The Influence of Probiotic Mouthwash on Genomic Profiles and Health of the Oral Human Microbiome

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Abstract
The human oral cavity is a complex ecosystem, with the oral microbiome playing a critical role in oral and systemic health. This study investigates the impact of LuvBiotics® Probiotic Lozenges on oral health markers, including gum bleeding, tooth plaque, and salivary pH. Conducted over a six-week period with five participants, this intervention study aimed to research the effect of oral probiotic supplements on these markers by methods such as 16S sequencing and regular health assessments. Initial findings indicated no significant difference in the reduction of gum bleeding and tooth plaque among most participants, except for individual variances that suggested a potential for probiotics to influence oral health. Studying the oral microbiome composition was not possible due to insufficient DNA isolation from tooth plaque samples, revealing the need for improved sampling methods of the oral microbiome. Overall, despite promising directions, the small sample size and short duration limit the results and their generalizability, and hence there is a need for further research with a larger sample size, a stronger focus on the oral microbial composition and corresponding DNA sampling, and extended duration of the intervention to more accurately assess the effectiveness of probiotic interventions in oral health care.

Introduction

The human oral cavity is a complex ecosystem that includes a diverse community of microorganisms, collectively known as the oral microbiome[1]. Contrary to popular beliefs, a healthy oral microbiome is not merely characterized by absence of bacterial, fungal or viral species, but rather by a positive (symbiotic) balance of mutualistic or commensal microbial species with concurrent absence of pathogenic species[1]. Such a 'balanced oral microbiome' together with several oral health markers (i.e., absence of gum bleeding[2], minimal amounts of tooth plague[3], and normally ranged salivary pH between 6.2-7.6[4]) characterizes oral health. The relevance of maintaining a healthy oral microbiome even extends beyond oral health, as research has established connections between oral microbiome dysbiosis and systemic diseases, including cardiovascular diseases[5], diabetes[6], and certain forms of cancer[7]. Thus, understanding the factors that influence the health of the oral microbiome is crucial for both oral and systemic health.

Microorganisms in the oral cavity engage in a symbiotic relationship that yields mutual advantages. The commensal bacterial populations are harmless and, among others, help prevent pathogenic species from attaching to the mucosal surfaces[8], inducing epithelial cells to create antimicrobial peptides[9], and promote organised cellular growth of the epithelial tissue in the oral cavity[10]. Within the oral microbiome, around 700 prokaryotic species have been currently identified, encompassing 185 genera across 12 phyla[1]. Of these 700 species, close to 54% have been formally described, while the remaining species are yet unnamed of uncultivated[1,11]. Described phyla include *Firmicutes*, *Fusobacteria*, *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chlamydiae*, *Chloroflexi*, *Spirochaetes*, *SR1*, *Synergistetes*, *Saccharibacteria* (TM7), and *Gracilibacteria* (GN02)[12]. Although a balanced oral microbiome is characterized by core similarities in relative occurrence of the latter species, more specific microbial diversity is unique to both the individual[13] and to different sites within the mouth[8]. For example, the tongue is rich with papillae and scant anaerobic locales, whereas the buccal and palatal mucosae are characterized by lower microbial diversity. Besides, the composition of the oral microbiome is dynamic[14], constantly influenced by various factors such as diet, hygiene, lifestyle, and the host's immune response.

Pathogenicity arises when bacteria circumvent the protective barrier formed by the commensals, leading to infections and diseases[15]. As such, species as *Streptococcus mutans* or *Porphyromonas gingivalis* may cause dental caries[16,17] and gingivitis[18] respectively. Simultaneously, such pathogenic bacterial species may cause

tooth plague by creating biofilms[19], as well as causing a drop in pH as a result of fermentable carbohydrate metabolism which causes enamel demineralization[20], or causing increased gum bleeding as a result of inflammatory responses[18]. Complications due to poor oral health can be prevented by maintaining a healthy basis of oral care[21]. Such routines include sufficiently brushing the teeth, flossing the teeth, and-or dietary measures in relation to sugar intake. On top of that, in recent years, the use of probiotics has emerged as a promising strategy to support the health of the oral microbiome[22].

