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A Chlorine Releasing Solution as an Alternative for Dental Biofilm Control

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Abstract

Objective: To evaluate the antibiofilm activity of a chlorine-releasing solution (CRS) and its *in vitro* effects on enamel color stability, surface roughness, and microhardness.

Methods: A mixed biofilm composed of *Staphylococcus aureus*, *Candida albicans,* and *Streptococcus mutans* was formed on standardized bovine enamel specimens (6 × 6 × 2 mm). After 48 h of maturation, the specimens were immersed for 5 min in CRS (Granudacyn, Mölnlycke Health Care), 0.12% chlorhexidine (CHX, positive control), or phosphate-buffered saline (PBS, negative control). Antibiofilm activity was determined by colony-forming unit counts (CFU/mL). For enamel property evaluation, new specimens were treated once daily for 30 s using immersion or spray protocols. Color changes (ΔE_{00}), roughness (ΔRa), and microhardness (ΔKHN) were assessed at 1, 7, 15, and 30 days.

Results: CRS significantly reduced microbial counts compared with PBS and showed activity comparable to CHX. Surface roughness remained stable across treatments, except for CHX spray at 30 days ($p < 0.05$). CRS immersion resulted in lower microhardness values than PBS but did not differ from CHX. At 30 days, CHX spray produced the highest microhardness reduction ($p < 0.05$). No significant differences in color were observed among treatments.

Conclusions: CRS demonstrated antimicrobial activity comparable to CHX without greater alterations in enamel surface properties under the tested conditions.

Keywords: Chlorine Compounds, Biofilms, Color stability, Surface roughness, Microhardness

Introduction

The oral microbiota comprises a complex community of microorganisms that colonize both hard surfaces, such as dental enamel, and soft tissues, including the oral mucosa and gingiva[1]. These microorganisms organize into structured biofilms embedded in an extracellular polymeric matrix, which enhances microbial virulence and increases tolerance to antimicrobial agents[2–4]. Although salivary flow connects different oral sites, biofilm communities function within distinct ecological niches defined by their composition and spatial organization[5].

Under physiological conditions, host factors and microbiota maintain oral homeostasis and colonization resistance[6,7]. However, environmental and systemic factors, such as frequent sugar intake or reduced salivary flow, promote dysbiosis and the development of dental caries[8]. Dental caries results from a biofilm-driven imbalance between demineralization and remineralization of tooth structure[9]. Acidogenic microorganisms metabolize carbohydrates and produce organic acids, lowering pH and promoting enamel mineral loss[10,11]. Although saliva buffers pH and aids mechanical clearance[12,13], its protective effect may be insufficient under cariogenic challenges[14].

Adjunctive antimicrobial strategies are therefore used to control biofilm and reduce microbial load[15–18]. Given the polymicrobial nature of oral biofilms, effective approaches should ideally exhibit broad-spectrum activity against both bacterial and fungal pathogens[19]. *Streptococcus mutans* is a primary cariogenic bacterium involved in enamel demineralization and biofilm formation, while *Candida albicans* can synergistically enhance its virulence within mixed biofilms. In addition, *Staphylococcus aureus* represents an opportunistic pathogen that may colonize the oral cavity, particularly under conditions of poor oral hygiene, systemic disease, or immunocompromise[20,21]. The oral cavity may serve as a reservoir for its persistence and potential dissemination. Therefore, evaluating antimicrobial strategies against a multispecies biofilm model may provide a more clinically relevant assessment of efficacy.

Chlorhexidine (CHX), is considered the gold standard antiplaque agent[22,23] due to its bacteriostatic and bactericidal activity. Nevertheless, prolonged use may cause adverse effects such as taste alteration, dental staining, and cytotoxicity effects[25-27]. These limitations have stimulated the search for alternative antimicrobial strategies that reduce biofilm while preserving the oral tissues.

Chlorine-releasing solutions (CRS) have been proposed as antimicrobial alternatives[28]. Sodium hypochlorite (NaOCl) is the most extensively studied compound due to its strong antimicrobial activity and clinical applicability[28]. In aqueous solution, NaOCl forms hypochlorous acid (HOCl), which dissociates into hypochlorite (OCl^-) and hydrogen ions (H^+)[28]. The antimicrobial activity of chlorine-based agents is mainly attributed to HOCl and OCl^-[28,29]. HOCl penetrates microbial cell walls and induces oxidative damage to essential cellular components. This mechanism resembles the oxidative defense system of macrophages, contributing to its antimicrobial effectiveness with comparatively lower cytotoxicity than NaOCl alone[28]. However, HOCl is inherently unstable and undergoes exothermic decomposition into

chlorine (Cl_2), oxygen (O_2), and hydrochloric acid (HCl)[30]. Therefore, it must be used in diluted and stabilized formulations. Its stability depends on storage conditions, including protection from light and low temperatures (<10 °C), as well as formulation in high-purity water with minimal reactive contaminants[31,32].

Stabilized NaOCl/HOCl/NaCl formulations are used for irrigation and wound cleansing. These solutions demonstrate antimicrobial activity and acceptable biocompatibility[28,29]. In Dentistry, most studies have focused on sodium hypochlorite as an endodontic irrigant rather than as a low-concentration antiseptic for routine biofilm control. However, Zwicker et al. (2023) evaluated a combined NaOCl/HOCl/NaCl solution as a mouthrinse in a randomized clinical trial[33]. The product significantly reduced total colony-forming units on oral mucosa and saliva within 1 minute, with effects persisting for up to 60 minutes. No adverse effects were reported. However, only total bacterial counts were assessed, without species-level differentiation[33].

Therefore, this study aimed to evaluate the antibiofilm action of a chlorine-releasing solution and its *in vitro* effects on enamel color stability, surface roughness, and microhardness. The null hypothesis was that CRS, regardless of the application method, would not produce significant differences in antimicrobial activity or enamel properties.

Materials and Methods

Sample size was calculated based on a pilot study (n = 2), considering anti-=biofilm activity, color stability, surface roughness, and microhardness outcomes. Power was set at 80 % and significance level at 5 % (https://www.openepi.com/). Eight specimens per group were required.

Bovine enamel fragments (6 mm high x 6 mm wide x 2 mm thick) were obtained using a low-speed diamond disc under water cooling (Isomet 1000, Isomet, Buehler, Lake Bluff, IL, EUA). Surfaces were flattened under refrigeration using SiC sandpaper with decreasing grit sizes (600, 1200, and 2000) in a metallographic mechanical polishing machine (Polipan-U, Panambra São Paulo, SP, Brazil) for 3 minutes at low speed and with a standard weight of 172 g for each grit. Baseline surface roughness was standardized with a maximum variation of 0.07 μm.

