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Effect of an essential oil-containing antimicrobial mouthrinse on specific plaque bacteria in vivo

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Abstract

Aim: This study was conducted to investigate the effect of rinsing with an essential oil-containing mouthrinse on levels of specific supra and subgingival bacteria in subjects with gingivitis.

Material and Methods: Fifteen subjects meeting entry criteria completed this randomized, controlled, double-blind, crossover study. Subjects were required to have $\geqslant$1000 target organisms per millilitre in pooled samples from two subgingival sites. Following sampling of supra and subgingival plaque, subjects began twice-daily rinsing for 14 days with either an essential oil-containing mouthrinse (Cool Mint Listerine® Antiseptic) or a negative control. Supra and subgingival plaque was again sampled on day 15, and the procedure repeated after a 1-week washout period with subjects using the alternate rinse.

Results: Compared with the negative control, the essential oil mouthrinse produced significant reductions in supragingival plaque levels of *Veillonella* sp., *Capnocytophaga* sp., *Fusobacterium nucleatum,* and total anaerobes ranging from 52.3 to 88.5% ($p<0.001$ except for *Veillonella*, $p=0.002$); respective reductions in subgingival plaque ranged from 54.1 to 69.1% ($p<0.001$).

Conclusions: Rinsing with the essential oil mouthrinse can have an impact on the subgingival plaque flora. This study provides additional evidence indicating that reduction in supragingival plaque can reduce levels of subgingival plaque.

Key words: antimicrobial activity; mouthrinse; oral anaerobes; subgingival plaque

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Conflict of interest and source of funding statement

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This study was supported by a grant to NJDS from the Pfizer Consumer Healthcare Division, which was subsequently purchased by the Johnson and Johnson Company. The study was designed, conducted, and analysed by the NJDS staff (Kenneth Markowitz, David Furgang, Deborah Goldsmith and Daniel Fine) and was completely under their supervision and control.

The antiplaque and antigingivitis effectiveness of mouthrinses containing antimicrobial agents has been well documented, and there exists a sound rationale for their inclusion in daily oral hygiene regimens (Barnett 2003, 2006). In particular, the effectiveness and safety of a mouthrinse containing a fixed combination of essential oils has been demonstrated in a number of long-term clinical trials, both with respect to reduction of existing supragingival plaque and gingivitis (Lamster et al. 1983) and inhibition of newly forming plaque and gingivitis (Gordon et al. 1985, DePaola et al. 1989, Overholser et al. 1990, Charles et al. 2001, 2004, Sharma et al. 2004). In these 6-month studies, rinsing with the essential oil mouthrinse produced supragingival plaque reductions ranging from 13.8 to 56.3% compared with the negative control. Furthermore, in a meta-analysis of 6-month studies of antiplaque and antigingivitis agents, results clearly support the significant antiplaque and antigingivitis efficacy of the essential oil mouthrinse (Gunsolley 2006).

While these clinical trials have generally considered gross plaque reductions, it is also of interest to investigate the effect of mouthrinses on specific plaque organisms that might play a role in disease aetiology. Laboratory

kill kinetics studies have shown the essential oil mouthrinse to be effective in killing a wide range of oral organisms, including pathogens and opportunistic bacteria (Ross et al. 1989). However, given our current understanding of the biofilm nature of plaque, it is clear that studies on planktonic organisms, while an indication of a formulation's range of bactericidal activity, are not necessarily predictive of clinical antiplaque activity because of the resistance to antimicrobial agents conferred by biofilm formation (Fine et al. 2001). Therefore, studies on the bactericidal activity of mouthrinse formulations against biofilm organisms in vivo are useful in helping to elucidate the mechanisms by which antimicrobial mouthrinses exert their antiplaque and antigingivitis effects. To date, relatively few clinical studies have been conducted to investigate the bactericidal activity of mouthrinse formulations against dental plaque biofilm. However, studies of the essential oil-containing mouthrinse have demonstrated significant reductions of odorigenic bacteria contained within the gingival crevice (Pianotti & Pitts 1978, Pitts et al. 1981, 1983) as well as significant reductions in interproximal plaque bacteria (Charles et al. 2000) and in bacteria contained within plaque on buccal tooth surfaces in which killing was demonstrated using a vital stain method (Pan et al. 2000). In addition, another clinical study directed specifically at *Streptococcus mutans* showed the essential oil mouthrinse to produce significant 69.9% and 75.4% reductions, respectively, of recoverable streptococci and *S. mutans* in plaque (Fine et al. 2000). There are few data available on the bactericidal effect on specific periodontopathogens contained within the gingival crevice.

