

Research Article

Antimicrobial and Substantivity Properties of Silver Nanoparticles against Oral Microbiomes Clinically Isolated from Young and Young-Adult Patients

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The dental plaque is an oral microbiome hardly associated to be the etiological agent of dental caries and periodontal disease which are still considered serious health public problems. Silver nanoparticles (AgNPs) have demonstrated to have good antimicrobial properties affecting a wide variety of microorganisms, including oral bacteria; however, there is no scientific information that has evaluated the antimicrobial effect of AgNPs against clinical oral biofilms associated with dental caries and periodontal disease. The aim of this study was to determine the antimicrobial and substantivity effects of AgNPs in oral biofilms isolated clinically from patients with dental caries and periodontal disease. Sixty-seven young and young-adult subjects with dental caries and periodontal disease were clinically sampled through the collection of subgingival dental plaque. The inhibitory effect of AgNPs was performed with standard microbiological assays by triplicate using two sizes of particle. Polymerase chain reaction (PCR) assay was used to identify the presence of specific bacterial species. All AgNPs showed an inhibitory effect for all oral biofilms for any age and, generally, any gender ($p > 0.05$); however, the effectiveness of the antimicrobial and substantivity effects was related to particle size, time, and gender ($p < 0.05$). The identified microorganisms were *S. mutans*, *S. sobrinus*, *S. sanguinis*, *S. gordonii*, *S. oralis*, *P. gingivalis*, *T. forsythia*, and *P. intermedia*. The AgNPs could be considered as a potential antimicrobial agent for the control and prevention of dental caries and periodontal disease.

1. Introduction

The maintenance of oral health is presented as a very important challenge for the dentistry field due to complex mechanisms associated with the prevention and control of several varieties of microorganisms included in the regular oral microbiota. The human body contains a wide variety of foreign inhabitants essential for maintaining health, even for controlling or preventing diseases. This totality of microorganisms, including genomes and ecosystems, involves the microbiome [1]. Particularly, the microbiota of the oral cavity are a complex and delicate ecosystem that maintains the adequate balance between the environment and the oral microbiome; however, the alteration of this balance could induce pathological conditions and disease [2, 3]. Studies have considered that the oral microbiome belongs to biofilms through the oral cavity, producing an equilibrated ecosystem facilitating oral health; therefore, particular ecological disturbs allow that pathogens can start and develop diseases [2, 4]. Two of the most prevalent and complex multifactorial oral diseases are dental caries and periodontal disease which are actually considered as serious public health problems among children, young, and adult subjects [5, 6]. Dental caries is a chronic infectious disease characterized by the presence of small roughed surface or demineralized subsurface that progresses to cavitation of hard tissues (enamel and dentin) reaching the pulpal chamber and producing swelling, acute-chronic abscess, and particular systemic and oral symptoms mainly pain [7], while periodontal disease is presented as a chronic, inflammatory, and infectious disease that affects the teeth and surrounding tissues causing, in the most severe cases, excessive gingival bleeding and inflammation, increased dental mobility, and finally, tooth loss [8]. Both oral conditions share a bacterial etiology “dental plaque.” Dental plaque (DP) is a complex natural microbiome with well-organized multispecies microorganism communities included in an extracellular matrix which is strongly attached to the oral surfaces [9].

The authors have suggested that various sophisticated characteristics of particular dental biofilm species and their role in the oral diseases, as well as microbiological mechanisms, could be involved in their high resilience to environmental change, reduced sensitivity to antimicrobial agents, selective bacterial adhesion for oral surfaces, polysaccharide matrix and particular bacteria [10], complex attachment mechanisms arranged into specific bacterial clusters [11, 12], genetic approaches identifying bacterial preferences for clusters [13, 14], suppression of acid-sensitive species through the increased acidogenic and acid-tolerant environments [15], persistence of more virulent species [16], communication to other biofilms through nutritional interactions, genetic exchange, quorum sensing signaling, and others [17]. Each specific dental biofilm represents a well-defined bacterial distribution and, consequently, particular conditions in the oral cavity. Early colonizers involved in dental biofilm are yellow, blue, and green complexes, secondary colonizers integrate the orange complex, and the most virulent bacterial strains are involved in the red complex such as *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*

[16]. Particularly for dental caries, the microbial species are strongly associated with the metabolism of carbohydrates and develop acidogenic and aciduric environmental conditions. These species include *Streptococcus mutans*, *Streptococcus sobrinus*, *Lactobacillus*, *Bifidumbacterium*, and *Scardovia* species, among others [18–20]. Many successful protocols for prevention and control of dental caries and the different stages of periodontal disease have been implemented [21–23]. However, the prevalence and incidence of these diseases in some countries have not significantly decreased [5, 6, 24], suggesting the exploration and application of new and novel antimicrobial agents that have the biomedical potential for the prevention and control of microorganisms involved. Nanotechnology has recently contributed to develop several antimicrobial agents with enough bactericidal properties for a wide number of biomedical applications [25, 26].

Silver nanoparticles (AgNPs) are one of the most relevant metallic nanoscale materials with a great antimicrobial properties in several types of microorganisms, including oral bacteria [27–30]. Studies have reported that the use of AgNPs in biomedical field, including dental applications, have the potential to combat the dental biofilm decreasing the prevalence of dental caries [31, 32], periodontal disease [30, 33], and other oral bacterial conditions [30, 34]; however, there is not enough information that has determined the antimicrobial capacity of AgNPs against clinical dental biofilms associated with dental caries and periodontal disease conditions. The aim of this study was to synthesize and characterize two sizes of AgNPs, to determine its growth inhibition ability and substantivity effect against dental biofilms isolated clinically from young and young-adult patients presenting active dental caries and periodontal disease, and to compare and correlate physical properties of AgNPs with general sociodemographic conditions from studied subjects.

2. Materials and Methods

2.1. Materials and Reagents. Silver nitrate (AgNO_3 , CTR Scientific), gallic acid ($\text{C}_7\text{H}_6\text{O}_5$, Sigma-Aldrich), sodium hydroxide (NaOH, Jalmek Scientific™, Mexico), ammonium hydroxide (NH_4OH , Jalmek Scientific™, Mexico), Muller-Hinton (MH, BD™ Difco™, USA), and 2% chlorhexidine solutions (CHX, Consepsis™, Ultradent) were obtained, used, and stored according to the manufacturer's recommendations. All used reagents were of analytical grades.

2.2. Synthesis of Silver Nanoparticles. Two different sizes of AgNPs were prepared following the method previously reported by Espinosa-Cristóbal et al. [29]. For the first sample, 0.01 M AgNO_3 was dissolved in 100 mL of deionized water for 5 min under magnetic stirring in a 250 mL reaction vessel. After that, 10 mL of deionized water with 0.1 g of gallic acid was added to the solutions; then, the pH was immediately adjusted using 1.0 M NaOH raising to 11. For the second sample, similar concentrations of AgNO_3 were used; however, the amount of gallic acid was changed by 0.5 g. The pH was finally adjusted with NH_4OH raising a

pH of 10. Both samples were continually stirred for 10 min at room temperature.