Bovine teeth were obtained from a certified commercial slaughterhouse operating under national animal welfare and sanitary regulations. As the samples consisted of post-mortem biological waste from animals slaughtered for food production, and no animals were used or sacrificed specifically for this study, formal ethical approval was not required according to institutional and national guidelines. All procedures were conducted in accordance with relevant regulations, and the study was reported in line with ARRIVE principles.

Antibiofilm activity

Candida albicans (ATCC 10231), *Streptococcus mutans* (ATCC 25175), and *Staphylococcus aureus* (ATCC 6538) were used to evaluate the *in vitro* antibiofilm activity of the chlorine releasing

solution (CRS). The biofilm formation protocol followed the methodology described by Vasconcelos et al. (2020)[34].

Each strain was cultured for 48 h on Petri dishes containing specific culture media: *C. albicans* on Sabouraud Dextrose (SD) agar, and *S. mutans* and *S. aureus* on Brain Heart Infusion (BHI) agar. A single colony of each strain was then transferred into the corresponding broth medium (SD or BHI) and incubated for 18–24 h at 37 °C to obtain cells in the exponential growth phase. The cultures were centrifuged at 4200 g for 5 min (Centrifuge 5430R, Eppendorf AG, Hamburg, Germany), and the resulting pellet was washed twice with 10 mL of phosphate-buffered saline (PBS).

To determine cell concentration, optical density was measured at 625 nm in a spectrophotometer. The bacterial suspensions were adjusted to 10^8 colony-forming units per milliliter (CFU/mL), with absorbance readings of 0.085 and 0.150 for *S. aureus* and *S. mutans*, respectively. Due to the variable morphology of the genus, *C. albicans* cell concentration was determined using a Neubauer counting chamber under a microscope at 100× magnification. Bacterial inocula were prepared at 1×10^7 CFU/mL, and *C. albicans* at 1×10^6 CFU/mL. All assays were performed using three independent biological replicates, each analyzed in technical triplicate (n = 9).

In a laminar flow cabinet (Pachane, Pa 400-ECO, Piracicaba, SP, Brazil), the specimens were randomly distributed in 24-well cell culture plates (Techno Plastic Products, Trasadingen, Switzerland). Each well received 1.5 mL of inoculated culture medium, except for the negative control group, which received 1.5 mL of sterile medium. The plates were incubated at 37 °C under agitation at 75 rpm in a bacteriological incubator (Shaker Incubator, Mod. CE-320, CienLab, Campinas, SP, Brazil), under microaerophilic conditions. After 90 minutes of incubation (adhesion period), the specimens were washed twice with PBS and transferred to plates containing sterile medium. The plates were then incubated again at 37 °C, under agitation at 75 rpm, for biofilm growth and maturation. After 24 h, half of the medium was replaced with fresh sterile medium.

After 48 h of incubation, the specimens were removed from the culture plates and rinsed to eliminate planktonic cells. They were then transferred to 12-well plates containing 4 mL of the following solutions: CRS: chlorine releasing solution (Granudacyn, Mölnlycke Health Care – Ingredients: water, sodium chloride, 50 ppm hypochlorous acid, and 50 ppm sodium hypochlorite), CHX: 0.12% chlorhexidine (positive control), PBS: Phosphate-buffered saline (negative control).

After 5 minutes of immersion, the specimens were removed and rinsed three times with PBS to eliminate residual agents. They were then transferred to test tubes containing 10 mL of PBS and sonicated for 20 minutes in an ultrasonic bath at 200 W and 40 kHz (Altsonic, Clean 9CA) to detach the remaining biofilm.

Antibiofilm activity was assessed by determining microbial load. The samples were vortexed in a mechanical shaker (Phoenix® – AP56), and serial dilutions from 10^{-1} to 10^{-7} were prepared. Aliquots of these dilutions were plated on SD agar (for *C. albicans*), Mannitol Salt Agar (for *S. aureus*), and Mitis Salivarius Agar (for *S. mutans*). Plates were incubated at 37 °C for 48 h in a microbiological incubator; S. *mutans* were incubated under microaerophilic conditions.

After incubation, colony counts were performed with a stereomicroscope (Nikon, model 86786, Tokyo, Japan). For CFU/mL calculation, the dilution containing 30 to 300 colonies was used, applying the following formula:

$$CFU/mL = \frac{number\ of\ colonies \times 10^{n}}{q}$$

where n is the absolute dilution factor (0, 1, 2, or 3) and q is the plated volume in mL. Values were expressed as $\log_{10}$ CFU/mL.

In addition to viability, the solutions' ability to remove biofilm was evaluated. After the hygiene protocol, the specimen surfaces were stained with Propidium Iodide and Syto 9 (Filmtracer™ LIVE/DEAD™ Biofilm Viability Kit; Molecular Probes, Inc., Eugene, OR, USA) for 15 minutes, protected from light. After incubation, specimens were rinsed twice with PBS and examined under an inverted epifluorescence microscope with appropriate filters at 630× magnification. ZEN 2.3 lite software (Carl Zeiss) was used to capture 20 random fields from each specimen surface and quantify the area covered by remaining biofilm (μm^2), both live and dead [Chart 1].

After assessing antibiofilm activity, new bovine enamel fragments were prepared as previously described and analyzed for color, roughness, and microhardness. In these tests, artificial saliva (pH 7), based on a modified Fusayama–Meyer solution[35], was used as the control group. Chlorhexidine was used as the positive control. Since its conventional application for biofilm control is by mouthrinse, both chlorhexidine and the experimental CRS solution (pH = 6.8, measured using a calibrated digital pH meter prior to the experiments) were tested using an immersion protocol. No pH adjustment was performed. However, considering the rational use of oral hygiene products and their potential environmental impact, both solutions were also evaluated using a spray application method.

All treatments were performed once daily for 30 seconds, regardless of the solution or application method. During immersion, the specimens were completely submerged in the solution under agitation (350 rpm)[6] . For the spray application, six sprays (1.5 mL in total) were applied onto the surface of each fragment.

After treatment, the samples were rinsed and stored in artificial saliva at 37 °C. Color, surface roughness, and microhardness measurements were repeated at 1, 7, 15, and 30 days after treatment, using the same parameters previously described. The resulting changes were then analyzed.

Color stability

The initial and final color of the enamel surface was recorded using a spectrophotometer (Easyshade, Vita Zahnfabrik, Bad Säckingen, Germany). This device contains three individual optical fibers that illuminate the dental enamel and two spectrophotometric sensors that numerically measure color.

