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MICROBIOLOGY

Sanitizing activity of silver nanoparticles synthesized with natural products on dairy industry surfaces

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ABSTRACT: Surface contamination by pathogenic and deteriorating microorganisms is a constant concern in the food industry. Therefore, in this study we evaluated the antimicrobial activity of silver nanoparticles (AgNPs), produced using the essential oil of *Lippia origanoides* Kunth and ethanolic extract of *Hymenaea martiana* Hayne, against bacteria used in milk processing. For this, AgNPs were synthesized and their antimicrobial activity was evaluated for minimum inhibitory and bactericidal concentrations, time-kill, interference on the biofilm, and application on the surfaces of different materials. AgNPs showed bacteriostatic and bactericidal activity against *Staphylococcus aureus* (ATCC 25923), *Staphylococcus aureus* (ATCC 33591), *Escherichia coli* (ATCC 25922), *Salmonella enterica* subsp. *enterica* serovar *Choleraesuis* (ATCC 10708), *Listeria monocytogenes*, *Escherichia coli*, *Salmonella* spp.,and*Pseudomonas aeruginosa*,and interfered with biofilm formation more than with consolidated biofilms. A 1hour period was sufficient to reduce the bacterial cells, whereas a Minimum Duration for Killing (MDK) of 99% was reached after approximately 30 min. AgNPs were effective against bacteria attached to stainless steel and polyethylene, but ineffective on tile surfaces. Thus, owing to the growing microbial resistance and the need to develop new products based on the concepts of green chemistry, these AgNPs are presented as a new possibility for cleaning processes in the food industry.
Key words: antibacterial, *Hymenaea martiana* Hayne, *Lippia origanoides* Kunth, nanotechnology, green synthesis.

Atividade saneante de nanopartículas de prata sintetizadas com produtos naturais sobre superfícies da indústria de laticínio

RESUMO: A contaminação de superfícies por micro-organismos patogênicos e deteriorantes é uma preocupação constante na indústria de alimentos. Dessa forma, o objetivo deste trabalho foi avaliar a atividade antimicrobiana de nanopartículas de prata (AgNPs) produzidas utilizando óleo essencial de *Lippia origanoides* Kunth e extrato etanólico da *Hymenaea martiana* Hayne, frente a bactérias de importância na contaminação em áreas de processamento do leite. Para isso, as AgNPs foram sintetizadas e sua atividade antimicrobiana foi avaliada através das concentrações inibitória e bactericida mínimas, tempo de morte, interferência sobre o biofilme, e por fim, a sua aplicação sobre superfícies de diferentes materiais. As AgNPs apresentaram atividade bacteriostática e/ou bactericida frente à *Staphylococcus aureus* (ATCC 25923), *Staphylococcus aureus* (ATCC 33591), *Escherichia coli* (ATCC 25922), *Salmonella enterica* subsp. enterica serovar *Choleraesuis* (ATCC 10708), *Listeria monocytogenes*, *Escherichia coli*, *Salmonella* spp. e *Pseudomonas aeruginosa*, e interferiram na formação do biofilme mais do que sobre o biofilme consolidado. O tempo de uma hora foi o suficiente para reduzir as populações bacterianas, enquanto o *Minimum Duration for Killing* MDK 99% foi atingido próximo a 30 minutos. As AgNPs foram eficientes contra bactérias aderidas ao inox e ao polietileno. Assim, tendo em vista a crescente resistência microbiana e o desenvolvimento de novos produtos baseados nos conceitos da química verde, essas nanopartículas de prata apresentam-se como uma nova possibilidade de uso nos processos de higienização da indústria de alimentos.
Palavras-chave: antibacteriano, *Hymenaea martiana* Hayne, *Lippia origanoides* Kunth, nanotecnologia, síntese verde.

INTRODUCTION

The dairy industry has promising potential, especially in low- and middle-income countries, where raising small ruminants is an important economic activity. However, investments are necessary to provide inputs, markets, research, and production infrastructure because these issues remain challenges for the sector (MILLER & LU, 2019).

