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Anti-Biofilm Action of Biological Silver Nanoparticles Produced by *Aspergillus tubingensis* and Antimicrobial Activity of Fabrics Carrying it

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Abstract: Biological silver nanoparticles (AgNPs) were synthesized using the marine endophytic fungus *Aspergillus tubingensis* and inhibited *Bacillus subtilis* biofilm formation at low concentrations. Cotton and polyester fabrics impregnated with AgNPs were analyzed by transmission electron microscopy (TEM), and the concentration of AgNPs in both fabrics was determined using inductively-coupled plasma atomic emission spectrometry (ICP-AES). The fabrics carrying the AgNPs inhibited the *Staphylococcus aureus* and *Escherichia coli* growth by 100%. Both fabrics impregnated one time with AgNPs inhibited yeasts' clinical species' growth, *Candida albicans*, *Candida glabrata*, and *Candida parapsilosis*, from 80.1% to approximately 98.0%. Besides the anti-biofilm effect, the AgNPs impregnation process on cotton and polyester fabrics was highly efficient, and both fabrics presented antimicrobial effects against clinically relevant bacteria and yeast species. The results evidence that functionalized textiles containing these biological AgNPs can play an essential role in combating pathogenic microorganisms. Thereby offering an alternative to design effective solutions, mainly for hospital garments and biomedical devices, to avoid microorganisms transmissions and hospital-acquired nosocomial infections.

Keywords: *Bacillus subtilis*; anti-biofilm; *Candida* sp.; antimicrobial activity; fabrics.

1. Introduction

Antimicrobial resistance has threatened the lives of a considered number of hospitalized patients worldwide [1]. Metallic nanoparticles (MNPs) possess invaluable applications across different sectors, and the application of silver nanoparticles (AgNPs), in particular, has proved to be advantageous due to their antimicrobial activity against several clinically relevant pathogens [2]. As part of the effort to combat hospital-acquired infections, a high priority topic nowadays, functional materials able to inhibit pathogens' growth are very relevant [3, 4].

While researchers have produced large knowledge about the synthesis of nanomaterials employing physical [5] and chemical [6] methods, high energy costs and the use of chemical

reagents in these conventional methods is a serious problem with high environmental impact [7]. Consequently, biological approaches provide a significant advantage as a means to obtain MNP. There are several options of eco-friendly methods to synthesize nanoparticles, and plants [8] and microorganisms [9, 10] have been the main source of bioactive molecules that can synthesize MNP through metal bioreduction, thereby decreasing their toxicity and bioavailability [11]. Although the synthesis of MNP using bacterial bioactive molecules is prevalent, fungi present numerous advantages related to the process and production of MNP.

Recently, there has been a significant increase in the number of studies of biological MNPs. However, despite our group's scientific contributions concerning the use of *Aspergillus tubingensis* to synthesize AgNPs, these nanoparticles' effects have not been comprehensively investigated to date.

Previously, we have demonstrated that AgNPs efficiently inhibit Gram-positive and Gram-negative bacteria when impregnated on fabrics at low concentrations [9].

The current study is describing the biosynthesis of AgNPs using the fungus *A. tubingensis* followed by the evaluation of cotton and polyester fabrics carrying these AgNPs. Moreover, this study brings new information through the evaluation of the bacterial biofilm inhibition and the fabrics' antimicrobial activity. The application of biological AgNPs, synthesized by *A. tubingensis*, with antibacterial and antifungal activity in textiles is an attractive purpose that can be explored for hospital garments to avoid microorganisms transmissions and hospital infections.

2. Materials and Methods

2.1. Fungus morphology analysis by scanning electron microscopy (SEM).

The fungus *A. tubingensis* was previously isolated from *Rizhophora mangle* following the method previously described [12] and deposited at the Oswaldo Cruz Institute Collection (IOC, Rio de Janeiro, RJ, Brazil) and at the Collection of Microorganisms for Biocontrol of Plant, Pathogens and Weeds from "Embrapa Recursos Genéticos e Biotecnologia (CENARGEN, Brasília, DF, Brazil)" under the number IOC 4684 and CEN1066, respectively. After the growth in potato dextrose agar (PDA), the fungus was fixed with a modified Karnovsky solution (paraformaldehyde at 2.5% and glutaraldehyde at 2.5% in 0.1 M cacodylate buffer (pH 7.2)) and $CaCl_2$ 0.001 M [13]. After the fixation, the cells were washed thrice with the same buffer for 15 min and dehydrated in a graded series of acetone (15%, 30%, 50%, 75%, 95%, 100%) for 15 min. The last step was repeated three times, and after that, the sample was subjected to critical point drying with CO_2 (Leica CPD 030). Samples were covered with a gold film (in Sputter EMITEC for 4 min under 25 mA) and examined by SEM (LEO Gemini 982, SEM at an accelerating voltage of 10 kV). Images were acquired by secondary electron analysis and analyzed concerning modifications or ultrastructure arrangements in the cell morphology.

