OSR
ORAL SCIENCES REPORTS

Original Article

Received: June 30, 2025
Revised: July 30, 2025
Accepted: September 18, 2025

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Oral Sci Rep. 2026; 47(1): 82-94

In Vitro Effects of Different Probiotic Mouthwash Formulations on the Growth of Oral Pathogens

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Abstract

Objectives: To investigate the effect of various probiotic mouthwash formulations on the quantity of oral pathogenic microorganisms.

Methods: This was a laboratory-based study testing four different mouthwash formulations: (1) 10% probiotic supernatant mouthwash, (2) 10% probiotic supernatant / 5% propolis mouthwash, (3) 10% probiotic supernatant / 1% cannabidiol mouthwash, and (4) 10% probiotic supernatant / 5% propolis / 1% cannabidiol mouthwash. A positive control (chlorhexidine mouthwash) and a negative control (0.9% saline solution) were also included. The inhibitory efficacy of these formulations was tested against two microorganisms: *Candida albicans* and *Streptococcus mutans*. Quantitative data were collected by counting colonies before and after treatment (CFU/mL). Descriptive statistics and One way ANOVA test were used for analysis ($p<0.05$).

Results: The results showed that each mouthwash formulation could inhibit both pathogenic microorganisms to varying degrees, with a greater effect observed on Streptococcus mutans than on *Candida albicans*. When the quantitative data from colony counts were subjected to statistical analysis, no statistically significant differences were found among the mouthwash formulations ($p>0.05$) in inhibiting both types of microorganisms. Notably, the mouthwash containing 10% probiotic supernatant, 5% propolis and 1% cannabidiol demonstrated a statistically significant inhibitory effect on *Streptococcus mutans* ($p<0.05$).

Conclusions: All four mouthwash formulations showed a tendency to inhibit both pathogenic microorganisms, although this was not statistically significant in all cases. The formulation containing 10% probiotic supernatant, 5% propolis, and 1% cannabidiol had the most favorable effect in inhibiting the cariogenic bacterium *Streptococcus mutans.*

Keywords: mouthwash, oral bacteria, probiotic

Introduction

Microorganisms, tiny living entities, have long been recognized as a cause of various human diseases. However, it's crucial to understand that not all strains of these microorganisms are problematic and solely responsible for illness. A familiar example is the human oral cavity, which harbors approximately 700 species of microorganisms[1], encompassing both pathogenic and non-pathogenic types. The unique environmental factors within the oral cavity create a distinct microbial community that interacts with one another, collectively known as the oral microbiota.

Under normal circumstances, even in the presence of potentially pathogenic strains, the oral microbiota does not cause disease in healthy individuals. In a healthy state (eubiosis), the oral microbiome is diverse and dominated by commensal species that contribute to homeostasis, maintain biofilm stability, and suppress the overgrowth of pathogens. Pathogenic microorganisms are present only in low numbers and are kept in check by microbial competition and host immune responses. In contrast, in disease-associated states (dysbiosis), microbial diversity often decreases, with a relative increase in pathogenic or opportunistic species, accompanied by metabolic changes that favor inflammation and tissue destruction.

Multiple factors—including the integrity of oral tissues, the composition of commensal microorganisms, dietary habits, oral hygiene practices, and systemic health—influence whether the oral microbiome remains in eubiosis or shifts toward dysbiosis. When conditions favor eubiosis, disease is unlikely to occur. However, significant alterations in these factors can disrupt microbial balance, leading to the proliferation of pathogenic microorganisms. Dysbiosis can initiate disease processes such as dental caries, periodontitis, cardiovascular disease, and diabetes.[2] While antibiotics are one way to manage these issues, their use can lead to other problems, including antibiotic resistance and a lack of specificity that may eliminate beneficial microorganisms. This has spurred the development of alternative approaches to combat pathogenic microorganisms by replacing them with non-pathogenic ones, a principle known as bacteriotherapy.[3] These non-pathogenic microorganisms, when consumed in sufficient quantities, confer health benefits and are termed probiotics.[4]

Probiotics have a wide range of applications in medicine, including dentistry, due to their antimicrobial and immunomodulatory properties. In the oral cavity, they can combat pathogens by interfering with biofilm formation, a key factor in the development of diseases such as dental caries and periodontitis, through competition for adhesion sites and nutrients.[5] Beyond their antimicrobial activity, probiotics also exert anti-inflammatory effects[6], which may help reduce undesirable post-surgical complications.[7,8] A derivative product, the cell-free supernatant containing bioactive compounds secreted by probiotics, namely bacteriocins, and has demonstrated benefits in reducing inflammation after oral surgery.[8] This supernatant also exhibits antibacterial effects[9] can inhibit *Streptococcus mutans (S. mutans)* by forming pores in the cell membrane, disrupting cell wall biosynthesis, and impairing biofilm development[10,11] through reduced glucan-mediated adhesion.

Another natural extract gaining significant attention for its medicinal properties is cannabis extract, classified as cannabinoids. These compounds can stimulate the endocannabinoid system (ECS) in the human body.[12] Plant-derived cannabinoids, introduced externally, are referred to as exogenous cannabinoids and include various types such as Tetrahydrocannabinol (THC), Cannabidiol (CBD), and Cannabigerol (CBG). These substances can act on cannabinoid receptors present in the human body, including immune cells. Their effects on the body are extensive, including pain reduction, anti-emetic properties, anticonvulsant effects, and anti-inflammatory actions through mechanisms that reduce the production of various pro-inflammatory cytokines.[13] In dentistry, they have been incorporated into various forms, such as tablets, capsules, topical oils, toothpastes, sprays, mouthwashes, chewing gums, and even dental filling materials.[13-15] This highlights the broad potential for developing these extracts for dental applications. With these reasons, Nisapa[16], incorporated CBD into mouthwash formulations and study how they can enhance anti-inflammatory property of the mouthwash by comparing the mouthwash containing 10% *Lactobacillus paracasei (L.paracasei)* supernatant with and without CBD at 0.25% ,0.5% and 1%. The result shows that the mouthwash with at least 0.5% CBD can significantly further reduce TNF-α production in human monocytic cell compared to the formular without CBD and the concentration that shows the most reduction is the one with 1% CBD.

