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Chlorhexidine oral rinses for symptomatic COPD: a randomized, blind, placebo-controlled preliminary study

Running title: Chlorhexidine oral rinses for symptomatic COPD

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Abbreviations

BCSS	Breathlessness, Cough, and Sputum Scale
CLIMB	chlorhexidine effect in the oral and lung microbiota study
COPD	chronic obstructive pulmonary disease
CRP	c-reactive protein
FEV1	forced expiratory volume in the first second
FEV1pp	forced expiratory volume in the first second percent predicted
MVAMC	Minneapolis Veterans Affairs Medical Center
SD	standard deviation
SE	standard error
SGRQ	St. George's Respiratory Questionnaire
WBC	white blood cell count

Abstract

Objectives: Determine the effect of twice-daily chlorhexidine oral rinses on oral and lung microbiota biomass and respiratory symptoms.

Setting: Single center.

Participants: Participants were aged 40-85 with COPD and chronic productive cough or COPD exacerbation within the last year. Exclusions included antibiotics in the previous 2 months and/or those with less than four teeth. Forty-four participants were recruited and 40 completed the study.

Intervention: Participants were randomized 1:1 to twice-daily 0.12% chlorhexidine oral rinses vs. placebo for two months along with daily diaries. SGRQ, blood tests, oral rinse and induced sputum were collected at randomization and the final visit.

Primary and Secondary Outcomes: Primary outcome was a change in oral and sputum microbiota biomass. Secondary outcomes included: sputum and oral microbiota Shannon and Simpson diversity and taxonomy; inflammatory markers; BCSS and SGRQ scores.

Results: Neither the oral microbiota nor the sputum microbiota biomass decreased significantly in those using chlorhexidine compared with placebo (oral microbiota mean $\log_{10}$ difference [SE] = -0.103 [0.23], 95% CI: -0.59, 0.38, p=0.665; sputum microbiota 0.80 [0.46], 95% CI: -0.15, 1.75, p=0.096). Chlorhexidine decreased both oral and sputum microbiota alpha (Shannon) diversity (linear regression estimate [SE] oral: -0.349 [0.091], p=0.001; sputum -0.622 [0.169], p=0.001). Chlorhexidine use did not decrease systemic inflammatory markers compared to placebo (CRP [chlorhexidine 1.8 ± 7.5 vs. placebo 0.4 ± 6.8, p=0.467], fibrinogen [22.5 ± 77.8 vs. 10.0 ± 77.0, p=0.406], or leukocytes [0.2 ± 1.8 vs. 0.5 ± 1.8, p=0.560]). Chlorhexidine use decreased St.

George's Respiratory Questionnaire scores compared to placebo (chlorhexidine -4.7 ± 8.0 vs. placebo 1.7 ± 8.9, p=0.032).

Conclusions: We did not detect a significant difference in microbiota biomass due to chlorhexidine use. Chlorhexidine decreased oral and sputum microbiota alpha diversity and improved respiratory health-related quality of life compared to placebo.

Clinical Trial Registration: ClinicalTrials.gov NCT02252588

Strengths and Limitations of this Study

- Using a randomized control design, this study will provide the first example of the effects of altering the oral microbiome in the setting of COPD.
- A study intervention that is simple, inexpensive, and has few side effects.
- Our study was limited by its relatively small sample size and single-center design.
- Other limitations include our inability to distinguish between live and dead bacteria in our samples.

Introduction

Chronic obstructive pulmonary disease (COPD) is the 3rd-leading cause of death worldwide and a significant cause of morbidity and mortality.[1] COPD symptoms such as chronic cough, sputum production, breathlessness, and wheezing lead to decreased quality of life. COPD exacerbations are a major cause of this morbidity. Medications such as bronchodilators and anti-inflammatory medications modestly reduce COPD exacerbations but have not effectively improved symptoms as assessed by health status. Approximately 50% of COPD exacerbations are attributed to bacteria[2, 3] and patients with COPD often remain colonized with bacteria in their lower respiratory tracts even during periods of stable disease.[3] These bacteria make up the lung microbiota. Recent evidence supports that the oral microbiota is the main source of the lung microbiota.[4, 5] The COPD lung microbiota also correlates with COPD exacerbation frequency.[6] No studies have yet been conducted that seek to alter the COPD microbiota biomass using common and safe medications with only mild side effects.

Chlorhexidine is a topical antiseptic that is FDA-approved for use as an oral rinse.[7] It binds to bacterial cell walls and exerts bacteriostatic and bacteriocidal effects; it is broadly active against Gram positive and Gram negative bacteria as well as yeasts. In oral rinses it reduces dental plaque, gingivitis, periodontitis, and decreases oral bacteria after dental extractions or trauma. In meta-analysis, chlorhexidine oral rinses have been shown to reduce the risk of ventilator-associated pneumonia.[8] It is well-tolerated, with known side effects consisting of mild oral discomfort, transient decrease in taste, and tooth discoloration (particularly with tea or coffee consumption).

Randomized controlled trials of chlorhexidine oral rinses for dental diseases have shown some possible decrease in oral bacterial biomass,[9, 10] decrease in specific oral pathogens,[10] and decreased alpha diversity of the oral microbiota.[11] Oral chlorhexidine use results in an immediate and sustained decrease in oral bacteria viability.[12]

There is compelling evidence that chlorhexidine oral rinses improve oral health and are safe and well-tolerated. The oral microbiota is the source of the lung microbiota likely due to microaspiration. Among those with COPD, the oral and sputum microbiota correlate with COPD exacerbation frequency.[6] Oral treatment with chlorhexidine alters the oral microbiota, which may subsequently alter the lung microbiota and COPD-related symptoms. Our primary aim was to determine the effect of twice-daily chlorhexidine oral rinses on oral and lung microbiota biomass in participants with COPD.

Methods

The chlorhexidine effect in the oral and lung microbiota study (CLIMB) is a randomized, blind, placebo-controlled, parallel-group preliminary study of the effects of chlorhexidine oral rinses on COPD. It was conducted at a single tertiary-care Veterans Affairs medical center (USA). Ethics approval was granted by the Minneapolis Veterans Affairs Medical Center (MVAMC) Institutional Review Board (#4526-A; ClinicalTrials.gov NCT02252588), all participants provided written consent, and all procedures adhered to the study protocol. A data monitoring

committee did not oversee the study. Protocol and additional methods are provided in an online data supplement.

Patient and public involvement

The design of this study was based on previous randomized clinical trials designed for COPD exacerbations. We further received input from expert clinicians and researchers within the COPD Clinical Research Network. Patients with COPD were not involved in the development of the protocol, but participant feedback was obtained during the study.

Study Protocol:

Eligible participants were invited to participate in the study and consisted of those age 40-85 years with a diagnosis of COPD and the presence or high likelihood of a chronic cough and sputum production. Participants were excluded if they were not fully recovered for at least 30 days from a COPD exacerbation or were treated with antibiotics in the last two months.

Participants were assigned (1:1) via a random number generator to receive either 15 mL of twice-daily 0.12% oral chlorhexidine rinses (PerioGard®)[7] or matched placebo mouth rinses for eight weeks. The pharmacist conducted the allocation and assignment and was the only staff member unblinded to study assignment.

At visit 1, participants provided medical history, performed spirometry, completed the St. George's Respiratory Questionnaire (SGRQ),[13, 14] were instructed on how to complete the

Breathlessness, Cough, and Sputum Scale (BCSS)[15] daily diaries, and provided blood, oral, and induced sputum samples prior to randomization. Oral and sputum sample volumes were recorded. Participants returned 8 weeks later to return BCSS diaries, complete the SGRQ, assess outcomes, and provide blood, oral, and sputum samples.

The clinical laboratories at the MVAMC determined WBC and differential, fibrinogen, CRP levels, and sputum gram stain and culture results. All oral rinses, sputum samples, and unused sterile water (control samples) were frozen immediately and until DNA extraction. 16S rRNA quantification and 16S rRNA V4 MiSeq sequencing was performed at the University of Minnesota Genomics Center as previously described.[16]

Outcomes and Power Analysis:

The primary outcome was change in oral and sputum microbiota biomass after 8 weeks of chlorhexidine vs. placebo use, compared to baseline values as assessed by 16S rRNA quantification. The primary outcome was chosen based on the mechanism of action of chlorhexidine, however sample size calculations were based on a change in alpha diversity (a secondary outcome) due to data availability at study initiation. At a sample size of 20 per group and across a plausible range of effect sizes, our study had 67-94% power to detect a change in alpha diversity associated with chlorhexidine use. Sample size calculations are available in the online supplement. Secondary outcomes included: sputum and oral microbiota Shannon and Simpson diversity; sputum and oral microbiota taxonomy; inflammatory markers (WBC, fibrinogen, and CRP); BCSS scores; SGRQ score; and assessment of adverse events.

Statistical Analysis:

Baseline variables were compared using Fisher's Exact Test for categorical variables or the Wilcoxon Two-Sample Test for continuous variables. Means are presented with standard deviations (SD); mean differences and parameter estimates are presented with their associated standard error (SE).

All analyses were performed using SAS version 9.4 (SAS Institute) and the intention-to-treat principle. A two-sided type I error of 0.05 was used. Correction of the Type I error rate for multiple testing was performed using the Step-down Bonferroni method.[17]

For the primary analysis of both normalized oral wash and normalized sputum biomass count, values were transformed to the $\log_{10}$ scale and the mean difference between treatment groups was compared using the two-sample t-test. A multiple imputation procedure was used to impute each unavailable sputum weight.

Linear regression was used to examine the effect of treatment group on the 8-week change in the Shannon and Simpson biodiversity indices, BCSS, SGRQ and inflammatory markers separately, with each model adjusted for the baseline value of the measure.

Subgroup analyses of participants who did not receive antibiotics during the study were also performed for the outcomes of biomass and biodiversity.

For taxa abundance analyses, treatment effects on abundance were examined by modeling the 8-week change using linear regression, adjusted for baseline count. Analysis was restricted to genera with <20% of values equal to zero. Fisher's Exact Test was used to determine the proportion with a genus detected at Week 8 vs. baseline compared between treatment groups. Results were corrected for multiple comparisons.

Results

CLIMB assessed 511 participants for eligibility, excluded 215 because they did not meet criteria, 252 declined to participate, and 44 were randomized to study medication. Participants were recruited between September 8, 2014 and May 30, 2019 and the study ended when 40 participants completed the 8-week study. Four participants (all randomized to chlorhexidine) discontinued the study, leaving 20 participants in each group who completed the study. One participant withdrew without using any study medication, while the other 3 were lost to follow up (Figure 1). The primary data analysis included all those who completed the study, with baseline and mid-study phone call data included for non-completers when available. A sub-analysis of the microbiota data was conducted after excluding samples obtained from participants who used antibiotics during the study period.

Of the 44 CLIMB participants, 41 (93%) were male and 42 (95%) were Caucasian. The mean age was 67.9 years and mean tobacco exposure was 58.2 pack-years. Most were former tobacco

users (31, 70%) and the remainder were current smokers. High blood pressure (31, 70%) and coronary artery disease (27, 61%) were reported by most participants. Mean FEV_1 % predicted (FEV_1pp) was 41.7% and the mean number of COPD exacerbations reported in the prior 12 months was 2.1. Baseline mean SGRQ score was 45.8. No baseline characteristics differed significantly by treatment group (Table 1).

The number of participants experiencing a COPD exacerbation or using an antibiotic or oral corticosteroid during the study period are presented in Table 2. Eight participants (3 in the chlorhexidine group, 5 in the placebo group) received antibiotics during the study; most but not all antibiotic use was for a respiratory indication. No participants experienced more than one exacerbation, more than one course of antibiotics, or more than one course of oral corticosteroids during the study.

Our primary outcome was a change in oral and sputum microbiota biomass during the study period as assessed by 16S rRNA copy numbers. Sputum production was heterogeneous across participants and samples, so sputum sample 16S copy numbers were normalized to (i.e., divided by) sputum sample mass. Oral sample size also varied due to variations in expectoration efficiency and were therefore also normalized to oral sample mass. Oral rinse samples were available for 40 participants (20 per group). There was a decrease in biomass in both groups; the mean ± SD changes were -0.24 ± 1.0 and -0.14 ± 0.32 in the chlorhexidine and placebo groups respectively (Table 3a). The mean difference between treatment groups (active-placebo) was not significant (mean diff [SE] = -0.103 [0.23], 95% Confidence Interval [CI]: [-0.59,

0.38], p=0.665). Very similar results were seen in the subgroup that did not use antibiotics during the study (N=32, mean diff [SE] = -0.07 [0.29], 95% CI: [-0.65, 0.51], p=0.808) (Table 3b).