2.3. Characterization of Silver Nanoparticles. Dynamic light scattering assay (DLS, Nanoparticle Analyzer, Nanoparticle SZ-100 series, HORIBA Scientific, Ltd., New Jersey, USA) operating with a DPSS laser at a wavelength of 532 nm using a scattering angle of 90 degrees, temperature of the holder 25°C, and dispersion medium viscosity 0.895 mPa/s for 60 seconds for each sample was performed to evaluate size and zeta potential. Transmission Electron Microscopy (TEM, Phillips CM-200) at an accelerating voltage of 25 kV determined the shape of particles; the elemental analysis was evaluated using the element energy dispersive spectroscopy (EDS) system (Team™ EDS System, EDAX).

2.4. Sample Collection. This study recruited young and young-adult patients who were included in this cross-sectional study. All of them were collected in the Admission Clinic of the Program in Dentist Surgeon at the Autonomous University of Juarez City, Mexico, between January and December 2018. An informed and voluntary written consent was obtained from patients prior to taking the samples according to the ethical guidelines of the Helsinki Declaration (2008). The protocol was approved by the Research Committee of the Biomedical Sciences Institute, Autonomous University of Juarez City (reference number 8072-1). A nonprobabilistic sampling was performed, and 62 patients were involved. The inclusion criteria were subjects from 18 to 40 years old and either gender and subjects who had brushed their teeth at least three hours before the sampling; those who received antibiotics during the last three months before starting the study and those who have any evident systemic disease were excluded.

2.5. Oral Biofilm Sampling. Dental plaque biofilm samples were taken from patients using sterilized wooden sticks through mechanical sweeping at the gingival sulcus (subgingival level) and the gingival margin (supragingival level) from interproximal sites of mandibular molars. Then, the samples were immediately cultured in Müller-Hinton broth (MH, BD™ Difco™, USA) at 37°C for 24 h. This procedure was always made on mornings to diminish the circadian rhythm effects.

2.6. Bacterial Suspensions. Once microorganisms into dental plaque samples had growth after 24 h, 100 µL of each bacterial sample was dispersed in 3 mL of phosphate buffer solution (PBS, pH 7.4) and analyzed by spectrometry (Eppendorf BioPhotometer Plus, Germany) using a wavelength of 550 nm. Finally, standardized suspensions containing 1.3×10^8 UFC/mL were obtained by spectrometry (Eppendorf BioPhotometer Plus, Germany) using a wavelength of 550 nm and an optical density of 0.126 according to the McFarland scale [29].

2.7. Antibacterial Assay. The antibacterial test used in this work was performed according to the study previously reported [29]. All dental plaque samples were cultures in Müller-Hinton broth (MH, BD™ Difco™, USA) by 18 h at 37°C before the test. Minimum inhibitory concentrations

(MIC) were determined by incubating each sample (patient) in 96-well microdilution plates; 200 µL of each AgNP dispersion was placed in the first column, and it was diluted 1:1 with MH medium (containing 2% of sucrose for oral bacteria) inoculated with standardized suspensions from each sample containing approximately 1.3×10^6 UFC/mL; finally, plates were incubated at 37°C for 24 h. After that, the last well that presented turbidity was considered as MIC. 2% chlorhexidine solutions (Consepsis™, Ultradent) were used as a control group using similar parameters for antimicrobial assay. All antibacterial tests were made in triplicate.

2.8. Substantivity Assay. Five new patients were randomly selected according to similar procedures described above. The new inclusion criteria were to have positive periodontal disease and active caries. The exclusion criteria were similar parameters from oral biofilm sampling. The presence of periodontal disease was to have at least one clinical oral sign associated with periodontal disease such as partial or total gingival inflammation, spontaneous or induced gingival bleeding, epithelial migration with bleeding, or tooth mobility with bleeding [35]. The presence of dental caries was made through clinical examinations on the continuity, cavitation, or demineralization on the topography of the dental enamel [36]. The dental plaque biofilm samples were obtained using similar criteria than the sample collection, oral biofilm sampling, and bacterial suspension sections. The substantivity assay was made according to parameters previously reported with some modifications [37]. 20 µL of each antimicrobial sample (AgNPs, chlorhexidine, and deionized water) and 100 µL of a standardized suspension (1.3×10^8 CFU/mL) of dental plaque biofilm previously taken were added to sterilized tubes containing 4 mL of MH broth with 2% sucrose. All of them were incubated at 37°C and analyzed using a UV-Vis spectrophotometer to determine the changes in optical density at 550 nm after 1, 6, 12, 24, and 48 h. The final concentrations for each AgNP and chlorhexidine samples were 5.1941 µg/mL and 97.08 µg/mL, respectively. The substantivity activity was determined through the changes in the absorbance values according to growth inhibition of dental plaque samples exposed and non-exposed to AgNPs. All procedures were made in triplicate.

2.9. Identification of Microorganisms by Polymerase Chain Reaction (PCR). Dental plaque sample of a randomly selected patient with the presence of dental caries and periodontal disease was analyzed to identify the main dental caries and periodontal disease-associated bacteria. The sample was inoculated in Müller-Hinton broth and incubated for 24 h at 37°C. Once centrifugated to obtain bacterial pellet at 12000 rpm for 10 min, it was washed in 1 mL of PBS (pH 7.4), resuspended into 200 µL of cell lysis buffer (1.0% Triton X-100, 20 mM Tris-HCl, 2 mM EDTA, pH 8.0), and incubated at 85°C for 10 min. DNA was extracted by phenol-chloroform purification and isopropanol precipitation method as previously described [38, 39]. DNA was rehydrated by Tris-EDTA, and the concentration obtained was determined by spectrophotometry (NanoDrop 2000; Thermo Scientific, Madison WI, USA). The presence of

TABLE 1: Oligonucleotides used in this study.