Propolis, a resinous substance produced by bees for hive construction. It contains bioactive materials, flavonoids and phenolic acids, exhibits antibacterial, antiviral, antifungal, antiparasitic, and antioxidant activities. It also suppresses inflammation by inhibiting prostaglandin synthesis. These numerous properties have led to its long-standing use in medicine.[17] In dentistry, propolis has been investigated for use on post-surgical oral wounds in the form of mouthwash. Results indicate that it promotes epithelialization and possesses analgesic and anti-inflammatory effects.[18,19] As propolis proved to be medically beneficial, it was added to mouthwash and had been studied by Suetrongtrakool[20], which the study shows that with addition of propolis to the mouthwash formulars, they can further reduce TNF-α production in human monocytic cell compared to the mouthwash formulars without propolis.

Building on the demonstrated anti-inflammatory effects of *L. paracasei* supernatant, further research has focused on developing novel mouthwash formulations. An initial clinical study successfully reduced inflammation and postoperative complications following impacted third molar extraction using *L. paracasei* supernatant alone.[8] Subsequent development aimed to enhance this anti-inflammatory potential by incorporating additional bioactive components. For example, studies by Nisapa[16] and Suetrongtrakool[20] formulated mouthwashes containing varying concentrations of CBD and propolis. Both investigations reported promising laboratory findings, showing that addition of effectively inhibited TNF-α production. Among the tested combinations, the most promising was the formulation containing 10% *L. paracasei* supernatant, 1% CBD and 5% propolis.

However, beyond their anti-inflammatory properties, these developed mouthwash formulations likely possess another unexamined benefit, their ability to combat oral microorganisms. This is because all the components of the studied mouthwashes have demonstrated antibacterial, and potentially antifungal activity. If further research confirms their ability to reduce the number of oral pathogens, it will signify that these new mouthwash formulations possess both anti-inflammatory and antibacterial properties. This would make them an attractive alternative mouthwash for practical use in alleviating disease and improving patients' quality of life. The objective of this research was to investigate the effect of various probiotic mouthwash formulations on the quantity of oral pathogenic microorganisms.

Materials and Methods

This study was a laboratory-based investigation testing four mouthwash formulations. These formulations were referenced from previous research by our group, which demonstrated their efficacy in both laboratory and clinical settings. A key component of these formulations is 10% *L. paracasei* probiotic supernatant.

Independent variables

The independent variables in this study were the different types of mouthwash formulations, which included:

* Negative Control: 0.9% Sodium Chloride solution

* Probiotic Supernatant Mouthwash (10% *L. paracasei* supernatants)

* Probiotic Supernatant Mouthwash with Propolis (10% *L. paracasei* supernatants + 5% propolis)

* Probiotic Supernatant Mouthwash with CBD (10% *L. paracasei* supernatants + 1% cannabidiol)

* Probiotic Supernatant Mouthwash with CBD and Propolis (10% *L. paracasei* supernatants + 1% CBD + 5% propolis)

Dependent variable

The dependent variable was the colony count of oral pathogens observed after culturing with the various mouthwash formulations.

Population and sample

The microorganisms used in this experiment were *S. mutans* and *Candida albicans*. These two species were selected based on a literature review which indicates that *S. mutans* can produce insoluble polymers called glucans, a crucial substance for biofilm adherence to tooth surfaces. Thus, it plays a significant role in biofilm formation, which acts as a reservoir for pathogens and can lead to various oral diseases.[21] Similarly, *Candida albicans* is a commensal organism that, while normally non-pathogenic, contributes to biofilm formation in conjunction with other bacteria, leading to dysbiosis and various diseases.[22,23] Therefore, in this study, both species were used as representatives of an early colonizing bacterium and an oppor-

tunistic oral fungus, respectively, to evaluate the antimicrobial efficacy of the mouthwash formulations.

Research procedures

The research process was divided into four main parts:

Part I: Microbial isolation and standard growth curve determination

Candida albicans were obtained from Department of Microbiology, Faculty of Medicine, Srinakharinwirot University and *S. mutans* were obtained from Department of Stomatology, Faculty of Dentistry, Srinakharinwirot University. Both microbial species were isolated, and their standard growth curves were established to standardize microbial concentrations prior to testing by adjusting their optical density (OD) using a spectrophotometer. Initially, both strains were stored at -80°C and cultured on solid media: *S. mutans* was grown on BHI agar at 37°C in 5% CO_2 for 24 hours, while *Candida albicans* was cultured on SDA agar at 28°C for the same duration. Individual colonies from these solid media were then inoculated into liquid media, with *S. mutans* being transferred into BHI broth and *Candida albicans* into SDB. The optical density of the cultures was measured at a wavelength of 600 nm and recorded hourly over a 12-hour period. These OD readings over time were used to construct standard growth curves (OD vs. time), which were subsequently analyzed to determine the mid-logarithmic growth phase for use in downstream experiments.