For the analysis of biomass in sputum samples, 5 chlorhexidine and 4 placebo participants were unable to provide sputum samples; 2 were unable at Baseline, 6 were unable at Week 8 (including the 4 withdrawals), and one was unable at both Baseline and Week 8. Sputum weight is required for biomass count normalization and among the remaining 35 samples, there were 11 missing sputum weight values (6 at Baseline, 5 at Week 8) among 4 placebo and 4 chlorhexidine participants. Table 3b shows the primary analysis results using a two-sample t-test with the normalized data available (N=27) and using a multiple imputation procedure to estimate the missing sputum weights (N=35). The two analysis methods provide similar results. Although we hypothesized that the estimate would be negative, indicating that the active group saw a larger decrease in biomass from Baseline to Week 8 than the placebo group, without imputation we see a non-significant effect in the opposite direction (mean $\log_{10}$ difference[SE] =0.80 [0.46], 95% CI = [-0.15, 1.75], p=0.096) and similarly with imputation (mean $\log_{10}$ difference[SE] = 0.70 [0.39], 95% CI = [-0.08, 1.47], p=0.078). These results were supported by the subgroup analyses of those without antibiotic use during the study. Although the p-value for the imputation analysis is significant (p=0.036) and the effect is not in the hypothesized direction, this result should be interpreted with caution due to the large number of tests reviewed here.

Linear regression was used to examine the 8-week change in each biodiversity index (Shannon and Simpson Indices) as a function of treatment group and adjusted for the value of the index at baseline (Table 4). As hypothesized, those in the chlorhexidine group saw, on average, a significant decrease in the diversity indices in comparison to those in the placebo group. For the oral wash samples, those in the treatment group had a coefficient of -0.349 (SE=0.091, adj. p=0.001) for the Shannon diversity index and -0.030 (SE=0.008, adj. p=0.001) for the Simpson diversity index. The results were similar for sputum samples: -0.622 (SE=0.169, adj. p=0.001) for the Shannon diversity index and -0.091 (SE=0.034, adj. p=0.0123) for the Simpson diversity index.

For the additional secondary outcomes, the effect of treatment group on the 8-week change was examined using linear regression, adjusted for the measure at baseline (Table 5). There was no significant difference between treatment groups over the 8-week study period in BCSS score (mean change in the chlorhexidine and placebo groups respectively ± SD: -0.3 ± 1.9 vs. -0.1 ± 1.5, estimate [95% CI] = -0.28 [-1.45, 0.89], p=0.630), CRP (1.8 ± 7.5 vs. 0.4 ± 6.8, 1.54 [-2.72, 5.80], p=0.467), fibrinogen (22.5 ± 77.8 vs. 10.0 ± 77.0, 20.19 [-28.52, 68.91], p=0.406), or leukocytes (0.2 ± 1.8 vs. 0.5 ± 1.8, -0.32 [-1.42, 0.78], p=0.560). Participants in the chlorhexidine group showed a significantly larger decrease in SGRQ total score when compared with the placebo group (mean change ± SD: -4.7 ± 8.0 vs. 1.7 ± 8.9, -6.22 [-11.87, -0.57], p=0.032). This difference was not evidenced in any one SGRQ domain.

In exploratory analyses, we evaluated the taxonomic composition of samples to assess for chlorhexidine-associated changes in the microbiota. Among sputum samples there were 42 genera. The results of the linear regression analyses showed that only *Corynebacterium* sequences were less abundant after chlorhexidine use compared with placebo (mean change in the chlorhexidine and placebo groups respectively ± SD: -197 ± 342 vs. 12 ± 337, estimate [95% CI] = -282 [-438, -126], adjusted p=0.0378). Among oral wash samples there were 43 genera. Only *Lachnoanaerobaculum* sequences were less abundant after chlorhexidine use compared to placebo (mean change in the chlorhexidine and placebo groups respectively ± SD: -313 ± 483 vs. 216 ± 509, estimate [95% CI] = -521 [-815, -226], adjusted p=0.043). Follow up analyses relying on the presence or absence of sequences (rather than relative abundance) produced similar results.

Very few adverse events were experienced over the course of the study (Table 6).

Discussion

In this preliminary study, twice-daily chlorhexidine oral rinses decreased oral and sputum microbiota alpha diversity and improved pulmonary disease-related quality of life compared to placebo among those with symptomatic COPD. Chlorhexidine oral rinses did not appear to decrease the oral or sputum microbiota biomass, our primary outcome, compared to placebo as assessed by normalized 16S rRNA quantitative PCR. Furthermore, during the 8-week

treatment period chlorhexidine did not appear to decrease systemic inflammation or COPD symptoms, as assessed by the BCSS, compared to placebo.

We chose a change in biomass as our primary endpoint as we hypothesized that twice daily chlorhexidine would have its largest effect on microbiota biomass. However, we did not detect a significant decrease in biomass as a result of chlorhexidine use utilizing quantitative PCR. Chlorhexidine is known to be bactericidal and previous work has identified a decrease in viable bacteria following chlorhexidine oral use compared to water. Our total DNA extraction technique coupled with PCR-based biomass determination is unable to distinguish between live and dead bacteria. It is therefore possible that chlorhexidine decreased the number of live bacteria in the oral and sputum microbiota, and that our PCR-based biomass determination technique was unable to distinguish between live bacterial biomass and dead bacteria. Furthermore, both groups experienced some decrease in biomass during the study period. Changes in dental care habits, including twice-daily oral rinsing with either study drug or placebo, may be responsible for this decrease.

Although total microbiota biomass did not appear to change, oral and sputum microbiota alpha diversity decreased as a result of chlorhexidine use. The healthy lung and oral microbiota generally demonstrate greater alpha diversity than the microbiota found in disease states such as COPD or cystic fibrosis. Whether this association is due to frequent use of antibiotics among those with chronic lung disease or due to the chronic lung disease itself remains unknown. Loss of alpha diversity due to chlorhexidine use may seem paradoxical given our current

understanding of the relationship between low alpha diversity and worsening lung symptoms, however the current disease model does not differentiate between alpha diversity *per se* and the mechanisms by which it may be manipulated. Loss of alpha diversity due to chlorhexidine use, antibiotic use, or chronic lung inflammation likely represent clinically distinct entities.

Use of chlorhexidine oral rinses vs. placebo did not result in decreased systemic inflammation as evidenced by CRP, fibrinogen and WBC values. These three systemic markers of inflammation are often elevated among those with symptomatic COPD. In light of our other findings linking chlorhexidine use to microbiota alterations and improved respiratory-related quality of life, we had expected that chlorhexidine use would lead to decreased systemic inflammation. It is possible that chlorhexidine use improved local inflammation (in the lungs or mouth) without resulting in systemic inflammatory changes. Sustained use over a longer time period may be needed in order to observe systemic anti-inflammatory effects.

Although chlorhexidine use did not result in significant changes to BCSS scores, respiratory health-related quality of life did improve with use of chlorhexidine oral rinses vs. placebo during the 8-week intervention. SGRQ scores improved significantly among the chlorhexidine group relative to the placebo group, with a mean improvement (4.7 points) that is clinically meaningful (minimum clinically important difference of 4 points). The SGRQ encompasses 3 sub-scores for activity, impacts, and symptoms. No sub-score reached statistical significance, indicating that chlorhexidine use improved quality of life broadly, and was not due to isolated improvements in one or two SGRQ sub-domains. Our data support the further study of

chlorhexidine oral rinses among symptomatic patients with COPD to improve respiratory health-related quality of life.

In an exploratory analysis of the effects of chlorhexidine use on the sputum and oral microbiota, the only genus-level changes in DNA abundance were a decrease in *Corynebacterium* in sputum and a decrease in *Lachnoanaerobaculum* in oral rinses. Chlorhexidine is known to broadly decrease the viability of bacteria and yeast. Our microbiota analysis techniques, which cannot differentiate between DNA from "live" or "dead" organisms, therefore may be relatively insensitive to the effects of chlorhexidine. We were unable to detect overall changes in bacterial biomass or broad changes to individual genera among those using chlorhexidine compared with placebo. It is possible that broader assessments of the community composition, such as alpha diversity, are better able to detect chlorhexidine-related changes.

Our preliminary study had several strengths and limitations. Its strengths include a study intervention that is simple, inexpensive, and has few side effects; the randomized and blinded nature of the study; and objective assessment of outcomes. Our study was limited by its relatively small sample size and single-center design. In addition, other limitations include our inability to distinguish between live and dead bacteria in our samples, incomplete sample weights, lack of assessment of local inflammation, and limited in-person follow up while on study drug. Future larger clinical trials will determine if the beneficial effects of chlorhexidine

oral rinses can be sustained among COPD subjects, and the biological mechanisms for these improvements in quality of life.

Although we did not find a difference in daily respiratory symptoms as measured with the BCSS, we found a significant improvement in quality of life as measured by the SGRQ. This potential discrepancy likely arose because BCSS focuses solely on respiratory symptoms, while the SGRQ also assesses the broader impacts of COPD symptoms on quality of life. There was no single domain within the SGRQ that drove this result, but there was improvement in both the impacts and symptoms domains. We propose that oral chlorhexidine rinses improve respiratory health-related quality of life by decreasing the number of live oral bacteria, altering the content of the live oral microbiota, or both. Changes to the oral microbiota may decrease the lung inflammation that occurs following aspiration or change the composition of the lung microbiota itself and lead to an improved sense of wellness.

An additional clinical trial is needed to confirm our clinical endpoint findings with a larger group of participants and evaluate the mechanistic links between chlorhexidine, viable bacterial biomass, the microbiota, and respiratory health-related qualify of life in symptomatic patients with COPD.

Our data indicate that the use of twice-daily chlorhexidine oral rinses among symptomatic patients with COPD improves quality of life. This was a secondary outcome in our study and

warrants validation in a larger clinical trial. Our intervention is relatively easy to implement, inexpensive, and well-tolerated.

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Study data were collected and managed using REDCap electronic data capture tools hosted at the University of Minnesota.[18, 19] REDCap (Research Electronic Data Capture) is a secure, web-based software platform designed to support data capture for research studies, providing 1) an intuitive interface for validated data capture; 2) audit trails for tracking data manipulation and export procedures; 3) automated export procedures for seamless data downloads to common statistical packages; and 4) procedures for data integration and interoperability with external sources.

Guarantor statement: Dr. Wendt is the guarantor of the content of the manuscript, including the data and analysis.

Author Contributions: AAP supervised research laboratory work, critically reviewed the data analyses, and wrote the first draft with input from AMF. AMF performed the statistical analyses with supervision from CSR, contributed to the first draft, and created the figure and tables. CSR supervised the statistical analyses and critically reviewed the manuscript. CHW obtained

funding, supervised subject recruitment, critically reviewed the data analysis, and critically reviewed the manuscript.

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Tables

Table 1. Baseline characteristics by treatment group

	Chlorhexidine	**Placebo**	**All Participants**
	Mean ± SD or N (%)	Mean ± SD or N (%)	Mean ± SD or N (%)
Number of Randomised Participants	**24**	**20**	**44**
Gender (% female)	2 (8.3)	1 (5.0)	3 (6.8)
Age (years)	67.6 ± 7.2	68.3 ± 6.0	67.9 ± 6.6
Race non-white	1 (4.2)	1 (5.0)	2 (4.5)
Season**			
Spring	3 (15.0)	6 (30.0)	9 (22.5)
Summer	7 (35.0)	4 (20.0)	11 (27.5)
Fall	7 (35.0)	6 (30.0)	13 (32.5)
Winter	3 (15.0)	4 (20.0)	7 (17.5)
Years smoked	40.8 ± 10.4	43.6 ± 10.3	42.0 ± 10.4
Current smoker	6 (25.0)	7 (35.0)	13 (29.5)
Pack Years	58.7 ± 32.9	57.6 ± 39.8	58.2 ± 35.8
SGRQ	49.2 ± 17.2	41.8 ± 12.3	45.8 ± 15.5
FEV_1 % predicted	39.9 ± 12.6	43.8 ± 11.1	41.7 ± 12.0
FVC % predicted	66.2 ± 14.8	71.4 ± 12.9	68.5 ± 14.1
COPD exacerbations (past 12 months)	2.3 ± 1.5	1.8 ± 1.0	2.1 ± 1.3
COPD hospitalizations (past 12 months)	0.5 ± 0.7	0.7 ± 0.7	0.6 ± 0.7

**Assigned to the season that covered >50% of the study period for a given participant.
Abbreviations: SD = Standard deviation; SGRQ = St. George's Respiratory Questionnaire; FEV_1 = Forced expiratory volume in one second; FVC = Forced vital capacity; COPD = Chronic obstructive pulmonary disease.

