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ANALYSIS OF THE *IN VITRO* ADHERENCE OF *Streptococcus mutans* AND *Candida albicans*

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ABSTRACT

The objective of the present study was to investigate the *in vitro* adherence capacity of *Streptococcus mutans* and *Candida albicans*. Adherence assays were conducted on dental surfaces and analyzed by scanning electron microscopy (SEM). Extracted human teeth were inoculated with *Streptococcus mutans* or *Candida albicans* and with both species simultaneously, and incubated at 37ºC for 21 days. Bacterial inocula had been obtained from saliva samples of children that had been colonized by both organisms. ATCC reference strains were used as controls. SEM analyses showed that the biofilm that covered the entire analyzed dental surface was more homogeneous inoculated with the two microorganisms simultaneously than with each species separately. In a second experiment, carried out with isolates that had shown the highest adherence the isolates were tested for adherence to high-density polyethylene substrates. The potentialization of the adherence capacity of *Streptococcus mutans* and *Candida albicans* when in association was confirmed.

Key words: Adherence, *Candida albicans*, *Streptococcus mutans*, biofilm

INTRODUCTION

The buccal microbiota plays an important role in maintaining oral health, as well as in the occurrence of diseases resulting from disturbance to this ecosystem. The organisms that compose this biota contribute to the development of the host immune system, allowing for a balanced colonization of a large variety of microorganisms. However, they also constitute a reservoir of microorganisms that can potentially invade other tissues of their host. The complexity of this ecosystem depends on the age and immunocompetence of the host, the availability of new niches, dietary changes, the quality and frequency of oral hygiene, and the exposure to fluoride (16,20).

One of the buccal diseases that reflects a lack of equilibrium of this microbiota is caries, which causes the destruction of dental mineralized tissues during its progression. Its main etiological agent is the bacterium *Streptococcus mutans*, which is capable of adhering to dental surfaces and initiating the formation of the cariogenic bacterial plaque. The metabolic system of this bacterium generates an acidogenic niche that exceeds the salivary buffering capacities, triggering progressive changes in the mineral layer of the dental surface (11,18).

The concentration of *S. mutans* is often high in the saliva of patients with elevated levels of dental caries. Several factors related to the oral environment are though to contribute to the establishment and multiplication of *S. mutans* (11,15).

Candida albicans can be found in several locations on the human body due to its opportunistic nature, at times invading tissues to cause candidosis when in association with predisposing factors. In addition, the capacity of tissue invasion and adhesion to artificial materials such as probes, latex catheters, and several types of plastic material are responsible

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for causing important infections (34). In the case of dental biofilms, its role in coaggregation with *S. mutans* during the mechanisms of microbial adherence to the dental surface has been the focus of research (2,13,26,32,35). In addition, the adherence to acrylic prostheses has been often investigated (7,19,21,22,29,31).

Candida albicans has been isolated in dental biofilms (2,8,31,32) and its role in microbial adherence to dental surfaces in coaggregation with *S. mutans* has been studied in recent years (35). The presence of *C. albicans* has already been recorded in caries lesions, as well as its capacity to invade mouth tissues and artificial materials such as acrylic, which is used in several types of dental prostheses (1,3,7,20,22,26).

The hypothesis of the association between *S. mutans* and *C. albicans* is based on their mechanisms of virulence and biochemical characteristics, as well as the endogenous and exogenous factors that cause the host to provide a buccal environment that favors the action of both microorganisms (1,23,35).

The analysis of the *in vitro* adherence of *S. mutans* and *C. albicans* can contribute to the understanding of the behavior of these organisms in the dental plaque. Thus, the present study reports on an *in vitro* adherence assay using human teeth as the adherence surface and inocula of *S. mutans* and *C. albicans* isolated from saliva samples of children colonized by both microorganisms, as well as reference strains. The goal of these assays is to investigate the adherence capacity of these species, both in isolation and in combination in dental biofilms. A second assay was carried out to assess the same phenomenon on high-density polyethylene surfaces.

