

In vivo antimicrobial effectiveness of an essential oil-containing mouth rinse 12 h after a single use and 14 days' use

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Abstract

Objectives: Two studies were conducted to determine the antimicrobial effect of rinsing with an essential oil-containing mouth rinse 12 h after a single rinse and 12 h after 2 weeks of twice daily rinsing, during the daytime and overnight.

Materials and Methods: These studies utilized a randomized, double-blind, controlled crossover design. Following baseline sampling of bacteria from supragingival plaque and the dorsum of the tongue, subjects began twice-daily rinsing with either an essential oil mouth rinse containing 0.09% zinc chloride (Tartar Control Listerine® Antiseptic) or a negative control rinse. Bacterial sampling was repeated 12 h after the first rinse, and again 12 h after the final rinse 14 days later. The sampling schedule was adjusted according to whether the study was investigating daytime or overnight activity. Samples were plated on Schaedlers medium (total anaerobes), Schaedlers Nalidixic/Vancomycin medium (Gram-negative anaerobes), and OOPS medium (volatile sulphur compound (VSC)-producing organisms). Inter-group log₁₀ transformed colony-forming units /ml counts from samples of supragingival plaque and tongue swabs on each of the three media were compared by analysis of covariance.

Results: The mean bacterial counts in subjects using the essential oil mouth rinse were significantly lower ($p \leq 0.005$) than mean counts in subjects using the control rinse in all the comparisons, i.e., tongue and supragingival plaque samples on each of three media at two sampling periods in the daytime and overnight study, respectively. Mean bacterial count percent reductions for plaque samples ranged from 56.3 to 95.3; percent reductions for tongue samples ranged from 61.1 to 96.1. There was a trend to higher reductions after 14 days' rinsing than after the initial rinse.

Conclusion: Rinsing with the essential oil mouth rinse can have long-lasting effects in reducing anaerobic bacteria overall as well as Gram-negative anaerobes and VSC-producing bacteria. The significant reductions in numbers of these bacteria produced by the essential oil mouth rinse, both in plaque and on the dorsum of the tongue, can play a key role in explaining the essential oil mouth rinse's effectiveness in reducing supragingival plaque and gingivitis as well as its effectiveness in controlling intrinsic oral malodor over prolonged periods.

Key words: antimicrobial activity; essential oil mouth rinse; oral anaerobes; selective media; volatile sulphur compounds

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Antimicrobial mouth rinses have a variety of therapeutic and cosmetic clinical uses, which are primarily dependent upon the ability of these products

to decrease the quantity and pathogenicity of the oral flora. A large body of clinical data exists to demonstrate the effectiveness of an essential oil-contain-

ing mouth rinse (Listerine® Antiseptic, Pfizer Inc., Morris Plains, NJ, USA) in helping to control supragingival plaque and gingivitis (Lamster et al. 1983,

Axelsson & Lindhe 1987, DePaola et al. 1989, Overholser et al. 1990, Charles et al. 2001, Sharma et al. 2002, Bauroth et al. 2003). In addition, this mouth rinse has been shown to have significant effectiveness in controlling intrinsic oral malodor, i.e., oral malodor resulting from the production of odorigenic compounds by oral bacteria (Pitts et al. 1981).

The broad, non-specific bactericidal activity of the essential oil mouth rinse has been clearly shown in both *in vitro* and *in vivo* studies. *In vitro* studies using a kill kinetic assay have demonstrated that the essential oil mouth rinse kills virtually all of a wide spectrum of oral Gram-positive and Gram-negative bacteria, opportunistic bacteria, and yeast within a 30 s exposure, both in the presence and absence of exogenous protein (Ross et al. 1989). Since it is now recognized that, as a biofilm, dental plaque provides a more rigorous challenge to antimicrobial agents than do organisms in planktonic form (Barnett 2003), studies have also been reported which demonstrate the essential oil mouth rinse to have significant bactericidal activity against biofilm bacteria using *in vitro* biofilm models (Pan et al. 1999, Fine et al. 2001). In addition, clinical studies have confirmed that this same bactericidal activity occurs *in situ*. These studies have shown significant killing of bacteria in dental plaque (Pianotti & Pitts 1978, Fine et al. 2000a, Pan et al. 2000), in saliva (Jenkins et al. 1994, DePaola et al. 1996), and on the dorsum of the tongue (Pitts et al. 1981, 1983).

