

Divergent Oral Microbiome after Rinsing with of H₂O₂, Chlorhexidine and Essential Oil Mouthrinses: a Proof of Principle Study

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Objective: To compare the effects on the oral microbiome after rinsing with hydrogen peroxide (H₂O₂), chlorhexidine and essential oil mouthrinses and identify formulations that suppress pathogenic bacteria while preserving beneficial species and maintaining oral microbial balance.

Methods: Twelve healthy volunteers were randomly assigned to three groups: H₂O₂, chlorhexidine and essential oil mouthrinse. Saliva samples were collected at three time points: before and 5 minutes and 1 hour after rinsing with mouthrinse. Microbiome composition was analysed using 16S rRNA gene sequencing.

Results: Alpha and beta diversity showed no statistically significant differences among time points. The genus-level microbiome composition remained relatively stable in the H₂O₂ and essential oil groups but changed significantly in the chlorhexidine group. In the H₂O₂ group, *Neisseria* decreased significantly, while *Actinomyces* increased. In the chlorhexidine group, *Porphyromonas*, *Veillonella*, *Streptococcus*, *Neisseria* and *Gemella* decreased significantly. In the essential oil group, *Leptotrichia* decreased, and *Haemophilus* increased significantly.

Conclusion: Essential oil mouthrinse and chlorhexidine exhibit stronger bacteriostatic effects against oral pathogens than H₂O₂. However, chlorhexidine may disrupt microbial equilibrium, whereas essential oil mouthrinse more effectively preserves a stable oral microbiome. Thus, essential oil mouthrinse could serve as a viable alternative to chlorhexidine for oral microbiome management, though its long-term efficacy requires further investigation.

Keywords: chlorhexidine, essential oil mouthrinse, microbial diversity, oral microbiome ecology, 16S rRNA sequencing

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The oral cavity harbours the second most diverse microbial ecosystem in the human body, surpassed only by the gut microbiota,¹ with over 700 bacterial species identified to date.^{2,3} This complex community plays a dual role in health and disease: while commensal species contribute to mucosal immunity and nutrient metabolism, pathogenic shifts in microbial composition are linked to localised infections and systemic conditions, including cardiovascular diseases, diabetes and even oesophageal cancer.^{2,4,5} Dental procedures, such as implant placement or surgical extractions, create transient open wounds, allowing oral pathogens to invade sterile sites and increasing the risk of local

infections, bacteraemia and distant complications like infective endocarditis.² The microbiome cultured from areas with delayed-onset infections after mandibular third molar extractions contained the oral flora.⁶ For instance, *Porphyromonas gingivalis* and *Streptococcus sanguinis*, common oral pathobionts, are implicated in post-surgical infections and systemic inflammation.^{2,5} Therefore, modulation of the oral microbiota and selective inhibition of microorganisms during such interventions are essential to reduce these risks.

In clinical practice, common treatments to eliminate oral flora include scaling and root planing, mouthrinses, dentifrices, local drug delivery at infection sites and systemic chemotherapeutic agents,³ with mouthrinse being more convenient and associated with fewer side effects. A wide range of mouthrinses are available to reduce oral microbial populations. In addition to clinically established chemical formulations such as chlorhexidine, numerous over-the-counter (OTC) options have emerged, including naturopathic mouthrinses, alternative formulations, biological and immunostimulant agents and nanotechnology-driven solutions. Essential oil mouthrinses are among the alternative options; however, with the exception of these, the clinical efficacy of OTC products lacks robust validation through high-quality evidence.⁷

Chlorhexidine mouthrinse reduces dental biofilm buildup significantly and is often used for plaque control.⁸ However, its use has been associated with several unpleasant side effects, including tooth and tongue discolouration, dry mouth and taste disorders.^{9,10-12} Importantly, as a broad-spectrum antimicrobial agent, chlorhexidine can limit the growth of opportunistic bacteria and pathogens, which may lead to dysbiosis.^{9,12} Essential oil mouthrinse has been considered an effective agent for plaque control without adverse effects such as pigmentation, taste changes or supra-gingival calculus formation, in contrast to chlorhexidine.¹³

Prior research has predominantly focused on short-term microbial reduction, neglecting the ecological consequences of prolonged mouthrinse use.^{2,14} One study focused on the effects of mouthrinse on individual flora or fungi, ignoring the fact that the oral microbiota functions as a whole.¹⁵ Other studies have examined the effects of mouthrinse on a single indicator such as pH,¹⁶ gingival bleeding index,¹⁷ full mouth-plaque score (FMPS), full-mouth bleeding score (FMBS)¹⁸ and individual microorganisms,¹⁹⁻²¹ without directly comparing the effects of clinically used mouthrinses on the overall oral microbiota.