MATERIALS AND METHODS

The present study has been approved by the Research Ethics Committee of the Institute of Health Sciences of the Universidade Federal do Paraná.

Yeasts and Bacterial strains

The *in vitro* adherence study using human teeth included 6 isolates of *S. mutans* (M1, M2, M3, M4, M5, and M6) and 6 isolates of *C. albicans* (C1, C2, C3, C4, C5, and C6). The isolates of *S. mutans* and *C. albicans* were obtained from saliva samples of children colonized by both species that were treated at the Odontopediatric Clinic of the Universidade Federal do Paraná, Brazil. ATCC reference strains of both species (*C. albicans*, 10123 and *S. mutans*, 25175) were also used.

Isolation and identification of microorganisms

The isolation of *C. albicans* was carried out by submitting the non-stimulated saliva samples to decimal dilutions of saline solutions (10^{-1} and 10^{-2}), inoculating them on Sabouraud Dextrose Agar, and incubating them at 30ºC for 48 h to 7 days. The isolation of *S. mutans* from the same samples was conducted by submitting the saliva samples to decimal solutions of peptone water (10^{-1}, 10^{-2}, and 10^{-3}), inoculated on Mitis Salivarius Agar (Difco) containing bacitracin (30 µg/mL) and potassium telurite (0.001%), and incubated in microaerophilia in GasPak jars at 37ºC for 48 h. Identification was carried out using morphological and biochemical traits, both for *S. mutans* (10,17,27) and for yeasts of the *Candida* genus (14,17,28). Molecular markers (27) were used to confirm the identity of the *S. mutans* isolates through specific PCR using primers (1-ACTACACTTTCGGGTGGCTTGG and 2-CAGTATAAGCGCCAGTTTCATC) and the isolates were maintained and preserved in glycerol at -80ºC. Isolates of *C. albicans* were confirmed using an automated system (Vitek®) of biochemical identification (14,28).

Inoculate preparation for the adherence assay on the dental surface

Inocula were prepared from 6 isolates of *S. mutans* and 6 isolates from *C. albicans* obtained with the abovementioned protocol, as well as from ATCC reference strains (*C. albicans* 10231 and *S. mutans* 25175). Isolates of *S. mutans* were cultured at 37ºC for 18 h and standardized to a cell suspension with an optical density of 2.1 x 10^9 cells/mL using spectrophotometry and counting of colony-forming units (CFUs) in TSB agar. Likewise, isolates of *C. albicans* were standardized to a final concentration of 2.1 x10^8 cells/mL, with a later colony count in Saboraud medium.

Tooth adherence assay

An adherence assay for *S. mutans* and *C. albicans* was developed based on Yoshiyasu (36). A total of 21 healthy premolar human teeth that had been extracted for orthodontic purposes were donated by the patients to be used in the adherence tests. The teeth were kept in saline solution, which was replaced weekly until the beginning of the experiments. Before inoculation, each tooth was cleaned mechanically using limestones and water, as well as a Robson brush mounted on a hand piece. Following the mechanical cleaning, each tooth was perforated using a round diamond drill (Sorensen nº2), creating a hole through which a piece of dental floss was inserted to facilitate their handling during the experiments. Each tooth was introduced into a test tube containing 9 mL of the Gibbons and Nygaard culture medium (9), and autoclaved at 1 atm and 121ºC for 20 min. One mL of either previously standardized inoculate was then added to the tube in the monospecific culture, whereas the combined culture included 1 mL of the standardized inoculum of each species.

Incubation lasted for 21 days, replacing of their culture medium every 48 h, followed each time by a check for contamination by optical microscopy using Gram staining (36). The presence of coaggregates in the medium, degree of

precipitation, and the intensity of the visible biofilm in the crown and the root were used as criteria to assess the adherence patterns of clinical isolates and reference strains of *C. albicans*, *S. mutans*, and both microorganisms in association (5).

