

An *In Vitro* Comparative Study Determining Bactericidal Activity of Stabilized Chlorine Dioxide and Other Oral Rinses

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Abstract

- **Objective:** The study was conducted to determine the bactericidal activity of a stabilized chlorine dioxide oral rinse (ClōSYS® Oral Rinse) compared to products currently available on the market.
- **Methods:** Oral bacteria associated with gingivitis and periodontitis were exposed to rinses for one minute and five minutes. The numbers of colony forming units per milliliter (CFU/ml) were measured prior to and following exposure to determine the bactericidal activity.
- **Results:** As expected, Listerine® and Crest® Pro-Health™ demonstrated complete kill on all bacteria exposed within one minute. Breath Rx® exhibited the weakest levels of bactericidal effects overall. ClōSYS and chlorhexidine rinses proved identical 100% kills against the periodontal pathogens at five minutes; in some cases, ClōSYS oral rinse achieved a higher kill at the one-minute mark over the chlorhexidine rinse.
- **Conclusion:** The results demonstrated that ClōSYS Oral Rinse has potential for providing a therapeutic benefit, making it an attractive option to induce compliance in patients concerned about taste and tooth discoloration during oral health therapy.

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Introduction

Gingivitis is the presence of gingival inflammation without loss of connective tissue attachment. The precursor to gingivitis is undisturbed dental plaque biofilms. Studies have shown that gingivitis will develop within 10–21 days if all oral hygiene practices are stopped and plaque is allowed to accumulate undisturbed. Gingivitis is preventable by routine oral care, but if untreated may lead to a severe gum disease known as periodontitis. Periodontitis refers to a disease condition in which supporting tissues of the teeth, such as connective tissue and bone, are destroyed by plaque-induced inflammation. The most common form, known as chronic periodontitis, affects approximately 20% of the US adult population. Recent studies using genomics, proteomics, and metabolomics have described complex models with multiple levels of interactions between cellular and molecular processes occurring from the lowest levels to the tissue levels where clinical disease is manifested.

Plaque is one of the most recognizable precursors initiating a cascade of immunological mediators which, based on a person's genetic profile and environmental factors, can elicit epigenetic responses and lead to the development of clinical oral disease. An important change in the mouth within plaque biofilm communities is the shift in the composition of the microflora due to many factors, which results in microbial communities dominated by anaerobic bacteria. Plaque can be considered as a characteristic biofilm with a highly structured, matrix-embedded, diverse microbial population with many various bacterial species. The volume and structure of the biofilm provides protection to bacteria housed within it, potentially reducing the efficacy of antimicrobials.

Treatment of both gingivitis and chronic periodontitis is designed to facilitate the frequent removal and disruption of plaque biofilms. For gingivitis, effective home oral hygiene practices on

a twice-daily basis usually provide a sufficient standard of care. Typical interventions usually include oral hygiene instructions followed by thorough subgingival debridement (*e.g.*, scaling and root planing). Prescription mouthrinses, such as those containing chlorhexidine gluconate, are effective treatments for gingivitis, but are not intended for long-term use, may stain teeth, and have an unpleasant taste. An example of a non-prescription mouthrinse is Listerine® (McNeal-PPC Inc., Morris Plains, NJ, USA), which has been granted the American Dental Association seal of approval as an antiplaque and antigingivitis product. However, the high alcohol content and harsh taste of the formulation can be unpleasant for some consumers. A stabilized chlorine dioxide rinse is commercially available (ClōSYS® Oral Rinse, Rowpar Pharmaceuticals Inc., Scottsdale, AZ, USA) that does not stain teeth or have a harsh taste, yet offers a similar antibacterial efficacy.

Stabilized chlorine dioxide is a compound with antimicrobial properties against oral bacteria.¹⁻⁴ Clinical and laboratory evidence suggest that stabilized chlorine dioxide oral rinse reduces the number of bacteria in the mouth, essentially eliminates oral malodor, reduces the signs of gum disease, and has bactericidal properties comparable to other products with additional consumer acceptability relating to its non-alcoholic and non-staining features. While the study reported here did not assess whitening, there are no reports in the literature of any staining or discoloration from use of this product.

