [8]. Thus, food-processing establishments must ensure product safety [28].

Surfaces used in poultry production include polyethylene, stainless steel, polypropylene, polyurethane, silicone, and glass [29]. Polyethylene is routinely used during food preparation in industrial plants and is also present in poultry farms, including in transportation crates [30, 31]. The ability of *Salmonella* to produce biofilms on polyethylene has been previously described [11]. Chemical compounds are routinely used in disinfection programs to reduce contamination. However, the role of the environment in the spread of antimicrobial resistance has been widely recognized [32]. Thus, research to identify and develop alternative methods for controlling pathogenic microorganisms in poultry environments has increased in recent years. Among these methods, nanoparticles are a promising alternative, which are gaining special interest [33].

The intrinsic properties of metallic nanoparticles, including AgNPs, are primarily associated with their size, composition, crystallinity, and morphology. Reducing the size of the metal to the nanoscale modifies its chemical, mechanical, electrical, structural, morphological, and optical properties. This is because nanomaterials have

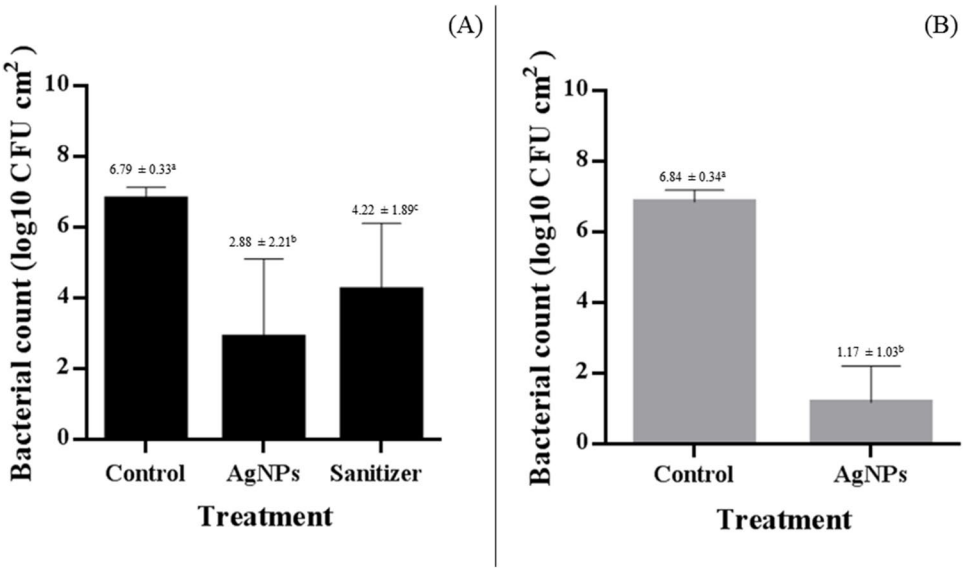

Fig. 4 Bacterial counts of *Salmonella* Enteritidis on polymeric surfaces according to the treatment (AgNPs or sanitizer[1]) after 10 (**A**) or 30 min (**B**) of contact. Different letters indicate significant difference in the same time of contact (Tukey test; $p < 0.05$). [1]Polyhexamethylene biguanide hydrochloride-based sanitizer associated with a quaternary ammonium compound

a considerable number of atoms on their surface, which leads to high surface reactivity [34]. The AgNPs synthesized in this study had a diameter of less than 10 nm, which is considered the optimal maximum value. They were dispersed in a liquid medium and presented a spherical shape. The characteristics observed in the nanoparticles synthesized in this study were in accordance with those recommended in the literature [15]. These characteristics are critical since they are considered ideal for the optimum performance of nanomaterials.

According to Elez et al. [17], AgNPs are promising candidates as replacements for antimicrobial agents owing to their intrinsic characteristics. Large-scale synthesis of AgNPs is simple, safe, and economical, and their surfaces can be easily modified. Another important characteristic of AgNPs is that bacterial resistance to them is rare [17, 35]. Of the evaluated strains, 50% were susceptible to 25 µg/mL of AgNPs. Previous studies have shown the antimicrobial effects of AgNPs at similar concentrations against foodborne pathogens, including *Salmonella* serotypes [17–19]. However, their antibiofilm activities against *S.* Enteritidis remain unclear. In addition, bacteria in biofilms are known to show increased resistant to antimicrobial compounds [36].

Our results demonstrated that AgNPs exhibited antibiofilm activity, aiding the removal of *S.* Enteritidis biofilms in HDPE coupons. Although both treatments (AgNPs and sanitizer) demonstrated antibiofilm activity, the bacterial count after treatment with nanoparticles was significantly lower than that after treating with the sanitizer. The instability of chemical compounds, decreased effectiveness in the presence of organic material, and decreased ability to penetrate the biofilm matrix are some factors that can result in resistance to conventional sanitizers, which are not observed in nanoparticles [37, 38].

For chemical compounds, the reduction in bacterial cells by chemical disinfectants is contact time dependent, and antibiofilm activity usually requires prolonged periods of contact [39]. The results also highlight the influence of contact time on the increased antibiofilm activity of nanoparticles [37]. To evaluate this effect, two contact times (10 and 30 min) were assessed in the present study. Ten minutes was selected to simulate the time required for operational procedures, as determined by Brazilian legislation [40, 41]. Therefore, both the compounds were tested at this contact time. As disinfectants must be removed after sanitization, they were not tested after 30 min. There is no legislation in Brazil regarding the presence of nanoparticles in food or animal production farms. Therefore, we tested the antibiofilm activity of AgNPs after 30 min of contact, to evaluate the effect of time on biofilm removal. For nanoparticles, the contact time influenced the bacterial count reduction, and after 30 min of contact, it was significantly lower than that after 10 min. Contact time plays a significant role in inhibiting pathogen growth [42]. According to the European Union standard EN 13,697:2001, sanitization procedures must achieve a reduction in bacterial counts of at least 4 $\log_{10}$ CFU cm^{-2} on surfaces that are in contact with food [43, 44]. In this study, the antibiofilm effect of AgNPs after 10 min of contact was close to this recommended value; however, after 30 min of contact, the bacterial reduction was more than the recommended value.

AgNPs exhibited antimicrobial and antibiofilm activity for the removal of biofilms produced by *S.* Enteritidis, demonstrating their potential as alternative antimicrobial agents. The bactericidal mechanisms of AgNPs are complex; hence, the risk of bacterial resistance is minimal, making nanoparticles a potential alternative for microbial control in the poultry chain.

Author contribution B. D. E., M. I. Q., T. Q. F., C. T. P. S., H. L. S. M., and V. P. N. conceived and designed the experiments. B. D. E., G. Z. C., and M. I. Q. performed experiments. B. D. E., K. A. B., and T. Q. F. analyzed the data. B. D. E., K. A. B., and T. Q. F. prepared the manuscript. All authors critically reviewed and approved the manuscript.

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Declarations

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