The objective of the controlled clinical study reported herein was to investigate the effect of 2 weeks' rinsing with the essential oil-containing mouthrinse on levels of representative periodontal disease-associated bacteria in supragingival plaque and adjacent subgingival plaque in subjects with gingivitis. The study used an adaptation of a model that had been previously shown to be capable of demonstrating specific antimicrobial effects of chemotherapeutic products (Fine et al. 2005, 2006). The bacteria investigated in the current study included *Capnocytophaga* sp., *Fusobacterium nucleatum, Veillonella* sp., and total anaerobes.

Material and Methods

This controlled clinical trial utilized a randomized, doubleblind, 2×2-crossover design. All 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.

Fifteen qualifying subjects were recruited to assure that 14 subjects would complete the study. The sample size of 14 was based on results of a pilot study and was calculated to provide at least 90% power to detect a statistically significant 50% reduction in the essential oil mouthrinse group compared with control of at least one of the four microbiologic variables. Subjects were required to satisfy inclusion and exclusion criteria as follows:

Inclusion criteria

- Males and females in good general health between the ages of 18 and 65.
- Willingness to comply with protocol requirements.
- Ability and willingness to read, understand, and sign the informed consent form after the nature of the study had been explained.
- Minimum of 16 natural teeth, including all molars and bicuspids free of restorations and not overlapped or crowded.
- Mild gingivitis (Modified Gingival Index $\sim$ 1.7 Lobene et al. 1986) and plaque accumulation (Plaque Index $\sim$ 1.5 Turesky et al. 1970)
- At least 1000 target organisms per milliliter sampled from two pooled subgingival sites, namely, *Veillonella* sp., *Fusobacterium nucleatum*, and *Capnocytophaga* sp.

Exclusion criteria

- History of significant adverse effects following use of oral hygiene products such as toothpastes and mouthrinses.
- History of rheumatic fever, heart murmur, mitral valve prolapse, or other conditions requiring antibiotic premedication before invasive dental procedures.
- Antibiotic therapy within 30 days before the screening and baseline examination.
- History of diabetes, hepatic or renal disease, heart disease, or other serious medical conditions or infectious diseases.
- Any site with a periodontal pocket >4.0 mm in depth.
- Fixed or removable orthodontic appliance, removable full or partial dentures, or any lip or tongue piercing.
- Participation in a dental clinical trial within the previous 30 days.

Subjects were given an ADA-accepted fluoride dentifrice and a soft-textured toothbrush for use during the week before baseline plaque sampling and throughout the remainder of the study. During this period, subjects continued their usual oral hygiene regimens. On the morning of the sampling visits, subjects abstained from all oral hygiene procedures as well as eating and drinking.

At baseline, supra and subgingival plaque on the mesial aspects of the maxillary left second molar and maxillary left second bicuspid was sampled. Supragingival plaque was sampled using a sterile Morse #00 detachable scaler, which was placed in a tube containing 1 ml of reduced transport fluid (RTF). The remaining supragingival plaque was removed and subgingival plaque was sampled by placing a medium paper point into each of the two gingival crevices for 10 s and the two samples then pooled in a tube containing 1 ml RTF. The microbial samples were processed as described below.

Subjects were assigned to one of two treatment sequences using a computer-generated random code. Following the baseline plaque sampling, subjects began rinsing with their randomly assigned treatment, either the essential oil-containing mouthrinse (Cool Mint Listerine® Antiseptic, Pfizer Consumer Healthcare, Morris Plains, NJ, USA) or a 5% hydroalcohol negative control rinse, in addition to their usual mechanical oral hygiene procedures. Subjects were instructed to rinse for 30 s with 20 ml twice daily for 14 days. The first rinse was conducted under supervision at the study site, with the remainder of rinses performed unsupervised at home. On the morning of the 15th day, subjects refrained from oral hygiene procedures as well as from eating and drinking and

returned to the study site for microbial sampling of the mesial aspects of the maxillary left first molar and first bicuspid. The sampling and handling of the samples were performed in the same manner as at baseline.

