# IN VITRO ANTIBACTERIAL ACTIVITY OF 12 COMMERCIAL MOUTHRINSE FORMULATIONS.

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A Thesis
Submitted to
the Temple University Graduate Board

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in Partial Fulfillment
of the Requirements for the Degree
MASTER OF SCIENCE

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by
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May, 2018

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ABSTRACT

*Objectives:* Mouthrinses are widely used by dental patients in daily oral hygiene practices. However, claims of antimicrobial effects are often made by mouthrinse manufacturers without substantiation by laboratory testing on complex oral microbial communities, such as the dorsal tongue microbiota, which are readily exposed in vivo to mouthrinse solutions, and contribute to the etiology of oral halitosis (bad breath). This study employed a modified Kirby-Bauer zone of inhibition test to comparatively examine the in vitro antimicrobial activity of 12 commercial mouthrinse formulations on a mixture of three bacterial species frequently isolated from the human tongue dorsum.

*Methods:* The 12 commercial mouthrinse formulations tested were 1.) Perio-Aid Treatment Mouthwash (Dentaid S.L., Cerdanyola del Vallès, Spain; containing 0.12% chlorhexidine gluconate and 0.05% cetylpyridinium chloride without alcohol), 2.) Paroex (Sunstar Americas, Inc., Schaumburg, IL; containing 0.12% chlorhexidine gluconate without alcohol), 3.) Peridex (3M ESPE Dental Products, St. Paul, MN, USA; containing 0.12% chlorhexidine gluconate plus 11.6% alcohol), 4.) Perio-Aid Maintenance Mouthwash (Dentaid S.L.; containing 0.05% chlorhexidine gluconate and 0.05% cetylpyridinium chloride without alcohol), 5.) Halita Mouthwash for Halitosis (Dentaid S.L.; containing 0.05% chlorhexidine gluconate, 0.05% cetylpyridinium chloride, and 0.14% zinc lactate without alcohol), 6.) Crest Pro-Health Clinical (Procter & Gamble, Cincinnati, OH, USA; containing 0.1% cetylpyridinium chloride without alcohol), 7.) Therasol (formerly from OraTec Corp., Manassas, VA; containing C31G complex of alkyl dimethyl amine oxide and alkyl dimethyl glycine plus 8% alcohol), 8.) Listerine Cool Mint Zero Alcohol (Johnson & Johnson Consumer, Inc., Skillman, NJ, USA;

containing essential oils 0.092% eucalyptol, 0.042% menthol, 0.06% methyl salicylate, and 0.064% thymol, without alcohol), 9.) PerioShield Oral Health Rinse (formerly from Sunstar Americas, Inc.; containing 0.2% delmopinol hydrochloride plus 1.5% alcohol), 10.) Listerine Cool Mint (Johnson & Johnson Consumer, Inc.; containing essential oils 0.092% eucalyptol, 0.042% menthol, 0.06% methyl salicylate, and 0.064% thymol, plus 21.6% alcohol), 11.) CloSys Antiseptic Oral Rinse (Rowpar Pharmaceuticals, Scottsdale, AZ, containing stabilized chlorine dioxide without alcohol), and 12.) PerioMed Antimicrobial Oral Rinse (3M ESPE Dental Products; containing 0.63% stannous fluoride without alcohol). *Streptococcus salivarius* subsp. *salivarius* ATCC 13419, *Veillonella atypica* ATCC 17744, and *Prevotella melaninogenica* ATCC 25845, which are among the most frequent bacterial isolates from the human tongue dorsum, were grown on enriched Brucella blood agar, comprised of 4.3% Brucella agar supplemented with 0.3% bacto-agar, 5% defibrinated sheep blood, 0.2% hemolyzed sheep red blood cells, 0.0005% hemin, and 0.00005% menadione. Pure cell suspensions of each species were adjusted to a 0.5 McFarland turbidity standard (approximately 1.5 x 108 CFU/ml), and combined equally into a standardized mixture. Undiluted 0.1 ml aliquots of the standardized bacterial mixture were spread with sterile cotton-tipped swabs onto non-selective enriched Brucella blood agar culture plates. After inoculation, four 7-mm diameter cylindrical wells were punched into each of the culture plates and filled with 60 µl of one of the commercial mouthrinse formulations, or sterile saline as a negative control. The inoculated culture plates were incubated upright in anaerobic jars containing 85% $N_2$-10% $H_2$-5% $CO_2$ at 37 °C for four days, after which the diameter of inhibition zones against the standardized bacterial mixture at each well was measured at three

locations to the nearest millimeter with a Boley gauge. Differences in mean bacterial inhibition zones (after subtraction of the agar well diameter) among the mouthrinse formulations and sterile saline were evaluated using a one-way analysis-of-variance and a post-hoc Tukey honestly significant difference test, with a Bonferroni adjustment for multiple comparisons. A *P*-value of $\leq 0.05$ was required for statistical significance.