The objective of this study was to compare *in vitro* bactericidal activity of a stabilized chlorine dioxide oral rinse to several other commercially available oral rinses. Oral bacteria associated with oral disease evaluated in this study included:

<i>Actinomyces viscosus</i>	<i>Actinomyces naeslundii</i>
<i>Streptococcus oralis</i>	<i>Streptococcus mutans</i>
<i>Enterococcus faecalis</i>	<i>Porphyromonas gingivalis</i>

Streptococcus sanguinis *Peptostreptococcus micros*
Actinomyces odontolyticus *Prevotella nigrescens*
Aggregatibacter actinomycetemcomitans

Additional bacteria tested not associated with oral disease, but pose as opportunistic pathogens to overall health, included *Klebsiella pneumonia* (nosocomial infections), *Pseudomonas aeruginosa* (nosocomial infections), *Staphylococcus aureus* (systemic infections), and *Haemophilus influenza* (systemic infections).

Data Assessment

Due to the very large number of organisms assessed and the kinetic approach, it would have been difficult to include multiple replicates and the differences are also large, and this was taken as a clear indicator of differences between the products studied without inferential statistics.

Materials and Methods

A time-kill bactericidal kinetics approach was used to determine bactericidal activity of each oral rinse. Fifty milliliters of nutrient broth were inoculated with bacterial stock (0.1 to 0.5 ml) and incubated according to culture requirements specific to each bacterial species being tested.

Following incubation, cells were harvested by centrifugation (7,500 × g) and re-suspended in Butterfield's Buffer. Absorbance was measured in a spectrophotometer at A660, and suspensions were adjusted to achieve the desired assay concentration (10⁷ CFU/ml) upon reference to predetermined standard curves for each organism. Culture purity was confirmed through gram staining of all cultures.

Standardized suspensions were exposed to each oral rinse product: ClōSYS, Listerine, Crest® Pro-Health™ (Procter & Gamble, Cincinnati, OH, USA); Breath Rx® (Discus Dental, Culver City, CA, USA); and Peridex® (3M ESPE, Panorama City, CA, USA). Bactericidal activity was determined as follows: One-hundred milliliters of each experimental rinse was pipetted into a separate sterile 250 ml beaker containing a stir bar, and placed on a stir plate with slow stirring. Ten milliliters of bacterial suspension were then added to the beaker and a timer was started.

One-milliliter samples were removed at one minute and at five minutes. Each sample was added to nine milliliters of Neutralizing Broth® (Neogen Corporation, Lansing, MI, USA) to stop the action of the rinse, and an additional 10⁻¹ dilution was made from these tubes. A sample at baseline was assayed for each mixture immediately after the bacteria were added to the Butterfield's Buffer (negative control).

After this procedure was performed for all rinses, each tube was spiral-plated onto appropriate media for each organism using an Autoplate 4000® system (Advanced Instruments Inc., Norwood, MA, USA). The agar plates were incubated for 24–96 hours in either 5% CO₂ or anaerobically (Coy Chamber). Determination of the numbers of viable bacteria in the assay tubes at the time points was completed following standard spiral-plating methodology. Raw counts were converted to log₁₀ values to equalize variances in the data.

Results

Table I shows the bactericidal activity (log reduction from baseline) observed with the different rinses at one and five minutes. The bactericidal data show that ClōSYS oral rinse exhibits strong bactericidal activity against a spectrum of oral bacteria and other opportunistic pathogens. With the exception of *Streptococcus oralis*, *Enterococcus faecalis*, *Streptococcus sanguinis*, and *Pseudomonas aeruginosa*, ClōSYS killed all bacteria in the assay. In most runs, bacteria were eliminated within the first minute of exposure.

In most cases the stabilized chlorine dioxide oral rinse, ClōSYS, completely eliminated bacteria within one minute; it completely killed *Actinomyces viscosus* within five minutes and reduced bacterial counts of *Streptococcus oralis*, *Enterococcus faecalis*, *Streptococcus sanguinis*, and *Pseudomonas aeruginosa*. Listerine showed complete kill on all bacteria within one minute and chlorhexidine within five minutes. Crest Pro-Health completely killed *Staphylococcus aureus* in five minutes and all others within the first minute. Breath Rx exhibited the weakest levels of bactericidal activity overall, showing complete kill on *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella nigrescens*, and *Haemophilus influenzae* within one minute, and *Streptococcus sanguinis* and *Pseudomonas aeruginosa* only after five minutes.