2.2. Silver nanoparticles biosynthesis.

The *A. tubingensis* fungus was grown in PDA at 28 °C for a week and used for the AgNPs biosynthesis, as previously reported [2]. The AgNPs biosynthesis was monitored using UV-VIS spectrophotometer (Agilent 8453) from 200 until 800 nm during 96 h, and the AgNPs were characterized using transmission electron microscopy (TEM), average diameter size

determination, size distribution, and zeta potential by photon correlation spectroscopy NanoSizer (Malvern Instruments Corp., Worcestershire, UK) [2].

2.3. Impregnation of silver nanoparticles on cotton and polyester fabrics by the Padding method.

To impregnate the AgNPs on cotton and polyester fabrics, the material was first washed, sterilized in an autoclave, and dried at room temperature (RT). Fabrics measuring 10 cm^2 were immersed into 10 mL of AgNPs dispersion until complete wetting and subsequently compressed between two rolling pins, using a rolling machine (Marcato, Padova, Italy). The fabrics were impregnated with AgNPs one, two, and four times and dried at RT protected from light for 24 h.

2.4. Concentration of silver in the AgNPs and on the impregnated cotton and polyester fabrics.

The silver concentration in the nanoparticles and on the impregnated fabrics was determined by inductively coupled plasma atomic emission spectrometry (ICP OES5100 VDV, Agilent Technologies, Tokyo, Japan) by comparison with a standard calibration curve prepared with silver, as previously reported [9].

2.5. Analysis of cotton and polyester fabrics morphology by SEM.

The morphology of the cotton and polyester fabrics with and without the AgNPs incorporation was analyzed using SEM and images were acquired (JEOL JSM - T300 microscopy, Tokyo, Japan) at 20 kV with secondary electron detectors. Samples were prepared using aluminum support (stubs) covered with a double-sided tape recovered with gold by 100 s. Images were recorded by a Proscan high-speed slow-scan CCD camera and processed in the Analysis 3.0 system.

2.6. Antimicrobial activity of fabrics carrying silver nanoparticles.

The antimicrobial activity assays were performed as previously reported [9]. To determine the antibacterial activity, the cotton and polyester fabrics (1 cm^2) carrying the AgNPs were incubated with suspensions of a mid-logarithmic phase culture of *Escherichia coli* (ATCC 25922) or *Staphylococcus aureus* (ATCC 25923) in a poor-broth nutrient medium (1 % bacto-tryptone and 0.5% (w/v) NaCl) in the concentration of 1×10^5 CFU/mL. Cultures were carried out in triplicate at 30 °C and 150 rpm, and the bacterial growth was determined after 24 h by the absorbance of the suspensions at 595 nm. Gentamicin at 8 and 16 μg/mL (17.0–34.0 μM) was used as a positive control, and untreated bacteria were used as the negative control. The percentage of bacterial growth inhibition was calculated by comparison with the negative control.

To evaluate the antifungal activity, fragments of the fabrics (1 cm^2) were placed in glass tubes with 0.8 mL of distilled sterile water and mixed for 10 min in a rotary stirrer (Labnet 211DS, Edison, New Jersey, USA) at 220 rpm. Each sample received 2.2 mL of potato dextrose (PD) broth and was incubated with 10 μL of a suspension of *Candida albicans* (ATCC 36802), *Candida glabrata* (IOC 4565), or *Candida parapsilosis* (IOC 4564) at 1.87 x 10^5 CFU/mL (prepared according to a standard curve previously established in our laboratory), at 32-35 ºC and 220 rpm for 24 h. After incubation, 10 μL of each suspension was serially diluted, and

each dilution was incubated in a dish plate containing PDA at 32 ºC for at least 48 h. The score of CFU/mL was counted and calculated by comparing it against the negative control.