Part II: Microbial exposure and viability assessment

Colonies from the initial solid media were inoculated into 50 mL Erlenmeyer flasks containing appropriate liquid media and cultured until *S. mutans* and *Candida albicans* reached their mid-logarithmic phase, as determined by previously established standard growth curves. The cultures were adjusted using a spectrophotometer at 600 nm to match McFarland standard 0.5 for *S. mutans* ($OD_{600} \approx 0.07$; ~1.5×10^8 CFU/mL) and McFarland standard 2.0 for *Candida albicans* ($OD_{600} \approx 0.5$; ~2×10^7 CFU/mL). For the direct contact test, 500 µL of each mouthwash formulation (Table 1), along with positive and negative controls, was transferred into 1 mL centrifuge tubes, followed by the addition of 500 µL of the adjusted microbial suspensions. This 1:1 mixing resulted in a final mouthwash concentration of 50% of the original formulation. The mixtures were vortexed thoroughly and incubated at 37°C for 1 minute to facilitate interaction. Following incubation, the mixtures were subjected to six 10-fold serial dilutions, reaching up to 10^{-6}. Aliquots of 100 µL from the 10^{-3}, 10^{-4}, and 10^{-5} dilutions for *Candida albicans* and from the 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} dilutions for *S. mutans* were spread onto solid culture media in duplicate and incubated for 24 hours. Colony counting was then performed to determine microbial viability under each condition.

Part III: Time-Dependent Microbial Exposure and Viability Assessment

This experiment further evaluated the antimicrobial effect of the selected mouthwash formulation containing 10% probiotic supernatant, 5% propolis, and 1% CBD against the pathogens that showed susceptibility in Part II. Microbial suspensions were prepared and adjusted to the same optical densities as described previously. 500 µL of mouthwash was mixed with 500 µL of microbial suspension in centrifuge tubes and incubated at 37°C for four exposure times: 0, 1, 2, and 3 minutes. Three replicate tubes per time point were prepared, and after each exposure, samples were serially diluted (up to 10^{-7}), plated in duplicate, and incubated for 24 hours. Colony-forming units were counted to assess microbial viability over time

Data collection and statistical analysis

Colony counting was performed, and the number of colonies in each sample was recorded. Counts with-

Table 1: Shows ingredients and volume of each mouthwash formular.

Ingredients	Volume (mL)				Function
	S	SC	SP	SCP	
0.9% Sodium Saccharin	0.33	0.33	0.33	0.33	Taste modifier
Lactobacillus paracasei supernatant	1	1	1	1	Active ingredient
Propolis	-	-	0.5	0.5	Active ingredient
Cannabidiol	-	0.1	-	0.1	Active ingredient
0.9% Normal saline solution	8.67	8.17	8.57	8.07	Solvent

in the readable range (30–300 colonies) were selected from all sets of duplicate plates prepared simultaneously. These values were compiled and averaged to represent the microbial load for each condition. Once all relevant data had been collected, statistical analysis was performed as described below:

Two preliminary assumptions for statistical analysis were tested:

* Normality of distribution using the Kolmogorov-Smirnov test.

* Homogeneity of variances using Levene's test.

If any of these tests yielded a *p*-value lower than the significance level (0.05), it indicated a violation of the assumption, necessitating the use of non-parametric statistics, specifically the Kruskal-Wallis test. However, if both tests yielded *p*-values higher than 0.05, then One-way ANOVA could be used. Statistical analysis was performed using GraphPad Prism version 10.4.2 (633)

Results

Based on experiments to establish the standard growth curves for both microbial species (Figure 1), *S. mutans* and *Candida albicans* reached their standard growth phases at 6 and 7 hours, respectively. This indicates that *Candida albicans* exhibits a slower growth rate compared to *S. mutans* bacteria.

The second part of the direct contact test involved evaluating the four mouthwash formulations against both microbial species, along with positive and negative control groups. Experiments were performed duplicate, and mouthwash concentrations were diluted 10-fold differently in each experimental set, as depicted in the laboratory testing framework image (Figure 2 and Figure 3).

The mouthwashes were mixed according to formular shown in Table 1. Then, pH of each formular were measured and recorded as shown in Table 2

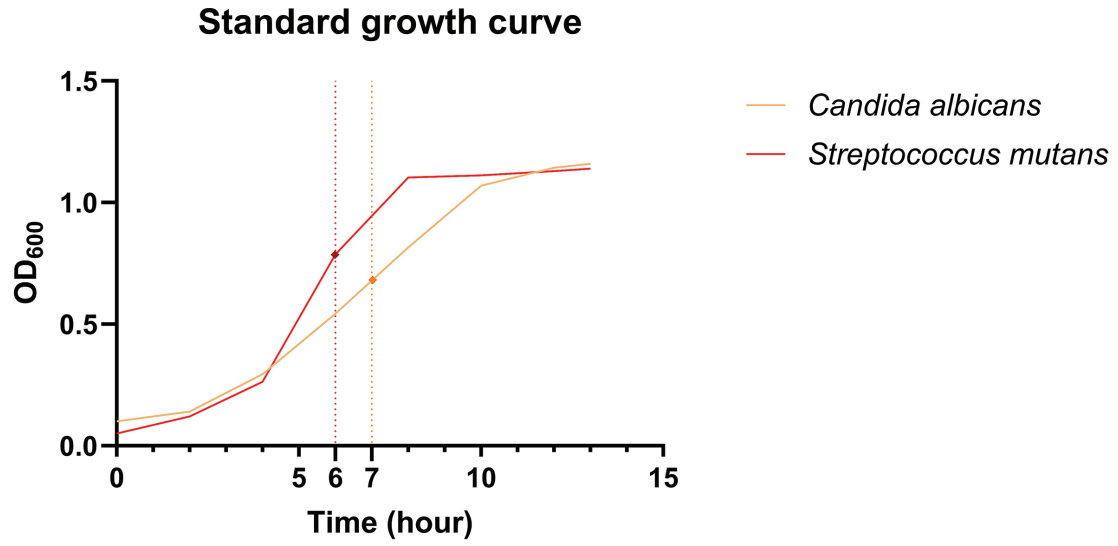

Figure 1: Illustrates the standard growth curves of these two oral pathogens.