Investigations into intrinsic oral malodor have revealed the important aetiological role of volatile sulphur compounds (VSC) and have identified the oral bacteria largely responsible for their production (Kleinberg & Codipilly 1995). These bacteria are primarily found on the dorsum of the tongue and in supragingival dental plaque. The development of culture media specific for VSC-producing bacteria (Turng et al. 1996) has advanced our ability to study mechanisms by which antimicrobial mouth rinses help to reduce and control oral malodor.

The studies reported herein investigated the antimicrobial effect of rinsing with an essential oil mouth rinse 12 h after a single rinse and 12 h after 2 weeks of twice daily rinsing, as determined by recoverable counts of bacteria from supragingival dental

plaque and tongue samples including VSC-producing bacteria. Because label directions specify rinsing in the morning and at night, two studies were conducted using the same protocol, one of which included a 12 h overnight period and the second of which included a 12 h period during the daytime, in order to determine effects of rinsing over a 24 h cycle.

Materials and Methods

These controlled clinical trials utilized a randomized, double-blind, 2 × 2 cross-over design. Subjects completed an informed consent form after the nature of the study was explained to them. The study protocol was reviewed and accepted by the Institutional Review Board of the University of Medicine and Dentistry of New Jersey.

Qualifying subjects were required to have a minimal plaque accumulation equivalent to a plaque index score of 1.5 (Turesky et al. 1970), no more than mild gingivitis equivalent to a gingival index of 1.5 (modified gingival index; Lobene et al. 1986) and no pockets greater than 4 mm. Subjects were given a standard ADA – accepted fluoride dentifrice and soft textured toothbrush for use 1 week prior to baseline bacterial sampling and for the duration of the study. For the daytime study, they reported to the clinical site for baseline sampling in the evening after refraining from any oral hygiene procedures during the previous 2 h. For the overnight study, they reported to the clinical site in the morning after refraining from eating, drinking, or oral hygiene procedures that morning. Supragingival plaque was collected from the buccal surfaces of the maxillary right quadrant teeth using a sterile Columbia 13/14 scaler, pooled, and placed in a tube containing 1 ml of sterile phosphate-buffered saline (PBS). In the same manner, plaque was collected from the mandibular right quadrant teeth and pooled separately. Prior to plaque collection, the teeth were isolated using cotton rolls and a saliva ejector was employed to help keep the sampled regions dry. Bacteria were then sampled from the right and left halves of the dorsum of the tongue using sterile cotton swabs. These tongue samples were collected by placing the cotton swab at the midline of the posterior region of the tongue and then rolling the swab toward the lateral border of the

tongue approximately four times from posterior to the tip. The swabs from the right and left sides of the tongue were placed in separate tubes containing 1 ml PBS.

Subjects were then randomly assigned to either an essential oil-containing mouth rinse group (Tartar Control Listerine® Antiseptic, Pfizer Consumer Healthcare, Morris Plains, NJ, USA) or a 5% hydroalcohol control group. The essential oil mouth rinse contained 0.09% zinc chloride as the anticalculus ingredient. Subjects continued to brush with the dentifrice provided. For the daytime study, subjects were instructed to brush with dentifrice, rinse thoroughly with water, and then rinse with 20 ml of their assigned rinse for 30 s at approximately 05:00 hours the next morning. They reported back to the clinical site at approximately 17:00 hours for microbial sampling. For the overnight study, subjects were instructed to brush with dentifrice, rinse thoroughly with water, and then rinse with 20 ml of their assigned rinse for 30 s at approximately 22:00 hours the same day. They reported back to the clinical site at approximately 10:00 hours the next morning for microbial sampling.