The pathogens *C. glabrata* (IOC 4565) and *C. parapsilosis* (IOC 4564) are clinically isolated strains from the Microbiology Department at Sao Paulo Federal University (Sao Paulo, Brazil) deposited at the Oswaldo Cruz Institute Collection (IOC, Rio de Janeiro, RJ, Brazil).

2.7. Anti-biofilm action of silver nanoparticles - analysis by SEM.

The bacterium *Bacillus subtilis* was cultivated on Müller-Hinton agar at 37 °C, and a suspension in PBS (pH 7.2), with an optical density of 1.0 at 600 nm, was prepared. To analyze the biofilm formation, 2 mL of this suspension was added to each well of a 12 well microplate containing sterile coverslips of 13 mm diameter in each well. Each well was treated with 10 µL of $AgNO_3$ or diluted AgNPs, resulting in final concentrations of 8, 16, and 32 µM, and the plate was incubated at 28 °C at 150 rpm for 24 h. Controls were performed with untreated bacterial cells, or bacterial cells treated with streptomycin/penicillin (2,500 UI/mL/2.5 mg/mL).

For the SEM, the *B. subtilis* was treated with 0.01, 0.1, 1, 10, and 50 µM of AgNPs or $AgNO_3$ as control. Samples were fixed and processed as described above and were dehydrated in a graded series of ethanol solution (30–100%). The critical point drying was performed with CO_2 in a CPD 030 equipment (Leica Microsystems, Heerbrugg, Switzerland), and samples were covered with a gold film and observed using SEM (FEI QUANTA 250 SEM, Netherlands) and accelerating voltage of 10 kV. Images were obtained by secondary electron analysis [14] and analyzed considering modifications or ultrastructure arrangements on the cell morphology.

3. Results and Discussion

3.1. A. tubingensis morphology by SEM.

Although the fungus *A. tubingensis* strain had been previously identified by molecular analysis [2], in this study, we are showing its morphology by SEM (Figure 1). The fungus isolates proliferated rapidly on PDA. The colonies were white initially, turned greyish, and finally black within 3-4 days. Conidiophores and conidia were present in a high amount, and there was high sporulation. The morphological characteristics are according to *Aspergillus* sp. previous identification [15].

Figure 1. Scanning electron microscopy of *A. tubingensis* (IOC 4684 / CEN1066). Samples were covered with a gold film and examined with an FEI QUANTA 250 SEM, an accelerating voltage of 5 kV. Bars are at 16 µm.

3.2. Silver nanoparticle formation, the concentration of silver in it, and in the impregnated cotton and polyester fabrics.

The formation of AgNPs was monitored by UV-Vis spectroscopy for at least 96 h. The brownish color formation, together with the surface plasmon resonance (SPR) band around 420-430 nm was observed and is evidence of AgNPs formation. Nanoparticles size observed employing TEM was 35 ± 10 nm, uniform and equitably distributed, as first reported [2] (data not showed).

The silver concentration present in the AgNPs determined by ICP OES was 93 ± 23 mg, corresponding to 86.2 ± 21.3% of the theoretical value (107.9 mg). This data is related to the fungal filtrate reaction's efficacy, with $AgNO_3$ showing a high reaction yield.

AgNPs incorporation on the fabrics was effective. However, there was a substantial difference in AgNPs impregnations between cotton and polyester. The percentage of AgNPs incorporated on the cotton fabric was 2.7%, 2.7%, and 12.5%, with one, two, and four impregnations, respectively.

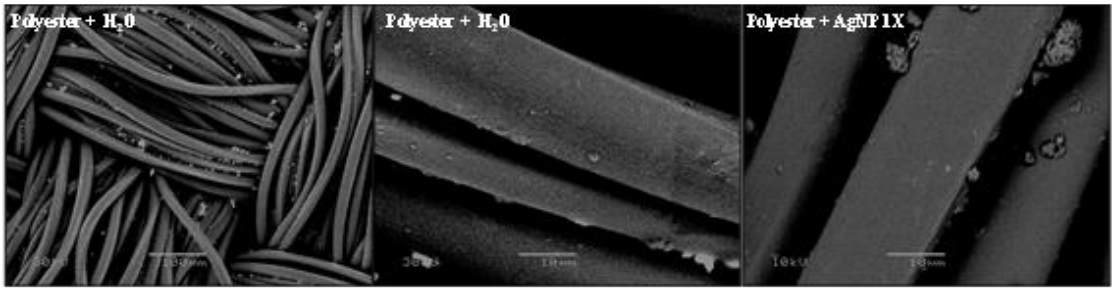

Figure 2. Scanning electron microscopy (SEM) images of the polyester fabrics without impregnation (polyester + H_2O) and impregnated with AgNPs by one and four times. Polyester + H_2O (100 and 10 μm); polyester + AgNPs 1x (10 μm).

