UNIVERSIDAD SAN FRANCISCO DE QUITO
USFQ

Colegio de Ciencias Biológicas y Ambientales

Evaluating Antimicrobial Activity of Silver Nanoparticles and their interaction with *Bursera graveolens* extract by green chemistry on foodborne bacteria

Dayanna Gabriela Cabascango Quishpe

Biotecnología

Trabajo de fin de carrera presentado como requisito
para la obtención del título de
Ingeniera Biotecnóloga

Quito, 20 de diciembre de 2023

UNIVERSIDAD SAN FRANCISCO DE QUITO

USFQ

Colegio de Ciencias Biológicas y Ambientales

HOJA DE CALIFICACIÓN

DE TRABAJO DE FIN DE CARRERA

Evaluating Antimicrobial Activity of Silver Nanoparticles and their interaction with *Bursera graveolens* extract by green chemistry on foodborne bacteria

Dayanna Gabriela Cabascango Quishpe

Nombre del profesor, Título académico Antonio Machado, PhD. en Ingeniería Biomédica

Quito, 20 de diciembre de 2023

© DERECHOS DE AUTOR

Nombres y apellidos: Dayanna Gabriela Cabascango Quishpe

Código: 00212968

Cédula de identidad: 1725209942

Lugar y fecha: Quito, 20 de diciembre de 2023

ACLARACIÓN PARA PUBLICACIÓN

Nota: El presente trabajo, en su totalidad o cualquiera de sus partes, no debe ser considerado como una publicación, incluso a pesar de estar disponible sin restricciones a través de un repositorio institucional. Esta declaración se alinea con las prácticas y recomendaciones presentadas por el Committee on Publication Ethics COPE descritas por Barbour et al. (2017) Discussion document on best practice for issues around theses publishing, disponible en http://bit.ly/COPETheses.

UNPUBLISHED DOCUMENT

Note: The following capstone project is available through Universidad San Francisco de Quito USFQ institutional repository. Nonetheless, this project – in whole or in part – should not be considered a publication. This statement follows the recommendations presented by the Committee on Publication Ethics COPE described by Barbour et al. (2017) Discussion document on best practice for issues around theses publishing available on http://bit.ly/COPETheses.

RESUMEN

Los alimentos contaminados por microorganismos son responsables de causar enfermedades a unos 600 millones de personas, 420.000 de las cuales mueren cada año en todo el mundo. Por ello, es necesario buscar nuevas formas de prevenir los alimentos contaminados para garantizar su seguridad. El uso de nanopartículas de plata (AgNPs) como agentes antimicrobianos es una herramienta que ha cobrado interés porque poseen características únicas, en particular una mayor superficie en relación con el volumen, lo que potencia sus interacciones con las moléculas. Estas propiedades hacen que las AgNPs sean eficaces en la inhibición del crecimiento bacteriano en diversos microorganismos sin toxicidad para el ser humano, lo que las hace muy ventajosas en aplicaciones antimicrobianas. En este estudio, se utilizaron AgNPs sintetizadas mediante química verde utilizando extracto de *Bursera graveolens* como un enfoque respetuoso con el medio ambiente, práctico y seguro. Se analizó la capacidad antibacteriana sobre bacterias patógenas alimentarias Grampositivas y Gramnegativas. Este trabajo demostró que las AgNPs verdes mostraban una inhibición superior de la biopelícula, especialmente frente a bacterias Gram negativas (>50% de inhibición). No se cumplieron las expectativas de actividad biocida debido a las limitaciones para diferenciar las bacterias viables de las no funcionales. No obstante, estos resultados sugieren que las AgNPs verdes son prometedoras para futuras aplicaciones de seguridad alimentaria.

Palabras clave: AgNPs, iones de plata, *Bursera graveolens*, bacterias transmitidas por alimentos, biofilm, MIC, MBC, microscopía de fluorescencia.

ABSTRACT

Food contaminated by microorganisms is responsible for causing disease in approximately 600 million people, 420,000 of whom die each year worldwide. Therefore, it is necessary to look for new ways to prevent contaminated food to ensure its safety. The use of silver nanoparticles (AgNPs) as antimicrobial agents is a tool that has become of interest because they possess unique characteristics, in particular a larger surface area relative to volume, which enhances their interactions with molecules. These properties make AgNPs effective in inhibiting bacterial growth in various microorganisms without toxicity to humans, which makes them very advantageous in antimicrobial applications. In this study, AgNPs synthesized by green chemistry using Bursera graveolens extract were used as an environmentally friendly, practical, and safe approach. Their antibacterial potential on Gram-positive and Gram-negative food pathogenic bacteria was analyzed. This work showed that green AgNPs displayed superior biofilm inhibition, especially against Gram-negative bacteria (>50% inhibition). Expectations for biocidal activity were not met due to limitations in differentiating viable but non-functional bacteria. Nonetheless, these findings suggest green AgNPs as promising for future food safety applications.

Keywords: AgNPs, silver ions, *Bursera graveolens*, foodborne bacteria, biofilm, MIC, MBC, fluorescence microscopy.

TABLE OF CONTENTS

TABLE INDEX

INDEX OF FIGURES

INDEX OF APPENDICES

INTRODUCTION

The consumption of food contaminated by pathogenic microorganisms is responsible for causing approximately 200 diseases in humans (Torgerson et al., 2015). According to the World Health Organization, this is a growing public health problem, especially in developing countries such as Ecuador because many of these diseases have a high morbidity and mortality rate (WHO, 2023). Food can be contaminated with pathogenic microorganisms by different factors such as the lack of good manufacturing practices in the production and transportation of food, the use of non-potable water to wash food (Kirk et al., 2017), environmental contamination at the time of preservation (Scherer et al., 2009) and the lack of effectiveness in the control systems of these for human consumption. For this reason, it is necessary to look for new ways to prevent contaminated food to ensure safety.

Over time, silver has been used as an antimicrobial agent, because several studies have demonstrated the high power of this metal to inhibit bacterial growth (Yaqoob et al., 2020). Currently, with the advances in nanoscience, the creation of silver nanoparticles has been developed, which are nanomaterials with sizes on the nanometer scale, specifically in the range of 1 to 100 nm (Bruna et al., 2021). Silver nanoparticles (AgNPs) are characterized mainly by their higher capacity and larger surface area when interacting with other molecules because of the area-volume ratio. In addition, they show unique catalytic, electrical, and optical properties compared to traditional silver. Specifically, AgNPs are promising antimicrobial agents because, according to a study by Yin and colleagues, these nanoparticles have three main mechanisms of action when interacting with bacterial cells. The first one is the rupture of the cell membrane because the silver ions have a positive charge, and the membrane has a negative charge facilitating the attraction. The second mechanism occurs inside the cell, as the nanoparticle will destroy

the electron transport chain leading to excessive production of reactive oxygen species which are toxic to the cell. In addition, nanoparticles can interact with DNA preventing replication and therefore cell multiplication (Yin et al., 2020). The advantages of using AgNPs are their high capacity to inhibit bacterial growth, they are not toxic to humans, and they work with a large variety of microorganisms (Bruna et al., 2021). For this reason, AgNPs are being used in the medical and food areas, incorporating them in surgical tools, equipment, and utensils in the food production area, etc. (Yaqoob et al., 2020).

However, the traditional synthesis of AgNPs depends on the use of environmentally toxic chemicals and solvents such as sodium borohydride (NaBH4) and sodium citrate (TSC) which in the long term can contaminate environmental waters and soils (Xu et al., 2020). In addition, their chemical manufacturing uses more complex and energy-intensive equipment. For this reason, the green synthesis of AgNPs has gained attention as it uses plant extracts as biological reducing agents. According to Deepa and colleagues, the stabilizing capacity of plant extracts is because of their antioxidant and antibacterial properties (Deepa et al., 2022). Plant extracts are biodegradable materials making the use of them for the synthesis of nanoparticles eco-friendly. Another advantage of using a green synthesis is that it represents a low cost, less energy, and is non-hazardous to manufacture (Wattimena et al., 2022).

Several studies have been described using plant extracts for the synthesis of AgNPs, for example, Mohammadi and colleagues used ginger extract (*Zingiber officinale*) where its nanoparticles showed a high antimicrobial capacity (Mohammadi et al., 2019). On the other hand, Premkumar and collaborators used Cinnamon extract (*Cinnamomum verum*) and the results showed great antibacterial capacity in bacteria and fungi (Premkumar et al., 2018). In this study, an extract of the plant *Bursera graveolens* commonly known as "Palo Santo" was used for the synthesis of green AgNPs. This plant

is commonly used for the manufacture of essential oils, repellents, food additives, and antimicrobial agents. Furthermore, according to Ganchala and colleagues, "Palo Santo" extract presents a high phytochemical activity due to the presence of n-terpenes, oleoresins, and lactones among others, which contribute to the stability of AgNPs (Ganchala et al., 2018).

