RESEARCH PAPER

In Vitro Antimicrobial Activity of a Novel Nano-Hydroxyapatite–Theobromine Fluoride-Free Mouthwash Formulated for Young Children

Dr. Mohamed Badhusha[1], **Dr. Dinesh Kumar**[1*]

[1*]Department of Pedodontics and Preventive Dentistry, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, Tamil Nadu, India

[1]Postgraduate Student (Dr. Mohamed Badhusha) | Email: 152311001.sdc@saveetha.com

[1*]Senior Lecturer (Dr. Dinesh Kumar) | Email: dineshkumarb.sdc@saveetha.com

***Corresponding Author:** Dr. Dinesh Kumar, Senior Lecturer, Department of Pedodontics and Preventive Dentistry, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, Tamil Nadu, India | Email: dineshkumarb.sdc@saveetha.com

Abstract

Background

Young children may have difficulty reliably expectorating mouthrinse, limiting the routine use of conventional fluoride mouthrinses in this age group. This creates a need for alternative fluoride-free formulations with potential preventive applications in pediatric oral care. Nano-hydroxyapatite (nHAP) and theobromine have been investigated as bioactive compounds with potential relevance to caries-preventive oral-care formulations; however, evidence regarding their combined use in a pediatric mouthwash remains limited.

Aim

To formulate a fluoride-free nano-hydroxyapatite–theobromine mouthwash and evaluate its in-vitro antimicrobial activity at different concentrations against selected oral microorganisms.

Methods

A fluoride-free mouthwash containing 5% w/v nano-hydroxyapatite and 0.5% w/v theobromine, together with xylitol and other formulation excipients, was prepared. The formulation was diluted to concentrations of 25, 50, and 100 µg/mL and evaluated against *Streptococcus mutans, Lactobacillus* sp., *Enterococcus faecalis, Staphylococcus aureus,* and *Candida albicans* using the agar well diffusion method. The assay was repeated three times using the same formulation batch. Antimicrobial activity was assessed by measuring the diameter of the inhibition zone.

Results

The nano-hydroxyapatite–theobromine mouthwash demonstrated measurable antimicrobial activity against all microorganisms tested. Increasing concentrations were associated with progressively larger inhibition zones. At 25, 50, and 100 µg/mL, respectively, the mean ± SD inhibition zones were 18.0 ± 1.0, 21.3 ± 1.5, and 36.3 ± 2.5 mm for *S. mutans*; 25.7 ± 2.5, 34.3 ± 2.5, and 43.0 ± 2.0 mm for *Lactobacillus* sp.; 25.3 ± 1.5, 36.7 ± 2.1, and 44.0 ± 2.6 mm for *E. faecalis*; 25.3 ± 3.8, 33.3 ± 3.1, and 37.3 ± 2.9 mm for *S. aureus*; and 18.0 ± 1.0, 20.3 ± 3.2, and 27.0 ± 3.0 mm for *C. albicans*. The Friedman test demonstrated a significant overall difference across the three concentrations for each microorganism ($\chi^2 = 6.00$, $p = 0.0498$).

Conclusion

The nano-hydroxyapatite–theobromine fluoride-free mouthwash demonstrated concentration-dependent in-vitro antimicrobial activity against the microorganisms evaluated, with the greatest inhibition consistently observed at 100 µg/mL. These preliminary findings support further investigation of the formulation as a potential fluoride-free adjunct for pediatric oral care.

Keywords Nano-hydroxyapatite; Theobromine; Fluoride-free mouthwash; Antimicrobial activity; Pediatric dentistry; Early childhood caries; Agar well diffusion; Oral microorganisms

How to cite this article: Mohamed Badhusha, DineKumar. In Vitro Antimicrobial Activity of a Novel Nano-Hydroxyapatite–Theobromine Fluoride-Free Mouthwash Formulated for Young Children. Int J Drug Deliv Technol. 2026;16(79s):1204-1210. DOI: 10.25258/ijddt.16.79s.198.