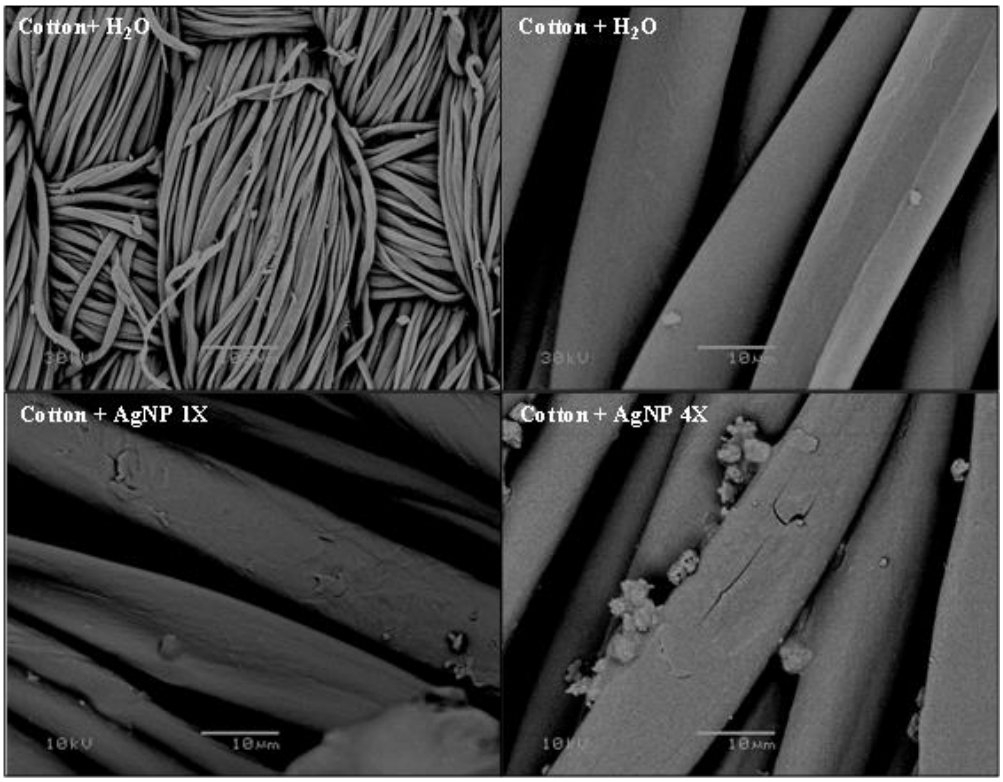

Figure 3. Scanning electron microscopy of the cotton fabrics without impregnation (cotton + H_2O) and impregnated with AgNPs by one and four times. Cotton + H_2O (100 and 10 μm); cotton + AgNPs 1x (10 μm); cotton + AgNPs 4x (10 μm).

For polyester fabric, the impregnation pointed out to uniform AgNPs incorporation reaching 9.9%, 9.3%, and 8.4% when impregnated one, two, and four times, respectively. As can be observed, there was no significant variation among the repetitions.

Figures 2 and 3 represent the SEM of polyester and cotton fabrics before and after incorporating AgNPs. It is possible to observe the AgNPs on the surface of the fabrics. It can be seen that the amount of AgNPs adhered to the cotton fabric surface that suffered four impregnations, as expected, is higher than other impregnation procedures.

Most likely, there was a saturation of the polyester fabric after the first impregnation. This was not observed on cotton, in which the most efficient impregnation process was repeated four times. The AgNPs adhered on the fabric's surface, mainly for the cotton fabric impregnated four times, as observed by SEM. The profile of AgNPs incorporation in cotton fabric is similar to that obtained using biological AgNPs from *Fusarium oxysporum* [16].