Silver nanoparticles synthesized by green chemistry using "Palo Santo" extract have the potential to be a great antimicrobial agent that can be used in the future in food safety (Ganchala et al., 2018). For this reason, it is important to analyze the antimicrobial activity of these AgNPs on foodborne bacteria. This work used Gram-positive bacteria such as *Staphylococcus aureus* ATCC 25923 and *Bacillus cereus* ATCC 11778 and Gram-negative bacteria such as *Escherichia coli* ATCC 25922 and *Salmonella enterica* Typhimurium ATCC 14028 because they are characterized by their potential to cause severe effects on human health if consumed in food (Kirk et al., 2017). The minimum inhibitory concentration (MIC) of Green AgNPs was analyzed for each bacterium, as well as their inhibitory antibacterial capacity in biofilm, and their biocidal capacity in biofilm with fluorescence microscopy (FM) analysis.

METHODS

Bacteria Isolates and Growth Conditions

Silver nanoparticles and their interaction with *Bursera graveolens* extract by green chemistry was studied for their antibacterial activities against some foodborne bacteria, more exactly *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 25922 and *Salmonella enterica* serovar Typhimurium ATCC 14028 from the bacterial collection of Microbiology Institute of the Universidad San Francisco de Quito (IM-USFQ). All bacterial species were stored at -80 °C and grown on Mueller-Hinton agar (MHA) medium at 37 °C 24 hours before each assay.

Minimum inhibitory concentration (MIC), minimum biocidal concentration (MBC)

The antimicrobial activity of three treatments was evaluated in the present study, more exactly (1) synthesis of silver nanoparticles combined with *Bursera graveolens* extract by green chemistry (AgNPs Green), (2) silver ions by themselves (15 mM silver nitrate solution, $AgNO_3$), and (3) *B. graveolens* (also known as "Palo santo") extract. The microdilution method for minimum inhibitory concentration (MIC) assays was performed, as described by Wiegand and colleagues (Wiegand et al., 2008), under the considerations established in the Clinical Laboratory Standards Institute (CLSI) (CLSI, 2022). The final concentrations of AgNPs Green and *Bursera graveolens* plant extract were tested at 1, 2.5, 5, 10, and 15 mM. For the final silver ions' concentrations, we analyzed at 0.1, 0.25, 0.5, 0.75, and 1 mM. Then, 10 µL of the alternative treatments at concentrated solutions and 190 µL of Mueller-Hinton broth (MHB) plus bacteria with a final concentration of $1x10^5$ colony-forming units (CFU)/mL were placed in a 96-well plate and incubated at 37°C for 20-24 hrs. Additionally, 200 µL of MHB medium plus bacteria was placed as a positive control and 200 µL of the medium as a negative control (sterility control). The results were measured using the Biotek Instruments ELx808IU

spectrophotometer at 570 nm (OD570) and the lowest concentration of each treatment without bacterial growth was classified as MIC (Macià et al., 2014). Finally, the minimum bactericidal concentration (MBC) was identified by removing 50 µL of each treatment into the 96-well plate and then placing 50 µL of resazurin (0.015%) incubating the 96-well plate at 37°C for 1-2 hours until a complete color change was observed in the positive control (i.e., resazurin blue to pink color). The MBC value was determined when the blue resazurin color remained unchanged indicating no metabolism and microbial death. All tests were performed at least 6 times and with two replicates.

Biomass evaluation in biofilm inhibition assays

To analyze the biofilm inhibition by each treatment, a final inoculum of $1x10^7$ CFU/mL of each bacterium was applied in 96-well plates, using a 0.5 McFarland turbidity standard ($1x10^8$ CFU/mL) as reference for the fresh bacterial colonies in saline solution and then diluted 1:10 in sterile Mueller Hinton Browth (MHB medium, as previously described (Atiencia-Carrera et al., 2022). In each 96-well plate, 200 µL of medium plus inoculum was added as a positive control, and 200 µL of strictly growth medium was added as a negative control (sterility control). Then, 190 µL of medium plus inoculum and 10 µL of each treatment were added at the previously established 1 and 2x MIC values (Sornsenee et al., 2021). The plates were then incubated for 20-24 hours at 37°C. After this period, each well was carefully washed twice with 200 µL of sterile phosphate-buffered saline (PBS) (Jara, 2022). Finally, the biofilm biomass in 200 µL of PBS was measured by optical density (OD) using the Elx808 spectrophotometer (BioTek, Winooski, USA) at 570 nm.

LIVE/DEAD staining in biofilm inhibition assays

Further characterization of the antibiofilm potential was analyzed in the best two treatments (AgNPs Green and Ag^+) for biomass inhibition using fluorescence microscopy

using LIVE/DEAD staining. After the biofilm inhibition assay was performed, any non-adherent cells were removed by two gentle washes with PBS (200 µL) and, thereafter, biofilms were scraped vigorously from the well in 200 µL of PBS finally adding 100 µL of each biofilm suspension on a sterile glass slide (Atiencia-Carrera et al., 2022; Castro et al., 2020). A working solution was prepared by adding 10 µL of SYTO® 9 stain and 10 µL of propidium iodide (PI) stain (FilmTracer™ LIVE/DEAD® Biofilm Viability Kit, Invitrogen, Carlsbad, California, USA) to 80 µL of distilled water (live/dead working solution). This working solution was stored at -20 °C. Then, 3 µL of live/dead working solution was added to each biofilm suspension and the samples were incubated for 30 minutes at room temperature without light. Then, fluorescence was visualized using the Olympus BX50 microscope at the final magnification of 1000x using oil immersion, as previously described by (Atiencia-Carrera et al., 2022). A minimum of 15 images were taken per sample/control on the glass slide. Finally, the percentage of live and dead cells was measured using ImageJ version 1.57 by Fiji (Schindelin et al., 2012). The results obtained were expressed as the number of cells ± standard deviation per cm^2 (N. of cells/cm^2 ± SD). Each experimental assay was performed by duplicate on different days.

Statistical Analysis

All data obtained from the MIC, biofilm inhibition, and LIVE/DEAD staining assays were statistically analyzed. Due to the non-normal distribution of the data, the Wilcoxon non-parametric test was performed to compare the difference between positive control and treated samples. Statistical analysis was performed using R studio version 4.0 (https://www.rstudio.com/products/rstudio/download/) using several R packages ("ggpubr", "rstatixs", "openxlsx" and the "tidyverse" set of packages). Finally, all p-values < 0.05 were considered significant.

RESULTS

Minimum inhibitory concentration (MIC) and minimum biocidal concentration (MBC) against foodborne pathogens

The results obtained from the treatments are displayed in Appendix A, where the antibacterial capacity of synthesis of silver nanoparticles combined with *Bursera graveolens* extract by green chemistry (AgNPs Green), silver ions (15 mM silver nitrate solution, $AgNO_3$), and *B. graveolens* (also known as "Palo santo") extract were evaluated. *B. graveolens* extract showed the lowest antimicrobial activity for all bacteria, with MIC and MBC values both defined at 15 mM (see Table 1), with a biomass inhibition percentage between 50 and 60% (Appendix A). Therefore, it was decided to discard this treatment for the analysis of biomass inhibition and fluorescence microscopy using LIVE/DEAD staining. Meanwhile, AgNPs Green showed different antimicrobial activity depending on the bacteria, where MIC and MBC values for *Bacillus cereus* ATCC 11778 and *Salmonella* Typhimurium ATCC 14028 were both defined at 2.5 mM showing growth inhibition percentages of 75.70 and 73.02% (Appendix A), respectively. In addition, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were more sensitive to this treatment showing a MIC value of 1 mM and a MBC value of 2.5 mM, as well asgrowth inhibition percentages of 85.84 and 93.30% (Appendix A), respectively.

Finally, the microbial activity obtained by silver ions was 78.26% of biomass growth inhibition and showed both MIC and MBC values at 2.5 mM in *S.* Typhimurium ATCC 14028. However, the preliminary evaluation for the remaining bacteria was not conclusive to define MIC values as previously realized with the other two treatments since the lowest concentration of 1 mM evidenced growth inhibition percentages between 71 and 92%, and, when resazurin was used for MBC evaluation, no metabolic activity was

observed suggesting that the minimum inhibitory and bactericidal concentrations for silver ions could be lower than 1 mM. To confirm both MIC and MBC values for silver ions' treatment, new concentrations were established between 0.1 and 1 mM, more exactly 0.1, 0.25, 0.5, 0.75, and 1 mM. The most sensitive microorganism to this treatment was *E. coli* ATTC 25922 showing both MIC and MBC values of 0.25 mM and a growth inhibition percentage of 91%. Regarding *B. cereus* ATCC 11778 and *S. aureus* ATCC 25923, both MIC and MBC values were 0.5 mM and the obtained growth inhibition percentages were 74.64 and 60.64% (Appendix B), respectively.

