**An in vitro microbial model for producing caries-like lesions on enamel**

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**Abstract**

This study aimed to develop a low cost in vitro viable microbiological model to produce biofilms to be used in dental researches. Single and multi-species biofilms of *S. mutans*, *S. sobrinus*, *S. mitis*, *S. salivarius*, *S. cricetus* and *S. sanguinis* were grown on bovine enamel slabs during 10 days, in a sterile brain-heart infusion broth, containing 5% sucrose and incubated at 37°C in an atmosphere of 10% CO₂. The slabs were transferred to a fresh medium at every 6, 12 or 24 hours. After the experimental period, enamel volume percent mineral was determined by cross-sectional microhardness. Caries-like lesions were found in all bacterial groups when compared with the control group. No statistical significant differences were found between *S. mutans* and *S. sobrinus* with respect of their cariogenicity or among the periods of medium change. However, it was found a statistical significant difference among the cariogenicity of *S. salivarius* and *S. sanguinis* (ANOVA followed by Tukey test). This model has successfully developed caries-like lesion on enamel and the medium can be changed at every 24 hours utilizing either *S. mutans* or *S. sobrinus*.

**Key Words:**

biofilms, microbiological, enamel, *S. mutans*, *S. sobrinus*
Introduction

There are a variety of model systems available that can be applied to study dental enamel caries process, each one presenting advantages and disadvantages\(^1\). Experimental chemical models such as pH cycling and immersion in acid medium are widely used to simulate cariogenic challenges\(^2\). The disadvantage of these models is that they do not simulate the real demineralization process of the oral environment due to the absence of microorganisms, consequently, concentrating on the physical-chemical aspects of enamel dissolution\(^3\). Another process of forming carious lesions involves bacterial models in which either planktonic bacteria or microorganisms organized in biofilms can be used. Studies have shown that planktonic microbial communities have different properties from microorganisms grown in a biofilm\(^4\). One major difference is that microorganisms growing on surfaces as biofilm are generally more resistant to antimicrobial agents than the same cells growing in conventional liquid media. This can be for a number of reasons, including the reduced penetration of the inhibitor into the biofilm (diffusion-reaction mechanism) and the slow growth rate and novel phenotype expressed by the attached cells\(^5\). An in vitro model that uses bacterial films is likely to be more representative than chemical or bacterial slurry systems, since dental caries is a bacterial disease and the bacteria which cause it are members of a biofilm community which may lead to altered metabolism compared with free-living microorganisms\(^6\). Bacterial models offer several advantages such as: (1) investigation of the etiology and prevention of carious lesions; (2) comparison of the cariogenic potential of different bacterial populations and (3) assessment of the cariogenicity of various diets\(^7\). Two bacterial in vitro models must be considered. One is known as the artificial mouth and provides a continuous or intermittent supply of nutrients to bacterial plaque or biofilms growing within an environment, which mimics the in vivo oral niches and habitats\(^8\). However, this in vitro model currently available, tends to require sophisticated laboratory equipment, presents frequent contamination problems and demands very high costs\(^9\). An alternative method for producing biofilms with lower costs and a good contamination control is a bacterial system involving a sequential batch culture technique, in which the samples are immersed in an enriched medium with microorganisms to evaluate the formation of caries lesions\(^10\). Growth of biofilms has been shown to occur via a sequence of colonization events in which initial adhesion to the enamel surface is followed by further bacterial-enamel binding, bacteria-bacteria interaction and growth\(^11\). The most common cariogenic bacteria associated with human dental caries are *Streptococcus mutans* and *Streptococcus sobrinus*\(^10\). Acidogenicity and aciduricity are important biochemical characteristics for cariogenicity of these microorganisms. The mutans streptococci have both these properties and are considered the most cariogenic group within the oral microbiota\(^10\). Regarding the disadvantages of the pH cycling and acid immersion models that do not allow a microbiological simulation of the oral environment and the difficulties and limitations of the artificial mouth method described above, the aim of this study was to present a low cost in vitro viable model utilizing a batch culture technique to produce biofilms to be used in dental researches.

Material and Methods

Experimental Design

A total of 120 bovine teeth free from macroscopic cracks were selected for this study. The teeth were stored in a 0.1% thymol solution pH 7.0 at 4°C for 30 days\(^11\). They had their roots removed (electrical cut BUEHLER-ISOMET) to obtain dental slabs (4 mm x 4 mm x 2 mm) from the vestibular surface of each tooth. The slabs were covered with nail varnish leaving exposed a 16mm\(^2\) enamel window. The fragments were autoclaved at 121°C for 20 minutes\(^12\) with neither interference on the enamel hardness\(^11\) nor on its demineralization pattern\(^6\). The teeth were randomly divided into 12 groups of 10 slabs each according to the type of microorganism and the period of change of the bacterial inoculation. The tooth slabs were attached to orthodontic wires, so as to leave the free enamel window to be immersed in the medium without touching the tube walls. The tubes were loosely closed to allow gas change with the environment and this complex was sterilized in autoclave.

