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Original Article

Effects of probiotic yogurt, casein phosphopeptide-amorphous calcium phosphate, and xylitol chewing gums on the salivary count of *Streptococcus mutans*: A single-blinded randomized controlled clinical trial

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ABSTRACT

Background: Dental caries is a preventable multifactorial disease, with *Streptococcus mutans* being suggested to be its primary pathogen. Our study aim was to compare the effects of three different low-cost and easy-to-use regimens with that of the gold standard (chlorhexidine [CHX] mouthwash) on the count of salivary *S. mutans* in dental students over 30 days.

Materials and Methods: In this single-blinded parallel randomized controlled clinical trial, a total of 120 dental students were included and randomly allocated into four intervention groups: (1) CHX mouthwash (control(, (2) probiotic yogurt, (3) casein phosphopeptide-amorphous calcium phosphate chewing gum, and (4) xylitol chewing gum. Salivary *S. mutans* counts were evaluated at baseline, 15 days, and 30 days after initiation of the study and compared at different times and among different groups using the repeated measures analysis of variance design analysis and least significant difference test with SPSS software version 20. The level of significance was determined to be 0.05.

Results: The microorganism count variable at baseline, first, and second follow-ups was significantly different for all groups except the probiotic yogurt group (P = 0.340). *S. mutans* count was significantly different when comparing the first follow-up and baseline values in the CHX and xylitol gum groups (P = 0.027, P = 0.037). When comparing the second follow-up with baseline values, a significant difference was observed in the xylitol gum group (P = 0.003).

Conclusion: Xylitol chewing gum seems to be a viable alternative to the gold standard (CHX mouthwash) in reducing the salivary count of *S. mutans*.

Key Words: Casein phosphopeptide-amorphous calcium phosphate nanocomplex, chlorhexidine, dental caries, probiotics, *Streptococcus mutans*, xylitol

INTRODUCTION

Dental caries is one of the most prevalent diseases with a considerable economic burden worldwide.[1]

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Its development has traditionally been attributed to the dynamic relationship between three indispensable factors, including dental plaque bacteria, dietary carbohydrates, and susceptible teeth.[2] Concerning the former, *Streptococcus mutans* (*S. mutans*) is suggested to be the primary pathogen of this multifactorial disease.[3] In assessing caries risk, many models incorporate assays to determine salivary levels of cariogenic microorganisms,[4] including *S. mutans*. *S. mutans* levels in saliva can reflect its levels in plaques.[5]

Increased accumulation of dental plaque puts individuals at higher risk of caries.[6] For many, achieving adequate levels of plaque control by mechanical methods, especially at the most caries-susceptible sites, is difficult. Thus, adjunctive use of products that contain antiplaque or antimicrobial agents seems beneficial to reduce plaque accumulation.[1,6] Chlorhexidine (CHX) is the gold standard for antiplaque agents. Despite desirable characteristics, CHX has several reported side effects limiting its long-term use.[7] Recently, there has been a continuing search for more efficient alternative agents and delivery methods.

Nowadays, chewing gum is viewed as a delivery method/system for oral health therapeutic agents. Concerning dental caries, xylitol and casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) are examples of these agents added to chewing gum. Further, gum chewing itself can aid in the control of dental caries by stimulating saliva flow, which ultimately neutralizes the drop in plaque pH after eating.[8,9]

Another factor attributed to chewing gum's ability of control caries is its noncariogenic sugar substitute content.[8] Xylitol is currently one of the most common polyols used in sugar-free chewing gums, which cannot be fermented by oral bacteria.[9] This five-carbon sugar alcohol promotes the remineralization process by increasing saliva flow and reducing the overall counts of *S. mutans* as well as its adhesion.[10,11]

CPP-ACP is an anticariogenic substance composed of casein (a milk phosphoprotein) and calcium phosphate which exerts its main effect by delivering bioavailable mineral ions, i.e. calcium and phosphate ions, to demineralized dental tissues.[12] Indeed, multiple phosphoryl residues in CPP stabilize calcium and phosphate ions in aqueous solutions. Thus, calcium phosphate ions can be released to maintain supersaturation levels which would promote the remineralization process.[13,14] In addition, CPP-ACP can reduce *S. mutans* count in dental biofilm.[15] Although CPP-ACP is contraindicated in patients with milk protein allergy, it can still be consumed by those with lactose intolerance, since it is lactose-free.[16]