*Results:* Perio-Aid Treatment exhibited significantly greater in vitro antimicrobial inhibition than the other tested mouthrinse formulations and sterile saline, followed in descending in vitro antibacterial activity by Paroex, Peridex, Perio-Aid Maintenance, Halita, Crest Pro-Health Clinical, Therasol, Listerine Cool Mint Zero Alcohol, and PerioShield. Listerine Cool Mint with alcohol, Closys, and PerioMed were not significantly different from sterile saline in antibacterial in vitro activity.

*Conclusions:* The mouthrinse containing 0.12% chlorhexidine gluconate plus 0.05% cetylpyridinium chloride (Perio-Aid Treatment) exerted the greatest in vitro inhibitory potential against a combination of three bacterial species frequently predominant on the human tongue dorsum. Significantly less antibacterial effects were found with chlorhexidine gluconate or cetylpyridinium chloride alone, or chlorhexidine gluconate at lower concentrations in combination with cetylpyridinium chloride. The relative lack of in vitro antibacterial activity of mouthrinses comprised of essential oils with alcohol, stabilized chlorine dioxide, or stannous fluoride raises questions about their potential clinical effectiveness against dorsal tongue surface biofilms and oral halitosis.

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# CHAPTER 1

## INTRODUCTION

Mouthrinses are widely used by dental patients in daily oral hygiene activities as adjuncts and/or substitutes to mechanical toothbrushing and flossing (Bakdash 1995). A large number of mouthrinse formulations are commercially available both over-the-counter and by prescription (Moran 2008).

Various claims of antimicrobial effects of mouthrinses are often asserted by manufacturers without substantiation by laboratory testing of the products on complex oral microbial communities, such as dental plaque biofilms. For example, the bottle label of one commercial mouthrinse says it "kills 99% of germs to improve breath" (Crest Pro-Health, Procter & Gamble, Cincinnati, OH, USA), while another states that it is "proven to kill millions of germs that cause bad breath on contact" (Listerine Cool Mint Zero Alcohol, Johnson & Johnson Consumer, Inc., Skillman, NJ, USA).

The dorsal tongue microbiota is a prime target in the oral cavity for antibacterial mouthrinses. In contrast to periodontal pockets where gingival crevicular flow prevents subgingival introduction of mouthrinses (Rams & Slots, 1996), the dorsal surface of the tongue is readily exposed to the effects of mouthrinses. Complex microbial communities grow on the tongue dorsum in the presence and absence of oral halitosis (bad breath). Using culture-independent molecular analysis of 16S rRNA genes recovered from DNA in tongue dorsum scrapings from 5 individuals with a normal breath status, and 6 persons with halitosis, Kazor et al. (2003) found *Streptococcus salivarius* to be the most predominant tongue organism in persons without halitosis. In comparison, statistically

significant increases in other species were found in halitosis samples, including *Solobacterium moorei*, *Fusobacterium periodonticum*¸ *Eubacterium sulci*, *Atopobium parvulum*, *Dialister* phylotype clone BS095, *Streptococcus* phylotype clone BW009, TM7 phylotype clone DR034, and *Cryptobacterium curtum* (Kazor et al. 2003). In a similar molecular analysis with a larger sample size (12 adults without halitosis, and 20 with halitosis), Riggio et al. (2008) also found *S. salivarius* predominant on the tongue dorsum in the absence of halitosis, along with *Veillonella dispar*, *Actinomyces odontolyticus*, *A. parvulum* and *Veillonella atypica*. In halitosis patients, *S. salivarius*, *Prevotella melaninogenica*, *Prevotella veroralis* and *Prevotella pallens* were the most commonly identified bacterial species (Riggio et al. 2008), supporting the notion that the predominant microbiota on the tongue dorsum is different between persons with and without halitosis (Kazor et al. 2003, Riggio et al. 2008). Similarly, Yang et al. (2013), with pyrosequencing of 16S rRNA bacterial genes, found *Prevotella tannerae*, *Leptotrichia wadei*, and *Haemophilus parainfluenzae* to be significantly associated with increases over a 3-day period in hydrogen sulfide levels in excreted mouth air.

However, in more recent analysis of the tongue dorsum microbiome with 16S amplicon sequencing, similar qualitative microbial compositions were found in persons with and without intra-oral halitosis, suggesting that quantitative rather than qualitative changes in the tongue dorsum microbiology are more important in the etiopathogenesis of oral malodor (Seerangaiyan et al. 2017).

