Brazilian Journal of Microbiology (2006) 37:20-25
ISSN 1517-8382

IN VITRO BACTERIAL PLAQUE SUPPRESSION AND RECOLONIZATION BY *S. MUTANS* AND *S. SOBRINUS*

Cássio Vicente Pereira[1]; Luciano José Pereira[2]; Reginaldo Bruno Gonçalves[3]; José Francisco Höfling[3]*

[1]Departamento de Biologia Oral, Universidade Sagrado Coração, Bauru, SP, Brasil; [2]Área de Diagnóstico Bucal, Universidade Vale do Rio Verde, Três Corações, MG, Brasil; [3]Laboratório de Microbiologia e Imunologia, Faculdade de Odontologia de Piracicaba, Universidade Estadual de Campinas, Piracicaba, SP, Brasil

Submitted: April 27, 2004; Returned to authors for corrections: August 23, 2004; Approved: February 15, 2006

ABSTRACT

The *in vitro* study of the interactions between *S. mutans* and *S. sobrinus* is important to determine the role of these microorganisms in the formation of biofilms on dental structures and their potential to induce carious lesions. The objective of this research was to study the suppression of bacterial plaque formation and its recolonization by rifampycin-resistant *S.mutans* and streptomycin-resistant *S. sobrinus*. To study the competitive relationship between these species, previously standardized strains were incubated in media containing different fermentable carbohydrates. At determined time intervals, samples were collected from mixed cultures of *S. mutans* and *S. sobrinus*, diluted and plated on BHI-agar containing rifampycin or streptomycin to determine the number of viable cells of each species by counting colony-forming units. In order to study the bacterial colonization process and *in vitro* recolonization of bacterial plaque, three experiments were performed: I – co-cultivation of *S. mutans* and *S. sobrinus*; II – inoculation of bacterial plaque pre formed by *S. sobrinus* with *S. mutans*; and III - bacterial plaque pre formed by *S. mutans* dispersed and plated on BHI- agar containing streptomycin or rifampicin to determine the number of viable cells for each species. The results indicated a predominance of *S. mutans* in relation to *S. sobrinus*, demonstrating the capacity of *S. mutans* to inhibit plaque formation by *S. sobrinus* and recolonize the surfaces.

Key words: *Streptococcus mutans*, *Streptococcus sobrinus*, suppression

INTRODUCTION

Streptococci of the mutans group are closely associated with dental caries, mainly those involving smooth surfaces. Production of acid and extracellular polysaccharides due to hydrolysis of sucrose facilitates their adhesion to tooth surfaces (5,21).

In relation to the importance of the mouth microbiota in the decay process, *S. mutans* and *S. sobrinus* are the most frequent species from the mutans group encountered in saliva. Some authors have reported (1,8) that these bacterial species present a high caries-inducing potential, enhancing the infection process when combined. The beginning and development of caries are determined by a host-parasite relationship, which can be altered by several environmental factors as demonstrated by *in vitro* models simulating the *in vivo* phenomenon in the mouth (9,20). The *in vitro* studies of the bacterial relationship based on the action of these microorganisms in the plaque formation on tooth surfaces and their potential to induce caries are in agreement with several results reported in the literature (3,8-10,14,15).

The aim of the present study was to evaluate the suppression of bacterial plaque formation and recolonization by *S. mutans* and *S. sobrinus*, through *in vitro* tests using streptomycin- and rifampicin-resistant strains, cultivated separately and in combination.

*Corresponding Author. Mailing address: Laboratório de Microbiologia e Imunologia, Faculdade de Odontologia de Piracicaba, UNICAMP, Av. Limeira, 901, Areião, Caixa Postal 052. 13414-903, Piracicaba, SP, Brasil. Tel.: (+5519) 3412-5322. Fax: (+5519) 3412-5218. E-mail: microbiologia@fop.unicamp.br

MATERIALS AND METHODS

Suppression of bacterial plaque formation

Fresh culture samples of rifampicin-resistant *S. mutans* 32K and streptomycin-resistant *S. sobrinus* 6715 previously standardized by Mc Farland's scale (tube 2) in BHI medium were used. The samples were donated by the Microbiology Laboratory of Chiba University – Japan. A 0.2 mL sample from each culture was added to a tube containing 5 mL of BHI medium supplemented with 10% fermentable carbohydrate, either sucrose, glucose, fructose or glucose (5%) + fructose (5%), and incubated at 37ºC for 24 h in a 10% CO_2 atmosphere (10). After 3, 6, 12 and 24 h, 0.1 mL of the mixture was submitted to decimal dilution from 10^{-1} to 10^{-4} in sterile saline (0.9% NaCl), and plated in BHI-agar medium with rifampicin (0.1 mg/mL) or streptomycin (1.0 mg/mL). The plates were incubated for 24 h at 37ºC and the number of colony-forming units for each species was determined.

