Oral Microbiology Immunology 2005: 20: 180–185
Printed in Singapore. All rights reserved

Oral Microbiology and Immunology

An *ex-vivo* multiplexed antibacterial test on oral microflora

Y. Yang, P. K. Sreenivasan
Colgate-Palmolive Company, Piscataway, NJ, USA

Yang Y, Sreenivasan PK. An ex-vivo *multiplexed antibacterial test on oral microflora. Oral Microbiol Immunol 2005: 20: 180–185.* © Blackwell Munksgaard, 2005.

Background/aims: Characterized clinical strains of oral bacteria are utilized to examine the antimicrobial efficacy of oral care formulations. A demonstration of antimicrobial effects of formulations on microbial samples obtained from the human mouth offers advantages, i.e. incorporates the considerable microbial diversity of this environment with bacteria harvested from naturally occurring biofilms to form the focus of this investigation.
Materials and methods: Samples of oral flora from each adult subject were briefly treated (2 min) with test and control formulations and plated on appropriate agar for multiplexed antimicrobial effects on functional groups of oral bacteria associated with specific conditions.
Results: *Ex-vivo* treatments of oral samples with the triclosan/copolymer dentifrice demonstrated significant dose-dependent antimicrobial effects compared with a control formulation on anaerobic and facultative oral bacteria from a group of 16 volunteers ($P < 0.05$) with reproducible effects observed in three separate trials ($P < 0.05$). Similarly, significant dose-dependent effects were noted with chlorhexidine mouthrinses ($P < 0.05$). A simultaneous assessment of the effects of these formulations on several functional classes of oral bacteria associated with specific oral conditions such as dental caries and oral malodor demonstrated multiplexed antimicrobial activity ($P < 0.05$).
Conclusions: A rapid procedure using small volumes of human oral samples and well-known formulations demonstrates *ex-vivo* multiplexed antimicrobial effects on functional groups of bacteria with both dentifrices and mouthrinses. Modifications of this approach may include antibacterial effects over time with formulations at clinically relevant concentrations, effects of novel agents or samples from subjects stratified on the basis of their clinical status.

Key words: biofilm; chlorhexidine; clinical tests; dental plaque; mouthrinse; saliva; tongue; triclosan

Prem K. Sreenivasan, Colgate-Palmolive Company, 909 River Road, Piscataway, NJ 08855, USA
Tel.: +1 732 878 6375;
fax: +1 732 878 6031;
e-mail: prem_sreenivasan@colpal.com
Accepted for publication December 8, 2004

The diverse microbial ecology of the human mouth includes large numbers of microorganisms in the saliva and those found in plaque, a complex, naturally occurring biofilm associated with teeth and the tongue (4, 8, 27). Clinical investigations have examined the relationship between oral organisms and the etiology of oral conditions such as periodontal disease, caries, and oral malodor. Based on clinical observations, current dental practices emphasize the control of oral organisms, particularly those found in the plaque, to maintain oral health (8, 27).

Efficacious formulations with anti-plaque effects serve as an important adjunct for the maintenance of oral health (8, 17, 27). To develop and validate these formulations, an extensive set of laboratory and clinical studies is required to examine attributes relevant to oral health (8). An important aspect of these tests is the demonstration of antimicrobial effects on laboratory and clinical strains of oral bacteria. These tests examine the effects of antimicrobials on a large set of bacteria in the planktonic (3, 13, 16) or biofilm mode of growth (8, 22), with recent studies comparing effects on bacteria in both modes (6). Most of the tests are modifications of methods available in the field of antibiotics (8, 15), with many tests typically determining the effects on one bacterial strain per test (3, 6, 8, 13, 15).

This investigation reports the development of a rapid *ex-vivo* antimicrobial test using oral samples derived from adult human volunteers. Subjects provided saliva samples by rinsing their mouth and samples were also obtained from tongue scrapings. The sampling regimen incorporates the inherent variability in the

numbers and density of the microorganisms among subjects (18). In contrast to laboratory cultures, organisms derived from natural habitats are more likely to demonstrate diverging physiological states reflecting the changing parameters of these environments such as nutrition, pH, and oxygen tension (4, 8, 27). Tests were conducted with small volumes of sample to examine the effects of oral hygiene formulations with common antimicrobial agents at concentrations typically found in the mouth during use (2, 31). The dose-dependent antimicrobial effects were also examined. Additionally, the tests were multiplexed, i.e. the tests examined antimicrobial effects on functional classes of oral organisms associated with specific oral conditions to rapidly screen several formulations.