Scanning electron microscopy

Each tooth was fixated in Karnovsky solution, washed three times using a 0.05 M sodium cacodilate buffer solution (pH 7.4), and then cut with Isomet Cut-off Rotary Instrument. Cuts were dehydrated in an increasing series of Acetone solutions (30%, 50%, 90%, 100% and 100%), dried under room temperature, and metallized using a MED 010 (Balzers®). Observations were carried out in a JSM-6360LV microscope at the Center for Electron Microscopy of the UFPR.

Assay of adherence on high-density polyethylene

Inocula were prepared from 3 isolates of *C. albicans* (C1, C2, and C4) and 3 isolates from *S. mutans* (M1, M2, and M4). These isolates were chosen based on qualitative differences in their adherence patterns according to Barbieri (5). In addition, ATCC reference strains of *C. albicans* 10231 and *S. mutans* 25175 were also included. Based on the protocol of Tamura *et al.* (34), isolates were cultivated at 37ºC for 18 h and their concentrations were standardized to 2.1 x 10^8 cells/mL using spectrophotometry. A total of 12 inocula were prepared using *S. mutans* (M), *C. albicans* (C), or both *S. mutans* and *C. albicans* (M+C). A total of 12 acrylic sheets with dimensions 0.5 cm X 2.5 cm X 0.5 mm were placed in individual test tubes containing 0.9 mL of Gibbons and Nygaard culture medium and 0.1 mL of the inoculate. Culture was carried out at 37ºC for different incubation periods (2, 12, 24, 48, and 72 h), with one repetition for each analyzed period.

After each incubation period, the high-density polyethylene sheet were washed three times using phosphate buffered saline (PBS) to remove weakly adhered cells. The fragments were then placed on a magnetic stirrer with two glass pearls for 30 s to detach the cells that had adhered to polyethylene fragment and to release them into a suspension. Each suspension sample was used to create three decimal dilutions in PBS (10^{-1}, 10^{-2}, and 10^{-3}), which were then plated on Petri dishes with Gibbons and Nygaard agar. The whole protocol was repeated twice. Plate culture was conducted at 37ºC for 48 h, followed by an estimated count of CFU/mL. This value was used as an approximation to the total adherence for each 1 cm^2 of the high-density polyethylene.

Statistical analysis of the adherence to high-density polyethylene

Average adherence values were assessed by an analysis of variance (ANOVA) according to a completely randomized design with two replicates and a factorial distribution of the treatments: monospecific culture of clinical isolates and reference strains of *S. mutans* (M), *C. albicans* (C) and the combined culture of both species (M+C) and culture times (2, 12, 24, 48, and 72 h). Multiple comparisons were processed using the orthogonal contrasts of the F test according to Hinkelmann and Kempthorne (12).

RESULTS

SEM analyses of the colonized teeth showed that, when the six isolates and reference strain of *S. mutans* were cultivated separately, the adherence to the dental surface was characterized by a thick compact biofilm, with tightly grouped cells of uniform size and morphology (Fig. 1A), although that pattern was not observed in some regions of the biofilm (Fig. 2A). When clinical isolates and reference strain of *C. albicans* were cultivated separately, the biofilm showed the presence of cellular morphology with a predominance of filaments (Fig. 1B). The biofilm formed by *C. albicans* under these conditions was characterized by a thinner aspect (Fig. 2B). However, there was greater uniformity in the biofilm, with all regions of the tooth being equally colonized (Fig. 1B). When both microorganisms were associated, the biofilm was more homogeneous, covering the entire dental surface (Fig. 2C). In addition, there was an extensive colonization of the cellular surface of the yeast by cells of *S. mutans*. An abundant amorphous substance was present in the interstices between the cells of both species (Fig. 1C). The cells of *C. albicans*, when in association with *S. mutans*, showed structures such as blastopores, pseudohyphae, and germ tubes covered by amorphous extra-cellular structures (Fig. 1D).

