ORIGINAL RESEARCH

The Evaluation of Antimicrobial Effects of Nanoemulsion against Cariogenic Bacteria in Plaque Samples of Pediatric Patients: An *Ex Vivo* Study

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Abstract

Objective: To evaluate the antimicrobial efficacy of a cetylpyridinium chloride (CPC)-based nanoemulsion (NE) against mutans streptococci (MS) and *Lactobacillus* (LB) isolated from pediatric dental plaque, and compare its performance with 0.12% chlorhexidine (CHX).

Materials and methods: A CPC NE was formulated and characterized using dynamic light scattering and zeta potential analysis. Antimicrobial activity was assessed by determining the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and time-kill kinetics. Plaque samples from 51 children (5–14 years) were randomly allocated to baseline, NE_{1000} (1:1000 dilution), or CHX treatment groups. Pre- and posttreatment bacterial counts (CFU/mL) were compared using nonparametric tests, with effect sizes and 95% confidence intervals (CIs) calculated.

Results: The NE exhibited a mean particle size of 97.8 nm and a zeta potential of +44 mV. NE demonstrated greater antimicrobial potency than CHX, achieving MIC and MBC values at 27–81-fold higher dilutions. NE_{1000} produced a mean reduction of 6.51×10^6 CFU/mL in MS (95% CI: 4.96×10^6 to 8.06×10^6; Cohen's d =1.63) compared with 5.55×10^6 CFU/mL for CHX (95% CI: 3.89×10^6 to 7.21×10^6; d =1.30). For LB, NE_{1000} achieved a mean reduction of 3,500 CFU/mL (95% CI: 2,576 to 4,423; d =1.47) versus 2,588 CFU/mL for CHX (95% CI: 1,465 to 3,711; d =0.89). Time-kill assays confirmed rapid activity, with greater than 85–95% bacterial reduction within 1 minute.

Conclusion: The CPC NE demonstrated significantly greater antimicrobial efficacy than chlorhexidine against pediatric plaque-derived MS and LB. Its rapid action and enhanced potency highlight its potential as an alternative antimicrobial agent in pediatric dentistry.

Keywords: Cetylpyridinium chloride, Chlorhexidine gluconate, *Lactobacillus*, Nanoemulsion, *Streptococcus mutans*.

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Introduction

Dental caries is one of the most prevalent chronic diseases affecting children worldwide. Despite the availability of preventive strategies, including fluoride use and oral hygiene measures, dental caries remains a significant public health concern—particularly in pediatric populations. The primary microbial agents associated with the initiation and progression of caries are mutans streptococci (MS) and *Lactobacillus* (LB) species, which thrive within dental plaque biofilms.[1] Biofilm-associated bacteria exhibit increased resistance to conventional antimicrobials due to their altered physiology and phenotypic adaptations.[2]

Commonly used antimicrobial agents, such as chlorhexidine (CHX), povidone-iodine, cetylpyridinium chloride (CPC), and triclosan, have shown efficacy in reducing the cariogenic bacterial load.[3] However, prolonged use of these agents has been associated with several undesirable effects, including tooth staining,[4] altered taste perception, mucosal irritation, and the potential for microbial resistance.[5]

Recent advances in nanotechnology have led to the development of nanoemulsions (NEs), which offer a promising alternative for targeted drug delivery and antimicrobial therapy. NEs are thermodynamically stable mixtures of oil, water, and surfactants, with droplet sizes typically below 100 nm. Their small particle size, increased surface area, and positive surface charge enhance their ability to penetrate biofilms and disrupt the membranes of microbial cells. Additionally, because NEs act through nonspecific mechanisms, the development of bacterial resistance is considered less likely.[6]

Cetylpyridinium chloride, a quaternary ammonium compound, has been widely used as an antiplaque agent. It has been FDA-approved and categorized under generally recognized as safe (GRAS). When incorporated into an NE system, CPC may exhibit enhanced antibacterial properties due to improved delivery and interaction with negatively charged bacterial surfaces and plaque biofilms.[7]

