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The effect of daily usage of Listerine Cool Mint® mouthwash on the oropharyngeal microbiome

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Abstract

Listerine® is a bactericidal mouthwash used to prevent oral health problems such as dental plaque and gingivitis. It is unknown whether it promotes or undermines a healthy oral microbiome. We aimed to assess if daily usage of Listerine Cool Mint® influenced the composition of the pharyngeal microbiome. The current microbiome substudy is part of the PreGo trial conducted in 2019-2020, wherein sixty-four MSM taking HIV PrEP were enrolled. In this crossover trial, participants were received 3 months of daily Listerine followed by 3 months of placebo mouthwash or vice versa. Oropharyngeal swabs were taken at baseline and after 3 months use of each mouthwash. DNA was extracted for shotgun metagenomic sequencing (Illumina Inc.). Non-host reads were taxonomically classified with MiniKraken and Bracken. The diversity indices alpha and beta diversity were compared between baseline and after each mouthwash use. Differentially abundant bacterial taxa were identified using ANOVA-Like Differential Expression analysis. Streptococcus was the most abundant genus in most samples (n=103, 61.7%) with a median relative abundance of 31.5% [IQR 20.6 to 44.8], followed by Prevotella (13.5%, [IQR 4.8 to 22.6]) and Veillonella (10.0%, [IQR 4.0 to 16.8]). Compared to baseline, the composition of the oral microbiome at the genus level (beta diversity) was significantly different after 3 months Listerine (P=0.006, pseudo-F = 2.29) or placebo (P=0.003, pseudo-F = 2.49, PERMANOVA) use. Fusobacterium nucleatum and Streptococcus anginosus were significantly more abundant after Listerine use compared to baseline. Listerine use was associated with an increased abundance of common oral opportunistic bacteria previously reported to be enriched in periodontal diseases, oesophageal and colorectal cancer, and systemic diseases. These findings suggest that the applications of Listerine mouthwash should be carefully considered.
Introduction

The oral cavity harbours the second most complex microbial community of the human body (1). Over 700 bacterial species have been detected, the majority belonging to the phyla Actinobacteria, Bacteroides, Firmicutes, Fusobacteria, Proteobacteria and Spirochaetes (2). Interestingly, distinct compositions are found at different niches, such as the soft palates, the tonsils, and the tongue (2,3). The composition and diversity of these bacterial species provides a defence against pathogenic microbes colonizing the same niche: a mechanism called colonization resistance (4). Several factors, such as antimicrobial use and poor oral hygiene, are known to reduce colonization resistance, promoting the growth of opportunistic pathogens (5,6). There is increasing recognition that two of the most common bacterial diseases in humans, dental caries and periodontal diseases, are caused by a dysbiotic state of the oral microbiome (7). Oral bacteria including Neisseria, Rothia and Actinomyces also play an important role in several other functions, such as the conversion of dietary nitrate to nitrite, which is a precursor to nitric oxide and is crucial for cardiovascular health (8–10). Consequently, it is important to determine which external factors drive oral dysbiosis. Lifestyle choices including dietary habits and smoking have, for example, been linked with oropharyngeal dysbiosis (11).

Bactericidal mouthwashes are widely used to prevent common oral health problems, including dental plaque and gingivitis (12,13). In addition, the use of antiseptic mouthwashes such as Listerine® has been proposed to treat and prevent oropharyngeal sexually transmittable infections (STIs) (14–19), as there is a growing body of evidence that the pharynx is an important reservoir of infection and transmission of certain STIs (20,21). The STI Neisseria gonorrhoeae is of particular interest since it often colonizes the oropharynx and responds less well to antibiotics in this anatomical site (22).
Despite the fact that frequent mouthwash use is commonly reported, there is no consensus as to whether it promotes or undermines a healthy oral microbiome (23,24). Two studies found that fluoride and Listerine-zero mouthwashes had only trivial effects on the oral microbiome (25,26). In contrast, two other studies found that chlorhexidine mouthwash use resulted in an altered oral microbiome, reduced plasma nitrite and increased blood pressure (27,28). To contribute to this evidence base we evaluated if the regular use of Listerine Cool Mint® mouthwash affects the composition of the oropharyngeal microbiome.

