

A Biofilm Assay to Study the Effects of Mouthrinse and Dentifrice Components

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ABSTRACT

We have developed an *in vitro* model to evaluate the ability of a mouthrinse or dentifrice slurry to inhibit the metabolic activity of a mixed cariogenic bacterial biofilm composed of *S. mutans*, *S. sobrinus*, and *L. casei*. A bacterial metabolic end product, lactic acid, was monitored over time to determine the effects active ingredients have on metabolism/regrowth of the biofilm. Briefly, a bacterial biofilm was formed in a plastic uncoated 12-well tissue culture plate. The biofilm was washed then starved and treated with a test solution or appropriate control for various periods of time. Subsequently, the biofilm was washed and incubated at 37°C in dilute buffered TSB (w/o dextrose) (pH 7.0) supplemented with 1% sucrose. A sample was collected at three hours after treatment and analyzed for lactic acid concentrations and compared to its control. Then the media was discarded and the biofilm was washed and incubated in TSB media (w/o dextrose) overnight in 5% CO₂ at 37°C to study the ability for regrowth. Next day, the biofilm was washed, starved and incubated in the diluted buffered TSB as above to determine the metabolic activity. A treatment-time course (5 sec. to 2 min.) study for Peridex[®] showed that a two minute exposure of treatment was required for complete inhibition of the metabolic activity of cells in the biofilm immediately after the treatment. However, the regrowth data revealed that a minimum of 30 sec. exposure was sufficient for complete inhibition. This behavior could be due to adsorption of chlorhexidine to the biofilm which acted as a reservoir providing long term antimicrobial effects, as explained in the literature. Non-ionic based and sodium alkyl sulfate-based chlorhexidine dentifrice slurries were also tested. An important aspect of this biofilm *in vitro* model is its flexibility and maneuverability in the evaluation of active ingredients in both rinse and dentifrice formulations, thereby providing a better understanding of the efficacy of oral formulations.

INTRODUCTION

Streptococcus mutans, *Streptococcus sobrinus*, and *Lactobacilli casei* have been implicated in the development of caries due to the ability of these organisms to convert sucrose to glucans, which aids in bacterial colonization of the tooth surface, and produce lactic acid through glycolysis

of sugar. Thus, the inhibition of bacterial biofilm metabolism has been the focus of this dental research for the prevention and cure of caries. The use of antimicrobials to reduce caries has been advocated for years based on the correlation between high levels of these cariogenic bacteria and an increased risk for caries. This study describes the development of methods by which solutions and dentifrice supernatants can be quickly screened for their ability to inhibit the metabolic activity based on lactic acid production by the bacteria present in a biofilm. Lactic acid production was determined by using flow injection analysis (FIA) with an immobilized lactate oxidase enzyme reactor and electrochemical detection of hydrogen peroxide based on the following enzymatic reaction:



PROTOCOL

Day 1

1.) Two ml of sterilized TSB w/o Dextrose plus 10% sucrose were added to each well of a 12 well tissue culture plate. Each well was inoculated with 120 µl of a mixed bacterial culture consisting of equal volumes of a suspension of *S. mutans*, *S. sobrinus* and *L. casei*. The plates were incubated for 16-18 hours in 5% CO₂ at 37° C to promote biofilm formation.

Day 2

2.) The plates were removed from the incubator and the biofilms were washed twice with sterile saline.
3.) To deplete the biofilm of endogenous reserves, 2 ml of sterile saline were added to each well and the plate was placed at 37° C for 15 min. on a platform rocker. The saline was removed and the step was repeated twice more, for a total starvation time of 45 min.

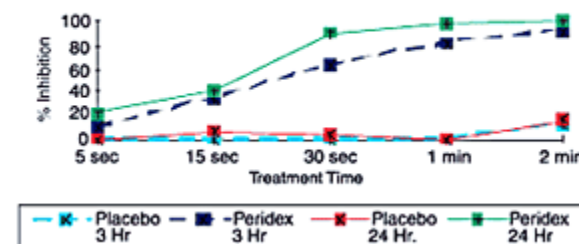
4.) Two ml of treatment or appropriate control was added to 3 wells, then the plate was placed on a titer plate shaker and shook for 1 min. (unless otherwise indicated) at room temperature. The treatments were poured off and the biofilm was washed 3 times with sterile saline.
5.) Two ml of sterile 1:10 dilution of TSB W/O Dextrose, with a final concentration of 1% sucrose and 25 mM phosphate buffer, pH 7.5, were added to each well and then the plate was placed on a platform rocker and incubated for 3 hr in 5% CO₂ at 37° C.
6.) After 3 hr., a 100 µl aliquot was transferred from each well into a sample vial for lactic acid measurements as an indicator of biofilm glycolysis.
7.) The remaining media was removed from the plate and the biofilm was washed three times with sterile saline. Two ml of TSB w/o dextrose were added to each well and the plate was placed back in an incubator overnight at 37° C in 5% CO₂.

Day 3

8.) For the 24 hour time-point, steps 2 and 3 were repeated to starve the biofilm and then steps 5 and 6 were repeated for lactic acid measurements.

RESULTS AND CONCLUSIONS

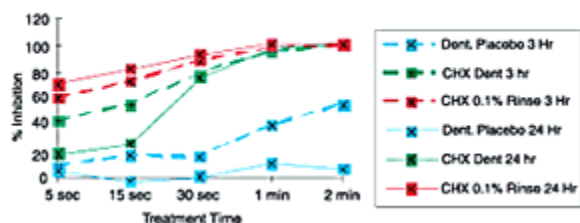
Figure 1: Efficacy of Peridex on Biofilm Metabolism Time Course Evaluation



-Greater than 30 sec exposure of Peridex® resulted in irreversible inhibition of glycolysis

-The assay is a useful tool to evaluate the effect of exposure time on efficacy of formulations

Figure 2: Efficacy of Chlorhexidine in a Non-Ionic Based Dentifrice - Time Course Evaluation



-Non-ionic detergents do not affect the activity of biofilm

-Efficacy of CHX is not affected by a non-ionic dentifrice matrix

-A 30 sec exposure resulted in irreversible inhibition of metabolism

Table 1. Efficacy of SAS-Based Dentifrice

Treatments	% Inhibition of Metabolism	
	3 Hr	24 Hr
Placebo Dentifrice	98	25
CHX-Dentifrice	77	21

-SAS can inhibit the metabolism for short periods

-Inhibition of metabolism by SAS is reversible

-CHX and SAS appear to interact antagonistically

Table 2. Effect of Ionic Detergents on Activity of Chlorhexidine

Treatment	% Glycolysis Inhibition	
	3 Hr	24 Hr
0.1% CHX	94	98
0.14% SAS	99	32
0.1% CHX + 0.14% SAS	32	15
0.1% CHX	96	99
0.28% SAS	100	30
0.1% CHX + 0.28% SAS	98	37
0.1% CHX	94	98
0.56% SAS	100	82
0.1% CHX + 0.56% SAS	100	47

-Data confirms loss of CHX activity is due to interactions with SAS

-Effect of SAS is reversible except at high concentrations

-CHX and SAS appear to interact antagonistically