Materials and methods

Chemicals and media

Buffers and chemicals for routine use were obtained from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise. Premade agar media obtained from Becton-Dickinson (Sparks, MD) were trypticase soy agar enriched with 5% sheep blood (BA) for the enumeration of all oral bacteria. Also obtained from Becton-Dickinson were agar media supplemented with 5% sheep blood and the selective agents phenylethyl alcohol or kanamycin, and vancomycin to isolate gram-positive and gram-negative bacteria, respectively (7). Prepared media obtained from Anaerobe Systems (San Jose, CA) were mitis-salivarius agar to enumerate streptococci and oral hydrogen sulfide (H_2S) producing organisms agar (OHO) to enumerate oral H_2S producing organisms (20). Another medium with casein as a protein source and supplements designed to enumerate oral proteolytic bacteria and referred to as the proteolytic organism's differentiation agar was prepared. A range of oral bacteria with proteolytic activity grow on this medium as demonstrated by a zone of clearing or a creamy colored or pigmented colony. Oral bacteria implicated in malodor such as *Fusobacterium nucleatum*, *Prevotella intermedia,* and *Prevotella nigrescens* produce characteristic reactions on this medium. Dehydrated Rogosa agar for the isolation of lactobacilli was obtained from Becton-Dickinson and reconstituted in accordance with the manufacturer's instructions. The agar media used to enumerate aciduric and iodophilic oral bacteria were prepared in accordance with published procedures (30, 32). In brief, agar at pH 5.5 was utilized to enumerate aciduric bacteria without preselection of acid adapted bacteria. Media supplemented with 5% sucrose were utilized for iodophilic bacteria. After incubation, iodophilic media were flooded with gram's iodine and colonies with a strong brown color enumerated as iodophilic.

Formulations tested

Test formulations obtained commercially included the triclosan/copolymer dentifrice with 0.3% triclosan and 2% gantrez (triclosan/copolymer dentifrice) and a fluoride dentifrice that served as a control (control dentifrice). The mouthrinses tested were a fluoride rinse with no additional antimicrobial agents and a rinse with 0.12% chlorhexidine. Both rinses are available commercially and are formulated with ethanol. Another rinse with 0.06% chlorhexidine was included and is identical to the 0.12% rinse except for the concentration of chlorhexidine.

Collection of oral microflora

Adult volunteers (18–65 years old) participated in studies that were conducted in accordance with procedures widely accepted for clinical trials (1). Subjects on prescription medications or on antibiotic therapy in the past month were excluded and the selected participants were informed of the test procedure. Volunteers accepted for tests were provided with a commercially available fluoride dentifrice and a soft-bristled toothbrush for their oral hygiene during the 1-week wash-out phase and for the duration of their participation in tests. In addition, volunteers discontinued the use of all other oral hygiene formulations including chewing gums, mints, etc. Groups of subjects were scheduled for studies and requested to discontinue oral hygiene procedures prior to their arrival at the dental clinic for sample collection. Based on availability, a few subjects were scheduled per day with sample collection completed by 9 AM. Commercially available drinking water (Poland Springs, Hollis, ME) was dispended in sterile tubes (10 ml) and subjects instructed to rinse for 10 s. The samples were collected in sterile tubes and are referred to as saliva rinse samples. Additional scrapings from the tongue were collected from subjects who were provided with a sterile brush (MasterAmp Buccal Swab Brushes, Epicenter, Madison, WI) and instructed to scrape the entire surface of the tongue. Tongue scrapings were pooled with the saliva rinse samples.

Preparation of dentifrices for tests

Test dentifrices (10 g) were weighed and mixed with water to obtain dentifrice to water ratios (w/w) of 1 : 2, 1 : 3, and 1 : 4. These mixtures were stirred to homogeneity on a magnetic stir plate at room temperature and the slurries centrifuged at 8,000 *g* for 10 min. The supernatants were decanted and used for tests.

Antibacterial tests

Tests with dentifrices

Oral microbial samples from each subject (1.9 ml) were incubated for 2 min at room temperature with 0.1 ml of each dentifrice concentration and 0.1 ml of phosphate buffered saline (PBS), as an untreated control. After incubation, these treatments were rapidly diluted (10-fold dilutions) in PBS and plated on appropriate agar as described for each test. Microorganisms growing on BA, mitis-salivarius, proteolytic, aciduric, iodophilic and OHO agars were enumerated after anaerobic incubation at 37°C for 4–7 days as described previously (7, 9, 13, 20, 26, 30, 32). Gram-positive and gram-negative bacteria were incubated at 37°C in anaerobic conditions in accordance with published procedures (7). Aerobic incubation at 37°C for 4–5 days was utilized for the enumeration of aerobic organisms on BA and lactobacilli on Rogosa agar (7).

Tests with mouthrinses

The tests with mouthrinses were similar to those described with dentifrices. Mouthrinses (0.1 ml) were incubated for 2 min with oral samples (1.9 ml) from each subject. These samples and an untreated control were diluted in PBS and plated on BA. Media were incubated under anaerobic conditions for bacterial enumeration as described above.