ClōSYS compared well with other rinses tested. Bactericidal activity for ClōSYS surpassed that of the positive control, chlorhexidine, in the first minute for *Actinomyces naeslundii* (involved in periodontal disease and endodontic infections) and *Peptostreptococcus micros* (found in periodontal disease). For *Streptococcus mutans* (which causes caries) and *Actinomyces viscosus* (involved with oral infections), the bactericidal activity was higher for chlorhexidine in a first-minute sample but, by five minutes, ClōSYS and chlorhexidine samples exhibited identical 100% killing of the test suspensions.

With *Porphyromonas gingivalis* (strong association with the progression of chronic periodontitis), *Actinobacillus actinomycetemcomitans* (etiologic agent for localized aggressive periodontitis), *Actinomyces odontolyticus* (associated with deep dental caries and chronic periodontitis), and *Prevotella nigrescens* (found in endodontic infections), both ClōSYS and chlorhexidine achieved a 100% kill in the first minute.

ClōSYS killed all bacteria in the study, with the exception of *Streptococcus oralis*, *Enterococcus faecalis*, *Streptococcus sanguinis*, and *Pseudomonas aeruginosa*. ClōSYS was observed to exhibit greater bactericidal activity compared to the other rinses against *Staphylococcus aureus*, which is not necessarily an oral pathogen but is the causative agent of staph infections, exhibiting 100% kill within the first minute of exposure.

Discussion

Bacteria in dental plaque are major etiologic agents initiating several oral diseases, including gingivitis and periodontitis. It is during the maintenance phase of oral hygiene that non-invasive over-the-counter products are especially useful in slowing down the formation of dental plaque biofilms on tooth surfaces and reducing the risk of oral disease. The bactericidal activity of stabilized chlorine dioxide oral rinse, ClōSYS, suggests that it has

Table I
Bactericidal Activity at One Minute and Five Minutes from Baseline (0 Minutes)

Bacteria		Log10 (CFU/ml)			Log10 Reduction (CFU/ml) From Baseline	
		Minutes			1	5
		0	1	5		
<i>A. viscosus</i>	Baseline					
	ClōSYS	6.064	3.778	0.000	2.286	6.064
	Listerine	6.064	0.000	0.000	6.064	6.064
	Crest Pro-Health	6.064	0.000	0.000	6.064	6.064
	Breath Rx	6.064	4.869	3.647	1.195	2.417
	Peridex	6.064	2.301	0.000	3.763	6.064
<i>S. oralis</i>	Butterfield's Buffer	6.064	5.893	5.967	0.171	0.097
	ClōSYS	7.033	6.957	6.802	0.076	0.231
	Listerine	7.033	0.000	0.000	7.033	7.033
	Crest Pro-Health	7.033	0.000	0.000	7.033	7.033
	Breath Rx	7.033	6.090	4.511	0.944	2.523
	Peridex	7.033	2.301	0.000	4.732	7.033
<i>E. faecalis</i>	Butterfield's Buffer	7.033	7.090	7.158	-0.056	-0.125
	ClōSYS	6.199	6.104	4.809	0.095	1.390
	Listerine	6.199	0.000	0.000	6.199	6.199
	Crest Pro-Health	6.199	0.000	0.000	6.199	6.199
	Breath Rx	6.199	6.086	5.953	0.112	0.246
	Peridex	6.199	5.064	0.000	1.134	6.199
<i>S. sanguinis</i>	Butterfield's Buffer	6.199	6.299	6.217	-0.100	-0.019
	ClōSYS	5.241	5.199	4.556	0.042	0.684
	Listerine	5.241	0.000	0.000	5.241	5.241
	Crest Pro-Health	5.241	0.000	0.000	5.241	5.241
	Breath Rx	5.241	3.881	0.000	1.360	5.241
	Peridex	5.241	0.000	0.000	5.241	5.241
<i>A. odontolyticus</i>	Butterfield's Buffer	5.241	5.255	5.394	-0.015	-0.154
	ClōSYS	6.225	0.000	0.000	6.225	6.225
	Listerine	6.225	0.000	0.000	6.225	6.225
	Crest Pro-Health	6.225	0.000	0.000	6.225	6.225
	Breath Rx	6.225	6.127	4.680	0.098	1.545
	Peridex	6.225	0.000	0.000	6.225	6.225
<i>A. actinomycetemcomitans</i>	Butterfield's Buffer	6.225	6.199	6.223	0.027	0.003
	ClōSYS	6.984	0.000	0.000	6.984	6.984
	Listerine	6.984	0.000	0.000	6.984	6.984
	Crest Pro-Health	6.984	0.000	0.000	6.984	6.984
	Breath Rx	6.984	0.000	0.000	6.984	6.984
	Peridex	6.984	0.000	0.000	6.984	6.984
<i>A. naeslundii</i>	Butterfield's Buffer	6.984	6.894	6.894	0.090	0.090
	ClōSYS	6.140	0.000	0.000	6.140	6.140
	Listerine	6.140	0.000	0.000	6.140	6.140
	Crest Pro-Health	6.140	0.000	0.000	6.140	6.140
	Breath Rx	6.140	5.477	4.358	0.663	1.782
	Peridex	6.140	3.204	0.000	2.936	6.140
<i>S. mutans</i>	Butterfield's Buffer	6.140	6.061	6.017	0.079	0.123
	ClōSYS	6.588	6.378	0.000	0.209	6.588
	Listerine	6.588	0.000	0.000	6.588	6.588
	Crest Pro-Health	6.588	0.000	0.000	6.588	6.588
	Breath Rx	6.588	6.253	6.190	0.335	0.397
	Peridex	6.588	0.000	0.000	6.588	6.588
<i>S. mutans</i>	Butterfield's Buffer	6.588	6.425	6.529	0.163	0.059