3.3. Antimicrobial activity of fabrics impregnated with AgNPs.

Previously, the antimicrobial activity of biogenic AgNPs produced by *A. tubingensis* on Gram-negative and Gram-positive bacteria and on several species of clinical strains of *Candida* sp. was reported, and the nanoparticles showed high antibacterial and antifungal effect [2].

In this study, both fabrics impregnated only once with AgNPs inhibited *S. aureus* and *E. coli* growth by 100%.

C. albicans is usually present in infections caused by yeasts, and due to that, both fabrics, cotton and polyester, carrying the AgNPs were first tested on this yeast species. The cotton impregnated with AgNPs one, two, and four times inhibited the *C. albicans* by 80.1, 80.6, and 83.5%, respectively, while the polyester fabric effect was similar, inhibiting 86.3, 85.1, and 76.2% of the yeast growth, respectively.

The antifungal effect of both fabrics carrying AgNPs impregnated one time was also observed on *C. parapsilosis* and C. *glabrata*, which were inhibited by 96.9 and 97.7%, respectively. On *C. albicans* and *C. parapisilosis*, the polyester fabric was more effective than the cotton and inhibited the growth efficiently by 86.3% and 97.7%. The inhibition induced by the cotton fabric carrying AgNPs on both yeasts was 80.1% and 96.9. The cotton fabric carrying the AgNPs was more effective against *C. glabrata* and inhibited the growth by 97.9%, whereas the polyester inhibition was 94.2%.

Both fabrics showed remarkable antimicrobial effects against clinically relevant pathogens. Cotton fabric carrying AgNPs in different concentrations presented similar activities against *C. albicans*, and the fabric impregnated four times (12.5% of AgNPs) showed slightly higher growth inhibition. The increase of 4.6 fold in the concentration of AgNPs from one to four impregnations was not relevant for improving the antifungal activity that increased only from 80.1 to 83.5%. Likewise, polyester fabric with similar percentages of impregnated AgNPs also presented high growth inhibition for all impregnations. Thus, in both cases, only one impregnation of the fabrics with AgNPs was enough for achieving the nanoparticles' concentration and provided the significant antifungal effect of AgNPs textiles. Moreover, both fabrics carrying the AgNPs impregnated once efficiently inhibited *C. parapsilosis* and *C. glabrata* growth. The data shows that functionalized textiles were efficient in killing the pathogens, despite carrying AgNPs in low percentage.

The current data corroborate previous data from our laboratory, which show that cotton and polyester fabric carrying biological AgNPs obtained from the fungus *Bionectria*

ochroleuca were very effective in inhibiting pathogenic yeasts and bacteria growth [9]. It is interesting to observe that the impregnation process by only one time is enough for obtaining fabrics carrying AgNPs in concentrations able to kill the yeasts. Besides, it is an advantage for future manufacturing. In a scale-up process, a small number of steps and a lower concentration of AgNPs for impregnation are advantageous to save both money and time, and the data presented here support this perspective.

3.4. Anti-biofilm activity of AgNPs.

The anti-biofilm activity of AgNPs was evaluated against *B. subtilis* exposed to AgNPs in concentrations between 0.01 to 50 µM (Figure 4). AgNPs at 8 µM inhibited the biofilm formation, but this effect did not increase when the bacteria were exposed to higher AgNPs concentration (Figure 5). $AgNO_3$ inhibited biofilm formation in all assayed concentrations as well as the antibiotic streptomycin/penicillin (2,500 UI/mL/2.5 mg/mL – data not showed) used as control.

It is known that biofilm is a reservoir for pathogens in the hospital environment and medical devices and should be carefully and entirely eliminated. Functionalization of materials with AgNPs by coating or impregnation is a promising alternative to avoid microorganisms dissemination and biofilm formation [17].

B. subtilis is a Gram-positive bacterium usually found in the environment; however, it can cause diseases in humans like diarrhea and urinary tract infections, and even fatal infections [18]. Considering its relevance, in this study, the AgNPs were evaluated on *B. subtilis* and showed an anti-biofilm effect, also observed in the SEM results.

