

Dental Whitening Effect Produced *In Vitro* by *Streptococcus oralis*

Robert T. Zahradnik¹, Emily McDonell¹, Charles H. Hillman² and Jeffrey D. Hillman¹

¹Orogenics, Inc., 13700 Progress Blvd., Alachua, FL 32615

²University of Illinois, Department of Kinesiology and Community Health Department,
Champaign, IL 61801

Running Title: Dental Whitening Effect by *S. oralis*

Corresponding Author:

Jeffrey D. Hillman, DMD, PhD
Orogenics, Inc.
13700 Progress Blvd.
Alachua, FL 32615
Telephone: 386-418-4018 ext. 224
Fax: 386-418-1660
Email: jhillman@orogenics.com

ABSTRACT

Aims : To conduct a laboratory study to evaluate the ability of a culture of a hydrogen peroxide-producing oral bacteria, *Streptococcus oralis* strain KJ3sm™, to whiten stained dental ceramic disks over a period of 4 weeks.

Methods and Results: A colorimeter was employed to measure changes in the lightness value of the stained ceramic disks at weekly intervals during the treatment phase. At the conclusion of the 4-week experimental period, a statistically significant whitening effect was observed from the 2-week point through week 4. The whitening effect was influenced by the presence of glucose and/or catalase in the incubation medium. A plot of the lightness values as a function of time demonstrated that this whitening effect did not plateau, indicating that maximum dental whitening effect of the *S. oralis* strain had not occurred within the timeframe of the study.

Conclusions: The *S. oralis* strain was able to significantly lighten stained dental ceramic disks *in vitro*. However, for maximum benefit, elected bacterial strains for use in tooth whitening procedures should be good hydrogen peroxide producers and exhibit a transient residence time on tooth surfaces that closely corresponds to the anticipated frequency of product application.

Significance and Impact of the Study: Oral care products that incorporate probiotic cultures of hydrogen peroxide-producing bacteria, such as *S. oralis*, may represent a safe and effective means of naturally whitening vital teeth.

Key Words: Probiotic, Teeth Whitening, Peroxide-Producing Bacteria, Dental Esthetics

INTRODUCTION

The use of tooth whitening techniques to improve the esthetics of the dentition has increased dramatically during the past two decades (Sarrett 2002). Tooth whitening procedures are typically based either on the use of peroxide bleaching agents to address stains below the tooth surface or abrasive agents to remove surface tooth stains.

Tooth discoloration varies in etiology and appearance. Such lifestyle and dietary choices as drinking coffee, tea, red wine and fruit juice, and utilizing tobacco products can produce tooth staining or discoloration (Hattab, Qudeimat et al. 1999; Watts and Addy 2001). Professional scaling and polishing of teeth or the use of whitening toothpastes can help to remove many types of surface stains, primarily through the action of their abrasive/polishing agents. However, for difficult sub-surface discoloration, peroxide bleaching methods have proven to be effective in whitening the treated tooth surfaces. These bleaching methods are based upon hydrogen peroxide applied directly to the tooth surface, or produced by a chemical reaction from carbamide peroxide or sodium perborate (Dahl and Pallesen 2003). Peroxide bleaching methods may be employed by a dental professional during a single or multiple visits, or by an individual using a special mouth tray or plastic polymer strip, loaded with a peroxide agent, over a treatment period that can extend for weeks or possibly months.

The success of a bleaching procedure depends on several factors, including the concentration of the peroxide agent, the ability of the agent to reach the stain-causing entity, and the duration and frequency that the peroxide agent is applied to the stained tooth surfaces. A large number of at-home tooth whitening products are available today and include toothpastes, mouthrinses, and chewing gums, as well as products that contain

a peroxide bleaching agent. These peroxide products typically consist either of trays, custom made by a dental professional, and filled with from 5 to 22% solution of carbamide peroxide, or over-the-counter (OTC) products containing carbamide peroxide or hydrogen peroxide in a pre-fabricated tray or in whitening strips (Gerlach, Gibb et al. 2000).