Adherence analysis on the acrylic surface used isolates M1, M2, and M4 of *S. mutans* and C1, C2, and C4 of *C. albicans*, which had been selected based on visualization of the adherence patterns using SEM and the presence metabolic products in the culture medium, as described in the Materials and Methods section. The results obtained from the tests of adherence on the high-density polyethylene models are presented in Fig. 3 (A, B), showing estimated adherence values in CFU/cm^2. No increase in the number of adhered viable cells was evident after 48h (Fig. 3A, B) except in the case of clinical isolates of *C. albicans* that had been cultivated separately (Fig. 3B).

There was considerable variation in adherence over in the 48 h period among the studied clinical isolates and reference strains. However, *S. mutans* in association with *C. albicans* had higher adherence than either species separately (Fig. 3A). A statistically significant increase in the adherence of *S. mutans* was observed in the association with *C. albicans* in relation to the monospecific treatment in the 48h period (p=0.0078, Table 1), where clinical isolates showed a higher number of adhered cells in relation to the reference strains of *S. mutans* (Fig. 3A, Table1).

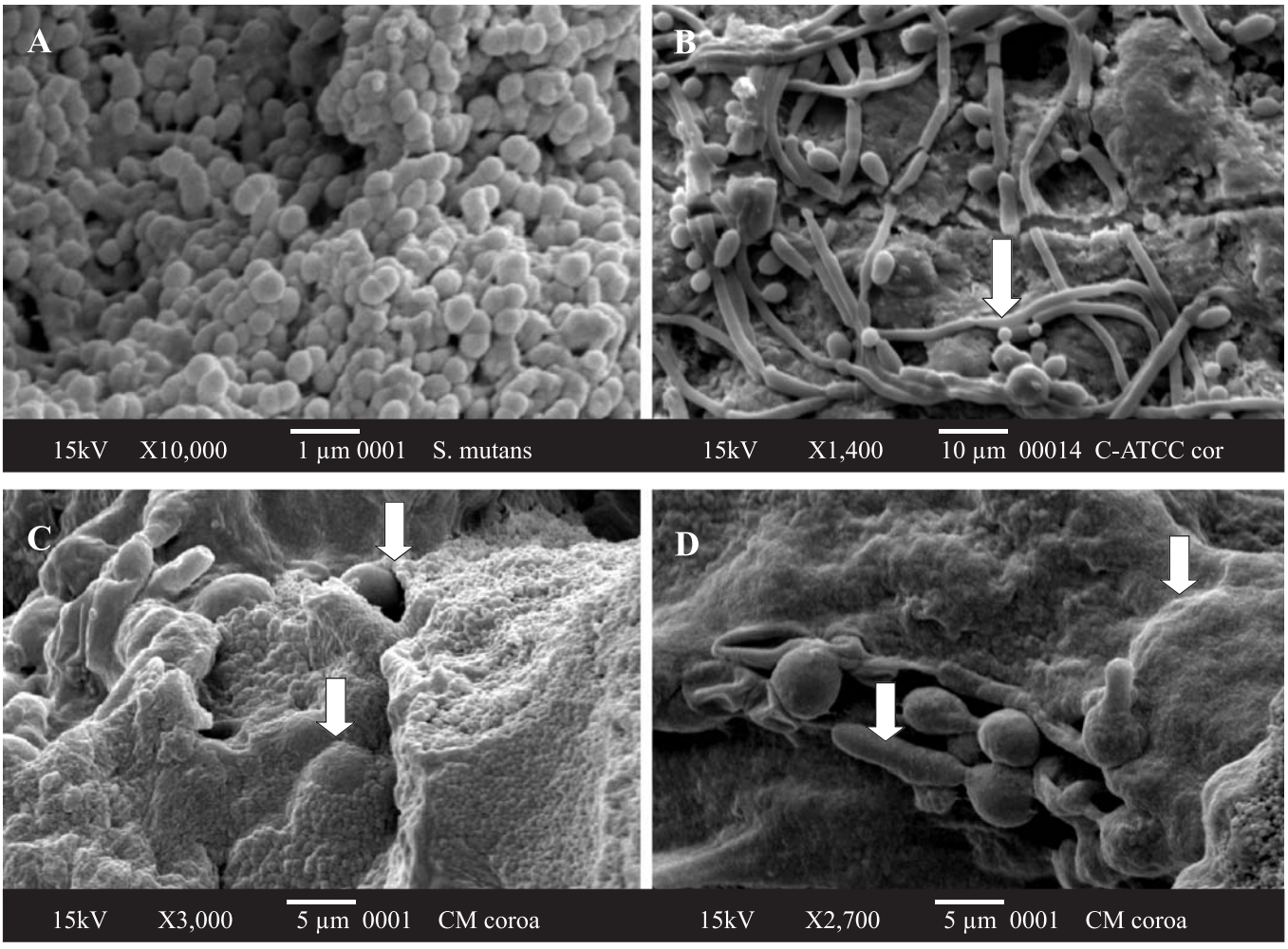

Figure 1. Scanning Electron Micrographs showing the *in vitro* adherence to the dental surface after 21 days of cultivation in Gibbons & Nygaard medium (9) **A** - Magnification of 10000x of adherence pattern of *S. mutans* cells on the dental surface showing tightly grouped cells of uniform size and morphology. **B** - Magnification of 1400x Adherence pattern of *C. albicans* cultured separately on the dental surface. Arrow = Filamentous morphology of cells showing budding with all regions of the tooth being equally colonized **C**- Magnification of 3000x *C. albicans* in association with *S. mutans* on dental surface. Arrows = Abundant amorphous extracellular structures are seen covering the surface of the yeast cells and providing support of adherence. **D**- Magnification of 2700x *C. albicans* in association with *S. mutans* on the dental surface. Arrow = Abundant amorphous extracellular structures are seen between cocci and yeast cells. Arrow = *Candida* cells exhibiting germ tubes.

Likewise, clinical isolates and reference strains of *C. albicans* showed a smaller number of viable adhered cells separately than in association (Fig. 3A). However, when cultivated separately, reference strains of *C. albicans* showed a larger number of adhered cells in relation to clinical isolates, particularly in the 48h period (Fig. 3B, Table 1).

C. albicans cultivated separately showed an increase in the number of viable adhered cells until 72 h (Fig. 3B), whereas *S. mutans* began declining after 48 h (Fig. 3A), both for reference and clinical strains.

DISCUSSION

Based on the SEM images, the predominant morphology of *C. albicans* cells when cultivated separately was the filamentous form (Fig. 1B), a pattern that could indicate that this situation favors cellular morphologies with higher capacity for tissue invasion, possibly due to the absence of competition as previously reported (33,35).

The combined colonization of *S. mutans* and *C. albicans* (both in the case of clinical isolates and reference strains)