Introduction

Early childhood caries (ECC) remains one of the most common oral diseases affecting children and is an important public health concern because of its potential consequences for pain, oral function, quality of life, and general well-being. [1] In India, a systematic review and meta-analysis of 71 studies involving 69,330 children reported a pooled ECC prevalence of 46.9%, highlighting the substantial burden of the disease in the pediatric population. [1] Dental caries is a biofilm-mediated, multifactorial disease in which microbial activity, dietary factors,

host susceptibility, and environmental conditions interact to produce an imbalance between demineralization and remineralization of dental hard tissues. [2] Cariogenic biofilms contain microorganisms capable of metabolizing dietary carbohydrates and producing acids that contribute to the initiation and progression of carious lesions. [2]

Streptococcus mutans is one of the microorganisms most frequently associated with dental caries and has been extensively studied because its presence and abundance in young children are associated with increased caries risk. [3] *Lactobacillus* species are also associated with caries progression because of their acidogenic and aciduric characteristics. [2] In addition, interactions between *S. mutans* and *Candida albicans* have been investigated in relation to the development of ECC and may contribute to a more cariogenic biofilm environment. [4] Therefore, approaches aimed at controlling cariogenic microorganisms and modifying pathogenic biofilm activity remain relevant to the prevention and management of ECC. [2]

Fluoride is an established caries-preventive agent, and fluoride mouthrinses have been evaluated extensively as an adjunctive method of caries prevention in children and adolescents. [5] A Cochrane systematic review including 37 trials and 15,813 participants found that supervised regular use of fluoride mouthrinse was associated with a substantial reduction in caries increment in permanent teeth. [5] However, the use of mouthrinse in young children requires consideration of their ability to control the rinse and expectorate it appropriately, particularly because swallowing may occur when rinsing skills are not yet well established. [6] This creates an interest in developing fluoride-free formulations that could potentially provide additional oral-health benefits for selected pediatric populations while avoiding dependence on fluoride as the active ingredient.

Hydroxyapatite (HAp) is a calcium-phosphate mineral closely related to the inorganic component of dental hard tissues and has consequently been investigated as a biomimetic material for oral-care applications. [7] Nano-hydroxyapatite (nHAp) has attracted particular attention because its small particle size provides a large surface area and may facilitate interactions with enamel and dentinal surfaces. [7] Experimental and clinical studies have investigated nHAp-containing products for remineralization and caries prevention, and a systematic review and meta-analysis reported evidence supporting its potential to reduce mineral loss and promote remineralization of early carious lesions. [8] More recent clinical evidence has also suggested that hydroxyapatite-containing oral-care products may have a caries-preventive effect, although the available studies differ in design, intervention, comparator, and outcome assessment. [9] Thus, while HAp-based fluoride-free formulations are promising, further well-designed studies are required to establish their effects in different clinical and experimental settings. [9]

The potential antimicrobial properties of HAp and nHAp are also relevant because disruption or control of cariogenic biofilms is an important component of caries prevention. [10] A recent systematic review found that HAp/nHAp demonstrated antimicrobial activity against several Gram-positive and Gram-negative bacteria as well as fungi. [10] However, the same review noted that evidence concerning the antibiofilm effects of nHAp remains limited, indicating that antimicrobial findings should be interpreted cautiously and supported by further experimental studies. [10]

Theobromine, a naturally occurring methylxanthine found predominantly in cocoa, has been investigated as another potential bioactive compound for dental applications. [11] Previous in-vitro research has suggested that theobromine may enhance enamel remineralization and increase enamel microhardness under demineralizing conditions. [11] However, findings have not been entirely consistent across experimental models, and some studies have reported limited or no significant effects on enamel demineralization or remineralization under specific experimental conditions. [12] A recent systematic review and meta-analysis reported that theobromine may increase enamel microhardness following demineralization, while also identifying differences between studies according to the methods and outcome measures used. [13] These findings suggest that theobromine remains a promising but incompletely characterized bioactive compound for dental applications.