Probiotics, which are beneficial microorganisms, have been shown to confer numerous health benefits when administered in adequate amounts. In the context of oral health, probiotic therapy aims to restore and maintain a healthy balance within the oral microbiome by enhancing the proliferation of beneficial microorganisms and inhibiting the growth of pathogenic species[22]. Studies have demonstrated the potential of probiotics in reducing the incidence of dental caries, alleviating the symptoms of gingivitis and periodontitis, and improving breath odor, highlighting their significance as an adjunctive tool for promoting oral health[22].

In this regard, species of the *Lactobacillus* genus as a probiotic are most often described due to several reasons[23]. First, species of the *Lactobacillus* genus adhere to the oral mucosa and tooth enamel[23], creating a barrier against pathogenic invaders. Besides, they produce a suite of antimicrobial substances, including bacteriocins, lactic acid, and hydrogen peroxide, which directly inhibit the growth of cavity-causing bacteria like *Streptococcus mutans*[23]. Additionally, the acidogenic nature of *Lactobacillus* helps maintain an acidic environment in dental biofilms, which can deter the colonization of harmful microbes[23,24]. Finally, *Lactobacillus* strains are adept at modulating the local immune response[25], thereby enhancing mucosal immunity and reducing inflammatory reactions often associated with periodontal diseases.

Currently, there are a few products on the market that include *Lactobacillus* species in a probiotic product destined to benefit oral health. In our intervention studies, we tested the LuvBiotics® Probiotic Lozenges. Their product claims to maintain healthier gums, restore the belance of oral flora, reduce incidence of mouth ulcers and accelerate the healing of such sores, prevent mycosis after antibiotics, and prevent tooth decay. LuvBiotics® uses their completely new and innovative 'Bionet' technology, which blends probiotics and xylitol with selected natural ingredients to deliver advanced dental hygiene. This technology is not yet described in literature, but the producers of LuvBiotics® claim that unlike most regular toothpastes which destroy most of the good and bad bacteria, bionet promotes growth of good bacteria to crowd out the bad, making it a kinder way to balance your oral microbiome. LuvBiotics claims to include a blend of 16 different live cultures in total, that either belong to *Lactobacilli*, *Bifidobacteria* and other specific species, selected based on bio-fermentation technology. The producers do not elaborate on the other bacterial species included in their product. Further, no literature was found on the exact efficiency of LuvBiotics® Probiotic Lozenges, but there are promising studies of similar products[26,27,28].

In this study, we test the effectiveness of administration of LuvBiotics® Probiotic Lozenges in a four-week administration phase. In this context, effectiveness of LuvBiotics® means that administration of the product as recommended by the producers causes (1) a decreased frequency of gum bleeding, (2) a reduced quantity of tooth plaque, (3) a restored salivary pH level between 6.2-7.6 if levels were high prior to administration, and (4) a positive shift in microbiome composition. The latter can be measured by 16s sequencing and a positive shift is regarded as an increase in relative abundance of beneficial species, such as *Lactobacillus* species, with a concurrent decreased relative abundance of pathogenic species, such as *Streptococcus mutans* and *Porphyromonas gingivalis*. The addition LuvBiotics® Probiotic Lozenge is hence expected to result in the abovementioned oral health benefits.

Methods

To measure the divergence between the oral microbiome before and after the use of probiotic mouthwash, several methods were conducted. The experiment included 5 participants. The experiment period consisted of 6 weeks in total: two weeks for the baseline period and four weeks for the use of the probiotic mouthwash. During these 5 weeks, pH, amount of plaque, and gum bleeding were regularly measured. After the baseline period and the experimental period, samples were taken for 16S sequencing.

LuvBiotics® Probiotic Lozenges

In this experiment, the lozenges from Luvbiotics were used. According to the information found on the Luvbiotics website[34], the Luvbiotics Lozenges contain Lactobacillus reuteri, xylitol, and natural ingredients

to promote live bacteria and balance the oral microbiome. The added xylitol, which is a natural sweetener, provides many dental benefits, such as reducing plaque build-up and tooth decay, and helps prevent cavities and gum diseases.

pH Measurements
Three times a week, on Tuesdays, Thursdays, and Sundays, pH was measured using a pH indicator strip from Macherey-Nagel REF 921 22, pH-fix 6.0-10.0. One hour after the last meal in the evening, 2 mL of saliva was captured using a small 12 mL tube. The pH strip was then placed in the tube and held there for more than 20 seconds. The color of the pH strip was compared to the indicator strip on the box.

Plaque Measurements
Tooth plaque was measured twice a week, on Wednesdays and Fridays. Plaque-search tablets from TePe were used according to the instructions on the packet. The number of teeth that were discolored blue or red was counted.