In vitro bacterial plaque colonization and recolonization

Fresh cultures of rifampicin-resistant *S. mutans* 32K and streptomycin-resistant *S. sobrinus* 6715 were standardized by the Mc Farland scale (tube 2) in BHI medium and used in the *in vitro* study of bacterial plaque colonization and recolonization. The tubes containing BHI medium supplemented with 10% fermentable carbohydrates – sucrose, glucose, fructose or glucose (5%) + fructose (5%) – were inoculated with *S. mutans* and *S. sobrinus* samples. A capillary tube, with one flame-closed end, was introduced into the tube, and the other end was modified (19) to promote the development of bacterial plaque. To facilitate the evaluation of the interaction between these species and to determine the colonization and recolonization potential (10) the procedures were divided in three distinct experiments.

Experiment I – *S. mutans* and *S. sobrinus*, 0.2 mL each, were transferred to a tube containing 5.0 mL BHI medium with 10% fermentable carbohydrate – sucrose, glucose, fructose or glucose (5%) + fructose (5%) – and the capillary tube. The tubes were incubated for 48 h at 37ºC in 10% CO_2.

Experiment II – The capillary tube containing the bacterial plaque formed by *S. sobrinus* during incubation in BHI medium as described above was transferred to a new tube containing fresh culture medium supplemented with 10% fermentable carbohydrate [sucrose, glucose, fructose or glucose (5%) + fructose (5%)]. *S. mutans* was then added and the culture tubes incubated for 48 h at 37ºC in 10% CO_2.

Experiment III – The procedure was similar to Experiment II: the capillary tube containing the plaque formed by *S. mutans* was transferred to a new culture tube containing fresh medium supplemented with 10% fermentable carboydrate [sucrose, glucose, fructose or glucose (5%) + fructose (5%)] and incubated further in the presence of added *S. sobrinus* for 48 h at 37ºC in 10% CO_2.

At the end of the experiments, the bacterial plaques were dispersed in sterile saline (0.9% NaCl) by means of a vibrator (Thornton – Marconi Ultrasound), then plated on BHI -agar medium containing streptomycin or rifampicin, and incubated for 24 h at 37ºC in 10% CO_2. The number of colony forming units per ml for each species was determined.

RESULTS

Fig. 1 shows that after 3h of incubation the number of *S. sobrinus* in the mixed colonies was greater than that of *S. mutans*. Afterwards, the number of *S. sobrinus* colonies decreased, and was lower than the number of CFU/mL of *S. mutans*. After 12 h of incubation, no viable *S. sobrinus* cells were detected.

As shown in Figs. 2, 3 and 4, the growth of *S. mutans* and *S. sobrinus* was similar regardless the substrate (glucose, fructose or glucose + fructose) added to the culture medium. The number of CFU/mL of *S. mutans* was greater than that of *S. sobrinus* for all incubation periods, in all culture media. After 12h of incubation, the samples obtained from the mixed culture of *S. mutans* and *S. sobrinus* in medium containing glucose, fructose or glucose + fructose, when plated on BHI – agar containing streptomycin, did not result in growth of *S. sobrinus*.

The growth interactions between *S. mutans* 32K and *S. sobrinus* 6715 in the *in vitro* colonization and recolonization process using media with different carbohydrate substrates added (Experiments I, II and III) are presented in Figs. 5, 6 and 7. According to these results, viable cells of *S. mutans* were shown to be present at the end of the incubation period while *S. sobrinus* decreased in number or was eliminated.

DISCUSSION

In the development of caries and formation of biofilms there are microbiotic interactions with the dental surface and among bacteria themselves and ecological changes caused by diet. Relevant physico-chemical characteristics and the composition of the plaque act together to initiate this pathology. These bacterial relationships that exist in the biofilm on the enamel surface are regulated by several factors such as the production of bacteriocins (antibacterial proteins that can interfere with the growth of other microorganisms, generally closely related bacteria). The bacteriocins produced by *S. mutans* are called mutacins, and these proteins are important for the establishment and balance of this species in dental plaque (7,15).