Results

Study population

In 2019-2020, 343 men who have sex with men (MSM) taking HIV pre-exposure prophylaxis were included in a randomized, placebo-controlled, crossover trial that assessed if the regular use of Listerine Cool Mint® could reduce the incidence of bacterial STIs (Preventing Resistance in Gonorrhoea [PReGo] study) (15,29). Sixty-four MSM participated in the present, prespecified sub study, which aimed to assess whether three months of daily use of Listerine Cool Mint® mouthwash affects the composition of the oropharyngeal microbiome. Oropharyngeal swabs were collected from the 64 participants at baseline, 3- and 6-months. Ten of the 64 participants discontinued the study early – mainly due to the COVID-19 pandemic (15). Five samples taken at baseline were excluded as they contained less than 200 non-host reads. As a result, 167 samples were available from 64 men, representing 59 samples at baseline, 54 samples after the Listerine Cool Mint® period and 54 samples after the placebo period (Fig. 1, Supplement 1). Baseline characteristics are summarized in Table 1, and the ingredients of the mouthwashes are provided in Supplement 2.
Figure 1. Flowchart of the study population showing the number of oropharyngeal samples taken at baseline and after Listerine Cool Mint® and placebo mouthwash use is shown.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Participants of whom at least 1 swab was included in this study (n = 59)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age category, n (%)</td>
<td></td>
</tr>
<tr>
<td>20-29</td>
<td>9 (15.3%)</td>
</tr>
<tr>
<td>30-39</td>
<td>25 (42.4%)</td>
</tr>
<tr>
<td>40-49</td>
<td>11 (18.6%)</td>
</tr>
<tr>
<td>50-59</td>
<td>10 (16.9%)</td>
</tr>
<tr>
<td>60-69</td>
<td>3 (5.1%)</td>
</tr>
<tr>
<td>70-79</td>
<td>1 (1.7%)</td>
</tr>
<tr>
<td>Used a mouthwash in the month before participation, n (%)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>30 (50.8%)</td>
</tr>
<tr>
<td>No</td>
<td>29 (49.2%)</td>
</tr>
</tbody>
</table>

Table 1. Baseline characteristics of the study population

The majority of the study participants, 63.9%, used their respective study mouthwashes daily for at least 75% of the three-months period (Table 2). Mouthwash adherence did not differ significantly between the placebo and Listerine Cool Mint® periods. Half (n=30, 50.8%) of the participants used a mouthwash of any kind in the month before the baseline visit.
Table 2. Mouthwash adherence in the Listerine Cool Mint® and placebo periods

<table>
<thead>
<tr>
<th></th>
<th>Listerine Cool Mint® (n = 54)</th>
<th>Placebo (n = 54)</th>
<th>Overall (n = 108)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 75% of study period</td>
<td>17 (31.5%)</td>
<td>16 (29.6%)</td>
<td>33 (30.6%)</td>
</tr>
<tr>
<td>≥ 75% of study period</td>
<td>36 (66.7%)</td>
<td>33 (61.1%)</td>
<td>69 (63.9%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>1 (1.9%)</td>
<td>5 (9.3%)</td>
<td>6 (5.5%)</td>
</tr>
</tbody>
</table>

Composition of the oropharyngeal microbiome

A median of 1,567,223 [IQR 325,232 to 2,652,550] sequencing reads were obtained per sample after quality filtering. In total, we observed 1,310 bacterial genera (consisting of 4,940 species) across all samples, with 249 genera remaining after filtering species with a relative abundance <0.1% on the sample level. Of these, eleven genera were present in at least 75% of the participants. *Streptococcus* was the most abundant genus in most samples (n=103, 61.7%) with a median relative abundance of 31.5% [IQR 20.6 to 44.8]. The subsequent most abundant genera and their median relative abundance were *Prevotella* (13.5%, [IQR 4.8 to 22.6]), *Veillonella* (10.0%, [IQR 4.0 to 16.8]), *Neisseria* (4.6%, [IQR 0.4 to 13.2]) and *Haemophilus* (2.6%, [IQR 1.0 to 6.6]) (Fig. 2A).

