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Antibacterial Efficacy of Hypochlorous Acid against *Streptococcus mutans* on Orthodontic Wires: An In Vitro Study

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Abstract

Aim
Streptococcus mutans plays a central role in the development of dental caries and white spot lesions in orthodontic patients. The prevention of biofilm formation on orthodontic appliances requires effective antimicrobial strategies. This in vitro study aimed to evaluate the antimicrobial efficacy of hypochlorous acid (HOCl) at concentrations of 0.25%, 0.75%, and 1.25% against *Streptococcus mutans* adhering to nickel–titanium (NiTi) and stainless steel orthodontic wires.

Methods
Orthodontic wire segments made of NiTi and stainless steel (n = 20) were inoculated with *Streptococcus mutans* (ATCC 25175) and exposed for 30 seconds to HOCl at three different concentrations. A 2% chlorhexidine solution served as the positive control, and sterile saline was used as the negative control. Antimicrobial activity was assessed using the agar diffusion method and turbidity-based bacterial growth evaluation following 24 hours of incubation at 37 °C. Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Statistical significance was set at $p < 0.05$.

Results
Hypochlorous acid at a concentration of 1.25% demonstrated the highest antimicrobial activity, producing significantly larger inhibition zones compared with 0.25% HOCl ($p = 0.021$), 0.75% HOCl ($p = 0.049$), and 2% chlorhexidine ($p=0.005$). No statistically significant differences were observed between 0.25% HOCl, 0.75% HOCl, and 2% chlorhexidine ($p > 0.05$). Stainless steel wires exhibited greater bacterial adhesion than NiTi wires. Sterile saline showed no antimicrobial effect.

Conclusion
Within the limitations of this in vitro study, hypochlorous acid at a concentration of 1.25% exhibited superior antimicrobial activity against *Streptococcus mutans* compared with 2% chlorhexidine. Lower HOCl concentrations (0.25% and 0.75%) demonstrated antimicrobial efficacy comparable to that of chlorhexidine, suggesting that reduced concentrations may be preferable in clinical practice to balance antimicrobial effectiveness while minimizing the potential risk of corrosion of orthodontic wires.

Keywords: Hypochlorous Acid, Orthodontic Wires, *Streptococcus Mutans*

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Background

One of the main challenges reported by orthodontic patients is the deterioration of oral hygiene associated with the use of both fixed and removable appliances, which can lead to a shift in the oral microbiota that may induce dental caries and periodontitis.(1) This observation is supported by previous studies showing an increase in the prevalence of cariogenic bacteria, particularly *Streptococcus mutans* and *Lactobacillus*, during and after orthodontic treatment involving acrylic removable appliances, bands, and metal or ceramic brackets (2–4).

Streptococcus mutans is a normal inhabitant of the oral cavity and is considered the most cariogenic of all oral streptococci, playing a key role in the initiation and progression of dental caries. Several factors contribute to bacterial adhesion, including surface charge, hydrophobicity, and surface roughness, which facilitate the formation of biofilms. Streptococcus mutans is a Gram-positive hydrophobic species, and bacteria in aqueous suspension typically carry a negative surface charge, increasing the likelihood of adhesion to orthodontic appliances and biofilm development (5).

Currently, various antimicrobial agents are available, including chlorhexidine (CHX) and cleaning tablets, for eliminating biofilms from retainers and orthodontic appliances. CHX is a broad-spectrum antimicrobial agent effective against *Streptococcus mutans*, *Escherichia coli, Streptococcus sanguinis, Candida albicans,* and *Staphylococcus aureus.* Studies have shown that orthodontic retainers disinfected with CHX mouthwash had significantly lower levels of *Streptococcus mutans* (3,6).

However, a microbiological study by Al-Lehaibi et al. (7) demonstrated variable inhibition outcomes of CHX against *Streptococcus mutans* and Lactobacillus species, possibly due to bacterial resistance mechanisms. Some orthodontic patients showed signs of CHX resistance, likely due to prolonged exposure, which may result in genetic changes favoring resistant microbial clones. Moreover, long-term CHX use has been associated with adverse effects such as tooth staining and taste alteration (8).