The present study aimed to explore the potential development of mouthrinses capable of selectively targeting and suppressing pathogenic bacteria such as *Streptococcus mutans* or *Porphyromonas gingivalis*, while maintaining a balanced oral microbiome that preserves beneficial commensal organisms. The authors compared the oral microbiome of samples collected after rinsing with hydrogen peroxide (H₂O₂), chlorhexidine and essential oil mouthrinse at different time points, and evaluated their effects on the oral microbiome.

Materials and methods

Participants

Saliva samples were collected from 12 adults (six men, six women) aged 24 to 26 years. Participants had no history of local oral trauma, surgery, injections, antibiotic use within the past 3 months, smoking or systemic disease. All participants underwent a thorough oral examination to confirm they were in good oral health and exclude existing inflammation before saliva collection. The study was approved by the Ethics Committee of Beijing Stomatology Hospital (approval number CMUSH-IRB-KJ-PJ-2022-01), and all participants provided voluntary written informed consent.

Sample collection

Participants were randomly divided into three groups: H₂O₂, chlorhexidine and essential oil mouthrinse. H₂O₂ (1.5%) was obtained from Jianning Pharmaceutical (Hebei, China), chlorhexidine mouthrinse (0.6 g chlorhexidine gluconate/500 ml) was purchased from Nanyue Pharmaceutical (Shenzhen, China) and essential oil mouthrinse containing 0.06% menthol and 0.05% eugenol was sourced from Carning Pharmaceutical (Dandong, China). Saliva samples were collected from participants in three groups at three time points: 5 minutes after rinsing with water (t0), and 5 minutes (t1) and 1 hour (t2) after rinsing with their respective assigned mouthrinse.

Participants rinsed with 20 ml mouthrinse for 60 seconds, three times. At each time point, 3 ml unstimulated saliva was collected in a sterile centrifuge tube. Two millilitres of saliva was transferred into a sterile cryogenic vial, centrifuged at 10,000 × g for 15 minutes; the supernatant was discarded, and the precipitate was stored at -80°C. The experiment was repeated three times.

DNA extraction

A frozen aliquot (200 mg) of each saliva sample was suspended in 250 µl guanidine thiocyanate, 0.1 M Tris (pH 7.5) and 40 µl 10% N-lauroyl sarcosine. DNA was extracted using the Qiagen QIAamp DNA Stool Mini Kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's instructions. DNA concentration and molecular weight were estimated using a Nanodrop instrument (Thermo Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively.

Library construction

The V3-V4 region of the bacterial 16S ribosomal RNA gene was amplified via polymerase chain reaction (PCR) (95°C for 2 minutes, followed by 25 cycles at 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds, with a final extension at 72°C for 5 minutes) using primers 338F 5'-ACTCCTACGGGAGGCAGCAG-3' and 806R 5'-GGACTACHVGGGTWTCTAAT-3', where the barcode is an eight-base sequence unique to each sample. PCR reactions were performed in triplicate using a 20-µl mixture containing 4 µl 5× FastPfu buffer, 2 µl 2.5 mM dNTPs, 0.8 µl of each primer (5 µM), 0.4 µl FastPfu polymerase and 10 ng template DNA.

Illumina MiSeq sequencing

Amplicons were extracted from 2% agarose gels, purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions, and quantified using QuantiFluor-ST (Promega, Madison, WI, USA). Purified amplicons were pooled in equimolar amounts and paired-end sequenced (2 × 250) on an Illumina MiSeq platform according to standard protocols.

Processing of sequencing data

Raw fastq files were demultiplexed and quality filtered using QIIME (version 1.9.1) with default parameters. Operational taxonomic units (OTUs) were clustered at a 97% similarity cutoff using UPARSE (version 7.1 <http://drive5.com/uparse/>) and chimeric sequences were removed using UCHIME. Taxonomy was assigned to each 16S rRNA gene sequence using the RDP Classifier (<http://rdp.cme.msu.edu/>) against the Silva (SSU123)16S rRNA database with a 70% confidence threshold. QIIME was used to calculate within-community (alpha diversity) and between-community diversity (beta diversity). Alpha diversity was evaluated using the Shannon index,

Chao1 index and observed OTUs index. A Wilcoxon signed-rank test was employed to identify significant differences between alpha diversity groups. Microbial diversity was visualised using principal coordinate analysis (PCoA) and calculated employing QIIME2. Weighted (quantitative) and unweighted (qualitative) UniFrac distance metrics were utilised to analyse oral microbiota diversity among the samples.

Results

Comparison of the alpha diversity of oral microbiota among different mouthwashes

The Chao, Shannon, observed species and coverage indices were used to evaluate the richness and diversity of the overall microbial community. The coverage indices of all samples were greater than 0.97, reflecting reliable results for microorganisms in all samples (Fig 1a). Comparative analysis of the differently timed samples showed that the Chao index, Shannon index and observed species at t0 in all three groups appeared to be higher than those at t1 and t2, but without significant statistical differences, although the three indexes presented larger differences between t0 and t1, and t2 in the chlorhexidine and essential oil groups (Fig 1b to d).