The most common side effects for bleaching procedures on vital teeth include transient tooth sensitivity and gingival irritation. Tooth sensitivity resulting from bleaching can generally be managed by bleaching less frequently or reducing the concentration of the bleaching agent or shortening the application time (Li 1996; Haywood 2000). Tooth sensitivity will typically disappear shortly after the bleaching process is terminated. Another common side effect of bleaching procedures is soft tissue irritation. This effect most commonly is caused by a poor-fitting bleaching tray or by applying an excessive amount of bleaching agent to the tray (Li 1996). In some instances, significant damage to enamel has been reported as a result of long-term or inappropriate use of home-care bleaching products (Cubbon and Ore 1991; Hammel 1998).

Recently, a probiotic mouthwash, ProBiora³, has been developed for use in maintaining oral health (Hillman, McDonnell et al. Submitted; Zahradnik, Magnusson et al. Submitted). The mouthwash contains three strains of naturally occurring oral bacteria, each with a specific function of maintaining a healthy oral environment. The probiotic strains consist of *Streptococcus oralis* KJ3smTM, *Streptococcus uberis* KJ2smTM, and *Streptococcus rattus* JH145TM. Interestingly, the *S. oralis* strain and, to lesser degree, the *S. uberis* strain produce hydrogen peroxide, and the mechanism of antagonistic activity

against periodontal pathogens has been demonstrated to depend on this metabolic activity (Hillman and Socransky 1982; Socransky, Haffajee et al. 1988).

Because of hydrogen peroxide production by *S. oralis* and *S. uberis*, it was proposed that the probiotic mouthwash may have an additional benefit of naturally whitening teeth over a treatment period of weeks or months. The present preliminary study was designed to test this hypothesis by exposing stained dental ceramic disks daily to fresh suspensions of *S. oralis* KJ3sm over a period of 4 weeks and monitoring the effectiveness of the treatment to whiten the stained ceramic disks.

MATERIALS AND METHODS

Ten dental ceramic disks (courtesy of Dr. Kenneth J. Anusavice, University of Florida, Gainesville, FL) were stained over an eight-week period with tea (Lipton) and chlorhexidine (0.12%, Hi Tech Phamacal Co., Amityville, NY). Each disk was placed in a 50 ml conical (Falcon) plastic test tube. Three ml of brewed tea (prepared by the addition of a family size tea bag to 200 ml of boiling water for 5 min) were added to cover the disks. After 24 hours of incubation at room temperature, the tea was removed by decanting, the disk was rinsed with 5 ml of tap water, and the tea solution was replaced with 3 ml of a commercial 0.12% chlorhexidine mouth rinse for 24 hours. Steps 2 and 3 were repeated for 4 weeks, Monday through Friday, and the disks remained in Friday's solution over the weekend. The surface color of the stained disks was quantitatively measured using a Chroma Meter CR-400 colorimeter (Minolta, Ramsey, NJ). The color or lightness values for the stained disks were generated directly by

placing the instrument's measuring head over the disks. Standard color plates were used to calibrate the colorimeter.

The treatment phase began one day after the final colorimeter readings were taken. *S. oralis* KJ3sm is a naturally occurring genetic variant of KJ3 (Hillman and Shivers 1988) that is resistant to 1 mg/mL of streptomycin. Nine separate cultures of KJ3sm, inoculated from starter plates, were grown in 30 ml of Todd Hewitt Broth (Becton, Dickinson Co., Sparks, MD) supplemented with 0.1% sodium bicarbonate/0.5% glucose/0.1% streptomycin sulfate in an environmental shaker (200 rpm) at 37°C. After overnight incubation, the cells were harvested by centrifugation at room temperature, washed once with 10 ml of Amies media (Amies 1967), and resuspended in 30 ml of Amies media with or without glucose (Sigma-Aldrich, Inc., St. Louis, MO) and catalase at a concentration of 1000 U/ml (Sigma-Aldrich, Inc.) as shown in Table 1. A medium control contained 30 ml of Amies medium with glucose and inactivated catalase but no cells. Where indicated, catalase was inactivated by heating in a boiling water bath for 5 minutes. The entire 30 ml aliquots described in step 2 and Figure 1 were added to 50 ml Falcon tubes containing 1 stained dental ceramic disk per tube. These treatment steps were repeated daily, Monday through Friday, for 4 weeks, and the disks remained in Friday's solution over the weekend.

The lightness value for each stained disk was measured weekly during the treatment phase, which was carried out over a 4-week period.