Biofilm inhibition with AgNPs Green treatment

The ability to inhibit biofilm formation of AgNPs Green was then evaluated by comparing the biomass produced from untreated samples (positive control) with treated samples subjected to two different concentrations (1x MIC and 2x MIC). AgNPs Green showed a different ability to inhibit biomass depending on the bacteria. As shown in Figure 1A, for *S. aureus* the capacity to inhibit biofilm was significantly lower when using a concentration of 1 mM (1x MIC) obtaining a percentage of biofilm formation of 54.45% when compared to the concentration of 2.5 mM (2x MIC), where 28.48% of biofilm formation was obtained (p <0.001). *E. coli* showed a different behavior with a biofilm formation rate of 32.99% when exposed to AgNPs Green concentration of 2.5 mM (2x MIC) and a biofilm formation rate of 30% at 1 mM concentration (1x MIC). Nonetheless, a statistical significance was observed (p <0.05). Likewise, *B. cereus* also evidenced similar biofilm inhibition ability at 2.5 and 5 mM Green AgNPs concentrations (1x and 2x MIC values, respectively) showing 61.04 and 62.59% biofilm formation rates, respectively. However, these data showed no statistically significant difference (p > 0.05) (Appendix B). Finally, *S.* Typhimurium demonstrated a similar biofilm inhibition when using 2.5 and 5 mM AgNPs Green concentrations (1x and 2x MIC values, respectively),

more exactly 43.24 and 44.82% biofilm formation rates, respectively. Also, no statistically significant difference between both concentrations was observed (p >0.05). Therefore, a high efficiency of AgNPs Green at 1x MIC value was obtained in biofilm inhibition assays, but these 1x MIC concentrations differed on the bacterium.

Biofilm inhibition with silver ions treatment

Regarding silver ions treatment, S. *aureus* exhibited a higher biofilm inhibition when 2x MIC value (1 mM) was applied in comparison to 1x MIC value (0.5 mM), demonstrating a biofilm formation rate of 63.85 and 83.81% (p <0.05), respectively, as shown in Figure 1B. Furthermore, *E. coli* showed a biofilm formation rate of 65.94 and 50.46% when using silver ions concentrations of 0.25 (1x MIC) and 0.50 mM (2x MIC, p <0.001), respectively. Similarly, *B. cereus* revealed a biofilm formation rate of 63.68 and 52.88% when exposed to 0.5 (1x MIC) and 1 mM (2x MIC, p <0.05; see Appendix C), respectively. Finally, *S.* Typhimurium also showed a higher biofilm formation at 2.5 mM (1x MIC, 40.06%) when compared to 5 mM silver ions concentration (2x MIC, 37.24%), however, no statistical difference was observed (p >0.05). Overall results suggested that 2 MIC values are more suitable to treat the foodborne pathogens in the present study, exhibiting an inhibition percentage range between 30 and 60% (see Appendix C).

Evaluation of biofilm inhibition with AgNPs Green and silver ions treatments by fluorescence microscopy using LIVE/DEAD staining

Lastly, we analyzed the number of total cells and live/dead cells with AgNPS Green and silver ions treatments on Gram-positive (*S. aureus* and *B. cereus*, see Figure 2) and Gram-negative foodborne pathogens (*E. coli* and *S.* Typhimurium, see Figure 3). The total number of cells significantly decreased in both treatments when compared to the control samples (no treatment) against all foodborne pathogens (p <0.05, see Table

2). When comparing between treatments, silver ions evidenced a higher reduction in the total cell amount among foodborne pathogens, except for *S. aureus*, but only *B. cereus* evidenced a statistical difference (p <0.01; see Table 3). However, regarding the number of dead cells, AgNPs Green treatment evidenced statistically higher dead cell percentages in *S. aureus* (15.49%) and *B. cereus* (47.41%) when compared to silver ions treatment (10.15 and 39.96%, respectively; p <0.01, see Table 3). Meanwhile, no statistical differences in dead cell percentages were found between treatments against *E. coli* and *S.* Typhimurium (Gram-negative pathogens; p >0.05, see Table 3). Finally, *S. aureus* and *E. coli* treated samples showed the lowest percentages of dead cells, more exactly 10.15 and 11.34%, respectively, exhibiting greater antimicrobial resistance to both treatments when compared to *B. cereus* and *S.* Typhimurium.

DISCUSSION

Silver nanoparticles (AgNPs) have attracted considerable attention recently because of their antimicrobial characteristics and potential uses in various fields (Pilaquinga et al., 2019; Tacconelli et al., 2018), such as food safety. The main mechanism of action of nanoparticles is based on the destruction of the bacterial cell membrane by penetrating them and causing toxicity. Previous studies have also demonstrated their high inhibitory biofilm capacity compared to traditional antimicrobials (Rabiee et al., 2022), for example, Mohammadi and colleagues used ginger extract (*Zingiber officinale*) where its nanoparticles showed a high antimicrobial capacity (Mohammadi et al., 2019). On the other hand, Premkumar and collaborators used Cinnamon extract (*Cinnamomum verum*) and the results showed great antibacterial capacity in bacteria and fungi (Premkumar et al., 2018). The increasing demand for future pharmaceutical applications using AgNPs has led to the exploration of green synthesis methods, which allow to obtain viable, eco-friendly, and non-toxic compounds (Srinatha Narayanaswamy, 2022). The present study demonstrated the antimicrobial efficiency of silver nanoparticles synthesized by green chemistry using *Bursera graveolens* (also known as "Palo santo") extract on foodborne pathogens, more exactly Gram-positive *Staphylococcus aureus* and *Bacillus cereus* as well as Gram-negative *Escherichia coli* and *Salmonella* Typhimurium, encouraging further studies for potential applications in the food control industry.

Overall results on the antibacterial activity of silver ions were higher than AgNPs Green through MIC and MBC assays, mainly for *S. aureus*, *B. cereus*, and *E. coli* pathogens. According to the study realized by Li and colleagues (Li et al., 2017), although silver ions have a similar mode of action to nanoparticles, their antimicrobial activity is higher agreeing with our preliminary planktonic evaluation. In addition, Kędziora and

colleagues suggested that silver ions show high antimicrobial activity even with low concentrations in accordance with the results obtained in this study where the minimum inhibitory concentration was lower than 1 mM (Kędziora et al., 2018). Moreover, their prominent antimicrobial activity could be explained due to the silver ions' ability to interact with the bacterial membrane leading to their accumulation and inducing the separation and/or destruction of the cytoplasmic membrane (Kędziora et al., 2018). However, *S.* Typhimurium showed a higher resistance to silver ions evidencing a MIC and MBC value of 2.5 mM. These results agree with the study performed by Estevez and colleagues, where the antimicrobial activity of Green AgNPs and silver ions was evaluated in the same *S.* Typhimurium ATCC 14028 strain (Estevez et al., 2021) demonstrating no growth inhibition at lower concentrations of silver ions than those performed in this study and concluding that this strain is sensitive to silver ions only at high concentrations. In fact, Lossaso and colleagues explained that *S.* Typhimurium strain ATTC 14028 possesses numerous resistance mechanisms that alter cell membranes being able to decrease the uptake of silver ions and pupping these ions out of the cell through several efflux pumps (Losasso et al., 2014).

Further antimicrobial evaluation was then realized by biofilm inhibition analysis through biomass, total cell amount, and live/dead cell characterization. It is important to mention that we discarded the *Bursera graveolens* extract due to the MIC and MBC values higher than 15 mM indicating its low antimicrobial effect when compared to the other two treatments. The lack of significant antimicrobial activity could be partially explained by several plant components and metabolites able to promote microbial growth, such as antioxidants, n-terpenes, oleoresins, and lactones among others (Ganchala et al., 2018). However, when this *B. graveolens* extract was used for the green synthesis of AgNPs, the bonded compounds helped to stabilize the silver particles through their

reducing activity by negatively charged metabolites and establishing their antimicrobial effect (Gurunathan et al., 2014).