In this production chain, some factors directly affect the final quality of dairy products, with surface contamination by pathogenic and/or spoilage microorganisms being one of the most critical issues. Thus, preventing initial microbial binding, eliminating

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microorganisms, and/or removing dead bacteria from materials in contact with food is considered one of the greatest and most crucial challenges in the food industry (SHARMA et al., 2022).

Given the need to prevent the compromise of raw materials and manufactured products, and, eventually, problems with consumer health, studies have focused on the development of products and technologies that favor the maintenance of food safety. Cleaning contaminated surfaces in the food industry requires new strategies that provide higher efficiency (DALLAGI et al., 2022).

From this perspective, silver nanoparticles obtained through green synthesis have gained prominence, especially because they prevent the use of chemical, toxic, and costly reagents, reduce waste production, and are considered environmentally friendly (GUIMARÃES et al., 2020). Thus, plants such as wild oregano (*Lippia origanoides* Kunth) and jatobá (*Hymenaea martiana* Hayne), which are examples of the diversity of the Brazilian flora, are potential species for use as silver bioreductive agents in the green synthesis of nanoparticles.

The essential oil of *L. Origanoides* has verified its antimicrobial action and potential for use in the development of alternative products, such as antiseptics and sanitizers, which can be used to control pathogenic strains in the production of animal source foods, especially in dairy production (ALMEIDA et al., 2021). Conversely, *H. martiana*, belonging to the family Fabaceae, is native to Brazil, and is a prominent tree in the Brazilian Caatinga biome. The extract of this plant contains flavonoids as the dominant constituent, which are strongly associated with its antimicrobial and antioxidant activities (VIEIRA et al., 2018).

Therefore, in this study we evaluated a new sanitizing technology for dairy surfaces based on the green synthesis of AgNPs, obtained from the essential oils of wild oregano and jatobá extracts, against bacteria of importance in the environmental contamination of food industry.

MATERIALS AND METHODS

The specimens of the two plant species used in this study were identified and herborized. The exsiccates were deposited at the Vale do São Francisco Herbarium (HVASF) and registered under voucher numbers HVASF 14578 (*Lippia origanoides* Kunth) and HVASF 16036 (*Hymenaea martiana* Hayne). Plant species and microorganisms were registered in SisGen - National System for the Management of Genetic Heritage and Associated Traditional Knowledge (protocol number: A6F8E32).

EO and CEE extraction

Leaves and inflorescences of *L. origanoides* were extracted immediately after sampling. Distilled water was added to the fresh plant material to extract the essential oil (EO) by hydrodistillation using a Clevenger apparatus, with extraction occurring at a temperature of 80 ºC for 2 hours.

The crude ethanolic extract (CEE) was obtained using the dry leaf power of *H. martiana* (45 °C for 72 hours), which was macerated with 95% ethanol followed by rotary evaporation. Four extractions were performed at 72-hour intervals.

Green synthesis and characterization of silver nanoparticles

The silver nanoparticles (AgNPs) were produced at the Laboratory of Impedance Spectroscopy of Organic Materials (LEIMO) at Federal University of Vale do São Francisco (UNIVASF), Juazeiro Campus. Synthesis was performed according to the methodology of GUIMARÃES et al. (2020) with modifications, using a green synthesis pathway employing the crude ethanolic extract of jatobá leaves (*H. martiana*) and the essential oil of wild oregano (*L. origanoides*) as bioreductive agents.

AgNPs were prepared in a neutral medium (pH 7) using CEE and in a basic medium (pH 11) using EO, controlled by the inclusion of NaOH (0.1 M) and HCl (0.1 M) in the aqueous solution. The reaction kinetics were monitored by UV-Vis spectroscopy, and characterization was performed by Dynamic Light.