Although nHAp and theobromine have each been investigated independently, there is limited evidence evaluating their combination in a fluoride-free mouthwash designed specifically for young children. The formulation investigated in the present study contains 5% w/v nHAp and 0.5% w/v theobromine together with xylitol and other excipients intended to produce a stable mouthwash formulation. The antimicrobial activity of the formulation was therefore evaluated against *S. mutans, Lactobacillus* sp., *Enterococcus faecalis, Staphylococcus aureus,* and *Candida albicans* using the agar well diffusion method. The mouthwash was evaluated at 25, 50, and 100 µg/mL to determine whether antimicrobial activity varied with concentration. The aim of the present study was to assess the in-vitro antimicrobial activity of a novel fluoride-free nano-hydroxyapatite–theobromine mouthwash at different concentrations against selected oral microorganisms.

Materials and Methods

Study design

An in-vitro experimental study was conducted to evaluate the antimicrobial activity of a newly formulated fluoride-free mouthwash containing nano-hydroxyapatite (nHAp) and theobromine. The antimicrobial activity of the formulation was assessed at three concentrations using the agar well diffusion method against selected bacterial and fungal microorganisms relevant to oral health.

Formulation of the fluoride-free mouthwash

A fluoride-free mouthwash formulation containing nano-hydroxyapatite and theobromine was developed for potential use in young children. The formulation contained 5% w/v nano-hydroxyapatite (20–50 nm) and 0.5% w/v theobromine. Xylitol (8% w/v) was incorporated as a sweetening/anticariogenic component, while glycerol (5% v/v) was used as a humectant. Hydroxyethyl cellulose (0.2% w/v) served as a suspension stabilizer and polysorbate 20 (0.1%) as a dispersing agent. Sodium citrate (0.15%) was included as a buffering agent, and the pH was adjusted to 6.9–7.0 using citric acid. Potassium sorbate (0.15%) and sodium benzoate (0.10%) were incorporated as preservatives. Sucralose (0.05%) and natural strawberry/banana flavour (0.1–0.2%) were added for palatability, and purified water was used as the vehicle to obtain a final volume of 100 mL.

Preparation of test concentrations

The prepared mouthwash formulation was diluted to obtain three test concentrations: 25, 50, and 100 µg/mL. These concentrations were selected to evaluate the concentration-dependent antimicrobial activity of the formulated mouthwash.

Test microorganisms

The antimicrobial activity of the formulation was evaluated against five microorganisms:

- *Streptococcus mutans*
- *Lactobacillus* sp.
- *Enterococcus faecalis*
- *Staphylococcus aureus*
- *Candida albicans*

These organisms were selected to represent microorganisms of relevance to the oral microbial environment, including cariogenic bacteria and an opportunistic fungal species. The organisms evaluated are documented in the experimental dataset.

Agar well diffusion assay

The antimicrobial activity of the experimental mouthwash was evaluated using the agar well diffusion method. Microbial cultures were prepared according to the laboratory protocol and inoculated onto the appropriate agar medium. Wells were subsequently prepared in the inoculated agar, and the respective concentrations of the mouthwash formulation were introduced into the wells.

The plates were incubated under the appropriate conditions for each microorganism. Following incubation, the diameter of the clear zone surrounding each well was measured in millimetres and recorded as the zone of inhibition.

The experimental data included inhibition-zone measurements for the three concentrations of the mouthwash against all five test microorganisms.

Experimental replication

The antimicrobial assay was performed three times using the same prepared formulation batch. The three datasets represented repeated measurements of the antimicrobial activity of the same formulation at 25, 50, and 100 µg/mL. The individual inhibition-zone measurements from the three experimental repetitions were used to calculate the mean and standard deviation for each microorganism and concentration.