The device tip has a diameter of 6 mm, matching the specimen size, ensuring consistent measurement area. The optical geometry simulates a 45/0 measurement with the specular component excluded to prevent interference from surface gloss[3].

The specimens were positioned on a standard white background inside a light booth with a gray backdrop and standardized D65 illumination[4]. The color measurements were obtained in the CIEDE2000 color system, which evaluates color coordinates along three dimensions: black–white luminosity (L*), green–red chromaticity (a*), and blue–yellow chromaticity (b*). The spectrophotometer emitted visible light (400–700 nm) onto the enamel surface and measured the reflected spectrum, allowing the recording of the L*, a*, and b* color coordinates.

The values of ΔE_{00} were calculated by the formula[36]: $\Delta E_{00} = \Delta E_{00} = (\Delta L/K_L \,.\, S_L) + (\Delta C/K_C \,.\, S_C)^2 + (\Delta H/K_H \,.\, S_H)^2 + R_T \,.\, (\Delta C/K_C \,.\, S_C)\ X\ (\Delta H/K_H \,.\, S_H)^{0.5}$, where ΔL*, ΔC* and ΔH* are the differences in brightness, chroma, and hue between two specimens and RT (rotation function) is a function that explains the interaction between chroma and hue differences in the blue region. SL, SC, and SH are the weighting functions for the luminance, chroma, and hue components, respectively. KL, KC, and KH are the parametric factors corresponding to the different visualization parameters, each set to 1. The perceptibility (0.8) and acceptability (1.8) limits were used to analyze differences in color between groups[37].

Surface roughness

The initial and final enamel surface roughness was measured using a profilometer (Model SJ-201P, Mitutoyo, Tokyo, Japan). Readings were taken over a distance of 3.2 mm, with a cutoff value of 0.8 mm and a speed of 0.5 mm/s. Three readings were performed at different locations on the enamel surface: one at the center, one 1 mm to the right, and another 1 mm to the left. The mean of these readings was taken as the initial surface roughness value for the samples.

Microhardness

The Knoop microhardness of the enamel surface was measured (Micro Hardness Tester HMV-2, Shimadzu, Tokyo, Japan), with a static vertical load of 25 g for 5 s. When activated, the indenter exerted a compressive force on the sample surface, resulting in an inverted pyramidal indentation. Enamel surface microhardness was determined by measuring the longest diagonal of the rhomboid indentation using the following formula: $KHN = 1.451\ F/d^2$, where KHN is the Knoop Hardness Number, *F* is the applied load (25 grams), and *d* is the length of the longest diagonal of the indentation. Five readings were performed: one central, one 1 mm to the right (upper and lower), and one 1 mm to the left (upper and lower). The mean of these five readings was considered the initial microhardness value.

The relative microhardness (ΔKHN) was calculated based on the relative differences from the initial values using the formula: $\Delta KHN = ((KHNf - KHNi) / KHNi) \times 100\%$, where KHNi represents the initial and KHNf the final microhardness values.

Data normality was assessed using the Shapiro–Wilk test, confirming normal distribution. CFU data were $\log_{10}$-transformed before statistical evaluation. Antimicrobial data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test ($\alpha = 0.05$). Color change (ΔE_{00}) was evaluated using two-way repeated-measures ANOVA, considering time and treatment as independent factors, followed by Tukey's post hoc test ($\alpha = 0.05$). Surface roughness (ΔRa) and relative microhardness (ΔKHN) were analyzed using two-way ANOVA (time × treatment), followed by Tukey's multiple comparison test ($\alpha = 0.05$).

Results

Antibiofilm activity

Mean $\log_{10}$ CFU/mL values are presented in Table 1. CRS significantly reduced microbial accounts compared to PBS ($p < 0.05$) and showed activity comparable to CHX ($p > 0.05$) for *Staphylococcus aureus*, *Streptococcus mutans*, and *Candida albicans*.

For *S. mutans*, CRS produced a 2 $\log_{10}$ reduction corresponding to an approximate 100-fold decrease in viable cells after 5 min of exposure. Reductions of approximately 1.9 $\log_{10}$ (80-fold) for *C. albicans* and 1.6 $\log_{10}$ (40-fold) for *S. aureus* were also observed. All treatments reduced microbial load compared with PBS. Viability staining results were consistent with CFU findings (Chart 1). The Chart 1 illustrates the photomicrographs and figure 1 showed the biofilm covered area (μm^2) of the biofilms treated with evaluated solutions, and revealed a higher presence of dead cells after using CRS.

Color stability

Mean ΔE_{00} values are presented in Table 2. No significant differences were observed between treatments at any time point ($p > 0.05$). Over time, only the CRS immersion showed a significant increase between days 1 and 7 ($p < 0.05$). No other significant differences over time were detected ($p > 0.05$).

Surface roughness

Mean ΔRa are presented in Table 3. No significant differences were observed between treatments and saliva at any time point ($p > 0.05$). At days 1 and 30, CRS immersion showed lower ΔRa values than CRS spray ($p < 0.05$). At 30 days, CRS immersion also differed from the other treatments ($p < 0.05$). No other significant differences among treatments were detected at the remaining time points ($p > 0.05$). Over time, only CHX spray exhibited a significant increase in ΔRa between days 1 and 30 ($p < 0.05$).

Microhardness

Relative enamel microhardness (ΔKHN) values are shown in Table 4. CRS immersion differed from saliva at all time points ($p < 0.05$), but did not differ from the other experimental treatments ($p > 0.05$), except for CHX spray at 30 days.

At 30 days, CHX spray showed the greatest reduction in microhardness ($p < 0.05$). At 1 and 7 days, CRS spray and both CHX treatments did not differ from saliva ($p > 0.05$), whereas at 15 and 30 days, all

treatments differed significantly ($p < 0.05$). At 30 days, CHX spray induced greater microhardness loss than both CRS applications ($p < 0.05$), while CHX immersion showed intermediate values.

Over time, all groups except CRS immersion exhibited significant reductions between days 1 and 30 ($p < 0.05$). For both spray protocols, differences were also observed between days 1 and 15 ($p < 0.05$). CHX treatments showed additional temporal differences as indicated in Table 3.

Discussion

Mechanical biofilm control remains the primary strategy for preventing caries and periodontal diseases. However, patient adherence is variable, and adjunctive antimicrobial approaches may be necessary in specific situations[5]. This study evaluated the antibiofilm activity of a chlorine-releasing solution (CRS) and its effects on enamel properties under controlled *in vitro* conditions. The null hypothesis that CRS, regardless of the mode of application, would not significantly affect either antimicrobial activity or enamel properties was rejected.