Primer	Sequence	Amplicon (bp)	Reference
<i>S. mutans</i>	F: GCACCACAACATTGGGAAGCTCAGTT R: GGAATGGCCGCTAAGTCAACAGGAT	433	Hoshino et al. [40]
<i>S. sobrinus</i>	F: TTCAAAGCAAGACCAAGCTAGT R: CCAGCCTGAGATTGAGCTTGT	328	Yoshida et al. [41]
<i>S. sanguinis</i>	F: GGATAGTGGCTCAGGGCAGCCAGTT R: GAACAGTTGCTGGACTTGCTTGTC	313	Hoshino et al. [40]
<i>S. salivarius</i>	F: GTGTTGCCACATCTTCACTCGCTTCGG R: CGTTGATGTGCTTGAAAGGGCACCCATT	544	Hoshino et al. [40]
<i>S. gordonii</i>	F: CTATGCGGATGATGCTAATCAAGTG R: GGAGTCGCTATAATCTTGTGTCAGAAA	440	Hoshino et al. [40]
<i>S. oralis</i>	F: TCCCGGTCAGCAAACCTCCAGCC R: GCAACCTTTGGATTTGCAAC	374	Hoshino et al. [40]
<i>P. gingivalis</i>	F: TGTAGATGACTGATGGTGAAAACC R: ACGTCATCCCCACCTTCTCTC	197	Amano et al. [42]
<i>T. forsythia</i>	F: GCG TATGTAACCTGCCCGCA R: TGCTTCAGTGTGAGTTATACC T	641	Ashimoto et al. [43]
<i>T. denticola</i>	F: TTATACCGAATGTGCTCATTTACAT R: TCAAAGAAGCATTCCCTCTTC TTCTTA	316	Watanabe and Frommel [44]
<i>P. nigrescens</i>	F: ATGAAAACAAAGGTTTTCCGGTAAG R: CCCACGTCTCTGTGGGCTGCGA	804	Ashimoto et al. [43]
<i>P. intermedia</i>	F: CAAAGATTCATCGGTGGA R: GCCGTCCTTATTGGAAG	375	Stubbs et al. [45]

TABLE 2: Characterization of silver nanoparticles.

AgNP (nm)	DLS diameter (nm)	Shape	Initial concentration ($\mu\text{g}/\text{mL}$)	Zeta potential \pm ZD* (mV)
10.2	10.2 \pm 0.7	Spherical	1070	-48.4 \pm 6.96
29.3	29.3 \pm 0.7	Spherical	1070	-52.6 \pm 8.51

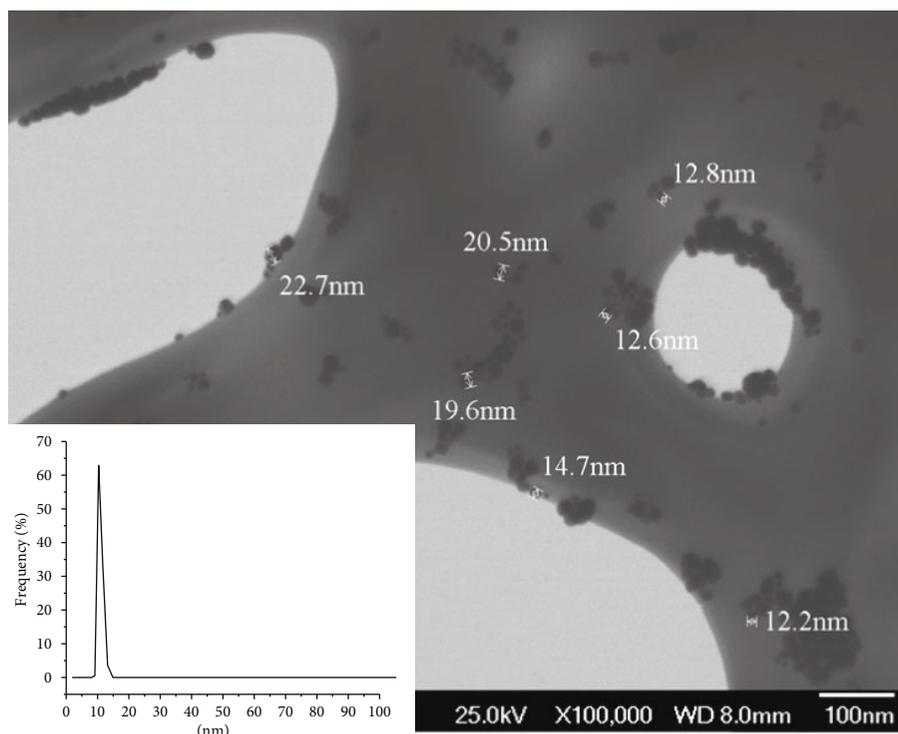
DLS: dynamic light scattering. *Zeta potential is expressed in average and zeta deviation.

Streptococcus mutans (*S. mutans*), *Streptococcus sobrinus* (*S. sobrinus*), *Streptococcus sanguinis* (*S. sanguinis*), *Streptococcus salivarius* (*S. salivarius*), *Streptococcus gordonii* (*S. gordonii*), *Streptococcus oralis* (*S. oralis*), *Porphyromonas gingivalis* (*P. gingivalis*), *Tannerella forsythia* (*T. forsythia*), *Treponema denticola* (*T. denticola*), *Prevotella nigrescens* (*P. nigrescens*), and *Prevotella intermedia* (*P. intermedia*) was detected by PCR. Bacterial DNA in the dental plaque sample were identified using specific primers described in Table 1. DNA amplification was performed with a thermal cycler (ProFlex Dual PCR System, Thermo Scientific) with specific cycling protocols for each set of primers [40–45]. Positive and negative controls were included in each PCR set. The PCR products were analyzed by electrophoresis in a 2% agarose gel-Tris-acetate EDTA buffer, and a 100 bp DNA ladder marker (New England Biolabs, Beverly, MA, USA) was used as a reference for molecular size [46]. Gels were stained with 0.5 μg of ethidium bromide/mL and observed under UV light (E-Gel Imager System with UV Base; Thermo Fisher Scientific, Life Technologies, Waltham, MA, USA).

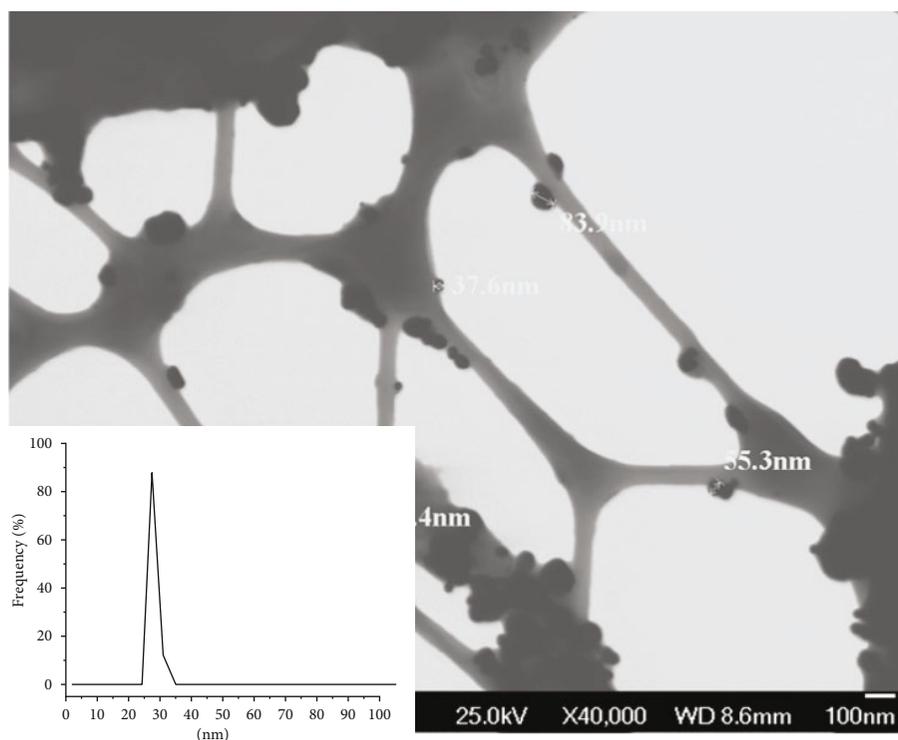
2.10. Statistical Analysis. All data, diagnosis, and microbiological evaluations were made by calibrated researchers and dental clinicians. Descriptive statistics for patients were expressed in frequencies and percentages. The differences between the frequencies according to the gender were analyzed using chi-square and age with the Mann-Whitney *U* test. All data of antimicrobial testing were expressed as the mean \pm standard deviation. Significant differences between antimicrobials, genders, optical density, and time were analyzed by Mann-Whitney *U* test for nonparametric values, and the correlation analysis was performed using Pearson's test. The applied statistics software was the StatView (SAS Institute Inc., v5.0.1, USA) and IBM-SPSS programs (IBM-SPSS, Statistics, v25, USA). Groups were considered significantly different when $p < 0.05$.