Statistical analysis

Bacterial counts (CFU/ml) from each subject following each treatment were transformed to $\log_{10}$ for analysis. The antimicrobial effects of different concentrations of formulations were examined by analysis of variance (ANOVA). Significant differences were analyzed by a generalized linear model with the two factors (subjects and treatments) as effects. Multiple comparison tests with contrasts examined the effects of specific concentrations. Studies determining the

effects of formulations on different types of bacteria were examined by ANOVA with *posthoc* comparisons by Tukey tests. Statistical analyses were conducted by SAS (SAS Institute, Cary, NC) with significance for all tests reported at $P < 0.05$.

Results

The *ex-vivo* effects of different concentrations of dentifrices on oral bacteria

Initial studies with 16 subjects compared the effects of different concentrations of dentifrices on oral bacteria. For the study, the effects of the triclosan/copolymer dentifrice and the control dentifrice were tested at three concentrations as described in Materials and methods and are referred to as series 1–3, respectively, in Fig. 1. Within each series, the concentration of the dentifrices were similar. The final triclosan concentrations in series 1, 2, and 3 were 50, 37.5, and 30 ppm, respectively. Within each series (series 1–3) treatment with the triclosan/copolymer dentifrice resulted in significantly lower recoveries of viable bacteria in comparison to the control ($P < 0.05$). The percent differences in viable bacteria between the control and the triclosan/copolymer dentifrice in series 1, 2 and 3 were 60%, 64%, and 72%, respectively, with *posthoc* analysis by multiple comparison tests indicating dose-dependent effects by the triclosan/copolymer dentifrice ($P < 0.05$).

The *ex-vivo* multiplexed effects of dentifrices on different classes of oral bacteria

The *ex-vivo* effects of dentifrices on different functional groups of oral bacteria were studied for the triclosan/copolymer dentifrice and the control dentifrice. The tests were conducted at the least concentration of both dentifrices from the section above (equivalent to 30 ppm triclosan).

Effects on gram-positive and gram-negative bacteria and bacteria implicated in halitosis

This experiment compared effects of the triclosan/copolymer and the control dentifrices on oral bacteria that included the anaerobic and facultative members, gram-positive and gram-negative bacteria. The effects on oral bacteria producing H_2S and proteolytic activity were also determined. The results (Fig. 2) from this study (samples from 14 subjects) demonstrate that treatment with the triclosan/copolymer dentifrice resulted in a significant decrease in each class of oral bacteria compared with the control dentifrice ($P < 0.05$); a 59%, 51%, 53%, 51%, and 68% percent decrease in anaerobic bacteria, gram-positive bacteria, proteolytic bacteria, bacteria producing H_2S and gram-negative bacteria, respectively.

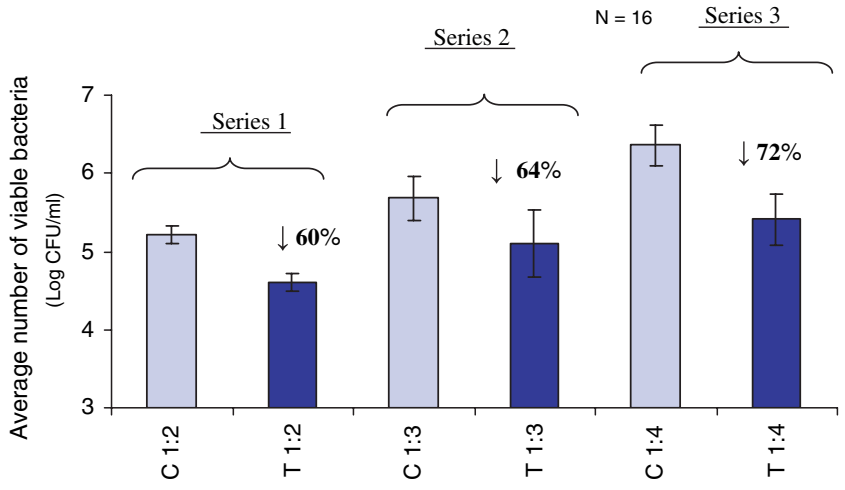

Fig. 1. The e*x-vivo* antimicrobial effects of different concentration of dentifrices on oral microbial samples from 16 subjects. The control dentifrice (C) and the triclosan/copolymer dentifrice (T) were tested. Shown in figure are the average numbers of viable bacteria (Log CFU/ml) following each treatment ± the standard error of the mean. Dentifrice concentrations within each series are similar. The applied dose of triclosan/copolymer in series 1 to 3 correspond to 50, 37.5 and 30 ppm respectively. Statistically significant results are indicated as * ($P < 0.05$).