Comparison of the beta diversity of oral bacterial communities among different samples

The authors used PCoA plotting based on weighted and unweighted UniFrac analyses to compare the overall bacterial community composition in the three groups at different times. No significant differences were observed between the samples collected at any of the three time points. No significant differences in weighted or unweighted UniFrac distances were observed among the samples collected at different time points after rinsing with the mouthrinse (Figs 2 and 3).

Differential oral macrobiotic profiles among different mouthwashes

Differential macrobiotic profiles of the samples collected from the three groups were examined based on the proportions of bacterial sequences at the phylum and genus levels. The top 50 microbiome profiles at the genus level of the three groups collected at three time points are shown using a heat map (Fig 4). In the H₂O₂ group, the species distribution of enriched bacteria changed slightly after mouthwash use. Among these, the

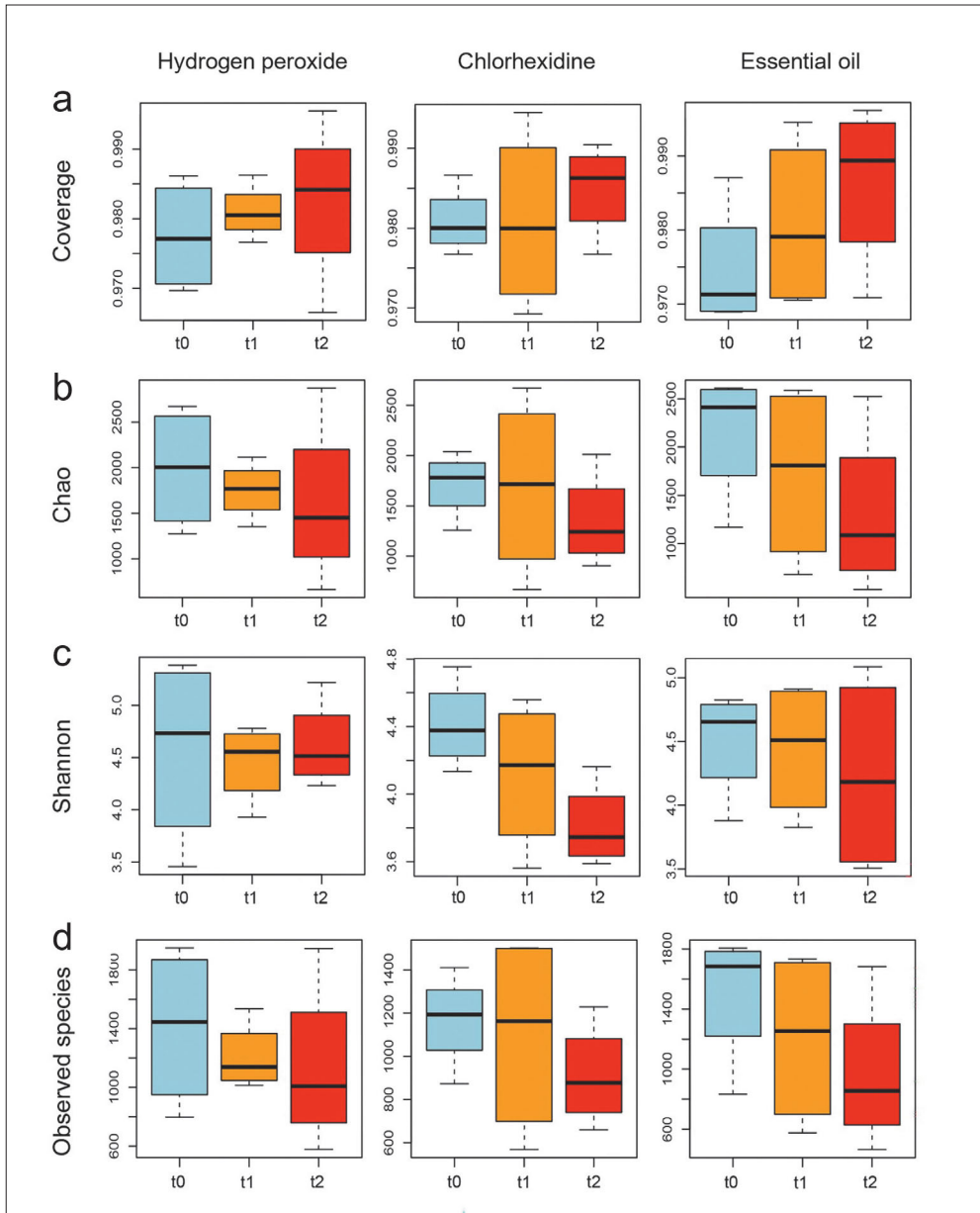
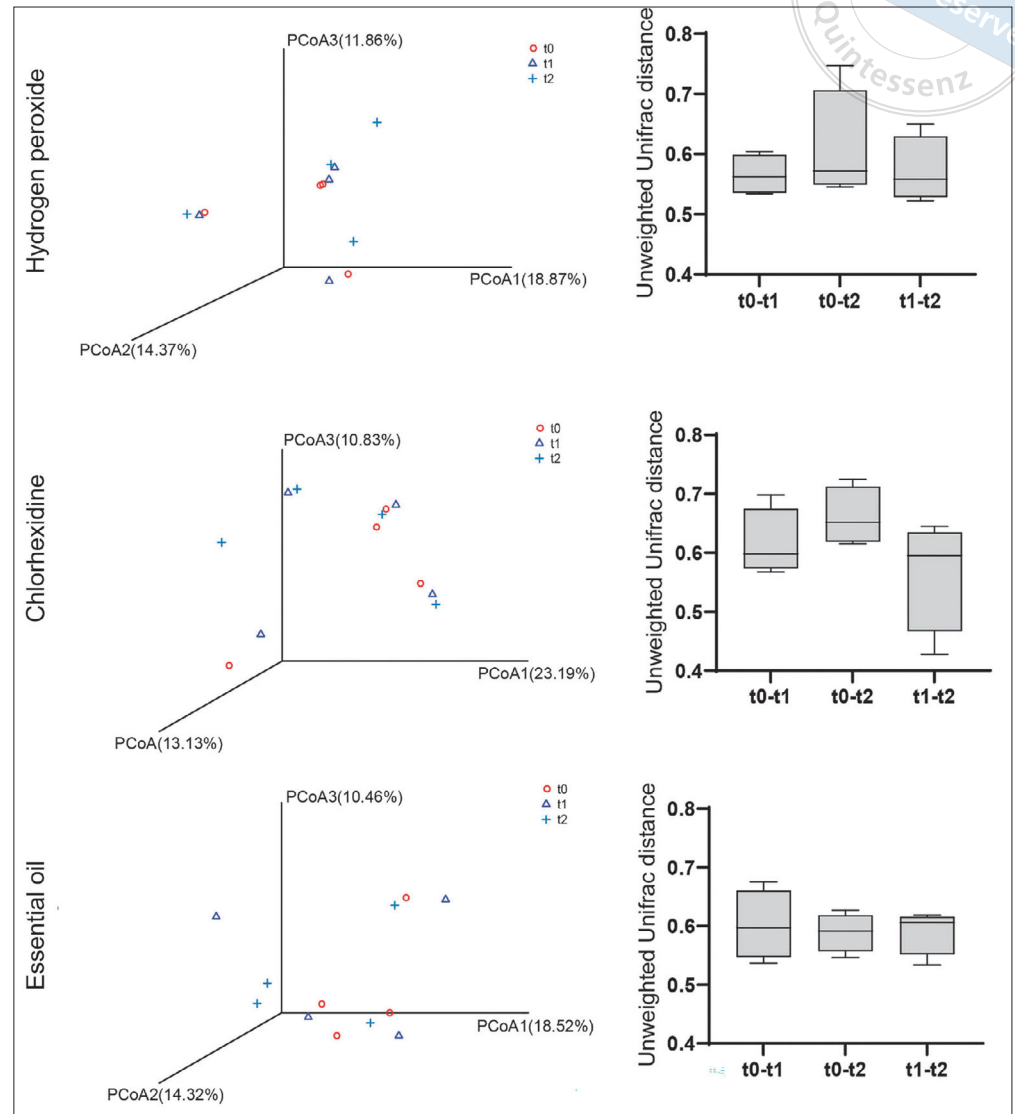


Fig 1a to d Comparison of alpha diversity of oral microbiota across different mouthrinses. The coverage indexes of all samples were greater than 0.97, indicating reliable microbial results across all samples (a). The Chao index, Shannon index and observed species at t0 were higher than at t1 and t2 across all three groups but were not statistically significant (b to d).

abundance of *Neisseria* decreased significantly, while that of *Rothia*, *Fusobacterium*, *Actinomyces* and *Capnocytophaga* increased significantly. In addition, there were several newly emerging bacteria among the 50 most enriched bacteria. In the chlorhexidine group, the number of enriched bacteria changed significantly after the use of mouthrinse, and the number of enriched bacterial species increased. Among these, the abundance of *Leptotrichia*, *Stomatobaculum*, *Rothia*, *Fusobacterium* and *Actinomyces* increased significantly, while that of *Neisseria*, *Haemophilus*, *Veillonella*, *Streptococcus*, *Gemella*

and *Porphyromonas*, which are the main periodontitis pathogens, decreased. In the essential oil mouthrinse group, there was no significant change in the species distribution of enriched bacteria before and after use of mouthrinse. The abundance of *Neisseria* and *Gemella* increased slightly; that of *Haemophilus* increased significantly, while that of *Leptotrichia* and *Prevotella*, other periodontitis pathogens, decreased. The changes in the top 50 microbiome profiles at the genus level from t0 to t2 are shown in Table 1.