A recent systematic review of NE-based oral mouthrinses found that they effectively control plaque and improve gum health; however, there is insufficient evidence to support their

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effectiveness in reducing cavities. The review stressed the need for more *ex vivo* and clinical studies.[8] It is also noted that bacteria from natural environments, such as dental plaque, differ significantly from standard laboratory strains, suggesting that using clinically relevant strains in antimicrobial studies could enhance the applicability of the results.[9]

The present *ex vivo* study aims to formulate a CPC-based NE and to evaluate and compare its antimicrobial efficacy against natural strains of *MS* and *LB* obtained from pediatric dental plaque samples, using minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and time-kill kinetics. The overarching objective is to assess the NE's potential as a more potent, safer, and effective alternative to 0.12% CHX in pediatric dental care, thereby addressing the current gap in the literature regarding the use of CPC NEs on clinically relevant pediatric strains.

Materials and Methods

This *ex vivo* study was conducted on patients attending the outpatient department in our tertiary care center in collaboration with a research laboratory. The institutional ethics committee board approved the study.

Preparation of Nanoemulsion

The preparation of antimicrobial NE was carried out in the nanotherapeutic and analytical chemistry department at a research laboratory. The NE had an oil phase (soybean oil), which was 25% v/v of total emulsion, CPC, 1% w/v, and a surfactant Triton X-100 (10% v/v) in 65 v/v deionized water. The solution was emulsified using a microfluidizer processor (M110, Microfluidics, Newton, MA). Two passes of emulsification were performed at 20,000 PSI and room temperature. The droplet size of NE was measured using a dynamic light scattering method (DLS) (Brookhaven Malvern`s instruments). All the ingredients used in NE are approved for over-the-counter human applications and are on the FDA-GRAS list. The ratio of ingredients used in the study closely matched that described by Baker et al.[10]

Plaque Sample Collection from Pediatric Patients

The sample size was calculated based on the primary outcome: the reduction in bacterial colony-forming units per milliliter (CFU/mL) of MS after treatment with nanoemulsion (NE_{1000}). To calculate sample size, a standard statistical formula based on pilot data values was used:

$$n = \left(\frac{(Z_{1-\alpha/2} + Z_{1-\beta}) \cdot \sigma}{\Delta} \right)^2$$

Where: $Z_{1-\alpha/2} = 1.96$ for a two-sided significance level of 0.05 and $Z_{1-\beta} = 0.84$ for 80% power.

Although the minimum sample size calculated was 35, to improve external validity and allow subgroup comparisons, a convenience sample of 51 patients was taken. Children aged 5–14 years were included in the study, providing sufficient statistical power to detect meaningful differences in antimicrobial efficacy.

Written informed consent was obtained from the parent/guardian, and assent was obtained from the child. All children participating in the study were instructed to refrain from brushing their teeth on the day of sampling and to maintain their regular dietary habits. The patient was asked to rinse their mouth with water to remove any food residue. Plaque samples were collected from all the smooth surfaces of the most posterior fully erupted mandibular molars on either the right or left side of the jaw by scraping with the tip of a sterile explorer.[11] The samples were immediately transferred to 500 µL of phosphate buffer solution.

Isolation of Mutans Streptococci and *Lactobacillus* from Plaque Samples

The plaque samples were diluted to 1 mL and mixed in a vortex spinning machine (Genei Vortexer) for 15 seconds, and serial dilutions of tenfold were made to 10^2 and 10^3 in phosphate-buffered isotonic saline. An aliquot of the sample (100 µL) was poured onto mitis salivarius bacitracin (MSB) agar for 24 hours to isolate *MS* and Mann Rogosa Sharpe (MRS) agar for 48 hours to isolate *Lactobacillus*.

Brain–heart infusion broth (BHI) was used to grow broth cultures of bacterial strains. The incubation period was 24 hours for *MS* and 48 hours for *LB* at 37°C.

The suspected colonies of MS and *LB* were further subjected to Gram staining and standard biochemical tests (e.g., catalase test, sugar fermentation) to confirm their species identity.

Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of Nanoemulsion

Minimum inhibitory concentration is the lowest concentration of an antimicrobial agent that inhibits the visible growth of a microorganism after overnight incubation. The assessors were blinded to the contents of the solutions and culture plates to eliminate bias.

The MICs were determined using a sterile 96-well microtiter plate (microtiter broth serial dilution method). The NE was serially diluted with sterile BHI using a total volume of 100 µL BHI broth per well. The optical density of each well was recorded as a control on the ELISA plate reader (Bi-Tek). A volume of 20 µL of prepared bacterial inocula with an optical density (OD) of 0.2–0.4 at 600 nm was inoculated per well. The bacterial count was adjusted to 2×10^8 CFU/mL per well. The optical density (OD) of each well was recorded after incubation periods of 24 hours and 48 hours, respectively, for *MS* and *LB* at 600 nm. The highest dilution with no visible bacterial growth and with minimum optical density was recorded as the minimum inhibitory concentration (*Note*: The readings of the control were subtracted from test values to record the actual change in optical densities).

An aliquot of 100 µL from the wells in which no visible growth was present was spread on bacterial culture plates and incubated. The highest dilution of the antimicrobial agent that kills more than 99.9% of the initial bacterial population is recorded as the MBC. Each set of procedures was repeated at least three times. The MIC and MBC were expressed as dilutional folds of NE. The BHI was taken as a negative control.

Time Kinetics of Killing of Bacteria

Time-kill kinetics were assessed to evaluate the rate of bacterial killing and the antimicrobial agents' ability to prevent regrowth. A 100 µL aliquot of pure bacterial culture (OD_{600} = 0.2–0.4, corresponding to ~2×10^8 cells) of *Streptococcus mutans* and *Lactobacillus* was added to 900 µL of NE at different dilutions (1:1, 1:500, 1:1000, 1:2000) or Chlorhexidine (control) in sterile Eppendorf tubes. Following inoculation, 100 µL samples were withdrawn at 1 minute, 15 minutes, and 30 minutes, immediately diluted in 900 µL of BHI broth in 12-well microplates, and incubated at 37°C for 24 hours and 48 hours. Bacterial growth was quantified by measuring the optical density (OD) at 600 nm using an ELISA plate reader. Each condition was tested in at least three independent experiments. Chlorhexidine served as a positive control.

Individual and Comparative Antibacterial Effects of Nanoemulsion (NE_{1000}, 1:1000 Dilution of Nanoemulsion) and 0.12% Chlorhexidine (CHX) on Dental Plaque Bacterial Counts of Mutans Streptococci and *Lactobacillus*

The plaque samples collected from each of the pediatric patients enrolled in the present study were randomly divided into three groups. The random allocation was based on a computer-generated sequence. Each of these groups was subjected to different treatment interventions:

- *Group A (baseline bacterial count)*: Measured volume of 100 µL of plaque samples from stock solutions was directly spread on the selective media for initial MS and *Lactobacillus* bacterial counts.
- *Group B* (*Nanoemulsion treatment for 1 minute*): 100 µL of plaque samples from stock solutions were mixed with 100 µL of nanoemulsion (NE_{1000}) in sterile Eppendorf tubes for 1 minute.
- *Group C* [*Chlorhexidine gluconate treatment for 1 minute (positive control)*]: 100 µL of plaque samples from stock solutions were mixed with 100 µL of 0.12% CHX digluconate in sterile microcentrifuge tubes (Eppendorf) for 1 minute, as in group B.

After 1 minute, the aliquot of samples (100 µL) was collected from groups B and C and diluted in BHI to a 1:9 dilution. A measured volume of the diluted sample (100 µL) was spread on selective media and incubated at 37°C for 24 hours (for MS) and 48 hours (for *Lactobacillus*) to promote colony formation. Two independent observers counted the CFUs/mL of the bacterial strain to prevent interobserver variation. The observers were blinded to the group allocation.