The microbiome composition of the 30 most abundant genera across all samples revealed that samples did not cluster based on the visit (month 0 (baseline), month 3 and month 6) or mouthwash use (baseline, Listerine Cool Mint® and placebo) (Fig. 2B).
Figure 2. Microbiome composition. A) Relative abundances of the eleven core genera present in at least 75% of the participants. B) Relative abundances of the 30 most abundant genera across all samples. Each column represents a sample (n=167 samples from 64 men), and each row represents a bacterial genus. The dendrogram above the heatmap shows the similarity of microbiota composition between samples, created by hierarchical clustering of Euclidean distances with Ward linkage. The metadata bars above the heatmap indicate visit (i.e., month 0 (baseline), month 3 or month 6) and mouthwash use (i.e., baseline, Listerine Cool Mint® or placebo).
Effect of mouthwash on the oropharyngeal microbiome

Bacterial alpha diversity of the oropharyngeal microbiome on genus level did not significantly change after 3 months’ use of Listerine Cool Mint® (median Shannon diversity index 1.88 [IQR 1.60 to 2.10], \( P=0.679 \)) or placebo (median Shannon diversity index 1.83 [IQR 1.68 to 2.05], \( P=0.660 \)) compared to baseline (median Shannon diversity index 1.88 [IQR 1.68 to 2.12], Fig. 3 A).

Permutational multivariate analysis of variance (PERMANOVA) revealed that compared to baseline, the composition of the oral microbiome at the genus level was significantly different after 3 months Listerine Cool Mint® (\( P=0.006 \), pseudo-\( F = 2.29 \)) or placebo (\( P=0.003 \), pseudo-\( F = 2.49 \)) use (Fig. 3B).

Groups had homogenous variances (multivariate analogue of Levene's test for homogeneity of variances \( P>0.05 \)). Adjustment for mouthwash adherence did not contribute significantly to the analysis.

Figure 3. Bacterial diversity and composition. A) Alpha diversity (Shannon index) of bacterial genera of oropharyngeal samples at baseline and after three months of daily Listerine Cool Mint® or Placebo use. B) Principal Component Analysis (PCA) of the oropharyngeal microbial community on centred log-ratio-transformed bacterial genera at baseline and after Listerine Cool Mint® or Placebo use. Visits (month 0, 3 or 6) are indicated by circles, squares, or diamonds, respectively.
**Differentially abundant taxa**

ANOVA-like Differential Expression Analysis (ALDEx2) of the 75% most prevalent taxa identified one phylum, one genus and two species that were differentially abundant following false-discovery rate (FDR) correction (Wilcoxon test $P<0.05$) (30). At phylum level, *Actinobacteria* were less abundant after three months of Listerine Cool Mint® use versus baseline (median centred log-ratio (CLR) transformed abundance at baseline 3.82 versus Listerine Cool Mint® 2.69, $P=0.005$, Fig4). On the other hand, the genus *Fusobacterium* and the species *Fusobacterium nucleatum* in particular, were more abundant after 3 months of Listerine Cool Mint® use compared to baseline (median *Fusobacterium nucleatum* CLR transformed abundance at baseline 0.16 versus Listerine Cool Mint® 2.19, $P=0.003$) (Fig. 4). In addition, *Streptococcus anginosus* was more abundant after 3 months of Listerine Cool Mint® use compared to baseline (median CLR transformed abundance at baseline 0.24 versus Listerine Cool Mint® 2.58, $P=0.004$) (Fig. 4). There were no differentially abundant taxa after placebo use compared to baseline and between the placebo and Listerine Cool Mint® groups. Differentially abundant taxa found by ALDEx2 were all confirmed by Analysis of Compositions of Microbiomes (ANCOM-II) (31).
Figure 4. A) Relative abundance of the core (prevalence ≥75%) bacterial phyla and genera at baseline and following 3 months of placebo or Listerine Cool Mint® mouthwash use. B) Relative abundance of bacterial species detected as differentially abundant by ALDEx2 (False Discovery Rate > 0.05) after Listerine Cool Mint® use (n=167 specimens from 64 men).
Discussion