As a result, alternative agents have been explored, including hypochlorous acid (HOCl), which is derived from sodium hypochlorite and is associated with fewer side effects (9). HOCl is naturally produced by the human immune system and exerts its antimicrobial effect by penetrating bacterial membranes, disrupting structure and metabolism, ultimately causing cell death (10). In addition to its antimicrobial activity, HOCl is biocompatible with oral tissues and has demonstrated anti-inflammatory effects on the oral mucosa. It interferes with protein synthesis in microbial cells, destroying them, and is considered safe for daily use, since it is naturally produced by neutrophils and macrophages during the respiratory burst in the phagocytosis of antigens (11,12)

In dentistry, this non-antibiotic antimicrobial agent has been used for root canal irrigation and in 0.05% mouthwashes to minimize bacterial and pathogen accumulation. In vitro studies have demonstrated that HOCl effectively eradicates microorganisms and disrupts the extracellular polymeric matrix of biofilms, a process similar to mechanical debridement (13).

Furthermore, HOCl at 180 ppm has been shown to be effective in cleaning biofilm-contaminated titanium implant surfaces, yielding results comparable to those of NaOCl and CHX; however, it requires four times the volume for similar outcomes (14). With the growing concern of antimicrobial resistance worldwide, HOCl has gained interest as a treatment option for biofilm-associated and viral infections (15). Tsai et al. (16) reported that HOCl exhibits concentration-dependent antimicrobial activity and biocompatibility. At 400 ppm, HOCl significantly reduced oral bacterial populations without adverse cytotoxic effects, making it a promising antiseptic. Additionally, the bacteriostatic effect was slightly greater with a 10-minute treatment compared to a 1-minute exposure.

Therefore, HOCl solution could be considered a potential non-antibiotic mouthwash for reducing gingivitis and plaque or as an irrigant in root canal therapy and peri-implantitis treatment.

Nevertheless, Lafaurie et al. (17) noted that HOCl exhibits lower antimicrobial inhibition against Gram-positive bacteria and higher efficacy against Gram-negative microorganisms. Tsai et al. (16) further emphasized that the antimicrobial effectiveness of HOCl depends on various factors, including pH, concentration, time, and volume. Increased volume, higher concentration, and extended contact time enhance bactericidal activity, with greater effects observed on Gram-negative oral bacteria than on Gram-positive species.

HOCl exhibits concentration-dependent antimicrobial effects and good biocompatibility. Lower concentrations have shown promising

antibacterial action with reduced cytotoxicity, while higher concentrations enhance bactericidal activity against biofilm-forming species commonly associated with orthodontic appliances. (10,12,13,16) Thus, the 0.25%, 0.75%, and 1.25% concentrations enable the evaluation of low, intermediate, and high levels within clinically relevant ranges, providing insight into a potential dose–response relationship.

Based on these findings, this study aimed to evaluate the antimicrobial effectiveness of hypochlorous acid at concentrations of 0.25%, 0.75%, and 1.25% against *Streptococcus mutans* strains associated with orthodontic appliances using microbiological assays. Both stainless steel and nickel-titanium (NiTi) wires were included in this study because they are widely used in orthodontic practice and present different surface properties that may influence *Streptococcus mutans* adhesion and biofilm development.

Methods

The present in vitro experimental study was conducted using *Streptococcus mutans* (strain ATCC 25175), obtained from the microbiology laboratory of the University of the Hemispheres, Quito, Ecuador (protocol No. CEUHE25-53). All procedures were carried out under controlled laboratory conditions at the Biohack UIO Laboratory to evaluate the inhibitory effect of hypochlorous acid (HOCl) at concentrations of 0.25%, 0.75%, and 1.25% on *Streptococcus mutans* adhering to orthodontic wires made of stainless steel and nickel-titanium (NiTi).

A total of 20 orthodontic archwire segments were used: 10 NiTi and 10 stainless steel wires (Orthometric, Marília, Brazil), each cut into 2-cm segments. The wire segments were experimentally contaminated with *Streptococcus mutans* and subsequently exposed to HOCl at three concentrations (0.25%, 0.75%, and 1.25%). A 2% chlorhexidine solution was used as a positive control and sterile saline as a negative control, following the methodology described by Portes Canongia et al. (18).