For the single-use 12 h sampling, supragingival plaque was harvested from the buccal surfaces of the maxillary left quadrant teeth and the right half of the tongue in the same manner as at baseline. The subjects contained their usual oral hygiene with the provided dentifrice and rinsed unsupervised twice daily for 30 s with 20 ml of their assigned mouth rinse for the next 13 days. Subjects returned for microbial sampling approximately 12 h after their final rinse, either at 17:00 hours (daytime study) or 10:00 hours (overnight study). For the 14-day use 12 h sampling, plaque was harvested from the buccal surfaces of the mandibular left quadrant teeth and the left side of the tongue.

Following a wash-out period of at least 7 days, the entire procedure was repeated with subjects using the alternative rinse. The oral soft tissues were examined prior to the start of the study and at each sampling period.

Microbiological procedures

The tubes of PBS containing the sampled supragingival plaque and the cotton swabs from the tongue were

subjected to brief periods of sonication over 30 s and then serially diluted in PBS to 10^{-4} . Dilutions of 10^{-2} and 10^{-4} were plated in duplicate on selective media using a spiral plater. The following media were used: Schaedlers medium (STA) to enumerate total anaerobic bacteria; Schaedlers Nalidixic/Vancomycin medium (SNV) to enumerate total Gram-negative anaerobes; and OOPS medium supplemented with 5% sheep's red blood cells (El-Halabi et al. 1999) to enumerate VSC-producing organisms (Turng et al. 1996). Both of the Schaedlers media were pre-reduced in an anaerobic chamber overnight. The inoculated plates were incubated anaerobically for 5–7 days at 37°C. Colony-forming units (CFU) were calculated from dilutions yielding at least 20 colonies per plate and not more than 20 colonies in the first sector. Total counts on STA and SNV media were determined using a CASBA 4 Counter (Spiral Systems, Bethesda, MD, USA). The OOPS plates were counted manually since even though the OOPS medium permits the identification of VSC-producing organisms by the presence of black lead sulphide precipitates within the colonies, other non-pigmented colonies can also grow on this medium. In all cases, the laboratory technician doing the counting did not have knowledge of the group to which the plates belonged. For the purpose of statistical analysis, the total counts were log transformed (base 10); duplicate counts were averaged prior to log transformation.

Statistical methods

For each of the two studies, a sample size of 16 evaluable subjects (eight per treatment sequence) was selected based on historical data and determined to provide at least 90% power to detect a 1-log difference between the two treatment groups. The efficacy variables consisted of the number of total recoverable bacteria on each of the three media at 12 h after a single use and at 12 h after 14 days' use.

Baseline demographic variables were summarized by treatment sequence. The treatment sequences were compared with respect to age using a one-way analysis of variance (ANOVA) with treatment sequence as a factor, and with respect to gender, race, and smoking status by means of a chi-squared test or Fisher's Exact Test (in the case of small cell sizes).

Inter-group comparisons of 12 h \log_{10} -transformed CFU counts from supragingival plaque and tongue swab samples on each of the three culture media were made using an analysis of covariance (ANCOVA) with the baseline counts for the corresponding period as a covariate and sequence, subject within sequence, period, and treatment as factors. Each comparison was performed at the 0.05 significance level using a two-sided test.

Results

Daytime study

Eighteen subjects were randomized; one subject left the study for personal reasons leaving 17 evaluable subjects. Demographic characteristics of the sub-

ject population are presented in Table 1. The subjects ranged in age from 30 to 56 years, with a mean age of 39.6 years. There were no significant differences in age, race, gender, or smoking status between the two treatment sequences.