Statistical Analysis

Analyses were performed using SPSS (IBM, version 21) software. The results were subjected to testing for normality using the Shapiro–Wilk test, which showed nonnormally distributed data. Thus, nonparametric tests, specifically the Wilcoxon matched-pairs (W) test and the Mann–Whitney U test, were performed to determine the level of significance. A two-sided ($\alpha = 0.05$) and $p < 0.05$ was considered statistically significant. Effect sizes were calculated to quantify the magnitude of antimicrobial reduction. Cohen's *d* (unpaired approximation) was computed using pooled standard deviation to allow comparison between NE_{1000} and CHX, and 95% confidence intervals (CIs) for mean differences were calculated using standard error estimates derived from group means and standard deviations.

Results

Characterization of Nanoemulsion

The prepared NE exhibited a mean particle diameter of 97.80 ± 0.48 nm, with a mean particle dispersion index (PDI) of 0.23, indicating a uniform particle distribution. The zeta potential was recorded at +44 mV, suggesting good stability of the NE dispersion (Fig. 1). The dynamic light scattering (Malvern instrument) method was used to record the size distribution, PDI, and zeta potential (Fig. 1). The data analysis was conducted using dispersion technology software (DTS).

Scanning electron microscopy (SEM) further confirmed the presence of spherical nanoparticles with a size range of 20–30 nm (Fig. 2).

Minimum Inhibitory Concentration and Minimum Bactericidal Concentrations

The NE demonstrated significantly higher antimicrobial potency than CHX. For MS, the MIC and MBC were recorded at 1:59049 and 1:6561 dilutions, respectively. For *Lactobacillus*, MIC and MBC values were observed at 1:19683 and 1:2187 dilutions, respectively

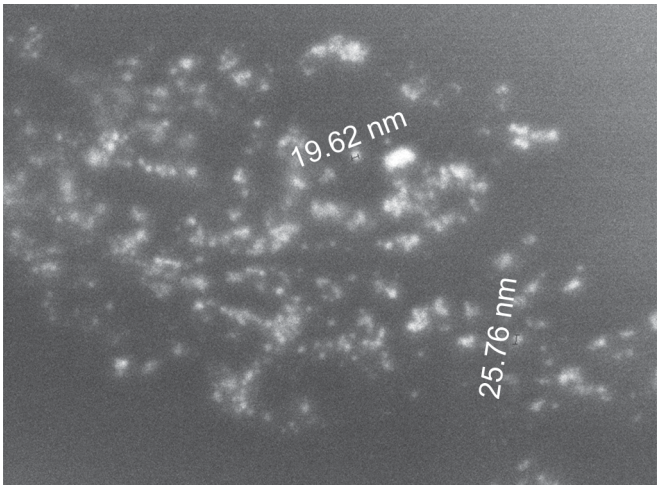

Fig. 2: Scanning electron microscopic examination of NE with particle size 20–30 nm

