

## *S. mutans* biofilm model to evaluate antimicrobial substances and enamel demineralization

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**Abstract:** The aim of this study was to validate a model of *S. mutans* biofilm formation, which simulated ‘feast-famine’ episodes of exposure to sucrose that occur in the oral cavity, showed dose-response susceptibility to antimicrobials and allowed the evaluation of substances with anticaries potential. *S. mutans* UA159 biofilms were grown for 5 days on bovine enamel slabs at 37°C, 10% CO<sub>2</sub>. To validate the model, the biofilms were treated 2x/day with chlorhexidine digluconate (CHX) at 0.012, 0.024 and 0.12% (concentration with recognized anti-plaque effect) and 0.05% NaF (concentration with recognized anti-caries effect). CHX showed dose-response effect decreasing biomass, bacterial viability and enamel demineralization ( $p < 0.05$ ). Whereas, 0.05% NaF did not show antimicrobial effect but had similar effect to that of 0.12% CHX decreasing enamel demineralization ( $p < 0.05$ ). The model developed has potential to evaluate the effect of substances on biofilm growth and on enamel demineralization.

**Descriptors:** *Streptococcus mutans*; Biofilms; Chlorhexidine; Sucrose; Dental caries.

### Introduction

Dental biofilm is an organized microbiologic community enclosed in a matrix of extracellular material and attached to dental surfaces.<sup>1</sup> Under some conditions, such as high carbohydrate consumption, the presence of a high amount of sugars can change the biochemical and microbiological composition of biofilm, leading to an increase in the proportion of pathogenic species and transforming healthy biofilm into cariogenic biofilm. Moreover, depending on the frequency this can lead to the formation and development of dental caries disease.<sup>2</sup>

In the oral cavity, the microorganisms in dental biofilm are exposed to large amounts of sugar during a short period of time and some microorganisms have the capacity to use these carbohydrates to produce acid, synthesize extracellular polysaccharides and store energy. After this rapid exposure to sugar, dental biofilm undergoes long periods of sugar starvation. These physiological conditions of bacterial growth are known as ‘feast or famine’ episodes<sup>3</sup> and can cause microbiological selection strategies that increase the proportion of acid-tolerant species such as *S. mutans* in biofilm.<sup>4,5</sup>

*S. mutans* are considered the most cariogenic microorganisms in

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dental biofilm due to their capacity to use dietary carbohydrates such as sucrose, to synthesize extracellular polysaccharides (EPS) and because of their acidogenic and aciduric properties.<sup>4</sup> EPS are important virulence factors of *S. mutans* because they promote bacterial adherence to the tooth surface,<sup>6,7</sup> contribute to the structural integrity of dental biofilms,<sup>8,9</sup> change the porosity of the biofilm<sup>10</sup> and consequently increase enamel demineralization.<sup>11</sup> Therefore, *S. mutans* biofilms have been used to evaluate their cariogenic properties due to difficulties of developing *in vivo* studies in controlled cariogenic situations.<sup>12</sup>

However, in most *S. mutans* biofilm models, the biofilm is grown under constant exposure to carbohydrates, which maintains the biofilm under constant acid stress, and does not simulate the “feast and famine” episodes of sugar exposure and pH-cycling that occur in the oral cavity.<sup>13,14,15</sup> In addition, these protocols do not use dental substrates to evaluate the effect of antimicrobial substances on dental demineralization caused by the attached biofilm. Furthermore, an important requirement of biofilm models is that they should show a dose-response effect against antimicrobial substances. With regard to oral biofilm models, chlorhexidine has been used as the ‘gold standard’ because it is considered the most efficient topical substance to reduce dental plaque, a type of biofilm.<sup>16</sup> Moreover, it is recognized that although fluoride is the most important anticaries substance,<sup>17</sup> its antibacterial effect is limited<sup>18</sup> and the model should simulate the main mechanism of action of fluoride on dental caries.

Therefore, the aim of this study was to validate a *S. mutans* biofilm model that simulates exposure to sucrose that occurs in the oral environment, and allows the evaluation of the effect of antimicrobial substances on biofilm formation and on enamel demineralization.