3. Results

3.1. Characterization of Silver Nanoparticles. The physical and chemical characteristics of AgNP are shown in Table 2. Uniform sizes, spherical shapes, and good particle



(a)



(b)

FIGURE 1: DLS and TEM results of AgNPs. (a) 10.2 nm; (b) 29.3 nm.

distributions were consistently observed for both AgNP samples (Figure 1). According to the DLS results, single, centered, and thin peaks were found in smaller (10.2 ± 0.7 nm) and

larger (29.3 ± 0.7 nm) AgNP samples, respectively. The zeta potential results indicate that both AgNP samples had negative values with acceptable electrical charges to

TABLE 3: Distribution of dental plaque samples from patients.

Patients	Frequency (%)	Age**	OD
Male	25 (40.3)	24.6 ± 4.5	0.061 ± 0.03
Female	37 (59.7)	21.9 ± 3.8	0.048 ± 0.02
Total	62 (100)	23.0 ± 4.3	0.053 ± 0.03

The age and optical density are expressed in average and standard deviation. * $p < 0.05$; ** $p < 0.01$.

prevent particle agglomeration (from -52 to -48 mV); however, the larger AgNP samples (-52.6 ± 8.51 mV) had slightly higher electrical charges than smaller Ag particles (-48.4 ± 6.96 mV).

3.2. Distribution of Patients. Table 3 shows the distribution of dental plaque samples used in this study from patients. A total of 62 dental plaque samples taken clinically from patients were included, in which 25 subjects were male (40.3%) and 37 females (59.7%) were equally recruited ($p > 0.05$); although young patients were basically sampled (23.0 ± 4.3 years old), the male subjects were statistically older compared to female patients ($p < 0.01$). The bacterial growth ability of oral dental plaque samples was analyzed using spectrometry finding statistically a similar bacterial growth capacity between male and female patients ($p > 0.05$).

3.3. Initial Bacterial Suspensions. The results of bacterial suspensions obtained previously to antibacterial assay are shown in Figure 2. The initial bacterial growth from male patients showed statistically similar absorbance values compared to female subjects (Figure 2(a)); however, female patients showed a significantly positive correlation (0.372 , $p < 0.05$) between age and initial absorbance values compared to men (0.106 , $p > 0.05$). These results suggest that the microorganisms involved in specific dental plaque biofilms had similar growth activity in regular conditions, which is gender independent; however, the microorganisms from older women demonstrated statistically to have better bacterial growth abilities according to the age, in comparison to male patients (Figures 2(b) and 2(c)); which could conclude for female patients only, bacterial growth activity depends on age.

3.4. Antibacterial Test. The results of the antibacterial assay are shown in Figure 3. The average of MIC was slightly higher for female compared to male patients, but no statistical differences were found (Figure 3(a)); however, microorganisms involved in the dental biofilm samples from woman patients seemed to have statistically more resistance to the larger AgNPs (29.3 nm) compared to men subjects (Figure 3(b)). On the other hand, smaller AgNP samples (10.2 nm) presented significantly better antimicrobial properties than larger AgNPs (29.3 nm); however, chlorhexidine samples had the highest levels of antimicrobial activity for any gender and AgNP samples (Figure 3(c)). Interestingly, when the growth inhibition activity of AgNPs was compared according to gender, smaller AgNP samples had statistically a similar antimicrobial ability for male compared to female subjects (Figure 3(d)). Although the chlorhexidine and

smaller AgNPs had the highest antimicrobial activity for both genders, results indicate that those variations in the levels of antimicrobial resistance to the AgNPs could be associated with particular characteristics presented by specific oral microorganisms involved in each type of gender. Figure 4 shows significant correlations between MIC values and age of male and female patients. The MIC values and the age of female and male patients for both sizes of AgNPs indicated no significant statistical correlation ($p > 0.05$); the results suggest that the Ag concentrations for both sizes of AgNPs are not influenced by the age of male or female patients maintaining a uniform antimicrobial activity at any age; however, male patients showed MIC values that could increase according to the age (Figure 4(d)).

3.5. Substantivity Assay. Table 4 shows the distribution of dental plaque samples taken from patients for the substantivity test. Five dental plaque samples taken clinically from patients were included, in which 2 subjects were men (40%) and 3 women (60%) were sampled. The included subjects were generally young-adult patients (35.8 ± 1.6 years old). The results of the substantivity activity are shown in Figure 5. As the results from antimicrobial assay, smaller AgNP samples had better inhibition growth activity compared to larger AgNPs and the control group despite its low Ag concentration ($5.1941 \mu\text{g/mL}$) ($p < 0.05$); moreover, the chlorhexidine solutions continued to be the most efficient antimicrobials ($p < 0.01$) for the inhibition of dental plaque even in that of smaller AgNPs although its concentration used ($97.08 \mu\text{g/mL}$) had been higher (Figure 5(a)). Also, it is very evident that larger AgNPs need more Ag concentrations to inhibit statistically the bacterial growth from dental biofilms compared to smaller particles (Figure 5(a)). On the other hand, it was observed that bacterial growth was gradually increased according to the time for all AgNP samples and the chlorhexidine solution with significant differences for each group (Figures 5(b) and 5(c)); interestingly, the smaller AgNPs had statistically more uniform growth inhibition activities during 1, 6, and 12 h starting at 24 h with the more significant bacterial growth compared to larger AgNPs and the control group (Figure 5(b)). Although smaller AgNPs demonstrated to have a well-sustained antimicrobial activity up to 12 h, chlorhexidine samples showed markedly the highest substantivity levels ($p < 0.05$). The correlation test indicated that all groups had significant and positive correlations according to the time suggesting that even the smaller (0.34 , $p < 0.01$) and larger AgNPs (0.58 , $p < 0.01$) as well as the chlorhexidine solution (0.33 , $p < 0.01$) had a limited antimicrobial activity that started to decrease significantly with the time, diminishing the ability to inhibit the bacterial growth from dental plaque samples (Figure 5(d)).