In this study, we assessed if daily usage of Listerine Cool Mint® mouthwash influenced the composition of the oropharyngeal microbiome. While bacterial alpha diversity did not significantly change, the composition of the oral microbiome was significantly different after Listerine Cool Mint® or placebo use compared to baseline. In particular, we found that Listerine Cool Mint® use was associated with an increased abundance of *S. anginosus* and *F. nucleatum*.

A number of studies have found that alcohol intake is associated with an increase in the abundance of *S. anginosus* and *F. nucleatum* (32–35). A clinical study from Japan that used real time PCR to quantify the relative abundance of streptococcal species in the saliva found that alcoholics had a 5-fold increased relative abundance of *S. anginosus* compared to four control populations (34). Furthermore, a case control study from Brazil found that alcoholics had higher mean counts of *F. nucleatum* in their subgingival swabs than non-alcoholics (32). The high concentration of alcohol in Listerine Cool Mint® (20%) thus provides a parsimonious explanation for the increased relative abundance of *S. anginosus* and *F. nucleatum* following the use of Listerine Cool Mint®. We did not however confirm the associations with real time PCR. In addition, within the *S. anginosus* species, multiple ribogroups exist, of which some are associated with invasive disease and others not (36). Using shotgun metagenomics data, we were unable to identify which specific ribogroup was elevated after the Listerine Cool Mint mouthwash use. None the less, these findings are worthy of further investigation.
Both organisms can cause severe invasive infections and have been linked to various types of cancer (37–39). *S. anginosus* is a member of the viridans streptococci, but unlike the other members of this group, *S. anginosus* should be considered a pathogen (40–42). It is typically responsible for suppurative, polymicrobial infections in the head, neck and abdomen. An important co-pathogen involved in these infections is *F. nucleatum*, which enhances abscess formation in combination with *S. anginosus* (43,44). In contrast, *F. nucleatum* is typically classified as an oral commensal, with the potential to cause opportunistic infections such as appendicitis and brain abscesses (45).

There is increasing epidemiologic, *in-vivo* and experimental evidence linking *S. anginosus* and *F. nucleatum* to colonic and oesophageal cancers, respectively (37–39,46,47). Alcohol consumption has been strongly linked to oral, oesophageal, colonic and other cancers (48,49). This effect is mediated in part by toxic metabolites of ethanol such as acetaldehyde (48,49). Much of this breakdown of ethanol into acetaldehyde is performed by particular bacterial (such as *S. anginosus*) and fungal species of the oral and intestinal microbiota (50). This is thought to explain why the concentration of acetaldehyde after alcohol consumption is typically an order of magnitude greater in the oral cavity than elsewhere in the body (51). This represents one pathway whereby microbes may mediate the risk between alcohol and cancer (48). In short, our findings corroborate those from other studies that have found that alcohol intake may increase the abundance of cancer-associated bacteria such as *S. anginosus* and *F. nucleatum* (32–35). Both pathways could explain the link that has been raised between extensive use of alcohol containing mouthwashes and oral cancer (52,53).
Another concerning finding was the decrease in the abundance of the phylum Actinobacteria after using Listerine Cool Mint®. Various Actinomyces species are part of the nitrate-reducing oral bacteria (54,55) which convert salivary nitrate to nitrite for further generation of the potent vasodilator nitric oxide (NO) (56,57). The nitrate-nitrite-nitric oxide pathway is an important mechanism linking the oral microbiome to cardiovascular health (56). While salivary and plasma levels of nitrate and nitrite greatly increase after nitrate ingestion, several studies have shown that prior mouthwash use dramatically reduces the oral conversion of nitrate (58–60). As a result, chlorhexidine and cetylpyridinium mouthwashes have been shown to increase blood pressure (59–61). This effect has not, however, been found after Listerine® or 0.35% povidone-iodine (59,61). Our findings provide potential further evidence of the detrimental effects of mouthwashes on the oral microbiome. However, this should be interpreted with caution as the prevalence and abundance of nitrate-reducing bacteria are considered to be influenced by individual factors such as age, sex, health and life-style, and we did not follow up on the blood pressure of our study participants (62).