Outcome measure

The primary outcome was the diameter of the zone of inhibition, expressed in millimetres (mm), produced by each concentration of the experimental mouthwash against the respective test microorganism.

Ethical considerations

As the present investigation was an in-vitro laboratory study involving microbial cultures and no human or animal subjects, human or animal ethical approval did not apply to this experiment. Any institutional laboratory biosafety or microbiological approval obtained for the work should be reported here.

Statistical Analysis

The diameters of the inhibition zones were recorded in millimetres (mm) for each microorganism at concentrations of 25, 50, and 100 µg/mL of the formulated mouthwash. The results were expressed as the mean ± standard deviation (SD) from three repeated assay measurements.

As the three concentrations were evaluated repeatedly using the same formulation batch, the observations were considered matched measurements. Therefore, differences in inhibition-zone diameter across the three concentrations were evaluated separately for each microorganism using the non-parametric Friedman test. The Friedman test was selected because of the repeated-measures structure and the small number of experimental repetitions. A two-sided p value <0.05 was considered statistically significant.

All analyses were performed separately for *Streptococcus mutans, Lactobacillus* sp., *Enterococcus faecalis, Staphylococcus aureus,* and *Candida albicans*. The 9-mm value reported as "Standard" in the original experimental dataset was excluded from the statistical analysis because it was not considered an experimental antimicrobial outcome.

Results

The formulated nano-hydroxyapatite–theobromine mouthwash exhibited measurable antimicrobial activity against all five microorganisms tested. The diameter of the inhibition zone increased with increasing mouthwash concentration across all organisms evaluated. The individual experimental measurements and the corresponding descriptive statistics are presented in Table 1.

	25 µg/mL	**50 µg/mL**	**100 µg/mL**	**Friedman p-value**
S. mutans	18.00 ± 1.00	21.33 ± 1.53	36.33 ± 2.52	
Lactobacillus sp.	25.67 ± 2.52	34.33 ± 2.52	43.00 ± 2.00	
E. faecalis	25.33 ± 1.53	36.67 ± 2.08	44.00 ± 2.65	**0.0498**
S. aureus	25.33 ± 3.79	33.33 ± 3.06	37.33 ± 2.89	
C. albicans	18.00 ± 1.00	20.33 ± 3.21	27.00 ± 3.00	

Table 1. Antimicrobial activity of the nano-hydroxyapatite–theobromine mouthwash at different concentrations.

For *Streptococcus mutans*, the mean inhibition zone increased from 18.00 ± 1.00 mm at 25 µg/mL to 21.33 ± 1.53 mm at 50 µg/mL and 36.33 ± 2.52 mm at 100 µg/mL. *Lactobacillus* sp. showed inhibition zones of 25.67 ± 2.52, 34.33 ± 2.52, and 43.00 ± 2.00 mm at 25, 50, and 100 µg/mL, respectively.

Similarly, the mean inhibition zone against *Enterococcus faecalis* increased from 25.33 ± 1.53 mm at 25 µg/mL to 36.67 ± 2.08 mm at 50 µg/mL and 44.00 ± 2.65 mm at 100 µg/mL. For *Staphylococcus aureus*, the corresponding inhibition zones were 25.33 ± 3.79, 33.33 ± 3.06, and 37.33 ± 2.89 mm. *Candida albicans* demonstrated inhibition zones of 18.00 ± 1.00, 20.33 ± 3.21, and 27.00 ± 3.00 mm at the three concentrations, respectively.

The 100 µg/mL concentration produced the largest mean inhibition zone for every microorganism tested. The greatest inhibition at this concentration was observed against *E. faecalis* (44.00 ± 2.65 mm), followed by *Lactobacillus* sp. (43.00 ± 2.00 mm), *S. aureus* (37.33 ± 2.89 mm), *S. mutans* (36.33 ± 2.52 mm), and *C. albicans* (27.00 ± 3.00 mm).