After a 1-week washout period, the entire procedure was repeated with subjects using the alternative rinse. For this leg, baseline plaque was sampled from the mesial aspects of the maxillary right second molar and second bicuspid and plaque on the 15th day was sampled from the mesial aspects of the maxillary right first molar and first bicuspid. A complete oral soft tissue examination was performed at each visit to monitor oral adverse events.

The sampling plan utilized was based on findings from a seven-person crossover pilot study investigating the reproducibility of results obtained when supra and subgingival plaque was sampled from contralateral pairs of maxillary molars and bicuspids at a 2-week interval. Results of the study indicated that bacterial counts at the two time points were reproducible and that differences were no higher than 3% for any of the microbes evaluated (total anaerobes, *F. nucelatum* and *Veillonella* sp.) when the data were log transformed.

Microbiological procedures

The pooled samples in RTF were dispersed by sonication for 20 s using a Branson 200 sonifier with cup horn attachment (Branson Ultrasonic, Danbury, CT, USA). The sonicated samples were then serially diluted in RTF and a 40 μl aliquot of each dilution was plated in duplicate using an Autoplate 4000 Spiral Plater (Spiral Systems, Bethesda, MD, USA) on the following media: ETSA agar for total anaerobe counts; CVE agar for enumeration of *F. nucleatum*; TBBP agar for enumeration of *Capnocytophaga* sp.; and *Veillonella* agar for enumeration of *Veillonella* sp. The plates were incubated at 37° under appropriate anaerobic or capnophilic atmospheric conditions for 2–7 days. Total anaerobes (ETSA plates) and *Capnocytophaga* sp. (TBBP plates) were numerated using a CASBA 4 automated plate counting system (Spiral Systems). The other plates were counted manually and the data entered into the CASBA 4 system for calculation of CFU/ml.

Statistical methods

Baseline demographic variables were summarized by treatment sequence. The efficacy variables consisted of the number (CFU/ml) of each of the following supra and subgingival plaque organisms after 14 days of rinsing: total anaerobes; *F. nucleatum*; *Capnocytophaga* sp.; *Veillonella* sp. CFU counts were $\log_{10}$ transformed for analysis.

The efficacy variables were summarized by period and treatment sequences. Treatment comparisons of the post-treatment log-transformed CFU counts for supra and subgingival organisms on each of the four culture media were made using analysis of covariance (ANCOVA). The model included the sequence, period, treatment, and baseline of each period as fixed effects and subject within sequence as random effect. Each comparison was performed using a two-sided test and the multiplicity issue was addressed by Bonferroni's method.

Results

The demographic characteristics of the subject population are presented in Table 1. The subjects ranged in age from 26 to 51 years, with a mean age of 38 years. There were four males and 11 females, of whom five were Caucasian, nine were Black, and one was Hispanic, and four were smokers. The two treatment sequences were comparable with respect to age, gender, race, and smoking status. The essential oil-containing mouthrinse produced significant reductions in levels of all the specific periodontopathogens studied in both supragingival and subgingival plaque. There were no lesions attributable to the mouthrinses observed during the study.

Table 1. Demographic characteristics

	Treatment sequence	
	AB	BA
N	8	7
Age (years)		
Mean	35.50	40.86
SD	7.96	6.18
Median	33.00	40.00
Minimum	26.00	33.00
Maximum	51.00	50.00
Gender		
Male	2 (25%)	2 (29%)
Female	6 (75%)	5 (71%)
Race		
White	2 (25%)	3 (43%)
Black	5 (63%)	4 (57%)
Hispanic	1 (13%)	0
Smoker		
No	6 (75%)	5 (71%)
Yes	2 (25%)	2 (29%)

A, negative control; B, essential oil-containing mouthrinse.