Fig 2 Comparison of unweighted UniFrac among different samples. PCoA plot based on unweighted UniFrac analysis comparing overall bacterial community composition in the three groups at different time points. No significant clustering was observed among samples collected at the three time points. Similarly, no significant differences in unweighted UniFrac distances were observed after rinsing with mouthrinses.



Effects of different mouthrinses on oral microbiota composition

To detect the effects of different mouthrinses on microbiota composition, the present authors further analysed the taxonomic profiles of oral microbiota with an abundance greater than 1% (Fig 5a). Taxonomic profiles of the samples from the three groups at different time points had the same microbiota composition with abundance greater than 1% at the genus level. The 14 major bacteria genera with the highest richness at the genus level were *Streptococcus*, *Neisseria*, *Haemophilus*, *Actinomyces*, *Leptotrichia*, *Prevotella*, *Prevotella_7*, *Porphyromonas*, *Rothia*, *Fusobacterium*, *Gemella*, *Capnocytophaga*, *Staphylococcus*

and *Veillonella* (Fig 5a). However, the proportions of the 14 major bacteria genera in each group differed. In the H₂O₂ group, the proportion of *Neisseria* decreased significantly, with a slight decrease in *Prevotella_7* and an increase in *Actinomyces*. In the chlorhexidine group, the proportions of most of the major bacterial genus changed. Among these, *Porphyromonas*, *Veillonella*, *Streptococcus*, *Neisseria* and *Gemella* decreased significantly. In the essential oil mouthrinse group, the proportions of most of the major bacterial genus remained unchanged, but *Leptotrichia* decreased significantly, whereas *Haemophilus* increased significantly (Fig 5b). Changes in oral microbiota with an abundance greater than 1% from t0 to t2 are shown in Table 2.