The results obtained from the bacterial suppression experiment shown in Figs. 1, 2 and 3 demonstrated similar behavior for *S. mutans* and *S. sobrinus* derived from mixed cultures during the incubation period, even though they showed a higher number of CFU/mL in medium with sucrose added when compared to the other carbohydrates. These data confirm other studies such as those of Ashley and Wilson (2), Brecx

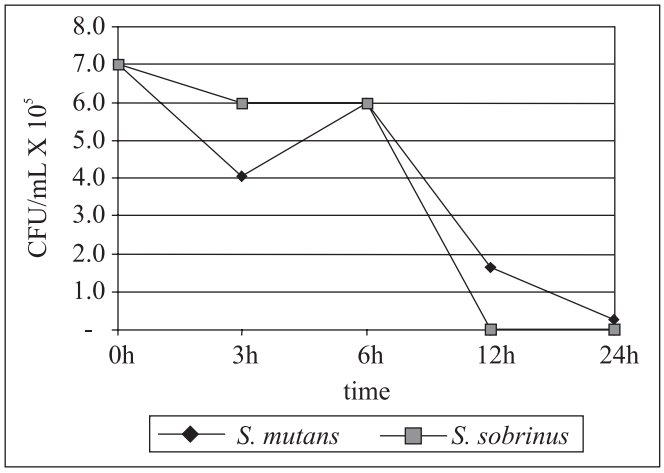

Figure 1. Number of CFU/mL of *S. mutans* and *S. sobrinus* in the mixed culture in medium containing 10% sucrose, during incubation at 37ºC.

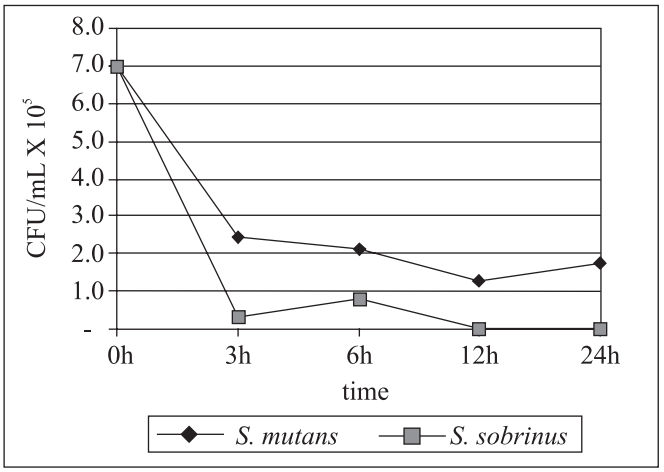

Figure 3. Number of CFU/mL of *S. mutans* and *S. sobrinus* in the mixed culture in medium containing 10% fructose, during incubation at 37ºC.

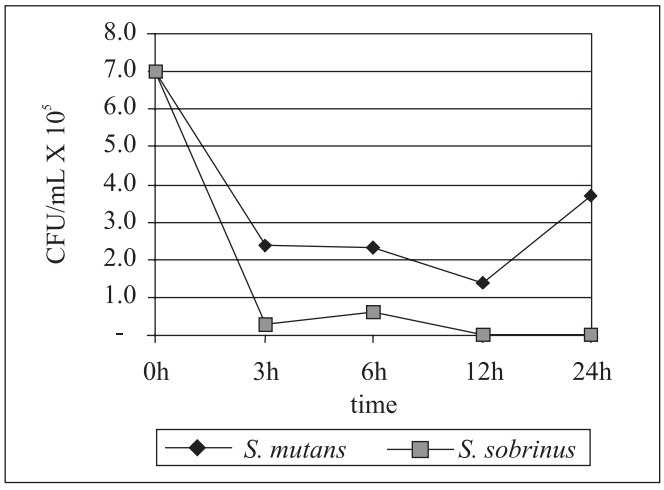

Figure 2. Number of CFU/mL of *S. mutans* and *S. sobrinus* in the mixed culture in medium containing 10% glucose, during incubation at 37ºC.