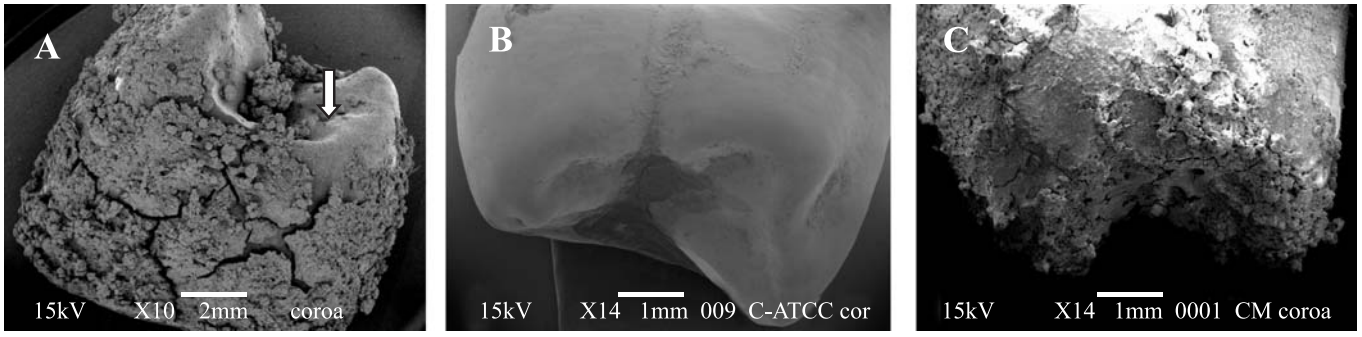

Figure 2. Scanning Electron Micrographs showing the *in vitro* adherence to the dental surface after 21 days of cultivation in Gibbons & Nygaard medium (9) A - Magnification of 10x of the thick compact biofilm formed by *S. mutans*, showing the detachment of the biofilm (arrow). B - Magnification of 14x, thin and weakly evident biofilm formed by *C. albicans*. C - Magnification of 14x, biofilm formed by *S. mutans* and *C. albicans*, with the entire dental surface covered by a homogeneous biofilm.

Table 1. P value of the orthogonal contrasts of the F test of the concentration of viable adhered cells on high-density polyethylene of clinical isolates and reference strain of *S. mutans* and *C. albicans* cultivated separately and in association.

Studied species	Time (h)				
	2	12	24	48	72
	Clinical isolates of *S. mutans*				
Isolates of *S. mutans* in association	0.2176	0.2622	0.2324	0.0078	0.0221
Reference strain of *S. mutans*	0.5393	0.3393	0.1818	0.0085	0.0343
Reference strain of *S. mutans* in association	0.2222	0.2417	0.1947	0.0001	0.0331
	Clinical isolates of *S. mutans* in association				
Reference strain of *S. mutans*	0.6171	0.3941	0.2674	0.0076	0.0013
Reference strain of *S. mutans* in association	0.6315	0.3741	0.2598	0.0042	0.6468
	Reference strain of *S. mutans*				
Reference strain of *S. mutans* in association	0.3222	0.3833	0.2899	0.4463	0.0087
	Clinical isolates of *C. albicans*				
Isolates of *C. albicans* in association	0.3865	0.6171	0.5485	0.0134	0.1797
Reference strain of *C. albicans*	0.3272	0.2899	0.4884	0.0239	0,1473
Reference strain of *C. albicans* in association	0.3125	0.3197	0.3482	0.0001	0.0237
	Clinical isolates of *C. albicans* in association				
Reference strain of *C. albicans*	0.2059	0.4028	0.6315	0.0043	0,7401
Reference strain of *C. albicans* in association	0.1573	0.6468	0.5485	0.0017	0.0309
	Reference strain of *C. albicans*				
Reference strain of *C. albicans* in association	0.3455	0.4583	0.5169	0.0001	0.0134

indicated the *in vitro* adherence of the biofilm throughout the entire dental surface, forming a network of cells of both microorganisms together with an amorphous substance that filled the space between cells (Fig. 1C, D). These observations are consistent with previous reports assessing the adherence of *C. albicans* in the presence of bacteria (4,30,35). These studies have shown that the associated microorganisms form an extensive network, such that the mature biofilm comprise all morphologies of the present microorganisms embedded in an exopolimeric matrix material.

The intensity of the colonization by *S. mutans* was more extensive when cultivated with *C. albicans*, indicating a possible

