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MICROBIOLOGY

Subinhibitory concentrations of silver nanoparticles and silver nitrate on the adaptative and cross-resistance to antibiotics on bovine mastitis pathogens

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ABSTRACT: *Biocides and/or antibiotics used in subinhibitory concentrations can promote the development of adaptive resistance or even cross-resistance in microorganisms. However, studies on these responses following silver treatments are scarce in the literature. Silver-based compounds, including silver nanoparticles (Ag-NPs), can be an alternative in the prevention and treatment of bovine mastitis. Thus, this research evaluated the effect of subinhibitory dosages of Ag-NPs and Ag^+ ions from silver nitrate ($AgNO_3$) on Staphylococcus aureus and Escherichia coli isolated from milk of cows with mastitis. Ag-NPs were synthesized by chemical reduction using $AgNO_3$ and sodium citrate and the minimum inhibitory concentration (MIC) of Ag-NPs and Ag^+ ions on the mastitis pathogens were determined. Isolates were exposed to subinhibitory concentrations of Ag-NPs or $AgNO_3$ for 10 consecutive days to verify the development of adaptive resistance evaluated by changes in the MIC values. The development of cross-resistance with antibiotics was also studied, being verified by comparing the sensitivity profile of treated cells with non-treated cells. $AgNO_3$ was more effective against all isolates. There was no change in the MIC values or in the antibiotic sensitivity profile for both bacteria following consecutive exposure to subinhibitory dosages of Ag-NPs or $AgNO_3$, indicating that silver was not able to select adaptive resistance or cross resistance to the tested antibiotics. The potential of silver presented by these results is favorable to the continuity of studies aiming to elaborate silver-based therapies for the treatment of bovine mastitis.*
Key words: *adaptive resistance, antibiotics, microbial resistance, Staphylococcus aureus, Escherichia coli.*

Concentrações subinibitórias de nanopartículas de prata e nitrato de prata na resistência adaptativa e cruzada com antibióticos em patógenos de mastite bovina

RESUMO: *Biocidas e/ou antibióticos em concentrações sub-inibitórias podem promover o desenvolvimento de resistência adaptativa ou mesmo resistência cruzada nos micro-organismos. Entretanto, estudos destas respostas após o tratamento com a prata são escassos na literatura. Compostos a base de prata, incluindo as nanopartículas de prata (Ag-NPs), podem ser uma alternativa na prevenção e/ou tratamento de mastite bovina. Assim, este trabalho objetivou determinar o efeito de doses sub-inibitórias de Ag-NPs e dos íons Ag^+ provenientes do nitrato de prata ($AgNO_3$) sobre isolados de Staphylococcus aureus e de Escherichia coli, provenientes de leite de vacas com mastite. As Ag-NPs foram sintetizadas por redução química utilizando $AgNO_3$ e citrato de sódio e a Concentração Mínima Inibitória (CMI) das Ag-NPs e íons Ag^+ nos patógenos da mastite foi determinada. Os isolados foram expostos a concentrações sub-inibitórias de Ag-NPs ou de $AgNO_3$ por 10 dias consecutivos para verificar o desenvolvimento de resistência adaptativa à prata pela mudança no valor da CMI, e de resistência cruzada com antibióticos pela mudança no perfil de sensibilidade em relação ao controle. $AgNO_3$ apresentou-se mais efetivo contra todos os isolados. Não foi verificada alteração no valor da CMI nem do perfil de sensibilidade aos antibióticos, indicando que não houve seleção de resistência adaptativa à prata e de resistência cruzada aos antibióticos pelos micro-organismos estudados. O uso potencial da prata apresentado nos resultados é favorável à continuidade dos estudos objetivando a elaboração de terapias à base de prata para o tratamento da mastite bovina.*
Palavras-chave: *resistência adaptativa, antibióticos, resistência microbiana, Staphylococcus aureus, Escherichia coli.*

INTRODUCTION

Mastitis, the inflammatory process of the mammary gland, is an important disease in dairy cattle and is characterized by decreased production and changes in the composition of milk (WANG et al., 2017), causing great losses to farmers and industries. In general, it is caused by infectious agents such as algae, yeast, mycoplasmas and, mainly, bacteria (SANKAR, 2016). The main treatment for mastitis is antibiotic therapy; however, this method has some limitations due to the increased occurrence

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of microbial resistance and possible presence of antibiotic residues in the milk, which is a serious public health problem. Therefore, alternative to antibiotics in the treatment of bovine mastitis have been suggested, including the use of nanoparticles, vaccines, bacteriophages, medicinal plants and bacteriocin (SANKAR, 2016; MUSHTAQ et al., 2018; LASAGNO et al., 2019; ALGHARIB et al., 2020).