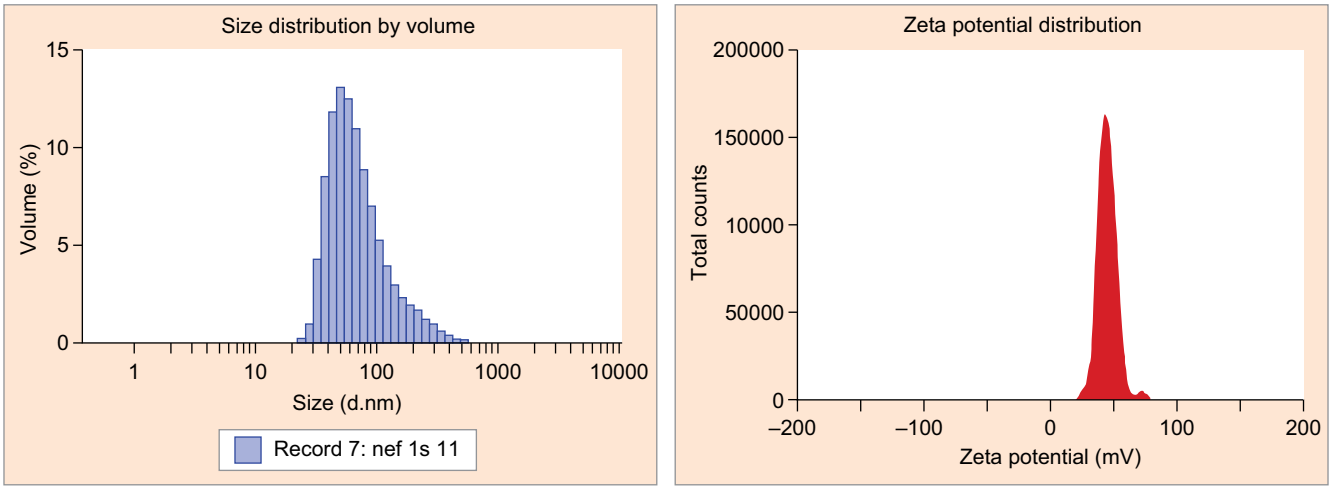

Fig. 1: Light scattering image showing particle size distribution and zeta potential of NE

(Fig. 3). These results indicate that the NE remained effective even at 27–81-fold higher dilutions compared with CHX.

Time Kinetics for Killing

The time-dependent bactericidal activity of the NE was evaluated at various dilutions (1:1, 1:500, 1:1000, and 1:2000). At a 1:1000 dilution (NE_{1000}), a rapid reduction in bacterial load was observed. Within 1 minute, the NE reached an 86.2% reduction in MS and a 96% reduction in *Lactobacillus*. Minimal differences were observed at 15 minutes and 30 minutes, suggesting that most bacterial inactivation occurred rapidly upon contact (Fig. 4).

Comparative Evaluation of Antibacterial Effects of Nanoemulsion (NE_{1000}) and 0.12% CHX on Mutans Streptococci and Lactobacilli in Plaque Samples

The bacterial colony count of MS at baseline (pretreatment) and after treatment intervention with NE_{1000} and CHX is shown in Table 1. As shown in Figure 5, following treatment, NE_{1000} produced a substantial reduction in MS counts, decreasing bacterial load by 6.51×10^6 CFU/mL from baseline (95% CI: 4.96×10^6 to 8.06×10^6), corresponding to a 90.8% reduction and a large effect size (Cohen's $d = 1.63$). Chlorhexidine (0.12% CHX) also reduced MS counts but to a lesser extent, with a mean reduction of 5.55×10^6 CFU/mL (95% CI: 3.89×10^6 to 7.21×10^6; $d = 1.30$). A direct comparison between the two interventions revealed that NE_{1000} achieved an additional reduction of 9.58×10^5 CFU/mL over CHX (95% CI: 1.93×10^5 to 1.72×10^6), indicating a moderate effect favoring the NE ($d = 0.49$).

For *Lactobacillus* species, NE_{1000} resulted in a mean reduction of 3,500 CFU/mL from baseline (95% CI: 2,576–4,423), corresponding to an 88.6% decrease and a large effect size ($d = 1.47$). CHX produced a reduction of 2,588 CFU/mL (95% CI: 1,465–3,711; $d = 0.89$). NE_{1000} demonstrated significantly greater antibacterial activity than CHX, reducing *LB* counts by an additional 912 CFU/mL (95% CI: 221–1,603; $d = 0.51$). Collectively, these findings confirm that the NE exhibits superior efficacy compared with chlorhexidine, even at higher dilutions.