Bacterial Preparation

The microorganisms used in this study were *Streptococcus mutans* (ATCC 25175), *Streptococcus sobrinus* (6715), the association of these two, *Streptococcus mitis* (ATCC 903), *Streptococcus salivarius* (ATCC 25975), *Streptococcus cricetus* (ATCC 19642) and *Streptococcus sanguinis* (ATCC 10556). The optical density of the culture was adjusted to obtain a standard amount of cells of approximately 2.15 x 10\(^6\) CFU/mL.

Biofilm Growth and Lesion Production

After sterilization, the dental slabs were removed from distilled water and immersed in sterile brain-heart infusion broth - BHI (Difco Lab. Detroit, USA) containing 5% sucrose (Synth, Labsynth, SP, Brazil)\(^8\). The BHI recipients were inoculated with 10 mL overnight cultures of *S. mutans*, *S. sobrinus*, and with 5mL of each of these species in the association group. The experiment lasted for a period of 10 days and the slabs were divided in groups as follows: groups C6, C12 and C24 were the controls immersed in a sterile medium without any...
bacterial inoculation; groups M6, M12 and M24 were immersed in a medium containing S. mutans; groups S6, S12 and S24 were immersed in a medium containing S. sobrinus and groups MS6, MS12 and MS24 were immersed in a medium containing equal amounts of both species (S. mutans and S. sobrinus). Bacterial inoculation of the groups M6, S6 and MS6 was performed at every 6 hours when the enamel slabs were transferred to a fresh new medium. For the groups M12, S12 and MS12, this inoculation was performed at every 12 hours and at every 24 hours for the groups M24, S24 and MS24 that were also transferred to a fresh new medium. All slabs had their medium changed at the same time to prevent any kind of contamination and were incubated at 37°C in an atmosphere of 10% CO₂ (Cole Parmer Instruments, USA). Contamination at test recipients was verified at each 24 hours by inoculation in BHI agar media (Merck, Darmstadt, Germany). Additionally, it was performed another experiment with the same methodology described above, except for the microorganisms and the pre-determined period of medium change that had already been tested. We chose to change the medium at every 24 hours since it did not show any statistical significant difference in the cariogenicity between the microorganisms in the previous trial. The bacteria chosen for the new groups were Streptococcus mitis, Streptococcus salivarius, Streptococcus cricetus and Streptococcus sanguinis, besides the control group with no bacteria. This new experiment was performed in order to verify if there were any cariogenicity differences between these microorganisms.

**Microhardness Assessment**

At the end of the experimental period, the tooth slabs were longitudinally sectioned through the center of the enamel area. One of its halves was embedded in epoxy resin, with the outer enamel surface perpendicular to the resin block surface. The slabs were serially polished with aluminum oxide disks of #400, #600 and #1200 grits, and a diamond paste of 200 µm. Indentation lengths were converted to Knoop Hardness Number and after to volume % mineral. After calculating volume percentage mineral values for each depth evaluated, mineral profiles, integrated area of mineral content were obtained for all groups.

**Statistical Analysis**

In order to assess the effect of microorganisms and the medium change on the cariogenicity of the in vitro model, the dependent variable volume % mineral versus micrometer was independently analyzed by analysis of variance (ANOVA). ANOVA was followed by Tukey test to evaluate the significance of all pair wise comparisons. The software SAS system (version 8.02, SAS Institute Inc., Cary: NC, 1999) was used and the significance limit was set at 5%.

**Results**

Table 1 shows that statistical significant differences were not found among any periods of evaluated medium changes (p > 0.05). S. mutans, S. sobrinus and the association of these microorganisms presented a statistical significant difference when compared to the control group (p < 0.01), regarding the enamel volume percent mineral. However, there was no statistical significant difference (p > 0.05) between them, with respect of their cariogenicity (table 1). Table 2 shows that Streptococcus mitis, Streptococcus salivarius, Streptococcus cricetus and Streptococcus sanguinis presented a statistical significant difference when compared to the control group (p < 0.01), considering the enamel percentage mineral volume. In addition, S. sanguinis group showed a statistically higher cariogenicity than S. salivarius group (p < 0.05).

**Discussion**

Chemical induction of caries by organic acids is one of the main approaches in clarifying the mechanisms involved in demineralization and remineralization of enamel, but direct acid exposure does not allow the bacterial interactions that characterize caries in vivo. In vivo caries studies have the advantage of including host factors involved in the natural caries process but have some fundamental limitations.

