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In vitro antimicrobial effects of two antihalitosis mouth rinses on oral pathogens and human tongue microbiota

Abstract: *Objectives:* The aim of the study was to compare the antimicrobial activity of a mouth rinse containing chlorhexidine and cetylpyridinium chloride (MR1) with a stannous fluoride-based mouth rinse (MR2) *in vitro*. *Materials and methods:* Samples of the tongues from 10 subjects with and 10 subjects without halitosis were inoculated on blood agar plates. The agar was perforated, and the cylindrical holes were filled either with mouth rinse MR1 or with mouth rinse MR2. After incubation, inhibition zones of the whole tongue microbiota and *Fusobacterium nucleatum* were measured. In addition, MR1 and MR2 were applied in a short interval killing test (SIKT) on four oral pathogens *Porphyromonas gingivalis*, *Prevotella intermedia*, *F. nucleatum* and *Aggregatibacter actinomycetemcomitans*. Total viable cell counts were made after two minutes of incubation with increasing concentrations of MR1 and MR2. *Results:* MR1 showed a significantly higher *in vitro* antimicrobial activity against the whole tongue microbiota and *F. nucleatum* than MR2 in both groups of subjects. In the SIK test, MR1 showed a significantly greater killing capacity than MR2. The results show that a mouth rinse with low concentrations of chlorhexidine and 0.05% cetylpyridinium chloride appears to be more effective in inhibiting growth of the human tongue microbiota *in vitro* than a fluoride/stannous fluoride-containing mouth rinse. *Conclusion:* This *in vitro* observation supports the use of chlorhexidine and cetylpyridinium chloride in the treatment of oral halitosis.

Key words: bacteria; halitosis; *in vitro*; mouth rinses

Introduction

Foul body odour, also known as kakidrosis (1–3), is a taboo in our society. Conditions that are associated with body odours are bromidrosis (the secretion of foul-smelling sweat), flatulence (excessive production of bowel gases) (4, 5) and halitosis (bad breath) (6, 7). One factor these conditions have in common is the essential role of bacteria in the aetiology of these conditions. Clinical surveys have shown that over 90% of all bad breath odours originate in the oral cavity, and this condition is called oral halitosis, bad breath or oral malodour (6, 8). If the origin of the source resides outside the oral cavity, it is named extra-oral halitosis (9).

The cause of halitosis is multi-factorial, but bacteria present in a biofilm at the dorsum of the tongue play an important role in the development of this condition (10). Some of these bacteria produce malodorous

volatile sulphur compounds (VSC), products of metabolic breakdown of the sulphur-containing amino acids methionine, cystine and cysteine, which are formed from protein hydrolysis of human tissues (11, 12). VSC are strongly associated with halitosis (13–15). McNamara *et al.* (13) observed that mainly Gram-negative microorganisms were responsible for VSC formation and were therefore considered the cause of bad breath. Yasukawa *et al.* (14) reported an increase in total VSC and organoleptic scores in subjects with increased numbers of *Treponema denticola* and *F. nucleatum* in tongue plaque, while the presence of both species in subgingival plaque did not show a gain of total VSC and organoleptic scores. Besides causing malodour, VSC in the oral cavity may play a role in periodontal disease (16–19). Exposure of methyl mercaptan and hydrogen sulphide to human gingival fibroblasts affects the protein and collagen metabolism in human gingival tissue (20).

Treatment of oral halitosis is based on reduction of the bacterial biofilm on the dorsum of the tongue. This is achieved by the removal of plaque deposits mechanically with toothbrush or tongue scraper and chemically by the use of a mouth rinse. Both interventions have been shown to reduce VSC levels in exhaled breath (12, 21–23). Mouth rinses containing zinc salts can act as a protective agent through binding of zinc to mucosal tissues thereby inactivating VSC and maintaining the permeability barrier (24). Zinc ions also directly bind volatile sulphur molecules and reduce levels of VSC (22). Chlorhexidine and cetylpyridinium chloride (CPC) are chemical compounds that derive their antimicrobial and anti-VSC characteristics from their ability to modify the hydrophobic regions of the bacterial cell walls (25). The effect of CPC is relatively small compared to that of chlorhexidine, but it is suitable for frequent and longer use (26). Stannous fluoride has shown activity against a range of oral microbes including streptococci as well as oral anaerobes (27). Although many mouth rinses are available, only a few have been specifically designed to combat intra-oral halitosis. The clinical effectiveness of antihalitosis oral rinses is based on antimicrobial activity and binding of VSC. Although clinical studies have been carried out, the antimicrobial activity has often not been established. The aim of this study was to study the antimicrobial activity of a chlorhexidine-containing (MR1) and a non-chlorhexidine-containing (MR2) mouth rinse. Two *in vitro* models were used to test the antimicrobial activity of the two mouth rinses: (i) by determining the growth inhibition of the whole human tongue microbiota and *F. nucleatum*, and (ii) by determining the short interval killing capacity of both mouth rinses on planktonic monocultures of four oral pathogens.