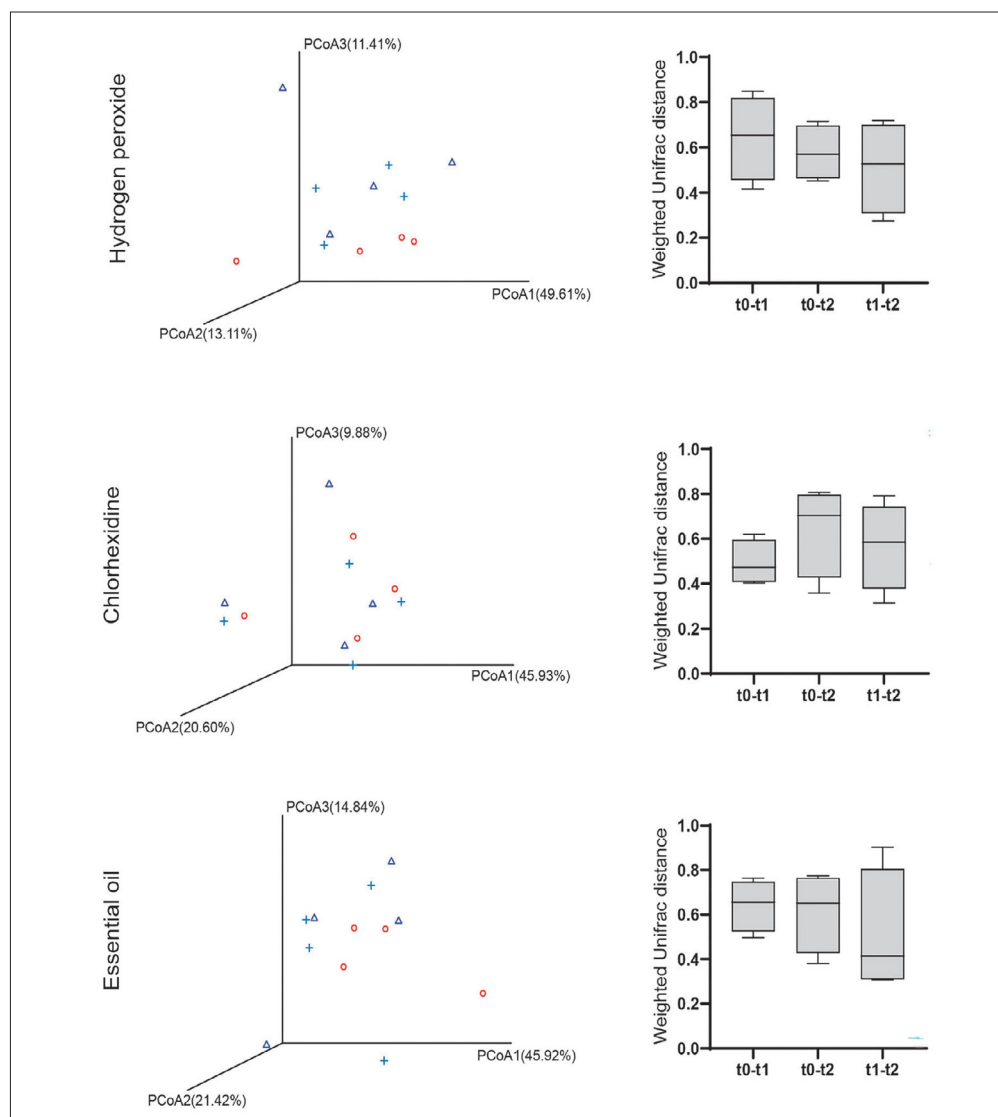


Fig 3 Comparison of weighted UniFrac among different samples in a PCoA plot based on weighted UniFrac analysis comparing overall bacterial community composition in the three groups at different time points. No significant clustering was observed among samples collected at the three time points. Likewise, no significant differences in weighted UniFrac distances were found after rinsing with mouthrinses.

Discussion

This study revealed distinct impacts of H₂O₂, chlorhexidine and essential oil mouthrinses on oral microbial ecology. Chlorhexidine potently suppressed *Porphyromonas*, *Veillonella*, *Streptococcus*, *Neisseria* and *Gemella* (genera associated with periodontal disease, pulpitis, periapical periodontitis, halitosis, recurrent aphthous stomatitis, dental caries,²²⁻²⁴ bacterial meningitis and infective endocarditis²⁵) but concurrently reduced microbial richness and disrupted the community structure, as evidenced by increased *Leptotrichia* and *Stomatobaculum*.

In contrast, essential oil selectively targeted *Leptotrichia* and *Prevotella* while preserving overall α -diversity and maintaining dominant taxa stability,

suggesting ecological compatibility in this study. The main components of the essential oil mouthrinse are mint and eugenol. Mint has been used in combination with traditional antibiotics for antibacterial properties.²⁶ Essential oil mouthrinse exhibits a function in *Pyococcus*, but its function in the oral microbiota when compared with other mouthrinses remains unclear. Thus, it is necessary to study the effects of various essential oil mouthrinses on the oral microbiota.

The present results are consistent with those of previous studies. H₂O₂ exhibited limited broad-spectrum efficacy, with minimal effects on microbial composition. In fact, H₂O₂ has an excellent oral bacterial reduction effect, but it needs long-term application.²⁷ Beta diversity analyses confirmed no significant temporal shifts in community structure post-rinsing across