The antibiofilm activity of AgNPs Green and silver ions was then evaluated. As expected, the antibiofilm activity of silver ions depended on their concentration, where higher silver ion concentrations evidenced lower biofilm formation rates among foodborne pathogens (Swidan et al., 2022). On the other hand, AgNPs Green showed a superior higher antibiofilm activity and demonstrated a superior biomass inhibition and equal or superior dead cell amount on treated-biofilm samples when compared to silver ions' treatment. When comparing 1x and 2x MIC values, biofilm inhibition by AgNPs Green showed no statistically significant difference for most studied bacteria, suggesting that a higher concentration should be applied in further application for this alternative treatment against biofilms. In fact, according to Rabiee and colleagues, it is recommended to use the higher inhibitory concentration because subinhibitory concentrations are known to induce biofilm structure modifications allowing biofilms to be more resistant against a secondary or recurrent AgNPs treatment (Rabiee et al., 2022). Moreover, Rabiee et al. (2022) reported that Gram-negative bacteria are usually more sensitive to AgNPs than Gram-positive bacteria, in agreement with the results of biofilm inhibition assays of the present study. Finally, the analysis by fluorescence microscopy using LIVE/DEAD staining showed that both treatments can statistically reduce the total cell amount during biofilm formation when compared to positive control and evidencing the highest dead cell rates against *B. cereus* (39.96-47.41%) and *S.* Typhimurium (36.61-37.15%). However, the remaining lower range of dead cells obtained during both treatments (10.15-15.49%) against *S. aureus* and *E. coli* suggested a bacteriostatic effect instead of bactericidal activity, as previously described (Rabiee et al., 2022). In fact, Mohanta and collaborators also evaluated the antimicrobial activity of AgNPs synthesized by green

chemistry using different plant extracts, such as ginger, and cinnamon among others, evidencing similar results (Mohanta et al., 2020). They performed minimum inhibitory concentration (MIC) analysis where it was found that *E. coli* growth inhibition occurred at a concentration of $60\,\mu g/ml$ AgNPs. In addition, they performed biofilm inhibition analysis where it was observed that AgNPs synthesized by green chemistry were able to inhibit biofilm formation of *S. aureus* and *E. coli* up to 99% depending on the plant extract used (Mohanta et al., 2020).

However, to the authors' best knowledge, this is the first study to characterize the antimicrobial activity through biomass and LIVE/DEAD assays of AgNPs using *Bursera graveolens* extract synthesized by green chemistry. The antibiofilm evaluation of the present study is in agreement with the results reported by Mohanta et al. (2020), highlighting the excellent antibacterial and antibiofilm effects against foodborne bacteria. Some authors postulated that AgNPs may be involved in the neutralization of adhesive components thus preventing the first step of biofilm formation (Gurunathan et al., 2014).

Finally, Kędziora and colleagues suggested that silver ions and probably silver nanoparticles often induced a "live but non-viable" state in bacterial cells (Kędziora et al., 2018) justifying their bacteriostatic effect. This means that the analysis with the dead/live kit could indicate that there were more live bacteria than dead with the treatments, but of these live bacteria perhaps not all are viable, meaning that they cannot multiply by cell division, due to the effect of the silver nanoparticles. So, further studies involving colony-forming unit (CFU) counting assays should be realized to confirm the increment of non-viable cells within the treated biofilm samples.

CONCLUSIONS

The present study demonstrated the high antibacterial capacity of silver nanoparticles synthesized by green chemistry using Bursera graveolens "Palo santo" extract. Initially, the MIC analysis indicated that silver ions were more effective compared to silver nanoparticles, however, when the biofilm inhibition capacity was analyzed, the green AgNPs presented a higher percentage of inhibition, especially in Gram-negative bacteria with a percentage of inhibition higher than 50%. A biocidal activity higher than the percentage obtained was expected, however, it should be considered that the analysis with the live/dead kit does not differentiate between bacteria that are alive but no longer viable because of the silver nanoparticles. These results indicate that green AgNPs are good candidates for future applications in food safety. Finally, further analysis should be performed on resistant bacteria and fungi to verify the effectiveness of the nanoparticles on other types of microorganisms, and biomass evaluations such as CFU counting assay should be performed to verify the viability of bacteria when subjected to AgNPs treatments.

TABLES

TABLE 1. Summary of the results obtained in the minimum inhibitory concentration (mic) and minimum biocidal concentration (mbc) assays against foodborne pathogens.

Treatment	**MIC; MBC (mM)**			
	Microorganisms			
	Gram-positive bacteria		**Gram-negative bacteria**	
	***S. aureus* ATCC 25923**	***B. cereus* ATCC 11778**	***E. coli* ATTC 25922**	***S.* Thypimurium ATCC 14028**
AgNPs Green	1; 2.5	2.5; 2.5	1; 2.5	2.5; 2.5
Silver ions	0.5; 0.5	0.5; 0.5	0.25; 0.25	2.5; 2.5
***Bursera graveolens* Extract**	>15; >15	>15; >15	>15; >15	>15; >15

Legend ―MIC: minimum inhibitory concentration, MBC: Minimum biocidal concentration for each bacterium. AgNPs green is the silver nanoparticles synthesized by green chemistry using the *Bursera graveolens* extract. Silver ions are found on their own without being in nanoparticle form.

TABLE 2. Initial evaluation of the results obtained by fluorescence microscopy using live/dead staining in the biofilm inhibition assays against foodborne pathogens.

FLUORESCENCE MICROSCOPY							
Total cells/cm^2							
Treatments	**Control**	**AgNPs Green**			**Silver ions**		
M.O	**Mean cm^2 (SD)**	**Mean cm^2 (SD)**	**Significant difference**	***p-value***	**Mean cm^2 (SD)**	**Significant difference**	***p-value***
S. aureus	2.03E+07 (6.14E+06)	5.10E+06 (3.39E+06)	Yes	0.00003	8.06E+06 (1.94E+06)	Yes	0.0000005
B. cereus	4.47E+06 (1.18E+06)	2.37E+06 (1.05E+06)	Yes	0.0001	1.81E+06 (1.18E+06)	Yes	0.000006
E. coli	1.08E+07 (9.79E+06)	4.52E+06 (2.79E+06)	Yes	0.03	3.52E+06 (2.02E+06)	Yes	0.016
S. Typhimurium	9.75E+06 (3.90E+06)	2.46E+06 (1.40E+06)	Yes	0.00005	5.29E+05 (3.28E+05)	Yes	0.0000007
Live cells/cm^2							
Treatments	**Control**	**AgNPs Green**			**Silver ions**		
M.O	**Mean cm^2 (SD)**	**Mean cm^2 (SD)**	**Significant difference**	***p-value***	Mean cm^2 (SD)	**Significant difference**	***p-value***
S. aureus	1.95E+07 (6.16E+06)	5.04E+06 (3.39E+06)	Yes	0.000003	6.81E+06 (2.55E+06)	Yes	0.0000006
B. cereus	2.87E+06 (7.28E+05)	1.25E+06 (1.63E+06)	Yes	0.005	1.09E+06 (4.72E+05)	Yes	0.006
E. coli	1.04E+07 (9.60E+06)	4.01E+06 (2.47E+06)	No	0.505	3.08E+06 (1.84E+06)	Yes	0.016
S. Typhimurium	8.59E+06 (4.28E+06)	2.37E+06 (1.38E+06)	Yes	0.0004	3.35E+05 (3.35E+05)	Yes	0.000000005
Dead cells/cm^2							
Treatments	**Control**	**AgNPs Green**			**Silver ions**		
M.O	**Mean cm^2 (SD)**	**Mean cm^2 (SD)**	**Significant difference**	***p-value***	**Mean cm^2 (SD)**	**Significant difference**	***p-value***
S. aureus	5.85E+04 (2.59E+04)	9.09E+05 (8.51E+05)	Yes	0.000442	1.25E+06 (1.46E+06)	No	0.812
B. cereus	1.60E+06 (3.35E+05)	1.80E+06 (1.18E+06)	Yes	0.016	7.24E+05 (1.18E+06)	No	0.63
E. coli	5.12E+05 (3.68E+05)	3.80E+05 (2.55E+05)	No	0.442	4.42E+05 (2.52E+05)	No	0.539
S. Typhimurium	9.49E+04 (9,16E+04)	1.16E+06 (6.66E+05)	Yes	0.00004	1.94E+05 (2.32E+04)	Yes	0.0002

Legend - Evaluation of green AgNPs and silver ions on Gram-positive and Gram-negative bacteria. For data evaluation, the Wilcoxon test for non-parametric data was performed using the R-studio program. The mean of each treatment and standard deviation were obtained. The p-value was used to compare whether there is a significant difference between the mean of the positive control and the treatment with AgNPs green and silver ions, for total, live, and dead cells. A p-value less than or equal to 0.05 indicates whether there is a significant difference between the pairs of variable levels.

TABLE 3. Statistical difference between the treatment of agnps green and silver ions using the wilcoxon test.