Our results are in line with a range of studies that evaluated the impact of mouthwashes other than Listerine® on the oral microbiome. Studies of mouthwashes containing chlorhexidine have reported major shifts in the microbiome of the saliva and tongue of healthy individuals and subgingival plaque samples of gingivitis cases (27,28,63). Similar to our findings, one study reported a decreased abundance of Actinobacteria and an increase of *F. nucleatum* after chlorhexidine use in subjects with experimental gingivitis (64). On the other hand, studies using fluoride containing mouthwashes have found only a minimal effect on the microbiome (25,65).
Different mouthwashes may thus have different effects on the composition of the oral microbiome. The only report we could find of a study evaluating the effect of Listerine mouthwashes on the oral microbiome found it had little effect. This study assessed the effect of 3 months’ daily Listerine Zero® use compared to baseline and found a small but significant increase in alpha diversity, no changes in beta diversity, and identified no differentially abundant taxa (26). Several factors may explain the differences between this and our study. First, Listerine® Zero is alcohol-free, which may explain why the noted effects of alcohol on the microbiome were not observed with this mouthwash. Second, our work differs from the Listerine® Zero study in that the latter only amplified variable region V4 of the 16S rRNA gene, which has very little discriminatory ability in comparison to shotgun metagenomic sequencing (66).

Surprisingly, we found significant changes in the microbiome after use of the placebo mouthwash. The placebo mouthwash may thus have had an unintended impact on the oral microbiota. This could be explained by the fact that the placebo mouthwash was noted to have a slight impact on the growth of *N. gonorrhoeae* after prolonged contact time (67). We did not test its effects on other bacterial species residing in the oral cavity.

Our study had several limitations besides those already mentioned. The oral microbiome was analysed by taking swabs of both tonsillar pillars and the posterior oropharynx. As microbial communities can significantly differ between sites, our results may not be representative for the entire oral cavity (68).
Also, samples were taken with rather long (3-month) intervals, adherence to the mouthwash was not 100%, we did not control for non-adherence in our analyses, and we did not confirm changes in abundance of species with a second method. Furthermore, as only MSM were included, our findings may not be generalisable to the general population. Finally, mouthwash use prior to study enrolment may have diminished the observed impact of our study mouthwashes.

In conclusion, this study indicates that daily usage of Listerine Cool Mint® is associated with changes in the oral microbiome that may not be beneficial to the host. Further research should assess the impact of alcohol-containing mouthwashes on the oral microbiome and link such impact to clinical outcome parameters. In the interim, our findings are a reminder that the regular exposure to antibacterial products may have unintended consequences.

Methods

Study population and sampling

Sixty-four MSM using HIV pre-exposure prophylaxis were recruited as part of the Preventing Resistance in Gonorrhoea (PReGo) clinical trial and participated in the current microbiome substudy (NCT03881007). The PReGo trial was a randomized, placebo-controlled, crossover study evaluating the use of Listerine Cool Mint® to prevent STIs, conducted at the Institute of Tropical Medicine in Antwerp, Belgium in 2019-2020. A detailed description of the design of the main study, including entry criteria and randomization strategy, has been published (15,29).
In brief, participants were randomized to start using Listerine Cool Mint® (Listerine Cool Mint® - placebo arm) or a placebo mouthwash (placebo – Listerine Cool Mint® arm) (Figure 1). They were asked to use each study mouthwash for three months and were instructed to gargle and rinse once daily for 1 min with 20 mL of the mouthwash. They were also asked to use the mouthwash before and after sex with their partner.