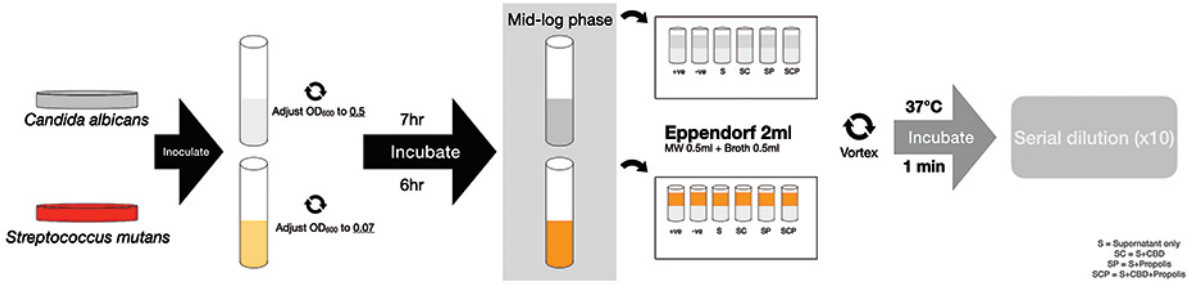

Figure 2: Shows laboratory investigation with direct contact technique.

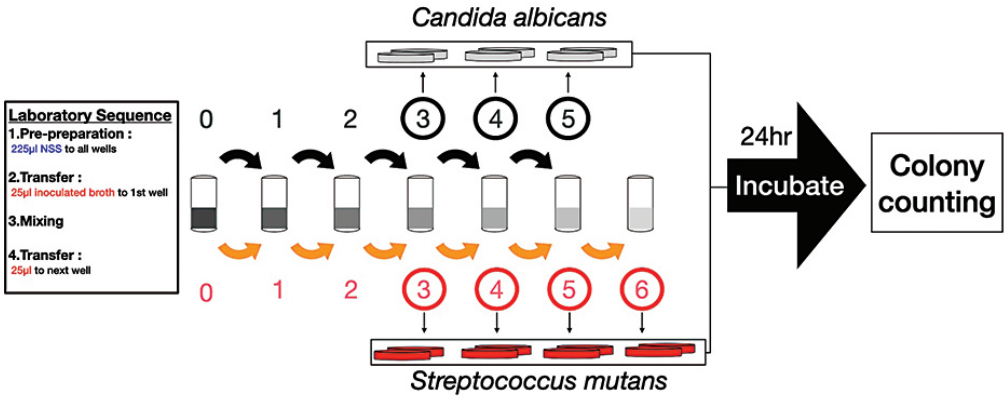

Figure 3: Shows laboratory investigation serial dilution (x10).

Table 2: Shows pH of each mouthwash formular.

Formular	pH
S	4.28
SC	4.33
SP	4.36
SCP	4.31

Table 3: Shows the inhibitory effect of different mouthwash formulas against both pathogens.

Formular	Mean colony (CFU/mL)	
	Candida albicans	Streptococcus mutans
Positive control	0	0
Negative control	1.33×10^7	2.57×10^8
S	1.24×10^7	3.61×10^8
SC	1.40×10^7	3.51×10^8
SP	1.27×10^7	3.14×10^8
SCP	9.30×10^6	1.95×10^8

*Positive control: 0.12% Chlorhexidine mouthwash, Negative control: 0.9% Normal saline solution, S: *Lactobacillus paracasei* supernatant only, SC: *Lactobacillus paracasei* supernatant + cannabidiol, SP: *Lactobacillus paracasei* supernatant + propolis, SCP: *Lactobacillus paracasei* supernatant + cannabidiol + propolis

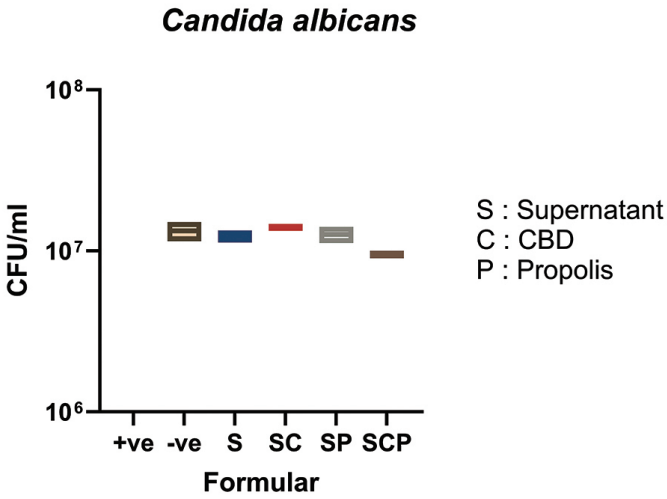

Figure 4: Shows illustration of the test results of different mouthwash formulas on the inhibition of *Candida albicans.*

The results from the direct contact test demonstrated varying inhibitory effects of each mouthwash formulation on the two tested pathogens. These findings are presented in Table 1, which shows the inhibition values of different mouthwash formulations on both pathogenic species.

When the obtained values were subjected to statistical analysis, based on the established hypotheses (H_0: The number of microbes cultured with various mouthwash formulations does not differ from the negative control; H_1: The number of microbes cultured with various mouthwash formulations differs from the negative control).