Some microorganisms are naturally resistant to certain antimicrobial agents, while others can develop mechanisms to protect themselves, such as adaptive resistance (GALÁN et al., 2013). Adaptive resistance occurs due to the microorganism's ability to adapt and modify its phenotype to develop resistance to certain stressors. It is characterized by an increase in bacterial resistance to inhibitory or lethal doses of an antimicrobial agent due to pre-exposure to a subinhibitory concentration that does not inhibit bacterial growth, but can activate certain mechanisms, resulting in greater resistance to this antimicrobial agent (PATEL & LEVITIN, 2014). Cross-resistance is another microbial response that happens when microorganisms pre-exposed to subinhibitory dosages of a single biocide become resistant to many other structurally and functionally unrelated antimicrobials (MAVRI & SMOLE MOŽINA, 2013).

Silver-containing compounds have been used for years due to their antimicrobial properties, and were widely used before the introduction of antibiotics in the early 20th century (MIRSATTARI et al., 2004). According to PAL et al. (2007), silver attacks microbial cells by multiple mechanisms, making them difficult to develop resistance, compared to conventional antibiotics that generally exert their antimicrobial effect at a specific target. According to these authors, microorganisms would only protect themselves from silver if they were able to develop multiple mutations simultaneously. In recent years, silver nanoparticles (Ag-NPs) have emerged as the most used antimicrobial nanomaterial in various consumer products such as textiles, personal care products, medical devices, dental and wound healing applications (EDWARDS-JONES, 2009; BALLOTTIN et al., 2017; BURDUȘEL et al., 2018). Thus, silver-based compounds, such as Ag-NPs, can be used as a relevant alternative in the prevention and/or treatment for bovine mastitis.

There are several studies in the literature reporting the effect of subinhibitory dosages of antibiotics and other biocides on the development of microbial resistance (OTTO et al., 2013; KUMARI et al., 2014; ROCH et al., 2014; BENGTSSON-PALME & LARSSON, 2016); however, studies on the effect of subinhibitory dosages of silver on this physiological response are scarce in the literature. In this context, the objective of this research evaluated the antimicrobial activity and development of adaptive and cross-resistance induced by subinhibitory concentrations of Ag-NPs and $AgNO_3$ in *S. aureus* and *E. coli* isolated from bovine mastitis.

MATERIALS AND METHODS

Antimicrobial agents

The Ag-NPs used in this study were synthesized by chemical reduction of silver nitrate ($AgNO_3$) (Sigma-Aldrich, USA) with sodium citrate (Sigma-Aldrich, USA), as previously described by Monteiro et al. 2009, with minor modifications. The synthesized Ag-NPs were characterized by UV-vis spectroscopy in a wavelength ranging from 290 to 700 nm using a quartz cuvette with 10 mm optical path (spectrophotometer model UV-1601 PC Shimadzu, Japan), transmission electron microscopy (TEM) (FEI Tecnai G2-20 SuperTwin, USA), dynamic light scattering (DLS) and zeta potential (ζ) (Zetasizer Nano ZS, Malvern Instruments, UK).

The antibiotic disks were purchased from Cefar (São Paulo, Brazil). The following antibiotics were used for *S. aureus*: cephalexin 30 µg (CFE), tetracycline 30 µg (TET), streptomycin 10 µg (EST), gentamicin 10 µg (GEN), ciprofloxacin 5 µg (CIP), and norfloxacin 10 µg (NOR) (SAEKI et al., 2011). For *E. coli*, ampicillin 10 µg (AMP), cephalexin 30 µg (CFE), ceftiofur 30 µg (CTF), enrofloxacin 5 µg (ENO), gentamicin 10 µg (GEN) and cotrimoxazole 25 µg (SUT) were used as suggested for the treatment of environmental mastitis (COSTA et al., 2014).