Statistical Analyses

The L value (lightness) was plotted as a function of time using the mean group values (\pm S.D.). Inter- and intra-group L values were compared for statistically

significant differences. A 4 (Treatments: A, B, C, D) x 5 (Time: Weeks 0, 1, 2, 3, 4) mixed-model repeated measures ANOVA was conducted with Greenhouse-Geisser correction to determine the differential influence of various treatments on L values across time. Follow-up analyses determined treatment effects at each time point. Post hoc Bonferroni-corrected t tests were used to determine significant differences among the treatments. All analyses used a family-wise alpha level after Bonferroni correction of $p = .05$.

RESULTS

The L value (lightness) was plotted as a function of time using the mean group values (\pm S.D.; Figure 1). The trend line for Group A (experimental group) data had a slope of 2.37, which was substantially greater than the slopes of the trend lines for Group B (catalase control group; slope, -0.54), Group C (glucose control group; slope, 0.52) and Group D (medium control group; slope, -0.84), indicating that disks in Group A were becoming lighter or whiter over time at a much faster rate than the disks in the other groups. At the conclusion of the 4-week experimental period, a lightening of the ceramic disks in Group A was readily apparent to the unaided eye (Figure 2).

The data was further analyzed as follows: *S. oralis*, strain KJ3sm, incubated in the presence of glucose and air (Group A) produced a significantly larger whitening effect on tea and chlorhexidine-stained ceramic disks at Weeks 2-4 than Control Groups B and D, and a significantly larger effect than Control Group C at Week 4. Inclusion of catalase in the medium (Group B), to enzymatically destroy hydrogen peroxide generated by KJ3sm, significantly reduced the whitening effect of KJ3sm. When glucose was omitted from the

incubation medium (Group C), a smaller whitening effect was observed than in Group A, and this difference became significant at the 4-week point. Medium supplemented with glucose and inactivated catalase (Group D) showed no tendency to produce lightening of the stained ceramic disks. The plot of L value as a function of time for Group A in Figure 1 did not plateau, indicating that maximum whitening effect from exposure of stained disks to the KJ3sm strain had not occurred within the timeframe of the study.

DISCUSSION

Over-the-counter (OTC) products are currently available that utilize carbimide peroxide or hydrogen peroxide either in prefabricated or user-modified trays (Li 1996) or incorporated into strips that users apply to their teeth (Gerlach, Gibb et al. 2000). This cosmetic procedure, however, is not currently recommended for everyone (Leonard, Haywood et al. 1997; Haywood 2000; Lee, Zhang et al. 2005; Tredwin, Naik et al. 2006), and is not without its side effects. Tooth sensitivity is the most common side-effect of bleaching, observed in 15% to 78% of the patients (Tam 1999; Dahl and Pallesen 2003). Gingival irritation during treatment is another commonly reported side effect (Leonard, Haywood et al. 1997; Tam 1999). In animal toxicity studies, it was reported that exposure of the gingiva to 1% hydrogen peroxide for 6 to 48 hours resulted in epithelial damage and acute inflammation of the subepithelial connective tissue (Martin, Bishop et al. 1968), and long-term application of 3% hydrogen peroxide in the hamster cheek pouch twice weekly resulted in inflammatory changes (Weitzman, Weitberg et al. 1986). Furthermore, surface alterations in enamel topography were routinely observed following tooth bleaching with 10% and higher concentrations of carbamide peroxide (Shannon,

Spencer et al. 1993; Bitter 1998; Oltu and Gurgan 2000). A possible clinical implication of this final finding may be that the teeth are more susceptible to extrinsic discoloration after bleaching due to increased surface roughness (Dahl and Pallesen 2003).

If we consider the above side effects of professional and at-home bleaching methods, it is reasonable to conclude that preparations with the lowest concentration of a bleaching agent would provide the maximum level of short and long-term safety for the user. Furthermore, since considerable care must be exercised by the user when self-applying bleaching trays and strips to teeth at home, time and effort would likely be minimized with the use of a more user-friendly delivery vehicle. It was hypothesized by the authors that the use of indigenous oral bacterial strains that are known to metabolically produce hydrogen peroxide, when delivered to the surfaces of teeth in a convenient mouthwash format, may represent a natural alternative for whitening teeth.