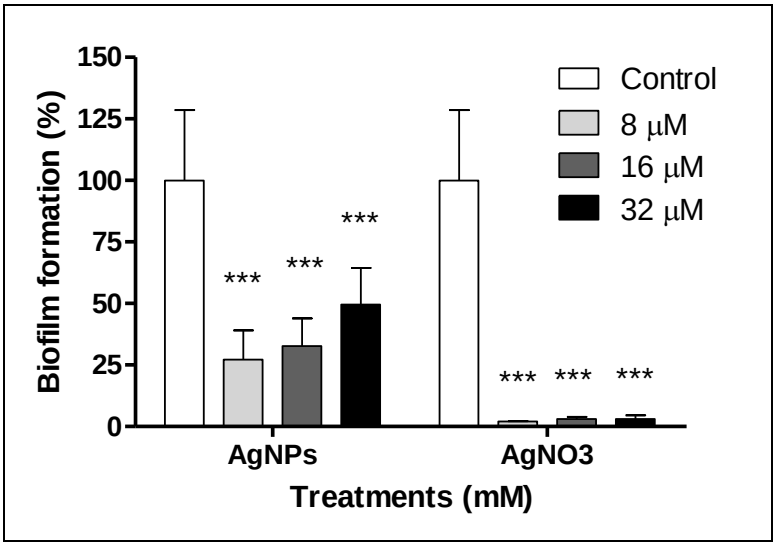

Figure 4. Biofilm formation for *B. subtilis* exposed to 8, 16 and 32 µM of AgNPs obtained from *A. tubingensis* and $AgNO_3$. Controls are only *B. subtilis* culture without treatment. Statistical analysis was performed using GraphPad Prism 5.0 software by two-way ANOVA / Bonferroni's multiple comparison tests. Differences in relation to the control values were considered statistically significant when $P < 0.05$.

Literature data highlights the efficient antibacterial activity of AgNPs, even at low concentrations. AgNPs obtained from aqueous and ethanolic extract of *Andrographis paniculata* stem showed antibacterial effect on *B. Subtilis* [19], and that from exopolysaccharide of the bacterium *Mesoflavibacter zeaxanthinifaciens* also showed an anti-biofilm effect on *B. Subtilis* [18].