Microorganisms	**Treatment**	**Total cells/cm^2**	**Live cells (%)**	**Dead cells (%)**	**Significant difference**	***p-value***
S. aureus	Positive Control	2.03E+07	95.90	4.48		
	AgNPs Green (2.5 mM)	5.10E+06	84.51	15.49	Yes	0.000398
	Silver ions (1 mM)	8.06E+06	89.85	10.15		
B. cereus	Positive Control	4.47E+06	64.12	35.88		
	AgNPs Green (2.5 mM)	2.37E+06	52.59	47.41	Yes	0.00117
	Silver ions (1 mM)	1.81E+06	60.04	39.96		
E. coli	Positive Control	1.08E+07	96.47	3.53		
	AgNPs Green (1 mM)	4.52E+06	88.66	11.34	No	0.933
	Silver ions (0.5 mM)	3.52E+06	87.47	12.53		
S. Typhimurium	Positive Control	9.75E+06	88.08	11.92		
	AgNPs Green (2.5 mM)	2.46E+06	63.39	36.61	No	0.371
	Silver ions (5 Mm)	5.29E+05	62.65	37.15		

Legend - Statistical difference between green AgNPs and silver ions treatment is shown by the Wilcoxon test. The p-value indicates whether there is a significant difference between green AgNPs and silver ions in total cells for each bacterium. A p-value less than or equal to 0.05 indicates whether there is a significant difference between the pairs of variable levels

FIGURES

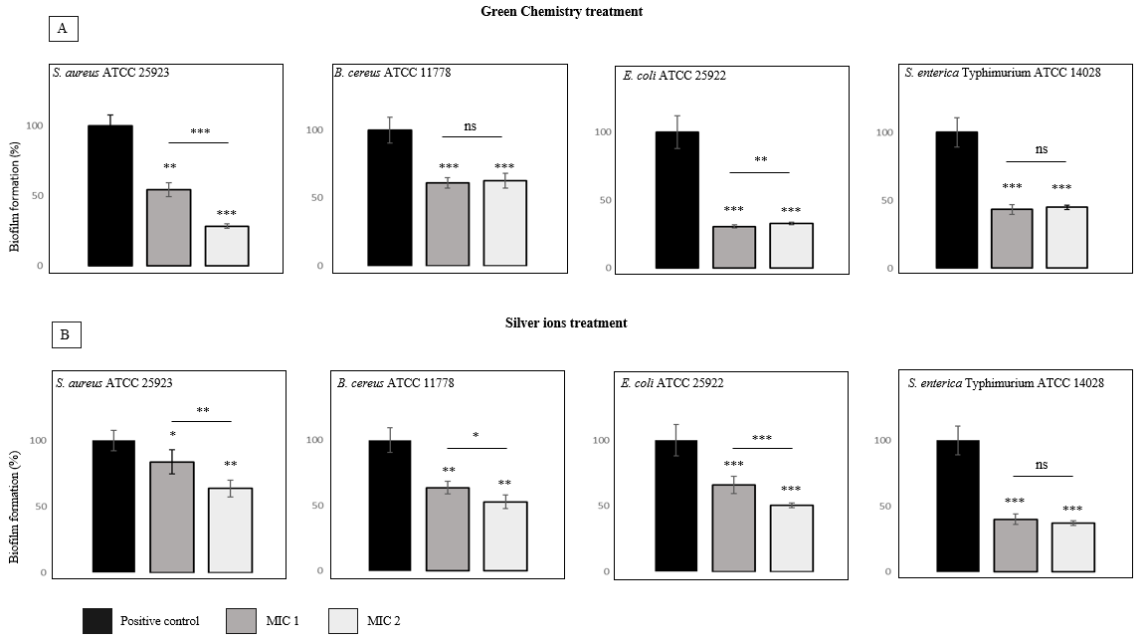

Figure 1. Illustrative representation of the results obtained by biomass evaluation in biofilm inhibition assays against foodborne pathogens using agnps green (a) and silver ions (b).

Evaluation of the biofilm inhibition capacity of Green AgNPs (A) and silver ions (B) on Gram-positive and Gram-negative bacteria. After 20 hours of incubation, the effect of two different concentrations of each treatment (MIC 1 and MIC 2) was compared with the positive control which is the bacterial inoculum without any treatment. The Wilcoxon test was used to evaluate if the difference between treatments is statistically different in comparison between the positive control (upper line) and between the concentrations (above each bar). * p <0.05; ** p <0.01; * * * p <0.001; **** p <0.0001, (ns) non-significant.

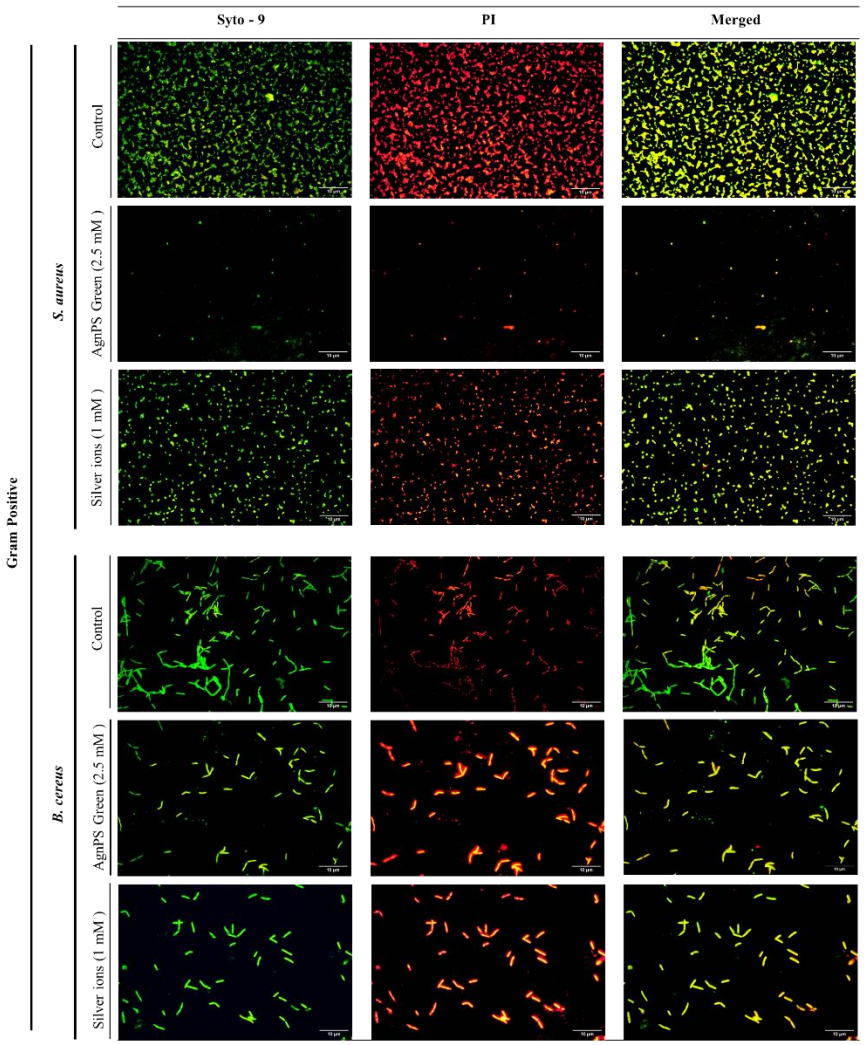

FIGURE 2. Illustrative representation of the results obtained by fluorescence microscopy using live/dead staining in the biofilm inhibition assays on gram-positive pathogens (*s. Aureus and b. Cereus*) with agnps green and silver ions treatments.

Gram-positive bacteria by fluorescence microscopy using the Live/Dead satining kit. Biofilm samples illustrated with an initial inoculum of 1E+8 CFU/mL (0.5 McFarland) were used to compare the total number of live and dead cells. An Olympus BX50 microscope with 100X magnification was used, images were obtained with AmScope software, and images were merged with ImageJ software.