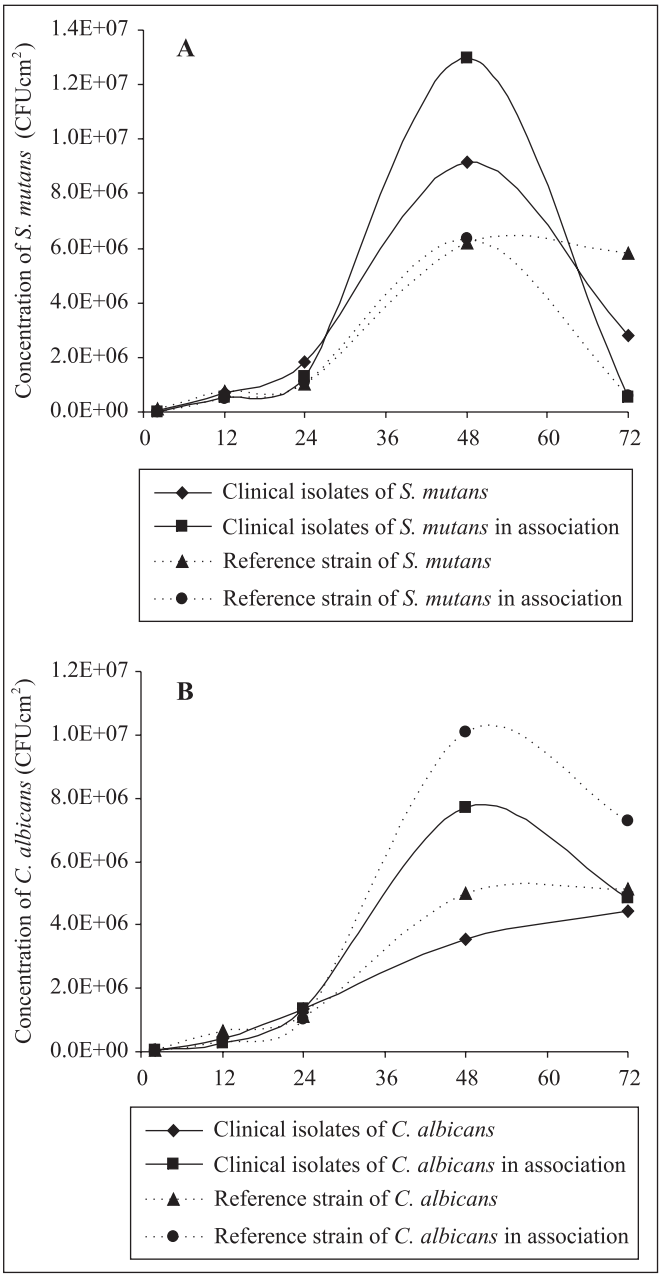

Figure 3. Median adherence value in CFUs/cm² of the concentration of viable adhered cells in the high-density polyethylene of clinical isolates and reference strain of *S. mutans* and *C. albicans* cultivated separately and in association.

facilitation mechanism during this association, where the yeast cells could be used by the bacteria as support for their adherence. The most common morphology of *C. albicans* when in association with *S. mutans* is the yeast-like form, with the formation of blastospores. This can be explained by the presence of *S. mutans* and its metabolic products, which would hamper the formation of the filamentous forms, although a few germinative tubes could be observed (Fig. 1D), as previously reported by Thein *et al.* (35).

According to Soll (33), *C. albicans* is capable of differentiation, showing yeast-like and filamentous developmental forms in association with both disease and commensalism, although the yeast-like form is more prevalent in the case of comensalism. Nikawa *et al.* (24) analyzed the co-adherence of *C. albicans* with oral bacteria and also found a predominance of blastospores during the co-adhesion with *S. mutans* in a culture medium supplemented with sucrose. These authors considered the coaggregation as a key factor in the ecology of oral biofilms. Due to the colonization and virulence factors of *C. albicans*, its presence in microbiotas with caries activity is expected given that it is an aciduric, dentinophilic microorganism with high adherence capacity (1). Given that *C. albicans* has efficient adherence mechanisms, its colonization of the oral environment might represent a predisposing factor favoring its interaction with *S. mutans*, as has been suggested by several authors (1,23,26,35). Most studies on the *in vitro* interaction between two microorganisms use either reference strains or isolates from different hosts (6,24-26). The use of isolates from hosts colonized by both microorganisms can be a starting point to future findings in specific populations.