As a result, rinsing with antimicrobial solutions to non-specifically reduce dorsal tongue bacterial populations may be beneficial in the prevention and treatment of intra-

oral halitosis originating from tongue coatings. Unfortunately, no specific mouthrinse formulation has been identified to date as the best for suppressing intra-oral bacterial populations while concurrently providing safe and acceptable side effects (Tartaglia et al. 2017). Since comparative studies examining the clinical in vivo efficacy of various mouthrinses against oral microbial populations are expensive to conduct, and difficult to standardize, it is rational to initially carry out more limited in scope in vitro studies to assess the antimicrobial activity of varying mouthrinse formulations. Similar to the concept underlying the use of in vitro susceptibility testing of bacterial pathogens with antibiotics (Stratton 2006), mouthrinses with no or minimal antibacterial activity under favorable in vitro growth conditions are unlikely to exhibit marked antibacterial effects in vivo in the oral cavity.

The Kirby-Bauer method is widely employed in the in vitro testing of antimicrobial agents against bacteria (Bauer et al. 1966). With this approach, disks with known amounts of antimicrobial agents are placed onto the surface of a culture medium inoculated with a lawn of bacteria, and after a suitable incubation period, diameters of areas around the disks showing no growth are measured, with larger diameters of microbial inhibition indicative of a greater antimicrobial effect for the tested agent (Barry et al. 1979). More recently, a modified Kirby-Bauer zone of inhibition test, where agar wells were punched into culture media and filled with mouthrinses in place of antimicrobial agent-impregnated surface disks, was used by Raangs et al. (2013) to assess the in vitro inhibition of mouthrinses against cultivable microorganisms derived from dorsal tongue samples.

Building upon this prior work, the purpose of the present research study was to evaluate, using the modified Kirby-Bauer zone of inhibition test of Raangs et al. (2013), the in vitro antibacterial activity of 12 commercial mouthrinses and sterile saline against a standardized mixture of three bacterial species frequently recovered from the human tongue dorsum (*Streptococcus salivarius* subsp. *salivarius*, *Veillonella atypica*, and *Prevotella melaninogenica*) (Kazor et al. 2003, Riggio et al. 2008, Seerangaiyan et al. 2017).

# CHAPTER 2

# MATERIALS AND METHODS

## Laboratory Facilities

All procedures in this study were performed using non-human and non-animal materials in the facilities of the Oral Microbiology Testing Service Laboratory located in the Temple University Maurice H. Kornberg School of Dentistry on the Temple University Health Sciences Center campus in Philadelphia, Pennsylvania. Since the data for the present study was non-clinical and laboratory-based, without any intervention or interaction with living individuals, and did not involve any identifiable private information, the research activity did not involve human subjects, as defined by United States Department of Health and Human Services regulations at 45 CFR part 46.116(f), and did not require a human subjects institutional review board approval, per a written determination issued by the Temple University Human Subjects Protections Institutional Review Board.

## Mouthrinses

A total of 12 commercial mouthrinse formulations were tested. The pH of each mouthrinse was first as measured with a laboratory pH meter (Accumet Basic AB15 Plus, Fisher Scientific, Waltham, MA, USA). The tested mouthrinse formulations and their pH values included 1.) Perio-Aid Treatment Mouthwash (Dentaid S.L., Cerdanyola del Vallès, Spain; containing 0.12% chlorhexidine gluconate and 0.05% cetylpyridinium chloride without alcohol), pH = 6.0, 2.) Paroex (Sunstar Americas, Inc., Schaumburg, IL; containing 0.12% chlorhexidine gluconate without alcohol), pH = 6.0. 3.) Peridex (3M

ESPE Dental Products, St. Paul, MN, USA; containing 0.12% chlorhexidine gluconate plus 11.6% alcohol), pH = 5.8, 4.) Perio-Aid Maintenance Mouthwash (Dentaid S.L.; containing 0.05% chlorhexidine gluconate and 0.05% cetylpyridinium chloride without alcohol), pH = 5.8, 5.) Halita Mouthwash for Halitosis (Dentaid S.L.; containing 0.05% chlorhexidine gluconate, 0.05% cetylpyridinium chloride, and 0.14% zinc lactate without alcohol), pH = 5.2, 6.) Crest Pro-Health Clinical (Procter & Gamble, Cincinnati, OH, USA; containing 0.1% cetylpyridinium chloride without alcohol), pH = 4.1, 7.) Therasol (formerly from OraTec Corp., Manassas, VA; containing C31G complex of alkyl dimethyl amine oxide and alkyl dimethyl glycine plus 8% alcohol), pH = 5.1, 8.) Listerine Cool Mint Zero Alcohol (Johnson & Johnson Consumer, Inc., Skillman, NJ, USA; containing essential oils 0.092% eucalyptol, 0.042% menthol, 0.06% methyl salicylate, and 0.064% thymol, without alcohol), pH = 4.7, 9.) PerioShield Oral Health Rinse (formerly from Sunstar Americas, Inc.; containing 0.2% delmopinol hydrochloride plus 1.5% alcohol), pH = 5.7, 10.) Listerine Cool Mint (Johnson & Johnson Consumer, Inc.; containing essential oils 0.092% eucalyptol, 0.042% menthol, 0.06% methyl salicylate, and 0.064% thymol, plus 21.6% alcohol), pH = 4.6, 11.) CloSys Antiseptic Oral Rinse (Rowpar Pharmaceuticals, Scottsdale, AZ, containing stabilized chlorine dioxide without alcohol), pH = 6.1, and 12.) PerioMed Antimicrobial Oral Rinse (3M ESPE Dental Products; containing 0.63% stannous fluoride without alcohol), pH = 3.8 (Figure 1).