Table 2. Exacerbations, antibiotic use, or systemic steroid use during the study, excluding those withdrawn prior to study completion

	Chlorhexidine	**Placebo**
	N(%)	N(%)
No. of Randomised Participants Assessed	**20**	**20**
COPD Exacerbation[1]	3 (15.0)	5 (25.0)
Systemic steroid use[2]	1 (5.0)	5 (25.0)
Antibiotic use[3]	3 (15.0)	5 (25.0)

[1]Self-reported COPD exacerbation (worsening of chronic respiratory symptoms) during the study. One placebo subject reported an exacerbation but deferred any therapy until after study completion.
[2]Self-reported use of systemic corticosteroids during the study for any indication.
[3]Self-reported use of systemic antibiotics during the study for any indication. One placebo subject took antibiotics for a non-respiratory reason.
Abbreviations: COPD = Chronic obstructive pulmonary disease.

Table 3a. Biomass (log_{10}) from oral wash and sputum samples by treatment group

	Oral Wash (log_{10} molecules/µL/mL)				Sputum (log_{10} molecules/µL/gram)			
	Chlorhexidine (N=24)		Placebo (N=20)		Chlorhexidine (N=24)		Placebo (N=20)	
	N	Mean (SD)	N	Mean (SD)	N	Mean (SD)	N	Mean (SD)
Biomass at Baseline	24	5.22 (0.58)	20	5.38 (0.48)	21	6.07 (1.15)	13	6.66 (0.59)
Biomass at Week 8	20	4.94 (0.96)	20	5.24 (0.34)	17	6.35 (1.04)	15	6.31 (0.86)
Change in biomass	20	-0.24 (1.00)	20	-0.14 (0.32)	15	0.42 (1.24)	12	-0.38 (1.13)
Excluding antibiotic use	Chlorhexidine (N=21)		Placebo (N=15)		Chlorhexidine (N=19)		Placebo (N=10)	
Biomass at Baseline	21	5.23 (0.61)	15	5.48 (0.48)	19	6.14 (1.18)	10	6.77 (0.55)
Biomass at Week 8	17	4.93 (1.04)	15	5.29 (0.37)	16	6.39 (1.06)	11	6.19 (0.97)
Change in biomass	17	-0.26 (1.09)	15	-0.19 (0.31)	14	0.45 (1.28)	9	-0.62 (1.20)

Abbreviations: SD = Standard deviation.

Table 3b. Biomass analysis results – Two-sample t-test on the log_{10} change

Two sample t-test on log_{10} change (CHL-PLA)	N TOT	N CHL	N PLA	Mean difference (SE)[1]	95% CI	t value	P-value
All participants with available data							
Oral Wash Samples	40	20	20	-0.103 (0.23)	(-0.59, 0.38)	-0.44	0.665
Sputum Samples							
No imputation	27	15	12	0.80 (0.46)	(-0.15, 1.75)	1.73	0.096
Imputation[2]	35	19	16	0.70 (0.39)	(-0.08, 1.47)	1.76	0.078
Excluding antibiotic use during study							
Oral Wash Samples	32	17	15	-0.07 (0.29)	(-0.65, 0.51)	-0.25	0.808
Sputum Samples							
No imputation	23	14	9	1.06 (0.53)	(-0.05, 2.17)	1.99	0.060
Imputation[2]	28	16	12	0.98 (0.47)	(0.06, 1.89)	2.09	0.036

[1] Chlorhexidine group change in biomass minus placebo group.

[2] Imputation refers to the use of multiple imputation techniques to impute the 11 missing sputum weights.

Abbreviations: TOT = total, CHL = Chlorhexidine, PLA = Placebo, SE = Standard error, CI = Confidence interval.

Table 4. Linear regression results of the effect of treatment group on the change in biodiversity

	Linear Regression			
Outcome	**Predictor**	**Estimate(SE)**	**Unadjusted P-value**	**Adjusted P-value[1]**
Shannon Diversity Index Change (Week 8- Baseline)				
Oral Wash (N=40)	Treatment Group[2]	-0.349 (0.091)	0.0005	0.0010
	Baseline Index	-0.197 (0.073)	0.0100	
Sputum (N=35)	Treatment Group	-0.622 (0.169)	0.0008	0.0010
	Baseline Index	-0.312 (0.111)	0.0083	
Simpson Diversity Index Change (Week 8- Baseline)				
Oral Wash (N=40)	Treatment Group	-0.030 (0.008)	0.0005	0.0010
	Baseline Index	-0.196 (0.114)	0.0938	
Sputum (N=35)	Treatment Group	-0.091 (0.034)	0.0123	0.0123
	Baseline Index	-0.109 (0.179)	0.5472	

[1]A Step-down Bonferroni p-value adjustment is made for the two comparisons (oral wash and sputum) within each Diversity Index.

[2]Treatment group is coded as Chlorhexidine = 1, Placebo = 0.

Table 5. Linear regression analysis of the effect of treatment group on secondary outcomes

		Chlorhexidine (N=20)		**Placebo (N=20)**	**Treatment Group[1]**	**P-value[2]**
Outcome: 8-week Change[3]	N	Mean ± SD	N	Mean ± SD	Estimate (95% CI)	
BCSS	19	-0.3 ± 1.9	18	-0.1 ± 1.5	-0.28 (-1.45, 0.89)	0.630
C-reactive Protein (mg/L)	20	1.8 ± 7.5	20	0.4 ± 6.8	1.54 (-2.72, 5.80)	0.467
Fibrinogen (mg/dL)	19	22.5 ± 77.8	20	10.0 ± 77.0	20.19 (-28.52, 68.91)	0.406
Leukocytes (K/cmm)	20	0.2 ± 1.8	19	0.5 ± 1.8	-0.32 (-1.42, 0.78)	0.560
SGRQ Total Score	20	-4.7 ± 8.0	20	1.7 ± 8.9	-6.22 (-11.87, -0.57)	0.032
Activity Domain	20	-0.5 ± 9.1	20	3.9 ± 12.9	-3.84 (-10.92, 3.24)	0.279
Impacts Domain	20	-5.4 ± 12.6	20	0.7 ± 10.0	-5.46 (-12.92, 1.99)	0.146
Symptoms Domain	20	-10.1 ± 15.2	20	0.8 ± 18.8	-6.81 (-17.82, 4.19)	0.217

[1] Treatment group is coded as Chlorhexidine = 1, Placebo = 0.

[2] The p-value is for the comparison of chlorhexidine vs. placebo.

[3] Each model is adjusted for the baseline value of each outcome.

Abbreviations: SD = Standard deviation; CI = Confidence interval; BCSS = Breathlessness, Cough and Sputum Scale; SGRQ = St. George's Respiratory Questionnaire.

Table 6. Adverse events by treatment group

	Week 4 Phone Call		Week 8 Visit	
	Chlorhexidine[1]	**Placebo**	**Chlorhexidine**	**Placebo**
Number of Randomized Participants	**23**	**20**	**20**	**20**
Irritation and/or sores of the lining of the mouth, N(%)	0 (0.0)	1 (5.0)	0 (0.0)	1 (5.3)
Allergic reaction (swelling, rash, or hives), N(%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Change in taste, N(%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Discoloration of teeth, N(%)	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.3)
Other side effects, N(%)[2, 3]	2 (8.7)	3 (15.0)	2 (11.1)	0 (0.0)

[1] Due to study withdrawals, adverse effects were assessed for 23 of 24 chlorhexidine participants at Week 4 and 20 of 24 at Week 8.

[2] Other adverse effects in the chlorhexidine group - dry mouth (1 patient at Week 4), feeling of loose teeth + cough + green tinged sputum (1 patient at Week 4), widening gaps in teeth (1 patient at Week 8) and blue tongue for 15-20 minutes after using drug (1 patient at Week 4), widening gaps in teeth (1 patient at Week 8) and blue tongue for 15-20 minutes after using drug (1 patient at Week 8).

[3] Other adverse effects in the Placebo group - increased congestion (1 patient at Week 4), sinus/nasal infection (1 patient at Week 4), and dry mouth/dry cough (1 patient at Week 4).

Figure Legend

Figure 1. CLIMB study consort diagram. CLIMB assessed 511 individuals for eligibility. Of these, 467 were excluded and 44 were randomized. Four participants (all assigned to the chlorhexidine group) discontinued the study. Forty participants completed the study.

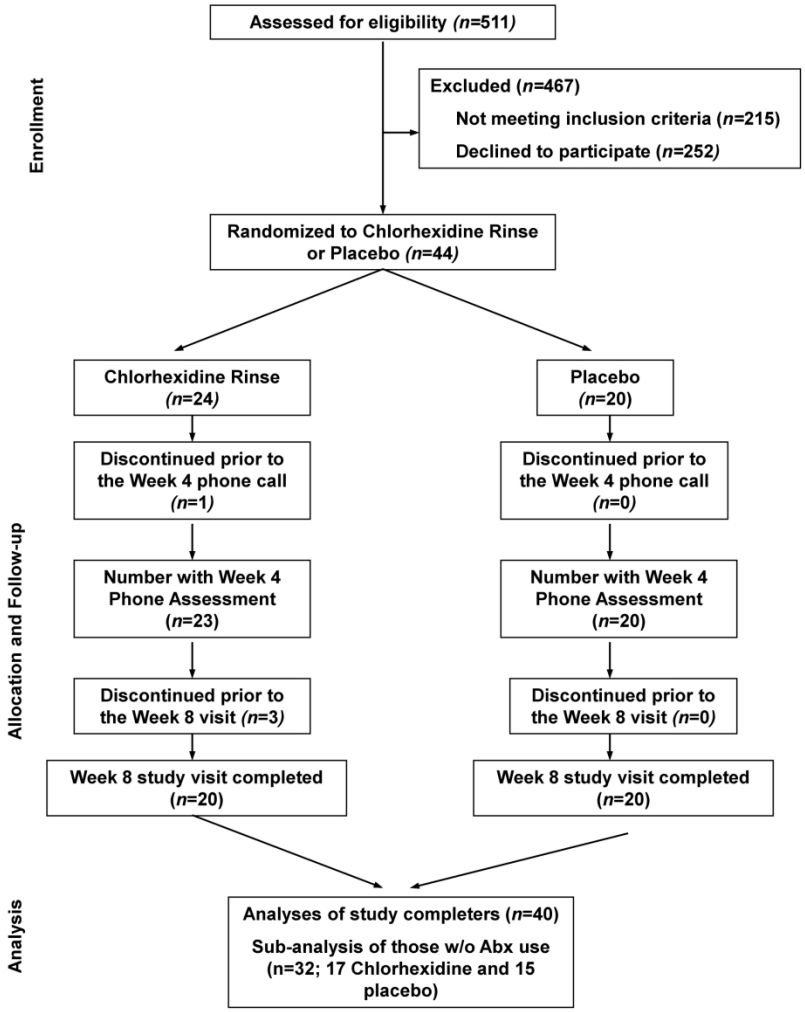

Figure 1. CLIMB study consort diagram. CLIMB assessed 511 individuals for eligibility. Of these, 467 were excluded and 44 were randomized. Four participants (all assigned to the chlorhexidine group) discontinued the study. Forty participants completed the study.

215x279mm (300 x 300 DPI)

Chlorhexidine oral rinses for symptomatic COPD: a randomized, blinded, placebo-controlled preliminary study

Alexa A. Pragman, Ann M. Fieberg, Cavan S. Reilly, and Chris H. Wendt

Online Data Supplement

Supplemental Methods

Study design

The chlorhexidine effect in the oral and lung microbiota study (CLIMB) is a randomized, blind, placebo-controlled, parallel-group preliminary study of the effects of chlorhexidine oral rinses on COPD. It was conducted at a single tertiary-care Veterans Affairs medical center (USA). Ethics approval was granted by the Minneapolis Veterans Affairs Medical Center (MVAMC) Institutional Review Board (#4526-A) and all procedures adhered to the study protocol (available in supplementary information).

Participants

Eligible CLIMB participants were those age 40-85 years receiving care at the MVAMC with a clinical diagnosis of COPD, a FEV_1/FVC ratio (post-bronchodilator) ≤ 70%, FEV_1 (post-bronchodilator) ≤ 65%, current or former smokers with lifetime cigarette consumption of ≥ 10 pack-years, presence of ≥ 4 natural teeth, and the presence of high likelihood of a chronic cough and sputum production defined as one of the following: 1) self-report of either cough or sputum production occurring “several days per week” or “almost every day”; or 2) a COPD exacerbation within the previous 12 months (defined as taking antibiotics and/or prednisone for respiratory symptoms, being hospitalized, or visiting the emergency department for respiratory illness). Participants were excluded if they were pregnant, not fully recovered for at least 30 days from a COPD exacerbation, treated with antibiotics (for any indication) in the last two months, had an active oral infection (e.g., dental abscess), currently used chlorhexidine oral rinses, had a known allergy or sensitivity to chlorhexidine, or used supplemental oxygen.