Bacterial strains and culture conditions

Experiments were carried out on eight *S. aureus* isolates obtained from the Mastitis Pathogens Culture Collection, maintained at Embrapa Dairy Cattle Research Center (Juiz de Fora, Minas Gerais, Brazil) and eight *E. coli*, belonging to the Bacterial Diseases Laboratory of the Veterinary Department of the Federal University of Viçosa (Viçosa, Minas Gerais, Brazil), all of them isolated from milk of cows with clinical mastitis. Stock cultures were kept at -80 ºC in microtubes containing Brain Heart Infusion broth (BHI) (Himedia, India) added of 20% glycerol. Before tests, all isolates were grown twice in BHI at 37 ºC for 18 h. Then, the bacterial suspensions were centrifuged at 4000 x *g* for 5 min at 4 ºC. The supernatant was discarded and the cells were washed twice in 10 mL 0.85% $m \cdot v^{-1}$ saline solution. The inoculum was prepared by directly suspending the

obtained pellet in saline solution. The absorbance was adjusted to the range of 0.08 to 0.10 at 625 nm using a spectrophotometer (Kazuaki IL227, China), which corresponds to the McFarland standard of 0.5, which contains approximately 1.0 x 10^8 CFU·ml^{-1}.

Minimum Inhibitory Concentration (MIC)

The MIC of Ag-NPs and $AgNO_3$ on the mastitis isolates was determined by broth microdilution test according to the methodology proposed by the Clinical and Laboratory Standards Institute (CLSI, 2003). For this purpose, serial dilutions ranging from 50 to 0.098 µg·ml^{-1} of Ag-NPs or $AgNO_3$ were made in Müller-Hinton broth (Himedia, India) in 96-wells polystyrene microtiter plates. Bacterial inoculum was prepared as previously described in order to obtain an initial concentration of approximately 5.0 x 10^5 CFU·ml^{-1} in each well. Plates were incubated in an automatic microplate reader (Multiskan GO 1510, Thermo Scientific, Finland) at 37 ºC for 18 h, with reading every 60 min. The MIC was determined as the lowest concentration in which there was no microbial growth after 18 h of incubation.

Adaptive resistance

Experiments were performed according to SANTANA et al. (2012), with minor modifications. To verify the development of adaptive resistance to Ag-NPs and $AgNO_3$, the bacteria were transferred 10 times, after incubation at 37 ºC for 24 h, in Müller-Hinton broth supplemented with Ag-NPs or $AgNO_3$ in subinhibitory concentration (0.25 x MIC). The same procedure was performed in Müller-Hinton broth without antimicrobials (control). After this procedure, bacteria adapted with Ag-NPs or $AgNO_3$ in subinhibitory dosages and non-adapted cells (control), were exposed to the previous inhibitory concentrations of these antimicrobials (MIC values) and growth was monitored by reading the optical density at 600 nm, at every 60 min, during 18 h at 37 ºC. Results obtained were compared to the previously performed with cells not exposed to subinhibitory dosages of Ag-NPs or $AgNO_3$.

Determination of cross-resistance with antibiotics

To verify the development of cross-resistance with antibiotics, the antibiogram was performed with cells adapted to subinhibitory dosages of Ag-NPs or $AgNO_3$ and cells not adapted (control). For this experiment, four isolates of *S. aureus* and four of *E. coli* were randomly selected. After pre-exposure of the bacteria for 10 consecutive days, in Müller-Hinton broth supplemented with Ag-NPs or $AgNO_3$ at subinhibitory concentration (0.25 x MIC), disk-diffusion sensitivity test was performed according to (CLSI, 2015) with adapted and non-adapted cells to verify changes in the antibiotic sensitivity profile. After sterilization at 121 ºC for 15 min, Müeller-Hinton agar (Himedia, India) was cooled to 45 ºC and 25 mL were distributed in 90 mm diameter Petri dishes, to ensure a uniform depth of approximately 4 mm. Bacterial inoculum, prepared as previously described, was spread on the agar surface and then, 6.35 mm diameter discs impregnated with the antibiotics were placed on the agar. Plates were incubated at 37 ºC for 18 h and the inhibition halos were determined using a millimeter rule. The bacteria were classified as sensitive (S) or resistant (R) to antibiotics, before and after the adaptation treatment according to the Sensifar and Multifar-cefar® manual.

Data analysis

The results of the halo sizes were subjected to analysis of variance (ANOVA), and the means compared by Tukey test at 5% probability. Statistical analyses were performed in the Statistical Analysis System (SAS Institute, North Carolina, USA), version 9.1, licensed to Federal University of Viçosa.