Materials and methods

Study population and methodology

The ethical committee responsible for clinical studies conducted by the University Medical Center Groningen approved the study protocol.

Twenty subjects were recruited for this study: 10 subjects without and 10 subjects diagnosed with intra-oral halitosis. The subjects with intra-oral halitosis were recruited from patients visiting a dental practice and had an organoleptic score ≥ 3 on a scale of 0–5 (22). The subjects without halitosis were volunteers from the dental department of the University Medical Center Groningen with an organoleptic score ≤ 1. None of the subjects suffered from severe or advanced periodontitis, but some showed moderate gingivitis.

Sampling and culturing

From each subject, a sample from the biofilm on the dorsum of the tongue was obtained by zigzag streaking with a sterile swab from the dorsal to the ventral site of the tongue while rotating the swab. The samples were transferred to vials containing 1.5 ml reduced transport fluid (28) and vortexed twice for 1 min with an interval of five minutes. After homogenization, tenfold dilution series were prepared in sterile saline, and aliquots of 100 µl of 10^{-3}, 10^{-4} and 10^{-5} dilutions were transferred to 5% sheep blood agar plates (Oxoid no. 2; Oxoid, Basingstoke, UK) supplemented with 5 mg l^{-1} hemin, 1 mg l^{-1} menadione) and spread using a sterile Drigalski loop to obtain homogeneous growth. After inoculation, four holes (diameter 7 mm) were punched in the agar plates and filled with either 60 µl of MR1 (in duplicate) (Halita®; Dentaid, Cerdanyola, Spain) or MR2 (in duplicate) (Meridol®; Gaba, Weesp, The Netherlands). The chemical compositions of the 2 rinses are summarized in Table 1.

60 µl of sterile saline (0.9% NaCl in water) was used as negative control and was tested in separate plates. Processing of the samples was performed in air. All experiments were performed in duplicate. Plates were incubated in jars in an atmosphere of 80% N_2, 10 H_2 and 10% CO_2 at 37°C for up to 5 days (Anoxomat; Mart Microbiology, Drachten, The Netherlands). Inhibition zones were measured after 3 and 5 days of incubation.

Table 1. **Chemical composition of the two mouth rinses used in this study**

MR1	MR2
Chlorhexidine digluconate 0.05%	Hydrochloric acid
Cetylpyridinium chloride 0.05%	Aqua
Zinc Lactate 0.14%	Zinc lactate
Aqua	Olaflur
Glycerin	Propylene glycol
Propylene glycol	Xylitol
Xylitol	Stannous fluoride 125 ppm
Digluconate	Amine fluoride 125 ppm
PEG-40 Hydrogenated castor oil	Hydrogenated castor oil
Gluconic acid	PEG 40
Sodium saccharin	Odium saccharin
Aroma	Aroma
CI 42090	CI 42051

Microbial inhibition zones

After 3 days of incubation, plates with semi-confluent growth were selected, and the diameter of the antimicrobial inhibition zones of the whole tongue microbiota was measured at three different positions at each of the two wells of MR1 and MR2. An average value was calculated from the six measurements of the two MR1 and the two MR2 zones.

Inhibition of monocultures of periodontal pathogens

To determine the antimicrobial activity of the two mouth rinses after a short exposure time, the short interval killing test (SIKT) was applied (29). Three strict anaerobic bacterial species *P. gingivalis* (Pg, ATCC 33277), *P. intermedia* (Pi, ATCC 25611) and *F. nucleatum* (Fn, ATCC 10953) and the capnophilic species *A. actinomycetemcomitans* (Aa, ATCC 33384) were cultured in brain heart infusion broth (BHI-2). The cultures were harvested at exponential growth and mixed to a total volume of 5 ml with increasing amounts of MR1 or MR2 ranging from 10%, 20%, 40% and 60% (v/v). The mixtures of mouth rinse and bacterial cells were incubated at 37°C by constant rotation. After incubation for 2 min, tenfold dilution series were prepared, and aliquots of 100 µl of the 10^{-5}, 10^{-6} and 10^{-7} dilutions were seeded on blood agar plates to determine total viable cell counts. Dilution series of cultures without mouth rinse were prepared, seeded and used as controls. Anaerobic incubation of the plates was performed as described. The survival of the bacteria after exposure to the MR1 and the MR2 mouth rinses was calculated as proportions of the control and expressed in percentages of survival. The experiments were performed five times in independent tests.