Probiotics have recently received increasing attention in the field of caries control. The Joint Food and Agriculture Organization/World Health Organization working group defines probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host."[17] Bacteria added to yogurt and fermented milk products are the most important sources of probiotics.[18] Many probiotic bacteria belong to the genera *Propionibacterium*, *Streptococcus*, *Lactobacillus,* and *Bifidobacterium*. Among bacteria known as probiotics, some genera of *Bifidobacterium* and *Lactobacillus* have been shown to inhibit the growth of *S. mutans*, thereby preventing or reducing dental caries by adhering to tooth surfaces, competing with cariogenic bacteria, and reducing acid production.[18,19] Thus, replacing routine consumption of nonprobiotics with probiotic dairy products containing microorganisms with such effects seems to be a viable alternative to other standard caries control strategies.

As the current literature, to our knowledge, lacks a direct comparison between CPP-ACP and xylitol chewing gums, probiotic yogurt, and CHX mouthwash (gold standard), this clinical study aimed to compare the effect of four different consumption regimens based on these agents on the salivary count of *S. mutans* over 30 days. The null hypothesis was that there would be no differences between the four regimens in reducing the salivary count of *S. mutans* within 30 days.

MATERIALS AND METHODS

This single-blind parallel randomized controlled clinical trial was conducted at the outpatient dental clinic of the Pediatric Dentistry Department, Faculty of Dentistry, during April and May 2017. Ethical clearance for this study was obtained from the Ethics Committee of the university (IR.mums.Sd.REC.1394.199), and all methods were performed in accordance with the Declaration of Helsinki on medical protocol and ethics. Written informed consent

was also obtained from all included participants. The protocol of this research was registered at the Iranian Registry for Clinical Trials (IRCT2016100730193N1, registered June 26, 2017). The Consolidated Standards of Reporting Trials checklist was used to report the present study [Figure 1].

Sample size

Sample size calculation was performed using NCSS software (NCSS 11 Statistical Software (2016). NCSS, LLC. Kaysville, Utah, USA). Using a confidence level of 99.5% and a power of 80%, it was determined that a minimum of 30 patients is required in each group.[20]

Participants and eligibility criteria

Study participants were recruited from volunteer undergraduate dental students. The inclusion criteria were as follows: (1) good general health, (2) good oral hygiene, (3) presence of at least 22 teeth, and (4) absence of active dental caries and chronic inflammation of the oral mucosa. The exclusion criteria were as follows: (1) systemic conditions compromising dental and periodontal