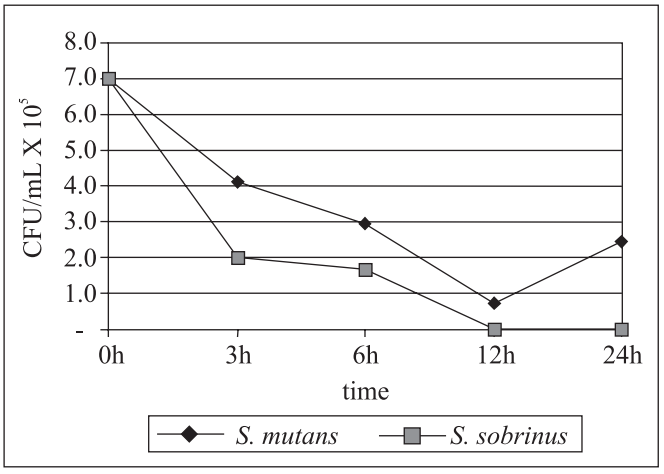

Figure 4. Number of CFU/mL of *S. mutans* and *S. sobrinus* in the mixed culture in medium containing glucose (5%) + fructose (5%), during incubation at 37ºC.

et al. (4), Macpherson and Dawes (12), Gibbons and Van Houte (6) and Margolis *et al.* (13), which determined sucrose to be the main substrate of the streptococcus mutans group. After a short incubation period (3 h), the mixed culture in medium containing sucrose or glucose (10%), fructose (10%) or glucose (5%) + fructose (5%) showed a greater number of CFU/mL of *S. sobrinus* compared to *S. mutans.* After a period of 12 h, there was no detection of viable cells of *S. sobrinus* in any of the culture media tested. These results are in agreement with Ikeda *et al*. (10), who showed that *S. sobrinus* was eliminated in mixed *in vitro* cultures by bacteriocins produced by *S. mutans*.

The data regarding bacterial plaque colonization and recolonization capacity of *S. mutans* and *S. sobrinus* strains are shown in Figs. 5, 6 and 7. These values indicate a higher number of viable cells of *S. mutans* in agreement with the results of Ikeda *et al*. (10) demonstrating the capacity of *S. mutans* to produce bacteriocins that inhibit the growth of other streptococcus of the mutans group and to recolonize the pre-formed bacterial plaque. These data suggest that *S. mutans* bacteriocins (mutacin) are able to eliminate sensitive plaque bacteria, resulting in greater colonization *in vivo*. Strains of the same *S. mutans* genotype show different mutacin profile,

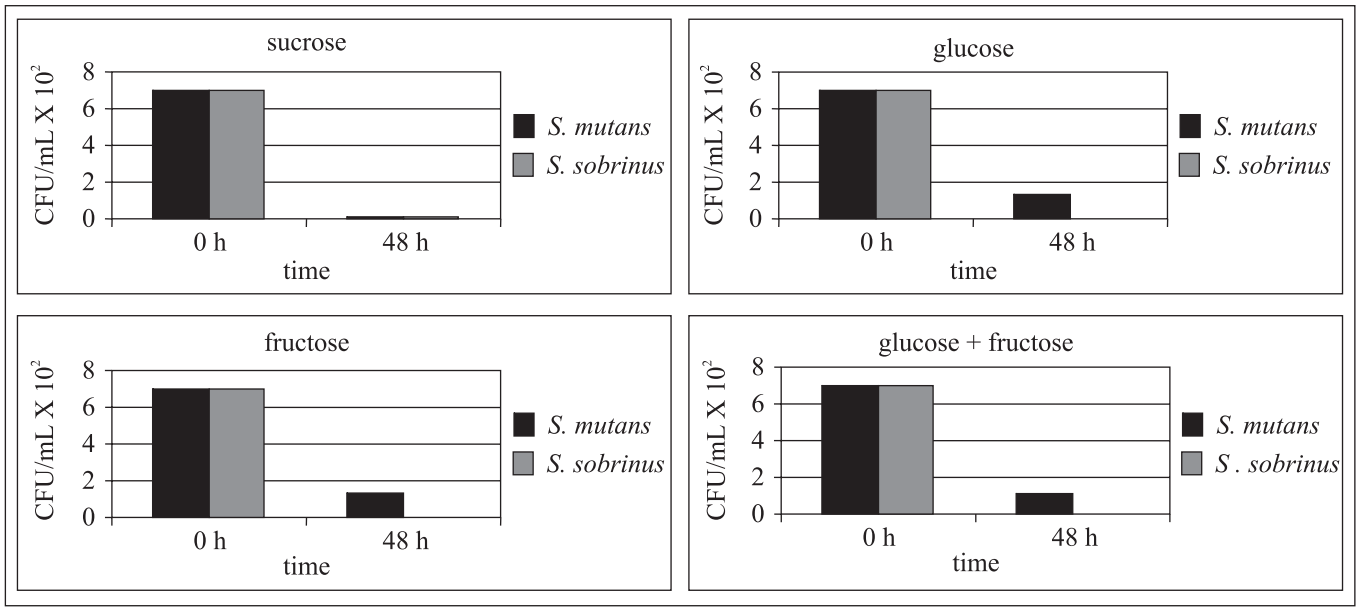

Figure 5. Number of CFU/mL X 10^2 obtained from the bacterial plaque formed by considered-culture of equal numbers of *S. mutans* and *S. sobrinus* in medium containing different substrates – sucrose (10%), glucose (10%), fructose (10%) and glucose (5%) + fructose (5%).