Figure 1. Containers of the tested mouthrinse formations: (A) Perio-Aid Treatment, (B) Paroex, (C) Peridex, (D) Perio-Aid Maintenance, (E) Halita, (F) Crest Pro-Health Clinical, (G) Therasol, (H) Listerine Cool Mint Zero Alcohol, (I) PerioShield, (J) Listerine Cool Mint (with alcohol), (K) CloSys, and (L) PerioMed

Microbial Species

*Streptococcus salivarius* subsp. *salivarius* ATCC 13419, *Veillonella atypica* ATCC 17744, and *Prevotella melaninogenica* ATCC 25845 were purchased from the American Type Culture Collection (Manassas, VA, USA). The three species were each grown on non-selective enriched Brucella blood agar (EBBA), comprised of 4.3% Brucella agar supplemented with 0.3% bacto-agar, 5% defibrinated sheep blood, 0.2% hemolyzed sheep red blood cells, 0.0005% hemin, and 0.00005% menadione (Slots et al. 1988). The EBBA inoculated plates were incubated at 37 °C for five days in a upright heated incubator (Caron, Marietta, OH, USA) in jars containing an 85% $N_2$-10% $H_2$-5%

$CO_2$ atmosphere introduced by an Anoxomat™ Mark II automatic jar evacuation-replacement system (Advanced Instruments, Inc., Norwood, MA, USA) (Brazier & Smith 1989) (Figure 2).

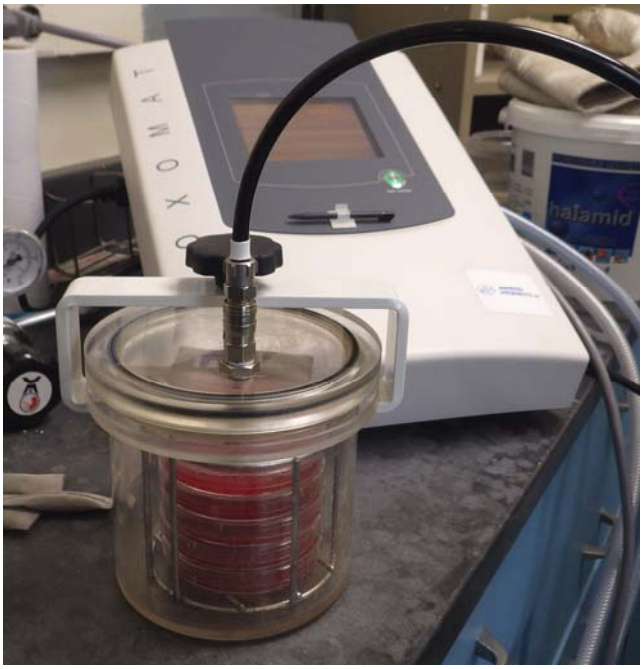

Figure 2. Anoxomat instrument introducing anaerobic incubation atmosphere into jar containing EBBA inoculated culture plates

Antibacterial Testing of Mouthrinse Formulations

After initial recovery and laboratory growth of the ATCC strains of *S. salivarius* subsp. *salivarius*, *V. atypica*, and *P. melaninogenica*, pure cell suspensions of each were created with sterile physiologic saline, adjusted to a 0.5 McFarland turbidity standard (providing approximately 1.5 x 108 CFU of the species/ml), and combined in equal volumes into a standardized mixture containing all three species at similar cell concentrations (Figure 3).