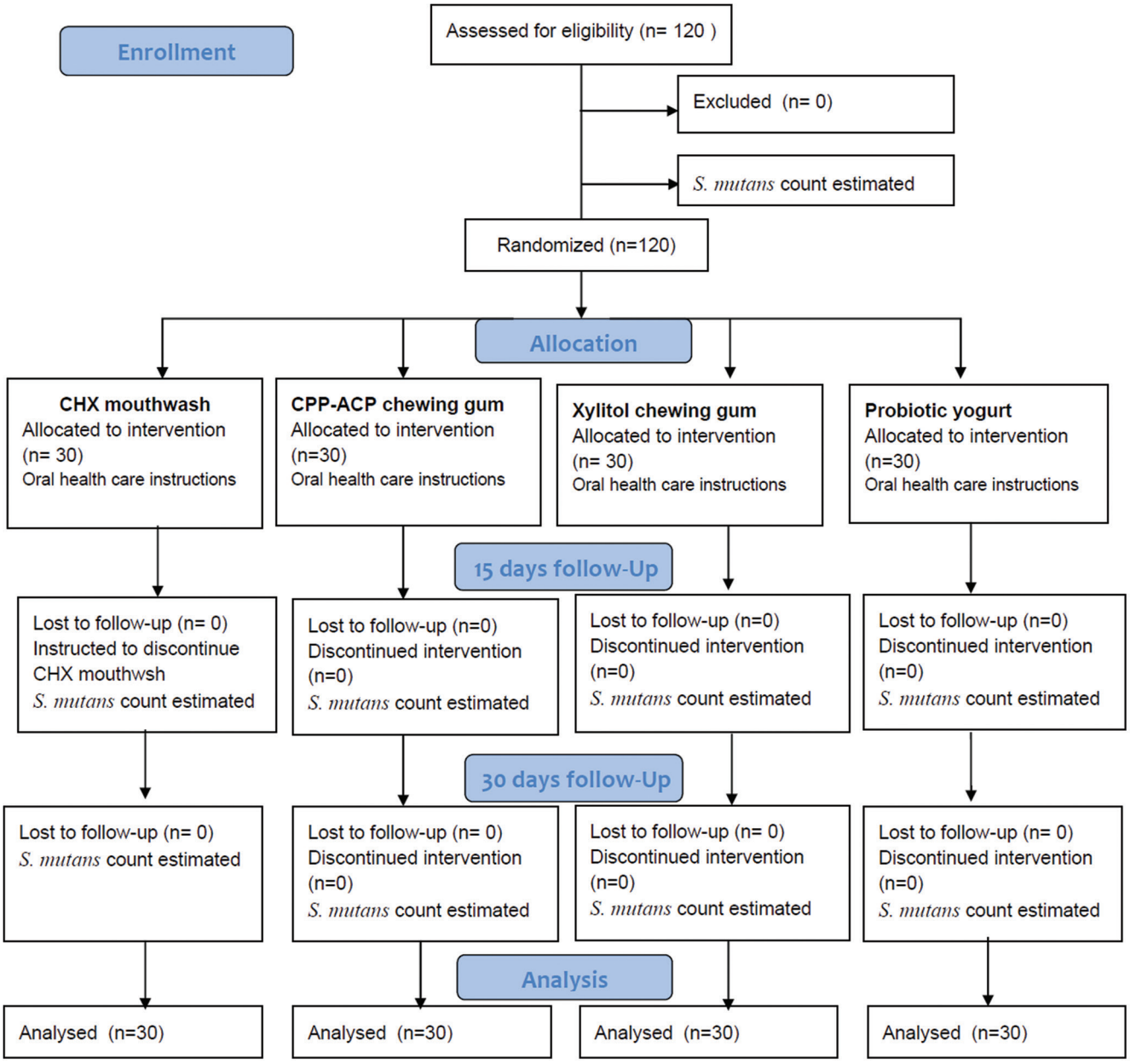

Figure 1: Consort flow diagram of the study. CHX: Chlorhexidine, CPP-ACP: Casein phosphopeptide-amorphous calcium phosphate, *S. mutans: Streptococcus mutans.*

health, (2) chronic antibiotic use, (3) antimicrobial mouthwash application in the past 3 months, (4) wearing orthodontic appliances, (5) consumption of saliva-affecting medications, (6) history of maxillofacial radiotherapy, and (7) allergy to milk proteins (including casein) or phenylketonuria.

Type of interventions

After a thorough explanation of the study and obtaining written consent, the included participants were asked to continue their routine oral hygiene activities throughout the entire next week while avoiding consumption of any probiotic products, mouthwashes, and chewing gums containing xylitol or CPP-ACP. They also refrained from eating, drinking, and chewing gums for 1 h before the next appointment scheduled at 8 a.m. 1 week later.

In the second session, approximately 1 cc of unstimulated saliva was collected in coded Falcon tubes, using the spitting method.[21] Briefly, the subjects should be sitting comfortably with eyes open and the head slightly forward, resting for 5 min; minimizing orofacial movements, saliva would be allowed to accumulate in the floor of the mouth and the subjects spit it out into the preweighed or graduated test tube every 60 s.[21] The samples were then sent to the microbiology laboratory of Ghaem Hospital to determine the count of *S. mutans* in the saliva of each individual.

Following saliva sampling, participants were randomly and equally assigned to four intervention groups (n = 30 per group) by block randomization method using a web-based randomization service (www.randomizer.org). An independent researcher not involved in the current project generated a list of random numbers kept by one investigator (MM) not directly involved in any clinical procedures until patient allocation. At the time of allocation, she provided one code at a time for the examiner (SA.S) who performed the first sampling. Given the type of interventions, participants could not be blinded in our study.

