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Chlorhexidine with or without alcohol against biofilm formation: efficacy, adverse events and taste preference

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Abstract: In recent years, different chlorhexidine formulations have been tested, including an alcohol-free alternative, but the effect of this solution on early biofilm formation is not clear. A crossover, randomized, double-blind clinical trial was conducted to evaluate the effect of two chlorhexidine solutions against supra- and subgingival biofilm formation (NCT#02656251). Thirty-five participants were randomized and asked to rinse twice daily with 15 ml of an alcohol-containing 0.12% chlorhexidine solution, an alcohol-free 0.12% chlorhexidine solution, or placebo. The study was conducted in three experimental periods of 4 days each, with a 10-day washout between the periods. All the experimental periods followed the same protocol, except that the solutions were switched. Biofilm distribution was evaluated every 24 hours by the Plaque-Free Zone Index, during 96 hours. Adverse events were self-reported and sensory evaluation was performed using a hedonic scale. Compared to the placebo, the chlorhexidine solutions resulted in a significantly higher number of surfaces free of plaque over 96 hours ($p < 0.01$), and were able to prevent subgingival biofilm formation ($p < 0.01$). The alcohol-free chlorhexidine solution was associated with a lower incidence of adverse events, compared with alcohol-containing chlorhexidine ($p < 0.05$); it also received better sensory evaluation and acceptance by trial participants, compared with the alcohol-containing chlorhexidine ($p = 0.007$), and had a similar inhibitory effect on the formation of supra- and subgingival biofilms.

Keywords: Chlorhexidine; Mouthwashes; Dental Plaque; Cross-Over Studies; Alcohols.

Declaration of Interest: The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

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Introduction

Various substances are used to control oral biofilm, including chlorhexidine, essential oils, triclosan, and cetylpyridinium chloride. Of these, chlorhexidine is certainly the most widely studied and efficient antimicrobial agent for the chemical control of dental biofilm, and is considered the gold standard with which all anti-biofilm agents should be compared[1, 2].

Both alcohol-containing and alcohol-free chlorhexidine solutions are sold worldwide. Ethyl alcohol is used in oral mouth rinses as a solvent and antiseptic agent[3]. In addition, alcohol is important to ensure product stability and activity, since it may prevent contamination of the solution[4].

Accordingly, alcohol-free chlorhexidine formulations often include other ingredients aimed at stabilizing and maintaining the sterility of the solution.

Arweiler et al. compared the antibacterial properties of a 0.2% alcohol-free chlorhexidine solution containing an anti-discoloration system with an established 0.2% alcohol-containing chlorhexidine solution[5]. The results suggested that the 0.2% alcohol-containing solution performed better in inhibiting biofilm regrowth and reducing bacterial vitality than the alcohol-free solution. Conversely, Lorenz et al. failed to demonstrate differences between alcohol-free and alcohol-containing rinses for both plaque and gingival bleeding index, in a 21-day experimental gingivitis study[6]. Other studies that compared alcohol-free and alcohol-containing chlorhexidine solutions, as adjuncts to tooth brushing for 4 weeks or more, failed to find differences in plaque and gingival bleeding index[7, 8].

It may be hypothesized that chlorhexidine may inhibit subgingival biofilm buildup by inhibiting supragingival biofilm formation. Maliska et al. described an index to evaluate the presence of early subgingival biofilm formation, and found that an alcohol-containing 0.12% chlorhexidine solution reduced the number of dental surfaces bearing subgingival biofilm more than tenfold, compared with placebo[9]. The same index was used to demonstrate that triclosan could postpone early subgingival formation in the interdental area[10].