3.6. PCR Assay. The identification of microorganisms by the PCR test is shown in Table 5. The presence of microorganisms related to dental caries was six bacterial strains in which five strains (*S. mutans*, *S. sobrinus*, *S. sanguinis*, *S. gordonii*, and *S. oralis*) present positive results (63.3%) and only one was absent (*S. salivarius*, 36.7%). For microorganisms associated with periodontal disease, five bacterial strains were

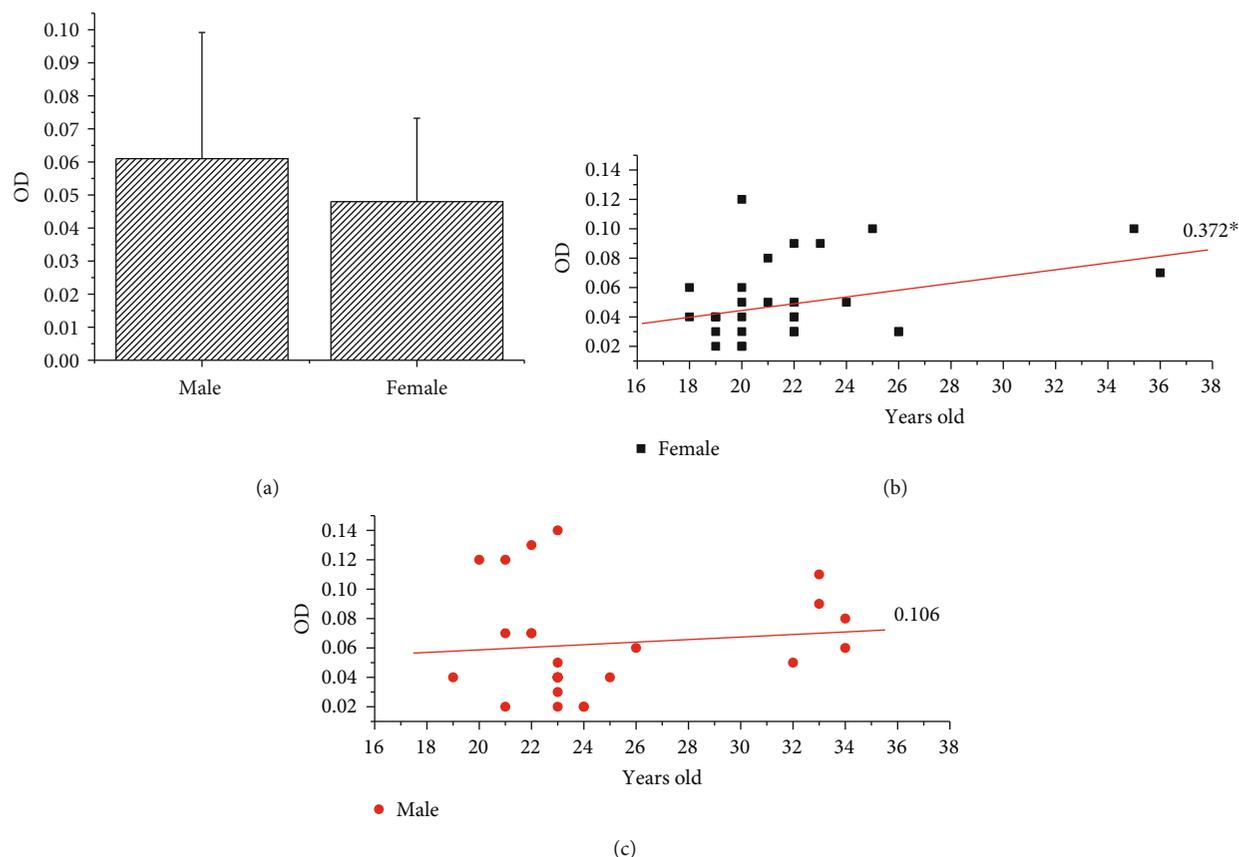


FIGURE 2: Initial bacterial suspensions before the antimicrobial test. * $p < 0.05$; ** $p < 0.01$.

included obtaining the presence (60%) of three strains (*P. gingivalis*, *T. forsythia*, and *P. intermedia*) and absence (40%) for 2 microbial species (*T. denticola* and *P. nigrescens*). In general, eleven bacterial strains were analyzed obtaining the presence of 8 species (72.7%) associated with dental caries and periodontal disease.

4. Discussion

This study determined that the AgNPs can significantly inhibit the bacterial growth of microorganisms involved in dental plaque biofilms taken clinically from patients associating the smaller AgNP samples with better antimicrobial properties compared to larger AgNPs acting significantly up to 12-24 h ($p < 0.05$). Although the chlorhexidine solutions were the best antimicrobial properties for the different microbiological evaluations; both sizes of AgNP samples demonstrated also to have significant antimicrobial activities against a wide variety of dental plaque samples from young and young-adult patients finding an acceptable bacterial growth inhibition according to the exposition time even with relatively low Ag concentrations. On the other hand, the substantivity assay indicated that smaller AgNP samples demonstrated to maintain better antimicrobial activity in bacterial samples from patients with active caries and periodontal disease up to 12-24 h compared to larger AgNPs and the control group ($p < 0.01$); however, the substantivity activity of the chlorhexidine was the best. PCR results support the presence

of various dental bacterial species associated with the beginning, development, and severity of dental caries and periodontal disease. In general, the AgNPs showed good bacterial inhibition activity when they were exposed to dental biofilms isolated clinically from young and young-adult patients with active dental caries and periodontal disease.

Several studies have consistently demonstrated the great antimicrobial activity and antiadherence properties of AgNPs against a wide variety of microorganisms, including oral bacteria; however, there are not enough works that have determined the bactericidal and the substantivity activities of AgNPs against oral biofilms taken clinically from young and young-adult patients with active caries and periodontal disease. Studies have reported that AgNPs covered human dentin and different alloy medical implants can significantly inhibit the biofilm formation on the surface of the dentin and the implants as well as control the bacterial growth around them [47, 48]. Also, the authors determined the antimicrobial and antibiofilm properties of AgNP (~17 nm) included in orthodontic appliances and standardized microbiocidal assays against *S. mutans*, *Lactobacillus casei* (*L. casei*), *Staphylococcus aureus* (*S. aureus*), and *Escherichia coli* (*E. coli*) as well as *in vitro* biofilms using cariogenic bacteria (*S. mutans*), suggesting the potential use to prevent the dental biofilm, decreasing the incidence of demineralization activities associated with caries during conventional dental treatments [49, 50]. A recent *in vitro* study reported the antimicrobial activity of AgNPs (30-50 nm) and chlorhexidine

