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Demineralizing potential of dental biofilm added with *Candida albicans* and *Candida parapsilosis* isolated from preschool children with and without caries

Thayse Caroline de Abreu Brandi [a], Maristela Barbosa Portela [b], Paula Moraes Lima [a], Gloria Fernanda Barbosa de Araújo Castro [a], Lucianne Cople Maia [a], Andréa Fonseca-Gonçalves [a, *]

[a] Departamento de Odontopediatria e Ortodontia, da Faculdade de Odontologia da Universidade Federal do Rio de Janeiro, Brazil
[b] Departamento de Clínica e Odontopediatria, da Faculdade de Odontologia da Universidade Federal Fluminense, Niterói, Brazil

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ABSTRACT

This study aimed to investigate the demineralizing potential of dental biofilm added of *Candida albicans* (CA) and *Candida parapsilosis* (CP), isolated from preschoolers with and without caries. Bovine enamel blocks (n = 48), with initial hardness = 341.50 ± 21,83 kg/mm^2 were fixed in 24 well plates containing culture media. A pool of children saliva (PHS) was the inoculum for biofilm formation in the presence or absence of isolated CA or CP in accordance with each group (G n = 8): G1 - PHS; G2 - PHS + CA isolated from children with caries; G3 - PHS + CP isolated from children with caries; G4 - PHS + CA isolated from children without caries; G5 - PHS + CP isolated from children without caries; and G6 - blank control. The plates were incubated at 37 °C for 5 days, with daily changes of culture media. The microhardness loss percentage (MHL%) of the blocks was calculated, taking in account the hardness values before and after the experiment. Dental biofilm became more cariogenic, independently of the isolated *Candida* species. The highest MHL% was observed in G4 (85.90 ± 8.72%) and G5 (86.13 ± 6.74%) compared to the others ($p < 0.001$): G1 (34.30 ± 14,30%) < G2 (59.40 ± 10.56%) and G3 (65.80 ± 6.36%) < G6 (13.68 ± 4.86%) ($p < 0.001$). *C. albicans* and *C. parapsilosis* isolates induced the demineralization of the dental enamel.

1. Introduction

Mutans streptococci, some species of Lactobacilli [1], Bifidobacteria [2] and Veillonellas [3] are considered the main microorganisms involved in the etiology of dental caries. However, the literature [4–6] shows that oral fungal microbiota also influences the cariogenic potential of dental biofilm.

Different species of yeast colonize the oral mucosa in balanced conditions of the ecosystem. Among them, *Candida albicans* is the species most commonly isolated from humans. They are considered inhabitant of normal human mucosa, but under special conditions such as low salivary flow, immunosuppression, medications and other, they become pathogenic, being capable of causing diseases such as oral candidiasis. Moreover, *Candida albicans* are acidogenic and heterofermentative, mainly in the presence of high carbohydrate concentrations [7]. Thus, this microorganism can participate in the tooth demineralization process and may represent an important pathogen involved in dental caries [5,8].

Although other *Candida* species, such as *C. parapsilosis*, represent less than 10% of the yeasts in the oral cavity [9], they are commonly found in preschoolers [10]. Thus, it is not least important to investigate their presence in dental biofilms, as well as their actual role in the development of dental caries. Biofilm models developed in *in vitro* studies that simulate the oral environment aim to investigate characteristics related to this disease, especially as regards the composition and cariogenicity of these biofilms [11,12], mainly when it is formed from material collected from individuals with or without caries [13]. However, little is known about the cariogenic biofilms containing isolates of *C. albicans* or *C. parapsilosis*, in the same context.

In this sence, the purpose of this study was to investigate the demineralizing potential of dental biofilm in the presence of

* Corresponding author. Universidade Federal do Rio de Janeiro, Departamento de Odontopediatria e Ortodontia, Rua Rodolpho Paulo Rocco, 325, CEP 21941-913 Rio de Janeiro, RJ, Brazil.
E-mail address: andreagantonio@yahoo.com.br (A. Fonseca-Gonçalves).

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C. albicans and *C. parapsilosis*, isolated from preschoolers with and without caries.

2. Materials and methods

2.1. Study design

This *in vitro* experimental, blind, randomized, controlled study was performed at Dental Research Laboratory of the School of Dentistry in the Federal University of Rio de Janeiro with the approval of the Local Ethics Committee (# 422,911).

Bovine enamel blocks (n = 48; mean initial hardness = 341.50 ± 21,83 kg/mm^2) were used as hosts for the formation of biofilm from a pool of human saliva (PHS), added or not of *C. albicans* or *C. parapsilosis*, isolated from the biofilm of preschool healthy children, with and without dental caries. These blocks were randomly distributed into 24 well microtiter plates containing culture medium (BHI, Difco, Sparks, USA) supplemented with 10% sucrose. Six different groups (G, n = 8) according to the type of inoculum used were evaluated in the study: G1 - PHS; G2 - PHS + *C. albicans* isolated from children with caries; G3 - PHS + *C. parapsilosis* isolated from children with caries; G4 - PH + *C. albicans* isolated from children without caries; G5 - PHS + C. *parapsilosis* isolated from children without caries; and G6 - blank control (no inoculation) (Fig. 1). After 5 days of incubation, blocks were removed from the wells, cleaned and subjected to final microhardness analysis by a single blinded investigator in order to calculate microhardness loss percentage (MHL%) of tooth enamel, translating the cariogenicity of each biofilm group.