There are a number of non-pathogenic bacterial species that are known to produce hydrogen peroxide, including *lactobacillus*, *bifidobacterium* and *viridans streptococcus* strains (Lahtinen, Jalonen et al. 2007; Saito, Seki et al. 2007). *Streptococcus oralis* KJ3sm was selected for testing in this *in vitro* whitening model because it has been shown in a clinical trial to be safe and well tolerated, with no user complaints of tooth sensitivity or gingival irritation throughout the 8-week treatment period involving twice daily product use (Zahradnik, Magnusson et al. Submitted). Furthermore, this strain has been reported to inhibit the growth of microorganisms known to be associated with periodontal diseases (Hillman, Socransky et al. 1985; Socransky, Haffajee et al. 1988), and is an excellent hydrogen peroxide producer (Hillman and Shivers 1988).

A colorimeter was employed in this *in vitro* study to quantitatively determine the extent of the whitening effect of KJ3sm under the various treatment conditions. A shade guide is typically used in clinical tooth whitening studies to measure the efficacy of a particular bleaching treatment. However, color matching using a shade guide can be subject to investigator variability and bias. Furthermore, the shade guide units are not evenly distributed in color space and are not as discriminating as the measured colorimeter parameters (Gegauff, Rosenstiel et al. 1993).

The results of the present study demonstrate that, in a laboratory model, the metabolically active, non-growing *S. oralis* KJ3sm strain, is capable of significantly lightening a tea and chlorhexidine stained dental ceramic disk in a time-dependent fashion. Importantly, the lightening of the stained disks did not plateau over the course of the 4-week study, which suggests that the maximum dental whitening effect of the KJ3sm strain had not occurred within the time frame of the study, and that additional stain removal may be possible with longer treatment times. The proposed mechanism for the observed whitening effect relates to the normal end product production of hydrogen peroxide by the KJ3sm strain. Peroxide is produced mainly from pyruvate by the pyruvate-oxidase activity in *S. oralis*, and it is excreted into the environment as an end product of sugar metabolism (Hillman and Shivers 1988). Hydrogen peroxide was shown in that study to be the mechanism used by *S. oralis* to inhibit the growth of periodontal pathogens. In the present study, the inclusion of active catalase in the incubation medium (Group B) demonstrated that hydrogen peroxide production is also the mechanism for the whitening effect observed with stained ceramic disks.

Peroxide production, and thus the whitening effect, is dependent on the presence of a metabolizable carbon source (e.g., glucose). Thus, a whitening effect would not be expected when the incubation medium does not contain a carbon source. The small whitening effect observed when glucose was omitted from the incubation medium (Group C) may be explained by residual peroxide production resulting from metabolism of stored carbohydrate, most likely in the form of intracellular polysaccharide. The incubation medium itself (Group D) showed no tendency to cause lightening of the ceramic disks.

The concentration of peroxide in contact with oral surfaces is much lower for a probiotic-based whitening mouthwash than for the current range of OTC peroxide-based products. The whitening process, therefore, would be expected to be slower. It would, however, most certainly be safer to oral tissues, and more widely applicable to population groups that are currently contra-indicated for office and at-home tooth whitening procedures. A probiotic-based whitening mouthwash also represents a more user-friendly delivery vehicle since it eliminates the need for using a mouth tray or polymer strip to limit contact of the whitening agent to only tooth surfaces. The ProBiora³ mouthwash that incorporates three natural oral bacterial strains into its formulation, including the *S. oralis* KJ3sm strain, has been clinically demonstrated to lower the levels of key dental and periodontal pathogens in the mouth (Zahradnik, Magnusson et al. Submitted). This *in vitro* study suggests a further benefit of tooth whitening may be possible with the use of the ProBiora³ mouthwash containing hydrogen peroxide-producing bacterial strains. Nevertheless, a human trial will be conducted to determine

the optimum dose and application frequency necessary to achieve a safe and acceptable level of teeth whitening *in vivo*.

ACKNOWLEDGEMENTS

We wish to thank Dr. Kenneth J. Anusavice, University of Florida, for the contribution of the dental ceramic disks used in this study. ProBiora³, KJ2sm, KJ3sm and JH145 are trademarks of Oragenics, Inc., Alachua FL.