The potential of an alcohol-free chlorhexidine solution to prevent subgingival biofilm buildup has not been reported in the literature. In addition to the positive effect of alcohol-free chlorhexidine solutions against supra- and subgingival biofilm formation, it is important to point out the adverse effects and taste preferences related to both solutions. The use of chlorhexidine is associated with adverse events such as staining of teeth, restorations, prosthetic appliances and even the tongue, as well as taste disturbances, supragingival calculus formation, and, possibly, reversible swelling of the lips or parotid glands, desquamation of the oral mucosa, urticaria, dyspnea, and anaphylactic shock[11,12]. Regarding taste, two studies showed that alcohol-free chlorhexidine solutions with cetylpyridinium chloride were more palatable than alcohol-containing chlorhexidine solutions[13,14], using a visual analogue scale. However, there is no information in the literature about taste preference for alcohol-free chlorhexidine solutions, as assessed by a specific scale designed to measure hedonic responses.

In this context, this study had two aims; first, to evaluate how well two commercially available 0.12% chlorhexidine solutions, one alcohol-containing and one alcohol-free, could inhibit supra- and subgingival biofilm formation, and, second, to evaluate self-reported adverse events and taste preferences related to both solutions. Our hypothesis is that using an alcohol-containing 0.12% chlorhexidine solution will result in a lower percentage of sites bearing supra- and subgingival biofilm, compared with using an alcohol-free 0.12% chlorhexidine solution.

Materials and Methods

Study design

A randomized, double-blind, three-way crossover trial was conducted among dental students from the Federal University of Rio Grande do Sul (UFRGS) School of Dentistry (Figure 1). The study protocol was approved by the Federal University of Rio Grande do Sul Human Research Ethics Committee (CAAE 40236514.3.0000.5347), and all the participants signed informed consent forms. The study was registered at http://www.clinicaltrials.gov (NCT#02656251).

Sample size calculation was performed in the GPower 3.1 software environment. According to data from the literature[5], a 37% reduction in biofilm formation was estimated by using an alcohol-free chlorhexidine solution, and a 73% reduction, by using an alcohol-containing solution, both compared with placebo, during a 96-hour period. The sample size was set at 29 subjects. Considering an attrition rate of 20%, 35 individuals were included.

Subjects

Thirty-five dental students (18 men and 17 women; mean age 22.5 ± 3.2 years) participated in the study. The inclusion criteria were: adults of either sex, aged 18 to 40 years, non-smokers, in good general health, with at least 20 natural teeth (excluding third molars), including anterior teeth, no restorations