RESULTS AND DISCUSSION

The dispersion of synthesized Ag-NPs presented typical surface plasmon resonance with a peak at 418 ± 3,2 nm confirming their formation (Figure 1A). The images observed by TEM (Figure 1B) revealed that the Ag-NPs are approximately spherical with size around 5 nm. The mean size determined by DLS and the ζ potential of Ag-NPs was 3.4 ± 1.2 and - 31.9 ± 8.6 mV, respectively.

Despite the high level of astigmatisms and low contrast of the TEM image, the presence of nanoparticulate material in the sample can be observed. These findings are supported by the results of DLS and the UV-vis peak, which is compatible with the silver nanoparticle plasmon resonance.

The MIC value of Ag-NPs was 50 µg·ml^{-1} for all eight *S. aureus* isolates and 25 µg·ml^{-1} for all eight *E. coli* isolates. Other studies have also reported greater efficiency of Ag-NPs against gram-negative bacteria than gram-positive, associated with the structural difference in the cell wall between these two groups of bacteria (KIM et al., 2007; VU et al., 2018). In a study conducted by FAYAZ et al. (2009) the MIC value was 80 µg·ml^{-1} and 65 µg·ml^{-1} for the gram-positive bacteria *S. aureus* and *Micrococcus luteus,* respectively, whereas the MIC was 30 µg·ml^{-1} and 35 µg·ml^{-1} for *E. coli* and *Salmonella* Typhi,

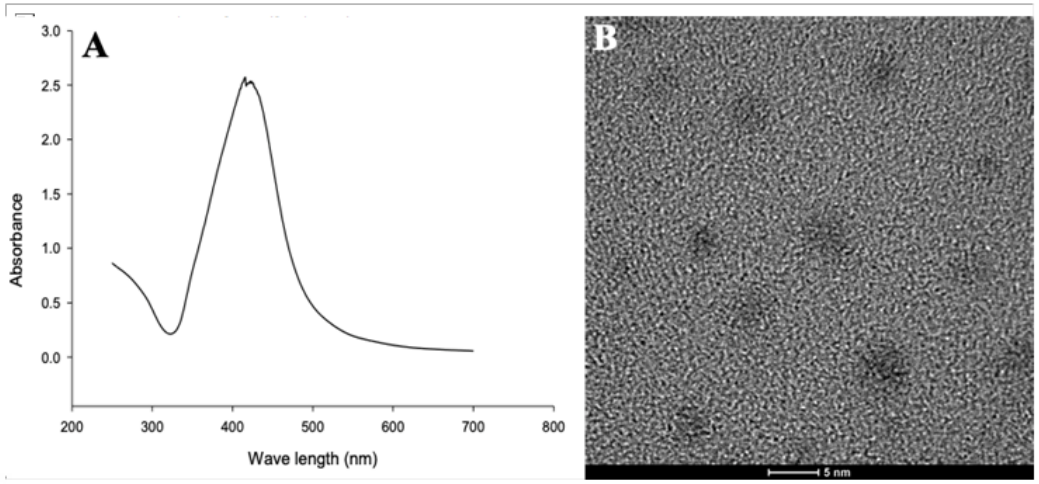

Figure 1 - Characterization of the synthesized silver nanoparticles. (A) UV-visible spectrum of the silver nanoparticles. (B) Transmission electron microscopy images of the silver nanoparticles.

respectively. The result highlighted the antimicrobial potential of Ag-NPs against *E. coli* since based in their major resistance in comparison with gram-positive bacteria, considering their cellular envelope differences, several strategies have been used on the gram-negative bacteria control (BREIJYEH et al., 2020). The MIC of $AgNO_3$ was 12.5 $\mu g \cdot ml^{-1}$ for both bacterial species. In a study performed by XIU et al. (2011), a greater efficiency of $AgNO_3$ in relation to Ag-NPs was observed against *E. coli*. These authors have attributed the higher toxicity of $AgNO_3$ in comparison with Ag-NPs to the greater bioavailability and potential for uptake of Ag^+ ions of the salt.

After adaptation of *S. aureus* and *E. coli* isolates following consecutive exposition to subinhibitory dosages of Ag-NPs or $AgNO_3$, the development of resistance to silver was evaluated by assessing changes in the MIC values. The adapted cells of *S. aureus* and *E. coli* did not grow when they were exposed to the previous inhibitory dosages of the antimicrobials (MIC) at 37 ºC for 18 h, indicating that both species did not develop adaptive resistance to silver.