## Material and Methods

### Experimental design

This *S. mutans* biofilm model was a modified version detailed by Koo *et al.*,<sup>13</sup> (2003) with the main modifications being the use of dental substrate and the simulation of ‘feast or famine’ episodes of

exposure to sucrose. Therefore, *S. mutans* UA159 biofilms were formed on saliva-coated bovine enamel slabs suspended vertically in ultrafiltered (10 kDa molecular weight cut-off membrane; Amicon) tryptone-yeast extract broth (UTYEB) at 37°C, 10% CO<sub>2</sub> for 5 days<sup>13</sup> and exposed 1 min, 8x/day to 10% sucrose. After 48 h, the growth of some biofilms (n = 4) was stopped (baseline) and the others were grown for another 3 days and treated 2x/day for 1 min with one of the following solutions: 1) 0.9% NaCl (Control, n = 4), 2) 0.012% chlorhexidine digluconate (CHX, n = 4), 3) 0.024% CHX (n = 4), 4) 0.12% CHX (n = 4) and 5) 0.05% NaF (n = 4). The biomass, viable bacteria and biochemical composition of all biofilm samples were determined. Besides, the mineral loss of enamel slabs was assessed. The pH of the culture media was determined daily as an indicator of biofilm acidogenicity. For statistical evaluation each biofilm was considered an experimental block.

### Enamel block preparation

Bovine incisor teeth, from which the roots were removed, were stored in 2% formol solution for a period of at least 30 days.<sup>19</sup> The dental crown was fixed in an acrylic base, and with the aid of two parallel disks spaced 4 mm apart, a longitudinal slice was obtained from the central part of the dental specimen. Using two parallel disks spaced 7 mm apart, this slice was transversally cut. The dentin of this 7 x 4 mm dental slab was completely worn in grinder machine (Phoenix Beta, Buehler, Lake Bluf, IL, USA) using 400-grit aluminum oxide abrasive paper. Enamel surfaces were polished, flattened and baseline enamel surface hardness was determined on the outer enamel surface by making 3 indentations, spaced 100 µm from each other, using a Knoop indenter with a 25 g load for 5 s and a microhardness tester coupled to FM-ARS 900 software (Future-Tech FM, Kawasaki, Japan). Slabs presenting hardness of 331.69 ± 13.81 kg/mm<sup>2</sup> were randomly divided into six groups (n = 4).

Each slab was individually placed in 1 ml of a solution containing 0.06 mM P<sub>i</sub> and 0.08 mM Ca<sup>++</sup> and sterilized by autoclaving (a previously standardized condition, data not published). The sterilized

slabs were anchored vertically on metal devices and suspended in a 24-well culture plate.

### Biofilm growth

UTYEB was used as culture media<sup>13</sup> and depending on the experimental phase, the media contained 1% glucose, 1% sucrose or 0.1 mM glucose as described as follows. *S. mutans* UA159 colonies were transferred to UTYEB containing 1% glucose and incubated at 37°C, 10% CO<sub>2</sub> to reactivate the microorganisms. The slabs on which human salivary pellicle was formed, were individually positioned in wells containing 2.0 ml of the inoculum and were incubated at 37°C, 10% CO<sub>2</sub> to allow bacterial adhesion on the acquired pellicle. All these procedures were carried out according to Koo *et al.*<sup>13</sup> (2003) but 8 h (previously standardized) after incubation the slabs were transferred to the fresh UTYEB containing 0.1 mM glucose (salivary basal concentration) and incubated for an additional 16 h at 37°C, 10% CO<sub>2</sub>. The next day, the biofilms on enamel slabs were transferred to fresh UTYEB containing 0.1 mM glucose and were exposed 8x/day for 1 min to 10% sucrose (containing 1.23 mM Ca, 0.74 mM P<sub>i</sub> and 0.023 µg F/mL, previously standardized) at predetermined times (8:00, 9:30, 11:00, 12:30, 14:00, 15:30, 17:00 and 18:30 h). This procedure was repeated for the next 3 days. After each sucrose exposure, the biofilms on enamel slabs were washed 3 times in 0.01% NaCl. The pH of the culture media was determined every 24 h and the media was replaced with a fresh solution.