Inter-group comparisons of \log_{10} transformed CFU/ml at 12 h after a single rinse are presented in Table 2. In all cases, mean bacterial counts after using the essential oil mouth rinse were significantly lower than mean counts after using the negative control rinse ($p < 0.001$). For supragingival plaque bacteria, percent reductions for the essential oil rinse compared with the control rinse ranged from 56.3% on OOPS medium to 87.7% on SNV medium. Corresponding percent reductions for tongue bacteria ranged from

Table 1. Demographic characteristics, daytime study (randomized subjects)

	Treatment sequence		p-value
	AB	BA	
N	9	9	
Age (years)			
Mean	41.3	37.8	0.393
SD	9.62	7.43	
Median	44	35	
Minimum	30	30	
Maximum	56	52	
Gender			
Male	3 (33.3%)	3 (33.3%)	0.999
Female	6 (66.7%)	6 (66.7%)	
Race			
White	8 (88.9%)	3 (33.3%)	0.063
Black	1 (11.1%)	4 (44.5%)	
Asian	0 (0.0%)	2 (22.2%)	
Smoker			
Yes	1 (11.1%)	1 (11.1%)	0.999
No	8 (88.9%)	8 (88.9%)	

A, negative control; B, essential oil mouth rinse with zinc chloride.

Table 2. Inter-group comparisons, daytime study – 12 h after single use (evaluable subjects)

Comparison	Difference in means	Percent reduction*	Standard error of difference	p-value
EO rinse <i>versus</i> control, STA medium				
Dental plaque samples (7.26 [†] <i>versus</i> 8.04)	–0.78	83.4	0.12	<0.001
Tongue swab samples (6.98 <i>versus</i> 7.97)	–0.99	89.8	0.11	<0.001
EO rinse <i>versus</i> control, SNV medium				
Dental plaque samples (6.44 <i>versus</i> 7.35)	–0.91	87.7	0.17	<0.001
Tongue swab samples (6.41 <i>versus</i> 7.22)	–0.81	84.5	0.14	<0.001
EO rinse <i>versus</i> control, OOPS medium				
Dental plaque samples (4.85 <i>versus</i> 5.21)	–0.36	56.3	0.07	<0.001
Tongue swab samples (4.76 <i>versus</i> 5.17)	–0.41	61.1	0.06	<0.001

*Percent reduction = $(1 - 10^{\text{diff}}) \times 100$, where diff is the difference in means in \log_{10} scale.

[†] \log_{10} CFU/ml adjusted means.

STA, Schaedlers medium; SNV, Schaedlers Nalidixic/Vancomycin medium; CFU, colony-forming units.

61.1% on OOPS medium to 89.8% on STA medium.

Inter-group comparisons of \log_{10} transformed CFU/ml at 12 h after 14 days' rinsing are presented in Table 3. The findings after 2 weeks of rinsing paralleled those after a single use, although the percent reductions were larger with the longer-term use. In all cases, mean bacterial counts after using the essential oil rinse were significantly lower than counts after using the negative control rinse ($p < 0.001$). For plaque bacteria, percent reductions for the essential oil rinse, compared with the control rinse, ranged from 72.5% on OOPS medium to 93.8% on SNV medium. Corresponding reductions for tongue bacteria ranged from 74.3% on OOPS medium to 93.8% on STA medium.

Overnight Study

Sixteen subjects were randomized; one subject was not compliant with mouth rinse use and was dropped from the study, leaving 15 evaluable subjects. Demographic characteristics of the subject population are presented in Table 4. The subjects ranged in age from 30 to 56 years, with a mean age of 39.8 years. There were no significant differences in age, race, gender, or smoking status between the two treatment sequences.

Inter-group comparisons of \log_{10} transformed CFU/ml at 12 h after a single rinse are presented in Table 5. As was the case in the daytime study, mean bacterial counts after using the essential oil mouth rinse were all significantly lower than mean counts after using the negative control rinse ($p \leq 0.005$). For supragingival plaque bacteria, percent reductions for the essential oil rinse compared with the control rinse ranged from 57.3% on OOPS medium to 86.2% on SNV medium. Corresponding percent reductions for tongue bacteria ranged from 76.0% on SNV medium to 84.5% on STA medium.