Despite growing interest in antimicrobial oral care agents, quantitative evidence regarding the balance between antibiofilm efficacy and preservation of enamel properties under repeated exposure remains limited. CRS not only reduced microbial counts compared with the PBS ($p < 0.05$) and showed activity comparable to chlorhexidine (CHX), but also showed a higher concentration of dead biofilm covered area. These findings are consistent with previous reports describing the antimicrobial properties of hypochlorous acid-based solutions[28,29]. Specifically, CRS reduced *S. mutans* by 2 $\log_{10}$ (~100-fold), *C. albicans* by 1.9 $\log_{10}$ (~80-fold), and *S. aureus* by 1.6 $\log_{10}$ (~40-fold) after 5 minutes of exposure. Cell viability assays confirmed these rapid bactericidal/fungicidal effects across all tested microorganisms. Although complete biofilm eradication is rarely achieved in multispecies oral biofilm models due to the structural protection provided by the extracellular matrix and microbial community interactions. In dental biofilm control studies, reductions between 1 and 2 $\log_{10}$ CFU/mL, can be considered important, as literature shows that this reduction means approximately 90–99% reduction in viable microorganisms[38].

The CRS used in this study contains water, sodium chloride (NaCl), 50 ppm hypochlorous acid (HOCl), and 50 ppm sodium hypochlorite (NaOCl)[6,7]. In aqueous solution, NaOCl partially hydrolyzes to form HOCl, which can further dissociate into hypochlorite ions (OCl^-) and hydrogen ions (H^+). OCl^- is highly reactive and particularly effective against bacterial spores under alkaline conditions, whereas HOCl, stable at acidic to neutral pH (≤7.0), exerts its bactericidal effect through oxidative damage to intracellular proteins, inhibition of DNA synthesis, lipid membrane oxidation, and suppression of ATP production[9-11]. The combination of these chlorine species provides CRS with rapid, broad-spectrum activity and strong anti-biofilm properties. Consistently, recent studies have shown that solutions combining HOCl, NaOCl, and NaCl are well tolerated in the oral cavity, produce rapid microbial reductions, and maintain their efficacy for up to 60 minutes after application, although their effects on enamel surfaces have not been previously reported[28,29].

While CHX is widely used for its antimicrobial properties, it has well-documented adverse effects, including reversible mucosal desquamation, taste alteration, increased supra-gingival calculus formation,

and higher cytotoxicity[15-17]. In contrast, CRS combines high antimicrobial efficacy with low toxicity, supporting its potential as a safe and effective alternative for biofilm control[8,9,22,39].

Mouthrinses must be safe for both oral soft tissues and hard tissues, such as enamel, highlighting the need to evaluate their effects on enamel's physical properties. However, evidence on the effects of CRS on enamel remains limited. Surface roughness is clinically important because increased roughness facilitates bacterial adhesion, promotes biofilm formation, and may cause irreversible damage to hard dental tissues[40]. Elevated roughness can also favor deposition of oral pigments, negatively affecting the esthetic appearance of enamel and restorations[41].

In this study, no significant differences in ΔRa were observed between the experimental solutions and saliva at any time point, indicating that none of the tested solutions induced clinically relevant alterations. CRS treatments behaved similarly to CHX, except at 30 days, when CRS immersion showed slightly negative ΔRa values (–0.01 μm), reflecting minimal, clinically irrelevant smoothing of the enamel surface, and significantly lower ΔRa compared with the other experimental solutions. At days 1 and 30, CRS immersion also exhibited significantly lower ΔRa values than CRS spray ($p < 0.05$), highlighting the influence of application mode on enamel-solution interactions. Importantly, CRS maintained stable surface roughness over time, with no significant changes observed from day 1 to day 30, regardless of the mode of application.

The slightly negative ΔRa values observed during CRS immersion likely reflect a subtle smoothing effect resulting from more homogeneous, continuous contact between the solution and the enamel surface. In contrast, spray application delivers discrete, intermittent exposures, which may generate localized interactions and slightly higher ΔRa values. All observed changes in surface roughness were minimal and far below the clinically significant threshold of ≥ 0.20 μm[23,24]. Given that the baseline surface roughness was standardized at 0.20 μm, these findings indicate that CRS, regardless of the mode of application, preserves enamel surface integrity and may be considered a safe alternative for clinical use with minimal impact on enamel roughness.

The evaluation of dental tissue microhardness provides a direct, non-destructive indication of enamel mineral content[25,26]. In this study, CRS immersion caused greater microhardness loss than saliva immersion at all time points ($p < 0.05$), but the values were comparable to those with CHX immersion ($p > 0.05$). Notably, CRS—irrespective of the mode of application—resulted in less microhardness loss than CHX spray after prolonged exposure ($p < 0.05$), suggesting that CRS may exert a less pronounced effect on enamel microhardness under repeated use.

The reduced microhardness observed with CHX may be associated with its cationic, dicationic nature, which has been reported to promote adsorption to negatively charged enamel surfaces[28,29] and to contribute to its substantivity (~4 h)[29,30]. However, the present study did not directly assess binding kinetics or retention mechanisms. It is possible that repeated applications over 15 or 30 days interfered with the protective and remineralizing effects of artificial saliva, which buffers pH and provides ions (Ca^{2+}, PO_4^{3-}, F^-) essential for enamel repair[25,31]. Additionally, CHX may interact with salivary proteins and inorganic

components, potentially influencing ion diffusion and enamel surface dynamics[32], although these interactions were not directly evaluated in this model.

CRS contains NaOCl and HOCl, compounds described as having proteolytic and tissue-dissolving activity[42]. Despite these characteristics, alterations in enamel microhardness induced by CRS were comparable to those observed with CHX at most time points and application modes, and were less pronounced than CHX spray at 30 days ($p < 0.05$). The slightly greater immediate effects observed with CRS immersion may be related to more uniform enamel exposure, whereas the spray protocol delivered shorter and discontinuous applications.

Over time, significant microhardness changes were observed for all treatments except CRS immersion ($p < 0.05$). The relative stability may be associated with differences in exposure pattern rather than intrinsic chemical aggressiveness. Spray applications of both CRS and CHX involve repeated short exposures, which could contribute to cumulative surface changes over time. CHX immersion also showed significant changes, consistent with its known substantivity, although this mechanism was not directly tested in the present study.

Overall, these findings suggest that CRS, particularly in immersion mode, was not associated with greater reductions in enamel microhardness when compared with CHX under the tested conditions. These results indicate that CRS may represent a comparable alternative to biofilm control, although further studies are necessary to clarify long term effects and underlying mechanisms.