2.2. Selection of preschoolers to obtain isolates of Candida species

Preschool children, who attended at the Babies Clinic of the School of Dentistry in the *Universidade Federal do Rio de Janeiro* were selected for the study and their parents consented to their participation. According to the eligibility criteria, these children should have good general health and not be making use of antimicrobials for a minimum period of 3 months.

A calibrated examiner (Kappa = 0.943), performed the clinical examination in the dental chair, using a mouth mirror and explorer, and the dmft was noted [14]. The children were classified according to the caries experience in: **caries free** (dmft = 0; without white spot lesion, and with the parent's report of a diet low in sucrose); or **caries activity** (dmft > 0, presenting necessarily decayed, missed or filled in at least one tooth; and with the parent's report of a diet rich in sucrose).

Of the total of included preschoolers (n = 11; average age of 4.13 ± 1,13 years), eight (4 with dmft = 0 and 4 with cavitated lesions, average dmft = 15.75 ± 1.25) were selected for biofilm collection to obtain isolates of *Candida* species; and the others three (dmft > 0), participated in the study only for unstimulated saliva collection which consisted in the pool to form biofilm on bovine enamel blocks.

2.3. Preparation of bovine enamel blocks

Bovine incisor crowns without decay, stains, cracks or other defects in the enamel were selected through a stereomicroscope (Zeiss - 475,200-9901, West Germany) with 40× magnification. From them, enamel blocks (4 × 4 mm^2) were cut. These blocks were then planned and polished with sandpaper 600 and 1200, respectively, in a Metallographic polisher (APL4, AROTEC, Cotia, SP) for further initial surface microhardness analysis.

2.4. Evaluation of the initial surface microhardness and selection of enamel blocks

Prior to the experiment, the surface microhardness test was performed for selection of bovine enamel blocks. For this analysis, microhardness tester (Buehler, 5104 MICROMET, 679-MIT4-00335, Yokohama, Kanagawa, Japan) with a Knoop diamond indenter type was used, under a load of 25 g for 10 s. Three indentations were made in the center of each specimen spaced from each other by 100 μm [15] yielding a value in kgf/mm^2 for each indentation.

The average of these three values represented the initial surface hardness of the sample. Forty-eight blocks, with initial average hardness of 341.5 ± 21.83 kgf/mm^2 were selected for microbiological experimental test. All samples were stored in humid environment with Milli-Q water until the start of the experimental phase.

2.5. Inoculum preparation

The inoculum used to form biofilm was made up of a pool of human unstimulated saliva collected from three children, added of isolates of *C. albicans* and *C. parapsilosis* of preschoolers with (n = 4) and without caries (n = 4).

Thus, 1 ml of saliva each of those three children was transferred to one tube, resulting in an inoculum with 3 × 10^8 CFU/ml of total microorganisms. This suspension was quantified the number of colonies of *Streptococcus mutans* (1.5 × 10^6 CFU/mL), *Lactobacillus* spp. (2 × 10^3 CFU/mL) and *Candida* spp. (3.5 × 10^2 CFU/mL).

The isolates of *Candida*, which were added to the pool, were obtained from the dental biofilm collected from the other 8 preschoolers. This biofilm was itself firmly adhered to the buccal surface of the maxillary anterior teeth and was collected with the help of an explorer. Immediately after collection, the biofilm (average wet weight = 0.90 ± 0.58 mg) of each subject was placed individually in sterile tubes. To this, 1000 μL of saline solution was added in each tube, and after stirring (2 min), aliquots (10^0 to 10^{-4} dilutions) were seeded with the aid of handles Drigalski in petri dishes containing CHROMagar (Microbiology, France).

After incubation of the plates for 48 h at 37 °C, colonies with different colors were definitely identified with the use of biochemical tests for observation of sugar assimilation and fermentation in accordance with the API 20C system (Biomerieux, Marcy L'Etoile, France). Isolates were then stored in Tryptic Soy Broth medium culture (TSB, Oxoid, Hampshire, England) plus 20% glycerol. The most prevalent isolates were selected: *C. albicans* (CA) found in all volunteers with caries and 2 volunteers without cavities; and *C. parapsilosis* (CP), found in 3 volunteers with caries and 2 volunteers without decay. Eight strains were selected randomly (2 CA and 2 CP from patients with caries, 2 CA and 2 CP from patients without caries) and reactivated on plates containing CHROMagar (Microbiology, France) for forming the inoculum which was added to the previously described saliva pool.

Thus, with the use of a loop, the colonies were transferred from the petri dish to glass tubes individually identified, containing BHI broth (Difco, Sparks, USA). Each resulting suspension was placed on a vortex for 15 s and the cell density adjusted using a spectrophotometer (Biospectro SP-220 UV-VIS spectrophotometer, Equip Ltda., Curitiba, Brazil) with reading at 625 nm, to obtain equivalent transmittance of a standard solution of McFarland 1.0 - about 3 × 10^8 CFU/mL [16].

2.6. Biofilm formation on enamel blocks

The enamel blocks were randomly fixed on polystyrene plates containing 24 wells (TPP, 24 Zellkultur Testplatte F) by means of sticky wax [17]. This system of plates/blocks was sterilized by