Inter-group comparisons of \log_{10} transformed CFU/ml at 12 h after 14 days' rinsing are presented in Table 6. In this study as well, the findings after 2 weeks of rinsing paralleled those after a single use, although with larger percent reductions. For all comparisons, mean bacterial counts after using the essential oil rinse were significantly lower than counts after using the negative control rinse ($p < 0.001$). For supragingival

Table 3. Inter-group comparisons, daytime study – 12 h after 14 days' use (evaluable subjects)

Comparison	Difference in means	Percent reduction*	Standard error of difference	p-value
EO rinse <i>versus</i> control, STA medium				
Dental plaque samples (7.08 [†] <i>versus</i> 8.27)	– 1.19	93.5	0.14	<0.001
Tongue swab samples (6.87 <i>versus</i> 8.08)	– 1.21	93.8	0.08	<0.001
EO rinse <i>versus</i> control, SNV medium				
Dental plaque samples (6.33 <i>versus</i> 7.54)	– 1.21	93.8	0.16	<0.001
Tongue swab samples (6.23 <i>versus</i> 7.41)	– 1.18	93.4	0.14	<0.001
EO rinse <i>versus</i> control, OOPS medium				
Dental plaque samples (4.72 <i>versus</i> 5.28)	– 0.56	72.5	0.05	<0.001
Tongue swab samples (4.64 <i>versus</i> 5.23)	– 0.59	74.3	0.05	<0.001

*Percent reduction = $(1 - 10^{\text{diff}}) \times 100$, where diff is the difference in means in \log_{10} scale.

[†] \log_{10} CFU/ml adjusted means.

STA, Schaedlers medium; SNV, Schaedlers Nalidixic/Vancomycin medium; CFU, colony-forming units.

Table 4. Demographic characteristics, overnight study (randomized subjects)

	Treatment sequence		p-value
	AB	BA	
N	8	8	
Age (years)			
Mean	40.5	39.1	0.770
SD	8.35	10.05	
Median	42	34.5	
Minimum	31	30	
Maximum	56	53	
Gender			
Male	4 (50.0%)	2 (25.0%)	0.608
Female	4 (50.0%)	6 (75.0%)	
Race			
White	6 (75.0%)	7 (87.5%)	0.999
Asian	2 (25.0%)	1 (12.5%)	
Smoker			
Yes	1 (12.5%)	1 (12.5%)	0.999
No	7 (87.5%)	7 (87.5%)	

A, negative control; B, essential oil mouth rinse with zinc chloride.

Table 5. Inter-group comparisons, overnight study – 12 h after single use (evaluable subjects)

Comparison	Difference in means	Percent reduction*	Standard error of difference	p-value
EO rinse <i>versus</i> control, STA medium				
Dental plaque samples (7.12 [†] <i>versus</i> 7.65)	– 0.53	70.5	0.12	<0.001
Tongue swab samples (6.98 <i>versus</i> 7.79)	– 0.81	84.5	0.10	<0.001
EO rinse <i>versus</i> control, SNV medium				
Dental plaque samples (5.96 <i>versus</i> 6.82)	– 0.86	86.2	0.11	0.001
Tongue swab samples (6.84 <i>versus</i> 7.46)	– 0.62	76.0	0.15	0.001
EO rinse <i>versus</i> control, OOPS medium				
Dental plaque samples (5.01 <i>versus</i> 5.38)	– 0.37	57.3	0.11	0.005
Tongue swab samples (4.99 <i>versus</i> 5.73)	– 0.74	81.8	0.13	<0.001

*Percent reduction = $(1 - 10^{\text{diff}}) \times 100$, where diff is the difference in means in \log_{10} scale.

[†] \log_{10} CFU/ml adjusted means.