Treatment comparisons of $\log_{10}$-transformed supragingival bacterial counts are presented in Table 2. In case of all four determinations, the mean bacterial counts after 14 days' use of the essential oil-containing mouthrinse were significantly lower than those after using the negative control rinse ($p < 0.001$ except for *Veillonella* with $p = 0.002$). Per cent reductions for the essential-oil containing mouthrinse *versus* negative control were 52.3, 74.1, 81.5, and 88.5 for *Veillonella* sp., *Capnocytophaga* sp., *F. nucleatum*, and total anaerobes, respectively (Fig. 1).

Treatment comparisons of $\log_{10}$-transformed subgingival bacterial counts are presented in Table 3. For subgingival bacteria, as well, the mean bacterial counts in all cases after 14 days' use of the essential oil mouthrinse were significantly lower than those after using the negative control rinse ($p < 0.001$). Per cent reductions for the essential oil containing mouthrinse *versus* negative control were 54.1, 63.5, 68.3, and 69.1 for *Veillonella* sp., *Capnocytophaga* sp., *F. nucleatum*, and total anaerobes, respectively (Fig. 2).

Discussion

This study demonstrated that levels of representative plaque organisms in both supra and adjacent subgingival plaque can be significantly reduced by 2 weeks' rinsing with an essential oil-containing antimicrobial mouthrinse. The bacteria studied were selected because they are readily cultivable and could be expected to be present in sufficient numbers in both the supra and subgingival plaque of subjects with mild gingivitis. Whereas previous studies have documented significant reductions in supragingival plaque overall with long-term use of the essential oil mouthrinse, this is the first study to investigate the effect of twice daily rinsing on levels of specific organisms associated with gingival diseases and to demonstrate a significant reduction of subgingival as well as supragingival levels of these organisms. The use

Table 2. Intergroup comparisons, supragingival organisms

Organism	Negative control rinse		Essential oil rinse	
	baseline	post-treatment	baseline	post-treatment
N	15	15	15	15
Capnocytophaga				
Mean (SD)	5.35 (0.12)*	5.28 (0.23)	5.37 (0.15)	4.73 (0.33)
Adjusted means		5.30		4.71
p-value†				<0.001
95% CI‡				0.38, 0.80
F. nucleatum				
Mean (SD)	6.50 (0.12)	6.37 (0.37)	6.50 (0.15)	5.64 (0.36)
Adjusted means		6.37		5.64
p-value				<0.001
95% CI				0.45, 1.01
Veillonella				
Mean (SD)	4.12 (0.11)	4.06 (0.18)	4.04 (0.12)	3.72 (0.22)
Adjusted means		4.05		3.73
p-value				0.002
95% CI				0.14, 0.50
Total anaerobes				
Mean (SD)	9.12 (0.16)	8.90 (0.43)	9.11 (0.15)	7.98 (0.45)
Adjusted means		8.91		7.97
p-value				<0.001
95% CI				0.60, 1.29

*Log_{10}-transformed CFU.
†*p*-value for difference between adjusted means, ANCOVA.
‡95% confidence interval (CI) for differences between adjusted means.