Effects on oral bacteria implicated in caries

Recent studies indicate the prevalence of mutans streptococci and significant numbers of several other types of bacteria in caries. These include bacteria that grow at low pH (aciduric) and bacteria that synthesize and store intracellular polysaccharides (iodophilic) (21, 24). A study with 10 subjects simultaneously determined the effects of dentifrices (the triclosan/copolymer and control) on several types of bacteria, i.e. anaerobic and aerobic bacteria, streptococci, iodophilic, acidophilic bacteria, and lactobacilli. The effects of the triclosan/copolymer dentifrice were significantly greater than the control dentifrice on each type of bacteria assessed ($P < 0.05$) (Fig. 3); a 66%, 89%, 85%, 73%, 90%, and 71% percent decrease in anaerobic bacteria, aerobic bacteria, mutans streptococci, iodophilic, acidophilic bacteria, and lactobacilli, respectively.

Statistical analysis of the results from the three *ex-vivo* studies comparing the triclosan/copolymer and the control dentifrices

The percent differences in the numbers of oral anaerobic bacteria recovered from each of the above three trials (with different groups of subjects) after treatment with the control dentifrice and the triclosan/copolymer dentifrice (at a concentration equivalent to 30 ppm triclosan) were analyzed by a paired *t*-test ($\alpha = 0.05$). Differences between dentifrices from each trial were significantly greater than zero (P-values < 0.01), i.e. the average number of bacteria recovered post-treatment with the control was always higher than with the triclosan/copolymer dentifrice .

To examine the overall reproducibility of the trials, a one-way ANOVA compared the percent differences of anaerobic bacteria after using the triclosan/copolymer and the control dentifrices in the three trials. No significant differences were found in the three groups of subjects (P-value $= 0.626$), indicating similar effects of the dentifrices in the three trials (Fig. 4). Further, analysis of the pooled differences between the dentifrices from the three trials demonstrates an average percent difference ± 95% confidence interval of 65.8% ± 11.0%, respectively, (95% confidence interval ranged from 54.8% to 76.8%).

The *ex-vivo* effects of mouthrinses

With oral samples from 13 subjects, the effects of the 0.06 and 0.12%

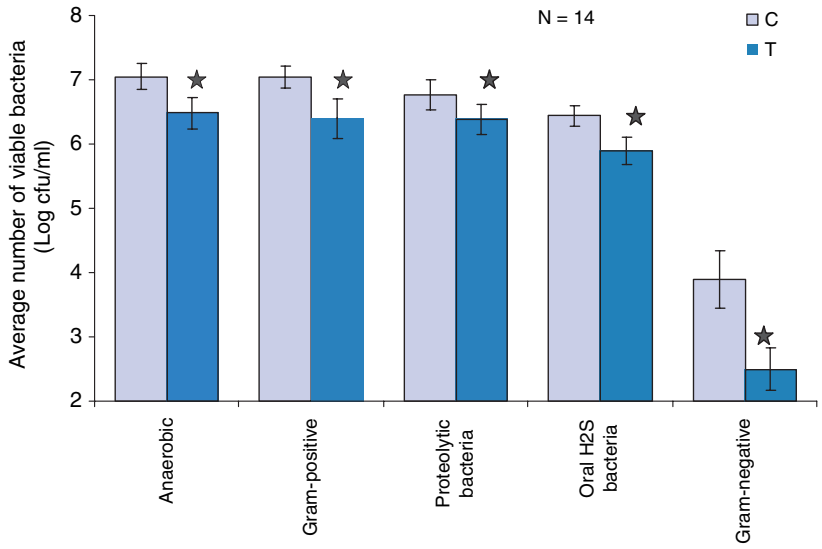

Fig. 2. The *ex-vivo* effects of control dentifrice (C) and triclosan/copolymer (T) on several classes of oral bacteria including those implicated in halitosis. Both dentifrices were applied at similar concentration and reflect 30 ppm of triclosan/copolymer in the applied dose. Shown in figure are the average numbers of viable bacteria (log CFU/ml) following each treatment ± the standard error of the mean from 14 subjects. Statistically significant results are indicated as * by Tukey *posthoc* analysis ($P < 0.05$).