As for *Candida albicans* (Table 3, Figure 4), despite results of Kruskal-Wallis analysis yield $p<0.05$, no statistically significant differences were found in Dunn's multiple comparison ($p>0.05$). (Table 4 and Table 5)

As for *S. mutans* (Table 3, Figure 5), despite Kruskal-Wallis analysis yield $p<0.05$, statistically significant differences were found only in comparison between positive control to mouthwash containing supernatant with cannabidiol and mouthwash containing supernatant and propolis. (Table 6 and Table 7)

The results indicated that the mouthwash formulations exhibited an inhibitory effect against *S. mutans*; therefore, this pathogen was selected for time-dependent exposure analysis

The final part of the study involved evaluating the mouthwash formulation containing 10% probiotic supernatant, 5% propolis, and 1% CBD against *S. mutans* at various time points. Laboratory testing framework are illustrated in Figures 6 and 7, and the results are shown in Table 8 and Figure 8.

When the obtained values were subjected to statistical analysis using one-way ANOVA, no statistically significant differences were found ($p>0.05$). (Table 9)

Table 4: Shows result of statistical analysis with Kruskal-Wallis test of *Candida albicans.*

Kruskal-Wallis test	
p value	0.0209
Exact or approximate *p* value?	Approximate
p value summary	*
Do the medians vary sig. ($p<0.05$)?	Yes
Number of groups	6
Kruskal-Wallis statistic	13.27

Table 5: Show Dunn's multiple comparison of *Candida albicans.*

Dunn's multiple comparisons test	**Mean rank diff.**	**Significant?**	**Summary**	**Adjusted *p* value**
+ve vs. -ve	-11.00	No	ns	0.1073
+ve vs. S	-9.000	No	ns	0.4164
+ve vs. SC	-11.00	No	ns	0.1073
+ve vs. SP	-9.000	No	ns	0.4164
+ve vs. SCP	-4.000	No	ns	>0.9999
-ve vs. S	2.000	No	ns	>0.9999
-ve vs. SC	0.000	No	ns	>0.9999
-ve vs. SP	2.000	No	ns	>0.9999
-ve vs. SCP	7.000	No	ns	>0.9999
S vs. SC	-2.000	No	ns	>0.9999
S vs. SP	0.000	No	ns	>0.9999
S vs. SCP	5.000	No	ns	>0.9999
SC vs. SP	2.000	No	ns	>0.9999
SC vs. SCP	7.000	No	ns	>0.9999
SP vs. SCP	5.000	No	ns	>0.9999

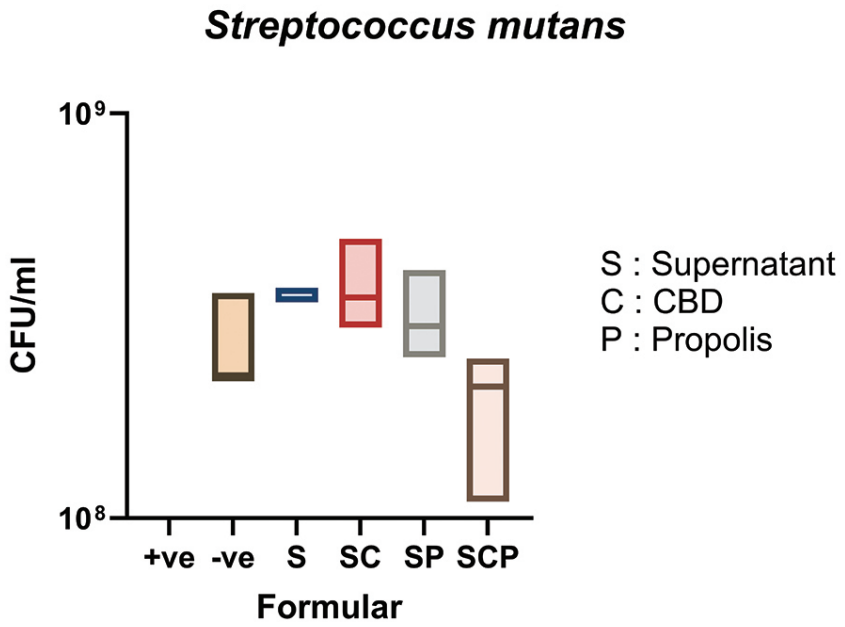

Figure 5: Shows illustration of the test results of different mouthwash formulas on the inhibition of *Streptococcus mutans*.

Table 6: Shows result of statistical analysis with Kruskal-Wallis test of *Streptococcus mutans*.

Kruskal-Wallis test	
p value	0.0030
Exact or approximate *p* value?	Approximate
p value summary	*
Do the medians vary sig. ($p<0.05$)?	Yes
Number of groups	6
Kruskal-Wallis statistic	17.93

Table 7: Shows Dunn's multiple comparisons test of *Streptococcus mutans*.

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary	Adjusted *p* value
+ve vs. -ve	-9.125	No	ns	>0.9999
+ve vs. S	-17.63	Yes	**	0.0062
+ve vs. SC	-14.75	Yes	*	0.0466
+ve vs. SP	-13.75	No	ns	0.0876
+ve vs. SCP	-4.750	No	ns	>0.9999
-ve vs. S	-8.500	No	ns	>0.9999
-ve vs. SC	-5.625	No	ns	>0.9999
-ve vs. SP	-4.625	No	ns	>0.9999
-ve vs. SCP	4.375	No	ns	>0.9999
S vs. SC	2.875	No	ns	>0.9999
S vs. SP	3.875	No	ns	>0.9999
S vs. SCP	12.88	No	ns	0.1477
SC vs. SP	1.000	No	ns	>0.9999
SC vs. SCP	10.00	No	ns	0.6748
SP vs. SCP	9.000	No	ns	>0.9999

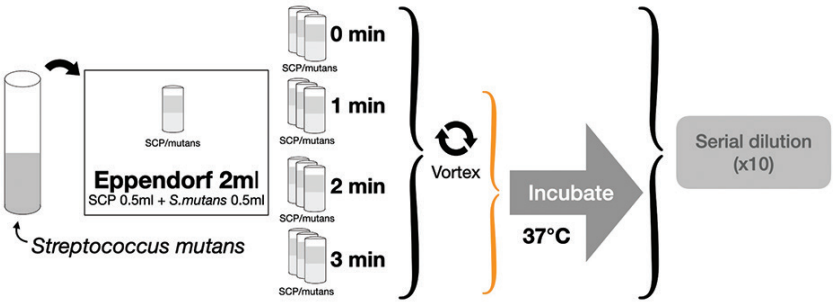

Figure 6: Shows laboratory sequences of various time-point tests.