Presence of chronic cough, chronic sputum production, or COPD exacerbation in the last 12 months were used to enrich the study population with participants able to produce sputum and to report respiratory symptoms. The presence of at least four natural teeth was used to maintain consistency of the oral microbiota across participants, as the edentulous oral microbiota is different from the dentate oral microbiota. Likewise, participants who had not completely recovered from a COPD exacerbation, used antibiotics in the last two months, or had a dental infection were excluded to ensure that microbiota samples were collected from participants at their baseline.

Participants were recruited from those visiting the Emergency Department or admitted to the hospital for a COPD exacerbation, and among those participating in COPD case management due to frequent COPD exacerbations. All participants provided written informed consent.

Randomization and masking

Participants were recruited by the study coordinator and randomly assigned (1:1) via a random number generator to receive either 15 mL of twice-daily 0.12% oral chlorhexidine rinses (PerioGard®)[1] or matched placebo mouth rinses for eight weeks. Randomization was not stratified. Matched placebo was compounded by the research pharmacist and consisted of sterile water with blue dye (FD&C#2), polysorbate, and sodium saccharin for flavoring. The pharmacist conducted the allocation and assignment and was the only one unblinded to study assignment. Study medications were dispensed directly to participants in identical opaque

bottles. Participants as well as those interacting with participants (study coordinator and investigators) were blinded to group assignment during the conduct of the study. Investigators adjudicated antibiotic use and exacerbations after unblinding, but these data were used for post-hoc subgroup analyses and not in the primary or secondary outcome analyses.

Procedures

At visit 1, participants provided details of their medical history, performed spirometry, completed the St. George's Respiratory Questionnaire (SGRQ), were instructed on how to complete the Breathlessness, Cough, and Sputum Scale (BCSS)[2] daily diaries during the study, and then provided blood, oral, and induced sputum samples prior to randomization. Blood samples were used to determine white blood cell count and differential, fibrinogen, and C-reactive protein (CRP). Oral and sputum samples were obtained after at least a two-hour fast. Oral samples were obtained by swishing 15-mL sterile water in the mouth for 30 seconds and then spitting the water into a sterile cup. Sputum induction was performed with nebulized 3% saline (0.9% saline if FEV_1 <35%) for up to 20 minutes. Nebulization was terminated when participants either expectorated a 5 mL sputum sample into a sterile cup, 20 minutes of induction had elapsed, or the peak flow dropped to ≤ 80% of the baseline value. Unused sterile water was collected for use as control samples in microbiota analyses. Oral and sputum sample volumes were recorded. Using sterile technique, sputum samples were divided for cell count and gram stain performed by the clinical microbiology laboratory and microbiota analyses (including biomass quantification).

Participants were instructed to swish 15 mL of the study medication (either 0.12% chlorhexidine or placebo) in their mouth for 30 seconds twice daily (morning and evening) followed by expectoration. The study mouth rinse was used twice daily for eight weeks. Participants were told to avoid routine dental clinic visits during the study period.

After four weeks of study mouth rinse use, the study coordinator conducted a mid-study phone call to assess for hospitalizations, Emergency Department visits, unplanned clinic visits, new medication use (including antibiotics), compliance with the study drug, BCSS diary completion, and to assess adverse events. Additional study drug was mailed to participants by the research pharmacist following this phone call.

Eight weeks after randomization participants returned for a second study visit. They were instructed not to use the study mouth rinse the morning of the visit. Participants returned completed BCSS diaries and used study medication bottles to the study coordinator. Participants again completed the SGRQ, completed questionnaires (assessing medication changes, hospitalizations, Emergency Department visits, unplanned clinic visits, new medication use), and provided samples (blood, oral rinses, and induced sputum) for biomarker and microbiota analyses.

The BCSS (a daily diary for tracking the severity of respiratory symptoms) was started on Day 1, the day of first treatment. Participants answered three symptom questions on a 0 to 4 scale and a total daily score was calculated from those answers. Baseline BCSS score was the average

of daily BCSS measurements from Days 1-7. Week 8 BCSS score was the average of daily BCSS measurements from Days 50-56.

The SGRQ is a self-administered questionnaire that measures pulmonary disease-related quality of life. It has been validated for use in many chronic lung diseases, including COPD.[3] The SGRQ is scored on a scale of 0 to 100, with 100 reflecting the most severe symptoms. The minimum clinically important difference in the SGRQ is widely accepted as being 4 units.[4]

The clinical laboratories at the MVAMC determined WBC and differential, fibrinogen, CRP levels, and sputum gram stain and culture results. All oral rinses, sputum samples, and unused sterile water (control samples) were frozen immediately and until DNA extraction. 16S rRNA quantification and 16S rRNA V4 MiSeq sequencing was performed at the University of Minnesota Genomics Center as previously described.[5]

Outcomes

The primary study outcome was change in oral and sputum microbiota biomass after 8 weeks of study medication use, compared to baseline values, in participants who used 0.12% chlorhexidine oral rinses vs. placebo as assessed by 16S rRNA quantification. To adjust biomass for the size of the sputum sample, raw counts were normalized by dividing by the sample volume or mass. Secondary outcomes (all compared to baseline values in participants receiving chlorhexidine vs. placebo) included: i) sputum and oral microbiota alpha diversity (as assessed by Shannon and Simpson diversity); ii) sputum and oral microbiota taxonomy; iii) inflammatory

markers (WBC, fibrinogen, and CRP); iv) BCSS scores (week 8 vs. week 1); v) SGRQ score; and vi) assessment of adverse events. Adverse events were assessed both during the mid-study phone call and at the second visit by assessing hospitalizations, new medication use, and death. Participants were asked specifically about known adverse events associated with chlorhexidine oral rinses (oral pain, decreased taste, and tooth discoloration) and open-ended questions about new symptoms.

Statistical analysis

The power analysis for the study examined the power to detect differences in lung microbiota diversity as measured by the Simpson (1-D) diversity measure, ranging from 0.22 to 0.32 between the treatment groups using a two-sample t-test with equal variances. Our data showed that age had a significant positive impact on Simpson diversity with a change in diversity of 0.34 and the averaged standard deviation in the Simpson measure among moderate and severely affected COPD patients of 0.281. If chlorhexidine were to have an effect size similar to the effect of age with 20 participants per group, there was 67%, 75%, 81%, 87%, 91%, and 94% power to detect a difference of 0.22, 0.24, 0.26, 0.28, 0.30, and 0.32 between treatment groups.

Baseline variables were compared using Fisher's Exact Test for categorical variables or the Wilcoxon Two-Sample Test for continuous variables. Means are presented with standard deviations (SD); mean differences and parameter estimates are presented with their associated standard error (SE).

All analyses were performed using SAS version 9.4 (SAS Institute) and the intention-to-treat principle. A two-sided type I error of 0.05 was used. Correction of the Type I error rate for multiple testing was performed for the endpoints that report results from both oral wash and sputum samples using the Step-down Bonferroni method.[6]

For the primary analysis of both normalized oral wash and normalized sputum biomass count, values were transformed to the $\log_{10}$ scale and the mean difference between treatment groups was compared using the two-sample t-test. Additionally, for the analysis of sputum biomass count, a multiple imputation procedure was used to impute each unavailable sputum weight (PROC MI with seed=501213, MCMC method, and acceptable value range of 0.01 to 2.5). For each of the 25 datasets created by the procedure, the normalized biomass (count/mass) was calculated, the values were transformed to the $\log_{10}$ scale, and a t-test was performed. Lastly, PROC MIANALYZE was used to obtain an estimate from the t-test that accounted for the variability in the imputed values.

Linear regression was used to examine the effect of treatment group on the 8-week change in the Shannon and Simpson biodiversity indices, BCSS, SGRQ and inflammatory markers separately, with each model adjusted for the baseline value of the measure.

Subgroup analyses of participants who did not receive antibiotics during the study were also performed for the outcomes of biomass and biodiversity.

For taxa abundance analyses, the number of sequences assigned to each genus were determined for each sputum and oral wash sample. Treatment effects on the abundance of each genera were examined by modeling the 8-week change using linear regression, adjusted for baseline count. We restricted the analyses to the genera with <20% of values equal to zero. In addition, the proportion with organisms detected at Week 8 was compared between treatment groups for each genus using Fisher's Exact Test. Results were corrected for multiple comparisons.

A data monitoring committee did not oversee the study. The study was registered in ClinicalTrials.gov as NCT02252588.

Role of funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

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CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	5
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	2
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	4-5
	2b	Specific objectives or hypotheses	5
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	5-6
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	6
Participants	4a	Eligibility criteria for participants	6
	4b	Settings and locations where the data were collected	6
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	5-7
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	7
	6b	Any changes to trial outcomes after the trial commenced, with reasons	NA
Sample size	7a	How sample size was determined	7
	7b	When applicable, explanation of any interim analyses and stopping guidelines	NA
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	6
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	6
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	6
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	6
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those	6

		assessing outcomes) and how	
	11b	If relevant, description of the similarity of interventions	NA
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	7-8
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	8
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	9
	13b	For each group, losses and exclusions after randomisation, together with reasons	9
Recruitment	14a	Dates defining the periods of recruitment and follow-up	9
	14b	Why the trial ended or was stopped	NA
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	9
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	9
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	10
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	10
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	11-13
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	13
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	16
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	16-17
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	16-17
Other information			
Registration	23	Registration number and name of trial registry	3
Protocol	24	Where the full trial protocol can be accessed, if available	3
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	facepage

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.

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Chlorhexidine oral rinses for symptomatic COPD: a randomized, blind, placebo-controlled preliminary study

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Primary Subject Heading:	Infectious diseases
Secondary Subject Heading:	Respiratory medicine
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Chlorhexidine oral rinses for symptomatic COPD: a randomized, blind, placebo-controlled preliminary study

Running title: Chlorhexidine oral rinses for symptomatic COPD

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Prior presentation: This work was previously presented virtually at IDWeek 2020 (October 21, 2020).

Abbreviations

BCSS	Breathlessness, Cough, and Sputum Scale
CLIMB	chlorhexidine effect in the oral and lung microbiota study
COPD	chronic obstructive pulmonary disease
CRP	c-reactive protein
FEV1	forced expiratory volume in the first second
FEV1pp	forced expiratory volume in the first second percent predicted
MVAMC	Minneapolis Veterans Affairs Medical Center
SD	standard deviation
SE	standard error
SGRQ	St. George's Respiratory Questionnaire
WBC	white blood cell count

Abstract

Objectives: Determine the effect of twice-daily chlorhexidine oral rinses on oral and lung microbiota biomass and respiratory symptoms.

Setting: Single center.

Participants: Participants were aged 40-85 with COPD and chronic productive cough or COPD exacerbation within the last year. Exclusions included antibiotics in the previous 2 months and/or those with less than four teeth. Forty-four participants were recruited and 40 completed the study.

Intervention: Participants were randomized 1:1 to twice-daily 0.12% chlorhexidine oral rinses vs. placebo for two months along with daily diaries. SGRQ, blood tests, oral rinse and induced sputum were collected at randomization and the final visit.

Primary and Secondary Outcomes: Primary outcome was a change in oral and sputum microbiota biomass. Secondary outcomes included: sputum and oral microbiota Shannon and Simpson diversity and taxonomy; inflammatory markers; BCSS and SGRQ scores.

Results: Neither the oral microbiota nor the sputum microbiota biomass decreased significantly in those using chlorhexidine compared with placebo (oral microbiota mean $\log_{10}$ difference [SE] = -0.103 [0.23], 95% CI: -0.59, 0.38, p=0.665; sputum microbiota 0.80 [0.46], 95% CI: -0.15, 1.75, p=0.096). Chlorhexidine decreased both oral and sputum microbiota alpha (Shannon) diversity (linear regression estimate [SE] oral: -0.349 [0.091], p=0.001; sputum -0.622 [0.169], p=0.001). Chlorhexidine use did not decrease systemic inflammatory markers compared to placebo (CRP [chlorhexidine 1.8 ± 7.5 vs. placebo 0.4 ± 6.8, p=0.467], fibrinogen [22.5 ± 77.8 vs. 10.0 ± 77.0, p=0.406], or leukocytes [0.2 ± 1.8 vs. 0.5 ± 1.8, p=0.560]). Chlorhexidine use decreased St.

George's Respiratory Questionnaire scores compared to placebo (chlorhexidine -4.7 ± 8.0 vs. placebo 1.7 ± 8.9, p=0.032).

Conclusions: We did not detect a significant difference in microbiota biomass due to chlorhexidine use. Chlorhexidine decreased oral and sputum microbiota alpha diversity and improved respiratory health-related quality of life compared to placebo.