Study groups are as follows:

- Group 1 (control): Students in this group were asked to use 5 mL of 0.2% CHX mouthwash (Vi-one, Rojin Cosmetic Co., Tabriz, Iran) once a day following toothbrushing at night for 15 consecutive days
- Group 2: These participants were asked to consume 200 g of low-fat probiotic yogurt (Kalleh, Dairy Products, Food processing, Iran) once a day for 30 consecutive days during lunch as a dessert
- Group 3: These participants were asked to chew CPP-ACP gum (Trident Sugar Free Gum with CPP-ACP, Chewing Gum, USA) 20 min three times a day after breakfast, lunch, and dinner and continue for 30 consecutive days
- Group 4: These students were asked to chew xylitol gum (Trident Sugar Free Gum with Xylitol, Chewing Gum, USA) 20 min three times a day after breakfast, lunch, and dinner and continue for 30 consecutive days.

The composition of materials used in this study is presented in Table 1.

To establish a similar plaque control regimen among participants, similar oral health-care instructions (toothbrushing 3 times/day for 3 min after each meal using the modified Bass technique) in addition to a toothbrush (Oral-B®, Medium Bristle, USA) and a toothpaste (Crest®, Sodium Fluoride Toothpaste, USA) were provided to each dental student. Individuals were asked not to make any other changes in their eating habits, except for those mentioned for each specific group.

The participants were asked to make no changes in their dietary habits.

The second and third saliva samples were performed 15 and 30 days later, respectively, in a similar manner. After 15 days, CHX mouthwash was discontinued due to its side effects, but oral hygiene was observed as with other groups.

Microbiological testing of saliva

Briefly, laboratory procedures of counting salivary *S. mutans* were blindly performed as follows: after stirring for 3–4 min using a vortex, the saliva samples were incubated at 37°C for 1 h and again stirred for another 3–4 min. Due to the large number of bacteria present in human saliva, a serial dilution method was employed to isolate *S. mutans* and 5 different dilutions (1/10, 1/20, 1/40/, 1/80, and 1/160) were prepared by adding 100 µL of saliva to 900 µL of sterile saline. Next, 20 µL of each dilution was inoculated on Mitis Salivarius Bacitracin Agar using the spread plate method. The plates were incubated in a candle jar (10% CO2) for 48 h at 37°C. Bacitracin in the culture medium would prevent the growth of other streptococci of normal oral flora. To detect *S. mutans*, first a Gram staining slide is prepared from the colonies grown on the mentioned medium,

Table 1: Composition of materials used in this study

Materials	Brand	Ingredients
Xylitol chewing gum	Trident Sugar Free Gum with Xylitol	Sorbitol, chewing gum base, xylitol, glycerin, natural and synthetic lubricants, acesulfame potassium, aspartame, BHT, mannitol, soy lecithin
CPP-ACP chewing gum	Trident Sugar Free Gum with CPP-ACP	Synthetic sweetener, flavors, CPP-ACP aspartame, acesulfame potassium, sorbitol, mannitol, maltitol, gum base, citric acid
Probiotic yogurt	Kalleh Dairy Products	Fresh cow's milk, skim milk, thermophilic starter, and probiotic starter (*Streptococcus thermophilus, Lactobacillus thermophilus, Lactobacillus acidophilus, Lactobacillus casei*, and *Bifidobacterium*)
CHX mouthwash	Vi-one CHX mouthwash	Thymol, xylitol, CHX 0.2%

CPP-ACP: Casein phosphopeptide-amorphous calcium phosphate; CHX: Chlorhexidine; BHT: Butylated hydroxytoluene

then Gram-positive cocci are selected, and the catalase test is performed for them. Catalase-negative, Gram-positive cocci were subjected to biochemical tests for identification. The biochemical results as mannitol positive, sorbitol positive, arginine negative, esculin bile positive and urea negative, Voges–Proskauer positive were considered *S. mutans* and were counted. Afterward, the number of colonies on each plate was counted. The number of colonies on each of the plates that can be accurately counted was multiplied by the dilution factor of the corresponding plate and the cultured volume and presented as colony-forming unit/mL.