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Table I (continued from previous page)
Bactericidal Activity at One Minute and Five Minutes from Baseline (0 Minutes)

Bacteria		Log10 (CFU/ml)			Log10 Reduction (CFU/ml) From Baseline	
		Minutes			1	5
		0	1	5		
<i>P. gingivalis</i>	ClōSYS	7.628	0.000	0.000	7.628	7.628
	Listerine	7.628	0.000	0.000	7.628	7.628
	Crest Pro-Health	7.628	0.000	0.000	7.628	7.628
	Breath Rx	7.628	0.000	0.000	7.628	7.628
	Peridex	7.628	0.000	0.000	7.628	7.628
	Butterfield's Buffer	7.628	7.631	7.669	-0.003	-0.041
<i>P. micros</i>	ClōSYS	6.621	0.000	0.000	6.621	6.621
	Listerine	6.621	0.000	0.000	6.621	6.621
	Crest Pro-Health	6.621	0.000	0.000	6.621	6.621
	Breath Rx	6.621	6.173	5.124	0.448	1.497
	Peridex®	6.621	3.380	0.000	3.241	6.621
	Butterfield's Buffer	6.621	6.674	6.573	-0.053	0.048
<i>P. nigrescens</i>	ClōSYS	6.314	0.000	0.000	6.314	6.314
	Listerine	6.314	0.000	0.000	6.314	6.314
	Crest Pro-Health	6.314	0.000	0.000	6.314	6.314
	Breath Rx	6.314	0.000	0.000	6.314	6.314
	Peridex	6.314	0.000	0.000	6.314	6.314
	Butterfield's Buffer	6.314	6.267	5.922	0.047	0.392
<i>K. pneumoniae</i>	ClōSYS	6.528	0.000	0.000	6.528	6.528
	Listerine	6.528	0.000	0.000	6.528	6.528
	Crest Pro-Health	6.528	0.000	0.000	6.528	6.528
	Breath Rx	6.528	5.831	4.982	0.697	1.546
	Peridex	6.528	0.000	0.000	6.528	6.528
	Butterfield's Buffer	6.528	6.613	6.615	-0.085	-0.087
<i>P. aeruginosa</i>	ClōSYS	5.851	3.301	2.903	2.550	2.948
	Listerine	5.851	0.000	0.000	5.851	5.851
	Crest Pro-Health	5.851	0.000	0.000	5.851	5.851
	Breath Rx	5.851	4.000	0.000	1.851	5.851
	Peridex	5.851	0.000	0.000	5.851	5.851
	Butterfield's Buffer	5.851	5.771	5.771	0.080	0.080
<i>S. aureus</i>	ClōSYS	5.380	0.000	0.000	5.380	5.380
	Listerine	5.380	0.000	0.000	5.380	5.380
	Crest Pro-Health	5.380	2.602	0.000	2.778	5.380
	Breath Rx	5.380	5.121	4.310	0.260	1.071
	Peridex	5.380	3.531	0.000	1.849	5.380
	Butterfield's Buffer	5.380	5.334	5.365	0.046	0.015
<i>H. influenzae</i>	ClōSYS	5.025	0.000	0.000	5.025	5.025
	Listerine	5.025	0.000	0.000	5.025	5.025
	Crest Pro-Health	5.025	0.000	0.000	5.025	5.025
	Breath Rx	5.025	0.000	0.000	5.025	5.025
	Peridex	5.025	0.000	0.000	5.025	5.025
	Butterfield's Buffer	5.025	5.064	4.991	-0.039	0.035