The evaluation of enamel color changes (ΔE_{00}) provides insight into the potential effects of antimicrobial solutions on dental aesthetics. NaOCl is considered the “gold standard” antimicrobial agent, but its adverse effects on color have been widely reported[43,44]. Evidence on the use of CRS as oral rinses is limited, with only one previous study available[33], which did not evaluate tooth color.

Therefore, the present study provides novel data on the effects of CRS on enamel color. In this study, no significant differences were observed between treatments at any time point ($p > 0.05$), indicating that both CRS and CHX, regardless of the mode of application, did not induce clinically relevant discoloration. Over time, only CRS immersion showed a significant increase in ΔE_{00} between days 1 and 7 ($p < 0.05$), with no further significant changes detected up to day 30. This suggests that initial interactions between CRS and the enamel surface may slightly alter color, but these effects do not progress with continued exposure.

The slight increase in ΔE_{00} observed for CRS immersion is likely due to the continuous, homogeneous contact of the solution with the enamel surface. NaOCl and HOCl in CRS may react with superficial organic components, such as the acquired pellicle or residual proteins[42], producing minor oxidation-related color changes. After this initial interaction, the surface likely reaches a chemical equilibrium, preventing further noticeable alterations over time.

With the exception of CRS spray at day 1, all ΔE_{00} values exceeded the perceptibility (0.8) and clinical acceptability (1.8) thresholds established in the literature[37]. The CRS spray at day 1 remained below the acceptability threshold, indicating minimal short-term impact on enamel color

It is important to consider that the perceptibility and acceptability thresholds proposed by Paravina et al[37]. are based on a 50:50% visual assessment criterion, meaning that 50% of observers would perceive or judge the color change as acceptable. Therefore, exceeding these thresholds does not necessarily imply clinically unacceptable discoloration under all conditions. Moreover, no statistically significant differences were observed among the treatment groups and the artificial saliva control, suggesting that the observed color changes may be associated with aging and storage conditions rather than with the intrinsic effect of the tested solutions.

Although chlorine-based solutions have been widely investigated in medical settings—particularly in wound care—demonstrating effective antibiofilm activity and favorable biocompatibility, their application in Dentistry remains limited. Most dental studies have focused on sodium hypochlorite as an endodontic irrigant rather than as a low-concentration antiseptic for routine biofilm control. The present study expands this field by demonstrating that a stabilized chlorine-releasing solution can reduce a multispecies oral biofilm while preserving enamel surface integrity over time. Considering the well-documented adverse effects associated with chlorhexidine, including staining and cytotoxicity, these findings suggest that CRS may represent a potential alternative for oral biofilm management, bridging an important translational gap between medical antisepsis and preventive Dentistry.

The limitations of the present study are inherent to its *in vitro* design, which does not fully reproduce the complexity of the oral environment. Important variables such as continuous salivary flow, dynamic pH fluctuations, biofilm formation, and interactions with daily oral hygiene habits were not simulated. The current experimental model represents a simplified *in vitro* system and does not replicate the structural and ecological complexity of mature multispecies oral biofilms. In particular, the synergistic and competitive microbial interactions that characterize polymicrobial communities *in vivo* were not evaluated. Therefore, the antimicrobial effects observed here should be interpreted within the context of a controlled experimental model, and further studies using more complex biofilm systems are warranted to better simulate clinical conditions. Additionally, the antimicrobial solutions were evaluated in isolation, without the influence of mechanical toothbrushing, which may significantly affect surface properties and color behavior of enamel under clinical conditions.

Bovine enamel was used as an experimental substrate. Bovine and human enamel share similarities in chemical composition and mechanical behavior; however, subtle structural and histological differences may influence mineral interactions and surface responses to antimicrobial agents[45-47]. For this reason, clinical extrapolation of these findings should be performed with caution. Nevertheless, the present study provides relevant preliminary evidence, and further *in situ* and *in vivo* studies are required to validate the effects of CRS under real oral conditions and to evaluate its interaction with routine mechanical cleaning procedures.

Conclusions

Within the limitations of this *in vitro* study, CRS demonstrated antimicrobial performance comparable to CHX, achieving meaningful reductions in multispecies biofilm viability without inducing greater alterations in enamel surface properties under the tested conditions. These findings suggest that CRS may represent a promising adjunctive option for oral biofilm control; however, further investigations using more complex biological models and clinical designs are necessary to confirm its effectiveness and long-term safety.