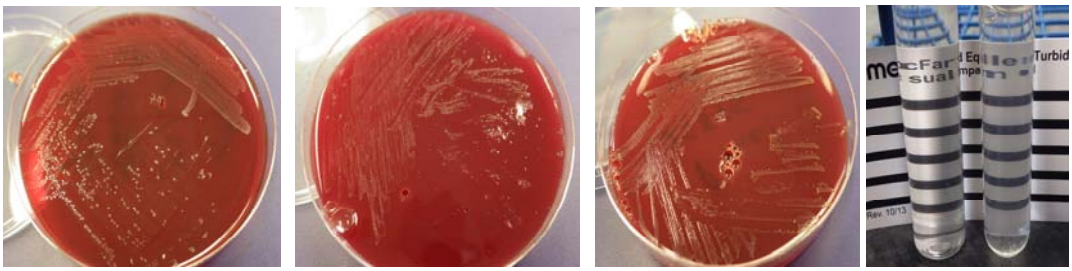

Figure 3. Growth of *S. salivarius* subsp. *salivarius*, *V. atypica*, and *P. melaninogenica* on EBBA culture plates (left to right, respectively), and standardized mixture containing all three species (right test tube in right image) compared to a 0.5 McFarland turbidity standard (left test tube in right image)

Undiluted 0.1 ml aliquots of the standardized bacterial mixture then were spread with sterile cotton-tipped swabs onto new EBBA culture plates. After inoculation, four 7-millimeter diameter cylindrical wells were punched into each of the culture plates and filled with 60 µl of one of the commercial mouthrinse formulations, or sterile saline as a negative control (Figure 4).

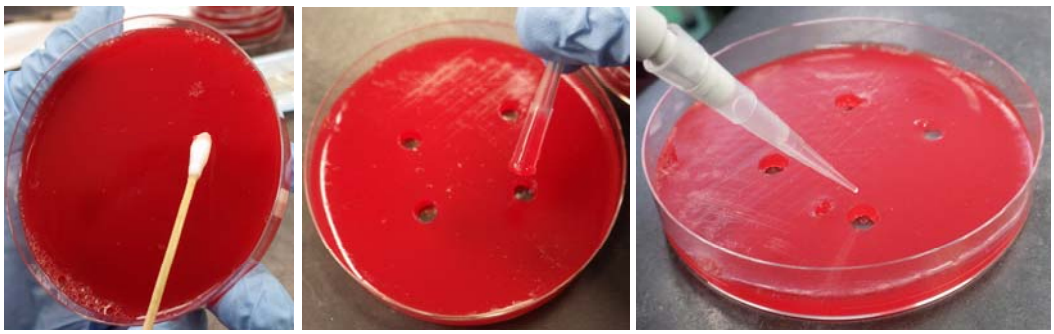

Figure 4. Inoculation of EBBA culture plates with standardized bacterial mixture (left), creation of cylindrical wells (center), and introduction of mouthrinse formulation (right)

The inoculated EBBA culture plates, with various mouthrinse formulations or sterile saline placed into quadruplet wells, were incubated upright in anaerobic jars

containing 85% $N_2$-10% $H_2$-5% $CO_2$ (Figure 1) at 37 °C for four days, after which the diameter of inhibition zones against the standardized bacterial mixture at each well was measured at three locations to the nearest millimeter with a Boley gauge. This provided a total of 12 bacterial inhibition zone measurements per mouthrinse formulation and sterile saline against the cultivated standardized bacterial mixture of *S. salivarius* subsp. *salivarius*, *V. atypica*, and *P. melaninogenica*.

Data Analysis

Mean and standard deviation (SD) values for the measured bacterial inhibition zones were calculated for each of the mouthrinse formulations and sterile saline. Differences in mean bacterial inhibition zones (after subtraction of the agar well diameter) among the mouthrinse formulations and sterile saline were evaluated using a one-way analysis-of-variance and a post-hoc Tukey honestly significant difference test, with a Bonferroni adjustment for multiple comparisons. A *P*-value of $\leq 0.05$ was required for statistical significance.

The PC-based STATA/SE 14.2 for Windows (StataCorp PL, College Station, TX USA) 64-bit statistical software package was used in the data analysis.

# CHAPTER 3

## RESULTS

Table 1 and Figure 5 present mean zones of bacterial inhibition attained by each of the 12 tested mouthrinse formulations and sterile saline.