Because the three concentrations were evaluated repeatedly using the same formulation batch, differences across concentrations were assessed using the Friedman test. A statistically significant overall difference across the three concentrations was observed for *S. mutans*, *Lactobacillus* sp., *E. faecalis*, *S. aureus*, and *C. albicans* (Friedman $\chi^2 = 6.00$, $p = 0.0498$ for each microorganism).

Microorganism	**F value**	**p-value**
S. mutans	88.79	**0.001**
Lactobacillus sp.	40.56	**0.001**
E. faecalis	58.24	**0.001**
S. aureus	10.50	**0.011**
C. albicans	9.66	**0.013**

Table 2. Statistical comparison of antimicrobial activity among different concentrations of the nano-hydroxyapatite theobromine mouthwash.

The consistent increase in inhibition-zone diameter from 25 to 100 µg/mL indicates a concentration-related pattern of antimicrobial activity under the conditions of the agar well diffusion assay. However, because only three repeated measurements from a single formulation batch were available, these findings should be considered preliminary and interpreted with caution.

Discussion

The present study evaluated the in-vitro antimicrobial activity of a fluoride-free mouthwash containing nano-hydroxyapatite (nHAp) and theobromine against five microorganisms relevant to the oral environment. The formulation produced measurable inhibition against all microorganisms tested, and the inhibition-zone diameter generally increased as the concentration increased from 25 to 50 and 100 µg/mL. This concentration-related pattern suggests that the formulation possesses antimicrobial activity under the conditions of the agar well diffusion assay. [14]

The greatest inhibition at 100 µg/mL was observed against *Enterococcus faecalis* and *Lactobacillus* sp., whereas *Candida albicans* showed the smallest inhibition zone at the same concentration. Differences in susceptibility between microorganisms are expected because antimicrobial responses can vary according to microbial cell structure, physiology, growth characteristics, and interaction with the tested formulation. [15] Recent evidence specifically examining HAp and nHAp has demonstrated antimicrobial activity against both bacterial and fungal organisms, although substantial heterogeneity exists between studies in terms of materials, concentrations, microorganisms, and experimental methods. [14]

The concentration-dependent increase observed in the present study is consistent with experimental evidence showing that hydroxyapatite can exhibit concentration-related antibacterial effects against cariogenic microorganisms. [16] In that study, increasing concentrations of synthesized hydroxyapatite produced greater antibacterial effects against *Streptococcus mutans* and *Streptococcus sobrinus*. [16] Although the present study used a different formulation and experimental protocol, the similar concentration-related pattern supports the possibility that increasing exposure to HAp-containing material may enhance microbial inhibition under in-vitro conditions. [16]

The antimicrobial activity observed in the present formulation may partly relate to the physicochemical characteristics of nHAp. Nano-hydroxyapatite has a chemical composition similar to the mineral phase of dental hard tissues and possesses surface properties that allow interaction with biological and microbial interfaces. [17] Previous research has demonstrated interactions between hydroxyapatite surfaces and *S. mutans*, suggesting that surface-mediated interactions may influence bacterial attachment and behavior. [17] However, the present study cannot determine the individual contribution of nHAp because nHAp alone was not included as a separate experimental group. [14]

Theobromine may also have contributed to the antimicrobial response observed in the present study. An in-vitro study evaluating theobromine against cariogenic microorganisms reported antimicrobial activity against *S. mutans* and *Actinomyces naeslundii*. [18] The authors observed reduced bacterial abundance following exposure to theobromine compared with the untreated control, supporting further investigation of the compound as a potential antimicrobial component in oral-care formulations. [18] These findings provide a possible biological basis for including theobromine in the present formulation, although the current experimental design does not allow the contribution of theobromine to be separated from that of nHAp or the other ingredients.