The experimental protocol consisted of three sequential phases:

• Verification of *Streptococcus mutans* adhesion to orthodontic wire segments

• Evaluation of the antimicrobial activity of the tested disinfectant solutions

• Assessment of the inhibitory effect of HOCl on *Streptococcus mutans* adhered to orthodontic wires

Preparation of Culture Medium and Test Tubes

Twenty test tubes were filled with brain heart infusion (BHI) broth to assess bacterial adhesion. BHI was prepared by dissolving 37 g of dehydrated powder in 1 liter of distilled water, according to the manufacturer's instructions. The preparation involved:

- Weighing 14.8 g of BHI for 400 mL of water
- Mixing until fully dissolved
- Distributing into test tubes
- Autoclaving at 121°C for 15 minutes
- Cooling before inoculation(19)

Wire Preparation and Sterilization

The 0.019″×0.025″ NiTi and stainless steel wires were used. Wires were sectioned into 2-cm segments, classified into:

- 10 test tubes with NiTi segments
- 10 test tubes with stainless steel segments

Each segment was immersed in BHI broth and sterilized before bacterial inoculation. (20,21)

Bacterial Activation and Inoculation

The *Streptococcus mutans* strain was activated in fresh sterile BHI medium (10 mL) and incubated at 37°C for 24 h. Bacterial growth was confirmed by visible turbidity, and the resulting suspension was homogenized and used to inoculate all the samples (20).

Each tube contained:

- 5 mL of sterile BHI broth
- 1 sterilized wire segment
- 0.1 mL of activated *Streptococcus mutans* culture

All the samples were incubated at 37°C for 24 h to allow bacterial adhesion to the wire surfaces.

Evaluation of Bacterial Growth

After incubation, the wires were removed using sterile forceps. To eliminate non-adherent bacteria, each segment was washed three times in sterile saline with gentle agitation. (20) The wires were then transferred to fresh BHI broth and incubated for another 24 h.

Due to increased turbidity, serial dilutions (10^{-5} and 10^{-6}) were prepared and plated on solid BHI agar using the surface spread technique with

Drigalski loops. Plate distribution was as follows:

- 9 Petri dishes for 10^{-5} NiTi
- 11 for 10^{-6} NiTi
- 8 for 10^{-5} stainless steel
- 10 for 10^{-6} stainless steel

The plates were incubated at 37°C for 24 h. Colonies were manually counted and reported as colony-forming units (CFU/mL) (19,22).

Gram Staining

Gram staining was performed to confirm the presence of Gram-positive cocci consistent with *Streptococcus mutans* morphology and to exclude potential contamination. The staining procedure was carried out according to standard microbiological protocols (19,22).

Antimicrobial Activity of Hypochlorous Acid

HOCl was synthesized in the laboratory by acidifying sodium hypochlorite (NaClO, 9.5–12%), following stoichiometric calculations to determine the reactant ratios. NaClO was mixed with a known concentration of organic acid under a fume hood, and after 30 minutes, the active chlorine content was measured. The solution was then buffered, stabilized, and transferred to a sealed high-density polyethylene container to ensure stability and reproducibility, in accordance with previously described protocols (23). Each concentration was freshly prepared to achieve the target levels of 0.25%, 0.75%, and 1.25%.

The 30-second exposure time was selected to simulate a clinically relevant short-term disinfection protocol, consistent with chairside rinsing or appliance immersion times commonly used in orthodontic practice. This choice also aligns with previous in vitro antimicrobial studies that evaluated the rapid activity of oxidizing agents such as HOCl and NaOCl, thereby ensuring comparability with existing literature and allowing for an assessment of HOCl efficacy under conditions that reflect realistic clinical exposure (12,16,21).

For the agar diffusion test, 16 BHI agar Petri dishes were seeded with Streptococcus mutans (lawn technique). Wells were created in the agar, and the following substances were applied:

- 0.25% HOCl (n=4)
- 0.75% HOCl (n=4)
- 1.25% HOCl (n=4)
- 2% chlorhexidine (n=3, positive control)
- Sterile saline (n=1, negative control)

The plates were incubated at 37°C for 24 h, and the diameter of the inhibition zones was measured to assess antimicrobial efficacy. (22,23)

Inhibitory Effect of HOCl on Streptococcus mutans in Orthodontic Wires

Sixteen sterile test tubes were prepared with 5 mL of BHI and divided into:

- 8 tubes with stainless steel wires
- 8 with NiTi wires

Each tube received 0.1 mL of activated *Streptococcus mutans* culture and was incubated at 37°C for 24 h.