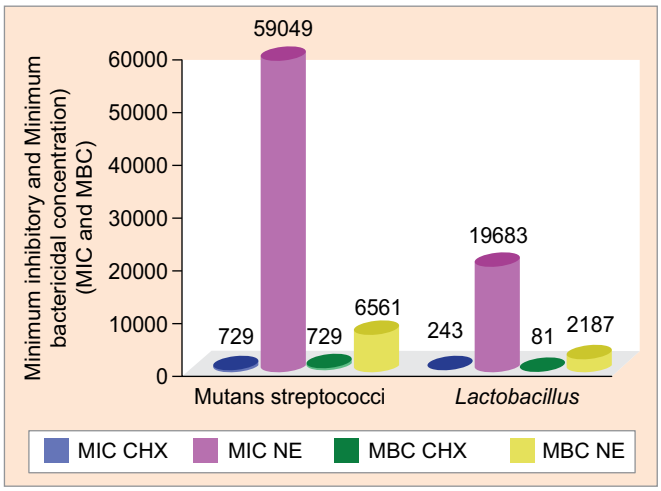

Fig. 3: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of chlorhexidine (CHX) and NE on MS and *Lactobacillus*

Discussion

This study demonstrated that a CPC-based NE exhibited potent antimicrobial activity against *S. mutans* and *Lactobacillus* from pediatric dental plaque, with efficacy at higher dilutions than CHX.

Nanoemulsion systems offer unique advantages due to their small particle size (typically below 100 nm), which facilitates improved penetration of biofilms and direct interaction with bacterial cell membranes. The pronounced surface charge (more than ± 30 mV) signifies strong repulsive forces, preventing aggregation and promoting stability.[12] In our formulation, the small mean particle size (97.8 nm) and high positive zeta potential (+ 44 mV) likely promoted stronger electrostatic binding to the negatively charged bacterial surfaces, thereby increasing cell disruption and death. The observed size discrepancy—97.80 nm (DLS) versus 20–30 nm (SEM)—is attributable to differences in measurement principles. DLS quantifies the hydrodynamic diameter, which encompasses the particle core, the solvation shell, adsorbed molecules, and any double-layer effects—essentially, how the particle behaves in suspension. Scanning electron microscopy (SEM), on the other hand, provides direct imaging of the dried particle core, typically under vacuum, giving a smaller—and more physically intrinsic—size.[13] Thus, the larger DLS measurement is expected when particles are characterized in their native, hydrated state. Similar observations were made by Li et al. in their *in vitro* and *in vivo* study, who reported that the CHX NE exhibited MIC and MBC against *S. mutans* at double-fold dilutions, demonstrating its ability to compromise biofilm integrity and reduce bacterial viability.[14]

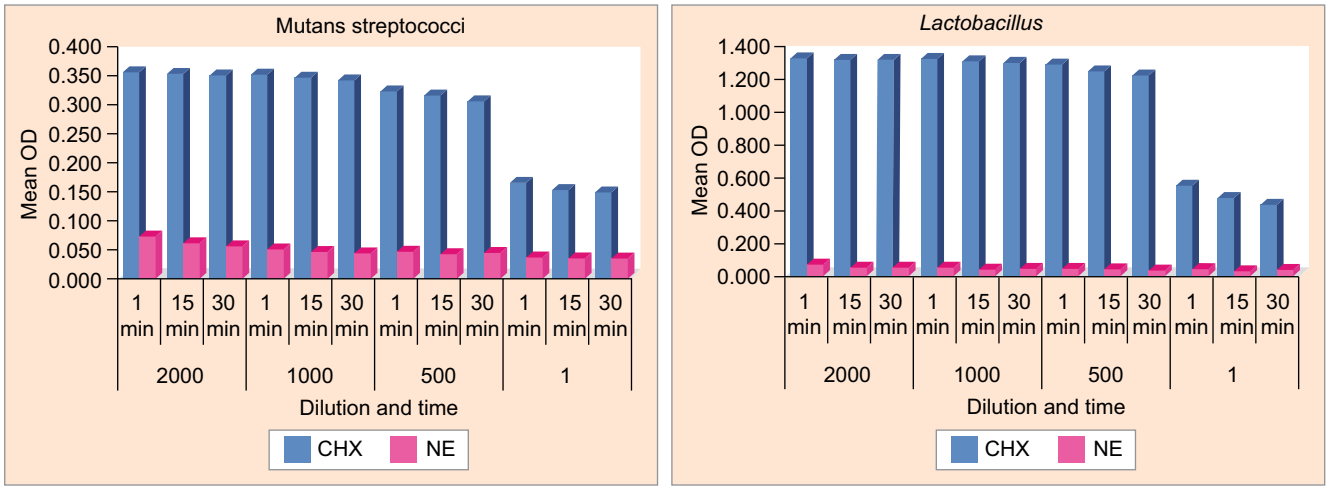

Fig. 4: Bar graph showing the kinetics of killing of chlorhexidine and NE against MS and *Lactobacillus*