The interpretation of theobromine in the present study should nevertheless remain cautious because the evidence for its dental effects is not completely consistent. [19] Previous investigations have reported effects of theobromine on enamel remineralization and microhardness, whereas other experimental models have not demonstrated a significant effect on enamel demineralization or remineralization. [19] A systematic review and meta-analysis also identified considerable variation among studies evaluating theobromine, suggesting that its effects may depend on concentration, experimental conditions, and the outcome assessed. [20] Therefore, the antimicrobial activity observed in the present study should not be interpreted as evidence that theobromine has an established clinical anticaries effect. [19]

The activity against *S. mutans* is particularly relevant to pediatric oral health because this organism has been consistently investigated in relation to ECC and cariogenic biofilm development. [21] However, the inhibition of microorganisms in an agar diffusion assay does not necessarily translate into equivalent activity against an established oral biofilm. [14] Dental caries develops within complex microbial communities, and biofilm architecture can substantially influence the susceptibility of microorganisms to antimicrobial agents. [22]

The agar well diffusion method provided an appropriate initial screening approach for determining whether the formulated mouthwash produced measurable antimicrobial activity. [23] However, inhibition-zone diameter is influenced not only by the antimicrobial properties of a test formulation but also by its ability to diffuse through the agar medium. [23] This limitation is relevant to the present formulation

because it contains suspended nano-hydroxyapatite together with viscosity-modifying and stabilizing components. Consequently, the inhibition-zone measurements should be interpreted as evidence of antimicrobial activity under the specific experimental conditions rather than as a direct measure of antimicrobial potency or minimum inhibitory concentration. [23]

The present study has several limitations. First, the antimicrobial assay was repeated three times using the same formulation batch, and therefore the repetitions do not establish reproducibility between independently prepared batches. [14] Second, the small number of repeated measurements limits the statistical power of the analysis and warrants cautious interpretation of the observed concentration-related differences. [24] Third, nHAp alone, theobromine alone, and the formulation vehicle were not evaluated as separate controls, preventing assessment of the contribution of individual ingredients to the antimicrobial activity. [14] Fourth, the agar diffusion model does not reproduce important characteristics of the oral environment, including saliva, acquired pellicle, dietary substrates, and multispecies biofilm structure. [22]

The findings should therefore be considered preliminary laboratory evidence rather than evidence of clinical effectiveness in children. Recent systematic reviews of hydroxyapatite-based fluoride-free oral-care products have reported promising caries-preventive findings but have also highlighted heterogeneity in study design, formulations, exposure periods, and clinical outcomes. [25] Evidence obtained from hydroxyapatite-containing toothpastes or other oral-care products cannot automatically be extrapolated to the present mouthwash because the delivery system, concentration, contact time, and intended population differ. [25]

Future studies should evaluate independently prepared formulation batches with an adequate number of biological replicates to establish reproducibility. [14] Determination of minimum inhibitory concentration and minimum bactericidal or fungicidal concentration would provide quantitative antimicrobial endpoints that complement the agar diffusion findings. [23] Biofilm-based experiments, particularly using *S. mutans* and multispecies oral biofilms, would provide additional information regarding the performance of the formulation under conditions that more closely resemble the oral environment. [14] Further experiments comparing the complete formulation with nHAp alone, theobromine alone, and the formulation vehicle would also help determine whether the observed antimicrobial effect is attributable primarily to one component or to the formulation as a whole. [14]

Overall, the nano-hydroxyapatite–theobromine mouthwash demonstrated measurable and concentration-related antimicrobial activity against the microorganisms evaluated in this preliminary in-vitro study. The findings are consistent with the growing experimental literature supporting further investigation of HAp/nHAp and theobromine as components of fluoride-free oral-care formulations. [14,18] However, the current findings should not be interpreted as evidence of clinical efficacy or as proof that the formulation can replace fluoride-based caries-preventive interventions. Additional microbiological, biofilm, physicochemical, safety, and clinical studies are required before its suitability for routine pediatric use can be established. [14,25]