### Treatments

The CHX solutions were prepared from 20% chlorhexidine digluconate (Sigma, Steinheim, Germany) using sterilized distilled water. The solution of 0.05% NaF was prepared and sterilized by autoclaving. The treatments were performed 2x/day, after the first and the last sucrose exposure of the day. After each treatment, the biofilms on enamel slabs were washed 3 times in 0.9% NaCl.

### Biofilm collection

After the assigned experimental time of biofilm growth, the enamel slabs containing the biofilms

were washed 3 times in 0.9% NaCl and individually transferred to microcentrifuge tubes containing 1 ml of 0.9% NaCl. The tubes were sonicated at 7 W for 30 s (Branson, Sonifier 50, Danbury, CT USA) to detach the biofilms formed on the slabs.<sup>20</sup> The slabs were carefully removed from the suspension and stored to determine enamel demineralization. Aliquots of the suspension were used to determine biofilm bacterial viability, biomass (dry weight and total soluble proteins) and polysaccharides.

### Biomass determination

Biofilm dry weight was determined according to Koo *et al.*<sup>13</sup> (2003) from 200 µl of the suspension. To determine total soluble protein,<sup>21</sup> 50 µl of the suspension was transferred to a microcentrifuge tube, to which the same volume of 2 M NaOH was added. The tube was vortexed and placed at 100°C at 15 min, centrifuged (10000 g for 10 min, 4°C) and the concentration of soluble protein was determined in the supernatant (DC Protein Assay, Bio-Rad, Hercules, Ca, USA).

### Bacterial viability

An aliquot of 100 µl of the suspension was diluted in 0.9% NaCl in series up to 10<sup>-7</sup> and 2 drops of 20 µl of each dilution were inoculated on BHI agar (BD, Sparks, USA) to determine the number of viable microorganisms.<sup>22</sup> The plates were incubated for 48 h at 37°C, 10% CO<sub>2</sub> (IG 150, Jouan incubator). CFU were counted and the results were expressed as CFU/mg of biofilm dry weight.<sup>20</sup>

### Polysaccharide analyses

From 100 µl of the suspension, insoluble and soluble extracellular polysaccharides were extracted according to Aires *et al.*<sup>20</sup> (2008) and analyzed for total carbohydrate according to Dubois *et al.*<sup>23</sup> (1956). The results were normalized by biofilm dry weight.

### Enamel demineralization assessment

At the end of each experimental phase, the enamel slab surface hardness (SH) was again measured. One row of three adjacent indentations spaced at 100 µm was made 100 µm from the three baseline

measurements. The mean values of the three baseline indentations and the three measurements after treatments were then averaged and the % SHL was calculated as follows:

$$\frac{(\text{baseline SH} - \text{SH after treatment}) \times 100}{\text{baseline SH}}$$

Surface hardness loss (SHL) was used as indicator of enamel demineralization.<sup>11</sup>

### Statistical analysis

The assumptions of equality of variances and normal distribution of errors were checked for all the response variables tested and those that did not satisfy these conditions were transformed.<sup>24</sup> The relationship between CHX concentrations and the variable evaluated was estimated by regression analysis. When significant correlation was found, the data were submitted to ANOVA followed by Tukey's test, with the exception of acidogenicity, which was analyzed by repeated measures. Original data were used with the exception of viable bacteria counts, which were transformed to log<sub>10</sub>. The software SPSS for Windows 15.0 (SPSS, Chicago, IL, USA) was used and the significance level was fixed at 5%.

### Results

A statistically significant linear effect was found between CHX concentration and biofilm dry weight, total soluble proteins and viable bacteria, and enamel demineralization (Table 1), showing dose-response effect for these variables. No effect

on polysaccharides was found.