Evaluation of the in vitro antimicrobial activity

The leaf extract of *H. martiana* was adjusted to a concentration of 25,000 µg/mL and diluted with 9.5% ethanol to obtain a stock solution. The final concentrations in the microdilution were 12,500; 6,250; 3,125; 1,562.5; 781.25; 390.6; 195.3 and 97.65 µg/mL. For the essential oil of *L. origanoides*, a stock solution was prepared by diluting the EO in methanol. The final concentrations were: 3,200, 1,600, 800, 400, 200, 100, 50, and 25 µg/mL.The concentrations

of the AgNP-J and AgNP-AP solutions were adopted as percentages of 50%, 25%, 12.5%, 6.25%, 3.125%, 1.562%, 0.781%, and 0.390%.

The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were determined following protocol M07-A9 (CLSI, 2012). The gentian violet assay (STEPANOVIĆ et al., 2007; MERINO et al., 2009) was employed to quantify the production of biofilm by the microorganisms used in this study. Biofilm production was classified according to the criteria described by STEPANOVIĆ et al. (2000). Test substances (CEE-J, EO-AP, AgNP-J,and AgNP-AP) were added to evaluate interference with biofilm formation and the consolidated biofilm, with a final concentration equivalent to ½ MIC of each isolate (JOHNSON et al., 2002; MERINO et al., 2009). Assays were performed in triplicate, and medium sterility and bacterial viability controls were used.

Time-kill

The inoculants were prepared at a concentration of $1.5x10^6$ colony-forming units (CFU/mL)based on the optical density (D.O) of each pre-inoculum and were subjected to two treatments: MH broth supplemented with AgNP-J and AgNP-AP at a concentration corresponding to 2xCBM of each isolate, and MH broth without the addition of the test substances (control). Soon after, the cultures were maintained at 37 °C and stirred at 180 rpm for 2 h (maximum evaluation time). During this period, an aliquot was withdrawn every 30 min for serial dilution and plated by the pour-plate method on MH agar (BARON et al., 2006). Time-kill curves were determined by counting colony-forming unitsat each interval.

Ex situ antimicrobial activity

The methodology proposed by ENGEL et al. (2017) was followed with a few adaptations using AISI 304 stainless steel, polyethylene, and tile surface replicates measuring 4 cm² and two bacterial strains, *Escherichia coli* and *Staphylococcus aureus* – ATCC 25923. The culture was adjusted to 10^6CFU, and replicates of the different materials were arranged in Petri dishes and immersed in 50 mL of the inoculum. The dishes were then incubated at 37 ºC for 24 h in a bacteriological incubator for biofilm formation. After the period described above, the replicates were washed with 20 mL sterile distilled water to remove non-adherent cells. Next, each replicate was immersed in 50 mL of the tested antimicrobial solution (AgNP-J, AgNP-AP, sodium hypochlorite as the positive control, and sterile distilled water as the negative control) for 30 and 60 min. Concentrations of the solutions with nanoparticles were prepared according to the MBC results (2x) and 200 ppm sodium hypochlorite. The control treatment was performed by immersing the test replicates in sterile distilled water, and counts were performed simultaneously, as described above.

After the contact time, the two replicates were washed again with 20 mL of sterile distilled water to remove any substances. After this step, the replicates were transferred to empty sterile Petri dishes, and then sterile swabs soaked withpeptone water (0.1%) were rubbed in an 'X' movement performed 10 times. Next, swab samples were diluted in peptone water (0.1%) to dilutions from 10^{-1} to 10^{-6}. The drop methodology was used in the assay, with 10 µl of each dilution being inoculated in triplicate in Plate Count Agar (PCA) and incubated at 37 °C for 24 hours, after which the colony forming units (CFU) were quantified.

RESULTS AND DISCUSSION

As shown in table 1, neither CEE-J nor EO-AP exhibited bactericidal activity against any of the bacteria evaluated. However, when nanoparticles were produced using these products, their ability to eliminate microorganisms was observed even at low concentrations. These results could be explained by the fact that nanotechnology makes plant components more bioavailable and potentially bioactive (GHOSH et al., 2022). The nanoparticles showed antimicrobial effects against both Gram-positive and Gram-negative microorganisms.