Conclusion

The fluoride-free nano-hydroxyapatite–theobromine mouthwash demonstrated measurable antimicrobial activity against *Streptococcus mutans, Lactobacillus* sp., *Enterococcus faecalis, Staphylococcus aureus,* and *Candida albicans* under the conditions of the agar well diffusion assay. The inhibition-zone diameter increased with increasing formulation concentration, with the highest mean inhibition observed at 100 µg/mL for all microorganisms tested. These findings provide preliminary in-vitro evidence supporting further investigation of the nano-hydroxyapatite–theobromine formulation as a potential fluoride-free adjunct for pediatric oral care. However, the findings should not be interpreted as evidence of clinical anticaries efficacy or as confirmation that the formulation can replace fluoride-based preventive measures. Further studies using independently prepared formulation batches, larger numbers of biological replicates, minimum inhibitory and bactericidal/fungicidal concentration testing, biofilm models, physicochemical characterization, and comprehensive safety evaluation are required before clinical application in young children can be considered.

References

1. Devan I, Ramanarayanan V, Janakiram C. Prevalence of early childhood caries in India: a systematic review and meta-analysis. *Indian J Public Health.* 2022;66(Suppl):S3-S11. doi:10.4103/ijph.ijph_1078_22. PMID: 36412465.

2. Meyer F, Schulze Zur Wiesche E, Amaechi BT, Limeback H, Enax J. Caries etiology and preventive measures. *Eur J Dent.* 2024;18(3):766-776. doi:10.1055/s-0043-1777051. PMID: 38555649.

3. Manchanda S, Sardana D, Peng S, Lo ECM, Chandwani N, Yiu CKY. Is *Mutans Streptococci* count a risk predictor of early childhood caries? A systematic review and meta-analysis. *BMC Oral Health.* 2023;23(1):648. doi:10.1186/s12903-023-03346-8. PMID: 37679718.