The interaction of *C. albicans* and *S. mutans* in a combined culture can be understood as mutualistic, given that both microorganisms seem to be favored. In particular, the amorphous substance observed in the interstices between cells seems to play an important role in this interaction. This observation might also explain the formation of aggregates in the culture medium during the combined culture on teeth. Branting *et al.* (6) studied the adherence behavior of *C. albicans* in association to *S. mutans* in acrylic surfaces and suggested that the presence of insoluble glucans produced by *S. mutans* could increase the adhesive capacity of the yeast in combined cultures. Nikawa *et al.* (24) also detected coaggregates composed of bacterial cells covering the surface of yeast cells using SEM, although in that case no adherence substrate was provided.

The *in vitro* adherence to high-density polyethylene observed in the present study confirmed the results from SEM, showing that the association favored the adhesion of both microorganisms. Previous studies demonstrate the benefits of microorganism interactions (4,8,16,29,30), although it is likely that antagonistic interactions also play an important role in mixed biofilm. In particular, the 48 h period was the most favorable to *S. mutans* (p-value=0.0078, Table 1). From that time onward, the number of adhered viable cells of *S. mutans* decreases substantially (Fig. 3A), whereas *C. albicans* still showed viable adhered cells until the 72 h period, as observed by Thein *et al.* (35). However, when in association, both species showed a decrease in the number of adhered cells after 48 h

(Fig. 3A, B). This pattern might have resulted from competition within the same niches, the physical presence of the bacterial, the effect of bacterial metabolites in the development of the biofilm, and the nutritional requirements of the component species, as suggested by Thein *et al.* (35).

Clinical isolates of *S. mutans* obtained from coinfected children showed a higher number of viable adhered cells in the polyethylene assay, when compared to the respective reference strain (Table 1). This difference indicates that the clinical isolates show considerable adherence potential, which is even higher when in association (Fig. 3A). A different behavior was found in the case of *C. albicans*, in which the adherence is lower in the clinical isolates in relation to the reference strains (Fig. 3B). These results indicate that coinfection indeed leads to an increase in the adherence potential. Moreover, there seems to be considerable variation among strains in their adherence capacity, particularly when comparing clinical and reference strains.

Although *in vitro* studies can help the understanding of these associations, the dynamics of this interaction in the oral environment in the presence of other microorganisms, the immunocompetence of the host and other factors such as diet and oral hygiene can play an important regulatory function (16), and can provide a prolific area for future research. According to our results, as well as previous studies, there is strong evidence that *C. albicans* might favor the *in vitro* adherence of *S. mutans* in the dental biofilms, thus favoring its colonization. Research in this area corroborates the suggestion by Moalic *et al.* (23) that the presence of *C. albicans* in the oral environment can be considered as an additional factor that needs to be taken into account in evaluating risks to caries.

RESUMO

Análise da aderência *in vitro* de *Streptococcus mutans* e *Candida albicans*

O objetivo deste trabalho foi investigar a capacidade de aderência *in vitro* de *Streptococcus mutans* e *Candida albicans.* Ensaios de aderência foram realizados *in vitro* na superfície dentária, com posterior análise por Microscopia Eletrônica de Varredura (M.E.V.). Dentes humanos extraídos foram inoculados com *Streptococcus mutans* e *Candida albicans*, além de ambas espécies em conjunto, e foram incubados a 37ºC por 21 dias. Os inóculos eram provenientes de amostras salivares de crianças colonizadas por ambos microrganismos. Como controles foram utilizadas linhagens de referência ATCC dos dois microrganismos. A análise por M.E.V. mostrou a formação de um biofilme que cobriu toda a superfície dentária analisada de forma mais homogênea quando incubados juntos do que separadamente. Um segundo experimento foi desenvolvido utilizando isolados mostrando maior aderência dos experimentos anteriores e cortes de polietileno de alta densidade como substrato. A potencialização da capacidade de aderência de *Streptococcus mutans* e *Candida albicans* em associação foi confirmada.

Palavras-chave: Aderência, *Candida albicans*, *Streptococcus mutans*, biofilme

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