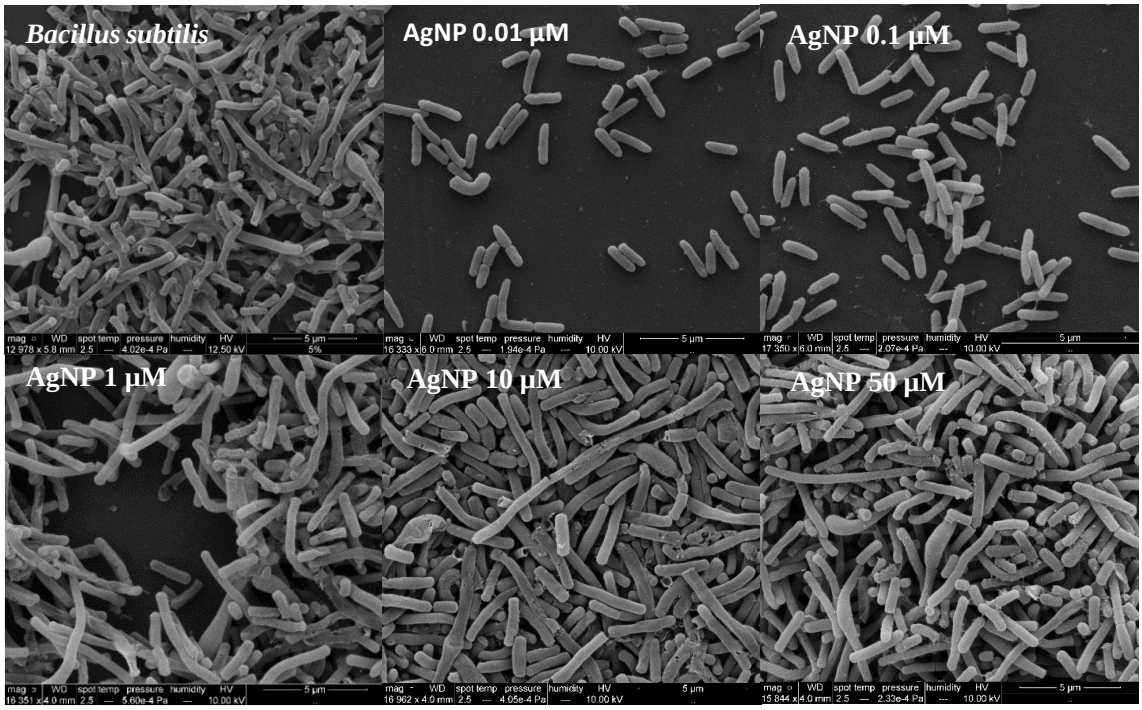

Figure 5. Scanning electron microscopy (SEM) for *B. subtilis* biofilm exposed to 0.01 until 50 µM of AgNPs were obtained from *A. tubingensis*. Samples were covered with a gold film and examined with an FEI QUANTA 250 SEM an accelerating voltage of 10 kV. Bars are at 5 µm.

The antimicrobial activity of nanoparticles is attributed to oxidative stress and the generation of reactive oxygen species (ROS) that cause disruption of microorganisms' membranes and their death [20]. Due to the small sizes, MNPs have a high surface area, and this property is advantageous for inhibiting biofilm formation, which promotes a direct interaction of nanoparticles and cell surfaces.

Biofilms are formed by the association of microorganisms able to maintain the communication and cooperation related to metabolism, gene expression, and virulence [21]. These mechanisms for survival have been adapted through the microorganisms' evolution, increasing their resistance to antimicrobial agents and protection from host defenses [22]. This interaction process among the cells can probably be modified by the presence of small nanoparticles that can penetrate the cells, interrupting communication and metabolism, inducing death as a result.

The biological AgNPs applied in this study have a size of 35 ± 10 nm (TEM) and positive zeta potential on their surface (+7.8) [2]. These properties are interesting to promote the interaction with bacterium cells. Although AgNPs showed an excellent antimicrobial activity and inhibited the biofilm formation at lower concentrations, unexpectedly, biofilm formation was not inhibited at higher concentrations. This result was observed in both analyses, and one hypothesis is that at higher concentrations, saturation and/or aggregation of the AgNPs may have occurred, which could not inhibit the cells' interactions, leading to biofilm formation. It is established that biofilms develop the extracellular matrix (ECM), which has a protective function in limiting the access of antimicrobial agents by physical contact or reducing the penetration rate and consequently the effectivity [23]. This is a reasonable explanation for the phenomenon observed here.

An extracellular polymeric substance such as nucleic acids, proteins, lipopeptides, and poly-gamma-glutamate (PGA) can be secreted by *B. subtilis* [24] and due to their tertiary structure and chemical properties, some of these compounds can react with metals. They can

be applied for bioremediation of contaminated soils [25]. Eymard-Vernain *et al.* [26] showed that synthetic AgNPs surrounded by polyvinylpyrrolidone (PVP) presented no significant effect on the intracellular physiology of *B. subtilis* during the stationary phase and that the secreted bioorganic molecules – PGA - interacted with the AgNPs. PGA may physically interact with the AgNPs by trapping them, thereby decreasing their bioavailability and, consequently, the biocidal effect [26]. It may have occurred when the *B. subtilis* tested herein was exposed to higher concentrations of AgNPs. Perhaps the most stressful condition had stimulated a higher PGA production (or any other specific proteins to resist environmental stresses), neutralizing the AgNP effect, allowing the bacterial growth, as observed when the bacteria were exposed to increasing concentrations of AgNP.

The effect of AgNPs on the biofilm observed in this study agrees with previous data showing that bacteria from mature bacterial biofilm [27, 28] or stationary phase [24] are more resistant to AgNPs. Although no doubt exists about the biological AgNPs anti-biofilm effect at low concentrations, additional studies are necessary to understand its interaction with *B. subtilis* biofilm in different concentrations.

4. Conclusions

This research showed that the impregnation of cotton and polyester fabrics with AgNP was very efficient, and both fabrics showed strong antimicrobial effects against clinically relevant pathogens and excellent antimicrobial activity of the free AgNPs against such pathogens.

Further research could be useful to determine the threshold of the inhibition of the microorganisms' growth by AgNPs, its use even at lower concentrations on fabrics, and better understand the process of antimicrobial and anti-biofilm effect.

It can be anticipated that the applications of functionalized textiles containing AgNPs will play a key role in the near future in combating pathogenic strains, thereby offering an alternative to design effective solutions against hospital-acquired nosocomial infections.

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Conflicts of Interest

The authors declare no conflict of interest.

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