Bacterial adhesion evaluation

Bacterial adhesion to orthodontic wires was assessed using a quantitative culture-based method. After 24 h incubation of wire segments with *Streptococcus mutans*, the specimens were rinsed three times in sterile saline to remove non-adherent bacteria and then transferred to fresh brain heart infusion (BHI) broth for an additional 24 hour incubation. Adhesion was quantified by evaluating bacterial growth through medium turbidity and by counting colony-forming units (CFU/mL) obtained from serial dilutions plated on BHI agar, allowing standardized indirect quantification of viable bacteria adherent to the wire surfaces.

After confirming bacterial adhesion, the wires were exposed to:

- HOCl (0.25%, 0.75%, and 1.25%)
- 2% CHX (positive control)
- Saline (negative control)

The exposure lasted 30 seconds. The wires were then transferred to new tubes with 5 mL of sterile BHI and incubated again at 37°C for 24 hour. (19,21)

Bacterial growth was evaluated based on the turbidity of the medium, indicating the effectiveness of each disinfectant concentration. (23)

Randomization and Blinding

All wire segments were randomly allocated to the experimental groups using a computer-generated randomization sequence to minimize allocation bias. In addition, the investigator responsible for measuring inhibition zones and evaluating bacterial growth was blinded to the group assignment during outcome assessment.

Sample Size Calculation

An a priori sample size calculation was performed using G*Power software (version 3.1.9.7; Heinrich Heine University, Düsseldorf, Germany). Based on previous study (25) assessing antimicrobial inhibition zones against *Streptococcus mutans*, a one-way ANOVA test was selected with an assumed effect size (f) of 0.40, a

significance level (α) of 0.05, and a statistical power of 80% (1-β = 0.80). The analysis indicated that a minimum total sample size of 20 specimens was required, corresponding to at least four samples per experimental group.

Results

In this study, normality was confirmed using the Shapiro–Wilk test (P>0.05 for all groups), which allowed the use of parametric tests.

According to Table 1 and Figure 1, the largest inhibition zone was observed in the group treated with 1.25% hypochlorous acid (HOCl), with a mean of 39.75 mm. This was followed by 0.75% HOCl (33.75 mm), 0.25% HOCl (32.75 mm), and 2% chlorhexidine (29.67 mm). As expected, the negative control group (sterile saline) showed no inhibition (0 mm).

One way ANOVA revealed significant differences among groups (p=0.000).

Table 1 : Descriptive statistics of inhibition zone diameters (mm) for different groups

Group	N	Mean	SD	Minimum	Maximum
2% chlorhexidine	3	29.67	2.52	27	32
0.25% HOCl	4	32.75	1.89	30	34
0.75% HOCl	4	33.75	2.87	30	37
1.25% HOCl	4	39.75	3.59	37	45
Saline solution	1	0.00	NA	0	0
One way ANOVA			P=0.000		

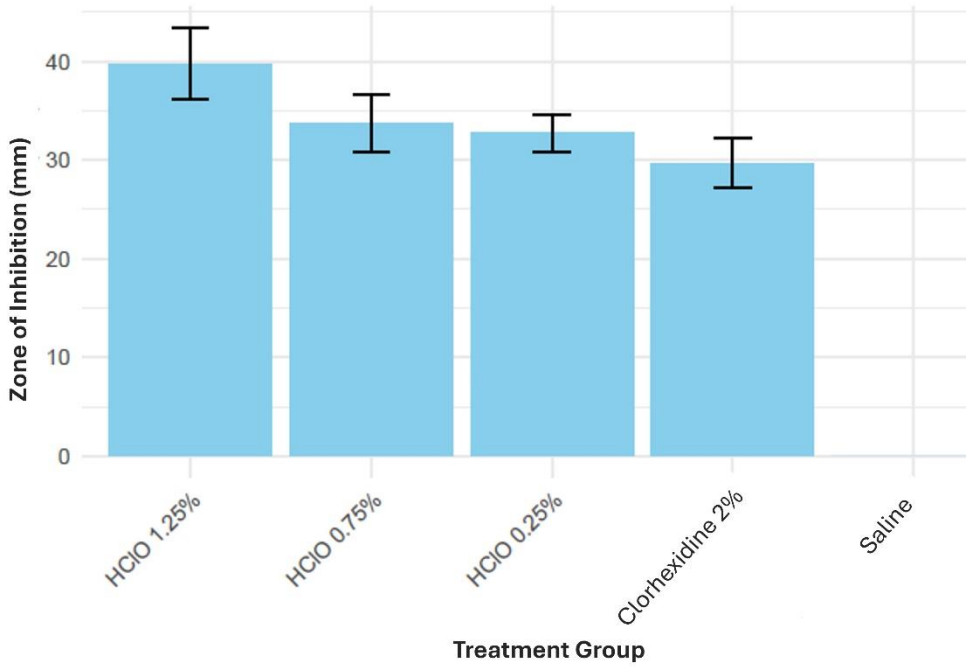

Figure 1 : Inhibition zones (mm) observed for each treatment group against *Streptococcus mutans.*