Table 1. Mean zones of bacterial inhibition ± SD, in millimeters, induced by 12 commercial mouthrinse formulations and sterile saline

| Mouthrinse formulation | Mean ± SD zone of bacterial inhibition, millimeters |
|---|---|
| Perio-Aid Treatment | 26.9 ± 9.5 |
| Paroex | 19.3 ± 3.2 |
| Peridex | 17.8 ± 1.7 |
| Perio-Aid Maintenance | 16.6 ± 3.8 |
| Halita | 14.6 ± 1.2 |
| Crest Pro-Health Clinical | 12.8 ± 4.2 |
| Therasol | 12.3 ± 3.1 |
| Listerine Cool Mint Zero Alcohol | 7.8 ± 2.5 |
| PerioShield | 5.4 ± 1.6 |
| Listerine Cool Mint (with alcohol) | 0.3 ± 1.2 |
| CloSys | 0 ± 0 |
| PerioMed | 0 ± 0 |
| sterile saline | 0 ± 0 |

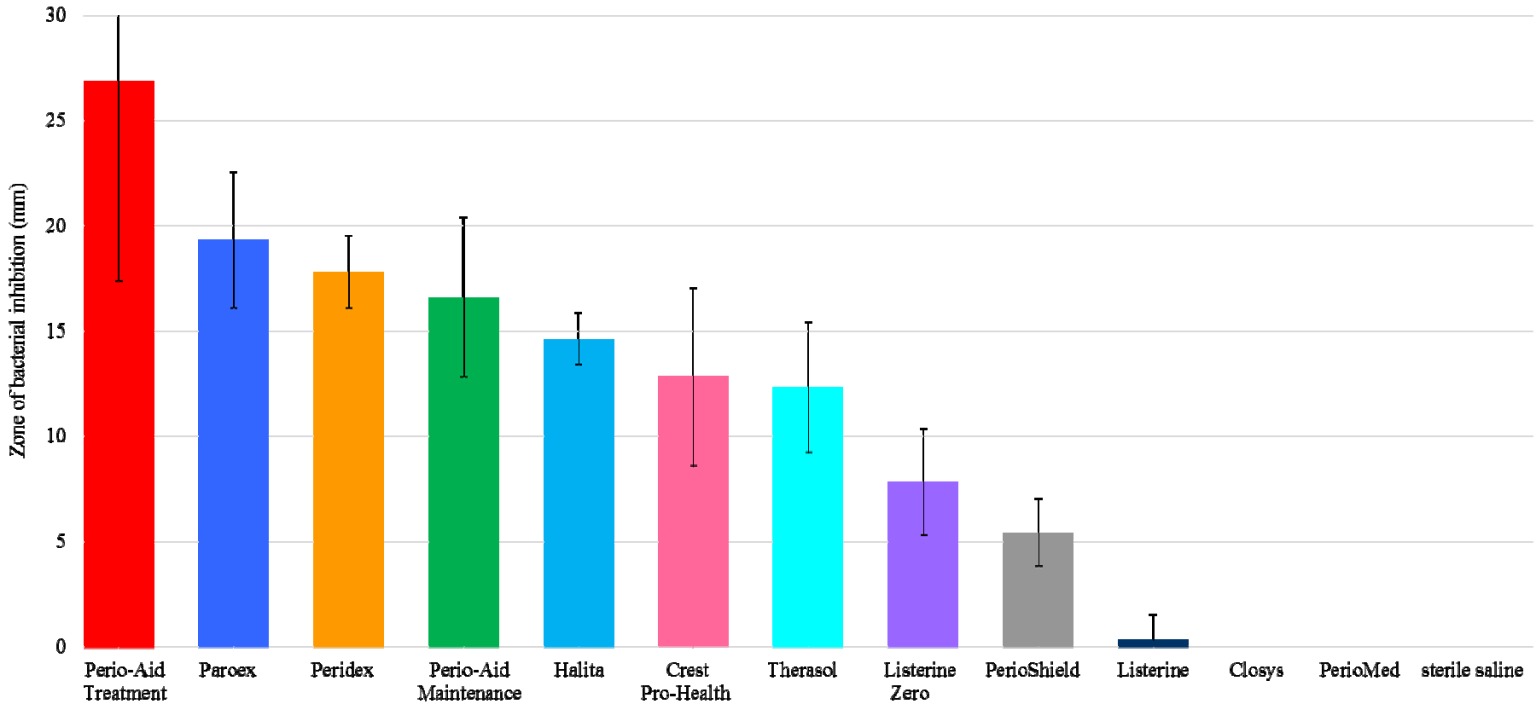


Figure 5. Mean zones of bacterial inhibition by mouthrinse formulations and sterile saline

Perio-Aid Treatment exhibited the greatest in vitro antimicrobial inhibition than the other tested mouthrinse formulations and sterile saline, followed in descending in vitro antibacterial activity by Paroex, Peridex, Perio-Aid Maintenance, Halita, Crest Pro-Health Clinical, Therasol, Listerine Cool Mint Zero Alcohol, PerioShield, and Listerine Cool Mint with alcohol. No in vitro antibacterial activity was found with Closys, PerioMed, and sterile saline (Table 1).

Figure 6 provides representative zones of antibacterial inhibition seen among the tested mouthrinse formulations and sterile saline.