Ingredients of the mouthwashes are provided in Supplement 2. Oropharyngeal swabs were taken by a study physician at the time of enrolment and after each mouthwash period by rubbing both tonsillar pillars and the posterior oropharynx with a dry FLOQSwab (COPAN, Brescia, Italy). All swabs were stored at -80°C within less than 4 hours and later transported on dry ice to the Laboratory of Medical Microbiology at the University of Antwerp for further processing. Prior to the collection of data and samples, participants provided informed consent. This study was approved by ITM’s Institutional Review Board (1276/18) and the Ethics Committee of the University of Antwerp (19/06/058). The research was performed in accordance with the relevant guidelines including the Declaration of Helsinki.

**Shotgun Metagenomic Sequencing**

Metagenomic DNA was extracted from oropharyngeal swabs using the FastDNA SPIN Kit (MP Biomedicals, Irvine, CA). Library preparation was done using the Nextera XT sample preparation and sequenced using Illumina NextSeq and Illumina MiSeq platforms using 2x150-bp and 2x250-bp paired-end reads, respectively (Illumina Inc., USA).

**Sequence and Data Analysis**

Sequence reads were trimmed for removal of low quality bases and sequences using Trimmomatic (v 0.39) whereafter host DNA was removed by mapping against the human
Non-host reads were then classified with MiniKraken2_v2_8GB (https://ccb.jhu.edu/software/kraken2) followed by abundance estimation with Bracken (v2.6.1) (72,73). Samples with less than 200 non-host reads were discarded and taxa identified as non-bacterial were removed.

Downstream analyses were performed in R (v. 4.1.2). Analyses were performed on taxa with a relative abundance above 0.1% per sample and analysed using the compositional data analysis approach as recommended for microbiome datasets (74). Normalization (relative abundance) and transformation (CLR-transformation) were done as applicable with the R package microbiome (v.1.16.0). Visualization of the microbiome composition was done with ggplot2 v.3.3.5 and ComplexHeatmap version 2.10.0 (75–77). Bacterial alpha diversity was assessed with the Shannon diversity index on genus level using the package phyloseq (78).

Changes in bacterial alpha diversity following mouthwash use were assessed using a mixed effect linear regression model with by-participant random intercepts. The overall oropharyngeal microbiome composition was visualized at baseline and after three months of Listerine Cool Mint® or placebo mouthwash use by principal component analysis (PCA) of CLR sequence data on genus level using the MicrobiotaProcess package v.1.7.8.992 (79).

Statistical differences between microbial composition at baseline and after the two study mouthwashes were analysed using permutational multivariate analysis of variance based on the Aitchison distance (Euclidean distances of CLR transformed sequence data) using the adonis function in vegan (v.2.5-7) with 999 permutations (80). Differentially abundant taxa after mouthwash use were identified using ANOVA-Like Differential Expression analysis (ALDEx2, v.1.26.0) on CLR transformed taxa present in at least 25% of samples with 128
Dirichlet Monte Carlo instances and a Wilcoxon signed-rank test following a Benjamini-Hochberg false-discovery rate (FDR, <0.05) correction (30).

Results were confirmed by the Analysis of Composition of microbiomes (ANCOM) tool with a cut-off value of 0.7 and FDR <0.05 (31). Both ALDEx2 and ANCOM indicated the genus *Ralstonia* to be differentially abundant between both mouthwash conditions and baseline (baseline – Listerine Cool Mint® and baseline – placebo). As this genus is a well-known laboratory contaminant, we decided to remove it from our dataset after filtering bacterial non-host reads (81). A sensitivity analysis including the genus *Ralstonia* gave similar results regarding alpha and beta diversity (Supplement 3). Sequences are publicly available in the NCBI Sequence Read Archive under the project accession number PRJNA862659.

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**Competing interests**

The authors declare that they have no conflict of interest.

**Data availability**

The datasets generated and/or analysed during the current study are available in the NCBI Sequence Read Archive under the project accession number PRJNA862659.

**Author contribution**
CK, JL, CVD, SB, TDB, SA and SMK conceptualized the study. JL, CVD, SB and BX conducted the bioinformatic analyses and wrote the first draft of the manuscript. All the authors contributed to the final draft.


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Supplementary Files

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- Supplementaryfile.pdf