References

1. Simões T M S, Neto J D A F, Ferreira A C D, de Sousa J A, de Medeiros C L S G,de Vasconcelos Catão M H C. (2020). Controle do biofilme oral e sua relação com a redução de infecções respiratórias em pacientes de UTI: uma revisão de ensaios clínicos. Research, Society and Development. 2020; *9*(10), e8339108594-e8339108594.
2. Coltro G M, Lima J. Z, Pejon O J Rodrigues V G S. Activation of organic compost with phosphoric acid and the effects on Pb and Cd adsorption.2023
3. Devigus A, Lombardi G. Shading Vita YZ substructures: influence on value and chroma, part I. International journal of computerized dentistry. 2004; *7*(3), 293-301.
4. Silame F D, Tonani R, Alandia-Roman C C, Chinelatti M, Panzeri H, Pires-de-Souza F C. Colour stability of temporary restorations with different thicknesses submitted to artificial accelerated aging. Eur J Prosthodont Restor Dent. 2013; *21*(4), 187-90.
5. Aherne O, Ortiz R, Fazli M M, Davies J R. Effects of stabilized hypochlorous acid on oral biofilm bacteria. BMC Oral Health. 2022; *22*(1), 415.
6. Wang L, Bassiri M, Najafi R, Najafi K, Yang J, Khosrovi B, Robson M C. Hypochlorous acid as a potential wound care agent: part I. Stabilized hypochlorous acid: a component of the inorganic armamentarium of innate immunity. Journal of burns and wounds. 2007; *6*, e5.
7. Chang B, Nerandzic M M, Kundrapu S, Sunkesula V C, Deshpande A, Donskey C J. (2013). Efficacy of dilute hypochlorite solutions and an electrochemically activated saline solution containing hypochlorous acid for disinfection of methicillin-resistant Staphylococcus aureus in a pig skin model. Infection Control & Hospital Epidemiology. 2013; *34*(11), 1231-1233.
8. Weiss S J, Lampert M B, Test S T. Long-lived oxidants generated by human neutrophils: characterization and bioactivity. Science. 1983; *222*(4624), 625-628.
9. Severing A L, Rembe J D, Koester V, Stuermer E K. Safety and efficacy profiles of different commercial sodium hypochlorite/hypochlorous acid solutions (NaClO/HClO): antimicrobial efficacy, cytotoxic impact and physicochemical parameters in vitro. Journal of Antimicrobial Chemotherapy. 2019; *74*(2), 365-372.
10. Wang L. Hypochlorous acid as a potential wound care agent. Journal of burns and wounds. 2007; *6*, 65-79.
11. da Cruz Nizer W S, Inkovskiy V, Overhage J. Surviving reactive chlorine stress: responses of gram-negative bacteria to hypochlorous acid. Microorganisms. 2020; *8*(8), 1220.
12. Sakarya S, Gunay N, Karakulak M, Ozturk B, Ertugrul B. (2014). Hypochlorous acid: an ideal wound care agent with powerful microbicidal, antibiofilm, and wound healing potency. Wounds. 2014; *26*(12), 342-350.
13. Castillo D M, Castillo Y, Delgadillo N A, Neuta Y, Jola J, Calderón J L, Lafaurie G I. Viability and effects on bacterial proteins by oral rinses with hypochlorous acid as active ingredient. Brazilian dental journal. 2015; *26*(5), 519-524.
14. Selkon J B, Cherry G W, Wilson J M, Hughes M A. (2006). Evaluation of hypochlorous acid washes in the treatment of chronic venous leg ulcers. Journal of wound care. 2006; *15*(1), 33-37.
15. Çoğulu D, Aşık A, Yılmaz Süslüer S, Yuecel Er C, Topaloğlu A, Uzel A, Gündüz C. In vitro analysis of various mouthwashes: cytotoxic, apoptotic, genotoxic and antibacterial effects. Clinical Oral Investigations. 2025; 29(4), 183.
16. Coelho A S, Laranjo M, Gonçalves A C, Paula A, Paulo S, Abrantes A M, Botelho M F. Cytotoxic effects of a chlorhexidine mouthwash and of an enzymatic mouthwash on human gingival fibroblasts. Odontology. 2020; 108(2), 260-270.
17. Supranoto S C, Slot D E, Addy M, Van der Weijden G A. The effect of chlorhexidine dentifrice or gel versus chlorhexidine mouthwash on plaque, gingivitis, bleeding and tooth discoloration: a systematic review. International journal of dental hygiene. 2015; 13(2), 83-92.
18. Kranz S, Guellmar A, Braeutigam F, Tonndorf-Martini S, Heyder M, Reise M, Sigusch B. Antibacterial effect of endodontic disinfections on Enterococcus faecalis in dental root canals—An in-vitro model study. Materials. 2021; 14(9), 2427.
19. Koo H, Andes D R, Krysan D J. Candida–streptococcal interactions in biofilm-associated oral diseases. PLoS pathogens. 2018; *14*(12), e1007342.
20. Khadka S, Khan S, King A, Goldberg L R, Crocombe L, Bettiol S. Poor oral hygiene, oral microorganisms and aspiration pneumonia risk in older people in residential aged care: a systematic review. Age and Ageing. 2021; *50*(1), 81-87.
21. Procopio S W, Tavares M C, Carrada C F, Ribeiro Scalioni F A, Ribeiro R A, Paiva S M. Perceptions of parents/caregivers about the impact of oral conditions on the quality of life of children and adolescents with autism spectrum disorder. Journal of Autism and Developmental Disorders. 2024; 54(11), 4278-4287.
22. Ortega-Peña S, Hidalgo-González C, Robson M C, Krötzsch E. In vitro microbicidal, anti-biofilm and cytotoxic effects of different commercial antiseptics. International Wound Journal. 2017. 14(3), 470-479.
23. Bollenl C M, Lambrechts P, Quirynen M. Comparison of surface roughness of oral hard materials to the threshold surface roughness for bacterial plaque retention: a review of the literature. Dental materials. 1997;13(4), 258-269.
24. Jones C S, Billington R W, Pearson G.J. The in vivo perception of roughness of restorations. British dental journal. 2004; 196(1), 42-45.

25. Ballal N V, Khandewal D, Karthikeyan S, Somayaji K, Foschi F. Evaluation of chlorine dioxide irrigation solution on the microhardness and surface roughness of root canal dentin. Eur J Prosthodont Restor Dent. 2015;23(4), 173-8.
26. Mandava J, Reddy Y S, Kantheti S, Chalasani U, Ravi R C, Borugadda R, Konagala R K. Microhardness and penetration of artificial white spot lesions treated with resin or colloidal silica infiltration. Journal of clinical and diagnostic research: JCDR. 2017; 11(4), ZC142.
27. Oliveira L D, Carvalho C A T, Nunes W, Valera M C, Camargo C H R, Jorge A O C. Effects of chlorhexidine and sodium hypochlorite on the microhardness of root canal dentin. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology. 2007; 104(4), e125-e128.
28. Ofek I, Beachey E H. Bacterial adherence. Advances in internal medicine. 1980;25, 503-532.
29. Jones C G. Chlorhexidine: is it still the gold standard?. *Periodontology 2000*. 1997; *15*, 55-62.
30. Toljanic J A, Hagen J C, Takahashi Y, Shapiro R D.. Evaluation of the substantivity of a chlorhexidine oral rinse in irradiated head and neck cancer patients. Journal of oral and maxillofacial surgery. 1992; 50(10), 1055-1059.
31. López-Galindo M, Atashkadeh W. Exploring potential salivary biomarkers for dental caries: a systematic review. Medicina Oral, Patología Oral y Cirugía Bucal. 2025; 30(6), e849.
32. Nancollas G H, Johnsson M A S. Calculus formation and inhibition. Advances in dental research. 1994; 8(2), 307-311.
33. Zwicker P, Freitag M, Heidel F H, Kocher T, Kramer A. Antiseptic efficacy of two mouth rinses in the oral cavity to identify a suitable rinsing solution in radiation-or chemotherapy induced mucositis. BMC Oral Health. 2023; 23(1), 176.
34. Lopes Vasconcelos G L, Almeida Curylofo P, Targa Coimbra F C, de Cássia Oliveira V, Paula Macedo A, Oliveira Paranhos H D F,Pagnano V O. In Vitro Antimicrobial Activity of Effervescent Denture Tablets on the Components of Removable Partial Dentures. International Journal of Prosthodontics. 2020; 33(3).
35. Hayran Y, Kuşcu S, Aydın A. Determination of streptococcus mutans retention in acidic and neutral pH artificial saliva environment of all-ceramic materials with different surface treatment. BMC Oral Health. 2025; 25(1), 7.
36. Sharm, G, Wu W, Dalal E N. The CIEDE2000 color-difference formula: Implementation notes, supplementary test data, and mathematical observations. Color Research & Application: Endorsed by Inter-Society Color Council, The Colour Group (Great Britain), Canadian Society for Color, Color Science Association of Japan, Dutch Society for the Study of Color, The Swedish Colour Centre Foundation, Colour Society of Australia, Centre Français de la Couleur. 2005; 30(1), 21-30.
37. Paravina R D, Ghinea R, Herrera L J, Bona A D, Igiel C, Linninger M, Mar Perez M D. Color difference thresholds in dentistry. Journal of Esthetic and Restorative Dentistry. 2015; 27, S1-S9.
38. Boyce J M, Pittet D. Guideline for hand hygiene in health-care settings: recommendations of the Healthcare Infection Control Practices Advisory Committee and the HICPAC/SHEA/APIC/IDSA Hand Hygiene Task Force. Infection Control & Hospital Epidemiology. 2002; 23(S12), S3-S40.
39. Sakarya S, Gunay N, Karakulak M, Ozturk B, Ertugrul B. Hypochlorous acid: an ideal wound care agent with powerful microbicidal, antibiofilm, and wound healing potency. Wounds. 2014; 26(12), 342-350.
40. Cross S E, Kreth J, Wali R P, Sullivan R, Shi W, Gimzewski J K. Evaluation of bacteria-induced enamel demineralization using optical profilometry. Dental materials. 2009; 25(12), 1517-1526.
41. Koc Vural U, Bagdatli Z, Yilmaz A E, Yalçın Çakır F, Altundaşar E, Gurgan S. Effects of charcoal-based whitening toothpastes on human enamel in terms of color, surface roughness, and microhardness: an in vitro study. Clinical Oral Investigations. 2021; 25(10), 5977-5985.
42. Erkal D, Çakmak Y E, Pistol A M, Er K. (2025). Pulp tissue dissolution capacities of two hypochlorite-based irrigants at different temperatures: an in vitro study. BMC Oral Health. 2025; 25(1), 1104.