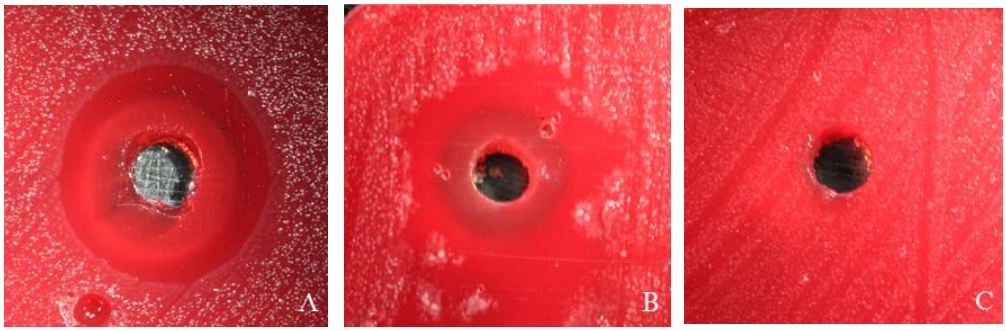


Figure 6. Representative bacterial inhibition zones associated with Peridex (A), Crest Pro-Health Clinical (B), and both Listerine Cool Mint with alcohol, and sterile saline (C)

Table 2 provides the outcome of statistical significance testing of differences in mean bacterial inhibition zones of the mouthrinse formulations and sterile saline with one-way analysis-of-variance and a post-hoc Tukey honestly significant difference test.

Table 2. *P*-values from statistical significance testing of differences in mean bacterial inhibition zones of the mouthrinse formulations and sterile saline with one-way analysis-of-variance and a post-hoc Tukey honestly significant difference test.

| | Perio-Aid Treatment | Paroex | Peridex | Perio-Aid Maintenance | Halita | Crest Pro-Health | Therasol | Listerine Cool Mint Zero | PerioShield | Listerine Cool Mint | Closys | PerioMed | sterile saline |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Perio-Aid Treatment** | - | | | | | | | | | | | | |
| **Paroex** | < 0.01 | - | | | | | | | | | | | |
| **Peridex** | < 0.01 | NS | - | | | | | | | | | | |
| **Perio-Aid Maintenance** | < 0.01 | NS | NS | - | | | | | | | | | |
| **Halita** | < 0.01 | NS | NS | NS | - | | | | | | | | |
| **Crest Pro-Health** | < 0.01 | < 0.01 | NS | NS | NS | - | | | | | | | |
| **Therasol** | < 0.01 | < 0.01 | < 0.05 | NS | NS | NS | - | | | | | | |
| **Listerine Cool Min: Zero** | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | NS | NS | - | | | | | |
| **PerioShield** | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | NS | - | | | | |
| **Listerine Cool Min:** | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.05 | - | | | |
| **Closys** | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.05 | NS | - | | |
| **PerioMed** | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.05 | NS | NS | - | |
| **sterile saline** | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.05 | NS | NS | NS | - |

Perio-Aid Treatment exhibited significantly greater in vitro antimicrobial inhibition than the other tested mouthrinse formulations and sterile saline, followed in descending in vitro antibacterial activity by Paroex, Perio-Aid Maintenance, Halita, Crest Pro-Health Clinical, Therasol, Listerine Cool Mint Zero Alcohol, and PerioShield (Table 2). Listerine Cool Mint with alcohol, Closys, and PerioMed were not significantly different from sterile saline in antibacterial in vitro activity (Table 2).

# CHAPTER 4

# DISCUSSION

The various mouthrinse formulations tested in this study were not equally active in inhibiting the in vitro growth of bacterial species that are frequently present in the human dorsal tongue microbiota. The mouthrinse containing 0.12% chlorhexidine gluconate plus 0.05% cetylpyridinium chloride (Perio-Aid Treatment) exerted the greatest in vitro inhibitory potential against the standardized bacterial mixture of *S. salivarius* subsp. *salivarius*, *V. atypica*, and *P. melaninogenica*. This finding is consistent with previous in vitro evaluations of a combined 0.12% chlorhexidine gluconate plus 0.05% cetylpyridinium chloride mouthrinse formulation by Herrera et al. (2003). In their study, 0.12% chlorhexidine gluconate plus 0.05% cetylpyridinium chloride demonstrated 100% efficacy and additive in vitro antibacterial activity against a panel of 20 putative oral microbial pathogens in a short interval (one minute) killing test assay, and showed better in vivo salivary bacterial count reductions after a one-minute rinse than chlorhexidine alone (Herrera et al. 2003). In comparison, significantly less antibacterial effects were found with chlorhexidine gluconate (Paroex, Peridex) or cetylpyridinium chloride (Crest Pro-Health Clinical) alone, or with chlorhexidine gluconate at lower concentrations than 0.12% in combination with cetylpyridinium chloride (Perio-Aid Maintenance, Halita).