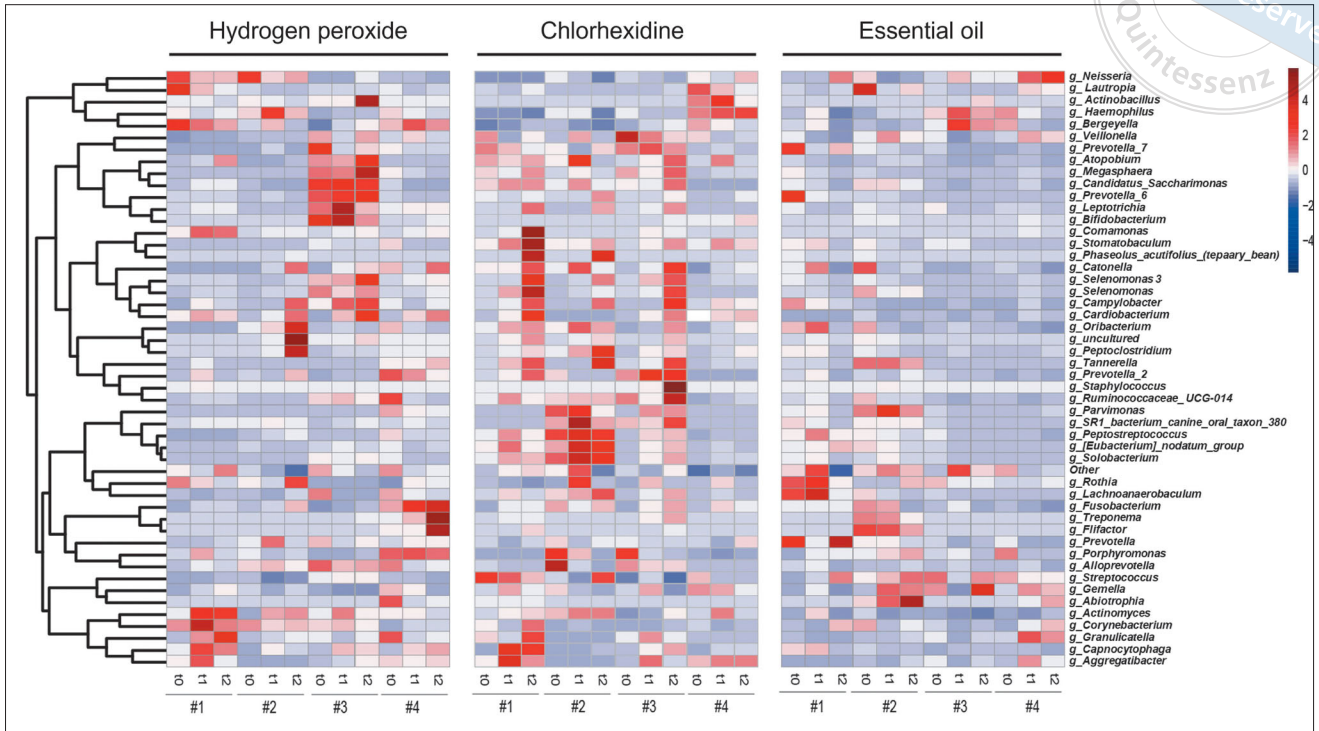


Fig 4 Differential oral microbiota profiles among different mouthrinses. A heatmap shows the top 50 microbiome profiles at the genus level across the three groups at three sampling time points. In the H_2O_2 group, *Neisseria* abundance significantly decreased, while *Rothia*, *Fusobacterium*, *Actinomyces* and *Capnocytophaga* significantly increased. In the chlorhexidine group, *Leptotrichia*, *Stomatobaculum*, *Rothia*, *Fusobacterium* and *Actinomyces* increased significantly, whereas *Neisseria*, *Haemophilus*, *Veillonella*, *Streptococcus*, *Gemella*, and *Porphyromonas* notably decreased. In the essential oil mouthrinse group, *Haemophilus* significantly increased, while *Leptotrichia* and *Prevotella* significantly decreased. #1, #2, #3 and #4 represent four individuals in each group. t0, 5 minutes after rinsing with water; t1, 5 minutes after rinsing with one of the three mouthrinses; t2, 1 hour after rinsing with mouthrinse.)

Fig 5a and b Effects of different mouthrinses on oral microbiota composition. Taxonomic profiles of oral microbiota at the genus level with relative abundance > 1%. The 14 most abundant genera were *Streptococcus*, *Neisseria*, *Haemophilus*, *Actinomyces*, *Leptotrichia*, *Prevotella*, *Prevotella_7*, *Porphyromonas*, *Rothia*, *Fusobacterium*, *Gemella*, *Capnocytophaga*, *Staphylococcus* and *Veillonella* (a). In the H_2O_2 group, *Neisseria* decreased significantly, with a slight decrease in *Prevotella_7* and an increase in *Actinomyces*. In the chlorhexidine group, *Porphyromonas*, *Veillonella*, *Streptococcus*, *Neisseria* and *Gemella* decreased significantly. In the essential oil mouthrinse group, *Leptotrichia* decreased significantly, whereas *Haemophilus* increased significantly (b).