43. Arruda C N F, Sorgini D B, Oliveira V D C, Macedo A P, Lovato C H S, Paranhos H D F O. Effects of denture cleansers on heat-polymerized acrylic resin: a five-year-simulated period of use. Brazilian dental journal. 2015; 26(4), 404-408.
44. Patel M P, Parmar V B, Rami D S, Trivedi V R, Rana D M, Bajania D N, Bajania D. Comparative evaluation of the effect of microwave, 1% sodium hypochlorite, and sodium perborate disinfection on the color stability of two nanoparticle-reinforced heat-polymerized PMMA denture base resins: an in vitro study. Cureus. 2024; 16(8).
45. Schilke R, Lisson J A, Bauß O, Geurtsen W. Comparison of the number and diameter of dentinal tubules in human and bovine dentine by scanning electron microscopic investigation. Archives of oral biology. 2000; 45(5), 355-361.
46. Attin T, Wegehaupt F J. Methods for assessment of dental erosion. Erosive tooth wear. From diagnosis to therapy. 2014; 25, 123-142.
47. Wiegand A, Attin T. Design of erosion/abrasion studies–insights and rational concepts. Caries research. 2011; 45(Suppl. 1), 53-59.

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Author contribution

All the authors contributed substantially throughout the drafting, data interpretation, and critical revision of the paper. Moreover, they approved the final version of the paper and agreed with all aspects of the work. Additionally, PYVT worked at all stages of the research; VCO worked on microbiology methodology, RGV helped with the methodology and writing; CNFA worked at all stages of the writing; FCP worked at all stages and supervised the research.

Data Availability Statement

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Author Disclosure Statement

No competing financial interests exist.

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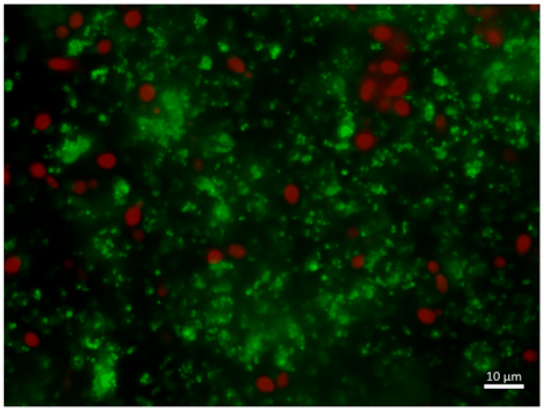
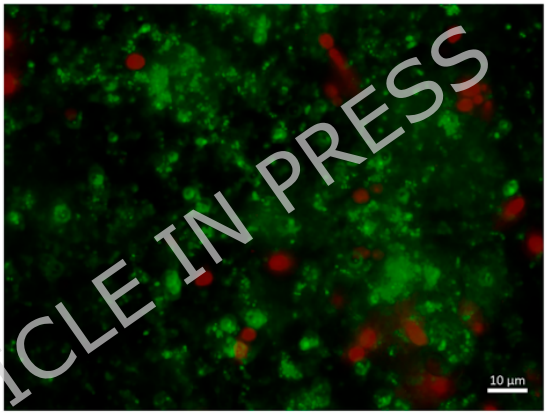
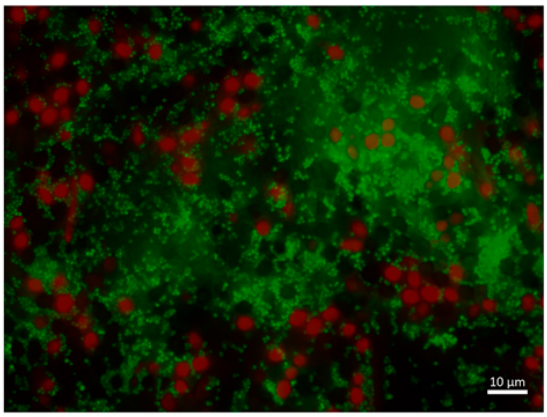
Chart