Table 1

GROUP	DISK NUMBER	TREATMENT CONDITIONS		
		KJ3sm ($\sim 10^9$ cfu/ml)	Glucose (0.5%)	Catalase (3000 U/ml)
A ¹	1,2,3	+	+	Inactivated
B ²	4,5,6	+	+	Active
C ³	7,8,9	+	-	Inactivated
D ⁴	10	-	+	Inactivated

¹ Experimental

² Catalase control

³ Glucose control

⁴ Medium control

Figure 1. L Value as Function of Time

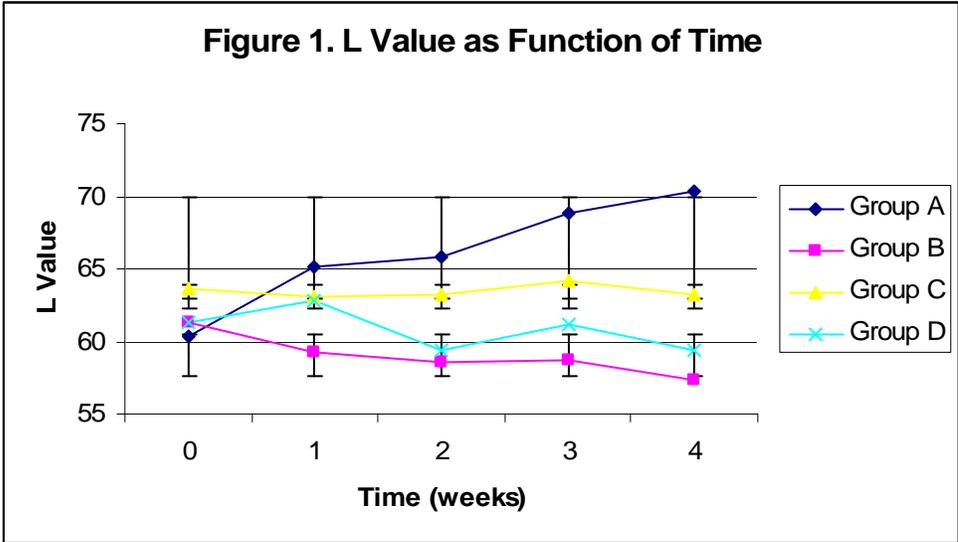
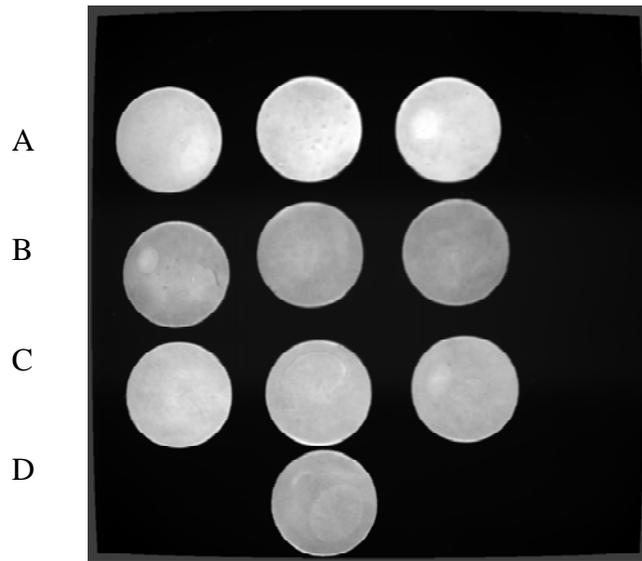


Figure 2.