The antibacterial activity of nanoparticles depends on various factors, such as their interaction with cell walls, which differs according to the nature of the bacteria (Gram-positive or Gram-negative) and the characteristics of each organism (GUIMARÃES et al, 2021). According to GHOSH et al. (2022), the mechanism of action of metallic nanoparticles stabilized with plant extracts is as follows: When added to the bacterial

Table 1 - Minimum nhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the substances CEE-J, EO-AP, AgNP-J, and AgNP-AP against standard and environmental strains.

Microorganisms		Tested substance							
		CEE-J µg/mL		EO-AP µg/mL		AgNP-J % v/v		AgNP-AP % v/v	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Standard strain	*S. aureus* (ATCC 25923)	3.125	3.125	1.600	1.600	12.5	12.5	12.5	12.5
	S. aureus (ATCC 33591)	1.562.5	6.250	800	800	12.5	12.5	25	25
	E. (ATCC 25922)	6.250	-	400	400	12.5	-	6.25	6.25
	Salmonella enterica (ATCC 10708)	12:500	-	-	-	6.25	6.25	6.25	6.25
Environmental strain	*L. monocytogenes*	-*	12.500	-*	-	-*	12.5	-*	25
	E. coli	-	-	-	-	12.5	12.5	12.5	12.5
	Salmonella spp.	12.500	-	-	-	12.5	12.5	12.5	12.5
	P. aeruginosa	1.562.5	1.562.5	-	-	1.562	3.125	3.125	3.125

CEE-J = Crude ethanolic extract of jatobá; EO-AP = Essential oil of wild oregano; AgNP-J = Silver nanoparticles with jatobá; AgNP-AP = Silver nanoparticles with wild oregano; - = No activity at the tested concentrations; - * = No MIC observed. Source: the authors.

culture medium, the particles are oxidized and emit metal ions, which increase reactive oxygen species (ROS) at the cellular level, leading to cellular lipid peroxidation, protein oxidation, DNA damage, and, finally, cell death.

The antimicrobial action of AgNPs synthesized from the essential oil of *L. origanoides* and the ethanolic extract of *H. martiana* are novel results because no research findings have been found using the same natural products in biosynthesis.

The effect observed in this study (Figure 1) against the strains was confirmed by the 100% microbial growth inhibition observed after one hour of contact with AgNPs. The results obtained from the time-kill curve provide a profile of antimicrobial activity over time and information on the extent of death, which can be employed to detect the presence of resistant subpopulations (SELESTINO NETA et al., 2017).

Based on the results found in table 2, it should be noted that the interference with biofilm formation, either by reducing or preventing its production, is a significant result for this study since, by having these abilities, the tested substances favor the non-existence of bacterial communities with protection advantages against sanitizing agents. In this scenario, preventing the formation of bacterial biofilms in food-related environments is the best strategy to eradicate them (COUGHLAN et al., 2016).

In the interference assay on consolidated biofilms (Table 3), AgNPs showed less efficiency compared to their use during biofilm formation. The cell density is higher when the biofilm is consolidated, thus making the process less reversible during this phase. The biofilm matrix is responsible for providing favorable shelter to microbial cells and promoting interactions, communication through molecular signals, and defense against hostile environments (LU et al., 2022). According to ZORE et al. (2020), microbial persistence in the environment depends not only on the strain but also on the surface.

Biofilm formation and the elimination of pathogenic microorganisms during the food processing stage is a relevant issue for food safety (ZHU et al., 2022) as the persistence of microorganisms in biofilms throughout the food processing chain offers risks to consumers' health (LING et al., 2020).

Microbial reduction on stainless-steel and polyethylene surfaces; although, the action of AgNPs and HS was satisfactory, restricted to zero the counts of *S. Aureus* and *E. coli*. The greatest reductions in *S. aureus* and *E. coli* on the tile surface (Table 4) were observed when they were in contact with sodium hypochlorite. Silver nanoparticles (AgNP-J and AgNP-AP) also reduced the adhesion.