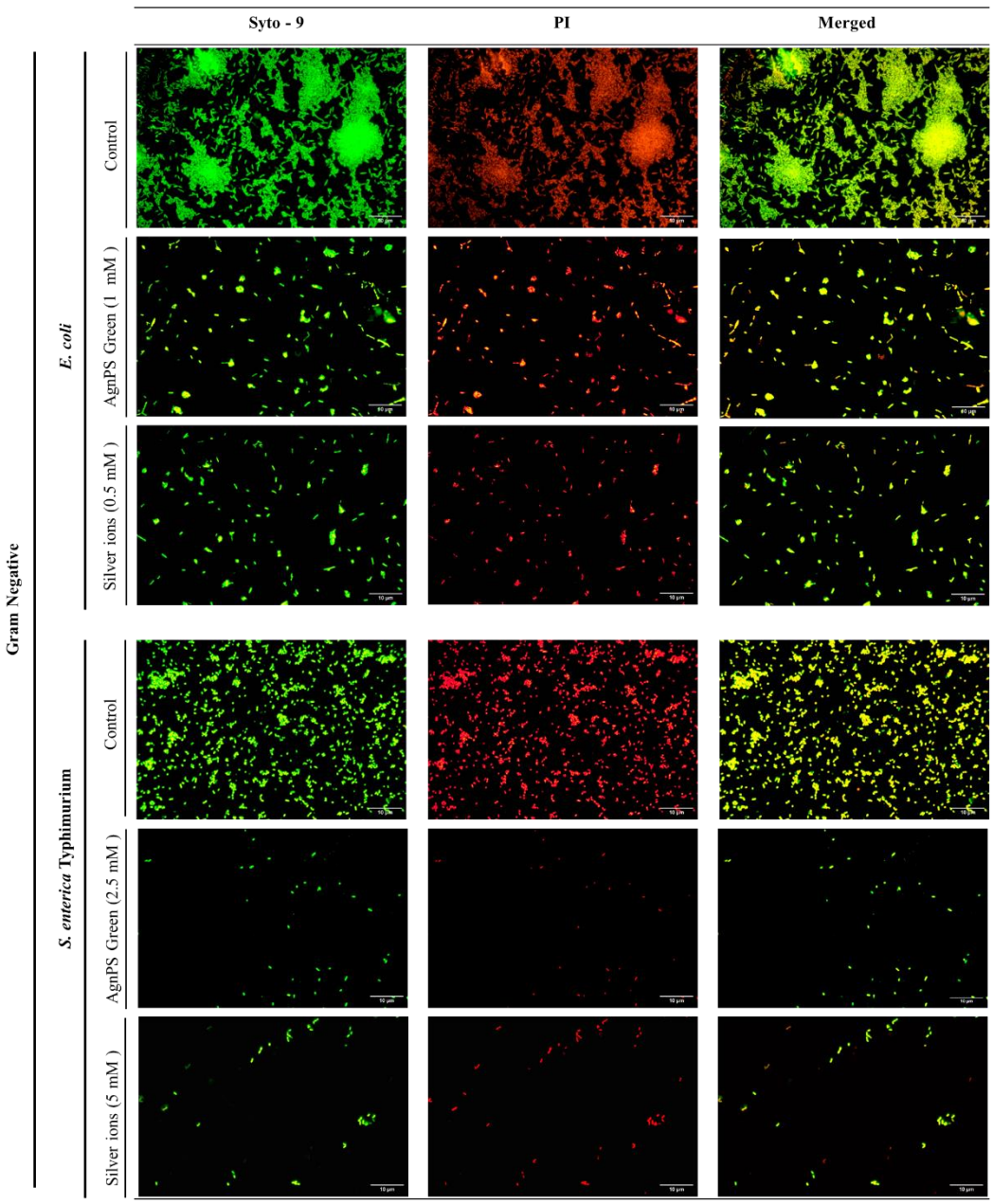

Figure 3. Illustrative representation of the results obtained by fluorescence microscopy using live/dead staining in the biofilm inhibition assays on gram-negative pathogens (*e. Coli* and *s.* Typhimurium) with agnps green and silver ions treatments.

Gram-negative bacteria by fluorescence microscopy using the Live/Dead kit. Biofilm samples illustrated with an initial inoculum of 1E+8 CFU/mL (0.5 McFarland) were used to compare the total number of live and dead cells. An Olympus BX50 microscope with 100X magnification was used, images were obtained with AmScope software, and images were merged with ImageJ software.

REFERENCES

Atiencia-Carrera, M. B., Cabezas-Mera, F. S., Vizuete, K., Debut, A., Tejera, E., & Machado, A. (2022). Evaluation of the biofilm life cycle between Candida albicans and Candida tropicalis. *Frontiers in Cellular and Infection Microbiology*, *12*. https://doi.org/10.3389/fcimb.2022.953168

Bruna, T., Maldonado-Bravo, F., Jara, P., & Caro, N. (2021). Silver Nanoparticles and Their Antibacterial Applications. *International Journal of Molecular Sciences*, *22*(13), 7202. https://doi.org/10.3390/ijms22137202

Cabezas-Mera, F. S., Atiencia-Carrera, M. B., Villacrés-Granda, I., Proaño, A. A., Debut, A., Vizuete, K., Herrero-Bayo, L., Gonzalez-Paramás, A. M., Giampieri, F., Abreu-Naranjo, R., Tejera, E., Álvarez-Suarez, J. M., & Machado, A. (2023). Evaluation of the polyphenolic profile of native Ecuadorian stingless bee honeys (Tribe: Meliponini) and their antibiofilm activity on susceptible and multidrug-resistant pathogens: An exploratory analysis. *Current Research in Food Science*, *7*, 100543. https://doi.org/10.1016/j.crfs.2023.100543

Castro, J., Rosca, A. S., Cools, P., Vaneechoutte, M., & Cerca, N. (2020). Gardnerella vaginalis Enhances Atopobium vaginae Viability in an in vitro Model. *Frontiers in Cellular and Infection Microbiology*, *10*(March), 1–9. https://doi.org/10.3389/fcimb.2020.00083

CLSI. (2022). *Performance Standards for Antimicrobial Susceptibility Testing - M100* (C. and L. S. Institute, Ed.; 32nd Editi). CLSI supplement M100.

Deepa, Ameen, F., Amirul Islam, M., & Dhanker, R. (2022). Green synthesis of silver nanoparticles from vegetable waste of pea Pisum sativum and bottle gourd Lagenaria siceraria: Characterization and antibacterial properties. *Frontiers in Environmental Science*, *10*. https://doi.org/10.3389/fenvs.2022.941554

Estevez, M. B., Casaux, M. L., Fraga, M., Faccio, R., & Alborés, S. (2021). Biogenic Silver Nanoparticles as a Strategy in the Fight Against Multi-Resistant Salmonella

enterica Isolated From Dairy Calves. *Frontiers in Bioengineering and Biotechnology*, *9*. https://doi.org/10.3389/fbioe.2021.644014

Ganchala, D., Gu.Coronado, J. L., Jara, E., Meneses, L., Granda, E., & Pilaquinga, F. (2018). Synthesis of silver nanoparticles functionalized with aqueous extract (Bursera graveolens) and antimicrobial evaluation in Escherichia coli, Staphylococcus aureus and Klebsiella pneumoniae. *Periódico Tchê Química*, *15*(29), 282–291. https://doi.org/10.52571/PTQ.v15.n29.2018.282_Periodico29_pgs_282_291.pdf

Gurunathan, S., Han, J. W., Kwon, D.-N., & Kim, J.-H. (2014). Enhanced antibacterial and anti-biofilm activities of silver nanoparticles against Gram-negative and Gram-positive bacteria. *Nanoscale Research Letters*, *9*(1), 373. https://doi.org/10.1186/1556-276X-9-373

Jara, N. (2022). *Analysis of the biofilm formation of Vibrio parahaemolyticus and Vibrio cholerae*. Universidad San Francisco de Quito.

Kędziora, A., Speruda, M., Krzyżewska, E., Rybka, J., Łukowiak, A., & Bugla-Płoskońska, G. (2018). Similarities and Differences between Silver Ions and Silver in Nanoforms as Antibacterial Agents. *International Journal of Molecular Sciences*, *19*(2), 444. https://doi.org/10.3390/ijms19020444

Kirk, M. D., Angulo, F. J., Havelaar, A. H., & Black, R. E. (2017). Diarrhoeal disease in children due to contaminated food. *Bulletin of the World Health Organization*, *95*(3), 233–234. https://doi.org/10.2471/BLT.16.173229

Li, W.-R., Sun, T.-L., Zhou, S.-L., Ma, Y.-K., Shi, Q.-S., Xie, X.-B., & Huang, X.-M. (2017). A comparative analysis of antibacterial activity, dynamics, and effects of silver ions and silver nanoparticles against four bacterial strains. *International Biodeterioration & Biodegradation*, *123*, 304–310. https://doi.org/10.1016/j.ibiod.2017.07.015

Losasso, C., Belluco, S., Cibin, V., Zavagnin, P., MiÄ• etiÄ‡, I., Gallocchio, F., Zanella, M., Bregoli, L., Biancotto, G., & Ricci, A. (2014). Antibacterial activity of silver nanoparticles: sensitivity of different Salmonella serovars. *Frontiers in Microbiology*, *5*. https://doi.org/10.3389/fmicb.2014.00227

Macià, M. D., Rojo-Molinero, E., & Oliver, A. (2014). Antimicrobial susceptibility testing in biofilm-growing bacteria. *Clinical Microbiology and Infection*, *20*(10), 981–990. https://doi.org/10.1111/1469-0691.12651

McQuin, C., Goodman, A., Chernyshev, V., Kamentsky, L., Cimini, B. A., Karhohs, K. W., Doan, M., Ding, L., Rafelski, S. M., Thirstrup, D., Wiegraebe, W., Singh, S., Becker, T., Caicedo, J. C., & Carpenter, A. E. (2018). CellProfiler 3.0: Next-generation image processing for biology. *PLOS Biology*, *16*(7), e2005970. https://doi.org/10.1371/journal.pbio.2005970