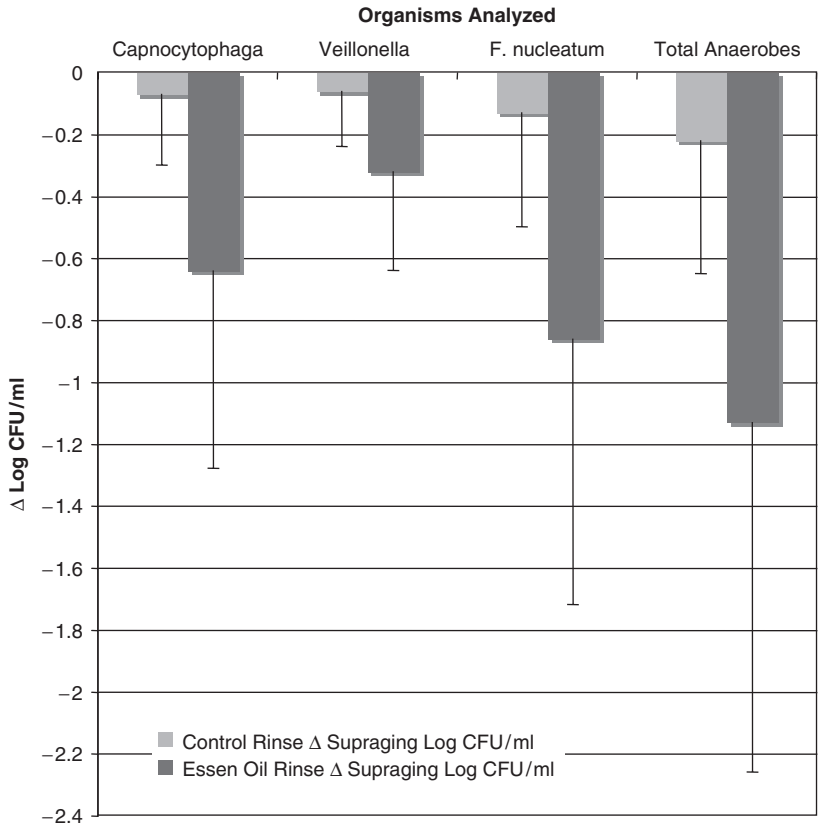

Fig. 1. Change in supragingival log CFU/ml of organisms tested after subjects rinsed for 2 weeks with control or essential oil mouthrinse. Figure shows reduction in total anaerobes and supragingival *Veillonella*, *Capnocytophaga*, *F. nucleatum* from basdeline in log-transformed counts. Total anaerobes were reduced from control by 88.5%. *Veillonella*, *Capnocytophaga*, and *F. nucleatum* were reduced by, 52.3%, 74.1%, and 81.5%, respectively. Reductions were statistically significant; *Veillonella* ($p \leqslant 0.002$), others ($p < 0.001$).

of a cross-over study design helped to minimize the impact of variability often seen in studies of oral bacteria since each subject serves as his or her own control. The per cent reductions of bacteria found in this study are consistent with those seen in the study investigating the effects of the essential oil-containing mouthrinse on streptococci and *S. mutans* (Fine et al. 2000) and those seen in the study of its in situ bactericidal activity determined using a vital stain method (Pan et al. 2000).

Although it is possible that, with rinsing, the mouthrinse was able to penetrate the subgingival domain to a limited extent, it is likely that the reduction in level of subgingival organisms was mediated primarily through an effect on supragingival plaque. Indeed, supragingival plaque seems to have a key role in the transfer of bacteria from the supra to the subgingival domain. The organisms chosen for evaluation in this study were selected because they have the ability to survive in both the supra and subgingival environment (Moore et al. 1987). Furthermore, *Veillonella* and *F. nucleatum* have been shown to occupy a prominent position in plaque derived from healthy, gingivitis, and periodontitis subjects (Fine et al. 1989). Supragingival plaque has been shown to harbour periodontopathogens and serve as a reservoir of these species for their spread to, or reinfection of, adjacent subgingival sites (Ximinez-Fyvie et al. 2000). Moreover, *Veillonella* and *F. nucleatum* provide key nutrients sources for fastidious subgingival plaque bacteria and thus removal of these organisms and their supply of nutrients can have a profound impact on the subgingival flora (Loesche 1968). In support of this hypothesis, studies have demonstrated that rigorous control of supragingival plaque can have a significant effect in reducing the level of putative pathogens subgingivally (Smulow et al. 1983, Dahlen et al. 1992, Katasnoulas et al. 1992, Hellstrom et al. 1996).

In conclusion, this study provides additional evidence that helps to elucidate the mechanism by which the essential oil-containing rinse significantly reduces levels of both plaque and gingivitis. By doing so, it adds to the scientific basis for the recommendation of the daily use of the mouthrinse in addition to mechanical oral hygiene procedures.