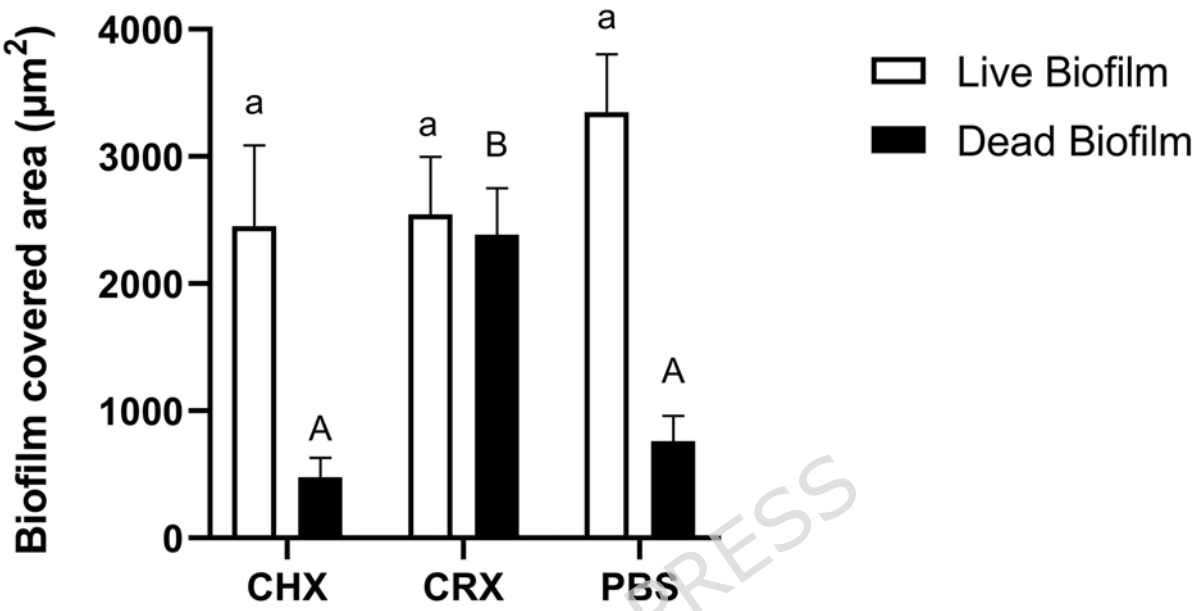
Chart 1 – Live/Dead Results – Fluorescence Microscopy

Protocol	Live/Dead Biofilm
PBS	
CHX	
CRS	

Green-stained cells are viable, and the cells marked in red or yellow are dead.

Figures

Figure 1. Biofilm covered area (μm²) for each evaluated solution



Uppercase letters indicate statistical differences in the dead biofilm, whereas lowercase letters indicate statistical differences in the live biofilm ($p<0.05$).

Tables

Table 1. Means (± standard deviation) of colony-forming unit counts (log_{10} CFU/mL) of *Staphylococcus aureus*, *Streptococcus mutans*, and *Candida albicans* after exposure to the different disinfectant treatments (one-way ANOVA, Tukey's test, $p < 0.05$).

Protocol	***S. aureus***	***S. mutans***	***C. albicans***
PBS (Negative Control)	6.22 ± 0.35 A	6.41 ± 0.73 A	3.79 ± 1.06 A
CHX (Positive Control)	5.29 ± 1.03 AB	4.75 ± 1.69 AB	3.07 ± 1.22 AB
CRS	4.83 ± 1.50 B	4.27 ± 2.20 B	1.97 ± 1.73 B

Table 2 Means and standard deviations of color changes (ΔE_{00}) in enamel submitted to different disinfectant solutions at 1, 7, 15, and 30 days (two-way repeated-measures ANOVA, Tukey's test, $p < 0.05$).

Time	Saliva Immersion	CRS Immersion	CRS Spray	CHX Immersion	CHX Spray
1 d	2.67 ± 1.88 aA	2.21 ± 2.47 aA	1.29 ± 0.74 aA	2.87 ± 4.35 aA	3.13 ± 3.51 aA
7 d	2.69 ± 2.87 aA	3.51 ± 3.33 aB	2.45 ± 2.05 aA	2.52 ± 4.11 aA	2.95 ± 2.57 aA
15 d	3.00 ± 1.99 aA	4.28 ± 3.10 aAB	2.07 ± 1.52 aA	2.75 ± 2.05 aA	3.22 ± 2.82 aA
30 d	2.67 ± 1.88 aA	3.29 ± 2.81 aAB	2.85 ± 2.70 aA	2.26 ± 2.26 aA	3.55 ± 3.30 aA

Different lowercase letters in the same row indicate significant differences between treatments. Different uppercase letters in the same column indicate statistically significant differences over time within the same treatment (two-way repeated-measures ANOVA, Tukey's test, $p < 0.05$).

Table 3. Means and standard deviations of surface roughness changes in enamel submitted to different disinfectant solutions at 1, 7, 15, and 30 days.

Time	Saliva Immersion	CRS Immersion	CRS Spray	CHX Immersion	CHX Spray
1 d	0.00 ± 0.01 abA	–0.02 ± 0.03 bA	0.02 ± 0.05 aA	0.00 ± 0.02 abA	0.00 ± 0.02 abA
7 d	–0.00 ± 0.01 aA	0.00 ± 0.03 aA	0.01 ± 0.03 aA	0.00 ± 0.02 aA	0.01 ± 0.02 aAB
15 d	–0.00 ± 0.01 aA	–0.00 ± 0.03 aA	0.01 ± 0.03 aA	0.00 ± 0.03 aA	0.02 ± 0.03 aAB
30 d	0.00 ± 0.01 abA	–0.01 ± 0.04 bA	0.02 ± 0.02 aA	0.01 ± 0.04 aA	0.02 ± 0.03 aB

Different lowercase letters in the same row indicate significant differences between treatments. Different uppercase letters in the same column indicate statistically significant differences over time within the same treatment (two-way repeated-measures ANOVA, Tukey's test, $p < 0.05$).

Table 4. Means and standard deviations of changes in enamel microhardness (ΔKHN) after exposure to different disinfectant treatments at 1, 7, 15, and 30 days.

Time	Saliva Immersion	CRS Immersion	CRS Spray	CHX Immersion	CHX Spray
1 d	–1.46 ± 5.01 aA	–24.54 ± 15.27 bA	–10.39 ± 25.13 abA	–18.02 ± 16.71 abA	–11.50 ± 15.07 abA
7 d	–0.86 ± 9.91 aAB	–25.33 ± 15.26 bA	–13.89 ± 26.04 abAB	–17.28 ± 15.29 abA	–14.49 ± 21.87 abA
15 d	0.66 ± 6.79 aAB	–24.92 ± 16.74 bA	–25.18 ± 24.74 bB	–28.90 ± 18.93 bAB	–33.86 ± 18.28 bB
30 d	–1.80 ± 9.24 aB	–23.91 ± 10.43 bA	–25.23 ± 19.68 bB	–35.34 ± 13.26 bcB	–45.71 ± 21.16 cC

Different lowercase letters in the same row indicate significant differences between treatments. Different uppercase letters in the same column indicate statistically significant differences over time within the same treatment (two-way repeated-measures ANOVA, Tukey's test, $p < 0.05$).