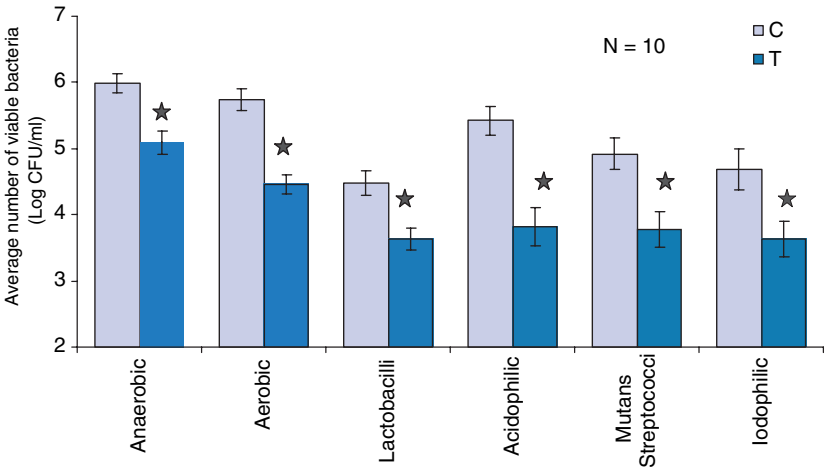

Fig. 3. The *ex-vivo* effects of dentifrices (C) and triclosan/copolymer (T) on several classes of oral bacteria including those implicated in caries. Both dentifrices were applied at similar concentration and reflect 30 ppm of triclosan/copolymer in the applied dose. Shown in figure are the average numbers of viable bacteria (log CFU/ml) following each treatment ± the standard error of the mean from 10 subjects. Statistically significant results are indicated as * by Tukey *posthoc* analysis ($P < 0.05$).

chlorhexidine mouthrinses were examined using the rinse without chlorhexidine as a control. The results (Fig. 5) demonstrate significant effects of both chlorhexidine rinses on oral anaerobic microflora in comparison to the control rinse ($P < 0.05$). A 77% and 58% decrease in anaerobic microflora were observed with the 0.12% and 0.06% chlorhexidine rinses, respectively, compared with the control rinse, representing statistically significant dose-dependent effects ($P < 0.05$). Additional parameters demonstrate the significant effects of these chlorhexidine rinses on mutans streptococci and oral bacteria producing H_2S compared to the control rinse ($P < 0.05$). A dose-dependent decrease in mutans streptococci with an 81% and 56% decrease in bacteria was observed with the 0.12% and 0.06% chlorhexidine rinses, respectively ($P < 0.05$). The 0.12% and 0.06% chlorhexidine rinses produced an 82% and 69% decrease in oral H_2S-producing bacteria, respectively; however, no significant dose-dependent effects were observed.

Discussion

Chlorhexidine and triclosan are broad-spectrum agents that find application in oral care formulations (8, 17, 19, 33). The significant effects of these formulations have been extensively documented in clinical studies, with improvements noted in several indices for oral health. The present investigations were conducted with these agents at different concentrations as model systems to develop and optimize test parameters with oral samples *ex-vivo*.

Test conditions including the period of bacterial contact with treatments and the concentrations for agents tested were standardized. Oral samples were treated for 2 min to reflect the 68–83 s of brushing reported in subjects (25). Triclosan concentrations for the *ex-vivo* tests ranged from 37.5 to 50 ppm. Previous studies report 19 ± 12 μg/ml triclosan in saliva 5 min postbrushing (2). Concentrations of chlorhexidine in *ex-vivo* tests are similar to previous clinical results describing salivary levels of chlorhexidine after treatments. Salivary levels of 20 μg/ml of chlorhexidine were reported 15 min after rinsing with a 0.1% chlorhexidine rinse (31). The *ex-vivo* tests with chlorhexidine were conducted with 60 μg/ml.

Procedures for sample collection from subjects were identical to preserve the diversity and individual variations in the prevalence of oral bacteria amongst subjects. Factors including food and host physiology influence variations in the composition of oral microflora (4, 8, 27) in addition to the clonal diversities in isolates of oral bacteria (*F. nucleatum*, *Streptococcus mitis*, *Eikenella corrodens, Actinomyces naeslundii, Actinobacillus actinomycetemcomitans*, and others) (10, 12, 14, 24). Differences in the antibiotic susceptibility of laboratory bacterial strains and those derived from natural environments (15) and in biofilms are known (11, 27). For example, a recent report indicates heterogeneity among isolates of *Stentophomonas maltophilia* from cystic fibrosis and non-cystic fibrosis patients along with variations in antibiotic susceptibility patterns in related or closely related clones from individuals (5). In this study, the antimicrobial effects of test and control formulations were examined on oral bacteria including samples from the tongue to include bacteria in a biofilm mode of growth. The advantages of *ex-vivo* tests include an examination for effects on the entire spectrum of oral flora incorporating natural flora and the diverse clones among