Clinical Trial Registration: ClinicalTrials.gov NCT02252588

Strengths and Limitations of this Study

- Using a randomized control design, this study will provide the first example of the effects of altering the oral microbiome in the setting of COPD.
- A study intervention that is simple, inexpensive, and has few side effects.
- Our study was limited by its relatively small sample size and single-center design.
- Other limitations include our inability to distinguish between live and dead bacteria in our samples.

Introduction

Chronic obstructive pulmonary disease (COPD) is the 3rd-leading cause of death worldwide and a significant cause of morbidity and mortality.[1] COPD symptoms such as chronic cough, sputum production, breathlessness, and wheezing lead to decreased quality of life. COPD exacerbations are a major cause of this morbidity. Medications such as bronchodilators and anti-inflammatory medications modestly reduce COPD exacerbations but have not effectively improved symptoms as assessed by health status. Approximately 50% of COPD exacerbations are attributed to bacteria[2, 3] and patients with COPD often remain colonized with bacteria in their lower respiratory tracts even during periods of stable disease.[3] These bacteria make up the lung microbiota. Recent evidence supports that the oral microbiota is the main source of the lung microbiota.[4, 5] The COPD lung microbiota also correlates with COPD exacerbation frequency.[6] No studies have yet been conducted that seek to alter the COPD microbiota biomass using common and safe medications with only mild side effects.

Chlorhexidine is a topical antiseptic that is FDA-approved for use as an oral rinse.[7] It binds to bacterial cell walls and exerts bacteriostatic and bacteriocidal effects; it is broadly active against Gram positive and Gram negative bacteria as well as yeasts. In oral rinses it reduces dental plaque, gingivitis, periodontitis, and decreases oral bacteria after dental extractions or trauma. In meta-analysis, chlorhexidine oral rinses have been shown to reduce the risk of ventilator-associated pneumonia.[8] It is well-tolerated, with known side effects consisting of mild oral discomfort, transient decrease in taste, and tooth discoloration (particularly with tea or coffee consumption).

Randomized controlled trials of chlorhexidine oral rinses for dental diseases have shown some possible decrease in oral bacterial biomass,[9, 10] decrease in specific oral pathogens,[10] and decreased alpha diversity of the oral microbiota.[11] Oral chlorhexidine use results in an immediate and sustained decrease in oral bacteria viability.[12]

There is compelling evidence that chlorhexidine oral rinses improve oral health and are safe and well-tolerated. The oral microbiota is the source of the lung microbiota likely due to microaspiration. Among those with COPD, the oral and sputum microbiota correlate with COPD exacerbation frequency.[6] Oral treatment with chlorhexidine alters the oral microbiota, which may subsequently alter the lung microbiota and COPD-related symptoms. Our primary aim was to determine the effect of twice-daily chlorhexidine oral rinses on oral and lung microbiota biomass in participants with COPD.

Methods

The chlorhexidine effect in the oral and lung microbiota study (CLIMB) is a randomized, blind, placebo-controlled, parallel-group preliminary study of the effects of chlorhexidine oral rinses on COPD. It was conducted at a single tertiary-care Veterans Affairs medical center (USA). Ethics approval was granted by the Minneapolis Veterans Affairs Medical Center (MVAMC) Institutional Review Board (#4526-A; ClinicalTrials.gov NCT02252588), all participants provided written consent, and all procedures adhered to the study protocol. A data monitoring

committee did not oversee the study. All data relevant to the study are included in the article. Protocol and additional methods are provided in an online data supplement, and the dataset is available in Dryad.[13]

Patient and public involvement

The design of this study was based on previous randomized clinical trials designed for COPD exacerbations. We further received input from expert clinicians and researchers within the COPD Clinical Research Network. Patients with COPD were not involved in the development of the protocol, but participant feedback was obtained during the study.

Study Protocol:

Eligible participants were invited to participate in the study and consisted of those age 40-85 years with a diagnosis of COPD and the presence or high likelihood of a chronic cough and sputum production. Participants were excluded if they were not fully recovered for at least 30 days from a COPD exacerbation or were treated with antibiotics in the last two months.

Participants were assigned (1:1) via a random number generator to receive either 15 mL of twice-daily 0.12% oral chlorhexidine rinses (PerioGard®)[7] or matched placebo mouth rinses for eight weeks. The pharmacist conducted the allocation and assignment and was the only staff member unblinded to study assignment. Study data were collected and managed using REDCap electronic data capture tools hosted at the University of Minnesota.[14, 15]

At visit 1, participants provided medical history, performed spirometry, completed the St. George's Respiratory Questionnaire (SGRQ),[16, 17] were instructed on how to complete the Breathlessness, Cough, and Sputum Scale (BCSS)[18] daily diaries, and provided blood, oral, and induced sputum samples prior to randomization. Oral and sputum sample volumes were recorded. Sputum production was heterogeneous across participants and samples, so sputum sample 16S copy numbers were normalized to (i.e., divided by) sputum sample mass. Oral sample size also varied due to variations in expectoration efficiency and were therefore also normalized to oral sample mass. Participants returned 8 weeks later to return BCSS diaries, complete the SGRQ, assess outcomes, and provide blood, oral, and sputum samples.

The clinical laboratories at the MVAMC determined WBC and differential, fibrinogen, CRP levels, and sputum gram stain and culture results. All oral rinses, sputum samples, and unused sterile water (control samples) were frozen immediately and until DNA extraction. 16S rRNA quantification and 16S rRNA V4 MiSeq sequencing was performed at the University of Minnesota Genomics Center as previously described.[19]

Outcomes and Power Analysis:

The primary outcome was change in oral and sputum microbiota biomass after 8 weeks of chlorhexidine vs. placebo use, compared to baseline values as assessed by 16S rRNA quantification. The primary outcome was chosen based on the mechanism of action of chlorhexidine, however sample size calculations were based on a change in alpha diversity (a secondary outcome) due to data availability at study initiation. At a sample size of 20 per group

and across a plausible range of effect sizes, our study had 67-94% power to detect a change in alpha diversity associated with chlorhexidine use. Sample size calculations are available in the online supplement, and a rarefaction curve is provided in Figure S1. Secondary outcomes included: sputum and oral microbiota Shannon and Simpson diversity; sputum and oral microbiota taxonomy; inflammatory markers (WBC, fibrinogen, and CRP); BCSS scores; SGRQ score; and assessment of adverse events.

Statistical Analysis:

Baseline variables were compared using Fisher's Exact Test for categorical variables or the Wilcoxon Two-Sample Test for continuous variables. Means are presented with standard deviations (SD); mean differences and parameter estimates are presented with their associated standard error (SE).

All analyses were performed using SAS version 9.4 (SAS Institute) and the intention-to-treat principle. A two-sided type I error of 0.05 was used. Correction of the Type I error rate for multiple testing was performed using the Step-down Bonferroni method.[20]

For the primary analysis of both normalized oral wash and normalized sputum biomass count, values were transformed to the $\log_{10}$ scale and the mean difference between treatment groups was compared using the two-sample t-test. A multiple imputation procedure was used to impute each unavailable sputum weight.

The primary data analysis included all those who completed the study, with baseline and mid-study phone call data included for non-completers when available. A sub-analysis of the microbiota data was conducted after excluding samples obtained from participants who used antibiotics during the study period.

Linear regression was used to examine the effect of treatment group on the 8-week change in the Shannon and Simpson biodiversity indices, BCSS, SGRQ and inflammatory markers separately, with each model adjusted for the baseline value of the measure.

Subgroup analyses of participants who did not receive antibiotics during the study were also performed for the outcomes of biomass and biodiversity.

For taxa abundance analyses, treatment effects on abundance were examined by modeling the 8-week change using linear regression, adjusted for baseline count. Analysis was restricted to genera with <20% of values equal to zero. Fisher's Exact Test was used to determine the proportion with a genus detected at Week 8 vs. baseline compared between treatment groups. Results were corrected for multiple comparisons.

Results

CLIMB assessed 511 participants for eligibility, excluded 215 because they did not meet criteria, 252 declined to participate, and 44 were randomized to study medication. Participants were recruited between September 8, 2014 and May 30, 2019 and the study ended when 40 participants completed the 8-week study. Four participants (all randomized to chlorhexidine) discontinued the study, leaving 20 participants in each group who completed the study. One participant withdrew without using any study medication, while the other 3 were lost to follow up (Figure 1).

Of the 44 CLIMB participants, 41 (93%) were male and 42 (95%) were Caucasian. The mean age was 67.9 years and mean tobacco exposure was 58.2 pack-years. Most were former tobacco users (31, 70%) and the remainder were current smokers. High blood pressure (31, 70%) and coronary artery disease (27, 61%) were reported by most participants. Mean FEV_1 % predicted (FEV_1pp) was 41.7% and the mean number of COPD exacerbations reported in the prior 12 months was 2.1. Baseline mean SGRQ score was 45.8. No baseline characteristics differed significantly by treatment group (Table 1).

The number of participants experiencing a COPD exacerbation or using an antibiotic or oral corticosteroid during the study period are presented in Table 2. Eight participants (3 in the chlorhexidine group, 5 in the placebo group) received antibiotics during the study; most but not all antibiotic use was for a respiratory indication. No participants experienced more than one exacerbation, more than one course of antibiotics, or more than one course of oral corticosteroids during the study.

Our primary outcome was a change in oral and sputum microbiota biomass during the study period as assessed by 16S rRNA copy numbers. Oral rinse samples were available for 40 participants (20 per group). There was a decrease in biomass in both groups; the mean ± SD changes were -0.24 ± 1.0 and -0.14 ± 0.32 in the chlorhexidine and placebo groups respectively (Table S1). The mean difference between treatment groups (active-placebo) was not significant (mean diff [SE] = -0.103 [0.23], 95% Confidence Interval [CI]: [-0.59, 0.38], p=0.665). Very similar results were seen in the subgroup that did not use antibiotics during the study (N=32, mean diff [SE] = -0.07 [0.29], 95% CI: [-0.65, 0.51], p=0.808) (Table 3).

For the analysis of biomass in sputum samples, 5 chlorhexidine and 4 placebo participants were unable to provide sputum samples; 2 were unable at Baseline, 6 were unable at Week 8 (including the 4 withdrawals), and one was unable at both Baseline and Week 8. Among the 35 sputum samples, there were 11 missing sputum weight values (6 at Baseline, 5 at Week 8) among 4 placebo and 4 chlorhexidine participants. Table 3 shows the primary analysis results using a two-sample t-test with the normalized data available (N=27) and using a multiple imputation procedure to estimate the missing sputum weights (N=35). The two analysis methods provide similar results. Although we hypothesized that the estimate would be negative, indicating that the active group saw a larger decrease in biomass from Baseline to Week 8 than the placebo group, without imputation we see a non-significant effect in the opposite direction (mean $\log_{10}$ difference[SE] =0.80 [0.46], 95% CI = [-0.15, 1.75], p=0.096) and similarly with imputation (mean $\log_{10}$ difference[SE] = 0.70 [0.39], 95% CI = [-0.08, 1.47],

p=0.078). These results were supported by the subgroup analyses of those without antibiotic use during the study. Although the p-value for the imputation analysis is significant (p=0.036) and the effect is not in the hypothesized direction, this result should be interpreted with caution due to the large number of tests reviewed here.

Linear regression was used to examine the 8-week change in each biodiversity index (Shannon and Simpson Indices) as a function of treatment group and adjusted for the value of the index at baseline (Table 4). As hypothesized, those in the chlorhexidine group saw, on average, a significant decrease in the diversity indices in comparison to those in the placebo group. For the oral wash samples, those in the treatment group had a coefficient of -0.349 (SE=0.091, adj. p=0.001) for the Shannon diversity index and -0.030 (SE=0.008, adj. p=0.001) for the Simpson diversity index. The results were similar for sputum samples: -0.622 (SE=0.169, adj. p=0.001) for the Shannon diversity index and -0.091 (SE=0.034, adj. p=0.0123) for the Simpson diversity index. Very similar results for both oral wash and sputum alpha diversity were seen in the subgroup that did not use antibiotics during the study, indicating that the decrease in diversity with chlorhexidine use was not related to antibiotic use (Table S2).