Data analysis

Data are expressed as mean (standard deviation, SD). The student's *t*-test (unpaired, two-tailed) was applied for assessing differences in the mean inhibition zones and the Mann–Whitney *U*-test (independent samples, two-tailed) for differences in inhibition of monocultures of periodontal pathogens between the two mouth rinses. $P < 0.05$ was regarded as significant.

Results

The negative controls with sterile saline produced no inhibition of the whole tongue microflora in any of the experiments. Mean inhibition zones of the two rinses on whole tongue microbiota from the 10 subjects with and 10 subjects without oral halitosis are shown in Table 2.

No differences between the mean inhibition zones after 3 and 5 days of incubation were observed. The growth inhibition zones of MR1 or MR2 on the whole tongue microbiota of the non-halitosis subjects did not differ from those of the halitosis subjects. MR1 showed a significantly higher *in vitro* antimicrobial activity against the whole tongue microbiota of both halitosis ($P < 0.0001$) and non-halitosis subjects ($P < 0.0001$) in comparison with MR2. Because a clear specific inhibition of *F. nucleatum* was observed, it was possible to measure the distance from the centre of the punch hole to the nearest *F. nucleatum* colonies. The specific mean inhibition zones of *F. nucleatum* were significantly greater by MR1 than by MR2 in both halitosis ($P = 0.0014$) and non-halitosis subjects ($P < 0.0001$) (Table 2).

Table 2. **Mean inhibition zones (mm) and standard deviations (SD) of 2 oral mouth rinses on whole tongue microbiota and *F. nucleatum* from the 10 subjects with and 10 subjects without halitosis**

	Non-Halitosis		Halitosis	
	MR1	MR2	MR1	MR2
Inhibition zone of whole tongue microbiota (mm)	17 (0.9)*	11 (0.8)	16 (1.2)*	11 (0.6)
Inhibition zone of *F. nucleatum* (mm)	9 (2.2)†	6 (1.2)	9 (1.6)†	6 (0.7)

*Significantly greater inhibition zone of MR1 than MR2, $P < 0.0001$.
†Significantly greater inhibition zone of MR1 than MR2, $P < 0.0014$.

The results of the SIKT test are shown in Figures 1a–d. Both mouth rinses showed a concentration-dependent antimicrobial activity. There were marked differences in the susceptibility of the tested species towards the two mouth rinses. *P. gingivalis* (Fig. 1a) was most susceptible to MR1; a significantly better killing was observed by MR1 at all concentrations tested ($P < 0.05$). At a concentration of 10% (v/v), 3% survival was observed for MR1 and 54% for MR2. No differences in survival were found for *P. intermedia* (Fig. 1b). Besides for *P. gingivalis*, differences between MR1 and MR2 were also observed for *F. nucleatum* (Fig. 1c) and *A. actinomycetemcomitans* (Fig. 1d). While almost complete killing was obtained with 40% MR1, MR2 showed significantly ($P < 0.05$) less killing effects on these two species with still some bacterial survival at 60%.

Discussion

In this study, the antimicrobial activity of two mouth rinses commonly used to treat oral halitosis was investigated *in vitro*. Two models were used to study the ability to affect the growth of whole tongue biofilm bacteria and monospecies cultures of oral pathogens. The rational for this study is based on the concept that oral halitosis has a bacterial aetiology. The rationale for the selected two mouth rinses was the fact that both products have been designed to combat intra-oral halitosis and claim antimicrobial activity. To test the antimicrobial effects of both mouth rinses, the growth inhibition capacity on whole tongue microbiota and the differential inhibition capacity of *F. nucleatum* sp. were tested. In addition, the short interval killing characteristics of both mouth rinses were studied using planktonic monocultures of four oral pathogens. The observation of similar growth inhibition zones of the tongue biofilm bacteria from subjects with and without halitosis can be explained by the non-specific antimicrobial effects of