Table 1: Bacterial colony count (CFU/mL) of MS and *Lactobacillus* after chlorhexidine and nanoemulsion (NE_{1000}) treatments

	Statistic	*Baseline*	*After CHX intervention*	*After NE_{1000} intervention*
Streptococcus counts	N	51	51	51
	Mean	7,170,588	1,617,647	6,58,824
	SD	5,508,077	2,507,166	1,222,322
	Min–max	(4×10^6)–(3×10^7)	0–1.2 $\times 10^7$	0–6 $\times 10^6$
Lactobacillus counts	Mean	3,951	1,363	451
	SD	3,296	2,426	677
	Min–max	1,000–15,400	0–12,900	0–2,500

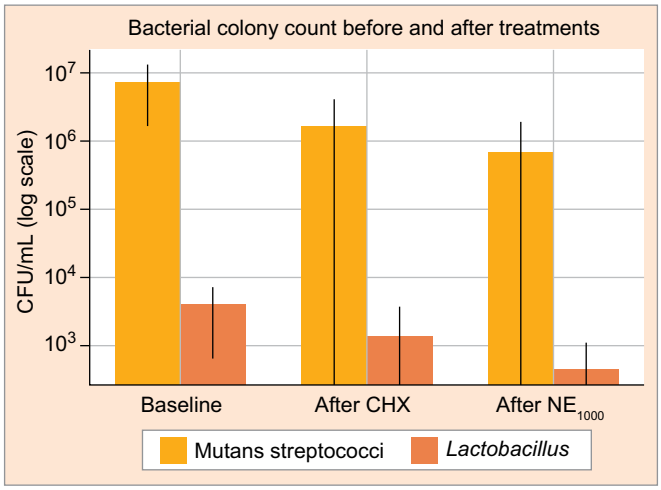

Fig. 5: The mean bacterial colony counts (CFU/mL) for MS and *Lactobacillus* before and after treatments with CHX and NE_{1000}, displayed on a logarithmic scale

Although CHX remains the gold standard among antiseptic mouthwashes for reducing gingival, periodontal, and caries-associated bacteria, its clinical use is limited by several drawbacks.[15] Notably, Ardila CM's review highlights that CHX and triclosan exhibit the highest rates of bacterial resistance in *S. mutans* and *Lactobacillus*, raising concerns about antimicrobial effectiveness over time.[16] CPC is an established quaternary ammonium compound with a more favorable side-effect profile. For example, Oo MMT et al. reported that a 0.05% CPC mouthwash resulted in significantly less staining and taste alteration, alongside better patient acceptance, when compared with a 0.12% CHX formulation.[17] These findings provided the rationale for developing a CPC-based NE for this study.

A particular strength of this study is the use of clinically derived bacterial strains rather than laboratory-adapted cultures. Microorganisms isolated directly from pediatric plaque are more representative of the oral environment, where bacteria are exposed to fluctuations in pH, nutrient levels, and oxygen availability. This enhances the translational relevance of the findings. To our knowledge, no study in the literature has evaluated the effect of a CPC-based NE on bacteria isolated from pediatric dental plaque samples. The age-group selected in our study was 5–14 years because at this age, new permanent teeth are erupting. The microbial ecology of dental plaque, particularly the prevalence of MS and *Lactobacillus*, is dynamic during this developmental stage.[18] Plaque sample was collected from the mandibular molars for the simple reason that they are the most neglected part of the dentition left by children and are most susceptible to plaque accumulation and caries. Also, to maintain uniformity and ease of collection.