For the additional secondary outcomes, the effect of treatment group on the 8-week change was examined using linear regression, adjusted for the measure at baseline (Table 5 and Table S3). There was no significant difference between treatment groups over the 8-week study period in BCSS score (mean change in the chlorhexidine and placebo groups respectively ± SD: -0.3 ± 1.9 vs. -0.1 ± 1.5, estimate [95% CI] = -0.28 [-1.45, 0.89], p=0.630), CRP (1.8 ± 7.5 vs. 0.4 ±

6.8, 1.54 [-2.72, 5.80], p=0.467), fibrinogen (22.5 ± 77.8 vs. 10.0 ± 77.0, 20.19 [-28.52, 68.91], p=0.406), or leukocytes (0.2 ± 1.8 vs. 0.5 ± 1.8, -0.32 [-1.42, 0.78], p=0.560). Participants in the chlorhexidine group showed a significantly larger decrease in SGRQ total score when compared with the placebo group (mean change ± SD: -4.7 ± 8.0 vs. 1.7 ± 8.9, -6.22 [-11.87, -0.57], p=0.032). This difference was not evidenced in any one SGRQ domain.

In exploratory analyses, we evaluated the taxonomic composition of samples to assess for chlorhexidine-associated changes in the microbiota. Among sputum samples there were 42 genera. The results of the linear regression analyses showed that only *Corynebacterium* sequences were less abundant after chlorhexidine use compared with placebo (mean change in the chlorhexidine and placebo groups respectively ± SD: -197 ± 342 vs. 12 ± 337, estimate [95% CI] = -282 [-438, -126], adjusted p=0.0378). Among oral wash samples there were 43 genera. Only *Lachnoanaerobaculum* sequences were less abundant after chlorhexidine use compared to placebo (mean change in the chlorhexidine and placebo groups respectively ± SD: -313 ± 483 vs. 216 ± 509, estimate [95% CI] = -521 [-815, -226], adjusted p=0.043). Follow up analyses relying on the presence or absence of sequences (rather than relative abundance) produced similar results.

Very few adverse events were experienced over the course of the study (Table S4).

Discussion

In this preliminary study, twice-daily chlorhexidine oral rinses decreased oral and sputum microbiota alpha diversity and improved pulmonary disease-related quality of life compared to placebo among those with symptomatic COPD. Chlorhexidine oral rinses did not appear to decrease the oral or sputum microbiota biomass, our primary outcome, compared to placebo as assessed by normalized 16S rRNA quantitative PCR. Furthermore, during the 8-week treatment period chlorhexidine did not appear to decrease systemic inflammation or COPD symptoms, as assessed by the BCSS, compared to placebo. Our preliminary study had limited statistical power to detect several of our secondary endpoints; therefore, our results cannot definitively exclude a relationship between chlorhexidine use and systemic inflammation or symptoms.

We chose a change in biomass as our primary endpoint as we hypothesized that twice daily chlorhexidine would have its largest effect on microbiota biomass. However, we did not detect a significant decrease in biomass as a result of chlorhexidine use utilizing quantitative PCR. Chlorhexidine is known to be bactericidal and previous work has identified a decrease in viable bacteria following chlorhexidine oral use compared to water.[12] Our total DNA extraction technique coupled with PCR-based biomass determination is unable to distinguish between live and dead bacteria. It is therefore possible that chlorhexidine decreased the number of live bacteria in the oral and sputum microbiota, and that our PCR-based biomass determination technique was unable to distinguish between live bacterial biomass and dead bacteria. Furthermore, both groups experienced some decrease in biomass during the study period.

Changes in dental care habits, including twice-daily oral rinsing with either study drug or placebo, may be responsible for this decrease.

Although total microbiota biomass did not appear to change, oral and sputum microbiota alpha diversity decreased as a result of chlorhexidine use. The healthy lung and oral microbiota generally demonstrate greater alpha diversity than the microbiota found in disease states such as COPD or cystic fibrosis.[21] Whether this association is due to frequent use of antibiotics among those with chronic lung disease or due to the chronic lung disease itself remains unknown. Loss of alpha diversity due to chlorhexidine use may seem paradoxical given our current understanding of the relationship between low alpha diversity and worsening lung symptoms, however the current disease model does not differentiate between alpha diversity *per se* and the mechanisms by which it may be manipulated. Loss of alpha diversity due to chlorhexidine use, antibiotic use, or chronic lung inflammation likely represent clinically distinct entities.

Use of chlorhexidine oral rinses vs. placebo did not result in decreased systemic inflammation as evidenced by CRP, fibrinogen and WBC values. These three systemic markers of inflammation are often elevated among those with symptomatic COPD.[22] In light of our other findings linking chlorhexidine use to microbiota alterations and improved respiratory-related quality of life, we had expected that chlorhexidine use would lead to decreased systemic inflammation. It is possible that chlorhexidine use improved local inflammation (in the lungs or

mouth) without resulting in systemic inflammatory changes. Sustained use over a longer time period may be needed in order to observe systemic anti-inflammatory effects.

Although chlorhexidine use did not result in significant changes to BCSS scores, respiratory health-related quality of life did improve with use of chlorhexidine oral rinses vs. placebo during the 8-week intervention. SGRQ scores improved significantly among the chlorhexidine group relative to the placebo group, with a mean improvement (4.7 points) that is clinically meaningful (minimum clinically important difference of 4 points). The SGRQ encompasses 3 sub-scores for activity, impacts, and symptoms.[16] No sub-score reached statistical significance, indicating that chlorhexidine use improved quality of life broadly, and was not due to isolated improvements in one or two SGRQ sub-domains. Our data support the further study of chlorhexidine oral rinses among symptomatic patients with COPD to improve respiratory health-related quality of life.

In an exploratory analysis of the effects of chlorhexidine use on the sputum and oral microbiota, the only genus-level changes in DNA abundance were a decrease in *Corynebacterium* in sputum and a decrease in *Lachnoanaerobaculum* in oral rinses. Chlorhexidine is known to broadly decrease the viability of bacteria and yeast.[12] Our microbiota analysis techniques, which cannot differentiate between DNA from "live" or "dead" organisms, therefore may be relatively insensitive to the effects of chlorhexidine. We were unable to detect overall changes in bacterial biomass or broad changes to individual genera among those using chlorhexidine compared with placebo. It is possible that broader assessments of the

community composition, such as alpha diversity, are better able to detect chlorhexidine-related changes.

Our preliminary study had several strengths and limitations. Its strengths include a study intervention that is simple, inexpensive, and has few side effects; the randomized and blinded nature of the study; and objective assessment of outcomes. Our study was limited by its relatively small sample size and use of a secondary endpoint to determine statistical power, our homogeneous patient population, and our single-center design. In addition, other limitations include our inability to distinguish between live and dead bacteria in our samples, incomplete sample weights, lack of assessment of local inflammation, and limited in-person follow up while on study drug. Future larger clinical trials will determine if the beneficial effects of chlorhexidine oral rinses can be sustained among COPD subjects, and the biological mechanisms for these improvements in quality of life.

Although we did not find a difference in daily respiratory symptoms as measured with the BCSS, we found a significant improvement in quality of life as measured by the SGRQ. This potential discrepancy likely arose because BCSS focuses solely on respiratory symptoms,[18] while the SGRQ also assesses the broader impacts of COPD symptoms on quality of life.[16] There was no single domain within the SGRQ that drove this result, but there was improvement in both the impacts and symptoms domains. We propose that oral chlorhexidine rinses improve respiratory health-related quality of life by decreasing the number of live oral bacteria, altering the content of the live oral microbiota, or both. Changes to the oral microbiota may decrease the lung

inflammation that occurs following aspiration or change the composition of the lung microbiota itself and lead to an improved sense of wellness.

An additional clinical trial is needed to confirm our clinical endpoint findings with a larger group of participants and evaluate the mechanistic links between chlorhexidine, viable bacterial biomass, the microbiota, and respiratory health-related qualify of life in symptomatic patients with COPD.

Our data indicate that the use of twice-daily chlorhexidine oral rinses among symptomatic patients with COPD improves quality of life. This was a secondary outcome in our study and warrants validation in a larger clinical trial. Our intervention is relatively easy to implement, inexpensive, and well-tolerated.

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Study data were collected and managed using REDCap electronic data capture tools hosted at the University of Minnesota. REDCap (Research Electronic Data Capture) is a secure, web-based software platform designed to support data capture for research studies, providing 1) an intuitive interface for validated data capture; 2) audit trails for tracking data manipulation and export procedures; 3) automated export procedures for seamless data downloads to common statistical packages; and 4) procedures for data integration and interoperability with external sources.

Guarantor statement: Dr. Wendt is the guarantor of the content of the manuscript, including the data and analysis.

Author Contributions: AAP supervised research laboratory work, critically reviewed the data analyses, and wrote the first draft with input from AMF. AMF performed the statistical analyses with supervision from CSR, contributed to the first draft, and created the figure and tables. CSR supervised the statistical analyses and critically reviewed the manuscript. CHW obtained

funding, supervised subject recruitment, critically reviewed the data analysis, and critically reviewed the manuscript.

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Role of the sponsors: The sponsors had no role in the design, conduct, analysis, or interpretation of the data.

Ethics statements

Patient consent for publication

Not required.

Ethics approval

Minneapolis Veterans Affairs Medical Center (MVAMC) Institutional Review Board (#4526-A)

Data availability statement:

Extra data can be accessed via the Dryad data repository at http://datadryad.org/ with the doi:10.5061/dryad.m0cfxpp37

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Tables

Table 1. Baseline characteristics by treatment group

	Chlorhexidine	**Placebo**	**All Participants**
	Mean ± SD or N (%)	Mean ± SD or N (%)	Mean ± SD or N (%)
Number of Randomised Participants	**24**	**20**	**44**
Gender (% female)	2 (8.3)	1 (5.0)	3 (6.8)
Age (years)	67.6 ± 7.2	68.3 ± 6.0	67.9 ± 6.6
Race non-white	1 (4.2)	1 (5.0)	2 (4.5)
Season**			
Spring	3 (15.0)	6 (30.0)	9 (22.5)
Summer	7 (35.0)	4 (20.0)	11 (27.5)
Fall	7 (35.0)	6 (30.0)	13 (32.5)
Winter	3 (15.0)	4 (20.0)	7 (17.5)
Years smoked	40.8 ± 10.4	43.6 ± 10.3	42.0 ± 10.4
Current smoker	6 (25.0)	7 (35.0)	13 (29.5)
Pack Years	58.7 ± 32.9	57.6 ± 39.8	58.2 ± 35.8
SGRQ	49.2 ± 17.2	41.8 ± 12.3	45.8 ± 15.5
FEV_1 % predicted	39.9 ± 12.6	43.8 ± 11.1	41.7 ± 12.0
FVC % predicted	66.2 ± 14.8	71.4 ± 12.9	68.5 ± 14.1
COPD exacerbations (past 12 months)	2.3 ± 1.5	1.8 ± 1.0	2.1 ± 1.3
COPD hospitalizations (past 12 months)	0.5 ± 0.7	0.7 ± 0.7	0.6 ± 0.7

**Assigned to the season that covered >50% of the study period for a given participant.
Abbreviations: SD = Standard deviation; SGRQ = St. George's Respiratory Questionnaire; FEV_1 = Forced expiratory volume in one second; FVC = Forced vital capacity; COPD = Chronic obstructive pulmonary disease.

Table 2. Exacerbations, antibiotic use, or systemic steroid use during the study, excluding those withdrawn prior to study completion

	Chlorhexidine	**Placebo**
	N(%)	N(%)
No. of Randomised Participants Assessed	**20**	**20**
COPD Exacerbation[1]	3 (15.0)	5 (25.0)
Systemic steroid use[2]	1 (5.0)	5 (25.0)
Antibiotic use[3]	3 (15.0)	5 (25.0)

[1]Self-reported COPD exacerbation (worsening of chronic respiratory symptoms) during the study. One placebo subject reported an exacerbation but deferred any therapy until after study completion.
[2]Self-reported use of systemic corticosteroids during the study for any indication.
[3]Self-reported use of systemic antibiotics during the study for any indication. One placebo subject took antibiotics for a non-respiratory reason.
Abbreviations: COPD = Chronic obstructive pulmonary disease.

Table 3. Biomass analysis results – Two-sample t-test on the log_{10} change

Two sample t-test on log_{10} change (CHL-PLA)	N TOT	N CHL	N PLA	Mean difference (SE)[1]	95% CI	t value	P-value
All participants with available data							
Oral Wash Samples	40	20	20	-0.103 (0.23)	(-0.59, 0.38)	-0.44	0.665
Sputum Samples							
No imputation	27	15	12	0.80 (0.46)	(-0.15, 1.75)	1.73	0.096
Imputation[2]	35	19	16	0.70 (0.39)	(-0.08, 1.47)	1.76	0.078
Excluding antibiotic use during study							
Oral Wash Samples	32	17	15	-0.07 (0.29)	(-0.65, 0.51)	-0.25	0.808
Sputum Samples							
No imputation	23	14	9	1.06 (0.53)	(-0.05, 2.17)	1.99	0.060
Imputation[2]	28	16	12	0.98 (0.47)	(0.06, 1.89)	2.09	0.036

[1] Chlorhexidine group change in biomass minus placebo group.