4. Lu M, Lin Z, Li Y, He Y. Roles of *Streptococcus mutans-Candida albicans* interaction in early childhood caries: a literature review. *Front Cell Infect Microbiol.* 2023;13:1186902. PMID: 37260705.
5. Marinho VCC, Chong LY, Worthington HV, Walsh T. Fluoride mouthrinses for preventing dental caries in children and adolescents. *Cochrane Database Syst Rev.* 2016;7(7):CD002284. doi:10.1002/14651858.CD002284.pub2. PMID: 27472005.
6. Adair SM. The role of fluoride mouthrinses in the control of dental caries: a brief review. Pediatr Dent. 1998 Mar-Apr;20(2):101-4. PMID: 9566013.
7. Bordea IR, Candrea S, Alexescu GT, et al. Nano-hydroxyapatite use in dentistry: a systematic review. *Drug Metab Rev.* 2020;52(2):319-332. doi:10.1080/03602532.2020.1758713. PMID: 32393070.
8. Wierichs RJ, Wolf TG, Campus G, Carvalho TS. Efficacy of nano-hydroxyapatite on caries prevention—a systematic review and meta-analysis. *Clin Oral Investig.* 2022;26(4):3373-3381. doi:10.1007/s00784-022-04390-4. PMID: 35103837.
9. Pawinska M, Paszynska E, Amaechi BT, Meyer F, Enax J, Limeback H. Clinical evidence of caries prevention by hydroxyapatite: an updated systematic review and meta-analysis. *J Dent.* 2024;151:105429. doi:10.1016/j.jdent.2024.105429. PMID: 39471896.
10. Dewi N, Gartika M, Gustiono D, Kurnia D, Cahyanto A. Antimicrobial and antibiofilm properties of hydroxyapatite/nano-hydroxyapatite in preventing dental caries: a systematic review. *Eur J Dent.* 2025;19(3):563-579. doi:10.1055/s-0045-1802568. PMID: 40311636.
11. Premnath P, John S, Manchu A, et al. Effectiveness of theobromine on enamel remineralization: a comparative in-vitro study. *Cureus.* 2019;11(10):e5686. doi:10.7759/cureus.5686. PMID: 31720155.
12. Amaechi BT, et al. The effect of theobromine on the in vitro de- and remineralization of enamel carious lesions. *J Dent.* 2021;107:103600. PMID: 34059300.
13. da Silva ARJ, dos Santos Gonçalves RI, de Vasconcelos Catão MHC. Theobromine for remineralization of white spot lesions on dental enamel: a systematic review and meta-analysis. *Oper Dent.* 2024;49(4):376-387. doi:10.2341/23-153-LIT. PMID: 3898793
14. Dewi N, Gartika M, Gustiono D, Kurnia D, Cahyanto A. Antimicrobial and antibiofilm properties of hydroxyapatite/nano-hydroxyapatite in preventing dental caries: a systematic review. *Eur J Dent.* 2025;19(3):563-579. doi:10.1055/s-0045-1802568. PMID: 40311636.
15. Murray PR, Rosenthal KS, Pfaller MA. *Medical Microbiology.* 9th ed. Elsevier; 2020.
16. Ibrahim AZ, et al. Antibacterial activity of microwave synthesized hydroxyapatite against cariogenic bacteria: a preliminary study. *Saudi Dent J.* 2024;36(8):1117-1122. PMID: 39176152. doi:10.1016/j.sdentj.2024.06.004.
17. Rölla G, Robrish SA, Bowen WH. Interaction of hydroxyapatite and protein-coated hydroxyapatite with *Streptococcus mutans* and *Streptococcus sanguis*. *Acta Pathol Microbiol Scand B.* 1977;85(5):341-346. PMID: 602782.
18. Rafiq IH, Dame-Teixeira N, Do T. The antimicrobial activity of theobromine against cariogenic microbes: an in vitro pilot study. *BDJ Open.* 2024;10(1):8. doi:10.1038/s41405-024-00190-y. PMID: 38302447. (PubMed)
19. Thorn AK, Lin WS, Levon JA, Morton D, Eckert GJ, Lippert F. The effect of theobromine on the in vitro de- and remineralization of enamel carious lesions. *J Dent.* 2021;107:103600. PMID: 34059300.
20. Malcangi G, Patano A, Morolla R, De Santis M, Piras F, Settanni V, Mancini A, Di Venere D, Inchingolo F, Inchingolo AD, Dipalma G, Inchingolo AM. Analysis of Dental Enamel Remineralization: A Systematic Review of Technique Comparisons. Bioengineering (Basel). 2023 Apr 12;10(4):472. doi: 10.3390/bioengineering10040472. PMID: 37106659; PMCID: PMC10135549.
21. Manchanda S, Sardana D, Peng S, Lo ECM, Chandwani N, Yiu CKY. Is *Mutans Streptococci* count a risk predictor of early childhood caries? A systematic review and meta-analysis. *BMC Oral Health.* 2023;23(1):648. PMID: 37679718. doi:10.1186/s12903-023-03346-8.
22. Meyer F, Enax J, Epple M, Amaechi BT, Simader B. Cariogenic biofilms: development, properties, and biomimetic preventive agents. *Dent J (Basel).* 2021;9(8):88. PMID: 34435948. doi:10.3390/dj9080088.
23. Holder IA, Boyce ST. Agar well diffusion assay testing of bacterial susceptibility to various antimicrobials in concentrations non-toxic for human cells in culture. *Burns.* 1994;20(5):426-429. PMID: 7999271. doi:10.1016/0305-4179(94)90035-3.
24. Vickers AJ. Underpowering in randomized trials reporting a small number of events. *J Clin Epidemiol.* 2003;56(5):430-435. PMID: 12821253.
25. Pawinska M, Paszynska E, Amaechi BT, Meyer F, Enax J, Limeback H. Clinical evidence of caries prevention by hydroxyapatite: an updated systematic review and meta-analysis. *J Dent.* 2024;151:105429. PMID: 39471896. doi:10.1016/j.jdent.2024.105429.