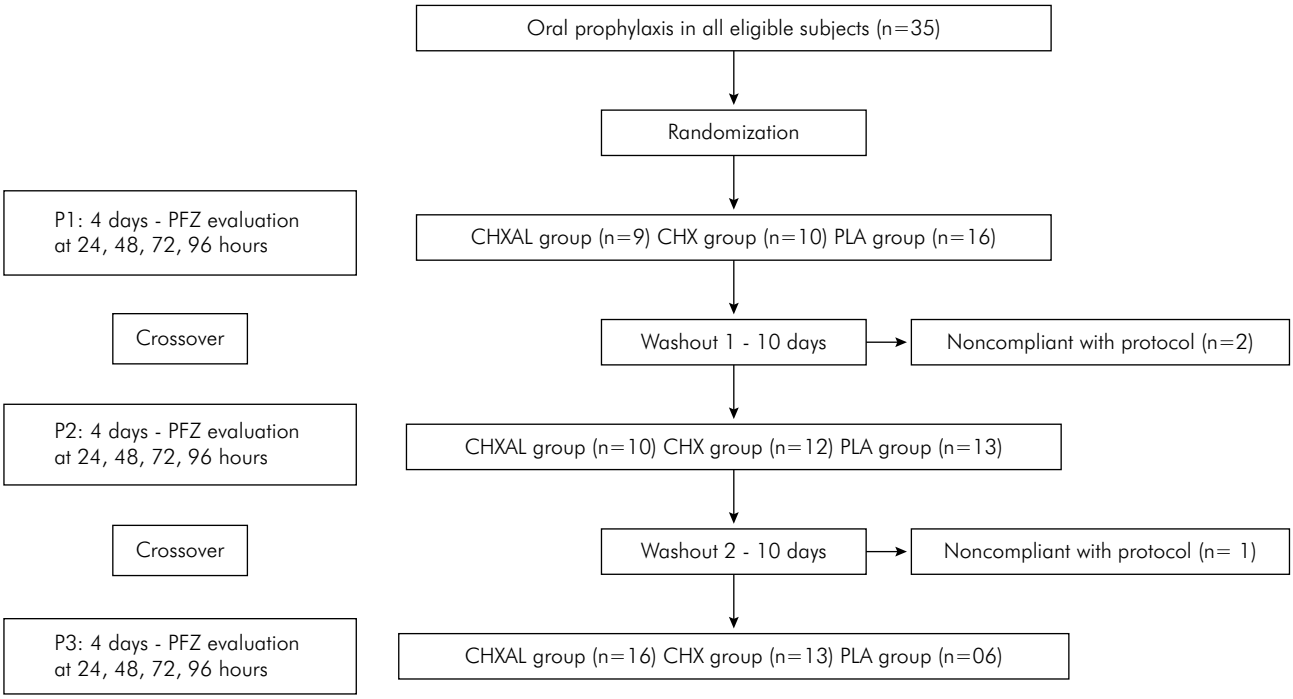

Figure 1. Study flow diagram. (P1, period one; P2, period two; P3, period three; PFZ, plaque free zone; PLA, placebo; CHXAL, alcohol-containing chlorhexidine; CHX, alcohol-free chlorhexidine).

on premolars, canines or incisors, no orthodontic appliances, anatomic irregularities, gingival recession, gingivitis[15] and/or periodontitis (no loss of attachment, no bleeding on probing), no antibiotic or anti-inflammatory therapy up to 30 days prior to the start of the study, no known allergy to the experimental product, no pregnant or breastfeeding mothers, and no tumors in the oral cavity.

Experimental procedures

This clinical trial took place from May to June 2015. The participants underwent testing during three experimental periods lasting 4 days each, with a 10-day washout between each period. All the subjects were randomly assigned across three different groups: Group A (CHXAL), an alcohol-containing 0.12% chlorhexidine solution (Periogard®, Colgate, São Paulo, Brazil); Group B (CHX), an alcohol-free 0.12% chlorhexidine solution (Periogard® Sem Álcool, Colgate, São Paulo, Brazil); and Group C (PLA), placebo. An investigator not involved in the clinical examination (FCM) performed randomization using the www.randomization.com website, and the randomization table was kept secret throughout the experimental period. The same investigator in charge of random allocation was also responsible for product distribution and identification. The experimental formulations were dispensed daily into individual 15-ml opaque plastic containers labeled with a six-digit alphanumeric code (to ensure allocation concealment). The first rinse was supervised every day; the participant rinsed immediately after biofilm formation was recorded. After twelve hours, the second rinse was performed at home. The examiner (GOS) and participants had no knowledge of what solution was being used in each experimental period. The placebo solution was prepared at a local compounding pharmacy (Farmácia Uso Indicado, Pelotas, RS, Brazil) to ensure utmost similarity of both chlorhexidine solutions in terms of taste, color, and smell, using the following formulation: 0.18% methylparaben, 6% mint flavor, 5% glycerin, and water and blue dye QSP.

Before the start of each experimental period, basic fuchsin (ReplanicT®, Iodontec, São Paulo, SP, Brazil) was applied with a small cotton ball to teeth #14-#24, as a plaque-disclosing agent. After 30 seconds, the participants rinsed their mouth with water, and the disclosed biofilm was removed with a rubber

cup (Microdont, São Paulo, Brazil), prophylactic paste (Pert X®, SSWhite, Rio de Janeiro, Brazil) and dental floss (Colgate Palmolive, São Paulo, Brazil). The subjects were then instructed not to brush or floss their upper incisors, canines or premolars, but were allowed to brush their lower teeth and upper molars with standard dentifrice (Sorriso®, Colgate Palmolive, São Paulo, Brazil). Moreover, they were instructed to rinse only with the substance provided by the study, every 12 hours, for 1 minute, using the entire amount contained in the container (15 ml), and to refrain from eating or drinking for 30 minutes after using the mouthwash.