marked bactericidal effects against oral bacteria associated with gingivitis and periodontitis, as well as other opportunistic pathogens. The results are consistent with previous studies evaluating a stabilized chlorine dioxide oral rinse against polymicrobial suspensions and biofilm environments.^{5,6}

ClōSYS products were first investigated for treating and managing periodontitis as an adjunctive therapy to scaling and root

planing. Two published clinical studies were performed to assess the effect of a stabilized chlorine dioxide oral rinse and toothpaste on both gingivitis and oral soft tissue. The research study called for subjects to use the rinse twice daily. Periodontitis and periodontal pockets were measured and evaluated. A significant percentage of the probe scores (67.4%) were reduced from ≥ 4 mm to ≤ 3 mm in an average of 3.4 months.¹

A second retrospective clinical study was performed to compare the number of bleeding on probing sites that measured 4 mm or more with the use versus the non-use of the ClōSYS oral rinse and toothpaste system. The rinse and toothpaste, used together between dental hygiene recall visits, showed an average reduction of bleeding sites of 71.85%. A small number of participants discontinued use of the products and exhibited increased bleeding sites by 157%. When the products were returned to use, bleeding was again reduced to 44%.²

Additionally, *in vitro* studies demonstrated ClōSYS oral rinse's microbicidal activity against various oral pathogens.^{3,4,7-9} These studies showed that ClōSYS oral rinse kills oral bacteria associated with the development and/or progression of oral diseases up to 99% in 10 seconds, and that the oral rinse is less toxic than chlorhexidine to human gingival cells *in vitro*. An additional study was conducted that showed infection control in the dental office by using the oral rinse as a lavage in ultrasonic scaling. This study establish that ClōSYS safely reduced the exposure to microbial aerosols by 60%.⁹

ClōSYS oral rinse exhibited less bactericidal activity against some of the test organisms than chlorhexidine and Listerine. However, it is difficult to make direct extrapolations as to the level of activity rinses would have on bacteria within plaque communities by the monoculture methods used in this study. This is an important first step, and one that provides valuable information as to relative levels of activity against oral bacteria in a very standardized laboratory setting. A bactericidal kinetics approach was undertaken to provide meaningful data as to short-term exposure of organisms to the rinses. It is clear that more complex laboratory assessments need to be done involving polymicrobial biofilms and a multiple exposure regimen. We have, in fact, done many of these experiments and have reported these findings at national meetings.^{4,9} Of course, well-designed clinical trials will need to be conducted to adequately assess the effect of these rinses on oral flora over a longer period of time. Then we will be able to discern truly clinically significant changes in the composition of flora that could cause oral disease.

With its strong performance against oral bacteria that are known to be etiological agents of infections of the periodontium and endodontium, as well as dental caries and staph infections, and its favorable behavior toward healing cells (fibroblasts), ClōSYS addresses a spectrum of clinical oral hygiene concerns.

Additionally, the agreeable taste and non-tooth-staining characteristics of ClōSYS, coupled with a lower cost and over-the-counter convenience compared to its prescription counterpart, encourage patients to be fully compliant. As a non-prescription oral rinse, ClōSYS not only meets and may surpass chlorhexidine in post-procedure applications, it also can be recommended for twice-daily regular oral care as a maintenance and preventive measure against oral diseases.

In conclusion, the stabilized chlorine dioxide oral rinse, ClōSYS, demonstrated strong bactericidal activity against oral bacteria that are associated with infections of the periodontium and endodontium, as well as dental caries and staph infections. It also overcomes the unpleasant taste and potential teeth staining characteristics that have traditionally hindered patient compliance in oral hygiene treatment. As a non-prescription oral rinse, ClōSYS affords lower cost and over-the-counter convenience for consumers.

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