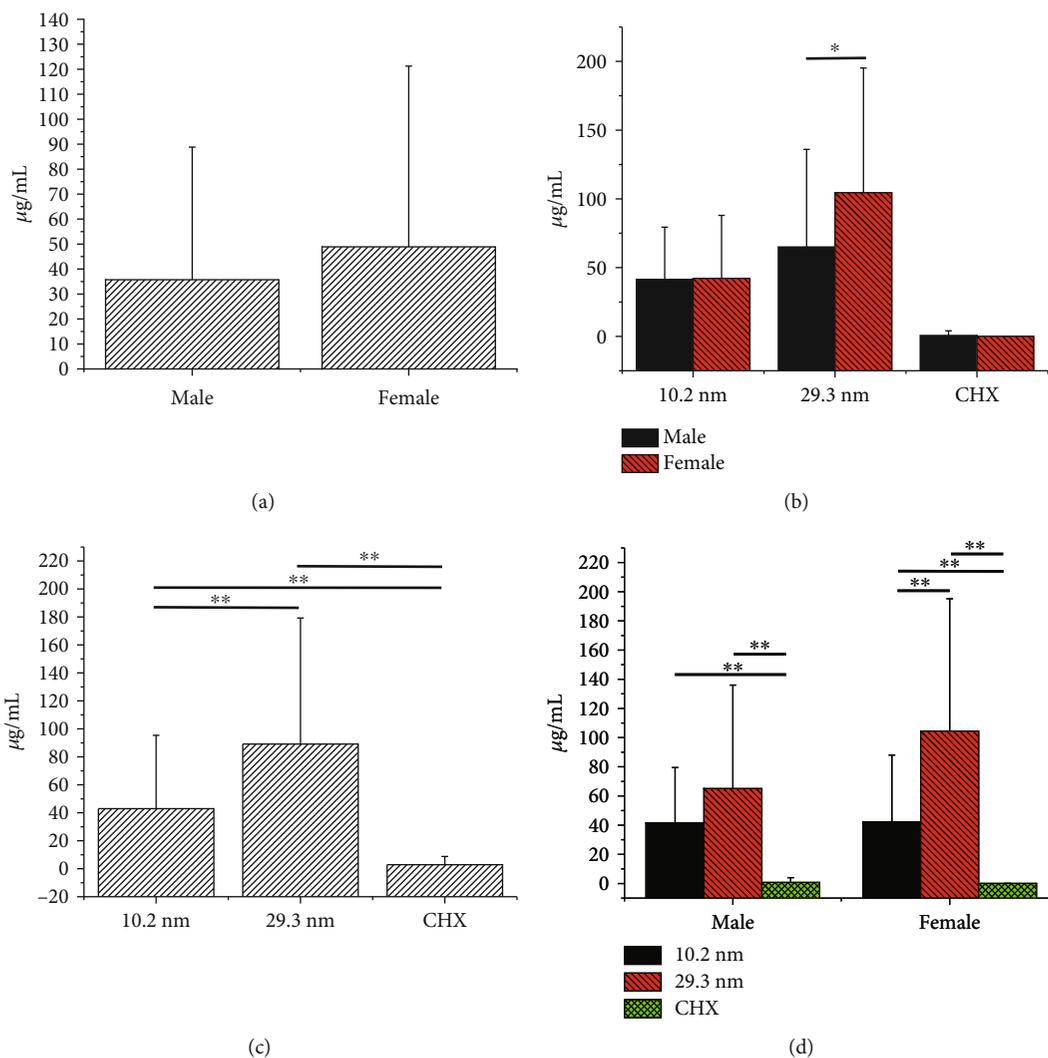


FIGURE 3: Antimicrobial assay for AgNP samples and gender. * $p < 0.05$; ** $p < 0.01$.

(main antimicrobial agent in dentistry) against different oral pathogenic bacteria (*S. mutans*, *S. oralis*, *Lactobacillus acidophilus*, *Lactobacillus fermentum*, and *Candida albicans*), determining good bactericidal and bacteriostatic properties of AgNPs for all microbial strains with concentrations less than five folds as compared to chlorhexidine supporting its potential and safe use in the dentistry field [51]. Besides, the authors recently reported the *in vitro* antibiofilm efficacy of biosynthesized AgNPs (10-20 nm) against three different pathogenic bacteria associated with endodontic failures and periodontal disease (*Enterococcus faecalis*, *Porphyromonas gingivalis*, and *Bacillus pumilus*); the minimum inhibitory concentrations were very narrow between the microorganisms (20 $\mu\text{g/mL}$ for *Bacillus pumilus* and 30 $\mu\text{g/mL}$ for *Enterococcus faecalis* and *Porphyromonas gingivalis*, respectively). This study concluded that those biosynthesized AgNPs showed to have a good antibiofilm activity to the growth control of pathogenic microorganisms associated with endodontic and periodontal problems [30, 52]. The results indicated and demonstrated that the AgNPs (10.2 and 29.3 nm) have good antimicrobial properties, which

inhibit the bacterial growth of microorganisms involved in dental biofilms taken clinically from young and young-adult patients with active caries and active periodontal disease; the results are statistically associating the antimicrobial efficacy of AgNPs with the particle size and, in some cases, the gender for larger AgNPs ($p < 0.05$). Also, both samples of AgNPs showed no significant correlations between the antimicrobial activity and age of patients indicating that the antimicrobial efficacy works independently of the age ($p > 0.05$). The AgNPs demonstrated statistically to have acceptable substantivity ability in periods of 1, 6, 12, 24, and 48 h compared to the control group determining significant associations related to the particle size and a time-dependent activity ($p < 0.05$). It is possible that particular factors related to the gender, presented in female patients (Figure 3(b)), are directly involved to promote more resistant antimicrobial activities to the AgNPs due to high concentrations of specific microorganisms, more incidence of dental caries, more genetic and hormonal variations, and differences among saliva flow compared to male patients [53]; but also, these biofilms might show particular microbial species