Statistical analysis

The primary outcome was to determine the effect of CPP-ACP and xylitol chewing gums, probiotic yogurt, and CHX mouthwash on salivary *S. mutans* over a 30-day period. Our secondary outcomes were to compare this effect at 15- and 30-day intervals (1) in each group and (2) between different groups.

The normality of data was assessed by the Kolmogorov–Smirnov test. Repeated measures analysis of variance (ANOVA) design analysis was used to compare the count of microorganisms at different sampling times. Statistical graphs and tables were used to describe the data; if the result of repeated measures design analysis was significant, the least significant difference (LSD) test was employed to determine a significant period of time. A pairwise comparison of groups and times was done with Bonferroni correction. The significance level of the tests was 0.05, and the software utilized was SPSS version 20 (IBM Corp, Armonk, NY, USA).

RESULTS

Overall, 120 dentistry students met the inclusion criteria and were included in this study, out of which 43 (35.83%) were male and 77 (64.16%) were female. The mean age of the participants was 23.88 ± 2.86 years (range: 20–36 years). All students were available for follow-up examinations at 15 and 30 days after the start of interventions [Figure 1].

The microbial count variable had a normal distribution. Results of ANOVA showed that all four groups at baseline were not significantly different in terms of microorganism count ($P = 0.124$).

The result of the one-way repeated measures ANOVA design test showed that the microbial count variable at baseline, first, and second follow-ups was significantly different for all groups except the probiotic yogurt group [Table 2].

The results of the LSD test showed that the microbial count was significantly different in comparison with the first follow-up and baseline values in CHX and xylitol gum groups ($P = 0.027$, $P = 0.037$). Comparing the second follow-up with baseline values, a significant difference was observed in the xylitol gum group ($P = 0.003$). There was a significant difference between the first and second follow-ups of the CPP-ACP gum group ($P = 0.038$) [Table 3].

The microbial count declined in the first follow-up in all groups except the CPP-ACP gum group. In the second follow-up, compared to the baseline, the microbial count decreased in all groups [Figure 2]. In the second follow-up, compared to the first follow-up, in all groups except the CHX group, there was a descending trend due to discontinuation of treatment after 15 days. At the end of the treatment period, in all groups, the microbial count diminished compared to the baseline [Figure 3].

In this study, no side effects were reported among participants in different groups.

DISCUSSION

In the present study, all groups except probiotic yogurt showed a significant reduction in salivary *S.*

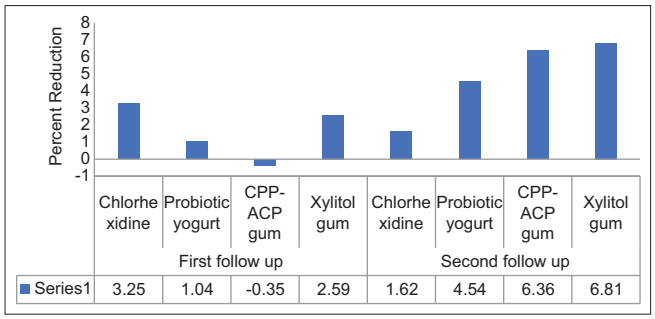

Figure 2: Percent reduction of *Streptococcus mutans* in first and second follow-ups compared to the baseline. CPP-ACP: Casein phosphopeptide-amorphous calcium phosphate.

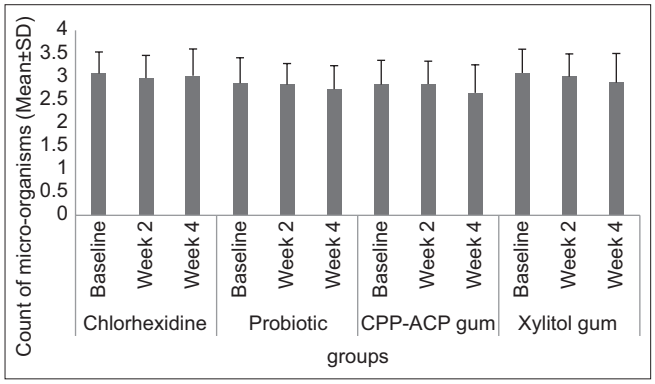

Figure 3: Logarithmic distribution of microbial count at baseline, 15 days, and 30 days later. CPP-ACP: Casein phosphopeptide-amorphous calcium phosphate, SD: Standard deviation.

mutans count at 30-day follow-up compared with baseline. Although in probiotic yogurt, a drop in the count of *S. mutans* compared to baseline values was observed, this reduction was not significant. Based on our results, it seems that xylitol chewing gum can be considered a viable alternative for CHX mouthwash for routine application.