According to ZORE et al. (2020), microbial persistence in the environment depends

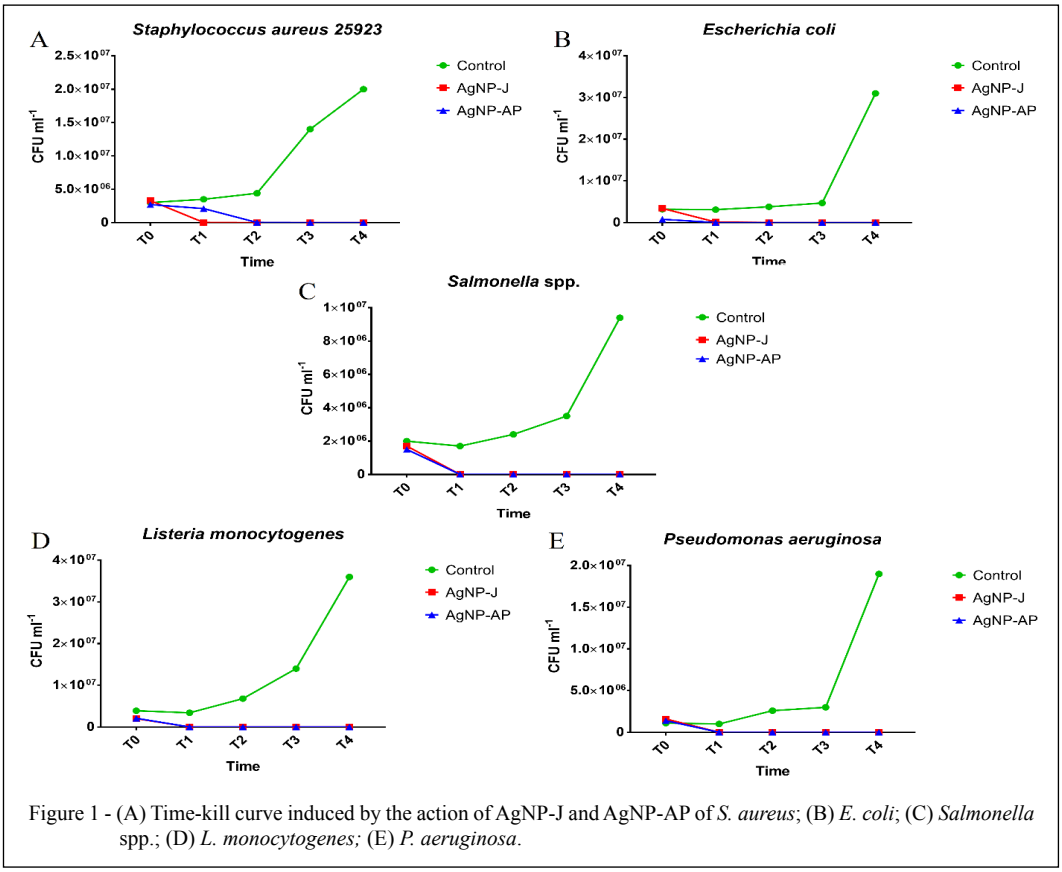

Figure 1 - (A) Time-kill curve induced by the action of AgNP-J and AgNP-AP of *S. aureus*; (B) *E. coli*; (C) *Salmonella* spp.; (D) *L. monocytogenes;* (E) *P. aeruginosa*.

not only on the strain but also on the surface. The physical and chemical properties of the equipment surface, such as the roughness, hydrophobicity, electrical charge, and conservation status, are also involved in this process (BERNARDES et al., 2012).

From this perspective, the results showed that under the conditions used in this study, 30 and

Table 2 - Quantification of production and interference of CEE-J, EO-AP, AgNP-J, and AgNP-AP on biofilm formation by standard and environmental bacterial strains.