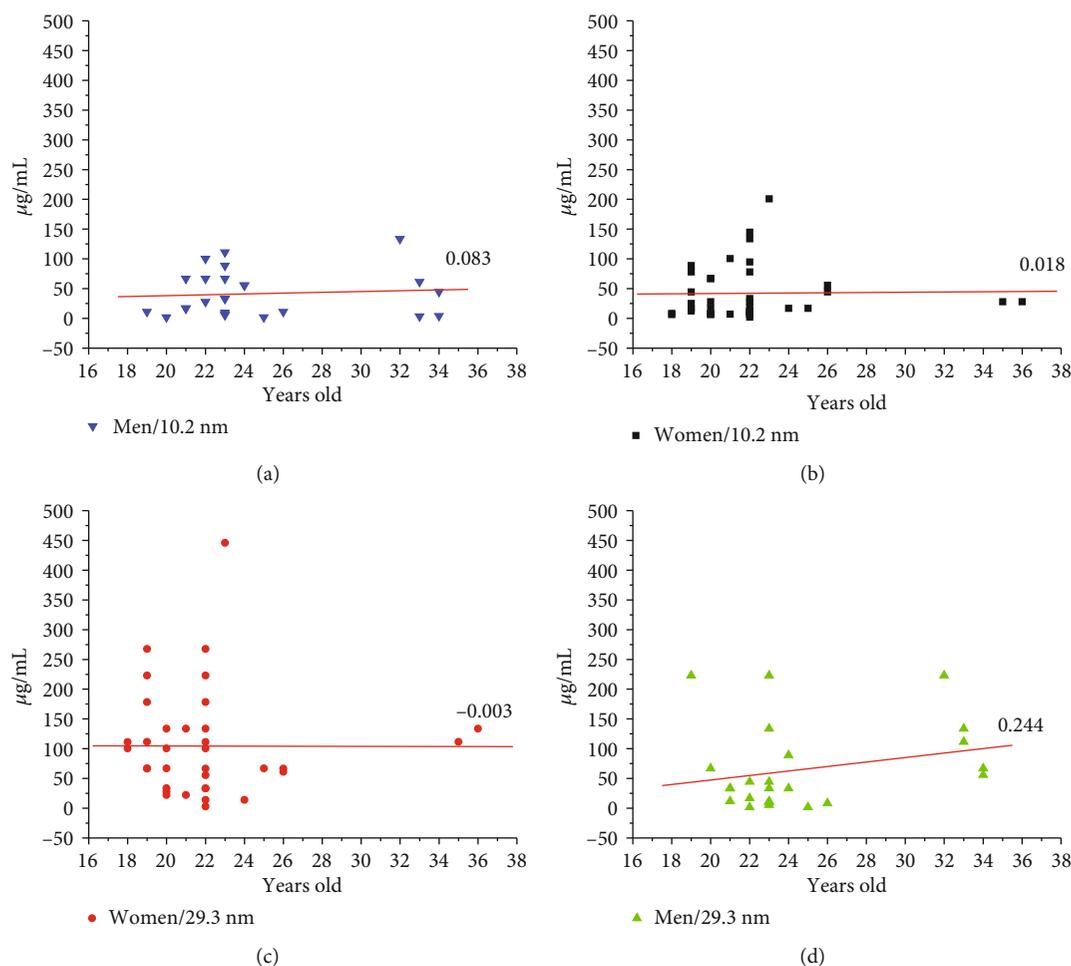


FIGURE 4: Significant correlations according to MIC values and age. * $p < 0.05$; ** $p < 0.01$.

TABLE 4: Distribution of dental plaque samples for the substantivity test.

Patients	Frequency (%)	Age
Male	2 (40)	22.5 ± 13.4
Female	3 (60)	44.6 ± 13.3
Total	5 (100)	35.8 ± 1.6

The age is expressed in average and standard deviation.

such as *S. parasanguinis*, *S. mitis*, and *S. oralis* for healthy patients and *Actinomyces*, *Lactobacillus*, *S. mutans*, and *S. sobrinus* for patients with dental caries [54, 55] in which some ability from bacterial strains associated with the formation of dental caries and periodontal disease to resist the antimicrobial effect of conventional antibiotics might also be involved [56, 57].

Although the action mechanisms of AgNPs are still under investigations, several studies have suggested that AgNPs, particularly smaller particles, released Ag ions and other physical properties such as electrical charge from particle surface (zeta potential) can have the ability to adhere to cell membrane and interact with amino, thiol, and hydroxyl

groups as well as DNA generating pores and damage in cell membrane by accumulation of AgNPs, cytoplasmic leakage, free radicals, reactive oxygen species (ROS), DNA damage, denaturation of proteins, and others, leading the cell death [29, 58, 59]. In the case of PCR results, it is very well known that particular bacterial strains are strongly associated with the beginning, development, and severity of dental caries and periodontal disease (gingivitis and periodontitis), respectively. Particularly, the streptococci group are the principal pathogens involved to the initial colonization, the incipient demineralization, and the formation of cavitated surfaces producing the dental caries; bacterial species such as *S. mutans*, *S. sobrinus*, *S. sanguinis*, *S. salivarius*, *S. oralis*, *S. gordonii*, *Lactobacillus*, and *Bifidobacteria* are commonly found in this demineralization process [15, 19, 20, 60]. The periodontal disease is divided according to the damage of the periodontal tissues: (1) gingival inflammation or gingivitis and (2) loss of periodontal tissues (alveolar bone and periodontal ligament, mainly) or periodontitis [61]. The most severe stage of periodontal disease is the periodontitis in which the bacterial pathogens involved are *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia* belonging to the red complex [14, 16, 39]; however, other bacterial species, such as *P. nigrescens* and *P. intermedia*,