We evaluated the effects of four different regimens on salivary *S. mutans* count. *S. mutans*, one of the primary etiological factors of dental caries, can form bacterial biofilms through its several glycosyltransferase enzymes that convert sucrose into a gel-like extracellular polymer glucan through cellular attachment to dental surfaces.[22] Microbial biofilms have a significant causative role in tooth caries.[23] Thus, reducing the number of free *S. mutans* in the oral cavity or disturbing the mechanisms resulting in biofilm formation might be possible strategies to prevent this infectious dental disease. Due to the easier sampling and process, saliva is preferred to plaque to estimate the count of this microorganism.[5]

Table 2: Comparison of treatment groups on microbial count by repeated measures design

Treatment groups	Microbial count index[b], mean±SD			*P*
	Baseline	15 days	30 days	
CHX[a]	3.07±0.463	2.97±0.491	3.02±0.581	0.021*
Probiotic yogurt	2.86±0.547	2.83±0.452	2.73±0.509	0.340
CPP-ACP gum	2.83±0.523	2.84±0.494	2.65±0.604	0.048*
Xylitol gum	3.08±0.516	3.00±0.491	2.87±0.630	0.001*
P	0.124[c]	0.537[d]	0.431[d]	

*Statistically significant; [a]The reference group; [b]The unit of measurement is log CFU/mL; [c]ANOVA; [d]Baseline Adjusted by ANCOVA. log CFU: Logarithm of colony-forming unit; SD: Standard deviation; CPP-ACP: Casein phosphopeptide-amorphous calcium phosphate; CHX: Chlorhexidine

Table 3: Binary comparison of microbial count measurement times by least significant difference test with Bonferroni correction

Comparison	CHX[a]	Probiotic yogurt	Gum CPP-ACP	Xylitol gum
Baseline				
First follow-up	0.027*	0.980	1	0.037*
Second follow-up	1	0.432	0.091	0.003*
First follow-up				
Second follow-up	1	0.963	0.038*	0.084

*Statistically significant; [a]The reference group. CPP-ACP: Casein phosphopeptide-amorphous calcium phosphate; CHX: Chlorhexidine

In the present study, the reduction in the bacterial count was not significant only in the probiotic yogurt group. The nonsignificant result can be attributed to the limited time probiotic bacteria were present in the oral cavity due to the consumption method employed for this group in our study.[18] It was shown that probiotics can decrease the count of *S. mutans* by reducing *S. mutans* virulence gene expression and producing cell adhesion inhibitors and antimicrobial compounds.[24,25] These bacteria can affect different aspects of the immune system such as humoral, cellular, and nonspecific immunity. For instance, increased production of secretory immunoglobulin A and cytokines has been shown during treatment with probiotics.[26,27] However, the literature regarding the effects of probiotic-containing products on salivary *S. mutans* count is inconsistent, probably due to different study designs/protocols, laboratory techniques, or, most importantly, types of probiotic bacteria. Dairy products are preferred over other products as vehicles for probiotics due to their acid-buffering capacity.[28] Among probiotics in the low-fat yogurt used in the present study, *Lactobacillus acidophilus, Lacticaseibacillus casei*, and *Bifidobacterium* spp. were proposed and used to achieve the effects of caries prevention, changes in the count of mutans

streptococci and lactobacilli as well as plaque pH control in previous studies.[28,29]

We used CHX mouthwash as a control in our study as this substance is considered the standard gold of antimicrobial agents against which the efficacy of other antimicrobial and antiplaque agents is assessed. The antibacterial properties of CHX can be explained by the attachment of positively charged molecules of CHX to the negatively charged bacterial cell wall and consequent osmotic balance disruption resulting in bacterial cell death.[30] The mouthwash we used in our study contained xylitol and thymol. In addition to optimizing the taste of mouthwash, xylitol increases saliva secretion, reduces acid secretion by bacteria, and increases remineralization of tooth enamel. It has also been shown that the antimicrobial effect of CHX in combination with thymol is greater against biofilm cultures of *S. mutans*.[31] In the second follow-up of treatment, compared to the first follow-up, in all groups except the CHX group, there was a diminishing trend due to cessation of treatment in this group after 15 days. According to articles, its usage for more than 2 weeks causes side effects such as discoloration of teeth and change in taste sensation.