Gum Bleeding Measurements
Gum bleeding was measured every day by flossing the teeth after standard oral care. Gum bleeding measurements were collected by filling in the spreadsheet with "yes" (for gum bleeding) and "no" (for no gum bleeding).

Sampling for 16S Sequencings
Participants were asked not to brush their teeth the morning before sampling to allow plaque buildup. Plaque was collected from the six front teeth using a sterile omniSwab from Qiagen. The swabs for the baseline experiment were diluted in 200 µl of PBS and directly flash-frozen in liquid nitrogen. The cups with the swabs were stored at -80 degrees Celsius until further processing. The swabs for the experimental period were diluted in 500 µl of PBS and proceeded for DNA extraction.

DNA Extraction
Before DNA extraction, the cup with the swab was vortexed for 2 minutes. After vortexing, the swab was removed from the cup using a sterile pipette. DNA extraction was performed using the QIAamp DNA Microbiome Kit. DNA extraction was carried out according to the QIAamp Microbiome protocol, except for the steps that required 20,000 x g centrifugation. The Thermospin pro did not reach 20,000 x g, so the max spin centrifugation of the Thermospin pro was used instead. After extraction of the DNA, the concentration of the DNA was measured using the broad-range Qubit. DNA was stored at -80 degrees Celsius until further processing.

Statistics
For the measurements of the pH values and the amount of teeth with plaque, a QQ plot was performed to assess if the data was normally distributed. To analyze if there was a significant difference between the measurements for each participant before and during the use of the probiotic tablets, a two-sample Mann-Whitney U-test for two medians was conducted. Both statistical functions were employed according to Apol[35].

Metagenomic profile analysis using bracken and kraken2

Kraken2:
During this experiment, the sequencing reads without host, obtained from our DNA extraction from both samples taken during the baseline and the four-week test period from all the five participants, were classified using the kraken2 software program to generate an output report which was later used for further analysis by bracken.

Bracken:
Bracken then used the output report generated from the kraken2 software program with the classified read counts and estimated the abundance of each taxon in the samples obtained from the five participants from the baseline and four-week test period. The report finally generated by bracken was visualized using some python libraries and pavian.

Results

The amount of tooth plaque was measured using plaque-search tablets from TePe. The number of teeth showing discoloration on the day of testing was recorded in an Excel file. Tooth plaque was measured both before and during the use of the probiotic tablet.

The results, expressed in the number of discolored teeth, show that the mean amount of plaque before the use of the probiotic tablets differed per participant. The lowest mean amount of tooth plaque before using the probiotics was observed in participant 5, with a mean of 4.8 teeth with plaque. The highest amount of teeth with plaque was observed for participant 3, with a mean of 6.8. The remaining participants had values ranging between 4.8 and 6.8 teeth with plaque.

During the use of the probiotic, tooth plaque was also measured. Over time, there was no difference in the amount of tooth plaque for each participant. However, by comparing the mean amount of teeth with plaque before and during the use of the probiotic mouthwash, it was observed that the mean amount of teeth with plaque during the use of the probiotic mouthwash was lower than before using the probiotic mouthwash for participants 2 and 3. The mean amount of teeth with plaque before using the probiotic was lower for participants 1, 4, and 5.

The results of the QQ plot showed that all the data was normally distributed. Due to the small sample size, a two-sample Mann-Whitney U-test for two medians was performed. The result showed a significant difference in tooth plaque for participant 3 ($p = 0.0359$). All the other participants showed no significant difference.

pH **Results**

Introduction:

The critical function of saliva in oral health is well-recognized, particularly its role in digestive processes and maintaining the structural integrity of the mouth through its acid-base balance. Daily activities, including consumption and interactions with oral microorganisms, can disturb this balance, making the understanding of salivary pH essential.36 To explore this, our study initiated with a survey among dental students to assess their knowledge of the role of salivary pH in oral health, highlighting the importance of educational programs in this area.

pH Analysis Section with Visual Aids:

The focus of this investigation is the effect of probiotic mouthwash on salivary pH levels, measured before and after its use. Initial pH readings exhibited minor individual differences, and subsequent measurements identified a significant increase in pH for only one participant, suggesting person-to-person variability in microbiome response to the probiotic mouthwash.