As regards the pH of the culture media (Graph 1), the groups did not differ statistically after 48 h of the biofilm growth ( $p > 0.05$ ). At 72 h of biofilm growth, the pH of media for the 0.12% CHX treatment was similar to that of the NaF 0.05% group ( $p > 0.05$ ) but higher than that of the other groups ( $p < 0.05$ ). At the 72 h and 120 h of biofilm growth, the pH of the NaF 0.05% was similar to that of the control group ( $p > 0.05$ ) but lower than that of the other groups ( $p < 0.001$ ).

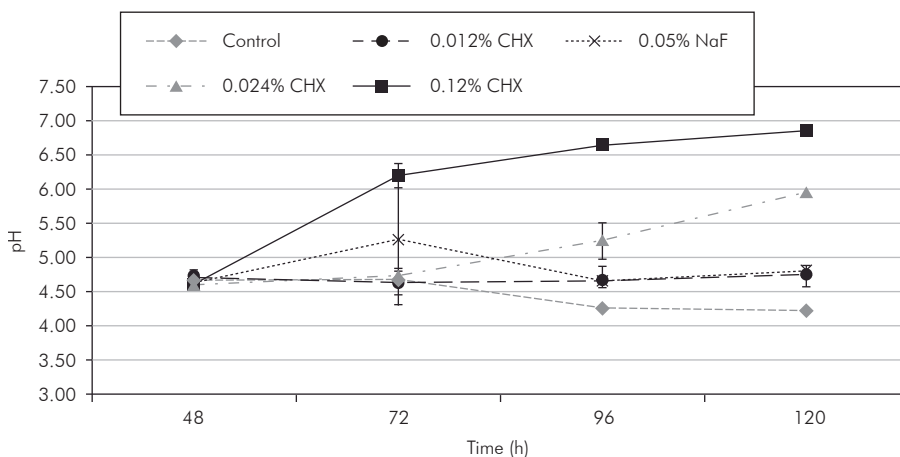
The dry weight and protein values (Graph 2) showed that the biomass of the biofilm treated with 0.12% CHX had lower values than those of the control ( $p = 0.001$  and 0.07, respectively) and it did not differ statistically from the baseline value ( $p = 0.11$  and 0.99, respectively). However, the values of the biomass of biofilm treated with 0.05% NaF did not

**Table 1** - Correlation (r) and significance (p) between chlorhexidine concentration (0 – 0.12%) and the response variables.

Variables	r	p
Dry weight	-0.808	< 0.001
Proteins	-0.767	0.001
Viable bacteria	-0.689	0.006
%SHL	-0.628	0.009
IEPS	0.148	0.615
SEPS	0.249	0.412

\*% SHL = Percentage of surface hardness loss. IEPS = Insoluble extrapoly-saccharides. SEPS = Soluble extrapolyssaccharides.

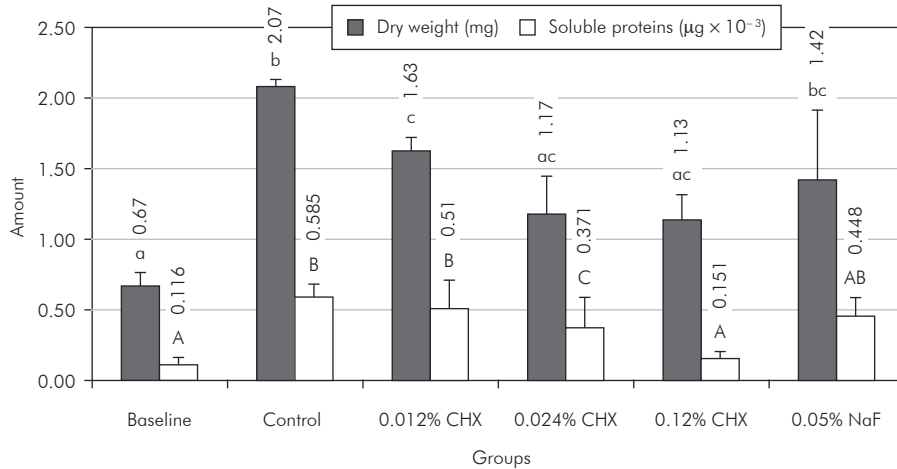
**Graph 1** - pH of the culture medium after 48 of biofilm growth in the absence of treatments and at each 24 h after beginning the treatments (72, 96 and 120 h data). The statistical significance among the treatments is described in the Results section ( $n = 4$ ).