REFERENCES

- Amies, C. R. (1967). "A modified formula for the preparation of Stuart's Transport Medium." Can J Public Health **58**(7): 296-300.
- Bitter, N. C. (1998). "A scanning electron microscope study of the long-term effect of bleaching agents on the enamel surface in vivo." Gen Dent **46**(1): 84-8.
- Cubbon, T. and D. Ore (1991). "Hard tissue and home tooth whiteners." CDS Rev **84**(5): 32-5.
- Dahl, J. E. and U. Pallesen (2003). "Tooth bleaching--a critical review of the biological aspects." Crit Rev Oral Biol Med **14**(4): 292-304.
- Gegauff, A. G., S. F. Rosenstiel, et al. (1993). "Evaluating tooth color change from carbamide peroxide gel." J Am Dent Assoc **124**(6): 65-72.
- Gerlach, R. W., R. D. Gibb, et al. (2000). "A randomized clinical trial comparing a novel 5.3% hydrogen peroxide whitening strip to 10%, 15%, and 20% carbamide peroxide tray-based bleaching systems." Compend Contin Educ Dent Suppl(29): S22-8; quiz S42-3.
- Hammel, S. (1998). Do-it-yourself tooth whitening is risky. U.S. News and World Report: 66.
- Hattab, F. N., M. A. Qudeimat, et al. (1999). "Dental discoloration: an overview." J Esthet Dent **11**(6): 291-310.
- Haywood, V. B. (2000). "Current status of nightguard vital bleaching." Compend Contin Educ Dent Suppl(28): S10-7; quiz S48.
- Hillman, J. D., E. McDonell, et al. (Submitted). A spontaneous lactate dehydrogenase deficient mutant of *Streptococcus rattus* for use as a probiotic in the prevention of dental caries.
- Hillman, J. D. and M. Shivers (1988). "Interaction between wild-type, mutant and revertant forms of the bacterium *Streptococcus sanguis* and the bacterium *Actinobacillus actinomycetemcomitans* in vitro and in the gnotobiotic rat." Arch Oral Biol **33**(6): 395-401.
- Hillman, J. D. and S. S. Socransky (1982). "Bacterial interference in the oral ecology of *Actinobacillus actinomycetemcomitans* and its relationship to human periodontitis." Arch Oral Biol **27**(1): 75-7.
- Hillman, J. D., S. S. Socransky, et al. (1985). "The relationships between streptococcal species and periodontopathic bacteria in human dental plaque." Arch Oral Biol **30**(11-12): 791-5.

- Lahtinen, S. J., L. Jalonen, et al. (2007). "Specific *Bifidobacterium* strains isolated from elderly subjects inhibit growth of *Staphylococcus aureus*." Internat J Food Micro **117**: 125-128.
- Lee, S. S., W. Zhang, et al. (2005). "Tooth whitening in children and adolescents: a literature review." Pediatr Dent **27**(5): 362-8.
- Leonard, R. H., Jr., V. B. Haywood, et al. (1997). "Risk factors for developing tooth sensitivity and gingival irritation associated with nightguard vital bleaching." Quintessence Int **28**(8): 527-34.
- Li, Y. (1996). "Biological properties of peroxide-containing tooth whiteners." Food Chem Toxicol **34**(9): 887-904.
- Martin, J. H., J. G. Bishop, et al. (1968). "Cellular response of gingiva to prolonged application of dilute hydrogen peroxide." J Periodontol **39**(4): 208-10.
- Oltu, U. and S. Gurgan (2000). "Effects of three concentrations of carbamide peroxide on the structure of enamel." J Oral Rehabil **27**(4): 332-40.
- Saito, M., M. Seki, et al. (2007). "A novel agar medium to detect hydrogen peroxide-producing bacteria based on the prussian blue-forming reaction." Microbiol Immunol **51**(9): 889-92.
- Sarrett, D. C. (2002). "Tooth whitening today." J Am Dent Assoc **133**(11): 1535-8; quiz 1541.
- Shannon, H., P. Spencer, et al. (1993). "Characterization of enamel exposed to 10% carbamide peroxide bleaching agents." Quintessence Int **24**(1): 39-44.
- Socransky, S. S., A. D. Haffajee, et al. (1988). "Associations between microbial species in subgingival plaque samples." Oral Microbiol Immunol **3**(1): 1-7.
- Tam, L. (1999). "The safety of home bleaching techniques." J Can Dent Assoc **65**(8): 453-5.
- Tredwin, C. J., S. Naik, et al. (2006). "Hydrogen peroxide tooth-whitening (bleaching) products: review of adverse effects and safety issues." Br Dent J **200**(7): 371-6.
- Watts, A. and M. Addy (2001). "Tooth discolouration and staining: a review of the literature." Br Dent J **190**(6): 309-16.
- Weitzman, S. A., A. B. Weitberg, et al. (1986). "Effects of hydrogen peroxide on oral carcinogenesis in hamsters." J Periodontol **57**(11): 685-8.

Zahradnik, R. T., I. Magnusson, et al. (Submitted). Preliminary assessment of safety and effectiveness in humans of ProBiora^{3TM}, a probiotic mouthwash.