Statistical Analysis:

Data were subjected to time series analysis, median value comparisons, normality testing, and quantile comparisons. Visual aids, such as Q-Q plots and distribution charts, are presented to illustrate pH fluctuations over the study period.

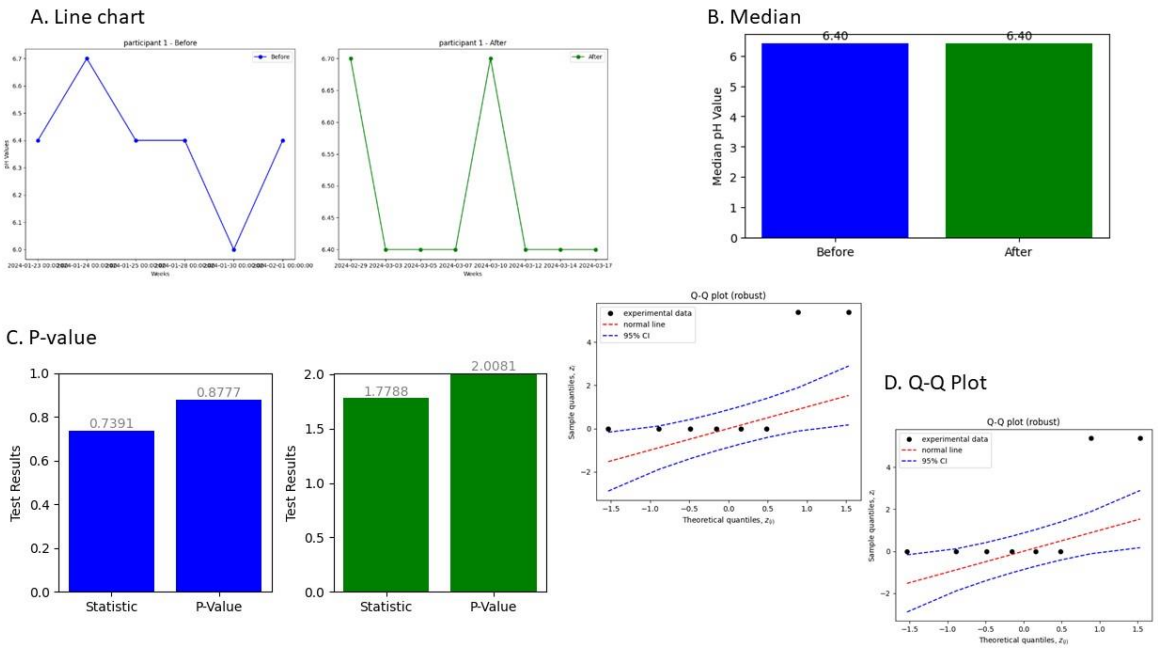

Figure 1: Visual Representations of Salivary pH Analysis in Probiotic Mouthwash Study

Panel A: Line charts showing salivary pH levels of a participant over consecutive weeks before and after probiotic mouthwash use, highlighting temporal trends and variations. Panel B: Comparative bar chart of the median pH value before and after intervention, demonstrating the central tendency of pH levels in the participant's saliva. Panel C: Statistical analysis bars illustrating the calculated test statistic and p-value, providing insight into the significance of the changes observed in pH levels. Panel D: Q-Q Plots assessing the normality of the pH data distribution against a theoretical normal distribution, with confidence intervals indicating the precision of the data fit.