Subsequently, post hoc Tukey tests (Table 2) showed that the 1.25% HOCl group produced significantly larger inhibition zones than:

- 2% chlorhexidine (P=0.005)
- 0.25% HOCl (P=0.021)
- 0.75% HOCl (P=0.049)

No significant differences were found between 2% chlorhexidine, 0.25% HOCl, and 0.75% HOCl.

These results confirmed that 1.25% HOCl was the most effective concentration for inhibiting *Streptococcus mutans*, outperforming both the lower concentrations of HOCl and the conventional antiseptic, chlorhexidine.

Table 2: Tukey test results

Group 1	Group 2	Mean difference	P-value	95% CI (lower bound)	95% CI (upper bound)	Significant
2% CHX	0.25% HOCl	3.08	0.628	-3.94	10.11	No
2% CHX	0.75% HOCl	4.08	0.380	-2.94	11.11	No
2% CHX	1.25% HOCl	10.08	0.005	3.06	17.11	Yes
2% CHX	Saline	-29.67	<0.001	-40.28	-19.05	Yes
0.25% HOCl	0.75% HOCl	1.00	0.985	-5.50	7.50	No
0.25% HOCl	1.25% HOCl	7.00	0.021	0.49	13.51	Yes
0.25% HOCl	Saline	-32.75	<0.001	-42.92	-22.58	Yes
0.75% HOCl	1.25% HOCl	6.00	0.049	0.00	11.99	Yes
0.75% HOCl	Saline	-33.75	<0.001	-43.92	-23.58	Yes
1.25% HOCl	Saline	-39.75	<0.001	-49.92	-29.58	Yes

Discussion

Orthodontic wires require chemical disinfection to reduce microbial adhesion, particularly by *Streptococcus mutans*, a key etiologic agent in dental caries and white spot lesions. (24) Therefore, this study aimed to evaluate the antimicrobial activity of hypochlorous acid at concentrations of 0.25%, 0.75%, and 1.25% against *Streptococcus mutans* strains adhering to orthodontic appliances. Previous reports have suggested that HOCl demonstrates higher efficacy in eliminating bacteria adhering to orthodontic surfaces. (25)

The inclusion of both wire types allowed comparison between two clinically relevant alloys. Stainless steel showed higher bacterial colonization, which can be attributed to its higher surface energy and roughness, whereas NiTi demonstrated lower adhesion, consistent with previous findings. (20,26)

According to Oliveira et al., (20) Streptococcus mutans exhibits greater adhesion to stainless steel wires than to NiTi wires, consistent with our findings. This behavior is attributed to factors such as the material's composition, surface energy, and roughness, all of which influence bacterial adhesion.

Abutayyem et al. (26) confirmed the increased bacterial adhesion to stainless steel due to higher surface porosity, which facilitates plaque accumulation, gingivitis, and enamel demineralization—especially in the presence of *Streptococcus mutans* and *Lactobacillus acidophilus*. (26) Nafarrate-Valdez et al. (24) also noted that orthodontic appliances (wires, brackets, and bands) undergo chemical changes when exposed to the oral environment, which favors the proliferation of acidogenic bacteria, such as Streptococcus mutans .

In the present study, the highest inhibition zones were recorded for 1.25% HOCl, indicating superior antimicrobial performance. This finding is supported by Tsai et al., (16) who demonstrated that increasing the HOCl concentration improves efficacy against Streptococcus mutans , depending on factors such as exposure time, pH, volume, and percentage concentration.

A significant difference was observed between 1.25% HOCl and 2% chlorhexidine (P=0.005), with the former exhibiting greater antibacterial activity. This finding aligns with those of Aherne et al. (28) who reported that HOCl disrupts bacterial membranes and oxidizes sulfhydryl groups in bacterial enzymes, thereby leading to cell damage.