Table 3. Intergroup comparisons, subgingival organisms

Organism	Negative control rinse		Essential oil rinse	
	baseline	post-treatment	baseline	post-treatment
N	15	15	15	15
Capnocytophaga				
Mean (SD)	4.70 (0.10)*	4.68 (0.22)	4.74 (0.11)	4.25 (0.27)
Adjusted means		4.68		4.25
p-value†				<0.001
95% CI‡				0.24, 0.63
F. nucleatum				
Mean (SD)	5.51 (0.10)	5.43 (0.23)	5.52 (0.11)	4.96 (0.33)
Adjusted means		5.45		4.95
p-value				<0.001
95% CI				0.29, 0.71
Veillonella				
Mean (SD)	3.75 (0.15)	3.74 (0.15)	3.75 (0.18)	3.40 (0.26)
Adjusted means		3.74		3.40
p-value				<0.001
95% CI				0.22, 0.46
Total anaerobes				
Mean (SD)	7.42 (0.14)	7.33 (0.27)	7.41 (0.12)	6.82 (0.25)
Adjusted means		7.33		6.82
p-value				<0.001
95% CI				0.31, 0.71

*Log_{10}-transformed CFU.
†*p*-value for difference between adjusted means, ANCOVA.
‡95% confidence interval (CI) for difference between adjusted means.

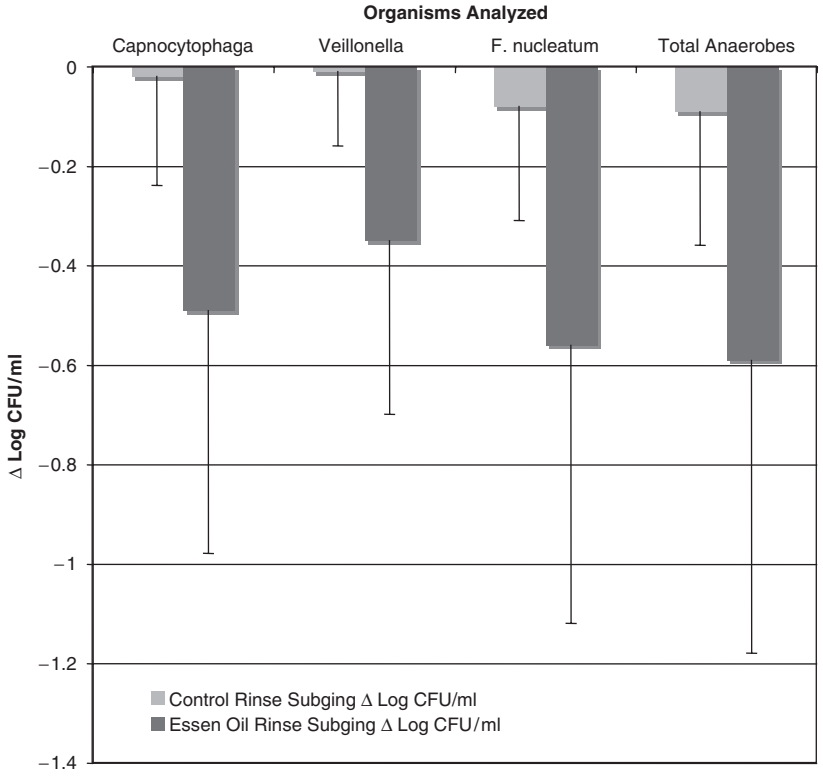

Fig. 2. Change in subgingival log CFU/ml of organisms tested after subjects rinsed for 2 weeks with control or essential oil mouthrinse. Figure shows reduction in total anaerobes and subgingival *Veillonella*, *Capnocytophaga*, *F. nucleatum* from control in log-transformed counts. Total anaerobes were reduced from baseline by 69.1%. *Veillonella*, *Capnocytophaga*, and *F. nucleatum* were reduced by 54.1%, 63.5%, and 68.3%, respectively. Reductions were statistically significant ($p<0.001$).

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Clinical Relevance

Scientific rationale for study: Supragingival plaque has been shown to be a reservoir for subgingival plaque organisms. This study investigated whether rinsing with an essential oil-containing antimicrobial mouthrinse can affect the composition of subgingival plaque.

Principal findings: Compared with the negative control, the essential oil mouthrinse produced significant reductions of subgingival as well as supragingival levels of the target organisms, *Fusobacterium nucleatum, Veillonella* sp., *Capnocytophaga* sp., and total anaerobes.

Practical implications: The results help to provide additional scientific support for inclusion of the mouthrinse in daily oral hygiene regimens for control of plaque and gingivitis.