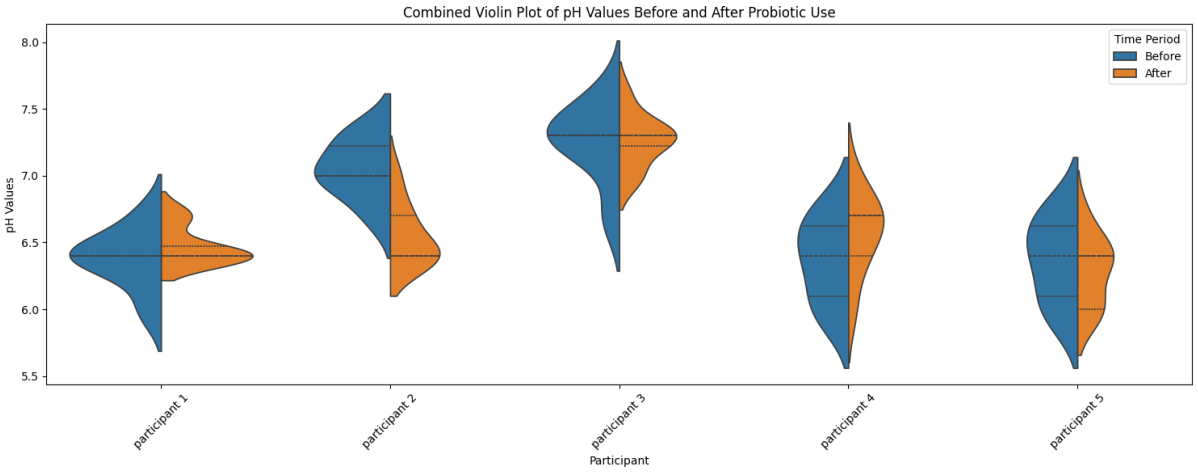

Figure B: Combined Violin Plot of pH Values Before and After Probiotic Use for All Participants. This plot visualizes the distribution and density of pH values for all participants, emphasizing the notable pH shift for Participant 2.

The survey of dental students underscored a considerable gap in the awareness of the specific pH levels associated with various oral health conditions. These educational insights, paired with the study findings, reveal a complex landscape of individual microbiome responses to probiotic interventions, which could be influenced by diet, genetics, and existing oral health.

It is important to note that our research, with its small sample size and lack of a control group, cannot provide conclusive evidence of the efficacy of probiotic mouthwash in modifying salivary pH. The significant pH change in one participant may indicate a potential for probiotics to influence oral health, but further research is needed to substantiate these findings.

Our study underlines the importance of understanding and maintaining the delicate acid-base balance in the oral cavity, potentially through the use of probiotics. However, the results also highlight the necessity for improved educational programs on oral pH within dental curricula. Future research should aim for larger sample sizes, include control groups, and consider additional variables to provide more definitive conclusions.

Results: Gum Bleeding

In the intervention study, distinct improvements in gum bleeding were observed across all participants. Participant 1 initially experienced multiple weekly episodes of gum bleeding, which reduced to three instances following probiotic intervention. Participant 2, who had isolated gum bleeding events prior to the intervention, reported complete cessation post-probiotic use. Participant 3 showed a decrease in gum bleeding from four incidents to two, indicating a reduction but not a complete cessation. Participant 4, who consistently experienced gum bleeding every week, noted a significant reduction to two episodes post-intervention. Finally, Participant 5, who occasionally had gum bleeding, reported no instances following the probiotic intervention, demonstrating the efficacy of the probiotic mouthwash in reducing gum bleeding among the participants.

The data indicates a general trend of reduced gum bleeding across all participants following probiotic use. While not all participants experienced complete cessation of bleeding, the reduction in frequency suggests potential benefits of probiotics in managing gum health. These findings align with existing literature that supports the role of probiotics in improving oral health parameters.

Statistical Analysis of Gum Bleeding:
The study recorded the total gum bleeding count and the proportion of days with gum bleeding before and after the introduction of the probiotic mouthwash across five participants.

Statistical Analysis Results:

The study showed that after using the probiotic mouthwash, all participants' gum bleeding metrics significantly improved. In particular, Participant 1 showed a drop in the overall number of gum bleeding episodes from six to three, along with a corresponding drop in the percentage of bleeding days from 37.5% to 10.71%. In participant 2, gum bleeding completely stopped; there were no more incidents, and the percentage of days with bleeding dropped from 12.5% to zero. Likewise, Participant 3 saw a drop in the number of gum bleeding episodes from four to two as well as a drop in the percentage of bleeding days from 25% to 7.14%. With a drop in the number of bleeding events from seven to two and a reduction in the percentage of bleeding days from 43.75% to 7.14%, participant 4 demonstrated a noticeable improvement. Additionally, participant 5 reported that there was no longer any gum bleeding at all, with two occurrences decreasing to zero and 12.5% of days having bleeding to zero. All these results point to a statistically significant decrease in the frequency and severity of gum bleeding in the participants as a result of using the probiotic mouthwash.

Discussion

Good health starts from a healthy mouth. This has been shown in several scientific research over time. This shows that improving the oral health of individuals can improve the quality of life of individuals and society [29]. Poor oral health has been linked to several diseases such as diabetes, cardiovascular diseases, Alzheimer's disease, dementia, elevated risk pregnancy, infertility amongst others.