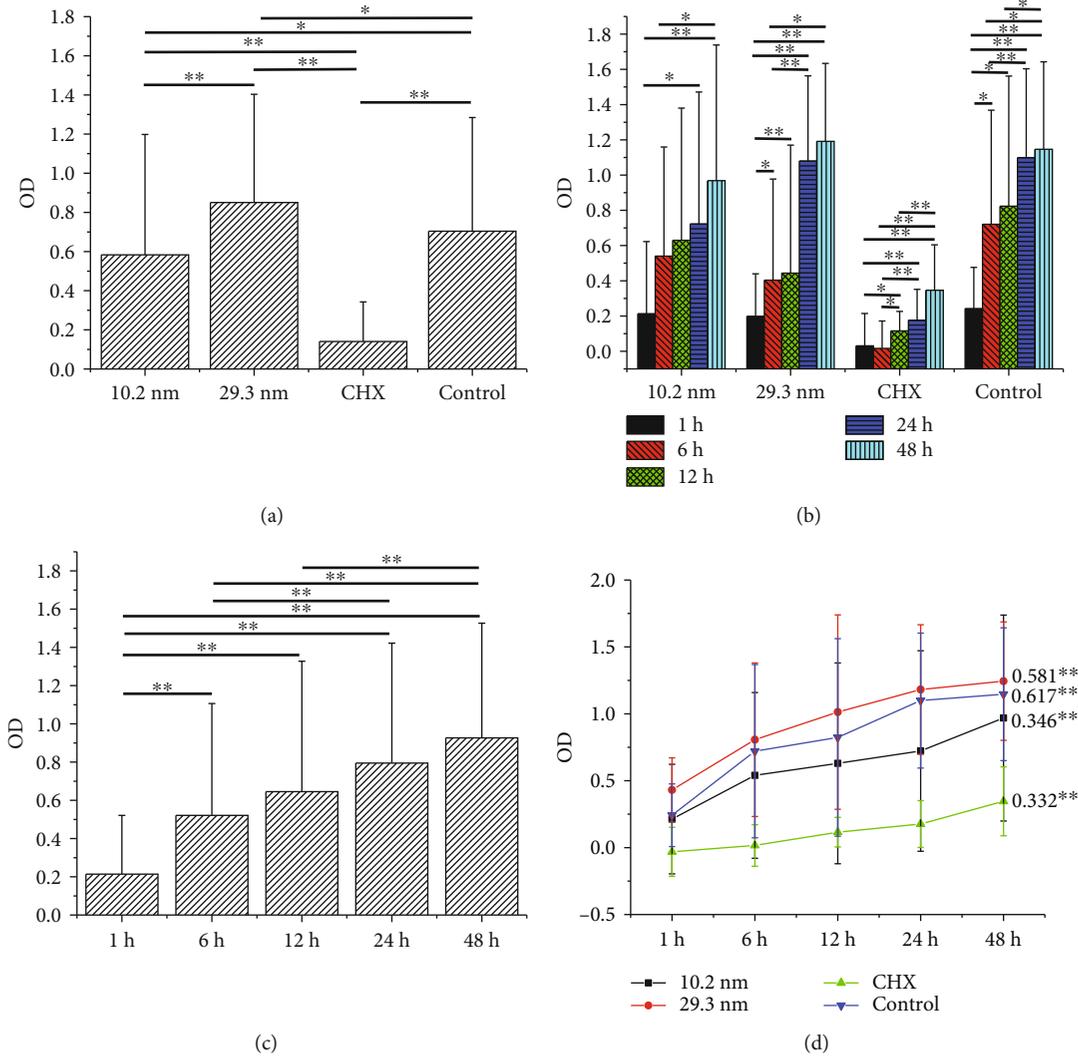


FIGURE 5: Substantivity assay for AgNPs and CHX. * $p < 0.05$; ** $p < 0.01$.

TABLE 5: Presence of oral bacteria related to dental caries and periodontal disease by PCR.

Microorganism	Presence
<i>S. mutans</i>	+
<i>S. sobrinus</i>	+
<i>S. sanguinis</i>	+
<i>S. salivarius</i>	-
<i>S. gordonii</i>	+
<i>S. oralis</i>	+
<i>P. gingivalis</i>	+
<i>T. forsythia</i>	+
<i>T. denticola</i>	-
<i>P. nigrescens</i>	-
<i>P. intermedia</i>	+

+ = positive; - = negative. PCR assay was made at least by triplicate for each strain.

are also related to specific stages and conditions of periodontal disease [62, 63]. PCR assay indicated the presence of various bacterial species associated with dental caries (*S. mutans*, *S. sobrinus*, *S. sanguinis*, *S. oralis*, and *S. gordonii*) and periodontal disease (*P. intermedia*), particularly periodontitis (*P. gingivalis* and *P. forsythia*), confirming the presence of cariogenic and periodontopathic bacterial species distributed into oral biofilm from adult patients. Standard antimicrobial assay is used containing 2% of sucrose which enhances the cariogenic power of microorganisms associated with dental caries because this compound is frequently present in our daily diet [36] facilitating the metabolization of fermentable carbohydrates from streptococci species [60] considering, from this point of view, a more realistic condition. It has been reported that biofilms can also show different microbiologic characteristics than planktonic strains due to the biofilms that have particular biochemical processes to interchange genetic material which might promote better bacterial metabolisms to survive to more extreme conditions [64]. One of them is the “quorum sensing” phenomena in which bacterial cells could have the ability to communicate precise

information through chemical signals permitting the regulation of gene expressions, low antibiotic penetration, the formation of multilayered defense, and other complex mechanisms for the survival of the biofilm [65]. Furthermore, it has been reported that the presence and distribution of oral microorganisms change according to the age of subjects due to low saliva production [66] affecting the regular oral functions of the oral cavity to reach an oral microenvironmental instability and cause some oral disease associated with bacterial biofilms [67].

The present study determined that the AgNPs can statistically have the same antimicrobial and substantivity effects for any age and gender; however, the larger AgNPs presented more limited properties for the female patients compared to the smaller particles even with the presence of sucrose that promote high bacterial growth conditions. These results might suggest that a more complex action mechanism of AgNPs could promote better antimicrobial and substantivity activities in which specific particle size or Ag ions might have an evident bactericidal effect and, at the same time, the ability to intervene in the production of extracellular polysaccharides (bacterial adhesion agents), such as glucans, fructans, teichoic and lipoteichoic acids, and fimbriae, interfering in specific metabolic processes for the development of streptococci groups mainly binding structures on surfaces and other bacteria [29, 47, 68]. Those results might also suggest that the action mechanism of AgNPs is presented as a complex activity where synergistically various combined mechanisms could be acting at the same time in the antimicrobial activity and substantivity effect of AgNPs against biofilm samples from patients with dental caries and periodontal disease affecting regular metabolic activities for bacterial growth, reproduction, bacterial cell adhesion, and, finally, the limited survival of the oral biofilm. Factors such as physicochemical properties of AgNPs (size, shape, concentration, distribution, administration, exposition time, and Ag ion release), particular microorganisms (type, variations in cell membrane, species, site of growth, concentration, standard/clinical strain, mutagenicity, and pathogenicity level), and specific conditions from patients (systemic and oral habits, sociodemographic conditions, presence of systemic and oral diseases, genetic, gender, age, and others) might directly play an important role in the effectiveness of the antimicrobial and substantivity properties of AgNPs. Although our results might suggest this possible hypothesis related to the action mechanism of AgNPs and the high potential to be a great antimicrobial alternative in the inhibition growth of oral biofilms from patients with active dental caries and active periodontal disease limiting the high frequency of the most common and infectious oral diseases associated with the presence of biofilms (dental caries and periodontal disease), more scientific works should undoubtedly investigate different physicochemical properties of AgNPs and other simulated oral environments, variations from their systemic and oral health as well as include other sociodemographic conditions from patients, and, finally, microbiological and molecular characterizations of the clinical biofilm samples isolated from patients according to the presence and distribution of the different species included in the sample

with any other oral or systemic disease considered as a public health problem.

5. Conclusions

The AgNPs used in this study demonstrated to have good antimicrobial and substantivity properties to inhibit the bacterial growth ability of various oral biofilms isolated from subjects with active dental caries and active periodontal disease. The main factors associated with the best effectiveness of the antimicrobial and substantivity activities of AgNPs were statistically in the smaller particles (10.2 nm), for short periods of time (1-24 h) and, in some cases, the gender (male patients); therefore, the chlorhexidine solution, as a gold standard agent, had the best antimicrobial and substantivity properties. Based our understanding, this is the first study that determined the antimicrobial and substantivity levels of two different sizes of AgNPs against biofilms taken directly from young and young-adult patients with active caries and active periodontal disease. Although it is still needed to make more scientific studies to understand the variations and the antimicrobial behavior of AgNPs, this study could suggest the use of AgNPs as a potential antimicrobial agent in the biomedical and stomatology fields for the prevention and control of dental caries and periodontal disease.

Data Availability

The data obtained from this study can be found in the research archives of the Master's Program in Dental Sciences of the Autonomous University of Ciudad Juarez and can be requested through the corresponding author.

Disclosure

The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Conflicts of Interest

The authors declare no conflict of interest.

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