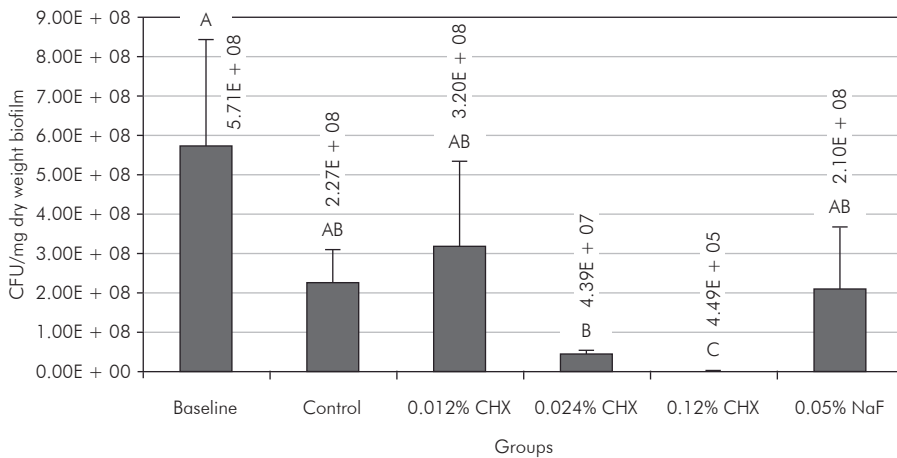
differ from those of the control ( $p = 0.209$  and  $0.758$ , respectively) but were higher than the baseline values ( $p = 0.001$  and  $0.045$ , respectively) (Graph 2).

The viable bacteria count in the biofilm (Graph 3), normalized by biofilm dry weight, was

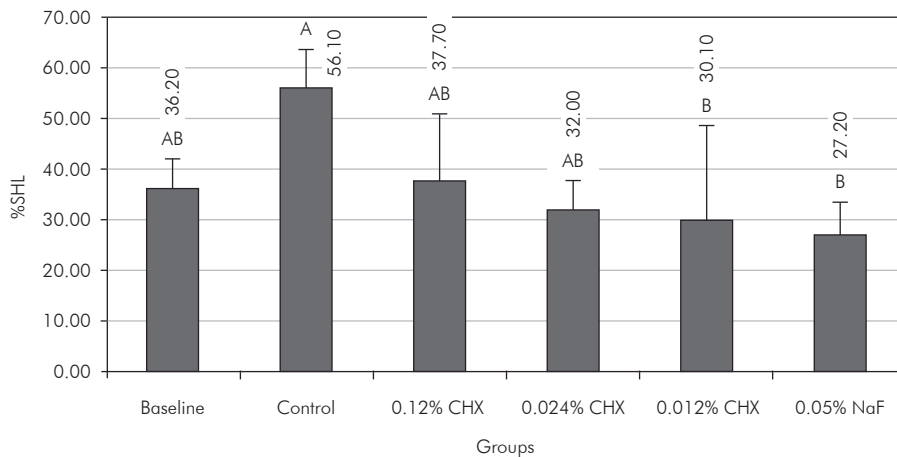
significantly lower ( $p < 0.001$ ) for the treatment with 0.12% CHX when compared with the baseline and the control group. However, the viable bacteria counts of biofilm treated with 0.05% NaF did not differ from those of the control and the baseline val-



**Graph 2** - Means of biofilm dry weight (mg) and amount of soluble proteins ( $\mu\text{g} \times 10^{-3}$ ) for the baseline and according to the treatments ( $n = 4$ ).