According to WHO, 2024, oral health is the state of the mouth, teeth and orofacial structures that enables individuals to perform essential functions such as eating, breathing, and speaking, and encompasses psychosocial dimensions such as self-confidence, well-being, and the ability to socialize and work without pain, discomfort, and embarrassment. Oral health is integral to general health and supports individuals in participating in society and achieving their potential.

Maintaining a healthy mouth requires mechanical oral health products such as toothpaste, dental floss, and interproximal brushes. However, several studies have shown that these products are not entirely effective in maintaining oral health, as many people struggle to use them effectively. Chemical control methods like mouthwashes were added to supplement mechanical oral health products, aiding, and enhancing the maintenance of a healthy mouth. Mouthwashes serve as therapeutic products for maintaining good oral health [32].

Considering the potential long-term effects of synthetic mouthwashes currently on the market, researchers are actively conducting research to find alternative therapies that can help balance and maintain the health of the oral microbiome. Probiotics have been one of the main focuses of many clinical trials to help improve on the genomic profiles, health, and the balance of the oral human microbiome. These probiotic mouthwashes contain living microbes such as lactobacilli or Bifidobacterium, which are considered a part of oral microflora and could reduce the level of Streptococcus mutans in saliva by several mechanisms such as the production of antimicrobial agents (lactic acid, hydrogen peroxide, and bacteriocins), modulating the inflammatory response, and competing with pathogens for adhesion surfaces.

This research investigates the effectiveness of Luvbiotics lozenges, a probiotic mouthwash on market, on improving the genomic profiles and health of the oral human microbiome. The efficacy of the probiotic mouthwash was tested using PH testing, classification of gum bleeding (frequency) and classification of plaque occurrence. In this research, an efficacious probiotic mouthwash means a mouth wash being able to improve the oral health parameters by reducing gum bleeding prevalence, decrease in tooth plaque accumulation, maintenance of normal salivary PH levels, and a positive shift in the oral microbiome composition.

After the experiment, results for both baseline period and the test period were analyzed using the appropriate statistical tests including Mann Whitney test, Anderson Darling test, QQ plot, Mcnemar's test, and Wilcoxon-sign-ranked test, for the various experimental protocols. No statistically significant difference was observed in the frequency of gum bleeding between the results obtained from the five participants during the baseline period and the four-week test period. For plaque occurrence and salivary PH, a significant difference was seen in only one participant out of the five participants. The statistically significant difference in the one participant for salivary PH protocol, could be due to a confounding factor, which is intake of foods containing probiotic during the baseline period and four-week test period. Two participants out of the five participants continued with their intake of foods containing probiotics [1]but was tracked and one of them had their salivary PH showing a statistically significant difference. The other participant whose plaque occurrence showed a statistically significant difference took no foods containing probiotics during the baseline and the test period. So the statistically significant difference observed in the salivary PH of the other participant who took in foods containing probiotic the entire baseline period and the four week test period, could be due to chance or due to the probiotic foods taken during the test and baseline period or the probiotic mouthwash was just effective for this particular participant due to factors not known.

The foremost limitations of this study were the small sample size, the short test period and using the probiotic lozenges once daily. Therefore, further research of a longer test period, larger sample size and using the probiotic lozenges twice daily are needed to assess the effectiveness of probiotic mouthwash in improving the genomic profiles, health, and the balance of the oral human microbiome.

Conclusion

According to the results from this research, it can be concluded that Luvbiotics probiotic lozenges can be considered the least effective solution for maintaining oral health. These results could be because of the limitation of the research, which is believed to be small sample size, the short test period and using the probiotic lozenges once daily. By adjusting these conditions, better results can be achieved in further research on this product. Probiotic mouthwash appears to be a preferable alternative to synthetic mouthwashes, which have been shown to have adverse effects in numerous studies. Probiotic mouthwash offers a safer option that reduces the risk of adverse effects and promotes good oral health, thereby potentially improving individuals' overall health.

Additional Information

Disclosures

Human subjects: Consent was obtained or waived by all participants in this study. Institutional Ethical.

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Supplementary Information: Division of Tasks

Delinyah	Wrote full abstract and introduction, gathered references 1 to 28
Lily	Wrote full discussion, conclusion & additional information, method (kraken2 and bracken), gathered references 29-33
Jetske	Wrote method and plaque results, gathered references 34-35
Maryam	Wrote method and pH results, gathered references 36
Dipto	Wrote Gum Bleeding and statistical analysis