Our findings align with and extend prior work on CPC NEs. Lee et al.[19] first demonstrated the potential of CPC-based NEs in reducing caries development in their *in vitro* pilot study, and Ramalingam et al.[7] later reported 27-fold dilutions for MIC values against laboratory-grown *S. mutans*. In the present study, the CPC NE exhibited superior potency, achieving MIC and MBC values at even greater dilutions (81-fold for *S. mutans* and 27-fold for *Lactobacillus*) compared with CHX, which exceeded the 27-fold dilutions reported by Ramalingam et al. for their CPC NE against laboratory-grown *S. mutans*.[7] This enhanced antimicrobial activity is primarily attributed to the significantly smaller mean particle diameter recorded in our formulation (97.80 ± 0.48 nm by DLS), compared with the 308 nm reported by Ramalingam et al.[7] The sub-100 nm size in our NE likely facilitated greater and faster penetration through the complex microbial structure of dental plaque and enhanced electrostatic interaction with bacterial membranes, thereby resulting in higher antimicrobial potency and rapid killing kinetics.

The rapid bactericidal activity observed, resulting in a reduction of *S. mutans* by more than 78% and a reduction of *Lactobacillus* of more than 90% within 1 minute, even at high dilutions, is another clinically relevant feature. This property is especially valuable in pediatric dentistry, where rinsing contact times are typically brief. Such swift action is consistent with other reports of NEs providing rapid microbial killing.[19]

Beyond CPC and CHX systems, a variety of herbal and essential oil-based NEs have also been investigated, though with mixed outcomes. Jeong J[20] reported that a cinnamon oil-based NE inhibited the maturation of bovine oral biofilms, but the effect was not found to be statistically superior to CHX. Similar findings were observed by Cho et al., who tested a NE of *Curcuma xanthorrhiza* oil.[21] Other herbal NEs have shown potential benefits; for example, one study demonstrated a reduction in the progression of artificial cavitated lesions, supporting a role in remineralization.[22] Haq N et al.[23] reported that a polyherbal mouthwash NE achieved MIC values at 50-fold dilutions against *S. mutans* compared with CHX. In contrast, a *Nigella sativa*–based NE exhibited antimicrobial activity against cariogenic bacteria, but was less effective than CHX.[24] Collectively, these findings highlight the potential of NE systems as antimicrobial delivery platforms, though outcomes vary widely depending on the active agent incorporated.

However, these promising findings should be interpreted with caution. The study employed an *ex vivo* design, which does not fully replicate the dynamic conditions of the oral cavity. Salivary flow, enzymatic activity, dietary influences, and multispecies biofilm interactions were not simulated, all of which may affect antimicrobial efficacy. Furthermore, the evaluation was limited to two key cariogenic species, whereas natural dental plaque involves complex microbial communities with competitive and synergistic relationships that could alter outcomes.

Conclusion

Within the limitations of the study, this study demonstrated that a CPC-based NE exhibits potent antimicrobial activity against MS and *Lactobacillus* derived from pediatric dental plaque, with significantly higher potency than 0.12% chlorhexidine even at greater dilutions. The NE acted rapidly within 1 minute, which is clinically advantageous for pediatric applications where brief exposure times are typical. While the results are promising, it is essential to note that the *ex vivo* design does not fully replicate the dynamic oral environment. Long-term *in vivo* studies are recommended to confirm the persistence of antimicrobial effects, reliability in diverse clinical populations, and potential side effects. Additionally, investigations into optimizing NE composition, flavoring, and delivery systems could enhance patient compliance, particularly in pediatric groups.

Clinical Significance

Nanoemulsions can provide similar antimicrobial activity to their conventional counterparts, but at higher dilutions, thus reducing the possibility of side effects such as antimicrobial resistance. The formulated NE has a reduced particle size, allowing for better penetration of the bacterial cell membrane. This NE demonstrates rapid bactericidal activity within 1 minute, which is advantageous for pediatric mouthrinses. CPC has GRAS status, supporting its potential for safe clinical translation. However, further *in vivo* and toxicity studies are needed.

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