[2] Imputation refers to the use of multiple imputation techniques to impute the 11 missing sputum weights.

Abbreviations: TOT = total, CHL = Chlorhexidine, PLA = Placebo, SE = Standard error, CI = Confidence interval.

Table 4. Linear regression results of the effect of treatment group on the change in biodiversity

	Linear Regression			
Outcome	**Predictor**	**Estimate(SE)**	**Unadjusted P-value**	**Adjusted P-value[1]**
Shannon Diversity Index Change (Week 8- Baseline)				
Oral Wash (N=40)	Treatment Group[2]	-0.349 (0.091)	0.0005	0.0010
	Baseline Index	-0.197 (0.073)	0.0100	
Sputum (N=35)	Treatment Group	-0.622 (0.169)	0.0008	0.0010
	Baseline Index	-0.312 (0.111)	0.0083	
Simpson Diversity Index Change (Week 8- Baseline)				
Oral Wash (N=40)	Treatment Group	-0.030 (0.008)	0.0005	0.0010
	Baseline Index	-0.196 (0.114)	0.0938	
Sputum (N=35)	Treatment Group	-0.091 (0.034)	0.0123	0.0123
	Baseline Index	-0.109 (0.179)	0.5472	

[1]A Step-down Bonferroni p-value adjustment is made for the two comparisons (oral wash and sputum) within each Diversity Index.
[2]Treatment group is coded as Chlorhexidine = 1, Placebo = 0.

Table 5. Linear regression analysis of the effect of treatment group on secondary outcomes

		Chlorhexidine (N=20)		**Placebo (N=20)**	**Treatment Group[1]**	**P-value[2]**
Outcome: 8-week Change[3]	N	Mean ± SD	N	Mean ± SD	Estimate (95% CI)	
BCSS	19	-0.3 ± 1.9	18	-0.1 ± 1.5	-0.28 (-1.45, 0.89)	0.630
SGRQ Total Score	20	-4.7 ± 8.0	20	1.7 ± 8.9	-6.22 (-11.87, -0.57)	0.032
Activity Domain	20	-0.5 ± 9.1	20	3.9 ± 12.9	-3.84 (-10.92, 3.24)	0.279
Impacts Domain	20	-5.4 ± 12.6	20	0.7 ± 10.0	-5.46 (-12.92, 1.99)	0.146
Symptoms Domain	20	-10.1 ± 15.2	20	0.8 ± 18.8	-6.81 (-17.82, 4.19)	0.217

[1] Treatment group is coded as Chlorhexidine = 1, Placebo = 0.
[2] The p-value is for the comparison of chlorhexidine vs. placebo.
[3] Each model is adjusted for the baseline value of each outcome.
Abbreviations: SD = Standard deviation; CI = Confidence interval; BCSS = Breathlessness, Cough and Sputum Scale; SGRQ = St. George's Respiratory Questionnaire.

Figure Legend

Figure 1. CLIMB study consort diagram. CLIMB assessed 511 individuals for eligibility. Of these, 467 were excluded and 44 were randomized. Four participants (all assigned to the chlorhexidine group) discontinued the study. Forty participants completed the study.

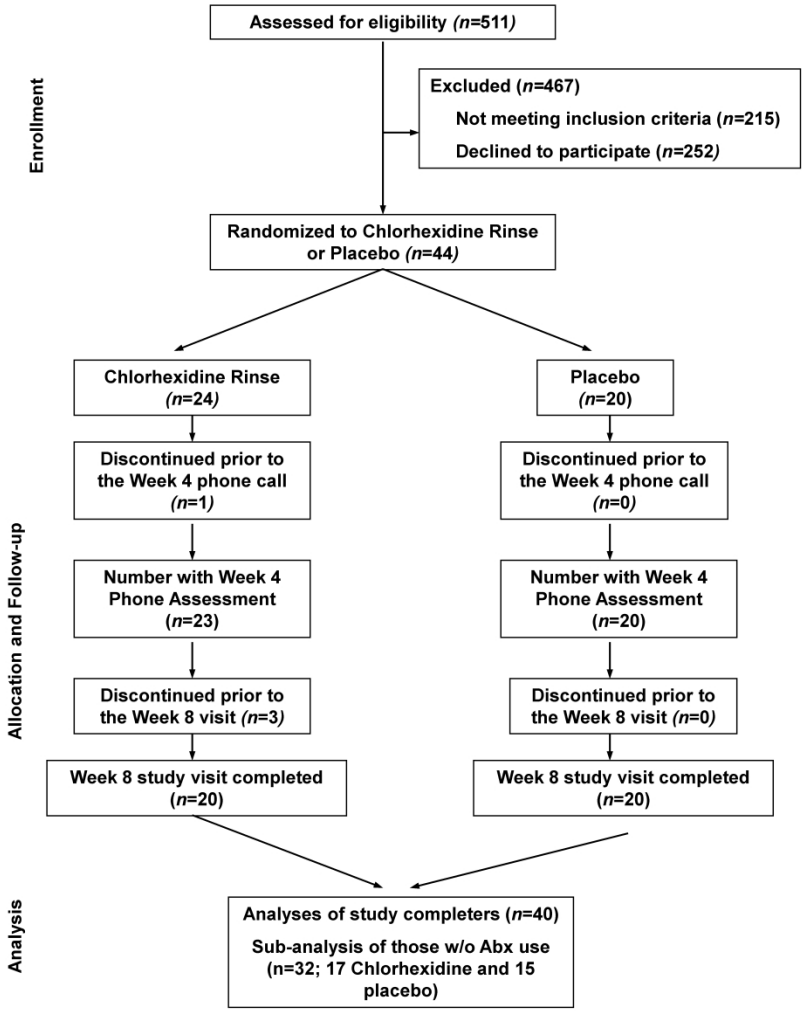

Figure 1. CLIMB study consort diagram. CLIMB assessed 511 individuals for eligibility. Of these, 467 were excluded and 44 were randomized. Four participants (all assigned to the chlorhexidine group) discontinued the study. Forty participants completed the study.

215x279mm (600 x 600 DPI)

Chlorhexidine oral rinses for symptomatic COPD: a randomized, blinded, placebo-controlled preliminary study

Alexa A. Pragman, Ann M. Fieberg, Cavan S. Reilly, and Christine H. Wendt

Online Data Supplement

Supplemental Methods

Study design

The chlorhexidine effect in the oral and lung microbiota study (CLIMB) is a randomized, blind, placebo-controlled, parallel-group preliminary study of the effects of chlorhexidine oral rinses on COPD. It was conducted at a single tertiary-care Veterans Affairs medical center (USA). Ethics approval was granted by the Minneapolis Veterans Affairs Medical Center (MVAMC) Institutional Review Board (#4526-A) and all procedures adhered to the study protocol (available in supplementary information).

Participants

Eligible CLIMB participants were those age 40-85 years receiving care at the MVAMC with a clinical diagnosis of COPD, a FEV_1/FVC ratio (post-bronchodilator) ≤ 70%, FEV_1 (post-bronchodilator) ≤ 65%, current or former smokers with lifetime cigarette consumption of ≥ 10 pack-years, presence of ≥ 4 natural teeth, and the presence of high likelihood of a chronic cough and sputum production defined as one of the following: 1) self-report of either cough or sputum production occurring “several days per week” or “almost every day”; or 2) a COPD exacerbation within the previous 12 months (defined as taking antibiotics and/or prednisone for respiratory symptoms, being hospitalized, or visiting the emergency department for respiratory illness). Participants were excluded if they were pregnant, not fully recovered for at least 30 days from a COPD exacerbation, treated with antibiotics (for any indication) in the last two months, had an active oral infection (e.g., dental abscess), currently used chlorhexidine oral rinses, had a known allergy or sensitivity to chlorhexidine, or used supplemental oxygen.

Presence of chronic cough, chronic sputum production, or COPD exacerbation in the last 12 months were used to enrich the study population with participants able to produce sputum and to report respiratory symptoms. The presence of at least four natural teeth was used to maintain consistency of the oral microbiota across participants, as the edentulous oral microbiota is different from the dentate oral microbiota. Likewise, participants who had not completely recovered from a COPD exacerbation, used antibiotics in the last two months, or had a dental infection were excluded to ensure that microbiota samples were collected from participants at their baseline.

Participants were recruited from those visiting the Emergency Department or admitted to the hospital for a COPD exacerbation, and among those participating in COPD case management due to frequent COPD exacerbations. All participants provided written informed consent.

Randomization and masking

Participants were recruited by the study coordinator and randomly assigned (1:1) via a random number generator to receive either 15 mL of twice-daily 0.12% oral chlorhexidine rinses (PerioGard®)[1] or matched placebo mouth rinses for eight weeks. Randomization was not stratified. Matched placebo was compounded by the research pharmacist and consisted of sterile water with blue dye (FD&C#2), polysorbate, and sodium saccharin for flavoring. The pharmacist conducted the allocation and assignment and was the only one unblinded to study assignment. Study medications were dispensed directly to participants in identical opaque

bottles. Participants as well as those interacting with participants (study coordinator and investigators) were blinded to group assignment during the conduct of the study. Investigators adjudicated antibiotic use and exacerbations after unblinding, but these data were used for post-hoc subgroup analyses and not in the primary or secondary outcome analyses.

Procedures

At visit 1, participants provided details of their medical history, performed spirometry, completed the St. George's Respiratory Questionnaire (SGRQ), were instructed on how to complete the Breathlessness, Cough, and Sputum Scale (BCSS)[2] daily diaries during the study, and then provided blood, oral, and induced sputum samples prior to randomization. Blood samples were used to determine white blood cell count and differential, fibrinogen, and C-reactive protein (CRP). Oral and sputum samples were obtained after at least a two-hour fast. Oral samples were obtained by swishing 15-mL sterile water in the mouth for 30 seconds and then spitting the water into a sterile cup. Sputum induction was performed with nebulized 3% saline (0.9% saline if FEV_1 <35%) for up to 20 minutes. Nebulization was terminated when participants either expectorated a 5 mL sputum sample into a sterile cup, 20 minutes of induction had elapsed, or the peak flow dropped to ≤ 80% of the baseline value. Unused sterile water was collected for use as control samples in microbiota analyses. Oral and sputum sample volumes were recorded. Using sterile technique, sputum samples were divided for cell count and gram stain performed by the clinical microbiology laboratory and microbiota analyses (including biomass quantification).

Participants were instructed to swish 15 mL of the study medication (either 0.12% chlorhexidine or placebo) in their mouth for 30 seconds twice daily (morning and evening) followed by expectoration. The study mouth rinse was used twice daily for eight weeks. Participants were told to avoid routine dental clinic visits during the study period.

After four weeks of study mouth rinse use, the study coordinator conducted a mid-study phone call to assess for hospitalizations, Emergency Department visits, unplanned clinic visits, new medication use (including antibiotics), compliance with the study drug, BCSS diary completion, and to assess adverse events. Additional study drug was mailed to participants by the research pharmacist following this phone call.

Eight weeks after randomization participants returned for a second study visit. They were instructed not to use the study mouth rinse the morning of the visit. Participants returned completed BCSS diaries and used study medication bottles to the study coordinator, who noted any remaining volume of study drug. Participants again completed the SGRQ, completed questionnaires (assessing medication changes, hospitalizations, Emergency Department visits, unplanned clinic visits, new medication use), and provided samples (blood, oral rinses, and induced sputum) for biomarker and microbiota analyses.

The BCSS (a daily diary for tracking the severity of respiratory symptoms) was started on Day 1, the day of first treatment. Participants answered three symptom questions on a 0 to 4 scale and a total daily score was calculated from those answers. Baseline BCSS score was the average

of daily BCSS measurements from Days 1-7. Week 8 BCSS score was the average of daily BCSS measurements from Days 50-56.