Microorganisms	Biofilm production	----------Interference on biofilm formation----------			
		CEE-J	EO-AP	AgNP-J	AgNP-AP
Staphylococcus aureus (ATCC 25923)	Moderate	No biofilm	Weak	Weak	Weak
Staphylococcus aureus (ATCC 33591)	Moderate	Weak	Weak	Weak	Weak
Escherichia coli (ATCC 25922)	Weak	No biofilm	No biofilm	No biofilm	No biofilm
Salmonella enterica subsp. *enterica* serovar *Choleraesuis (*ATCC 10708)	Weak	No biofilm	No biofilm	Weak	Weak
Listeria monocytogenes	Weak	Weak	Weak	Weak	No biofilm
Escherichia coli	No biofilm	Weak	No biofilm	No biofilm	No biofilm
Salmonella spp.	Weak	Weak	No biofilm	No biofilm	No biofilm
Pseudomonas aeruginosa	Weak	Weak	Weak	Weak	No biofilm

CEE-J = Crude ethanolic extract of jatobá; EO-AP = Essential oil of wild oregano; AgNP-J = Silver nanoparticles with jatobá; AgNP-AP = Silver nanoparticles with wild oregano. Source: The authors.

Table 3 - Percentage of interference of the substances CEE-J, EO-AP, AgNP-J, and AgNP-AP on consolidated biofilm.

Microorganisms	----------Interference on consolidated biofilm----------			
	CEE-J (%)	EO-AP (%)	AgNP-J (%)	AgNP-AP (%)
Staphylococcus aureus (ATCC 25923)	145.8	83.5	120.0	154.4
Staphylococcus aureus (ATCC 33591)	151.7	89.7	104.7	175.8
Escherichia coli (ATCC 25922)	72.7	128.6	142.8	142.5
Salmonella enterica subsp. *enterica* serovar *Choleraesuis* (ATCC 10708)	59.5	99.3	100.7	109.8
Listeria monocytogenes	59.3	95.9	116.4	200.0
Escherichia coli	62.3	99.3	106.8	140.7
Salmonella spp.	70.3	113.4	109.3	101.1
Pseudomonas aeruginosa	89.4	116.8	108.7	110.5

>100: Increased the biofilm; < 100: Reduced the biofilm; CEE-J = Crude ethanolic extract of jatobá; EO-AP = Essential oil of wild oregano; AgNP-J = Silver nanoparticles with jatobá; AgNP-AP = Silver nanoparticles with wild oregano. Source: the authors.

60 min were not sufficient for *S. aureus* and *E. coli* to form biofilms on stainless steel, polyethylene, and tile surfaces. However, the initial adhesion was sufficient to favor the contamination of the tested surfaces and to evaluate the bacterial reduction capacity of AgNPs.

CONCLUSION

The AgNPs showed antibacterial and antibiofilm activity, eliminating bacteria adhered to stainless steel and polyethylene surfaces, materials that are usually part of the composition of utensils, and equipment involved in milk processing. Tile surfaces deserve greater attention during the cleaning stages because of higher bacterial adhesion and inefficiency of the antimicrobials tested.

Considering the growing microbial resistance and the development of new products based on the principles of green chemistry, the improvement and incorporation of these nanoparticles as components in the formulation of cleaning products in the food industry is essential to increase cleaning efficiency, constituting a potential technology for use in the dairy industry.

Table 4 - Number of *Staphylococcus aureus* and *Escherichia coli* adhered to the tile surfaces after immersion in different antimicrobials.

	Treatment	30 min of immersion (log CFU/cm^2)	Log reduction	60 min of immersion (log CFU/cm^2)	Log reduction
S. aureus	ADE (Control)	1.43		1.44	
	HS	0.97	0.46	0.9	0.54
	AgNP-J	1.17	0.26	1.2	0.24
	AgNP-AP	1.17	0.26	1.04	0.4
E. coli	ADE (Control)	1.18		1.4	
	HS	1	0.18	0.87	0.53
	AgNP-J	1.32	- 0.14	1.37	0.03
	AgNP-AP	1.37	- 0.19	1.31	0.09

ADE = sterile distilled water; HS = sodium hypochlorite; AgNP-J = silver nanoparticles with jatobá; AgNP-AP = silver nanoparticles with wild oregano. Source: the authors.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTIONS

MMS was the executor and main author of the article; MLG and HPO participated in the synthesis of nanoparticles; LET and LSB isolated environmental microorganisms; BWCN participated in the laboratory analysis; DSR participated in the biofilm analysis; RMP and MMC supervised the research and drafted the article.

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