The teeth were evaluated for biofilm accumulation at 24, 48, 72, and 96 hours during the first observation period (P1). Experimental teeth were rinsed with a water spray, and basic fuchsin was applied topically for 30 seconds, followed by further spray rinsing and gentle air-drying. A hectograph copying pencil was used to mark two reference landmarks on the buccal gingiva, 1 to 2 mm apical to the gingival margin, thus splitting the buccal surface vertically into thirds: mesiobuccal, buccal, and distobuccal. The mesiobuccal and distobuccal surfaces were considered proximal, whereas the free surface of the tooth was defined as buccal. The three thirds thus marked on each tooth were then ready for evaluation by the examiner. Biofilm accumulation was recorded using a classification system that describes the process from biofilm formation to elimination of the plaque-free zone (PFZ), as proposed by Maliska et al. (2006). In brief, the parameter consists of a three-point scale, whereby a score of 0 corresponds to absence of biofilm, score 1 corresponds to presence of biofilm and a PFZ, and score 2 indicates presence of biofilm and absence of any PFZ. Before applying this index, the examiner was trained and calibrated by an experienced investigator (PW) to measure the above criteria. Examiner reproducibility was tested with the kappa statistic. The interexaminer and intraexaminer kappa coefficients were 0.85 and 0.81, respectively.

At the end of the first 96-hour experimental period, dental prophylaxis was performed and a 10-day washout period was allowed to elapse. The study continued with the second (P2) and third (P3) experimental periods, during which the participants rinsed with a solution different from that used in P1, as indicated in the randomization table (Figure 1). Throughout the study, volunteers were asked to report any adverse effects that emerged. At the end of each experimental period, a hedonic (facial) scale[16] was used to evaluate perceived palatability. The 9-point hedonic scale is a balanced bipolar scale with a neutral central point. A "smiley face" accompanies each of the nine categories, and the subject is asked to record the expression that best corresponds to the taste perceived during the use of each solution. The degree of palatability (like/dislike) was then recorded.

Statistical analysis

The absolute and relative frequencies of each PFZ Index score were calculated for each group. Conversion of scores 0 and 1 to score 2 in each experimental period was calculated individually for the proximal and buccal thirds, to assess subgingival biofilm formation. Comparisons were performed with the Friedman and McNemar tests, and Bonferroni correction was applied in cases of multiple comparisons. Subgingival biofilm formation was the primary outcome, whereas supragingival biofilm formation, adverse events and taste preference were secondary outcomes.

Three individuals did not comply fully with the study protocol, but were evaluated during the entire study period. Two participants used amoxicillin during the study to treat tonsillitis. The third subject used an alcohol-containing 0.12% chlorhexidine solution after third molar extraction surgery, during the interval between P2 and P3, thereby not complying with the protocol-defined washout period between use of alcohol-containing versus alcohol-free chlorhexidine solutions.

All the data presented considered the intention-to-treat analysis with 35 participants (Tables 1 and 2; Figure 2). For the purpose of evaluating the carryover effect, the authors compared the percentage frequency of PFZ Index score 1 at the 48-hour time point, during each experimental period, using the Friedman test. The number of individuals with presence/absence of adverse events occurring at any point during the study was compared using Cochran's Q and the McNemar test.

Table 1. Intention-to-treat analysis of the percentage of scores 0, 1 and 2 for all tooth surfaces, with placebo, alcohol-containing 0.12% chlorhexidine solution and alcohol-free 0.12% chlorhexidine solution (n = 35).