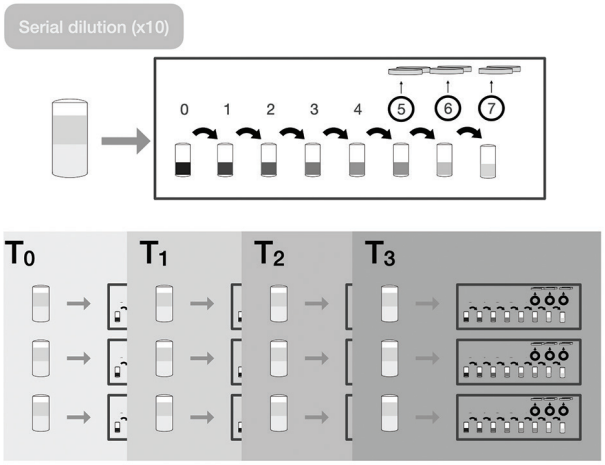

Figure 7: Shows laboratory sequences of serial dilution (x10) after various time-point tests.

Table 8: Shows the test results of different time of contact of mouthwash containing 10% probiotic supernatant, 5% propolis and 1% cannabidiol on the inhibition of *Streptococcus mutans.*

Time (minute)	Mean *Streptococcus mutans* (CFU/ml)
0	3.49×10^8
1	3.70×10^8
2	4.15×10^8
3	3.48×10^8

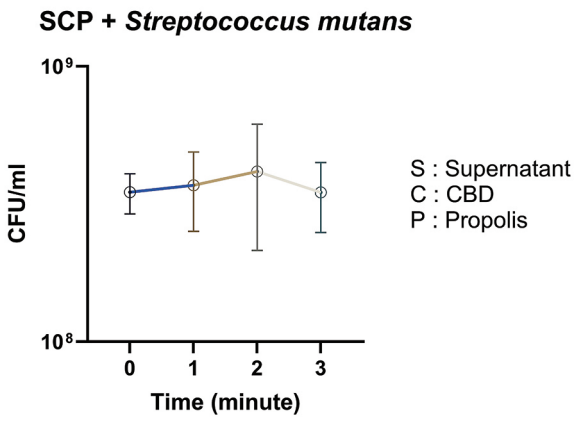

Figure 8: Show a declining trend of bacterial count after exposure to the mouthwash in longer time.

Table 9: Show results of statistical analysis with one-way ANOVA test of *Streptococcus mutans* in various time-point experiments.

ANOVA summary	
F	0.3483
p value	0.7907
p value summary	ns
Significant diff. among means ($p<0.05$)?	No
R squared	0.04965

Discussion

This study was a laboratory-based investigation designed to address a research gap identified by our research group, which aims to extend the findings from the Center of Excellence in Medical Probiotics. Previous research, both *in vitro* and clinical, has demonstrated the anti-inflammatory benefits of *L. paracasei* MSMC39-1 probiotic supernatant.[7,8] Building upon previously established beneficial proportions, this study aimed to explore additional advantages beyond inflammation reduction, specifically focusing on the potential interference or inhibition of oral pathogenic microorganisms. Two common oral pathogens were selected for testing: *S. mutans,* a bacterium associated with dental caries, and *Candida albicans*, a fungal species.

Several interesting points emerged from the experimental results. Firstly, a clear trend and distinctions were observed when analyzing the raw data. For instance, the probiotic supernatant mouthwash at 10%, cannabidiol at 1%, and propolis at 5% formulations showed the greatest reduction in microbial load among the four tested formulations. Furthermore, the positive control group exhibited no growth of either microbial species. However, when the same data set was subjected to statistical analysis, the tests show non-normal distribution of data for both pathogens, so non-parametric statistics were selected. Even Kruskal Wallis test yield significant differences in either pathogen (*Candida albicans p*-value:0.0209, *S. mutans p*-value:0.0030) but when the data were subjected Dunn's to multiple comparison, *Candida albicans* shows no pair of significant difference while *S. mutans* shows two pair of significant difference, which are positive control to mouthwash with only probiotic supernatant and positive control to mouthwash containing supernatant and CBD. This discrepancy may be attributed to statistical reason, an insufficient sample size, precluding robust statistical conclusions. Therefore, increasing the sample size in future experiments could potentially normalize data distribution, allowing for the application of more reliable parametric statistical methods. Another possible reason is short duration of the direct contact test, 1 minute, which may be too short for any mechanism to occur biologically. This duration was chosen based on manufacturer recommendations for mouthwash rinsing, typically ranging from 30 seconds to 1 minute. However, in real-world practice, patients often rinse for less time than recommended.[24] Therefore, even if longer exposures show significant pathogen reduction, these findings may have limited practical relevance if the mouthwash is not retained in the oral cavity long enough. Nonetheless, most commercial mouthwashes are marketed as supplemental cleansing products rather than long-term therapeutic agents, unlike the mouthwash formulated in this study. In clinical use, detailed instructions from dentists could help ensure adequate rinsing time to maximize efficacy.