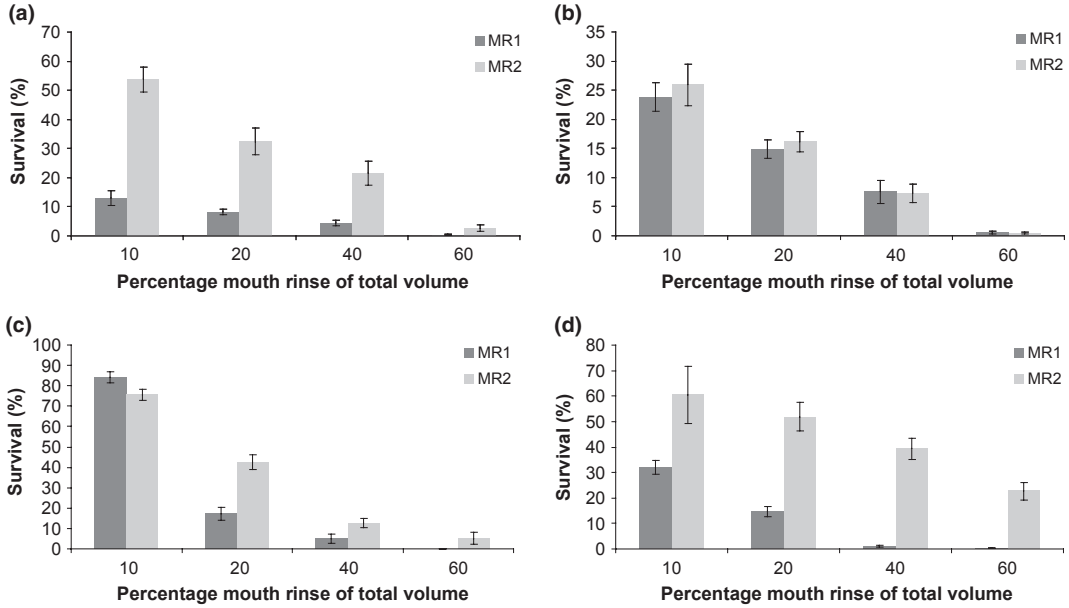

Fig. 1. In this short interval killing test (SIKT), an increasing volume (v/v) of antimicrobial agent is added to a bacterial suspension in BHI broth. After 2 minutes of exposure, the percentage of surviving bacterial cells relative to the initial inoculum was determined. On the *x*-axis, the percentage (v/v) of mouth rinse relative to the total volume is depicted. (a) *P. gingivalis*. Growth inhibition of MR1 is significantly greater at all concentrations ($P < 0.01$). (b) *P. intermedia*. Growth inhibition of MR1 and growth inhibition MR2 are not significantly different ($P > 0.05$). (c) *F. nucleatum* Growth inhibition of MR1 is significantly greater at 20%, 40% and 60% concentrations ($P < 0.01$). (d) *A. actinomycetemcomitans*. Growth inhibition of MR1 is significantly greater at all concentrations ($P < 0.01$).

chlorhexidine and CPC in MR1 and of amine fluoride/stannous fluoride in MR2. In both experiments, MR1 showed a significantly higher antimicrobial effect than MR2. The strong effect of MR1 on *F. nucleatum* is also noteworthy. This species has been implicated in the aetiology of intra-oral halitosis by several authors (14, 30, 31), and this observation can explain the effect of MR1 on organoleptic and objective halitosis scores (22, 32).

The greater antimicrobial effect of MR1 was also apparent in the short interval killing experiment. In this experiment, the killing potential of an antimicrobial agent is tested after a contact time of only 2 min. This information may be relevant because rinsing or gargling is usually performed for a short time. At low concentration, MR1 showed a significant killing effect on *P. gingivalis*, a species that is a strong producer of VSC (33, 34) and frequently detected on the dorsum of the tongue in oral halitosis patients (35). MR1 showed stronger killing effects in comparison with MR2 for three of the four pathogens tested. Antihalitosis rinses have multiple actions to reduce oral bad breath. Capturing the produced VSC by zinc lactate is one mode of action, and reducing the VSC-producing bacterial load on the tongue dorsum is a second mode of action. Only the antimicrobial capacity of both mouth washes was tested in this study. MR1 is one of few products that have been tested for its antihalitosis activity in a double-blind, placebo-controlled study in patients with overt oral halitosis, and has shown clinical efficacy (22). Treatment with MR1 has demonstrated reduction in VSC values and scored high on sensory tests in a comparative study (36). Furthermore, MR1 has recently been used as a reference in a study with MR2 (37).

Conclusions

On the basis of the observation made in this *in vitro* study, we conclude that an oral mouth rinse containing a low concentration of chlorhexidine and 0.05% CPC is more effective than an oral mouth rinse containing fluoride/stannous fluoride, in inhibiting tongue microbiota *in vitro* of both healthy subjects and patients with oral halitosis. Also, short contact time of a chlorhexidine-containing mouth rinse results in killing of oral Gram-negative VSC-producing anaerobes.

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Conflict of interest

The authors declare that they have no conflict of interests.

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