As presented in table 1, all *S. aureus* isolates were sensitive to the tested antibiotics, before and after the adaptation treatment with subinhibitory dosages of the antimicrobials, except for the isolates 4051 and 4075, which were resistant to tetracycline before and after adaptation. Resistance to tetracycline by *S. aureus* isolates has been reported in the literature. COSTA et al. (2013) reported variation in the resistance profile of *S. aureus* exposed to some products routinely used in the treatment of bovine mastitis, including tetracycline. In a study by CARVALHO et al. (2018), 33.33% of *S. aureus* isolated from raw milk were resistant, 44,44% sensitive and 22.22% showed intermediate resistance to tetracycline.

There was as a significant reduction ($P < 0.05$) in the diameter of the inhibition halo of isolate 4236 following adaptation to $AgNO_3$; however, this reduction was not sufficient to change the sensitivity profile of that bacterial isolate to the antibiotic, maintaining the status of sensitive (Table 1). Therefore, it is important to note that there was no change in the sensitivity profile of *S. aureus* isolates to the antibiotics tested after exposure to subinhibitory dosages of Ag-NPs or $AgNO_3$, indicating that these isolates did not develop cross-resistance to the tested antibiotics.

In a research carried out by BEHIRY et al. (2012), *S. aureus* strains isolated from bovine mastitis were exposed to subinhibitory concentrations of commercial disinfectants based on chlorhexidine digluconate or nonoxynol-9 iodide complex (iodophor), used in the cleaning of the ceilings. The authors observed that the majority of the isolates (90 %) developed resistance (increased MIC) to iodophor, after ten repeated passes in subinhibitory concentrations of this antimicrobial. However, only

Table 1 - Diameter of the inhibition zone (mm) and standard deviation of non-adapted *Staphylococcus aureus* isolates (Control) and adapted to subinhibitory doses of silver nanoparticles (Ag-NPs) and silver nitrate ($AgNO_3$), profile of sensitivity (P), being sensitive (S) or Resistant (R).

Isolates	CEF 30 µg	P*	TET 30 µg	P	EST 10 µg	P*	GEN 10 µg	P	CIP 5 µg	P	NOR 10 µg	P
----------Antibiotics----------												
----------Control----------												
3984	30.7 ± 2.5 [a]	-	25.8 ± 7.7 [a]	S	19.3 ± 2.6 [a]	-	19.0 ± 0.4 [a]	S	25.0 ± 2.9 [a]	S	21.0 ± 1.2 [a]	S
4051	33.8 ± 3.6 [a]	-	11.3 ± 2.6 [a]	R	10.0 ± 0.0 [a]	-	21.0 ± 0.5 [a]	S	25.0 ± 1.7 [a]	S	22.0 ± 0.7 [a]	S
4075	32.5 ± 0.5 [a]	-	8.0 ± 3.0 [a]	R	8.0 ± 0.8 [a]	-	21.0 ± 0.5 [a]	S	31.0 ± 0.3 [a]	S	27.0 ± 1.8 [a]	S
4236	35.0 ± 0.5 [a]	-	23.7 ± 9.6 [a]	S	15.0 ± 2.0 [a]	-	19.7 ± 0.5 [a]	S	32.0 ± 1.8 [a]	S	26.0 ± 7.2 [a]	S
----------Cells adapted to silver nanoparticles (Ag-NPs)----------												
3984	28.8 ± 0.8 [a]	-	24.0 ± 5.5 [a]	S	16.0 ± 1.5 [a]	-	16.0 ± 1.8 [a]	S	25.0 ± 1.3 [a]	S	21.0 ± 0.3 [a]	S
4051	26.0 ± 5.5 [a]	-	10.0 ± 0.8 [a]	R	9.0 ± 0.5 [a]	-	21.0 ± 2.5 [a]	S	30.0 ± 3.8 [a]	S	24.0 ± 2.7 [a]	S
4075	39.0 ± 2.0 [a]	-	11.0 ± 1.0 [a]	R	8.0 ± 0.3 [a]	-	19.0 ± 0.8 [a]	S	29.0 ± 4.0 [a]	S	25.0 ± 4.8 [a]	S
4236	35.8 ± 0.8 [a]	-	33.0 ± 0.2 [a]	S	19.0 ± 0,0 [a]	-	20.0 ± 0.0 [a]	S	33.5 ± 3.5 [a]	S	33.0 ± 2.5 [a]	S
----------Cells adapted to Silver Nitrate ($AgNO_3$)----------												
3984	30.5 ± 3.8 [a]	-	29.0 ± 1.8 [a]	S	18.0 ± 0.3 [a]	-	20.0 ± 0.5 [a]	S	24.0 ± 0.5 [a]	S	22.0 ± 1.3 [a]	S
4051	33.2 ± 3.8 [a]	-	10.0 ± 2.8 [a]	R	10.0 ± 0.5 [a]	-	19.0 ± 0.2 [a]	S	25.0 ± 0.5 [a]	S	24.0 ± 2.3 [a]	S
4075	32.5 ± 0.5 [a]	-	12.0 ± 0.8 [a]	R	9.0 ± 0.8 [a]	-	20.0 ± 0.2 [a]	S	25.0 ± 0.0 [a]	S	22.0 ± 0.0 [a]	S
4236	32.8 ± 3.1 [a]	-	27.0 ± 4.8 [a]	S	13.0 ± 1.8 [a]	-	18.0 ± 0.0 [b]	S	28.5 ± 1.5 [a]	S	26.0 ± 1.3 [a]	S