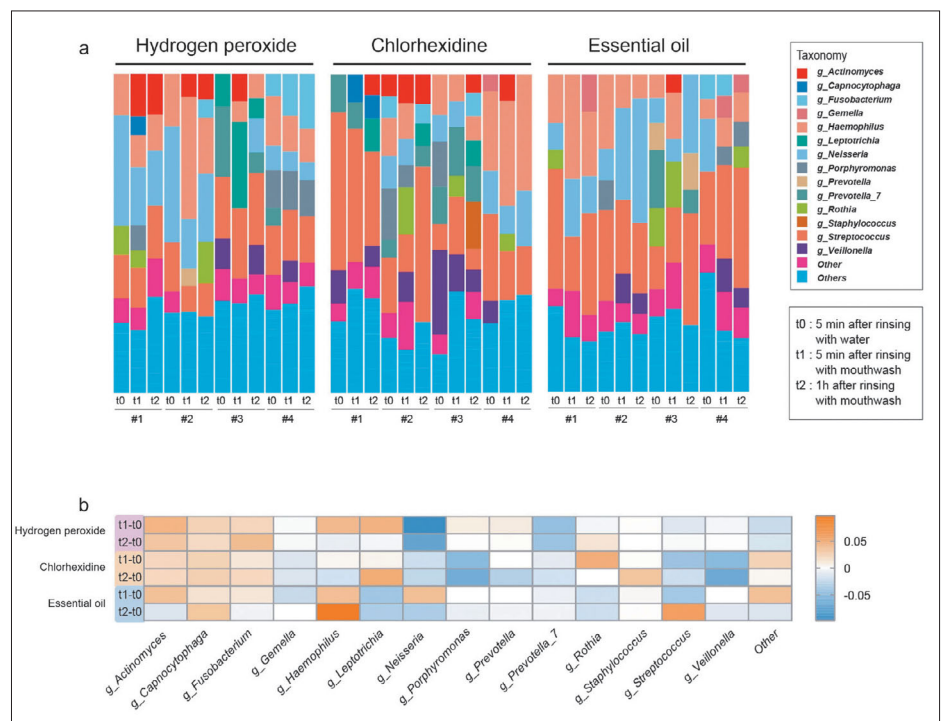




Table 1 Changes in top 50 microbiome profiles at the genus level from t0 to t2.

Mouthrinse	Increase from t0 to t2	Decrease at from t0 to t2
H ₂ O ₂	<i>Rothia</i> , <i>Fusobacterium</i> , <i>Actinomyces</i> , <i>Capnocytophaga</i>	<i>Neisseria</i>
Chlorhexidine	<i>Leptotrichia</i> , <i>Stamatobaculum</i> , <i>Rothia</i> , <i>Fusobacterium</i> , <i>Actinomyces</i>	<i>Neisseria</i> , <i>Haemophilus</i> , <i>Veillonella</i> , <i>Streptococcus</i> , <i>Gemella</i> , <i>Porphyromonas</i>
Essential oil	<i>Haemophilus</i>	<i>Leptotrichia</i> , <i>Prevotella</i>

Table 2 Changes in the oral microbiota with abundance greater than 1% from t0 to t2.

Mouthrinse	Increase from t0 to t2	Decrease at from t0 to t2
H ₂ O ₂	<i>Actinomyces</i>	<i>Neisseria</i>
Chlorhexidine	No	<i>Porphyromonas</i> , <i>Veillonella</i> , <i>Streptococcus</i> , <i>Neisseria</i> , <i>Gemella</i>
Essential oil	<i>Haemophilus</i>	<i>Leptotrichia</i>

groups, implying that H₂O₂ did not change the oral microbiome profile and maintained the dynamic equilibrium of the oral ecosystem. The present data showed that H₂O₂ has an obvious inhibitory effect on *Neisseria*. As a pathogenic species of the *Neisseria* genus, *N. meningitidis* can cause bacterial meningitis by migrating from the oral cavity.²⁸ Thus, H₂O₂ may have potential applications in the prevention of stomatogenous meningitis.

These findings highlight the trade-off between the antimicrobial potency of chlorhexidine and ecological disruption. Conversely, essential oils balance pathogen control and microbiome resilience. The activity of H₂O₂ against *Neisseria* warrants further investigation for targeted clinical applications.

The present study has certain limitations. The small cohort size (n = 12) and short-term observation period (1 hour post-rinsing) restrict the generalisation to diseased populations or long-term outcomes. Therefore, validation in larger cohorts with longitudinal designs is necessary. In addition, the molecular mechanisms underlying the selective antibacterial activity of various essential oil mouthrinses remain to be determined.

Future research should evaluate the applicability of mouthrinses across diverse clinical scenarios, such as preventing peri-implantitis, controlling infections following tooth extraction and managing oral microbiomes in patients with systemic diseases. Additionally, integrating meta-transcriptomics can reveal functional shifts in the microbiome, resulting in a salivary microbial and human meta-transcriptomic signature of microbial metabolic pathways and functional modules after the use of different mouthrinses.²⁹ Overall, these efforts will advance personalised oral care strategies, preserve ecological balance in the oral cavity and provide critical insights for guiding the development of tailored mouthrinses.

Conclusion

This study evaluates the antimicrobial efficacy and ecological impacts of H₂O₂, chlorhexidine and essential oil mouthrinses. Essential oil mouthrinses may serve as an ecologically balanced alternative to chlorhexidine for oral microbiome management, whereas H₂O₂ niche inhibition highlights its potential for targeted applications, warranting further validation in diverse clinical settings and longitudinal studies.

Conflicts of interest

The authors declare no conflicts of interest related to this study.

Author contribution

Drs Lin HE and Zheng HE designed the study; Drs Lin HE, Zheng HE, and Yi ZHANG drafted the manuscript; Drs Lin HE, Fei WU and Hui LIU performed the experiments; Drs Lin HE, Zheng HE and Lei HU analysed the data; Drs Yi ZHANG, Xing Gang LIU, Dong Xiang ZENG, Rui Qi SHAO, Lei HU and Qing Song JIANG revised the manuscript. All authors read and approved the final manuscript.

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