Variable	PLA	CLXAL	CLX
24 hours			
0	34.2	79.9	76.2
1	65.8	20.1	23.8
2	0	0	0
		*	*
48 hours			
0	9.9	65.5	54.9
1	89.9	34.4	45.1
2	0.4	0.1	0
		* #	* #
72 hours			
0	3.5	47.4	42.7
1	80.9	51.7	56.9
2	15.6	0.8	0.4
		* #	* #
96 hours			
0	3	33.7	36.7
1	69.8	63.3	61.7
2	27.2	3	1.6
		*	*

PLA, placebo; CLXAL, alcohol-containing chlorhexidine; CLX, alcohol-free chlorhexidine.*Statistically significant difference from placebo (Friedman and McNemar tests, $p < 0.01$); # Statistically significant difference between the chlorhexidine solutions (Friedman and McNemar tests, $p < 0.01$).

Table 2. Absolute frequency of adverse events during use of the tested solutions (n = 35).

Adverse event	PLA	CLXAL	CLX
Absence	23	15	25
Presence	12	20*	10

PLA, placebo; CLXAL, alcohol-containing chlorhexidine; CLX, alcohol-free chlorhexidine; * Statistically significant difference between chlorhexidine solutions (Cochran's Q and McNemar tests $p = 0.013$).

Hedonic scale scores were converted into numeric values ranging from 1 ("dislike extremely") to 9 ("like extremely"). These were used to calculate the mean palatability scores for each solution, which were then compared by analysis of variance (ANOVA) with Tukey's test. The crossover study design was considered in the analysis. PASW Statistics version 18.0 software (SPSS Inc., Chicago, IL, USA) was used, and the level of significance was set at 5%.

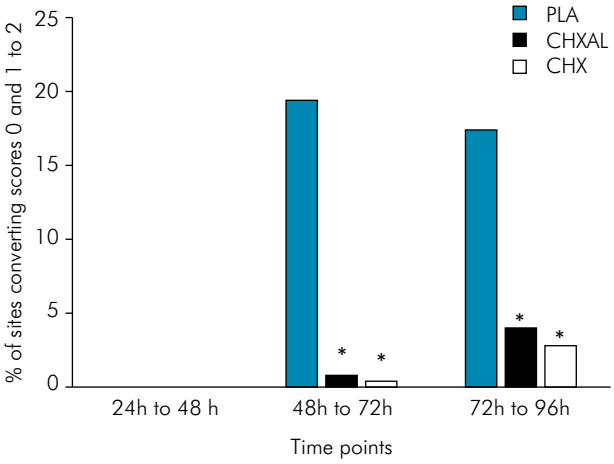

Figure 2. Analyses of the conversion of scores 0 and 1 into score 2, for all dental surfaces, stratified by solution (PLA, placebo; CHXAL, alcohol-containing chlorhexidine; CHX, alcohol-free chlorhexidine) and time point of analysis (n = 35). * Statistically significant difference from placebo (Friedman and McNemar tests, $p < 0.01$).

Results

Both chlorhexidine solutions, regardless of the presence of alcohol, were able to maintain a significantly higher number of surfaces free of biofilm at 24 hours, compared with PLA ($p < 0.01$) (Table 1). At this time point, 79.9% and 76.2% of the surfaces had a PFZ Index score of 0, when using CHXAL and CHX solutions, respectively, whereas only 34.2% of the surfaces had a score of 0 in placebo-treated participants. At the 48-hour and 72-hour time points, use of a CHXAL solution resulted in significantly less supragingival biofilm formation, compared with the CHX solution (34.4% versus 45.1%; $p < 0.01$). At 96 hours, there was no statistically significant difference in the distribution of scores 0, 1, and 2, between both chlorhexidine solutions tested. The results of the per-protocol analysis were identical to those found in the intention-to-treat analysis, except for the 24-hour time point, at which the CHXAL solution was found to maintain a significantly higher proportion of surfaces free of biofilm (80.2% versus 75.5%; $p < 0.01$). The carryover effect analysis indicated no statistically significant difference in the percentage of scores obtained in each experimental period for each solution, indicating no carryover effect during the study.