Mohammadi, M., Shahisaraee, S. A., Tavajjohi, A., Pournoori, N., Muhammadnejad, S., Mohammadi, S. R., Poursalehi, R., & Delavari H, H. (2019). Green synthesis of silver nanoparticles using *Zingiber officinale* and *Thymus vulgaris* extracts: characterisation, cell cytotoxicity, and its antifungal activity against *Candida albicans* in comparison to fluconazole. *IET Nanobiotechnology*, *13*(2), 114–119. https://doi.org/10.1049/iet-nbt.2018.5146

Mohanta, Y. K., Biswas, K., Jena, S. K., Hashem, A., Abd_Allah, E. F., & Mohanta, T. K. (2020). Anti-biofilm and Antibacterial Activities of Silver Nanoparticles Synthesized by the Reducing Activity of Phytoconstituents Present in the Indian Medicinal Plants. *Frontiers in Microbiology*, *11*. https://doi.org/10.3389/fmicb.2020.01143

Pilaquinga, F., Morejón, B., Ganchala, D., Morey, J., Piña, N., Debut, A., & Neira, M. (2019). Green synthesis of silver nanoparticles using Solanum mammosum L. (Solanaceae) fruit extract and their larvicidal activity against Aedes aegypti L. (Diptera: Culicidae). *PLoS ONE*, *14*(10), 1–13. https://doi.org/10.1371/journal.pone.0224109

Premkumar, J., Sudhakar, T., Dhakal, A., Shrestha, J. B., Krishnakumar, S., & Balashanmugam, P. (2018). Synthesis of silver nanoparticles (AgNPs) from cinnamon against bacterial pathogens. *Biocatalysis and Agricultural Biotechnology*, *15*, 311–316. https://doi.org/10.1016/j.bcab.2018.06.005

Rabiee, N., Ahmadi, S., Akhavan, O., & Luque, R. (2022). Silver and Gold Nanoparticles for Antimicrobial Purposes against Multi-Drug Resistance Bacteria. *Materials*, *15*(5), 1799. https://doi.org/10.3390/ma15051799

Scherer, K., Mäde, D., Ellerbroek, L., Schulenburg, J., Johne, R., & Klein, G. (2009). Application of a Swab Sampling Method for the Detection of Norovirus and Rotavirus on Artificially Contaminated Food and Environmental Surfaces. *Food and Environmental Virology*, *1*(1), 42–49. https://doi.org/10.1007/s12560-008-9007-0

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., & Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. *Nature Methods*, *9*(7), 676–682. https://doi.org/10.1038/nmeth.2019

Sornsenee, P., Chatatikun, M., Mitsuwan, W., Kongpol, K., Kooltheat, N., Sohbenalee, S., Pruksaphanrat, S., Mudpan, A., & Romyasamit, C. (2021). Lyophilized cell-free supernatants of *Lactobacillus* isolates exhibited antibiofilm, antioxidant, and reduces nitric oxide activity in lipopolysaccharide-stimulated RAW 264.7 cells. *PeerJ*, *9*, e12586. https://doi.org/10.7717/peerj.12586

Srinatha Narayanaswamy, R. B. , R. K. K. J. S. K. M. R. M. A. P. (2022). A Comprehensive Review on the Antimicrobial and Photocatalytic Properties of Green Synthesized Silver Nanoparticles. *Letters in Applied NanoBioScience*, *12*(4), 140. https://doi.org/10.33263/LIANBS124.140

Swidan, N. S., Hashem, Y. A., Elkhatib, W. F., & Yassien, M. A. (2022). Antibiofilm activity of green synthesized silver nanoparticles against biofilm associated

enterococcal urinary pathogens. *Scientific Reports*, *12*(1), 3869. https://doi.org/10.1038/s41598-022-07831-y

Tacconelli, E., Carrara, E., Savoldi, A., Harbarth, S., Mendelson, M., Monnet, D. L., Pulcini, C., Kahlmeter, G., Kluytmans, J., Carmeli, Y., Ouellette, M., Outterson, K., Patel, J., Cavaleri, M., Cox, E. M., Houchens, C. R., Grayson, M. L., Hansen, P., Singh, N., … Zorzet, A. (2018). Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *The Lancet Infectious Diseases*, *18*(3), 318–327. https://doi.org/10.1016/S1473-3099(17)30753-3

Torgerson, P. R., Devleesschauwer, B., Praet, N., Speybroeck, N., Willingham, A. L., Kasuga, F., Rokni, M. B., Zhou, X.-N., Fèvre, E. M., Sripa, B., Gargouri, N., Fürst, T., Budke, C. M., Carabin, H., Kirk, M. D., Angulo, F. J., Havelaar, A., & de Silva, N. (2015). World Health Organization Estimates of the Global and Regional Disease Burden of 11 Foodborne Parasitic Diseases, 2010: A Data Synthesis. *PLOS Medicine*, *12*(12), e1001920. https://doi.org/10.1371/journal.pmed.1001920

Wattimena, S. C., Ririmasse, V., Killay, A., & Patty, P. J. (2022). Kinetics of Formation and Characterization of Green Silver Nanoparticles of Ficus variegata Leaf Extract. *Jurnal Kimia Sains Dan Aplikasi*, *25*(1), 34–40. https://doi.org/10.14710/jksa.25.1.34-40

WHO. (2023). *Foodborne diseases*. https://www.who.int/health-topics/foodborne-diseases#tab=tab_1

Wiegand, I., Hilpert, K., & Hancock, R. E. W. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols*, *3*(2), 163–175. https://doi.org/10.1038/nprot.2007.521

Xu, L., Wang, Y.-Y., Huang, J., Chen, C.-Y., Wang, Z.-X., & Xie, H. (2020). Silver nanoparticles: Synthesis, medical applications and biosafety. *Theranostics*, *10*(20), 8996–9031. https://doi.org/10.7150/thno.45413

Yaqoob, A. A., Umar, K., & Ibrahim, M. N. M. (2020). Silver nanoparticles: various methods of synthesis, size affecting factors and their potential applications–a review. *Applied Nanoscience*, *10*(5), 1369–1378. https://doi.org/10.1007/s13204-020-01318-w

Yin, I. X., Zhang, J., Zhao, I. S., Mei, M. L., Li, Q., & Chu, C. H. (2020). The Antibacterial Mechanism of Silver Nanoparticles and Its Application in Dentistry . *International Journal of Nanomedicine*, *Volume 15*, 2555–2562. https://doi.org/10.2147/IJN.S246764

APPENDIX

APPENDIX 1. SUMMARY OF THE RESULTS OBTAINED IN MINIMUM INHIBITORY CONCENTRATION (MIC) ASSAYS IN EACH ALTERNATIVE TREATMENT AGAINST FOODBORNE PATHOGENS.

Treatment	Microorganisms	Type	Bacterial Growth Inhibition (%)	Bacterial Growth (%)	Standard Deviation (%)	*p*-values
AgNPs Green	*S. aureus* ATCC 25923	Positive Control	0.000	100.000	3.417	
		Sample 1 mM	86.981	13.019	11.395	0.0060
		Sample 2.5 mM	85.841	14.159	12.191	0.0060
		Sample 5 mM	84.043	15.957	2.006	0.0060
		Sample 10 mM	82.816	17.184	8.935	0.0060
		Sample 15 mM	75.627	24.373	6.299	0.0060
	B. cereus ATCC 11778	Positive Control	0.000	100.000	5.475	
		Sample 1 mM	23.565	76.435	2.716	0.0010
		Sample 2.5 mM	75.700	24.300	6.529	0.0002
		Sample 5 mM	77.623	22.377	5.490	0.0002
		Sample 10 mM	70.254	29.746	3.057	0.0006
		Sample 15 mM	62.447	37.553	2.778	0.0006
	E. coli ATTC 25922	Positive Control	0.000	100.000	3.862	
		Sample 1 mM	93.414	6.586	7.771	0.0030
		Sample 2.5 mM	93.300	6.700	2.721	0.0030
		Sample 5 mM	93.166	6.834	4.517	0.0030
		Sample 10 mM	91.443	8.557	1.984	0.0070
		Sample 15 mM	89.739	10.261	1.219	0.0070
	S. Typhimurium ATCC 14028	Positive Control	0.000	100.000	5.027	
		Sample 1 mM	36.226	63.774	12.835	0.0020
		Sample 2.5 mM	73.015	26.985	3.852	0.0020
		Sample 5 mM	74.083	25.917	6.987	0.0020
		Sample 10 mM	70.540	29.460	1.647	0.0020
		Sample 15 mM	70.443	29.557	3.844	0.0020
Silver ions	*S. aureus* ATCC 25923	Positive Control	0.000	100.000	3.417	
		Sample 1 mM	87.682	12.318	9.253	0.0060
		Sample 2.5 mM	86.411	13.589	4.770	0.0060
		Sample 5 mM	86.279	13.721	1.917	0.0060
		Sample 10 mM	84.438	15.562	9.217	0.0060
		Sample 15 mM	85.359	14.641	3.169	0.0060
	B. cereus ATCC 11778	Positive Control	0.000	100.000	5.475	
		Sample 1 mM	71.617	28.383	3.068	0.0010
		Sample 2.5 mM	71.852	28.148	6.318	0.0010
		Sample 5 mM	75.915	24.085	3.329	0.0010
		Sample 10 mM	76.622	23.378	13.873	0.0010