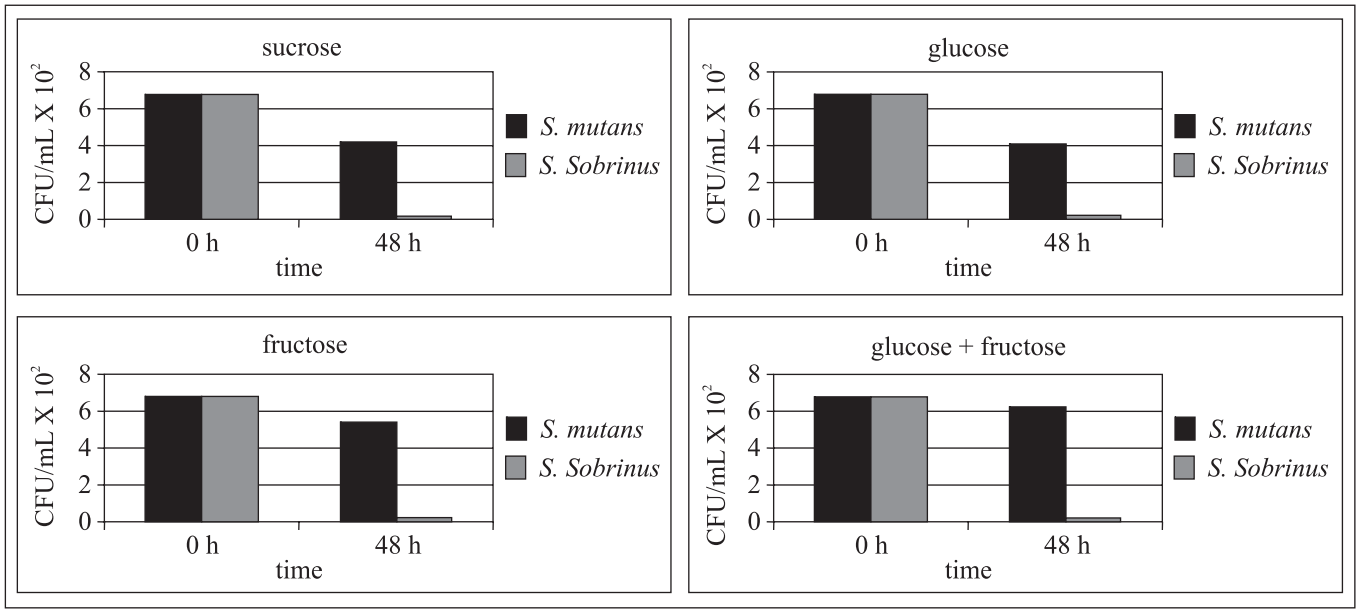

Figure 6. Number of CFU/mL X 10^2 obtained from the bacterial plaque, pre-formed by *S. sobrinus* and inoculated with *S. mutans* samples, cultivated in medium containing different substrates – sucrose (10%), glucose (10%), fructose (10%) and glucose (5%) + fructose (5%).

suggesting a high degree of interstrain diversity. Mutacin production seems to be of clinical importance in the colonization of *S. mutans* and is highly diversified in the *S. mutans* species (11-13).

In conclusion, *Streptoccoccus mutans* showed a higher number of cells after short or long incubation periods when cultivated with *Streptococcus sobrinus* indicating its inhibition and recolonization capacity *in vitro*.