The conversion of scores 0 and 1 to score 2, representing subgingival biofilm formation, is represented in Figure 2. Between the 24- and 48-hour time points, there was no significant difference among the three solutions. In the periods between 48 and 72 hours, and between 72 and 96 hours, a lower percentage of surfaces converted to score 2 when using the chlorhexidine solutions, regardless of the presence of alcohol, as compared with the placebo. Moreover, the differences were significant ($p < 0.01$) in both periods. These findings were similar in the intention-to-treat and per-protocol analyses (data not shown).

The adverse events most frequently reported by the participants were burning sensation in the mouth, bitter taste after rinsing, and taste disturbance. Twelve individuals reported the presence of these events while using the PLA solution, 10 subjects reported adverse events with the CHX solution, and 20 subjects reported adverse events after using the CHXAL solution (Table 2). The frequency of adverse events was significantly higher when using the CHXAL versus the CHX solution (57.1% versus 28.6%; $p = 0.013$).

Regarding the sensory evaluation, the CHXAL solution performed worst, with the lowest palatability scores. The mean hedonic scale score was 5.77 (±1.63) for PLA, 5.21 (±1.84) for the CHX solution, and 4.37 (±2.13) for the CHXAL solution. The difference between CHXAL and the other two solutions was statistically significant ($p = 0.007$).

Discussion

This study aimed at evaluating the ability of two commercially available 0.12% chlorhexidine solutions to prevent biofilm formation, and showed that both CHXAL and CHX formulations had a significant inhibitory effect on supra- and subgingival biofilm formation compared to PLA. Taste preferences and adverse effects were also assessed, and the CHX solution was found to be more palatable and produce fewer adverse effects, compared with the CHXAL solution.

The results of this study demonstrated that use of a chlorhexidine solution, regardless of the presence of alcohol, significantly prevented supragingival biofilm formation for 96 hours. Our results are corroborated by Lorenz et al., who compared two alcohol-free 0.2% chlorhexidine solutions and one alcohol-containing chlorhexidine solution in an experimental gingivitis model[6]. The authors demonstrated that a statistically significant lower biofilm accumulation resulted when chlorhexidine was used in comparison to the placebo, regardless of the presence of alcohol in the chlorhexidine solutions. Zimmer et al. and Leyes Borrajo et al. found similar results[7,8]. However, tooth brushing was allowed in both studies, thus preventing direct comparison of their results with ours.

On the other hand, the present findings diverge from the results obtained by Arweiler et al., who observed a greater reduction in the amount and extent of supragingival biofilm after using an alcohol-containing versus alcohol-free chlorhexidine solution[5]. It should be noted that the alcohol-free chlorhexidine solution tested in their study contained an anti-discoloration system designed to reduce the staining potential. This anti-staining system can interact with chlorhexidine and reduce its efficacy against biofilm formation[17,18], thus partially explaining the weaker anti-biofilm activity of their tested alcohol-free chlorhexidine solution. Systematic reviews could add important information on this topic, but none are available in the literature to date. The small number of studies comparing alcohol-containing and alcohol-free chlorhexidine solutions in terms of their ability to prevent supragingival biofilm formation, and the different methodologies used in these studies, make a systematic review unfeasible at this time.

In this study, the aim was to evaluate the early alterations in biofilm formation and in the dentogingival area obtained by using both chlorhexidine solutions, during 96 hours. This is an established model to study early biofilm formation[19, 20,21], based on the fact that higher increments in biofilm growth are observed in the first 4 days after the absence of oral hygiene, and that lower increments in the biofilm occur after the fourth day[22,23,24]. Moreover, the plaque index used in this study incorporates this concept, and was designed to assess early biofilm formation[9]. It should be mentioned that the results of the present study should be interpreted within this scenario, and should be not directly extrapolated to using alcohol-free and alcohol-containing chlorhexidine in clinical situations, where long-lasting plaque accumulation periods occur.