		Sample 15 mM	62.666	37.334	12.326	0.0010
	E. coli ATTC 25922	Positive Control	0.000	100.000	3.862	
		Sample 1 mM	92.955	7.045	2.348	0.0030
		Sample 2.5 mM	93.223	6.777	5.871	0.0070
		Sample 5 mM	93.759	6.241	3.815	0.0030
		Sample 10 mM	93.823	6.177	12.413	0.0070
		Sample 15 mM	93.146	6.854	3.225	0.0070
	S. Typhimurium ATCC 14028	Positive Control	0.000	100.000	5.027	
		Sample 1 mM	37.326	62.674	9.538	0.0003
		Sample 2.5 mM	78.257	21.743	4.140	0.0030
		Sample 5 mM	80.586	19.414	1.826	0.0020
		Sample 10 mM	82.722	17.278	7.621	0.0020
		Sample 15 mM	83.498	16.502	6.299	0.0020
***Bursera graveolens* Extract**	*S. aureus* ATCC 25923	Positive Control	0.000	100.000	3.417	
		Sample 1 mM	30.168	69.832	11.689	0.0060
		Sample 2.5 mM	20.451	79.549	11.162	0.0140
		Sample 5 mM	32.141	67.859	2.451	0.0060
		Sample 10 mM	35.955	64.045	2.316	0.0060
		Sample 15 mM	52.715	47.285	12.615	0.0060
	B. cereus ATCC 11778	Positive Control	0.000	100.000	5.475	
		Sample 1 mM	-24.781	124.781	2.395	0.0010
		Sample 2.5 mM	-31.552	131.552	8.734	0.0010
		Sample 5 mM	-29.503	129.503	3.428	0.0005
		Sample 10 mM	5.546	94.454	14.395	0.4120
		Sample 15 mM	60.546	39.454	6.176	0.0050
	E. coli ATTC 25922	Positive Control	0.000	100.000	3.862	
		Sample 1 mM	25.357	74.643	0.446	0.0030
		Sample 2.5 mM	19.155	80.845	1.204	0.0030
		Sample 5 mM	25.568	74.432	1.179	0.0030
		Sample 10 mM	31.311	68.689	2.459	0.0030
		Sample 15 mM	28.842	71.158	2.884	0.0030
	S. Typhimurium ATCC 14028	Positive Control	0.000	100.000	5.027	
		Sample 1 mM	43.749	56.251	6.887	0.0020
		Sample 2.5 mM	48.262	51.738	5.468	0.0020
		Sample 5 mM	43.749	56.251	6.215	0.0020
		Sample 10 mM	49.136	50.864	14.070	0.0070
		Sample 15 mM	53.019	46.981	3.649	0.0020

Legend – Evaluation of Minimum inhibitory concentration of green AgNPs, *Bursera graveolens extract* and silver ions on Gram-positive and Gram-negative bacteria. For data evaluation, the Wilcoxon test for non-parametric data was performed. The percentage of Bacterial growth and growth inhibition for each treatment and standard deviation were obtained. The p-value was used to compare whether there was a significant difference between the positive control and the treatment with different concentrations. A p-

value less than or equal to 0.05 indicates whether there is a significant difference between the pairs of variable levels.

APPENDIX 2. ADDITIONAL RESULTS IN MIC AND MBC ASSAYS FOR THE SILVER IONS' TREATMENT AGAINST FOODBORNE BACTERIA.

Treatment	Microorganisms	Type	Bacterial Growth Inhibition (%)	Bacterial Growth (%)	Standard Deviation (%)	*p*-values
Silver ions	*E. coli* ATCC 25922	Positive Control	0.000	100.000	10.684	
		Sample 0.1 mM	11.754	88.246	7.225	0.059
		Sample 0.25 mM	91.148	8.852	10.452	0.001
		Sample 0.5 mM	92.323	7.677	3.427	0.001
		Sample 0.75 mM	93.330	6.670	6.400	0.0009
		Sample 1 mM	93.238	6.762	5.080	0.001
	S. aureus ATCC 25923	Positive Control	0.000	100.000	5.679	
		Sample 0.1 mM	-10.575	110.575	2.884	0.022
		Sample 0.25 mM	3.606	96.394	2.396	0.552
		Sample 0.5 mM	60.673	39.327	2.665	0.008
		Sample 0.75 mM	81.969	18.031	2.573	0.008
		Sample 1 mM	86.021	13.979	7.794	0.000116
	B. cereus ATCC 11778	Positive Control	0.000	100.000	6.757	
		Sample 0.1 mM	-10.021	110.021	1.924	0.024
		Sample 0.25 mM	-4.791	104.791	6.153	0.315
		Sample 0.5 mM	74.648	25.352	5.014	0.006
		Sample 0.75 mM	77.750	22.250	4.327	0.006
		Sample 1 mM	80.260	19.740	6.443	0.006

Legend – Evaluation of Minimum inhibitory concentration of silver ions on *E. coli*, *S. aureus*, and *B. cereus*. For data evaluation, the Wilcoxon test for non-parametric data was performed. The percentage of Bacterial growth and growth inhibition for each treatment and standard deviation were obtained. The p-value was used to compare whether there was a significant difference between the positive control and the treatment with different concentrations. A p-value less than or equal to 0.05 indicates whether there is a significant difference between the pairs of variable levels.

APPENDIX 3. SUMMARY OF THE RESULTS OBTAINED IN BIOFILM INHIBITION ASSAYS AGAINST FOODBORNE BACTERIA.

Treatment	Microorganisms	Type	Biofilm inhibition (%)	Biofilm formation (%)	Standard deviation (%)	*Control vs. Treated sample p*-values	MIC 1 vs. MIC 2 sample p-values
AgNPs Green	*S. aureus* ATCC 25923	Positive Control	0.00	100.00	7.80		
		Sample 1 mM	45.55	54.45	9.17	0.001	
		Sample 2.5 mM	71.60	28.40	5.25	0.0006	0.000989
	B. cereus ATCC 11778	Positive Control	0.00	100.00	9.46		
		Sample 2.5 mM	38.96	61.04	6.52	0.000852	
		Sample 5 mM	37.41	62.59	8.70	0.000899	0.595
	E. coli ATTC 25922	Positive Control	0.00	100.00	11.89		
		Sample 1 mM	69.29	30.71	3.95	0.000579	
		Sample 2.5 mM	67.01	32.99	3.12	0.000574	0.003
	S. Typhimurium ATCC 14028	Positive Control	0.00	100.00	10.83		
		Sample 2.5 mM	56.76	43.24	8.44	0.000611	
		Sample 5 mM	55.18	44.82	3.86	0.000616	0.563
Silver ions	*S. aureus* ATCC 25923	Positive Control	0.00	100.00	7.80		
		Sample 0.5 mM	16.19	83.81	10.87	0.012	
		Sample 1 mM	36.15	63.85	9.95	0.002	0.008
	B. cereus ATCC 11778	Positive Control	0.00	100.00	9.46		
		Sample 0.5 mM	36.32	63.68	7.66	0.004	
		Sample 1 mM	47.12	52.88	9.63	0.004	0.033
	E. coli ATTC 25922	Positive Control	0.00	100.00	11.89		
		Sample 0.25 mM	34.06	65.94	9.73	0.000931	
		Sample 0.5 mM	49.54	50.46	3.62	0.000621	0.000626
	S. Typhimurium ATCC 14028	Positive Control	0.00	100.00	10.83		
		Sample 2.5 mM	59.94	40.06	10.08	0.000931	
		Sample 5 mM	62.76	37.24	4.80	0.000899	0.167

Legend – Evaluation of Biofilm inhibition of green AgNPs, *Bursera graveolens extract*, and silver ions on Gram-positive and Gram-negative bacteria. For data evaluation, the Wilcoxon test for non-parametric data was performed. The percentage of Biofilm inhibition and growth inhibition for each treatment and standard deviation were obtained. The p-value was used to compare whether there is a significant difference between the positive control and the treatment with MIC 1 and MIC 2. Also, the p-value was used to compare the difference between the two concentrations for each treatment. A p-value less than or equal to 0.05 indicates whether there is a significant difference between the pairs of variable levels.