Another crucial aspect involves the numerous factors influencing pathogen inhibition experiments, such as the inherent characteristics of the microorganisms, the mechanisms of killing/inhibition, the environmental conditions conducive to microbial growth, the duration of exposure to the mouthwash, and the concentration of the different mouthwash formulations. Nevertheless, the experimental design for this study was based on previous research where these formulations were developed and shown to reduce oral inflammation. This reliance on prior formulations might be a limitation, as it does not encompass scenarios where increasing the concentration of certain or all active ingredients in the mouthwash could alter their antimicrobial efficacy, especially at this short duration of time. This observation also applies to the experimental duration, which might have been either sufficient or insufficient for the active ingredients in the mouthwash to exert their full effects on the pathogens. Consequently, a subsequent experiment was designed to test the most

promising formulations over three intervals of time to investigate the trend of microbial reduction with increased exposure time. Although the results of this extended testing do not show district declining trend, the growth of the mouthwash-treated bacteria is diminished compared to the growth of bacteria in mid-logarithm phase which is shown in first part of the studied. These results were subjected to one-way ANOVA. The analysis yield *p*-value of 0.3483, hence no significant differences were found.

An additional relevant factor is the pH of the various mouthwash formulations. All our formulations contain Lactobacillus probiotic supernatant, which retains some acidity due to acid production by Lactobacillus bacteria. Therefore, pH measurements were conducted to clarify any relationship between pH and antimicrobial/antifungal properties. The results shown in Table 2 indicate that the pH values of all formulations were very similar, ranging narrowly from 4.28 to 4.36. This suggests an insignificant role of pH in the observed antimicrobial and antifungal effects. Our findings closely resemble those of Rossoni[25], who evaluated the influence of acids produced by Lactobacillus strains on *S. mutans* and found that all strains exhibited similar acidogenic activity, with no significant pH difference compared to the control, suggesting that pH change may have no effect. Conversely, Lin[26], found that adjusting the pH of Lactobacillus supernatant removed the inhibitory effect of some strains on *S. mutans*, suggesting that growth and biofilm formation are likely inhibited by low pH. Importantly, with the addition of key components such as CBD and propolis, a reduction in colony counts was observed. This indicates that other substances or mechanisms are involved in creating an environment less conducive to microbial growth.[12,13,16,18]

Our mouthwash has a pH below 4.5, which is lower than the critical pH for enamel demineralization; however, this value is comparable to that of several commercially available mouthwashes. In a survey of 47 products, B.W.M. van Swaaij *et al.,*[27] reported that 20 mouthwashes (43%) had pH values below 5.5, including 10 that contained fluoride. Some formulations may be intentionally acidic, as a mildly low pH can facilitate the conversion of hydroxyapatite (HA) to the more acid-resistant fluorapatite (FA) in the presence of fluoride. In another word, our mouthwash might be beneficial to the FA conversion process if used in presence of fluoride which is typically abundantly retained in oral cavity after brushing with fluoride-containing toothpaste.

Regarding the proposed mouthwash which shows best performance, the one with all the ingredients, the result is reasonable and predictable as the formular contains most active ingredients, *L. paracasei* supernatant, CBD and propolis. Although the study cannot reveal what mechanism behind the result or whenever the effect observed is increment of three substances acting independently or synergically, the result shows that they work best together. According to prior studies, *L. paracasei* cell-free supernatant (CFS) contains various bioactive compounds, including bacteriocins, organic acids, and enzymes, which exert antimicrobial and anti-inflammatory effects. The CFS has been sh own to inhibit the growth and biofilm formation of oral pathogens such as *S. mutans* by disrupting cell membranes and interfering with adhesion mechanisms.[25,26] When combined with propolis and cannabidiol, which also target microbial membranes and modulate host inflammation[12,13,18,19], the supernatant may enhance the overall antimicrobial efficacy of the formulation. This multi-targeted approach could provide broader-spectrum pathogen suppression while reducing potential cytotoxicity to beneficial oral microbiota. The complementary actions of these agents suggest a potentially synergistic effect that warrants further exploration in oral health applications

Despite the lack of statistically significant differences, the raw data clearly indicate a trend of reduced microbial load after a single exposure to mouthwash. This information is highly valuable for the development of mouthwash formulations, as it suggests the mouthwash possesses antimicrobial properties. Continued use could potentially reduce the oral microbial load, thereby contributing to the prevention of pathogen-induced diseases. Furthermore, unlike the positive control (0.12% chlorhexidine mouthwash, broad-spectrum antiseptic agent), these novel mouthwash formulations do not eliminate all microorganisms. Complete eradication, if used long-term in real-world scenarios, could disrupt the delicate balance of oral microbiota. Therefore, these new mouthwash formulations may offer a superior long-term option for reducing oral inflammation without disturbing the oral microbial balance.

Conclusions

The four mouthwash formulations demonstrated a trend towards inhibiting both tested pathogenic microorganisms, although this trend was not statistically significant. The formulation containing 10% probiotic supernatant, 5% propolis, and 1% CBD showed the most promising results in inhibiting the cariogenic bacterium *S. mutans*.

Conflicts of Interest

The authors declare no conflicts of interest.