<table>
<thead>
<tr>
<th>Group</th>
<th>6h</th>
<th>12h</th>
<th>24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9093.9 ± 823.4 a</td>
<td>7948.8 ± 922.0 a</td>
<td>7089.8 ± 1273.1 a</td>
</tr>
<tr>
<td>S. mutans</td>
<td>4521.0 ± 1599.6 b</td>
<td>5035.0 ± 1868.0 b</td>
<td>3957.4 ± 468.4 b</td>
</tr>
<tr>
<td>S. sobrinus</td>
<td>4568.5 ± 1046.8 b</td>
<td>4276.2 ± 985.0 b</td>
<td>3936.7 ± 780.6 b</td>
</tr>
<tr>
<td>S. mutans/S. sobrinus</td>
<td>4831.2 ± 1827.1 b</td>
<td>3813.3 ± 734.3 b</td>
<td>4509.4 ± 1882.3 b</td>
</tr>
</tbody>
</table>

* Means followed by distinct letters are statistically different (p < 0.05). No differences were observed between the three different periods of medium changes. Lower case letters show differences among the microorganisms.
oral environment is difficult to control and varies greatly with intraoral location over time and between different persons\textsuperscript{17}. Bacteria in dental biofilms metabolize carbohydrates to the acids that cause dental caries. Laboratory models of this process are potentially valuable in understanding the mechanisms involved, in developing and testing procedures to combat and prevent caries\textsuperscript{18}. A useful in vitro model should have the following characteristics: ease of sterilization of the different components, ability to manipulate model components under sterile conditions, ease of access to test specimens, reproducibility of experiments and optimal simulation of the oral environment\textsuperscript{19}. Bacterial systems where the mixed natural microbiota are controlled by in vitro environment and nutrient conditions provide a means for studying complex microbial ecosystems such as dental biofilm and its effect on the development of dental caries\textsuperscript{20}. An in vitro model system using bacterial films is likely to display less inherent variability since variables such as fluid flow, carbohydrate intake and bacterial population composition can be controlled more accurately in vitro\textsuperscript{1}. Moreover, an in vitro model that uses bacterial films is likely to be more representative than chemical systems, since dental caries is a bacterial disease and the bacteria which cause it are members of a biofilm community which may lead to altered metabolism compared with free-living organisms\textsuperscript{1}.

One of the main advantages of using this in vitro model to produce caries lesions is the presence of an experimental tooth enamel surface that is freely exposed to the bacterial challenge, the low cost of the system and the possibility of controlling contamination, by monitoring the medium at each 24 hours by inoculation in BHI agar media. The disadvantage is that this model does not mimic the diverse conditions present in the oral cavity, such as presence of saliva, antimicrobial proteins and enzymes, absence of the remineralization period, all of which may affect dental caries development\textsuperscript{20}.

Simple monobacterial biofilm models have been developed, for example using Streptococcus mutans\textsuperscript{18}. Defined-species biofilm consortia, although simpler than in vivo, have the advantage of allowing detailed control and study of the properties of the individual bacterial species present\textsuperscript{18}. Even in batch culture, oral multi-species consortia develop complex biofilms on enamel that can induce carious lesion similar to those in vivo\textsuperscript{21} as shown in a study that the bacterial model produced caries-like lesions similar to those found with purely chemical systems\textsuperscript{22}.

The results of this study confirm that this microbiological model was able to produce caries lesions in all tooth slabs in all periods of medium changes, but did not find any differences between the cariogenicity of the microorganisms. This is in accordance with studies that did not find differences in cariogenicity between S. mutans and S. sobrinus with respect of enamel lesions in rats\textsuperscript{23} or in vitro\textsuperscript{24}. In creating laboratory caries models based on biofilms of selected species of bacteria, the properties of the particular strain of each specie selected will determine the activities of the biofilm and hence the outcome of the experiment\textsuperscript{16}. Thus the distinction of the species to promote caries in this bacterial system may be important because the two microorganisms chosen display differences in initial colonization, virulence mechanisms\textsuperscript{10} and also because S. mutans and S. sobrinus can coexist in fairly close proximity to one another in small dental sites\textsuperscript{25}. Studies have shown that young children with both S. mutans and S. sobrinus in their saliva had significantly more dental caries than those with either S. mutans or S. sobrinus alone\textsuperscript{26}. In an animal model, it has been suggested that S. sobrinus could be more acidogenic than the other species of mutans streptococci\textsuperscript{27}. On the other hand, some authors showed that the animals infected with S. sobrinus strains generally showed lower caries scores than those infected with S. mutans strains\textsuperscript{28}. There was a tendency for S. sanguinis to be more cariogenic compared to S. mitis, S. salivarius and S. cricetus as revealed in the second experiment. This is in accordance with studies that show that some mitis group streptococci may also contribute significantly to the pool of acids produced in dental biofilm at low pH\textsuperscript{29} and other authors who showed that S. sanguinis produce significant amounts of acids\textsuperscript{30}.

In conclusion, this model has successfully developed caries lesion on enamel and since there were not statistically significant differences between the periods of medium changes, or at the bacteria strains used to produce the demineralization, the most appropriate period to changing the medium would be at every 24 hours using either S. mutans or S. sobrinus.

Acknowledgements

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References


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<thead>
<tr>
<th>Table 2 - Percentage of mineral volume (%) X micrometer (mean ± SD) according to four different microorganisms.</th>
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<tbody>
<tr>
<td><strong>Group</strong></td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>S. mitis</td>
</tr>
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<tr>
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<td>S. sanguinis</td>
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