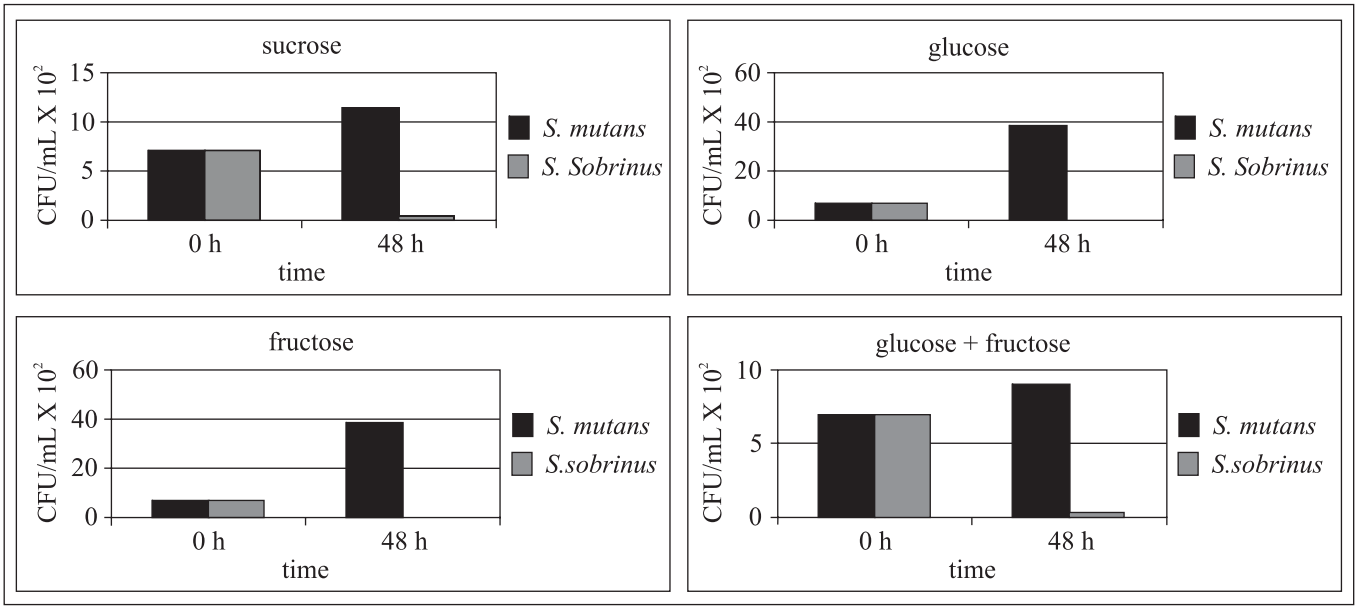

Figure 7. Number of CFU/mL X 10^2 obtained from the bacterial plaque, pre - formed by *S. mutans* and inoculated with *S. sobrinus* samples, cultivated in medium containing different substrates – sucrose (10%), glucose (10%), fructose (10%) and glucose (5%) + fructose (5%).

RESUMO

Supressão e recolonização de placa bacteriana por *S. mutans* e *S. sobrinus in vitro*

O estudo *in vitro* das interações entre *S. mutans* e *S. sobrinus* pode ser importante na determinação do papel desses microrganismos na formação de biofilmes nas estruturas dentais e seu potencial em induzir lesões cariosas. O objetivo da presente pesquisa foi estudar a supressão da formação da placa dental e sua recolonização por *S. mutans* rifampicina-resistentes e *S. sobrinus* estreptomicina-resistentes *in vitro*. Para avaliar as relações de competitividade entre essas espécies, cepas que foram previamente padronizadas foram incubadas em meio de cultura contendo diferentes carboidratos fermentáveis. Em intervalos de tempo determinados, amostras de *S. mutans* e *S. sobrinus* foram coletadas a partir de culturas mistas, diluídas e semeadas em placas com meio BHI-ágar contendo rifampicina ou estreptomicina para determinação do número de células viáveis de cada espécie por contagem de unidades formadoras de colônia. Para a avaliação da colonização bacteriana e recolonização da placa bacteriana *in vitro*, três experimentos foram realizados: I – co-cultivo de *S. mutans* e *S. sobrinus*; II – inoculação de *S. mutans* em placa bacteriana pré-formada por *S. sobrinus*; e III – placa bacteriana pré-formada por *S. mutans* dispersada e plaqueada em meio BHI-ágar contendo estreptomicina ou rifampicina para determinação do número de células viáveis para cada espécie. Os resultados indicaram uma predominância de *S. mutans* em relação ao *S. sobrinus*, demonstrando a capacidade do *S. mutans* em inibir a formação de placa por *S. sobrinus* e recolonizar a superfície dentária.

Palavras-chave: *Streptococcus mutan*, *Streptococcus sobrinus*, supressão

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