Of particular importance in the present study was the finding that Listerine Cool Mint with alcohol, Closys, and PerioMed were not significantly different from sterile

saline in antibacterial in vitro activity against the standardized bacterial mixture of *S. salivarius* subsp. *salivarius*, *V. atypica*, and *P. melaninogenica*. It is not clear as to why these mouthrinses, particularly Closys, failed to exert any discernable antibacterial effects in the present study, in contrast to other investigations on stabilized chlorine dioxide rinses (Drake & Villhauer, 2011). Some of their components may have experienced binding to or neutralization by culture media constituents, or the concentration of active agent in the mouthrinses may have been below inhibitory levels needed to impede growth of the microbial species encompassed in the standardized bacterial mixture. Another possibility is variability in the manufacture and storage of the mouthrinses, where it is possible that some batches of the agent may possess different properties, particularly if some are exposed to excessive temperature changes during product shipment and/or storage prior to commercial sale. Future studies should examine additional bottles of the mouthrinses obtained from different locations to test the variation in antimicrobial effects across varying commercially-distributed containers. The relative lack of in vitro antibacterial activity seen in the present study by these mouthrinses raises questions about their potential clinical effectiveness against dorsal tongue surface biofilms and oral halitosis.

Several other mouthrinse formulations revealed relatively modest antibacterial activity as compared to chlorhexidine-containing rinses, including Therasol, Listerine Cool Mint Zero Alcohol, and PerioShield, consistent with prior studies (Brecx et al. 1990, Moran et al. 1992, Netuschil et al. 1995, Yesilsoy et al. 1995, Renton-Harper et al. 1996).

The limitations of the present study need to be appreciated. The three bacterial species selected to comprise the standardized mixture tested in the in vitro assay, *S. salivarius* subsp. *salivarius*, *V. atypica*, and *P. melaninogenica*, are not necessarily representative of the tongue dorsum microbiome as a whole, or the microbiota at other oral sites, but are frequently among predominant species isolated from tongue scrapings (Kazor et al. 2003, Riggio et al. 2008, Seerangaiyan et al. 2017). Other oral microorganisms associated with dental caries, periodontal diseases, peri-implant mucositis/peri-implantitis, and oral mucosal infections may demonstrate different susceptibilities to the tested mouthrinses than was found in the present study with the standardized bacterial mixture.

The effects of saliva and gingival crevicular fluid on the antibacterial effects of the tested mouthrinses were not evaluated. Proteins in salivary secretions and gingival crevicular fluid may bind to some mouthrinses, such as those with chlorhexidine, and markedly reduce their antimicrobial activity (Rams & Slots, 1996).

It is not known to what extent the present in vitro laboratory findings correlate with clinical in vivo efficacy of the tested mouthrinses in altering the tongue dorsum microbiota and intra-oral halitosis originating from tongue coatings. Since mouthrinses are most commonly used clinically by patients for approximately one minute or less in vivo (Herrera et al. 2003), it is not clear that the extended exposure time between mouthrinses placed into agar wells and the surrounding lawn of the standardized bacterial mixture (4 days of incubation time), appropriately reflects real-world mouthrinse-bacterial population interactions seen in vivo in the oral cavity. Alternatively, it may

have been more appropriate to neutralize the mouthrinses in agar wells after one minute or less of exposure to the lawn of *S. salivarius* subsp. *salivarius*, *V. atypica*, and *P. melaninogenica*, similar to what was done in test tube studies of mouthrinse antibacterial activity by Herrera et al. (2003). Additional in vitro and in vivo research is indicated to address these limitations.

# CHAPTER 5

# CONCLUSIONS

The mouthrinse containing 0.12% chlorhexidine gluconate plus 0.05% cetylpyridinium chloride (Perio-Aid Treatment) exerted the greatest in vitro inhibitory potential against a combination of three bacterial species frequently predominant on the human tongue dorsum. Significantly less antibacterial effects were found with chlorhexidine gluconate or cetylpyridinium chloride alone, or chlorhexidine gluconate at lower concentrations in combination with cetylpyridinium chloride.

The relative lack of in vitro antibacterial activity of mouthrinses comprised of essential oils with alcohol, stabilized chlorine dioxide, or stannous fluoride raises questions about their potential clinical effectiveness against dorsal tongue surface biofilms and oral halitosis.

The findings from this in vitro study may be used to identify mouthrinse formulations more likely to demonstrate in vivo antibacterial activity against complex oral communities, such as may be found on the dorsum of the human tongue, and clinically benefit patients.

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