This was the first study to compare alcohol-containing and alcohol-free chlorhexidine solutions in terms of their ability to prevent subgingival biofilm formation. It is widely known that an important correlation exists between supra- and subgingival biofilm for the establishment and progression of periodontal disease[25]. Studies such as this one are important, because they can add to current knowledge concerning the development and prevention of periodontitis. Our study demonstrated that both chlorhexidine solutions were able to prevent subgingival biofilm formation during a 96-hour period, compared with PLA. These results corroborate those reported by Maliska et al., who compared an alcohol-containing chlorhexidine solution with a placebo, and who used the same measuring system employed in this study[9]. The percentage of PFZ Index score 2 (representing the presence of subgingival biofilm) at 96 hours after using CHXAL was 2.8% in the present study, versus 3.4% in the Maliska et al. study[9]. The inhibitory effects observed for the CHXAL and CHX formulations broaden the known scope of the inhibitory effects of chlorhexidine, by providing evidence on its ability to inhibit subgingival biofilm formation.

The present study included an evaluation of adverse events. Use of the CHXAL solution was associated with a significantly higher prevalence of adverse effects, compared with the CHX or PLA solutions. It is interesting to note that the subjects reported the presence of adverse effects with the PLA solution, at a prevalence rate similar to that reported with the CHXAL solution. Apart from the presence of alcohol in CHXAL, both experimental solutions had similar formulations. All three solutions used a mint-flavored sweetener. The burning sensation reported by the participants, particularly associated with the use of PLA and CHXAL, could be associated with the presence of this additive, and, in turn, may be mitigated in the CHXAL solution. In contrast with our results, a study by Quirynen et al. reported a similar incidence of adverse events between alcohol-containing and alcohol-free chlorhexidine formulations[14]. Two other studies evaluating adverse events compared two alcohol-free chlorhexidine solutions[26, 27], thus precluding direct comparison with the results of this study.

Participants also showed differences in acceptance of the three formulations. Again, CHXAL received a significantly worse assessment than either the CHX formulation or PLA. This finding is corroborated by other studies that also reported better palatability of alcohol-free formulations[13,14]. This was the first study in the literature to use a hedonic scale to assess taste preference for chlorhexidine solutions. Hedonic scales provide an evaluation that involves both sensory and cognitive processes, with several reference points that can be selected by participants (from "like extremely" to "dislike extremely"), rather than a single quantitative measurement, as provided by the visual analogue scale[28]. Considering that the aims of this study included comparing the taste preference of each of the three solutions, the hedonic scale was chosen because it enables predicting the level of acceptance of each solution, taking both sensory and hedonic perceptions into consideration[29,30].

The present findings should be interpreted in the context of some limitations. In crossover studies, results during the second series may be influenced by a residual effect associated with the first experimental series (carryover effect). In the present study, the interval between sets was 10 days. This is considered a sufficient washout interval, as accepted by several authors[5, 9, 31], and data analysis showed no carryover effect. The loss of participants during the study is an additional disadvantage of this experimental design. Since three participants broke the study protocol, both per-protocol and intention-to-treat analyses were performed to determine the impact of protocol deviations on the results. The participants of the study were dental students, and the results may have been influenced by their knowledge. On the other hand, adherence to the study protocol and motivation were necessary throughout the trial, characteristics inherent to this group of participants.

The results of this study provide additional evidence regarding the inhibitory effects of both chlorhexidine solutions, including their ability to inhibit formation of subgingival biofilm. Furthermore, the CHX formulation was associated with a lower incidence of adverse events and better acceptance among the participants. Future studies should be designed to confirm or refute these findings,

and long-term clinical trials should be considered to evaluate the consequences of inhibiting early subgingival biofilm formation on attachment loss.

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