References

1. Chugh P, Dutt R, Sharma A, Bhagat N, Dhar MS. A critical appraisal of the effects of probiotics on oral health. J Funct Foods. 2020;70:103985.
2. Maier T. Oral microbiome in health and disease: Maintaining a healthy, balanced ecosystem and reversing dysbiosis. Microorganisms. 2023;11(6):1453.
3. Huovinen P. Bacteriotherapy: the time has come. BMJ. 2001;323(7309):353-4.
4. Mohd Fuad AS, Amran NA, Nasruddin NS, Burhanudin NA, Dashper S, Arzmi MH. The mechanisms of probiotics, prebiotics, synbiotics, and postbiotics in oral cancer management. Probiotics Antimicrob Proteins. 2023;15(5):1298-311.
5. Homayouni Rad A, Pourjafar H, Mirzakhani E. A comprehensive review of the application of probiotics and postbiotics in oral health. Front Cell Infect Microbiol. 2023;13:1009606.
6. Vincenzi A, Goettert MI, Volken de Souza CF. An evaluation of the effects of probiotics on tumoral necrosis factor (TNF-α) signaling and gene expression. Cytokine Growth Factor Rev. 2021;57:27-38.
7. Ladda B, Theparee T, Chimchang J, Tanasupawat S, Taweechotipatr M. *In vitro* modulation of tumor necrosis factor α production in THP-1 cells by lactic acid bacteria isolated from healthy human infants. Anaerobe. 2015;33: 109-16.
8. Banjonjit S, Taweechotipatr M, Rungsiyanont S. Effect of probiotic *Lactobacillus paracasei* on tumor necrosis factor-alpha level in gingival crevicular fluid of patients undergoing impacted third molar removal. J Oral Sci. 2022;64(3):185-9.
9. Wu CC, Lin CT, Wu CY, Peng WS, Lee MJ, Tsai YC. Inhibitory effect of *Lactobacillus salivarius* on *Streptococcus mutans* biofilm formation. Microb Pathog. 2018;121:258-64.
10. Cotter PD, Hill C, Ross RP. Bacteriocins: Developing innate immunity for food. Nat Rev Microbiol. 2005;3(10):777-88.
11. Darbandi A, Asadi A, Mahdizade Ari M, Ohadi E, Talebi M, Halaj Zadeh M, *et al*. Bacteriocins: properties and potential use as antimicrobials. J Clin Lab Anal. 2022;36(1):e24093.
12. Nagarkatti P, Pandey R, Rieder SA, Hegde VL, Nagarkatti M. Cannabinoids as novel anti-inflammatory drugs. Future Med Chem. 2009;1(7):1333-49.
13. Lowe H, Toyang N, Steele B, Bryant J, Ngwa W, Nedamat K. The current and potential application of medicinal cannabis products in dentistry. Dent J (Basel). 2021;9(9):106.
14. Vasudevan K, Stahl V. Cannabinoids infused mouthwash products are as effective as chlorhexidine on inhibition of total-culturable bacterial content in dental plaque samples. J Cannabis Res. 2020;2(1):20. doi: 10.1186/s42238-020-00027-z.
15. Ercan N, Erdemir EO, Ozkan SY, Hendek MK. The comparative effect of propolis in two different vehicles; mouthwash and chewing-gum on plaque accumulation and gingival inflammation. Eur J Dent. 2015;9(2):272-6.
16. Nisapa N, Taweechotipatr M, Rungsiyanont S. The development of probiotic mouthwash formulation with cannabis extracts on the reduction of proinflammatory cytokine: TNF-alpha level. In: The 13th Hatyai National and International Conference; 2022 Dec 1-3; Hatyai, Thailand. Hatyai University; 2022. p. 848-61.
17. Zulhendri F, Lesmana R, Tandean S, Christoper A, Chandrasekaran K, Irsyam I, *et al*. Recent update on the anti-inflammatory activities of propolis. Molecules. 2022;27(23):8473.
18. Zulhendri F, Felitti R, Fearnley J, Ravalia M. The use of propolis in dentistry, oral health, and medicine: a review. J Oral Biosci. 2021;63(1):23-34.
19. Magro-Filho O, de Carvalho AC. Topical effect of propolis in the repair of sulcoplasties by the modified kazanjian technique. cytological and clinical evaluation. J Nihon Univ Sch Dent. 1994;36(2):102-11.
20. Suetrongtrakool N, Taweechotipatr M, Rungsiyanont S. Effectiveness of probiotic mouthwash containing cannabis extracts and propolis on the reduction of tumor necrosis factor-alpha inflammatory cytokine. Procedia Multidiscip Res. 2023;1(7):484-90.
21. Chen X, Daliri EB, Kim N, Kim JR, Yoo D, Oh DH. Microbial etiology and prevention of dental caries: exploiting natural products to inhibit cariogenic biofilms. Pathogens. 2020;9(7):531.
22. Peterson SN, Snesrud E, Liu J, Ong AC, Kilian M, Schork NJ, *et al.* The dental plaque microbiome in health and disease. PLoS One. 2013;8(3):e58487.
23. Peres MA, Macpherson LMD, Weyant RJ, Daly B, Venturelli R, Mathur MR, *et al*. Oral diseases: a global public health challenge. Lancet. 2019;394(10194):249-60.
24. Phillips TR, Fairley CK, Maddaford K, Trumpour S, Wigan R, Bradshaw CS, *et al.* Duration of gargling and rinsing among frequent mouthwash users: a cross-sectional study. BMJ Open. 2020;10(9):e040754. doi: 10.1136/bmjopen-2020-040754.
25. Rossoni RD, Velloso MDS, de Barros PP, de Alvarenga

JA, Santos JDD, Santos Prado ACCD, *et al*. Inhibitory effect of probiotic lactobacillus supernatants from the oral cavity on *Streptococcus mutans* biofilms. Microb Pathog. 2018;123:361-7.
26. Lin X, Chen X, Chen Y, Jiang W, Chen H. The effect of five probiotic lactobacilli strains on the growth and biofilm formation of *Streptococcus mutans*. Oral Dis. 2014;20(4): e128-34.
27. van Swaaij BWM, Slot DE, Van der Weijden GA, Timmerman MF, Ruben J. Fluoride, pH value, and titratable acidity of commercially available mouthwashes. Int Dent J. 2024;74(2):260-7.