The SGRQ is a self-administered questionnaire that measures pulmonary disease-related quality of life. It has been validated for use in many chronic lung diseases, including COPD.[3] The SGRQ is scored on a scale of 0 to 100, with 100 reflecting the most severe symptoms. The minimum clinically important difference in the SGRQ is widely accepted as being 4 units.[4]

The clinical laboratories at the MVAMC determined WBC and differential, fibrinogen, CRP levels, and sputum gram stain and culture results. All oral rinses, sputum samples, and unused sterile water (control samples) were frozen immediately and until DNA extraction. 16S rRNA quantification and 16S rRNA V4 MiSeq sequencing was performed at the University of Minnesota Genomics Center as previously described.[5]

Outcomes

The primary study outcome was change in oral and sputum microbiota biomass after 8 weeks of study medication use, compared to baseline values, in participants who used 0.12% chlorhexidine oral rinses vs. placebo as assessed by 16S rRNA quantification. To adjust biomass for the size of the sputum sample, raw counts were normalized by dividing by the sample volume or mass. Secondary outcomes (all compared to baseline values in participants receiving chlorhexidine vs. placebo) included: i) sputum and oral microbiota alpha diversity (as assessed by Shannon and Simpson diversity); ii) sputum and oral microbiota taxonomy; iii) inflammatory

markers (WBC, fibrinogen, and CRP); iv) BCSS scores (week 8 vs. week 1); v) SGRQ score; and vi) assessment of adverse events. Adverse events were assessed both during the mid-study phone call and at the second visit by assessing hospitalizations, new medication use, and death. Participants were asked specifically about known adverse events associated with chlorhexidine oral rinses (oral pain, decreased taste, and tooth discoloration) and open-ended questions about new symptoms.

Statistical analysis

The power analysis for the study examined the power to detect differences in lung microbiota diversity as measured by the Simpson (1-D) diversity measure, ranging from 0.22 to 0.32 between the treatment groups using a two-sample t-test with equal variances. Our data showed that age had a significant positive impact on Simpson diversity with a change in diversity of 0.34 and the averaged standard deviation in the Simpson measure among moderate and severely affected COPD patients of 0.281. If chlorhexidine were to have an effect size similar to the effect of age with 20 participants per group, there was 67%, 75%, 81%, 87%, 91%, and 94% power to detect a difference of 0.22, 0.24, 0.26, 0.28, 0.30, and 0.32 between treatment groups.

Baseline variables were compared using Fisher's Exact Test for categorical variables or the Wilcoxon Two-Sample Test for continuous variables. Means are presented with standard deviations (SD); mean differences and parameter estimates are presented with their associated standard error (SE).

All analyses were performed using SAS version 9.4 (SAS Institute) and the intention-to-treat principle. A two-sided type I error of 0.05 was used. Correction of the Type I error rate for multiple testing was performed for the endpoints that report results from both oral wash and sputum samples using the Step-down Bonferroni method.[6]

For the primary analysis of both normalized oral wash and normalized sputum biomass count, values were transformed to the $\log_{10}$ scale and the mean difference between treatment groups was compared using the two-sample t-test. Additionally, for the analysis of sputum biomass count, a multiple imputation procedure was used to impute each unavailable sputum weight (PROC MI with seed=501213, MCMC method, and acceptable value range of 0.01 to 2.5). For each of the 25 datasets created by the procedure, the normalized biomass (count/mass) was calculated, the values were transformed to the $\log_{10}$ scale, and a t-test was performed. Lastly, PROC MIANALYZE was used to obtain an estimate from the t-test that accounted for the variability in the imputed values.

Linear regression was used to examine the effect of treatment group on the 8-week change in the Shannon and Simpson biodiversity indices, BCSS, SGRQ and inflammatory markers separately, with each model adjusted for the baseline value of the measure.

Subgroup analyses of participants who did not receive antibiotics during the study were also performed for the outcomes of biomass and biodiversity.

For taxa abundance analyses, the number of sequences assigned to each genus were determined for each sputum and oral wash sample. Treatment effects on the abundance of each genera were examined by modeling the 8-week change using linear regression, adjusted for baseline count. We restricted the analyses to the genera with <20% of values equal to zero. In addition, the proportion with organisms detected at Week 8 was compared between treatment groups for each genus using Fisher's Exact Test. Results were corrected for multiple comparisons.

A data monitoring committee did not oversee the study. The study was registered in ClinicalTrials.gov as NCT02252588.

Role of funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Table S1. Biomass (log_{10}) from oral wash and sputum samples by treatment group

	Oral Wash (log_{10} molecules/µL/mL)				Sputum (log_{10} molecules/µL/gram)			
	Chlorhexidine (N=24)		Placebo (N=20)		Chlorhexidine (N=24)		Placebo (N=20)	
	N	Mean (SD)	N	Mean (SD)	N	Mean (SD)	N	Mean (SD)
Biomass at Baseline	24	5.22 (0.58)	20	5.38 (0.48)	21	6.07 (1.15)	13	6.66 (0.59)
Biomass at Week 8	20	4.94 (0.96)	20	5.24 (0.34)	17	6.35 (1.04)	15	6.31 (0.86)
Change in biomass	20	-0.24 (1.00)	20	-0.14 (0.32)	15	0.42 (1.24)	12	-0.38 (1.13)
Excluding antibiotic use	Chlorhexidine (N=21)		Placebo (N=15)		Chlorhexidine (N=19)		Placebo (N=10)	
Biomass at Baseline	21	5.23 (0.61)	15	5.48 (0.48)	19	6.14 (1.18)	10	6.77 (0.55)
Biomass at Week 8	17	4.93 (1.04)	15	5.29 (0.37)	16	6.39 (1.06)	11	6.19 (0.97)
Change in biomass	17	-0.26 (1.09)	15	-0.19 (0.31)	14	0.45 (1.28)	9	-0.62 (1.20)

Abbreviations: SD = Standard deviation.

Table S2. Linear regression results of the effect of treatment group on the change in biodiversity – subgroup of participants who did not use antibiotics during the study (N=32)

	Linear Regression			
Outcome	Predictor	Estimate(SE)	Unadjusted P-value	Adjusted P-value[1]
Shannon Diversity Index Change (Week 8- Baseline)				
Oral Wash (N=32)	Treatment Group[2]	-0.369 (0.097)	0.0007	0.0014
	Baseline Index	-0.168 (0.080)	0.0446	
Sputum (N=28)	Treatment Group	-0.675 (0.192)	0.0017	0.0017
	Baseline Index	-0.455 (0.132)	0.0020	
Simpson Diversity Index Change (Week 8- Baseline)				
Oral Wash (N=32)	Treatment Group	-0.026 (0.008)	0.0021	0.0042
	Baseline Index	0.052 (0.135)	0.7021	
Sputum (N=28)	Treatment Group	-0.087 (0.031)	0.0097	0.0097
	Baseline Index	-0.680 (0.188)	0.0013	

[1]A Step-down Bonferroni p-value adjustment is made for the two comparisons (oral wash and sputum) within each Diversity Index.

[2]Treatment group is coded as Chlorhexidine = 1, Placebo = 0.

Table S3. Linear regression analysis of the effect of treatment group on secondary outcomes

		Chlorhexidine (N=20)		Placebo (N=20)	Treatment Group[1]	P-value[2]
Outcome: 8-week Change[3]	N	Mean ± SD	N	Mean ± SD	Estimate (95% CI)	
C-reactive Protein (mg/L)	20	1.8 ± 7.5	20	0.4 ± 6.8	1.54 (-2.72, 5.80)	0.467
Fibrinogen (mg/dL)	19	22.5 ± 77.8	20	10.0 ± 77.0	20.19 (-28.52, 68.91)	0.406
Leukocytes (K/cmm)	20	0.2 ± 1.8	19	0.5 ± 1.8	-0.32 (-1.42, 0.78)	0.560

[1] Treatment group is coded as Chlorhexidine = 1, Placebo = 0.
[2] The p-value is for the comparison of chlorhexidine vs. placebo.
[3] Each model is adjusted for the baseline value of each outcome.
Abbreviations: SD = Standard deviation; CI = Confidence interval.

Table S4. Adverse events by treatment group

	Week 4 Phone Call		Week 8 Visit	
	Chlorhexidine[1]	Placebo	Chlorhexidine	Placebo
Number of Randomized Participants	**23**	**20**	**20**	**20**
Irritation and/or sores of the lining of the mouth, N(%)	0 (0.0)	1 (5.0)	0 (0.0)	1 (5.3)
Allergic reaction (swelling, rash, or hives), N(%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Change in taste, N(%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Discoloration of teeth, N(%)	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.3)
Other side effects, N(%)[2, 3]	2 (8.7)	3 (15.0)	2 (11.1)	0 (0.0)

[1] Due to study withdrawals, adverse effects were assessed for 23 of 24 chlorhexidine participants at Week 4 and 20 of 24 at Week 8.
[2] Other adverse effects in the chlorhexidine group - dry mouth (1 patient at Week 4), feeling of loose teeth + cough + green tinged sputum (1 patient at Week 4), widening gaps in teeth (1 patient at Week 8) and blue tongue for 15-20 minutes after using drug (1 patient at Week 4), widening gaps in teeth (1 patient at Week 8) and blue tongue for 15-20 minutes after using drug (1 patient at Week 8).
[3] Other adverse effects in the Placebo group - increased congestion (1 patient at Week 4), sinus/nasal infection (1 patient at Week 4), and dry mouth/dry cough (1 patient at Week 4).

Figure S1. Rarefaction curve.

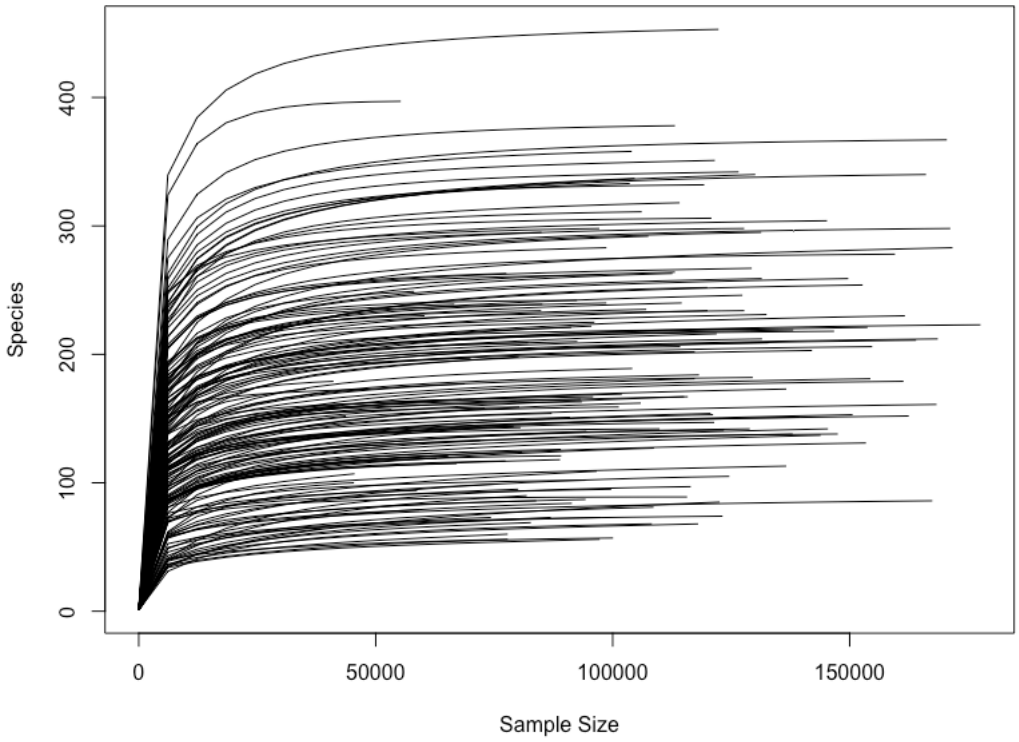

Figure S1. Rarefaction curve. Each sample is represented by a line which illustrates the number of species identified within a subset of sequences taken from that sample. Horizontal asymptotes indicate that additional sequences obtained from that sample are unlikely to identify additional species.

References

1. https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=&ved=2ahUKEwj5uKeM3IzsAhXYWc0KHS4nCHYQFjAAegQIBBAB&url=https%3A%2F%2Fwww.accessdata.fda

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6. Holm S. A simple sequentially rejective multiple test procedure. *Scand J Stat.* 1979;6:65-70.

CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	5
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	2
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	4-5
	2b	Specific objectives or hypotheses	5
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	5-6
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	6
Participants	4a	Eligibility criteria for participants	6
	4b	Settings and locations where the data were collected	6
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	5-7
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	7
	6b	Any changes to trial outcomes after the trial commenced, with reasons	NA
Sample size	7a	How sample size was determined	7
	7b	When applicable, explanation of any interim analyses and stopping guidelines	NA
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	6
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	6
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	6
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	6
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those	6

		assessing outcomes) and how	
	11b	If relevant, description of the similarity of interventions	NA
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	7-8
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	8
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	9
	13b	For each group, losses and exclusions after randomisation, together with reasons	9
Recruitment	14a	Dates defining the periods of recruitment and follow-up	9
	14b	Why the trial ended or was stopped	NA
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	9
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	9
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	10
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	10
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	11-13
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	13
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	16
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	16-17
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	16-17
Other information			
Registration	23	Registration number and name of trial registry	3
Protocol	24	Where the full trial protocol can be accessed, if available	3
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	facepage

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.