STA, Schaedlers medium; SNV, Schaedlers Nalidixic/Vancomycin medium; CFU, colony-forming units.

plaque bacteria, percent reductions for the essential oil rinse, compared with the control rinse, ranged from 63.7% in OOPS medium to 95.3% in both the

STA and SNV media. Corresponding reductions for tongue bacteria ranged from 85.9% in OOPS medium to 96.1% in STA medium.

Table 6 Inter-group comparisons, overnight study – 12 h after 14 days' use (evaluable subjects)

Comparison	Difference in means	Percent reduction*	Standard error of difference	p-value
EO rinse versus control, STA medium				
Dental plaque samples (6.68 [†] versus 8.01)	-1.33	95.3	0.10	<0.001
Tongue swab samples (6.68 versus 8.09)	-1.41	96.1	0.15	<0.001
EO rinse versus control, SNV medium				
Dental plaque samples (5.67 versus 7.0)	-1.33	95.3	0.14	<0.001
Tongue swab samples (6.47 versus 7.65)	-1.18	93.4	0.09	<0.001
EO rinse versus control, OOPS medium				
Dental plaque samples (4.88 versus 5.32)	-0.44	63.7	0.06	<0.001
Tongue swab samples (4.82 versus 5.67)	-0.85	85.9	0.17	<0.001

*Percent reduction = $(1 - 10^{\text{diff}}) \times 100$, where diff is the difference in means in log₁₀ scale.

[†]Log₁₀ CFU/ml adjusted means.

STA, Schaedlers medium; SNV, Schaedlers Nalidixic/Vancomycin medium; CFU, colony-forming units.

Discussion

The main point of the clinical studies reported herein was to demonstrate that rinsing with the essential oil mouth rinse containing zinc chloride could have long-lasting effects in reducing anaerobic bacteria overall as well as Gram-negative anaerobes and VSC-producing bacteria both in supragingival plaque and on the tongue. Significant reductions were seen 12 h after just a single rinse, with a trend to higher reductions after 14 days' use. The findings were similar whether the 12-h period studied was during the daytime or overnight. The authors recognize that statistical significance does not automatically imply clinical significance. However, when the results of this study are compared with results on the effects of the essential oil mouth rinse on malodor reported in other studies (Pianotti & Pitts 1978, Pitts et al. 1983) the inference of a clinical effect appears to be justified.

The percent reductions observed in dental plaque were consistent with those found in a previous study investigating the effect of an essential oil mouth rinse on levels of total recoverable streptococci and *Streptococcus mutans* in dental plaque (Fine et al. 2000a). In that study, subjects rinsed twice daily with either an essential oil mouth rinse without zinc chloride (Listerine® Antiseptic, Pfizer Inc.) or a negative control rinse for 12 days. Compared with the negative control, the essential oil mouth rinse produced respective 69.9% and 75.4% reductions in total streptococci and *S. mutans* recoverable from dental plaque. The finding that the essential oil mouth rinse can significantly reduce in vivo levels of both Gram-positive and

Gram-negative bacteria reflects the broad spectrum of activity of the mouth rinse, which has been demonstrated by the wide range of microorganisms quickly killed in kill-time determinations and by activity in minimum inhibitory concentration assays (Ross et al. 1989). The crossover design (Fine et al. 2000b) of this study, in which each subject served as his or her own control, helped to minimize the impact of the variability often seen in studies of oral bacteria.

The enriched indicator media, OOPS media-containing haemin, menadione, reduced glutathione and lead citrate as an indicator for sulphide-producing organisms focussed on bacteria associated with oral malodor, especially VSC-producing organisms (Persson et al. 1990). The significant reductions in numbers of these bacteria produced by the essential oil mouth rinse, both in supragingival plaque and on the dorsum of the tongue, can play a key role in explaining the essential oil mouth rinse's effectiveness in reducing supragingival plaque and gingivitis as well as its effectiveness in controlling intrinsic oral malodor over prolonged periods.

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