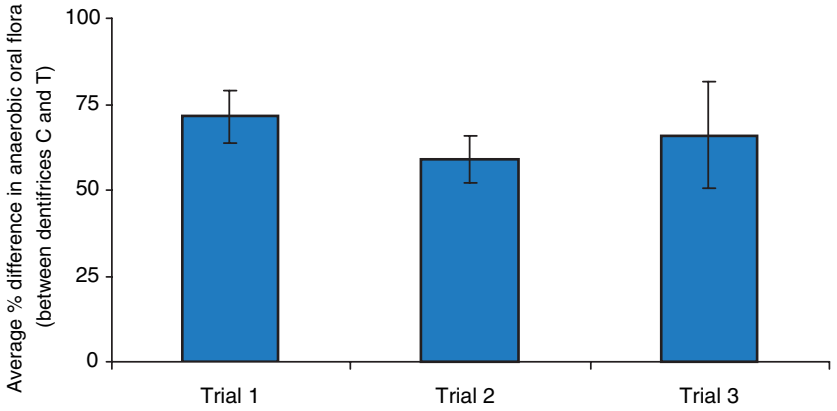

Fig. 4. A comparison of the percent differences between dentifrices (C) and triclosan/copolymer (T) on oral anaerobic bacteria in three studies. Both dentifrices were applied at similar concentration and reflect 30ppm triclosan/copolymer in the applied dose. Shown in figure are the average percent differences between C and T ± standard error of the mean from each study.

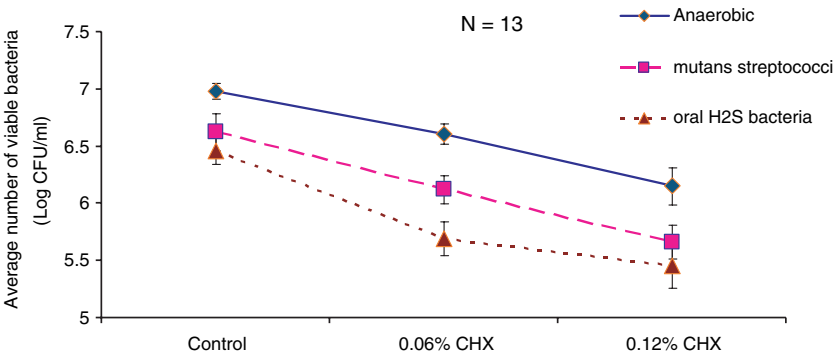

Fig. 5. The *ex-vivo* effects of chlorhexidine mouthrinses (0.06% and 0.12%) and a control rinse on several classes of oral bacteria. Shown in figure are the average numbers of viable bacteria (log CFU/ml) following each treatment ± the standard error of the mean from 13 subjects.

individuals with the bacteria recovered after treatment available for microbial identification. Further modifications include e*x-vivo* tests with samples from individuals screened on the basis of their clinical status or microbial profiles. It is unlikely that laboratory methods for assessing antimicrobial effects would sufficiently replicate this range of variables for the growth of oral microorganisms as found in samples obtained from the human mouth.

Microbial culture-based isolation of microflora examined the effects of treatments on functional groups of organisms associated with specific oral conditions for multiplexed antimicrobial effects. This approach is in contrast to that determining the effects on selected bacteria such as *Streptococcus mutans, Porphyromonas gingivalis* or *A. actinomycetemcomitans* (3, 6, 8, 13). The multiplexed concept was examined with microbiological procedures based on previous studies for the recovery of several classes of oral bacteria, including anaerobic, aerobic oral flora, gram-positive, gram-negative bacteria, and oral bacteria implicated in malodor and caries (8, 17, 27, 30, 32). Also included was an agar medium to enumerate proteolytic bacteria. Oral bacteria metabolize proteins and peptides to produce malodorous volatile sulfur, compounds which are implicated in malodor (17). Casein is a primary substrate in the proteolytic medium and the inclusion of this agar enabled comparisons with anaerobic bacteria and those producing H_2S.

Initial studies with the triclosan/copolymer dentifrice demonstrated a significant dose-dependent effect on oral anaerobic microflora compared with a commercial fluoride dentifrice. Further tests at the lowest concentration of these dentifrices indicate that the triclosan/copolymer dentifrice consistently caused significant decreases in anaerobic bacteria in three separate trials. These observations are relevant to the overall reproducibility of the tests, and indicate that, on average, treatment with the triclosan/copolymer dentifrice resulted in a 65% decrease of bacteria compared with treatment with the control formulation in the three studies. In a previous clinical trial, there was a 49% decrease in salivary microflora along with effects on bacteria producing H_2S in subjects provided with the triclosan/copolymer dentifrice (28). An extensive series of clinical trials have conclusively demonstrated the effects of triclosan/copolymer on supragingival plaque, gingivitis, and malodor (33). Further, reductions in subgingival microflora, including *P. gingivalis, P. intermedia,* and *A. actinomycetemcomitans,* have been reported in periodontitis-susceptible subjects provided with the triclosan/copolymer dentifrice (23). *Ex-vivo* results on different classes of microorganisms are consistent with these earlier clinical results. Gram-positive and gram-negative bacteria were reduced by 51% and 68%, and microorganisms implicated in caries and malodor by 66–90% and 51–53%, respectively.