The xylitol group displayed a decrease in the number of salivary *S. mutans*. It was shown that such decreased bacterial count following xylitol use is associated with a reduction of *S. mutans* in dental plaque.[32] Inside the bacterial cytoplasm, xylitol is phosphorylated into xylitol-5-phosphate, which inhibits bacterial growth and ultimately leads to bacterial cell death by interfering with glycolysis and adenosine triphosphate as well as acid production. The effect of xylitol on bacteria may be influenced by the form of xylitol used. The use of xylitol in foods as a sugar substitute does not reduce *S. mutans*, but chewing gum and candy forms have more beneficial effects due to direct and constant contact with the tooth surface.[32] The inhibitory effect of xylitol gums on the number of mutants has also been shown in various studies.[8,26,33,34]

Our results supported the relative superiority of CPP-ACP chewing gum over the CHX mouthwash in reducing salivary *S. mutans*; therefore, the hypothesis of this study was rejected. The antimicrobial activity of CPP-ACP can be shown by various mechanisms that reduce the adhesion of *S. mutans* to the teeth: (i) the antiplaque effect of CPP-ACP and salivary calcium; (ii) the ability of the CPP-ACP to bond twice the bacterial cell affinity for calcium increasing the concentration of extracellular calcium, which may have bactericidal or bacteriostatic effects; and (iii) alteration of the surface properties of enamel in the presence of CPP-ACP, which reduces *S. mutans* adhesion.[35] Similarly, a recent randomized clinical trial found that daily consumption of CPP-ACP chewing gum significantly reduced *S. mutans* levels in saliva, and this effect was statistically greater than that of xylitol chewing gum.[36] The same results, however, were not achieved after applying the paste form of CPP-ACP.[37,38] This can be attributed to the type of study design, the type of comparison reference group, or the route of application of the material.

Another finding encountered in the CPP-ACP group was its delayed effect on reducing *S. mutans*. This can be explained by the fact that CPP-ACP acts as a reservoir for calcium, phosphate, and fluoride ions, which enables the slow release of these ions over a longer period of time.[39] Such delayed effect needs further investigation in future studies.

We instructed participants not to alter their eating habits, as the salivary *S. mutans* count for each individual was compared to their baseline. Moreover, we aimed to avoid making drastic changes in eating habits to ensure feasibility for participants.

Recruiting dental students as study participants had several advantages including unrestricted access to them which resulted in no dropout in any groups throughout the study. Further, controlling interfering factors (e.g., brushing method and oral hygiene regimen) was possible. On the other hand, higher oral health knowledge of the students may affect their performance; hence, extrapolating these results to ordinary people should be done with caution. Other limitations of our study were the impossibility of blinding participants and not having a no-intervention group. The latter was attempted to overcome by matching the study groups. Furthermore, in this study, we did not evaluate the durability of different materials, so future studies are suggested to evaluate the durability of each of these materials. A considerable strength of this study was employing practical delivery methods of the test agents. Nonetheless, the same method could not be applied to all groups due to the unavailability of commercial products at the time of the study. This, in turn, could affect our results, especially in the probiotic group as mentioned earlier. Thus, for future studies, we

strongly recommend using other delivery forms of probiotics with prolonged contact time in the oral cavity, such as chewing gum or lozenges. If not feasible, altering the pattern of consumption, for instance swishing and swallowing the probiotic dairy products, might be another viable alternative. We also encourage evaluating these four agents/regiments used in our study in children and other age groups. Future research can evaluate the different forms and methods of using these materials through various study designs.

Based on the results of this study, it can be concluded that significant salivary *S. mutans* count reduction at 30-day follow-up was observed in all groups except probiotic yogurt compared with baseline. In addition, xylitol chewing gum can be an alternative to CHX mouthwash, which comes with a variety of side effects.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or nonfinancial in this article.

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