**Graph 3** - Means of viable bacteria (CFU/mg dry weight) in the biofilms grown for 48 h in the absence of the treatments (baseline) and after 3 days of treatments described ( $n = 4$ ). Data were transformed by  $\log_{10}$ .



**Graph 4** - Means of enamel demineralization (%SHL) after 48 h of biofilm growth in the absence of the treatments (baseline) and after 3 days of the treatments described. ( $n = 4$ ).

[Vertical bars denote standard deviations, and significant differences among the groups are indicated by different letters ( $p < 0.05$ ).]

ues ( $p = 0.998$  and  $0.511$ , respectively).

As regards enamel demineralization (Graph 4), 0.12% CHX and 0.05% NaF significantly reduced the %SHL when compared with the control ( $p = 0.036$  and  $0.017$ , respectively), but these treatments did not differ between them ( $p = 0.99$ ).

## Discussion

Biofilm models are important tools to evaluate the biochemical and microbiological composition of biofilm formed under different conditions or the changes caused on the substratum surface on which the biofilm is attached. Therefore, the conditions of biofilm formation and the substratum used must be as close as possible to those of real life.

The improved model of *S. mutans* biofilm growth was validated and dose response effect of CHX on *S. mutans* biofilm was shown for most variables (Table 1). Therefore, the model is sufficiently sensitive to show biofilm and enamel demineralization changes in the presence of antimicrobial substances. The treatment 2x/day with 0.12% CHX showed a bactericidal effect, killing a large proportion of the viable bacteria in the biofilm (Graph 3), decreasing the biofilm capacity to produce acids (Graph 1), avoiding the increase in biofilm mass (dry weight and total proteins) (Graph 2) and consequently, the enamel demineralization process was stopped (Graph 4). This effect may be attributed to the ability of the CHX molecule to bind to the negatively charged bacterial cell surface, alter and disrupt the integrity of the cell membrane, causing bacterial death.<sup>25,26</sup> The concentration of 0.012% CHX had a bacteriostatic effect, not interfering in the viable bacteria counts, but affecting the acid production level, which was lower than that of the control group, but it was not able to prevent enamel demineralization. At this sublethal stage, the effects of CHX are reversible; removal of excess CHX by neutralizers allows the bacterial cell to recover.<sup>26</sup> This implies that the structural damage caused by 0.012% CHX was less than that caused by 0.024% and 0.12% CHX. The results found with the use of this model are supported by a clinical trial showing that 0.12% CHX is more effective in reducing *S. mutans* CFU than lower concentra-

tions.<sup>27</sup>

As opposed to CHX, fluoride did not show any effect on biofilm formation based on biomass (Graph 2) and viable bacteria counts (Graph 3). It also did not inhibit sucrose fermentation since the pH of the media did not differ when compared with the control (Graph 1), nevertheless, it reduced enamel demineralization (Graph 4). The findings are supported by the knowledge that at least 10 ppm of fluoride constantly in the media is necessary to inhibit sugar fermentation.<sup>28</sup> In this model, simulating the clinical use of mouthrinse, although the biofilm was treated with 225 ppm of fluoride, the duration time of treatment was only 1 min. Although 0.05% NaF showed absence of antimicrobial effect, it reduced enamel demineralization, suggesting that the main effect of F on caries control is physicochemical.<sup>29,30</sup>

Moreover, the findings suggest that CHX did not have a direct effect on the synthesis of extracellular polysaccharides (Table 1). This result is apparently in disagreement with Koo *et al.*<sup>13</sup> (2003) but it reflects the way in which the results were expressed. Koo *et al.*<sup>13</sup> (2003) expressed the results in amount of polysaccharides found in the biofilm and in the present study the results were normalized by biofilm dry weight. Since the biofilm weight increased (Graph 2) but the viable bacteria decreased (Graph 3), the reduction in the amount of EPS should be attributed to bacterial death and not to a specific effect of CHX inhibiting the synthesis of extracellular polysaccharides.

In conclusion, the results suggest this improved *S. mutans* model can be used to test the effect of antimicrobial agents on biofilm growth and on enamel demineralization.

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