Another area investigated was the effects of mouthrinses in the *ex-vivo* tests. Procedures amenable to an assessment of both dentifrices and mouthrinses enable an assessment of different delivery systems. Based its extensive use for oral applications, chlorhexidine was chosen as the antimicrobial agent for the mouthrinses. Tests conducted with two concentrations of chlorhexidine (0.06% and 0.12%) have previously been reported as within the plateau range for dose-dependent effects in clinical studies (26 and references therein). Additionally, our previous efforts have examined the dose–response of chlorhexidine in laboratory and clinical studies (29). In this investigation the effects of 0.06% and 0.12% chlorhexidine mouthrinses were compared with a control rinse. Some studies comparing different chlorhexidine mouthrinses have utilized saline as a control (13); however, for the present study, the control comprised a mouthrinse without chlorhexidine. In the *ex-vivo* studies, the chlorhexidine rinses demonstrate a dose-dependent effect on oral anaerobic microflora compared to the control rinse and corroborate previous results examining effects on salivary bacteria in samples obtained after rinsing (29). The 0.12% chlorhexidine rinse resulted on average in a 77% decrease on anaerobic bacteria compared to the control rinse, comparing favorably with previous clinical results that demonstrate an approximately

80% reduction in anaerobic salivary bacteria at 5 min postrinsing (13). Other investigations with chlorhexidine rinses (formulated with 0.12% or 0.2% chlorhexidine) also document substantial effects (80–99% reductions) on anaerobic salivary bacteria. However, these studies included oral prophylaxis or examination after several days of rinsing (26 and references therein).

The dose-dependent effects of chlorhexidine on oral streptococci were tested, based on previous reports examining the effects of chlorhexidine on oral streptococci (19). Confirmation of the present results in clinical studies may facilitate the development of additional intervention strategies without the side-effects observed with long-term use of chlorhexidine. The *ex-vivo* tests also determined the effects of the chlorhexidine rinses on oral bacteria producing H_2S and implicated in malodor and were done on the basis of the significant inhibiting effects of chlorhexidine on oral malodor in clinical studies (17). The two chlorhexidine rinses demonstrated significant effects on H_2S-producing bacteria compared with the control and are similar to results from a clinical study that determined the effects on salivary H_2S-producing bacteria (29).

In summary, the advantages of the *ex-vivo* procedure include tests with small volumes of oral samples for simultaneously examining several formulations that may be available as dentifrices or mouthrinses on bacterial samples from volunteers incorporating diverse populations of bacteria. Modifications of the *ex-vivo* procedure might include tests with samples from specific niches of the human mouth or samples from subjects selected on the basis of their clinical status. The *ex-vivo* procedure may aid in the initial screening of new investigational agents.

Acknowledgments

The authors gratefully acknowledge Dr. C. Lewus for constructive comments on the manuscript. Statistical analyses by L.R. Mateo, V. Galicia and C. Kloos, Technology Statistics is gratefully acknowledged.