This study aligns with recent research highlighting alternative antimicrobial agents for controlling *Streptococcus mutans*, a key pathogen involved in dental caries and biofilm formation on orthodontic appliances. Kashi et al. (29) demonstrated that natural compounds can effectively inhibit *Streptococcus mutans* growth and biofilm development through multiple mechanisms, including disruption of bacterial metabolism and suppression of acid production. Although hypochlorous acid is not a natural compound, it similarly exhibits strong antimicrobial activity by damaging bacterial cell membranes and interfering with essential cellular functions. The comparable effectiveness of hypochlorous acid observed in the present in vitro study supports its potential as an efficient agent for reducing microbial colonization on orthodontic wires and preventing enamel demineralization during orthodontic treatment.

Although 2% chlorhexidine is widely used in clinical settings, it exhibited lower efficacy than 1.25% HOCl in our study. Similar results were reported by Koluman etal.(30) who highlighted that while CHX acts more slowly, HOCl—produced via the acidification of H2O2 or NaClO—shows rapid and effective antimicrobial action. Almhöjd et al. (31) further confirmed the limited effectiveness of CHX under certain conditions compared to oxidizing

agents.

Interestingly, 0.25% and 0.75% HOCl exhibited antimicrobial activity comparable to that of 2% CHX, with no significant differences (P>0.05). As Millones-Gómez et al. (25) noted, the antimicrobial efficacy of HOCl is concentration-dependent and can match that of CHX in eliminating both Gram-positive and Gram-negative bacteria under specific conditions.

Since 0.25% and 0.75% HOCl achieved inhibitory effects similar to 2% CHX, lower concentrations of HOCl may be advisable in orthodontics to reduce potential corrosion of metal wires. According to Kumrular et al., (32) stainless steel and titanium-based orthodontic appliances are susceptible to corrosion in acidic environments, particularly due to chloride ions (Cl^-), which may compromise wire integrity over time.

This study emphasizes the importance of selecting suitable antimicrobial agents for disinfecting orthodontic wires, particularly in preventing the colonization of Streptococcus mutans, a significant contributor to dental caries and enamel demineralization during orthodontic treatment. The results confirm that hypochlorous acid, particularly at 1.25% concentration, exhibits superior antimicrobial efficacy compared to the conventional antiseptic 2% chlorhexidine, supporting its potential as an alternative disinfectant in orthodontics.

The concentration-dependent nature of HOCl's antimicrobial action suggests that even lower concentrations (0.25% and 0.75%) may offer adequate inhibition while minimizing the risks of corrosion to orthodontic alloys. This is particularly relevant in clinical practice, where maintaining wire integrity and preventing microbial biofilm formation are both critical to treatment success and patient oral health.

Moreover, considering the rising concern over bacterial resistance and the adverse effects associated with prolonged chlorhexidine use, HOCl presents a biocompatible, effective, and safer alternative for routine antimicrobial protocols in orthodontic care. However, future in vivo studies are necessary to assess long-term efficacy, safety, and potential effects on materials and oral tissues.

Limitations

This in vitro study does not fully reflect the complexity of the oral environment, where saliva, dietary factors, and microbial interactions may influence antimicrobial efficacy. Only *Streptococcus mutans* was evaluated, while orthodontic biofilms are polymicrobial. In addition, the short exposure time assessed only immediate antimicrobial effects, and the long-term impact of repeated HOCl use on bacterial recolonization and wire surface integrity was not investigated. Therefore, further in vivo studies are required to confirm the clinical relevance and material safety of HOCl in orthodontic practice.

Conclusion

Under the conditions of this in vitro study, HOCl at a concentration of 1.25% demonstrated the highest antimicrobial activity against *Streptococcus mutans*, showing significantly greater inhibition than 2% chlorhexidine and the lower HOCl concentrations tested. Lower bacterial adhesion was observed on NiTi wires, whereas stainless steel wires demonstrated higher bacterial colonization. No statistically significant differences were observed between 0.25% or 0.75% HOCl and 2% chlorhexidine, suggesting comparable antimicrobial activity within the limitations of the present experimental model. Although these lower concentrations produced smaller inhibition zones than 1.25% HOCl, they still exhibited measurable antibacterial effects against *Streptococcus mutans*. Sterile saline showed no antimicrobial activity and served as an appropriate negative control. Overall, the findings suggest that HOCl, particularly at a concentration of 1.25%, may have potential as an antimicrobial agent for orthodontic wire disinfection. However, further investigations evaluating biofilm behavior, corrosion potential, cytotoxicity, and clinical performance are required before clinical application can be recommended.

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None

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Data Availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request

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