CEF: cephalexin (30 µg); TET: Tetracycline (30 µg); EST: Streptomycin (10 µg); GEN: gentamicin (10 µg); CIP: ciprofloxacin (5 µg); NOR: Norfloxacin (10 µg). Same letters in the same column do not differ statistically at the 5% level by the Tukey test.

No classification established in the reference manual.

one isolate developed resistance to chlorhexidine digluconate. They also did not observe the development of simultaneous resistance to antibiotics.

All *E. coli* isolates were sensitive to the antibiotics tested before and after the adaptation treatment and there was no statistical difference ($P > 0.05$) in the size of the halos (Table 2). In a study conducted by COSTA et al. (2014), a high sensitivity of *E. coli* isolates from mastitis to the same antibiotics was also observed. As observed for *S. aureus*, all *E. coli* isolates did not develop cross-resistance to antibiotics after adaptation in subinhibitory dosages of Ag-NPs or $AgNO_3$, as there was no change in the sensitivity profile in comparison to the control. Similar results were reported by OLIVEIRA et al. (2017), in which the adaptation of bacteria to subinhibitory concentration of resveratrol did not promote the development of homologous resistance to resveratrol or cross-resistance with benzalkonium chloride or with the tested antibiotics.

In a study conducted by CAPITA et al. (2014), it was reported that the adaptation of *E. coli* ATCC 12806 to subinhibitory concentrations of trisodium phosphate, sodium nitrite and sodium hypochlorite promoted the development of acquired resistance to such biocides, in addition to reducing sensitivity to a range of antibiotics, especially, aminoglycosides, cephalosporins and quinolones, in relation to unexposed cells. Sodium nitrite caused a greater increase in cross-resistance compared to sodium hypochlorite and trisodium phosphate. These results showed that the use of these antimicrobials in subinhibitory concentrations can represent a serious public health problem, which did not occur with the use of Ag-NPs or $AgNO_3$. However, it is important to highlight that since the development of resistance may vary among different species as well as among different serotypes of the same species, tests including other bacterial species and other strains should be performed, as suggested by SOUMET et al. (2016).

CONCLUSION

The silver nanoparticles (Ag-NPs) synthesized in this research were more effective against ***E. coli*** than ***S. aureus***. Conversely, silver nitrate ($AgNO_3$) showed a similar effect against the two bacterial species studied, with lower MIC value compared to Ag-NPs. The adaptation of *S. aureus* and *E. coli* isolates to subinhibitory concentrations of Ag-NPs or $AgNO_3$ for 10 consecutive days did not promote the adaptative resistance to silver or cross-resistance to antibiotics as there was no change in the

Table 2 - Diameter of the inhibition zone (mm) and standard deviation of non-adapted Escherichia coli isolates (Control) and adapted to subinhibitory doses of silver nanoparticles (Ag-NPs) and silver nitrate ($AgNO_3$), profile of sensitivity (P), being sensitive (S) or Resistant (R).