References

1. Addy M. Evaluation of clinical trials of agents and procedures to prevent caries and periodontal disease: choosing products and recommending procedures. Int Dent J 1995: **45**: 185–196.
2. Afflitto J, Fakhry-Smith S, Gaffar A. Salivary and plaque triclosan levels after brushing with a 0.3% triclosan/copolymer/NaF dentifrice. Am J Dent 1989: **2**: 207–210.
3. Botelho MG. Fractional inhibitory concentration index of combinations of antibacterial agents against cariogenic organisms. J Dent 2000: **28**: 565–570.
4. Bowden GHW, Hamilton IR. Survival of oral bacteria. Crit Rev Oral Biol Med 1998: **9**: 54–85.
5. Canton R, Valdezate S, Vindel A, Sanchez Del Saz B, Maiz L, Baquero F. Antimicrobial susceptibility profile of molecular typed cystic fibrosis *Stenotrophomonas maltophilia* isolates and differences with non-cystic fibrosis isolates. Pediatr Pulmonol 2003: **35**: 99–107.
6. Decker E, Weiger R, von Ohle C, Wiech I, Brecx M. Susceptibility of planktonic versus attached *Streptococcus sanguinis* cells to chlorhexidine. Clin Oral Invest 2003: **7**: 98–102.
7. Difco and BBL. Manual for Microbiological Culture Media. Sparks, MD: Becton-Dickinson and Co., 2003.
8. Fine DH. Chemical agents for the prevention and regulation of plaque development. Periodontol 2000 1995: **8**: 87–107.
9. Fine DH, Furgang D, Bonta Y, DeVizio W, Volpe AR, Reynolds H, et al. Efficacy of a triclosan/NaF dentifrice in the control of plaque and gingivitis and concurrent oral microflora monitoring. Am J Dent 1998: **11**: 259–270.
10. Fujise O, Chen W, Rich S, Chen C. Clonal diversity and stability of subgingival *Eikenella corrodens*. J Clin Microbiol 2004: **42**: 2036–2042.
11. Gilbert P, Maria-Litran T, McBain AJ, Rickard AH, Whyte FW. The physiology and collective recalcitrance of microbial biofilm communities. Adv Microb Physiol 2002: **46**: 202–256.
12. Haraldsson G, Holbrook WP, Kononen E. Clonal persistence of oral *Fusobacterium nucleatum* in infancy. J Dent Res 2004: **83**: 500–504.
13. Herrera D, Roldán S, Santacruz I, Santos S, Masdevall M, Sanz M. Differences in antimicrobial activity of four commercial 0.12% chlorhexidine mouthrinse formulations: an *in vitro* contact test and salivary bacterial counts study. J Clin Periodontol 2003: **30**: 307–314.
14. Hohwy J, Reinholdt J, Kilian M. Population dynamics of *Streptococcus mitis* in its natural habitat. Infect Immun 2001: **69**: 6055–6063.
15. Isenberg HD. Antimicrobial susceptibility testing: a critical evaluation. J Antimicrob Chemother 1988: **22** (Suppl. A): 73–86.
16. Katsura H, Tsukiyama R, Suzuki A, Kobayashi M. *In vitro* antimicrobial activities of bakuchiol against oral microorganisms. Antimicrob Agents Chemother 2001: **45**: 3009–3013.
17. Loesche WJ, Kazor C. Microbiology and treatment of halitosis. Periodontol 2000 2002: **28**: 256–279.
18. Mager DL, Ximenez-Fyvie LA, Haffajee AD, Socransky SS. Distribution of selected bacterial species on intraoral surfaces. J Clin Periodontol 2003: **30**: 644–654.
19. Marsh PD. Antimicrobial strategies in the prevention of dental caries. Caries Res 1993: **27** (Suppl. 1): 72–76.
20. Minah GE, Turng. BF. Microbiological and ecological aspects of oral malodor. Res Adv Microbiol 2000: **1**: 43–59.
21. Munck A, Bonacorsi S, Mariani-Kurkdjian P, Lebourgeois M, Gerardin M, Brahimi N, et al. Genotypic characterization of *Pseudomonas aeruginosa* strains recovered from patients with cystic fibrosis after initial and subsequent colonization. Pediatr Pulmonol 2001: **32**: 288–292.
22. Roberts SK, Wei GX, Wu CD. Evaluating biofilm growth of two oral pathogens. Lett Appl Microbiol 2002: **35**: 552–556.
23. Rosling B, Dahlen G, Volpe A, Furuichi Y, Ramberg P, Lindhe J. Effect of triclosan on the subgingival microbiota of periodontitis-susceptible subjects. J Clin Periodontol 1997: **24**: 881–887.
24. Ruby JD, Li Y, Luo Y, Caufield PW. Genetic diversity of *Actinomyces naeslundii* genospecies 2 in mother–child pairs. Arch Oral Biol 2003: **48**: 851–855.
25. Saxer UP, Barbakow J, Yankell SL. New studies on estimated and actual toothbrushing times and dentifrice use. J Clin Dent 1998: **9**: 49–51.
26. Sekino S, Ramberg P, Uzel NG, Socransky S, Lindhe J. Effect of various chlorhexidine regimens on salivary bacteria and *de novo* plaque formation. J Clin Periodontol 2003: **30**: 919–925.
27. Socransky SS, Haffajee AD. Dental biofilms: difficult therapeutic targets. Periodontol 2000 2002: **28**: 12–55.
28. Sreenivasan P. The effects of a triclosan/copolymer dentifrice on oral bacteria including those producing hydrogen sulphide. Eur J Oral Sci 2003: **111**: 223–227.
29. Sreenivasan P, Gittins E. Effects of low dose chlorhexidine mouthrinses on oral bacteria and salivary microflora including those producing hydrogen sulfide. Oral Microbiol Immunol 2004: **19**: 309–313.
30. Svensäter G, Borgström M, Bowden GHW, Edwardsson S. The acid-tolerant microbiota associated with plaque from initial caries and healthy tooth surfaces. Caries Res 2003: **37**: 395–403.
31. Tsuchiya H, Miyazaki T, Ohmoto S. High-performance liquid chromatography analysis of chlorhexidine in saliva after mouthrinsing. Caries Res 1999: **33**: 156–163.
32. van Ruyven FO, Lingstrom P, van Houte J, Kent R. Relationship among mutans streptococci, 'low-pH' bacteria, and iodophilic polysaccharide-producing bacteria in dental plaque and early enamel caries in humans. J Dent Res 2000: **79**: 778–784.
33. Volpe AR, Petrone ME, De Vizio W, Davies RM, Proskin HM. A review of plaque, gingivitis, calculus and caries clinical efficacy studies with a fluoride dentifrice containing triclosan and PVM/MA copolymer. J Clin Dent 1996: **7** (Suppl.): S1–S14.