Isolates	AMP 10 µg	P	CEF 30 µg	P	CTF 30 µg	P	SUT 25 µg	P	ENO 5 µg	P	GEN 10 µg	P
----------Antibiotics----------												
----------Control----------												
1	23.0 ± 1.0 [a]	S	23.7 ± 2.2 [a]	S	28.0 ± 2.0 [a]	S	27.0 ± 3.0 [a]	S	29.0 ± 3.0 [a]	S	18.0 ± 1.0 [a]	S
7	21.0 ± 3.0 [a]	S	20.2 ± 1.9 [a]	S	27.7 ± 3.0 [a]	S	27.0 ± 3.0 [a]	S	31.0 ± 1.0 [a]	S	19.0 ± 1.0 [a]	S
19	20.0 ± 2.0 [a]	S	21.8 ± 2.1 [a]	S	29.0 ± 1.1 [a]	S	27.0 ± 1.0 [a]	S	29.0 ± 1.0 [a]	S	20.0 ± 1.0 [a]	S
23	25.0 ± 1.0 [a]	S	22.5 ± 0.7 [a]	S	28.5 ± 2.1 [a]	S	26.0 ± 1.0 [a]	S	31.0 ± 2.0 [a]	S	19.0 ± 1.0 [a]	S
----------Cells adapted to silver nanoparticles (Ag-NPs)----------												
1	24.0 ± 2.0 [a]	S	25.0 ± 1.0 [a]	S	28.0 ± 2.0 [a]	S	26.8 ± 3.3 [a]	S	30.0 ± 1.0 [a]	S	18.0 ± 2.0 [a]	S
7	20.0 ± 3.0 [a]	S	22.0 ± 1.0 [a]	S	25.0 ± 2.0 [a]	S	25.5 ± 3.0 [a]	S	31.0 ± 1.0 [a]	S	18.0 ± 1.0 [a]	S
19	21.0 ± 1.0 [a]	S	24.0 ± 4.0 [a]	S	29.0 ± 1.0 [a]	S	27.3 ± 1.8 [a]	S	31.0 ± 1.0 [a]	S	21.0 ± 1.0 [a]	S
23	25.0 ± 2.0 [a]	S	25.0 ± 1.0 [a]	S	28.0 ± 3.0 [a]	S	27.8 ± 2.3 [a]	S	31.0 ± 2.0 [a]	S	20.0 ± 2.0 [a]	S
----------Cells adapted to Silver Nitrate ($AgNO_3$)----------												
1	23.0 ± 2.0 [a]	S	23.0 ± 2.0 [a]	S	29.0 ± 1.0 [a]	S	30.0 ± 1.0 [a]	S	33.0 ± 2.0 [a]	S	20.0 ± 0.0 [a]	S
7	21.0 ± 4.0 [a]	S	20.0 ± 2.0 [a]	S	25.0 ± 6.0 [a]	S	26.0 ± 1.0 [a]	S	32.0 ± 2.0 [a]	S	18.0 ± 1.0 [a]	S
19	21.0 ± 2.0 [a]	S	22.0 ± 2.0 [a]	S	28.0 ± 1.0 [a]	S	28.0 ± 0.0 [a]	S	28.0 ± 3.0 [a]	S	19.0 ± 2.0 [a]	S
23	24.0 ± 1.0 [a]	S	23.0 ± 1.0 [a]	S	30.0 ± 2.0 [a]	S	27.0 ± 2.0 [a]	S	33.0 ± 1.0 [a]	S	19.0 ± 1.0 [a]	S

AMP: ampicillin; CEF: cephalexin; CTF: ceftiofur; SUT: cotrimoxazole; ENO: enrofloxacin; GEN: gentamicina.
Same letters in the same column do not differ statistically at the 5% level by the Tukey test.

MIC values or in the sensitivity profile of the isolates to the tested antibiotics, in relation to the control.

These results reinforce the potential use of silver on the mastitis-associated bacteria. Further experiments should be performed in order to evaluate the effect of silver nanoparticles (Ag-NPs) and Ag+ ions *in loco* against mastitis disease. Also, combined effect of the Ag-NPs with other antimicrobials would amplify treatment possibilities.

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DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

We have no conflict of interest to declare.

AUTHORS 'CONTRIBUTIONS

All authors contributed to the design and writing of the manuscript. All authors critically reviewed the manuscript and approved the final version.

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